Association for Medicinal and Aromatic Plants of Southeast European Countries
(AMAPSEEC)

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Introductory notes

The Third Conference on Medicinal and Aromatic Plants of Southeast European Countries (3rd CMAPSEEC) was held from the 5th till the 8th September 2004, in the Congress Centre of the Slovak University of Agriculture (SUA) in Nitra, Slovakia. Leadership in the organisation of the 3rd CMAPSEEC, which is official meeting of the Association for Medicinal and Aromatic Plants of Southeast European Countries (AMAPSEEC) has been given with trust to our colleagues and members from Slovakia, Dr. Miroslav Haban and Dr. Ivan Šalamon, which were expressing interest for such organisation even after the end of the first of our conferences. Their nomination of the Slovak University of Agriculture (SUA, Nitra) for the host of the 3rd Conference was fully accepted and approved by all relevant managing bodies of the AMAPSEEC. Under their supervision, as well as supervision of Prof. Imrich Okenka PhD, Rector of the Slovak University of Agriculture (SUA) in Nitra, and the AMAPSEEC, five working committees were formed with the aim to facilitate conduction of the all activities at the Third Conference. Composition of all these committees can be found in the written materials from the Conference, or at the AMAPSEC's Internet site www.amapseec.org. Book of Abstracts from the 3rd CMAPSEEC, distributed to participants during the registration (just before the start of the Conference) contained abstracts of 14 invited lectures, 23 short lectures and 140 posters. In preparation of 177 abstracts for the 3rd CMAPSEEC 389 authors from 25 countries gave their contribution, while the most significant majority of all announced contribution was very efficiently presented (about 85%).

The 3rd CMAPSEEC was organised in very good agreement with previous decisions, coming out from the 2nd Conference and the 3rd Member's meeting of the AMAPSEEC. During four working days, through the main conference program, participants have an extraordinary opportunity to be informed about the actual efforts in the field of MAPs in the SEE-region and wider. According to the number and quality of presented contributions, the Third Conference was very successful. Subsequently, main and additional program surely fulfilled expectations of the majority of participants. Purpose of keeping such conferences was, once again, fully approved.

One of the most important conclusions given at the closing ceremony was to prepare Proceedings from the 3rd CMAPSEEC. This task has been assign to Dr. Miroslav Haban from the SUA (Nitra), who was one of the leaders in the organisation of the conference, kept in Nitra. Two years later, he transfer preparation of Proceedings from the 3rd CMAPSEEC to the Association, which took initiative to accomplish that task. In meantime, plenty of our colleagues decided to publish full papers of their contribution elsewhere. Finally, almost 28 months after the 3rd CMAPSEEC, we decided to publish these proceedings, to fulfil our task and this way to save the part of heritage from our conferences.

Editors
Belgrade, December 31, 2006

Association for Medicinal and Aromatic Plants of Southeast European Countries (AMAPSEEC), Tadeuša Košćuška 1, Belgrade, Serbia
Belgrade, December 31, 2006

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[PL-04]

CULTIVATION AND INDUSTRIAL PROCESSING OF MAP

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ABSTRACT

The increase of MAP use in European market is not restricted only to pharmaceutical sector but also cosmetics, speciality foods, nutraceuticals and other uses. Till late 80ties of 20th century most southeastern European (SEE) countries were suppliers of MAP raw material harvested in the nature. But also cultivation took part in most of them.

In documents about biodiversity of MAP cultivation is now generally accepted as one of ways of biodiversity conservation. In last ten years the demand for high quality raw material with known provenance and traceability increased. This is achieved only by cultivation. The result is development of Guidelines for good agricultural practice in MAP cultivation. They are connected with GLP and GMP practices, EU and WHO regulations. Besides big pharmaceutical companies in developing economies of SEE countries small and medium size enterprises should take a leading role in innovative herbal products. Companies producing semi products as extracts, essential oils and special plant products should be equally promoted. SEE countries have tradition, knowledge and natural sources to promote sustainable cultivation of MAP and harmonise it with regulatory issues to make the raw material, processing and end products safe, efficient and of high quality.
Key words: medicinal and aromatic plants, cultivation, processing, South East European countries.

INTRODUCTION

We still witness demand increase for MAP on European market. Besides pharmaceutical sector also cosmetics, speciality foods, nutraceuticals other innovative uses are implemented. Till late 80ties of the last century most South East European (SEE) countries were considered only as suppliers of MAP raw material. It was either harvested in the nature or cultivated. In documents about biodiversity and gene resources management of MAP cultivation is now generally accepted as one of the ways of biodiversity conservation. The contribution will review general aspects of MAP cultivation implementing them into the area of South-East European countries which are members of our society. Advantages, disadvantages, and future tasks for this area to gain importance as a main supplier of high quality raw material, semi-products and end products from MAP will be discussed.

ADVANTAGES

The territories of South-East European countries offer a wide range of pedoclimatic conditions. They range from high mountainous conditions of Carpathian Mountains, Dinara range, Rodopi and Stara planina to lowland represented by Panonian and Romanian basins. Coastal conditions are present on Mediterranean, Adriatic, Aegean and Black sea coasts. This allowed numerous plant species to find their optimal range in the nature. Some important centres of biodiversity are placed in mentioned area. The result is very rich natural flora including wild growing MAP. Almost all countries of the region report over 3000 species of higher plants and from those 12 -20% traditionally or potentially useful as MAP species.

Wide biodiversity resulted in intense tradition of use of MAP in the region in the past. In all cultural environments of the region MAP were included in folk believes, legends, superstitions, as a symbols of wealth, health and longevity. Although for centuries MAP collection was the most important source of raw material for selfmedication, traditional folk medicines, pharmacies, galenic laboratories and later also for developing pharmaceutical industry, a tradition of maintaining MAP as garden plants was alive. From this tradition the modern introduction of MAP into field cultivation began in the region at the end of 19th century in Bulgaria and beginning of 20th century in Hungary and during first half of the 20th century in other countries. We can see that tradition of MAP cultivation as a raw material is strong and long lasting in the region. Combined with wild collected herbs it represented significant source of income for country economies of that time. Only 15 years old literature about MAP trade and production explicitly named Bulgaria, Yugoslavia, Albania and other countries of this region as main suppliers of raw material of MAP for developed countries of Europe and America.

Traditional knowledge about the use of MAP from folklore was a catalyst for establishment of institutions that helped introducing MAP into field cultivation, development new cultivars by breeding and developed techniques of cultivation. Almost in every country one or more institutions has activities concerning MAP production. Let

us mention just some o f them: Institute in Budakalasz Hungary, Institute J. Pančić in Serbia and Montenegro, Institute in Kazanlk Bulgaria, Institute of Hop Research and Brewing in Slovenia and others. They are involved in breeding, introduction and development of new cultivation and harvest techniques. Breeding programs have a long tradition in SEE countries. They resulted in many species of MAP in the sort lists. Especially Hungarian, Bulgarian and Yugoslav lists are to be mentioned. In former days breeding was motivated more by optimizing the yield and properties of the plants. Breeding programs are now going on with focus on species interesting for their features for industrial use and resistance to diseases. More and more endangered species are bred from conservation motives as well. Extensive conservation programs are the source of new genetic potential for breeders and scientists.

Experiences in building machines for mechanized harvesting and primary production gathered trough the decades of successful development are valuable bases for small business, which can offer machines and equipment also to countries outside the region. A lot of solutions can be adopted from tobacco industry that is traditional in many countries of the region.
In many cases a lot of innovation resulted from this work. Especially in the domain of mechanization, harvesting and drying processes. One of the examples are harvesters for chamomile for which the basic technique evolved in this area and are used also in Italy and in other parts of Europe.
The quality of the knowledge in the region is recognized also in the other parts of the world. Scientists from institutes and universities involved in MAP research from South Eastern European countries are active members of different associations dealing with MAP research and cultivation as Geselschaft für Arzneipfranzenforschung (GA), International Society of Horticultural Sciences (ISHS), World Health Organisation (WHO) and others.

STATUS OF CULTIVATION IN SEE COUNTRIES

Status of MAP cultivation in national agricultures is very different from county to country. In general three basic attitudes toward this sector can be defined. In countries as Hungary, Bulgaria or SCG they are considered as a real part of agriculture sector. Also in Czech Republic and Slovakia decision-makers have recognised the importance of MAP cultivation. In Slovakia a program of medicinal, aromatic and spicy plants cultivation was adopted as a priority in their agriculture. In the other hand we have countries with small MAP cultivation where the status is not so clear. In this countries decision-makers decided that cultivation is not a priority as in Macedonia or Albania where natural sources seem to be still sufficient. In case of Slovenia and Croatia they just ignore this sector with excuse it is too small. But economical interest for alternative crops is growing in all countries which joined the EU. The conditions for agriculture changed a lot and the problem with small family farms occurred. With proper development, organisation and cooperation whit interested users the cultivation of MAP could be a solution for those small farmers.

Regarding cultivation again three basic types of MAP cultivation can be defined. Large-scale production is a feature of lowland regions and has a long tradition there. It originates from large scale production in the past when the land was private and later was collectivised and brought under cultivation by cooperatives. It requires use of all cultivation techniques, predominantly done by mechanisation. Those conditions forced people to use a lot of pesticides. In the flow of time the regulations forced them to reduce their use. These products were either dried or transported to distillation or extraction industry. The market for these products is big.

Medium scale production was possible also in valleys and places where fields were smaller. In this case predominantly dried herbs were produced, since the quantities were too small for real industry. This type enables application of some practices of sustainable agriculture. The market is slightly restricted.

Small-scale cultivation is typical for premountainous regions. In this type of cultivation many species are cultivated on small areas. Produce is dried and packaged for small consumers on farm. In many cases this type is combined with certain extent of wild collection. It enables biodynamic or organic growing and boutique production. It fills the niche markets as organic shops, different events and promotions.

Almost in every country of the region some kind of regulative for quality of raw material was introduced in the past. Some of them are very old and do not follow new developments in this field. To achieve recognition on the international market harmonisation with EU regulative should be done. The regulative for quality of MAP raw material and herbal semi products is getting higher every year. Quality of cultivated plants is easier to achieve since optimisation of all levels of cultivation and processing is applicable. Traditional growers who have old technologies or capacities should combine their experience and new knowledge to gain better success.

Important issue in this aspect is the proposal of the guidelines of GAP for MAP presented in European parliament in 1998. From that time more and more producers and also buyers introduce them into a daily practice. The idea of assurance quality of raw material and traceability is tightly connected to higher standards in industrial processing and manufacturing of herbal end products. A lot of knowledge needed for establishment of GAP originates from South East European area and many experts were active in completing guidelines. Now there is a growing need for introduction of specific GAP practices in cultivation of different MAP regarding their requirements and environmental possibilities. It is necessary to establish new criteria of »technical ripeness« for MAP used as a raw material for special products as antioxidants, enzymes, oils, dyes, flavonoids etc. The importance of GAP was worldwide recognised with inclusion into WHO monographs.

INDUSTRIAL PROCESSING

In former days the industry in almost all member states was state owned, centrally managed and efficient for that type of economy. In transition companies were faced with changes in economical situation. They had to be turned to private or the state maintained a part of ownership. But big changes in internal organisation were necessary. Those who survived these changes now function on higher standards. In transition period some medium sized companies emerged as spin off or change of organisation from cooperatives. Those function on the bases of knowledge and experience and are more flexible. In many South-East European countries, companies process cultivated and wild collected material. In many cases only drying and packaging in form of tea mixtures is done. Some of them produce also extracts and essential oil by distillation. Pharmaceutical, food and cosmetics companies use raw material and semi products. In transition countries there is a big opportunity for development of small and medium businesses producing herbal products of higher added value. In this situation also companies in partnership with institutions or universities were founded. The sector is not restricted only to traditional products. More and more innovative techniques and uses of semi products from MAP give good opportunity. Different new techniques as supercritical liquid carbon dioxide extraction enable new products. The use of herbal semi products is getting novel possibilities as food supplements, processing additives, colorants, aromatics and antioxidants. In some cases producers of those materials gained leading role in European area, as it is the case of small company Vitiva in Slovenia which is leading in plant antioxidants extraction.

All industrial production is aiming to be organised under certain standards as GMP or GLP and introducing also HCCP system of assurance and ISO standards for organisation and environmental safety. Only with this high traceability and assurance of quality is guaranteed. Some companies or private firms in the region still have problems in achieving quality standards in accordance with EU regulative, but in the future when all the states join EU these will be a must. Those who are still in the waiting room must use the time to harmonise national standards with standards EU.

FUTURE TASKS

Imperatives for increasing the cultivation of MAP in the region are development and introduction of new, more demanded crops into cultivation, development of new cultivars of higher quality and high resistance to diseases, optimisation of cultivation, plant protection, and primary processing. This will enable to introduction of environmental friendly high scale production of MAP. Also GAP for specific plants and growing conditions should be developed in connection whit introduction of quality requirements for raw material for extraction of active compounds (antioxidants, flavonoides, xantophyl) for speciality uses.

Adoption of quality standards in accordance with EU and other potential users is inevitable for all the sectors of cultivation and processing of MAP. Very important feature is also better flow of information about markets in the region as it is done by ETZ in European Union.

In the future industry should give more interest to communication with growers and scientists to exchange information about their requirements and new perspective products. Participation in financing projects of breeding and introduction with goal to achieve better quality and desired quantity of traceable raw material produced under GAP.

Last but not least important task is informing the decision-makers and general public about positive effects of introducing MAP into cultivation as a substitute for non-economical crops and a measure of conserving biodiversity.

CONCLUSION

In conclusion we can say that South East European countries have good combination of traditional perception about MAP, experience in cultivation, industrial processing and scientific knowledge and potentials to develop their own products based on sustainable cultivated herbs. With innovative view and knowledge of quality requirements the products will gain recognition they deserve. Let countries of South East Europe be no more just a source of raw material but producers of high quality medicinal, condiment, food supplements and other products from MAP. Countries of the region did already contribute to common European knowledge in the past and should in the future have again a leading role. Existing knowledge, experience, tradition in MAP cultivation and industrial processing is comparable advantage and a base for development of new technologies and end products. They can path new ways into a future of MAP research, conservation, cultivation, processing and utilisation in the region of South East Europe.

[PL-04]

[PL-10]

QUALITY CONTROL OF MEDICINAL AND AROMATIC PLANTS AND THEIR PRODUCTS

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SUMMARY

Quality control (QC) of medicinal and aromatic plants (MAPs) and their products assumes procedure of determination and comparison of certain (selected) characteristics of these products, with requirements which can be supplied by different sources (pharmacopoeias, ISO and other standards, etc.). Number of mentioned characteristics, which should be determined by QC, is strictly defined by requirements taken from selected source. Since MAPs are roughly always used as raw material for production of different products, such as phytomedicines, cosmetics, beverages or food products, quality requirements may vary in some extent. It should be noticed that requirements given by pharmaceutical industry are surely the most sophisticated and the most restrictive. QC report is always detrimental in making decision about further destiny of tested MAPs and related products. In the final instance, reason of existence of QC is to protect consumers, as well as producers, processors and traders. Stable and homogeneous production and processing of MAPs assume implementation of certain generally adopted practices (GAP, GCP, GSP, GMP, GLP, etc.), approaches (such as HACCP), and standards (for example, ISO 9000), as the main precondition. Main effort in Europe in this field is focused to harmonisation and improvement of national and common policies, as well as foundation on new international standards [such as ISO 22000 (FSM)]. The actual procedure for marketing authorisation of herbal medicinal products, according to the newest directives supplied by EU will be briefly explained.
Keywords: quality control (QC), medicinal and aromatic plants (MAPs), MAP products, standards, legislation.

INTRODUCTION

In the literature, plenty of rather different definitions of the term quality control (QC) can be found. In one of those wide accepted (ISO 9000/2000), QC is defined as a set of activities or techniques whose purpose is to ensure that all quality requirements are being met. In order to achieve this purpose, processes are monitored and performance problems are solved. According to recommendation supplied by the same source, quality assurance (QA) is defined as a set of activities whose purpose is to demonstrate that an entity meets all quality requirements. QA activities are carried out in order to inspire the confidence of customers and managers, confidence that all quality requirements are being met. From these two definitions difference between QC and QA is very clear, so these terms can not be mixed. Moreover, in such constellation is obvious that QC presents only one component of the QA.

Subject of the QC in our case are medicinal and aromatic plants (MAPs) in the field (from seeds and seedlings) till harvesting, dried plant parts (whole, cut or pulverised), liquid, semisolid and solid drugs/plant isolates, such as extracts (tinctures, fluid, soft and dry extracts), essential and fatty oils, balsams, resins, etc., including single constituents, herbal medicinal products (teas and tea mixtures, capsules, tablets dragees, powders, sprays, ointments, creams, gels, liquid formulations), as well as all other MAP based products.

Since MAPs are used for production of different products, such as phytomedicines, cosmetics, beverages or food products, quality requirements may vary in some extent. Set of characteristics, which should be determined by QC is strictly defined by requirements taken from selected source (pharmacopoeias, ISO[1] and other standards, etc.). Quality requirements given by pharmaceutical industry are definitely the most sophisticated and the most restrictive ones. From these reasons, just these demands will be in this article put into the focus of our considerations. These are mostly based upon our knowledge in the field of pharmacognosy, which is compounded from several disciplines, such as pharmacobotany, phytochemistry and phytotherapy. In such an approach subject of the QC would be limited only to herbal medicinal products, or phytomedicines. According to ESCOP[2], phytomedicines, or herbal medicinal products, are medicinal products containing as active ingredients only plants, parts of plants or plant materials, or combination thereof, whether in the crude or processed state. This definition assumes that plant materials include juices, gums, fixed and essential oils, and any other directly derived crude plant product, but not chemically defined isolated constituents, either alone or in combination with plant material. Moreover, phytomedicines may contain excipients of plant or other origin.

[1] ISO = International Organisation for Standardisation (see Internet site www.iso.org).
[2] ESCOP = European Scientific Co-operative on Phytotherapy (see Internet site www.escop.com).

Quality of each final product is provided by the standard quality of raw material, through the application of standardised production process and processing equipment. Similar situation is with herbal drugs, extracts and phytopreparations. It means that quality of phytomedicines depend greatly upon quality of herbal raw materials, which should be standardised. In this place appears several problems: how to organise production of herbal drugs of standard quality (by collection and/or growing of plants), and how to control their quality. There is lot of factors affecting to the quality of herbal drugs. Genetic ones are surely the most important (species, variety, chemotype, etc.), followed by ecological factors. From the general point of view, these are climatic and soil factors and after that scientific farming measures, which are applying during the process of production in the plantations.

After collecting or harvesting of MAPs, all other steps, such as primary processing (transportation and cleaning up), secondary processing (drying, cutting, packing and storing), as well as duration and conditions of storing, affecting to quality of herbal drugs and related products. From these reasons, QC should be conducted after each of mentioned steps of processing.

The aim of this contribution is to point at the parameters, which are important for the evaluation of the quality of herbal drugs and related isolates and phytopreparations. Through determination of these parameters for a lot of samples, limits for every single drug could be determined, defining their quality. Beside the list of QC parameters, methods for their determination should be harmonised and sharply determined too. This enables and makes easier further control, because of use of harmonised, even identical QC requirements. The most detailed data about identification, quality and use contain monographs on herbal drugs, given in the frame of national pharmacopoeias, and different standards, such as ISO is. Very useful for the specifications of certain herbal drug or extracts are internal data supplied by producer, especially in the case of non-official drugs.

QUALITY CONTROL OF PHYTOMEDICINES

General considerations (status and registration)

Status of herbal drugs, isolates, and their products, as well as their distribution and use is defined in the world in different ways. The most often herbal products are classified as foods, dietary products and medicines. In the EU phytoremedies are classified in the category of over the counter (OTC) products. EU developed branched legislative environment and harmonised regulations about medicinal plant products, consisting of several important directives, regulations, decisions and communications, which can be found all at appropriate official Internet site (http://pharmacos.eudra.org/f2). Actual regulations permit registration of phytoremedies in three different ways, as herbal remedies accepted on the base of scientific standards (1°), as traditional herbal remedies (2°), and as dietary products (3°). In the first instance (1°), registration process is the same as for the common medicines. Instead of clinical trials, registration of traditional remedies (2°), as prove of efficacy, assumes evidence that product is used in Europe more then 30 years, without evident adverse effects. In some of EU countries, assessment of registration documentation on the bases of bibliographic data, is not permitted, what makes difficult mutual recognition of registration permits in all members of EU. Last mentioned possibility of registration, as dietary products (3°), is rather new in the EU, and not yet quite clearly defined. In certain EU countries (Holland and Great Britain, for example), and especially USA, smaller part of herbal drugs is registered as OTC products. On the basis of The Dietary Supplement Health and Education Act launched in 1994 and formation of Commission on Dietary Supplement Labels in 1995, the majority of these preparations are distributed as dietary supplement. At the beginning of 1997, EMEA[3] founded Herbal Medicinal Products Working Party, composed of experts, representatives of individual countries, European Commission, European Parliament and European Pharmacopoeia, which in 1999 became permanent working part of the EMEA. The aim of this group was development of criteria for approval of the quality, efficacy and safety of herbal drugs. Huge number of directives and guidelines has changed. For example, one of these is Quality of Herbal Remedies from 1989, now Quality of Herbal Medicinal Products [1,2]. Mentioned working party of EMEA will evaluate number of ESCOP monographs and publish their final review as core of summarised product characteristics (Core-SPCs).

From the other side, in the year 2000, FDA[4] published Guidelines for Industry, which is giving unique regulatory and scientific definition for herbal medicinal products, as well as directions for development of herbal medicines [3]. In 1996 WHO[5] published Guidelines for Assessment of Herbal Medicines [4], aiming to present basic criteria for development and harmonisation of the quality standards in the production and trade of

[3] EMEA = The European Agency for Evaluation of Medicinal Products (see Internet site www.emea.eu.int/).
[4] FDA = U.S. Food and Drug Administration (see Internet site www.fda.gov).
[5] WHO = World Health Organisation (see Internet site www.who.int/en).

phytomedicines. Last years, WHO and FDA put consideration of biopharmaceutical characterisation of herbal drugs into the focus. Aiming to makes easier evaluation of safety of herbal drugs, FDA established particular system of reporting on the drug quality, Drug Quality Reporting System (DQRS). DQRS reports being classified according to their source, type of problems and corrective measures are an integral part of FDA MedWatch programme [5]. Recommendation supplied by FIP[6] and EMEA, point at that bioequivalency of herbal drugs is close related with their quality [6,7]. Categories of extracts in phytomedicines, requiring biopharmaceutical characterisation, are listed [8].

Note on the evaluation of safety and efficacy of phytomedicines

Nevertheless to the quality, use of phytomedicines can not be considered without their efficacy and safety. This means that each of these components (quality, safety and efficacy) should be considered as indivisible part of basic requirements needed for the issuing marketing authorisation for the certain phytomedicine. From this reason, today, of the huge importance are data unified through WHO monographs[7] and EMEA monographs (EMEA, *ad hoc* Working Group on Herbal Medicinal Products). ESCOP monographs[8], as well as monographs created by Commission E[9], should be mentioned too. These contain data on the safety, efficacy and doses of herbal drugs, but not about their quality [9,10,11,12,13].

Quality and quality control of herbal drugs

Majority of general data on the QC parameters of MAPs and related products, could be found in Directives of EU, more or less harmonised national laws about production and trade of medicines, medical products and

[6] FIP = International Federation of Pharmacist (see Internet site www.fip.org).

[7] World trend of increase of herbs and herbal preparation in official and traditional medicine forced legislators and medical experts to reconsider status and legislation and manner of evaluation of these products. In 1991, WHO published Guidelines for the Assessment of Herbal Remedies, whose main purpose was to help nations, WHO members in establishing of similar laws criteria and procedures for the evaluation of the quality, safety and efficacy of herbal products. Regarding the safety of these preparations, recommendations directing to acceptance that long lasting historical use of the plant in traditional medicine present prove of security, if contemporary research does not oppose these findings. Regarding the efficacy of these preparations, recommendation assumes specification of indications for the product. In the case of traditional preparations, requirements for testing depend upon declared indication [9]. WHO prepared and published monographs on medicinal plants promoting international harmonisation of the use and quality control of MAPs, which are used as a model for development of national regulations. Each monograph consists of two parts. The first part is giving pharmacopoeia's QC requirements, including botanical aspect, identification tests, requirements regarding to foreign matter, chemical tests and main chemical constituents. The second one, founded on comprehensive reviewing of data from the literature, is giving detailed information on the pharmacology, contraindications, adverse effects, and dosing. Till this time the WHO published three volumes consisting of 89 such monographs (Appendix 1).

[8] In West European countries, phytotherapy and conventional medicine are mutually interlaced. Study conducted in 1991 revealed presence of about 1400 herbal drugs on EU market. From that number, 150 was used in more then a half of EU member countries. From this reason, herbal drugs are huge problem for harmonisation. Legislation dealing with herbal remedies varies even between country members of EU. In certain countries phytopreparations are part of conventional medicine, whilst in others they have status of dietary products, or food supplements [9]. Aiming to harmonise way of the scientifically based evaluation of phytopreparations and define criteria for the marketing authorisation, in 1989 majority of EU countries with few others has formed ESCOP [14]. In the period 1996-1998 ESCOP published 59 monographs on medicinal plants in the form of standardised dossiers for drugs, known as SPC (Summary of Product Characteristics). From the difference of pharmacopoeia's monographs, which are giving standards for determination of identity and quality, ESCOP's monographs are dealing with therapeutic aspects of herbal preparations. Each monograph contains detailed information about herbal raw material, major chemical constituents, as well as data on pharmacology, pharmacokinetics, toxicology and use. On the bases of these data, recommendations for doses, contraindications and adverse effects are given [15]. Latest edition contained 80 such monographs (Appendix 2).

[9] The most complete set of data dealing with safety and efficacy of herbal drugs and phytopreparations can be found in monographs of the Commission E, German expert's commission for medicinal plants and herbal remedies. Bearing in mind remarkable roll of phytomedicines in Germany, it was quite natural to expect from this country to develop mechanisms to assure safe use and efficacy of these preparations. Commission E was formed in 1978 and its activities were conducted under the supervision of the Federal Department for Health and later Federal Institute for Medicines and Medicinal Products on the consideration and approval of safety and efficacy of all medicines. Activity of the Commission E was cancelled 1994, upon the acceptance of new legislation. In this period Commission E scientifically evaluated about 360 plants, and published 384 monographs as official records. Monographs were created on the basis of bibliographic data, with the aim to inform users and elevate registration of herbal preparations. Although these monographs can not be used as guidelines for additional information, because they did not contain references, they are based on the comprehensive review of scientific and historical data, as well as interdisciplinary expertise of commission members. Commission reviewed in details data on the safety of use, trying to assure reasonable security, when the preparations are used in recommended dose, contraindications and other warnings and regulations specified in monographs. Regarding the efficacy, Commission made positive evaluation always when scientific data were giving sensible verification for certain historical traditional use of selected plant. Negative evaluation were made in the cases, where proves of efficacy were not available, or when potential benefit was lower than potential risk. Furthermore, commission published negative monographs for medicinal plants without clinical and pharmacological studies, or where acceptable proves of efficacy in traditional and empirical medicine were not recorded. Monographs about herbal drug and their preparations are published without recommendations about dosing. Monographs include nomenclature, part of plant used, chemical composition, range of application, contraindications, adverse effects, incompatibility with other medicines, dosing, mode of administration and activity of the plant. These monographs presenting the most precise available information about safety and efficacy of medicinal plants and phytopreparations at the moment they were published. From this reason, these monographs should be recommended to all those being interested in phytotherapy [9,13]. Complete list of all these monographs is given in Appendix 3.

related accompanying legislation. The latest downloadable revision of complete and updated data can be easily accessed at the address http://pharmacos.eudra.org/f2.

Before processed or used for the preparation of certain phytomedicines, identity of each herbal drug should be confirmed, nevertheless to declared composition on the package. During the acceptance and before use, identification and QC procedure should be conducted. Identification of herbal drug could be achieved by correlation of data obtained by:

- macroscopic analysis (organoleptic inspection),
- microscopic analysis [inspection under magnifying glass (6-10-fold magnification, scanning microscopy and inspection of histological cross-sections)],
- chemical analysis (chemical tests and TLC).

In Table 1, the list of parameters is given, on the bases of whose values quality of herbal drugs can be evaluated. All listed parameters are very well known and detrimental in the characterisation of herbal drugs. Till this time, the biggest problem was lack of limits (even in pharmacopoeias) for each of these parameters for each official drug. For example, do not all of monographs of selected pharmacopoeia contain data on total ash in drug, somewhere data on the extractive matter are given and somewhere not. However, newer pharmacopoeias and other monographs on herbal drugs are more complete, and data about quality becoming much more harmonised. More difficult for solving is the problem of the quality of number of non-official drugs, having huge commercial and application value. In such cases, the most important are criteria supplied by ISO, or as internal standards or specifications of different producers. In the past, quality of drugs was evaluated on the basis of certain general parameters. Gradually, along with new knowledge about chemical nature of active ingredients, mechanisms of action, and analytical techniques, the need, but also possibility of determination of active substances, appeared as necessity, as the most important and essential measure of the quality of herbal drugs.

Values of general parameters, which can be decisive in the evaluation of the quality and usability of herbal drugs, extracts and phytopreparations are presented in monographs of individual drugs in pharmacopoeias and certain other standards (ISO). These data most often can be found in the chapter dealing with definition, properties, or identification and testing. However, methods and regulations for determination of these parameters are common (and rarely specific for selected herbal drug), and given in the frame of general part of pharmacopoeia, or as separate standards. There is a plenty of examples of such non-specific methods, such are these for determination of foreign matter, total ash, ash insoluble in hydrochloric acid, swelling number, loss of drying, relative density, microbiological quality, etc.

For certain general testing, for example, determination of the quality regarding to the content of heavy metals, pesticide residues, radioactivity, microbiological quality, or presence of mycotoxins, in corresponding regulations (pharmacopoeias, and other standards), common values are given (Table 2). However, even now, for some of these parameters, limits for single herbal drugs are being defined. Thus, in the future, these values will be the part of the each of such monographs. Example for this are defined limits for much higher number of heavy metals in individual herbal drugs in *The National Formulary* [16].

Essential measure of the quality of drug is the presence and quantity of constituents responsible for it's therapeutic use. When these constituents are known, but problems and difficulties of identifying and determining them too, then the quantity of other selected constituent (marker substance) is used for the keeping up with persistence of the quality of drug.

For identification of active substances in herbal drugs, extracts and phytopreparations, along with physico-chemical methods, biological screening is also used. Majority of modern pharmacopoeias and other regulations, in the frame of herbal drug monographs is giving, as the example, typical fingerprint chromatogram, obtained by thin-layer chromatography (TLC), gas chromatography (GC), or high-performance liquid chromatography (HPLC). Actual efforts are directed to total standardisation of the quality of herbal drugs, related isolates and phytopreparations, what means quantitative analysis of active ingredients (see Table 1). There are still few different ways to define quantity of active ingredients, especially in the case of extracts and phytopreparations. Sometimes, for quantification of active substances in herbal drugs, extracts and phytopreparations sophisticated analytical techniques, such as EPR and NMR can be used. However, for routine control, the most often used are volumetric methods, electrochemical methods, spectrophotometric methods, chromatographic methods, capillary electrophoresis, immunological methods (RIA, ELISA), biological methods, hydrodistillation and determination of physical and chemical constants. Use of other methods should be assumed as acceptable, only if these methods are validated according to direction supplied by CPMP/ICH/281/95[10].

[10] EMEA/CPMP/ICH/281/95 (1995): Note for Guidance on Validation of Analytical Procedures: Methodology.

Table 1. Quality control of herbal drugs

Plant drug type	General testing*	Additional testing
Dried plant parts (*in toto*, cut or pulverised)	▪ organoleptic properties ▪ foreign matter ▪ sieving test ▪ moisture content ▪ ash content ▪ content of in acid insoluble ash ▪ sulphate residue content ▪ extractive matter content	1. presence of active substances 2. quantity of active substances A. total content of active substances, calculated as dominant, or the most active ingredient B. quantity of single constituents, within the complex of active substances C. content of selected marker substance
Powdery drugs	+ ▪ particle size distribution	
Liquid and liquid to solid drugs	+ ▪ physical constants ▪ chemical constants ▪ solubility ▪ boiling point ▪ solidification point ▪ melting point	

* In the frame of general testing of herbal drugs microbial quality, heavy metal content, presence (absence) of fumigants, pesticide residues, radioactivity, micotoxins, as well as presence of insects and their undeveloped forms, should be determined too (see Table 2).

Quality control of extracts

Extracts are concentrated products of liquid, semisolid or solid consistence. The most often extracts are prepared from dried chopped plant material. Before the extraction process, plant material can be treated in to inactivate enzymes, or remove fats or chlorophyll.

For the production of extracts, procedures based on maceration, percolation, and similar techniques can be used. The most often used solvent is ethanol, but other solvents can be also used. Selection of solvent depends upon the nature of constituents, which should be transferred into extract. Producer's declaration should contain right composition of the solvent used in the extraction process. Nowadays, super-critical fluid extraction is also becoming more and more of interest for industrial processing of MAPs.

Quality of product (extract) depends on plant material, solvents, herbal drug to solvent ratio, technology used in the extraction process, and further treatment of the extract. Processes of extraction, further finishing, storing and keeping of the extracts, should be conducted according to the principles of Good Manufacturing Practice (GMP), where all applied procedures should be standardised and validated.

Standardisation procedure for extract consists of several common steps. After the quantification of active ingredient, extract is adjusted to contain active ingredient(s) (or marker compound) in desired concentration by adding inert material or other extract. Depending on pharmacological activity of constituents, approach to standardisation could be different. Thus, as standardised extract of glossy buckthorn (*Frangulae extractum siccum standardisatus*) any extract containing between 15 % and 30 % of total glucofrangulins should be accepted, assuming that this content can not differ more then ± 10 % from that declared. When working with ingredients of strong pharmacological activity, and small therapeutic width, standardised extract should contain exact quantity of active substances, within very narrow limits. Thus, *Belladonnae folium extractum siccum standardisatus* contain 0.95 % to 1.05 % of total alkaloids, calculated as hyosciamin. Application of modern analytical techniques enables quantification of each of single constituents of an extract, offering one quite new approach in the quality of standardisation of extracts and related products.

QC of final product (extract), as well as, control of semi-finished products, obtained in different stages of extraction process, is required too. Moreover, data on stability of herbal extracts are required more then earlier.

Data, which can be used for evaluation of the quality of herbal extracts, are given in Table 3. It should be noticed that, along with common parameters, presence of impurities, which could be find in such products, should be sharply defined. These impurities include residual solvents and, sometimes, impurities, which such solvents can contain, too. Limits given by an internal standard permit the presence of residual solvents in the following manner: ethanol 0.5 %, methanol 0.05 %, acetone 0.02 %, n-butanol 0.02 % and heptane 0.02 % [11].

Table 2. Residues in herbal drugs

Parameter	Values taken from references 12, 13, 17 and 18	
Heavy metal content	Lead: max. 5 mg/kg [13] Cadmium: max. 0.2 mg/kg [13] (linseed, hawthorn blossom, yarrow herb max. 0.3 mg/kg) [13] (birch leaf, willow bark, St. John's wort herb max. 0.5 mg/kg) [13] Mercury: max. 0.1 mg/kg [13]	
	Values for fruits, vegetables, and grain: Lead: max. 0.5 ppm (max 1.2 ppm in leafy vegetables) [12] Cadmium max. 0.1 ppm in leafy vegetables and grains [12] max. 0.05 ppm in root-like vegetables [12] Mercury: max. 0.035 ppm (grains) [12]	
Fumigants	Ethylene oxide: max. 1.0 ppm	
Radioactivity	370-600 Bq/kg (^{134}Cs and ^{137}Cs)	
Pesticide residues (max.)	Alachlor 0.02 ppm Aldrin 0.05 ppm Azinphos-methyl 1.0 ppm Brompropylate 3.0 ppm Cypermethrin 1.0 ppm DDT 1.0 ppm Deltamethrin 0.5 ppm Diazinone 0.5 ppm Dichlorvos 1.0 ppm Dithiocarbamates 2.0 ppm Endosulfan 3.0 ppm Endrin 0.05 ppm Ethion 2.0 ppm Fenitrothion 0.5 ppm Fonophos 1.5 ppm Fosalone 0.1 ppm	Heptachlor 0.05 ppm Hexachlorbenzene 0.1 ppm HCH 0.3 ppm Chlordan 0.05 ppm Chloropyriphos 0.2 ppm Chlorpyriphos-methyl 0.1 ppm Quintozen 1.0 ppm Lindan 0.6 ppm Malathion 1.0 ppm Metidathion 0.2 ppm Parathion 0.5 ppm Parathon-methyl 0.2 ppm Permethrin 1.0 ppm Pyrimiphos-methyl 4.0 ppm Pyrethrins 3.0 ppm
Microbial quality	For herbal drugs or preparation subjected to thermal treatment before use, or used externally [12]: Aerobic bacteria: max. 10^7/g Yeast and moulds: max. 10^4/g *Escherichia coli*: max. 10^2 /g *Enterobacter*: max. 10^4/g *Salmonella*: must not be present For herbal drugs and preparation used orally, without previous treatment [12]: Aerobic bacteria: max. 10^5/g Yeast and moulds: max. 10^3/g *Escherichia coli*: max. 10^1/g *Enterobacter*: max. 10^3/g *Salmonella*: must not be present	
	Herbal preparations classify in category 4. [17] a. Preparation requiring addition of boiling water before use [17] Aerobic bacteria: max. 10^7/g, ml Yeast and moulds: max. 10^5/g, ml *Escherichia coli*: max. 10^2 /g, ml b. Remaining preparations [17] Aerobic bacteria: max. 10^5/g, ml Yeast and moulds: max. 10^4/g, ml *Enterobacter*: max. 10^3/g, ml *Escherichia coli*: must not be present in 1.0 g (ml) *Salmonella*: must not be present in 10.0 g (ml)	
Micotoxin content	Aflatoxin B1: 2 μg/kg Total aflatoxins B1, B2, G1, G2: 4 μg/kg	
Presence of insects	Insects, their eggs, and larvae should be absent	

As in the case of herbal drugs, parameters dealing with content of heavy metals, pesticides, radioactivity, mycotoxins, microbial quality, etc., should be controlled too (Table 2). Important detail is that, for the extraction, plant material containing higher quantity of moulds, than permitted for herbal drugs, can be officially used, giving extract of acceptable microbial quality [11,13]. Such an approach is quite inapplicable in the case of pesticides, heavy metals and certain other contaminant, because the reconcentration caused by the extraction process. Subsequently, these contaminants would be present in the product (extract) in higher concentration then in the starting raw material.

In Table 3, few different ways of presentation of additional parameters of the quality of herbal drugs are given. However, nowadays, the most appreciated manner is that, where quantity of active substance, group of substances (or selected marker constituent) is determined. For this purpose, similar, or adequately modified, methods, as those used for determinations in herbal drugs are used. If these (methods) are not pharmacopoeia's ones, they should be validated according to directions supplied by mentioned CPMP/ICH/281/95.

Table 3. Quality control of herbal extracts

Type of extract	General testing*	Additional testing
Fluid extract (1:1) *Extractum fluidum* (1:1)	▪ organoleptic properties ▪ dry residue ▪ relative density ▪ content of ethanol ▪ content of methanol ▪ content of 2-propanol	1. presence of active substances 2. quantity of active substances A. ratio drug : extract B. ratio drug : *vehikulum* C. dry residue D. total content of active substances, calculated to dominant or the most active constituent E. quantity of single constituents in the complex of active ingredients F. quantity of the marker substance
Soft extract *Extractum spissum* (≥ 70% of dry matter)	▪ organoleptic properties ▪ dry residue	
Dry extract *Extractum siccum* (≥ 95% of dry matter)	▪ organoleptic properties ▪ extractive matter ▪ dry residue	
Tincture (1:10; 1:5) *Tinctura* (1:10; 1:5)	▪ organoleptic properties ▪ dry residue ▪ relative density ▪ content of ethanol ▪ content of methanol ▪ content of 2-propanol	

* For all types of extracts microbial quality, heavy metal content, presence (absence) of fumigants, pesticide residues, radioactivity, micotoxins, as well as presence of insects and their undeveloped forms, should be determined too (see Table 2).

It should underline that plant extracts is being used not only for production of phytomedicines, but in cosmetic, food and tobacco processing industry, as well as, industry of non-alcoholic and alcoholic beverages, too. Depending upon the final purpose of plant extracts, quality requirements can differ, as in the case of essential and fatty oils, balsams, resins, and similar. Subsequently, attention should be paid while defining quality control parameters, their values and limits, as well as in the quality control process.

At the end, it should be point out once again, that declaration of one extract should contain all the data connected with identity of plant species, relevant data about processing technology and quality of product. Scientific name of plant, plant organ used, solvent used for the extraction, conditions of the extraction, type of extract, drug to extract ratio, or the concentration of active matter, should be precisely given.

Quality control of essential oils

During the last decade, shape of quality requirements for the essential oils changed significantly, following achievements reached by modern analytical techniques and especially gas chromatography (GC). Along with basic physico-chemical parameters, usage of older volumetric chemical methods for determination of certain oil characteristics and thin-layer chromatography (TLC), analysis of chromatographic profile (GC) of the essential oil sample became one of the most important parts of the QC report. One of the most important collections of general quality requirements for the essential oils can be found in ISO standards given by Technical Committee 54. Actual list consists of about 130 standards describing requirements for about 90 different oils. Remaining are dedicated mostly to the general and specific methods of the characterisation of oils. Complete list of all these ISO standards can be easily accessed through the Internet (see site www.iso.org). List of quality requirements and appropriate methods described in few other relevant sources, such as European Pharmacopoeia, is longer,

but list of oils is rather short, leading down to the most common essential oils. Requirements needed for correct usage of essential oils in certain particular field, for example, in food products, alcoholic and soft drinks, requires additional data which can be found only in appropriate specific literature [19].

QC of essential oils requires confirmation of identity, determination of basic physico-chemical characteristics, certain classical chemical determinations, usually quantification of the most abundant (or specific) constituents (or evaluation of chromatographic profile) and eventually some highly specific tests, as well as additional purity tests.

For confirmation of identity (identification) of an essential oil, along with organoleptic properties (appearance, odour and taste determination), TLC, GC and IR-spectroscopy can be used. Depending on the type of the oil for the sample following basic physico-chemical parameters can be determined: relative density, refractive index, optical rotation, solubility in ethanol, residue on evaporation, melting point, freezing point and flash point. The most usual classical chemical tests are determinations of acid value, ester value (before and after acetylation) and carbonyl value. From the other side, GC is the most frequently used technique for determination of the most abundant and/or other constituents of interest. Utilisation of GC-profiles in the evaluation of the quality of these products, nowadays, presents almost unavoidable step. Information obtained in such a way, in return, is surely the most valuable and the most important part of the report dealing with the quality of oil. Characterisation of oils requires usage of other analytical techniques (such as HPLC or UV-VIS spectroscopy) too, but in significantly lesser extent. Finally, there is a small set of methods used for the testing of purity of essential oils. These methods are used for determination of water, foreign esters, and fatty oils and resinified essential oils in essential oils.

Quality control of phytopreparations

Quality of monocomponent teas corresponds to the quality defined for appropriate single herbal drugs. Control leads down to checking of mass of the package, and to determination of parameters defining herbal drug quality.

In the case of multicomponent mixtures, prepared according to direction supplied for cut herbal drugs (tea mixtures), their control leads down to checking of mass of the package, identification of declared constituents, and determination of the mass ratio between these constituents.

In phytomedicines, formulated as higher dosage forms, content of active constituents could be defined in different ways (Table 4). Quality control for such products, assume checking of the mass of the package, i.e. single dose of the formulation, as well as the checking of all the other parameters defining one pharmaceutical form. Qualitative and quantitative analysis of the active ingredients (or marker constituent) of the products, should be surely accomplished. For this purpose, similar, or adequately modified, methods, as those used for determinations in herbal drugs are used. If these (methods) are not pharmacopoeia's ones, they should be validated according to directions supplied by CPMP/ICH/281/95.

When phytopreparation consists of the mixture of plant extracts, the most often is very difficult to accomplish quantitative analysis of the active ingredients of every single extract. For such products, producers are supplying very often-typical fingerprint chromatogram or spectrum of the product, acquired under sharply defined conditions, which could be used in the laboratory for the identification purposes. Such an approach could be also used in the quantitative analysis of the product.

Table 4. Quality control of phytomedicines

Formulations	General testing	Additional testing
Capsules, tablets, dragee, granulate, powder, spray, ointment, cream, gel, liquid formulations	▪ declared mass of package, or single doses ▪ parameters defining selected pharmaceutical form ▪ stability of product ▪ microbial quality ▪ heavy metals content ▪ residues of pesticides ▪ radioactivity ▪ micotoxins	1. presence of active ingredient 2. quantity of active ingredient a. quantity of standardised extract or other plant based component b. total active substances (for example flavonoids, valepotriates, or phenols in the essential oil) c. quantity of active ingredient (for example, hypericin, senosid B), quantity of marker substance 3. presence and quantity of auxiliary substances

Today is very common to declare auxiliary substances used for the preparation of the formulation. Quality control is covering identification and sometimes quantification of these constituents in formulated products. Moreover, results of testing of stability of herbal drugs, extracts and phytomedicines, become today common part of the registration documentation for these products. Such testing for now requires tracking of the stability of product (as is), but of the single constituents of formulation too. Subsequently, certain fingerprint techniques are very often used for these purposes. Procedure is defined through the certain EU Directives (Eudra/Q/92/021 and CPMP/QWP/556/96).

At the end it should be point out that attention was lately directed to interaction of herbal extracts with those substances being used in phytomedicine formulations. Besides, cumulative effects and possible interaction between different active ingredients of the plant origin, as well as those coming out from synthesis (synergism and antagonism), are coming into the focus of the different studies, aiming to provide safety and approve combined therapy. These problems must not be forgotten during the formulation of the product, its control and therapeutic use.

CONCLUSION

As always in the past, QC in reality will reflect balance between needs and possibilities. Growing trend in increase of demands regarding the quality requirements will continue, requiring better prerequisites in the field of QC of MAPs and related products. Share of expenses, needed for QC in total price of such products, will also increase. Since every possible approach, aiming to improve efficacy and safety of herbal medicinal products should surely take into account precious research, as well as strong QC support needed, further separation among producers, to those producing row materials and finish products, respectively, could be expected.

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Appendix 1. List of WHO monographs on selected medicinal plants [20,21,22]

Published in Volume 1 (28)	Published in Volume 2 (30)	Published in Volume 3 (31)
Bulbus Allii cepae	*Radix Althaeae*	*Fructus Ammi majoris*
Bulbus Allii sativi	*Herba Andrographidis*	*Fructus Ammi visnagae*
Aloe	*Radix Angelicae sinensis*	*Fructus Anethi*
Aloe Vera gel	*Flos Calendulae*	*Aetheroleum Anisi*
Radix Astragali	*Flos Caryophylli*	*Fructus Anisi*
Fructus Bruceae	*Rhiz. Cimicifugae racemosae*	*Semen Armeniacae*
Radix Bupleuri	*Folium cum Flore Crataegi*	*Flos Arnicae*
Herba Centellae	*Radix Eleutherococci*	*Folium Azadirachtae*
Flos Chamomillae	*Aetheroleum Eucalypti*	*Oleum Azadirachtii*
Cortex Cinnamomi	*Folium Eucalypti*	*Flos Carthami*
Rhizoma Coptidis	*Cortex Frangulae*	*Stigma Croci*
Rhizoma Curcumae longae	*Folium et Cortex Hamamelidis*	*Fructus Foeniculi*
Radix Echinaceae	*Semen Hippocastani*	*Radix Gentianae luteae*
Herba Echinaceae purpureae	*Herba Hyperici*	*Radix Gentianae scabrae*
Herba Ephedrae	*Aeth. Melaleucae alternifoliae*	*Gummi Gugguli*
Folium Ginkgo	*Folium Melissae*	*Radix Harpagophyti*
Radix Ginseng	*Aetheroleum Menthae piperitae*	*Rhizoma Hydrastis*
Radix Glycyrrhizae	*Folium Menthae piperitae*	*Radix Ipecacuanhae*
Radix Paeoniae	*Folium Ocimi sancti*	*Aetheroleum Lavandulae*
Semen Plantaginis	*Oleum Oenotherae biennis*	*Flos Lavandulae*
Radix Platycodi	*Rhizoma Piperis methystici*	*Strobulus Lupuli*
Radix Rauwolfiae	*Cortex Pruni africani*	*Gummi Myrrha*
Rhizoma Rhei	*Cortex Rhamni purshianae*	*Herba Passiflorae*
Folium Sennae	*Flos Sambuci*	*Testa Plantaginis*
Fructus Sennae	*Radix Senegae*	*Radix Rehmanniae*
Herba Thymi	*Fructus Serenoae repentis*	*Fructus Schisandrae*
Herba Valerianae	*Fructus Silybi mariae*	*Radix Scutellariae*
Rhizoma Zingiberis	*Herba Tanaceti parthenii*	*Radix cum Herba Taraxaci*
	Radix Urticae	*Semen Trigonellae foenugraeci*
	Folium Uvae ursi	*Cortex Uncariae*
		Fructus Zizyphi

Appendix 2. List of ESCOP monographs [14]

Absinthii herba	*Ginkgo folium*	*Plantaginis ovatae testa*
Agni casti fructus	*Ginseng radix*	*Polygalae radix*
Allii sativi bulbus	*Hamamelidis aqua*	*Primulae radix*
Aloe capensis	*Hamamelidis cortex*	*Psyllii semen*
Althaeae radix	*Hamamelidis folium*	*Rhamni purshiani cortex*
Anisi fructus	*Harpagophyti radix*	*Rhei radix*
Arnicae flos	*Hederae helicis folium*	*Ribis nigri folium*
Betulae folium	*Hippocastani semen*	*Rosmarini folium*
Boldi folium	*Hyperici herba*	*Rusci rhizoma*
Calendulae flos	*Juniperi pseudo-fructus*	*Salicis cortex*
Carvi fructus	*Lichen islandicus*	*Salviae folium*
Centaurii herba	*Lini semen*	*Sennae folium*
Chelidonii herba	*Liquiritiae radix*	*Sennae fructus acutifoliae*
Cimicifugae rhizoma	*Lupuli flos*	*Sennae fructus angustifoliae*
Cinnamomi cortex	*Matricariae flos*	*Serenoae repentis (Sabal) fructus*
Crataegi folium cum flore	*Meliloti herba*	*Solidaginis virgaureae herba*
Curcumae longae rhizoma	*Melissae folium*	*Tanaceti parthenii herba*
Cynarae folium	*Menthae piperitae aetheroleum*	*Taraxaci folium*
Echinaceae pallidae radix	*Menthae piperitae folium*	*Taraxaci radix*
Echinaceae purpureae herba	*Myrrha*	*Thymi herba*
Echinaceae purpureae radix	*Myrtilli fructus*	*Trigonellae foenugraeci semen*
Eleutherococci radix	*Ononidis radix*	*Urticae folium/herba*

Eucalypti aetheroleum	*Orthosiphonis folium*	*Urticae radix*
Filipendulae ulmariae herba	*Passiflorae herba*	*Uvae ursi folium*
Foeniculi fructus	*Piperis methystici rhizoma*	*Valerianae radix*
Frangulae cortex	*Plantaginis lanceolatae folium/herb*	*Zingiberis rhizoma*
Gentianae radix	*Plantaginis ovatae semen*	

Appendix 3. List of Commission E monographs [9,23]

[without label = positive monograph; S = drug used exclusively in mixtures; NS = negative monograph of drug used exclusively in mixtures; NO = negative monograph of drug of unapproved efficacy; NR = negative monograph of drug of documented or suspicious risks in use]

Absinthii herba	*Echinaceae purpurae radix*[NS]	*Piceae turiones recentes*
Achillea millefolium	*Eleutherococci radix/ Eleutherococcus senticosus*	*Pimpinellae herba*[NO]
Aconitum napellus[NR]	*Ephedrae herba*	*Pimpinellae radix*
Adonidis herba	*Equiseti herba*	*Pini aetheroleum*
Agni casti fructus	*Eschscholzia californica*[NS]	*Pini turiones*
Agrimoniae herba	*Eucalypti aetheroleum*	*Piperis methystici rhiz./Kava-kava*
Alchemilla alpinae herba[NO]	*Eucalypti folium*	*Plantaginis lanceolatae herba*
Alchemillae herba	*Euphrasia officinalis*[NO]	*Plantaginis ovatae semen*
Allii cepae bulbus	*Faex medicinalis*	*Plantaginis ovatae testa*
Allii sativi bulbus	*Farfarae flos/herba/radix*[NR]	*Podophylli peltati rhizoma/resina*
Aloe	*Farfarae folium*	*Pollen*
Althaeae folium	*Filipendula ulmaria*	*Polygalae radix*
Althaeae radix	*Foeniculi aetheroleum*	*Polygoni avicularis herba*
Ammeos visnagae fructus[NR]	*Foeniculi fructus*	*Populi cortex/folium*[NS]
Anethi fructus	*Foenugraeci semen*	*Populi gemma*
Anethi herba[NO]	*Fragariae folium*[NO]	*Potentillae anserinae herba*
Angelicae fructus/herba[NR]	*Frangulae cortex*	*Primulae flos*
Angelicae radix	*Fraxinus excelsior*[NO]	*Primulae radix*
Anisi fructus	*Fucus*[NR]	*Pruni spinosae flos*[NO]
Anisi stellati fructus	*Fumariae herba*	*Pruni spinosae fructus*
Antennariae dioicae flos[NO]	*Galangae rhizoma*	*Psyllii semen*
Apium graveolens[NR]	*Galangae rhizoma*	*Ptychopetali lignum*[NO]
Armoraciae rusticanae radix	*Galegae officinalis herba*[NR]	*Pulmonariae herba*[NO]
Arnicae flos	*Galeopsidis herba*	*Pulsatillae herba*[NR]
Artemisia vulgaris[NR]	*Galii odorati herba*[NO]	*Quercus cortex*
Asparagi herba[NO]	*Gelsemii rhizoma*[NS]	*Raphani sativi radix*
Atropa belladonna	*Gentianae radix*	*Ratanhiae radix*
Aurantii flos[NO]	*Ginkgo folium*[NO]	*Rauwolfiae radix*
Aurantii pericarpium	*Ginkgo biloba folium, extractum siccum* (35-67:1) acetone-water	*Rhamni cathartici fructus*
Avenae fructus[NO]	*Ginseng radix*	*Rhamni purshianae cortex*
Avenae herba[NO]	*Graminis flos*	*Rhei radix*
Avenae stramentum	*Grindelia herba*	*Rhododendri ferruginei folium*[NS, NR]
Balsamum peruvianum	*Guajaci lignum*	*Rhoeados flos*[NO]
Balsamum tolutanum	*Gypsophilae radix*	*Rosae flos*
Bardanae radix[NO]	*Hamamelidis folium et cortex*	*Rosae fructus*[NO]
Barosmae folium[NO]	*Harpagophyti radix*	*Rosae pseudofructus*[NO]
Basilici aetheroleum[NS]	*Harunganae madagascariensis cortex et folium*	*Rosae pseudofructus cum fructibus*[NO]
Basilici herba[NR]	*Hederae helicis folium*	*Rosmarini folium*
Berberis vulgaris[NO]	*Helenii radix*[NR]	*Rubi fruticosi folium*
Betulae folium	*Helichrysi flos*	*Rubi fruticosi radix*[NO]
Boldo folium	*Hepatici nobilis herba*[NR]	*Rubi idaei folium*[NO]
Borago[NR]	*Herniariae herba*[NO]	*Rubiae tinctorum radix*[NR]
Bromelainum	*Hibisci flos*[NO]	*Rusci aculeati rhizoma*
Bryoniae radix[NR]	*Hippocastani cortex/flos*[NS]	*Ruta graveolens*[NR]
Bursae pastoris herba	*Hippocastani folium*[NO]	*Sabal fructus*
Cacao semen[NS]	*Hippocastani semen*	*Saccharomyces cerevisiae*

Cacao testes[NR]	*Hyoscyami folium*	*Salicis cortex*
Cajeputi aetheroleum[NS]	*Hyperici herba*	*Salviae folium*
Calendulae flos	*Hyssopus officinalis*[NO]	*Sambuci flos*
Calendulae herba[NO]	*Iridis rhizoma*[NO]	*Saniculae herba*
Calluna vulgaris[NO]	*Juglandis folium*	*Santali lignum rubrum*[NO]
Capsicum (capsaicin free)[NO]	*Juglandis fructus cortex*[NR]	*Saponariae herba*[NR]
Capsicum	*Juniperi fructus*	*Saponariae rubrae radix*
Cardamomi fructus	*Lamii albi flos*	*Sarsaparillae radix*[NR]
Cardui mariae fructus	*Lamii albi herba*[NO]	*Scillae bulbus*
Cardui mariae herba[NO]	*Laminariae stipites*[NR]	*Scopoliae rhizoma*
Caricae fructus[NO]	*Lavandulae flos*	*Secale cornutum*[NR]
Caricae papayae folium[NR]	*Lecithinum ex soja*	*Selenicereus grandiflorus*[NO]
Caricis rhizoma[NO]	*Ledi palustris herba*[NR]	*Senecionis herba*[NR]
Carvi aetheroleum	*Leonuri cardiacae herba*	*Sennae folium*
Carvi fructus	*Levistici radix*	*Sennae fructus*
Caryophylli flos	*Lichen islandicus*	*Serpylli herba*
Castaneae folium[NO]	*Lini semen*	*Sinapis albae semen*
Centaurea cyanus[NO]	*Liquiritiae radix*	*Solidago*
Centaurii herba	*Luffa aegyptiaca*[NO]	*Sorbi aucupariae fructus*[NO]
Chamomillae romanae flos[NR]	*Lupuli strobulus*	*Spinaciae folium*[NO]
Chelidonii herba	*Lycopi herba*	*Stramonii folium/semen*[NO]
Chrysanthemum vulgare[NR]	*Malvae arboreae flos*[NO]	*Strychni semen*[NR]
Cichorium intybus	*Malvae flos*	*Symphyti herba/folium*
Cimicifugae racemosae rhizoma	*Malvae folium*	*Symphyti radix*
Cinchonae cortex	*Manna*	*Syzygii cumini cortex*
Cinnamomi cassiae cortex	*Marrubii herba*	*Syzygii cumini semen*[NO]
Cinnamomi ceylanici cortex	*Mate folium*	*Taraxaci herba*
Cinnamomi flos[NR]	*Matricariae flos*	*Taraxaci radix cum herba*
Citri sinensis pericarpium	*Meliloti herba*	*Terebinthina Laricina*
Cnici benedicti herba	*Melissae folium*	*Terebinthinae aetherol. rectificatum*
Coffeae carbo	*Menthae arvensis aetheroleum*	*Thymi herba*
Colae semen	*Menthae piperitae aetheroleum*	*Tiliae flos*
Colchicum autumnale	*Menthae piperitae folium*	*Tiliae folium*[NO]
Colocynthidis fructus[NS, NR]	*Mentzelia cordifolia*[NO]	*Tiliae lignum*[NO]
Condurango cortex	*Menyanthis folium*	*Tiliae tomentosae flos*[NO]
Convallariae herba	*Myristica fragans*[NR]	*Tormentillae rhizoma*
Coriandri fructus	*Myrrha*	*Tropaeolum majus*[S]
Crataegi flos[NO]	*Myrtilli folium*[NR]	*Turnera diffusa*[NO]
Crataegi folium[NO]	*Myrtilli fructus*	*Urticae herba/folium*
Crataegi folium cum flore	*Nasturtii herba*	*Urticae radix*
Crataegi fructus[NO]	*Niauli aetheroleum*	*Usnea species*
Croci stigma[NR]	*Oleae folium*[NO]	*Uvae ursi folium*
Cucurbitae peponis semen	*Oleandri folium*[NR]	*Uzarae radix*
Curcumae longae rhizoma	*Olivae oleum*[NO]	*Valerianae radix*
Curcumae xanthorrhizae rhizoma	*Ononidis radix*	*Verbasci flos*
Cymbopogon species[NR]	*Origani vulgaris herba*[NO]	*Verbenae herba*[NO]
Cynarae folium	*Origanum majorana*[NR]	*Veronicae herba*[NO]
Cynoglossi herba[NR]	*Orthosiphonis folium*	*Vincae minoris herba*[NR]
Cytisi scoparii flos[NS]	*Paeonia*[NO]	*Violae odoratae flos*[NS]
Cytisi scoparii herba	*Papainum crudum*[NO]	*Violae tricoloris herba*
Delphinii flos[NR]	*Passiflorae herba*	*Visci albi fructus*[NO]
Droserae herba	*Petasites hybridus/folium*[NR]	*Visci albi herba*
Dryopteris filix-mas[NR]	*Petasitidis rhizoma*	*Visci albi stipites*[NO]
Dulcamarae stipites	*Petroselini fructus*[NR]	*Yohimbehe cortex*[NR]
Echinacea angustifolia/pallida[NO]	*Petroselini herba/radix*	*Zedoariae rhizoma*[NO]
Echinacea pallidae radix	*Phaseoli fructus sine semine*	*Zingiberis rhizoma*
Echinaceae purpureae herba	*Piceae aetheroleum*	

[PL-10]

[PL-11]

INFLUENCE OF SOIL CHARACTERISTICS AND NUTRIENT SUPPLY ON MEDICINAL AND AROMATIC PLANTS

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ABSTRACT

Soil characteristics and nutrients supply are of great importance for growth and development of MAPs. Apart from this, soil factors are also responsible for the final quality of MAPs. Physical characteristics of soil in great incidence determine suitability for growing particularly of species that are grown for their roots. Soil reaction (pH) and parent material are the most responsible factors moderating the content and availability of mineral elements in the soil. In soils with high pH reaction and high content of lime, availability of most mineral elements decreases, what usually causes chlorosis and retards development of certain susceptible plants. On the contrary, in strong acid soils, the availability of mineral elements increases so, that it can cause harmful accumulation of some heavy metals, especially in plant species that are prone to such phenomenon.

Dose of the applied macronutrients (in form of fertilisers) in MAP production depends primarily on the content of these nutrients in the soil as well as on the requirements of grown MAP species. Nitrogen has the greatest influence on growth and development of the aboveground part of plant (the yield) and in certain cases also has a positive influence on plant quality. However, too much of N in the soil may cause negative effects in species that are grown for their flowers or their roots (delay in flowering and vigorous development of the herb instead of desirable root development). Potassium most often has positive effects on development of roots, while phosphorus has positive influence on development of generative organs and stimulation of flowering. Such a global division has an array of deviations in practice, depending on numerous other ecological and physiological factors, among which the most important one is availability of the other nutrients in the soil (Ca, Mg, B, Fe, Zn, and Mn).

Last decades, under the strong anthropogenic influence (industrial centres, highways, Cu, Zn and Pb smelters, etc), some soils have been additionally loaded with heavy metals (Pb, Cd, Cr, Ni, Hg) as well as with some other potentially harmful substances (organic waste). This problem may be solved by avoiding such localities, weather it is a word about growing or collection of MAPs from the nature. Strongly defined principles of GAP and/or organic collection and production of MAP, promote avoidance of any kind of pollution. Consequently, it is expected in the future that these kinds of MAP production and/or exploitation from the nature will be intensified.
Key words: medicinal and aromatic plants, soil characteristic, nutrients supply, heavy metals.

INTRODUCTION

Soil characteristics and nutrients supply are of great importance for growth and development of any plant species. Apart from the influence on the yield of medicinal and aromatic plant species, soil factors are also responsible for their final quality. However, some other factors, such as climate, genetic characteristics of a plant species (Nemeth, 2000) etc, also determine rate of development and final quality of the obtained row material, in each particular case. Therefore, it is sometimes hard to say how much certain plant property is influenced just by the soil factors and how much it is a word about the influence of some other factors. Due to a fact that the total soil fertility is actually a resultant of mutual interaction between all of them, it is hard to clearly split their influences into the separate ones. This is even more complicated, if we take in consideration that the plant itself, with its very diverse, genetically determined, physiological mechanisms shows specific response to all ecological factors, so as to the soil itself. These, all together, bring about very complex situation in the nature, where certain plant's responses hold strictly local characteristics.

Due to this reason, the data obtained from the experiments, weather they are carried out at open field or in controlled conditions, have relatively limited significance. Yet, even in such conditions, up to now, many authors tried to summarise the obtained results giving them wider meaning. Some of such results will be presented in this review, including our own experimental results obtained from a number of geo-morphological entities at Balkan Peninsula.

SOIL CONDITIONS

Physical characteristics of soil (texture, porosity, air and water capacity, and water permeability) have great influence on growth of species particularly those grown for their roots (such as angelica and marsh mallow). It

was generalised that the medium loam or sandy loam soils, with medium humus content are suitable for valerian (*V. officinalis*) cultivation. The advantages of a loam soil are obvious; the plant development is more intense, the number of leaves increases and plants become from 20 to 25 cm higher. Positive changes are also observed in the manner of root formation; number of primary roots is higher and the total root biomass increased in about 100 % (Berbec, 1965 - cited by Bernath, 1997). Importance of soil characteristic on production of valerian was also presented by Bernath *et al*., 1973. The root production of valerian plants was 151.6 g in sandy soil, 156,6 g in loam, while only 91,5 – 137,3 g in clay rich soils.

In addition to this, there is opinion that the plants grown on soils of lighter texture are less prone to plant diseases. In combination with high altitude of locality, these two environmental factors lead to decrease in virulence of pathogenic fungi causing anthracnose on St. John's Worth (Gaudin *et al*., 2002). This disease can destroy the perennial St. John's worth culture already in the first year, and this mainly happens on heavy soils and in damp regions (Bomme, 1997).

Soil pH reaction and parent material are the most responsible factors moderating the content and availability of mineral elements in the soil. In soils with high pH value and high content of lime, availability of most mineral elements decreases, what usually causes chlorosis and retards development of certain plants (arnica). On the contrary, in strong acid soils, the availability of mineral elements increases so that can cause harmful accumulation of heavy metals (Cd, Ni, etc.), especially in those plant species that are prone to such phenomenon (St. John's worth, yarrow, etc). Plants respond differently in different pH of the soil. Authors Dachler and Pelzman (1999) state in their work some MAP species with their different soil pH preferences:

- Species that may be grown on soils rich in lime (high pH values): *Foeniculum vulgare, Coriandrum sativum, Lavandula* sp.*, Origanum majorana, Calendula officinalis, Salvia officinalis, Thymus vulgaris, Hysopus officinalis.*
- Species that may be grown on soils poor in lime (low pH values): *Epilobium parviflorum, Plantago lanceolata, Arnica* sp., *Digitalis purpurea*, *Centaurea cyanus*
- Species that may be grown equally on both, alkaline and acid soils: *Chamomilla recutita, Carum carvi, Malva mauritanica, Papaver somniferum, Linum usitatissimum, Achillea millefolium.*

NUTRIENT SUPPLY

Content of nutrients in plants and total soil fertility are very specific properties that vary in wide diapason. Therefore, for successful management of nourishment during MAP production, it is very important to be informed about the condition of the main nutrients in the soil. Dose of the macronutrients that are to be applied in form of fertilisers depends primarily on the content of these nutrients in arable layer of the soil, as well as on requirements of particular plant species grown. Harmonisation of the soil fertility potential and particular medicinal plant nutrient requirements is of great importance, regarding the opportunity to make a better use of lower fertility soils just by making selection of right plant species. Nutrient quantities taken out from the soils by the yield of some MAP species are presented in table 1.

Table 1. Main macronutrients quantities taken out from the soils by the yield of some MAP species (Bomme and Nast, 1998 – part)

Species	Plant part	Yeld of fresh biomass t/ha	N kg/ha	P_2O_5 kg/ha	K_2O kg/ha	MgO
Calendula officinalis	flower	5	15	6	23	2
Chamomilla recutita	flower	4	17	8	22	1
Levisticum officinalis	root	12	26	18	29	10
Urtica dioica	root	8	30	16	41	8
Valeriana officinalis	root	15	44	28	62	9
Tanacetum parthenium	flowering herb	12	54	19	96	6
Angelica arhangelica	root	20	60	46	130	18
Ehinacea angustifolia	root	15	69	21	76	21
Satureja hortensis	flowering herb	12	84	18	95	11
Origanum majorana	herb at beginning of flowering	20	96	29	118	14
Althea officinalis	root	15	99	45	108	31
Hypericum perforatum	flowering herb	20	105	40	119	13
Borago officinalis	flowering herb	70	108	33	306	12
Melissa officinalis	herb	30	146	41	228	27
Inula helenium	root	30	150	64	227	20
Mentha x piperita	herb	40	167	45	220	31
Salvia officinalis	herb	35	172	38	216	43

Depending on data presented in table 1. for the nutrients that are taken up from the soil by the yield and depending on fertility of the soil itself, doses of macronutrients that are to be applied can be easily calculated for each particular case. It should be also taken in consideration that remaining plant parts that don't represent the yield also consume certain, often considerable, amount of nutrients from the soil. From the aspect of the management of soil fertility, it is important to return by plowing the plant parts following the harvest back to the soil (Radanovic and Nastovski, 2002).

Plant that are grown for flower take up from the soil relatively modest quantities of the nutrients so they could also be successfully grown at low fertility soils, with minimal application of fertilisers (chamomile). On the contrary, high nutrient demanding crops (peppermint, lemon balm) are better to be grown on high-fertility soils. Speaking generally, species grown for their aboveground parts and leaves only, respond positively on **nitrogen nutrition**. Nitrogen has the greatest influence on growth and development of the aboveground part of plant (the yield) and in certain cases also has a positive influence on plant quality. In one of our experiments (Radanovic *et al*., 2000a), conducted in moderately humoglay soil of South Banat (Serbia), fertilisation with Nitrogen showed to have weak influence on the increase of yield in the first year of sage cultivation. Increase of sage yield achieved at dose N_1 was only 6.2%. Higher average increase has been achieved in the second year, at N_2 dose above 14%. Similar result was presented by Röhricht *et al*. (1996), in the experiments with sage. The plants of density 32 000 pl./ha (0,9 X 0,35 m) achieved significant yield increase with application of 100 kg N/ha in comparison to control treatment (without N fertilization), but only in the second year of investigation.

In species grown for seed production, the influence of N on agricultural behavior of MAP species may be very different. Increasing of nitrogen rates caused no uniform trend in plant height, but slight increase in TSM (thousand seed mass). As it can be observed in table 2, an increase was up to 150 kg/ha in *Sinapis alba* grown on chernozem in Austria. The highest yields were reached with 100 kg N/ha. Fat content dropped from 28,8 % (no application of nitrogen) to 24, 6 % (200 kg N/ha) and subsequently the highest fat yield was achieved with 100 kg N/ha (Dachler, 1992).

Table 2. The influence of increasing nitrogen rates on some agronomical and quality traits of mustard (*Sinapis alba*).

N-rate kg/ha	Plant height cm	TSW g	Yield kg/ha	Fat content % of D.M.	Fat yield kg/ha
0	162	5.79	1669	28.8	480
50	165	6.06	1824	27.9	508
50+50	163	6.10	2019	26.5	535
75+75	170	6.26	2009	25.2	506
100+100	166	5.76	1932	24.6	475
LSD 5%			245		

Peppermint, well know as a species that responds positively on stronger nutrition and rich soils, in lot of experiments proved to achieve the highest yield of leaves and essential oils with application of 150 kg N/ha (Singh, 1989; Maksimovic *et al*., 2000; Piccaglia and Marotti, 1989). Experiments were carried out on soils of good fertility. In the case of soils of lower fertility the yield of peppermint will also increase but with application of higher doses (200 and more kg N / ha). On the contrary, on rich nitrogen soils optimal yields may be achieved with application of lower nitrogen doses (ca. 100 kg/ha).

Stinging nettle due to its nitrophilic affinity is often a subject of investigations regarding nitrogen and particularly nitrate metabolism. In 3 years experiments, an increase of nitrogen fertilisation in each year achieved significant increase of DM production of both, leaves and stems. In the first year of cultivation development was postponed and effect of nitrogen was not distinct comparing to the other two years. In the second and the third year, starting with application of 80 kg N/ha annually, the yield increased with each higher level of nitrogen applied, showing statistical significance (Weiß, 1993).

However, too much of nitrogen may, in some cases, cause negative effects. High doses of nitrogen fertiliser to a nitrophilic species like stinging nettle was likely to influence nitrate content in the herb. In these experiments highest nitrogen input always caused highest nitrate nitrogen content in plant. In the first year average nitrate N contents influenced by increased nitrogen rates ranged from 200 - 900 ppm in leaves and 1100 - 5700 ppm in stems. It means that the separation of steams from the herb, which is common practice, decreases nitrate content of the drug considerably. What is even more interesting is that the content of nitrate nitrogen has been greater in each following harvest obtained (there were 4 harvests in one year). In the primary growth and first re-growth, an average of 160 ppm nitrate N in leaves and 1130 ppm in stems was found. In the second and third re-growth

nitrate N contents increased significantly up to an average content of 950 ppm in leaves and 3150 ppm in steams. This increasing nitrate contents in the latest harvests may be explained, to the some extent, by reduced intensity of sun radiation and also by fact that in the second and in the third re-growth physiologically younger plants, which contain of higher content of nitrate N in comparison to the older plants, were harvested.

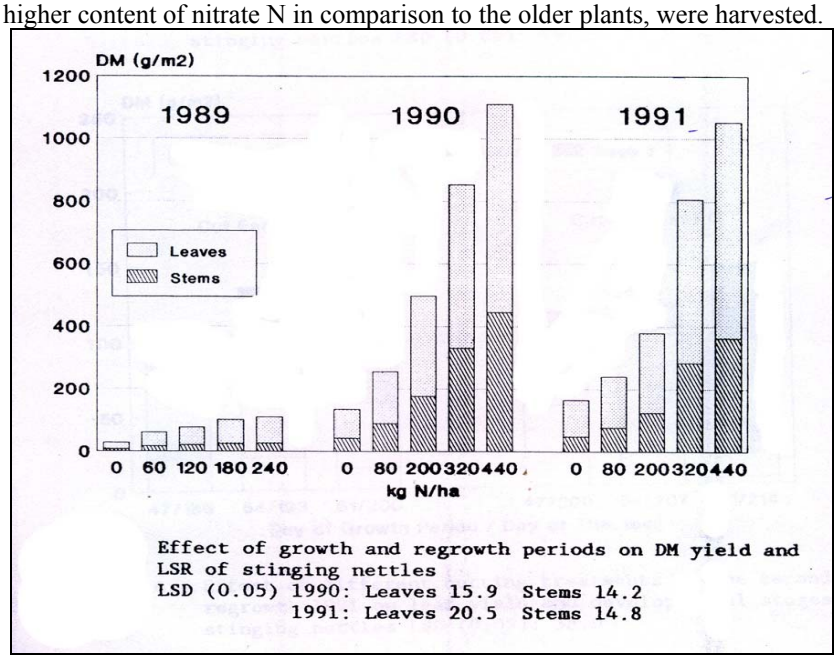

Chart 1. Source: Weiß, 1993.

Too much of nitrogen in nutrition may cause some negative effects in species that are grown for their flowers (*Chamomilla recutita*) or their roots (*Echinacea angustifolia*). Actually, excess of nitrogen in the soil may cause postponement in flowering and vigorous development of aboveground part of the plants instead of desirable root development. Franke and Schenk (1999 - cited by Berti *et al*., 2002) reported a slight increase in root yield of *Echinacea pallida* when nitrogen was added. Fertilisation with N caused decrease in the concentration of active compound echinosides. Echinoside content was 1,16% without and 0,94% with application of nitrogen.

Plant nutrition with **phosphor and potassium** is much more dependent on the level of these nutrients in the soil, since the available forms of P and K are more stable categories then it is a case with available forms of nitrogen in the soil. Nutrition of MAPs with P and K deserves more attention in case of soils that are poor in these two elements, while in case of better supplied soils, fertilisation should not exceed quantities taken up by the plant yield. It is well-accepted opinion that potassium most often has positive effects on development of MAP cultures grown for their roots (angelica, valerian, coneflower).

Fertilisation of *Echinacea purpurea* plants indicates that in absence or at low levels of nitrogen fertilisation (0 and 50 kg/ha) the addition of 60 and 120 kg K_2O / ha increased aboveground plant parts, flower heads and root yield (Shalaby *et al*., 1997). Another report indicated that the highest aerial biomass and root yield in *E. purpurea* was obtained with 100 kg / ha of nitrogen and constant rates of phosphorus and potassium. Phosphorus has a positive influence on development of generative organs and it stimulates flowering. Azizi *et al*. (2002), obtained higher quantities of flowers and higher content of hypericin in St. John's worth by the application of 100 and 200 kg of P in comparison to control treatments (without P application).

Such a global division has an array of deviations in practice, depending on numerous other ecological and physiological factors, among which the most important one is availability of other nutrients in the soil (Ca, Mg, B, Fe, Zn, and Mo) (Radanović *et al*., 1998, Antić-Mladenović *et al*., 2000). Bernath (1997) in his work presents that CaO in loam and $CaCO_3$ in sandy – clay soil accelerate the growth of roots of *Valeriana officinalis*. Fertilisation with calcium also improved root quality, increasing the utilisable part of rootlets by 75.2% - 78.8%. Positive effect of Ca application at very acid soils may have for its consequence diminishing of the Cd content in herb of St. John's worth (Radanović *et al*., 2004).

PROBLEM OF HEAVY METALS IN MEDICINAL PLANTS

There are two major reasons why it is important to study heavy metals in the environment:

- some of them (Fe, Mn, Cu, Zn, Co, Cr, Mo) are essential for metabolism of humans, animals and plants;

- all of them, both essential and non-essential (Pb, Ni, Cd, As, Hg) can cause toxic effect to humans, animals and plants if found in high concentrations.

Since heavy metals enter into food chain mainly throughout the plants, primarily interest is to study and understand their availability in the soil and uptake by plants. Heavy metal content in medicinal plants is a subject of great interest, published in many papers (Schneider and Marquard, 1995; Röhricht *et al*., 1996; Chizzola, 1998; Radanovic *et al*.1998; 2000b; 2001; Antic-Mladenovic *et al*., 2000). Soil is main source of heavy metals for terrestrial plants. Availability and uptake of heavy metals from a soil are affected by several factors, which do not exclude each other:

1. Origin of heavy metals in a soil
In the soils where heavy metals dominantly originate from geochemical sources, their great proportion is associated with the chemically most resistant soil fraction, made of different minerals (silicates, oxides), which, therefore, has the little environmental concern. Opposite to that, substantial proportion of heavy metals from anthropogenic sources is associated with more labile chemical compounds and forms, which constitute important pool of their potentially available and available forms in the soil.

It is also important that, some of the anthropogenic sources of heavy metals, besides leading to soil contamination with heavy metals, at the same time also cause contamination of plants by deposition of the air particles. Zeljazkov and Nilsen (1996) have found highly polluted medicinal plants of geni *Mentha* and *Lavandula* from air deposits in a vicinity of NFMC (Non – Ferrous Metals Combine near Plovdiv) in Bulgaria. Pollution of plants with Pb, Zn and Cd were recorded at the different distances from combine. The highest heavy metals content was found at 400 m, at 3 km it was five times lower, while at the distance of 10 km no pollution has been recorded. The authors found extremely high concentrations of Pb and Cd in the aboveground organs of two cultivars of lavender. As concentrations of those elements in root tissue were much lower, existence of aerosol pollution in the area near smelter was confirmed. This example shows that, although in a vicinity of metal's smelters soil contain high level of heavy metals, contamination of plants via airpollution remains as the most significant source of heavy metals in such area. It is interesting to mention that essential oil has the same level of heavy metals as found at control location, which implies to a possible way of utilisation of raw material from contaminated areas.

2. Soil properties, such as: reaction (pH), redox-potential, content and form of organic matter, content and form of clay minerals, etc. affect heavy metal chemical behaviour in a soil. Among all of this factors, soil reaction is probably the most important one, since availability of all heavy metals, with the exception of Mo, increase as pH decrease. The investigation conducted at about twenty locations in Serbia, Montenegro and Bosnia and Herzegovina (Radanović *et al*., 2002), revealed that heavy metals content in species *Hypericum perforatum* growing wild, among the other factors, greatly depends on soil pH (Chart 2). In *H. perforatum*, decrease of soil pH below 5.5 has been followed by an increase of nickel concentrations above it natural level in plant material (up to 5 mg/kg). High correlation coefficient, with negative sign, between soil pH and Ni content in the herb, confirms that this is not just a coincidence, but comes as the result of higher solubility and availability of nickel in acid conditions. Cadmium availability also increases rapidly with the increase of soil acidity. It is found that pH of 6.5 represents upper limit of soil pH, above which cadmium concentrations do not exceed permissible limit values.

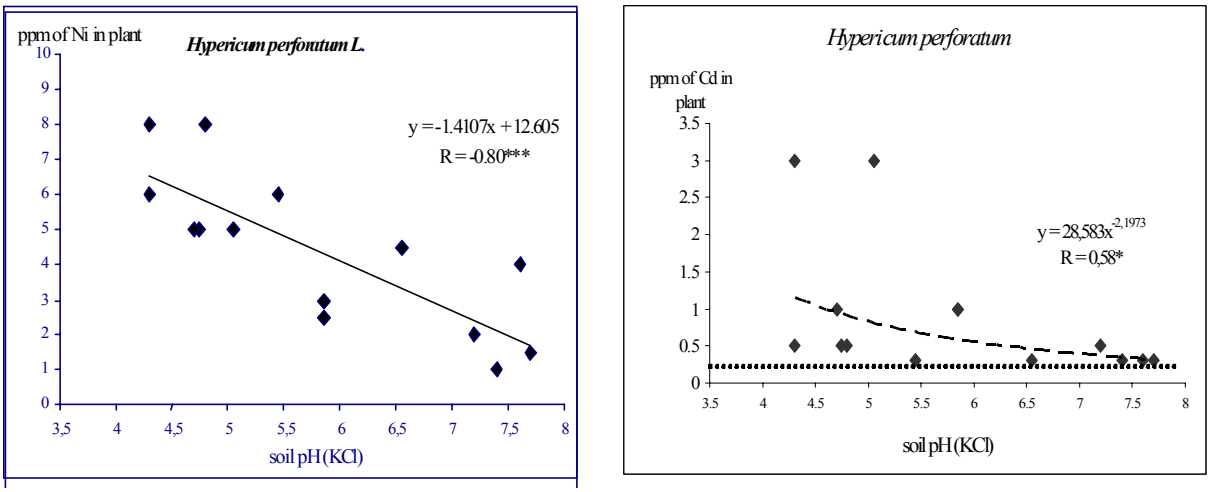

Chart 2. Content of Ni and Cd in *H. perforatum* at different soil pH (Radanović *et al*., 2002)

Bingel *et al*. (1998) analyzed Cd content in species *Hypericum perforatum* and F*agopyrum esculentum,* grown in acid soil, containing elevated total Cd (1,78 mg/kg). After the liming, soil pH was increased from 5.6 to 7.6, what caused decrease of Cd content in plants. But, Cd level in St. John's worth flowers was still above permitted value of 0.5 mg/kg. Similar was found for *F. esculentum.* These examples imply that in the conditions of high total Cd in the soil, increase of soil pH was not sufficient to prevent high Cd uptake by *H. perforatum* and *F. esculentum.*

3. Genetic characteristics of plant species are the next factor that affects heavy metal's uptake. It is recognised that some species have the ability to accumulate significant quantities of heavy metals. Some of them are found among the medicinal and aromatic plants. Those are *Hypericum perforatum* L., *Linum usitatissinum* L., *Achillea millefolium* L *Calendula officinalis* L., *Origanum majorana* L., *Chamomilla recutita* (L.) Raushert. (Hasselbach, 1992. cit. by Röhricht *et al*., 1997), *Betula folium, Darura stramoium fructus, Salix cortex, Crategi folium cum flore.*

Since elevated concentrations of heavy metals in plants may, through food chain, endanger humans and animals health, majority of countries has legislative regulations for heavy metal's thresholds in environment, soil, slugs and plant material. In Germany, maximum permitted concentrations of heavy metals in MAPs have been regulated by the Law (Steinhoff, 1998; Marquard and Schneider, 1998). Special interest is paid to permitted concentrations of Cd, since some MAPs are prone to increase assimilation of that element. Cd uptake by different medicinal plants has been the scope of numerous investigations and literature reports.

Marquard and Schneider (1998) investigated Cd content in different MAP species collected from loesial soil that have contained 0.13 mg/kg of total Cd. The analysis reveled that fourteen of twenty-four analysed species contained less than 0.2 mg Cd/kg, while Cd content in *Melissa officinalis* was equal to permissible limit. The five species contained between 0.2 and 0.5 mg Cd/kg and just three of them contained above 0.5 mg Cd/kg (Chart 3). The question is would the Cd content be less then 0,2 mg/kg in the mentioned fourteen species in the case of higher Cd content in a soil?

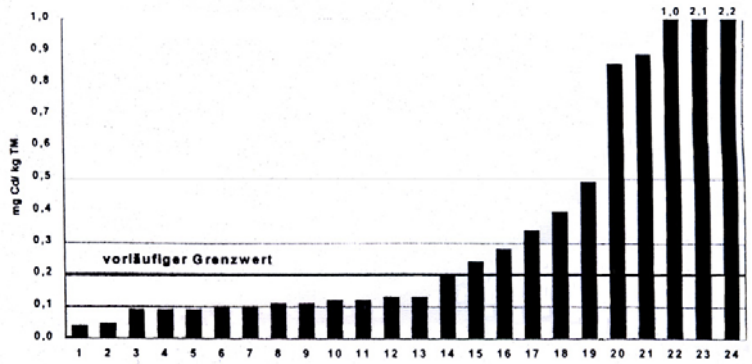

Chart 3. Cd content in different medicinal plants (Marquard and Schneider, 1998)

[Legend: 1. *Lavandula* sp., 2. *Artemisia absinthium*, 3. *Mentha pip*-»Bulgarien«, 4. *Salvia officinalis*, 5. *Hysopus officinalis*, 6. *Mentha p.*-»Polymentha«, 7. *Ruta graveolens*, 8. *Origanum vulgare*, 9. *Achillea millefolium*, 10. *Symphytum officinale*, 11. *Mentha p.*-»Mitcham«, 12. *Digitalis sp.*, 13. *Satureja montana*, 14. *Melissa officinalis*, 15. *Marubium vulgare*, 16. *Chamomilla recutita*, 17. *Thymus vulgaris*, 18. *Potentilla erecta*, 19. *Althea officinalis*, 20. *Hypericum* sp., 21. *Artemisia dracunculus* D, 22. *Artemisia dracunculus* R, 23. *Hypericum perforatum*, 24. *Viola tricolor*]

Radanovic *et al*. (2004) investigated the content of Cd, Ni, and Pb in some MAPs grown on distric acid soil, formed over serpentine of mountainous region of Serbia. Due to intact nature and absence of industrial pollution, this region may be very attractive for organic MAP wild collection. However, content of some heavy metals above maximally allowed concentration warns at possible problems that may be encountered in terms of the final quality of MAP row material. Content of Cd in the analysed MAP species was in the range 0,0 – 4,0 mg/kg, at 0,8 mg/kg of soil Cd with medium acid soil reaction (pH/KCl = 5,0). More than 0,2 mg Cd/kg was found in the following species: *Tanacetum larvatum, Gentiana lutea, Plantago lanceolata,* while the highest concentrations were found in species belonging to genus *Hypericum*.

The data obtained for Ni (Radanovic *et al*., 2004) showed that total Ni in the soil varied in very wide range within very small land area (60-1300 mg/kg within 3 – 4 ha), which is typical for serpentine soils. Consequently, Ni content in plants also varied in wide interval (2.1 to 93 mg/kg). This means that Ni content in plants is the result of both, high soil Ni and acid soil reaction. Content of Pb in the soil samples were within its level for unpolluted soils, so its content found in MAPs has also been within permitted level, despite of acid soil reaction.

It was found that some MAP species have the ability to keep Cd in root tissue and restrain its translocation to upper organs; in *Mentha piperita* and *Salvia officinalis* grown at soil containing from 0,16 – 1,0 mg Cd / kg, content of Cd in herb stayed below 0,2 mg/kg (Bingel *et al*., 1998). But, distribution of heavy metals between organs within same species might be a matter of cultivar characteristic. Some cultivars of *Linum usitatissimum* (Marquard and Schneider 1998) keep greater proportion of Cd in root and straw, while smaller proportion is being translocated to seed. But, there are also cultivars where Cd is, after adsorption from a soil, mostly being distributed toward upper organs, straw and seeds. Differences between cultivars also exist regarding their ability to adsorb Cd from a soil in a first place.

As it has already been mentioned, St. John worth has the ability to accumulate Cd from a soil, which represents practical problem during its growth. Well-selected land, having low total Cd and neutral reaction, is one of possible ways to prevent accumulation of high Cd quantities in an herb. The other way is breeding towards selections of genotypes with lower accumulation ability (Marquard and Schneider, 1998). Reducing heavy metal adsorption by medicinal plants through selection of genotypes with lower ability for adsorption is, without any doubt, very desirable process. Remaining question is how realistic are the expectations for the investments in that kind of breeding, since very narrow market for medicinal plants' seeds. Nevertheless, genetic research is certainly a good way to overcome a problem of high accumulation of heavy metals in medicinal and aromatic plants.

Another question is why studding heavy metals in medicinal plants since they make a very small proportion of human dietary? Importance of this investigation lies in two facts: 1) natural habitats of most of the medicinal plants are found in hilly and mountain regions. Soils from such regions are usually formed over ores deposits, and, therefore, may contain elevated concentrations of heavy metals; 2) mountain soils are usually characterised by acid soil reaction, which are the environmental conditions when solubility, availability and uptake of heavy metals become higher (Jakovljević *et al*., 2002).

Therefore, medicinal plants collected from the wild should be tested for heavy metal content. In the light of our new knowledge, it wouldn't be too pretentious to say that medicinal plants for a wide commercial exploitation should be, if possible, grown as regular agricultural crops at previously well selected land, regarding heavy metals content in the soil, soil properties and potential anthropogenic influence. Thus, agro-technique has to be chosen in a way that prevents heavy metals adsorption from a soil during growth. Great number of factors has an effect on quantities of heavy metals that could be adsorbed from a soil. Choice of growing site can also greatly contribute to either increase or decrease of heavy metal content in plant material. For growing of sensitive species, land should be carefully chosen to have low heavy metal content and to be maintained by liming.

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[PL-12]

PREHISTORIC USE OF MEDICINAL AND AROMATIC PLANTS IN THE SOUTHEAST PART OF THE PANNONIAN PLAIN

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SUMMARY

Prehistoric use of medicinal and aromatic plants in the southeast part of the Pannonian plain have been investigated by archaeobotanists at archaeological sites: Feudvar (Bronze and Iron Age) and Židovar (Bronze Age). Silent witnesses of plant husbandry and gathering economy, carbonised seeds and fruit stones, lay scattered in the cultural layers of prehistoric settlements. Traditional herbal medicine is still using some plants which were found during archaeological excavations: wall germander *Teucrium chamaedrys* L., vervain *Verbena officinalis* L., high mallow *Malva sylvestris* L., dwarf mallow *Malva neglecta* Wallr., black henbane *Hyoscyamus niger* L., St. John's wort *Hypericum* sp., white mallow *Althaea officinalis* L., mint *Mentha* sp. and camomile *Matricaria* sp.

Prehistoric use of medicinal and aromatic plants in the southeast part of the Pannonian plain have been investigated by archaeobotanists at archaeological sites: Feudvar (Bronze and Iron Age), Gomolava (Neolithic, Eneolithic, Iron Age), Gradina/Bosut (Iron Age), Kalakača (Iron Age), Opovo (Neolithic), Petrovaradin (excavation in progress), Starčevo (Neolithic) and Židovar (Bronze Age). Just for illustration here is included the most recent chronological division of prehistoric periods for Vojvodina stated by P. Medović (verbal report): Palaeolithic (60000-9000 BC), Mesolithic (9000-6300 BC), Neolithic (6300-3200 BC), Eneolithic (3200-2000 BC), Bronze Age (2000-950 BC), Iron Age (950 BC to the break of the eras). Silent witnesses of plant husbandry and gathering economy, carbonised seeds and fruit stones, lay scattered in the cultural layers of prehistoric settlements. Macrofossil plant remains consist mostly of cultivated plants (cereals, pulses and oil/fibre plants) and accompanying weeds. Woody plants used for building and other technical purposes, wild fruits, vegetables and spices complete the spectrum of ancient settlements plant economy. Based on archeobotanical evidence from rich assemblages obtained by water flotation at site Židovar, A. Medović (2003) divides findings in several groups with regard to historical background and potential use of plants. These innovated and completed results are presented in Table 1.

Table 1. Židovar, Vojvodina, Serbia & Montenegro. Macrofossil plant remains (charred seeds and fruits). List of all species. Bronze Age, Vattina-Culture (r: ‹ 0,5 %; +:› 0,5% ‹ 1,0 %).

Taxon	n	n %	frequency	frequency %
Cereals				
Triticum aestivum	1526	12	18	86
T. aestivum, rhachis	1	r	1	5
Triticum monococcum	505	4	20	95
T. monococcum, rhachis, glumellae	1177	9	21	100
Hordeum vulgare vulgare	197	1	18	86
Triticum dicoccon	176	1	15	71
T. dicoccon, rhachis, glumellae	219	2	20	95
Panicum miliaceum	15	r	4	19
Triticum spelta	8	r	4	19
T. spelta, rhachis, glumellae	3	r	3	14
Cerealia indeterminata	214	2	18	86
Pulses				
Lens culinaris	167	1	14	67
Pisum sativum	48	r	12	57
Vicia ervilia	3	r	3	14
Leguminosae sativae indeterminatae	2	r	2	10
Oil plants				
Camelina sativa	1198	9	14	67
Lallemantia iberica	6	r	1	5
Wild fruits and nuts				
Sambucus ebulus	103	+	20	95
Quercus sp.	29	r	12	57
Prunus spinosa	12	r	7	33

Pyrus sp.	12	r	1	5
Vitis vinifera sylvestris	10	r	4	19
Fragaria vesca	5	r	3	14
Corylus avellana	2	r	2	10
Prunus sp.	2	r	2	10
Cornus mas	1	r	1	5
Prunus fruticosa	1	r	1	5
Prunus non *spinosa*	1	r	1	5
Possible medicinal plants				
Verbena officinalis	23	r	4	19
Teucrium chamaedrys-Type	15	r	9	43
Malva sp.	2	r	2	10
Hyoscyamus niger	1	r	1	5
Weeds and synanthrope vegetation				
Chenopodium (polyspermum)	7211	54	21	100
Bromus arvensis	38	r	14	67
Galium spurium	29	r	14	67
Polygonum convolvulus	26	r	10	48
Solanum nigrum	23	r	10	48
Agrostemma githago	19	r	6	29
Lolium sp., "small seeded"	14	r	9	43
Polygonum aviculare	12	r	7	33
Setaria viridis	12	r	4	19
Bromus secalinus	10	r	5	24
Bromus sp., "long seeded"	9	r	7	33
Bromus arvensis/secalinus	7	r	1	5
Echinochloa crus-galli	5	r	3	14
Chenopodium hybridum	3	r	3	14
Digitaria sanguinalis	2	r	2	10
Mentha sp.	2	r	1	5
Prunella vulgaris	2	r	2	10
Rumex sp.	2	r	2	10
Silene-Type	2	r	2	10
Agrimonia eupatoria	1	r	1	5
Anthemis-Type	1	r	1	5
Bupleurum rotundifolium	1	r	1	5
Convolvulus arvensis	1	r	1	5
Euphorbia helioscopia	1	r	1	5
Matricaria sp.	1	r	1	5
Portulaca oleracea	1	r	1	5
Rosa sp.	1	r	1	5
Rumex crispus-Type	1	r	1	5
Sherardia arvensis	1	r	1	5
Urtica dioica	1	r	1	5
Plants of wetlands				
Carex, subgen. Eucarex	1	r	1	5
Cladium mariscus	1	r	1	5
Other plants remains				
Trifolium-Type	45	r	11	52
Poaceae	33	r	13	62
Lamiaceae	23	r	7	33
Cyperaceae	11	r	3	14
Polygonaceae	11	r	7	33
Asteraceae	2	r	2	10
Rosaceae	2	r	1	5
Brassicaceae	1	r	1	5
Unindentified	1	r	1	5
Total taxa/total samples	**73**		**21**	
Total sum	**13254**	**100**		

Archaeobotanical findings from Židovar are similar to the results obtained from Feudvar. Table 2. contains a database of possible drug or medicinal plants. Complete data information are published by Borojević (1991), Kroll and Borojević (1988), Kroll (1990a, 1990b, 1991a, 1991b, 1992, 1995, 1998).

Table 2. Feudvar, Vojvodina (Serbia): list of possible medicinal plants and synanthrope vegetation

Taxon	n
Possible medicinal plants	
Teucrium chamaedrys -Type	1682
Verbena officinalis	288
Malva	133
Malva sylvestris	10
Malva neglecta	3
Hyoscyamus niger et similes	79
Hypericum	42
Althaea officinalis	14
Synanthrope vegetation/other possible medicinal plants	
Galium spurium et similes	1248
Polygonum aviculare	1993
Adonis	106
Agrimonia eupatoria	213
Plantago lanceolata	349
Allium	17
Galium	65
Geranium	15
Mentha	
Urtica dioica et similes	14
Verbascum	30
Brassica nigra	4
Cichorium intybus	2
Papaver dubium-Type	4
Ranunculus acris-Type	9
Ranunculus	7

The existence of medicinal plants in prehistory as such can be hardly proven. Parts of many plant species were gathered and used for diverse purposes that we will probably never be able to find out. A concentrated find of seeds of many-seeded goosefoot *Chenopodium polyspermum* L. in the fortified settlement of Feudvar is a nice example for this. However, today's traditional herbal medicine is still using some plants which were found during archaeological excavations: wall germander *Teucrium chamaedrys* L., vervain *Verbena officinalis* L., high mallow *Malva sylvestris* L., dwarf mallow *Malva neglecta* Wallr., black henbane *Hyoscyamus niger* L., St. John's wort *Hypericum* sp., white mallow *Althaea officinalis* L., mint *Mentha* sp. and camomile *Matricaria* sp. The list of possible drug/medicinal plants can be extended with poppy *Papaver somniferum* L. - in the prehistory common cultivated oil plant. The hemp *Cannabis sativa* L. was archaeobotanicaly recorded in the central part of the Pannonian plain in Iron Age. The inhabitants of Feudvar were using barley *Hordeum vulgare* L. and emmer *Triticum dicoccon* Schrank, an prehistoric wheat-crop, for making beer. Use of hops *Humulus lupulus* L. as beer-spice was not proven. Dill *Anethum graveolens* L., wild carrot *Daucus carota* L., wild celery *Apium graveolens* L. and wild parsnip *Pastinaca sativa* L. are species that could have been used for spicing seasoning food.

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[PL-12]

[L-06]

PHYTOCHEMICAL RESEARCH ON *ORIGANUM VULGARE* L. (LAMIACEAE) FROM ROMANIA

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ABSTRACT

The chemical composition of *Origanum vulgare* L. (Lamiaceae) from Romania was studied. In *Origani herba* harvested from two sites (Valea Drăganului – Cluj County and Tg. Jiu – Gorj County) the content of essential oil, flavonoids, anthocyanins, phenylpropanoid compounds and tannins were performed. The TLC and GC-MS analysis of the essential oil showed the absence of phenolic compounds (thymol and carvacrol). In the essential oil native from Cluj County 40 components were identified whereas in that of Gorj County only 35. The main 7 components are similar (sabinene, α-pinene, δ^4-carene, trans-β-ocimene, β-caryophyllene, α-humulene, germacrene-D) and represents 71.84% of total amount in Cluj County, respectively 55.9% in Gorj County. In addition in *Origani herba* 0.58% essential oil, 1.36 flavonoids, 5.1% phenylpropanoid compounds, 46 mg/100 g anthocyanins and 4.56% tannins were determined. By mean of TLC, two major flavonoidal fractions and three phenylpropanoic compounds were emphasised whereas by HPLC 15 components were emphasised and the caffeic acid, γ-cumaric acid, luteolin and apigenin as hydrolysed product were identified.
Key words: *Origanum vulgare*, essential oil, phytochemistry, medicinal plant.

INTRODUCTION

Origanum vulgare L., is a perennial, herbaceous plant, the single wild spread species in Romanian flora, the common infraspecific taxa being *O. vulgare var. barcense* (Simk.) Hay [Flora RPR]. For medicinal purposes is used "*Origani herba*" with the following therapeutic indications: in the respiratory infections, expectorant in bronchitis and as cough-suppressing agent, spasmolytic, stimulant, tonic of CNS, antimicrobial and antifungal [4, 2]. In the chemical composition of *Origani herba* were found: essential oil (0.15–1%), phenylpropanoid compounds, flavonoids, tannins, triterpenic compounds [4]. In Romania *Origani herba* is valuated in *Antibronchitics* and *Sedative teas* (Plafar) and for preparation of *Galov* and *Sedocalm* (Plantavorel) tablets [7].

Our researches are focused on the following purposes: to establish the chemical composition of *Origanum vulgare* from Romania and especially for the essential oil and the content of phenolic compounds in this oil. These because the Romanian treatises and books show a high content of phenolic compounds (thymol, carvacrol) without any experimental research, the content being assumed from literature that are referring to the other countries, especially of Southern Europe. In contrast, our former research (1978) [21] showed 0–10% thymol, and more recently was found only 2.41% [18] and 26% [15]. Thus, according to Romanian authors, the content of phenolic compounds in essential oil of *Origanum vulgare* is 15% [5], 90% [11], 56-68% [4], 45-75% [9]. The flavonoids of *Origanum vulgare* from Romania were studied by Matei [14] and Antonescu [1]. According to Bruneton [2] in the research of the essential oil of *Origanum species*, for avoiding the confusions it is very important to know: the origin of plant material, the identity of species and of infraspecific taxa and the accurate scientific name.

In the last years in SE Europe were studied the different taxa of *Origanum* by Kokkini *et al*. (1994), Ravid and Putievski (1986), Danin *et al*. (1997), Droushions (2001), and Horvath (2001).

The most important species of *Origanum* used as medicinal drug or spice are the following:

- *O. vulgare* ssp. *viride* (Briss) Hayak (South of Italy);
- *O. heracleoticum* L. var. *carvacroliferum* (Greece, 50-70% phenols);
- *O. hirtum* from Yugoslavia, Greece, Creta (60-85% phenols);
- *O. creticum* (Greece) having the highest production as spice for pizza;
- *O. onites* L. (Turkey);
- *O. smirnaceum* L. (Turkey) – 25-45% carvacrol;
- *O. compactum* Benth. – 60-70% phenols;
- *O. maru* (Cyprus) – 70-80% thymol and carvacrol.

Other species belonging to another genera or family but having the same trading name of oregano may be *Coridothymus capitatus* (L.) Reich. (Lamiaceae) from Spain (67-79% phenols) and *Lipia graveolens* H.B.K. (Verbenaceae) from Mexico [2].

MATERIAL AND METHODS

Origani herba was harvested from Valea Drăganului (Cluj County) and Tg. Jiu (Gorj County) in July 2003, during blossom period and the material was dried at room temperature (humidity = 11.2%). The essential oil was extracted and isolated by water steam distillation in Neo-Clevenger apparatus (FR X).
The quantitative determination of the other active principles (flavonoids, anthocyanins, tannins and phenylpropanoids compounds) was made by spectrophotometer methods according to Romanian and European Pharmacopoeia). The qualitative analysis of the essential oil was performed by TLC and GS-MS [20]. The flavonoids and phenylpropanoids compounds were analysed by TLC on silica gel and microcrystalline cellulose, and by HPLC [22, 21].

For GS/MS analysis of essential oil the following apparatus and techniques were used [20]: HP 5890 Gas chromatograph with HP 5972 MSD mass detector; capillary column HP 5-MS with the stationary phase 95% methylpolysiloxan and 5% phenylpolysiloxan, of 30 m length with inner diameter 0.25 mm; thickness of the stationary phase was 0.25 μm. Helium was used as carrier gas, at the flow rate of ml/min (split ratio 1:1). Oven temperature was programmed from 60° to 240°C, at the rate of 3° C/min. The identification of the components was done by two means, by comparison of MS of each compound with the database (Wiley Library), and on the basis of comparison of the relative retention indices.

RESULTS AND DISCUSSIONS

Table I: The content in active principles of *Origani herba*.

Active principles	Content
Essential oil	0.58 ml/100 g
Flavonoids	1.36% (in rutoside)
Anthocyanins	46 mg/100 g (in cyaniding HCl)
Phenylpropanoids comp.	5.10% (in caffeic acid)
Tannins	4.56% (in pyrogalol)

According to the TLC and GC-MS analyses, the essential oil of *Origanum vulgare* gathered from Romania does not contain phenolic compounds (thymol or carvacrol). For comparative purposes, we analysed in addition by chromatographic methods the essential oil of *Thymus vulgaris* (*Aetheroleum Thymi*) and essential oil from commercial product "**Oregano**" (imported in Romania) respectively.

The TLC qualitative analysis showed that the thymol is present both in *Aetheroleum Thymi* and essential oil of *Oregano* (Rf = 0.80, red color).

The gas chromatography analyses highlighted the thymol absence in essential oil of *Origanum vulgare* harvested from two different sites (Cluj County and Gorj County) [Fig. 1, 2].

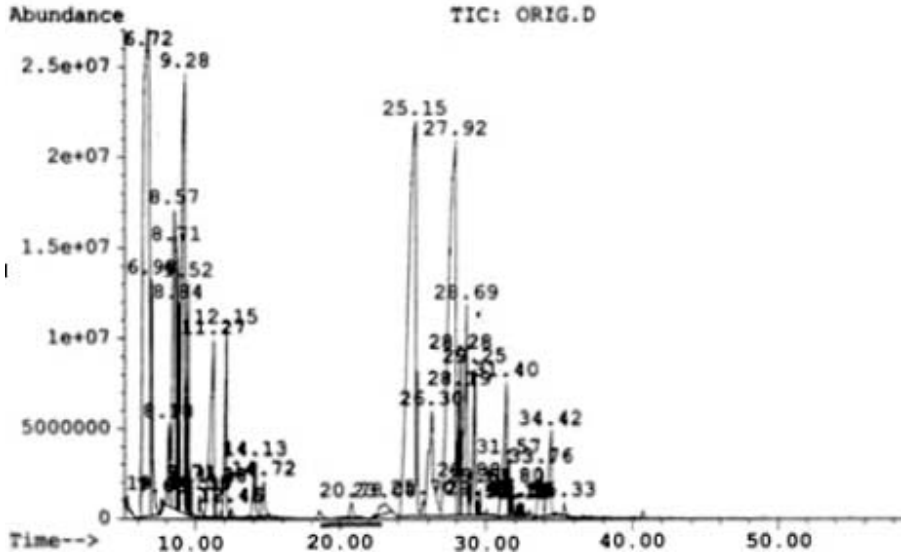

Fig. 1: Gas chromatogram of essential oil of *Origanum vulgare* (Cluj County).

The thymol is present only in essential oil of *Thymus vulgaris* at RT= 20.54, [Fig. 3] but in essential oil of *Oregano* together with thymol can be also found carvacrol with RT= 20.87-21.15 (closed to thymol RT) [Fig. 4].

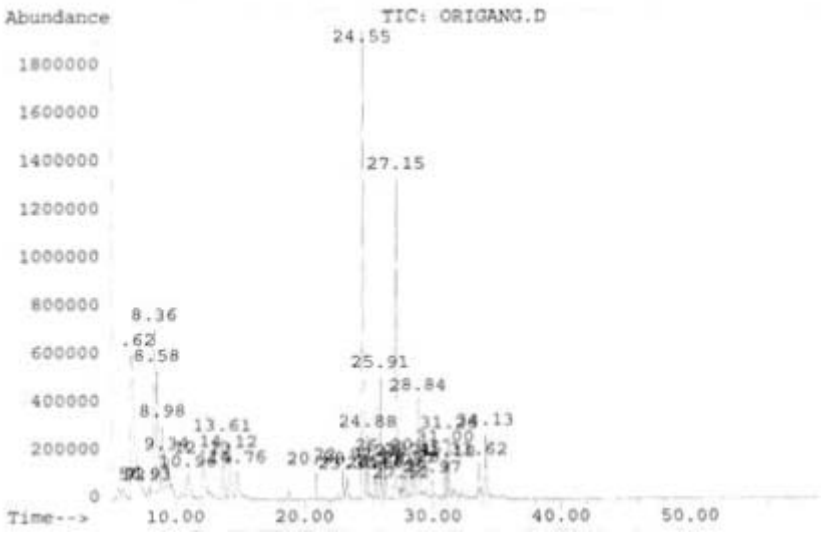

Fig. 2: Gas chromatogram of essential oil of *Origanum vulgare* from Gorj County.

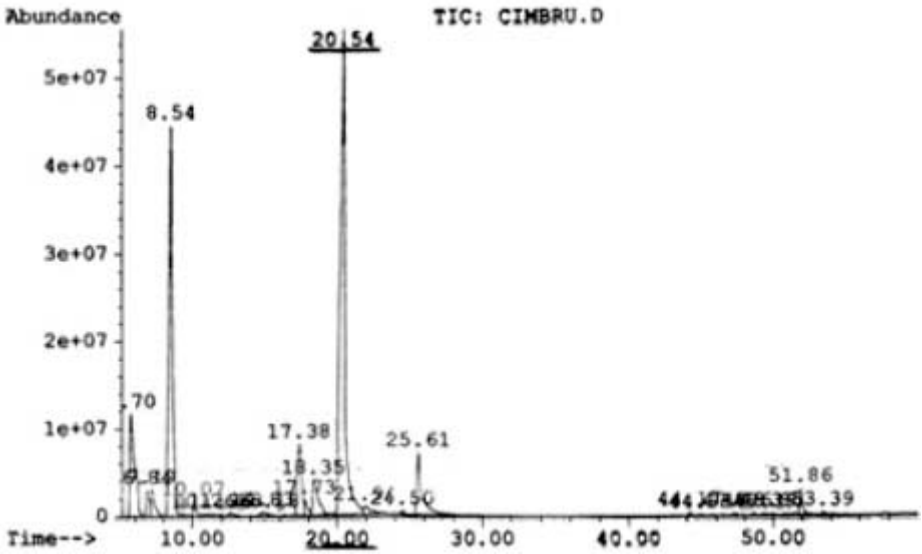

Fig. 3: Gas chromatogram of essential oil of *Thymus vulgaris* (*Aethroleum Thymi*).

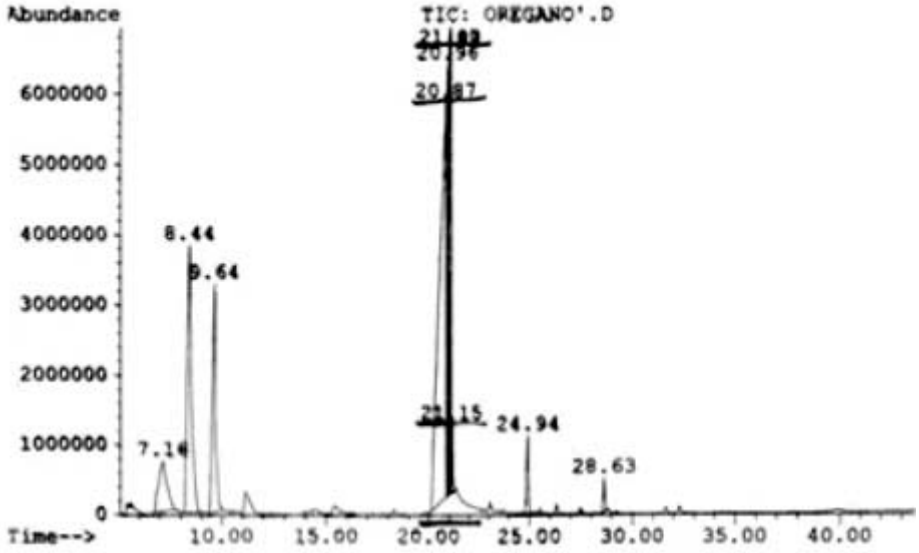

Fig. 4: Gas chromatogram of essential oil of "Oregano".

Thymol and carvacrol has been identified by mass spectrometry in two samples of essential oil (*Aetheroleum Thymi* and *Aetheroleum Oregani*) [Fig. 5].

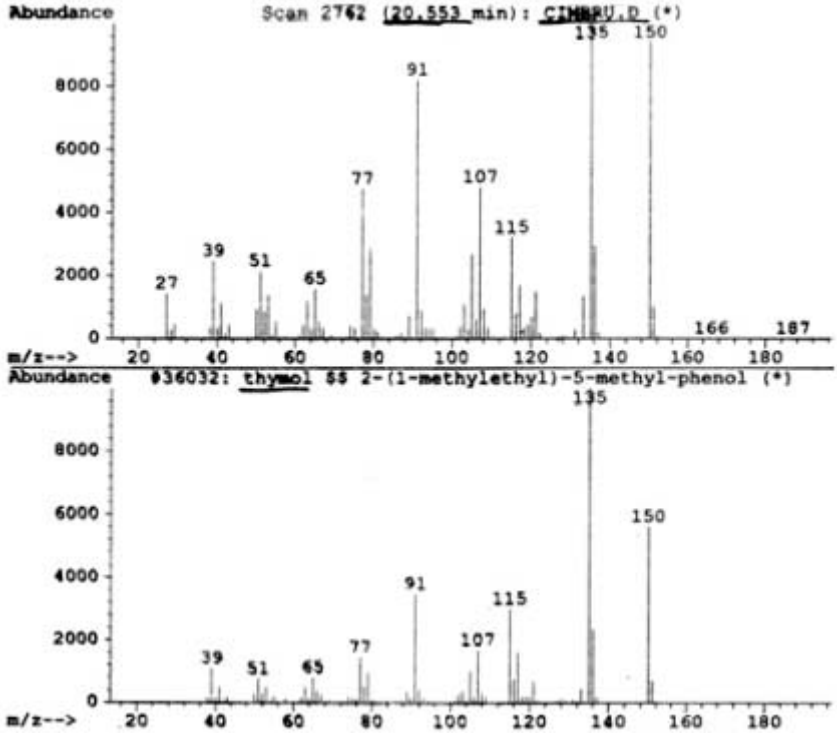

Fig. 5: MS of component with RT=20.553 from the essential oil of *Thymus vulgaris* (up) and of thymol (below).

In the essential oil of *O. vulgare* from Cluj County were emphasized and identified 40 components whereas in that of Gorj County 35 components were emphasized and identified, the main component being presented in Table II.

Table II: The main components from essential oil of *O. vulgare* identified by GC-MS.

RT	Components	Content, %	
		Cluj County sample	Gorj County sample
6.72	sabinene	18.13	14.18
6.98	myrcene	2.14	-
8.36	1,8-cineol	-	9.16
8.57	α-pinene	5.83	0.82
8.71	δ^4-carene	2.26	6.30
9.29	trans-β-ocimene	8.97	3.86
11.27	linalool	3.63	-
25.15	β-caryophyllene	17.81	17.12
26.30	α-humulene	3.21	3.25
27.91	germacrene D	15.63	10.37
28.69	α-farnesene	3.25	-
28.84	cadinene	-	3.33
31.00	1,6-germacradien-5-ol	-	1.23
31.40	germacrene-4-ol	2.56	-
34.13	α-cadinol	-	2.16

There it could be appreciated that the essential oils of *Origanum vulgare* from these two sites have the same main compounds: 71.84% (in Cluj) and 55.9% (in Gorj) respectively from total. The seven main components are the same: sabinene, α-pinene, δ^4-carene, trans-β-ocimene, β-caryophyllene, α-humulene and germacrene D.

In the essential oil of *O. vulgare* from Gorj County there appear different compounds such as 1,8-cineol (9.16%), cadinene (3.33%), α-cadinol (2.16%) and 1,6-germacradien-5-ol (1.23%). The compounds that can be found only in essential oil from Cluj County are myrcene (2.14%), linalool (3.63%), α-farnesene (3.25%) and germacren-4-ol (2.56%).

The qualitative TLC analysis of flavonoids showed that in *O. vulgare* 2 glycosides and 3 phenylpropanoic compounds were recorded, but these are different from rutoside, hyperoside and chlorogenic acid. Only the caffeic acid was identified by this technique [Fig. 6a]. By two-dimensional TLC on microcrystalline cellulose two flavonoid glycosides (yellow spots), two anthocyanosides (in red) and many phenylpropanoic compounds (in green-blue) were found.

The preparative TLC allowed the separation of flavonoids from phenylpropanoic compounds [Fig. 6b]. The major hydrolysis products were identified as luteoline along the apigenin but the quercetol and kaempherol are absent. The hydrolysis of whole extract gave a great number of phenylpropanoic compounds (in green-blue) [Fig. 6c].

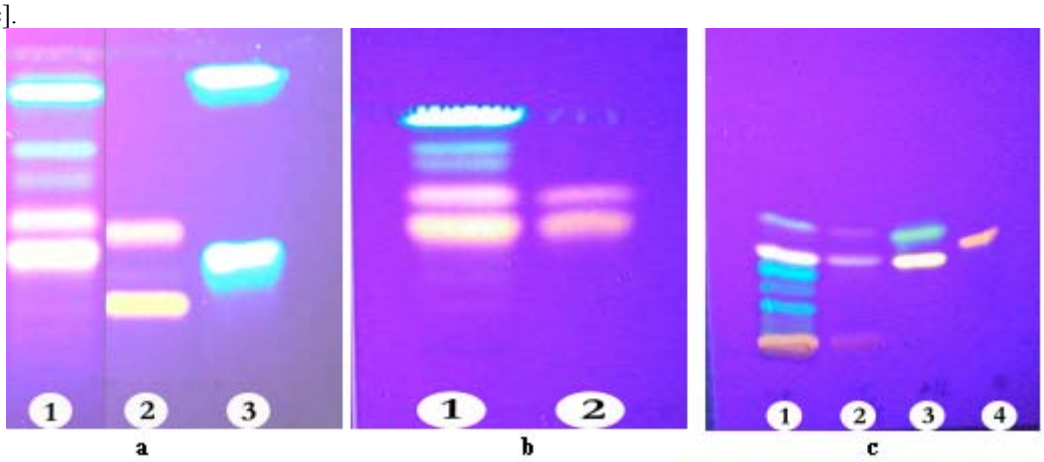

Fig. 6 a: TLC of whole extract of *O. vulgare* (1) hyperosid and rutoside (2), chlorogenic and caffeic acid (3); **b:** TLC of whole extract (1) and purified flavonoid (2) of *Origanum vulgare*; **c**: TLC of hydrolyzed products of *O. vulgare* extract (1), purified flavonoid (2), apigenin and luteolin (3) and quercetol (4).

The HPLC of whole metanolic extract showed 15 components and 16 in the hydrolised sample. We have compared the RT of separated components with 19 standard substances.

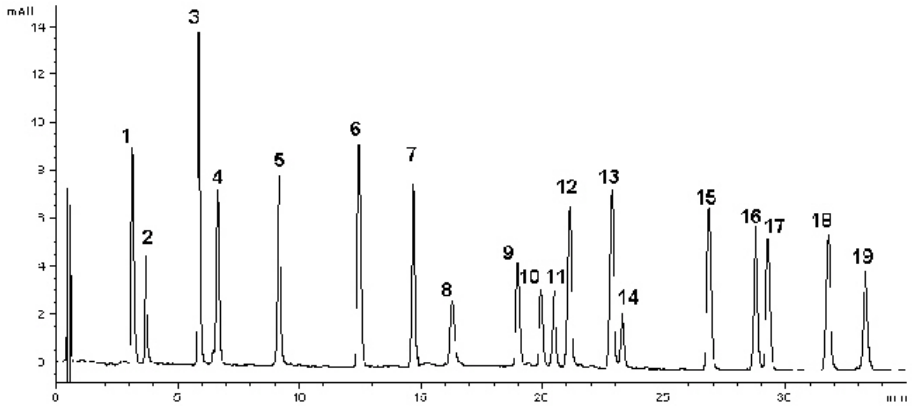

Fig. 7: HPLC for standard substances (see Tab. III).

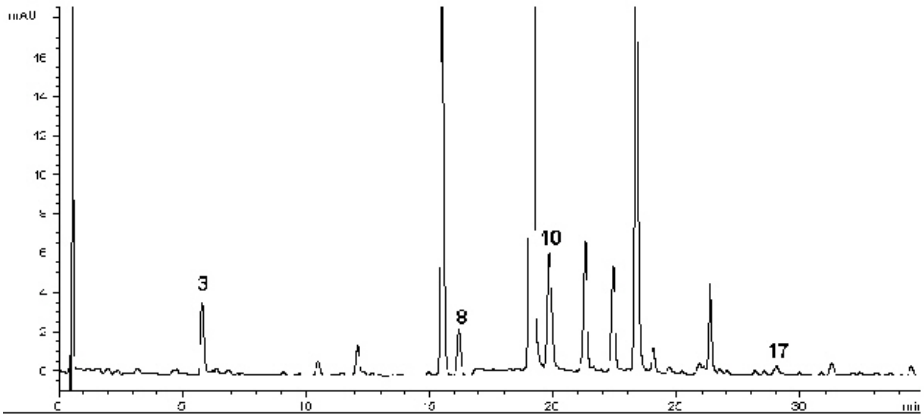

Fig. 8: HPLC of hydrolysed products of *Origanum vulgare* (see Tab. III).

Table III: RT of standard substances and hydrolytic product identified in *Origanum vulgare*.

		Whole extract	
RT	Component	Area	Conc. (µg/ml)
3.13	Caftaric acid		0.039
3.67	Gentisic acid		0.023
5.85	Caffeic acid	31.700	0.713
6.63	Clorogenic acid		0.050
9.15	p-cumaric acid		0.010
12.43	Ferulic acid		0.026
14.67	Sinapic acid		0.006
16.2	Cicoric acid	21.600	0.485
18.96	Hyperozid		-0.006
19.90	Isoquercitrin	42.000	3.257
20.40	Rutozid		-0.017
21.10	Miricetol		-0.010
22.80	Fisetin		-0.017
23.26	Quercitrin		-0.004
26.82	Quercetol		0.032
28.74	Patuletin		0.014
29.24	Luteolin	5.200	0.206
31.73	Kaempferol		0.042
33.24	Apigenin		0.045

The caffeic acid, cichoric acid, isoquercitrine and traces of free luteoline were identified in whole extract. In the hydrolysed extract the caffeic acid, p-cumaric acid, luteoline and apigenine were identified.

CONCLUSIONS

In the phytochemical studies on *Origanum vulgare* from wild flora of Romania (two stations),
-we determined the content of essential oil, flavonoids, anthocyanins, phenylpropanoic compounds and tannins;
-a qualitative analysis of essential oil and flavonoids was performed;
-by TLC, GC/MS analyses we found that in essential oil of *O. vulgare* gathered from Cluj and Gorj counties, the phenolic compounds (thymol or carvacrol) are absent;
-in the essential oil of both samples of *O. vulgare* the main components are sabinene, α-pinene, Δ-4-carene, trans-β-ocimene, β-caryophyllene, α-humulene and germacrene D, but there are a few qualitative and quantitative differences;
-*Origani herba* from Romania contains 1,36% flavonoids, 46 mg% anthocyanosides, 0,47-0,58% essential oil, 5,10% phenylpropanoic compounds and 4,56% tannins.

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[L-06]

[L-07]

THE CHANGES IN APIGENIN, LUTEOLIN AND THEIR -7-O-GLUCOSIDES CONTENT DURING *HERBA MILLEFOLII (ACHILLEA COLLINA* BECKER EX. RCHB. VAR. ´ALBA´) ONTHOGENESIS

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ABSTRACT

The spasmolytic and antioxidant activity of *Achillea collina* Becker ex. Rchb. var.´Alba´ (yarrow) was attributed to flavonoids. The screening of flavonoids content in ten developmental stages of yarrow flowering top *(Herba millefolii*) is the aim of this paper. The methanol extract of plant material (tops in stages from the beginning of flower bud differentiation, through full flowering, until the stage of ripe seeds) was used for HPLC analysis with column Nova-Pak C_{18} and acetonitrile/0,1% TFA as the mobile phase with gradient program. Apigenin, luteolin and their 7-O-glucosides were found as the main flavonoid constituents in all developmental stages of drug. Luteolin, apigenin and apigenin-7-O-glucosid amount have a similar trend during onthogenesis - their content is fluently increased until full flower phase and after than turns down. The maximal amount of these flavonoids reached about 600 $\mu g.g^{-1}$ of dry material. The luteolin-O-glucosid on the other hand has the highest content at the stage of small flower bud - it is more than 900 $\mu g.g^{-1}$ of dry material. The curve of luteolin-O-glucosid amount decrease later on and second top is reached together with the others flavonoids, at the full flower phase on the level 600 $\mu g.g^{-1}$of dry material.
Key words: *Achillea* L., flavonoids, apigenin, luteolin, HPLC.

INTRODUCTION

Yarrow (*Achillea* L.) is one of most asked stocks of pharmaceutics, cosmetic and food industry. On human organism it has influence as antispasmodic, amarum, stomachic, carminative and cholagogum. The main effective substance of yarrow drug is essential oil but antispasmodic activity is due flavonoids content. Antioxidant activity of flavonoids is also indisputable (Rice-Evans *et al*. 1997; Bors *et al*. 1990; Pulido *et al*. 2000, etc.). Their presence and efficiency in some *Achillea* L. species have been demonstrate already in 1961 (Hörhammer) and later on confirmed by many authors (Valant-Vetschera 1987; Schultz, Albroscheit 1988, etc.).

Problematic of yarrow flavonoids content was already discussed in literature several times but information about flavonoids of *Achillea collina* Becker ex. Rchb. var. *´Alba´*, one of the most pharmaceutical effective taxa of *Achillea* genus, is currently missing. It is commonly known that above all flavons (luteolin, apigenin and their derivatives) are present in yarrow plants. Informations about their amount are unfortunately very differ from each other (Chandler *et al*. 1982; Franzén 1988) and dynamics of their production during plants ontogenesis was not solved until now at all. The very fact, exact information about effective compounds producing, is important for producers and processing industry to optimise harvesting period of pharmaceutic drugs. The aim of this work is to carry out primary study of flavons content variability in *Achillea collina* Becker ex. Rchb. var ´Alba´ tops during plant ontogenesis.

MATERIAL AND METHODS

Plant material: The *Achillea collina* Rchb. ´Alba´ plants cultivated at Faculty of Horticulture of Mendel University of Agriculture and Forestry in Lednice were used in this experiment. The flowering tops with stems about 30 cm long - "*herba millefolii*" - were harvested from 3 years old overgrowth, dried up under laboratory temperature conditions and ground with a laboratory grinder according Czech Pharmacopoeia (ČsL 1987) into fine parts. The content of flavonoids was determined in the following 10 developmental stages of plants (Fig.1): I. beginning of flower bud differentiation; II.flower buds are partly developed, green, flower top is covered by compact hairs; III. flower buds developed, green; ray and tubular flowers both undistinguished, hidden in buds; IV. flower buds developed; ray flowers start to jet out from the buds, tubular flowers still hidden in buds; V. inflorescences partly developed; tubular and ray flowers flowering at <50% of inflorescences; VI. inflorescences partly developed; tubular and ray flowers flowering at >50% of inflorescences; VII. fully developed inflorescences; tubular and ray flowers flowering at 100% of inflorescences; VIII. shedding of blossoms; tubular and ray flowers getting gradually brown; leaves remain still green; IX. inflorescences almost brown; seed formation; leaves decreasing, getting brown; inflorescences and leaves completely brown and almost dry; ripe seeds.

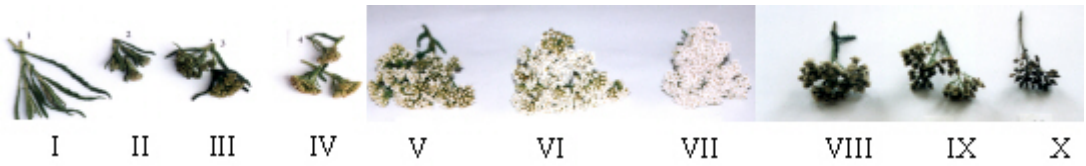

Figure 1. Ten different stages of plants development

Extraction: Flavonoids content was established in their glycoside forms and therefore non-hydrolysed plant extracts was prepared: 0,25 g pulverised drug was extracted in ultrasonic bath in 1,875 ml 80% MeOH during 15 min and extract was filtrated by 0,2 µm PTFE filter.

HPLC analysis: MeOH flavonoids extract was analysed by HPLC system Waters 600E with reverse phase column Nova-Pak C_{18} (3,9 x 150 mm, 4 µm) (Waters chromatography, Milford, MA). Mobile phase acetonitrile - 0,1% TFA (trifluoracetic acid) was used for flavonoids separation in the column together with gradient program: linear 5-25% /30 min, 25-30% / 5 min a 30-50% / 2 min, and next 3 min washing with 50% acetonitrile in 0,1% TFA) at flow rate 1,0 $ml.min^{-1}$. Flavonoid compounds spectra coming throw the column was on-line screened by PAD detector Waters TM996 in the wavelength range 190-900 nm. Peak purity, identification, integration and calibration of flavonoids were proved by software Waters Millenium^{32} version 3.05.01 and commercial available flavonoid standards (Apin Chemicals, Ltd., Abingdon, Oxon, UK).

Recalculation per dry matter: All data about flavonoid content in yarrow drug are recalculated per plant dry matter. Plant material (air-dried under laboratory conditions) is heated in an oven at 105ºC for 4 hours – after this manipulation the weight of plant mass remains constant (to ČsL 1987). This dry matter makes about 80 – 90% of air-dried material.

Statistical evaluation: Each measurement was made in two parallel replications and one-factor analysis of variance at the significance level $\alpha = 0,05$ was used for statistical evaluation of data.

RESULTS AND DISCUSSION

Results obtained by HPLC analysis of *Achillea collina* Rchb. ´Alba´ have confirmed that flavones and their derivatives are the major flavonoids in yarrow drug. Flavones apigenin, luteolin (Fig. 2) and their –7-O-glucosides are on HPLC chromatograms visible as ones of the highest peaks (Fig. 3).

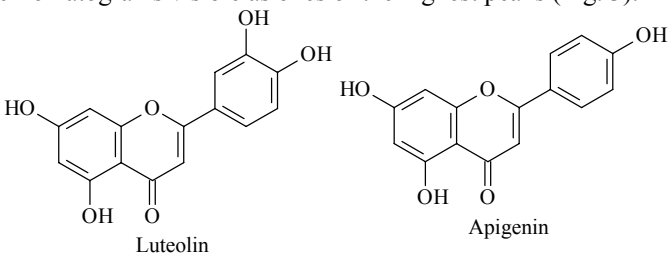

Figure 2. Structural formulas of luteolin and apigenin

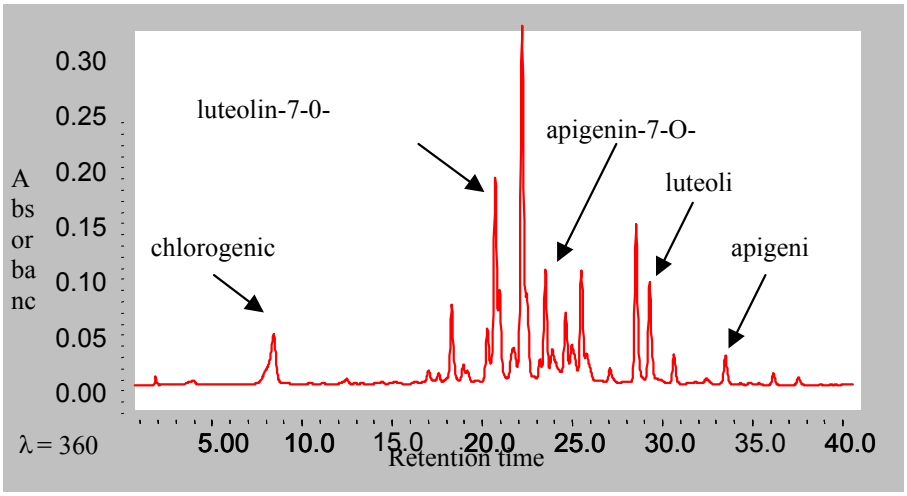

Figure 3. Typical HPLC chromatogram of MeOH extract of yarrow

From the viewpoint of these major flavonoids producing dynamics is interesting that flavonoids are by the plants generally synthesised very similar way as the essential oils (Ćerenaj *et al*. 1983) – flavonoids amount is from the stage of flowers differentiation beginning continuously increasing until the stage of full flowering, where is maximal flavonoids content reached. This maximal content value is about 0,6 – 0,7 $mg.g^{-1}$ of dry plant material in *millefolii herba* (Tab. 1.).

Table 1. Maximal content of apigenin, luteolin and their -7-O-glucosides in *Achillea collina* Rchb. ´Alba´

Flavonoid	Maximal content ($mg.g^{-1}$)	In develop. stage
luteolin-7-O-glucosid	0,923 ± 0,001	II
luteolin	0,613 ± 0,054	VII
apigenin-7-O-glucosid	0,656 ± 0,043	VII
apigenin	0,601 ± 0,049	VII

During fade away and seed ripening the flavonoids content is decreased. There is only one exception in flavonoids, which this experiment had been focused on – it is luteolin-7-O-glucosid what reached absolute content maximum at the beginning of flower differentiation. In full flowering phase luteolin-7-O-glucosid content has the second maximum (0,715 $mg.g^{-1}$) but it does not achieved first one (0,923 $mg.g^{-1}$) (Fig. 4). All four evaluated flavonoids showed in their content between individual yarrow developmental stages statistically significant different.

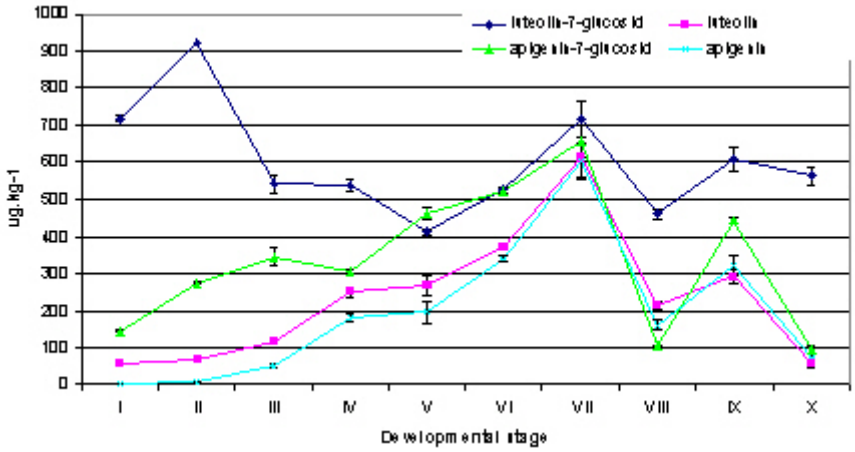

Figure 4. The content of major flavones in *Achillea collina* Rchb. ´Alba´ flowering tops

In comparison of simple and derive flavonoid forms ratio it was find out that apigenin and apigenin–7-O-glucoside are very well balanced whereas luteolin-7-O-glucoside content prevail over luteolin. This different is stronger mainly at flowering beginning and seed ripening period. However luteolin preference as an enzymatic flavone glycolysis substrate is not proved in literature.

Some tested yarrow drugs have contained also minority amount of rutine (quercetin-3-O-rutinosid). Rutine traces were found in yarrow HPLC chromatograms independent on developmental stage of plants. Presence of quercetine and kaempferol, which are sometimes also alluding to be found in yarrow according literature (Valant, 1978; Krenn, 1998), was not proved.

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[L-07]

[L-08]

A COMPARATIVE STUDY OF SOME VERONICA SPECIES

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ABSTRACT

We have tried to establish some criteria to avoid the substitution of *Veronica officinalis* (common speedwell) with other species of the *Veronica* genus, especially *Veronica chamaedrys* (germander speedwell), widely spread and without therapeutic action. We have studied the differential histological, anatomical and phytochemical characters, for the two species. A rapid method for the identification of the two species is the TLC for flavonoids and phenylpropanioide compounds. We have done also a HPLC study, which has permitted the detection of acteoside in *Veronica chamaedrys* and isoacteoside in *Veronica officinalis*. In order to confirm the supposed hypocholesterolaemic effect of *Veronica officinalis* (used in ethnopharmacy like a hypocholesterolaemic agent) we have done an experiment in two steps for the two species of *Veronica*: *Veronica officinalis* and *Veronica chamaedrys*. *Veronica officinalis* in the diet showed no significant effect on the levels of cholesterol and triglycerides in the serum of the cholesterol free diet animals. *Veronica officinalis* had also a lowering effect on triglycerides and cholesterol level in the serum of high cholesterol diet animals.
Key words: *Veronica officinalis*, *Veronica chamaedrys*, hypocholesterolaemic effect, flavonoids, phenyl-propanoide compounds.

INTRODUCTION

The *Veronica* genus belongs to the *Scrophulariaceae* family and includes 41 species in the Romanian flora (6). It is different from the others genus of the family by the number of stamens (only two) and the number of petals (four) with irregular symmetry. From the 41 species we can remark with demonstrate phytotherapeutic activity *Veronica officinalis* (common speedwell) (3). *Veronica officinalis* is a low, perennial herb, up to 20 cm in height, with hairy, opposite leaves and pale blue flowers, in erect, axillaries racemes. The fruits are flats hearts shaped capsules (4).

Veronica officinalis has a specific areal (in lightwoods, especially in the mountain) and it is often substituted with another species of *Veronica* genus especially with *Veronica chamaedrys*, widely spread and without therapeutic effects.

MATERIAL AND METHODS

The comparative morpho-anatomical study was done by scanning and optic microscopy. For the powdered medicinal products, the differentiation between the two species can be evaluated by TLC, for flavonoids and phenylpropanoide compounds in the following conditions (5):
Test solution: to 0.5 g of the powdered drug in a 25 ml flask add 5 ml methanol and heat on a steam bath for 10 minutes; after cooling filter and complete at 5 ml with methanol.
Reference solution: dissolve 1 mg acteoside, 3 mg hyperoside, 3 mg rutoside, 1 mg caffeic acid, and 1 mg chlorogenic acid in 10 ml methanol.
Stationary phase: silica gel GF 254 Merck.
Mobile phase: acetic acid R: formic acid R: water R: ethyl acetate R=7:7:14:72.

After drying the plate should be sprayed with 1% diphenylboryloxyethylamin R solution in methanol R. The chromatogram should be examined at 365 nm after 10 minutes.

To confirm the TLC, for the phenyl-propanoide compounds, a HPLC was done in the following conditions (2).
Test solution: to 1.00 g of the powdered drug in a 25 ml flask add 10 ml of 70 per cent V/V solution of alcohol R and shake for 30 minutes. Filter and rinse flask and filter with two 5-ml portions of 70 per cent V/V solution of alcohol R. Evaporate the combined filtrates to dryness *in vacuo*. Dissolve the residue in 25 ml of 70 per cent V/V solution of methanol R.
Reference solution: dissolve 1.0 mg of acteoside R in 2.0 ml of methanol R.
Column: material: stainless steel,
Stationary phase: octadecylsilyl silica gel for chromatography R (5 µm)

Size: L=0.25m; diameter=4.6 mm
Temperature: 20 ^{o} C
Mobile phase: phosphoric acid R: acetonitrile R: water R= 0.07:18.0:72.0
Flow rate: 1.5 ml/min
Type: isocratic
Detector: spectrophotometer at 340 nm
Injection volume: 20 µl.

In order to confirm the supposed hypocholesterolaemic effect of *Veronica officinalis* – used in ethnopharmacy like a hypocholesterolaemic agent – we have done an experiment in two steps for the two species of Veronica: *Veronica officinalis* and *Veronica chamaedrys* (1). In the first step we have studied comparatively the effect of infusion, extract and powered plant suspension to the level of serum cholesterol and triglycerides to the cholesterol free diet animals. In the second experiment the animals were fed with a high cholesterol diet (1.4 g lard/day/animal) in order to induce hypercholesterolemia, and we also give to the animals the powered plant (0.14 g /day/animal). In both experiments we used adult male Wistar rats (approximately 200 g) for 4 weeks.

RESULTS AND DISCUSSION

After the scanning microscopy study we can determinate the longitudinal diameter of stomata, the length of non-glandular trichomes and their diameter at the base and on the top. The results are shown in table 1. By optic microscopy we can determinate the palisade ratio, the stomata index and the vascular islands. The results of this study are shown in table 2. The images obtained by scanning microscopy are shown in Fig. 1 for *V. officinalis* and in Fig. 2 for *V. chamaedrys*.

Table 1. Biometrics data obtained by scanning microscopy

Characters / Species	*Veronica officinalis*	*Veronica chamaedrys*
Longitudinal diameter of stomata	16.8 µm	39.3 µm
The length of non-glandular trichomes	591µm	572 µm
Trichomes diameter at the base and on the top	61.7 µm (base) 39 µm (top)	26.8 µm (base) 10 µm (top)

Table 2. Biometrics data obtained by optical microscopy

Characters / Species	*Veronica officinalis*	*Veronica chamaedrys*
Stomata index	58	70
Vascular island	3,5	5
Palisade ratio	10.75	20

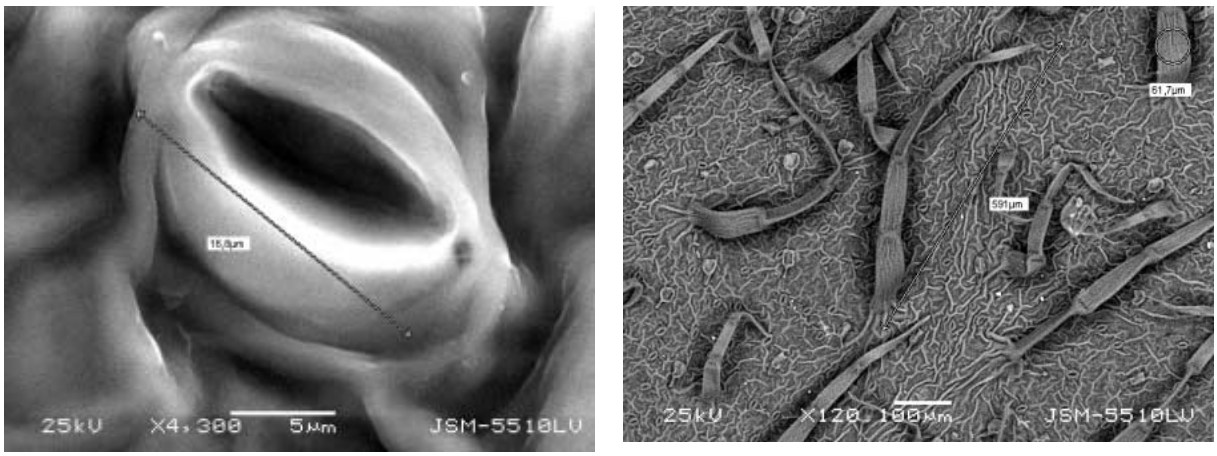


Figure 1: Stomata and protector trichomes of inferior epidermis of *Veronica officinalis*

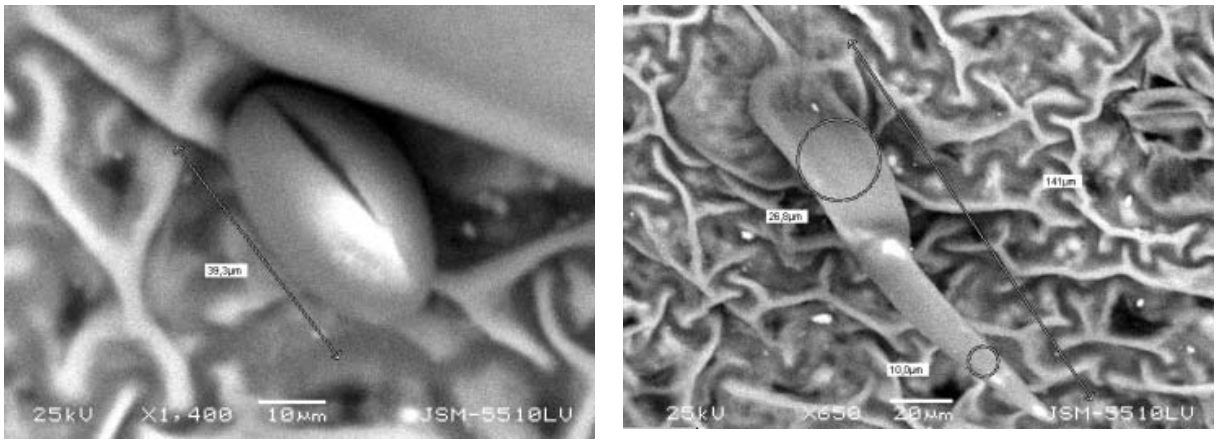
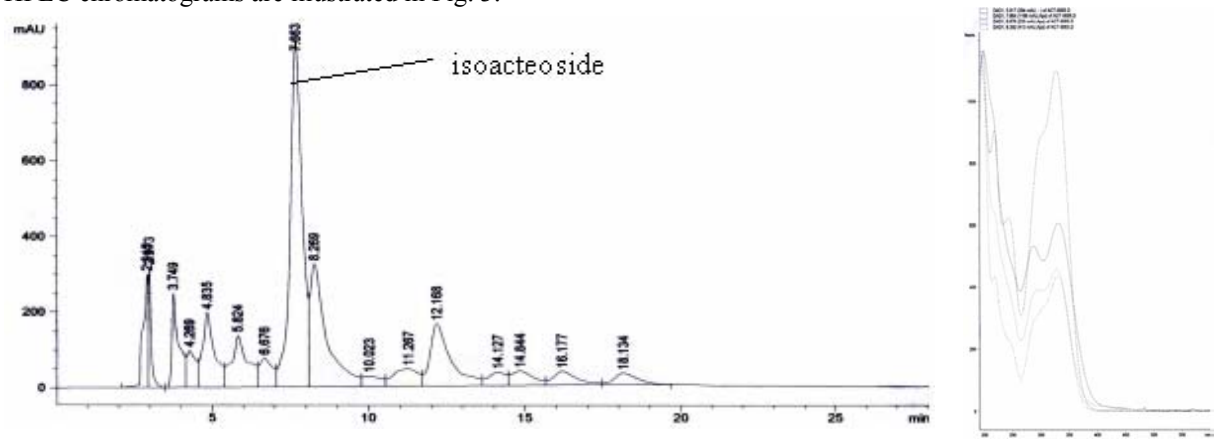
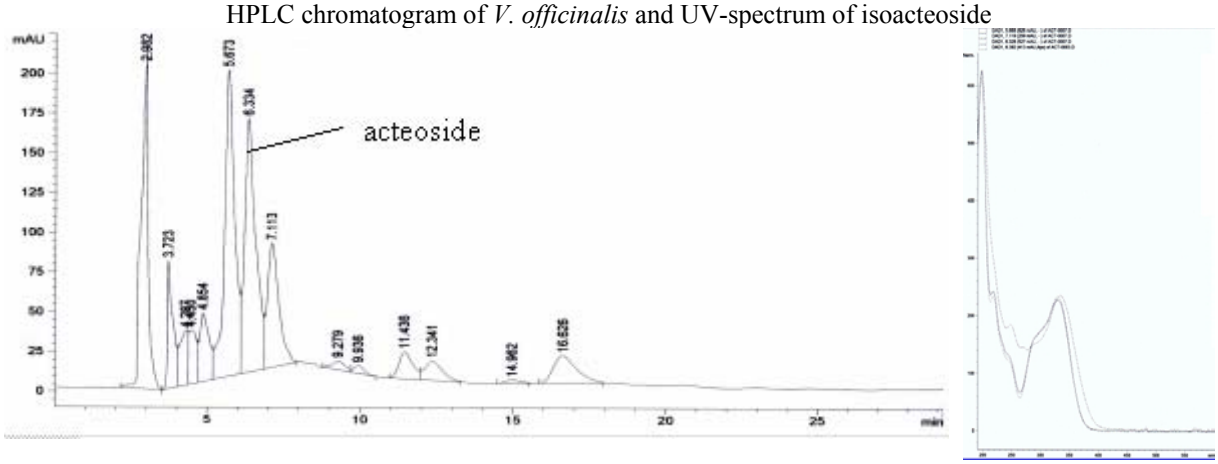


Figure 2. Stomata and protector trichomes of inferior epidermis of *Veronca chamaedrys*

The TLC for flavonoids and phenylpropanoide compounds is the easiest way to differentiate the two species of *Veronica* because *V. chamaedrys* does not show the orange spots of flavonoids (Rf=0.36; and Rf=0.58) and they appear in the *V. officinalis*; acteoside (blue spot, Rf=0.65) is present in *V. chamaedys* and absent in *V. officinalis*; caffeic acid (Rf=0.95) is present in *V. officinalis* and absent in *V. chamaedrys*.
The HPLC study has shown the presence of acteoside in *V. chamaedrys* and isoacteoside in *V. officinalis*. The HPLC chromatograms are illustrated in Fig. 3.



HPLC chromatogram of *V. officinalis* and UV-spectrum of isoacteoside



HPLC chromatogram of *V. chamaedrys* and UV-spectrum of acteoside

Fig. 3: HPLC of *Valeriana officinalis* and *Valeriana chamaedrys.*

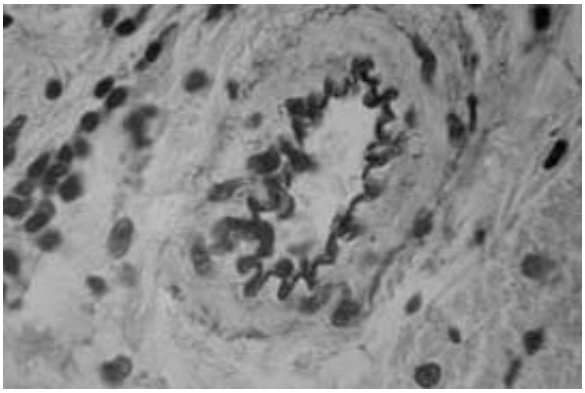
After the morphological and chemical studies we have done a pharmacological experiment to compare the hypocholesterolaemic effect of *Veronica officinalis* and *V. chamaedrys,* because in ethnopharmacy is used like a hypocholesterolaemic agent only *V. officinalis* and it is often substituted with *V. chamaedrys*. After the four weeks of the second step of the experiment in which we have fed the rats with lard we obtained the following results for the cholesterol and triglycerides serum level (Table 3).

Table 3: Cholesterol and triglycerides serum levels for the fours groups for the second experiment and their statistic interpretation by t-Student test

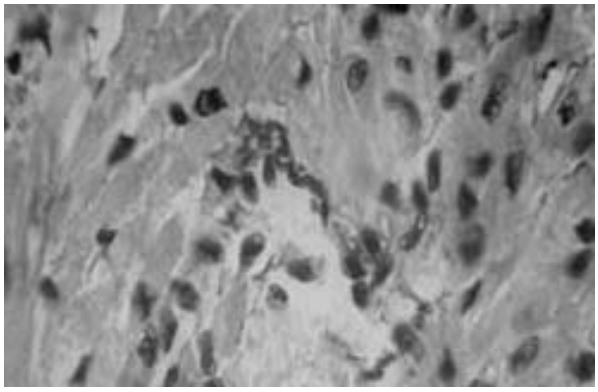
Groups \ *Parameters*		*means± SEM*	
		Cholesterol (mg/ml)	Triglycerides (mg/ml)
I	Control Group	281.444±74.208	46.000±14.733
II	Lard Group	357.556±69.938	77.333±21.251
III	Lard + V. officinalis	298.9±41.289	67.800±13.234
IV	Lard + V. chamaedrys	355.8±32.991	109.333±15.854

„t"Student Test		
Groups	*p (cholesterol)*	*p (triglycerides)*
I-II	0.239281	0.255753
I-III	0.782227	**0.010943**
I-IV	0.232402	0.287524
II-III	0.32383	0.244993
II-IV	0.975109	0.701857
III-IV	0.320757	0.058814

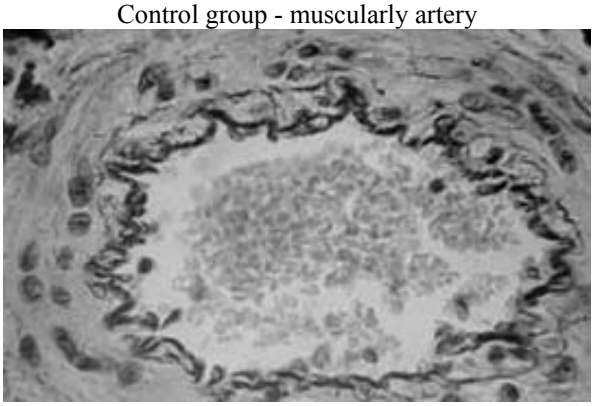
The "T Student" test shows statistic significance only when we compare the first and the third group. After the determination of serum cholesterol and triglycerides for the retro orbital artery, the rats were killed and we analysed by optic microscopy the components of walls of coronary artery for the fours groups of the experiment and we obtained the following results (Fig. 4).



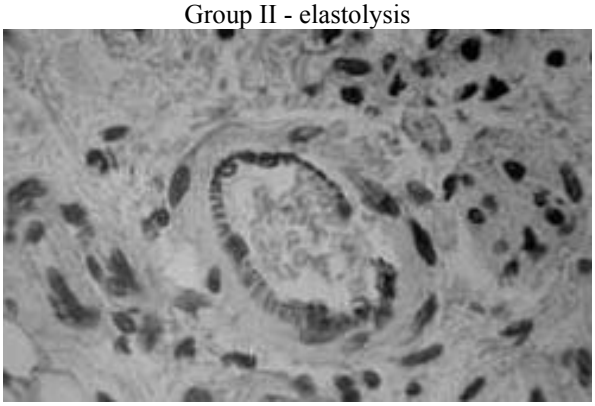
Control group - muscularly artery



Group II - elastolysis



Group III – muscularly artery



Group IV – elastolysis

Fig. 4: The artery wall for the four groups of the experiment

CONCLUSIONS

- A rapid and easy method for the identification of the two species of *Veronica* is the TLC for flavonoids and phenylpropanoide compounds.
- We have done also a HPLC study, which has permitted the detection of acteoside in *Veronica chamaedrys* and isoacteoside in *Veronica officinalis*.
- *Veronica officinalis* in the diet showed no significant effect on the levels of cholesterol and triglycerides in the serum of the cholesterol free diet animals.
- *Veronica officinalis* also had a lowering effect on triglycerides and cholesterol level in the serum of high cholesterol diet animals.

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[L-08]

[L-10]

STUDIES ON *SCOPOLIA CARNIOLICA* JACQ. FROM THE SPONTANEOUS FLORA AND *IN VITRO* CULTURES

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ABSTRACT

Scopolia carniolica Jacq. (*Solanaceae*) is a herbaceous perennial plant that grows at the basis of the forests in the Central and South-East Europe. In Romania it has few areals in the spontaneous flora and from some of them the plant is harvested in order to be used in the industrial extraction of scopolamine and atropine, alkaloids that are present in the rhizomes (*Scopoliae rhizoma*). Our study concerned two directions: the analysis of the vegetal mass harvested from the spontaneous flora and the study of possible *in vitro* cultivation for the protection of the environment from the excessive harvesting.

A phytochemical analysis of the medicinal product harvested in three different regions from the Romanian spontaneous flora was made. It was investigated for its alkaloid content by TLC-densitometry and titrimetry. The alkaloid content was established at 0.26-0.39% (g/g d.w.), the atropine and scopolamine being in 1: 0.43 ratio. The study of *in vitro* cultures concerned the possibility of cultivation, the optimal culture medium and conditions for the plant growth and the alkaloid biosynthesis. Cultures of regenerated plants, calus and adventitious roots were obtained and investigated; only roots, adventitious or from plantlets, could synthesise tropane alkaloids. The atropine and scopolamine content was determined by HPLC. The adventitious root cultures developed in Gamborg medium could produce higher levels of alkaloids (atropine and scopolamine) than the other types of culture; the biosynthesis increased in the presence of putrescine. The atropine synthesis was favorised by the light-dark exposure, while the scopolamine one was favorised by the dark exposure.
Key words: *Scopolia carniolica*, atropine, scopolamine, root cultures.

INTRODUCTION

Scopolia carniolica Jacq., the scopola, is a perennial herbaceous plant from the *Solanaceae* family, spread in Central and South-East Europe. In Romania it grows in few areas at the basis of the Carpathian forests. In some of these areas, the rhizomes of the plant (*Scopoliae rhizoma*) are gathered in order to be used for the industrial extraction of atropine and scopolamine; sometimes the excessive gathering reduces dramatically the plant population in the region. The references in Romanian literature show no data about the alkaloid content in the medicinal product from the Romanian flora [5,12,13], that is why we have initiated the study of the rhizomes gathered from the spontaneous flora in three different regions.

Along with this analysis we have initiated *in vitro* cultures of *S. carniolica*, studying the possibility of *in vitro* cultivation and the favourable conditions both for the development of cultures and for the alkaloid biosynthesis. Cultures of regenerated plants, calus and adventitious roots were obtained [6,7]. Samples of calus, roots, stems and leaves from regenerated plants and also adventitious roots developed in liquid medium were analysed regarding the alkaloid biosynthesis. Among those, only root samples (adventitious or from plantlets) seemed to synthesise atropine and scopolamine, according to the fact that generally undifferentiated cell cultures do not produce these compounds efficiently because their synthesis is linked to root differentiation; therefore, root cultures are preferred [2,14,18]. The actual study was conducted on adventitious root cultures, aiming to establish the favourable medium and conditions for the alkaloid biosynthesis.

One of the strategies used for increasing the yield of the desired compounds is the exogenous supply of a biosynthetic precursor to the culture medium. In the case of tropane alkaloids, most of the experiments were performed using cell suspensions induced from various *Solanaceae* species; precursors of hyoscyamine and scopolamine were added in their culture medium [16,18]. Therefore, putrescine was used in our study as a precursor and the influence of its add in the culture medium towards the alkaloid biosynthesis was investigated.

MATERIAL AND METHODS

Plant material. The rhizomes were gathered from three different regions: Remeţi (Maramureş), August 1999 (sample I); Săcuieu (Cluj), may 2000 (sample II); Răstolița (Mureş), May 2001 (sample III). They were thoroughly cleaned, cut in discs, dried at room temperature and then powdered.

Root cultures. Adventitious root cultures of *S. carniolica* were established from rhizogenic callus grown on Murashige and Skoog (MS) [11] solid medium containing 1.0 mg l^{-1} NAA or 1.0 mg l^{-1} IBA [6]. The adventitious roots were maintained in MS and in Gamborg et al. [8] (B5) liquid media, both containing 3% sucrose and 1.0 mg l^{-1} IBA. The inoculum used was about 700 mg fresh weight (40 mg dry weight) per 50 ml of fresh medium, after which the flask was incubated on 100 rpm rotary shaker (in the dark regime (total dark), as well as in a light/dark regime (photoperiod regime: 16 h light (38 µmol/m^2/s) and 8 h dark)), at 25±1 ºC. (RMD: roots cultivated in MS medium, in total dark regime; RML: roots cultivated in MS medium, in dark/light regime; RBD: roots cultivated in B5 medium, in total dark regime; RBL: roots cultivated in B5 medium, in dark/light regime). The root cultures were subcultured at 2-week intervals. The analyses were performed on 14-culture day's samples. In addition, for other samples, cultivated on B5 medium in the same light or dark regime, putrescine was added in the culture fresh medium before autoclavation, in a concentration of 1 mM. The root samples were gathered and analysed (along with witness samples without putrescine) at 7, 14, 21 and 28 days after inoculation.

Alkaloids extraction and analysis.

TLC analysis. It was performed on dry mass (rhizomes and adventitious roots) for the detection of the alkaloids, according to Romanian Pharmacopoeia X [19] and Stahl [15] techniques: 0.6 g powder was shaken with H_2SO_4 0.05 M for 15 min, then filtered in a bolting funnel. 1.0 ml concentrate ammonium hydroxide were added and then shaken twice with 10 ml ether. The ether phase was filtered through a filter containing anhydrous sodium sulphate and evaporated at dryness. The residue was dissolved into 0.5 methanol. Pre-coated plates: kieselgel 60 G (Merck). Mobile phase: acetone:water:concentrate ammonia (90:7:3). Standards: atropine sulphate 0.450% (w/v) and scopolamine bromide 0.030% (w/v) in methanol. Detection: the layer was dried at 105^{o}C for 15 min then Dragendorff reagent [10] was pulverised. The chromatograms for the rhizomes were analysed by *densitometry means*: Desaga densitometer, reflexion technique at 510 nm wavelength.

Titrimetric analysis. It was performed for the quantitative determination of the total alkaloid content in the rhizomes, according to Ph. Eur. [20] technique: 10.0 g powdered plant material was thoroughly homogenised with 0.75 ml concentrate ammonia, 1.5 ml methanol and 4.5 ml ether. After 12 h it was extracted with ether in a Soxhlet device for 4 h. The extract was shaken twice with H_2SO_4 0.25 M. The pH of the aqueous solution was set at 9-10 with concentrate ammonia, then it was extracted with chloroform. The chloroform solution was dried and the rest was kept at 105^{o}C for 1 h, then dissolved into 5 ml chloroform. 20 ml H_2SO_4 0.01 M was added and the remaining acid titrated with NaOH 0.02 M.

HPLC analysis. Tropane alkaloids were extracted from dry mass (80ºC, 24 h) of adventitious roots. For the quantitative analysis of atropine and scopolamine, 0.1 g powder dry weight was shaken 10 min, in a Vortex-Genie 2 device, with 1.5 ml 1 M H_2SO_4. 0.5 ml concentrated ammonia was added and then shaken for 5 min with 10 ml $(C_2H_5)_2O$. The sample was centrifuged at 4500 rpm for 5 min. The residue was dissolved into 0.2 ml phosphate buffer (40 mM, pH = 2.3). 5 µl were injected in a HPLC HP Series system with Zorbax SB C18 column (3.0 mm i.d. ×100 mm). The mobile phase ((16:84 v/v) CH_3OH : KH_2PO_4 40 mM, pH = 2.3 with 85% H_3PO_4) was eluted with 1 ml/min flow rate at 45^{o}C. The alkaloids were detected using UV detector at 210 nm. The system was calibrated with standard atropine sulphate and scopolamine bromide over the concentration range 1 - 50 µg/ml.

RESULTS AND DISCUSSION

Atropine (Rf = 0.3) [19] and scopolamine (Rf = 0.8) [19] were identified by TLC in the rhizome samples gathered from the spontaneous flora, by the same Rf value as standards of atropine sulphate and scopolamine bromide. The atropine/scopolamine ratio was established by densitometry means at 1/0.43, so atropine was found in almost double amount compared to scopolamine. The total alkaloid content in the three samples was determined by titrimetric means and it is shown in Table 1.

Table 1: Results of titrimetric determination of total alkaloids

Sample	I	II	III
Total alkaloid content (g/g% w/v)	0.26	0.35	0.39

The differences between samples were considered to be due to the pedoclimatic differences and to the gathering period. Also, the first sample came from an area from which the rhizomes were gathered yet for several years, the sample consisting of young rhizomes. The total alkaloid amount found is comparable with the amount indicated in literature for the rhizome of *S. carniolica* [1,3,4,9,17].

For the *in vitro* cultures, the first study aimed to establish the influence of the culture medium and of the light conditions over the alkaloid (atropine and scopolamine) biosynthesis.

In adventitious root samples cultivated on MS medium atropine (Rf = 0.3, identical with standard) and atropamine (in the inferior half of the chromatogram [15]) were identified, while in the samples grown on B5 medium atropine, atropamine, tropine (on the start line [15]) in traces and scopolamine (Rf = 0.8, identical with standard) were identified. The amount of atropine and scopolamine was determined by HPLC (Fig. 1).

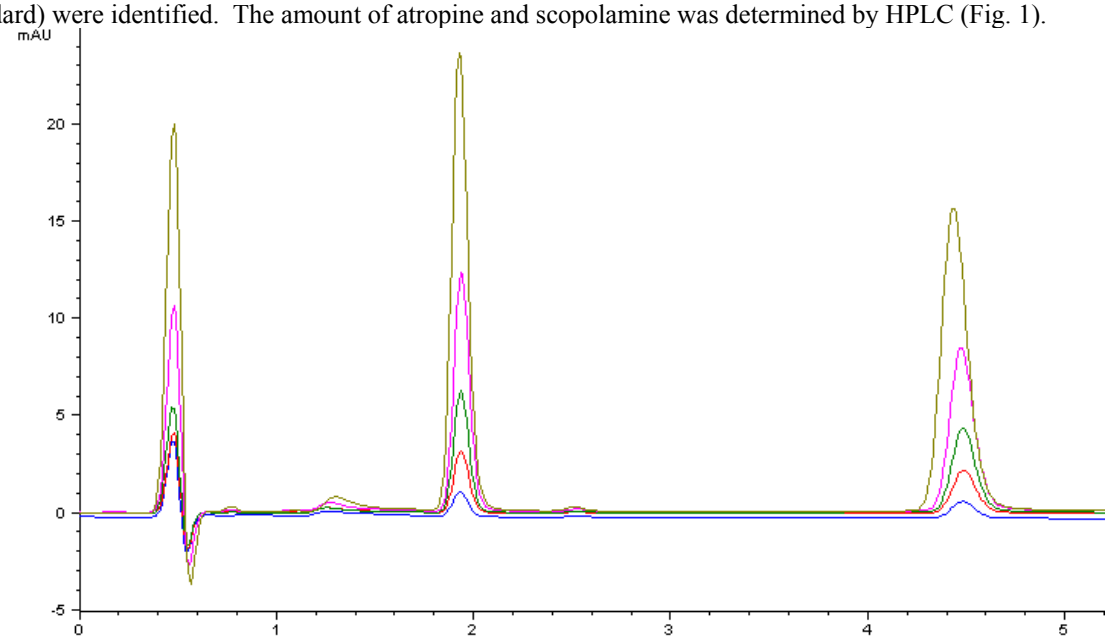

Figure 1: Standard calibration of the HPLC system: scopolamine bromide (Rt = 1.94 min) and atropine sulphate (Rt = 4.90 min)

The basal medium composition and light proved to have a major influence on alkaloid, (scopolamine and atropine) production (Fig. 2). Thus, comparatively with roots grown in MS medium, those in B5 medium accumulate a much larger scopolamine and atropine amount. This first experiment also showed that, after 14 culture days, the total amount of tropane alkaloids (scopolamine + atropine) accumulated in roots grown in the dark does not significantly differ from that accumulated in roots grown in a light/dark regime.

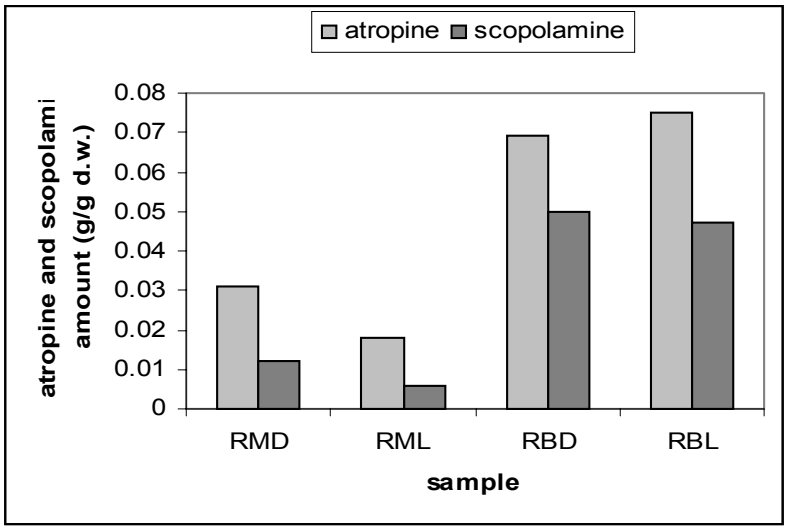

Figure 2: Atropine and scopolamine amount in samples cultivated in MS and B5 media

The influence of putrescine on the alkaloid biosynthesis was studied on samples grown only on B5 medium. TLC analysis identified atropine (Rf identical with standard) and scopolamine (Rf identical with standard) in all samples and also traces of atropamine (inferior half of the chromatogram) [15] and tropine (on start line) [15]. The amount of atropine and scopolamine increased in time from the 7th day up to the 28th one (Fig. 3). For the

dark exposed samples, the amount of atropine in both witness and putrescine samples increased up to day 14 (up to double quantity) followed by a plateau stage. The scopolamine amount in witness sample reached a plateau stage after day 21, while in putrescine sample it had a spectacular and constant increase (even seven times more scopolamine in day 28 compared to day 7). The addition of putrescine increased the amount of scopolamine more than twice compared to witness samples. For the light exposed samples, the amount of atropine slowly increased, reaching a plateau between days 14 and 21, followed by a decrease in witness samples. The scopolamine amount in the witness samples had the same increase-decrease aspect as atropine one, while in putrescine samples it constantly increased.

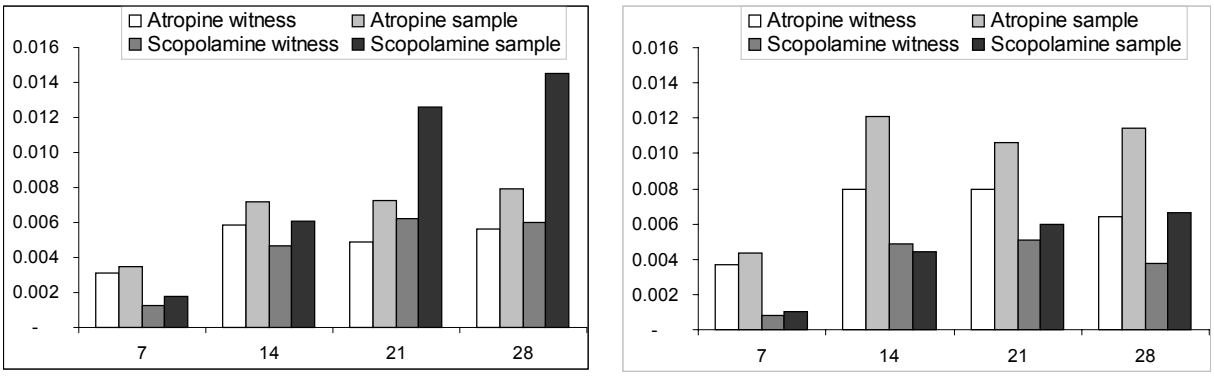

Figure 3: The amount of atropine and scopolamine (g/g% d.w.) in witness samples and in putrescine ones per days of culture. Left: dark exposure; right: light exposure

The amount of atropine was considerably higher compared to the scopolamine one in the light exposed samples, especially in those with putrescine. The amount of scopolamine, on the contrary, showed to by higher in the dark exposed samples, especially in those with putrescine. The prevalence of atropine in light exposed samples is similar to the one found in the product gathered from the spontaneous flora.

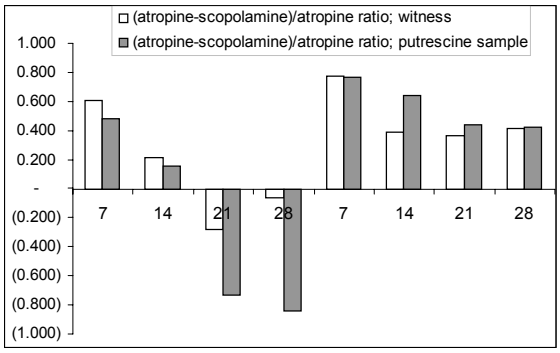

Figure 4: The variability of (atropine-scopolamine)/scopolamine ratio in time. Left: dark exposure; right: light exposure

We found interesting that in dark exposed samples, the constantly increasing amount of scopolamine overtook the amount of atropine, both in witness and putrescine sample, after day 21 (Fig. 4). We can say that the light conditions can influence the alkaloid biosynthesis, conducting mostly to atropine or to scopolamine.

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MORPHOGENETICAL AND BIOCHEMICAL STUDIES OF *MENTHA VIRIDIS* L. IN *IN VITRO* CULTURES

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ABSTRACT

The morphogenetic reaction of shoot nodes and tips was unusually uniform on numerous tested hormonal formulae in the respect of giving rise to neoplantlets. Only when we introduced 2.4-D (single or combined with citokinins) into the culture medium, the nodes, the fragments of internode, leave and root produced a friable, low proliferating, cream coloured callus tissue which does not differentiate and finally degenerates. Neoplantlet accommodation to the septic medium was easily achieved in a hydroponics system and the regenerants had no significant losses when transferred in field. Phytochemical qualitative analyses showed that the polyphenols are represented by a small number and the flavones are absent within all the 'in vitro' samples. The number of triterpene fractions varied with the hormonal formula. Most of the fractions in the volatile oil of the in vitro regenerated neoplantlets were identified on B_{02} and BG_1 mediums.
Key words: *Mentha*, morphogenetic reaction, phytochemical tests.

INTRODUCTION

Active principles of *Mentha viridis* (synonymous to *M. spicata*) have a choleretic and cholagogue, stomahic, sedative, anti-inflammatory, cicatrisant, anti-catharral, anti-diarrhoeic, anti-spastic, sudorific, diuretic etc, effects (1,4-8). Mint leaves contain volatile oil (about 1-3% in dry leaves), tannins (3.5-4.5%), polyphenolic acids, flavonosides, pentacyclic triterpenes, monoterpenil-glucosides, carotenoides etc. Volatile oil is mostly represented by carvone (55-65%, even up to 80%), trans-tujanol-4 (20%), menthone, menthofuran, α- and β-pinene, camphene, myrcene, α-phellandrene, β-caryophyllene, borneol, carveol, pulegone, dihydrocarvone, 1,8-cineol etc. (2,3,5,8).

Considering this species' pharmaceutical and cosmetical importance, we considered it's useful to know its *in vitro* behaviour and the possibility of elaborating a micropropagation technology. This technology would be extremely useful to breed some possible valuable genotypes on the respect of production, productivity of active principles, resistance to some environmental factors. We also aimed to point out the influence of hormonal content on the capacity to syntethyze some specific active principles *in vitro* and also determine their content and spectrum for the field-grown regenerants.

MATERIAL AND METHODS

The biological material (*Mentha viridis* L.) originates from Chalkidiki (Greece) where some plants were taken from and cultivated in soil pots, then submitted to field conditions. Shoot tips and upper stem nodes were used for *in vitro* culture initiation. The explants were sterilised with a chloramine – T solution (5 %) for 25 minutes and were inoculated on basic hormone-free Murashige-Skoog (1962) medium, or on MS supplemented with 0.2 mg/l BAP. The cultures were placed in 100 ml Erlenmeyer vials (type B). The carbon source in the nutritive medium was represented by saccharose (25 g/l). In order to solidify medium we used 8.5 g/l agar-agar. Culture incubation was accomplished in the Genetics Laboratory of 'Stejarul' Research Centre of Piatra Neamt at about 23-25° C and 2500 lux under continuous illumination. The explants inoculated on MS hormone-free medium generated single-stem neoplantlets with small thin leaves but very strong roots. These sterile neoplantlets were then used as explants source to test the morphogenetic reaction on varied hormonal formulae of MS medium. Our test results are displayed in Table 1.

During our researches we also ran the qualitative analysis (by TLC) of some classes of active principles on the biological material provided by *in vitro* cultures on certain hormonal formulae, previously submitted to dichloro-methane and methanol extractions. We tested the flavonic, polyphenolic, triterpenic fractions and also those present in volatile oil. As standards for polyphenols we used catechyne, chlorogenic, ferulic and cumaric acids; for flavones: quercetol, ruthoside, apigenine, lutheoline; for triterpenes: cholesterole, β-sitosterole, stigmasterole, oleanolic and ursolic acid, and for volatile oil: borneol, thymol, menthol and linalool. These tests' results are presented in Table 2.

RESULTS AND DISCUSSION

With very few exceptions, shoot tips and nodes with leaves' inoculation on the hormonal formula we tested led to neoplantlet generation, but their strengthness, growth speed and shoot and root dimensions in a certain period of time were different (Table 1). For example, when shoot tips and nodes were cultivated on MS supplemented with 0.2-0.5 mg/l BAP, they produced neoplantlets with more vigorous shoots and roots than in the case of BAP associated with IAA or IBA, the roots being long and fascicled. The basal node gave 1 to 3 shoots. The leaves are generally thin, goffered, especially those proximate to the shoot base. As previously shown, BAP associated with IAA caused neoplantlet generation from nodes and shoot tips, with a lower shoot growth rate than on BAP medium and bearing smaller leaves; the stem, sometimes coloured in pink to violet hues, provides up to 4 shoots of varied dimensions at the basal node. Some vials containing this medium formula contained neoplantlets that differed by their aspect, meaning more turgescent stems and leaves, many short roots fasciculated from their nodes up, thicker internodes, smaller leaves and obvious multiple shooting. The phenomenon of adventitious rooting from upper nodes was sporadically noticed. In the presence of IBA neoplantlets beared up to 8 shoots at the stem basal node, a growth rate superior to previously tested hormonal formula, neoplantlet roots being fascicled, long, sometimes with secondary branches. When BAP was put together with NAA, the neoplantlets generated by nodes and shoot tips were vigorous, providing 1 to 4 shoots at base, these roots being also long and fascicled. A very efficient medium formula in obtaining vigorous neoplantlets contained kinetin and NAA, these plantlets being characterised by stronger roots, in fascicles from the node that was connected to the nourishing medium.

The explants (nodes and shoot tips) were also tested on medium formulae comprising only auxines, the morphogenetic reaction being also neoplantlet formation. It was ascertained that if the medium contained 2 mg/l IAA, IBA or NAA, the explants' reaction was similar, meaning they provided neoplantlets with fascicled roots, but their shoots beared small, frail leaves. The medium with IBA and NAA generated up to 4 shoots on the basal node, while on a nutritive medium supplemented with IAA generally produced only one stem. We noticed that on a culture medium with BAP and giberelic acid (GA) the rooting was less intense than on the other hormonal formula previously mentioned, but the multiple shooting was stimulated. The nodes and shoot tips provided neoplantlets even on hormone-free MS medium, a node comprising one or two shoots, the leaves had a larger limb, with fewer but longer roots having short secondary branches that confer them a comb aspect. The surprise element on all these formula was root vigour and growth (in a period of 30 days they exceed frequently 10 cm in length), in a striking contrast with the aspect of leaves (which are often fragile). Probably this impressing root development makes the process of neoplantlet adaptation to septic environment easy to go, contrary to leaf system, which has a slower development.

Internodes or root fragments inoculated on MS supplemented with 2,4-D or BAP together with 2,4-D provided a cream callus, friable on its entire surface, with an average proliferation speed (the one in the internode displayed a higher proliferation speed). On a medium containing only 2,4-D, callus generation was a bit more intense than in the case of using BAP. Transferring this callus on medium formula comprising cytokinins or auxins did not cause neither cell differentiation nor cell proliferation. Generally within one month this callus turned brown and degenerated.

Even though the neoplantlets obtained display a feeble aspect with small thin leaves, their accommodation to septic environment in a hydroponic system did not cause any problem. This adaptation process is more efficient and shorter at lower and quite constant temperature (about 20 to 22°C). The regenerants' transfer in field didn't also encounter problems even though it was done in summertime, an important aspect being considered: the plants were very well watered during the first ten days after field transplant.

Phytochemical qualitative tests showed that a small number represents polyphenols. Chlorogenic acid was found in neoplantlets obtained on B_{02}, KN, KN_1 and N_2, and caffeic and ferulic acids are absent on KN medium. Flavones are absent within all the analysed samples. The number of triterpenic fractions varied with the hormonal formula: the most of the fractions were found on B_{02} and KN, and the fewest on A_2. The biological material comprised β-sitosterole, oleanolic and ursolic acid on each hormonal formula. Most of the fractions in the volatile oil of the *in vitro* regenerated neoplantlets were identified on B_{02} (8) and BG_1 (7), (Table 2). All the biological material contained menthol, excepting the one generated on A_2.

CONCLUSIONS

1. *In vitro* culture initiation at *Mentha viridis* can be successfully accomplished using (as explants) shoot tips and nodes from the upper stem parts of the greenhouse or laboratory cultivated plants which were sterilised

for 25 minutes in chloramine-T solution (5%) and inoculated on Murashige-Skoog hormone-free medium or supplemented with small amounts of BAP (0.2 mg/l).

2. This species has an unusually plain *in vitro* behaviour. Nodes and shoot tips'inoculation on media comprising cytokinins or auxins (or their combinations) provided neoplantlets of varied basal branching degree, shoots of various dimensions (with a high growth rate) and leaves that are often small, with thin limbs but bearing a very strong root system. The adventitious rooting from superior stem nodes was evident on many formulae. Only on a medium comprising 2.4-D caulogenesis and rhysogenesis were inhibited, a compact cream-greenish callus was provided on a medium with BAP and giberelic acid the rooting was less present, but an increased multiple shooting occurred.
3. The fragments of internodes and roots provided a cream, friable callus with an average proliferation speed on MS medium supplemented with 2,4-D or with 2,4-D combined with BAP. Its differentiation when transferred on other hormonal formulae was not successful as it degenerated in about 30 days of culture.
4. TLC analyses of some *in vitro* obtained samples on some hormonal formulae showed that flavones are absent, polyphenols are represented by a small amount and the number of triterpenic fractions varied with hormonal formula. Most fractions of volatile oil were evidenced on B_{02} and BG_1 mediums.
5. The *in vitro* obtained neoplantlets were easily adapted to septic conditions in hydroponic system (registering small losses of 5 to 10 %) in quite constant conditions and lower temperatures (about 20^o C). Field potting and rooting had favourable results.

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Table 1. Morphogenetic reaction of some explants of *Mentha viridis* in *in vitro* culture

Var	The explant	Hormonal formula	Growth regulators (mg/l)							Morphogenetic reaction and proliferation speed
			BAP	GA	IAA	IBA	Kin	NAA	2.4-D	
1	Node and shoot tip	A	-	-	2.0	-	-	-	-	Neoplantlets (++) with 1 basal shoot, long and thin roots (+++), thin and waved leaves
2	"	B	0.2 – 0.5	-	-	-	-	-	-	Neoplantlets (++) with 1 – 3 basal shoots, with very strong roots (+++), sometimes with secondary branches, sporadically (+) adventitious roots from the upper stem nodes
3	"	BA	1.0		0.5					Vigorous neoplantlets (+++), with thicker and turgescent leaves, thicker internodes, short fascicled roots (+++), multiple shooting (+), adventitious roots (+)
4	"	BB	1.0			0.5				Neoplantlets (++) with a fragile aspect, bearing 1-4 shoots of varied dimensions at base, sometimes multiple shooting (+), long, strong, fascicled roots (+++)
5	"	BG	1.0	0.5						Multiple shooting (+++), neoplantlets (sporadically) (+)
6	"	BN	1.0					0.5		Vigorous neoplantlets (+++) with 1-4 shoots at base, long fascicled roots (+++)
7	"	D							2.0	Cream, friable callus (++) surrounding the node; shoot development completely inhibited
8	"	IB				2.0				Neoplantlets (+++) with 1-4 basal shoots, long fascicled roots (+++), small thin leaves
9	"	KN					1.0	0.5		Neoplantlets (+++) with 1-4 basal shoots of varied dimensions, bearing longer and thicker roots (+++), adventitious roots sporadically (+)
10	"	MS								Neoplantlets (+++) with 1-2 basal shoots, leaves with larger limb, long but fewer roots (++) bearing secondary branches; sporadically adventitious roots (+)

Table 2. The spectrum of some active principles identified by TLC in *Mentha viridis* neoplantlets regenerated on certain hormonal formulae

Var.	Hormonal formula	Polyphenolic fractions	Flavonic fractions	Triterpenic fractions	Volatile oil fractions
First experience					
1	MS	3	-	6	6
2	BA_1	4	-	6	5
3	BA_2	3	-	6	5
4	BN_1	4	-	7	5
5	BG_1	3	-	7	7
6	BB_2	3	-	7	6
7	KN_1	4	-	8	6
8	A_2	2	-	5	5
Second experience					
1	MS	3	-	7	6
2	B_{02}	5	-	10	8
3	KN	1	-	8	6
4	KN_1	4	-	8	6
5	N_2	5	-	9	6
6	A_2	2	-	5	5

[L-11]

[L-20]

MISTLETOE (*VISCUM ALBUM* L.) BERRIES EXTRACTS IN PREVENTION OF *IN VIVO* CCl_4-INDUCED OXIDATIVE STRESS – BIOCHEMICAL STUDY

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ABSTRACT

Mistletoe (*Viscum album* L.) is well-known as a medicine from ancient times. There are few reports on hypotensive, immunomodelling and anticancer potential of mistletoe extracts, but there are no or very few reports on it's *in vivo* antioxidative properties. In this paper effects of different extracts obtained from mistletoe berries on some biochemical parameters in rats liver homogenate and blood hemolysate were examined. 10% ethanol solutions of ether (Et_2O), chloroform ($CHCl_3$), ethylacetate (EtOAc), n-butanol (nBuOH) and aqueous (H_2O) extracts of berries were used. Liver and blood were used for determination of the following enzymes: xanthine oxidase (XOD), catalase (CAT), peroxidase (Px), glutathione peroxidase (GSHPx), as well as reduced glutathion content (GSH) and intensity of lipid peroxidation (LPx). Almost all examined extracts of *Viscum album* berries showed significant influence on biochemical markers of oxidative stress, changing their activity and thus exhibiting possible antioxidative or prooxidative potential. We concluded that some of the examined ethanol solutions of different extracts of mistletoe berries showed some very good potential antioxidative and hepatoprotective properties.

INTRODUCTION

One of the paradoxes of life on the Earth is that oxygen is necessary for living of aerobic organisms. On the other hand, increased concentration of oxygen and it's metabolites (reactive oxygen species-ROS) may cause a number of diseases. Enviromental contamination, modern lifestyle, food rich in hormones and aditives, etc., are just some of the causes of increased production of reactive oxygen species and development of oxidative stress. Oxidative stress plays an important role in patogenesis of many diseases and disorders, such as carcinoma, diabetes, Alzheimers, Parkinsons, cardiovascular diseases, etc. The central processes are changes of cell membrane properties and functions (fluidity, selective permeability, membrane enzymes activity, …). During evolution, aerobic organisms developed different types of antoxidative protection – enzymatic and non-enzymatic. Thus, in healthy cells, oxidative stress and antioxidative protection are in constant equilibrium. In the last decade, significant attention is focused on active principles from plants as potential antioxidants.

Mistletoe (*Viscum album* L.) contains viscotoxins, phenylpropanes, lignans, flavonoids (Chou *et al*., 1999; Wollenweber *et al*., 2000), biogenamines, polisaccharides and lectins, which have cytostatic effects (Hoffman, 1990) and immunomodelling potential (Fischer *et al*., 1997; Stein *et al*., 1998). Isolated viscotoxins show hypotensive effects (British Herbal Pharmacopoeia 1983, 1989). During some preliminary analysis, we concluded that mistletoe contains significant amount of flavonoids and phenolic compounds. Considering well known antioxidative properties and hepatoprotective effects of flavonoids, the effects of different extracts obtained from mistletoe berries on some biochemical parameters in rats blood hemolysate and liver homogenate were examined.

MATERIALS AND METHODS

Preparation of Viscum album L. berries extracts: Mistletoe berries were dried in air and grounded in a mixer. Finely powdered material was subjected to extraction with 70% methanol solution (MeOH). After removal of MeOH under reduced pressure, the aqueous phase was successively extracted with four solvents of increasing polarity; ether (Et_2O), chloroform ($CHCl_3$), ethylacetate (EtOAc) and n-butanol (n-BuOH).The residue was the aqueous extract. All five extracts (Et_2O, $CHCl_3$, EtOAc, n-BuOH, H_2O) were evaporated to dryness and after that dissolved in 50% ethanol to make 10% solutions to be used in the experiment.

Biochemical tests: Albino "Wistar" rats of both sexes (250g), that were used in this experiment, were devided into groups of five animals in each. The first was control group. The second was the negative control in which animals were treated only with CCl_4. Next five groups (3-7) have been receiving pure ethanol solutions of different fractions (Et_2O, $CHCl_3$, EtOAc, nBuOH and H_2O) for seven days, while the oter five groups (8-12) received carbon-tetrachloride (CCl_4) in olive oil (1:1) on the eight day beside the *Viscum album* extract. Twenty-four hours after intoxication with CCl_4, animals were sacrified. Livers were removed and blood samples were

collected and further used for determination of the following enzymes: xanthine oxidase (XOD), catalase (CAT), peroxidase (Px), glutathione peroxidase (GSHPx), glutathione reductase (GSHR), as well as reduced glutathion content (GSH) and intensity of lipid peroxidation (LPx).

RESULTS AND DISCUSSION

Results of the investigation, presented in tables 1 - 4, showed some very interesting points concerning changes of activity of examined enzymes, both their increase/decrease of activity after administering pure extracts of *Viscum album* L. berries and after intoxication with CCl_4. All results were obtained by measurement of five samples and they are certainly statistically significant. Intensity of LPx in rats blood was increased after application of each extract of mistletoe berries, as well as combination of extracts and CCl_4. Et_2O and $CHCl_3$ extract inhibited LPx in rats liver, EtOAc extracts haven't influenced LPx, while nBuOH extract increased the process of membrane peroxidation. Since CCl_4 is known as a strong prooxidant and hepatotoxic agent, LPx value was increased in a very high degree, but all applied *Viscum* extracts turned this value to the control level. Having this in mind, we may conclude here that mistletoe berries extracts could show protective effect. As for blood CAT activity, it was reduced by two extracts ($CHCl_3$ and nBuOH fraction). Application of CCl_4 haven't changed those values a lot. Neither extracts nor carbon-tetrachloride showed statistically significant changes of catalase activity in rats' liver homogenate. Activity of plasma XOD was extremly increased upon treatment with *Viscum* extracts and CCl_4 as well, probably due to release of this enzyme from liver, where XOD level was slightly lower with extracts in comparison to negative control. Increased Px levels point to the presence of increased concentration of certain ROS types in blood, but on the other hand Px levels were lower in the presence of extracts than in CCl_4 control group. The content of GSH has not been changed upon teatment with mistletoe extracts, but it has been significantly reduced after intoxication with CCl_4. Combination extract/CCl_4 increased the GSH content. Et_2O and EtOAc extract increased, while polar nBuOH and H_2O extract decreased levels of GR in rats liver (table 4). Treatment with CCl_4 caused small increase of GR activity. Having in mind that GR catalyses the reaction of GSH regeneration, it could be concluded that mistletoe berries extracts exhibited certain protection degree, or didn't change GR status in rats liver. GSHPx activity in hepatocytes, was increased a lot both upon treatment with mistletoe extracts and CCl_4, that was in accordance with the results obtained with the rest of "GSH enzymes".

Resuming all obtained results it could be concluded that in most cases, ethanol solutions of different extracts of mistletoe berries exhibited certain level of "antioxidative" and "hepatoprotective" properties. For getting the more detailed insight in possible antioxidative effects of *Viscum album* L. berries, further analysis and fractinations of active principles are necessary.

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Table 1. Effect of misletoe extracts on some biochemical parameters of oxidative stress in rats blood hemolysate

	Control	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5
CAT	0,5288±0,04	0,9614±0,12	0,1328±0,01	0,5365±0,02	0,1966±0,01	0,5384±0,02
XOD	0,8089±0,03	10,011±0,57	4,918±0,23	6,554±0,10	6,054±0,17	8,958±0,49
Px	0,2792±0,01	0,9842+0,07	0,9554±0,08	6,798±0,13	4,909±0,08	4,708±0,08
GSHPx	0,7724±0,04	0,9595±0,08	0,9694±0,03	1,952±0,23	1,789±0,14	4,247±0,23
GR	1,384±0,03	1,486±0,08	0,7490±0,03	0,7077±0,01	0,6127±0,04	2,043±0,04
GSH	1,451±0,04	1,113±0,02	1,123±0,04	1,115±0,05	1,075±0,05	1,084±0,05
LPx	0,0286±0,00	0,0677±0,00	0,1021±0,00	0,0618±0,00	0,0640±0,00	0,0529±0,00

Activities of CAT, XOD, Px, GSHPx and GR are expressed in μmol/ml erythrocytes.
Amount of GSH is expressed in μmol/ml erythrocytes.
Intensity of LPx is expressed in μmol MDA/ml erythrocytes.

Table 2. Effect of different misletoe extracts on CCl_4 – induced oxidative stress in rats blood hemolysate.

	Control+CCl_4	Extract 1+CCl_4	Extract 2+CCl_4	Extract 3+CCl_4	Extract 4+CCl_4	Extract 5+CCl_4
CAT	0,5144±0,03	0,6948±0,02	0,0850±0,01	0,4158±0,02	0,4112±0,01	0,4712±0,04
XOD	0,9982±0,04	7,204±0,34	4,032±0,26	2,966±0,27	5,503±0,36	6,098±0,17
Px	0,435±0,01	1,374±0,01	1,186±0,07	5,565±0,08	3,650±0,05	3,606±0,08
GSHPx	0,4859±0,005	0,5179±0,03	2,257±0,07	0,8452±0,05	0,8465±0,01	3,487±0,16
GR	6,199±0,04	0,8650±0,02	0,7875±0,01	0,7467±0,01	0,7297±0,03	0,9531±0,04
GSH	1,004±0,03	0,9955±0,17	1,100±0,03	1,099±0,01	1,091±0,03	1,115±0,07
LPx	0,0250±0,00	0,0607±0,00	0,1275±0,01	0,0683±0,00	0,0858±0,00	0,0571±0,00

Activities of CAT, XOD, Px, GSHPx and GR are expressed in μmol/ml erythrocytes.
Amount of GSH is expressed in μmol/ml erythrocytes.
Intensity of LPx is expressed in μmol MDA/ml erythrocytes.

Table 3. Effect of misletoe extracts on some biochemical parameters of oxidative stress in rats liver homogenate.

	Control	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5
CAT	1,589±0,13	1,8138±0,02	2,045±0,16	1,947±0,26	2,128±0,11	1,348±0,04
XOD	3,968±0,60	15,68±1,19	12,90±0,69	11,38±0,40	48,17±1,55	10,75±0,54
Px	0,0552±0,01	0,0739±0,00	0,1234±0,01	0,0537±0,00	0,1214±0,01	0,0468±0,00
GSHPx	12,31±0,51	26,50±1,38	35,19±0,63	30,86±1,10	34,66±0,45	20,13±0,99
GR	5,294±0,63	12,26±0,44	10,87±0,80	4,672±0,40	2,448±0,15	7,828±0,07
GSH	1,185±0,17	4,515±0,09	4,389±0,11	1,089±0,02	1,670±0,09	0,372±0,01
LPx	0,3909±0,01	0,2168±0,01	0,2096±0,01	0,3935±0,01	0,4338±0,01	0,4370±0,02

Activities of CAT, XOD, Px, GSHPx and GR are expressed in nmol/mg protein.
Amount of GSH is expressed in nmol/mg protein.
Intensity of LPx is expressed in nmol MDA/mg protein.

Table 4. Effect of different misletoe extracts on CCl_4 – induced oxidative stress in rats liver homogenate.

	Control+CCl_4	Extract 1+CCl_4	Extract 2+CCl_4	Extract 3+CCl_4	Extract 4+CCl_4	Extract 5+CCl_4
CAT	1,421±0,07	2,082±0,26	1,781±0,13	1,737±0,32	1,756±0,13	1,648±0,23
XOD	16,13±0,73	22,59±0,82	21,13±0,83	7,430±0,67	40,48±0,93	22,30±1,04
Px	0,0558±0,00	0,0626±0,00	0,1116±0,01	0,1805±0,02	0,6975±0,05	0,0977±0,01
GSHPx	13,87±0,65	22,08±0,60	15,96±0,27	32,93±0,84	64,84±3,48	22,85±1,05
GR	5,983±0,23	9,905±0,57	8,845±0,63	2,751±0,23	5,552±0,29	4,354±0,32
GSH	1,094±0,07	4,256±0,11	5,011±0,26	0,4294±0,02	0,8056±0,05	0,4793±0,02
LPx	0,6127±0,04	0,2631±0,01	0,2571±0,00	0,4774±0,03	0,4137±0,02	0,3445±0,03

Activities of CAT, XOD, Px, GSHPx and GR are expressed in nmol/mg protein.
Amount of GSH is expressed in nmol/mg protein.
Intensity of LPx is expressed in nmol MDA/mg protein.

Legend:
Extract 1 – 10% solution of Et_2O fraction in 50% ethanol
Extract 2 – 10% solution of $CHCl_3$ fraction in 50% ethanol
Extract 3 – 10% solution of EtOAc fraction in 50% ethanol
Extract 4 – 10% solution of nBuOH fraction in 50% ethanol
Extract 5 – 10% solution of H_2O fraction in 50% ethanol

[L-20]

[P-015]

THE MEDICINAL PLANTS OF TARNAVE PLATEAU ROMANIA

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ABSTRACT

The paper presents the medicinal plants identified on Tarnave Plateau Transsylvania. In order to appreciate as correct as possible the genefond of medicinal plants presenting each species is accompanied by ecological, chorological, coenological features, on the affiliation to different bioform groups information and values of ecological index UTR (humidity, temperature, soil reaction), and chromosome number (2n). An analysis of the plants according with the dominant active principles of medicinal plants used in traditional medicine and phytotherapy was made. We necessarily considered the medicinal flora to be known in this part of the country in order to turn to good account their phytotherapeutic benefits.
Key words: medicinal plants, Tarnave Plateau.

INTRODUCTION

The Târnave Plateau flora can be characterised by a great diversity of vegetable taxons as a result of localisation the area but mostly of variety on relief forms (hills, floods, plateau), of different exposures and inclination of the slopes. Beside this the heterogeneity of the soils can be added.

The research area has been studied geographically, geologically and ethnographically for ages but these information has been accompanied by floristical and vegetation studies as well. The first wide floristical writings on Târnave Plateau are due to botanist of Sighişoara J.G. Baumgarten (1765-1843), who in the floristic work of Transilvania "*Enumeratio stirpium Magno Transsilvaniae Principatui*", Vindobonae, edited in Vienna in 1816 refers to Târnave Plateau flora.

In Soó Rezsö's work "*Podromus Florae Terrae Siculorum*" (1940), there are a lot of data concerning some botanists' mentions on Târnave hydrographic basin flora: Lengyel Géza, Mathé Imre, E.I.Nyárády etc. We mention as well Kacso Albert's doctorate dissertation held at the Faculty of Pharmacy from Tg-Mureş "*Plantele medicinale şi aromatice din Valea Târnavei Mici*" which proves to be important for the inventory of medicinal and aromatic plants of wild flora and in initiating cultures of some therapeutic species, in evaluating vegetable resource reserves pharmaceutically interested.

The summary of medicinal plants was elaborated by researches made in several localities: Abuş, Adămuş, Apold, Bahnea, Bălăuşeri, Bezid, Biertan, Brădeni, Buneşti, Călimăneşti, Cerghid, Chibed, Corneşti, Crăieşti, Criş, Daia, Daneş, Fântânele, Fiser, Floreşti, Ghindari, Herepea, Hoghilag, Iacobeni, Idrifaia, Jibert, Laslea, Mica, Merghindeal, Mihai Viteazu, Mureni, Nou Săsesc, Rondola, Saschiz, Săcădat, Sângiorgiu de Pădure, Sovata, Stejărenii, Şaeş, Şarpatoc, Târnăveni, Viişoara, Viscri, Vulcan.

MATERIAL AND METHODS

This work was made on the basis of the researches accomplished during 2000-2004 as well as of the biographic data. Both the system of classification accepted and the plants nomenclature has been adjusted according to the following authors' publications N. Boşcaiu (1971), E. Oberdorfer (1970), W. Adler, K. Oswald, R. Fischer (1994), Flora României (1952-1976), Flora Europaea (1964-1980). By making these researches we adopted right nomenclatural solution after the International Code of Botanical Nomenclature (Code de Tokyo, 1993).

RESULTS AND DISCUSSIONS

The floristic inventory includes 240 species of plants, divided in 65 families (Table1). The most representative are the following families: *Rosaceae* (23 sp.), *Asteraceae* (23 sp.), *Lamiaceae* (22 sp.), *Apiaceae* (14 sp.). *Scrophulariaceae* (14 sp.), *Fabaceae* (10 sp.), *Brassicaceae* (8 sp.) etc.

Table 1. Families and number of medicinal plants species identified in Târnave Plateau

Family	Sp. no.	Family	Sp.no.	Family	Sp. no.
Alliaceae	1	*Eleagnaceae*	1	*Orchidaceae*	3
Apiaceae	14	*Equisetaceae*	1	*Papaveraceae*	2
Apocynaceae	2	*Euphorbiaceae*	1	*Pinaceae*	1
Araceae	1	*Fabaceae*	10	*Plantaginaceae*	3
Araliaceae	1	*Fagaceae*	3	*Poaceae*	1
Aristolochiaceae	1	*Fumariaceae*	5	*Polygalaceae*	3
Asclepiadaceae	1	*Gentianaceae*	3	*Polygonaceae*	7
Asparagaceae	3	*Geraniaceae*	2	*Primulaceae*	4
Asteraceae	23	*Grossulariaceae*	1	*Ranunculaceae*	7
Berberidaceae	1	*Hypericaceae*	2	*Rhamnaceae*	2
Betulaceae	2	*Iridaceae*	2	*Rosaceae*	23
Boraginaceae	4	*Juglandaceae*	1	*Rubiaceae*	4
Brassicaceae	8	*Lamiaceae*	22	*Rutaceae*	1
Cannabaceae	1	*Liliaceae*	1	*Salicaceae*	5
Caprifoliaceae	3	*Loranthaceae*	1	*Scrophulariaceae*	14
Caryophyllaceae	1	*Lycopodiaceae*	1	*Solanaceae*	5
Celastraceae	2	*Lythraceae*	1	*Tiliaceae*	3
Colchicaceae	1	*Malvaceae*	7	*Urticaceae*	1
Convolvulaceae	2	*Melanthiaceae*	1	*Valerianaceae*	1
Cornaceae	2	*Menyanthaceae*	1	*Verbenaceae*	1
Cuscutaceae	1	*Oleaceae*	1	*Violaceae*	1
Dryopteridaceae	1	*Onagraceae*	4		
				TOTAL	240

The analysis the **ecological categories** of medicinal species in Târnave Plateau shows the ecological particularity of ecosystems belonging to this area as a result of edapho-climatical factors complex. UTR (humidity, temperature, soil reaction) edapho-climatical factors were taking into consideration. By studying species needs for humidity we notice that most of the species are mesophilous (40,83%) and xero-mesophilous (35,41%). Regarding temperature, the preponderance of the micro-mesothermophilous (61,66%), T4 (15,83%), eurythermics (13,75%) can be observed. Speaking of soil reaction, we can find out that the most numerous are the low acid neutrophilous (42,91%), euryionics (29,16%) and acid-neutrophilous (20,83%).

Bioforms analysis is a major element in characterising flora. The high percent of hemicryptophytes (47,5%) indicates that the research field belongs to temperate climate areas. These are followed by therophytes (20,83%). Concerning **floristic elements** the preponderance of eurasiatics (26,66%), europeans (11,66%), europeo-caucasians (9,16%) and paleotemperates (12,91%) species are shown.

Caryological information is extremely important (Fedorov A., 1969; Löve A., Löve D., 1974; Májovsky J., Murin J.A.,1987). Out of the whole medicinal species 47,91% are diploids species, older, which provides a favourable genetic potential to future phytoevolution, 47,08% polyploids ensuring species with a stronger resistance against ecological conditions tending to extremes and 4,16% are diplo-polyploids.
We necessarily considered the medicinal flora to be known in this part of the country in order to turn to good account their phytotherapeutic benefits (Ciulei I., Grigorescu E., Stănescu U.,1993; Eşianu S., Csedö C.,1999; Istudor V.,1998,2001; Rácz G., Laza A., Coiciu A., 1970; Farmacopeea Românăm 1993).

By studying medicinal plants with their **chemical composition** (Table 2, Figure 1) we can notice that most of the plants include tannins (15,126%), volatile oils (11,344%), alkaloids (10,924%), flavons (9,243%), mucilage's (7,983%), saponins (7,563%) etc.

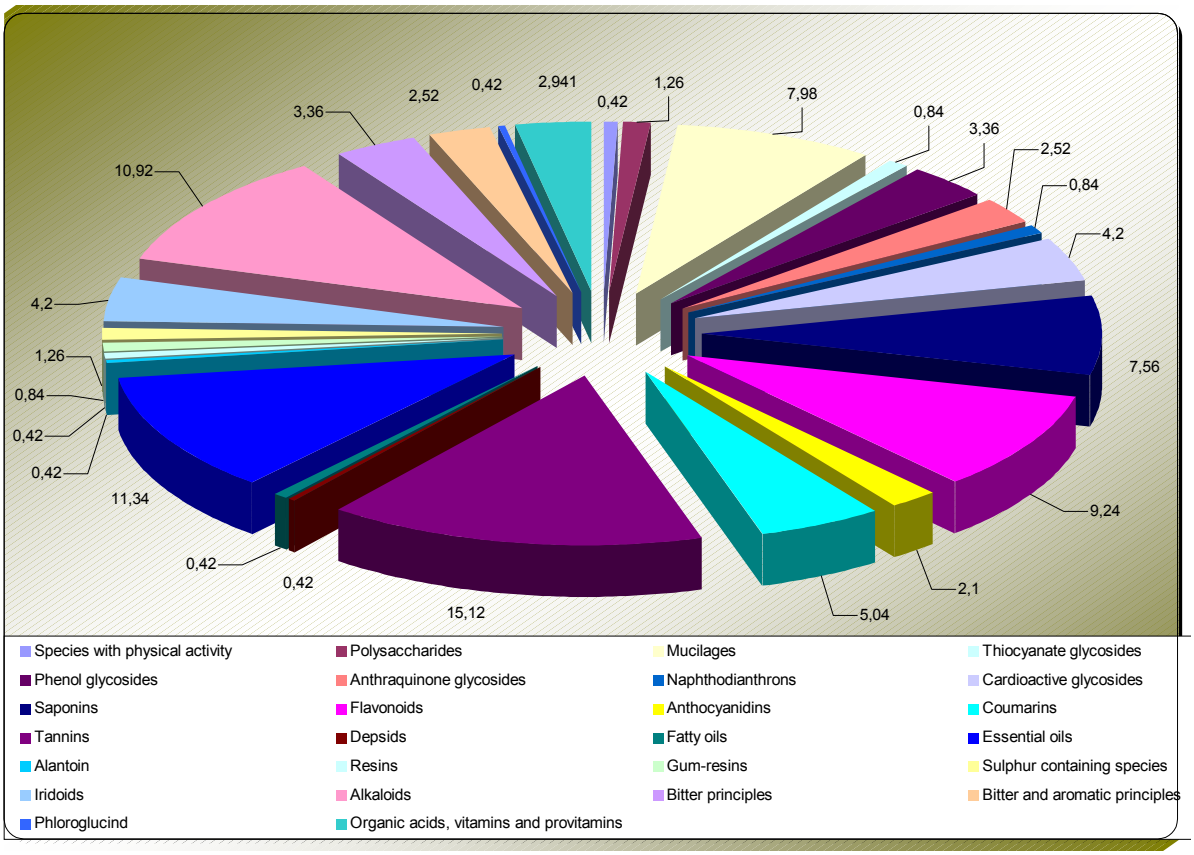

Figure 1. The dominant active principles of medicinal plants used in traditional medicine and phytotherapy

CONCLUSIONS

It is necessarily to establish the quantity of vegetable genefond therapeutically used so that the medicinal plants are protected. This can be done by making local inventory with plants, because this work shows us the right territorial spreading of medicinal plants in a very tight bound with the diversity of phytocoenosis in which they exist and with the ecological factors.

Figure 2. *Adonis vernalis*

Table 2. The dominant active principles of medicinal plants used in traditional medicine and phytotherapy

The dominant active principles	Species	Medicinal vegetal products
Species with physical activity	*Lycopodium clavatum*	*Sporae*
Polysaccharides	*Arctium lappa*	*Radix*
	Elymus repens	*Rhizoma*
	Inula helenium	*Radix*
Mucilages	*Alcea pallida*	*Flos et folium*
	Althaea officinalis	*Radix et folium*
	Anchusa officinalis	*Flos et folium*
	Hibiscus trionum	*Herba*
	Lavathera thuringiaca	*Radix*
	Malva alcea	*Flos et folium*
	Malva neglecta	*Flos et folium*
	Malva sylvestris	*Flos et folium*
	Orchis militaris	*Tuber*
	Orchis morio	*Tuber*
	Orchis purpurea	*Tuber*
	Plantago lanceolata	*Folium*
	Plantago major	*Folium*
	Plantago media	*Folium*
	Tussilago farfara	*Folium*
	Verbascum lychnitis	*Flos*
	Verbascum phlomoides	*Flos*
	Verbascum phoeniceum	*Flos*
	Verbascum thapsus	*Flos*
Thiocyanate glycosides	*Brassica nigra*	*Semen*
	Sisymbrium officinale	*Herba*
Phenol glycosides	*Filipendula ulmaria*	*Flos*
	Populus nigra	*Gemma*
	Populus tremula	*Gemma*
	Pyrus pyraster	*Folium*
	Salix alba	*Cortex*
	Salix caprea	*Cortex*
	Salix cinerea	*Cortex*
	Viburnum opulus	*Cortex*
Anthraquinone glycosides	*Frangula alnus*	*Cortex*
	Rhamnus cathartica	*Cortex*
	Rumex acetosa	*Herba*
	Rumex acetosella	*Herba*
	Rumex conglomeratus	*Rhizoma*
	Rumex crispus	*Rhizoma*
Naphthodianthrons	*Hypericum maculatum*	*Herba*
	Hypericum perforatum	*Herba*
Cardioactive glycosides	*Adonis vernalis* (fig.2)	*Herba*
	Asparagus officinalis	*Radix*
	Convallaria majalis	*Herba*
	Digitalis grandiflora	*Folium*
	Erysimum cheiranthoides	*Herba*
	Erysimum odoratum	*Herba*
	Euonymus europaea	*Cortex*
	Euonymus verrucosa	*Cortex*
	Helleborus purpurascens	*Rhizoma et radix*
	Leonurus cardiaca	*Herba*
Saponins	*Anagallis arvensis*	*Herba*
	Bellis perennis	*Flos*
	Bupleurum falcatum	*Radix*
	Eryngium campestre	*Herba*

	Eryngium planum	*Herba*
	Equisetum arvense	*Herba*
	Hedera helix	*Herba*
	Mercurialis annua	*Herba*
	Ononis arvensis	*Radix*
	Ononis spinosa	*Radix*
	Polygala comosa	*Herba*
	Polygala major	*Herba*
	Polygala vulgaris	*Herba*
	Primula veris	*Rhizoma cum radicibus*
	Ranunculus ficaria	*Radix*
	Saponaria officinalis	*Radix*
	Solidago virgaurea	*Radix*
	Viola tricolor	*Herba*
Flavonoids	*Anthriscus cerefolium*	*Herba et fructus*
	Anthriscus sylvestris	*Herba et fructus*
	Betula pendula	*Folium*
	Bidens tripartita	*Herba*
	Capsella bursa-pastoris	*Herba*
	Crataegus laevigata	*Folium, fructus et flos*
	Crataegus monogyna	*Folium, fructus et flos*
	Dictamnus albus	*Radix*
	Eupatorium cannabinum	*Rhizoma et radix*
	Hieracium pilosella	*Herba*
	Linaria vulgaris	*Herba*
	Polygonum hydropiper	*Herba*
	Prunus avium	*Stipites*
	Robinia pseudacacia	*Flos*
	Sambucus nigra	*Flos*
	Veronica beccabunga	*Herba*
	Veronica chamaedrys	*Herba*
	Veronica officinalis	*Herba*
	Veronica persica	*Herba*
	Veronica spicata ssp. orchidea	*Herba*
	Vincetoxicum hirundinaria	*Radix*
	Viola tricolor	*Herba*
Anthocyanidins	*Centaurea cyanus*	*Flos*
	Consolida regalis	*Flos*
	Papaver rhoeas	*Flos*
	Ribes nigrum	*Fructus*
	Rosa gallica	*Flos*
Coumarins	*Cruciata laevipez*	*Herba*
	Fraxinus excelsior	*Folium*
	Galium mollugo	*Herba*
	Galium odoratum	*Herba*
	Galium verum	*Herba*
	Heracleum sphondylium	*Radix, folium et fructus*
	Medicago falcata	*Herba*
	Medicago lupulina	*Herba*
	Medicago sativa	*Herba*
	Melilotus officinalis	*Flos et herba*
	Pastinaca sativa	*Radix*
	Pimpinella saxifraga	*Radix*
Tannins	*Agrimonia eupatoria*	*Herba*
	Alchemilla xanthochlora	*Herba*
	Anthyllis vulneraria	*Flos*
	Castanea sativa (subspont.)	*Folium*

	Cornus mas	*Cortex*
	Cornus sanguinea	*Cortex*
	Corylus avellana	*Folium*
	Cuscuta europaea	*Herba*
	Epilobium angustifolium	*Herba*
	Epilobium hirsutum	*Herba*
	Epilobium parviflorum	*Herba*
	Erodium cicutarium	*Herba*
	Fragaria vesca	*Folium*
	Fragaria viridis	*Folium*
	Geranium robertianum	*Herba*
	Geum urbanum	*Rhizoma*
	Juglans regia (subspont.)	*Folium*
	Lysimachia nummularia	*Herba*
	Lysimachia vulgaris	*Herba*
	Lythrum salicaria	*Herba*
	Polygonum aviculare	*Herba*
	Polygonum bistorta	*Rhizoma*
	Potentilla anserina	*Herba*
	Potentilla arenaria	*Rhizoma*
	Potentilla argentea	*Rhizoma*
	Potentilla erecta	*Rhizoma*
	Potentilla recta	*Rhizoma*
	Potentilla reptans	*Rhizoma*
	Prunus spinosa	*Flos, fructus*
	Prunus tenella	*Semen*
	Quercus petraea	*Cortex*
	Quercus robur	*Cortex*
	Salix alba	*Cortex*
	Salix caprea	*Cortex*
	Salix cinerea	*Cortex*
	Sanguisorba officinalis	*Herba*
Depsids	*Cichorium intybus*	*Herba et radix*
Fatty oils	*Oenothera biennis*	*Semen*
Essential oils	*Achillea millefolium*	*Flos*
	Acorus calamus	*Rhizoma*
	Anthemis tinctoria	*Flos*
	Arnica montana	*Flos*
	Asarum europaeum	*Rhizoma*
	Carum carvi	*Fructus*
	Iris pseudacorus	*Rhizoma*
	Matricaria recutita	*Flos*
	Melissa officinalis	*Folium*
	Mentha aquatica	*Folium*
	Mentha arvensis	*Folium*
	Mentha longifolia	*Folium*
	Mentha verticillata	*Folium*
	Nasturtium officinale	*Folium*
	Nepeta nuda	*Summitates*
	Origanum vulgare	*Herba*
	Peucedanum officinale	*Rhizoma*
	Peucedanum oreoselinum	*Rhizoma*
	Pimpinella saxifraga	*Radix*
	Pinus sylvestris	*Turiones*
	Thymus glabrescens	*Herba*
	Thymus pannonicus	*Herba*
	Thymus pulegioides	*Herba*
	Tilia cordata	*Flos*

	Tilia plathyphyllos	*Flos*
	Tilia tomentosa	*Flos*
	Valeriana officinalis	*Radix*
	Xanthium strumarium	*Herba*
Alantoin	*Symphytum officinale*	*Radix*
Resins	*Humulus lupulus*	*Strobuli*
Gum-resins	*Calystegia sepium*	*Herba*
	Convolvulus arvensis	*Herba*
Sulphur containing species	*Allium ursinum*	*Bulbus*
	Alliaria petiolata	*Herba*
	Armoracia rusticana	*Radix*
Iridoids	*Ajuga genevensis*	*Herba*
	Ajuga reptans	*Herba*
	Euphrasia rostkoviana	*Herba*
	Euphrasia stricta	*Herba*
	Lamium album	*Herba et flos*
	Sambucus ebulus	*Radix, flos, fructus*
	Stachys germanica	*Herba*
	Stachys officinalis	*Herba*
	Stachys recta	*Herba*
	Verbena officinalis	*Herba*
Alkaloids	*Berberis vulgaris*	*Cortex*
	Chamaespartium sagittale	*Herba*
	Chelidonium majus	*Herba*
	Clematis recta	*Folium*
	Clematis vitalba	*Folium*
	Colchicum autumnale	*Semen*
	Conium maculatum	*Fructus*
	Corydalis cava	*Tuber*
	Corydalis solida	*Tuber*
	Cynoglossum officinale	*Radix*
	Datura stramonium	*Folium*
	Echinops sphaerocephalus	*Flos*
	Echium vulgare	*Herba*
	Fumaria officinalis	*Herba*
	Fumaria schleicheri	*Herba*
	Fumaria vaillantii	*Herba*
	Genista tinctoria	*Herba*
	Hyoscyamus niger	*Folium*
	Scopolia carniolica	*Rhizoma*
	Senecio jacobaea	*Herba*
	Senecio vulgaris	*Herba*
	Solanum dulcamara	*Stipes*
	Thalictrum minus	*Herba*
	Veratrum album	*Rhizoma*
	Vinca herbacea	*Herba*
	Vinca minor	*Herba*
	Viscum album	*Stipes*
Bitter principles	*Ballota nigra*	*Herba*
	Centaurium erythraea	*Herba*
	Gentiana asclepiadea	*Radix*
	Gentiana cruciata	*Radix*
	Glechoma hederacea	*Herba*
	Marrubium vulgare	*Herba*
	Menyanthes trifoliata	*Folium*
	Taraxacum officinale	*Radix et herba*
Bitter and aromatic principles	*Artemisia absinthium*	*Herba*
	Artemisia pontica	*Herba*

	Artemisia vulgaris	*Herba*
	Teucrium chamaedrys	*Herba*
	Tanacetum corymbosum	*Flos*
	Tanacetum vulgare	*Herba*
Phloroglucinol	*Dryopteris filix-mas*	*Rhizoma*
Organic acids, vitamins and provitamins	*Daucus carota*	*Radix*
	Hippophae rhamnoides	*Fructus*
	Physalis alkekengi	*Fructus*
	Rosa canina	*Fructus*
	Rubus idaeus	*Folium*
	Sorbus aucuparia	*Fructus*
	Urtica dioica	*Folium*

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[P-019]

PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS USEFUL AS THE MARKERS OF SUSCEPTIBILITY OF MEDICINAL AND AROMATIC PLANTS TO STRESS FACTORS

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ABSTRACT

The aim of this work was to show the usefulness of two popular physiological methods: electrolyte leakage assay (EL) and potential quantum efficiency of photosystem 2 (F_v/F_m), as the markers of the plants' condition at water stress (drought and waterlogging) and thermal stress (chill). Experiments performed on sage, lemon balm, basil and maize revealed that electrolyte leakage is a useful tool in such studies. F_v/F_m parameter should be used with some restrictions, however, because its changes may reflect both destructive influence of stress conditions on PS 2 and the acclimatory processes involved in light energy dissipation (e.g. carotenoid formation). For this reason the monitoring of the light regime and additional measurements are recommended.
Key words: stress, membrane permeability, electrolyte leakage, F_v/F_m.

INTRODUCTION

Physiological methods may give to the researchers involved in the studies on medicinal and aromatic plants the ability to assess the condition of plants influenced by non-optimal environmental factors. In this paper we will show the usefulness of two popular methods. One of them is electroconductivity test performed as estimation of the permeability of cell membranes. This test is invasive, and the obtained result is called electrolyte leakage parameter [Dexter *et al*. 1932]. The second one is a parameter of chlorophyll *a* fluorescence, signed F_v/F_m obtained by the assay with various fluorometers. In a popular opinion, the less F_v/F_m is the worse condition of plants. This value, so-called potential quantum efficiency of photosystem 2 (PS 2) [Lichtenthaler and Wellburn 1983, Björkmann and Demmig-Adams 1994], provide the information about the status of PS 2 and, in consequence, about the intensity of light phase of photosynthesis. As the biomass production and essential oil content depend on photosynthetic activity, F_v/F_m seems to be serviceable in studies on medicinal and aromatic plants [Bączek-Kwinta 2001, Bączek-Kwinta and Seidler-Łożykowska 2004].

The aim of our work is to show the usefulness of EL and F_v/F_m as the markers of the degree of the influence of stress factors such as water stress (drought and waterlogging) and thermal stress (chill) on various species. All these factors are common for many European countries where the cultivation of medicinal and aromatic plants is very popular.

MATERIALS AND METHODS

Experiment 1

Plant material. Plants of sage (*Salvia officinalis* L.), lemon balm (*Melissa officinalis* L.) and basil (*Ocimum basilicum* L.) obtained from the local herb producer (Swedeponic Poland) were grown in plastic pots containing organic soil, peat and sand (2:1:1, v/v), in vegetative chamber at the temperature of 22/17°C (day/night), photoperiod 14/10 h (day/night), and relative humidity ca. 50%. Plants at their vegetative phase were divided into 4 groups: control one, subjected to drought and waterlogging, and chilled one. Each group consisted 7-10 plants (biological replicates). Control and water stressed plants were grown in vegetative chamber at 22/17°C (day/night), whereas chilled plants (15/10°C) were kept in a naturally-illuminated, air-conditioned greenhouse. Other parameters were unified for all groups. The treatments were implemented for 10 days.

Methods and equipment. F_v/F_m parameter was obtained *via* the measurement of chlorophyll *a* fluorescence (PSM fluorometer; *BioMonitor AB*, Umeå, Sweden) of the youngest developed leaves of 7-10 plants. The parameter F_v/F_m was obtained after dark adaptation for 15 min. The saturating irradiance intensity was 600 μmol (quantum) m^{-2} s^{-1}.

Statistics. The statistical significance of differences was evaluated by variance analysis followed by the Duncan's multiple range test.

Experiment 2

Plant material. Maize caryopses (*Zea mays* L., local hybrid KOC 9431 obtained from *Nasiona Kobierzyc*, Poland) were sown into plastic pots (volume 2000 cm^3) with a mixture of organic soil, peat, and sand (3:1:1, v/v). Preliminary growing was performed in air-conditioned greenhouse with thermoperiod 20/17°C (day/night), photoperiod 16/9°C (day/night), air humidity *ca*. 60 %, and additional lightening during cloudy days. Plants at the phase of fully developed first leaf were divided into hardened (15°C day/night, for 7 days) and non-hardened group (20/17°C, day/night), kept in vegetative chambers. After that, chilling (5°C; vegetative chamber) was performed on plants of both groups (n=7-10).

Methods and equipment. F_v/F_m parameter was obtained as described at Experiment 1. Electrolyte leakage was measured as the tissue electroconductivity according to Dexter *et al*. (1932) with some modifications, on leaf discs of the area of 0,75 cm^2 with a conductometer with automatic temperature compensation (CC-315 Elmetron, Poland). Plant material was washed with deionized water, closed in tubes with 15 cm^3 of deionized water and shaken for 24 hours. Conductance was measured (L_{s1}), then samples were boiled at 100°C for 15 min, shaken for 24 hours and the assay was repeated (L_{s2}). To eliminate the effect of the boiling on the tubes, the conductance of deionized water was also measured, both at the beginning of analysis (L_{w1}) and during the second analysis of plant samples (L_{w2}). Electrolyte leakage was calculated as a percentage of total electrolyte content according to the equation:
$EL= [(L_{s1} - L_{w1})/(L_{s2} - L_{w2})]\cdot 100$ %
Increased percentage means more severe membrane dysfunction [Creencia and Bramlage 1974].
Pigment composition was assayed spectrophotometrically according to Lichtenthaler and Wellburn [1983] (spectrophotometer LKB Biochrom Ultrospec II, UK).

Statistics. All analyses were performed on the 3rd leaf of detached plants (n=7-10), after 7-d chilling. The statistical significance of differences was evaluated by variance analysis followed by the Student's t-test.

RESULTS

Experiment 1
Plants of all studied species subjected to drought revealed retarded growth when compared to the control ones (Fig. 1). Both wilting and desiccation were visible. These effects were accompanied by a decrease of F_v/F_m (Fig. 1); the lowest in lemon balm (97% of control), the biggest in basil (16%).
One of the symptoms of the influence of waterlogging on lemon balm and sage were diminished stem growth (Fig. 1). Control and waterlogged basil plants had their stems of similar length. F_v/F_m did not reflect these relations: its values obtained for stress-treated lemon balm and basil were lower than in the control, and in case of sage no change was noticed.
Chill also retarded the elongation processes of sage and basil stems, although chill-treated lemon balm plants were higher than those of control. Interestingly, plants of all species, also that of basil (chill-sensitive) had their F_v/F_m similar to the control (lemon balm, basil) or even higher (sage).

Figure 1. The influence of stress factors on sage (*Salvia officinalis* L.), lemon balm (*Melissa officinalis* L.) and basil (*Ocimum basilicum* L.). Mean values of F_v/F_m (n=7-10) are given together with the results of statistical analysis. Various letters mean differences significant according to Duncan's test; $p = 0.05$.

Experiment 2
F_v/F_m of chill-hardened plants was ca. 50% of the control ones, although these seedlings did not show the dysfunction of membranes, estimated as electrolyte leakage. EL of hardened seedlings was higher than that of non-hardened. The content of carotenoid in leaves, both pure concentration and that related to chlorophylls, was higher in leaves of hardened plants (all data in Table 1). This was also visible directly on leaves, which were more yellow on hardened than on control plants. Similar data and the discolouration of leaves was obtained in case of another hybrid [Bączek-Kwinta, 2002]. These data are not presented.

Table 1. Effect of chilling (5°C, 7days) on physiological and biochemical parameters of maize (*Zea mays* L.) seedlings. All differences between means of hardened and non-hardened groups were significant at $p = 0.05$ (Student's t-test).

Parameter	Hardened (15°C, day/night,7 days)	Non-hardened (20/17°C)
EL [%]	13,8	17,4
F_v/F_m	0,108	0,297
Carotenoid content μg cm^{-2}(leaf)	0,40	0,33
Carotenoid/chlorophyll ratio μg CAR μg^{-1}CHL	0,25	0,20

DISCUSSION

The growth response of studied species for drought stress reflects the importance of available water for turgorescence-promoting cell elongation (Schulze 1986). However a drop in expenditure on energy for growth, and its allocation for specific protectants synthesis together with repairing processes, has been postulated as a prerequisite for survival (Muller *et al.* 1997, İnci *et al.* 1998), we must remember that 10-day soil drought is a long-term stress. This was visible on plants of all species also as desiccation of leaves and stems.
Waterlogging influences the roots *via* anoxic conditions, because oxygen diffusion in water is 10000 times slower than in the air (Armstrong 1979). This influences the whole metabolism resulting in various perturbations (Dennis *et al.* 2000). One of them is growth restriction, as observed in our experiment. Various plant species are differentiated in their response and susceptibility to flooding. Our data confirm this opinion, and we may estimate the susceptibility in descending order: lemon balm> sage> basil. It seems to be interesting that growth response is irrespective to quantum efficiency of PS 2, and this effect would be studied in the future.
Basil belongs to the species very sensitive to chill. For this reason we decided to set the growth temperature not to be lethal (15/10°C), but to influence the plants. These thermal conditions were probably closer to optimal ones for lemon balm, and this was visible as better growth of these plants than that of considered as the control ones. To some extent this was reflected by F_v/F_m ratio – chilled: 0,770, control: 0,730, however the difference between these means were not significant. We suppose that the main factor differing F_v/F_m in control and chilled plants was not the temperature, but the light regime. Chilled plants, grown in the greenhouse, were adapted to solar light spectrum and photosynthetic photon flux density (PPFD) varied from 250 up to 1500 microeinstein. The other plants, kept in growth chambers, were illuminated with artificial light of PPFD ca. 500 microeinstein.

Maize originates from tropical and subtropical zones, and this predisposes this species to chilling injury [Lyons *et al*. 1964, Stamp 1987]. The primary effect of low temperature on plants is membrane dysfunction meant as increased permeability, leading to the leakage of native cellular elements from the cells and to influx of various intracellular particulates [Wilson and Crawford 1974]. Chill-hardening is the process which may occur in the field. Prior to the period of severe chill, the temperature may lower to non-lethal level. Our study revealed that such thermal hardening diminished membrane dysfunction assayed as electrolyte leakage. Interestingly, potential quantum efficiency of PS 2 was also lower in leaves of hardened plants, which may suggest some perturbations in photophosphorylation. Higher carotenoid content may partly explain low F_v/F_m ratio. Plants produce these pigments for protection from excessive light, especially interacting with other unfavourable environmental factors, chill in this case [Haldimann 1997]. The more energy is dissipated as heat, the less is utilized in photosynthetic machinery.

The first conclusion drawn from both experiments is that electrolyte leakage is a useful tool for estimation of condition of plants during various stress conditions. This may be confirmed by our other works performed on various species and in environmental factors [Skrudlik *et al*. 2000, Bączek-Kwinta et al. 2003, Bączek-Kwinta and Kościelniak 2004].

In opposite to EL, F_v/F_m parameter should be used with some restrictions. One should be aware that the decline of F_v/F_m value may reflect destructive influence of studied factor on PS 2. However, in case of low-temperature stress the explanation may be also the acclimatory processes leading to light energy dissipation. The response of

plants may rely on the light regime, influenced by growth conditions (growth chamber or the greenhouse, but also the sun-exposed or shaded area). This method is also not recommended in studies of yielding mechanisms [Czyczyło-Mysza 2002]. For these reasons other physiological (e.g. water content, relative water content and gas exchange), and biochemical assays (e.g. carotenoid content) should be implemented to obtain the full information about plant's status.

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[P-019]

[P-020]

INDIRECT CAULOGENESIS AT *CHRYSANTHEMUM MORIFOLIUM* RAMAT

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ABSTRACT

The technologies of vegetative multiplication *via callus* offer opportunities for obtaining and propagation of superior genotypes. This paper aims to study the *in vitro* behaviour of single-node stem segments excised from two cultivars of Chrysanthemum morifolium Ramat: "Escorte" and "La Cagouille", belonging to the collection of Botanical Garden from Iasi (Romania). It were obtained and multiplicated the calli of *Chrysanthemum morifolium* Ramat, which have been subjected to regeneration. The process of callus formation was favoured by relatively high citokinine (2 mg/l BAP) concentrations in the MS medium. This callus was generally friable, green-yellow in colour. After six weeks of incubation, one of these callus masses formed adventitious shoot buds from cells close to the callus surface. The capacity of shoot differentiation was observed on MS medium with 2mg/l BAP and 0,002 mg/l NAA. Shoots of 2-3 cm, elongated *in vitro* were rooted on the MS medium in the absence of growth regulators. The autonomous regenerated plants displayed a vigorous growth and adaptive capacity by transferring on soil.
Key words: *Chrysanthemum morifolium* Ramat, callus, organogenesis.

INTRODUCTION

The technologies of vegetative multiplication *via callus* offer opportunities for obtaining and propagation of superior genotype {1}, {2}, {3}. This paper aims to study the *in vitro* behaviour of single-node stem segments excised from two cultivars of *Chrysanthemum morifolium* Ramat: "Escorte" and "La Cagouille", belonging to the collection of Botanical Garden from Iasi (Romania). The micropropagation of *Chrysanthemum morifolium* Ramat was achieved through tissue culture technique and involved callus induction followed by shoot multiplication, rooting and establishment of plantlets in soil.

MATERIAL AND METHODS

Explants consisting in single-node stem segments were cultured on MS medium with 1 mg/l 2,4 D and 2 mg/l BAP. The shoot fragments were sterilised by sinking in Na hypochlorite 3% for 7-8 minutes. The explants were rinsed several times wits distilled sterile water. The capacity of shoot differentiation was observed on MS medium with 2 mg/l BAP and 0,002 mg/l NAA. Shoots of 2-3 cm, elongated *in vitro* were rooted on the MS medium in the absence of growth regulators. The regenerated plants were transferred *ex vitro* to soil and the first few days were protected with a transparent cover and watered with water at room temperature. The culture substrate that has been used consisted of a mixture of soil. This mixture has been sterilised by autoclaving.

RESULTS AND DISCUSSIONS

It were obtained and multiplicated the calli of *Chrysanthemum morifolium* Ramat, which have been subjected to regeneration. The process of callus formation was favoured by relatively high citokinine (2 mg/l BAP) concentrations in the MS medium and a photoperiode of 12/12. The calli developed after two weeks of culture were compact in texture and brownish in colour Following the subculturing of the 30 day-old callus resulted in the development at the initial culture of some callus islands having a green-yellowish colour and a disperse structure. At the level of these calli, organogenic structures occurred sporadically.

This callus was generally friable, green-yellow in colour. After six weeks of incubation, one of these callus masses formed adventitious shoot buds from cells close to the callus surface (photo 1 and 5). The capacity of shoot differentiation was observed on MS medium with 2mg/l BAP and 0,002 mg/l NAA. Shoots of 2-3 cm, elongated "in vitro" were rooted on the MS medium in the absence of growth regulators. The both cultivars responded in a similar way (photo 2, 3 and 6).

The autonomous regenerated plants displayed a vigorous growth and adaptive capacity by transferring on soil. This step was performed by transferring the plants on soil and covering the aerial parts with transparent plastic for maintaining high humidity (photo 4 and 7).

CONCLUSIONS

1. The capacity for shoot differentiation depended on concentration of cytokinin in the shoot-induction medium and age of the callus cultures.
2. For root induction, shoots were excised and transferred to MS medium lacking growth regulators.
3. The two cultivars of *Chrysanthemum morifolium* Ramat. regenerated *in vitro* displayed a vigorous growth capacity to the natural environment.

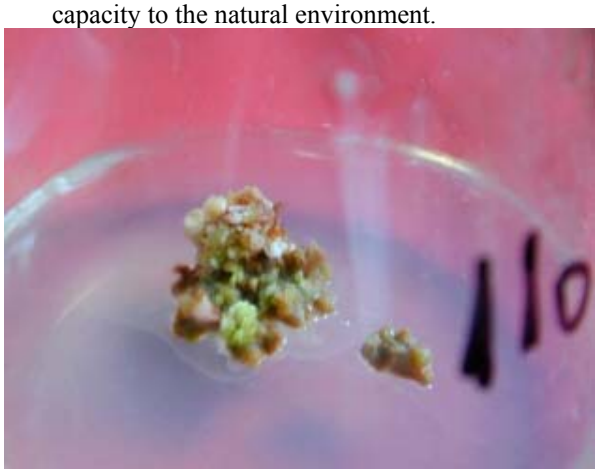

Photo 1. Callus induction from single node stem explants (La Cagouille)

Photo 2. Stages of "in vitro" caulogenesis (La Cagouille)

Photo 3. Shoots differentiation from calli (La Cagouille)

Photo 4. Plants regenerated *via callus* (La Cagouille)

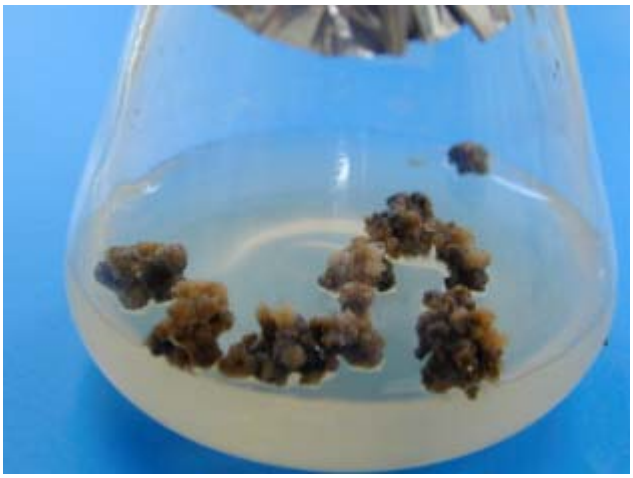

Photo 5. Callus induction from single node stem explants at *Chrysanthemum morifolium* Ramat (Escorte)

Photo 6. Shoots differentiation from callus at *Chrysanthemum morifolium* Ramat (Escorte)

Photo 7. Plants regenerated "via callus" at *Chrysanthemum morifolium* Ramat (Escorte)

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[P-020]

[P-039]

COMPARATIVE PHARMACOGNOSTICAL RESEARCH ON *ACHILLEA MILLEFOLIUM* L. AND *A. DISTANS* W. *ET* K. FROM ROMANIA

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ABSTRACT

Achillea distans W. *et* K. (*Asteraceae*) may substitute, in mountain regions, the officinal *Achillea millefolium* L. (yarrow), the former having a greater high leaves and inflorescences. *Achillea distans* was not investigated till now in Romania and our preliminary results have indicated some important differences. For chemical characterisation of the two species the comparative qualitative and quantitative analysis were made. The polyphenolic compounds (flavonoids, caffeic acid derivatives), were analysed, quantitatively by a spectrophotometric method and qualitatively by high performance liquid chromatography (HPLC), before and after hydrolysis, for both species. Caffeic acid, chlorogenic acid, p-cumaric acid, luteoline and apigenine were identified. By the microbiological tests, the essential oils showed a better activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans*. The anti-inflammatory action was also tested by the plethysmometric method and the *A. distans* tincture showed a significant activity.
Key words: *Achillea millefolium*, *Achillea distans*, polyphenols, anti-inflammatory activity.

INTRODUCTION

The species of *Achillea* genus (*Asteraceae*) are widespread in Europe, North Africa and Asia and in North America. Several species of *Achillea* are found in Romanian flora, but only *Achillea millefolium* flowers (*Millefolii flos*), official in Romanian Pharmacopoeia 10th edition [5], are used for medical purposes.

Achillea millefolium (yarrow) is one of the oldest medicinal plants, known and used for its anti-inflammatory, spasmolytic, carminative, antimicrobial, diuretic, expectorant, haemostatic, antipyretic, sedative and antirheumatic properties. The main therapeutic uses of pharmaceutical preparations from yarrow are digestive, respiratory, central nervous system, genito-urinary, dermatologic diseases, where it can be administered both internally and externally [2,4].

Achillea distans W. et K. (mountain yarrow) is one of the species which can substitute *Achillea millefolium*, being present also in hill and mountain areas, often alongside *Achillea millefolium*. The two species are similar regarding flowers and inflorescences, but *Achillea distans* is taller and presents longer and wider leaves.

Our research focused on analysis of polyphenolic compounds (flavonoids, caffeic acid derivatives), from *Achillea millefolium* and *Achillea distans* and also on testing antimicrobial and anti-inflammatory activities of the two species.

By means of GC/MS we have identified the main 30 components of the essential oils; the most interesting observation was the very low concentration of chamazulene and bisabolol in the *Achillea distans* essential oil, important compounds for the pharmacological activity of this vegetable product [3].

MATERIAL AND METHODS

Analysis of polyphenolic compounds: flavonoids and caffeic acid derivatives
Quantitative analysis of flavonoids and caffeic acid derivatives were performed spectrophotometrically and results were expressed in rutoside (flavonoids) and in caffeic acid (caffeic acid derivatives) [5]. Quantitative analyses were performed using an UV-VIS spectrophotometer Jasco V530.

Polyphenolic compounds were analysed by HPLC, using Agilent 1100 Series chromatograph, and Zorbax SB-C18 reverse-phase analytical column (100 mm × 3,0 mm, 3,5 µm particles), at 48°C. Two mobile phases were used, methanol and buffer solution containing 40 mM KH_2PO_4, and adjusted to pH=2,3 with 85% phosphoric acid. During the run concentration of methanol was increasing from 5% to 42% over the first 35 minutes,

followed by isocratic elution with 42% methanol over the next 3 minutes. The flow rate was 1 ml/min and data were collected at 330 nm. Injection volume was 10µl.

Sample preparation
Dried vegetal product was extracted in Soxhlet with chloroform, then with methanol; the residue was dissolved in hot water; successive extractions in separation funnel with ethyl ether, ethyl acetate and n-butanol were performed. The methanolic solutions of the reuniting residues were analysed by HPLC. In order to study the flavonoidic aglycons, a hydrolysis with 2N HCl was performed.

Testing of antimicrobial activity
Antimicrobial activity of essential oils from *A. millefolium* and *A. distans* inflorescence, was tested by a difusimetric method. The following bacteria were used: *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and a fungus *Candida albicans* [1].

Testing of anti-inflammatory activity
General anti-inflammatory activity was evaluated by rat paw oedema test, induced by kaolin 10% - plethysmometric method - using phenylbutasone as control. The tested samples were made of the tinctures (1:10 in ethanol 70%) obtained from *A. millefolium* and *A. distans* by maceration.

Solutions were administered orally, 1 h prior to the induction of the acute inflammation with kaolin 10%. Inflammation was evaluated at 2h, 4h and 24 h after induction, the following parameters being calculated: mean value of inflammatory oedema (X), standard error, percentage of inhibition of inflammatory oedema compared to the witness lot with the following formula: % inhibition = (1-X substance/X witness) x 100.

RESULTS AND DISCUSSION

The flavonoids concentrations expressed in rutoside were 0,08 % in *Achillea millefolium* and 0,33 % in *Achillea distans.* The caffeic acid derivatives concentrations expressed in caffeic acid were 0,32 % in *Achillea millefolium* and 1,67 % in *Achillea distans*.

Table 1. Constituents determined by HPLC

No.	Rt	Compounds	*A. millefolium* (before/after hydrolisis), mg/100 g	*A. distans* (before/after hydrolisis), mg/100 g
1	5.85	caffeic acid	2.80 / 27.67	2.43 / 68.50
2	6.63	chlorogenic acid	32.30 / 31.80	104.50 / 98.40
3	9.15	p-cumaric acid	- / 7.57	- / 14.30
4	12.43	ferulic acid	- / -	3.07 / 3.27
5	29.24	luteoline	13.43 / 28.97	36.20 / 101.3
6	33.24	apigenine	6.20 / 33.00	9.20 / 31.50

Caffeic acid, chlorogenic acid, luteoline and apigenine were identified in *Achillea millefolium* extracts. After hydrolysis, a significant increase in caffeic acid concentration was observed (as a result of the hydrolysis of its esters), and also cumaric acid appeared; luteoline and apigenine quantities increased, as aglycones obtained by hydrolysis of flavonoid-O-glucosydes (Table 1).

The remanence of some flavonoidic glycosides in the chromatogram of hydrolised extract is explained by the fact that these are C-glycosides (vitexine, orientine), which cannot be hydrolised in the conditions mentioned above (Fig. 1, 2).

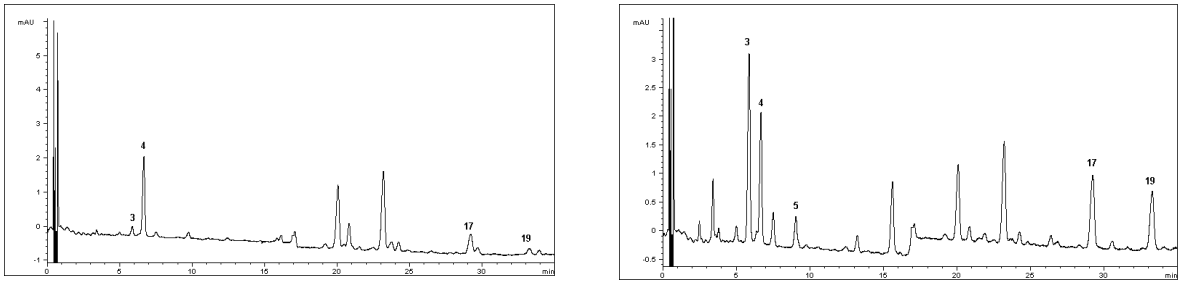

Fig. 1. HPLC chromatograms of *A. millefolium* extracts before (left) and after hydrolysis (right).

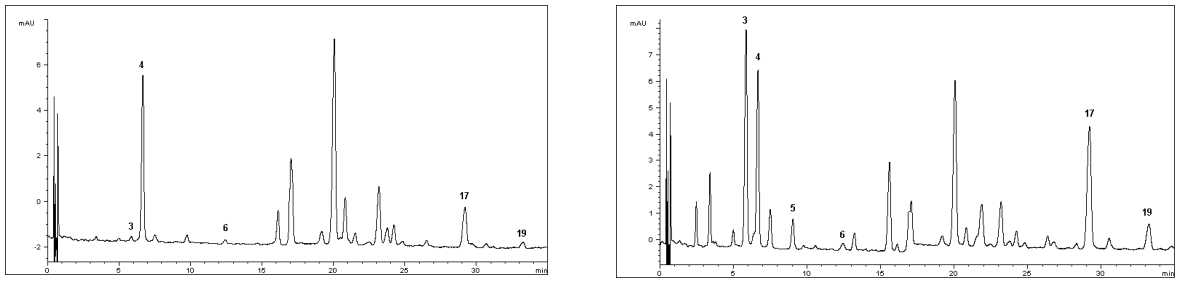

Fig. 2. HPLC chromatograms of *A. distans* extracts before (left) and after hydrolysis (right). **Standards**: caffeic acid (3), chlorogenic acid (4), p-cumaric acid (5), ferulic acid (6), luteoline (17), apigenine (19).

Testing of antimicrobial activity

The most significant activity for the essential oil from *A. millefolium* was detected on *Bacillus subtilis, Staphylococcus aureus, Candida albicans* and for the essential oil from *A. distans,* on *Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus.*

Table 2. Antimicrobial activity of *A. millefolium* and *A. distans* essential oils

No.	Microorganism	*A. millefolium* – inhibition area diameter	*A.distans* – inhibition area diameter
1	*Escherichia coli*	5 mm	8 mm
2	*Bacillus subtilis*	15 mm	30 mm
3	*Enterococcus faecalis*	5 mm	20 mm
4	*Staphylococcus aureus*	15 mm	30 mm
5	*Pseudomonas aeruginosa*	-	-
6	*Candida albicans*	15 mm	8 mm

Diameter: 10-15 mm - weak effect, 15-25 mm - significant effect, >25 mm- strong effect.

Results were confirmed also by determination of the minimum inhibitory concentration (MIC), and the higher concentration of essential oil from *A. millefolium* was required for achieving of an antibacterial effect.

Testing of antiinflammatory activity

Table 3. Antiinflammatory activity of the tinctures from *A. millefolium* and *A. distans*

Lot No.	Sample	Dose (mg/kg)	Oedema 2 h X ± s.e.	Oedema 4 h X ± s.e.	Oedema 24 h X ± s.e.	% of oedema inhibition (2 h)	% of oedema inhibition (4 h)	% of oedema inhibition (24 h)
1	Distilled water	-	1.18 ± 0.09	1.52 ± 0.1	1.12 ± 0.12	-	-	-
2	Phenylbutasone	50	0.75 ± 0.05	1.91 ± 0.06	0.86 ± 0.09	36.44	40.13	23.21
3	*A. millefolium*, tinct.	500	0.68 ± 0.05	0.82 ± 0.07	0.84 ± 0.09	42.37	46.05	25
4	*A. distans*, tinct.	500	0.74 ± 0.07	0.73 ± 0.12	0.86 ± 0.03	37.28	51.97	23.21

The phenylbutasone lot presents an important reduction of inflammatory oedema at 2h, 4h and 24 h, confirming the anti-inflammatory activity of this compound.

The *A. millefolium* lot presents also a reduction of inflammatory oedema, the percentages of oedema inhibition being significant at 2h and 4h after induction.

The *A. distans* lot presents a reduction of inflammatory oedema especially at 4h after induction, the percentage of inhibition being superior even to phenylbutasone and also to the *A. millefolium* lot. Percentages of inhibition of inflammatory oedema are similar to phenylbutasone and inferior to the *A. millefolium* lot, at 2h and 24h after kaolin administration.

The tinctures contain polyphenolic compounds (flavonoids, polyphenolcarboxylic acids), terpenes, tannins. The higher anti-inflammatory activity of *A. distans* tincture could be explained by a higher concentration of polyphenolic compounds. The azulenes with anti-inflammatory activity are not present in the tinctures.

CONCLUSIONS

►Comparative phytochemical and pharmacologic studies were performed on *A. millefolium* and *A. distans.*
►Some flavonoids and polyphenol-carboxylic acids were identified by HPLC, in higher concentrations after hydrolysis; *A. distans* possessing a higher concentration of these active substances.

►Microbiological studies revealed a superior antimicrobial activity of essential oils from *A. distans* against *B. subtilis, Enterococcus faecalis* and *Staphylococcus aureus*.
►General anti-inflammatory activity of *A. distans* was significant, polyphenols being responsable for this effect.
►Although *A. distans* does not contain azulenes, it turned out to possess good anti-inflammatory and antimicrobial properties, due to a higher content of polyphenols.
►Research will continue to focus on therapeutic uses of the two species, considering the chemical composition differences.

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[P-039]

[P-042]

COMPARATIVE STUDY OF VOLATILE CONSTITUENTS AND ANTIMICROBIAL ACTIVITY OF *JUNIPERI FRUCTUS* SAMPLES FOR PHARMACEUTICAL USE

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ABSTRACT

Juniperi fructus is a medicinal product, which contains volatile oils known for its diuretic effect; particularly terpinen-4-ol, may cause an increase in urine volume without a loss of electrolytes such as potassium. Chemical studies of volatile fractions, realised upon 5 types of medicinal tea, *Juniperi fructus*, were based on the isolation, identification and quantitative evaluation of volatile oils. Qualitative and quantitative characterisation of these five volatile samples were performed by GC and GC/MS techniques. It can be observed from the quantitative results that there is a comparative significant variation in the content of volatile fractions isolated form juniperberry (2,4-5,0%). In all investigated samples a relative high content of terpinen-4-ol was detected. These five volatile samples exhibited strong antibacterial activities, particularly against *B. subtilis* and *B. cereus*; moderate antibacterial activities against *Sarcina* and *Klebsiela*. The most resistant bacteria was *S. aureus*. Different antimicrobial activities of the examined essential oils seem to be due to the differences in the oil composition.
Key words: juniperberry oil, GC/MS, antibacterial activities.

INTRODUCTION

Juniperi fructus (*Juniperus communis* L., *Cupressaceae* fam.) is a well known medicinal product, which contains volatile oils known for its diuretic effect; particularly terpinen-4-ol, may cause an increase in urine volume without a loss of electrolytes such as potassium [1, 3, 5]. The dried fruits are used in chronic bladder and kidney infections with atony, for edema caused by renal suppression, renal congestion, amenorrhea or dysmenorrhea, digestive atony, arthritis, gout and sciatica. The ability to relieve the pain of inflammation in arthritis, gout and other diseases may be due to its prostaglandin-inhibiting action. Juniper is used as a flavour in gin [2, 4]. In this paper we tried to reveal some differences in chemical composition and antimicrobial activity of volatile oils obtained from 5 types of *Juniperi fructus*, medicinal tea, which are commercialised in pharmacies from different suppliers. The samples were codified and given numbers from J1 to J5.

MATERIAL AND METHODS

Chemical studies of volatile fractions, realised upon 5 types of medicinal tea, *Juniperi fructus*, were based on the isolation, identification and quantitative evaluation of volatile oils. The volatile oil was isolated by steam distillation in Neo-Clevenger apparatus.
Qualitative and quantitative characterisation of isolated five volatile oil samples was performed by GC and GC/MS. An analytical system, consisting of HP 5890 Series II GC and HP 5971 MSD, was used. GC/MS analysis of the essential oil samples was performed on HP-5MS capillary column (25 m x 0.25 mm x 0.25 μm) coated with cross-linked methyl silicone gum. Carrier gas was helium at the flow rate of 1 ml/min. Temperature program: 40°C held for 5 min, then heated up to 260 °C at 10 °C/min, and for 5 additional minutes kept at this temperature. The temperature of injector was 250 °C and of MS interface 280 °C.

Identification of each individual compound was made by comparison of their retention times with those of the authentic samples, in the same operating conditions, and by computer searching, matching mass spectral data with those held in the computer library. For these purposes Wiley 275.L library, operating with collection of 135.720 mass spectra, was used.

Antimicrobial tests were performed using diffusimetric method based on the diffusion of the tested volatile oils on the gelose surface. To determine the antibacterial and antifungal activity the 5 volatile oils were tested against *S. aureus, Sarcina, B. subtilis*, *B. cereus, E. coli, Klebsiella, Acinetobacter* and *Candida albicans*.

RESULTS AND DISCUSSION

The quantitative results showed that there is a comparative significant variation in the content of volatile fractions isolated from juniperberries (2,4-5,0%) (Table1). The GC/MS analysis of those five volatile oils allowed the identification of over 70 components (Table 2). In all investigated samples a relative high content of

terpinen-4-ol was detected, but with significant quantitative variation. The same situation was revealed for α-pinene, β-caryophyllene, γ-elemene and other important constituents. The sabinene was found in relative small amounts, in all samples, that indicates the maturity of juniperberries. Those five volatile samples exhibited strong antibacterial activities, in particularly against *B. subtilis* and *B. cereus*; moderate antibacterial activities against *Sarcina* and *Klebsiela*. The most resistant bacteria was *S. aureus* (Table 3).

Table 1: The content of volatile oil in juniperberries samples

Juniperi fructus	Oil content (ml/100 g)
Sample J1	2,4
Sample J2	3,7
Sample J3	4,0
Sample J4	2,8
Sample J5	5,0

Table 2: Composition of different samples of juniperberries essential oil

RT	Constituents	Sample J1	Sample J2	Sample J3	Sample J4	Sample J5
6.77	α-pinene	23.74	26.75	23.01	16.97	21.65
6.86	δ^4-carene	1.31	n.i.	n.i.	0.98	n.i.
6.95	verbenene	1.37	n.i.	n.i.	n.i.	n.i.
7.30	sabinene	6.41	9.02	9.19	4.06	8.32
7.35	β-phellandrene	n.i.	n.i.	n.i.	n.i.	n.i.
7.55	myrcene	6.72	8.77	10.50	7.11	9.36
7.79	α-phellandrene	0.49	0.16	0.24	0.40	0.20
7.97	α-terpinen	1.04	0.69	0.96	1.91	1.04
8.27	limonene	9.60	5.73	5.60	6.67	4.91
8.70	γ-terpinene	2.36	1.06	2.04	2.70	1.62
8.79	trans-sabinene hydrate	0.13	0.24	n.i.	n.i.	n.i.
9.19	α-terpinolene	1.94	1.02	1.57	n.i.	1.21
9.62	β-thujone	0.19	n.i.	n.i.	0.34	n.i.
9.80	α-campholenal	n.i.	0.85	0.77	n.i.	0.76
9.94	terpinene-1-ol	n.i.	n.i.	n.i.	0.34	n.i.
10.14	camphor	n.i.	n.i.	n.i.	n.i.	0.96
10.15	dihydrocarvone	n.i.	n.i.	n.i.	1.86	n.i.
10.22	cis-verbenol	n.i.	0.68	0.40	n.i.	n.i.
10.83	terpinen-4-ol	12.43	3.52	6.90	12.69	7.18
10.95	α-terpineol	n.i.	0.53	0.53	1.89	1.11
11.04	myrtenol	0.94	0.49	0.40	n.i.	n.i.
11.23	verbenon	0.25	0.84	0.71	1.19	n.i.
11.64	cuminal	0.64	n.i.	0.18	n.i.	0.15
11.67	carvone	n.i.	0.15	n.i.	0.36	n.i.
11.89	piperitone	0.30	n.i.	n.i.	0.22	n.i.
12.32	bornyl acetate	n.i.	0.65	0.46	0.55	0.42
12.33	estragole	1.35	n.i.	n.i.	n.i.	n.i.
12.45	2-carene	n.i.	n.i.	n.i.	0.34	n.i.
12.93	myrtenol	0.33	0.20	n.i.	0.29	0.15
13.28	α-cubenene	0.75	0.82	0.90	0.75	0.92
13.36	α-longipinene	0.13	n.i.	0.17	0.21	n.i.
13.68	α-copaene	1.15	0.47	0.49	0.50	0.47
13.95	β-elemene	0.95	1.76	2.21	1.06	1.85
14.11	α-elemene	0.14	0.20	0.15	n.i.	0.17
14.23	junipene	n.i.	0.14	0.40	0.37	0.15
14.43	β-caryophyllene	1.53	2.88	2.72	1.98	4.45
14.58	γ-elemene	1.75	4.33	5.51	3.52	6.06
14.72	trans-β-farnesene	0.45	0.74	0.77	0.68	n.i.
14.93	α-humulene	1.21	2.21	1.96	1.54	2.79

15.32	germacrene D	1.25	5.23	5.47	3.91	5.91
15.32	β-selinene	0.53	n.i.	n.i.	n.i.	n.i.
15.43	α-muurolene	n.i.	n.i.	n.i.	1.26	1.53
15.47	α-gurjunene	0.94	1.54	1.53	0.51	0.62
15.61	α-amorphene	0.47	n.i.	0.97	n.i.	0.65
15.77	δ-cadinene	1.20	3.17	2.52	2.33	2.16
15.94	β-gurjunene	0.26	n.i.	0.50	n.i.	n.i.
16.00	epizonarene	0.33	n.i.	n.i.	0.48	n.i.
16.06	γ-selinene	n.i.	0.43	0.38	n.i.	n.i.
16.27	germacrene B	0.38	0.40	0.35	0.62	0.56
16.57	spathulenol	0.92	2.25	1.08	2.70	0.89
16.60	caryophyllene oxide	0.95	n.i.	0.63	n.i.	0.64
16.81	alloaromadendrene	0.27	1.03	0.46	0.67	0.40
17.09	cadina-1,4-diene	n.i.	0.41	0.31	n.i.	n.i.
17.28	valencene	0.75	1.44	n.i.	1.51	1.28
17.48	α-cadinol	0.67	1.61	1.09	1.82	1.36
17.80	vulgarol B	0.38	n.i.	0.27	0.44	n.i.
17.98	juniper camphor	n.i.	n.i.	0.27	0.69	0.36
21.12	cembrene	0.05	0.49	0.07	0.21	0.15

n.i. - not identified

Table 3: Antibacterial properties of juniperberry volatile oil (inhibition zone diameter in mm)

Sample	*S. aureus*	*Sarcina*	*B. subtilis*	*B. cereus*	*E. coli*	*Klebsiella*	*Acinetobacter*	*Candida*
J1	12	15	22	30	7	0	15	30
J2	7	0	9	10	0	0	7	14
J3	10	0	6	10	0	7	9	0
J4	9	0	18	14	7	7	10	8
J5	30	22	24	28	0	6	7	0

CONCLUSIONS

The chemical composition and antimicrobial properties of essential oils obtained from five pharmaceutical samples of *Juniperi fructus* were examined.

The essential oils were isolated by steam *distillation* and analyzed by GC/MS. The most important identified compounds, well known for their antimicrobial effects were α-pinene, β-carophyllene, limonene, myrcene, terpinen-4-ol, camphor (as antibacterial) and terpinolene and spatulenol (as antifungal).

The microbiological study revealed for J1 and J4 samples a significant antimicrobial activity on the tested strains, particularly against *B. subtilis, B. cereus, Sarcina, Acinetobacter*. Sample J1and J2 exhibited considerably stronger antifungal activity against *Candida albicans.*

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[P-053]

GROWING RESULTS OF SOME MAP SPECIES AT MOUNTANEOUS REGION OF SERBIA

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ABSTRACT

The aim of this investigation was to test possibilities of growing of following MAP species in mountainous region: *Arnica montana, Arnica chamissonis, Gentiana lutea* L., *Angelica archangelica* L. *and Origanum vulgare* L. Testing was conducted in ecological conditions of mountain Tara (1004 m a. s. l., g. latitude 43°$_N$, 53', 41" and g. longitude 19°$_E$, 33', 41") in order to introduce these MAP species into culture and to organize production of seeds and planting material in ecologically non-contaminated environment. The experiments started in autumn 2001., and lasted up to 2005., and they were conducted at small scale in order to select appropriate plants for given growing conditions. Special attention was devoted to introduce into culture ***Arnica montana*** and ***Arnica chamissonis*** (both species not present in Serbian flora) and to solve the optmal mode of nursery plant production for **Yellow Gentian** (without hormonal treatment). Out of MAP crpps commonly grown in Serbia, the best results in climatic conditions of mountain Tara reached Angelica and Oregano. The yield of **Angelica** was 8778 kg/ha of fresh roots (dry 2090 kg) with 0,7% of essential oil. In the first growing year the yield of **Oregano** was 411 kg/ha and in the second one 2833 kg/ha of dry herb. All tested MAP species gave acceptable results regarding development of their aboveground plant parts so it can be concluded that these crops may be successfully grown in agro-ecological conditions that offers mountain Tara. In following period investigation on economical parameters in order to estimate growing thrift are should be faced.
Key words: growing in mountainous region, *Arnica montana*, *Arnica chamissonis*, Yellow Gentian, Angelica, Oregano

INTRODUCTION

Recently, growing of medicinal plants becomes more and more rentable job so that development of this kind of occupation for most producers becomes increasingly attractive. Here, the special attention attracts development of MAP production in mountaneous region with its several advantages. As a rule, these regions are out of reach of antropogenuous polution so they can satisfy even the most rigid criteria of ecological – biological production. The results obtained in this investigation may find wider application and may decrease depopulation mountain regions in Balkan countries.

Mountain Tara is situated at the most western part of Serbia and it spreads over the region limited by the course of river Drina, between Višegrad and Bajina Bašta. In geo-morphological sense, there are few mountainous plateaus at Tara, ranging from 900 to 1250 meters above sea level that seem to be appropriate for cultivation of some montaneous medicinal and aromatic plant (MAP) species.
The aim of this investigation was to test possibilities of growing of some MAP species in order to introduce them into culture in ecologically non-contaminated environmental conditions of mountain Tara as well to organize production of their reproductive material (seeds and nursery plants).

MATERIAL AND METHODS

The experiments started in autumn 2001, and lasted up to the end of 2005 (table 1).
MAP species:
MAP species selected for investigation are presented in table 1. Only two of them, *Arnica montana* L and *Arnica chamissonis* L , do not belong to Serbian Flora.
Cultivation conditions:
Experimental fields were established at locality Kaluđerske bare (1004 m a. s. l., g. latitude 43°N, 53', 41" and g. longitude 19°E, 33', 41"), in the forest tree nursery of National park "Tara". The main soil and climate features of this locality are given in table 2.
The experimental plots of *Arnica chamissonis, A. montana,* Angelica and Oregano were fertilized with NPK (15-15-15) fertilizers in dose 600 kg/ha before planting, with further nitrogen application (150 kg/ha - KAN 27% N) following transplantation of nursery plants.
Weeding was carried out mechanically several times during vegetation period.
The size of experimental fields varied among the crops from 2 -15 are (0,02 – 0,15).

Table 1. MAP species cultivated on Tara mountain during the experimental period 2001 – 2005.

Common name	Latin name	Cultivated for:	Production of reproductive plant material	Experimental year
Arnica	*Arnica montana* L	flower		2003 – 2005
Arnica	*Arnica chamissonis* L	flower		2003 – 2005
Yellow Gentian	*Gentiana lutea* L.	root	seeds nursery plants	2002 – 2005
Angelica	*Angelica archangelica* L.	roots	seeds	2001 – 2003
Oregano	*Origanum vulgare* L.	herb		2003 – 2004

Table 2. Main features of the climate and soil on experimental locality Kaluđerske bare.

	Mountain Tara, locality Kaluđerske bare (1004 m a. s. l.)	
Climate	Annual T/Precipitation	6,1 ºC / 900 mm
	IV-IX T/Precipitation	11,9 ºC / 448 mm
Soil *(Distric cambisol)*	pH (H_2O)	5,5
	% Clay	7,0 %
	% Humus	5,0 %
	P_2O_5 mg/100g	5,3 mg/100g
	K_2O mg/100g	20,0 mg/100g

Measurements:
Fresh and dry yields of Arnica flower, Angelica root and Oregano herbae (upper 25 cm of plant) were observed, and the obtained row plant material was evaluated for its quality in the Laboratory for Quality Control of Institute for Medicinal Plant Research „Dr J. Pančić“, Belgrade.
Rate of Yelow Gentian seed germination was estimated every year in the spring and development of nursery plants were monitored through all phases of nursery plants development in the I vegetation.

EXPERIMENTAL PRODUCTION (2001 – 2005)

Based on five-years experimental period in production of five MAP species, in this paper there are presented the most important results regarding possibility of their cultivation on mountain Tara. The review includes short description of each crop and results of yields obtained in experimental production.

ARNICA – *Arnica montana* L. and *Arnica chamissonis* L.

Arnica is herbaceous perennial plant. The plant height ranges from 30 – 60 cm. One or two pairs of leaves form a flat rosette. From the center of rosette rises a round and hairy stalk that ends in 1-3 flower stalks, bearing each one orange-yellow daisylike blossom (flowers in *A. montana* are 5 -8 cm in diameter while in *A. chamissonis* they are smaller). In the nature, Arnica grows on poor acid soils of montaneous pastures, above 1000 m a.s.l. The main quantitues of this herb are still provided from the nature, deriving from Balkan, Romania, Spain, and Switzerland (Lange, 1998).
Arnica thrives the best on wet, well drained and fertile soils, pH ranging 5,5 – 6,5, without limestone. Bears full light but it prefers partial shade.
Arnica montana is difficult to be grown and is often replaced by *A. chamissonis* whose growing is quite easier. Chemical properties of *A. chamissonis* are not identical to those of *A. montana*, so *A. chamissonis* is not accepted in all countries for its medicinal purposes as it is a case with its common use in cosmetic industry.
Following constituents are present in Arnica: flavonoids, sesquiterpene lactones, coumarines, essential oils, tannins, etc. The flowers are said to contain more arnicin then rhiyome, but no tannin. Helenalin and dihydrohelenalin produce anti-inflammatory and analgesic effect. Its main external use: treatments of bruises and sprains, rheumatic pain, phlebitis, skin inflammations. Its use in pharmaceutical industry all over the World is in continuous increase so the shortage of this herbal drug is confirmed for a range of years. Since it thrives only in montaneous environmental conditions its cultivation is limited and in most contries is ussually conducted on small soil surfaces. Our experiments conducted on Tara mountain had for a main aim introduction of these two species into culture in order to satisfy domestic demands.

Experimental production of *Arnica montana* L. and *A. chamissonis* L. at Tara mountain (2003 -2005.)
Our experiments with two Arnica species (*A. montana* and *A. chamissonis*) started in 2003, with nursery plants production.

Summer nursery plants production showed satisfactory results only in case of *A. chamissonis*. Seeds were sown in June in open beds and in Octobar 2003. 3600 nursery plants were transplanted in 700 m^2 of open field (70 x 25-30 cm, crop density applied 515 plts / 100^2 m)

Since preliminary investigations showed that summer nursery plants production in case of *A. montana* was not succesful, the nursery plants were produced in greenhouse conditions, in containers, during the winter period February – April, 2003.). The 720 nursery plants were transplanted in 200 m^2 of open field by the end of May (70 x 40 cm). The plants of *A. montana* flowered for the first time in the next year vegetation (2004). The yields of both Arnica species, obtained in the I and the II vegetation are presented in table 3.

Table 3. Yields of *Arnica montana* and *A. chamissonis* obtained in experimental production on Tara mountain (2004 and 2005.).

2004. **I year of exploitation**		**Harvest / date**							**Fresh flower yield in all harvests (kg)**	**Dry flower yield in all harvests (kg)**	**Dry lower yield (kg/ar)**
		I 6.7.	**II 14.7.**	**III 20.7.**	**IV 26.7.**	**V 04.8.**	**VI 13.8.**	**VII 20.8.**			
***A. chamissonis* established X/2003**	**kg**	-	8,6	12,9	12,6	11,6	10,8	8,6	**65,1**	**13,0**	**1,864**
	%	-	13,2	19,8	19,5	17,8	16,6	13,2	***100,0***		
***A. montana* established V/2003**	**kg**	0,95	0,84	0,94	0,65	-	-	-	**3,38**	**0,6**	**0,3**
	%	28,1	24,9	27,8	19,2	-	-	-	***100,0***		

2005. **II year of exploitation**		**Harvest / date**					Ukupno kg svežeg cveta	Suvog cveta	
		I 14.6	II 17.6.	III 20.6.	IV 23.6.	V 29.6.		ukupno kg	kg/aru
***A. chamissonis* established X/2003**	**kg**	23,2	26,5	46,5	25,0	1,6	**122,8**	**24,0**	**3,43**
	%	18,9	21,6	37,8	20,4	1,3	***100,0***		
***A. montana* established V/2003**	**kg**	9,3	6,2	18,6	6,3	1,5	**41,9**	**6,8**	**3,40**
	%	22,2	14,8	44,4	14,9	3,7	***100,0***		

Arnica chamissonis had much faster development and reached significantly higher dry flower yields already in the first year of exploitation in comparison to dry flower yields of *A. montana*. *A. montana* reached its aboundant flower yields in the second year (table 3.). Yields in the II year of exploitation were identical in both species, but in comparison to results (5 kg and more of dry flower per ar) obtained in Germany (Bomme et al., 1995) and Finland (Galambosi, 2004), they were quite lower. We assume that the main reason for the lower yields might be in lower plant density applied, esspecially in case of *A. montana* (cca. 360 plts/ar), which was for 2,5 – 3 lower then it is reported in the literature (Munoz, 1987; Dachler und Pelzman, 1999).

Table 4. Content of total sesquiterpene lactones in dry flowers of two cultivated Arnica species, in their second year of exploitation (2005.).

Year	Vrsta	Harvest / date	Total sesquiterpene lactones (*expressed as helenalin tiglate*) in dry Arnica flowers (%)
2005.	*A. chamissonis* established X / 2003.	I harvest / 14. 06.	**0,76**
		III harvest / 20. 06.	**0,39 – 0,48**
		V harvest / 29. 06	**0,24 – 0,40**
	A. montana established V / 2003.	I harvest / 14. 06.	**1,25**
		III harvest / 20. 06.	**1,21**
		V harvest / 29. 06	**1,26**

Chemical evaluation of of produced row material - herbal drug *Arnicae* flos - produced in our experiments included: determination of content of total sesquiterpene lactones (expressed as helenalin tiglate, calculated with reference to the dried drug), according to European Pharmacopoeia (suppl. 2000), and the results are presented in table 4.
In comparison to *A. montaa*, content of total sesquiterpene lactones in *A. chamissonis* was much variable between the harvests. In general, content of total sesquiterpene lactones in *A. montana* was about 2,5 times higher then the value *A. chamissonis* (table 4.).

Photo 1. *A. chamissonis* in the II year of exploitation, Tara (2004.)

Photo 2. *A. chamissonis* in the II year of exploitation, Tara (2005.)

Photo 3. *A. montana* in the I year of exploitation, Tara (2004.).

Photo 4. *A. montana* in the II year of exploitation, Tara (2005.).

Table 5. Testing of Yellow Gentian nursery plants production in open beds, mountain Tara (2002 – 2004).

Seed collection	**Set up of experiment**	**Pre-sowing seed treatment**	**Date of sowing and chronology of observations during nursery plants sprouting and development**
August,2002. (experimental plantation at mountain Suvobor)	November 2002 - August, 2003	Sown without pre-sowing treatment Seed hibernated during the winter in open beds In March, 2003, PVC foil positioned over the open beds (low tunnel)	**13th November, 2002. – sowing** 15th April, 2003. Germination and beginning of sprouting (rate of germination estimated as ca. 30%) 21st May 2003. Phase of the first pair of permanent leaves 4th Jun 2003. Phase of two pairs of permanent leaves 8th July 2003. Three pairs and development of fourth pair of leaves 15th August 2003. Rosette composed of 6-7 pairs of leaves
August, 2003. (experimental plantation at mountain Suvobor)	November, 2003 – September, 2004.	Sown without pre-sowing treatment Seed hibernated during the winter in open beds 10th March, 2003 PVC foil positioned over the open beds (low tunnel)	**13th November, 2003 – sowing** 20th April, 2004 germination and beginning of sprouting 10th May 2004, phase of first pair of cotyledon leaves (sprouting rate estimated as ca. 35 %). 26th May 2004, first pair of permanent leaves 1st July 2004 - phase of the second pair of permanent leaves 14th July 2004 – beginning of development of third pair of leaves 13th August 2004 – development of fourth pair of leaves 15th September, 2004 - rosette composed of 5-6 pairs of leaves
`August, 2004. (experimental plantation at mountain Suvobor)	November, 2004 – September, 2005	Sown without pre-sowing treatment Seed hibernated during the winter in open beds 10th March, 2003. PVC foil positioned over the open beds (low tunnel)	**30th November, 2004 – sowing** 13th April, 2005 – beginning of germination 27th April, 2005 – beginning of sprouting (rate of germination estimated as ca. 40 – 50 %) 14th May, 2005 phase of first pair of cotyledon leaves 2nd June 2005 – phase of the first pair of permanent leaves 21st July 2005– development of third pair of leaves 4th August, 2005 phase of third pair of permanent leaves 6th September, 2005 - rosette composed of 4-6 pairs of leaves 29th September, 2005 - rosette composed of 5-7 pairs of leaves

YELLOW GENTIAN (*Gentiana lutea* L.)

Yellow Gentian (*Gentiana lutea*) is a herbaceous perennial and grows in mountainous regions, on meadow and open slopes from the Pyrenees to the Carpathian mountains, and from Alps to the Balkan peninsula and Anatolia. In many countries a general decline in population size dedicated to commercial exploitation has lead to Red Data Book (in Romania, Portugal, Bulgaria, Bosnia and Herzegovina, Albania, Germany, Czech Republic, Poland etc.).

The dry root of Yellow Gentian contains bitter substances which stimulate the digestive system and are used in a great variety of phytomedicines and other herbal preparations. The root of Yellow Gentian is the base of various bitters, the most famous being the "Enzian schnapps". Although cultivation takes place, the plant is only grown on an area of about 150 ha in total, above all in France, but also in Bavaria, Austria and Italy.
In Serbia, Yellow Gentian grows wild solely in mountainous regions (800 – 2500 m a.s.l.) and it may be found on following mountains: Stara mountain, Zlatibor, Tara, Kopaonik, Suvobor, Povlen, etc. Its natural stands in Serbia are soils of different types, formed equally on calcareous parent material and serpentine (Radanović et al., 2005b). Due to excessive exploitation from the nature its survival in the nature is endangered. Orientation to large-scale production of this plant species both, protection of natural resources and satisfaction of market demands may be provided. Regarding successful cultivation of Yellow Gentian production of good quality nursery plants may be considered for a main limitation (especially its seed dormancy). In the literature few methods of stratification in order to overcome Yellow Gentian seed dormancy are described (Fritz und Franz, 1980; Barralis et al., 1986; Dachler und Pelzman, 1999; Radanović et al., 2005a).

Experimental production of Yellow Gentian (*Gentiana lutea* L.) on mountain Tara (2002 – 2005.)

In attempt to find out the simplest and the cheapest procedure for Yellow Gentian seed stratification and nursery plant production for agro-ecological conditions of mountain Tara, during experimental period 2002 – 2005., a modified method of nursery plant production in open beds was tested (modification of procedure described by Fritz, D., and Franz, C., 1980.). The modified method involves following activities: seeds are sown in open beds each year in November, at 100 m^2 (3 g of seeds per m^2). Seeds were exposed to local climatic conditions and seedling emergence usually started in April, while the nursery plants in open beds developed during entire vegetation (Photo 5.). Nursery plants were ready to be transplanted in autumn (September) or in spring, following year (April – May). Results of experimental Yellow Gentian nursery plant production on mountain Tara in 3-years period are summarized in table 5.

Diagram 1. Height and duration of snow cover during the winter 2002 / 2003 for locality Tara (MS Zaovine)

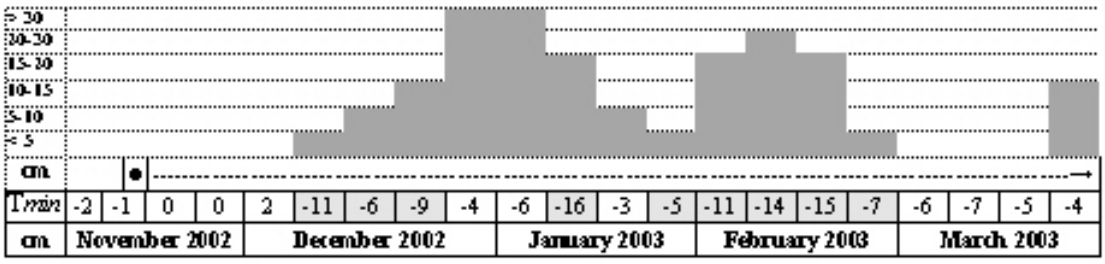


Diagram 2. Height and duration of snow cover during the winter 2003 / 2004, Tara mountain (MS Zaovine)

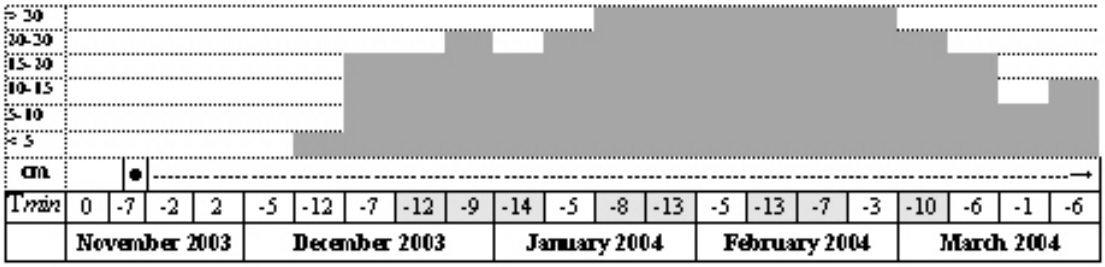
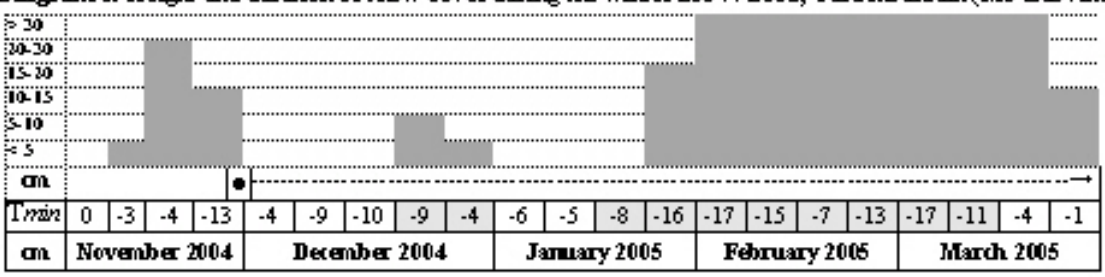


Diagram 3. Height and duration of snow cover during the winter 2004 / 2005, Tara mountain (MS Zaovine)



Legend:

Snow cover in cm

Tmin
- -3 - minimal air temperature (°C) in stated period
- -3 - minimal air temperature (°C) during period when maximal diurnal temperature did not reach 0 °C.
- ● - sowing date of Yellow Gentian seed

Taking in consideration that the snow cover is important for seed stratification and hibernation of Yellow Gentian seeds in the nature, data on snow cover and minimal temperatures during three winter periods are presented in diagrams 1-3. As it can be seen from diagrams, the snow cover in all years was continuously present longer then 70 days period, thus provided favourable temperature and moisture preconditions for breaking seed dormancy during 9-10 weeks, as stated in the literature that it is essential for inducing Yellow Gentian seed germination (Fritz und Franz, 1980; Barralis et al., 1986). Low temperatures without snow cover present in short-termed period, in January, 2005. (diagram 3.) did not influence seed germination (30-40%, table 5.). However, this phenomenon caused harm on one-year old nursery plants that were not transplanted in autumn but left to be transplanted following year (photo 7). Since the same situation (frost occurrence) obviously may jeopardize nursery plant production, one-year old nursery plants left in open beds during winter period have to be protected by covering with straw layer or similar organic material.

Following this method of nursery plant production, producers may obtain already in September, next year cca. 40 – 50 % nursery plants of very good quality (photo 6), ready to be transplanted in order to establish plantation of Yellow Gentian. Remaining 50 % nursery plants, that are too small to be transplanted should be left in open beds one year more. Up today, more then 50 000 Yellow Gentian nursery plants, produced according to described methodology, were transplanted at many localities of mountainous region of Serbia.

Photo. 5. Yellow Gentian nursery plants in open beds, July, 2005.

Photo. 6. Different size of Yellow Gentian nursery plants, September, 2005.

Photo 7. Harm of Yellow Gentian nursery plants caused by frost in January 2005 (dry winter period)

Photo 8. Yellow Gentian nursery plants that are left in open beds, II growing year (2005).

ANGELICA - *Angelica archangelica* L.

Angelica is biennual herbaceous plant. In the first growing year develops only rosette while in the second one it develops flowering stalk. It is a robust plant, 2 m high. It is grown for its root which contain following biologically active compounds: essential oils, coumarins, furocoumarins, flavonoids, etc.
Angelica succeeds the best in hilly-mountainous region, 600 - 2000 meters above sea level. It has moderate requirements towards light and heat and it is cold tolerant plant. It grows well in regions with average

precipitation per year is over 1000 mm and average T 8 - 11 °C (Stepanović and Radanović, 2005). It prefers deep soils of good structure, with high content of organic metter and good water-air regime.
The exploitation of root is in the phase of techological maturation, i.e. at the end of vegetation, since it contains the highest % of essential oils and dry metter. In agroecological conditions of mountain Tara it is in October. The average yield of dry root ranges 2 - 3 t / ha.

Experimental production of *Angelica archangelica* L.at Tara mountain (2001 -2003.)
Experimental production with *Angelica archangelica* L. started with nursery plants production. Seeds of Angelica were sowen in open beds, in summer 2001. (end of June). Experimental field of 16 are was established in October.

Root exploitation was conducted in the second half of Octobar and at bigining of November, 2002. Yields of obtained fresh and dry Angelica roots are presented in table 6.

Dry Angelica root contained 0,7% essential oil, what meets local quality normativs (Yugoslav standards - min. 0,5% of essential oil).

Angelica seed production was organized in 2003., and it was also successful, with the obtained yield of 18,5 kg of seeds per are.

Photo 9. Experimental production of *Angelica archangelica L.*, Tara (2002).

Photo 10. *Angelica* seed production, Tara (2002).

Table 6. Yields of *Angelica archangelica L.* root, Tara (2002.).

Angelica root yields					
Experimental filed – 0,16 ha [kg]		Average yield per plant [g]		Yield per ha [kg]	
fresh	dry	fresh	dry	fresh	dry
1407	335	187,6	44,0	8778	2090

OREGANO (*Origanum vulgare* L.)

Oregano (*Origanum vulgare* L.) is perrenial, herbaceous, semi-shrab plant species. Aboveground part is composed of great number of flowering shoots. In the nature, this plant species grows up to 50 cm, while the cultivated plants is much robust and have greater number of flowering shoots, and they grow up to 80 m. During winter period, the aboveground plant part freeze and dissappear, and in the following spring new shoots sprout. Oregano plantation lasts 4-6 years.
It is a plant of worm climate and prefers sunny terreins. It can be grown in all regions of Serbia, at all soil types, althought the best are deep fertile soils of good texture.
In the first growing year, the obtained yields range 1000 - 2000 kg/ha of dry aboveground plant parts, while in the second one 3000 - 4000 kg/ha. Oregano also contains 0,15 - 1,00% essential oil.

Experimental production of Oregano (*Origanum vulgare* L.), Tara 2003– 2004.
Oregano experimental production was established with cca. 3000 nursery plants at 9 are, in the third decade of May, 2003. Nursery plant production was organized in plastichouse of the Institute "Dr Josif Pančić", Belgrade, during the spring, 2003. Due to high temperatures and period of drought following transplantation, as well during the entire summer, only 60 % of transplanted plants reached the end of vegetation. In the autum 2003., empty places were filled with new nursery plants produced in open bads, during the summer.

In the first vegetation, 2003., Oregano plants had, by the end of August, 5 to 7 flowering stalks and were in the phase of fool blossom. The harvest was conducted 1. of September, and the obtained yield was 411 kg/ha. Such a low yield was a consequence of reduced crop density. By the end of second vegetation, plants reached their full haight of cca. 80 cm. Harvest of Oregano plants took place also in phase of full blossom, in August, 2004 (15.VIII). The obtained yields from both growing years are presented in table 7.
The yield of Oregano obtained in the second vegetation is more authentic since it reflects the yield obtained from plantation of 100 % crop density, with notification that 40% of plants used to fulfill empty places in plantation were actually in their first vegetation.

Photo 11. Oregano in the I growing year, Tara (2003).

Photo 12. Oregano in the II growing year, Tara (2004).

Table 7. The yields of Oregano in both growing vegetations, Tara (2003. and 2004).

Vegetation	Harvest / Date	Experimental field [ha]	Dry herb yield [kg]	Dry herb yield [kg/ha]
I	I / 01.IX.2003.	0,09	37	411
II	I / 15.VIII.2004.	0,09	255	2833

CONCLUSIONS

All tested aromatic and medicinal plant species gave acceptable results regarding development of their aboveground plant parts as well as seed bearing. The obtained data points out that these cultures may be successfully grown in agro-ecological conditions that offers mountain Tara. In following period investigation on economical parameters in order to estimate growing thrift are going to be faced.
Special attention was devoted to solve the production of planting material of **Yellow Gentian** (*Gentiana lutea* L.). Number of different methods for seed germination has been tried out, with sowing of seeds at beginning of winter in open beds proved to be the best one (Radanović et al., 2005a). Also, production of both Arnica species in this region is possible, and the demanded quality is also achived. However, the obtained yield is still to be improved (first of all by optimiyation of crop dencity).

ACKNOWLEDGEMENT

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[P-053]

[P-057]

FIRST REPORT ON PHYTOPLASMA DISEASE IN PURPLE CONEFLOWER IN SERBIA

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ABSTRACT

Echinacea purpurea and *E. angustifolia* at plantations located in Pančevo and Indjija vicinity (Serbia) showed symptoms of purplish-reddening and yellowing of leaves and stalks, redness or appearance of yellowish coloration of older leaves and different types of deformations such as shortening of internodia and stunting, proliferation and greening of floral elements in infected plants. Infected plants do not produced seeds. By the electron microscopy of ultra thin cross-section of conductive vessels of diseased plants presence of phytoplasmas was recorded in phloem tissues. This is the first report of phytoplasma disease of coneflower, as well as in medicinal plants at all in Serbia
Keywords: *Echinacea purpurea*, *E. angustifolia,* disease, phytoplasma.

INTRODUCTION

Species belonging to the genus *Echinacea* have been introduced from North America. Plantations of *Echinacea purpurea* and *E. angustifolia* were founded in Serbia in last seven years. In our country, as well as in other European countries, these plants are not present in spontaneous flora. These medicinal plants express antiseptic and antiviral activity. From these reasons they are used in healing colds, flu and other respiratory and similar infections.

After the second year of growing of *Echinacea spp.* at plantations located in Pančevo and Indjija vicinity, specific symptoms were registered, which included redness or appearance of yellowish coloration of older leaves and different types of deformations of infected plants. Similar symptoms were described on *Echinacea* sp. in Canada (1-3), and attributed to aster yellow phytoplasma. Phytoplasmas lack cell walls, usually spheroid to ovoid or irregularly tubular to filamentous (4). As the obligate parasites they are present in phloem sieve tubes of many different plant species (5). As disease of *Echinacea* sp. in Serbia was spread quickly, resulting in deterioration of more then 30 % of infected plants, the identification of causal agent was needed. It was conducted by the use of electron microscopy.

MATERIALS AND METHODS

This examination has been conducted in the plantation in Pačevo and Indjija from 2001 to 2004. The attention was paid to the symptom development and aetiology. We followed visible pathological changes from their appearing until some infected plant dry up and died prematurely or till the end of vegetation.

From diseased plants samples for histological and citopathological survey of zone of conductive vessels by electron transiet microscopy were taken. Preparations for electron microscopical ivestigation were done by the procedure of Hopkins *et al.* (6). Identification of pathogen was done according to its morphological characteristics.

RESULTS AND DISSCUSSION

Symptoms become noticeable by early June and usually occur on two to three-year-old plants. Diseased *Echinacea angustifolia* are stunt, with shortened internodia and purplish-reddening leaves and stalks Individual or all stalks of the same plant showed these symptoms (Fig.1). Flowers on such plants are smaller and do not produce seeds.

Fig. 1. Healthy and diseased *E.angustifolia* plants showing stunting and purplish-reddening leaves and stalks (right); diseased plant alone (left)

Affected plants of *Echinacea purpurea* generally show yellowing in the early stages of disease development (Fig. 2 a, b). Symptoms of leaf reddening, plant stunting and proliferation of axillary shoots appear as the disease progresses. Infected plants got bunchy and have witches-broom appearance (Fig. 2 c, d).

Fig. 2. Yellowing the leaves of *Echinacea purpurea* in the early stages of disease development (a and b); leaf reddening, plant stunting and proliferation of axillary shoots resulting in witches-broom appearance (c and d)

Infected leaves are smaller and subjected to drying and further deterioration. From leaves, disease attacks stems causing shortening of internodia and reduction of development. Whole plant appears bushy. At the end necroses of diseased plants have occurred. Typical symptoms are in blossoms, where proliferation and virulescence of crown leaves taking place. In mid-summer, when *Echinacea* species typically reaches its flowering stage, floral malformation involving virescence (greening) and phillody (conversion of floral parts to leaves) are prominent. Small secondary, sterile floret tissue from the original flower heads and consequently, they fail to produce seeds.

Since flower parts are changed into vegetative leaves and sterile florets in the infected plants, no seed are produced. Therefore, the disease has a tremendous impact on Echinacea. Almost all infected plants died (Fig. 3 a-d).

By the electron microscopy of ultra thin cross-section of conductive vessels of diseased plants, presence of phytoplasmas was recorded in phloem tissues. A large number of phytoplasma bodies are usually present in the sieve tube cells of affected leaves and phylloid flower pedicels (Fig. 4). Most of phytoplasma bodies are spherical to oval, without cell walls, surrounded with single membrane. It indicates that phytoplasma might be considered as the casual agent of redness of coneflowers.

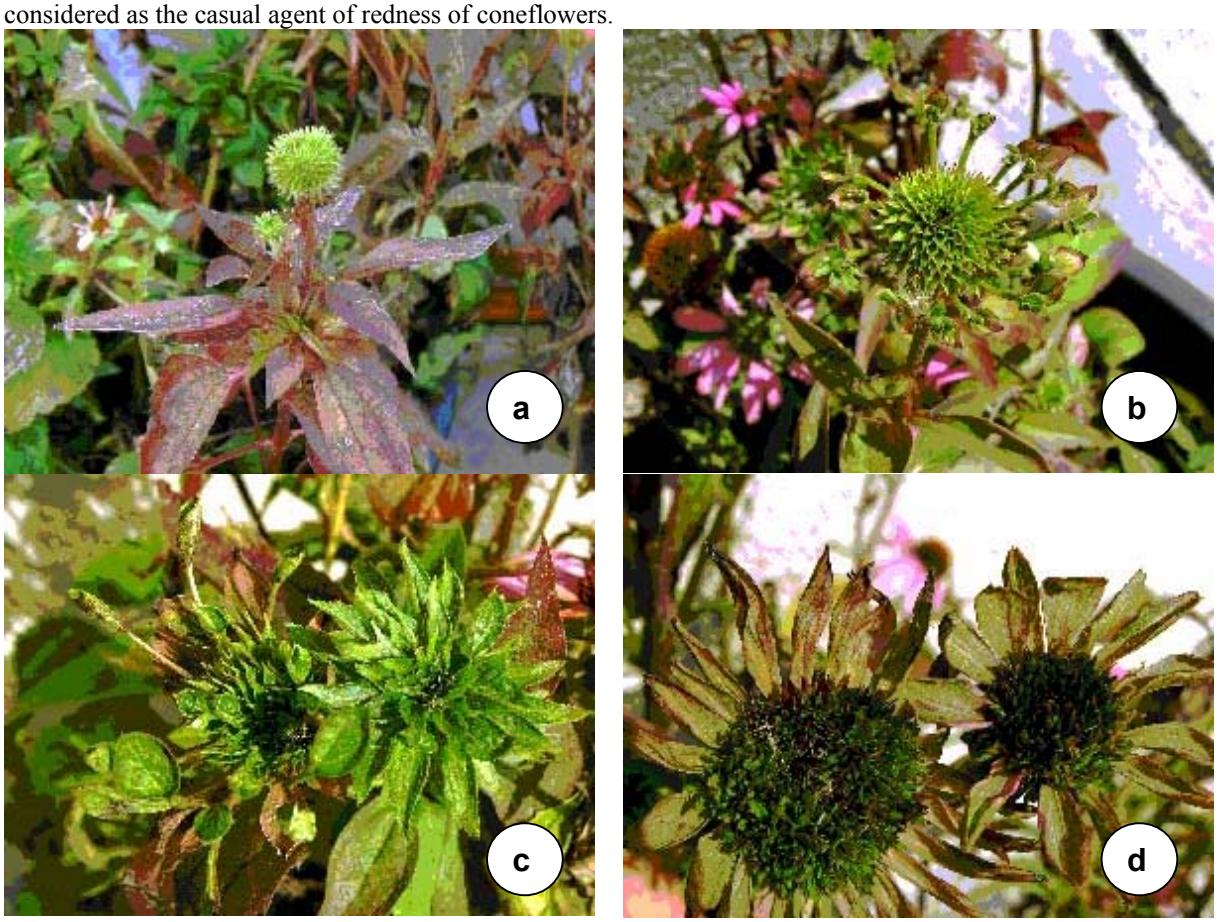

Fig. 3. Infected flower of *Echinacea purpurea* with symptom of virescence (a), distoration and virescence of ray and disk florets (b, c); malformation of floral organs (virescence and phyllody) (d)

As the cause of diseases of *Echinacea* species in Serbia, *Sclerotinia sclerotiorum*, *Alternaria spp.*, *Botrytis cinerea*, *Fusarium spp.*, *Pythium spp.* and *Rhizoctonia solani* have been recorded (7). These symptoms were very similar to those described by Hwang *et al*. (1), Khadhair *et al.* (2) and Chang *et al.* (3) who identified aster yellows phytoplasma as the causal agent of the disease. According to information of Martini (8), who took samples of diseased *Echinacea* species Pancevo, tipical disease symptoms in Serbia were caused by stolbur type phytoplasma. This is the first report of phytoplasma disease of coneflower, as well as in medicinal plants at all in Serbia. The disease severity and production loses requires further, more detailed study of biology and epidemiology of the desease, inluding molecular techniques for the identification purposes.

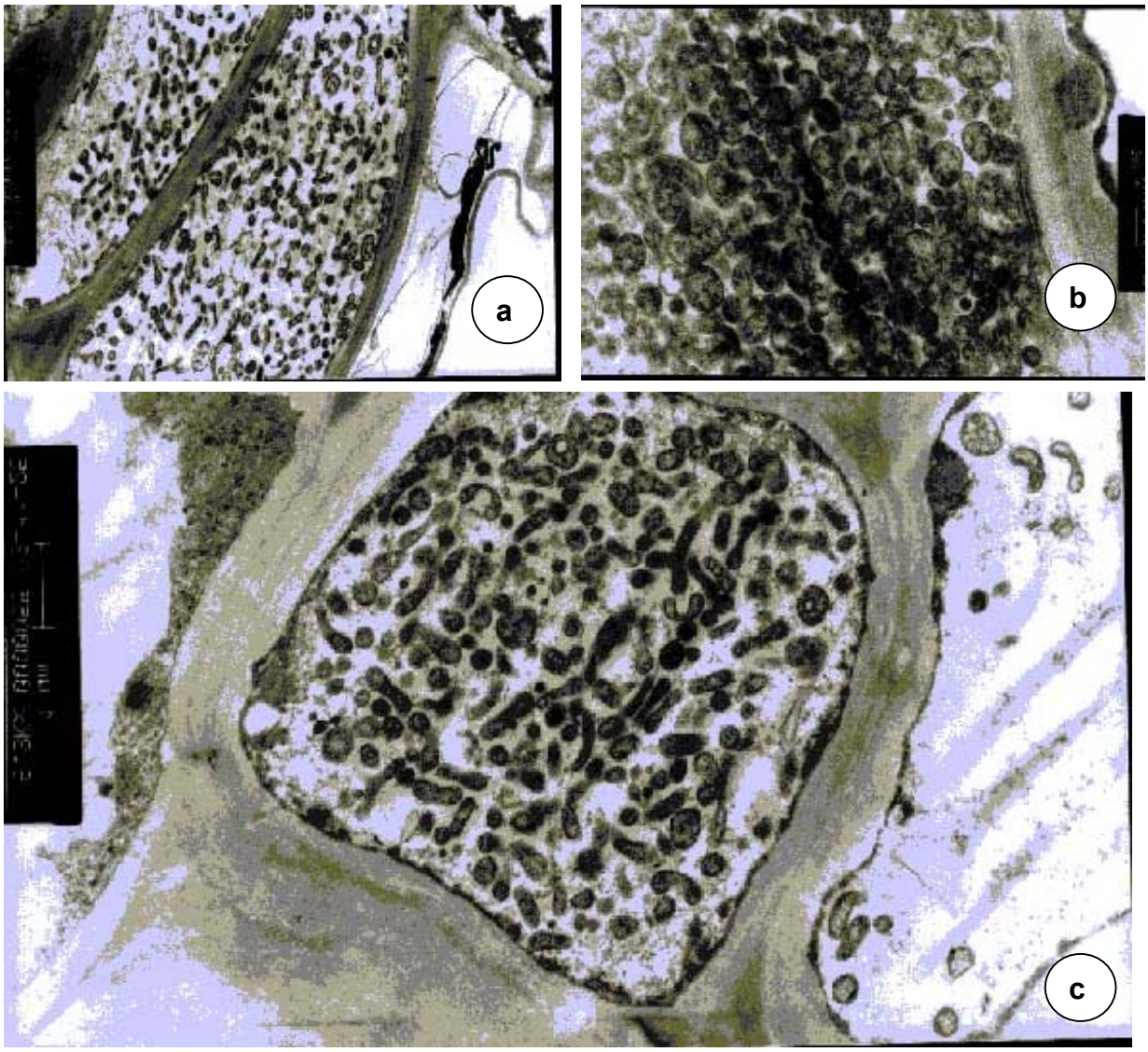

Fig. 4 – Electron micrograph of ultra-thin section of sieve elements of the *Echinacea purpurea* leaf (a, b) and *E. angustifolia* stalk (c) shows a large number of phytoplasma bodies with polymorphic shape.

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[P-057]

[P-061]

INFLUENCE OF STANDARDIZED *HYPERICUM PERFORATUM L.* ALCOHOLIC EXTRACT ON ACUTE GASTRIC ULCERATIONS IN RAT

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ABSTRACT

Hypericum perforatum L. extracts exhibit anti-depressive action in clinical and experimental circumstances. In addition, favorable effects were observed in human and animal gastric ulcerations. In order to investigate the supposed anti-ulcerous effect we have used an alcoholic extract of *Hypericum perforatum* L. in a reserpine-induced ulcer model in rats. Albino, male Wistar-Bratislava rats were randomized and divided into 5 groups. All animals received reserpine i.p., 5 mg/kg. Group I took distilled water through gavages, 15 minutes before and 4 hours after reserpine. Group II was treated similarly with ethanol 30%, groups III, IV and V received different concentrations of *Hypericum perforatum* L. extract: 1:8, 1:4 and respectively 1:2. Administered volume was 1 mL/kg. After 8 hours from the reserpine injection, all animals were sacrificed. Stomachs were observed, and number and severity of ulcers was evaluated. *Hypericum* extracts have differentiated effects according to their concentration, at an 1:8 dilution we have observed an aggravation, the 1:2 dilution has a protective effect.

Key words: *Hypericum perforatum*, reserpine, gastric ulcerations, rat.

INTRODUCTION

Hypericum perforatum L. *(*St John's Wort) extracts are widely used in depression treatment (11,14,22), and a number of indicative behaviour-tests on animals show antidepressant effect (8,16). *Hypericum perforatum* extracts (HP) show similar action to tricyclic antidepressants. Both classes inhibit neuronal uptake of biogenic amines and causes sub chronic down-regulation of beta-adrenergic receptors (16, 20). Commonly encountered is also the anti-ulcerous effect observed in animals and humans as well, supported probably by the same pharmacological mechanisms of activation of central and peripheral adrenergic and serotoninergic structures (4,13,19). In present paper we investigate the effect of an alcoholic extract of *Hypericum perforatum* (HE) using a reserpine-induced experimental model of gastric ulcer.

MATERIAL AND METHOD

Biological material. We worked on male, albino Wistar Bratislava rats, weighting 90-120 g from the *Cluj-Napoca University of Medicine and Pharmacy Breading Center.* Animals were kept at constant temperature, natural light/dark cycle, standard alimentation, water *ad libitum*. Five groups of eight animals were formed by randomization.

Experimental protocol. Before the induction of ulcers, animals were fasted 12 hours, than weighted. All groups received reserpine intraperitoneally (i.p.), 5 mg/kg. To group I was administered through gavages tube, 1 mL/kg distilled water, 15 minutes before, and 4 hours after reserpine. Group II was treated similarly with ethanol 30% w/v, groups III, IV and V received through gastric tube standardized alcoholic extract in three dilutions: 1:8, 1:4 and 1:2. Animals were sacrificed at 8 hours after reserpine injection. Stomach was opened along the great curvature, and mucosa was examined using 5 x magnifying glasses. General aspect of mucosa, hemorrhage, number and surface of ulcerations was considered. Gastric ulcerations were differentiated upon surface (diameter) in large (>1mm), confluent (>3 mm), and localization (antrum, the rest of glandular region). Finally 4 types of ulceration resulted (see table I and II). The severity of ulcerations was appreciated according to a scale between 0 and 4 (ulcer index - UI). The protection ratio (PR) was calculated according to the formula:

$$PR\% = \frac{UIcontrol - UItreated}{UIcontrol} \times 100$$

Statistical analysis. Results were processed using ANOVA test, followed by the multiple comparison Student's "t" test. Null hypothesis was rejected at $p < 0.05$. Statistical significance for PR was considered for values outside (–) 33% → (+) 33% range (4)

Drugs and substances.
1. Reserpine (Raunervil® – Sicomed, injectable solution, 2.5 mg/mL)
2. Standardized alcoholic extract of *Hypericum perforatum* (HE) 30% (hypericin 0.1%, flavonoids, as rutoside, 1%).
Ethical issues
This study was conducted in accordance with the Helsinki declaration on animal studies.

RESULTS AND DISCUSSION

Gastric hemorrhages are present in 87, 5% of the animals treated with distilled water. All the other groups have a 100% incidence of hemorrhages, more abundant in groups treated with alcohol and HE 1:8 (table I).
Concerning ulceration incidence, the only difference we have observed it was between the groups II and V for confluent ulcers, and II vs. III for antrum ulcers (table I).
Total number of ulcers and number of large ulcers does not differ in groups with distilled water and alcohol. The group treated with 1:8 HE has significantly larger number of ulcers than the ethanol group (table II). Ulcer index (UI) is also increased, and PR presents a significant aggravation (table III).
Contrarily, HE 1:2, administered through gastric tube, decreases the total number and the number of large ulcerations in comparison with the ethanol group (table II). In the HE 1:2 group, no confluent ulcers were observed. UI is lower, and protection ratio is significantly larger compared to the ethanol group (table III).

Table I . The incidence of gastric ulcerations

No. cr.	Groups	%				
		TU	LU	CU	AU	H
I	Reserpine - water (RW)	87.5	75	37.5	62.5	87.5
II	Reserpine – ethanol (RE)	100	87.5	62.5	25	100
III	RE + Hypericum (H) 1:8	100	100	87.5	87.5	100
IV	RE + Hypericum (H) 1:4	100	100	75	62.5	100
V	RE + Hypericum (H) 1:2	87.5	87.5	0	50	100

Legend: TU - total ulcerations; LU - large ulcerations; CU - confluent ulcerations;
A - antral ulcers; H - hemorrhage.
Only RE vs RE+H 1:2 ($p = 0.0256$) for CU and E vs H 1:8 ($p = 0.0406$) for AU as significant differences in Fisher exact probability test were seen.

Table II. The number of gastric ulcerations

No. cr.	Groups	UI ($\bar{x} \pm e.s.$)	
I	Reserpine - water (RW)	2.13 ± 0.45	
II	Reserpine – ethanol (RE)	2.31 ± 0.33	
III	RE + Hypericum (H) 1:8	3.13 ± 0.18	
IV	RE + Hypericum (H) 1:4	2.38 ± 0.35	
V	RE + Hypericum (H) 1:2	1.64 ± 0.36	
Comparison		''t'' Student (p)	Rp (%)
RW vs. RE		0.741	(-) 8.82
RE vs. RE + H 1:8		**0.048**	**(-) 35.135**
RE vs. RE + H 1:4		0.898	(-) 2.7
RE vs. RE + H 1:2		0.189	**(+) 67.05**

HE treatment has divergent results. Indeed, hemorrhage intensity, number of ulceration and severity is increased at the HE 1:8 groups. Higher concentration (1:2) decreases all parameters of ulcer genesis. There is little evidence in the literature regarding effects of *Hypericum perforatum* in animal gastric ulceration and human peptic ulcer.
In stress induced ulcers in mice, HP has an adaptative action. Gastro-protective effect is more emphasized in chronic stress, than in acute (13). Oral administration of absolute ethanol causes fast, extended lesions on gastric mucosa. Dry and alcoholic extracts of HP limit this damaging effect in a dose-dependent manner. Anti-ulcerous effect was ascribed to flavonoids and tannins (5). In the popular medicine, *Hypericum perforatum* is used as symptomatic treatment of nonspecific gastric dyspepsia (2,22) and gastric ulcer (22,23). Clinical observation on a limited number of alcoholic subjects, with peptic ulcer showed moderate effects (12). In this context, we can mention the antibacterial effect HP *in vitro* on *Helicobacter pylori* cultures (17,23).

Table III. The severity of gastric ulcerations

No cr.	Groups	Number of ulcerations ($\bar{x}$ ± e.s.)			
		Total	Large	Confluent	Antral
I	Reserpine - water (RW)	17 ± 4.67	9.38 ± 2.51	1.13 ± 0.58	2.63 ± 1.22
II	Reserpine – ethanol (RE)	18.88 ± 2.56	9.13 ± 1.94	1.25 ± 0.59	0.5 ± 0.38
III	RE + Hypericum (H) 1:8	39.13 ± 7.45	20.25 ± 2.78	2.5 ± 0.91	1.63 ± 0.53
IV	RE + Hypericum (H) 1:4	15.38 ± 2.88	11.63 ± 2.67	2 ± 0.89	3 ± 1.24
V	RE + Hypericum (H) 1:2	7.43 ± 1.66	4.86 ± 1.22	0	2 ± 1
	RW vs. RE	0.728	0.938	0.882	0.119
	RE vs. RE + H 1:8	0.022	0.005	0.267	0.107
p	RE vs. RE + H 1:4	0.378	0.461	0.493	0.074
	RE vs. RE + H 1:2	0.003	0.096	0.053	0.163

Anti-ulcerous effect is correlated with the antidepressant effect of *Hypericum perforatum* preparations. Tricyclic antidepressants and monoamine-oxidase inhibitors (IMAO) reinforce central and peripheral monoaminergic mechanisms. In this way, they protect gastric mucosa in ulcer experimental models (4,19). *Hypericum perforatum* extracts have a weak IMAO-like activity, probably irrelevant for antidepressant effects (1,13,16,20,21), but surely act on neuronal uptake of biogenic amines. On synaptosomes isolated from different parts of the brain, HP blocks pre synaptic reuptake of noradrenaline, serotonin, and dopamine (3,8,24). This effect appears to be different from selective inhibitors of serotonin reuptake (7), which inactivates the specific carrier of catecholamines and serotonin. HP acts non-specific, augmenting intracellular sodium concentration (20,24). Lack of specificity over intracellular transport of biogenic amines is associated also with the inhibition of GABA and glutamic acid reuptake by the same sodium-dependent mechanism (20). In addition, HP extracts can release glutamate from neuronal structures. By these effects on the neurotransmitter transport (24), or independently (6,10,18), HP components modulate the activity of some receptors: down regulation of beta-1 adrenergic receptor and up regulation of postsynaptic 5HT1A, 5HT2 and blockage of $GABA_A$ and glutamate NMDA receptors (16).

Contribution of HP components in carrying out of these effects is not established with certitude. Less recent bibliography suggests antidepressant effect to hypericin (14). Recent data brings strong evidence on hyperforin (15,18,25) and adhyperforin (9) participation at neurotransmitter reuptake inhibition.

Our data obtained on experimental ulcer model evidences discordant effect: aggravation in low-, and protection at high concentrations of HE. This action drives us to look for components present high concentrations in the extract. HE used in our experiments contain 0.2% hypericine. Usually, hyperforine is found in HE extracts in concentrations of 5-6%, so can contribute to the gastro-protection.

CONCLUSIONS

- *Hypericum perforatum* 30% alcoholic extract (HE) has divergent effect on the reserpine-induced ulcer in rat.
- HE aggravates in low-, and protects in high concentrations the gastric mucosa exposed to the injuring effect of reserpine.
- Inhibition of monoamine reuptake activity conferred by HE can be important for the gastro-protective effect at high concentrations.
- Ulcer aggravation by low concentration of HE cannot be explained based on the mechanisms of action of HP described so far.

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BIOLOGICAL ACTIVITY OF LINALOOL

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ABSTRACT

Essential oils of different medicinal and aromatic plants expresses broad spectrum of biological activities to different bioagents (microbial, insects etc.). Along with essential oils, we tested pure terpenoids (linalool, camphor, α- and β-thujone, 1,8-cineole, myrcene). The results of the antibacterial activity assays of linalool on different Gram-positive and Gram-negative bacterial strains indicated that this terpenic alcohol possesses very strong activity. In antifungal investigation macro- and microdilution method were used in order to determine the minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC). MIC in microdilution assay was 2.0-6.0 µl/ml (2.0-5.5 µl/ml), the same as in macrodilution assay 3.0-7.0 µl/ml (2.0-6.5 µl/ml). Thus, it could be concluded that linalool possesses strong antifungal activity. Antigenotoxic potential of linalool was evaluated with short-term bacterial tests in *Escherichia coli* K12, repair proficient SY252 strain, and *Salmonella typhimurium* TA100 strain. Antimutagenic activity was detected by inhibition of UV-induced mutations. The results indicate the significant antimutagenic effect of linalool (60%). Literature data shows that linalool exhibits antioxidative properties with no cytotoxic effect on mammalian cells [Deans S.G. *et al*., 1993; Rosa M.D. *et al*., 2003]. In our investigation we included examination of insecticidal, antifeeding, contact and digestive activity of linalool on caterpillars (L_2) of *Limantria dispar* L. in the laboratory conditions. It was found that this terpenic alcohol expressed very low toxicity (contact and digestive activity) but significant antifeeding activity on caterpillars of *Limantria dispar*. Antimicrobial and antimutagenic properties of linalool, as well as its antifeeding activity toward insects, makes possible use of this terpenic alcohol for control of several important bioagents realistic.

Key words: linalool, antimicrobial activity, antifeeding activity, bioagents.

INTRODUCTION

Plant extracts and essential oils are nowadays studied more and more because of their use in plant protection as alternative tools to the broad use of conventional pesticides. The finding that some plants possess secondary metabolites with antimicrobial activity and could prevent colonisation and nutrition of most herbivorous insect, resulted in considerable increase of empirical studies of relationships between plants and bioagents (such as bacteria, fungi, herbivorous insects). Final aim of these efforts is revelation of natural products of the plant origin suitable for their practical use in the field of plant protection. The lack of high toxicity to harmful and beneficial organisms is an advantage of such substances in the preserving biocenosis. These substances are believed to function principally in ecological roles, serving as herbivore-feeding deterrents, antifungal defenses and pesticides [Langenheim, 1994]. The expanding literature on the possibility of use of these natural compounds is reviewed and our interest is focused to the effects of essential oil and monoterpenes, for which are proved that are active against many insects and microorganisms, to these harmful bioagents. Linalool is the principal component of many essential oils known to possess several biological activities, attributable to this monoterpene. This monoterpene compound, prevalent in essential oil of plant species traditionally used as sedatives, has been characterised as anticonvulsant in several experimental models. Linalool inhibits the binding of [^{3}H]glutamate and [^{3}H]dizocipline to brain cortical membranes, indicating a participation of the glutameric transmission in its mechanism of action [Brum *et al*., 2001]. It was also shown that linalool play a major role in the anti-inflammatory activity expressed by the essential oils containing it [Peana *et al*., 2002]. Linalool was reported to have a vasodilatory effect on blood vessels and has been proposed as an antineoplastic and antiviral agent for veterinary administration. It has been characterized as moderately to severely irritating to rabbit skin, but non-irritating and non-sensitizing in human subjects. Linalool appears to be readily absorbed after oral administration with excretion of 93% (urine-55%, feces-15% and expired air-23%) occurring within 72 hours in male Wistar rats [SEAC, 1993].

MATERIAL AND METHODS

Test for antifungal activity

The fungi used in this study were: *Alternaria alternata* (ATCC 13963), *Aspergillus niger* (ATCC 6275), *A. ochraceus* (ATCC 12066), *A. versicolor* (ATCC 11730), *A. flavus* (ATCC 9170), *A. terreus* (ATCC 16792), *Penicillium ochrochloron* (ATCC 9112), *P. funiculosum* (ATCC 10509), *Cladosporium cladosporoides* (ATCC 13276), *Trichoderma viride* (IAM 5061), *Fusarium tricinctum* (CBS 514478), *Phomopsis helianthi* (ATCC 201540) and five dermatomycetes: *Microsporum canis*, *Epidermophyton floccosum*, *Trichophyton rubrum*, *T. mentagrophytes* and *T. tonsurans*. The moulds were from Mycoteca of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research, Belgrade. Dermatomycetes were isolated directly from the patients at the Centre for Preventive Medicine of the Military Medical Academy (Belgrade). The fungi were maintained on potato dextrose agar (PDA), malt agar (MA) and Sabourad-dextrose agar (SDA). The cultures were stored at +4ºC and sub cultured once a month [Booth, 1971].

Macrodilution method

The minimal inhibitory concentration (MIC) of component investigated necessary for the complete inhibition of mycelia growth of the fungal strain was determined. Different concentrations of component were diluted in Petri dishes with malt agar (MA). All fungal species were tested in triplicate. Petri dishes with ethanol and bifonazole were used as a control. The tested fungi were inoculated at the centre of the plates. Plates were incubated for three weeks at room temperature and after this period MIC was determined.

Microdilution method

The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (V/V). The spore suspension was adjusted with sterile saline to a concentration of approximately $1.0 \cdot 10^5$ in a final volume of 100 µL per well. Inoculates were stored at +4ºC for further use. Dilutions of inoculates were cultured on solid MA to verify the absence of contamination and to check the validity technique using 96-well microtitre plate [Hanel, 1988; Daouk, 1995]. The microplates were incubated for 72 h at 28ºC. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations, which completely inhibited fungal growth (MICs). MFCs were determined by serial subcultivation of a 2 µL into microtitre plates containing 100 µL of broth per well and further incubation for 72 h at 28ºC. Linalool was dissolved in ethanol.

Test for determination of antigenotoxic potential

Media and growth conditions

The bacteria were cultured overnight in LB medium (5 g NaCl, 10 g bacto-tryptone, 5 g yeast-extract, 1000 ml distilled water) at 37ºC with aeration.

UV-induced mutations in *E. coli* SY252*argE3* strain

The overnight culture of *E. coli* SY252*argE3* strain was washed by centrifugation and resuspended in 0.01M $MgSO_4$ giving a similar titre. UV-irradiation was carried out with a germicidal lamp (Camag) with a maximum output at 254 nm. Cells were irradiated at a thickness of less than 1 mm. Samples (0.1 mL) of UV-irradiated cells, appropriately diluted for determination of cellular viability and two-fold diluted for determination of mutagenesis, were spread on SEM agar plates with and without linalool. The number of Arg^+ revertants and viable cells was determined after incubation at 37 ºC for 72 h.

Ames test – *Salmonella* mutagenicity test

In 100 ml of molten Top agar (0.6% Difco agar and 0.5% NaCl) 10 ml 0.5 mM biotin solution was added. 2 ml of Top agar prepared on this way was distributed into 13·100 mm capped culture tubes and held at 45 ºC in a heating block. In Top agar 0.1 ml of fresh overnight culture (non- or UV-irradiated) of the tester strain was added and 0.3 ml S9 mix. The compound is tested with or without S9 mix and both positive and negative control plates are included in the assay. All components were mixed by vortexing in the soft agar for 3 sec at low speed and then pour onto a minimal glucose agar plate, which contain different concentrations (in duplicate) of linalool. Plates were inverted and placed in dark, vented incubator at 37 ºC for 48 h.

S9 fraction

S9 fraction was isolated from the liver of Albino Wister male rats (170-180 g) induced with phenobarbital/β-naphthoflavone [Ong *et al*., 1980]. S9 mix contained 4% (v/v) S9 fraction, 33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose-6-phosphate and 4 mM NADP in 0.1 M phosphate buffer pH 7.4.

Test for determination of the effect on the second instar larvae of *Lymantria dispar* L.

The effect of linalool on the second instars larvae of *Lymantria dispar*, was investigated in three concentrations 0.05%, 0.10% and 0.50%, with solution prepared by dissolving linalool in 96% ethanol, in four replications. In the evaluation of contact toxicity 0.3 ml of test solution was deposited onto the bottom of Petri dishes (R=9 cm), dried about 20 min at 21˚C and then 10 larvae were put per repetition. In the determination of digestive toxicity and antifeedancy of linalool small branches of *Prunus cerasifolia* L. (20 cm long with uniform leaf mass) were used. Branches were put into flask with water and then fixed into the pots with sand. Leaf mass was treated by spraying. When the deposit dried (about 20 minutes), glass cylinders were put on for the isolation of the treatments and then 10 second instar larvae per replication were put in. These tests were set in four repetitions too. The experiments lasted 4 days (96 h), in a micro climatic chamber supplied with Danfoss EKH 20 operational system. Optimal climatic conditions for *L. dispar* development were t=27 ˚C ± 1 ˚C, RH=65±5% and neon diffuse light with about 30160 cd with 12 h of light/dark.
Contact toxicity was evaluated after 24 and 48 h, while experiment for determination of digestive toxicity and antifeedancy lasted 4 days and assumed evaluation of effects after every 24 h. Experimental data were processed by ANOVA (average value, mean deviation, LSD and Duncan's multiple range test). In Duncan's test descending order was used. The highest differences from average, detected by statistical testing, were marked with **a**, the next lower with **b** etc. Mortality was corrected by Abbott's formula [Abbott, 1925]. Antifeedancy (antifeedant index) was calculated by the formula given by Mordue and co-workers [Mordue *et al*., 1995].

RESULTS AND DISCUSION

The results of the antibacterial activity assays of linalool on different Gram-positive and Gram-negative bacterial strains indicated that this activity was very strong. In antifungal investigations we used the macro- and microdilution method in order to determine MIC and MFC values. MIC in microdilution assay were 2.0-6.0 µl/ml (2.0-5.5 µl/ml), the same as in macrodilution assay 3.0-7.0 (2.0-6.5 µl/ml)(Table 1), so we can conclude that linalool possesses strong antifungal activity.

Table 1. MIC and MFC of linalool obtained by macrodilution and microdilution method

Micromycetes	**Macrodilution method**	**Microdilution method**	
	MIC µl/ml	**MIC µl/ml**	**MFC µl/ml**
Alternaria alternata	**3.0**	**3.0**	**3.0**
Aspergillus niger	**6.5**	**5.0**	**6.5**
A. ochraceus	**5.0**	**5.0**	**5.0**
A. versicolor	**5.0**	**5.0**	**5.0**
A. flavus	**5.0**	**5.0**	**5.0**
A. terreus	**6.5**	**5.0**	**6.5**
Cladosporium cladosporoides	**3.0**	**3.0**	**3.0**
Fusarium tricinctum	**3.0**	**3.0**	**3.0**
Penicillium ochrochloron	**7.0**	**6.0**	**7.0**
P. funiculosum	**5.0**	**5.0**	**5.0**
Phomopsis helianthi	**2.0**	**2.0**	**2.0**
Trichoderma viride	**5.0**	**5.0**	**5.0**
Trichophyton mentagrophytes	**3.0**	**3.0**	**3.0**
T. rubrum	**4.0**	**4.0**	**4.0**
T. tonsurans	**4.0**	**4.0**	**4.0**
Microsporum canis	**5.0**	**5.0**	**5.0**
Epidermophyton floccosum	**5.0**	**5.0**	**5.0**

In earlier investigation linalool showed better antifungal potential in comparison with other alcohol structural isomer, because of two double bonds that provide increasing the antifungal activity. The same studies showed that, among five monoterpenoids (cineole, citral, geraniol, linalool and menthol), linalool was the most effective in terms of antibacterial activity.
It was shown that linalool is not considered a human carcinogen, a developmental toxicant or mutagenic. In similar mutagenicity/genotoxicity assay linalool hasn't express mutagenicity in S*almonella typhimurium* TA100 and TA98 strains. Linalool elicited negative results in a carcinogenicity assay (in study which examined the hepato-carcinogenic potential) [SEAC, 1993].
In our investigation, linalool reduced UV-induced mutagenesis in both prokaryotic test systems at the same level. The antimutagenic activity was proportionally higher (stronger) with applied concentration and was 69% in *Escherichia coli* SY252 at maximal tested concentration (20 µl/plate) (Table 2). Because *Salmonella*

typhimurium TA100 strain possess *rfa* mutation which cause partial loss of the lipopolysaccharide barrier that coast the surface of the bacteria and increases permeability to large molecules, we applied smaller concentration in that prokaryotic system in comparison with *E. coli*. It was obtained similar extent of mutagenesis inhibition in *S. typhimurium* TA100 with or without metabolic activation (S9 fraction). At maximal concentration (5 µl/plate) linalool inhibited UV-induced mutagenesis with 70% without S9 and 77% with S9 fraction (Table 3). This great extent of inhibitory effect of linalool on mutagenesis indicates it's significant antimutagenic potential.

Table 2. Effect of linalool on UV-induced mutagenesis in *E. coli* SY252

Linalool (µl/plate)	**Viable cells**		**Arg$^+$ revertants**		**% I**
	-UV	**+UV**	**-UV**	**+UV**	
0	127	653	33	286	
Ethanol	146	742	27	242	
1.0	158	443	24	167	**31**
2.5	254	500	22	153	**37**
5.0	121	514	25	130	**46**
10	115	322	25	114	**53**
20	108	420	34	74	**69**

UV-dose was 24 J/m^2

Table 3. Effect of linalool on spontaneous and UV-induced mutagenesis in *S. typhimurium* TA100

Linalool (µl/plate)	**-S9**		**%I**	**+S9**		**%I**
	-UV	**+UV**		**-UV**	**+UV**	
0	122	478		134	673	
Ethanol	122	538		124	618	
0.25	110	409	**26**	90	487	**21**
1	124	269	**50**	126	339	**45**
2	128	192	**64**	143	307	**50**
3	128	189	**65**	154	213	**66**
5	111	163	**70**	164	143	**77**

UV-dose was 6 J/m^2

The latest investigation included the effect of linalool on the second instar larvae (L_2) of *Lymantria dispar* L. The results of investigation of contact toxicity of (+)- and (±)-linalool on inert glass surface proved that, 24 h after the treatment, it had low contact toxicity to the larvae (L_2) in all investigated concentrations (Table 4). After 48 h, the toxicity increased, and the highest effect of (+)-linalool (60.00%) was obtained at the lowest concentration of 0.05%, and 57.0% for (±)-linalool. According to Duncan's test, very significant differences were registered between this variants and untreated control and control treated with 96% alcohol.

Table 4. Contact toxicity of linalool for the second instar *Lymantria dispar* L. larvae, on glass medium

Variants	**Conc.**	**Mortality after 24 h**		**Mortality after 48 h**	
	%	**Mean, %**	**Corrected, % (K=100)**	**Mean, %**	**Corrected, % (K=100)**
(+)-linalool	0.05	15.00a	600.00	60.00a	1200.00
(+)-linalool	0.10	12.50a	500.00	22.50bc	450.00
(+)-linalool	0.50	17.50a	700.00	37.50ab	750.00
(±)-linalool	0.05	7.50a	300.00	37.50ab	750.00
(±)-linalool	0.10	20.00a	800.00	40.00ab	800.0
(±)-linalool	0.50	22.50a	900.00	57.50a	1150.0
Control alcohol	96.00	5.00a	200.00	5.00c	100.00
Control non treated	0.00	2.50a	100.00	5.00c	100.00
	LSD_{005}=20.27 LSD_{001}=27.59		LSD_{005}=23.62 LSD_{001}=32.14		

The results of evaluation of digestive toxicity for the second instars larvae (L_2) of *L. dispar* showed that within first 24 h mortality was not recorded (Table 5). After 48 h, (+)-linalool in concentration of 0.50% caused 30%

mortality of larvae. After 96 h, the most efficient variants were (+)-linalool with 50.00% and (±)-linalool with 35.00% of dead larvae.

Table 5 Digestive toxicity of linalool for the second instar *L. dispar* L. larvae, on treated leaves

Variants	Conc.	Mean % of dead larvae			
	(%)	24 h	48 h	72 h	96 h
(+)-linalool	0.05	0.00	15.00ab	17.50bc	22.50bc
(+)-linalool	0.10	0.00	10.00b	15.00bc	15.00bc
(+)-linalool	0.50	0.00	30.00a	42.50a	50.00a
(±)-linalool	0.05	0.00	5.00b	10.00bc	12.50bc
(±)-linalool	0.10	0.00	15.00ab	25.00b	35.00ab
(±)-linalool	0.50	0.00	10.00b	20.00bc	25.00bc
Control alcohol	96.00	0.00	7.50b	7.50bc	7.50c
Control non treated	0.00	0.00	2.50b	2.50c	2.50c
			LSD_{005}=15.1 LSD_{001}=20.5	LSD_{005}=17.5 LSD_{001}=23.8	LSD_{005}=21.9 LSD_{001}=29.7

P. cerasifolia leaf mass damage caused by the second instar larvae (L_2) of *L. dispar*, 24 h after treatment, was insignificant in all treated variants with (+)- and (±)-linalool, and it was the highest on untreated one (Table 6). Significant increase in leaf mass damage was registered in control (untreated 21.25% and that with ethanol 8.13%), 48 h after treatment, while in all treated groups the damage was still insignificant. Until the end of the experiment, the damage was considerably higher only in the untreated control variant.

Table 6 Protective effect of linalool for the leaf mass attacked by second instar larvae

Variants	Conc.	Mean % leaf mass damage			
	(%)	24 h	48 h	72 h	96 h
(+)-linalool	0.05	1.00b	3.13a	3.38a	3.50a
(+)-linalool	0.10	0.50ab	2.88a	3.00a	3.25a
(+)-linalool	0.50	0.75ab	2.63a	3.13a	3.75a
(±)-linalool	0.05	0.25a	1.88a	2.63a	3.00a
(±)-linalool	0.10	0.75ab	4.00a	4.88ab	5.25a
(±)-linalool	0.50	1.00b	2.25a	3.38a	3.75a
Control alcohol	96.00	2.00c	8.13a	10.13b	16.25b
Control non treated	0.00	5.00d	21.25b	33.75c	51.25c
		LSD_{005}=0.57 LSD_{001}=0.78	LSD_{005}=6.14 LSD_{001}=8.36	LSD_{005}=5.29 LSD_{001}=7.20	LSD_{005}=7.66 LSD_{001}=10.43

CONCLUSIONS

It can be concluded that linalool possesses strong antimicrobial activity, better than commercial antimicrobial synthetic agents used in this experiment. Besides, linalool showed neither contact nor digestive toxicity to the chosen test insect, but only antifeedant activity. Established strong antigenotoxic effect of linalool indicates that its use is not harmful for people and environment. All these results, makes possible use of this terpenic alcohol for control of several important bioagents realistic.

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[P-064]

CYTOGENETICS AND MORPHO-ANATOMY IN *STEVIA REBAUDIANA* BERTONI

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ABSTRACT

Stevia rebaudiana is an herb from the *Asteraceae* family, indigenous from the higher elevations of Northern Paraguay near the Brazilian borders. Due to non-caloric sweeteners extracted from its leaves, mainly stevioside, this plant has gained importance as a crop for the pharmaceutical and food industries. *Stevia* is diploid and has 11 chromosome pairs, which is characteristic for most of the South American members of the genus. Chromosome's number in *Stevia rebaudiana* is identified as 2n=22. There are three morphological types of chromosomes – m, sm and M. Chromosomes arm ratio was found from 1.00 to 2.45. The chromosomal length is comprised between 1.70 and 3.76 μm. We consider *Stevia rebaudiana* karyotype as asymmetric. The anatomical structure of *Stevia rebaudiana* vegetative organs frame to the general plans structure of the *Asteraceae* in general. The opposite leaves disposition is considerate like a peculiarity, because the most *Composite* species has alternate leaves. In *S. rebaudiana*, functional glandular trichomes are already present at early stages of leaf development of the leaves.
Key word: *Stevia rebaudiana*, caryotype, alternate leaves, glandular trichomes.

INTRODUCTION

Stevia rebaudiana Bertoni is one of 154 members of the genus *Stevia* and one of only two that produce sweet steviol glycosides. It is native to the valley of the Rio Monday in highlands of Paraguay, where it grows in sandy soils near streams. *Stevia* was first brought to the attention of Europeans in 1887 when M.S. Bertoni learned of its unique properties from the Paraguayan Indians and Mestizos. In 1980 Zaidan *et al*., obtain the stevioside from leaf (the most productive organ), stem and inflorescence.

The histo-anatomical studies regarding *Stevia rebaudiana* Bertoni are not extended in literature. Monteiro *et al*., 2001 investigate the secretory hairs of this species, using histochemical methods to underlining the composition of secretion products. In a previous work (Toma and Zbughin, 2003) we investigate the structure of the vegetative organs in *Stevia rebaudiana* cultivated *in vitro.*

Studies on karyotype have a well-known importance for species characterization. This kind of works is interesting and of great value because the chromosomal formula is useful in order to establish taxonomic position of each species and to chose the best way for selection. On the other hand it is possible to use karyotype information to understand the speciation process inside a genus or family.

MATERIALS AND METHODS

Cytogenetic study

When roots of regenerated plantlets were reached 5-10 mm in length, these were treated with 0.2% colchicines for 2 hours, at room temperature. Roots were kept for other two hours in distilled water.
Fixation was done for about 16 hours in ethyl alcohol / acetic acid (3:1) mixture, at room temperature.
For hydrolysis of vegetal material was used HCl 50% for 6-7 min, at room temperature. The staining was realized with Carr reactive, according literature (Campeanu *et al*., 2003).
The slides were prepared according squash method. Microscopy was carrying out using 100x objectives, with a Nikon Eclipse 600 light microscope. Photos were taken with a Nikon CoolPix 950 digital camera, at 1600x1200 dpi resolution. All images were processed with Adobe Photoshop software.
Homologous chromosome groups were settled in accordance with the rapport between long arm and short arm, the mitotic index, the difference between arms and with the relative length of the chromosomes.

Histo-anatomic study

Vegetative organs of *Stevia rebaudiana* Bertoni cultivated in Iassy Botanical Garden represent the studied material. The vegetal material were fixed and conserved in ethanol 70% or in a special mixture (ethylic alcohol: acetic acid: formol - 1:1:1). The sections were made after paraffin including. All samples were dehydrated through a tertiary butyl alcohol series, embedded in paraffin and then sectioned (Johansen, 1940). Serial transactions were cut at 12 μm thickness on a rotary microtome and colored either with fastgreen and saphranyne or with red-ruthen and methyl-blue. After that, the sections were fixed in Canada balsam. From the mature organs freehand

sections was performed. They were colored with iod-grun and carmine red. The photos were made after the obtained permanent preparates using a Novex (Holland) microscope and a Minolta photo camera.

RESULTS

Glands are initiated very early in leaf development (Pl. I, Photo 1). After enlarging and protruding above the leaf surface, a single protodermal cell undergoes an anticlinal, symmetric division, resulting in the trichome with two cells. Both cells divide periclinally and four cells results. From periclinal divisions six eight and ten cells result in the process of the development of the glandular hair. These cells do not appear to divide synchronously in the passage from one stage to another. Monteiro *et al*. (2001) underlined this aspect too.
The serial transections made in the length of the caulinar apex demonstrated the opuses disposition of the foliar primordial (Pl. I, Photo 2).
At the level of the caulinar apex, the transversal section has a circular-elliptical contour, the stem structure being represented by a meristematic homogenous tissue, which forms the tunic and corpus; both of them have isodiametricall cells with a polygonal contour without meatus between them. On the apex flanks are observed two pairs of foliar primordia and another two pairs of younger leaves. The tector and secretory hairs could be observed.
In the third superior stem part the next anatomical features could be observed (Pl. I, Photo 4): the tector and secretory hairs are rare; the sclerenchyma fibers are thin and unlignified walls; in the central cylinder, the vascular tissue has primary structure.
In the third basal part of the stem the epidermis is covered with a striate cuticle; the tector and secretory hairs are rare. The fibres are numerousness in the secondary xylem (Pl. II, Photo. 1).
At the stem basis the secondary structure results from both cambium and phelogen activity. The phelogen arise in the hypodermic position and produce a small quantity of cork and phelloderm (Pl. II, Photo.2). The epidermis is exfoliated and some lenticels could be observed. The cambium produces an external discontinuous zone of secondary phloem and an internal thick ring of secondary xylem. Against the phloemic ring a sclerenchyma sheath is visible.

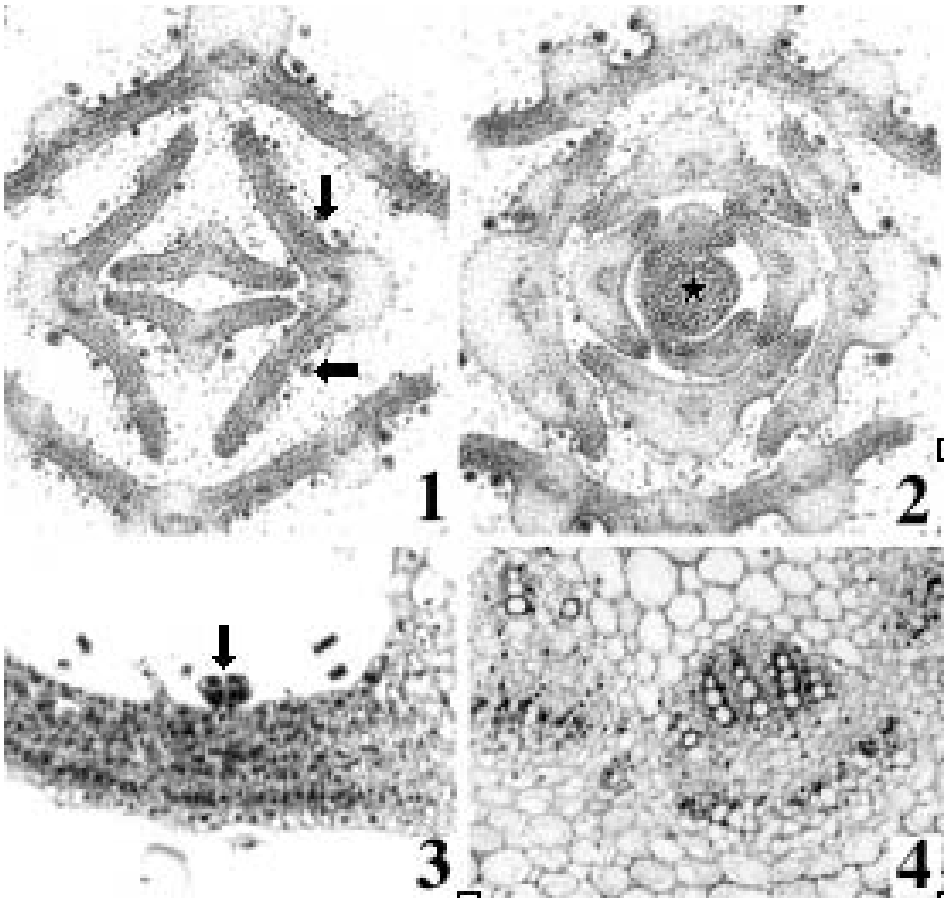

Plate I. Photo 1: Cross-section from the foliar primordial and young leaves (x100); Photo 2: Cross section from the caulinar apex (x100); Photo 3: Cross-section from a young leaf (x100); Photo 4: Cross-section from the young stem (vascular bundles with primary structure could be observed) (x 200) (glandular hairs-arrows, caulinar apex-aster).

The trans-sections from the primordia have semicircular or elliptic shape (Pl. II, photo 3). The mesophyll is homogenous, with isodiametrical cells. In the next stage in primordial development the middle vein become prominent at the lower side and contain a procambium girdle. In the epidermis long, pluricellular and uniseriate tector hairs and short secretory hairs could be observed. The young leaves middle vein is prominent at the both leaf sides. Under the epidermis collenchyma girdles are visible. In the vascular bundles the thick-walled and lignified xylem vessels are already developed; the assimilatory tissue is almost compact (Pl. I, Photo 3)

Differentiation into the typical palisade parenchyma and the spongy parenchyma takes place due to the uneven growth rates of the different cell layers in the developing leaf. The activities of cell division and cell elongation occur in the different layers at different times. Additionally, the cells expand into different directions. The mature leaf has palisade parenchyma at both surfaces (Pl. II, Photo 4).

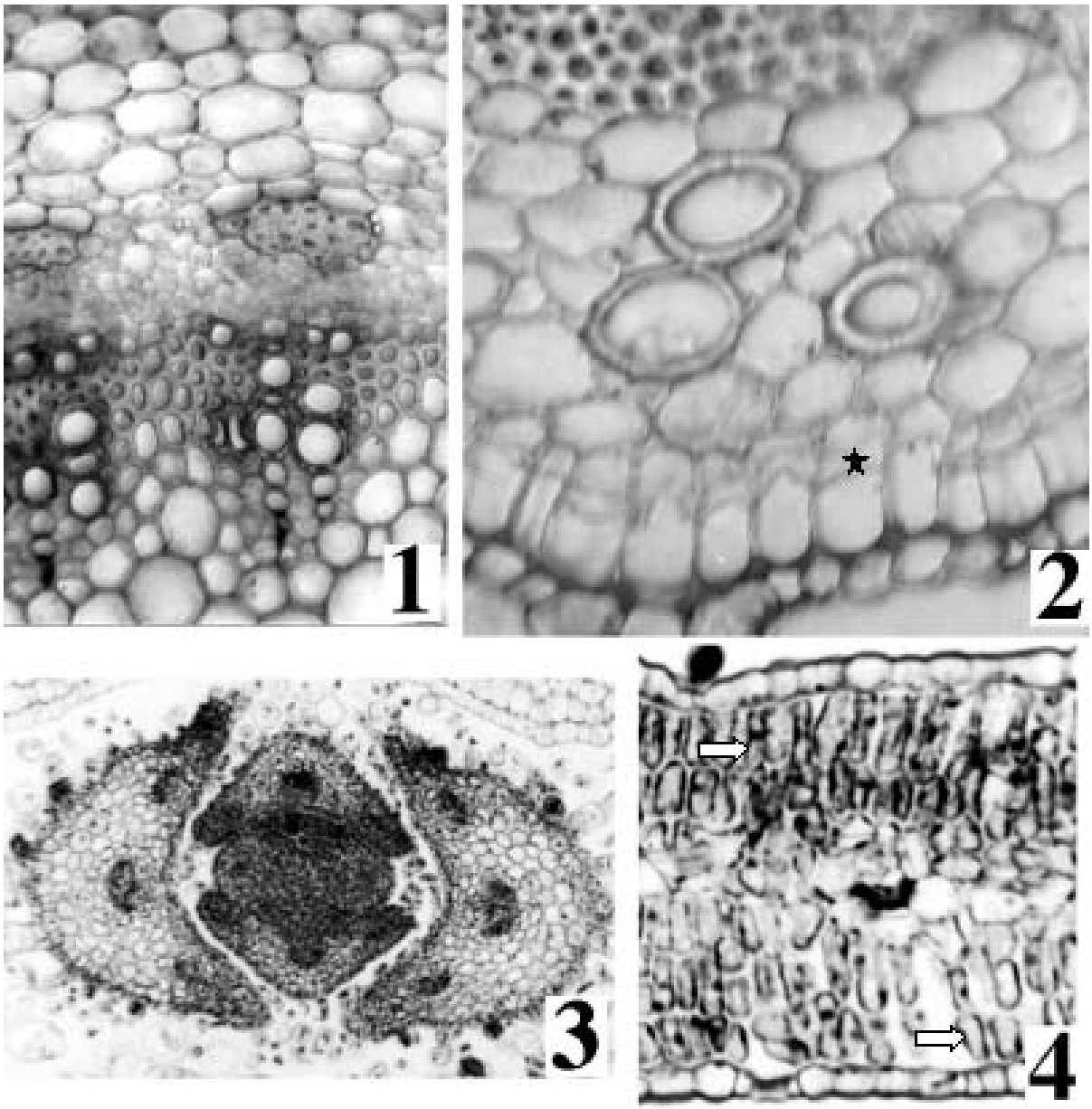

Plate II. Photo 1: Cross-section from the third basal part of the stem (vascular bundles with secondary structure could be observed) (x200); Photo 2: Cross-section from the basal part of the stem (the phelogen-aster) (x400); Photo 3: Cross-section from an axilar bud (leaf primordial could be observed) (x200); Photo 4: Cross-section from the mature leaf (palisade parenchyma – arrow – under both epidermis could be observed) (x200).

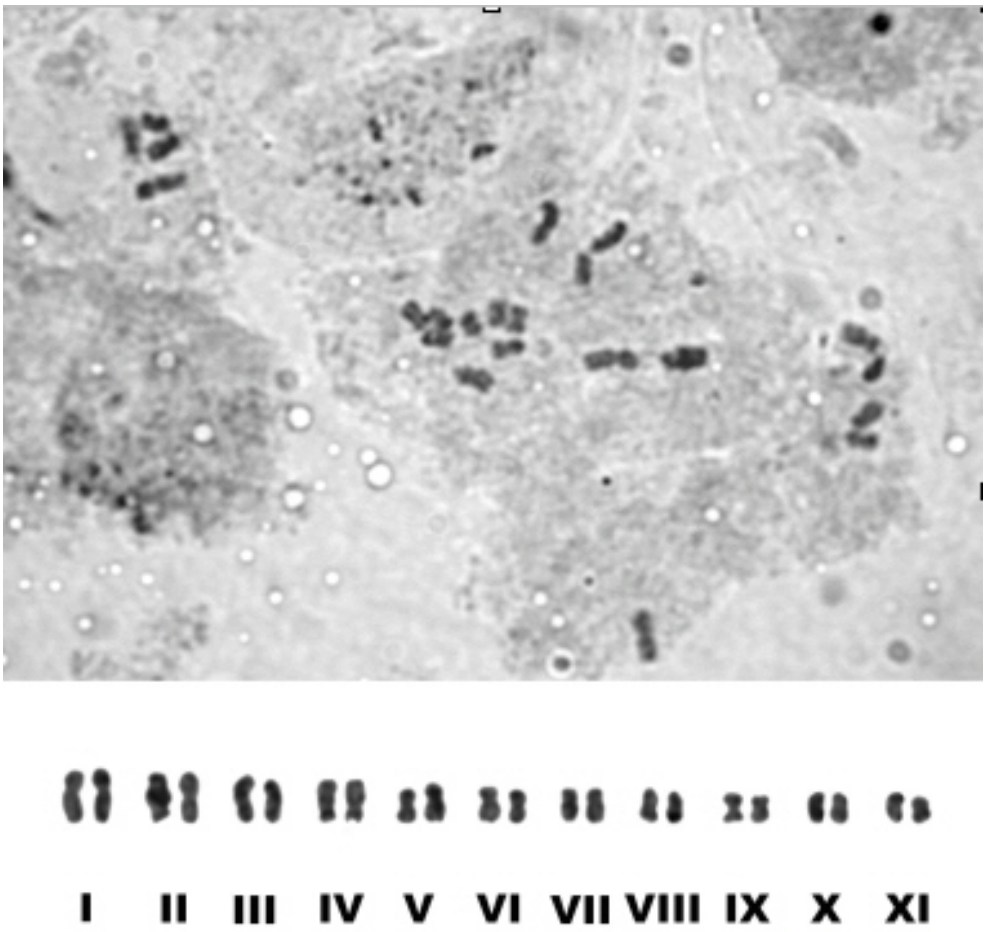

Plate III. Photo 1: Metaphase (x1000) and karyotype in *Stevia rebaudiana*

This is typical for plants of dry habitats. The palisade parenchyma located under the lower epidermis is less developed (1 cells layer) than that located under the upper epidermis (3 cells layers). The leaf has a bifacial heterofacial dorsiventral structure. Between the veins little vascular bundles, rely on the epidermis trough collenchyma girdles could be observed.

Chromosomes number in all analyzed metaphases (Pl. III) were found to be 2n=22.

We settled 11 chromosomes pairs arranged into decrease order of their total length media. As we can observe in Table 1, total length media were found from 3.76 μm (first chromosome pair) and 1.70 μm (last pair). Variability limits were close enough that confirms the authenticity of homologues chromosomes pair's establishment.

The difference between arms of the chromosomes ($L_a - S_a$) was 0.74 (pair IV) and 0.16 (pair X). For pair IX the difference was 0.0 that indicates the equality of arm length.

The arms ratio (L_a / S_a), essential for establishment of homologues, was found between 1.00 (pair IX) and 2.45 (pair IV). Centromeric index was between 28.88 (pair IV) and 50.73 (pair II). Relative length of the 11 chromosomes pairs were from 13.71 μm (pair I) to 6.19 μm (pair XI). The 22 chromosomes from *Stevia rebaudiana* were grouped into three morphological types: m (pairs II, III, V, VII, VIII, X and XI), sm (pairs I, IV and VI) and M (pair IX).

CONCLUSIONS

A particular feature of *Stevia rebaudiana* as a member of *Asteraceae* family is the opposite leaves disposition. These were demonstrating by histo-anatomical methods. In *S. rebaudiana*, fully differentiated functional glandular trichomes are already present at early stages of leaf development. They are numerousness in the top of the plant and decrease significant at the basis of the stem and in mature leaves. Biseriate glandular trichomes of the other species of *Asteraceae* were previously reported (Werker and Fahn, 1982, for *Inula crithmoides* and *I. graveolens*, Werker *et al*., 1994 for *Artemisia dracunculus*, Toma and Gostin, 2000, for *Chrysanthemum cinerareifolium*).

The diploid chromosome number in *Stevia rebaudiana* was found to be 2n=22. The chromosomes were short; their total length media were between 3.76 and 1.70. The chromosomes belong to three morphological types: m, sm and M. According Stebbins (1974) we consider that *S. rebaudiana* has an evolved, asymmetric karyotype.

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COMPARATIVE KARYOTYPE ANALYSIS IN MEMBERS OF APIACEAE (UMBELLIFERAE) FAMILY

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ABSTRACT

Umbelliferae family is represented on Earth by approximately 3500 species from 220 genuses [Minosuke, 1979]. In Romania there are 137 species from Umbelliferae [Kovacs *et.al*., 1973]. A large number of species from this family were used as food due to their nutritive properties (the content of sugars, proteins, lipid, vitamins), but also as medicinal plants with stimulatory, aromatics and antibiotics effects [Standley and Williams, 1966].
Chromosome's number in all members of Umbelliferae family investigated, were 2n=22, as in literature [Standley and Williams, 1966, Subramanian, 1986]. In *Petroselinum sativum* Hoffm. and *Anethum graveolens* L. we establish two morphological chromosomes types – **m** and **sm**. In *Coriandrum sativum* L. we have identified three chromosomes types (**sm**, **st** and **T**).
Key words: *Petroselinum sativum*, *Anethum graveolens*, *Coriandrum sativum*, karyotype, Umbelliferae.

INTRODUCTION

The Umbelliferae comprise a plants family with species which are distributed in most parts of the world, although are more commonly found in temperate regions. When botanists of the 16th century were classifying plants, the Umbelliferae was the first family to be recognised and systematically studied [Minosuke, 1979].
Although there are several species which are shrubs and small trees occurring mainly in South America, most of the umbellifers are herbaceous, annuals, biennials or perennials. Only a few genera of umbellifers are grown as ornamental garden plants, but many members of this family have practical uses. Carrots (*Daucus carota*) and parsnips (*Pastinaca sativa*), species are produced on a large scale as major root crops. In the past, species of *Lomatium*, which is the largest genus of umbellifers in the U.S.A., have provided staple foods for groups of Indians in the west of the country and also in Canada. Stems or leaves of other plants may also be used for food i.e. lovage (*Levisticum officinale*), angelica (*Angelica archangelica*), and celery (*Apium graveolens*) [Minosuke, 1979]. Culinary herbs include chevril (*Anthriscus cerefolium*), fennel (*Foeniculum vulgare*) and parsley (*Petroselinum crispum*). Spices as well as essential oils are derived from fruits and seeds. Examples include coriander (*Coriandrum sativum*), dill (*Anethum graveolens*), cumin (*Cuminum cyminum*), caraway (*Carum carvi*) and anise (*Pimpinella anisum*). Anise is also regularly used in the production of alcohol beverages as a flavouring substance. The most well-documented poisonous species is hemlock (*Conium maculatum*) [Standley and Williams, 1966]. Some other members of this family were commonly used in medieval times to cure ailments ranging from memory loss to eczema and gout. Dill (*Anethum graveolens*) and anise (*Pimpinella anisum*) are still commercially grown to produce crops for medicinal use.
Studies on karyotype have a well-known importance for species characterization. This kind has a great value because the chromosomal formula is useful in order to establish taxonomic position of each species, to choose the best way for selection and to to understand speciation process.

MATERIALS AND METHODS

The investigated species were *Petroselinum sativum* Hoffm., *Anethum graveolens* L. and *Coriandrum sativum* L. Germination of the seeds was performed into Petri dishes, on filter paper moistured with distilled water, at 22 ± 2°C. When roots were reached 5-10 mm in length, these were treated with 0.2% colchicine for 2 hours, at room temperature. Roots were kept for other two hours in distilled water. Fixation was done for about 16 hours in ethyl alcohol / acetic acid (3:1) mixture, at room temperature. For hydrolysis of vegetal material was used HCl 50% for 10 min, at room temperature. The staining was realized with Carr reactive, according literature [Cimpeanu *et al*., 2003; Das and Mallick, 1989; Ma *et al*., 1984; Pogan *et.al*., 1986; Poggio *et al*., 1994 and Stepanov and Muratova, 1995]. The slides were prepared according squash method. Microscopy was carrying out using 100x objectives, with a Nikon Eclipse 600 light microscope. Photos were taken with a Nikon CoolPix 950 digital camera, at 1600x1200 dpi resolution. All images were processed with Adobe Photoshop software.
Homologous chromosome groups were settled in accordance with the rapport between long arm and short arm, the mitotic index, the difference between arms and with the relative length of the chromosomes [Stepanov and Muratova, 1995 and Subramanian, 1986].

RESULTS AND DISCUSSIONS

***Petroselinum sativum* Hoffm.**

Chromosomes number in all analyzed metaphases were found to be 2n=22. From the best metaphase (Fig.1) we settled 11 chromosomes pairs arranged into decrease order of their total length media. As we can observe in Table 1, total length media were found from 3.33μm (first chromosome pair) and 2.40 μm (last pair). Variability limits were close enough that confirms the authenticity of homologues chromosomes pair's establishment. The difference between arms of the chromosomes ($L_a - S_a$) was 0.30 (pair III) and 0.00 (pairs V and X). The arms ratio (L_a / S_a), essential for establishment of homologues, was found between 1.00 (pairs V and X) and 1.22 (pair III). Centromeric index was between 49.83 (pair V) and 43.87 (pair III). Relative length of the 11 chromosomes pairs was from 10.43 (pair I) to 7.01 (pair XI). The 22 chromosomes from *Petroselinum sativum* Hoffm. were grouped into two morphological types: **M** (two pair) and **m** (nine pairs).

***Anethum graveolens* L.**

Chromosomes number in all analyzed metaphases were found to be 2n=22. From the best metaphase (Fig.2) we settled 11 chromosomes pairs arranged into decrease order of their total length media. As we can observe in Table 2, total length media (long arm + short arm + centromer) were found from 2.96 μm (first chromosome pair) and 1.81 μm (last pair). Variability limits were close enough that confirms the authenticity of homologues chromosomes pair's establishment. The difference between arms of the chromosomes ($L_a - S_a$) was 0.72 (pair I) and 0.03 (pair X). The arms ratio (L_a / S_a), essential for establishment of homologues, was found between 1.00 (pairs III and IX) and 1.66 (pair I). Centromeric index was between 61.14 (pair I) and 49.62 (pair III). Relative length of the 11 chromosomes pairs was from 10.80 (pair I) to 6.67 (pair XI). As in *Petroselinum sativum* Hoffm., the 22 chromosomes from *Anethum graveolens* L. were grouped into two morphological types: **M** (pairs III and IX) and **m** (nine pairs).

***Coriandrum sativum* L.**

Chromosomes number in all analyzed metaphases were found to be 2n=22. From the best metaphase (Fig.3) we settled 11 chromosomes pairs arranged into decrease order of their total length media. As we can observe in Table 3, total length media (long arm + short arm + centromer) were found from 2.88 μm (first chromosome pair) and 1.66 μm (last pair). Variability limits were close enough that confirms the authenticity of homologues chromosomes pair's establishment. The difference between arms of the chromosomes ($L_a - S_a$) was 1.15 (pair I) and 1.67 (pair III), bigger than the values from other investigated Umbelliferae. The arms ratio (L_a / S_a), essential for establishment of homologues, was found between 2.37 (pair I) and 10.76 (pair IX). Centromeric index was between 77.61 (pair V) and 91.47 (pair IX). Relative length of the 11 chromosomes pairs was from 12.99 (pair I) to 7.48 (pair XI). In *Coriandrum sativum* L. we were be able to identified next chromosomes types: **sm** (pair I) with r=2.37; **st** (pairs II, IV, V, VI, VII and VIII), with r between 3.84 and 6.16; **T** (pairs III, IX, X and XI) with r between 7.21 and 10.76.

In all investigated species of Umbelliferae chromosomes number was found to be 2n=22. On *Petroselinum sativum* Hoffm. and *Anethum graveolens* L. we establish two morphological chromosomes types – **m** and **sm**. The karyotype of these species is symmetric and less evolved. In *Coriandrum sativum* L. we have identified three chromosomes types (**sm**, **st** and **T**) and we consider that this specie has an evolved, asymmetric karyotype.

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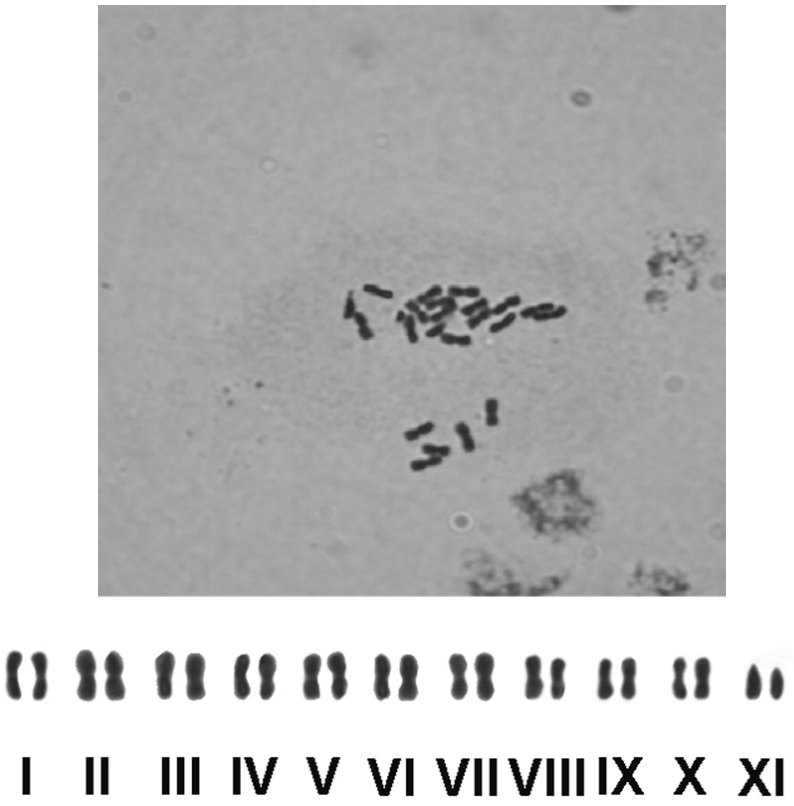

Fig. 1. Metaphase (up) and karyotype (down) in *Petroselinum sativum* Hoffm.

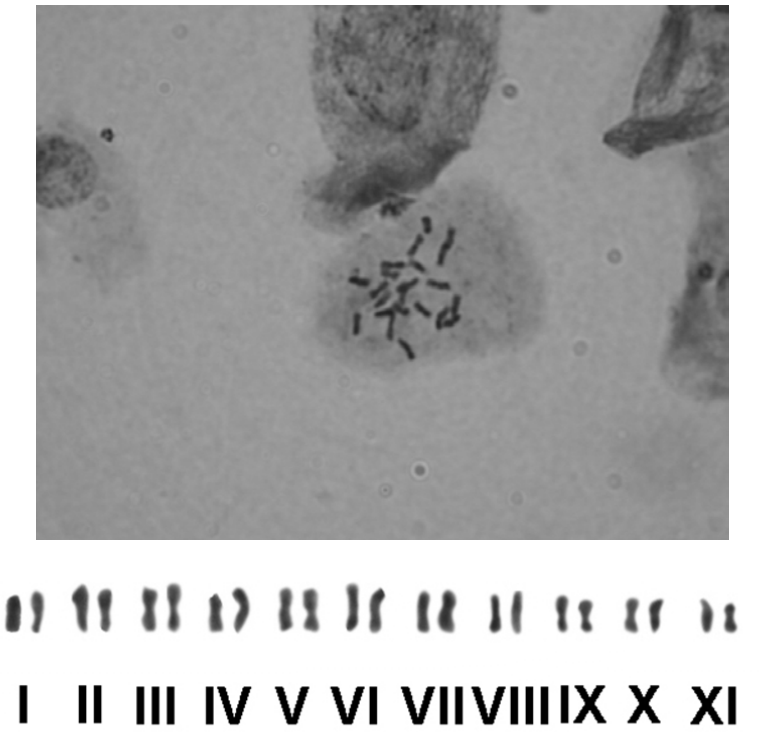

Fig. 2. Metaphase (up) and karyotype (down) in *Anethum graveolens* L.

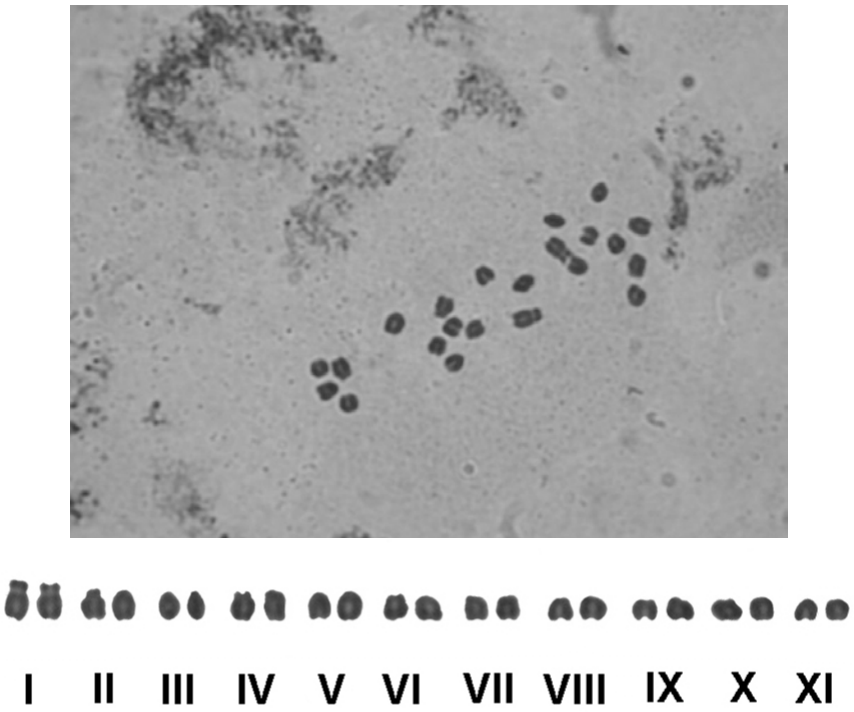

Fig. 3. Metaphase (up) and karyotype (down) in *Coriandrum sativum L.*

Table I. The chromosome traits at *Petroselinum sativum* Hoffm. (2n=22)

Chromosomes		Total length		Long arm (L_A)		Short arm (S_A)		Arm ratio		Arm sum (μm)	Arm difference (μm)	Centro-merique index	Relative length
Pairs	Types	(μm)	variabil. limits	(μm)	variabil. limits	(μm)	variabil. limits	L_A/S_A	variabil. limits				
I	m	3.33	3.33-3.33	1.75	1.75-1.75	1.51	1.51-1.51	1.15	1.15-1.15	3.26	0.24	45.34	10.43
II	m	3.18	3.18-3.18	1.58	1.57-1.60	1.51	1.45-1.57	1.04	1.01-1.08	3.09	0.07	47.48	9.96
III	m	3.10	3.03-3.18	1.66	1.66-1.66	1.36	1.36-1.36	1.22	1.22-1.22	3.02	0.30	43.87	9.71
IV	m	3.06	3.03-3.09	1.54	1.51-1.57	1.51	1.51-1.51	1.01	1.00-1.03	3.05	0.03	49.34	9.58
V	M	3.03	3.03-3.03	1.51	1.51-1.51	1.51	1.51-1.51	1.00	1.00-1.00	3.02	0.00	49.83	9.49
VI	m	2.90	2.87-2.93	1.46	1.45-1.48	1.44	1.42-1.45	1.02	1.02-1.02	2.90	0.02	49.65	9.08
VII	m	2.89	2.87-2.92	1.51	1.51-1.51	1.36	1.36-1.36	1.11	1.11-1.11	2.87	0.15	47.05	9.05
VIII	m	2.79	2.72-2.87	1.52	1.48-1.57	1.25	1.21-1.30	1.21	1.20-1.22	2.77	0.27	44.80	8.74
IX	m	2.76	2.75-2.78	1.45	1.45-1.45	1.31	1.30-1.33	1.10	1.09-1.11	2.76	0.14	47.46	8.64
X	M	2.64	2.57-2.27	1.27	1.27-1.27	1.27	1.27-1.27	1.00	1.00-1.00	2.54	0.00	48.10	8.27
XI	m	2.24	2.21-2.27	1.18	1.15-1.21	1.01	0.96-1.06	1.16	1.14-1.19	2.19	0.17	45.08	7.01

Table II. The chromosome traits at *Anethum graveolens* L. (2n=22)

Chromosomes		Total length		Long arm (L_A)		Short arm (S_A)		Arm ratio		Arm sum (μm)	Arm difference (μm)	Centro merique index	Relative length
Pairs	Types	(μm)	variabil. limits	(μm)	variabil. limits	(μm)	variabil. limits	L_A/S_A	variabil. limits				
I	m	2.96	2.87-3.03	1.81	1.81-1.81	1.09	1.06-1.12	1.66	1.61-1.70	2.90	0.72	61.14	10.80
II	m	2.82	2.78-2.87	1.48	1.45-1.51	1.24	1.13-1.36	1.19	1.11-1.28	2.72	0.24	52.48	10.29
III	M	2.66	2.60-2.72	1.33	1.30-1.36	1.33	1.301.36	1.00	1.00-1.00	2.66	0.00	50.00	9.71
IV	m	2.64	2.57-2.72	1.38	1.35-1.41	1.20	1.14-1.26	1.15	1.14-1.16	2.59	0.18	49.62	9.63
V	m	2.61	2.60-2.62	1.51	1.51-1.51	1.06	1.06-1.06	1.42	1.42-1.42	2.57	0.45	57.85	9.52
VI	m	2.54	2.51-2.57	1.48	1.42-1.54	1.04	1.03-1.06	1.42	1.33-1.52	2.52	0.44	58.26	9.27
VII	m	2.49	2.48-2.50	1.28	1.27-1.30	1.21	1.21-1.21	1.05	1.04-1.07	2.49	0.07	51.61	9.05
VIII	m	2.45	2.42-2.48	1.46	1.45-1.48	0.88	0.87-0.90	1.65	1.61-1.70	2.34	0.58	59.59	8.94
IX	M	2.33	2.24-2.42	1.16	1.12-1.21	1.16	1.12-1.21	1.00	1.00-1.00	2.32	0.00	49.78	8.50
X	m	2.09	2.06-2.12	1.06	1.06-1.06	1.03	1.00-1.06	1.03	1.00-1.06	2.09	0.03	50.71	7.63
XI	m	1.81	1.75-1.87	0.93	0.90-0.96	0.87	0.84-0.90	1.06	1.06-1.07	1.79	0.06	51.38	6.66

Table III. The chromosome traits at *Coriandrum sativum* L. (2n=22)

Chromosomes		Total length		Long arm (L_A)		Short arm (S_A)		Arm ratio		Arm sum (μm)	Arm difference (μm)	Centromerique index	Relative length
Pairs	Types	(μm)	variabil. limits	(μm)	variabil. limits	(μm)	variabil. limits	L_A/S_A	variabil. limits				
I	sm	2.88	2.87-2.90	1.99	1.96-2.03	0.84	0.81-0.87	2.37	2.33-2.41	2.83	1.15	69.09	12.99
II	st	2.22	2.21-2.24	1.87	1.87-1.87	0.34	0.33-0.35	5.50	5.34-5.66	2.21	1.53	84.23	10.01
III	T	2.20	2.20-2.21	1.94	1.93-1.96	0.26	0.26-0.27	7.21	7.18-7.25	2.20	1.67	88.18	9.92
IV	st	2.16	2.15-2.18	1.69	1.66-1.72	0.44	0.43-0.45	3.84	3.82-3.86	2.13	1.25	78.24	9.74
V	st	2.10	2.09-2.12	1.63	1.60-1.66	0.37	0.40-0.42	3.91	3.95-4.00	2.00	1.26	77.61	9.47
VI	st	1.84	1.84-1.84	1.58	1.57-1.59	0.25	0.25-0.26	6.16	6.11-6.21	1.83	1.33	85.86	8.29
VII	st	1.82	1.81-1.84	1.54	1.54-1.54	0.25	0.25-0.25	6.16	6.16-6.16	1.79	1.29	84.61	8.20
VIII	st	1.80	1.80-1.81	1.49	1.48-1.50	0.28	0.28-0.28	5.31	5.28-5.35	1.77	1.21	82.77	8.11
IX	T	1.76	1.75-1.75	1.61	1.60-1.63	0.15	0.15-0.15	10.76	10.66-10.86	1.76	1.46	91.47	7.93
X	T	1.73	1.72-1.75	1.58	1.57-1.59	0.15	0.15-0.15	10.53	10.46-10.60	1.73	1.43	91.32	7.80
XI	T	1.66	1.66-1.66	1.48	1.48-1.48	0.15	0.15-0.15	9.89	9.89-9.89	1.63	1.33	89.15	7.48

[P-067]

COMPARATIVE ANATOMY OF SOME SPECIES FROM *EQUISETUM* GENUS

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ABSTRACT

In this paper different aspects of histo-anatomical structure of 4 species of *Equisetum* genus (*E. arvense* L., *E. telmateia* Ehrh., *E. fluviatile* L. and *E. ramosissimum* Desf.) was studied. *Equisetum* aerial parts are traditionally used in poor digestion, to improve bile flow and as a liver protective. It is also a diuretic used in water retention and arthritis. The structural features are correlated with the environmental conditions and functionality of the vegetative organs. The epidermis has cells with thick walls at the internodal level and with thin walls at the nodal one. The aeriferous canals from middle cortex are a commune feature of this genus. They missing only in median and superior internodes of *E. fluviatile*; on the other hand, at this species the central aeriferous cavity is very large. The central cylinder is usually delimited by a casparyan endodermis. In *E. fluviatile* this endodermis is missing, but a layers of orderly cells (with thick external and lateral walls) surrounded each vascular bundles (possible with value of endodermis, with the role to control the water flux in the stem).
Key words: palisade parenchyma, aeriferous cavity, endodermis.

INTRODUCTION

The 15 living species of the genus *Equisetum* comprise the plants commonly known as horsetails. The genus name is derived from the Latin *equis*, meaning horse and *seta*, meaning bristle, in reference to the coarse black roots of *Equisetum fluviatile* which resemble a horse's tail. The genus *Equisetum* is the only remaining representative of the once abundant and diverse subdivision Sphenophytina.

All *Equisetum* species are herbaceous perennials. The plants consist of upright aerial stems, which arise from a very extensive underground rhizome system (Hauke, 1963). The upright aerial stems exhibit a monopodial branching pattern, having one main axis of growth. *Equisetum* species also have small microphyllous leaves that are arranged in true whorls and the leaves of each whorl are fused together to form a cylindrical sheath around each node. *Equisetum* species grow in wet places such as moist woods, ditches, wetlands, and in road fill where sufficient groundwater is available.

Equisetum arvense is an excellent genito-urinary system astringent. It may be applied to such conditions as urethritis or cystitis with haematuria, reducing haemorrhage and healing wounds thanks to the high silica content. The diuretic action is thought to be due partly to the flavonoids and saponins. Correct identification of this plant is important since other species of Equisetum contain toxic alkaloids, and excessive doses of *E. arvense* can themselves lead to symptoms of poisoning.

The histo-anatomical features of these species are important to identify the fragments of the organs in medicinal preparates, when the whole plant is not available. The structure of the aerial stem of *Equisetum hyemale* with a special overlook on the endodermis was described by Speck *et al*. (1998). Other structural aspects concerning different *Equisetum* genus was investigated by different authors: Hauke, 1954 (stomatal apparatus), Gifford, 1993 (development of root apical meristem), Kuriyama *et al.*, 1990 (histology and development of sporofitic shoot in *E. arvense*), Niklas, 1987 (the epidermis of the *Equisetum* rhizome).

MATERIAL AND METHODS

The material (aerial organs of 4 *Equisetum* species) was collected in 2003 and 2004 from Ceahlau Mountains and Potoci (Research Station). Fixation and conservation of the material was made in 70% ethylic alcohol; after that, the vegetal material was sectioned by chryotome. The obtained sections were coloured either with green-iod and ruthenium red. The permanent preparations have been analysed on a Novex (Holland) microscope and photographed with a Minolta camera.

RESULTS AND DISCUSSION

Equisetum arvense

This species produces aerial branching systems of two types; the branching is dimorphic. Very early in the spring, brownish fertile stems break through the soil and bear terminal cones. These cones are made up of

sporangiophores, and the fertile stems are ephemeral. Later in the spring, the same rhizomes produce green, vegetative branching systems. These systems have whorls of scale leaves, and branches of several orders arise in whorls.

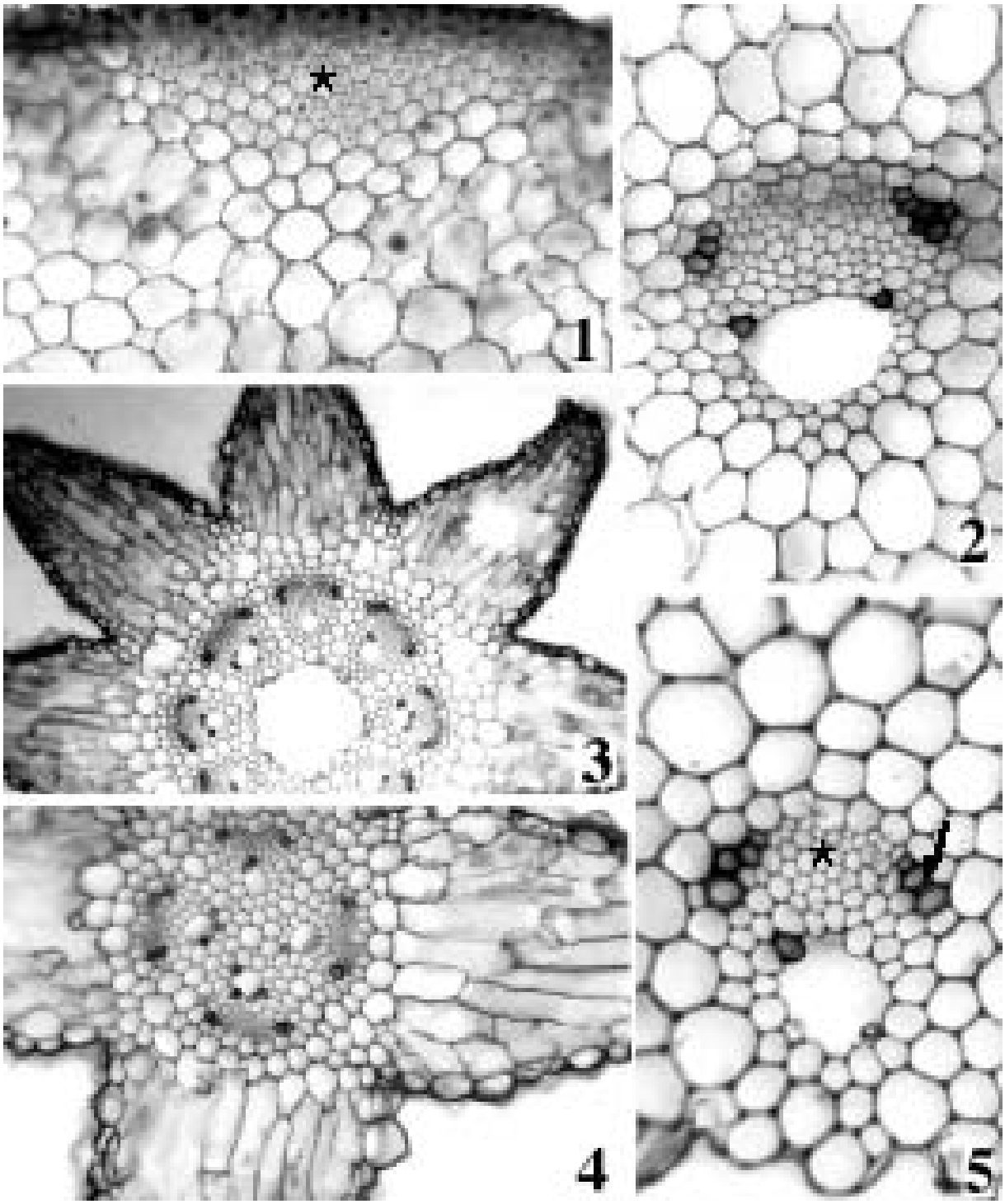

Plate I. *Equisetum arvense*: Photo 1. Cross-section from the stem (collenchyma-aster) (basal part) (x200); Photo 2. Cross-section from the stem (basal part) (carnal canal-arrow) (x200); Photo 3. Cross-section from the top of the stem (x100); Photo 4. Cross-section from the branch (x100); Photo 5. Cross-section from the branch (xylem-arrow, phloem-aster) (x200).

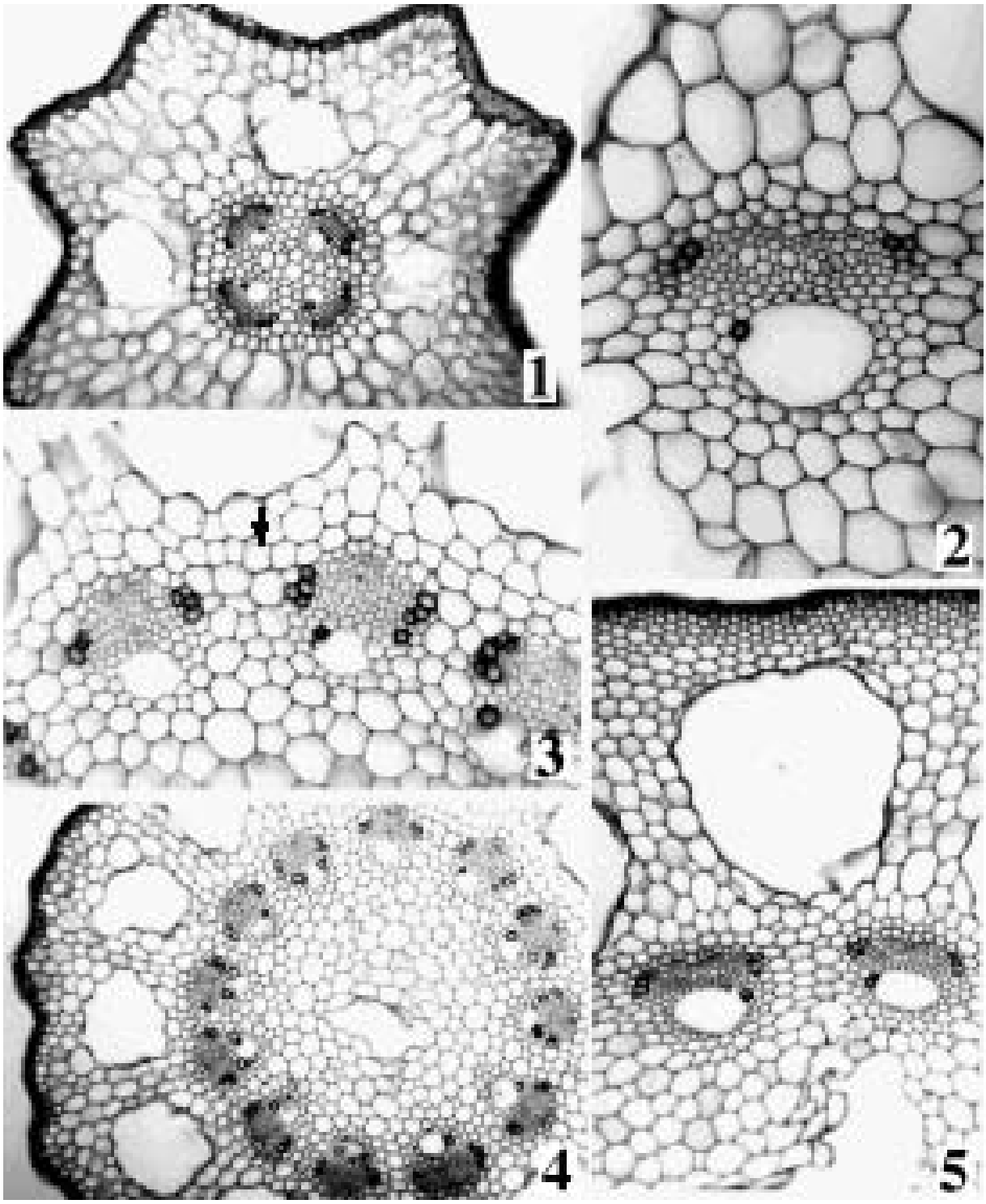

Plate II. *Equisetum telmateia*: Photo 1. Cross-section from the branch (x100); Photo 2. Cross-section from branch (x200); Photo 3. Cross-section from the top of the stem (endodermis-arrow) (x200); Photo 4. Cross-section from the top of the stem (x100); Photo 5. Cross-section from the stem (basal part) (x100).

The base of the sterile stem have circular shape with ribs; in the ribs collenchyma and assimilatory tissues could be observed (Pl. 1, Photo 1); the cortex have three zones: an external one, middle one (with aeriferous canals) and an internal one finished with an endodermis. The vascular bundles are formed from phloem, xylem vessels and an aeriferous cavity (vascular canal) witch is caused by the destruction of protoxylem during elongation (Pl. I, Photo 2).

The top of the sterile stem (Pl. I, Photo 3) the contour presents 8 very prominent ribs; in the ribs a well-developed palisade parenchyma could be observed; in the middle cortex small aeriferous canals are present. In the central cylinder are 8 vascular bundles (with 7-10 metaxylem vessel and 1-3 protoxylem vessels).

The branches have a square-rhombic profile, with very prominent ribs (Pl. I, Photo 4). The vascular bundles have small carinal (vascular) canals (Pl. I, Photo 5).

Equisetum telmateia

Basis of the stem (sterile) – the contour in cross-section is quite circular; the epidermic cells has thick and lignified walls (Pl. II, Photo 5); the cortex is divided in an external part (cells with thick walls), a middle part (with aeriferous canals) and an internal one finished with a primary endodermis. Speck *et al*., (1998) spotlight the mechanical role of the external cortex named by him as hypodermal sterome. The vascular bundles (18-19) are formed from phloem (sieve cells) and few xylem vessels located near the aeriferous lacuna. In the principal stem the chlorenchyma is missing. In the top of the stem the cortical and carinal canals are well represented, but the medular lacuna is missing. The branches have a circular shape with 4-5 ribs in cross-section (Pl. II, Photos 1, 2). In the external cortex palisade parenchyma could be observed.

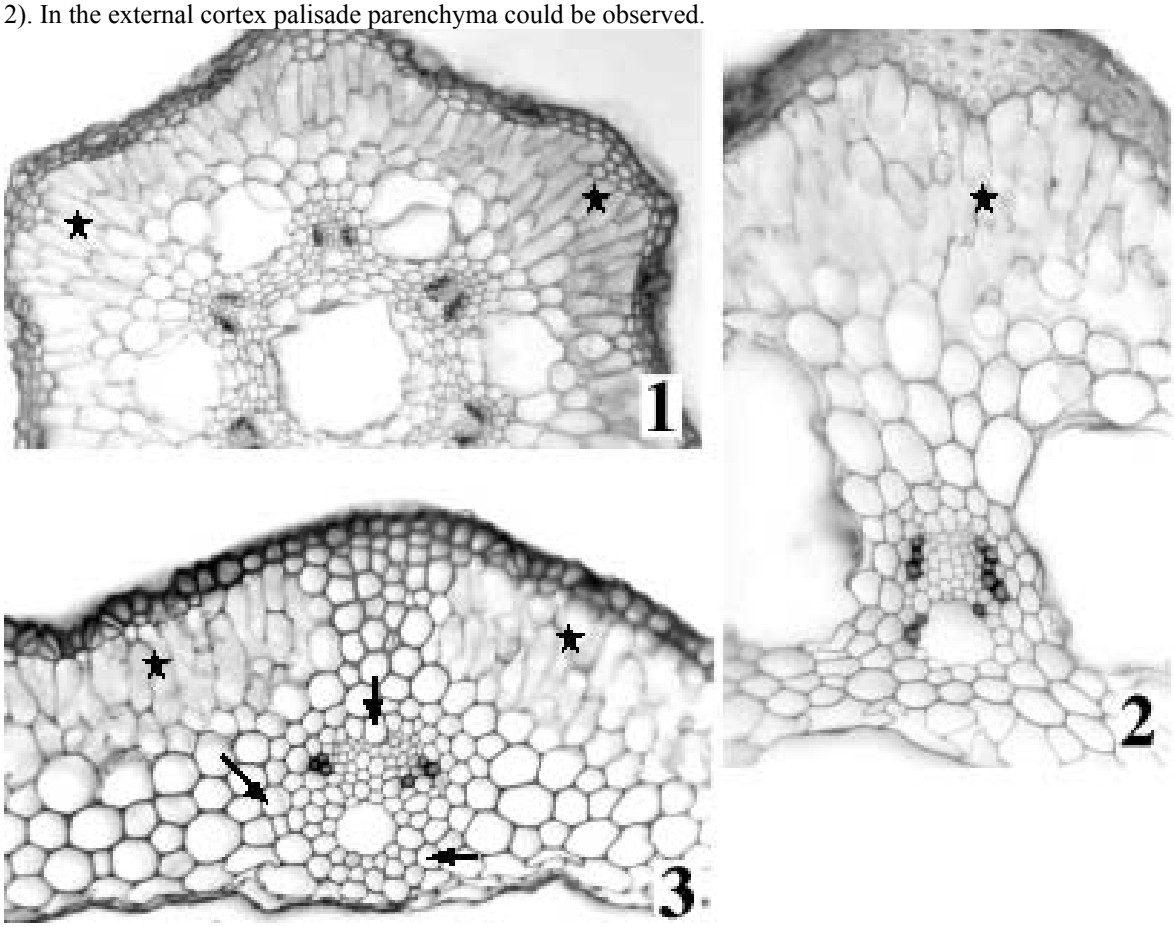

Plate III. *Equisetum ramosissimum*: Photo 1. Cross-section from the branch (x200); Photo 2. Cross-section from the stem (x400); *Equisetum fluviatile*: Photo 3. Cross-section from the stem (endodermis-arrows) (basal part) (x200) (palisade parenchyma in all photos-aster).

Equisetum fluviatile

At the basis of the stem the external cortex is thin, middle cortex has aeriferous canals; the vascular bundles are surrounded by primary endodermis (Pl. III, Photo 3). Middle of the stem - the contour is circular with low prominent ribs; the external cortex have assimilatory tissue, the middle cortex with aeriferous canals is missing; the vascular bundles (18-20) are connected with the epidermis through some wide sclerenchyma girdles. In the top of the sterile stem, the external cortex has assimilatory tissues; the vascular bundles are small, with few xylem vessels. The branches have an aster shape; with well develop palisade parenchyma, without aeriferous canals and with a large pith lacuna.

Equisetum ramosissimum

The stem have a circular shape with small ribs in cross-section (Pl. III, Photo 2); the epidermal cells have thick walls; a continuous collenchyma ring in sub epidermal position could be observed at the basis of the stem. The assimilatory tissue is present in the external cortex (in the ribs). The vascular bundles have 4-5 xylem vessels

disposed near the phloem. In cross-section the branches have a circular shape, with 6 ribs. The collenchyma ring is less developed, but the palisade parenchyma is well represented (Pl. III, Photo 1).

CONCLUSIONS

The external cortex contain palisade parenchyma in *E. arvense, E. ramosissimum* and in superior internodes of *E. fluviatile*. In *E. telmateia* principal axis the palisade tissue is missing; it is present in lateral branches. The vascular bundles are formed by phloem (sieve cells), well developed in all analysed species and few xylem vessels (tracheids) localized near the aeriferous lacuna from the bundle or from a part and another of the phloem (*E. ramosissimum*). The structural features of these species could be useful in determination of powders compositions.

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[P-067]

[P-074]

INVESTIGATION OF MISTLETOE (*VISCUM ALBUM* L.) EXTRACTS FROM PLUM HOST TREE CYTOTOXICITY – EHRLICH CARCINOMA

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ABSTRACT

Viscum album L. has been known as a secondary medicament. Mistletoe extracts are widely used in therapy of hypertension today, and have been shown to possess significant antitumour activity, *in vivo*, against certain carcinoma types. In this paper effects of different aqueous extracts obtained from mistletoe from plum tree on, *in vivo*, cancer prevention and inhibition of carcinogenesis of Ehrlich carcinoma were examined. Animals used in experiments (MNRI mice) were divided into groups. The first was control group (mice with transplanted Erlich carcinoma), the other groups received standardized aqueous extracts of mistletoe leaves collected from plum host tree as a late therapy, prevention and early therapy. We have observed significant reduction of cancer incidence in all groups that received mistletoe extracts in comparison to control. Carcinogenesis of Erlich breast carcinoma was inhibited almost 100% in male animals that received mistletoe extracts as a prevention, and approx. 85% in female animals. We also observed significantly reduced number of carcinoma cells in animals with developed carcinoma and high cytotoxicity of mistletoe extracts expressed through high percentage of damaged carcinoma cells.

Keywords: *Viscum album*, cytotoxicity, antitumour, Ehrlich carcinoma

INTRODUCTION

Since the earliest of times, mistletoe (*Viscum album* L.) has been one of the most magical, mysterious and sacred plants in nature, and used as a secondary medicament. Mistletoe exhibits a number of pharmacological activities: nervine, hypotensive, cardiac depressant, possibly anti-tumour (Hoffman, 1990), vasodilatator, slowing and steadying an excessive heart rate, relaxant, diuretic, stimulant, etc (Mills, 1994). Today, mistletoe extracts are widely used in therapy of hypertension (British Herbal Pharmacopoeia 1983, 1989) and have been shown to possess significant antitumour activity, *in vivo*, against Lewis lung carcinoma, colon adenocarcinoma 38 and C3H mammary adenocarcinoma 16/C. Main groups of compounds found in mistletoe are lectins, viscotoxins, flavonoids (Chou *et al*., 1999; Wollenweber *et al*., 2000), phenylcarbonic acids, polysaccharides, alcaloids, biogenamines (feretylamine, tyramine, hystamine, acetylholine), tannins, terpenoids, saponnins, phytosterols, aminoacids, vitamins, etc. Some of those compounds are proved to have cytotoxic effects and immunomodelling potential (Fischer *et al*., 1997; Stein *et al*., 1998).

Considering well known properties of some polyphenols found in *Viscum album* L., the effects of aqueous extract obtained from mistletoe leaves from plum as host tree on, *in vivo*, cancer prevention and inhibition of carcinogenesis of Erlich carcinoma was examined. The aim of the study was to follow the modulation of antitumour activity of aqueous extract of *Viscum album* leaves from plum tree on mice with ascit form of Erlich carcinoma (EAC), determine the number of carcinoma cells and their vitality (cell damage degree as a result of potential cytotoxic activity of examined mistletoe extract), as well as determination of biochemical parameters (markers of oxidative stress) in blood and liver.

MATERIALS AND METHODS

Preparation of *Viscum album* extract: Dried leaves of *Viscum album* L., collected from plum as a host tree, were used for preparation of *Viscum album* extract. Active components (polyphenols, lectins and viscotoxins) were extracted with boiling water in order to get primary infuse. After purification and dilution of the infuse, pure aqueous extract of *Viscum album* leaves was obtained. Extract was kept in the freezer (4-8ºC). Prepared extract was given to animal's i.p.

Animals used in experiments (MNRI mice) were divided into groups. The first was control group (mice with transplanted Erlich carcinoma), the second was the group in which animals were treated for 7 days with standardized aqueous extract of mistletoe leaves collected from plum host tree 5 days after Erlich carcinoma was transplanted. The third group received extract (i.p.) as a potential prevention of carcinoma 7 days before carcinoma was transplanted. The last group received extracts and carcinoma was transplanted at the same time. After 12 days, animals were sacified, ascites of carcinoma were collected and analysed. Also liver was removed and blood samples collected for further analysis. After the isolation of the carcinoma cells, their weight have

been determined, as well as their number and percentage of damaged cells. Vitality of the cells have been determined after colouring with tripanblue.

RESULTS AND DISCUSSION

Results obtained in this experiment showed a few interesting points. All results were obtained by measurement of 5 samples and they are certainly statistically significant. As it can be seen from the Figure 1, rather strong reduction of cancer incidence was observed in all groups that have received mistletoe extracts in comparison to Ehrlich control. Differences among male and female animals were also significant. Mistletoe extract used in experiment showed much better results in male mice in comparison to females. As it was expected, number of Ehrlich (breast) carcinoma cells was lower in male animals than in female animals in Ehrlich control group. Quantity of ascites was significantly reduced in mice of both sexes that received *Viscum* extract, both as a prevention (group III) or as a therapy (groups IV and V). Cell weight (expressed in mg/ml ascites) was also much lower in animals treated with the *Viscum* extract than in Ehrlich control group. Reduction in cell weight ranges from 83,3% in mice treated with *Viscum* extract five days after EAC transplantation to 66,4% in animals with administered mistletoe extract and carcinoma transplanted at the same time. Carcinogenesis of Erlich breast carcinoma was inhibited almost 100% in male animals that received mistletoe extracts as a prevention, and approx. 85% in female animals. Decrease in cell number was highest in male mice that received Viscum extract as a prevention, almost 95%, and in females with the same experimental protocol, approx. 60%. We also observed significantly reduced number of carcinoma cells in animals with developed carcinoma, that ranges from 83% for males to 60% for females. Differences in the results obtained with the extracts as a prevention or therapy are much more significant in male animals.

Furthermore, possible high cytotoxicity of mistletoe extracts expressed through high percentage of damaged carcinoma cells was noticed in the second group of measurements. Results are presented in Figure 2. The highest percentage of damaged carcinoma cells was observed in the fourth male group, with animals that received *Viscum* extract as a therapy (five days after the EAC transplantation), that means 21% of damaged cells in comparison to 8,9% in control group. Female animals showed better response to mistletoe therapy in the fifth group, where extract was given at the moment carcinoma was transplanted, approx 19%. All presented properties of the *Viscum album* extract from plum as a host tree might be due to the presence of lectins and viscotoxins that have already proved their cytotoxicity. In addition to these results, some biochemical markers of oxidative stress were also analysed in blood and liver, and showed significant decrease after the treatment with *Viscum* extract. Since polyphenols are known to be responsible for possible antioxidative properties of plant extracts, we may conclude that they might play an important role in prevention and inhibition of EAC development as well.

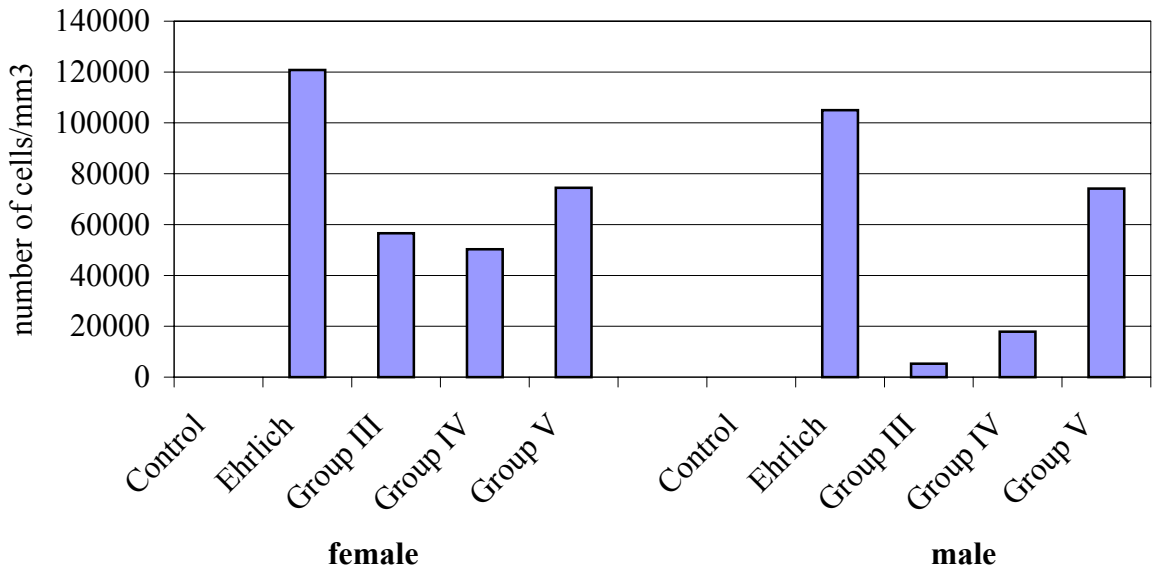

Figure 1. Effects of *Viscum album* L. leaves aq. extract from plum tree on number of Ehrlich carcinoma cells.

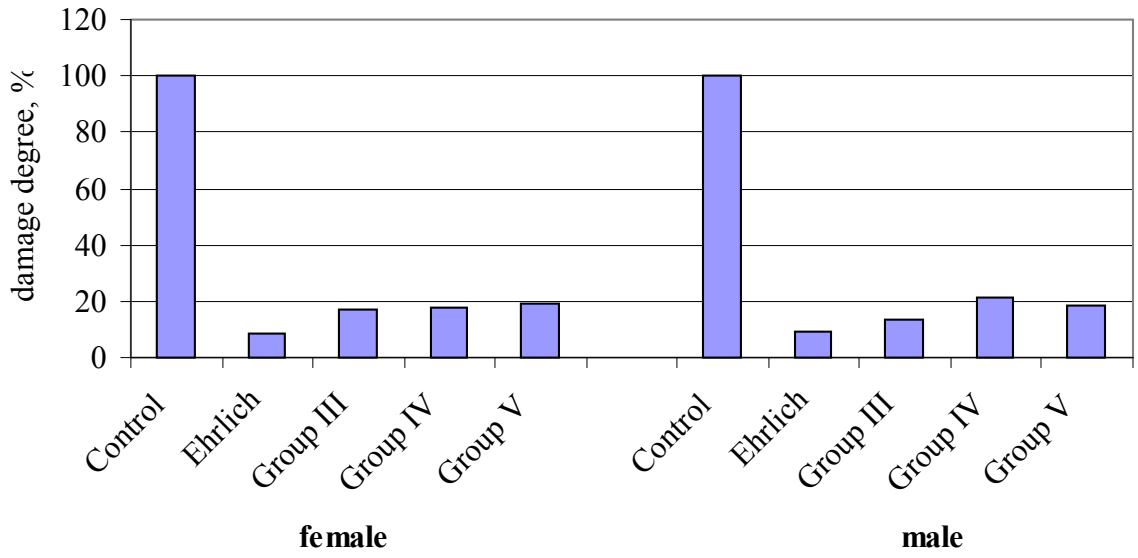

Figure 2 Effects of *Viscum album* L. leaves aq. extract from plum tree on Ehrlich carcinoma cell damage degree.

ACKNOWLEDGEMENTS

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[P-074]

[P-109]

NURSERY PLANTS PRODUCTION OF FEVERFEW - *Tanacetum parthenium* (L.) Schultz-Bip. (*Asteraceae*)

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ABSTRACT

Increasing demand for rich source and standardized quality row material of Feverfew imposes necessity to develop appropriate growing techniques and introduce this plant species into culture.
Four high parthenolide populations* selected for further work on the basis of criteria important for cultivation came out as a result of preliminary investigations. In two-year experiments two models of nursery plant production have been investigated; winter production in green house conditions (January - March) and summer production in the open environment (July - September).
In both models of nursery plants production, stage of germination occurred 5-6 days and stage of seedlings 7-8 days following the sowing. First pair and the third pair of true leaves have completed their formation depending on climatic conditions; in winter production in 20-22 days and in 39-40 days respectively following the sowing while in summer production in 15-16 days and in 30-34 days, respectively. The nursery plants reached its appropriate stage for transplantation (five pairs of true leaves), in winter production 56-67 days and in summer production 47-51 days following the sowing. The height of ready nursery plants and their overall appearance also showed differences between the examined models of production, the winter ones' leaf petioles being in general more longer then in summer ones that looked more compact. In general, summer production gave nursery plants of better quality, in shorter period of time and with lower production expenses.
Key words: Feverfew, models of nursery plants production.

INTRODUCTION

Tanacetum parthenium (L.) Schultz-Bip. *(Asteraceae)*, belongs to genus *Tanacetum* L. It is well-know by its common name Feverew. Interest in this plant species has been recently renewed since it is believed that its active compound sesquiterpene lactone parthenolide (Johnson *et al*., 1985; Murphy, 1988), found in the leaves and flowers of this plant, also has beneficial effects in prophylactic treatments of migraine (Barsby *et al*., 1993). An increasing demand for standardized quality of Feverfew row material imposes necessity to develop appropriate growing techniques and introduce this plant species into culture.
The aim of this experiment was to determine optimal model of nursery plant production for two optional periods (winter and summer) for Feverfew crop establishment in agro-ecological conditions of Serbia, what will present a major steps in process of its introduction into cultivation and will certainly greatly contribute to development of its growing technology.

MATERIAL AND METHODS

Four high parthenolide accessions, TP4, TP9, TP10 and TP12*, selected for further work on the basis of criteria important for cultivation, came out as a result of preliminary investigations (Nastovski et al, 2002). In two-year experiments, conducted during the period 2002 - 2004, both models of nursery plant production, the summer and the winter production, have been investigated.

The seeds used in experiments were collected in summer 2002 and 2003, from mother plants grown in isolation. Testing of seeds in laboratory conditions of the Institute for Medicinal Plant Research "Dr. J. Pančić", showed that the percentage of total seed germination (recorded 21st day following the sowing) for seeds started in summer ranged 53.0 - 98.1 % while for the same seeds started 6 months later (winter production) ranged 22.3 - 67.3 %**,** depending on accession. Prior to be sown, the seeds in all experiments were preventively treated with 0.1% solution of Benomyl. Production of nursery plants for all experiments was organized in multi-cell containers, with the single unit volume of ca. 90 ml, staffed with appropriate commercial substrate.

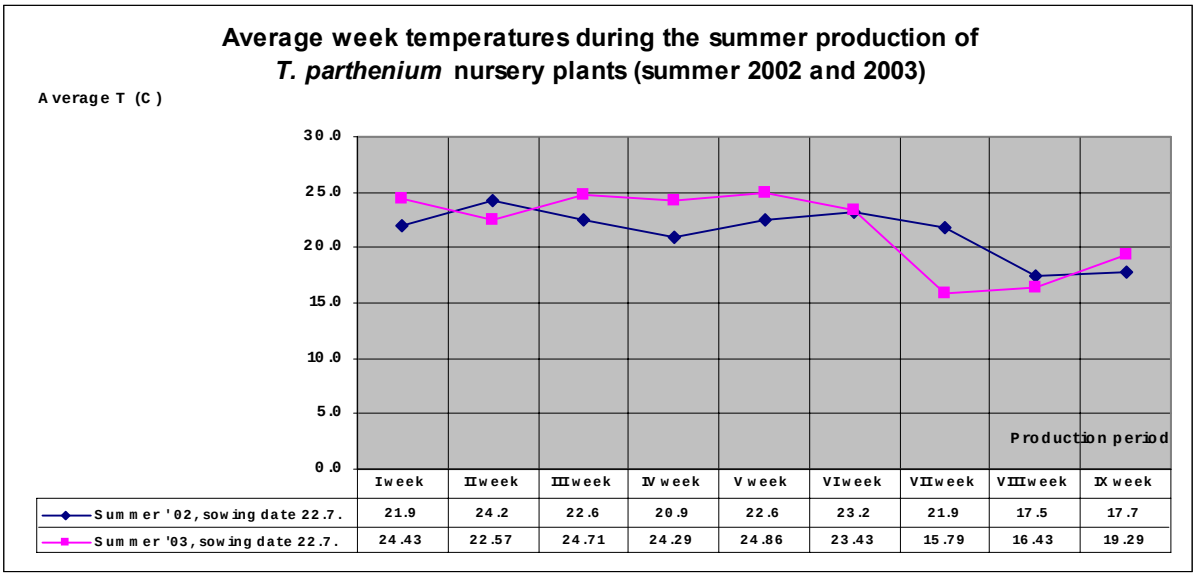
Summer production of nursery plants

Summer production of nursery plants was conducted both years in the open environment, at experimental filed of the Institute of Medicinal Plant Research - locality Pančevo (South Banat), in following periods: 22.7.-

*__TP4__ (Biologisch Dynamische Zaadteelt, *De Bolster*, Kielwindeweer-Gr, Holand), __TP9__ (Botanical Garden of Pisa (805/3), Italy), __TP10__ (Horizon Herbs, Williams, Oregon, USA), __TP12__ (Richter′s - The Herb Specialists Goodwood, (S2480-AA), Ontario, Canada.

1.9.2002., and 22.7. - 27. 8. 2003. The substrate used was intended for summer nursery plant production and it had following characteristics: N 50-300 mg / L, P_2O_5 80 – 300 mg / L, K_2O 80 - 400 mg / L, salts < 1,5 g / L, pH / $CaCl_2$ 5,0 – 6.5. The nursery plants were subjected to the influence of day/night temperatures (Chart 1.), but were left in semi-shade to be protected from high sun radiation. Moisture of the substrate was maintained at optimal level by repeated watering. Nursery plants received in stage of 3 pairs of true leaves one portion of additional liquid fertilization with 0.5% water solution of commercial liquid fertilizer, applied in dose of 100 ml /L of the substrate (fertilizer composition: N 12%, P_2O_5 4%, K_2O %, and micronutrients).

Diagram 1. Comparative presentation of average week temperatures during summer nursery plant production



Winter production of nursery plants

Experiments were conducted both years in greenhouse conditions of the Faculty of Agriculture, University of Belgrade, in following periods: 27. 1. - 24. 3. 2003; and 29.1. - 1. 4. 2004. Maximal diurnal T in greenhouse conditions, during winter production of nursery plants in 2003, ranged 22 - 24.5° C and the minimal T ranged 18.5 - 20° C, while in 2004 they ranged 17- 20° C and 20 - 28°C, respectively.

The seeds were sown at the surface of commercial substrate intended for use in winter nursery plants production and it had following characteristics: mixture of 70% white and 30% black peat, N 70 - 150 mg /L, P_2O_5 80 – 180 mg / L, K_2O 140 – 220 mg / L, salts 0.5 - 1.1 g / L, pH / $CaCl_2$ 5.2 - 6.0, super fine structure.

Moisture of the substrate was maintained at optimal level by repeated watering. Nursery plants received one portion of additional liquid fertilization with 0.5% of the same commercial liquid fertilizer applied in same dose and same stage of development as explained for summer nursery plants production.

Nursery plants development was monitored in all experiments and changes were recorded in regular periods, starting with days to germination, via stages of appearance of the first pair of cotyledon leaves when the seedlings were 1 cm high, appearance of the first pair of true leaves, appearance of the three pairs of true leaves, up to the stage of five pairs of fully formed true leaves. Height of nursery plants was also measured, but only in the last two stages of development (stage of appearance of 3 pairs and full development of 5 pairs of true leaves). All measurements were conducted in 10 replications and data were analyzed for statistical significance (ANOVA, T-test).

RESULTS AND DISSCUSSION

Differences in tempo of development between tested accessions ranged from 2-8 days, depending of stage of development; 3 of 4 accessions expressed similar tempo (2-3 days difference), while the fourth one expressed a bit greater difference.

Table 1 Development of summer nursery plants production of 4 selected (high parthenolide) accessions of Feverfew (*Tanacetum parthenium*), tested in two experimental years, 2002 and 2003.

Trial	Feverfew accessions	Sowing date	Germi-nation	Appearance of the I pair of cotyledon leaves (1 cm)	Appearance of the I pair of true leaves	Appearance of the III pair of true leaves		Fully developed five pairs of true leaves (ready for transplantation)	
			days	Days	days	days	height (cm)	days	height (cm)
Summer , 2002	TP4	22.7.	5	9	13	20	2.75	48	9.84
	TP9	22.7.	5	10	16	25	1.81	51	7.15
	TP10	22.7.	5	9	13	20	2.85	49	10.19
	TP12	22.7.	6	10	14	21	2.45	50	9.80
	LSD 5%						***0.48***		***0.71***
Summer , 2003	TP4	22.7.	5	9	12	19	2.61	47	9.51
	TP9	22.7.	6	10	16	26	1.59	52	6.54
	TP10	22.7.	5	9	12	20	2.50	50	10.91
	TP12	22.7.	5	9	13	23	2.25	49	9.17
	LSD 5%						***0.41***		***0.64***

In both models of nursery plants production, stage of germination occurred 5-6 days and stage of seedlings 7-8 days following the date of sowing. The first pair of cotyledon leaves was formed in 9-10 days in summer production and in 10-12 days in winter nursery plants production. The first and the third pair of true leaves appeared during the period 12-16 and 19-26 days following sowing in summer production, and 14-21 and 29-34 days in winter production, respectively (tables 1 and 2.). The nursery plants reached its appropriate stage for transplantation (five pairs of permanent leaves) in winter production 56-67 days and in summer production 47-52 days following the date of sowing (tables 1 and 2).

Table 2 Development of winter nursery plants production of 4 selected (high parthenolide) accessions* of Feverfew (*Tanacetum parthenium*) tested in two experimental years, 2002 and 2003.

Trial	Feverfew accessions	Sowing date	Germi-nation	Appearance of the I pair of cotyledon leaves (1 cm)	Appearance of the I pair of true leaves	Appearance of the III pair of true leaves		Fully developed five pairs of true leaves (ready for transplantation)	
			days	days	Days	days	height (cm)	days	height (cm)
Winter 2003	**TP4**	27.11.	5	9	13	31	2.84	56	10.6
	TP9	27.11.	5	10	16	34	2.11	67	8.06
	TP10	27.11.	5	9	13	31	2.71	58	9.69
	TP12	27.11.	6	10	14	32	2.62	61	9.58
	LSD 5%						***0.36***		***0.53***
Winter 2004	**TP4**	29.11.	5	9	12	30	2.75	55	10.21
	TP9	29.11.	6	10	16	32	2.23	65	7.81
	TP10	29.11.	5	9	12	29	2.65	57	10.28
	TP12	29.11.	5	9	13	30	2.58	58	9.81
	LSD 5%						***0.29***		***0.61***

Differences in height of nursery plants of different accessions expressed significance; accession TP9 show to be significantly lower in comparison to other 3 accessions (in summer production it ranged 27-34% while in winter one it ranged 18-22% in comparison to average values for remaining 3 accessions).
Differences in tempo of development of accessions between two tested years, within the same model of nursery plant production expressed in days required to achieve certain stages of development, showed not to be significant. The measured differences in height of accessions between two different years in all observed stages also did not show to differ significantly.
Differences in tempo of development of accessions between different models of nursery plant production prove to be statistically significant. Development in summer production was more rapid then in winter production. Differences in tempo of development became noticeable starting from the appearance of the first pair of true leaves (3 days) being more apparent in later stages of development: in stage of 3 pairs of true leaves as well as in stage of transplantation (5 fully developed true leaves) 10 days. Totally, difference in number of days required for nursery plants to reach proper stage for transplantation accounts for ca.10 days in favor to summer production (tables 1 and 2).
The height of ready nursery plants and their overall appearance also showed differences between the examined models of production. According to average values, winter nursery plants were in about half of centimeters higher then the summer ones, and the winter nursery plant leaf petioles were in general more longer then in summer ones, which also looked more compact.

CONCLUSION

In general, summer production gave nursery plants of better quality, in shorter period of time and with lower production expenses. Possible disadvantage of summer production might be observed in attack of leaf by aphids (*Aphis fabae* Scop., *Cryptosiphum* sp.) (Kostić et al, 2003) and snails that feed on young leaves, what contribute to necessity of greater patience in monitoring and maintenance during the summer nursery plant production. Possible disadvantage of winter nursery plant production may be observed, apart from the higher production costs, as well in longer period of production in comparison to the summer production, and even longer if we take in consideration that the plantlets need to be subjected to hardening, prior to be transplanted into the open field.

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SOME ASPECTS CONCERNING THE GAMMA RAY INDUCED EFFECTS IN SUCCESSIVE GENERATIONS OF WITHANIA SOMNIFERA DUN

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ABSTRACT

Gamma ray treatment in M_1 and M_3 with doses ranging between 1 and 15 Krad enhanced plant variability concerning: height, branching, individual biomass, fruit number and biomass per plant. In M_4 generation (2003), under the soil and climatic conditions of Piatra Neamt, at the moment of plant harvesting, we noted that root length at irradiated plants ranged between 19.5 and 31.0 cm, compared to 22.6 cm in control (C) , stem length between 43.2 and 88.2 cm (C=71.4 cm), number of stem basal branches - from 2.11 to 2.66 (C=2.66%), individual plant biomass varied between 91.2 and 337 g (C=223.8 g), individual root mass from 14.5 to 46.3 g (C=30.0 g), fruit number per plant from 53.0 to 302.6 g (C=179.7 g), and fruit biomass per plant 15.4-78.6 g (C=42.4 g).
The highest values of the tested parameters were registered at the variants treated with 1 and 3 Krad. Flavon content in herb ranged between 0.991 and 2.439% d.m. (C=1.657%) and the polyphenols between 0.087 and 0.202 % (C=0.265%). Several individuals valuable for *Withania somnifera* melioration were selected and registered high values for all the tested parameters.
Key words: *Withania somnifera*, gamma ray effects.

INTRODUCTION

The species *Withania somnifera* drew our attention due to some information displayed in literature, according to which this plant comprises a number of active principles acting as: cleanser, analgesic, diuretic, anti-inflammatory, antihelmintic, antibiotic, aphrodisiac, antipyretic, nervous system tonic, immunomodulator etc. (1,2,6,8,13-15,18). Modern research pointed out the presence in herb of some steroidic lactons - witanolides, some of them (witaferine A, witanolides D and E) with anti-tumoral effects (6,16). In this respect some chemotypes were isolated depending on the species' spreading area. Antitumoral activity of some witanolides determined some researchers take to their synthesis (7,17). It was also established that *Withania somnifera* drug contains alkaloids, tannins, polyphenols, flavons, glycosides, volatile oil, fatty acids, polysaccharides (6,19) etc, all these compounds conferring the above-mentioned effects. Sytoindosides with an antistress and adaptogene action were extracted from the seeds.

The pharmaceutical value of *Withania somnifera* species determined us to take it into conventional and *in vitro* cultures and use mutagens to diversify biological material in order to isolate valuable genotypes useful to plant adaptation to soil and climate conditions from Romania. Biological material for *in vitro* culture initiation originated from the Botanical Garden of Chisinau. Some of our previous tests (before the year 2000) rendered evident the morphogenetic reaction of *Withania somnifera* explants on varied hormonal formulae of MS medium and we perfected an *in vitro* multiplying technique of this plant and how to provide planting material for its cultivation (9, 10, 12). Since 1999 we began to study morpho-physiological and biochemical effects induced by gamma – ray treatment in successive generations (M_1 and M_3). Some of these results were already published (11). In this paper we'll present some general statements on the efficiency of this procedure of inducing variability at *Withania somnifera* species pointed out on the effects obtained in M_4 (2003).

MATERIAL AND METHODS

To accomplish experimental mutagenesis test, lots of *Withania somnifera* seeds, taken from *in vitro* regenerants, were irradiated with gamma – ray doses of 1 to 15 Krad and were cultivated in Candesti (Vrancea County). During the 3rd generation we used a new treatment of the same irradiation doses (1, 3, 5, 8, 10, 12 and 15 Krad, irradiation flow of 0.50 Krad per minute). The investigations for $M_2 – M_4$ generations were done in Piatra Neamt (Neamt county). The cultures were submitted to specific maintenance operations. At the end of vegetative period (usually the first decade of October) the plants were harvested (each of the 8 variants separately) and submitted to biometric measurements (number of basal stem branches, root and stem length, plant biomass, root biomass, number of fruit per plant and their biomass) and to some biochemical analyses (qualitative and quantitative evaluation of flavones and polyphenols in herb and in roots). Biometric measurements' results were statistically processed. We proceeded in a similar way for M_4 generation. Seeds belonging to the 8 variants (average samples used for each variant) were incorporated in soil on April 21st 2003 on an experimental plot of 'Stejarul' Research

Centre of Piatra Neamt. The plants were cultivated in rows, with 60 cm between rows and 30 cm between plants of the same row. Plant harvesting was proceeded on October 27th and they were submitted to the biometric measurements previously mentioned.

Average samples of herb per each experimental variant were tested qualitatively and quantitatively on the respect of flavones and polyphenols. Three individuals were selected for each variant, the criterion being the high values of morpho-physiological parameters tested, which were analyzed separately, including their content of polyphenols and flavones. The dosage of flavones and polyphenols was done according to the method described by the Romanian Pharmacopoeia (volume IX), the flavones being expressed in ruthoside and the polyphenols – in caffeic acid. The results are presented in table 1 and in the enclosed figures.

RESULTS AND DISCUSSION

Previous investigations showed that gamma – ray treatment in M_1 diminished the values of all the tested physiological parameters, this reduction being increased by higher irradiation doses. The variability of the analyzed indices was high or very high. It was ascertained that during the month of August polyphenols and flavones content in herb is obviously higher than at the moment of plant harvesting (in October), the doses of 3 and 7 Krad showing a stimulating effect on biosynthesis of these compounds. Also, polyphenol and flavone content in roots is lower than in herb. In M_2 the plants registered higher values of root and stem length, root and stem biomass, but lower values of number and biomass of fruit compared to M_1 generation. Important differences between the content of polyphenols and flavones of the 2 generations were noticed. Thus, in M_2 only the flavone content in herb was higher than in M_1. This could be explained through the influence of agro-meteorological conditions that were different for the plants during the two generations. A remarkable matter is that the inhibitory effect of high doses of gamma rays on some of the tested physiological parameters persists even in the second generation.

A new gamma – ray treatment in M_3 had a different effect compared to the irradiation during the first generation. The values of some physiological indices such as: root and stem length, number of basal branches and plant biomass, were superior to those registered after applying of the first mutagen treatment. This result may be explained by an increased radioresistance of the biological material due to repeated irradiation. We noticed that fruit number per plant and fruit biomass were much inferior to those of M_1 generation and the values of the analyzed indices at control plants in M_3 differ from the ones in M_1. That led us to the conclusion that the differences between the values registered in the two generations are also caused by the varied agro-meteorological conditions of the two areas of cultivation. It was still noticed that, compared to M_1, the lower doses (1 to 5 Krad) stimulated some of the analyzed parameters. The content of flavones and polyphenols in herb was inferior to the values present in previous generations. In exchange, high values of polysaccharides were rendered evident.

We'll present a detailed situation of what we found in M_4 generation. The mutagene treated *Withania somnifera* plot was cultivated on the same ground as M_3. The data shown in table 1 prove that root length of irradiated plants ranged between 19.5 cm (3 Krad) and 31 cm (15 Krad), while the control had 22.6 cm, these limits being similar to previous generations. An important fact is that the inhibitory effect of the 12 and 15 Krad doses on some morpho-physiological indices persists in M_4. As a matter of fact very few plants outlived the doses exceeding 12 Krad and judging by the results from M_1 and M_3, we estimated a DL_{50} somewhere between 10 and 12 Krad. It's intriguing that root length is very close in between the doses of 1 to 10 Krad (x=29-31 cm) and much higher than in control plants. The parameter 'root length' displays a great variability (including the control plants) and wasn't much influenced by the irradiation dose received in previous generations.

Average root biomass had approximately the same behaviour, when irradiated, as root length. Higher values of root biomass were present at the individuals of the variant irradiated with 3 Krad (x=46.3 g, while the control had x=30 g). The individuals of the variant treated with the doses: 1, 5 and 8 Krad registered superior values of root biomass compared to control plants (table 1). Parameter 'root mass' is much variable than 'root length'. The parameter 'stem length' had a similar manifestation, registering its maximal and minimal values at the same doses: x=88.2 cm (3 Krad) and x=43.2 cm (15 Krad). For the doses ranged between 1 and 8 Krad the plants showed superior values of stem length compared to control plants. An important aspect was that the plants treated with doses that increased stem growth generally displayed a lower variability of this parameter than control plants, (table 1). Concerning the number of branches at stem base, there are small, insignificant differences between the irradiated plants and the control ones. This parameter's variability is, for a change, very high (s% exceeds 30).

Another parameter tested was 'total plant biomass'. On this respect plants originating in the seeds of M_3 irradiated with doses ranged between 1 and 8 Krad have a bigger biomass than control ones. Thus, the plants treated with 1 and 3 Krad had an average biomass superior with 48.3-50.5% to control plants (x=337 and 332 g, compared to 223.8 g at control). Doses exceeding 10 Krad decrease biomass accumulation in plants, with 16 to 60% (table 1). The variability of this parameter is very high, including control plants that is why a rigorous selection is needed to make this parameter uniform at high values.

Table 1 – The values of some physiological and biochemical indices in gamma rays treated plants of *Withania somnifera* (M_4)

Vr	Irradiation dose	Root length (cm)	Stem length (cm)	No branches /plant	Plant biomass	Root biomass	No fruits/plant	Fruits biomass (g)	Flavones (ruthoside g% d.m.)	Polyphenols (caffeic ac. g% s.n.)
1	Control	22.6	71.4	2.7	223.8	30.0	179.7	42.4	1.657	0.265
	1-1	33.0	82.0	3.0	712.0	-	628.0	115.0	1.170	0.220
	1-2	37.0	93.0	2.0	315.0	-	204.0	51.0	1.514	0.234
	1-3	32.0	86.0	3.0	305.0	-	218.0	60.0	1.371	0.165
2	1 Krad	29.0	83.6	2.4	337.0	40.4	302.6	78.6	1.184	0.202
	2-1	40.0	110.0	2.0	670.0	-	794.0	245.0	1.310	0.242
	2-2	35.0	89.0	3.0	785.0	-	539.0	145.0	2.126	0.242
	2-3	32.0	194.0	4.0	470.0	-	335.0	105.0	1.898	0.331
3	3 Krad	31.0	88.2	2.4	332.0	46.3	219.4	55.3	0.871	0.141
	3-1	35.0	89.0	3.0	566.0	-	315.0	100.0	1.586	0.221
	3-2	39.0	103.0	3.0	850.0	-	1054.0	290.0	1.280	0.166
	3-3	29.0	95.0	3.0	920.0	-	831.0	245.0	1.366	0.355
4	5 Krad	30.0	79.6	2.3	238.6	38.2	141.9	34.5	0.991	0.087
	4-1	31.0	77.0	3.0	465.0	-	388.0	105.0	1.250	0.242
	4-2	33.0	93.0	3.0	432.0	-	440.0	100.0	2.030	0.169
	4-3	35.0	83.0	2.0	634.0	-	743.0	160.0	1.974	0.249
5	8 Krad	29.8	84.4	2.1	251.0	34.6	119.4	30.2	1.652	0.158
	5-1	30.0	84.0	4.0	440.0	-	305.0	80.0	1.419	0.233
	5-2	35.0	100.0	3.0	472.0	-	110.0	26.0	1.053	0.243
	5-3	29.0	87.0	3.0	515.0	-	408.0	95.0	1.965	0.425
6	10 Krad	29.4	67.3	2.7	187.4	27.0	66.9	15.4	1.630	0.178
	6-1	27.0	79.0	3.0	335.0	-	240.0	40.0	1.471	0.216
	6-2	26.0	85.0	4.0	410.0	-	181.0	48.0	1.657	0.303
	6-3	34.0	98.0	3.0	975.0	-	758.0	200.0	1.238	0.294
7	12 Krad	23.5	53.2	3.0	135.0	18.7	53.0	16.7	2.439	0.241
	7-1	32.0	49.0	3.0	170.0	-	84.0	10.0	2.232	0.403
	7-2	26.0	67.0	2.0	335.0	-	139.0	40.0	2.643	0.380
	7-3	26.0	75.0	4.0	530.0	-	277.0	75.0	1.296	0.230
8	15 Krad	19.5	43.2	2.7	91.2	14.5	71.2	17.0	1.275	0.086
	8-1	27.0	71.0	3.0	255.0	-	77.0	25.0	1.395	0.281
	8-2	32.0	68.0	3.0	340.0	-	347.0	85.0	1.562	0.290
	8-3	31.0	75.0	4.0	390.0	-	187.0	35.0	2.108	0.307

There are great differences concerning fruit number and fruit biomass per plant at the 8 variants.The biggest average fruit number per plant was encountered at the individuals belonging to the variant irradiated with 1 Krad (x=302.6, compared to 179.7 fruit per plant at control). The dose of 3 Krad (x=219.4 fruit/plant) had a positive effect on the above mentioned parameter. In the case of the other variants, although the plants were irradiated during the previous generation, the number of fruit per plant decreases with irradiation dose increase. The lowest value of the tested parameter was found at the 12 Krad dose, where the average number of fruit per plant is more than 3 times smaller than at control plants and about 6 times smaller than at the variant treated with 1 Krad, (Table 1).

Generally, there is a correlation between fruit number per plant and fruit biomass per plant. Thus, the highest value of fruit biomass was pointed out at the variant treated with 1 Krad (x=78.6 g) and the lowest at the 10 Krad dose (x=15.4 g), while at control plants the average fruit biomass per plant was 42.4 g. These two parameters' variability is extremely high and, in case of processing fruits and seeds from this species, the selection would be difficult and time-consuming.

Considering flavones and polyphenols content in herba (at the end of the vegetation season) the values are quite similar to those in M_3. Average content of flavones per variant ranged between 0.871% (3 Krad) and 2.439% (12 Krad), while the control registered 1.657%. Average content of polyphenols per variant oscillated between 0.086% (15 Krad) and 0.241% (12 Krad) while the control plants' level was 0.265% (table 1). It would have been interesting to analyze and turn to good account the biological material also on the respect of other specific active principles shown in the first chapter, but unfortunately we couldn't find the proper means.

Biometrical measurements and single biochemical tests led to the isolation of some individuals with high values of some parameters. Considering the root length, the next individuals stood out: 2-1, 2-2, 3-1, 3-2, 4-3 and 5-2; for stem length: 2-1, 2-3, 3-2, 5-2 and 6-3; for the number of basal stem branches: 2-3, 5-1, 6-2, 7-3, 8-3; for plant biomass: 2-1, 2-2, 3-2, 3-3, 4-3, 6-3 and 7-3; for the number of fruit per plant and their biomass: 2-1, 3-2, 3-3, 4-3, 6-3; for flavone content in herb the individuals 2-2, 4-2, 7-1, 7-2 and 8-3, and for the polyphenols content 3-3, 5-3, 7-1, 7-2, 8-3 (Table 1). The individuals with high values for more than one of the tested parameters are: 2-1, 2-2, 3-2, 3-3, 4-3 and 6-3. These genotypes are to be checked during to the next generations to follow the maintenance of high value indices and, if they prove to be useful, they will be brought to culture.

CONCLUSIONS

The investigations concerning the effects induced in M-4 generation by the successive gamma ray treatment (1-15 Krad) on some morpho-phsiological and biochemical indices in *Withania somnifera* Dun showed that:

1. Irradiation doses exceeding 10 Krad inhibited plant growth, biomass accumulation in roots and stems, fruit number and biomass, even in M_4 generation, while low doses, especially 1 Krad, stimulated all these processes. One couldn't point out an obvious connection between the irradiation dose and the polyphenols and flavons content in herb;
2. Gamma – ray determined an evident enhancing of plant variability; within each variant there have been isolated genotypes of *Withania somnifera* valuable on the respect of plant dimensions, the biomass of some vegetative and reproductive organs, the content of polyphenols and flavones in herb;
3. Future investigations will aim proving the stability of characters of some valuable selected genotypes, for their multiplying and use in culture and the dosage of other specific active principles for these species.

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PERSISTENCE OF DDVP IN STORED MEDICINAL PLANTS

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ABSTRACT

Nuvan 500-EC was used in combating moving forms of stored product pests in ready made herbal drugs, set for further processing and packing, aiming to resolve whether these products could be treated in such a way but keeping level of DDVP residues within acceptable boundaries. Treatment was performed employing ULV-technique by the use of 10 ml of formulated product per 100 m^3. Samples of selected herbal drugs from three uncovered boxes, hawthorn, senna, and mint, were taken before and 3, 7 and 14 days after treatment, and subjected to determination of DDVP residues. Extraction of herbal drug samples (10-15 g) was performed with dichloromethane (100 ml) at laboratory temperature during 24 hours. After filtration and rinsing to collect 100 ml of corresponding dichloromethane extracts, these extracts were evaporated to approximately 2 ml, resulting residues were transferred to narrow medium size vials, dissolved in dichloromethane to 5 ml and analysed by GC/FPD. On the basis of preliminary GC-testing (GC/FID, GC/MS and GC/FPD), contents of DDVP residues in processed samples were determined from the calibration curve (GC/FPD), covering range between 0.4-80 ng/g of DDVP. Residues of DDVP in hawthorn, senna and mint, ranging between 3.1-4.8, 0.1-0.2 and 4.9-28.2 ppb, respectively, were recorded in treated samples (recovery: >98%). Since upper level of residues, after 14 days was lower then 5 ppb in all cases, performed treatment could be marked as convenient and quite acceptable for common practice.
Key words: DDVP, persistence, residues, medicinal plants.

INTRODUCTION

Use of pesticides in protection of medicinal and aromatic plants (MAPs) in the field, from different weeds, pests and diseases, as well as processed herbal drugs and related products, is almost unavoidable measure, whose conduction obligatory affects to the quality of plants and their products. Since cultivation of MAPs assumes small-scale agricultural production, majority of producers of pesticides is not interested to register their products for the use in these plants. From the other side, use of pesticides in world proportions appearing to be under the severe and progressive restrictions. Hundreds of pesticides are already excluded from the market, while for many others could also be expected to be banned very soon. Artistic interpretation of recently launched term organic production makes any idea on the application of pesticide challenging. Subsequently, application of insecticides for extermination of different communal and stored product pests in MAP processing facilities should be carefully and quite seriously considered.

In this article, use of one still not banned organophosphorous insecticides (DDVP, dichlorvos) for the extermination of pests in MAP processing section of Institute for Medicinal Plant Research “Dr. Josif Pančić” in Pančevo, was thoroughly reconsidered. Study was focused to evaluation of potential negative effects of such treatment to the quality of raw materials, semi-finished and finished product, which are processing in this section.

EXPERIMENTAL

Experiment was set in the Unit for processing of medicinal and aromatic plants in Pančevo. In this unit herbal drugs, ready made for preparation and packing of finished products (mainly single teas and tea mixtures), are stored in wooden boxes, which are covered during the procedure of insect’s extermination in the working area. Standard operational procedure (SOP) for the treatment assumes that boxes should be covered to provide protection of herbal drugs of pesticide residues. The aim of the experiment was to check level of the pesticide residues in the plant material, which could be expected in the case of ignoring mentioned SOP in the part dealing with required closing (covering) of boxes. To acquire this data, SOP was temporary modified. Treatment was performed employing ULV-technique, by the use of 10 ml of formulated product per 100 m^3. Samples of

selected herbal drugs from three uncovered boxes, hawthorn, senna, and mint, were taken before and 3, 7 and 14 days after treatment, and subjected to determination of DDVP residues.

Sample preparation
Extraction of herbal drug samples (10-15 g) was performed with dichloromethane (100 ml) at laboratory temperature during 24 hours. After filtration, rinsing (to collect 100 ml of corresponding dichloromethane extracts) and drying (Na_2SO_4), these extracts were evaporated to approximately 2 ml, resulting residues were transferred to narrow medium size vials, dissolved in dichloromethane to 5 ml and analysed by GC/FPD.

Preliminary GC-testing
In the frame of preliminary testing of suitability of available analytical systems for the quantification of DDVP residues in prepared sample solutions, three gas chromatographs, with three different types of detectors were put in operation: HP 5890 Series II GC, equipped with split-splitless injector, HP-5 capillary column (25 m·0.32 mm·0.53 μm) and flame ionisation detector (FID), HP G1800C Series II GC-EID, equipped with split-splitless injector, HP-5MS capillary column (30 m·0.25 mm·0.25 μm) and mass spectrometric detector (MSD), and finally Shimadzu GC-8A analytical system, consisting of packed column injector, short packed column (1.5 m·2 mm), filled with 5% OV-210 on Gas Chrom Q (80-100 mesh), flame photometric detector (FPD) and attached to SP 4270 electronic integrator.

During preliminary testing DDVP analytical standard was characterised by GC/FID and GC/MSD, too. The aim of this testing was to confirm purity of analytical standard by GC/FID and to select from EI-MS of DDVP ions which could be successfully used for quantification purposes in the case of GC/MSD (in SIM mode). Simultaneously, because of complexity of matrixes, sample solutions were analysed in all three modes of detection to select the best detector and chromatographic condition for DDVP residue determination in them.

Calibration
For the preparation of calibration curve eight standard solutions, containing 0.05, 0.30, 0.40, 0.50, 1.00, 2.00, 5.00 and 10.00 μg/ml of DDVP in dichloromethane were consecutively injected (five repetitions). Amount of DDVP injected ranged between 0.4 to 80 ng. Referent substance used for preparation of standard solutions obtained from the company Zorka - Zaštita bilja (Šabac) contained 96.95% of DDVP (GC/FID).

Recovery
Along with measurement of DDVP in selected samples, effect of matrixes on the quantification, as well as recovery was also evaluated. Recovery was determined at the level of concentrations of 0.1 and 0.5 ppm of DDVP. For this purpose appropriate volume of standard solution was added to untreated sample of each analysed herbal drug, which is afterwards processed and prepared for GC analysis in already described manner.

Quantification
On the basis of preliminary GC-testing (GC/FID, GC/MS and GC/FPD), contents of DDVP residues in processed samples were determined by external standard method, from the calibration curve (GC/FPD), covering range between 0.4-80 ng/g of DDVP. In this part, peak areas were used as a base for the quantification.

RESULTS AND DISCUSSION

During the preliminary testing of standard solution by GC/MSD, very clean chromatogram of DDVP was recorded (Figure 1). Purity of standard DDVP was 96.95% (GC/FID). Simultaneously, analysis of mass spectrum of DDVP (Figure 2) showed that it contains few peaks suitable for DDVP quantification in selected ion monitoring mode (SIM).

From the other side, gas chromatography followed with flame-photometric detection (GC/FPD), gave the most appreciated preliminary results: the highest sensitivity and selectivity, along with the lowest interference's of tested matrixes. From these reasons GC/FPD (filtering for phosphorus) was selected as a technique of choice for the quantification of DDVP in tested herbal drug samples by external standard calibration method.

Figure 1. Typical gas chromatogram (GC/MSD) of DDVP standard solution (preliminary testing)

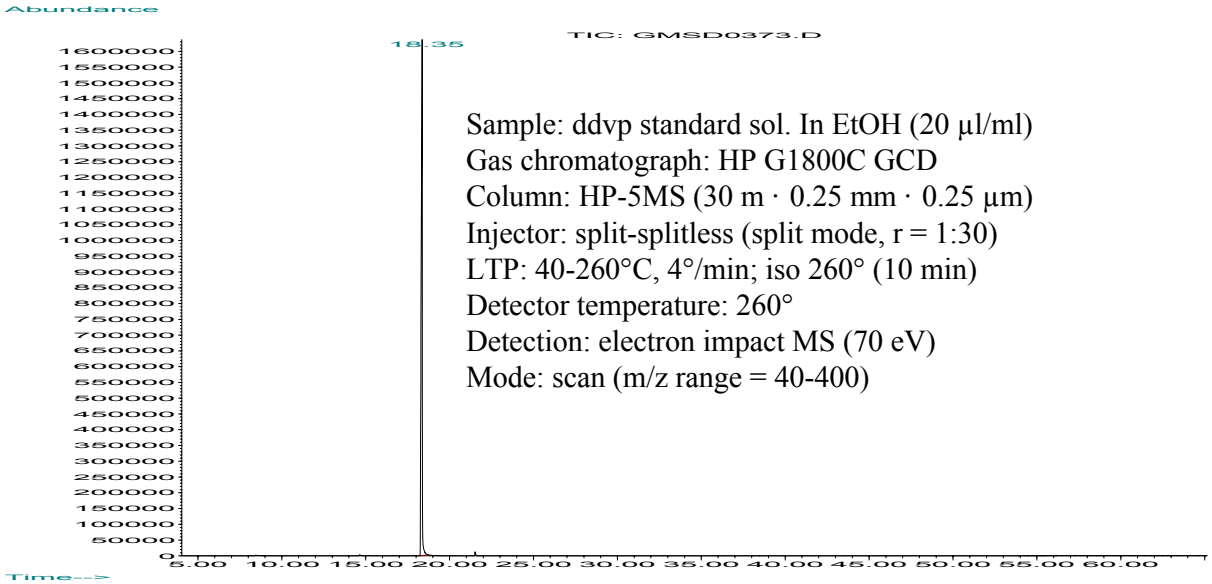
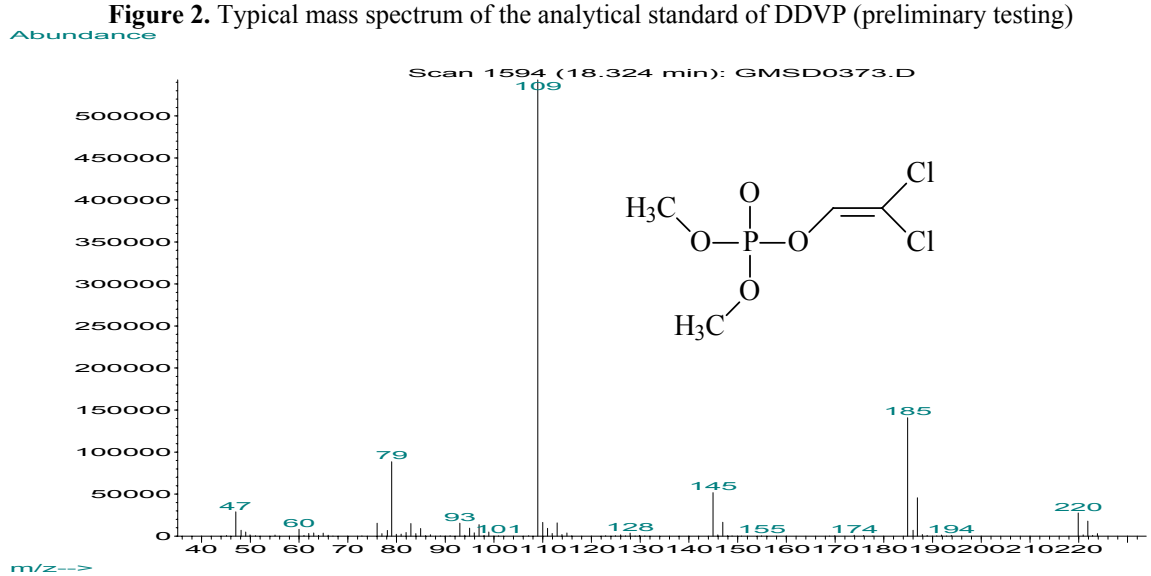


Figure 2. Typical mass spectrum of the analytical standard of DDVP (preliminary testing)



Obtained calibration curves expressed perfect linearity in the range of concentrations tested (Figures 3 and 4). Thanks to the chosen analytical technique (GC/FPD, P-mode), interference of matrixes to the results of DDVP measurements in the samples was relatively weak. Simultaneously, recovery at the level of concentrations tested was >98%. Selectivity, sensitivity and reproducibility were more then acceptable for expected quantification.
Untreated samples of senna, hawthorn, and mint did not contain DDVP. It's content in all other samples ranged between 0.14 and 28.22 ppb. The biggest values were found in mint, 28.22 ppb, 7.33 ppb and 4.89 ppb, 3, 7 and 14 days after treatment, respectively. All hawthorn samples contained less then 5 ppb of DDVP. The lowest values, however, were recorded in senna (less then 0.3 ppb) in all cases. According to regulation, supplied by respectable and actual sources, level of DDVP residues in herbal drugs is limited to 1.0 ppm (1-4). Established concentration of DDVP in our samples was at least 30 to 3000 times lower then the mentioned limit.

Subsequently, drastic modification of starting SOP for combating movable forms of stored product pests did not make treated herbal drugs unusable.

Figure 3. Response vs. injected amount of DDVP (GC/FPD, P-mode)

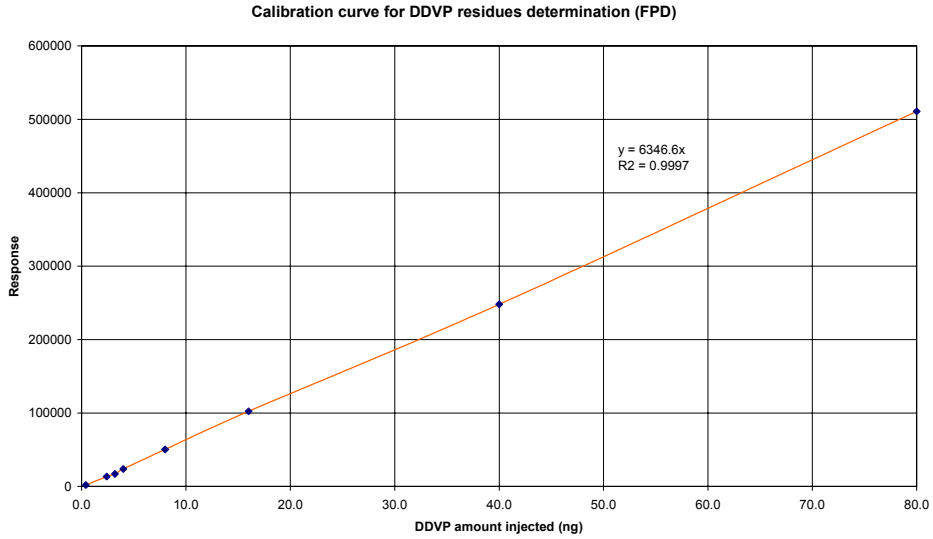
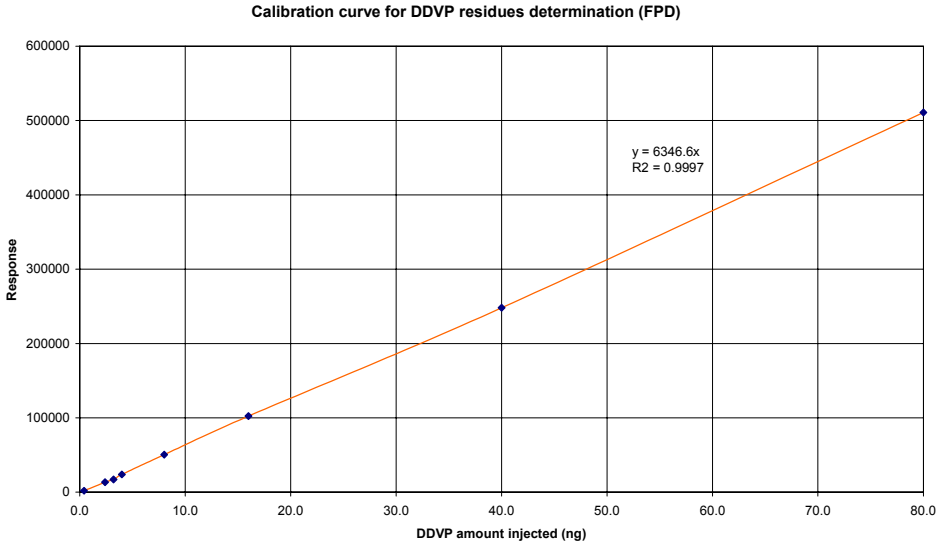


Figure 4. Response vs. concentration of DDVP (GC/FPD, P-mode)



CONCLUSION

DDVP could be safely used in combating moving forms of stored product pests and other insects, which could be found in facilities for processing and packing of MAPs. Along with proper use of this insecticide, which assumes correct conduction of carefully developed SOP and respecting of the whole spectrum of available toxicological data, treatment with DDVP will not affect to the quality of confined raw materials, semi-finished, or finished products. In the context of the latest list of 110 banned pesticides in EU, actual conclusion could be of the great importance for the common practice.

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DISEASES OF PURPLE CONEFLOWER SEEDS

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ABSTRACT

Alternaria alternata, *Fusarium oxysporum*, *Fusarium proliferatum*, *Epicoccum purpurascens*, *Pythium spp.*, *Rhizoctonia solani*, *Penicillium spp.*, *Aspergillus spp.* and *Botrytis cinerea* were identified on the coneflower (*Echinacea purpurea* and *E. angustifolia*) seeds in 2002 and 2003. There were no difference in mycopopulations between two *Echinacea* species and both years of investigation.
Key words: Echinacea, seed, mycoflora.

INTRODUCTION

Quality of seed (Fig 1), as plant reproductive material, has a huge influence to the yield of crops. As the seeds of *Echinacea* are rich in proteins, carbohydrates and mineral substances, it is a suitable medium for development of microorganisms, especially fungi that are being predominant pathogens.

Fig. 1. The healthy seeds (left) and seedlings (right) of *Echinacea purpurea* on wet filterpaper

Purple coneflowers have been growing in plantation on the experimental field of Institute for Medicinal Plant Research »Dr Josif Pančić« in Pančevo, as well as, in co-operation at locality of Stara Pazova since 1998. As the purple coneflowers are the new growing medical plant in Serbia, as well as its mycoflora, it wa sneccassary to ivestugate the influence of seed mycoflora on development of these medical plants in the field. Because of that, the mycoflora of *Echinacea* spp. seeds was investigated and monitoring of the appearance of the disease was carried out, especially in the early stages of plant development. Results obtained through the two years study of *Echinacea purpurea* and *E.angustifolia* seed mycoflora are presented here.

MATERIAL AND METHODS

For this purpose the seed samples of *Echinacea purpurea* and *E. angustiifolia* harvested in 2001 and 2002 from commercial fileld at Institute for Medicinal Plant Research "Dr. Josif Pancic" were analysed on the presence of pathogenic fungal flora. Analysis of the health status of *Echinacea* seed was done by incubation of seeds at filter paper and incubation of seeds on potato dextrose agar (PDA) and water agar with leaf piece of carnation (CLA).

Four hundred seeds (4 trials, each with 100 seeds) from each seed portion were sterilised (NaOCl) for 3 minutes and then rinsed with sterile water and transferred to Petri dishes 15 cm in diameter. Fifty seeds from each seed portion were transferred to PDA medium following the seed surface sterilisation. After the eight-day incubation, at 25°C, parts of mycelia taken from well-developed colonies were transferred to the PDA in order to be further examined.

Morphological examination of the isolated fungi was conducted at monosporial cultures. Following phenomena were monitored: speed of the growth at PDA at 25°C, nature of aerial mycelia, presence of pigmentation,

appearance of conidiophores and conidia, manner of conidia formation, production of chlamidospores, sometimes sclerocia, and formation of stroma. In every isolate 100 conidia were measured.

Identification of the present fungi was done on the basis of morphological and growing properties of the examined isolates, and classical as well as recent literature was used. The obtained results were processed with the use of analysis of variance (Snedecor and Cochram, 1967).

RESULTS AND DISCUSION

The fungi from nine taxa were registered on seeds of *E. purpurea* and *E. angustifolia* in both years (Tab. 1). The presence of fungi leaded to morfological changes of the seeds, such as discloration, necroses, deformations and roting, so the percentage of seed germination was decreased. Some of these symptoms are characteristic for the presence of the fungus from genui *Fusarim* and *Alternaria*. The most present fungi were from following taxa: *Alternaria* and *Fusarium.* However, it should be stressed that there were no distinctive differences between the yield of 2002 and 2003, as well as between examined cultivars of *Echinacea*. Altough the percentage of seeds infected by some fungi was not high, such seeds could serve as inocula source for inoculation in the field.

Table 1. Fungal flora of coneflower seeds (*Echinacea purpurea* and *E. angustifolia*) in 2002 and 2003.

Plant species	Fungal species	Infected seeds (%)	
		2002	2003
E. purpurea	*Alternaria alternata*	2	17
	Aspergillus spp.	-	2
	Penicillium spp	2	1
	Fusarium oxysporum	15	5
	Fusarium proliferatum	5	3
	Pythium spp.	2	-
	Rhizoctonia solani	-	1
	Botrytis cinerea	2	2
	Epicoccum purpurescens	2	3
E. angustifolia	*Alternaria alternata*	7	9
	Aspergillus spp.	2	-
	Penicillium spp.	-	-
	Fusarium oxysporum	2	2
	Fusarium proliferatum	3	3
	Pythium spp.	2	1
	Rhizoctonia solani	2	1
	Botrytis cinerea	3	-

Alternaria alternata was present in high percentage on *E. purpurea* seeds in 2003, as the dark brown mycelia and conidia formed in large chains (Fig. 2).

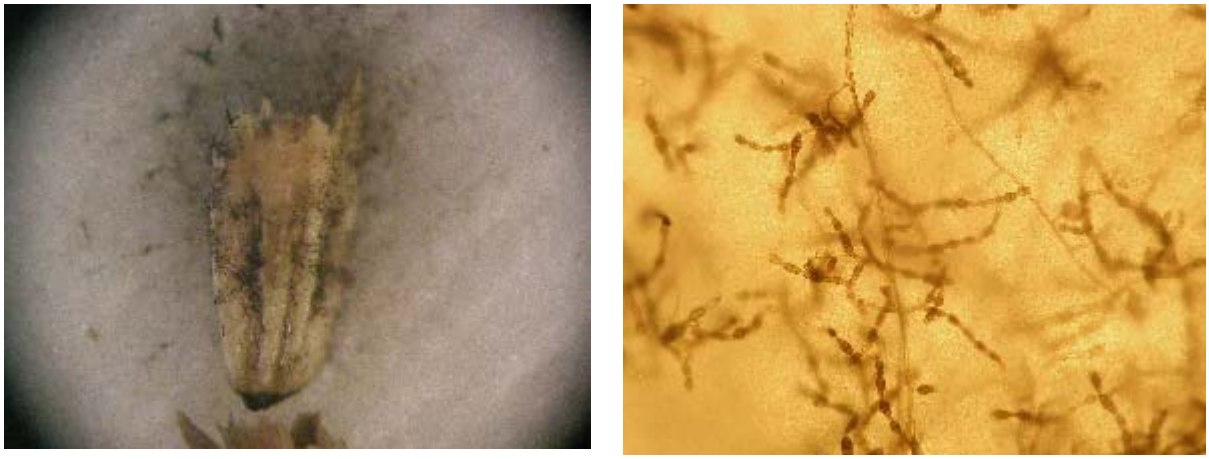

Fig. 2. The *Echinacea purpurea* seed covered with brown mycelia (left), conidia of *Alternaria alternata* (right).

On infected seeds *Fusarium oxysporim* produce sparsa to abudant growth, covering part or whole seed (Fig. 3) with white mycelium. Accoeding to Burgess *et al.* (1) this fungus has highly valiable colony moprphology. In general, the aerial mycelium first appears white, and then may change to a variety of colors, ranging from violet to dark purple. Filter paper under infected seeds became violet blue. On coleoptils, tip or roots and stalk of seedlings, brown necrotic lesions sometimes appeared.

Microconidia are one or two celled, and oval to elliptical, formed in false-heads on short monophialides, and are the type of spore most abundantly and frequently produced. Macroconidia, three to five celled, are usually formed in pale orange sporodochia. Chlamydospores are produced either terminally or intercalary on older mycelium. These spore are either one or two celled (Fig. 3).

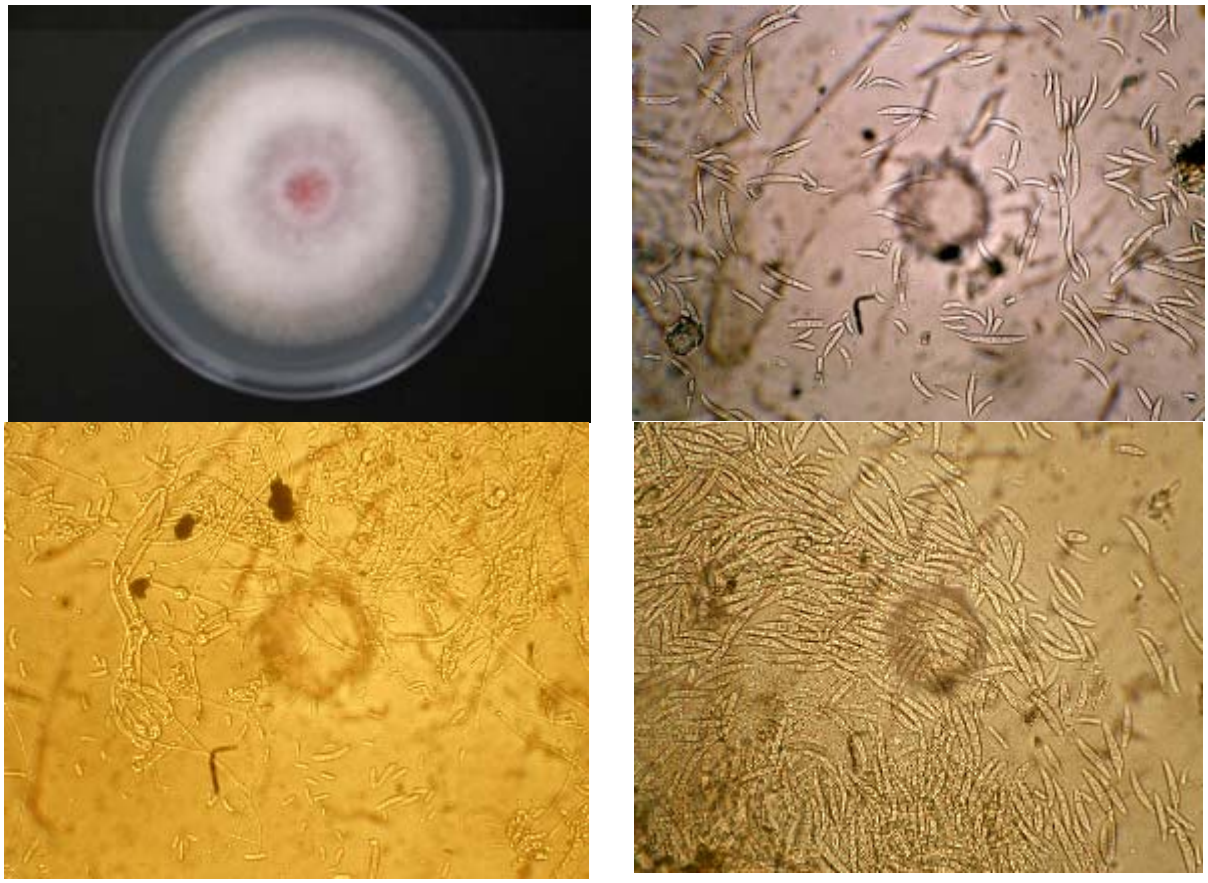

Fig. 3. *Fusarium oxysporum*. Colony on PDA (upper left), micro i macro conidia (upper right), microconidia i chlamidosporae (down left), microconidia formed in sporodochia (down right).

Fusarium proloferatun produces white to dirty white mycelium on the seeds (Fig. 4). On PDA aireal mycelium floccose, white to grayish-white, becoming violet is formed. Pigmentation in the agar is variabile. When the mycelium is removed a violet color may be seen on filter paper. Microconidia are formed abundantly in chains from polyphialides or monophialides on PDA after 2-3 days. Microconidia also formes false-heads. They are clavate, usually one to dwo celled. Long, usually 3-5 septated, thin walled macroconidia are produced in pale orange sporodochia (Fig. 5).

Botrytis cinerea produced long, brown conidiophores which repeatedly brached and beargreyish brown clusters of conidia. Conidia ellipsoidal or obovoid, one-celled, colorless to pale brown, smooth. On PDA micelim withish, becaming pale mouse gray, forming abundantly dark brown, almost black sclerotia after two weeks (Fig. 6).

Among the nine isolated fungi from *E. purpurea* and *E. angustifolia* seeds, only *Fusarium oxysporum* and *F. proliferatum* could cause economic losses by reducing seedlings' growth if they are present in seed endosperm. The rest of present fungi (*Epicoccum purpurascens*, *Pythium spp.*, *Rhizoctonia solani*, *Penicillium spp.*) and *Aspergillus spp.* are less important. The resalts of this investigation show that seed mycoflora of *Echinacea* species is very similar to those of same other aromatic plants (2-10).

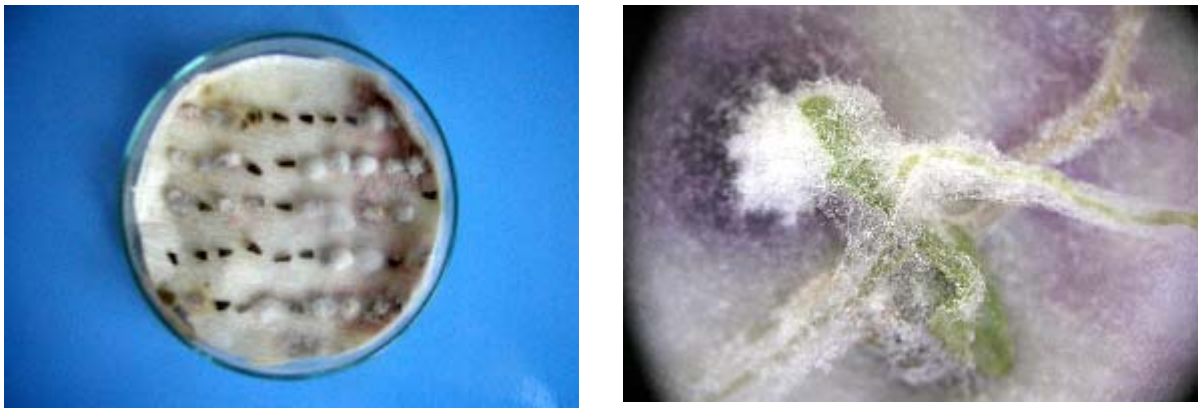

Fig. 4. *Fusarium proliferatum*. Infected seeds on wet filter paper (upper left and right).

Fig. 5. *Fusarium proliferatum*. Colony on PDA (left), microconidia in chains and false heads (right).

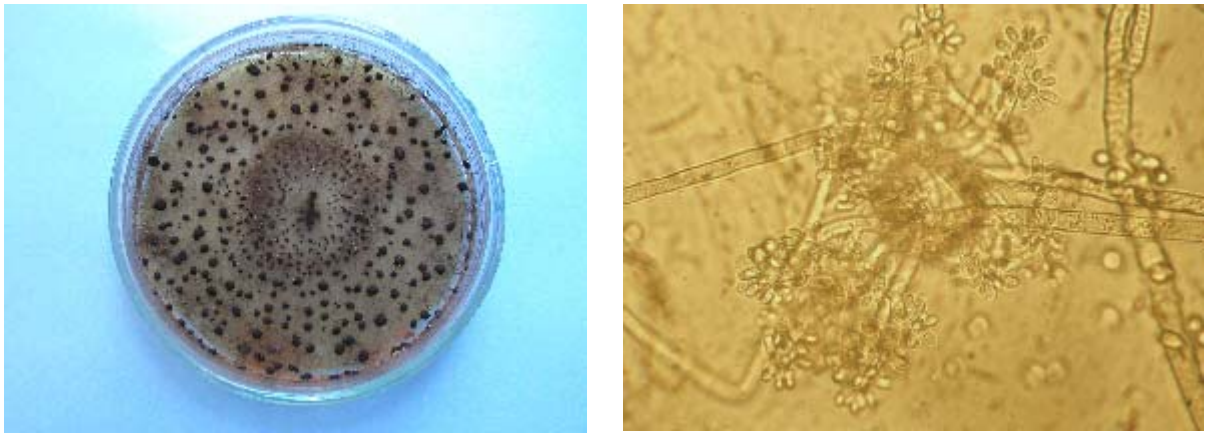

Fig. 6. *Botrytis cinerea*. Colony on PDA with numerous dark sclerotia (left), conidiophores with conidia (right).

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CAN HERBS BE INDICATORS OF AEROSOL POLLUTION WITH HEAVY METALS?

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ABSTRACT

Some herbs are well known as indicators of heavy metal pollution of soils. We tested indicator herbs as possible indicators of aerosol pollution. St. John's Worth (*Hypericum perforatum*), plantain (*Plantago lanceolata*) and dandelion (*Taraxacum officinale*) were exposed in the pot experiment on seven different locations. Three of them on impact area of Thermal Power Plants in Slovenia, three on less polluted areas and one on heavily polluted ex lead smelting area. Heavy metal concentrations (Cd, Pb, As, Hg) were determined by ICP-MS and AAS on two different depths of substrate and in roots as well as in over ground parts of herbs. Measurements showed that only on polluted areas differences in heavy metal accumulation where evident. Accumulation of Pb and Cd through the leaf surface was the most prominent. The extent of accumulation of Pb was Plantain > St. Johns Worth > Dandelion, and for Cd St. John's Worth > Dandelion > Plantain, respectively. Accumulations of As and Hg were under limit of detection. Results confirmed that indicator herbs could be useful for accession of Pb and Cd aerosol pollution.
Key words: Aerosols heavy metal pollution, Indicator herbs, *Hypericum perforatum, Plantago lanceolata, Taraxacum officinale*

INTRODUCTION

Some herbs are well known as indicators of heavy metal pollution. Test plants can be used for localizing emission sources and effects of emissions or aimed release of compounds either at acute or chronic exposure (Markert et al 2003, Kabelitz, 1998). Previous study in the Šalek Valley confirmed that dandelion and St. John's Worth were the most sensitive plants for Cd accumulation in comparison to forage, plantain and some other medicinal plants (Kugonič and Rode, 2000; Kugonič and Rode, 2002). In the meantime, significant higher concentration of Cd in surface layer was found (Kugonič *et al*. 2003). All this facts show possibility that aerosol pollution is the source of this type of contamination. With our present study we tested indicator herbs as possible indicators of aerosol pollution with heavy metals.

MATERIALS AND METHODS

St. John's Wort (*Hypericum perforatum*), plantain (*Plantago lanceolata*) and dandelion (*Taraxacum officinale*) were used as the most known plant indicators for heavy metal pollution. They were exposed in the pot experiment on eight different locations. Three of them were on impact area of Thermal Power Plants in Slovenia (the Šalek Valley, the Zasavje region, the Ljubljana region), three on less polluted areas without of sources of emission (Prekmurje region, Kočevje region, the Upper Savinja region) and one location on heavily polluted area as a result of Pb smelting activities in the past (the Upper Meža river Valley) (Figure 1).

Plants were sown and grown in containers. Nine seedlings of each plant species were exposed for 5 months on each location. At the end of the vegetation season roots as well as aboveground parts of each plant were sampled. Half of each above ground plant sample was washed by distilled water to remove possible aerosols deposits. Plant tissue samples were dried at 36°C and ground in an agate mortar. Heavy metal concentrations (Cd, Pb, As, Hg) were determined by ICP-MS and AAS after appropriate digestion of plant tissue samples. The same analyses were performed on samples of substrate at two different depths in container (0-5 cm and 5-20 cm).

RESULTS AND DISCUSSION

Analytical results of plant material from old lead smelting area in the Upper Meža Valley confirmed that absorption of Pb takes place trough leave surface. The extent is different regarding plant species: plantain > St. John’s Worth > dandelion, respectively. Differences between absorption trough leave surface at other locations were not significant between tested species. Absorption of Pb through roots was low and did not show any significant differences between locations nor tested plants (Figure 2).

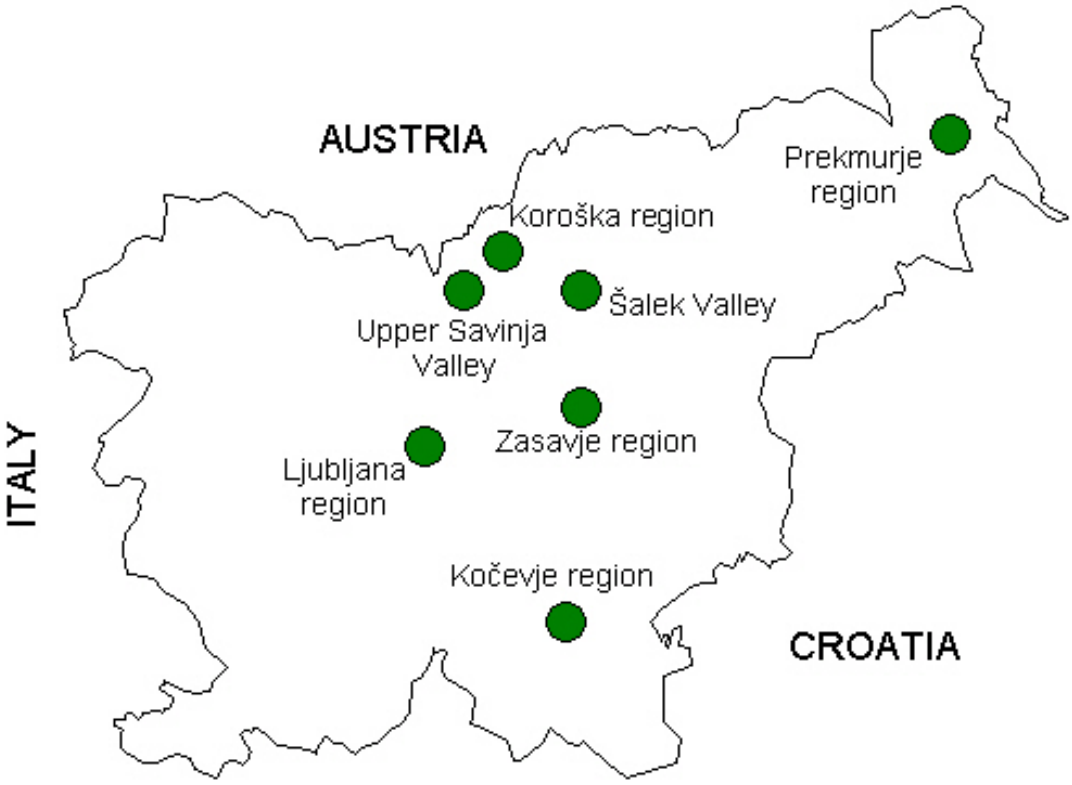

Figure 1: Map of Slovenia with areas where the study was carried out.

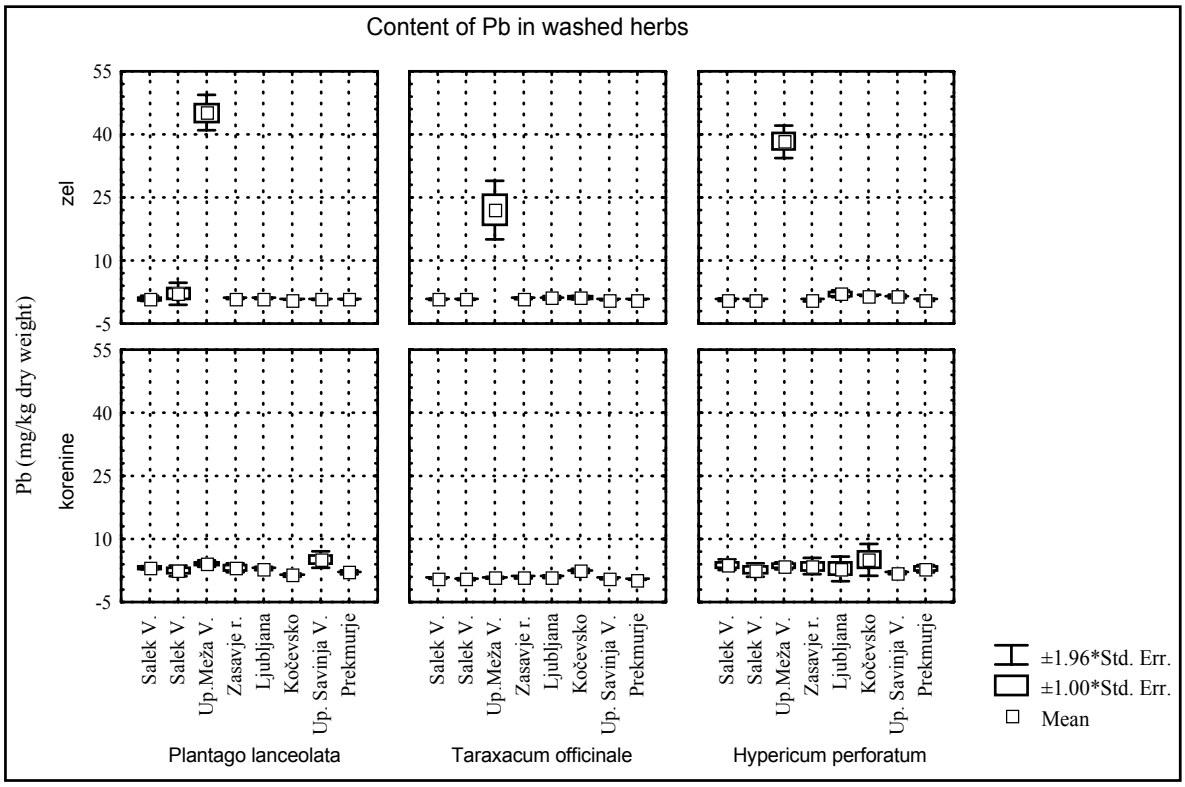

Figure 2: Pb content in the leaves and roots of tested plants in pot exposition experiment.

Accumulation of Cd was more heterogeneous regardless if it occurred on the leave surface or in the roots. The content of pollutant was always higher in the aboveground parts than in the roots. The most prominent plant regarding accumulation of Cd in the Upper Meža Valley was St. John's Worth (Figure 3). Accumulation of Cd through the leaf surface was: St. Johns Worth > Dandelion > Plantain, respectively.

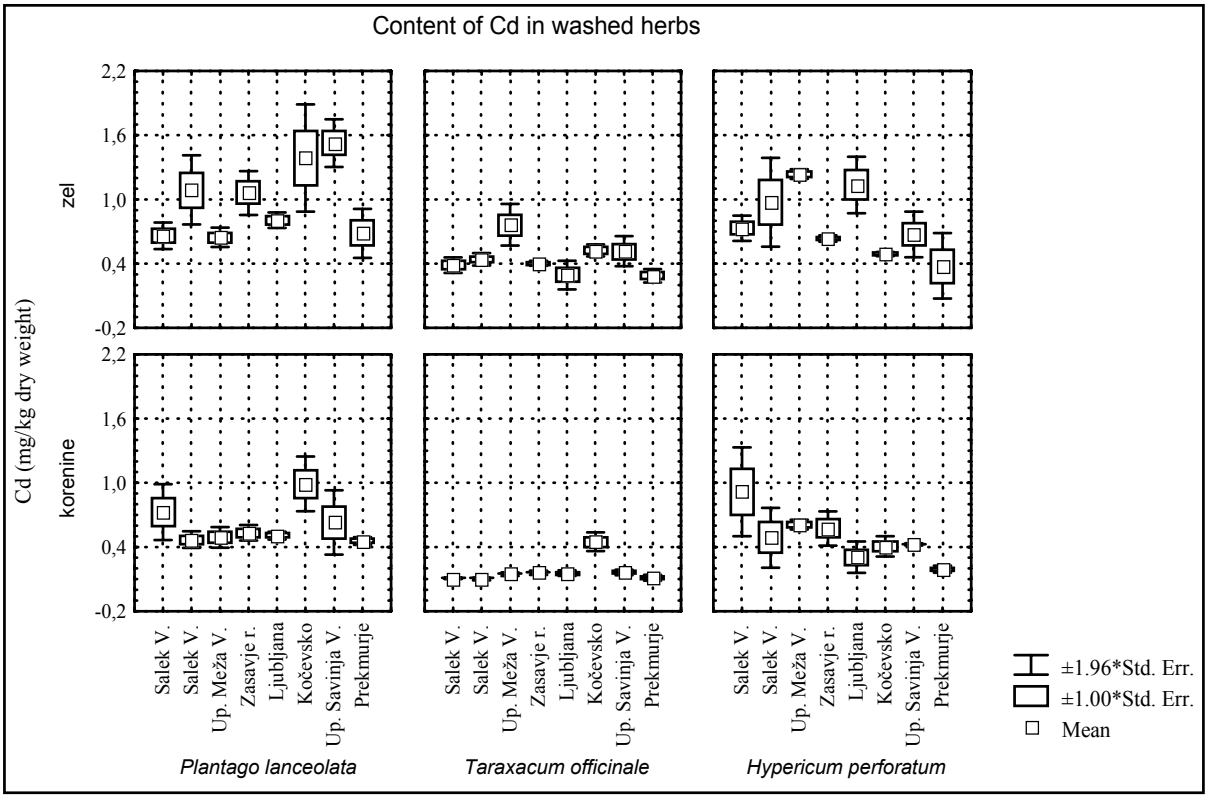

Figure 3: Cd content in the leaves and roots of tested plants in pot exposition experiment.

Low accumulation, under detection limits of used methods for As and Hg did not allow us to do any comparisons between different plant parts, species or exposition points regarding these pollutants.
Results of correlation tests between pollutant contents in the plant and substrate showed no significance in the case of Cd accumulation. No correlation could be shown between Pb accumulation in the roots of different test plants. But we confirmed correlation between content of Pb in the aboveground parts of all test plants and upper layer of the substrate (Figure 4). This confirmed that majority of accumulated heavy metals were introduced by aerosol emissions.

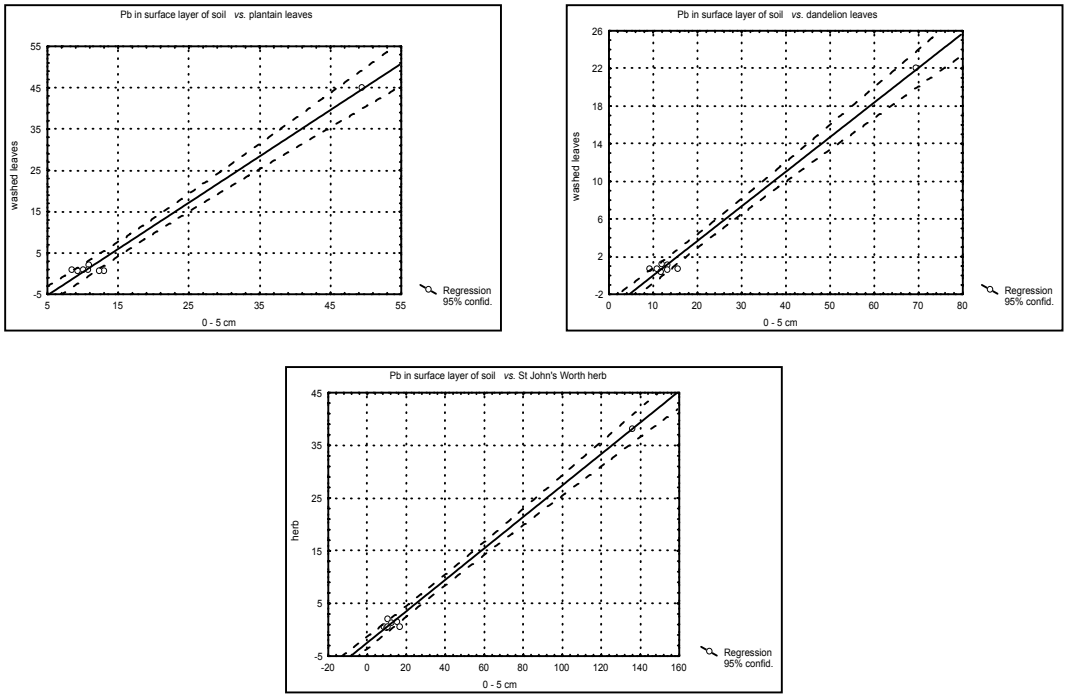

Figure 4: Correlations between the Pb content in herbs *vs.* surface layer of soil.

Analytical results from the Upper Meža Valley significantly influenced positive correlation between content of Pb and Cd in aboveground plant parts. But also without these data there is significant correlation between normal and washed leaves from other exposition points. The most prominent is correlation in St. John's Worth (Cd: R = 0,955; Pb: R = 0,928), in plantain (Cd: R = 0,942; Pb: R = 0,881) and lower in dandelion (Cd: R = 0,785; Pb: R = 0,571). It is a confirmation of pollution with emission of aerosol parts from the environment (Figure 5).
Results and statistical evaluation of the data confirmed that exposure in ex lead smelting area resulted in higher content of pollutants from aerosols, especially Pb and Cd. Specimens from all other locations showed lesser burden and comparable contents of pollutants. The variability of pollutant content was greater in the upper layer of substrate than in the lower one. This confirms that the pollution was airborne and related to ecological condition at places of exposition.

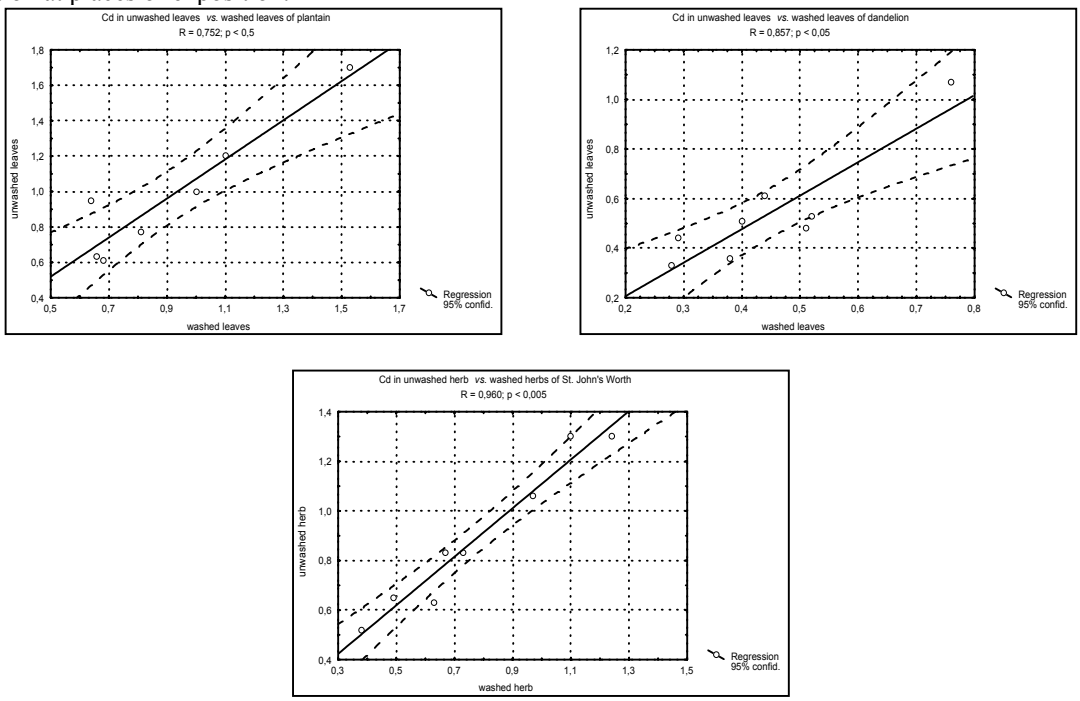

Figure 5: Correlations between the Cd content in washed *vs.* unwashed aboveground parts of herbs.

Results confirmed that indicator herbs could be useful for assessing of Pb and Cd pollution of aerosol deposits.

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THE INFLUENCE OF FUNGICIDAL TREATMENT ON GERMINATING POWER OF SEEDS OF ST. JOHN´S WORT (*HYPERICUM PERFORATUM* L.)

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ABSTRACT

Growing of St. John's wort (*Hypericum perforatum* L.) is limited by the infestation of stands by some diseases, which cause wilting and dying-off of plants. The fungus *Colletotrichum gloeosporoides* Penz. Sacc. is the most serious cause of St. John's wort anthracnosis. It is present on seeds and reduces both germinating power and germination rate of seedlings (Ondřej, Odstrčilová, 1999, 2000). In the laboratory experiment with St. John's wort seeds, the influence of disinfecting agents and selected fungicides to the occurrence of bacteria and *Penicillia sp*. was investigated. Further, the germinating power of treated seeds was determined. The presence of bacteria was constrained due to these fixtures: chloramine, carboxine + thiram. The occurrence of moulds of the *Penicillium sp*. was limited by nearly all preparations except one. None of the used fixtures reduced greatly the germinating power of seeds, except "sodium hyperchlorite 5%".
Key words: St. John´s wort, seed disinfecting, fungicides, germinating power.

INTRODUCTION

Within the framework of the project Innovation of growing technologies of some medicinal, aromatic and root plants we tried to study possibilities of limitation of the occurrence of diseases by the treatment of seed material of St. John's wort with some fungicides. Approximately 60 batches of St. John's wort seeds were obtained within the period of 2001-2003. Some seeds were collected in nature while others were purchased as commercial seeds from different producers and sellers.

MATERIAL AND METHODS

Seeds of three different provenience were treated with four disinfecting agents and five fungicides. The occurrence of *Colletotrichum gloeosporoides* Penz. Sacc. conidium in water used for washing of seeds was followed in 10 seed batches.

Preparations used	**Disinfecting agent**	**Fungicide**
	1 Untreated control	6 Carboxin + Thiram
	2 Ethanol (70%)	7 Carbendazim
	3 Ethanol (96%)	8 Dimetomorph + Mancozeb
	4 Sodium hyperchlorite 5%	9 Carbendazim + Flusilazole
	5 Chloramine	10 Carbendazim + Epoxinazole

Seed material used:
1. Cv. Hypera (obtained from Mendel Univ. of Agriculture and Forestry, Brno)
2. Commercial seed from Želešice
3. Cv. Hypera (obtained from SEVA FLORA Valtice

Experiments were performed under laboratory conditions. Treated seeds were placed on agar in the thermostat (20°C) and the occurrence of microorganisms on seeds was checked after four and seven days. The germination power of treated seeds was evaluated as well. Data about the occurrence of bacteria, fungi of the genus *Penicillium* and germinating power of three seed batches are presented in Tabs. 1-3. Number of *Colletotrichum gloeosporoides* Penz. Sacc. conidia are presented in Tab. 4.

RESULTS AND DISCUSSION

Preparations Chloramine and Carboxin + Thiram inhibited significantly the growth of bacteria and nearly eliminated the growth of moulds of the *Penicillium* sp. genus. Fungicides containing carbendazim, carbendazim + flusilazole and carbendazim + epoxinazole as effective agents inhibited the growth of mould but more or less promoted the growth of bacteria. The preparation containing dimetomorph + mancozeb as the effective agents did not inhibited the growth of moulds and only slightly suppressed the growth of bacteria. None of preparations under study did significantly reduced the germination power of seeds; sodium hyperchlorite (5%) was the only exception and the germinating capacity of individual seed batches was reduced to 31%, 16% and 28%, respectively.

Table 1. Effect of disinfecting agents and fungicides on the occurrence of bacteria on seeds of St. John's wort (*Hypericum perforatum* L.) (%)

Treatment	Origin of seed material		
Disinfecting agent	**1**	**2**	**3**
1 Untreated control	21.7	22.3	39.3
2 Ethanol (70%)	2.9	11.0	7.0
3 Ethanol (96%)	9.3	9.3	19.0
4 Sodium hyperchlorite (5%)	0	0	0
5 Chloramine (5%)	6.3	4.7	1.7
Fungicide			
1 Untreated control	21.7	22.3	39.3
6 Carboxin + Thiram	6.7	12.0	27.7
7 Carbendazim (0.1%)	43.0	50.0	59.0
8 Dimetomorph + Mancozeb (0.5%)	11.3	37.0	39.7
9 Carbendazim + Flusilazole (0.5%)	19.3	31.3	42.0
10 Carbendazim + Epoxinazole (0.4%)	54.7	32.3	46.0

Table 2. Effect of disinfecting agents and fungicides on the occurrence of fungi of the genus *Penicillium* on seeds of St. John's wort (*Hypericum perforatum* L.) (%)

Treatment	Origin of seed material		
Disinfecting agent	**1**	**2**	**3**
1 Untreated control	5.7	31.7	8.3
2 Ethanol (70%)	16.0	10.3	0
3 Ethanol (96%)	2.7	12.3	3.3
4 Sodium hyperchlorite (5%)	0.3	0.7	0
5 Chloramine (5%)	0.3	0	2.3
Fungicide			
1 Untreated control	5.7	31.7	8.3
6 Carboxin + Thiram	0	0	0.3
7 Carbendazim (0.1%)	0.7	2.3	0
8 Dimetomorph + Mancozeb (0.5%)	30.7	20.0	9.7
9 Carbendazim + Flusilazole (0.5%)	1.0	0	0
10 Carbendazim + Epoxinazole (0.4%)	0	0	0

Table 3. Germinating power of St. John's wort (*Hypericum perforatum* L.) seed after the treatment with disinfecting agents and fungicides (%)

Treatment	Origin of seed material		
Disinfecting agent	**1**	**2**	**3**
1 Untreated control	78	71	76
2 Ethanol (70%)	76	69	76
3 Ethanol (96%)	78	72	67
4 Sodium hyperchlorite (5%)	31	16	28
5 Chloramine (5%)	79	83	71
Fungicide			
1 Untreated control	78	71	76
6 Carboxin + Thiram	80	66	72
7 Carbendazim (0.1%)	77	63	75
8 Dimetomorph + Mancozeb (0.5%)	80	80	78
9 Carbendazim + Flusilazole (0.5%)	75	62	78
10 Carbendazim + Epoxinazole (0.4%)	74	94	72

Table 4. Occurrence of *Colletotrichum gloeosporoides* conidia on some samples of St. John's wort seeds

Sample	Number of *Colletotrichum gloeosporoides* Penz. Sacc conidia
1 Želešice 1997	94
13 Slavice 2000	0
16 Mikulov 2000	0
23 Želešice 2000	3
26 Valtice 2000	0
49 Želešice 2001 (dry plants)	30
50 Želešice 2001 (fresh plants)	26
56 Želešice 2002 locality Sadová	2
57 Želešice 2002 locality U dálnice	43
58 Želešice 2002 locality Urbanova Zahrada garden	28

The occurrence of *Colletotrichum gloeosporoides* conidia on seeds was different and depended on the provenience of seed material. In five samples, the numbers of conidia ranged from 0 to 3 while in four they ranged from 26 to 43. In one sample there were 94 conidia on one seed.
The effect of disinfecting agents and of some fungicides on the occurrence of bacteria and moulds of *Penicillia sp.* was studied in laboratory experiments with seeds of St. John's wort (*Hypericum perforatum* L.). The germinating power of treated seeds was evaluated as well. The following preparations inhibited the occurrence of bacteria: Chloramine and Carboxin + Thiram. The occurrence of mould was limited by nearly all preparations (Dimetomorph + Mancozeb 0.5% was the only exception. With exception of sodium hyperchlorite (5%) none of the preparations used did markedly reduced the germination power of seeds. Conidia of *Colletotrichum gloeosporoides* occurred at most on Sample 1 (Želešice 1997).
The aforementioned data indicate that seeds originating from natural resources are less infested by *Colletotrichum gloeosporoides,* and it is suitable to treat them with and disinfecting agent and/or fungicide when used for further cultivation.

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THE YIELD OF LEMON BALM (*MELISSA OFFICINALIS* L.) GROWN IN SLOVAKIA

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ABSTRACT

Therapeutic effects of lemon balm (*Melissa officinalis* L.) are described mainly due essential oil and therefore many studies have been directed to the analysis of volatile substances obtainable from the plant. Lemon balm var. Citra was used as a testing material. Harvesting of the plant material has been carried out during whole vegetation period. Essential oil was obtained by water steam distillation in a special apparatus at conditions given in the Pharmacopoeia Slovakia (PhS 1). During the years 2002-2003 these experimental factors were tested: the influence of the way of planting or sowing, applied nutrition and harvest time on the reached level of herbage crop and quality of air-dried drug. The main goal of the research will be the optimization of these bonds: plant – agro ecological conditions – crop – quality of the product – economic effect.
Key words: lemon balm, essential oil, drug quality, cv. Citra.

INTRODUCTION

One of the important species grown in agro-ecological conditions in Slovakia is lemon balm (*Melissa officinalis* L.) which is used as a basic raw material for the production of herb tea, phytopharmaceuticals and cosmetics (Habán, 2004). For this reason we decided to study productive characteristics of the cover grown in warm agro climatic conditions (macoregion).

MATERIALS AND METHODS

Plant material

During the experimental field work we used plant material of lemon balm (*Melissa officinalis* L.) cultivar ´Citra´ (Czech Republic).

Characteristics of the locality

The area in which the experimental field is situated belongs geomorphologic to "Žitavská pahorkatina" (hills) as a part of "Podunajská nížina" (lowland). The experimental field is owned by the University Farm of SUA in the village of Kolíňany. This locality belongs to a maize production type and barley subtype.

Climatic conditions

The area where experimental work was carried out can be divided according to agro-climatic conditions into:

Macro-area – warm with temperature sum t > 10 ^{o}C; within 3,100 – 2,400 ^{o}C**,**
Area – mostly warm with temperature sum t > 10 ^{o}C, within 3,000 – 2,800 ^{o}C**,**
Sub-area – very dry with rate of the climatic irrigation indicator in VI – VIII months = 150 mm**,**
Small area – mostly mild winter with average absolute minimum T_{min} = -18° to -21 ^{o}C

Average air temperature – the highest average temperature (23.1 °C) per month was recorded in July 1994 and a year temperature (11.1°C) in 1994 and 2000. The lowest temperature per month (-3.2 °C) was in January 2000 and a year temperature (9.0 °C) in 1996. The average air temperature between years 1961-1990 was 9.8 °C (within 8.4-10.8 °C), between years 1991-2000 it was 10.2°C (within 9 -11.1 °C).

Rainfalls – average total rainfalls per year in period 1961-1990 were 532.5 mm (350-761 mm), whilst in period 1991-2000 were 539 mm (436-680 mm). While the average rainfalls were not very different, the average air temperature in the last 10 years differs in 0.4 °C. The highest total rainfall per month occurred in September 1998 (149.6 mm), the lowest in February 1998 (0.0 mm). Total rainfall a year changed from 436.1 mm (1991) to 680.4 mm (1995).

The way of setting the covers - the polyfactor field experiment was set and experimentally watched within two vegetative years (2002, 2003). The experiment was held in three individual blocks. The structure of experimental parts was as follows:

A – Cultivar
A1 – Citra
b – The way of setting of the experiment via seeds
b1 – via seeds
b2 – via seedlings
c – Fertilizing
c1 – non-manure
c2 – manure
r – Repeating of the experiment
r_1 – 1 repetition
r_2 – 2 repetitions
r_3 – 3 repetitions

Crop evaluation by quantitative ecology methods
Reached crops were evaluated by analysis of plants structure with the use of quantitative ecology methods, i. e. the quantity of dry biomass to area unit. Particular pickings of herbs were realized on productive fields by destructive methods. The area of 1 m^2 was marked in the cover. The stems of the plant were cut by scissors. The picking of samples was done at three places in one area. The mass of fresh phytomass was weighed in laboratory conditions. After weighing the samples were dried in a dark room and after that in well-aired laboratory drying room. The gained dry biomass was weighed on scales.

RESULTS AND DISCUSSION

The result shows that the highest crop was marked in the cover of fertilized plants (405 $g.m^{-2}$, A_2b_2) in 2003. The lowest crop was marked in the non-fertilized cover set directly from seeds. Statistic evaluation of the dry herbage crop shows high the influence of the way of the setting of the cover as well as application of manure (table 1). The influence of vegetative year on the level of crops where there was a great (145.6 $g.m^{-2}$ $^{++}$) was also statistically determined.
From the result on bee seen above had that the manuring and climatic conditions in the highest influence on the amount of crop. The vegetative year also influenced the amount of dry herbage crop in our experiment. In spite of the highest crops were in 2003, this year was characterized by rainfall deficiency

Table 1: Dry herbage crop of lemon balm [$g.m^{-2}$] in the years 2002 and 2003

The way of setting the cover	Manure	Year 2002 (T_1)	Year 2003 (T_2)	Average (x) 2002 -2003
Direct-seeds (A_1)	without manure (b_1)	47.9 (r_1)	176.0 (r_1)	111.95
		43.7 (r_2)	207.0 (r_2)	125.4
		49.7 (r_3)	187.0 (r_3)	118.4
		x 47.1	x 190.0	118.6
	with manure (b_2)	141.5 (r_1)	275.0 (r_1)	208.3
		163.0 (r_2)	320.0 (r_2)	241.5
		172.5 (r_3)	305.0 (r_3)	238.8
		x 159.0	x 300.0	229.5
Seedlings (A_2)	without manure (b_1)	178.4 (r_1)	273.0 (r_1)	225.7
		190,0 (r_2)	258,0 (r_2)	224,0
		183.8 (r_3)	260.0 (r_3)	222.0
		x 159.0	x 263.6	223.8
	with manure (b_2)	178.0 (r_1)	375,1 (r_1)	276.6
		196.0 (r_2)	395.4 (r_2)	295.7
		191.2 (r_3)	444,5 (r_3)	317.9
		x 188.4	x 405.0	296.7

The results reported by Vaverková *et al*. (2003) correspond with our results. The authors who dealt with productive characteristics of the lemon balm cover (Kišgeci *et al*., 1987; Stepanović, 1998; Habán *et al*., 2004) report the herbage crop from 150 to 250 g m^{-2}. The herbage crops of lemon balm grown in warm agri-climatic

conditions within our experiment researched the amount of 41,7 g m^{-2} (non-manure) to 405 g.m^{-2} (manured, planted by seedlings).
Lemon balm (*Melissa officinalis* L.) cultivar ´Citra´ is suitable for growing in a warm agriclimatic macro-area. This was approved by the results of a two-year experiment in 2002 and 2003. According to the presented results we recommend to continue the research to the influence of intensification factors (the way of setting the cover, nutrition, manuring etc.) on the content of effective elements in the grown plants.

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EFFECT OF YEAR AND HARVEST TIME UPON YIELD AND ESSENTIAL OIL CONTENT OF MOUNTAIN SAVORY (*Satureja montana* L.) CULTIVATED IN SERBIA

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ABSTRACT

A selected line of mountain savory was analysed in two experiments performed in four replications on the experimental field of Bački Petrovac (84 m altitude). In the first experiment (ten year period) dry herb yield was between 2.0 and 4.7 t, i.e. 3.2 t/ha on the average while essential oil content in herb was between 0.8 and 1.3 %, i.e. 1.0 %, as dependent on year of growing. Carvacrol ranged from 55 to 73 %, i.e. 62 % on ten year average. The second experiment showed that investigated traits were strongly dependent on harvest time. Significantly higher dry herb yield (3.7 t/ha) and essential oil content (1.17 %) was obtained in the third harvest time. Cultivation of the perennial mountain savory characterized by the economic yield, high content of essential oil, and high oil carvacrol, in particular, in Serbia may be recommended.
Key words: *Satureja montana* L., growing year, harvest time, herb yield, essential oil content, Serbia.

INTRODUCTION

Varying quality of the raw material of wild medicinal plants collected from different sites has been reported. On the contrary, cultivation of wild species under controlled conditions ensures standard quality of the raw material. By applying this method it is possible to protect the most endangered species. These investigations include cultivation of medicinal species in continental lowlands, mountain, and coastal regions as well as certain specific areas like "Deliblato Sands" (Tucakov, 1969, 1970; Bože *et al*., 1997). An introduction of certain wild species is firmly connected with selection and breeding of genotypes with high yield and content of active substances (Adamović, 1982, 1998a, 1998b; Adamović *et al*., 1993). A high content of essential oil as related to a greater gland number in cultivated plants was reported (Janjatović *et al*., 1995).

Mostly wild populations of *Satureja* species have been examined in Serbia. Variability of their morphological and chemical features has been broadly discussed while their cultivation was much less investigated (Pavlović *et al*., 1987; Pekić *et al*., 1992; Slavkovska *et al*., 1993; Palić and Gašić, 1993).

Therefore, the aim of the present paper was to investigate the possibilities of growing of *Satureja montana* L. in the Serbian lowland, i.e. its plantation longevity and also effect of harvest time upon yield and essential oil. Literature data show that this perennial species may be successfully cultivated, whereas yield and quality parameters by years are missing (Stepanović, 1983; Adamović, 1995).

MATERIAL AND METHODS

The four replication experiments with the selected line of mountain savory were conducted on the experimental field of Bački Petrovac (84 m altitude). Basic plot area was 5,1 m^2. Planting row distance of 50 cm and distance between plants in row of 30 cm using standard agrotechnic measures were applied (Stepanović, 1983).

Experiment 1

In the period 1988-1997, dry herb yield and essential oil content were analysed without irrigation. Crop was harvested at flowering stage.

Experiment 2

Plant material was harvested every 15 days, from May 10 to July 10, indicated as 1, 2, 3, 4, and 5 in Table 2. Essential oil content was determined after Ph. Jug. IV and carvacrol content using gas chromatography. The obtained results were processed by the variance analysis.

RESULTS

Experiment 1

The lowest dry herb yield was recorded in the first, whereas the highest in the second year of growing while somewhat smaller values were obtained in the following years. In the sixth year of growing dry herb yield

increased to 3.6 t/ha while between the seventh and tenth year it was 2.6-3.0 t/ha (Tab. 1). In ten year period, dry herb yield was between 2.0 and 4.7 t, i.e. 3.2 t/ha on the average. The highest essential oil content in herb was recorded in the eighth, whereas the smallest in first and ninth cultivation years (Tab. 1).

Table 1. Yield and essential oil content of *Satureja montana* L. in ten years period of growing

Year	Dry herb yield, t/ha	Essential oil, %
I	2.0	0.75
II	4.7	1.13
III	4.4	1.10
IV	4.1	0.98
V	3.1	1.05
VI	3.6	0.83
VII	2.6	0.93
VIII	2.7	1.31
IX	2.7	0.82
X	3.0	1.11
Average	**3.2**	**1.00**
LSD 5 %	**0.6**	**0.14**
1 %	**0.8**	**0.19**

Essential oil content in dry herb ranged from 0.8 to 1.3 %, making up 1.0 % on ten year average. Carvacrol content in essential oil was between 55 and 73 %, i.e. 62 % on ten year average. Essential oil yield as related to year ranged from 14 to 43 kg/ha, i.e. 29 kg on the average (Adamović, 2001). Although certain yield reduction in later years was recorded, its level was still economically sounded.

Experiment 2

The highest yield of dry herb was obtained in the third harvest term (3.7 t/ha). When this value is compared with others, the obtained differences were significant or highly significant (Tab. 2). Also, content of essential oil was the highest in the third term of harvest (1.17 %) but these differences were significant comparing only with fourth and fifth terms (Tab. 2). Carvacrol content was between 57 and 65 %.

Table 2. Yield and essential oil content of *Satureja montana* L. in five harvesting time

Harvest time	Dry herb yield, t/ha	Essential oil, %
1	2.5	1.15
2	2.8	1.14
3	3.7	1.17
4	3.2	0.86
5	3.3	0.83
LSD 5 %	**0.3**	**0.16**
1 %	**0.5**	**0.22**

CONCLUSION

The experiments with mountain savory show that this perennial species may be successfully cultivated at least ten years under given ecological conditions of Serbia. Significant variation of yield and essential oil content by years was recorded. In addition, investigated traits were strongly influenced by harvest time. High yield of dry herb and essential oil, and content of essential oil and carvacrol were obtained.

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[L-18]

CHEMICAL COMPOSITION OF ESSENTIAL OIL FROM PELARGONIUM RADENS AND EFFECTS IT PRODUCES UPON MICROORGANISM CULTURES

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ABSTRACT

Samples of *Pelargonium radens* (H.E. Moore) have been taken before the flowering period, for anatomical identification of the secretory structures and essential oil extraction and analysis. The essential oil of *P. radens* before the flowering period has been found in amount of approximately 0,33% from the fresh material weight. Essential oil was extracted by steam distillation using a modified Clevenger apparatus, whereas the chemical composition was analysed by GC/MS, and for *P. radens* it was found to have 64 components. The main components of *P. radens* essential oil are: citronelol: 28,7%; menthone 27,3%; citronelyl formate 8,4%, β-eudesmol 4,81%, phenylethyl caproate 1,6%, geraniol 1,6%, isomenthone 1,58%, as well as other components, in a concentration of less than 1,5%. The microorganisms tested were strains of *Escherichia coli*, G(-) and *Staphylococcus aureus,* G(+) using the antibiogramme method. Essential oil was tested in a concentration of 1000 ppm and 500 ppm, dissolved in DMSO. The effect of DMSO was tested and was found null. At these concentrations, the essential oil of *P. radens* was found to have no effect upon *E. coli* and inhibitory effect upon *S. aureus*.

Key words: *Pelargonium radens*, essential oils, chemical composition, effects upon bacteria.

INTRODUCTION

Pelargonium radens (H.E. Moore) belongs to Geraniaceae family and it is used mainly for ornamental purposes, as well as its aromatic properties [1], the last being the main criterion for the choice of this species. The present study intended to cover various aspects concerning the *P. radens* essential oil, as: anatomical secretory structures, extraction efficiency, chemical composition and effects upon microorganisms. The purpose of the present study was to outline contingent aromatherapeutical properties [2] of essential oil extracted from *P. radens*, based on the aspect of its contingent antimicrobial effect, as well as to observe correlation between physiological parameters [3] and essential oils effects.

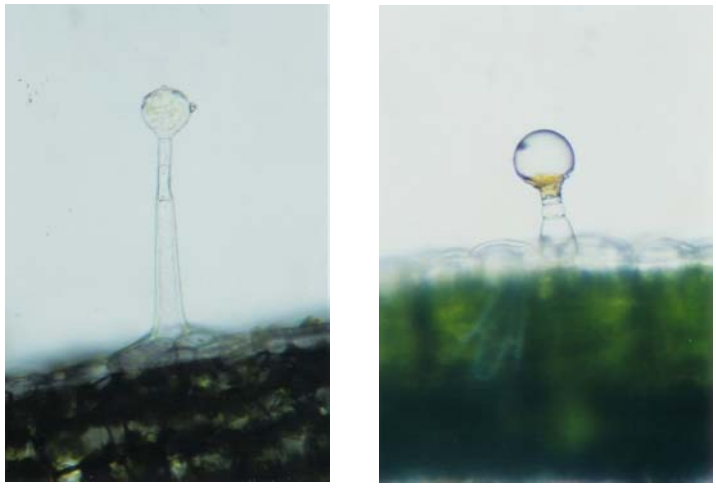
MATERIALS AND METHODS

Materials used consisted of *P. radens* plants taken from the collection of "Anastasie Fatu" Botanical Garden greenhouse, as well as microbial strains of *Escherichia coli* and *Staphylococcus aureus* respectively, taken from the collection of Biological Research Institute of Iasi. Samples have been taken before the flowering period. As far as the employed methods are concerned, they have been carried out following the aspects shown below:

1. The biological material was multiplied using the cutting technique, within the "Anastasie Fatu" Botanical Garden greenhouse; cuttings were planted directly on a soil mix made of 1,5-2 parts leaf earth, 1 part ground peat and 0,25 parts gardening soil.
2. In order to identify the secretory structures for the essential oils, there have been made cross sections through leafs, which have been analysed with a 20x magnifying lens microscope. The anatomical identification of the secretory structures was carried out within the Anatomy and Morphology laboratory from the Biology Faculty, "Al. I. Cuza University" of Iasi.
3. The extraction of the essential oils has been done by steam distillation, using a modified Clevenger apparatus. The report vegetal mass/ water was approximately 1:3; extraction time was approximately 3 hours. Extraction was made at the Plant Physiology Laboratory, within the Horticulture Faculty of the Agronomical Science and Veterinarian Medicine University, Bucharest.
4. The components of the essential oils have been studied by the gas-chromatographic method, using a GC/MS Agilant 6890. The chemical analysis was made also at the Plant Physiology Laboratory, within the Horticulture Faculty of the Agronomical Science and Veterinarian Medicine University, Bucharest.
5. The antimicrobial effect of *P. radens* essential oil was studied using the antibiograme difusimetrical method [4]. Oil was diluted with DMSO and placed upon paper discs and inside glass cylinders.

RESULTS

The secretory structures of the *P. radens* essential oil were found to be multiple celled glandular hairs located both in upper and in lower epidermis. The length of the hairs is variable, according with the hair foot component cells (**Figure 1 a, b**).



a b

Figure 1. Secretory structures of *P. radens* essential oils (multiple celled glandular hairs); longer hair foot (a); shorter hair foot (b).

Extraction efficiency of *P. radens* essential oil was found to be approximately 0,33% before the flowering period and its chemical composition revealed an amount of 64 components. The main components have been identified and they are illustrated in **Diagram 1**.

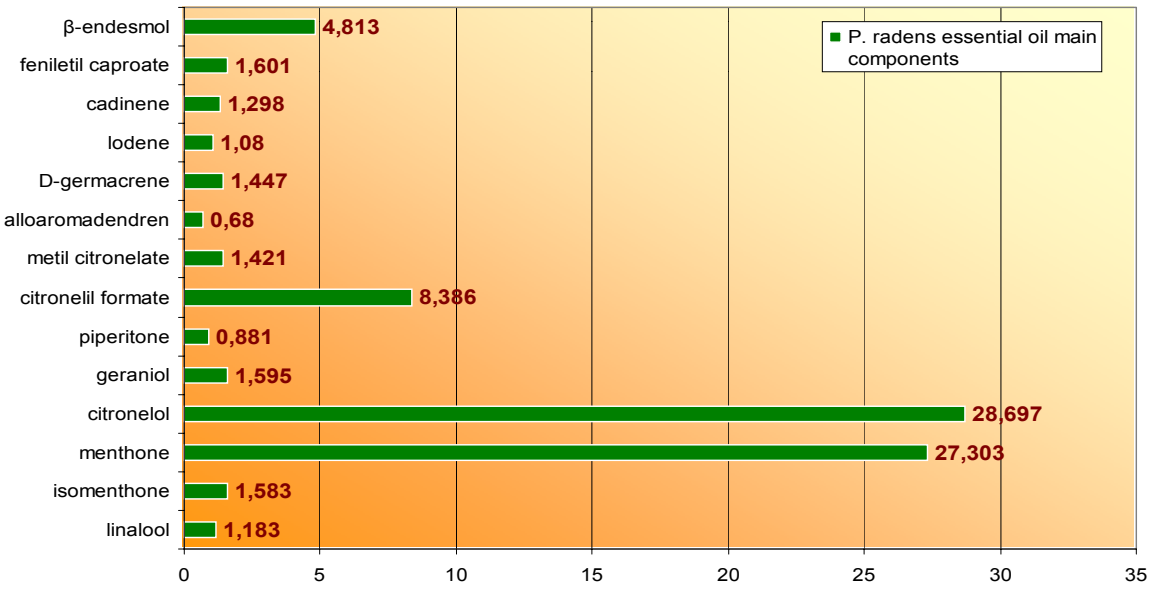


Diagram 1. *P. radens* essential oil main components (%)

The effect of DMSO upon the microbial cultures was null, for both *E. coli* and *S. aureus* (**Figure 2 a, b**)

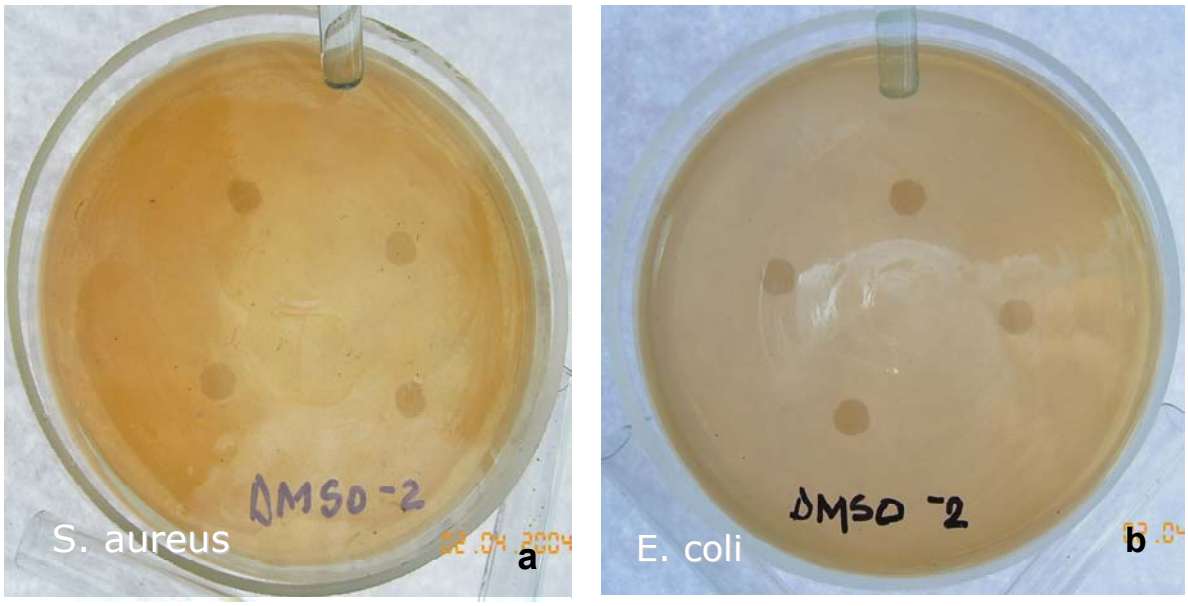


Figure 2. Effect of solvent (DMSO.) upon: (a) *Staphylococcus aureus* strains and (b) *Escherichia coli* strains.

The effect of *P. radens* essential oil is null upon *E. coli* strains (**Figure 3 a, b**) using both paper discs (**Figure 3,** left) and glass cylinders (**Figure 3,** right).

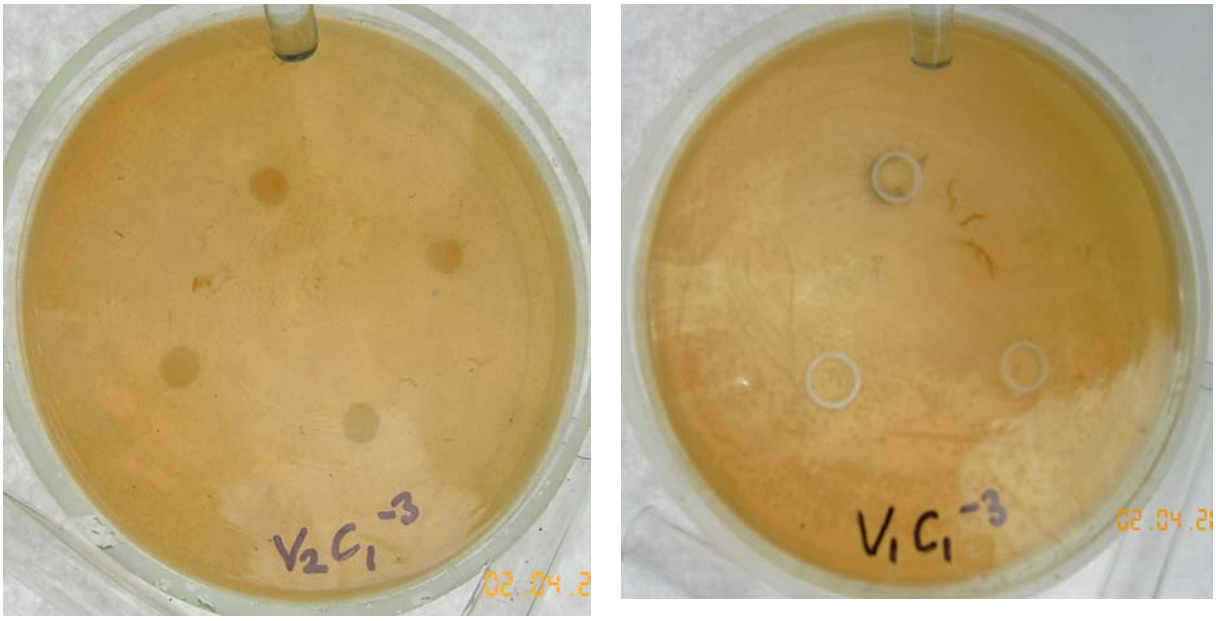


Figure 3. Effect of *P. radens* essential oil upon *Escherichia coli* strains using paper discs (left) and glass cylinders (right).

The effect of *P. radens* essential oil can be noticed upon *S. aureus* strains using paper discs (**Figure 4,** left). Inside glass cylinders there are no microbial colonies (**Figure 4,** right).

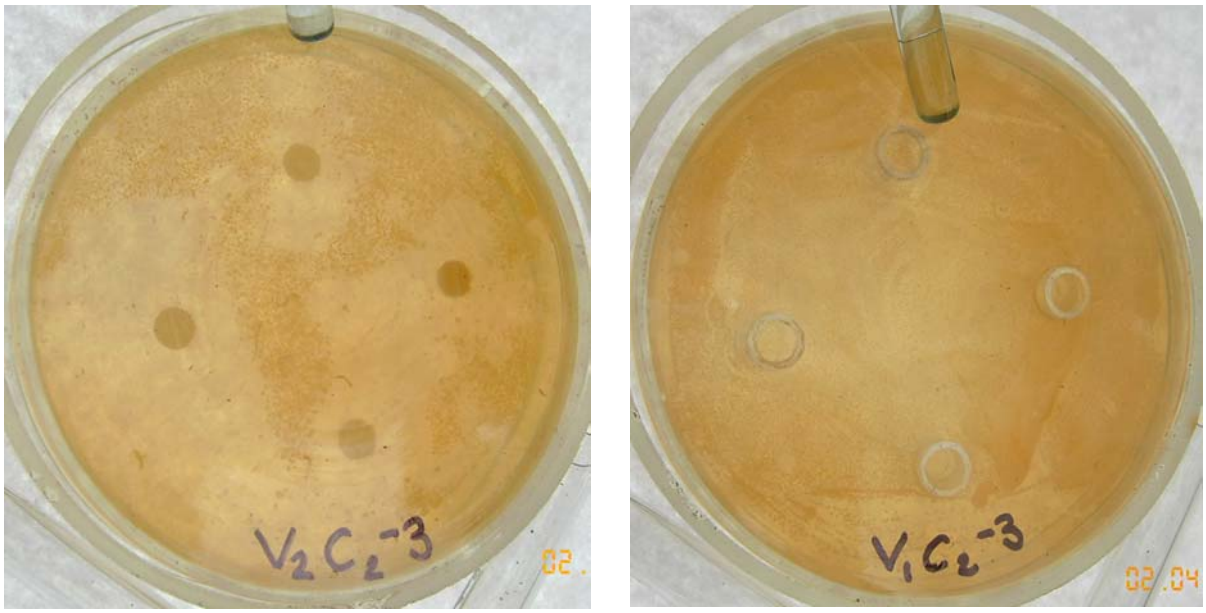


Figure 4. Effects of *P. radens* essential oil upon *Staphylococcus aureus* strains using paper discs (left) and glass cylinders (right).

CONCLUSIONS

- The secretory structures for the *P. radens* essential oil are multiple celled glandular hairs, located both in the upper and in the lower epidermis.
- Extraction efficiency of *P. radens* essential oil was found to be approximately 0,33% before the flowering period and its chemical composition revealed an amount of 64 different components.
- The solvent used for the essential oil (DMSO) has no influence upon the microbial cultures.
- *P. radens* essential oil has no influence upon *E. coli* strains and has inhibitory effects upon *S. aureus* strains.
- Further studies are required for the establishing of the minimum inhibitory concentration of the essential oil, in the case of *S. aureus*.

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[L-18]

[P-002]

THE DYNAMICS OF BIOCHEMICAL AND PHYSIOLOGICAL PARAMETERS FOR SPECIES OF PELARGONIUM CULTIVATED IN IASI BOTANICAL GARDEN

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ABSTRACT

There have been studied three species of *Pelargonium*: *P. zonale* (L.), *P. radens* (H.E. Moore), *P. fragans* (Willd). Samples have been taken in two different ontogenesis periods, before and during flowering period, respectively. The biochemical and physiological parameters studied have been the quantity and quality of the essential oils, photosynthesis and pigments. The extraction of the essential oils has been done by steam distillation using a modified Clevenger apparatus, whereas components have been studied by the gas chromatographic method. The essential oils of *P. zonale* have been found in amount of approximately 0,1%, with 49 components during the period before flowering, and approximately 0,15% with 161 components during the flowering period. For *P. radens* there has been extracted an amount of approximately 0,33% essential oils with 64 components during the period before flowering and approximately 0,38% and 44 components during the flowering period. *P. fragans* was found to have approximately 0,16% essential oils with 11 components before flowering and 0,23% amount with 69 components during flowering period. The photosynthesis process has been less intense before the flowering period, for all three species. For *P. zonale* the photosynthetic pigments have been found in larger amounts before the flowering period, comparing to the other of species studied.
Key words: *Pelargonium*, biochemical and physiological parameters, essential oils, photosynthesis, pigments.

INTRODUCTION

The species of *Pelargonium* [3] used for the present study have been chosen considering their more or less odorant properties. Used mainly as ornamental plants, we were interested in their potential aromatherapy properties, derived from the presence and chemical composition of essential oils.

We were interested in the chemical composition of essential oils [1] during the three ontogenetic periods: before, during and after flowering process. For this purpose there has been made an experimental lot of 15 plant individuals for each of the species investigated, which were cultivated in Iasi Botanical Garden. Up to date, there have been taken two series of samples, before and during flowering period, respectively. Plant metabolism is directly related to the physiological parameters, influencing on the other hand the biochemistry of the plant, essential oils including [2].

MATERIALS AND METHODS

Biological material

The biological material used for the present study consists of three species of *Pelargonium*: *Pelargonium zonale* (L.), *Pelargonium radens* (H.E. Moore), and *Pelargonium fragans* (Willd). The experimental lot consisted in 15 individuals for each of the species investigated, which were cultivated in Iasi Botanical Garden. Up to date, there have been taken two series of samples, before and during flowering period, respectively. Before taking the samples for the extraction of the essential oils, plants' heights have been measured and leaves have been counted, for a further contingent correlation with other biochemical and physiological parameters.

Methods

1. The multiplication of the biological material consisted of cuttings, planted directly on a soil mix made of 1,5 – 2 parts leaf earth, 1 part ground peat and 0,25 parts of gardening soil.
2. The extraction of the essential oils has been done by steam distillation using a modified Clevenger apparatus. The report vegetal mass/ water was approximately 1:3; extraction time was approximately 3 hours.
3. The components of the essential oils have been studied by the gas chromatography, using a GC/MS Agilant 6890.
4. The intensity of the photosynthetic process has been determined in vivo for two of the species investigated: *P. zonale* and *P. radens*, with a portable apparatus LC4L. For P. fragans, the leaf area did not allow a reading of this parameter.
5. Photosynthetic pigments have been determined spectrophotometrically, in acetone extracts.

RESULTS

The quantitative dynamics of the essential oils is given by the extraction efficiency, which was found to have the values shown in **Table 1** and illustrated in **Diagram 1**.

Table 1. Extraction efficiency values for the essential oils of *P. zonale, P. radens* and *P. fragans* before and during the flowering period.

Ontogenetic period	Extraction efficiency (%)		
	P. zonale	*P. radens*	*P. fragans*
Before flowering period	0.10	0.33	0.16
During flowering period	0.15	0.38	0.23

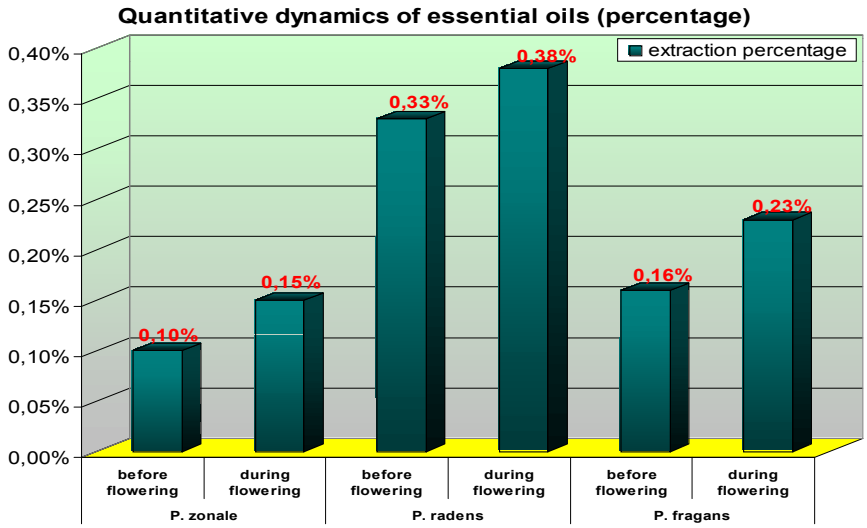

Diagram 1. Quantitative dynamics of the essential oils (percentage)

The qualitative dynamics of the essential oils is given by the number of components, which were found as shown in **Table 2** and illustrated in **Diagram 2**, for each of the species investigated, in the two ontogenetic periods.

Table 2. Number of components for the essential oils of *P. zonale, P. radens* and *P. fragans* extracted before and during the flowering period.

Ontogenetic period	Number of components		
	P. zonale	*P. radens*	*P. fragans*
Before flowering period	49	64	11
During flowering period	161	44	69

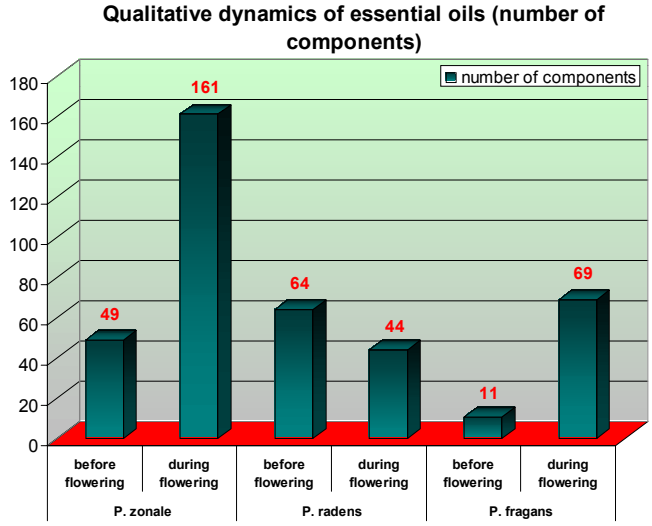

Diagram 2. Qualitative dynamics of the essential oils (number of components)

The main components of the *P. zonale* essential oil extracted before the flowering period and during the flowering period have been identified and they are shown in **Diagram 3** and **Diagram 4**, respectively. **Diagram 5** is a comparative representation of the percentage difference between the common main components found in the two ontogenetic periods.

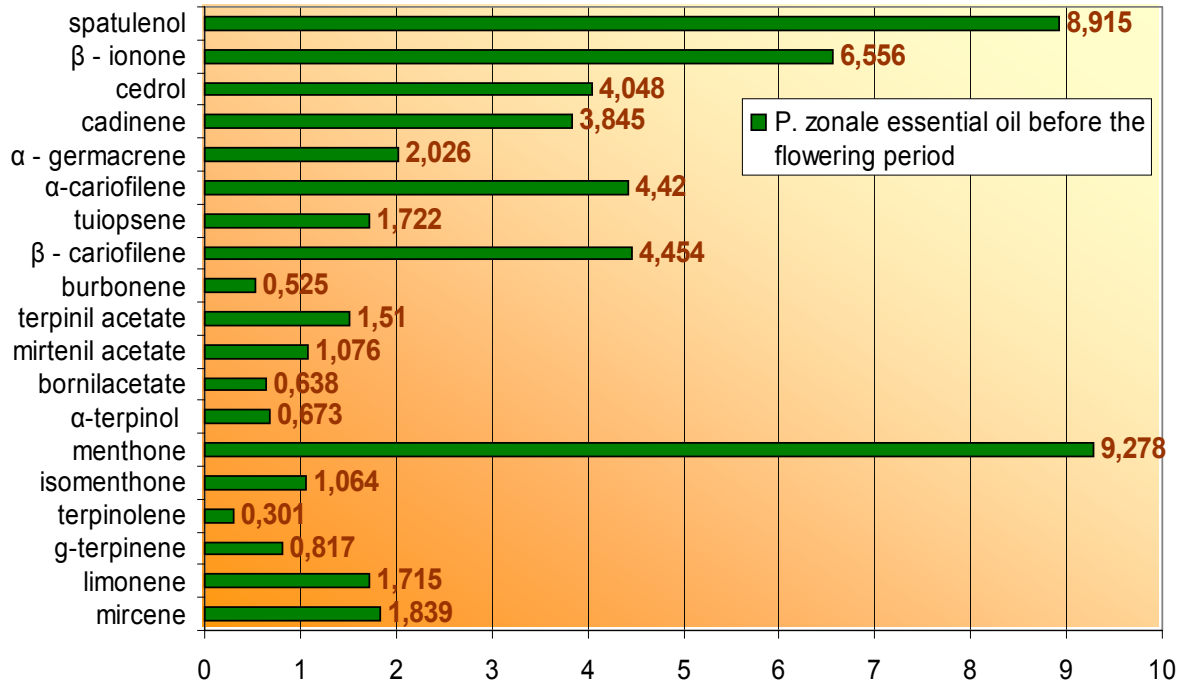

Diagram 3. Main components of *P. zonale* essential oil extracted before the flowering period

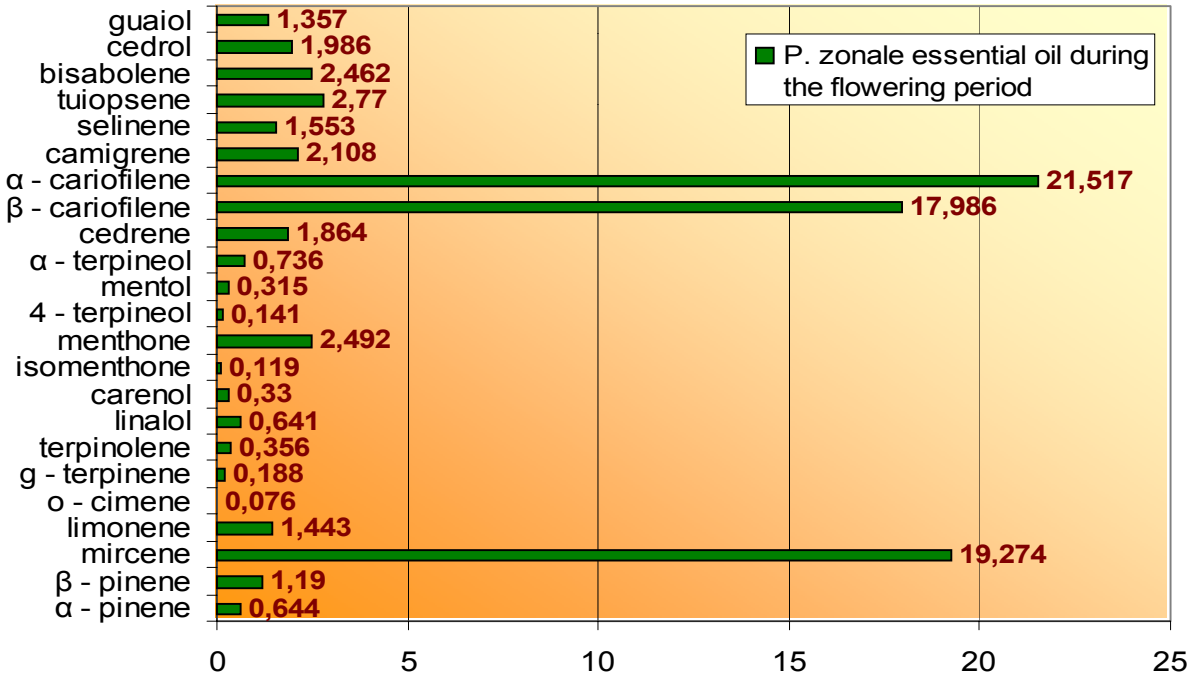

Diagram 4. Main components of *P. zonale* essential oil extracted during the flowering period

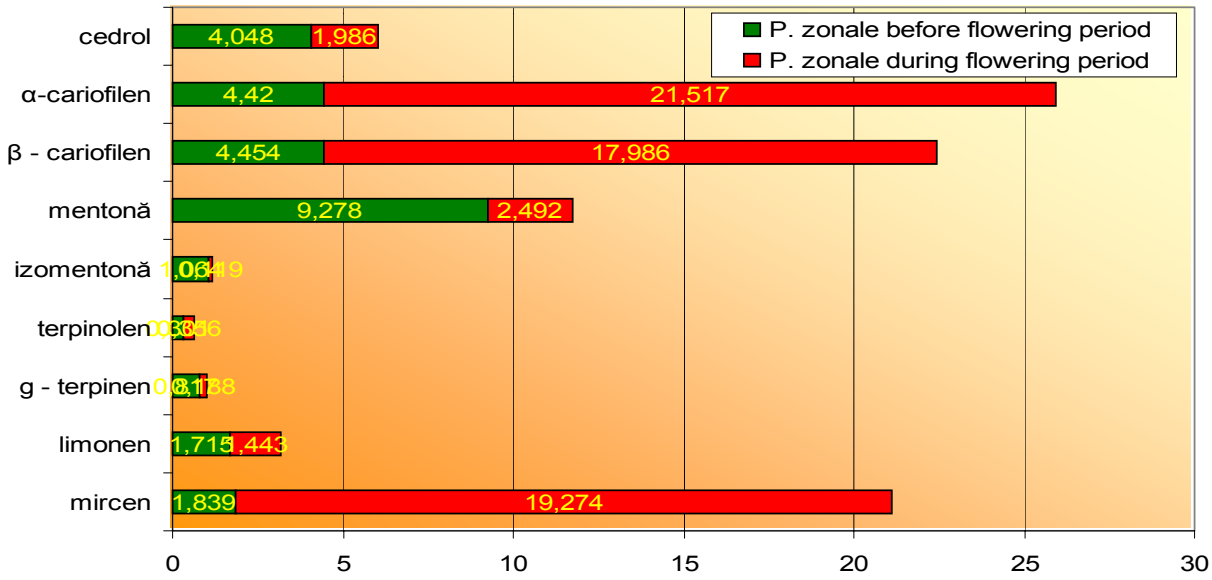

Diagram 5. Difference between the main components found in *P. zonale* essential oils extracted before and during flowering period (%)

The dynamics of photosynthetic pigments found in the leafs of the investigated species is illustrated in **Diagram 6** and values are shown in **Table 4.**

Table 4. Photosynthetic pigments found in leaves of *P. zonale, P. radens* and *P. fragans* during the two ontogenetic periods.

Species	before flowering period			during flowering period		
	Chlorophyll a	Chlorophyll b	Carotenoids	Chlorophyll a	Chlorophyll b	Carotenoids
P. zonale	2,177184	0,757763	0,000716	1,310286	0,475604	0,000451
P. radens	1,308879	0,452474	0,000412	1,371064	0,495493	0,000470
P. fragans	0,924729	0,357613	0,000317	0,998127	0,380239	0,000360

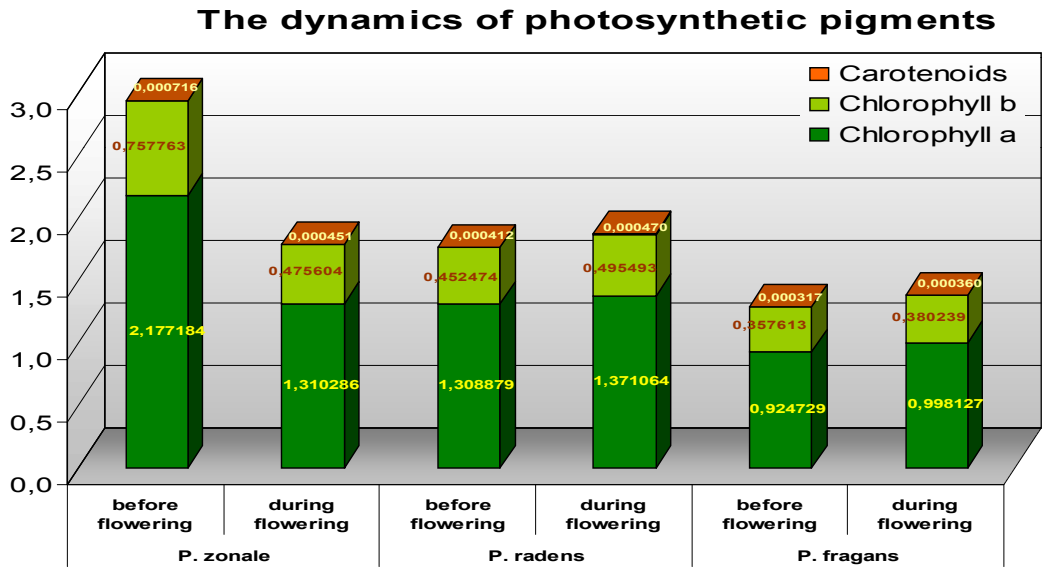

Diagram 6. The dynamics of photosynthetic pigments found in leafs of *P. zonale, P. radens* and *P. fragans* before and during the flowering period.

CONCLUSIONS

- The extraction efficiency has different values, according with the species and with the ontogenetic period; for all three species, the extraction efficiency is better during the flowering period.
- The number of components of the essential oils extracted is larger during the flowering period for the essential oils extracted from *P. zonale* and *P. fragans*, and lower for *P. radens*.
- The chemical composition of *P. zonale* essential oils extracted in the two different ontogenetic periods differs both qualitatively, as well as quantitatively. There are different components in the oils extracted in the two ontogenetic periods, whereas the common components for the two oils have been found in different percentages.
- For all three species, the amount of pigments (mg/ g fresh material) was found larger during the flowering period.

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[P-002]

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