REVIEW

by DSc Panteley Petrov Denev – Prof. https://ras.nacid.bg/dissertation-preview/23412

of a dissertation for the award of the educational and scientific degree "Doctor" in: field of higher education 4. Natural Sciences, Mathematics and Informatics professional field 4.2 Chemical Sciences doctoral program "Organic Chemistry" Author: Miglena Zlatkova Milusheva Topic: Synthesis of new antispasmodics affecting memory functions in experimental animals scientific supervisors: Assoc. Prof. Dr. Stoyanka Atanasova

Assoc. Prof. Dr. Iliyana Stefanova-Kancheva

General description of the submitted materials

I have been appointed as a member of the scientific jury by order No. PD-22-1134 of the Rector of Plovdiv University "Paisii Hilendarski" (from 15.05.2025) for the procedure of the defense of the dissertation on the topic: "Synthesis of new antispasmodics affecting memory in experimental animals" for the acquisition of the educational and scientific degree Ph.D. in the field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.2 Chemical Sciences, doctoral program Organic Chemistry. The author of the dissertation is Miglena Zlatkova Milusheva, a full-time PhD student at the Department of Organic Chemistry with scientific supervisors Assoc. Prof. Dr. Stoyanka Nikolova Atanasova from Plovdiv University "Paisii Hilendarski" and Assoc. Prof. Dr. Iliyana Dimitrova Stefanova-Kancheva from Medical University - Plovdiv.

The set of materials for electronic media presented by Miglena Zlatkova Milusheva is in accordance with Art. 36 (1) of the Regulations for the Development of the Academic Staff of the University of Plovdiv, includes the following documents:

- application to the Rector of the University of Plovdiv for a procedure for opening the defense of the dissertation;

- CV in European format;

- Report from the Department of Organic Chemistry, to open the procedure and with a preliminary discussion of the dissertation;

- dissertation;
- Abstract of a Thesis for the Educational and Scientific Degree "Doctor";
- list of scientific publications on the topic of the dissertation;
- copy of scientific publications;
- list of noted citations;
- Declaration of originality and authenticity of the attached documents.

The doctoral student has attached 3 scientific publications, 5 participations in research projects, 9 participations in international conferences, 8 participations in national conferences.

Brief biographical data of the doctoral student

Miglena Milusheva completed her secondary education at the Romain Rolland Foreign Language High School in Stara Zagora, with a specialization in English and German languages. She obtained a Bachelor of Science in Medical Chemistry in 2012 and a Master of Science in Food Chemistry in 2016 at the Faculty of Chemistry at the Paisii Hilendarski University of Plovdiv. She has gained practical and professional experience as a clinical laboratory assistant and chemist at the Independent Medical Diagnostic Laboratory "Caridad", as well as as a laboratory assistant and junior technologist at "Mondi Stamboliyski" LTD, where he independently performs analyses of production raw materials, intermediate waste products and qualification of final production: sampling, sample preparation and analysis, calculation and presentation of results in an appropriate form using titrimetry, gravimetric analyses, spectrophotometry, and exercises control over the production of cellulose based on analytical data. Since May 2021, she has been an assistant professor at the Medical University of Plovdiv, where she conducts laboratory exercises in "Chemistry" for students of Medicine and Dentistry in both English and Bulgarian at the Department of Bioorganic Chemistry.

Relevance of the topic and appropriateness of the set goals and objectives

It is known that the modern lifestyle is characterized by its dynamism, nervous tension, fast and irrational nutrition, inadequate rest and relaxation. These factors often cause functional disorders of the gastrointestinal tract, including irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), which continue to represent a serious clinical challenge. Antispasmodics are most often used for the treatment and therapy of these conditions. They exert their effect through direct relaxation of smooth muscle, modulation of calcium channels and anticholinergic activity. Despite their long-term use, existing antispasmodic drugs often show insufficient effectiveness or cause adverse reactions, which necessitates the search for new drug candidates with improved pharmacological profiles.

The relevance of the dissertation work "Synthesis of new antispasmodics affecting memory functions in experimental animals" by doctoral student Miglena Z. Milusheva lies in the fact that she presents original data on the synthesis, characterization and biological evaluation of new hybrid derivatives of anthranilic acid as potential antispasmodics with additional modulation of memory functions in experimental animals.

Among the promising directions is the development of hybrid molecules combining different pharmacophoric elements in order to optimize biological activity and safety. Anthranilic acid is a known structural unit in a number of bioactive compounds, which defines it as a suitable basis for the synthesis of new derivatives with a potential anti-inflammatory effect. 2-Phenylethylamines, on the other hand, are precursors of serotonin and, in this regard, can serve as a good basis for the synthesis of antispasmodics. In this regard, the PhD student was interested in combining the two pharmacophore residues to obtain new hybrid molecules as analogues of existing drugs. This expands the range of available structural analogues, supports the study of structure-biological action relationships, and serves as a starting point in the creation of new drugs as an alternative to the therapeutics used in practice.

Knowledge of the problem

PhD student Miglena Z. Milusheva has conducted a critical review of 394 research articles, conference papers, and regulatory documents relevant to the topic of the dissertation. Most of them have been published in the last 15 years.

The dissertation review accurately and clearly presents the current state of the scientific literature on inflammatory bowel diseases and drugs aimed at the multiple symptom complex of irritable bowel syndrome. Particular attention is paid to anthranilic acid and its derivatives, examining the properties, biochemistry and synthesis of anthranilic acid and its derivatives as substances with anti-inflammatory activity. The following are analyzed: hybrid molecules as types and anti-inflammatory agents; experimental models in the preclinical evaluation of bioactive substances; the importance of animal models in drug development, as well as ethical aspects and alternatives to animal experiments.

The contribution to the educational part of the doctoral program is the correct assessment, discussion, and summary of correctly cited scientific facts and the identification of problems that justify the

clearly formulated main goal. The literature review enables the doctoral student to orient herself correctly in the topic and to highlight the essential problems, both in a scientific and scientifically applied aspect. The research tasks set are specific, feasible, and correctly arranged, and their solution builds the experimental part of the dissertation work.

Research methodology

In accordance with the analysis of the literature data and the summarized conclusions made, the main goal of the dissertation work is formulated: to synthesize new derivatives of anthranilic acid as potential candidates for antispasmodics and substances affecting memory functions in experimental animals.

To achieve this goal, specific tasks have been set: *in silico* assessment of the biological activity and potential toxicity of new hybrid molecules; synthesis of a starting hybrid molecule combining pharmacophoric residues of anthranilic acid and substituted 2-phenylethylamines; preparation of amides of the hybrid molecule in order to expand the scope of the reaction and create derivatives with improved antispasmodic and anti-inflammatory activity; determination of the *ex vivo* antispasmodic, *in vitro* and *ex vivo* anti-inflammatory activity of the obtained compounds in comparison with known antispasmodics such as mebeverine and anti-inflammatory agents such as diclofenac, aspirin, ibuprofen and prednisolone; assessment of the antimicrobial activity of the synthesized compounds; determination of the effect of the substances on memory functions in experimental animals.

To achieve the set goals, a large set of characteristic methods for the synthesis of initial and modified hybrid molecules, methods for *in vitro* and *ex vivo* determination of their anti-inflammatory, antispasmodic, and antimicrobial activity on experimental animal models, tissues and materials, as well as methods for *in vivo* determination of the influence on motor activity and cognitive abilities of rats were used.

The main part of the research on the dissertation work, including the synthesis and purification of the compounds, computer modeling and determination of in vitro anti-inflammatory activity, was carried out at the Faculty of Chemistry of Plovdiv University "Paisiy Hilendarski". The experimental determination of the *ex vivo* antispasmodic activity and anti-inflammatory activity and *in vivo* the influence on memory functions was carried out at the Medical University - Plovdiv. The studies on antimicrobial activity were carried out at the University of Food Technologies in Plovdiv. The NMR measurements were made at Sofia University "St. Kliment Ohridski", Sofia. The HRMS spectra were recorded at the Medical University of Sofia. The presented results and the scientific analysis of these results are original, performed with the necessary repeatability and are of a very good quality and scientific level.

Characteristics and evaluation of the PhD thesis

The PhD thesis is presented on 170 standard typewritten pages and contains 32 figures, 12 tables, and 13 diagrams. 394 literature references are cited. The construction of the PhD thesis meets the requirements, containing the necessary main parts: Title page, Content, Introduction (2 pages), Literature review (48 pages), Goal and objectives, Results and Discussion (60 pages), Materials and Methods, described as an experimental part (28 pages), Conclusions, Contributions and Literature references, which follow a logical sequence, allowing the PhD student to research and analyze the problems with the selected scientific methods.

The PhD thesis is written in a very good professional-scientific language, with good style, conciseness, and clarity. The material is easy to read and perceive. The individual parts are well balanced in volume.

The section "Experimental part" describes precisely the main methods that were applied in the implementation of the tasks set in the dissertation: synthetic methods for obtaining the starting hybrid molecules and their diamide derivatives, experimental animal models, tissues and materials, the

method for *ex vivo* determination of antispasmodic activity, methods for *in vitro* and *ex vivo* determination of anti-inflammatory activity, the method for *in vitro* determination of antimicrobial activity, methods for *in vivo* determination of the influence on motor activity and cognitive abilities of rats.

The structure of the newly synthesized compounds was proven using ¹H-NMR and ¹³C-NMR spectra recorded on a Bruker Avance III HD 500 spectrometer at 500 MHz (¹H-NMR) and 125 MHz (¹³C-NMR) and room temperature (295 K), Infrared spectra were recorded on a FT-IR spectrometer VERTEX 70 (Bruker Optics), melting points were determined with a Kruss M5000 apparatus (A. Krüss Optronic GmbH), HRESIMS spectra were obtained on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific, Inc.) with HESI-II. For screening of biological effects, pharmacokinetic properties, and toxicity, the following freely available software products were used: PASS Online, SwissADME, ProTox-II, OSIRIS20.

The PhD student has skillfully selected and mastered a large number of modern methods, applicable and in full accordance with the set goal and tasks of the PhD thesis. Miglena Milusheva has acquired the necessary methodological experience in conducting the learned experiment, summarizing the reliability of the obtained results, and deducing the revealed regularities.

Contributions and significance of the thesis

The results of the PhD thesis have, above all, scientific and applied value. The dissertation presents original data on the synthesis of new hybrid derivatives of anthranilic acid, their characterization, and their biological evaluation as a possible application for antispasmodics, possessing additional modulation of memory functions in experimental animals. New hybrids of anthranilic acid have been synthesized, the antispasmodic activity of which has been measured. The results showed that for five of them, this activity is better than that of the known antispasmodic mebeverine. The anti-inflammatory activity of all synthesized compounds has been assessed by in vitro and ex vivo methods. All studied compounds show promising protection against the in vitro determined thermal denaturation of albumin, with the strongest activity demonstrated by the compounds 2-amino-N-phenylethylbenzamide and N-phenylethyl-2-(2-phenylacetamido)benzamide. In the study of antispasmodic activity in influencing memory functions of experimental animals, the compound showed a significant effect on the processes of memory and learning. Very good antimicrobial activity the was established for compound 2-amino-N-(3-chlorophenylethyl)benzamide against the pathogenic fungi Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum and Rhizopus spp. and moderate activity against the yeasts Candida albicans, Saccharomyces cerevisiae. Moderate sensitivity to the hybrid 2-amino-N-(3-chlorophenylethyl) benzamide and its diamide derivatives was shown by the studied strains of Gram-positive bacteria Listeria monocytogenes, Enterococcus faecalis, Micrococcus luteus, Gram-negative bacteria Salmonella enteritidis, Salmonella typhimurium, Klebsiella pneumoniae, Escherichia coli, and Pseudomonas aeruginosa.

The structure-biological activity relationship was determined for each of the groups of synthesized compounds.

The following contributions of a scientific nature can be mentioned:

• Small molecules as antispasmodics were designed, and synthetic approaches for their preparation were found.

• The influence of the antispasmodic activity on the introduction of various substituents was theoretically studied.

• Clear relationships between the molecular structure and biological activity were found for the synthesized compounds, which can serve as a basis for future rational design of biologically active compounds.

Scientific and Applied Contributions:

• Using the proposed synthetic method, 18 compounds were obtained, of which 16 represent new structures, not previously published in the scientific literature.

• The antispasmodic activity of all 18 synthesized compounds was measured and for five of them this activity was better than that of the known antispasmodic mebeverine.

• A degree of biological activity was proven, characterizing one of the hybrids as a substance with functional characteristics of an antispasmodic with anti-inflammatory action, affecting memory functions in experimental animals.

The established and proven results provide a solid foundation for continuing the doctoral student's future research activities.

Assessment of publications on the dissertation work

The results of the PhD thesis are presented in three scientific papers, which are published in refereed journals and indexed in world-renowned databases. All publications are in scientific journals, reflected in the world databases in quartile Q1, which covers the minimum scientific requirements for the professional field 4.2. Chemical Sciences. The total impact factor is 13.4. The results obtained on the presented scientific papers have found a response in the chemical literature, and in connection with them, 22 independent citations have been found in the databases Scopus and/or Web of Science.

The materials from the publications are included in the PhD thesis. The PhD student has presented a list of participations in 10 international and 8 national scientific conferences. In the scientific publications, the doctoral student Miglena Milusheva is the lead author, which shows that she is the main executor of the scientific experiment.

The PhD student is also included in two projects funded by the National Science Foundation of the Ministry of Education and Science, and one within the framework of the DUEcoS project at the Paisii Hilendarski University, the topic of which is related to the dissertation development. She has twice won a competition under the National Program "Young Scientists and Postdoctoral Fellows - 2" - young scientists module, selection 2022 and 2024.

In connection with the conduct of a procedure for acquiring an educational and scientific degree "doctor" at the Paisii Hilendarski University of Plovdiv, Miglena Zlatkova Milusheva has signed a "Declaration of Originality and Credibility", in which she declares that the results and contributions to the scientific production that she presents in the procedure are original and are not borrowed from research and publications in which she has no participation, and the information presented in the form of copies of documents and publications, personally compiled references, etc. corresponds to the objective truth.

I declare that I have not found any evidence of plagiarism.

These data give me reason to claim that the presented scientific works meet both the national scientometric requirements according to the Law for the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for the Implementation of the Law, and the relevant Regulations of the Paisii Hilendarski Plovdiv University for obtaining the educational and scientific degree "doctor".

Abstract

The abstract is 36 pages long, contains 18 figures, 4 tables and 2 diagrams, which correctly reflect the main results and contributions of the dissertation work and meets the specific requirements of the Paisii Hilendarski Plovdiv University.

CONCLUSION

The PhD thesis contains both scientific and applied scientific results that represent an original contribution to science and meet all the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for the Implementation of the Law, and the relevant Regulations of the Paisii Hilendarski Plovdiv University.

The thesis shows that the PhD student, Miglena Zlatkova Milusheva, possesses in-depth theoretical knowledge and professional skills in the scientific specialty of Organic Chemistry, demonstrating qualities and skills for independent conduct of scientific research.

Considering the relevance and significance of the topic of the dissertation, the abstract, the fulfillment of the set goal, the scientific and applied scientific results contained therein, which represent an original contribution to science, I kindly recommend to the scientific jury to award the educational and scientific degree "doctor" to Miglena Zlatkova Milusheva in the field of higher education: 4. Natural Sciences, Mathematics and Informatics, professional field 4.2. Chemical Sciences, scientific specialty "Organic Chemistry".

07.06.2025

Reviewer:

Prof. DSc. Panteley Denev