Phytochemical Composition of Arnicae flos from Wild Populations in the Northern Area of the Romanian Eastern Carpathians

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The aim of our ongoing study is to assess the quality of the autochthonous plant material of Arnica montana L. species from the northern area of the Romanian Eastern Carpathians. This is the first report for the phytochemical features for the wild populations in this area. The first qualitative assessments were performed by means of High Performance Thin Layer Chromatography (HPTLC). The High Performance Liquid Chromatography (HPLC) analysis highlighted a total content in sesquiterpene-lactones of 0.86-1.36%, while the total content in flavonoids and phenolic acids was 1.08-1.50%, 0.86-1.27% respectively.

Keywords: Sesquiterpene-lactones, Phenolic acids, Flavonoids, HPTLC, HPLC

Arnica montana L. (Asteraceae family) is a herbaceous perennial species, frequent in meadows poor in nutrients, distributed from the south of Norway and Latvia to the south of Portugal, and the north of Carpathians [1]. Two subspecies were identified, montana found throughout the distribution range, except Portugal, and atlantica from south-west France to south of Portugal [1, 2]. A. montana is a medicinal plant with traditional use in Europe for treating blunt injuries and rheumatic disorders [3]. For therapeutic purposes the flower heads (Arnicae flos) are mainly used.

In addition to the studies on the anti-inflammatory activity, several studies have focused on in the last decade the applicability of helenalin, one of the main sesquiterpene-lactone in A. montana, in the treatment of cancer and autoimmune diseases [4, 5].

A. montana was common in mountain meadows, but in the last years it was observed the rapid decline of the species as a result of habitat fragmentation, grassland degradation and excessive harvesting. Consequently, the species is considered one of the most threatened grassland species in Central Europe [6].

In Romania, A. montana is considered rare or vulnerable [7-9], yet, Romania is one of the main European countries that provides plant material on the world market. In 2003, 28 tonnes of flower heads were harvested in Romania [10], while the total amount of dried flower heads traded annually in Europe was estimated to 50 t [11].

In this context, it is necessary to perform a phytochemical screening on the wild population of A. montana in order to define the phytochemical peculiarities of the autochthonous plant material.

The main classes of phytochemical compounds in A. montana are represented by the sesquiterpene-lactones, flavonoids and phenolic acids.

The main sesquiterpene-lactones isolated from the flower heads of A. montana are helenalin, 11α,13-dihydrohelenalin and their esters [12, 13].

The anti-inflammatory activity of A. montana extracts is mainly due to the sesquiterpene-lactones, while the flavonoids and phenolic acids are responsible for the antioxidant and antimicrobial activities [14, 15]. The following flavonoids and phenolic acids were reported for A. montana: Quercetin 3-O-β-D-glucoside, Patuletin 3-O-β-D-glucoside, Kaempferol 3-O-β-D-glucoside, Kaempferol 3-O-β-D-glucuronide, 6-Methoxykaempferol 3-O-β-D-glucoside, Hipsidulin, Quercetin 3-O-gluconic acid, Chlorogenic acid, 3,5-Dicaffeoylquinic acid, 1-Methoxy-oxaloyl-3,5-dicaffeoylquinic acid, 4,5-Dicaffeoylquinic acid [15, 16, 17, 18].

The sesquiterpene-lactones in A. montana extracts are responsible for the anti-inflammatory activity. The mechanism of action involves the inhibition of pro-inflammatory cytokines interleukin-1, TNF-α (Tumor Necrosis Factor alpha) and the translocation of NF-kB (Nuclear Factor kappa B) and NF-AT (Nuclear Factor of Activated T-cells) which are involved in the inflammatory processes [13, 19, 20].

Helenalin, one of the main sesquiterpene-lactones in A. montana, suppresses the translocation of NFATc2 (Nuclear Factor of Activated T-cells, cytoplasmic 2) in the CD4+T cells, thus having immunosuppressive properties, with applicability in the treatment of autoimmune diseases [5]. In addition, in vitro studies showed the anti-cancer potential of helenalin, by its selective inhibition of human telomerase and due to its high cytotoxicity [4, 21].

Experimental part
Materials and methods

Chemicals
Neutral aluminum oxide (Sigma-Aldrich), ethyl acetate (Merck-Millipore), methyl chloride (Merck-Millipore), formic acid (Merck-Millipore), 2-aminoethylidiphenyl-borinate (N.P.) (Fluka), PEG 400 (Sigma-Aldrich), methanol (Merck-Millipore), acetone (Merck-Millipore), have been used as they have been delivered by the suppliers (analytical purity).

Plant material
A. montana flower heads were harvested in full flowering stage from 4 wild populations in the northern are of the Romanian Eastern Carpathians, Suceava county, in June 2012 and June 2013. The plant material was force dried at 40°C. The four A. montana sites differed in their features...
regarding the altitude, exposure, anthropization degree, slope, the use of the land, characteristic specific for the mountain landscape. The samples that were subjected to the phytochemical analysis are presented in table 1.

**Sample preparation**

**Sesquiterpene-lactones**

The extracts where prepared according to the European Pharmacopoeia [22], using santonin as internal standard. 1.00 g of grounded plant material was introduces into a 250 mL round-bottomed flask, 50 mL of a mixture of equal volumes of methanol and water was added and it was heated under a reflux condenser in a water bath at 50-60°C for 30 min, stirred continuously. The solution was allowed to cool and filtered through a filter paper. This procedure was repeated three times. To the combined filtrates 3.00 mL of the internal standard solution was added and then it was concentrated to 18 mL under reduced pressure. The roud-bottomed flask was rinsed with water and diluted, with the washing to 20.0 mL. The solution was transferred to a chromatography column EXtrelut NT20. The extract was allowed to stand for 20 min, stirred continuously. The solution was heated under reflux condenser in a water bath at 50-60°C for 30 min, after which we switched back to the initial conditions for 10 min.

**Flavonoids and phenolic acids**

The extracts were subject to HPLC analysis (Agilent, Zorbax SBC18, 3 x 150 mm, 5 μm), flow 1 mL/min.; injection volume 10 μL; DAD detection; mobile phase acetonitrile (A) and sodium acetate buffer (2mM), pH=3.5 (B); gradient: 2-14-20-30-25% solvent A for 0-20-40-50-60 min, after which we switched back to the initial conditions for 10 min.

**Results and discussions**

The list of samples and their harvested location is given in table 1.

The first qualitative assessments for the flavonoids and phenolic acids were performed by means of High Performance Thin Layer Chromatography (HPTLC).

The HPTLC fingerprint (fig. 1) for the analysed samples highlighted the presence of 3 yellow-orange spots corresponding to the flavonoids luteolin-7-O-glucoside (Rf = 0.47), isoquercitrine (Rf = 0.44) and hyperoside (Rf = 0.63) and the presence of the phenolic acids (blue spots) isochlorogenic acids (Rf = 0.70 – 0.85), cyanaré (Rf = 0.50) and chlorogenic acid (Rf = 0.63).

**HPTLC analysis**

The initial qualitative assessments of the flavonoids and phenolic acids were achieved by HPTLC. **Stationary phase** HPTLC 20X10cm, silica gel 60 F254 plates (Merck); **mobile phase**: anhydrous formic acid, water, and ethylacetate (10:10:80, v/v); **development distance**: 7 cm; **derivationatization**: NP solution (10 g/L, in ethylacetate) and PEG solution (Macrogel 400, 50 g/L, in dichloromethane); **visualization**: 366 nm.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample code</th>
<th>Harvest time</th>
<th>Population</th>
<th>GPS Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O1-12</td>
<td>June 2012</td>
<td>Ortodoxia</td>
<td>47°19'34.92&quot; N, 25°28'40.60&quot; E</td>
</tr>
<tr>
<td>2</td>
<td>O2-12</td>
<td>June 2012</td>
<td>Ortodoxia</td>
<td>47°19'12.43&quot; N, 25°28'38.9&quot; E</td>
</tr>
<tr>
<td>4</td>
<td>A2-12</td>
<td>June 2013</td>
<td>Arini</td>
<td>47°18'50.36&quot; N, 25°25'44.79&quot; E</td>
</tr>
</tbody>
</table>

**Table 1**

**LIST OF THE ANALYSED SAMPLES**
equivalents and the total content in phenolic acids in caffeic acid equivalents. All values are expressed as % d.w.

The analysis of the Arnicae flos samples revealed interpopulational variation in phytochemical profile for both investigated vegetation seasons (fig. 4).

The total content in sesquiterpene-lactones varied from 0.86 to 1.36 % d.w. while the total content in flavonoids was ranging from 1.08 to 1.50%. The phenolic acids were found in the range of 0.86-1.27 %.

For the vegetation season 2012, population O1 had the highest content of sesquiterpene-lactones (1.36 %) and the lowest content in flavonoids (1.08 %) and phenolic acids (0.86 %). Population O2 registered the highest phenolic acids content (1.27 %) while the sesquiterpene-lactones and flavonoids were in relatively high values. Population A1 had the highest content in flavonoids (1.50 %). For the population A2, the total contents of sesquiterpene-lactones, flavonoids and phenolic acids were within the average.

For the vegetation season 2013, the sesquiterpene-lactones content varied from 0.99 to 1.22 %, with values slightly higher than for the plant material harvested in 2012. The total content of flavonoids registered values of 0.89-1.37 % and the total phenolic acids content was of 0.93-1.56 %.

The population O2 had the highest content in sesquiterpene-lactones (1.22 %), while the population A1 had the highest content in flavonoids (1.37 %) and phenolic acids (1.56 %).

The content in biological active compounds was higher for the vegetation season 2013 for all analysed compounds and the values were more homogenous.

The interpopulational variation in the total content of sesquiterpene-lactones, flavonoids and phenolic acids may be explained by the genetic diversity and the pedo-climatic peculiarities in the wild populations. Literature data showed that the phytochemical profile of A. montana is influenced both by the biotic and abiotic characteristics in the wild habitats [16, 24, 25].

The total content of sesquiterpene-lactones is higher than the minimum content indicated by the European Pharmacopoeia (0.4 %) and is comparable with the content cited in the literature 0.40-1.55 % [3, 26, 27]. For the Arnicae flos originating from the Apuseni Mountains, Romania, the sesquiterpene-lactone content was of 0.74-1.08 % [28]. Seemann and colab. obtained a total content in sesquiterpene-lactones of 0.59-1.10 % for samples collected in the Alps in Germany, with a significant higher content for the samples collected from the foothills compared with the other Arnica sites [28].

The total content of flavonoids and phenolic acids is comparable with the values cited in the literature: 0.60-2.44% for the flavonoids and 1.03-1.88 % for phenolic acids [3, 15, 29].

The genetic diversity in the wild populations of A. montana may serve as a selection pool for the identification, isolation, preservation and propagation of chemotypes with
optimum phytochemical profiles depending on the final use of the plant material. *A. montana* extracts have a wide range of uses, as anti-inflammatory agent, in cosmetics and in homeopathy [30, 31]. Thus, if the extracts are used in the treatment of inflammatory disorders the plant material should have high sesquiterpene-lactones content. Yet, if the plant material is used in cosmetics, the content in flavonoids and phenolic acids is also of high importance.

Conclusions

We reported for the first time on the sesquiterpene-lactone, flavonoids and phenolic acids total content in *Arnicae flos* for *A. montana* wild populations in the northern area of the Romanian Eastern Carpathians.

The preliminary study should be further extended by assessing the plant material harvested in minimum 3 consecutive years in order to obtain a clearer image on the phytochemical diversity of the *A. montana* wild populations in the target area and to establish the sources of the variations in the phytochemical profile and by performing HPLC-MS analysis for a more thorough characterization of the extracts and extractive fractions which can be used in phytotherapy and medicine.

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