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***IN VIVO* AND *IN VITRO* PHYTOCHEMICAL DIVERSITY
OF SOME POPULATIONS OF *ARNICA MONTANA* L. IN THE
ROMANIAN EASTERN CARPATHIANS**

SUMMARY OF DOCTORAL THESIS

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Iași, 2014

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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 variety of the vegetable world, by the bio-resources offered by this one, establishes a domain of priority study to identify and to isolate new biologically active compounds, including the evaluation of their actions.

To complement the progress made in the development of synthetic components, medicinal and aromatic plants are an important source of raw material for numerous uses.

Together with revealing the health associated with the indiscriminant use of products based on synthetic compounds, there was a rise in the general awareness on the advantages of using natural products as an alternative.

As for the economic importance of medicinal and aromatic plants, as part of the variety of the vegetable world, it derives from the phenotypic expression of genetic and, implicitly, phytochemical diversity, which makes them useful for metabolic peculiarities of biosynthesis of biologically active principles with applicability in the food, pharmaceutical, cosmetic industry, herbal medicine, agriculture (as bio-pesticides), veterinary medicine, but also their morphological features as decorative plants.

Today, for the approximately 50,000 species of plants used in phytotherapy and medicine, the share of the use of culture raw materials in the pharmaceutical industry is very low, which raises issues of sustainability.

Vegetal resources in the mountain areas are a valuable source of raw material, since pedo-climatic peculiarities give distinct biosynthetic potential with adaptive significance for plants. This is reflected in the quality of plant material, in terms of the content of biologically active principles. The lack of major sources of pollution in mountain rural areas is also an advantage in promoting plant bioresources for product development for therapeutic and cosmetic use.

In the above context, *A. montana* is one of the species of traditionally used medicinal plants for 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 of the *Asteraceae* family with traditional medicinal use in Europe, arnica extract is used for its phyto topical anti-inflammatory action.

The phytocomplex specific to *A. montana* includes sescviterpenic lactones, mainly helenalin, dihidrohelenalin and esters, phenolic acids and flavonoids, respectively.

Sescviterpen-lactones have an anti-inflammatory effect, owing to different mechanisms of action: inhibiting the production of pro-inflammatory cytokines IL-1

(interleukin-1), TNF- α (tumor necrosis factor alpha) and nuclear translocation of nuclear factor NF- κ B (nuclear factor kappa B) and NF-AT (nuclear factor of activated T cells) involved in inflammatory processes. In addition, for the plant itself, sesquiterpenic lactones represent adaptive significance as a response to biotic medium action, with defensive role.

Phenolic acids and flavonoids have antioxidant, cytoprotective and anti-inflammatory activity. However, they have an adaptive role as response to environmental factors, primarily high temperature variation and high UV radiation especially characteristic to mountainous areas.

Also, *A. montana* is used in human and veterinary homeopathy, as well as in a wide range of products in the cosmetics industry.

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. The species *A. montana* can be a therapeutically valuable bio-resource with multiple applications.

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.

Romania is one of the main European countries, providing plant material of *A. montana* on the global market. In Romania, the plant material is collected mainly from natural habitats. In 2001, 28 tons of arnica flowers were collected in Romania, of which 90% were exported.

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 Eastern Carpathians the species is mentioned only in studies of flora.

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 this paper is the study of phytochemical diversity *in vivo* and *in vitro* of natural populations of arnica in the Eastern Carpathians, with implications in developing strategies for sustainable exploitation of native biological material in the current climate conditions characteristic to the area studied. Developing tissue culture and plant cells *in vitro*, is an alternative to conventional exploitation by isolation and propagation of chemotypes with

well-defined characteristics based on phytochemical screening, namely, the production of biologically active principles by biotechnological methods.

In accordance with the intended purpose, we concentrated on the following *specific objectives*:

1. Evaluation of bio-productive potential in natural populations of *A. montana* in northern Eastern Carpathians, based on measurements of biomass indicators;
2. Assessing the potential for biotechnological development through various techniques;
3. The phytochemical characterization of plant material collected from natural populations and obtained from plant tissue cultures *in vitro*.

Thus, biology, biotechnology and phytochemistry interdisciplinary studies have been developed, grouped into three major components: biology, with inter-connected aspects, phytochemistry, and biotechnology.

Studies on vegetation and abundance of this species in the areas investigated were made at the Botanical Garden “Anastasiu Fatu”, Romanian Flora and Vegetation Section, University “Alexandru Ioan Cuza” - Iasi. Phytochemical evaluation of plant material and cultures of plant tissues and cells *in vitro* have been developed at the Institute of Biotechnology, University of Applied Sciences in Zurich, Switzerland. Research on bioproductivity as biomass indices, chemical analysis of soil samples and initiation of tissue culture *in vitro* were performed at the Centre for Biological Research “Stejarul” in Piatra Neamt, a branch of the National Institute of Research and Development for Biological Sciences in Bucharest.

The study area is the northern Eastern Carpathians - in the commune Dorna Arini, Suceava County, which is considered, in the study conducted, as a pilot area for the development of a model, in general, for the Eastern Carpathians.

The study is included in the current research on the species *A. montana* at national and European level, with contributions to a number of aspects of fundamental and applied research. This study integrates research on traditional exploitation of the species, namely by *in vitro* tissue culture techniques, based on phytochemical evaluations.

Studies on the abundance of this species in the areas specified, biomass and content of biologically active principles in the whole plant, according to types of organs, were made in the context of specific environmental factors (dependent or independent variables) in the areas investigated.

Evaluation of some biomass indicators and phytochemical content (biologically active principles), of the above-ground parts - flowers and herba, as well as the underground parts -

rhizomes and roots (average sample), was aimed at the analysis of the bioproductive potential for the integral multiple purposes, such as: phytotherapy, human and veterinary homeopathy, cosmetics, etc.

The results of research undertaken are applicable to conservation strategies and sustainable development – preservation of native germo-plasm with its morpho-phytochemical characteristics and sustainable exploitation in the context of competitive markets, the quality certification of plant material - raw material.

The present thesis is divided into two parts: ***Theoretical considerations*** and ***Personal contributions***, resulting from documentary and experimental study developed and integrated in this work within the context of current national and international trends.

The *first part* of the thesis (***Theoretical considerations***) takes into account the critical analysis of available literature including the latest published information on the subject matter. The first part consists of a single chapter - ***Biology, ecology and biotechnological exploitation of the species Arnica montana L.***, where issues relevant to this study are pursued:

- the current state of research on the species, where the activities that were the subject of this study were developed;
- the taxonomy and chorology of genus *Arnica*, as reported in *Flora Europaea*;
- the biology and ecology of the species, identifying ecological indicators and biotic interactions affecting the distribution and abundance of species in natural habitats;
- the economic importance of the species residing in the biological activity of the phytocomplex inherent to the species and related to its traditional exploitation and new trends;
- the biotechnological exploitation of medicinal and aromatic plant species – types of *in vitro* cultivation of plant cells and tissues and established systems for biotechnological approach of biologically active principles.

The *second part* of the thesis - ***Personal contribution***, is structured in ***four chapters*** where the research methodology, optimized and developed and the original results of experiments conducted in order to achieve objectives can be found.

Chapter II presents the research methodology optimized and developed to achieve assessments of areas under study addressed in this thesis:

- analysis of weather and climate data for the area under study;
- chemical analysis of soil samples;

- phytocenologic studies in areas with *arnica* investigated;
- evaluation of the abundance and bioproductivity in the areas studied;
- determination of the biologically active principles content – qualitative determinations by High Performance Thin Layer Chromatography (HPTLC) and quantitative determinations by High Performance Liquid Chromatography (HPLC);
- achievement of the protocol for tissue and plant cells culture *in vitro* for the three techniques used.

Chapter III presents aspects of general characterization of the natural, specific area investigated and results of studies on the abiotic environmental component, the vegetation in the five areas investigated, i.e., the evaluation of abundance and bioproductivity in terms of biomass over three successive growing seasons (2012 -2014).

Chapter IV dwells on the evaluation of the development potential of plant tissue and cell cultures *in vitro* – the implementation of micropropagation protocol, evaluation of cultivation conditions on the calusogenic potential of explants tested and analysis of the development potential of plant cell cultures in suspension, in order to apply the biotechnological approach to the conservation and recovery of the species.

Chapter V presents the results of a comparative study of phytochemical diversity in *A. montana* on types organs taken from natural populations (*in vivo*) during two consecutive growing seasons and tissues from plants obtained by *in vitro* culture. The phytochemical study aimed at assessing the content of flavonoids, phenolic acids and sescviterpenic lactones.

Studies conducted in accordance with the scientific objectives of the thesis have led to relevant conclusions basic and applied research:

Investigations conducted in the activities carried out in the field have resulted, in the case of *A. montana*, in the identification of five natural areas in northern Eastern Carpathians – commune of Dorna Arini (Suceava County) and in the biological characterization of plants in these areas.

The data from the chemical evaluation of soil samples analysed, namely the study of vegetation in the areas investigated, fall within marker values of *Arnica montana* characteristic habitats; any variation may be due to differences on a number of factors specific to each area examined: slope, exposure, altitude, degree of human intervention, the type of land use.

Bioproductivity data, in indicators of biomass for the study area, have witnessed inter-population variations - the same growing season and intra-population - during the three growing seasons, under the weather and climatic conditions during the study interval (2012-2014).

With respect to the inflorescences collection strategy, particularly for the sample consisting in the first inflorescence, there were higher amounts of dry matter calculated for 100 calatides compared with the mean sample (terminal inflorescence and lateral inflorescences), the variation range being larger for values from fresh substance. A flowering rate heterogeneity has also been noted, which could be explained by the different degree of development of the populations in each area as a result of genetic determinism and microclimate parameters in each area.

Determinations for total plant, by types of organs, showed a higher amount of dry substance for above-ground parts (flowers and herba), correlated with lower amounts of dried underground parts (rhizomes and roots - mean sample) for all areas or vegetation seasons investigated.

The biotechnological approach to the exploitation of the phytochemical diversity of *A. montana* species in experimental conditions, aimed at developing an integrated study on three levels – micro-propagation by multiplying bud strains (cloning *in vitro* by means of tissue cultures), the callus culture to obtain some varieties with specific morphological and physiological features optimal for the production of inocula for the development of cell culture in suspension.

Regenerants obtained by micropropagation, in rosette phase, for the two experimental variants were the object of the phytochemical comparative study to assess qualitative and quantitative biological active principles versus plant material collected from natural populations.

The phytochemical analysis of plant material samples taken from *in vitro* regenerated plants led to the phytochemical evaluation for secondary metabolites under controlled culture conditions compared to the results obtained for natural populations (*in situ*) whose physiological and metabolic processes are subject to variations in the soil and climatic conditions.

By achieving the callus culture, an experimental protocol was developed for testing the culture media or explant sources, for the isolation and testing of variants of the callus with optimal characteristics for their use as inoculum in the development of cell suspension

cultures. They aim at assessing the potential for growth and biosynthesis of secondary metabolites of the biological material originating from northern Eastern Carpathians.

Intermediate results were obtained for plant cell suspension culture and investigation is ongoing to complete the experimental series, in order to develop new research directions.

Phytochemical analysis by the chromatographic methods used provided complex information on the quality of plant material collected from natural areas investigated and obtained by biotechnological techniques.

Qualitative assessment of plant extracts by thin-layer chromatography methods showed the presence of phenolic acids (iso chlorogenic acids, cynarin and chlorogenic acid) in all samples, and flavonoids (luteolin-7-O-glucoside, isoquercitrin and hyperoside) only in samples of inflorescences and herba. In the inflorescence samples, the presence of a green spot, corresponding to astragalin, was observed.

The results obtained by HPTLC were used as reference in the selection of calibrators for quantitative measurements made by HPLC, laying the basis for further qualitative phytochemical studies.

Quantitative analysis of total sesquiterpene lactone content showed their presence only in the parts of the plant above ground.

Comparative analysis of the content of the sesquiterpene lactone, depending on the type of organ, showed the highest content in inflorescence samples, followed by samples of leaves; flowering stem samples had significantly lower values.

Total quantities of sesquiterpene lactones of all analyzed inflorescences samples were higher than the upper limit of the European Pharmacopoeia, comparable with data reported in the literature for natural populations of *A. montana* in Italy, Spain and Germany.

Phytochemical screening revealed variations in the content of biologically active principles for the natural areas investigated, under the weather and climate conditions recorded during the study interval (2012 - 2014), showing variations in growth seasons (solar radiation, temperature, precipitation and relative humidity).

In the case of herba samples (rosettes), obtained by plant tissue culture *in vitro*, the phytochemical analysis showed the presence of phenolic acids and sesquiterpene lactones. The flavonoids in the plant material (rosettes) from the *in vitro* culture were below the limit of detection, unlike natural plant material taken from areas.

For sesquiterpene lactones, the experiments showed narrower limits of variability in samples of culture *in vitro* compared to *in vivo* samples, compared to the limits of phenolic acids in the same samples.

In the experiment carried out, plants regenerated *in vitro* showed a different picture for the total content of phenolic acids and sesquiterpene lactones. The total content of phenolic acids was higher in samples taken from the herba samples of regenerants acclimated *ex vitro* as compared to samples taken from the *in vitro* herba culture; the sesquiterpene lactone content was higher in samples taken from the *in vitro* herba culture in comparison to samples taken from herba samples of regenerants acclimated *ex vitro*.

The higher content of phenolic acids in herba samples taken from regenerants acclimated *ex vitro* compared with samples taken from *in vitro* herba culture - after the rooting phase, this may be associated with the accommodative response of plantlets from the *in vitro* culture conditions to the *ex vitro* stage or the different degree of maturation of the plant material analyzed.

The study conducted in order to achieve this doctoral thesis presents original results with scientific, fundamental and applied relevance, in the conservation and sustainable exploitation of the species *A. montana* in the Eastern Carpathians. Evaluation of the quality and availability of native plant material gives added value marketing strategies, as the scientific basis leads to the reduction of vulnerability on a competitive international market.

Dissemination of results through publications:

STEFANACHE C.P., DANILA D., NECULA R., VANTU S., GILLE E. 2012. *In vivo* and *in vitro* phytochemical studies of *Arnica montana* L. species from Romanian Eastern Carpathians, *Acta Horticulturae* (ISSN 0567-7572) **955**: 143-148.

ŞTEFANACHE C.P., TĂNASE C. 2014. The influence of biotic interactions on the distribution and abundance of *Arnica montana* L. species in natural habitats. *Acta Horticulturae* (ISSN 0567-7572) (accepted for publication).

STEFANACHE C. P., PETER S., MEIER B., DANILA D., TANASE C., WOLFRAM E. 2014. Phytochemical composition of *Arnicae flos* from wild populations in the northern area of the Romanian Eastern Carpathians. *Revista de Chimie* (accepted for publication).

STEFANACHE C.P., TANASE C., WOLFRAM E., DANILA D. 2014. *In situ* and *in vitro* comparative study on the bioproductivity of *Arnicae folium et caulis* from the northern area of the Romanian Eastern Carpathians. *Journal of Plant Development*, **21**: 3–12.