

## REVIEW

by Assoc. Prof. Iliya Dimitrov Kostadinov, MD, PhD

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On PhD thesis for awarding the educational and scientific degree 'Doctor'

**Professional direction:** 7.3 "Pharmacy"

**Field of higher education:** 7. "Healthcare and sports"

**Doctoral program:** "Pharmacology (including Pharmacokinetics and Chemotherapy)"

**Author of PhD thesis:** Kristina Yulianova Stavrakeva, Master Pharmacist - full-time PhD student

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

**Topic:** "Study of Biological Effects of Methanolic Extract of *Micromeria Frivaldszkyana* (degen) Velen. (Lamiaceae)".

**Scientific supervisors:**

Assoc. Prof. Elisaveta Apostolova, PhD; Head of Department of Pharmacology, Toxicology and Pharmacotherapy, Faculty of Pharmacy, Medical University of Plovdiv

Prof. Anelia Bivolarska, MD, PhD; Head of Department of Medical Biochemistry, Faculty of Pharmacy, Medical University of Plovdiv

### 1. General Presentation of the Procedure and the PhD student

Based on the Decision of the meeting of the Faculty Council at the Faculty of Pharmacy, Medical University of Plovdiv (Protocol No. 8/29.10.2024) and by Order of the Vice Rector of Science and Research Activities of the Medical University of Plovdiv No. P-946/25.02.2025, I have been appointed as a member of the scientific jury in the procedure for awarding the educational and scientific degree "Doctor" to Kristina Yulianova Stavrakeva. Furthermore, based on Protocol No. 1/27.02.2025, I have been assigned to prepare a review on the aforementioned procedure.

The presented set of materials, both in paper and electronic formats, complies with the requirements specified in Art. 70 (1) of Section I. Acquisition of educational and scientific degree "Doctor" at MU-Plovdiv; Regulations of MU-Plovdiv from 28.01.2021. The materials include the following documents:

- application to the Rector of MU-Plovdiv for the disclosure of the PhD thesis defense procedure;
- curriculum vitae in European format, signed by the PhD student;
- a notary certified copy of Master's degree diploma;
- an order from the Rector for enrollment of Kristina Yulianova Stavrakeva as a full-time doctoral student No. P-3651/11.12.2023; an order from the Vice Rector of Science and Research Activities for deduction with the right of public defence for up to one year No. P-899/31.10.2024;
- an order from the Vice Rector of Science and Research Activities for conducting an exam from the individual plan (No. P-2105/20.06.2024) and a corresponding protocol for passing the doctoral minimum in the specialty;
- protocol No. 10/04.10.2024 of the Extended Departmental Council regarding the preliminary discussion of the PhD thesis and the decisions made regarding the disclosure of the procedure and the composition of the scientific jury;
- PhD thesis (132 pages);
- author's abstract (in Bulgarian and English) - 61 pages;
- a list of scientific publications related to the dissertation topic – 3 full-text articles;
- copies of scientific publications;
- a list of 3 participations in scientific forums;
- a certificate for 152 credits obtained from the group study plan;
- a declaration of originality and authenticity of the attached documents;
- other documents related to the procedure, including an order from the Vice Rector of Science and Research Activities No. P-1018/01.04.2024 for second scientific supervisor; an order from the Vice Rector of Science and Research Activities No. P-1017/01.04.2024 for defining the topic of the PhD thesis.

Kristina Yulianova Stavrakeva has submitted 3 full-text articles. I have no objections to the documents provided.

## **2. Brief Biographical Information about the PhD student**

Kristina Julianova Stavrakeva was born on October 21, 1996, in Plovdiv, Bulgaria. She completed her secondary education in 2015 at the "Sts. Cyril and Methodius" High School of Humanities in Plovdiv. In the same year, after successfully passing an exam, she obtained a C1-level certificate in English proficiency (IELTS – Advanced C1). From 2017 to 2022, she studied Pharmacy at the Medical University of Plovdiv. In 2022, Kristina Stavrakeva graduated with a Master's degree in Pharmacy and acquired the professional qualification of "Master Pharmacist." She briefly worked as a Master Pharmacist at the "Zapad" pharmacies in Plovdiv.

Kristina Julianova Stavrakeva has been enrolled as a full-time doctoral student in doctoral program in "Pharmacology (including Pharmacokinetics and Chemotherapy)" with a duration of three years, starting from December 11, 2023 (Order No. R-3651/11.12.2023 from the Rector of MU-Plovdiv). Thanks to her theoretical knowledge and practical experience, organizational skills, and responsibility toward assigned tasks, Kristina Stavrakeva successfully

completed the preparation of her PhD thesis ahead of schedule. As a result, she was granted the right to defend her dissertation within one year, starting from October 29, 2024 (Order No. R-899/31.10.2024 from the Rector of Science and Research Activities of MU-Plovdiv). On January 21, 2025, after successfully passing a competitive exam, Kristina Stavrakeva was appointed as an Assistant Professor at the Department of Pharmacology, Toxicology, and Pharmacotherapy, Faculty of Pharmacy, Medical University of Plovdiv. As a doctoral student she studied at the Doctoral School of MU-Plovdiv and acquired 152 credits, exceeding the mandatory minimum of 129. She is proficient in English at the C1 level.

Kristina Stavrakeva possesses skills in using Windows, Microsoft Office, the statistical software package SPSS v.19, and the pharmacy inventory management system PharmaStar. The doctoral student has completed courses on "Arterial Blood Pressure," "Inhalation Techniques," and "Blood Glucose" at the Pharma Sim Center, Medical University of Plovdiv. She has also conducted training on the use of a digital learning platform "Body Interact" for the development of clinical thinking and demonstration of clinical cases. She is a member of the Bulgarian Pharmaceutical Union and the Bulgarian Society of Pharmacology, Clinical Pharmacology and Therapeutics.

### **3. Relevance of the Topic and Suitability of the Established Aim and Objectives.**

Medicinal plants are a rich source of biologically active compounds with diverse chemical structures, offering significant potential for the development of herbal medicinal products and dietary supplements. Numerous drugs have been developed from secondary metabolites found in medicinal plants. In this context, investigating the therapeutic effects of isolated plant-derived chemical compounds with biological activity presents an opportunity for the development of new drugs. Moreover, biologically active substances in plants occur in unique combinations, both qualitatively and quantitatively, which can lead to favorable therapeutic effects with a lower risk of toxicity and adverse reactions. This highlights the growing interest in recent years in studying the effects of plant extracts that contain combinations of biologically active compounds.

The PhD thesis of Kristina Stavrakeva addresses a relevant problem related to the study of the chemical composition and pharmacological effects of an extract from *Micromeria frivaldszkyana*. The genus *Micromeria* *Benth* includes approximately 70 species, four of which are found in Bulgaria. Some *Micromeria* species have been used in traditional medicine and culinary practices in the regions where they are native. Experimental studies have demonstrated that these species possess anti-inflammatory, antioxidant, anticholinesterase, antimicrobial, and analgesic effects. *Micromeria frivaldszkyana* is an endemic plant species of this genus in Bulgaria, and therefore, research on its phytochemical composition and biological activity remains limited. Existing scientific literature reports data on its phytochemical composition, antimicrobial activity, and antioxidant effects, but no studies have explored its toxicity or other therapeutic properties. This underscores the originality and novelty of the study being conducted.

The current PhD thesis investigates the phytochemical composition, including the content of secondary metabolites, in *Micromeria frivaldszkyana* collected from a specific region of the country. Based on available literature data regarding the biological activity of compounds found in the extract, the PhD student has chosen to study certain pharmacological effects. The results about the biological activity of the extract provide the scientific foundation

for further comprehensive pharmacological studies on this plant, as well as the potential standardization and application of its extracts in the treatment of various diseases.

The relevance of the dissertation is determined not only by the research object but also by the competently selected spectrum of pharmacological effects studied. Pain, inflammation, and dementia remain unresolved issues in modern pharmacotherapy, despite significant progress in these areas. The potential to supplement conventional treatments with plant-based products is promising. Additionally, it is important to note that environmental factors significantly influence the formation and quantity of secondary metabolites in plants. In this context, the dissertation emphasizes the therapeutic potential of a medicinal plant endemic to Bulgaria, collected from a specific geographical region.

Of particular interest is the study of the hepatoprotective effect. The role of free radicals and oxidative stress in the initiation and development of liver damage is well known. Natural antioxidants play a key role in preventing free radical-mediated liver damage by influencing various pathophysiological mechanisms. Numerous hepatoprotective products are derived from plant sources. The results obtained form the basis for the future development of hepatoprotective drugs based on *Micromeria frivaldszkyana* extracts.

#### **4. Knowledge of the Problem**

Kristina Stavrakeva demonstrates excellent knowledge of the topic addressed in the PhD thesis. The literature review is competently and comprehensively written, and supported by an extensive range of references. It spans 41 pages and is illustrated with 22 figures, which significantly enhance the presentation and understanding of the information. The literature analysis shows that the doctoral student is well-versed in the subject of the dissertation and is capable of summarizing and analyzing data from a large number of scientific sources.

The literature review consists of seven sections, which logically follow one another. In the first section, the PhD student provides detailed information on the botanical characteristics and distribution of *Micromeria frivaldszkyana*. The use of *Micromeria* species in traditional medicine and the scientific data on their biological activity are analyzed. In the following section, Kristina Stavrakeva presents data on the phytochemical composition of *Micromeria frivaldszkyana* and the biological activity of rosmarinic acid and hesperidin - compounds found in significant amounts in the plant. This information supports the candidate's choice of pharmacological effects to investigate, as the plant has not been widely studied, and there is limited information on its biological activity. The third section of the review covers oxidative stress and antioxidants, explaining their importance for health, methods for testing antioxidant activity, and the role of antioxidants in preventing chronic diseases. It also reviews available data on the antioxidant activity of *Micromeria frivaldszkyana*, comparing it with other plants in the same genus. The fourth section focuses on methods for testing analgesic activity in experimental settings, while the fifth focuses on the mechanisms of the inflammatory response, including mediators, cells involved in it, experimental models for studying inflammation, and the anti-inflammatory properties of plants from the *Lamiaceae* family, which includes *Micromeria frivaldszkyana*.

The next section examines how oxidative stress and inflammation contribute to nervous system damage, explores the neuroprotective effects of flavonoids, and reviews methods for studying memory and learning in experimental conditions. The last section of the review addresses models of experimentally-induced hepatotoxicity, markers of liver injury, and the

hepatoprotective potential of *Micromeria* plants and their bioactive compounds. Based on her strong knowledge of experimental models, Kristina Stavrakeva selects those with high predictive value and strong relevance for the design of her dissertation.

The presented literature review demonstrates that the PhD student has an excellent understanding of the data relevant to the topic, knows how to analyze and apply it in formulating the objectives, selecting appropriate methods, and interpreting the results obtained.

The review concludes with a concise yet comprehensive summary that justifies the choice of *Micromeria frivaldszkyana* for pharmacological study in the dissertation and outlines the key areas of research—investigating the plant's chemical composition, acute toxicity, analgesic, anti-inflammatory, memory-enhancing, anxiolytic and hepatoprotective effects.

## **5. Research Methodology**

The study design is described in details. The selected methods are modern, reliable, and appropriate for achieving the aim of the study. They are described in detail and precisely, which guarantees reproducibility of the obtained results. A broad range of *in vivo* and *in vitro* methods were employed, which reflects the comprehensive practical training of the doctoral student. The analysis of secondary metabolites in *Micromeria frivaldszkyana* was performed using liquid chromatography-mass spectrometry (LC-MS), while primary metabolites were analyzed using gas chromatography-mass spectrometry (GC-MS). *In vivo* experiments were conducted on a large number of laboratory animals, which were pre-treated for 7 or 14 days, indicating a labor-intensive and extensive experimental approach. The PhD student used a wide range of behavioral and other methods to evaluate analgesic (hot plate test, analgesimeter), anti-inflammatory (plethysmometer), anxiolytic, and memory-enhancing effects (elevated plus maze, activity cage for locomotor activity, two-way active avoidance, step-through passive avoidance test, novel object recognition test, and Y-maze). Two liver damage models were employed (paracetamol and tert-butyl hydroperoxide-induced hepatotoxicity). Serum markers of liver function (AST, ALT, alkaline phosphatase, bilirubin) were measured using a spectrophotometric method. The ELISA method was used to measure markers for oxidative stress (8-hydroxy-2'-deoxyguanosine, malondialdehyde), antioxidant defense (catalase, reduced glutathione), and pro-inflammatory cytokines (TNF- $\alpha$  and IL-6) in liver tissue homogenates.

The data obtained were analyzed using appropriate statistical methods with the IBM SPSS 19.0 software.

## **6. Characterization and Evaluation of the PhD Thesis**

The PhD thesis is structured according to the requirements for the educational and scientific degree "Doctor". It is laid out on 132 standard typewritten pages, with a well-balanced distribution across its sections - introduction (1 page), literature review (41 pages), aim and objectives (1 page), materials and methods (13 pages), results and discussion (38 pages), conclusions (1 page), and scientific contributions (1 page). The bibliography spans 22 pages and includes references to 257 authors (97 from the last ten years). The dissertation is illustrated with 45 figures and 12 tables.

The aim of the study is clearly and precisely defined. Kristina Stavrakeva has outlined 7 specific objectives to achieve the study's aim.

The "Results" and "Discussion" sections are integrated into a single section. A precise and detailed description of the results for each objective is followed by their analysis and

discussion of the potential mechanisms underlying the observed pharmacological effects. This section is illustrated with 23 figures and 12 tables. The presentation of the results logically follows the objectives of the PhD thesis and is divided into two main parts: phytochemical composition and laboratory tests.

In the first part, the results of the analysis of primary and secondary metabolites in the studied extract are described. A total of 123 secondary metabolites were identified, primarily flavonoids and their glycosides. The highest levels in the samples were registered for linarin and its derivatives, quinic acid, and derivatives of quercetin, kaempferol, naringenin, and apigenin. Significant amounts of rosmarinic acid were also detected. The data are compared with available information on the phytochemical composition of *Micromeria frivaldszkyana* and other species within the genus.

The second part is divided into six subsections, corresponding to respective objectives of the PhD thesis. The first subsection presents the results of the acute toxicity experiments. No lethality or toxicity was observed at doses up to 5 g/kg. Based on these results, Kristina Stavrakeva selected the doses for the *in vivo* experiments. The next two subsections present the results of the evaluation of analgesic and anti-inflammatory effects. No analgesic effect was found, but a reduction in carrageenan-induced edema was observed within the first 3 hours after its injection. The PhD student proposes a possible explanation for the observed anti-inflammatory effect, based on the biological activity of the secondary metabolites identified in the extract. The fourth subsection describes the results of experiments on the effect of the extract on memory functions and anxiety. No memory-enhancing effect was found, but a potential anxiolytic effect was noted.

The following subsection describes changes in liver function markers, oxidative stress, antioxidant defense, and pro-inflammatory cytokines in rats using two experimental models of hepatotoxicity. In the paracetamol-induced hepatotoxicity model, the extract was found to reduce levels of transaminases, malondialdehyde, and 8-hydroxy-2'-deoxyguanosine, while tissue levels of reduced glutathione were elevated. Significantly lower concentrations of TNF- $\alpha$  were observed in the liver. In the butyl hydroperoxide-induced liver damage model, the extract reduced levels of transaminases and malondialdehyde. Kristina Stavrakeva provides a thorough discussion of the potential mechanisms behind the observed changes, based on the biological activity of the compounds in the extract and the data on its antioxidant action.

The conclusion summarizes the main results of the study on the extract of *Micromeria frivaldszkyana* and the relationship between its phytochemical composition and the observed pharmacological effects.

Kristina Stavrakeva formulated three conclusions regarding the phytochemical composition of the extract and five conclusions regarding the studied biological activities. These conclusions are consistent with the experimental results and fully align with the objectives of the study.

## **7. Contributions and Significance of the PhD Thesis for Science and Practice**

The contributions of the PhD thesis are divided into 2 categories: contributions with scientific and theoretical significance and contributions with scientific and practical significance. From a theoretical perspective, it is significant that, for the first time, the acute oral toxicity and a comprehensive metabolic analysis of the methanol extract from the endemic plant species *M. Frivaldszkyana* of our country has been studied. A high content of phenolic

acids and flavonoids was found, which likely determines the observed biological activities of the extract. The contributions with practical significance hold high scientific value. For the first time, the anti-inflammatory and potential hepatoprotective effect of the methanol extract of *M. Frivaldszkyana* has been established. These findings provide a scientific foundation for future research on the possible application of the extract, in an appropriate pharmaceutical form, as adjunctive therapy to conventional anti-inflammatory and hepatoprotective drugs.

I acknowledge the contributions of the PhD thesis.

#### **8. Evaluation of the Publications of the PhD Student**

Kristina Stavrakeva has submitted 3 full-text articles and 3 participations in scientific forums on the topic of the dissertation. Two of the full-text articles have been published in scientific journals indexed in international scientific databases - one in a Bulgarian journal indexed in Scopus (Acta Medica Bulgarica) and one in a foreign journal indexed in Web of Science (International Journal of Molecular Sciences, IF<sub>2023</sub>=4,9). The third article was published in scientific volume from an international scientific conference held in Bulgaria. Among the listed scientific forums, one took place abroad, while two were held in Bulgaria. In all of the publications and two of the scientific presentations, Kristina Stavrakeva is the first author.

The PhD student's publication activity demonstrates her ability to analyze, summarize and present data from experimental studies in publications and scientific forums. The number and type of publications meet the requirements for obtaining the educational and scientific degree "Doctor" according to the Act on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Implementation, and the Regulations of MU-Plovdiv.

#### **9. Personal Impressions and Personal Contribution of the PhD Student**

I have known Kristina Stavrakeva since her participation in the competition for a full-time PhD position at the Department of Pharmacology, Toxicology, and Pharmacotherapy, Faculty of Pharmacy, Medical University of Plovdiv. She possesses strong theoretical and practical training. Kristina Stavrakeva is responsible, organized and demonstrates analytical thinking. Based on my personal impressions, the PhD thesis, the author's abstract, the presentation during the preliminary defense, as well as the scientific publications and participation in scientific forums, in most of which Kristina Stavrakeva is the first author, I am convinced that the research conducted, the results obtained, and the formulated conclusions and contributions are the personal achievements of the PhD student.

#### **10. Author's abstract**

The author's abstract of the dissertation is structured according to the established requirements and spans 61 pages. It includes 21 figures and 5 tables, and sufficiently reflects the main content of the dissertation, including the methods used, the results obtained, the discussion, the formulated conclusions and the scientific contributions.

#### **11. Critical Remarks and Recommendations.**

I have no critical remarks or recommendations.

#### **12. Recommendations for the Future Use of the Dissertation Contributions and Results.**

My recommendation is to continue research on the pharmacological activity of the extract from *M. Frivaldszkyana* and to proceed with publishing the results in journals indexed in globally recognized databases.

## CONCLUSION

The PhD thesis of Kristina Yulianova Stavrakeva addresses an important and relevant topic. The studies on the chemical composition and biological activity of the extract of *Micromeria frivaldszkyana* are innovative and provide solid scientific foundation for further research and potential clinical application. The PhD student demonstrates exceptional knowledge of the researched problems and utilizes a broad range of contemporary *in vitro* and *in vivo* scientific methods to achieve the objectives of the study. The results obtained are the outcome of extensive scientific research that is precisely planned and conducted. They are documented in a precise and thorough manner. The contributions of the PhD thesis are original and significant for the advancement of science in the field studied, as well as for its practical application.

Kristina Yulianova Stavrakeva is an established specialist in pharmacology, possessing deep theoretical knowledge and practical training. She also demonstrates the ability to independently plan and conduct scientific research.

The dissertation fully complies with the requirements set out in the Act on Development of the Academic Staff in the Republic of Bulgaria (ADASRB), the Regulations for its implementation and the Regulations of the Medical University of Plovdiv. The presented materials and the dissertation results are fully aligned with the specific requirements outlined in the Regulations of the Medical University of Plovdiv for the implementation of the ADASRB.

I confidently provide my **positive assessment** of the research presented in the peer-reviewed dissertation, including the author's abstract, the results obtained, and the scientific contributions. I recommend that the esteemed members of the scientific jury award the educational and scientific degree "Doctor" to Kristina Yulianova Stavrakeva in the doctoral program "Pharmacology (including Pharmacokinetics and Chemotherapy)", professional direction: 7.3 "Pharmacy", and field of higher education: 7. "Healthcare and sports".

17.03.2025

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

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(Assoc. Prof. Iliya Kostadinov, MD, PhD)