Composition, antioxidant and antimicrobial activity of the essential oil of *Achillea collina* Becker growing wild in western Romania

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Abstract
The investigation of the chemical composition, antioxidant and antimicrobial properties of the essential oil of *Achillea collina* Becker growing wild in western Romania was the aim of this study. The chemical composition of the essential oil was evaluated by GC–MS. The major compounds identified were chamazulene (38.89%), germacrene D (12.90%), β-caryophyllene (11.52%) and β-pinene (10.66%). The antimicrobial activity was assessed by the diffusimetric method against seven common food-related bacteria. No effects were observed against *Clostridium perfringens* and *Streptococcus pyogenes*. The antioxidant activity was evaluated using the DPPH test, the essential oil (IC_{50} = 25.03±0.12 μg/ml) demonstrated a stronger scavenging effect than BHA and lower than that of ascorbic acid and propyl gallate. The results reveal strong antimicrobial and antioxidant properties of the essential oil tested and contribute to future research to find new sources of natural antiseptics and antioxidants: a viable and safe alternative to reduce the use of synthetic additives.

Keywords: *Achillea millefolium* ssp. *collina* Becker, essential oil, GC–MS analysis, antimicrobial activity, antioxidant activity.

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The genus *Achillea* belongs to Asteraceae family (Compositae), this large family is represented in the Romanian spontaneous flora by 23 species and 10 varieties or subspecies, spread across all types of landforms [1]. Yarrow inflorescences (Romanian name: *coada șoricelului*) are known as a widely used Romanian traditional remedy, with antispasmodic, bitter tonic and antihemorrhagic actions [2].

The whole plant contains essential oil (EO), but for its isolation the inflorescences are preferred [3], the minimum EO content in the dried plant product being 2 mL/kg [4]. The major components of the yarrow EO are chamazulene, sabinene, germacrene D, β-pinene, 1,8-cineole, linalool, α- and β-thujone, *cis*- and *trans*-β-ocimene, myrcene, camphor, ascaridole, β-caryophyllene, *p*-cymene, bornyl acetate, camphene, limonene, γ-terpinene, caryophyllene oxide, α-phellandrene, β-eudesmol and α-bisabolol [5–9].

According to the European Pharmacopoeia 7.0 (Ph. Eur. 7.0) [4], the content of chamazulene in yarrow, dried plant, must be minimum 0.02%. However, the accumulation of azulenogenic compounds is not a common characteristic of all members of the genus *Achillea*, this property being closely related to the chromosome number in the plant [10]. According to Nemeth and Bernath [10], the accumulation of chamazulene is a characteristic of the members of the group *Millefolium*, in particular of *Achillea asplenifolia* Vent, *Achillea roseo-alba* Ehrend. and *Achillea collina* Becker, native species in the Romanian wild flora [1].

The increasing trend in the last decades for the applications of EOs in the food and pharmaceutical industries prompted the investigation on the biological activities of the members of the genus *Achillea*. Various studies have reported notable antimicrobial properties of extracts and EOs obtained from various species of *Achillea* [5,11–13], together with a good antioxidant potential [5,11,14,15]. Although these results suggest the potential applications as antioxidant or antiseptic for the yarrow EO, it is currently only used in the food industry for flavoring certain alcoholic beverages [16].

To date, based on our knowledge, only the chemical composition of the EO isolated from *Achillea collina* Becker originating in Romania was the subject of a small number of studies [17,18], while information on the *in vitro* antimicrobial and antioxidant activity has not been reported. The purpose of this study is to investigate the chemical composition, antimicrobial properties and antioxidant potential of the EO isolated

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by steam distillation from *A. collina* Becker growing wild in western Romania, in order to identify new sources of natural additives (antioxidants and antiseptics) with applicability in the food and pharmaceutical industries.

MATERIALS AND METHODS

Collection of raw material

The used inflorescences of *A. collina* Becker were collected in July 2012, in the Hunedoara county – the Orăştioara de Sus commune, village of Ludeştii de Jos (45°43'5"N 23°10'21"E), at their maximum flowering stage. A voucher specimen (V.FPT-278) was deposited in the Herbarium of the Faculty of Pharmacy, Victor Babeș University of Medicine and Pharmacy, Timișoara, Romania. After harvesting the material was dried in natural conditions (away from direct sunlight) and stored in double paper bags at temperatures of 3–5 °C.

Isolation of the essential oil

The dried plant material was subjected to steam distillation, according to the method previously described by Craveiro [19]. The EO was separated from water by decantation, dried over anhydrous sodium sulfate and stored for analysis in hermetically sealed amber glass vials at a temperature of 4 °C.

Physical analysis

The specific gravity and the refractive index of the EO were measured according to the method described by the Food Chemical Codex [20]. To determine the specific gravity a 2-mL Gay-Lussac pycnometer (Duran) was used, and a DR6100 digital refractometer (Krüss Optronic GmbH, Germany) was used for the refraction index. The tests were performed in triplicate at the temperatures of 20 °C (refraction index) and 25 °C (specific gravity), respectively.

Free radical-scavenging activity: DPPH assay

The radical scavenging activity was determined by the DPPH assay, as previously described by Brand-williams [21]. Briefly, 3 mL of methanolic stock solution of yarrow oil (1 mg/mL) were prepared, and then diluted to different concentrations (0.01–0.5 mg/mL). 0.5 mL of each diluted sample was mixed with 5 mL methanolic solution of DPPH 0.06 mM. The mixtures were shaken and held in the dark for 15 min. The same procedure was repeated for butylated hydroxylanisole (BHA), propyl gallate, and ascorbic acid (Sigma-Aldrich Chemie GmbH), used as positive controls. The decrease in the DPPH absorbance was measured at 517 nm using a Cecil UV/Vis spectrophotometer (model CE 7200, Milton, England). The methanolic solution of DPPH 0.06 mM was used as negative control and methanol (99.8%) as blank. The DPPH free radical inhibition as a percentage (*I%*) was calculated according to the following equation:

$$I\% = 100(A_{blank} - A_{sample}/A_{blank}) \quad (1)$$

where A_{blank} is the absorbance of the control, and A_{sample} is the absorbance of the test sample. Each test was performed in triplicate. IC_{50} was obtained using the BioDataFit 1.02 software (Chang Broscience Inc, Castro Valley, CA, USA).

Gas chromatography–mass spectrometry

The oil samples were analyzed by gas chromatography with a HP6890 gas chromatograph, coupled with a HP 5973 mass spectrometer. The gas chromatograph has a split/splitless injector and a Factor Four™ VF-35ms capillary column, 35% phenylmethyl phase, 30 m×0.25 mm, 0.25 µm film thickness. The gas chromatography conditions include a temperature range of 50 to 250 °C with a slope of 4 °C/min, with a solvent delay of 5 min. The temperature of the injector was maintained at 250 °C. The inert gas was helium at a flow of 1.0 mL/min, and the volume of injected sample in the splitless mode was 2 µL. The MS conditions were the following: ionization energy, 70 eV; quadrupole temperature, 100 °C; scanning velocity, 1.6 scan/s; weight range, 40–500 amu. The percent composition of the essential oils was calculated. The qualitative analysis was based on the percent area of each peak of the sample compounds. The mass spectrum of each compound was compared with the mass spectrum from the spectra library NIST 98 (USA National Institute of Science and Technology software).

Determination of antimicrobial activity

The antimicrobial activity was determined against seven common food-related bacteria: *Shigella flexneri* (ATCC 12022), *Klebsiella pneumoniae* (ATCC 13882), *Salmonella typhimurium* (ATTC 14028), *Staphylococcus aureus* (ATCC 25923), *E. coli* (ATCC 25922), *Streptococcus pyogenes* (ATTC 19615) and *Clostridium perfringens* (ATCC13124), using the diffusimetric method [22]. Briefly, a suspension of the tested microorganism (10^6 cells mL^{-1}) was spread on the solid media plates (Mueller–Hinton agar). The paper discs, 6 mm in diameter (Whatman No. 1), impregnated with 20µL EO were placed in the centre of the plates. A disc containing 10 µL of sterile broth medium was used as the negative control and as positive control was used rifampicin (5 µg/disk) (Oxoid, UK). After 1 h at room temperature to allow the EO to diffuse across the surface, the plates were sealed with sterile parafilm and incubated at 37 °C for 24–48 h. After incubation, the diameters of the inhibition zones were measured (in mm). Each test was performed in triplicate on at least three separate experiments. The results are presented as means ± *SD*.

Statistical methods

Data were expressed as means and standard deviations. One-way ANOVA test (Bonferroni correction) was used to assess the mean differences of continuous measurements between groups. StataIC 11 statistical software (StataCorp. LP, College Station, TX, USA, version 2009) was used for data analysis. A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

The physical properties and yield of the EO of *A. collina* Becker are shown in Table 1. In the analyzed sample thirty components were identified, representing 99.92% of the EO analyzed (Table 2). The major components were chamazulene (38.89%), germacrene (12.90%), caryophyllene (11.52%) and β-pinene (10.66%), suggesting that the EO analyzed belongs to

Table 1. Yield, physical properties and antioxidant activity of the essential oil of A. collina Becker grown in western Romania; values are mean values and standard deviations (SD, n = 3)

Parameter	Essential oil	Ascorbic acid	BHA	Propyl gallate
Yield, %	0.47	–	–	–
Refractive index (20 °C)	1.515±0.001	–	–	–
Specific gravity (25 °C)	0.912±0.000	–	–	–
DPPH, IC_{50} / μg mL^{-1}	25.03±0.12	23.56±0.12	35.04±0.15	2.1±0.13

Table 2. Composition of the essential oil obtained from A. collina Becker grown in western Romania; compounds are listed in the order of elution from the VF 35 MS column

No.	Compound	R.T. / min	% Of total
1	α-Thujene	5.454	0.16
2	α-Pinene	5.689	2.40
3	Camphene	6.253	0.10
4	Sabinene	6.806	4.87
5	β-Pinene	6.970	10.66
6	α-Terpinene	7.830	0.15
7	Limonene	8.104	0.63
8	1,8-Cineole	8.586	3.99
9	γ-Terpinene	9.004	0.27
10	Terpineol	9.544	0.17
11	Chrysanthenol	11.817	1.43
12	Camphor	12.605	0.25
13	Borneol	12.649	0.06
14	α-Pinocarvone	13.045	0.30
15	*p*-Menth-1-en-8-ol	13.128	0.74
16	Verbenyl acetate	13.700	0.29
17	Lavandulyl acetate	14.655	0.79
18	α-Copaene	16.007	0.10
19	β-Bourbonene	16.359	0.56
20	β-Elemene	16.483	0.39
21	2-Methylbicyclo[4.3.0]non-1(6)-ene	16.559	1.13
22	1*H*-Cycloprop[e]azulene	16.882	0.07
23	Caryophyllene E	17.405	11.52
24	β-Farnesene	17.775	0.73
25	Germacrene D	18.921	12.90
26	γ-Cadinene	19.755	0.38
27	Naphthalene, 1,2-dihydro-3,5,8-trimethyl	20.513	1.02
28	1*H*-Cycloprop[e]azulen-4-ol	21.753	2.51
29	Caryophyllene oxide	21.794	2.46
30	Chamazulene	25.754	38.89
Identified from total area			99.92

the chamazulene chemotype. At the same time, the presence in the composition of the tested EO of a high content of β-pinene, besides chamazulene, points to tetraploid species [5]. Compared with the results obtained, Gherase [17] reports a content below 53% chamazulene in the EO of the same species in Romania, while Nemeth [18] identifies a content of 57.7% chamazulene in the analyzed samples from Romania. The presence of chamazulene as the major compound was also recorded in oils obtained from *A. collina* Becker from Hungary (30.5–67.1%) [18] and Serbia (19.42%) [5], respectively.

The antioxidant capacity of the yarrow EO and the three standard references used, BHA, propyl gallate and ascorbic acid, was determined by the DPPH assay (Table 1). The yarrow EO demonstrated stronger scavenging effects than BHA and lower than that of ascorbic acid and propyl gallate. The literature in this area contains little data on the active compounds responsible for the antioxidant capacity of EOs. However, a number of studies report the antioxidant potential of chamazulene, the constituent with the highest share in the composition of the EO analyzed in this study [15,23,24]. Previously, Bozin [5], seeking to identify the most active constituents of the EO isolated from *A. collina* Becker, responsible for the radical scavenging capacity, indicated chamazulene, the mixture of mono- and sesquiterpene hydrocarbons and camphor. At the same time, a series of studies reported the interdependence between the antioxidant capacity and the presence of phenolic compounds in the composition of extracts of *A. collina* Becker [14,25], their accumulation being influenced by climatic conditions [25].

The results of antimicrobial activity determined by the diffusimetric method (Table 3 and Figure 1) demonstrate that the EO analyzed inhibits most strongly the

Table 3. Antimicrobial activity of the A. collina Becker essential oil, and rifampicin as positive control; inhibitions are expressed as diameter of inhibition zone in mm and include the diameter of the paper disc (6 mm). Data distributions were expressed as mean values and standard deviations (SD, n = 9). Rifampicin (5 µg/disk) was used as positive control; n.a.: no activity

No.	Test microorganism	Antioxidant	
		EO	Rifampicin
1.	*Shigella flexneri* (ATCC 12022)	11.05 (0.13)	14.05 (0.1)
2.	*Klebsiella pneumoniae* (ATCC 13882)	10.94 (0.21)	14.04 (0.06)
3.	*Salmonella typhimurium* (ATTC 14028)	10.14 (0.19)	14.02 (0.07)
4.	*Staphylococcus aureus* (ATCC 25923)	8.98 (0.13)	14.09 (0.08)
5.	*Escherichia coli* (ATCC 25922)	12.97 (0.19)	18.05 (0.07)
6.	*Streptococcus pyogenes* (ATTC 19615)	n.a.	13.03 (0.07)
7.	*Clostridium perfringens* (ATCC13124)	n.a.	12.06 (0.06)

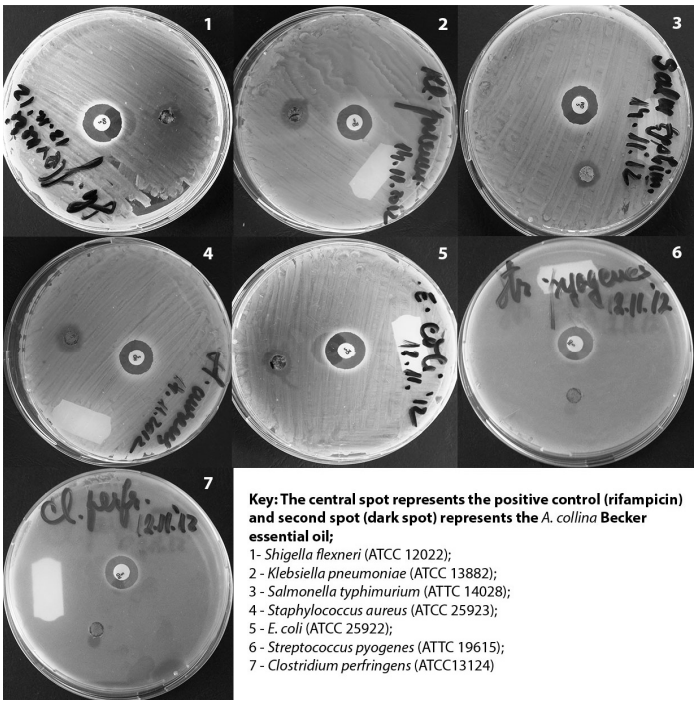

Figure 1. Agar plates showing the growth inhibition zones by A. collina Becker EO against bacterial strains tested.

development of *E. coli*, followed by *Shigella flexneri* > *Klebsiella pneumoniae* > *Salmonella typhimurium* > *Staphylococcus aureus*. There was a statistically significant difference between the mean values of the analyzed groups ($p < 0.0001$, one-way ANOVA test). The pairwise comparisons showed statistical differences between all groups ($p < 0.001$), except *Shigella flexneri* – *Klebsiella pneumoniae*. No effects were observed against *Clostridium perfringens* and *Streptococcus pyogenes*.

The antimicrobial activity of the analyzed oil is comparable to that reported for the EO of *A. collina* Becker originating from Serbia, except in the case of *Streptococcus pyogenes*, on which the latter exerts a strong inhibitory effect [5]. A possible explanation for the antimicrobial activity recorded could be the inhibitory effects exhibited by the major constituents of the EO analyzed: chamazulene [5], along with caryophyllene [26,27] and β-pinene [28]. Also noteworthy is the presence of certain minor components of the EO, known for their strong antimicrobial activity, such as limonene, α-pinene, 1,8-cineole, etc [28,29]. However, various studies have reported, in addition to the synergistic effect of minor components in the chemical composition of EOs, also additive and antagonistic effects, respectively [29–31]. These findings confirm the assumption that along with the major compounds, the total composition should be taken into consideration because of the synergistic role of the constituents, which can modify the biological activity of the oil [10].

CONCLUSIONS

We have investigated the chemical composition, antimicrobial properties and antioxidant potential of the EO of *A. collina* Becker growing wild in western Romania. Thirty components were identified by GC–MS analysis, chamazulene (38.89%), germacrene D (12.90%), β-caryophyllene (11.52%) and β-pinene (10.66%) being the major constituents. The analyzed sample showed comparable scavenging effects with the most used food antioxidants (BHA, propyl gallate and ascorbic acid) and inhibits foodborne pathogens such as: *E. coli*, *Shigella flexneri*, Klebsiella pneumoniae, *Salmonella typhimurium* and *Staphylococcus aureus*. The study complements the existing data in the literature on the biological activity of the EO isolated from *A. collina* Becker and contributes to future research to find new sources of natural antiseptics and antioxidants: a viable and safe alternative to reduce the use of synthetic additives.

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IZVOD

SASTAV, ANTIOKSIDATIVNA I ANTIMIKROBNA AKTIVNOST ETARSKOG ULJA DIVLJE HAJDUČKE TRAVE *Achillea collina* Becker POREKLOM IZ ZAPADNE RUMUNIJE

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Cilj ovog rada bio je da se ispita hemijski sastav, antioksidativna i antimikrobna svojstava etarskog ulja divlje hajdučke trave *Achillea collina* Becker poreklom iz zapadne Rumunije. Hemijski sastav etarskog ulja određen je GC–MS analizom. Kao glavni sastojci identifikovani su: kamazulen (38,89%), germacren D (12,90%), β-kariofilen (11,52%) i β-pinen (10,66%). Antimikrobna aktivnost testirana je difuzionim metodom na sedam vrsta bakterija, koje se mogu naći u hrani. Nije zapažen antimikrobni efekat prema vrstama bakterija *Clostridium perfringens* i *Streptococcus pyogenes*. Antioksidativna aktivnost je određena DPPH testom, koji je pokazao, da etarsko ulje (IC_{50} = 25.03±0.12 μg/ml) ima jači efekat sakupljanja slobodnih radikala od BHA, ali manji od askorbinske kiseline i propil galata. Rezultati su pokazali, da ispitivano etarsko ulje poseduje jaka antimikrobna i antioksidativna svojstva, što omogućava da se u budućim istraživanjima pronađu nova prirodna antiseptična i antioksidativna sredstva: održiva i bezbedna alternativa da se smanji primena sintetičkih aditiva.

Ključne reči: *Achillea millefolium* ssp. *collina* Becker • etarsko ulje • GC–MS analiza • antimikrobna aktivnost • antioksidativna aktivnost