COMPARATIVE PHYTOCHEMICAL EVALUATION IN SEVERAL
ACHILLEA SPECIES FROM ROMANIA

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Abstract
The aim of our study was to perform a comparative phytochemical evaluation in four Achillea species, including two subspecies, from the Romanian spontaneous flora. Essential oils, flavonoids and polyphenolcarboxilic acids were determined by chromatographic and spectrophotometric methods. Essential oil content varied between 0.24 - 0.40%, but only Achillea millefolium oil complied with the pharmacopoeial requirements regarding the chamazulene content. Amongst the other taxa, only Achillea stricta exhibited low amounts of chamazulene (2.41%). Overall, average content of flavonoids and phenolcarboxilic acids were registered in our taxa, with apigenin and luteolin as main flavonoids. All the investigated taxa contained clorogenic acid as well as caffeic acid but only in its esterified form. Based on essential oil composition, two infraspecific chemical taxa, Achillea distans subspecies distans and alpina, were first described. This is also the first record of the chemical composition of the essential oil in Achillea nobilis ssp. neilreichii.

Rezumat
Studiul constă în investigarea fitochimică a patru specii de Achillea, incluzând două subspecii, din flora spontană a României. Uleiurile volatile, flavonoidele și acizi polifenolcarboxilici au fost determinați prin metode cromatografice și spectrototometrice. Conținutul de ulei volatil a variat între 0.24 - 0.40%, dar numai specia Achillea millefolium a prezentat un conținut de azulene conformat cerințelor compendiale. Acest compus a mai fost identificat în cantităte foarte redusă (2.41%) doar la specia Achillea stricta. Conținutul de flavonoide și acizi polifenolcarboxilici poate fi apreciat ca un conținut mediu, flavonoidele reprezentative fiind apigenina și luteolină. În toate speciile a fost identificat acidul clorogenic, iar acidul cafeic doar sub formă esterificată. Pe baza compoziției uleiului volatil au fost desemnate pentru prima dată doi taxoni chimici infraspecifici aparțînînd celor două subspecii de Achillea distans. Compoziția chimică a uleiului esențial al speciei Achillea nobilis ssp. neilreichii este raportată pentru prima dată.

Keywords: Achillea, essential oils, total flavonoids, phenolcarboxilic acids

Introduction
The genus Achillea, generally known as “yarrow”, belongs to the richest and youngest evolutionary genera of the Asteraceae family [28]. About 140 perennial herbaceous species with worldwide distribution have been recognized in this genus [36]. Numerous species of the genus have been used traditionally as antibacterial, anti-inflammatory, vermifuge, analgesic and expectorant etc. [4, 24, 31]. Its richness in bioactive compounds has made this genus a promising candidate for future research and potential development of new drugs [16]. There are still numerous studies investigating the secondary metabolites of Achillea species known to be rich mainly in essential oils, sesquiterpene lactones, diterpenes, triterpenes, lignans, flavonoids and phenolic acids besides some other groups of compounds such as amino acids, fatty acids, alkane and inulin [3, 12, 20, 36]. In the Flora of Romania, the genus Achillea is represented by 24 species and 18 Achillea hybrids [28]. Among these, Achillea millefolium is the only recognised medicinal species registered in the Romanian Pharmacopoeia X-th edition (FR X) [38]. In this species, the whole aerial parts (Millefolii herba) or only the inflorescences (Millefolii flos) are used. Its pharmacological actions arise from various groups of compounds such as chamazulene and prochamazulenes (anti-inflammatory), betonicine (haemostatic), flavonoids, azulenes (spasmolytic) [26]. The most important compound in the oil is chamazulene [20], while among phenolic compounds, caffeic acid has both phytotereapeutic and chemotaxonomical importance [21, 25, 30, 32, 35]. In this context our purpose was to investigate the potential use as medicinal species of 5 Achillea taxa from Romania including: Achillea millefolium L., Achillea stricta Gremli, Achillea nobilis L. ssp.
The plant material consisting of inflorescences has been dried at 25°C, ground into a fine powder and subjected to the investigation of bioactive compounds. Gas-chromatographic analysis. The extraction and quantification of the essential oils from dried inflorescences was made in a Neo-Clevenger apparatus [38] and analysed further by GS-MS. The gas-chromatographic analysis has been performed after the method described by Popovici MP et al. [25]. Wiley Library was used as reference database. Spectrophotometric method. Quantitative determination of the total flavonoids and of polyphenocarboxylic acids was done by the spectrophotometric method indicated in the FR X [38] and expressed as g rutin per 100 g dry weight and polyphenocarboxylic acids were determined with Arnow reagent and expressed as g caffeic acid per 100 g dry weight. HPLC analysis. The qualitative analysis for both classes of compounds was performed by the HPLC method described by Popovici MP et al. [23]. For the preparation of un-hydrolysed samples, the plant material was extracted at 60°C on a water bath with 70% ethanol for 30 minutes. The supernatant was recovered after filtration. For the preparation of hydrolysed samples, the supernatant previously obtained was submitted to acid hydrolysis (HCl, 2N) on a water bath at 80°C for 60 minutes. Statistical analysis. The statistical significance of data was determined by one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) test (P ≤ 0.05) using SPSS program ver. 17.0 (SPSS Inc., Chicago, USA) [29].

Results and Discussion

The rapidly growing demand for medicinal plants correlated with habitat loss, are putting pressure on many species. Thus, we face not only to lose known medicinal plants but also plants with until-now-unknown properties and potential sources of health promoting compounds [11]. Yarrow plants have been shown to contain significant level of essential oils as well as flavonoids and phenolic acids with proven role as health-promoting chemicals showing a broad-spectrum inhibitory activity over micro-organisms, as demonstrated by the in vitro tests against several types of human pathogens [6, 33]. Therefore, exploring the phytochemical profile of lesser-known taxa such as A. distans ssp. alpina and A. nobilis ssp. neilreichii, is important to support their sustainable exploitation for the pharmaceutical industry.

While previous studies were limited to a confined group of specific compounds (only prochamazulenes) [14], or were restricted to a particular taxonomic group of Achillea species [8], our investigations not only contribute to a more complex characterization of volatile oils in 5 different taxa but also to the identification of two infra-specific taxa based on volatile oils composition in A. distans.

In our study, the essential oils content of the investigated Achillea species varied between 0.24 - 0.40 mL/100 g, without significant differences between them (Table II). The overall essential oil content corresponds to the Romanian Pharmacopoeia requirements [38], as FR X request only a minimal content of essential oils (0.2%). According to the European Pharmacopoeia 9th ed. [39], the Achillea millefolium Milefolii herba should contain a minimum of 2 mL/kg essential oil (dried drug) and proazulenes, expressed as chamazulene (C18H16, Mr 184.3) a minimum of 0.02 percent (dried drug). Thus, the differences between the required standards for essential oil and chamazulene content between the Romanian [38] and European [39] Pharmacopoeia may result from the fact that FR X refers to the inflorescences (Millefolii flos) while the European Pharmacopoeia [39] refers to Millefolii herba of Achillea millefolium.

Our results are also in agreement with the essential oil concentrations reported in the existing literature for other Achillea species. Thus, essential oil content was reported to vary between 0.1 to 1.35% dry weight depending on plant genotype and other conditions [17, 19]. The azulene content (chamazulene), the most...
plants were reported to contain mainly proazulene sesquiterpenes, which are transformed, as a result of hydro-distillation, mainly to chamazulene (up to 25%); while the main substances found in hexaploid plants were camphor (18%), sabinene (12%), 1,8-cineole (10%), etc., and the main substance found in octaploid plants was linalool 2 [26]. The essential oil of tetraploid Achillea species has been reported to contain up to 50% chamazulenes while diploid, hexaploid and octaploid species, may contain little to no chamazulenes [10, 18, 20, 22]. Our results are thus endorsed by the ploidy levels reported in literature for these species. Thus, species as A. nobilis reported as diploid and A. distans reported as hexaploid [1, 7, 9] are lacking chamazulene, while A. stricta reported as octoploid [1] contains low amounts of this compound. For A. millefolium, four ploidy levels have been detected (diploid, tetraploid, hexaploid and octoploid) [7, 15]. Considering these aspects we speculate that our chamazulene rich (25.26%) population of A. millefolium might be tetraploid. The first investigations regarding the content in the major proazulene (pro-chamazulene) of Achillea species from Romania were performed by Kotilla E [14] and Gherase F et al. [8].

Table II

The content in essential oils and chamazulene

| Species                  | Essential oil concentration (mL/100 g dry weight) | Essential oil colour | Chamazulene content of the essential oil (%) |
|-------------------------|-----------------------------------------------|---------------------|---------------------------------------------|
| A. millefolium          | 0.40 ± 0.10°                                   | dark blue           | 25.26 ± 0.25°                              |
| A. stricta              | 0.24 ± 0.12°                                   | pale blue           | 2.41 ± 0.15°                               |
| A. nobilis ssp. neilreichii | 0.27 ± 0.11°                                 | colourless          | -                                           |
| A. distans ssp. distans | 0.40 ± 0.20°                                  | colourless          | -                                           |
| A. distans ssp. alpina  | 0.25 ± 0.12°                                  | colourless          | -                                           |

Labelling columns not connected by the same letter are significantly different at P ≤ 0.05, based on a Tukey’s honestly significant difference test.

GC-MS analysis in A. millefolium revealed 34 essential oil components with: β-pinene (32.11%), chamazulene (25.26%), trans-caryophyllene (9.15%), eucalyptol (6.58%), germacrene D (5.59%) and α-pinene (3.24%) as major compounds. A. stricta essential oil contained 22 components of which the most predominant were: β-pinene (13.51%), eucalyptol (12.92%), camphor (11.14%), bornyl acetate (11.11%), α-pinene (4.29%), trans-caryophyllene (4.25%) and chamazulene (2.41%). The A. nobilis ssp. neilreichii oil comprised 48 compounds with five major components: lavandulyl acetate (2.50%), geranyl acetate (3.19%), caryophyllene oxide (3.79%), veridiflor (4.09%), p-cymene (2.96%) and valeranone (1.44%), while two components remained unidentified with Rf 12.79 (21.20%) and Rf 13.81 (21.27%). A. distans ssp. distans oil revealed 18 components with the following main compounds: α-thujone (33.31%), β-thujone (25.52%), sabine (15.60%), eucalyptol (9.05%) and camphor (2.59%). 36 compounds were separated in A. distans ssp. alpina essential oil and the main components were: eucalyptol (20.97%), sabine (6.37%), β-fenchol (5.52%), camphor (4.94%), borneol (4.48%), β-caryophyllene (3.00%), bornyl acetate (2.61%), valaranone (2.30%), α-pinene (1.15%) and gamma-terpinene (1.01%).

Regarding the essential oil composition of A. distans subspecies, it’s important to underline the fact that the two major components α- and β-thujone (53.83%) that are present in A. distans ssp. distans oil are completely lacking from A. distans ssp. alpina. These two subspecies show infra-specific variations in the chemical composition of the essential oil. It was shown that these subspecies also reveal significant morphological differences [30]. The literature survey revealed a high variability of Achillea species essential oil profile [12, 13, 20, 36].

Flavonoid and phenolic compounds were detected at varying concentrations across the analysed species (Table III). Our results show significant differences (P ≤ 0.05) in the total flavonoids content between the analysed species, while no significant differences were registered for polyphenolcarboxylic acids. The inflorescences of the investigated Achillea species contained significant amounts of total flavonoids ranging from 1.48% in A. stricta to 2.93% in A.
The taxa investigated in our study showed an average content of caffeic acid derivatives varying between 0.25% in A. stricta and 0.33% in A. distans ssp. alpina (Table III). Thus, lower amounts of caffeic acid derivatives were registered in our species investigated in the present study compared with those reported for other Achillea species (41.48 mg/g) [3].

Table III

Flavonoids and polyphenolcarboxylic acids content in 5 Achillea taxa

| Species          | Flavonoids (g% rutin) | Polyphenolcarboxylic acids (g% caffeic acid) |
|------------------|-----------------------|---------------------------------------------|
| A. millefolium   | 1.66 ± 0.12a          | 0.33 ± 0.14a                                |
| A. stricta       | 1.48 ± 0.15b          | 0.25 ± 0.12b                                |
| A. nobilis ssp. neilreichii | 2.13 ± 0.11c     | 0.33 ± 0.09d                                |
| A. distans ssp. distans | 2.52 ± 0.11b | 0.29 ± 0.10b                                |
| A. distans ssp. alpina | 2.93 ± 0.13b | 0.33 ± 0.11e                                |

Labelled columns not connected by the same letter are significantly different at P ≤ 0.05, based on a Tukey’s honestly significant difference test.
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Conflict of interest
The authors declare no conflict of interest.

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