https://doi.org/10.31925/farmacia.2023.4.15 **ORIGINAL ARTICLE**

PHYTOCHEMICAL INVESTIGATION, ACUTE TOXICITY, ANTIOXIDANT AND ANTIDIABETIC ACTIVITY OF EXTRACTS FROM AERIAL PARTS OF FOUR WILD GROWING *ACHILLEA* SPECIES

MILENKO RISTIĆ [1], BILJANA DEKIĆ [1], VIDOSLAV DEKIĆ [1], MILOŠ STANOJEVIĆ [2]*, SLAVIŠA MILOŠEVIĆ [2], MARIJA AKSIĆ [1], SUZANA JANIĆEVIĆ [1]

[1]*University of Priština in Kosovska Mitrovica, Faculty of Sciences and Mathematics, Department of Chemistry, Lole Ribara 29, 38220 Kosovska Mitrovica, Serbia*
[2]*University of Priština in Kosovska Mitrovica, Faculty of Sciences and Mathematics, Department of Biology, Lole Ribara 29, 38220 Kosovska Mitrovica, Serbia*

**corresponding author: milosstanojevic655@gmail.com*

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Abstract

Plants from the genus *Achillea* are rich in biologically active ingredients and are traditionally known for their healing potential. In this research, the phytochemical profile was investigated, the content of total phenolics and flavonoids was determined and examined the acute toxicity, antioxidant and antidiabetic activity of ethanolic, ethyl acetate and chloroform extracts of *Achillea abrotanoides* (Vis.) Vis., *Achillea chrysocoma* Friv., *Achillea grandifolia* Friv. and *Achillea millefolium* L. Tested extracts showed the presence of different groups of phytochemicals, while the high content of total phenolics and flavonoids was quantified, especially in the ethanolic and ethyl acetate extracts. The acute toxicity was examined in the *Artemia salina* model, where the extracts did not show significant toxicity after an incubation period of 24 hours, while after 48 hours, strong acute toxicity was recorded in chloroform extracts of *A. millefolium* and *A. chrysocoma*. Extracts were tested for antioxidant activity using the DPPH test, as well as antidiabetic activity based on their ability to inhibit the α-amylase. Ethanol and ethyl acetate extracts of *A. abrotanoides* and *A. millefolium* showed a great ability to reduce DPPH radicals, while the same extracts accomplished a significant inhibitory potential on α-amylase. Also, a correlation was established between the content of total phenolics and flavonoids with the antioxidant activity of the extracts.

Rezumat

Plantele din genul *Achillea*, bogate în principii biologic active, sunt cunoscute în mod tradițional pentru potențialul lor curativ. În cadrul acestui studiu, au fost caracterizate fitochimic și evaluate din punct de vedere al toxicității acute, al acțiunii antioxidante și al activității antidiabetice extractele etanolice, de acetat de etil și de cloroform ale speciilor *Achillea abrotanoides* (Vis.) Vis., *Achillea chrysocoma* Friv., *Achillea grandifolia* Friv. și *Achillea millefolium* L. Toxicitatea acută a fost examinată pe modelul *Artemia salina*, unde extractele nu au prezentat o toxicitate semnificativă după 24 de ore, în timp ce după 48 de ore, s-au observat efecte toxice marcante pentru extractele cloroformice de *A. millefolium* și *A. chrysocoma*. Extractele au fost testate pentru activitatea antioxidantă folosind-se testul DPPH, precum și pentru activitatea antidiabetică bazată pe capacitatea lor de a inhiba α-amilaza. Extractele de etanol și de acetat de etil din *A. abrotanoides* și *A. millefolium* au demonstrat o mare capacitate de reducere a radicalilor DPPH, în timp ce aceleași extracte au realizat un potențial inhibitor semnificativ asupra α-amilazei. De asemenea, s-a stabilit o corelație între conținutul în fenoli totali și flavonoide și activitatea antioxidantă a extractelor.

Keywords: *Achillea* species, phytochemicals, toxicity, antioxidant, antidiabetic

Introduction

Genus *Achillea* belongs to the family Asteraceae. This family encompasses around 2500 species that thrive in almost all climates around the world, with the exception of Antarctica [1]. They mostly occur in temperate and subtropical zones and are most abundant in dry habitats. Genus *Achillea* counts around 110 - 130 species which are widespread in Asia and Europe, while some of them grow in North America [2]. This indicates their exceptional ecological adaptability, from deserts to aquatic habitats and from sea level to high mountains. Among the plants of this genus, there is a significant number of rare, endemic species with very narrow areas of distribution [3]. Certain species have completely isolated small populations that are not numerous enough for chemical and pharmaceutical research.

The plants of this genus are widely used in both traditional and official medicinal practices, and they display considerable antimicrobial, antioxidant, anti-diabetic, antihypertensive, sedative, anti-inflammatory,

antinociceptive, cytotoxic, estrogenic and antispasmodic properties [4-11]. Certainly, one of the most famous plants from the genus *Achillea* is *A. millefolium* L., which is widely used in traditional medicine around the world and which undoubtedly has become a cosmopolitan species [12, 13]. Phytochemical research has revealed that many compounds of this genus are very biologically active, and in particular, phenolics and flavonoids are the most important secondary metabolites, which have been proven to possess a wide range of pharmacological activities [14, 15]. These activities mostly arise as a result of the synergistic effect of phenolics and flavonoids, as well as other groups of secondary metabolites that are present in plants of this genus. Therefore, there is a growing interest in the use of these plants in the pharmaceutical industry and phytomedicine, especially when it comes to protection against various types of diseases. Despite the intensive studies of the species of the genus *Achillea*, there are still gaps in the knowledge of the chemical composition and understanding of their physiological action.

In continuation of previous research [16-18], this study was aimed at examining the presence of certain groups of secondary metabolites, phenolics and flavonoid content, acute toxicity, antioxidant and antidiabetic activity of ethanolic, ethyl acetate and chloroform extracts of *A. abrotanoides* (Vis.) Vis., *A. chrysocoma* Friv., *A. grandifolia* Friv. and *A. millefolium* L. Here, for the first time, the results of phytochemical research and biological activity of extracts of species *A. chrysocoma*.

Materials and Methods

Chemicals. All chemicals and solvents in this work were p.a and HPLC purity, supplied by Sigma Aldrich (St. Louis, USA), Merck (Darmstadt, Germany) and J.T. Baker (Deventer, Netherlands) and were used as received.

Plant material. Aerial parts of all investigated *Achillea* species were collected in the same stage of vegetation (blooming stage) from wild populations growing on Šara mountain, Serbia (Table I). The plant material was identified by PhD. Miloš Stanojević (Department of Biology, Faculty of Sciences and Mathematics, University of Priština in Kosovska Mitrovica, Serbia). Immediately after collection, the aerial part of the plants was dried for 15 days in a shady place. After drying, and just before further processing, the plant material was ground in a mill, and then the required amount was taken for extract preparation.

Table I

Species, coordinate, altitude and time of plant material collection

***Achillea* species**	**Latitude (N)**	**Longitude (E)**	**Altitude (m)**	**Date**
A. abrotanoides	42°15′69″	20°99′75″	2050	August 2019
A. chrysocoma	42°19′18″	21°07′32″	2230	July 2018
A. grandifolia	42°20′77″	20°94′38″	1175	June 2018
A. millefolium	42°20′08″	20°93′68″	1420	June 2019

Extracts preparation. The Soxhlet extraction method was used for the preparation of extracts. Ten grams of dried and ground plant material were extracted with 150 mL of 96% ethanol (ET), ethyl acetate (EA) and chloroform (CH). The extraction was performed for 4 hours at the boiling temperature of the solvent. After the extraction was completed, the extracts were filtered and evaporated to dryness on a *vacuum* evaporator. For analysis, the dry remains of the extracts were dissolved in methanol or dimethyl sulfoxide (DMSO).

Phytochemical screening. For preliminary phytochemical screening, the isolated plant extracts were tested for the presence of certain groups of secondary metabolites, namely: alkaloids, phenols, flavonoids, saponins, tannins, terpenoids, steroids and coumarins, according to previously defined methods [19, 20].

Total phenolics content. The concentration of total phenolics content in the tested extracts was determined with the Folin-Ciocâlteu reagent according to a previously published method, with minor changes [21]. The Folin-Ciocâlteu reagent (1 mL) was added to test tubes containing 150 µL of tested extracts (1000 µg/mL), and after one minute, 2 mL of 20% Na_2CO_3 solution was added. All test tubes were shaken well and then incubated at 23°C for 40 minutes. The absorbance was measured at 765 nm (LLG UniSPEC 2 spectrophotometer), and the total phenolics content in the samples was calculated from the equation obtained from the gallic acid calibration curve and presented in gallic acid equivalents as mg GA/g of dry extract.

Total flavonoid content. The total content of flavonoids was determined using the well-known method with aluminium chloride, with minor modifications [22], in the following order: 500 µL of methanol, 150 µL of 1 M K-acetate solution, 150 µL of 10% $AlCl_3$ solution and 3 mL of distilled water were added to 250 µl of the tested extracts (1000 µg/mL). The tubes were shaken well, and after an incubation period of 40 minutes at 23°C, the absorbances were measured at 415 nm (LLG UniSPEC 2 spectrophotometer). The flavonoid content was calculated from the rutin calibration curve equation and expressed in rutin equivalents, as mg RU/g of dry extract.

Acute toxicity: Artemia salina model. For determination of the acute toxicity of extracts, the *Artemia salina* lethality assay was performed. About 5 g of lyophilized cysts of *A. salina* were put in 800 mL of artificial

seawater. This mixture (at 25°C) was constantly illuminated and aerated. After 48 hours most of the cyst was transformed. Extracts were dissolved in DMSO so their concentration was 1000, 500 and 10 μg/mL. In Petri dishes containing the extract solutions and artificial seawater, 20 hatched nauplii were transferred. DMSO is used as a negative, while different concentrations of sodium dodecyl sulfate (SDS) served as a positive control. Dead shrimp were counted after 24 and 48 hours and the percentage of mortality was expressed. The LC_{50} values (μg/mL) were calculated by using probit regression analysis.

Antioxidant activity. Determination of the antioxidant activity of the tested extracts by the DPPH (1,1-diphenyl-2-picrylhydrazyl) test was done spectrophotometrically, with minor changes [23]. A solution of DPPH was prepared in methanol with a final concentration of 0.1 mM, while the concentrations of the extract solutions were obtained by serial dilution and ranged from 6.25 to 400 μg/mL. The procedure consisted of adding 2 mL of DPPH solution to test tubes containing 200 μL of the tested extracts. The test tubes were shaken and left to stand for 30 min in the dark at room temperature. After 30 min of staying in a dark place, the absorbance of the solutions was measured at 517 nm (LLG UniSPEC 2 spectrophotometer) against the blank. The same procedure was repeated for ascorbic acid solutions (6.25 - 200 μg/mL), which is a known antioxidant and was used as a positive control. The free radical scavenging activity (RSA) was calculated as the ratio of the decrease in the absorbance of the DPPH solution after the addition of the extract solutions and the absorbance of the DPPH solution to which the extract solutions was not added, according to the formula (1):

$$\text{RSA}\ (\%) = [(A_c - A_s)/A_c] \times 100, \quad (1)$$

where: A_c is the absorbance of the DPPH solution to which no extract solutions were added (negative control) and A_s is the absorbance of the DPPH solution after the addition of the extract solutions. Based on the RSA (%) values, IC_{50} values (concentration at which 50% of the radicals are neutralized) were determined using regression analysis (function of RSA (%) depending on the extract concentrations), and the result was expressed in μg/mL.

Antidiabetic activity: α-amylase inhibitory activity. The assay of α-amylase inhibition was determined by the method based on the use of 3,5-dinitrosalicylic acid (DNSA), with minor modifications [24]. Extracts were dissolved in a minimal amount of 10% DMSO and then diluted with phosphate buffer (pH = 6.9) to a final concentration ranging from 100 to 1000 μg/mL. Then, 300 μL of samples in test tubes were incubated with 200 μL of α-amylase (1 U/mL dissolved in 0.1 M phosphate buffer, pH 6.9) for 10 minutes at 37°C. After that, the reaction was started by the addition of 100 μL of starch solution (0.5%) and incubated again for 5 minutes. After the time elapsed, the reaction was stopped by the addition of 300 μL of DNSA reagent (8 mL of 5.3 M Na-K-tartrate in 2 M NaOH and 20 mL of 96 mM 3,5-dinitrosalicylic acid diluted with 12 mL of distilled water). The contents of the test tubes were then thermostated at 95°C for 15 minutes, and after cooling, diluted with 10 mL of distilled water. The absorbances of the solution were measured at 540 nm (LLG UniSPEC 2 spectrophotometer). Acarbose solution (100 - 500 μg/mL) was used as a positive control, for which the same procedure was applied. The percentage of enzyme inhibition of α-amylase was calculated using equation (2):

$$\%\ \text{inhibiton of α-amylase} = [(A_c - A_s)/A_c] \times 100, \quad (2)$$

where: A_c is the absorbance of the solution to which no extracts were added (negative control) and A_s is the absorbance of the extract solutions. Using regression analysis, the IC_{50} values (concentration of the tested sample required to inhibit 50% of enzyme activity) was obtained and the result was expressed in μg/mL.

Statistical analysis. Experiments were performed in triplicate. Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-factor analysis of variance (ANOVA). Values $p < 0.05$ were considered statistically significant. Statistical analysis was performed using GraphPad Prism ver. 7.00 and MS Excel 2016 software packages. Agglomerative hierarchical cluster analysis (AHC) was performed using the MS Excel plug-in program XLSTAT ver. 2015.5.01, where Pearson difference and Euclidean distance were applied.

Results and Discussion

The yield of extracts. The results of the extraction yield of the plant species *A. abrotanoides*, *A. chrysocoma*, *A. grandifolia* and *A. millefolium* are shown in Table II and expressed as percentages (%) of extracts obtained *per* 100 g of dry plant material. The obtained extract yield results ranged from 1.87% to 20.80%, with ET extract yields being 7.21 - 20.80%, EA 3.11 - 5.41% and CH 1.87 - 6.26%. The highest extraction yield was with ET extract of *A. abrotanoides* (20.80%), while the smallest was in CH of *A. chrysocoma* (1.87%). The solubility and extraction of phytochemicals depend on the interaction with other components in the sample and the polarity of the used solvent. The effectiveness of the extraction depends on many factors such as the selectivity of solvents, pH, temperature, initial plant material, as well as the extraction techniques themselves [25]. According to the obtained results, the total dry matter of the extract is higher when ethanol is used for extraction compared to ethyl acetate and chloroform. The reason for this is most likely the lower selectivity of ethanol as a solvent in which the largest number of components was dissolved.

Table II

Yields of isolated extracts

Achillea species	The yield of extract (%, w/w)		
	ET	EA	CH
A. abrotanoides	20.80	5.41	6.26
A. chrysocoma	12.61	3.11	1.87
A. grandifolia	7.21	3.25	4.60
A. millefolium	10.21	4.02	2.25

ET – ethanol; EA – ethyl acetate; CH – chloroform

Phytochemical screening. The results of the preliminary qualitative analysis of the tested extracts of selected *Achillea* species for the presence of certain groups of secondary metabolites are shown in Table III. According to the results, a rich variability of secondary metabolites can be observed depending on the type of extract and plant species. Overall, all ET extracts of *A. abrotanoides*, *A. chrysocoma*, *A. grandifolia* and *A. millefolium* showed the presence of the highest number of secondary metabolites, followed by EA and CH extracts. Common to all extracts of the four *Achillea* species is the presence of tannins, polyphenols and flavonoids, except for tannins for the CH extract of *A. grandifolia*. If we consider each *Achillea* species individually, *A. millefolium* and *A. abrotanoides* showed the highest number of phytochemicals in all extracts. CH extracts of *A. grandifolia* and *A. chrysocoma* proved to be the poorest in the composition of phytochemicals. Phytochemical screening of selected groups of secondary metabolites in different extracts of *A. millefolium* has been the subject of several studies [26-28]. Considering that the extracts were isolated by different techniques, our results are somewhat in agreement with the results of these studies.

Table III

Preliminary phytochemical analysis of ET, EA and CH extract of four selected *Achillea* species

Secondary metabolites	*A. abrotanoides*			*A. chrysocoma*			*A. grandifolia*			*A. millefolium*		
	ET	EA	CH	ET	EA	CH	ET	EA	CH	ET	EA	CH
Tannins	+	+	+	+	+	+	+	+	–	+	+	+
Phenols	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	–	+	–	–	+	–	+	–	–	+	+
Glycosides	+	+	+	+	+	–	+	–	–	+	–	+
Alkaloids	+	–	–	+	–	–	+	+	–	–	+	+
Saponins	–	–	–	–	+	–	–	+	+	+	–	–
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
Coumarins	+	+	–	+	+	–	+	–	–	+	–	–
Terpenoids	–	+	–	+	–	–	+	–	–	+	+	–

ET – ethanol; EA – ethyl acetate; CH – chloroform; (+) – presence; (–) – absence

To group the tested extracts according to similarity based on the presence of certain groups of secondary metabolites, the results obtained by AHC analysis are presented in a dendrogram (Figure 1). The analysis grouped the extracts into three clusters: C1, C2 and C3. Cluster C3 contains only one extract, CH of *A. grandifolia*. This is expected, considering that this extract shows the presence of the smallest number of groups of secondary metabolites, i.e., the presence of phenols, saponins and flavonoids, and no similarity with the extracts of other examined species. Within cluster C2 there are two subclusters. Each subcluster includes ET and EA extracts of all investigated species. The first subcluster consists of EA of *A. chrysocoma* and ET of *A. millefolium* for which the common absence of steroids and alkaloids. The greatest similarity is shown by ET of *A. chrysocoma* and *A. grandifolia* since they show the presence of the same groups of secondary metabolites. These extracts along with EA of *A. abrotanoides* form the second subcluster of cluster C2. The first cluster C1 consists of ET, EA and CH extracts of the investigated species. Within this cluster, three subclusters were distinguished. ET of *A. abrotanoides* and CH of *A. millefolium* form one subcluster, CH of *A. abrotanoides* and *A. chrysocoma* second subcluster, while the third subcluster consists of EA of *A. grandifolia* and *A. millefolium*.

Total phenolics content. The results of spectrophotometric determination of total phenolics content in the extracts of four selected *Achillea* species are given in Table IV. According to the obtained results, it can be seen that the examined extracts have a significant content of total phenolics, ranging from 56.57 ± 0.23 to 233.67 ± 0.19 mg GA/g. From all tested extracts, ET of *A. abrotanoides* contains the highest amount of total phenols (233.67 ± 0.19 mg GA/g), while CH of *A. grandifolia* contains the smallest (56.57 ± 0.23 mg GA/g). From the EA extracts, the highest content of total phenols in the amount of 166.28 ± 0.19 mg GA/g was recorded in *A. millefolium*, followed by *A. abrotanoides* (156.07 ± 0.32 mg GA/g). From the CH extracts, the highest amount of total phenols of 95.88 ± 0.11 mg GA/g was found in *A. millefolium*, and then in *A. chrysocoma* with a value of 85.81 ± 0.26 mg GA/g. If we compare the content of total phenolic compounds in all extracts, the highest amount of total phenols was recorded in ET, followed by EA and finally CH extracts. Of all four species, *A. millefolium* is certainly one of the most studied so far in terms of determining the amount of total phenolics in different extracts. Research by other authors indicates that ethanolic

extracts of *A. millefolium* contain a significant amount of total phenolics [29-31], which is slightly lower than our results.

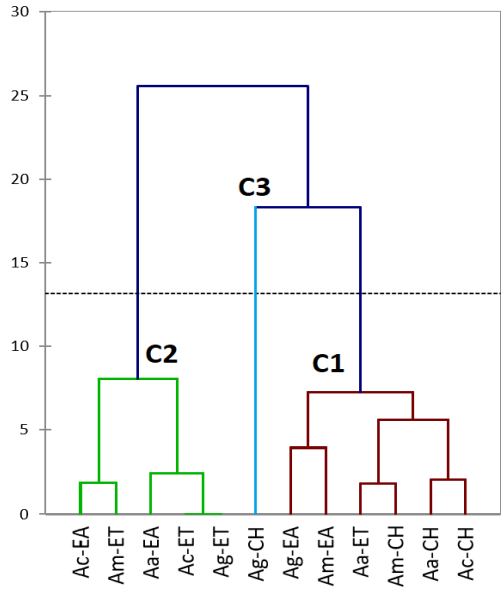

Figure 1.
Dendrogram of preliminary phytochemical screening of extracts of selected *Achillea* species. Three clusters were found: C1, C2 and C3. Aa – *A. abrotanoides*; Ac – *A. chrysocoma*; Ag – *A. grandifolia*; Am – *A. millefolium*; ET – ethanol; EA – ethyl acetate; CH – chloroform

The highest amount of total phenolics was recorded in the ethanolic extract of *A. millefolium* obtained by ultrasonic assisted extraction [29], and our results are in agreement with this study, where a slightly higher amount of total phenolics was recorded in our extract. On the other hand, the amount of total phenolics found in the ethyl acetate extract of *A. millefolium* [32], is lower than our result obtained for the same extract. Examination of extracts of species *A. abrotanoides*, *A. chrysocoma* and *A. grandifolia* in terms of determining the amount of total phenolics is very scarce, and there are almost no literature data based on which the obtained results could be compared.

Total flavonoid content. The results of spectrophotometric determination of total flavonoid content in the extracts of four selected *Achillea* species are given in Table IV. Based on the obtained results, it can be seen that the examined extracts contain smaller amounts of total flavonoids than phenols, ranging from 18.84 ± 0.19 to 168.56 ± 0.11 mg RU/g. Of all ET and EA extracts, the highest amount of total flavonoids (168.56 ± 0.11 and 102.11 ± 0.24 mg RU/g, respectively) was found in *A. abrotanoides*, while in CH, the highest amount was recorded in *A. chrysocoma* (38.22 ± 0.16 mg RU/g). With a value of 18.84 ± 0.19 mg RU/g, the CH extract of *A. grandifolia* showed the lowest amount of total flavonoids. Compared to the content of total flavonoids in the ethanolic extracts of *A. millefolium* [29, 31], which were obtained by different extraction methods, our extract was slightly richer in flavonoids. Also, the ethyl acetate extract of *A. millefolium* was poorer in the amount of total flavonoids compared to our extract [32]. Flavonoids, as a group of phenolic compounds, due to their wide distribution in *Achillea* species, have been presented as the principal bioactive constituents [4].

Table IV
Total phenolics and total flavonoid content in extracts of four selected *Achillea* species

***Achillea* species**	**Extract**	**Total phenolics content (mg GA/g)**	**Total flavonoid content (mg RU/g)**
A. abrotanoides	ET	233.67 ± 0.19	168.56 ± 0.11
	EA	156.07 ± 0.32	102.11 ± 0.24
	CH	79.81 ± 0.21	25.79 ± 0.17
A. chrysocoma	ET	195.38 ± 0.11	121.66 ± 0.25
	EA	134.20 ± 0.18	98.06 ± 0.06
	CH	85.81 ± 0.25	38.22 ± 0.16
A. grandifolia	ET	131.58 ± 0.36	83.60 ± 0.11
	EA	98.63 ± 0.15	31.87 ± 0.32
	CH	56.57 ± 0.23	18.84 ± 0.19
A. millefolium	ET	205.66 ± 0.15	115.23 ± 0.25
	EA	166.28 ± 0.19	96.74 ± 0.10
	CH	95.88 ± 0.11	35.21 ± 0.32

ET – ethanol; EA – ethyl acetate; CH – chloroform

Acute toxicity: Artemia salina model. Plant extracts were tested for their acute toxicity in the brine shrimp *A. salina* model. Based on the percentage of mortality of *A. salina* nauplii, after incubation periods of 24 and 48 hours with the tested extracts (Figure 2), the results of the toxicity are shown in Table V. After 24 hours, the CH extract of *A. millefolium* showed the greatest toxicity with an LC_{50} value of 424.62 µg/mL, followed by CH of *A. chrysocoma* and ET of *A. millefolium* (LC_{50} values of 676.08 and 916.22 µg/mL, respectively). All other extracts, after 24 hours, were with an LC_{50} value above 1000 µg/mL. The high toxicity of CH of *A. millefolium* and CH of *A. chrysocoma* towards brine shrimp was observed after 48 hours of exposure, with minimal LC_{50} values of 34.75 and 94.84 µg/mL, respectively. Expressed toxicity,

after 48 hours, was also shown by ET of *A. millefolium*, with an LC_{50} value of 211.35 µg/mL. With the exception of the mentioned extracts, a significant mortality rate (more than 50% after 48 h), was showed the EA of *A. grandifolia* and ET of *A. abrotanoides* (LC_{50} values of 676.08 and 712.85 µg/mL, respectively). Examination of the toxicity of extracts of *A. millefolium* toward the nauplii of *A. salina* was the subject of other studies [33, 34]. The acute toxicity of the chloroform extract of *A. millefolium* was proven [33], which is in agreement with our results. Comparing the LC_{50} value of the extract of the mentioned species obtained in this study, in our case this value is higher, after 24 hours.

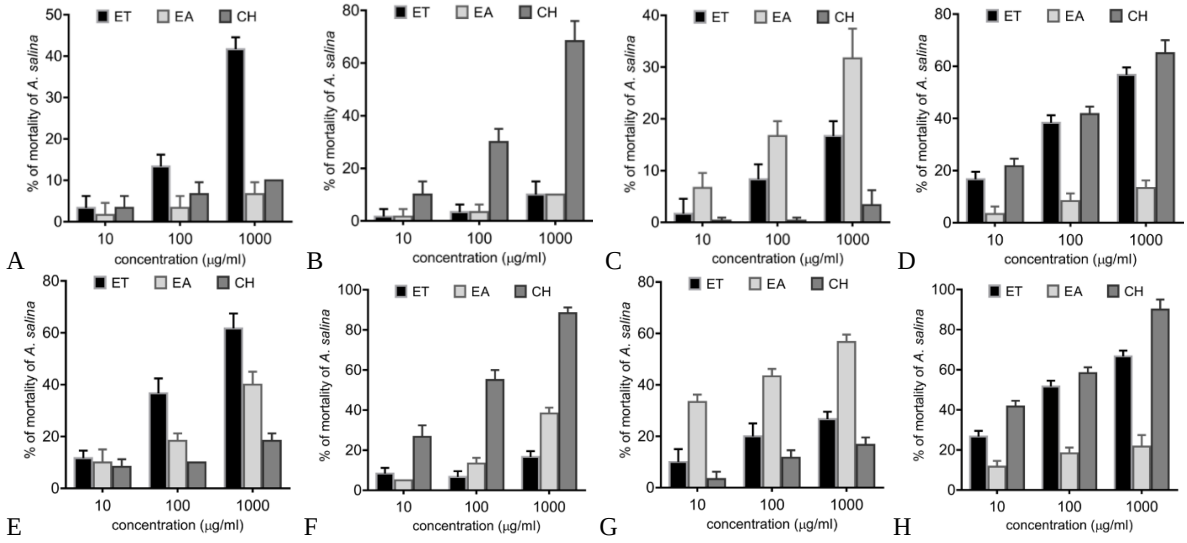

Figure 2.

Mortality (%) of brine shrimp *A. salina* caused by ET, EA and CH extracts of A – *A. abrotanoides*; B – *A. chrysocoma*; C – *A. grandifolia*; D – *A. millefolium* after 24 hours; and E – *A. abrotanoides*; F – *A. chrysocoma*; G – *A. grandifolia*; H – *A. millefolium* after 48 hours. ET – ethanol; EA – ethyl acetate; CH – chloroform

Table V

Acute toxicity of extracts of four selected *Achillea* species in the *Artemia salina* model (after 24 and 48 h)

***Achillea* species**	**Extract**	**LC50 (µg/mL)**	
		24 h	**48 h**
A. abrotanoides	ET	> 1000	712.85
	EA	> 1000	> 1000
	CH	> 1000	> 1000
A. chrysocoma	ET	> 1000	> 1000
	EA	> 1000	> 1000
	CH	676.08	94.84
A. grandifolia	ET	> 1000	> 1000
	EA	> 1000	639.73
	CH	> 1000	> 1000
A. millefolium	ET	916.22	211.35
	EA	> 1000	> 1000
	CH	424.62	34.75

ET – ethanol; EA – ethyl acetate; CH – chloroform

Based on previously published studies of toxicity and LC_{50} values, plant extracts were classified according to two scales, Meyer's and Clarkson's [35, 36]. If the LC_{50} value is below 1000 µg/mL the extracts are toxic and above 1000 µg/mL they are non-toxic, according to both scales. Additionally, Clarkson's scale is divided into extracts with low (500 - 1000 µg/mL), moderate (100 - 500 µg/mL) and high (0 - 100 µg/mL) toxicity. Based on the results and observations of all tested extracts after a 24-hour incubation, the CH of *A. millefolium* showed moderate, while the CH of *A. chrysocoma* and the ET of *A. millefolium* showed low toxicity. After 48 hours, both of CH from *A. millefolium* and *A. chrysocoma* exhibited high toxicity. Also, the ET of *A. millefolium* showed moderate, while the EA of *A. grandifolia* and the ET of *A. abrotanoides* showed low toxicity. All other extracts that had LC_{50} values above 1000 µg/mL toward brine shrimp, after 48 hours, were previously defined as being non-toxic.

Antioxidant activity. The antioxidant activity of the examined extracts was performed by the DPPH radical neutralization method. This method is based on the fact that the odd electron of the nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine [37]. This activity is manifested by a colour change from purple to yellow, which is monitored spectrophotometrically [38]. The results of testing the antioxidant activity of the extracts are shown in Table VI, where the obtained IC_{50} values range from 20.84 ± 0.05 to 396.96 ± 0.43 µg/mL. The ET extract of *A. abrotanoides* showed the strongest antioxidant activity and the highest level of DPPH radical neutralization with an IC_{50} value of 20.84 ± 0.05 µg/mL, followed by ET of *A. millefolium* (23.96 ± 0.23 µg/mL) and ET of *A. chrysocoma* (29.27 ± 0.32 µg/mL). The weakest antioxidant potential with the highest IC_{50} values has

exhibited all extracts of the species *A. grandifolia*. Among the EA extracts, the highest antioxidant activity was recorded in *A. millefolium* and *A. abrotanoides* (IC_{50} values of 31.11 ± 0.05 and 35.11 ± 0.11 µg/mL, respectively), while from CH a high degree of DPPH radical neutralization showed *A. millefolium* and *A. chrysocoma* (IC_{50} values of 72.96 ± 0.15 and 80.22 ± 0.19 µg/mL, respectively). Examination of the antioxidant activity of different extracts of *A. millefolium* found a significant antioxidant potential in neutralizing DPPH radicals [29, 31, 32, 39], while the ethanol extract obtained from the vegetative aerial parts of *A. abrotanoides* showed a higher antioxidant ability than chloroform [40].

Table VI

Antioxidant and α-amylase inhibitory activities of extracts of four selected *Achillea* species

***Achillea* species/standard**	**Extract**	**DPPH scavenging activity, IC_{50} (µg/mL)**	**α-amylase inhibition, IC_{50} (µg/mL)**
A. abrotanoides	ET	20.84 ± 0.05	108.11 ± 0.19
	EA	35.11 ± 0.11	128.97 ± 0.26
	CH	91.71 ± 0.22	379.11 ± 0.12
A. chrysocoma	ET	29.27 ± 0.32	209.25 ± 0.14
	EA	37.68 ± 0.25	212.35 ± 0.32
	CH	80.22 ± 0.19	478.31 ± 0.21
A. grandifolia	ET	287.22 ± 0.27	265.29 ± 0.11
	EA	325.49 ± 0.25	650.89 ± 0.29
	CH	396.96 ± 0.43	425.47 ± 0.17
A. millefolium	ET	23.96 ± 0.23	101.26 ± 0.32
	EA	31.11 ± 0.05	189.62 ± 0.05
	CH	72.96 ± 0.15	308.98 ± 0.12
Ascorbic acid		18.69 ± 0.22	–
Acarbose		–	83.25 ± 0.12

ET – ethanol; EA – ethyl acetate; CH – chloroform

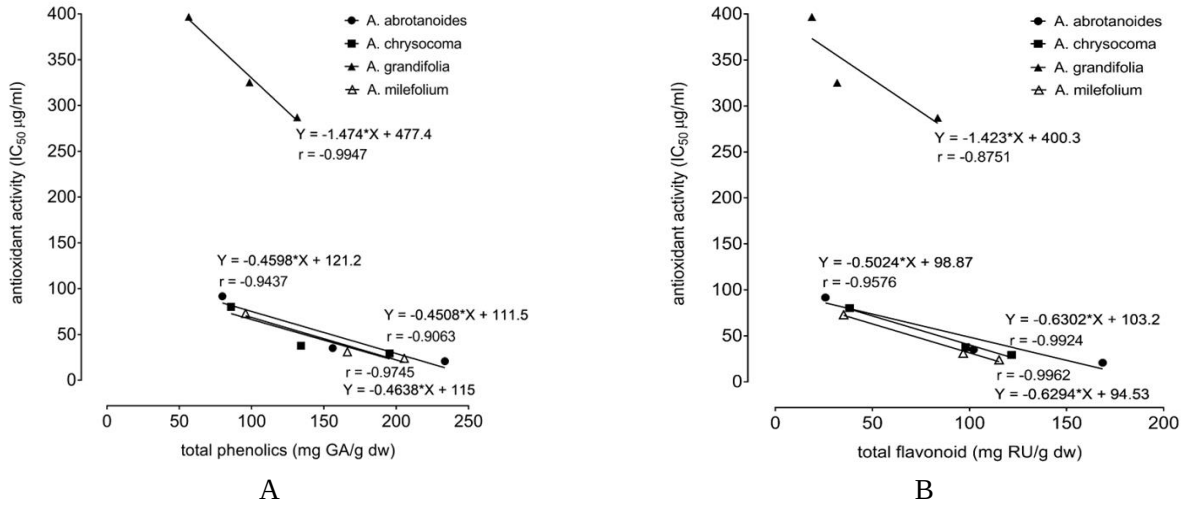

Figure 3.

Graphs of linear correlation between A – the amount of total phenolics and antioxidant activity; B – the amount of total flavonoids and antioxidant activity

The antioxidant potential of different plant species can be attributed to the presence of phenolic compounds since it has been established that there is a correlation between polyphenols and flavonoids with antioxidant activity [41]. Figure 3 shows a linear correlation between the amount of total phenolics and flavonoids with the antioxidant activities of the extracts. Considering that a lower IC_{50} value indicates a higher antioxidative activity, it can be observed that with an increase in the content of phenolics and flavonoids, the activity against DPPH radicals increases, i.e., the IC_{50} value decreases. The linear correlation in all extracts is therefore negative and very strong. In the case of total phenolics and antioxidant activity, the correlation coefficients (r) are -0.9437 (*A. abrotanoides*), -0.9063 (*A. chrysocoma*), -0.9947 (*A. grandifolia*), -0.9745 (*A. millefolium*), while in the case of total flavonoids and antioxidant activity are -0.9576 (*A. abrotanoides*), -0.9924 (*A. chrysocoma*), -0.8751 (*A. grandifolia*) and -0.9962 (*A. millefolium*). In our case, observing the correlation coefficients, the antioxidant activity can be related to the high content of total phenolics and flavonoids in the extracts, where these groups of compounds probably are the most responsible for the pronounced antioxidant capacity.

Antidiabetic activity: α-amylase inhibitory activity. Alpha-amylase is an enzyme that hydrolyses 1-4 glycosidic bonds, *i.e.*, an enzyme that breaks down polysaccharides, such as starch and glycogen, into disaccharides. Inhibition of this enzyme slows down the digestion of starch and consequently reduces the concentration of glucose in the bloodstream. Therefore, the use of preparations or herbal secondary metabolites that lower the level of glucose in the blood after a meal and affect the reduction of oxidative stress could be of importance in the prevention of the onset or supplementary therapy of diabetes [42-44].

The results of testing the inhibitory activity of extracts against α-amylase are shown in Table VI, where all extracts in the tested concentration range inhibited 50% of α-amylase activity. ET extracts of *A. millefolium* and *A. abrotanoides* showed the strongest α-amylase inhibition potential, with IC_{50} values of 101.26 ± 0.32 and 108.11 ± 0.19 μg/mL, respectively. These values were slightly higher than in the case of acarbose, a specific α-amylase inhibitor, whose inhibitory potential was determined under identical experimental conditions. Further in terms of efficiency comes the ET extract of *A. chrysocoma* (209.25 ± 0.14 μg/mL). A slightly weaker, but still significant ability to inhibit α-amylase was observed in EA extracts of *A. millefolium*, *A. abrotanoides* and *A. chrysocoma*, with IC_{50} values of 128.97 ± 0.26, 189.62 ± 0.05 and 212.35 ± 0.32 μg/mL, respectively, while all CH extracts showed a low ability to inhibit the mentioned enzyme. The weakest ability to inhibit α-amylase, with relatively high IC_{50} values, was observed in all extracts of species *A. grandifolia*. The results of previous research showed that the ethyl acetate extract of *A. millefolium* has a remarkable α-amylase inhibitory potential [32], which is in agreement with our results obtained for this plant species.

Conclusions

The results of preliminary phytochemical characterization of the extracts of examined species of the *Achillea* genus showed variability in the composition and bioactivity, which is dependent on the polarity of the solvents used during the extraction. In the examined extracts, a significant content of total phenolics and flavonoid compounds was determined, whereby the ethanolic and ethyl acetate extracts were richer in these compounds than the chloroform extracts. The extracts of *A. abrotanoides* and *A. millefolium* contained the highest amount of phenolics and flavonoids. Strong toxicity against *A. salina* showed chloroform extracts of *A. milefolium* and *A. chrysocoma*. Excellent antioxidant potential of all extracts was observed, with emphasis on ethanol and ethyl acetate of *A. abrotanoides* and *A. millefolium*. Pronounced antioxidant activity can be attributed to the high content of phenolics and flavonoid compounds in extracts, which is also confirmed by the strong correlation between these variables. A significant effect of extracts on the inhibition of α-amylase was also determined, whereby the inhibitory activity of ethanol extract of *A. millefolium* and *A. abrotanoides* stands out from the others. The obtained results suggest that the extracts of the investigated species represent a good source of bioactive compounds and a good basis for further *in vivo* research, to confirm the biological potential of these species and use them in the best possible way for the treatment of certain medical conditions. In this sense, the obtained results certainly represent a contribution to modern phytotherapy and medicine.

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Conflict of interest

The authors declare no conflict of interest.

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