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IN SITU AND *IN VITRO* COMPARATIVE STUDY ON THE BIOPRODUCTIVITY OF *ARNICAE FOLIUM ET CAULIS* FROM THE NORTHERN AREA OF THE ROMANIAN EASTERN CARPATHIANS

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Abstract: *Arnica montana* L. is an important plant bioresource, being traditionally used as medicinal plant, for which the scientific and economic interest remains at a high level. The aim of our study was to evaluate the bioproductivity for *Arnicae folium et caulis* in terms of biomass and content in biological active compounds originating from the wild populations and *in vitro* experimental cultures – under controlled environment. *In situ* biomass production registered variations, probably due to the different environmental features and also to the management strategies for each site. The development of *in vitro* cultures had as purpose the evaluation of the biomass production and production of plant material for the phytochemical screening. The *Arnicae folium et caulis* samples harvested from the wild populations have a higher content in phenolic acids than the *Arnicae flos* from the same area, but the total content in sesquiterpen-lactones was significantly lower. In contrast, the samples originating from the *in vitro* cultures had a low content in phenolic compounds and a high content in sesquiterpen-lactones, comparable with *Arnicae flos*.

Keywords: *A. montana*, biomass, phenolic acids, flavonoids, sescviterpen-lactones, HPTLC, HPLC

Introduction

In the current context of the development of basic research in the field of life sciences, with applicability in the improvement of the quality of the life, the plant species diversity, by the bio-resources offered by it, establishes a domain of priority study to identify and to isolate new biologically active compounds, including the evaluation of their activity.

Plant resources in the mountain areas are a valuable source of raw material, since pedo-climatic peculiarities give distinct biosynthetic potential with adaptive significance for the plants. This is reflected in the quality of plant material, in terms of the content of biologically active principles.

In this context, *Arnica montana* L. is one of the species of traditionally used medicinal plants for which the scientific and economic interest remains at a high level and is aimed at conservation and sustainable issues. *A. montana* is a perennial herbaceous species

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of the Asteraceae family with traditional medicinal use in Europe, arnica extract being used topically for its anti-inflammatory action.

The phytocomplex specific to *A. montana* includes sescviterpenic lactones, mainly helenalin, dihidrohelenalin and esters, phenolic acids and flavonoids, respectively. The phytochemical studies envisaged the characterization of the biological active compounds content for *A. montana* plant material harvested from wild populations and experimental cultures [AIELLO & al. 2012; GANZERA & al. 2008; NIKOLOVA & al. 2013; SCHMIDT & al. 1998] and the identification of the environmental factors that influence the content in biological active substances [ALBERT & al. 2009; SEEMAN & al. 2010; SPITALER & al. 2008].

Sescviterpen-lactones have an anti-inflammatory effect, through different mechanisms of action involved in inflammatory processes [LYSS & al. 1998]. Phenolic acids and flavonoids have antioxidant, cytoprotective and anti-inflammatory activity [ZHELEVA-DIMITROVA & BALABANOVA, 2012; WOERDENBAG & al. 1994].

In addition, for the plant itself, these biological active compounds present adaptive significance with defensive role for *A. montana* plants as a response to biotic and abiotic stress, characteristic for the mountain areas.

The species *A. montana* can be a therapeutically valuable bio-resource with multiple applications. Studies in the last decade have opened new perspectives on the applicability of arnica extract and helenaline in the treatment of some types of cancer and autoimmune diseases [HUANG & al. 2005; BERGES & al. 2009]. Also, *A. montana* is used in human and veterinary homeopathy, as well as in a wide range of products in the cosmetics industry.

The over-exploitation of the species *A. montana* and the environmental changes have led to problems of sustainability; the species has special conservation status in most areas of distribution in Europe, in Romania being considered rare or vulnerable [OLTEAN & al. 1994; NEGREAN, 2001].

Romania is one of the main European countries, providing plant material of *A. montana* on the global market. Although the interest in the species *A. montana* has remained high worldwide, the species is still poorly capitalized in Romania, for both economic and scientific purposes. Thus, there is a lack of comprehensive studies integrated into the current status of the species in natural populations, i.e., strategies for conservation and recovery by creating and promoting local products. Most studies in Romania concentrated on the Apuseni Mountains, while in the Romanian Eastern Carpathians the species is mentioned only in studies of flora [ZAMFIRESCU, 2006; MICHLER, 2007; MARDARI, 2008].

Evaluation of the quality and availability of native plant material gives added value to marketing strategies, as the scientific basis leads to the reduction of the vulnerability on a competitive international market.

The aim of our study was to evaluate the bioproductivity for *Arnicae folium et caulis* in terms of biomass and content in biological active compounds originating from the wild populations from the northern area of the Romanian Eastern Carpathians and *in vitro* experimental cultures – under controlled environment. The results of the *in situ* and *in vitro* studies may be used in cultivation strategies. Considering that the cultivation of the species involve high costs, and the flower heads production lowers after the 4th year, the exploitation of the underground parts of the plant may lead to the fulfilment of the

investment plan [PLJEVLJAKUSIC & al. 2012]. Thus, based on the phytochemical screening, *Arnicae folium et caulis* can be also promoted as raw material.

The assessments on *Arnicae flos* are the object of a parallel study [STEFANACHE & al. 2013], being well documented in literature since *Arnicae flos* is the official drug included in the European Pharmacopoeia. Currently, in human and veterinary homeopathy it is used only the *Arnicae folium et caulis*, respectively *Arnicae radix.*

Materials and methods

Plant material

Within the study 5 natural populations of *A. montana* (Ortoaia 1 – O1, Ortoaia 2 – O2, Arini 1 – A1, Arini 2 – A2 and Arini 3 – A3) from the northern area of the Romanian Eastern Carpathians, Neamt County, Dorna Arini commune, during the vegetation seasons 2012 and 2013. The plant material which was the object of this study consists of *Arnicae folium et caulis* samples collected from plants harvested from the 5 natural areals mentioned above (*in situ*), respectively from plant regenerated through the *in vitro* plant tissue culture, before and after the stage of acclimatization to the *ex vitro* environment (*in vitro* and *ex vitro*).

Assessment of the bioproductivity in terms of biomass

In situ studies

The plant material samples were harvested during the vegetation seasons 2012 and 2013, in the second half of June, in the stage of full flowering. The samples were dried at room temperature in the shadow, for about 1 week.

The assessment of the bioproductivity, in terms of biomass, was determined for an average sample of 25 plants. For this samples the fresh biomass, dry biomass and the drying ratio was determined. The drying ratio was calculated by dividing the fresh biomass to the dry biomass.

In vitro studies – development of the plant tissue culture

For the development of the plant tissue culture, the explants consisted of sterile plantlets, obtained from the achenes harvested from the natural populations Ortoaia 1 and Arini 3. The culture media used within this experiment are presented in the Tab. 1.

Culture initiation. The plantlets were placed on the MS basal media (M1) [MURASHIGE & SKOOG, 1962]. For the shoot inducing and growth variant of the MS supplement with the phytohormone BAP (6-benzylaminopurine) were used: 0.5 mg/l (M2) and 1.0 mg/L BAP (M3).

Subcultivation. The first subcultivation was performed after 8 weeks from the culture initiation, the following subcultivations being performed every 4–6 weeks depending on the multiplication and growth rates.

Rhisogenesis. The new formed shoots were isolated and transferred on MS culture medium, without phytohormones (M4 and M5).

Acclimatization. After the shoots developed a vigorous root system, they were transferred in pots with a mixture of soil and perlite (1:1), placed in an environment with high and constant humidity.

Tab. 1. Culture medium variants used for in the different stages of the *in vitro* plant tissue culture

Culture stage	Medium variant (code)	Components					
		mg/L				g/L	
		Macroelements	Microelements	Vitamins	BAP	Sucrose	Agar
Initiation	**M1**	MS	MS	MS	-	30	8
Multiplication	**M2**	MS	MS	MS	1.0	30	8
	M3	MS	MS	MS	0.5	30	8
Rhisogenesis	**M4**	MS	MS	MS	-	25	8
	M5	MS	MS	MS	-	25	7.5

Assessment of the bioproductivity in terms of content in biological active compounds

Sample preparation

Sesquiterpen-lactones. The extracts where prepared according to the European Pharmacopoeia, using santonin as internal standard. The percentage content of total sesquiterpene lactones, expressed as dihydrohelenalin tiglate, was calculated using the formula given in the European Pharmacopoeia.

Phenolic acids and flavonoids. Several extraction methods were tested, an optimum recovery of the compounds being obtain when implementing the protocol developed by ALBERT & al. (2009).

Phytochemical analysis

HPTLC analysis

The qualitative assessments for the flavonoids and phenolic acids were achieved by means of HPTLC. *Stationary phase* HPTLC 20x10 cm, silica gel 60 F_{254}, plates (Merck); *mobile phase:* anhydrous formic acid, water, ethylacetate **(**10:10:80, V/V/V); *development distance:* 7 cm; *derivatization:* NP solution (10 g/L, in ethylacetate) and PEG solution (Macrogol 400, 50 g/L, in dichloromethane); *vizualization:* 366 nm.

HPLC analysis

Sesquiterpene-lactones. The extracts were subjected to HPLC analysis (Nucleodur 100-5 C18 EC, 4 x 125 mm, 5 µm), flow 1 mL/min; injection volume 20 µL; 225 nm detection; mobile phase water + 1 mL/L phosphoric acid (A) and methanol (B); gradient 55-50-40-35-15-55% solvent A for 0-1.55-9.45-15.95-16.95-18.45-20.95 min (Fig. 1).

Phenolic acids and flavonoids. The extracts were subjected to HPLC analysis (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 (2 mM), 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 (Fig. 2).

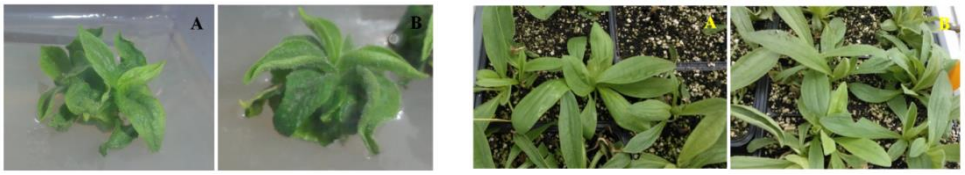

Fig. 1&2. Multiplication and growth, experimental variant O1 (**A**) and A3 (**B**), 2 weeks after subcultivation, respectively 20 days after the transfer in the *ex vitro* environment

Results and discussion

Assessment of the bioproductivity in terms of biomass

In situ studies

The *in situ* assessment of the bioproductivity in terms of biomass highlighted inter-populational variability for the same vegetation season, as well as the inter-populational variability registered during the 2 vegetations seasons (Fig. 1).

For the dry weight – *Arnicae folium et caulis*, the highest values were registered for Ortoaia 1 population in 2012, and for the Ortoaia 2 population in 2013. Although for the vegetation season 2012 Ortoaia 1 population registered the highest values, for the vegetation season 2013 it registered the lowest values compared with the other wild populations. From the field observations during 5 consecutive years we observed that Ortoaia 1 population has been established relatively recent after the deforestation of the area. In the first stage (first 2–3 years) the species had optimum developing conditions with low competition rate with other oligotrophic plant species and especially shrub species (*Vaccinium* sp.). Having a higher growth rate than the woody species, the *A. montana* population developed more quickly and spread in this area. In the second stage, when the woody species developed more and had a substantial covering percentage, the *A. montana* population declined due also to the high competition rate (for space and nutrients). This situation was not observed in the meadows that are properly managed by the owners (removing the woody plant species and mowing in order to obtain hay).

The inter-populational variability registered during the 2 vegetations seasons may also be explained through the meteorological peculiarities of each season.

The drying ratio for *Arnicae folium et caulis* had values of 4.02 to 5.69, varying both between the 5 populations in the same vegetation season and within the same population during the 2 vegetation season, fact that can be explained by the different water content in the plants and different relative air humidity at the time of harvest. The assessment of the drying ration is important in order to develop harvest strategies.

In vitro studies – development of the plant tissue culture

After the transfer of the plantlets on the shoot inducing medium, in the 22^{nd} – 29^{th} day new formed shoots were observed. After the first 2 subcultivations the multiplication rate was of 3 new shoots/explants. By reducing the BAP concentration from 1.0 mg/L (M2) to 0.5 mg/L (M3) both the multiplication and elongation were stimulated (Fig. 2). SMURMACZ-MAGDZIAK & SUGIER (2012) obtained similar results by reducing the BAP concentration. After 2–3 subcultivations on M3 we obtained an average multiplication rate of 7–8 new shoots per explant, with a maximum of 12–13 new shoots per explant.

After about 6 to 8 weeks, 95 % of the shoots developed a vigorous root system, being transferred for the acclimatization stage in pots in an environment with high and constant humidity. After 2 weeks the pots were transferred in a glasshouse, a survival rate of 90 % being obtained for both experimental variants (Fig. 3).

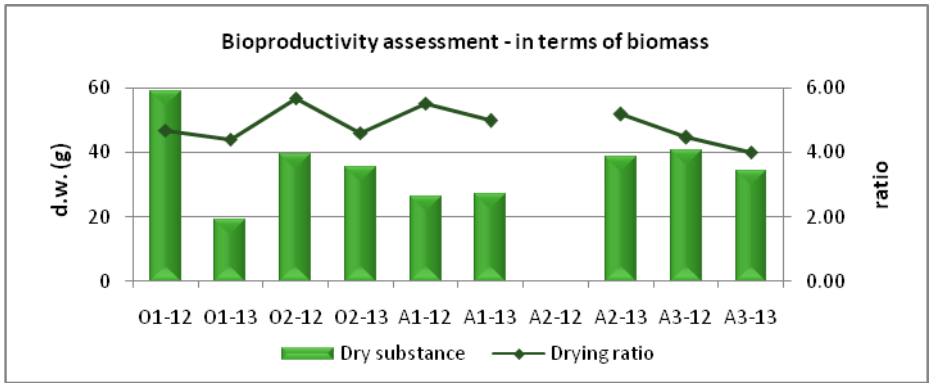

Fig. 3. Bioproductivity (in terms of biomass) assessment of the *Arnicae folium et caulis* harvested from the 5 studied wild populations during the vegetation seasons (2012 and 2013): dry substance (d.w. – dry weight) and drying ratio

Assessment of the bioproductivity in terms of content in biological active compounds

Qualitative assessments

The qualitative analysis of the phenolic acids and flavonoids contents was performed by means of High Performed Thin Layer Chromatography (HPTLC).

The phytochemical analysis of the *A. montana* samples harvested from the studied natural populations, highlighted the presence of the phenolic acids (isochlorogenic acids, Rf = 0.70 – 0.85; cynarine, Rf = 0.50 and chlorogenic acid, Rf = 0.63) and of flavonoids (luteolin-7-O-glicoside, Rf = 0.47; isoquercitrine, Rf = 0.44 and hyperoside, Rf = 0.40) (Fig. 4).

The analysis differentiated on the type of organs (leaves and stems) performed for the plant material harvested in 2013 had as purpose the assessment of the optimum raw material type depending on the final use of the plant material. The phenolic acids were present both in *Arnicae folium* and *Arnicae caulis*, while the flavonoids were present in all *Arnicae folium* samples and only in some *Arnicae caulis* samples. Thus, the HPTLC fingerprint of *Arnicae caulis* samples showed a narrower phytochemical spectrum than the *Arnicae folium*. In addition, the size and intensity of the spots corresponding to the flavonoids were lower in *Arnicae caulis* samples, which correlate with a lower content of this compounds (Fig. 5).

For the samples originating from the *in vitro* culture, harvested both before and after the stage of acclimatization to the *ex vitro* environment (*in vitro* and *ex vitro*), the HPTLC fingerprint highlighted only the presence of phenolic acids, the phytochemical spectrum being narrower when compared with the samples harvested *in situ* (Fig. 5).

Quantitative assessments

The quantitative phytochemical analysis, performed by means of High Performance Liquid Chromatography (HPLC), envisaged the total content in phenolic acids (expressed in caffeic acid equivalents), flavonoids (expressed in rutin equivalents) and sesquiterpen-lactones (expressed in dihydrohelenalin tiglate). All values are expressed as % dry weight.

In the *Arnicae folium et caulis* samples harvested from the natural populations the phenolic acids were found in total amounts of 1.01–3.02%, and the flavonoids in total amounts of 0.11–0.77%. For the samples originating from the *in vitro* culture, the total content in phenolic acids was of 0.05–0.23% for the samples harvested before the acclimatization stage (*in vitro*) and of 0.54–0.82% in the samples harvested after the acclimatization stage (*ex vitro*). Thus, it was observed, for the *in vitro* experimental variants, a significantly lower amount of phenolic acids, the flavonoids being under the detection limit, confirming the HPTLC analysis (Fig. 7).

The total content in sesquiterpen-lactones content, for the *in situ* samples varied from 0.08 to 0.33%, being under the lower limit of 0.4% stated by the European Pharmacopoeia for *Arnicae flos.* For the samples originating from the *in vitro* experimental variants the total content in sesquiterpen-lactones was of 1.15–1.29% for the samples harvested before the acclimatization stage (*in vitro*) and of 1.15–1.38% in the samples harvested after the acclimatization stage (*ex vitro*), being above the lower limit stated by the European Pharmacopoeia and comparable with the total content in the *Arnicae flos* samples harvested from the same area or presented in literature: 0.40–1.55% d.w. [SEEMAN & al. 2010; AIELLO & al. 2012].

SCHMIDT & al. (1998) obtained, for samples of leaves harvested from a convential culture, a total content in sesquiterpen-lactones of about 0.1%, while in the samples originating from the *in vitro culture* the total content was about 1.3%, with qualitative traits similar with the ones of the flower heads samples.

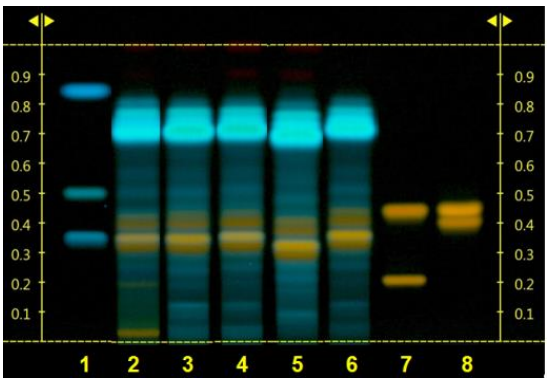

Fig. 4. HPTLC chromatogram for phenolic acids and flavonoids for samples harvested from the populations of Ortoaia and Arini (2012): 1. *Caffeic acid* + *Cynarine* + *Chlorogenic acid*; 2. O1FC – *Arnicae folium et caulis*, O1; 3. O2FC – *Arnicae folium et caulis*, O2; 4. A1FC – *Arnicae folium et caulis*, A1; 5. A2FC – *Arnicae folium et caulis*, A 2; 6. A3FC – *Arnicae folium et caulis*, A3; 7. *Isoquercitrine* + *Rutin*; 8. *Luteolin-7-O-Glucoside* + *Hyperoside*

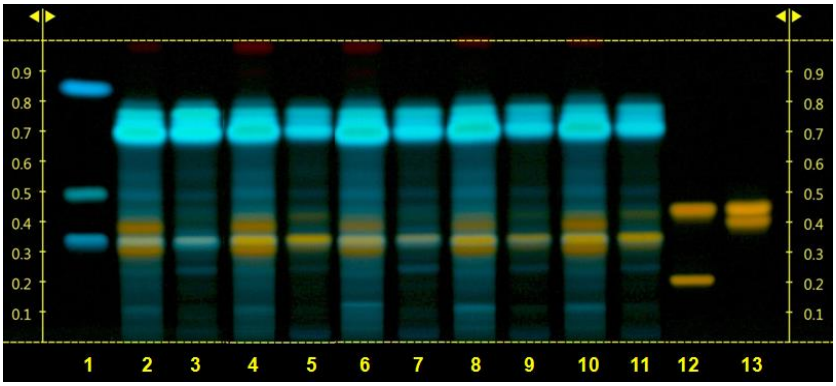

Fig. 5. HPTLC chromatogram, for phenolic acids and flavonoids for samples harvested from the populations of Ortoaia and Arini (2013): 1. *Caffeic acid* + *Cynarine* + *Chlorogenic acid*; 2. O1F – *Arnicae folium*, O1; 3. O1C – *Arnicae caulis*, O1; 4. O2F – *Arnicae folium*, O2; 5. O2C – *Arnicae caulis*, O2; 6. A1F – *Arnicae folium*, A1; 7. A1C – *Arnicae caulis*, A1; 8. A2F – *Arnicae folium*, pop. A2; 9. A2C – *Arnicae caulis*, A2; 10. A3F – *Arnicae folium*, A3; 11. A3C – *Arnicae folium*, A3; 12. *Isoquercitrine* + *Rutin*; 13. *Luteolin-7-O-Glucoside* + *Hyperoside*

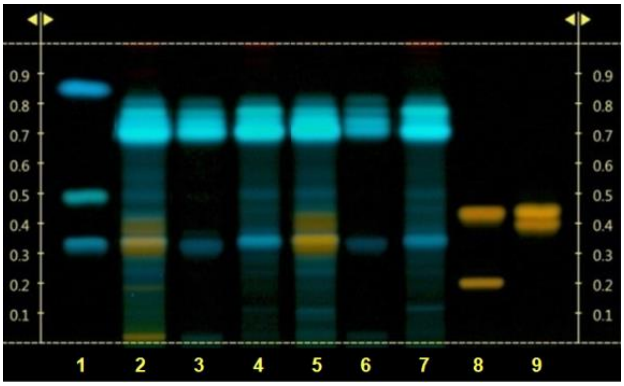

Fig. 6. HPTLC chromatogram for phenolic acids and flavonoids for samples harvested from *in situ* and from the *in vitro* plant tissue culture (experimental variants O1 and A3): 1. *Caffeic acid* + *Cynarine* + *Chlorogenic acid;* 2. O1FC12 – *Arnicae folium et caulis*, O1; 3. O1FCIv – *Arnicae folium et caulis*, *in vitro* plants, O1; 4. O1FCEv – *Arnicae folium et caulis*, *ex vitro* plants, O1; 5. A3FC12 – *Arnicae folium et caulis*, A3; 6. A3HIv – *Arnicae folium et caulis*, *in vitro* plants, A3; 7. A3FCEv – *Arnicae folium et caulis*, *ex vitro* plants, A3; 8. *Isoquercitrine* + *Rutin*; 9. *Luteolin-7-O-Glucoside* + *Hyperoside*

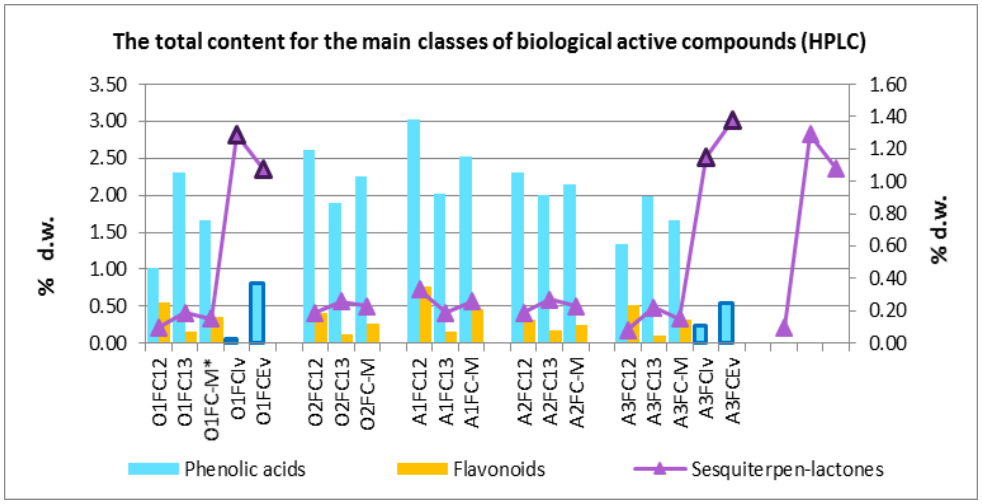

Fig. 7. The total content for the main classes of biological active compound: phenolic acids (exp. in caffeic ac. equiv), flavonoids (exp. in rutin equiv.) and sesquiterpen-lactones (exp. in dihydrohelenalin tiglate), in the samples harvested from the natural populations of Ortoaia and Arini in 2012 and 2013 (*in situ*) and from the *in vitro* experimental variants (*in vitro* and *ex vitro*); * mean value for 2012 and 2013.

Conclusions

The *in situ* biomass production and the content in biological active compounds is fluctuant due to the different environmental features and also to the management strategies for each site, parameters that can be partially controlled under cultivation conditions.

The *Arnicae folium et caulis* samples harvested from the wild populations have a higher content in phenolic acids than the *Arnicae flos* from the same area, but the total content in sesquiterpen-lactones was significantly lower. Thus, based on these results and on further studies regarding the qualitative featrures, *Arnicae folium et caulis* can be considered as a raw material for extractive fraction enriched in phenolic compounds.

In contrast, the samples originating from the *in vitro* cultures had a low content in phenolic compounds and a high content in sesquiterpen-lactones, comparable with *Arnicae flos* – above the lower limit stated in the European Pharmacopoeia. Thus, besides the applicability of the biotechnological methods in the production of planting material and in conservation strategies, these methods can be used in order to produce raw material in a controlled environment.

The origin of the plant material (*in situ* or *in vitro*) can be selected depending on the final use of the raw material.

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