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COMPARATIVE HPLC-MS ANALYSIS OF PHENOLICS FROM *ACHILLEA DISTANS* AND *ACHILLEA MILLEFOLIUM* AND THEIR BIOACTIVITY

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ABSTRACT. The purpose of this study was to characterize the polyphenolic composition of *Achillea distans* and *Achillea millefolium* and to evaluate their antioxidant and antimicrobial activities. The total polyphenolic content was determined spectrophotometrically. The phenolic compounds were identified and quantified by HPLC-MS. The extracts were screened for antioxidant activities using two *in vitro* assay models: DPPH radical scavenging assay and hemoglobin ascorbate peroxidase activity inhibition (HAPX) assay. A method based on electron paramagnetic resonance (EPR) polyphenolic radicals' detection was also described. The antimicrobial activity was determined using the disc diffusion method. For the species of *Achillea*, the phenolic profile showed the presence of two phenolic acid derivatives (chlorogenic and *p*-coumaric acids), four flavonoid glycosides (hyperoside, isoquercitrin, rutin and quercitrin) and four free flavonoids (quercetin, patuletin, luteolin and apigenin), in different concentrations. The evaluation of antioxidant activity indicated that *A. millefolium* extract has more antioxidant than *A. distans*, related with the polyphenolic total content. Both extracts showed activity against all tested bacterial species. These results suggest that the two native species of *Achillea* can be used as a potential source of polyphenolic compounds with bioactive properties for cosmetic and medicinal applications.

Keywords: *Achillea, phenolics, HPLC-MS, antioxidant and antimicrobial activities.*

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INTRODUCTION

The Achillea genus (within the *Asteraceae* family), commonly referred to as yarrow, contains ~ 130 flowering plants throughout the world [1-3] - most commonly in arid and semi-arid subtropical and lower-temperate regions. *Achillea* flowers contain sesquiterpene lactones, essential oil (azulene), flavonoids, phenolic acids determined by different chromatographic and spectroscopic methods: GC-MS, GCQ, HPTLC, RP-HPLC, HPLC-MS, NMR[4-13]. Several species of this genus have for a long time been used to treat wounds, bleedings, inflammation, respiratory infections, skin conditions, spasmodic diseases and dyspepsia, and are at present times employed in the medicinal and cosmetic industry, especially as they are also useful sources of metabolites, which known to display tonic, anti-inflammatory, anti-spasmodic, cytotoxic, diuretic or antimicrobial effects [1,310,12,14-16]. Phenolic acids such as present in these species are known for their potential protective role against oxidative damage diseases (coronary heart disease, stroke, and cancers) [17]. In Romania, there are 23 *Achillea* species and 10 varieties or subspecies [2]. *Achillea distans* Waldst. & Kit. ex Willd. (Alps yarrow) an alpino-carpatho-balkan type is often found alongside with the medicinal and officinal species *Achillea millefolium* (yarrow) with natural pink flowers. It causes the hybridization phenomenon's of the officinal species or the adulteration of the vegetal product *(Millefoliiflos*). Thus, a broader knowledge of the chemical composition of the species becomes an important aim. So far, studies have shown that the essential oil of *A. distans* has a poor quality. This is why the evaluation of the polyphenolic profile that may become a favorable argument for the medicinal use of *A. distans*.

The aim of this work was to analyze the phenolic compounds from two Romanian *Achillea* species and to investigate their antioxidant and antimicrobial properties, for a better chemical characterization and exploitation of these medicinal plants.

RESULTS AND DISCUSSION

HPLC Analysis of Polyphenolics

HPLC coupled with MS allows a simultaneous analysis of different classes of polyphenolics by a single pass column (the separation of all examined compounds was carried out in 35 min). The concentrations of identified polyphenolic compounds in the analyzed samples are presented in Table 1. The HPLC chromatograms of the *A. distans* and *A. millefolium* samples are presented in Figures 1 and 2, respectively.

Chlorogenic acid was found in *Achillea distans* and *Achillea millefolium* extracts in similar amounts (cca. 230 mg/100 g). Four flavonoid glycosides, hyperoside, isoquercitrin, rutin and quercitrin were identified and quantified (Table 1). Hyperoside and isoquercitrin were identified in both extracts, but they were in too low concentration to be quantified (< 0.02). *A. distans* was distinctly the richer species in rutin and quercitrin (with 31.43 mg/100 g and 18.313 mg/100 g, respectively). Four flavonoid aglycones (quercetin, patuletin, luteolin and apigenin) were found in these two extracts, but in varying amounts. Thus, A*. millefolium* contained larger quantities of quercetin, luteolin and apigenin than *A. distans, while* patuletin was quantified only in *A. distans* (0.21 mg/100 g). Concerning the phenolic compounds identification, the results obtained in the present study are in agreement with previously published data on the two species from other areas [7-811-12].

Considering the 19 standard compounds used in this study, some other peaks were not identified.

Therefore can be concluded that these two *Achillea* species can be considered important sources of chlorogenicacid.

Table 1. Phenolic compounds in *Achillea s*pecies
(mg/100 g plant material)

Polyphenolic compounds	**m/z value**	**tR ± SD (min)**	***A. distans***	***A. millefolium***
Chlorogenic acid	353	6.43 ± 0.05	233.75 ± 6.24	231.71 ± 8.28
p-Coumaric acid	163	9.48 ± 0.08	<0.02	0.50± 0.09
Hyperoside	463	19.32 ± 0.12	<0.02	<0.02
Isoquercitrin	463	20.29 ± 0.10	<0.02	<0.02
Rutin	609	20.76 ± 0.15	31.43 ± 3.53	3.52 ± 0.67
Quercitrin	447	23.64 ± 0.13	18.31 ± 0.68	8.21± 1.78
Quercetin	301	27.55 ± 0.15	1.38 ± 0.61	1.60 ± 0.19
Patuletin	331	29.41 ± 0.12	0.21 ± 0.08	<0.02
Luteolin	285	29.64 ± 0.19	50.24 ± 4.75	54.67 ± 3.32
Apigenin	279	39.45 ± 0.15	13.22 ± 1.75	25.96 ± 1.03

Note: NF - not found, below limit of detection. Values are the mean ± SD (n = 3).

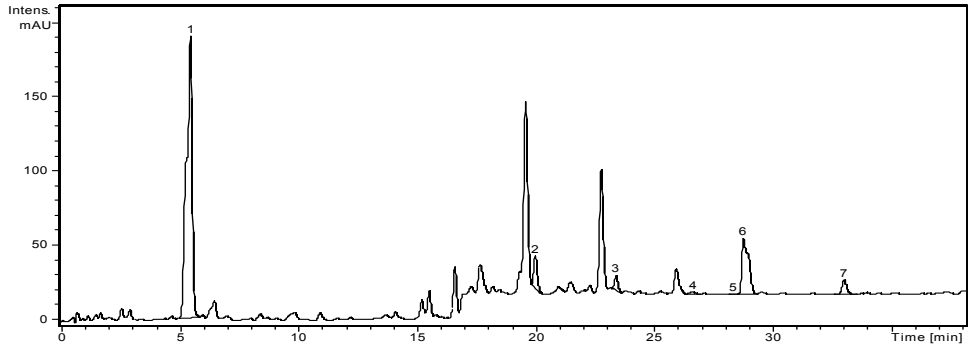

Figure 1. HPLC chromatogram of *A. distans*

Notes: The identified compounds: 1, chlorogenic acid; 2, rutin; 3, quercitrin; 4, quercetin; 5, patuletin; 6, luteolin; 7, apigenin.

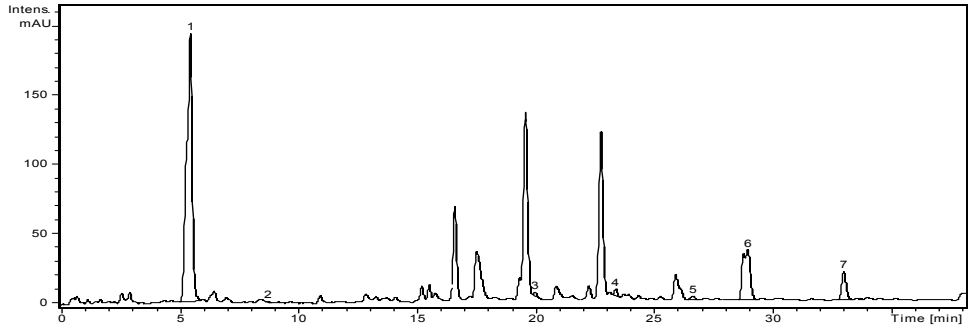

Figure 2. HPLC chromatogram of *A. millefolium*

Notes: The identified compounds: 1, chlorogenic acid; 2, *p*-coumaric acid; 3, rutin; 4, quercitrin; 5, quercetin; 6, luteolin; 7, apigenin.

Polyphenolic content and the antioxidant activity

The *A. millefolium* ethanolic extract contained a higher amount of polyphenols and flavonoids (38.12 and 22.21 mg/g, respectively) than the *A. distans* counterpart (32.56 and 19.47 mg/g, respectively), as illustrated in Table 2. Comparing the phenolic content, these species was poorer than the Italian same species [7].

The ethanolic extracts were also screened for their antioxidant activities using two *in vitro* assay models: DPPH bleaching method and hemoglobin ascorbate peroxidase activity inhibition (HAPX).

Table 2. The content of polyphenols (TPC, flavonoid) and the results of antioxidant activity for *Achillea* extracts

Samples	TPC (mg GAE/g)	Flavonoids (mg RE/g)	DPPH IC_{50} (µg/mL)	HAPX (mg RE/g)
A. distans	32.56 ± 1.42	19.47 ± 1.53	150.30 ± 7.69	0
A. millefolium	38.12 ± 2.00	22.21 ± 1.96	102.84 ± 1.15	724 ± 143
Quercetin	-	-	5.47 ± 0.16	-

Each value is the mean ± SD of three independent measurements. GAE: Gallic acid equivalents; RE: rutin equivalents.

Judging on the lower the IC_{50} value (i.e,more powerful antioxidant capacity [18]. *A. millefolium* (IC_{50} = 102.84 µg/mL), showed a slightly higher radical scavenging activity than *A. distans* (IC_{50} = 150.30 µg/mL). The results are in linewith the phenolic contents listed in Table 2. Compared to the methanolic extract of *A. millefolium from* Turkey, the ethanol extracts of Romanian *A. millefolium* showed lower antioxidant capacity [4].

The HAPX assay monitors the ability of the antioxidants present in the extracts to inhibit the oxidation of hemoglobin by hydrogen peroxide. This reaction occurs normally in the body and can be accelerated under certain stress conditions generating a high valent iron species – ferryl. The method was proposed to be more physiologically relevant for the evaluation of antioxidant capacity, compared to chemical-based methods (ABTS, DPPH, TBARS, etc) [19]. The *A. millefolium* extract exhibits an important capacity to quench the free radical generated in hemoglobin after exposure to hydrogen peroxide, while *A. distans* does not have any effect. This is despite the relatively similar TPC, ABTS and DPPH values, and suggests that certain *A. distans* components may act as prooxidants when confronted with a real-world protein reaction, offsetting the inherent antioxidant effects of the already demonstrated polyphenols and flavonoids.

Direct detection of free radical

Treatment of natural polyphenolic-rich extracts with alkali in presence of molecular oxygen is known to generate semiquinone anion radicals via autooxidation – allowing for a possible fingerprinting of the extracts as well as for correlation of the intensity of the signal with other chemical parameters, such as concentration of the EPR-activated antioxidants [19] The EPR spectra recorded 2 min after alkali treatment of the *Achillea* extracts display a hyperfine structure at the room temperature and show the same line shape for both extracts, with a pattern very similar to quercetin and other polyphenols as chlorogenic acid, isoquercitrin, quercytrin and hyperoside (data not shown).

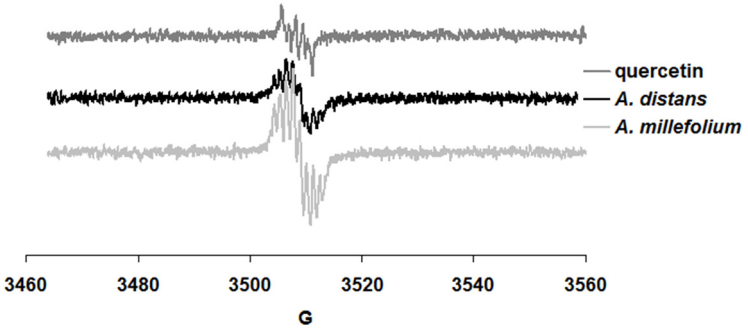

Figure 3. The EPR spectra of quercetin (2 mM) and *Achilea* extracts diluted 10 times and treated with NaOH, in ethanol 90%.

In vitro antimicrobial activity

The ethanolic extracts of *A. distans* and *A. millefolium* were investigated for their *in vitro* antimicrobial properties against four bacteria species and one fungus (Table 3).

Table 3. Antimicrobial activity of *Achillea* extracts

Samples	Inhibition zone diameter (mm)				
	S. aureus	*L. monocytogenes*	*E.coli*	*S. typhimurium*	*C. albicans*
A. distans	12	14	11	11	6
A. millefolium	12	12	10	10	6
Gentamicin	19	18	22	18	-
Fluconazole	-	-	-	-	25

Gentamicin (10µg/well) and Fluconazole (25 µg/well) were used as positive control.

The two extracts were found to be active against the gram-positive bacteria and inactive on the *C. albicans*. Both studied samples showed a low antibacterial action against all bacterial strains tested. However, *A. distans* extract was slightly more active against *L. monocytogenes*, *E. coli* and *S. typhimurium*. Our findings are in agreement with those of other authors [3,4,12,14].

CONCLUSIONS

The phenolic content, antioxidant and antimicrobial activities of *Achillea millefolium* and *Achillea distans* are reported, thus complementing the scientific information concerning the bioactivity and polyphenolic composition of *Achillea* species. This study shows that the phenolic profiles of the both *Achillea* were

generally similar, varying only in the amounts found. The results of the antioxidant assays as well as the phenolic contents indicate that *A. millefolium* extract is clearly more antioxidant than *A. distans* extract. The antimicrobial assays reveal that *Achillea* extracts were effective against tested bacteria. Accordingly, these two indigenous species of *Achillea* may be considered important sources of polyphenols with bioactive properties, justifying their pharmaceutical use.

EXPERIMENTAL SECTION

Plant material and extraction procedure

The flowers of *A. millefolium* (Voucher No. 952) and *A. distans* (Voucher No. 951) were collected in 2013 from the spontaneous flora in Cluj and Maramures counties, in the blossom period. Voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy of the Faculty of Pharmacy, Cluj-Napoca, Romania.

The plant material was reduced to a proper degree of fineness. 5.0 g of the material was extracted with 50 mL of 70% ethanol (Merck, Darmstadt, Germany), for 30 min on a water bath, at 60 °C. The samples were then cooled down and centrifuged at 4,500 rpm for 15 min, and the supernatant was recovered [12,18,20-21].

HPLC–MS analysis

HPLC–MSanalysis was performed on an Agilent 1100 HPLC Series system (Agilent, USA) using the chromatographic conditions previously described [12,18,20]. Quantitative determinations were performed using an external standard method; retention times were determined with a standard deviation ranging from 0.04 to 0.19 min. For all compounds, the accuracy was between 94.1.3% and 105.3%. Accuracy was checked by spiking samples with a solution containing each standard compound in a 10 µg/mL concentration. In all analyzed samples the compounds were identified by comparison of their retention times and recorded electrospray mass spectra with those of standards in the same chromatographic conditions. Calibration curves in the 0.5-50 mg/mL range with good linearity ($R^2 > 0.999$) for a five points plot were used to determine the concentration of polyphenolics in plant samples.

Determination of phenolic compounds content

The total polyphenolic content (TPC) of the extracts were determined using the Folin-Ciocalteu method, with a calibration curve of gallic acid ($R^2 = 0.999$), the absorbance being measured at 760 nm [12,21,22,23]. The spectrophotometric aluminum chloride method was used for flavonoid determination

and the absorbance was measured at 430 nm [21]. Total flavonoid content values, expressed as rutin equivalent (RE), were determined using a calibration curve based on rutin (R^2 = 0.999).

DPPH bleaching assay

For the DPPH assay, 2.0 ml of methanolic DPPH solution (0.25 mM) were added to 2.0 ml of extract solution (or standard) in ethanol at different concentrations (12.5-100 µg/mL). After 30 minutes of incubation at 40°C in a thermostatic bath, the decrease in the absorbance was measured at 517 nm. The percent DPPH scavenging ability was calculated as: DPPH scavenging ability = $(A_{control} - A_{sample})/A_{control} \times 100$, where $Abs_{control}$ is the absorbance of DPPH radical and methanol (containing all reagents except the sample) and Abs_{sample} is the absorbance of DPPH radical and sample extract. Afterwards, a curve of % DPPH scavenging capacity *versus* concentration was plotted and IC_{50} values were calculated. IC_{50} denotes the concentration of sample required to scavenge 50% of DPPH free radicals. Quercetin was used as positive control [12,18,20,23].

HAPX (hemoglobin/ascorbate peroxidase activity inhibition) assay

The hemoglobin ascorbate peroxidase activity assay (HAPX) was described in detail in [19,24]. The reaction was monitored at 405 nm, where all the changes are due to the hemoglobin transformation. Met hemoglobin (6 µM) was added to a mixture of ascorbate (120 µM), peroxide (700 µM) and extracts (5 µl), in acetate buffer, pH 5.5 to start the reaction. An increase in the inhibition time denotes a good antioxidant capacity of the tested extract which acts in competition with the ascorbate. The percentage of the inhibition time for each case was converted to rutin equivalents using a calibration curve (R = 0.98) with rutin standard solutions of 0-1.5 mM.

Free radical generation experiment

For the EPR experiment the extracts were diluted 10 times in 90% ethanol, followed by the treatment with 5 mM NaOH (yielding a pH of 11.7). A low quantity (100 µl) of sample was rapidly transferred to a glass capillary EPR tube. The capillary was placed in the holder of a Bruker ELEXSYS E-580 spectrometer with continuous wave at X band (~ 9.4 GHz). The spectra were measured at room temperature with the following parameters: modulation frequency 100 kHz, microwave power 9.6 mW, modulation amplitude 0.5 G, center field 3514 and sweep field 100 G [19].

Determination of antimicrobial activity

The disc-diffusion assay was employed on the ethanolic extracts of *A. distans* and *A. millefolium* against a panel of microorganisms including gram-positive and gram-negative bacteria: *Listeria monocytogenes* (ATCC 13076), *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Staphilococcus aureus* (ATCC 49444), and *Candida albicans* (ATCC 10231). All microorganism strains were distributed by MicroBioLogics®. Each microorganism was suspended in Mueller Hinton (MH) broth and diluted approximately to 10^6 colony forming unit (cfu)/mL). They were "flood-inoculated" onto the surface of MH agar and MH Dextrose Agar (MDA) and then dried. Six millimetres diameter wells were cut from the agar using a sterile cork-borer and 15 µL of each extract were delivered into the wells. The plates were incubated at 37° C and the diameters of the growth inhibition zones were measured after 18 h. Gentamicin and Fluconazole were used as standard drugs. The controls were performed with only sterile broth and with only overnight culture and 10 µL of 70% ethanol. The clear halos greater than 10 mm were considered as positive results [18,25].

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