

OPINION

by Prof. Dr. Snezha Zlateva Zlateva, MD
MU "Professor Dr. Paraskev Stoyanov" - Varna

of a dissertation for the award of the educational and scientific degree 'doctor'
professional field 7.3. "Pharmacy"
doctoral program "Pharmacology /incl. pharmacokinetics and chemotherapy"

Author: MA Pharm. Kristina Yulianova Stavrakeva

Form of doctoral studies: full-time

Department: "Pharmacology, Toxicology and Pharmacotherapy", Faculty of Pharmacy,
Medical University of Plovdiv

Topic: "Study of biological effects of methanol extract of *Micromeria frivaldszkyana* (Degen)
Velen. (Lamiaceae)"

Scientific supervisor: Assoc. Prof. Elisaveta Apostolova, MD and Prof. Dr. Anelia Bivo-larska,
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1. General presentation of the procedure and the doctoral student

The presented set of materials on electronic media is in accordance with Art. 70 (1) of Section I. Acquisition of the educational and scientific degree "DOCTOR" and the scientific degree "DOCTOR OF SCIENCES" at MU-Plovdiv; Regulations of MU-Plovdiv dated 28.01.2021 and includes the following documents:

- Application to the Rector of MU-Plovdiv for opening the procedure for defending a dissertation
- CV in European format with the signature of the doctoral student
- Notarized copy of a higher education diploma
- Orders for enrollment in doctoral studies
- Order for conducting an exam from the individual plan and a corresponding protocol for a passed exam or doctoral minimum in the specialty
 - protocol of the department council for preliminary discussion of the doctoral thesis and the decisions made to open a procedure and to form a scientific jury
- dissertation
- abstract

- list of scientific publications on the topic of the dissertation
- copies of scientific publications
- list of participation in scientific forums
- list of noted citations
- declaration of originality and authenticity of the attached documents
- other documents related to the course of the procedure

The doctoral student has attached 3 publications.

Notes and comments on the documents: The documents are well formatted and presented.

Emphasis on biographical data in relation to the procedure.

KRISTIN YULIANOVA STAVRAKEVA was born on 21.10.1996 in the city of Plovdiv. She graduated from the Medical University of Plovdiv, Faculty of Pharmacy with a Master of Pharmacy degree in 2022. She started working at Pharmacies Plovdiv. Enrolled as a full-time doctoral student at the Faculty of Pharmacy of the Medical University of Plovdiv with Order No. R-3651/11.12.2023.

From 21.01.2025, he started working at the Medical University - Plovdiv, Faculty of Pharmacy, Department of "Pharmacology, Toxicology and Pharmacotherapy" as an assistant professor, participating in the teaching, research and development activities of the department, conducting practical classes with students, conducting colloquiums, participating in examination sessions, and participating in the development of the research and development activities of the department.

2. Relevance of the topic

Plants from the Lamiaceae family, genus *Micromeria*, are found in many places in Europe and people use them for a number of ailments. Specifically, the plant *Micromeria frivaldszkyana* is endemic to Bulgaria, but there are very few scientific publications about its toxicity, composition of biologically active substances and what exactly its benefits for humans would be.

According to literature data, the species *Micromeria* possesses active substances of interest for medicine. The doctoral candidate Stavrakeva sets herself specific tasks to experimentally study *Micromeria frivaldszkyana* in Bulgaria, specifically the qualitative and quantitative composition of its active substances and whether they possess acute toxicity, whether they have analgesic, anti-inflammatory, hepatoprotective effects and whether they affect cognitive functions.

These are current diseases in Bulgaria, from which many people suffer. In this sense, I believe that the conducted study and the specific tasks are current and the evidence is convincing, both in a scientific and theoretical aspect, and the fact that the Bulgarian *Micromeria frivaldszkyana* will be of interest in developing new medications or nutritional supplements.

3. Knowing the problem

Pharmacist Kristina Yulianova Stavrakeva is well aware of the need to study and use more plants as a source of biologically active substances in order to reveal their therapeutic potential.

In the Literature Review, she presents detailed information about the entire genus *Micromeria*, the general characteristics, botanical features and distribution of the different species of plants in this genus, their use in traditional medicine, phytochemical composition and the main bioactive compounds in the different species of this genus.

The standardized and validated *in vivo* methods (animals) used to evaluate bioactive compounds in terms of antioxidant activity, analgesic effect, influence on various types of inflammation, and influence on liver and cognitive functions are discussed in detail.

The doctoral candidate Kristina Yulianova Stavrakeva creatively evaluates the literary material. She assesses *Micromeria frivaldszkyana* as an endangered species in Bulgaria, which is why this plant species has remained unexplored.

The scarce information on the composition is a prerequisite for a more in-depth study of the plant's metabolites in order to establish a relationship between the phytochemical composition and its biological activity, as well as the safety profile.

Pharmacist Kristina Stavrakeva presents in detail what is known and discovered about plants of the genus *Micromeria*, their activity and the precise tests that would reveal the properties of the Bulgarian plant *Micromeria frivaldszkyana*.

4. Research Methodology

The selected research methods allow to achieve the set goal of studying the chemical composition and biological effects of an extract from the above-ground parts of the Bulgarian endemic plant *Micromeria frivaldszkyana* and to obtain an adequate answer as to whether it possesses analgesic, anti-inflammatory, antioxidant, neuroprotective (cognition) and toxin properties.

5. Characteristics and evaluation of the dissertation work and contributions

The dissertation is very well structured, the material is presented on 132 pages in total, and includes 8 chapters (INTRODUCTION, LITERATURE REVIEW, PURPOSE AND OBJECTIVES, MATERIALS AND METHODS, RESULTS AND DISCUSSION, SUMMARY, CONCLUSIONS, CONTRIBUTIONS and BIBLIOGRAPHY).

The INTRODUCTION presents the various species of plants of the genus *Micromeria* and their use in folk medicine as antirheumatic, antiseptic, antimicrobial, antioxidant, gastroprotective, hepatoprotective, anti-inflammatory, anticholinesterase, central nervous system (CNS) stimulant, and general tonic.

The introduction informs us about the Bulgarian endemic species, which is not well studied. The doctoral candidate Kristina Y. Stavrakeva believes that her study will enrich the information about the phytochemical content of *Micromeria frivaldszkyana*, increase knowledge about its composition and help characterize its biological potential.

Pharmacist Stavrakeva believes that the discovery of unknown properties of the Bulgarian endemic species of the plant will determine its suitability for use and whether it can serve as a source for synthesizing new medicines or food supplements.

THE LITERATURE REVIEW is structured in 7 subchapters.

Subchapter 1 provides a general description of *Micromeria frivaldszkyana* with its botanical features and distribution, and the importance of the plant in traditional medicine and modern science.

Subchapter 2 presents the phytochemical composition of *Micromeria frivaldszkyana*, describes the main bioactive compounds, and pays special attention to those compounds in the plant that have antioxidant and anti-inflammatory properties.

Subchapter 3 theoretically presents the importance of antioxidants for health: protection against oxidative stress and cellular damage. Theoretically, a review of methods for assessing antioxidant activity and antioxidant enzymes and markers is provided. An analysis of the available literature data on the antioxidant potential of the methanol extract of *Micromeria frivaldszkyana*: comparison with other species of the Lamiaceae family is made. Brief information is given on the pathogenetic role of free radicals in a number of chronic diseases of social importance and the benefit of the application of anti-oxidants for their prevention.

Subchapter 4 discusses pain and its pathogenesis, describing in detail the *in vivo* methods used to evaluate the analgesic effect of chemical compounds in pain caused by mechanical, chemical, thermal and cold stimulus.

Subchapter 5 describes the pathogenesis of the inflammatory process, the patterns and chemical markers for the anti-inflammatory properties of the other plants from the Lamiaceae family (thyme, mint, rosemary, almond and others) and notes the lack of such for the Bulgarian endemic plant *Micromeria frivaldszkyana*.

Subchapter 6 discusses the impact of oxidative stress on neurodegenerative diseases and inflammation in the brain, which affect cognitive functions (learning, memory, attention, perception, language, intelligence). Results from previous studies on the effects of bioactive plant extracts on memory and behavior, as well as *in vivo* models for studying the impact on cognitive functions, are presented.

Subchapter 7 discusses cellular, molecular and biochemical changes in liver injury, the parameters measured during injury, the importance of oxidative stress and the therapeutic potential of some of the chemical compounds found in *Micromeria frivaldszkyana*. *In vivo* models for experimental chemical, drug-induced and nonsteroidal anti-inflammatory drug-induced hepatotoxicity are reviewed.

SUMMARY. Finally, a summary of the literature data is made, from which the goal and objectives logically follow.

GOAL AND OBJECTIVES. The goal is clearly defined, and the tasks for achieving it are 7: obtaining a methanol extract from the aerial parts of *Micromeria frivaldszkyana* (Lamiaceae); determining the chemical composition of the extract; acute toxicity; comparative study of the analgesic and anti-inflammatory effects of the extract and standard rosmarinic acid in rats; comparative study of the effects of the extract on learning and memory processes in rats and study of the hepatoprotective effect of the extract in models of hepatotoxicity in rats.

MATERIALS AND METHODS. Plant material was used from aboveground parts of *Micromeria frivaldszkyana* collected in June (during full flowering) of the 2019-2020 vegetation

period from the Bulgarka Nature Park, floristic region Stara planina (middle) in the area of Mount Shipka.

The primary metabolites were analyzed by GC-MS, and the specialized (secondary) metabolites, water-methanol extracts by UPLC-MS-MS. The experiments with the obtained primary and secondary metabolites from *Micromeria frivaldszkyana* were conducted on male Wistar rats and were carried out in compliance with the requirements of the Helsinki Convention and Regulation No. 20 of November 1, 2012.

The animals are distributed in cages in groups of 8 according to the requirements of the Bulgarian Food Safety Agency (BFSA) with permit No. 352/ 30 May 2023 and with protocol No. 6/05 October 2023 from the Ethics Committee at MU-Plovdiv. The standards for working with animals are complied with.

Methods for determining acute toxicity were used; the extract was tested for analgesic activity using the "hot plate" thermal stimulus test and the "Analgesimeter" mechanical stimulus test. The anti-inflammatory activity was studied in a rodent hind paw inflammation model.

To assess the impact on cognitive functions, locomotor activity is examined; recognition reflex; two-way active avoidance test; step-through passive avoidance test; step-down passive avoidance test; spatial working memory (Y-maze), anxiety behavior using an elevated cross maze (X-maze) and a recognition memory test (new object recognition test) are examined.

The method for studying the hepatoprotective effect is in a model of hepatotoxicity induced by paracetamol and t-BHP, by determining markers of liver function in serum, oxidative stress, antioxidant defense and levels of pro-inflammatory cytokines in liver homogenate.

The results of the experiments are processed with the SPSS 19.0 program.

RESULTS AND DISCUSSION.

The phytochemical composition, primary metabolites in plants that are not related to their biological activity, lipids and secondary metabolites whose biological activity was analyzed were determined.

According to the dissertation, for the first time, a toxicity study of an extract of *M. frivaldszkyana* in vivo has been carried out. The results show a lack of toxicity after oral administration to male Wistar rats. Experiments continue with doses of 250 and 500 mg/kg, which are 1/10 and 1/20 of the determined LD50 (5000 mg/kg b.w. in this case).

In a test with mechanical paw pressure (analgesimeter) and in a test with thermal pain stimulus, the plant extract did not show analgesic activity after 14 days of application.

Резултатите от приложението на метанолов екстракт от *Micromeria frivaldszkyana* при оток на задна лапа индуциран от карагенин при плъхове, приложен перорално за 14 дни, намалява отока на задна лапа по време на ранната фаза на възпаление (първи до трети час след инжектиране на карагенин).

The results of the application of methanol extract of *Micromeria frivaldszkyana* in carrageenan-induced hindpaw edema in rats, administered orally for 14 days, reduced hindpaw edema during the early phase of inflammation (first to third hour after carrageenan injection). This result, the dissertationist argues, may be associated with reduced free radical production, reduced levels of proinflammatory cytokines, nitric oxide, and reduced COX-2 activity. The dissertationist attributes the recorded anti-inflammatory activity to the antioxidant properties of the extract.

The results of the study of the extract of *Micromeria frivaldszkyana* on cognitive functions show a trend for increased motor activity compared to the control group and at a dose of 500 mg/kg, the X maze test showed a potential anxiolytic effect after 14 days of administration in rats. For the other active learning methods, there was no statistically significant difference in the latency time of the treated groups compared to the control.

A study of the hepatoprotective effect of the methanol extract of *M. frivaldszkyana* suppressed the production of free radicals (reduced markers of oxidative stress), but did not increase the activity of antioxidant enzymes.

SUMMARY. It includes a summary of the overall analysis of the methanol extract of *M. Frivaldszkyana*, which proves the presence of: primary metabolites (sucrose, glucose, mannose, fructose, polyphenols and sugar alcohols in the largest amount); lipids of which triacylglycerols are the most abundant; inorganic elements (K, Mg, Zn and Ca in large quantities); secondary metabolites (flavonoids and polyphenols linarin, chlorogenic and rosmarinic acid, rutin, eupatorin, kaempferol-3-O-rutinoside and apigenin).

The doctoral candidate summarizes the *in vivo* results, the most important of which are the lack of acute toxicity in male Wistar rats at oral administration of doses up to 5000 mg/kg bw;

The biological activity found is summarized: After 14-day oral administration of 250, 400 and 500 mg/kg bw of the *M. frivaldszkyana* extract, the anti-inflammatory potential was revealed in a model of carrageenan-induced hind paw edema in rats. Pharmacist Stavrakeva points out that the observed anti-inflammatory activity of the extract is associated with the high concentrations of flavonoids and phenolic compounds (chlorogenic and rosmarinic acid, rutin, eupatorin, kaempferol-3-O-rutinoside and apigenin).

K. Stavrakeva points out that the established hepatoprotective effect in hepatotoxicity induced by paracetamol and t-BHP is due to the antioxidant activity of flavonoids in the extract of *M. frivaldszkyana*.

CONCLUSIONS. They are correct, derived from the results of the experiments.

Regarding the phytochemical composition of the plant:

1. 83 compounds were identified by GC-MS analysis, classified as amino acids, organic acids, sugars and sugar alcohols.

2. A total of 163 lipid compounds, distributed into 10 classes, were identified by lipidomic study of the non-polar fraction.

3. 192 compounds were detected – 123 identified and 69 unknown compounds by UPLC-MS-MS analysis of samples of methanol extract of *M. frivaldszkyana*. The secondary metabolites with the highest concentrations are flavonoids, mainly flavonoid glycosides. Rosmarinic acid is among the most significant substances detected.

Regarding the studied biological activities:

1. The methanol extract of *M. frivaldszkyana* did not cause toxic effects when administered orally to rats at doses up to 5000 mg/kg body weight.

2. The plant extract did not show analgesic effect in tests with mechanical and thermal pain stimulus.

3. The methanol extract of *M. frivaldszkyana* in all three tested doses has an anti-inflammatory effect in a model of acute exudative inflammation induced by carrageenan.

4. The application of methanol extract of *M. frivaldszkyana* does not increase spatial working and episodic memory in native rats, but based on its phytochemical composition such an effect could be expected in models of impaired memory.

5. The methanol extract of *M. frivaldszkyana* affects oxidative stress in experimental models of hepatotoxicity primarily by suppressing the production of free radicals (reduced markers of oxidative stress), rather than by increasing the activity of antioxidant enzymes. This determines its primarily preventive role in liver damage.

CONTRIBUTIONS. They are of scientific-theoretical and scientific-applied significance. The contributions are derived from the very well-conducted study of the extract of *M. frivaldszkyana* from Bulgaria and the identified primary and secondary metabolites, lipid compounds and trace elements.

The experiments conducted with animals to establish the biological activity of the plant are very well conducted, the effects of different doses have been evaluated, the exact biomarkers have been analyzed. The statistical processing of the data, together with the very well-designed graphs, convince us with great categoricity of the presence or absence of the supposed biological effects.

Contributions of scientific and theoretical significance are: 1. Establishing for the first time the lack of acute toxicity of methanol extract of *M. frivaldszkyana* after oral administration in rats and 2. Proving for the first time all metabolites, the most important of which are the high content of phenolic acids and flavonoids, potentially responsible for the observed biological activities.

Contributions of scientific and applied importance are the demonstration of the hepatoprotective and anti-inflammatory effect of methanol extract of *M. Frivaldszkyana*.

These contributions give significance to the study for science and practice, because they theoretically enrich the knowledge about this plant, found in many places in Bulgaria, especially with the evidence of its composition, lack of toxicity and the possibility of its use for creating hepatoprotective and anti-inflammatory medications or nutritional supplements, which is a prospect for further research.

BIBLIOGRAPHY. It includes 257 sources, only 2 of which are in Cyrillic. The cited publications are contemporary studies.

6. Assessment of the publications and the personal contribution of the doctoral student

In summary, three publications are presented in which pharmacist K. Stavrakeva is the first author. In two publications she is part of a team, one international, and the other is of Bulgarian scientists. In one publication she is an independent author. All articles reflect different aspects, which are then reflected in the dissertation. The work of Stavrakeva in an international team, which reflects the quality and quantity of active compounds in the Balkan species *Micromeria*, is most highly appreciated. This publication (*International Journal of Molecular Sciences*. 2024, May 15) has an impact factor IF 2022 - 5.6, Q1.

The classification is as follows:

articles 3; reports -3. By importance - articles in publications with impact factor 1; plenary reports - 2 issues; articles in refereed international journals - 1 issue, articles in national journals - 2 issues; reports at international scientific conferences abroad – 1 issue (poster); reports at international scientific conferences in Bulgaria – 1 issue; reports at national scientific conferences, sessions and seminars – 1 issue; reports at a university in English - MU Plovdiv – 1 issue.

The personal participation of the doctoral student in the conducted dissertation research is evident, as the formulated contributions and obtained results are his personal merit and that of the two scientific supervisors.

I have no critical remarks.

I recommend that the research be deepened in order to connect this serious scientific work with the real use of the endemic Bulgarian plant Frivaldskieva Micromeria Frivaldszkyana as a medicinal remedy.

I believe that well-mastered in vivo methods are a good prerequisite for doctoral candidate Kristina Stavrakeva to deepen her scientific interests in searching for and revealing the healing potential of other plants from Bulgaria.

7. Abstract

The abstract is presented on 61 pages, well structured and illustrated with 45 figures and 12 tables. It meets the requirements for structuring, which includes INTRODUCTION, PURPOSE AND OBJECTIVES, MATERIALS AND METHODS, RESULTS AND DISCUSSION, SUMMARY, CONCLUSIONS, CONTRIBUTIONS, LIST OF SCIENTIFIC PUBLICATIONS AND COMMUNICATIONS RELATED TO THE DISSERTATION WORK and ACKNOWLEDGEMENTS.

CONCLUSION

The dissertation contains scientific, scientifically-applied and applied results, which represent an original contribution to science and meet all the requirements of the law on the development of the academic staff in the republic of Bulgaria, the regulations for the implementation and the regulations of MU - PLOVDIV.

The presented materials and dissertation results fully comply with the specific requirements adopted in connection with the Regulations of the Medical University - Plovdiv for the implementation of the Law.

The dissertation shows that the doctoral student, Master of Pharmacy Kristina Yulianova Stavrakeva, possesses in-depth theoretical knowledge and professional skills in the scientific specialty 7.3. Pharmacy, demonstrating qualities and skills for independent conduct of scientific research.

Due to the above, I confidently give my positive assessment of the conducted research, presented by the above-reviewed dissertation, abstract, achieved results and contributions, and

I propose to the esteemed scientific jury to award the educational and scientific degree of 'doctor' to mag. pharm. Kristina Yulianova Stavrakeva in professional field 7.3. "Pharmacy", doctoral program "Pharmacology / incl. pharmacokinetics and chemotherapy"

17.03.2025

Prepared the opinion: ...

Заличено на основание
Чл.5 §1, б."В" Регламент (ЕС)2016/679

Prof. Dr. Snezha Zlateva, MD