Variability and biologically active components of some Lamiaceae species

Summary of Ph.D. Thesis

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1. Introduction

Numerous species of the family Lamiaceae which are rich in volatile oils are of great importance as herbs and spices. As regards their morphology and chemical compositions, a substantial proportion of these plants are polymorphic. Such diversity raises important theoretical and practical issues. It can be challenging to classify the species, basic research for the purposes of variety breeding is incomplete, and quality assurance is also often problematic. Consistent with these features, the variability in essential oil composition may alter the gastronomic value of the spices and modify the physiological effects of the herbs in the event of medicinal use. Therefore, it is easy to understand why complex explorative pharmacological studies of the active constituents are playing an increasingly important role over and above chemical and analytical evaluations.

Practical evaluations of numerous Lamiaceae species have brought various classes of constituents other than essential oils into focus. These include hydroxycinnamic acid derivatives (mainly rosmarinic acid) as representatives of natural antioxidants, and also triterpene carboxylic acids, characterized by physiological activity.

In this context, complex research into the family Lamiaceae was begun some decades ago as a cooperative program in which several Hungarian research centers are engaged in the evaluation of medicinal plants. Participating in this research, I have focused on hyssop and origanum, two herbs of diverse appearance and chemical constitution, in order to study their variability and constituent characteristics. The initial results of this research activity were discussed in detail in my thesis for the degree of University Doctor, while those of the continued studies are presented in this Ph.D. thesis.

As regards my experiments with both species, I participated in the basic research preceding variety breeding, and working together with researchers at the Department of Medicinal and Aromatic Plants of Corvinus University (the former University of Horticulture), focusing on variety breeding; I was mainly engaged in chemical analyses.

2. Aims of the study

- Hyssop - *Hyssopus officinalis* L.

  The hyssop cultivar registered in Hungary as the variety Kékvirágú is far from being uniform in terms of the colour of the corolla and the chemical composition of its volatile oil. This issue gave rise to further studies of variety breeding. The basis of selection was the composition of the uniform blue colour of the corolla, a high volatile oil content and a definite essential oil composition.

  The primary aim of our study was

  - a thorough evaluation of the strain and progeny lines originating from different hyssop populations
  - the follow-up of the selected characteristics in progeny generations.

- Origanum - *Origanum vulgare* L.

  As regards origanum, no cultivar of this plant is available in Hungary, making it an up-to-date issue to come up with basic research on different origanum populations to achieve results with the potential of utilization for variety breeding.

  Aims of our study:

  - determining the amount and composition of the essential oil of several oregano populations,
  - performing stability studies on the essential oils,
  - completing a stock evaluation for the selection of favourable chemotypes,
  - evaluating the characteristics of the Mediterranean subspecies *O. vulgare* subsp. *hirtum* rich in essential oil, grown under the climate of Hungary.

- Exploring the relationship between the dynamics of accumulation of rosmarinic acid and the phenological stage, for both species.
- Elaborating methodology for parallel determination of the oleanolic acid (OA) and ursolic acid (UA) contents of Lamiaceae species and drawing chemotaxonomic conclusions from the results.
- Studying biological, e.g. antimicrobial and antioxidant activities.

3. Materials and methods

3.1. Plant material

The plant material was gathered from the perennial populations of *Hyssopus officinalis* L., *Origanum vulgare* subsp. *vulgare* L. and *Origanum vulgare* subsp. *hirtum* (Link) Ietswaart cultivated at the Experimental Station of the Faculty of Horticultural Science of Corvinus University of Budapest at Soroksár and at the experimental field of the Institute of Ecology and Botany of the Hungarian Academy of Sciences at Vácrátót, Hungary.

3.2. Chemical analysis

The evaluation of the active agents included the measurement and characterization of the essential oil, rosmarinic acid and oleanolic acid/ursolic acid content of the aerial part.

The essential oils were gained by Water Steam Distillation (WSD) for 2 h in a Hungarian Pharmacopoeia distillation apparatus. The oils were analysed by GC and GC-MS techniques. Identification of the compounds was based on comparisons with published MS data and a computer library search (the database was delivered together with the instrument) and also on the comparison of their Kovats retention indices with those of authentic references and with literature values.

The identification was confirmed with the aid of authentic samples. Rosmarinic acid contents were measured using TLC-densitometry.

UA and OA contents were measured in parallel after the preparation of derivatives of the original compounds, using GC analysis with an internal standard.

3.3. Measurements of Activity

Antimicrobial activity was evaluated by the MIC value and by the agar-well diffusion method. The determination of inhibition-zones, together with positive control tests was performed.

Antioxidant activities were screened by TLC combined with DPPH detection. Enzyme-independent lipid-peroxidation (LPO) was assayed on standard ox-brain homogenate.

4. Results and discussion

4.1. Preliminary studies

The first steps in our studies were the evaluation of available plant stocks and screening of inhomogenous and selected plant populations grown in Hungary as a result of international seed exchange from various botanical gardens. Differences in genotypes, corolla colour, and essential oil content and composition were considered crucial.

For our further experiments, defined in the study plan, we used the following selected taxons:

- *H. officinalis* ssp. *officinalis*
  - Magyar Kékvirágú (Medicinal Plant Research Institute, Budakalász), and its offspring selected on the basis of the blue colour of the corolla
  - Német hyssop, agricultural plant (Müggenburg GmbH, gene bank stock)
  - Bolgár hyssop (Pharmaplant)
- *O. vulgare* L.
  - subsp. *vulgare*
  - subsp. *hirtum*, selected progeny line
  - subsp. *hirtum* (domestic plant population grown in Hungary as a result of international seed exchange)

4.2. Hyssop

4.2.1. Morphological studies

Morphological studies of hyssop starting in 1998 were followed by the screening of plant height, inflorescence length, corolla colour and earliness. Comparison of the data on selected stocks of the parent plants and those of the offsprings revealed that the selected characteristics of the 2- and 3-year-old offsprings were as good as or even better than those of the parent plant. The appearance of the purple colour of the corolla was consistent, whereas the earliness of flowering was only modest among the offspring.

Comparison of the basic stocks of domestic Magyar and Német hyssop types suggests that the different patterns of the beginning of flowering is a phenological

mark. During the 3 years of our experiments (1998–2000), the Német hyssop type was observed to flower 1 or 2 weeks earlier than the Magyar type.

4.2.2. Studies of the essential oils

For the accumulation of essential oils, the proportional weights of the leaves, the stem and the inflorescence are crucial factors. Therefore, besides measuring the total weight, we also measured the proportional weight of each plant organ in the various phenological states studied. As the stem, which constitutes a substantial percentage of the total weight, contains no essential oil, its precise proportion within the drug is of particular importance.

The series of studies (1998–2001) using average samples of hyssop revealed the variations in essential oil content as a function of the phenological stage. In contrast with the common view that the essential oil content reaches its maximum at full flowering, our results showed that this maximum appears as early as at the beginning of the differentiation of the inflorescences. This holds true for all the 3 hyssop types examined. The essential oil content decreases dramatically in the ripe fruit, whereas it increases again in the plants of the second growth. In the event of reflorescence, the essential oil content of the plant reached the level measured at the time of the first florescence. The tendency of the essential oil to accumulate did not change in the F3 progeny generation. From a comparison of the 3 types of hyssop studied, it can be concluded that the Magyar type contains the highest amount of essential oils.

A significant fluctuation of the essential oil content was seen in plants grown in different years. According to the literature the essential oil content of hyssop may vary by as much as 50% from year to year. As concerns the generations, the plants in 1998–1999 had a low essential oil content, while a significant increase was observed in 2000–2001.

The qualitative composition of the essential oil content is a significant chemical hallmark. With regard to the information mentioned above, the ISO standard and the former Hungarian standards, we consider isopinocamphone and pinocamphone (50% together) and β-pinene to be significant constituents of the essential oil. Limonene is considered another characteristic hallmark. Thus, for control of the qualitative composition of the essential oil, we examined the presence of these 4 constituents and measured their absolute and relative quantities.

We found isopinocamphone to be the major constituent of the Magyar hyssop sample throughout the 3 years of our experiments (1998–2000), and its amount showed no substantial alterations in the vegetation period. In the Német hyssop isopinocamphone, whereas in the Bolgár hyssop pinocamphone was the main component. The sum of the quantities of isopinocamphone and pinocamphone met the standard requirements in all cases.

The composition of the essential oil content as regards the 4 main components was the same in the progeny generation as in the parental generation described above.

The comparison of these 3 types of hyssop furnishes evidence of a differing chemosyndrome line. We can conclude that the chemical fingerprints determined at the same periods of time reflect different genetics manifesting as different phenotypes. This is supported by the fact that the experimental samples of all three types of hyssop were collected from plants harvested in parallel to one another. Thus, the factors inducing or influencing chemosyndrome lines were the same for all the plants studied. The fingerprint of the Magyar hyssop is uniform in the selected plants and in their offspring, and no phenological correlations were revealed. All samples had isopinocamphone as the main constituent. In contrast, the ratio of pinocamphone/isopinocamphone changed over time in the vegetation period in the Német hyssop. The Bolgár hyssop is characterized by a predominance of pinocamphone. A novel finding of our team is that the increase in pinocamphone content is accompanied by a decrease in a relative amount of isopinocamphone during vegetation. This change is most pronounced in those plants that have pinocamphone as the main constituent. In our case this was the most marked in the Bolgár hyssop sample.

Our previous studies identified 4 characteristic chemotypes on the basis of the qualitative variations of the main constituents of the essential oils of hyssop. The chemotype containing a substantial amount (>30%) of limonene is a novum in the literature.

As a result of this complex selection research, we have a candidate variant of *Hyssopus officinalis* that has an uniform appearance and essential oil content, and additionally has an unique high essential oil production among European plants. Our

research has produced the basic material for the drug acknowledged by quality assurance.

4.3. Origanum

4.3.1. Studies of the essential oils

The two subspecies of *Origanum* studied, namely *O. vulgare* subsp. *vulgare* and *O. vulgare* subsp. *hirtum,* exhibited a striking difference in the essential oil content. While *O. vulgare* subsp. *vulgare* had an essential oil content of only approximately 0.2%, the selected *O. vulgare* subsp. *hirtum* displayed a high essential oil content throughout the period of our measurements (4.3% in 2000, and 5.3% in 2001). These findings are consistent with the literature data. This indicates that selection of the genotype of *O. vulgare* subsp. *hirtum* and introduction of its cultivation in Hungary would be successful, as the high essential oil content of the Mediterranean plant remains characteristic even under the circumstances present in Hungary.

The composition of the essential oil isolated from *O. vulgare* subsp. *vulgare* differed from that isolated from the subspecies *hirtum*. While *p*-cymene (13–21%) and the sesquiterpenes (22–24%) predominated in the former case, the subspecies *hirtum* proved to be a carvacrol-type subspecies with 64–76% carvacrol content.

4.3.2. Stability studies of the essential oils

Stability studies of the essential oils aimed to reveal how the circumstances of storage affect the composition of the essential oils. We found that storing the drug itself for 1 year did not produce a significant change in the composition of the essential oils. However, after storing the distilled essential oil frozen for 1 year a significant change of the monoterpene phase was revealed, while the composition of the sesquiterpene phase remained relatively stable.

Our stability studies have provided valuable pieces of information for quality assurance.

4.3.3. Stock evaluation for selection of chemotypes

Within the scope of our basic studies serving variety breeding, we screened some previously unstudied inhomogenous stocks of two *O. vulgare* subspecies. We aimed to find individual plants with a high essential oil and rosmarinic acid content, as well as to identify plants with an essential oil composition of significant diversity.

Individual plants of *O. vulgare* gathered in the same time frame had a low essential oil content, ranging from 0.07 to 0.3%. The RA content ranged from 0.3 to 0.95%. Individual plants of *O. vulgare* subsp. *hirtum* had a substantially higher essential oil content, ranging from 1.42 to 6.35%, and their RA content ranged from 1.41 to 2.06%. The individual plants differed significantly in terms of essential oil and RA contents.

The data on the RA contents of the individual plants of *O. vulgare* throughout the 3-year period of our experiments provided an interesting picture. In the second year (2003), the RA content was substantially higher in almost every plant examined, which is presumably a result of year-specific effects. Such a relationship was not observed for the essential oil content in the same period.

As regards the composition of the essential oils isolated, all the individual plants of *O. vulgare* subsp. *hirtum* were characterized by a predominance of carvacrol, which belongs in the chemical group described by Pasquier. The contents of carvacrol (low, intermediate or high) and of other major constituents (*p*-cymene and *γ*-terpinene) were considered as criteria for selection.

The compositions of the essential oils isolated from the plants selected in 2002 only partially met our expectations in the following 2 years. Although the predominance of the main components remained consistent, the proportional quantities of the individual components did not meet the expectations on several occasions. This deviation was especially striking for the essential oils isolated from plants with a low carvacrol content. In contrast with what we had expected, the carvacrol content rose in these cases, while the amount of *γ*-terpinene (its precursor) decreased. This phenomenon may reflect the changes in the climatic conditions.

Similarly as in the case of *O. vulgare* subsp. *hirtum*, we strived to select individual plants of *O. vulgare* with as diversified an essential oil composition as possible for microbiological studies

4.4. Quantitative determination of rosmarinic acid

As for the essential oil content, differentiation according to plant organs is a substantial factor for the determination of RA content.

Our measurements of the RA content in different plant organs of hyssop and origanum revealed a significant accumulation of this component in the leaves and the flowers together. In contrast, the stem had a very low RA content (<0.1%).

We extended our studies to the whole vegetation period of the plants to determine when the RA content reaches its maximum. The dynamics of accumulation described in the first 3 years of our experiments indicates a trend towards the RA content reaching its non-outstanding maximum by the time of flowering. An occasional increase was also detected in the spring and autumn sprouts. We obtained similar results for the progeniy generations

In conclusion, being familiar with the optimal ratios of plant organs and the dynamics of the accumulation of the essential oils is of particular imporatnce for chosing the proper time for plant collection.

4.5. Elaborating a method for the parallel measurement of oleanolic acid/ursolic acid contents

UA and OA are present in most of the genera belonging in the subfamily Nepetoideae. However, there is a scarcity of literature data on their typical quantities or their quantities relative to each other. As these triterpene carboxylic acids exert valuable physiological effects, filling this gap is a priority. In view of their similar chemical structures, they can not be measured by TLC-densitometry. We therefore elaborated a novel method to measure the UA and OA contents in parallel. After the preparation of derivatives of the original compounds, GC analysis with an internal standard allows the selective measurement of each compound. We screened a large number of samples by using this method, including the measurement of the OA/UA contents of samples of the *Hyssopus* and *Origanum* species discussed in the present thesis.

Both hyssop and origanum samples typically contain UA in an amount much higher than that of OA. A substantial difference between the two species is that hyssop contains higher amounts of both triterpene carboxylic acids. Studies of numerous *Lamiaceae* species revealed that these two triterpenoid acids are present in all of the taxa investigated. However, there are significant quantitative differences between the two subfamilies. The subfamily Lamioideae appears to be poorer in both OA and UA than the subfamily Nepetoideae. Mean values for the Lamioideae were

OA: 0.012% and UA: 0.023%, while those for the Nepetoideae were OA: 0.263% and UA: 0.638%.

4.6. Studies of physiological effects

4.6.1. Correlations between essential oil composition and antimicrobial activity

Four samples of essential oils of different composition isolated from *O. vulgare* subsp. *hirtum* in 2004 were used for microbiological studies. Approximately 97.26–99.35% of the constituents of these 4 samples were identified. The components present in the highest concentrations were carvacrol (61.72–84.21%) *γ*-terpinene (1.35–15.98%) and *p*-cymene (4.17–9.50%).

Antimicrobial activity was tested on Gram-positive and Gram-negative (proton pump-positive and negative) bacterial strains and *C. albicans* strains, using antibiotics as positive controls. At the same time, we separately analyzed the antimicrobial activities of the isolated conpounds (*α*-pinene, sabinene, *β*-pinene, *α*-terpinene, *p*-cymene, limonene, *γ*-terpinene, borneol, thymol, carvacrol and *β*-caryophyllene). All these experiments were carried out at the Department of Medical Microbiology and Immunobiology at the University of Szeged.

With an agar diffusion method and a positive control, the MIC values revealed that the presence of carvacrol is always essential for the antimicrobial effect, although other compounds also exerted minor antimicrobial activity.

4.6.2. Correlations between rosmarinic acid content and antioxidant activity

Using TLC and a DPPH reagent to detect the extracts of hyssop and origanum mass samples, we found that the extracts had antioxidant properties. After some preliminary experiments, we carried out pharmacological studies in cooperation with the Department of Pharmacodynamics and Biopharmacy at the University of Szeged, using 70% MeOH extracts of several hyssop and origanum plants to evaluate the relationship between their RA content and antioxidant activity. All extracts of origanum inhibited lipid peroxidation in a concentration-dependent manner.

Despite its substantially lower RA content (0.3–0.4%), the antioxidant activity of the hyssop extract was similar to that of the origanum extract, which contains a relatively high amount of RA (0.93–1.41%). This suggests that, besides the crucial role of RA, other constituents of hyssop also contribute to the antioxidant activity. This issue needs to be explored in future complex studies.

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List of publications

The thesis is based on the following publications:

1. **Veres K,** Varga E, Dobos Á, Hajdú Zs, Máthé I, Pluhár Zs, Bernáth J:
   Investigation on Essential Oils of *Hyssopus officinalis* L. populations
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