STATEMENT

by Assoc. Prof. Iliya Dimitrov Kostadinov, MD, PhD
Department of Pharmacology and Clinical Pharmacology, Faculty of Medicine, Medical University of Plovdiv

Regarding

PhD thesis of Milena Todorova Salbashian for awarding the educational and scientific degree 'Doctor'

Professional field: 7.1 "Medicine"
Field of higher education: 7. "Healthcare and sports"
Doctoral program: "Pharmacology (including Pharmacokinetics and Chemotherapy)"
Author of PhD thesis: Milena Todorova Salbashian - doctoral student on a self-study basis
Department: "Pharmacology, Clinical Pharmacology and Therapeutics", Medical University „Prof. Dr. Paraskev Stoyanov" - Varna
Topic: "Pharmacological study of behavioral effects of biologically active substances of plant origin in experimental animal models of depression"
Research Supervisor: Prof. Stefka Vasileva Valcheva-Kuzmanova, MD, Ph.D, D.Sc.; Department of Pharmacology, Clinical Pharmacology and Therapeutics, Medical University „Prof. Dr. Paraskev Stoyanov" - Varna
Consultant: Prof. Roman Emilov Tashev, MD, Ph.D, D.Sc.

Based on the Decision of the meeting of the Faculty Council at the Faculty of Medicine, Medical University „Prof. Dr. Paraskev Stoyanov" – Varna (Protocol № 75/02.11.2022) and by Order of the Rector of the Medical University „Prof. Dr. Paraskev Stoyanov" – Varna № P-109-441/11.11.2022, I have been appointed as a member of the scientific jury in the procedure for awarding the educational and scientific degree "Doctor" to Dr. Milena Todorova Salbashian. On the basis of a decision from the first absentee meeting of the Scientific Jury held on 15.11.2022, I have been appointed to prepare a statement on the above-mentioned procedure.

Dr. Milena Todorova Salbashian has submitted all the necessary documents in accordance with the requirements of the Act on Development of the Academic Staff in the Republic of Bulgaria (ADASRB), the Regulations for its implementation and the Regulations of Medical University „Prof. Dr. Paraskev Stoyanov"–Varna. I have no objections to the documents.

Brief biographical and professional notes

Milena Todorova Salbashian was born on 02/04/1966. She studied medicine at Medical University of Varna. In 1999, she graduated and obtained the educational and qualification degree "Master" in the specialty "Medicine" with the professional qualification "Physician". After graduation, she worked at the Department of Epidemiology, Hygiene and Epidemiologic Inspection, Dobrich (2000 – 2002). From 2002 to 2005, she worked in an individual medical practice for primary care in the city of Dobrich. In the period 2005-2013, Milena Salbashian worked as a medical assistant, phlebotomist, successively at the Adult Geriatric Institute, Florida, USA (2005-2007), EZ Clinical Laboratories, NJ, USA (2007-2009) and Monmouth Medical Center St. Barnabas Health System, NJ, USA (2009-2013). She is fluent in English and Russian and has skills in working with Microsoft office, CorelDraw, GraphPad, InSta. The doctoral student is a member of the Bulgarian Scientific Society of Pharmacology, the Bulgarian Scientific Society of Clinical Pharmacology and Therapy and the Bulgarian Medical Association. From September 2015 to the present, Milena Salbashian is an assistant professor at the Department of Pharmacology, Clinical Pharmacology and Therapeutics, Medical University „Prof. Dr. Paraskev Stoyanov"-Varna. Dr. Milena Salbashian has been enrolled as a doctoral student on a self-study basis in doctoral program of "Pharmacology (including Pharmacokinetics and Chemotherapy)" (order No. P-109-127/01.04.2019 of the Rector of MU-Varna). A protocol for successfully passing the doctoral minimum is presented. Based on order P-109-441/11.11.2022 of the Rector of MU-Varna, Dr. Milena Todorova Salbashian was deducted with the right of public defence of her PhD thesis.

Relevance of the topic

The presented PhD thesis is an original research on the behavioral effects of *Aronia melanocarpa* fruit juice and the phenolic acids found in it (chlorogenic, ferulic and gallic) in two models of depression in experimental animals - bilateral ovariectomy and bilateral olfactory bulbectomy. In the first model, the effects of chokeberry fruit juice and chlorogenic acid on locomotor activity, anxiety, depressive behavior and pain sensitivity are investigated. The effects of three phenolic acids on learning, memory and anxiety are studied in bulbectomized rats. A wide range of pharmacological effects have been recorded in behavioral tests – sedative, anxiolytic, antidepressant, analgesic and memory enhancing. The relevance of the topic is determined by the high prevalence and great medical and social significance of the diseases in which the studied substances can have a favorable therapeutic effect.

Anxiety disorders (panic disorder, generalized anxiety disorder, post-traumatic stress disorder, etc.) are among the most common mental disorders (based on population-based studies about 33.7% of people have suffered from such a condition at least once in their lifetime). The prevalence of these disorders is high in working-age population. Depression is another significant health problem. Globally, it is estimated that 5% of adults suffer from depression, and it can affect people of all ages. Depression increases the risk of other chronic socially significant diseases. Pharmacotherapy of anxiety and depressive disorders is associated with high risk of adverse drug reactions (including drug dependence e.g., with benzodiazepine anxiolytics) and drug interactions. In addition, some patients lack a satisfactory therapeutic effect from conventional drug therapy. Therefore, the research on new treatment strategies is necessary. The study of medicinal plants and their biologically active substances is a prerequisite for the development of medicinal products with a complex mechanism of action and a reduced risk of adverse drug reactions. Studies on the anxiolytic and antidepressant effect of *Aronia melanocarpa* fruit juice and its phenolic acids in experimental models of depression will reveal future perspectives for their clinical application in the therapy of depression and anxiety disorders.

Dementia (Alzheimer's disease, frontotemporal dementia, diffuse Lewy body disease, vascular dementia, etc.) is a significant health problem. The incidence of dementia is

progressively increasing as the life expectancy of the population increases. Worldwide there are an estimated 35.6 million people with dementia. By 2050 it has been estimated that this number will rise to more than 115 million. Dementia leads to deterioration of the quality of life and permanent disability of patients. Along with reduced cognitive functions, patients with dementia often suffer from depressive symptoms and anxiety. Chlorogenic, ferulic and gallic acids, in addition to their memory-improving effect, also have an anxiolytic effect. This would be beneficial in the treatment of neuropsychiatric symptoms that accompany dementia.

The treatment of pain is another relevant topic related to the current PhD thesis. According to some studies, chronic pain affects about 30% of people worldwide. Anxiety, insomnia, depressive symptoms can be both result and cause of chronic pain. Despite the progress in the pharmacotherapy of pain, its treatment is still an incompletely solved problem. Existing analgesics and adjuvants often do not lead to complete pain control, and on the other hand, their use is associated with a serious risk of adverse reactions. An important advantage of medicinal plants and their biologically active compounds is that they have various pharmacological effects which would affect anxiety and depressive symptoms that accompany chronic pain.

The present study has important scientific and practical significance. It would contribute to a more complete pharmacological characterization of *Aronia melanocarpa* fruit juice, chlorogenic, ferulic and gallic acids. This could be the scientific basis for further research on the therapeutic use of these compounds as an adjunctive therapeutic strategy in the treatment of depression, anxiety disorders, dementia and pain.

Knowledge of the problem

Dr. Milena Salbashian demonstrates excellent knowledge of the problem that is subject of the PhD thesis. The literature review of the dissertation is laid out on 54 pages and is based on above 300 sources. It is illustrated with 1 table and 16 figures. This greatly facilitates the perception of the presented information. The analysis of literature shows that the doctoral student knows the problem that is subject of the dissertation and is able to focus on unresolved issues, summarize and analyze the data from a large number of scientific sources.

The literature review is competently and comprehensively written. The scientific information is presented in 3 sections, which logically follow one another. In the first section of the review, the botanical characteristics of *Aronia melanocarpa*, the chemical composition of its fruits and the content of polyphenols in them are discussed. Detailed information on the chemical nature of the compounds that are supposed to be mainly involved in the biological activity of *Aronia melanocarpa* fruit juice (polyphenols) is also presented. These data support the doctoral student in the selection of phenolic acids, whose biological effects are analyzed in the current PhD thesis. In the second section of the literature review, data on the pharmacokinetics of polyphenolic compounds are discussed. The information about the oral absorption of these biologically active compounds and their ability to cross the blood-brain barrier is important for the design of the current PhD thesis. In this way, the doctoral student justifies the oral administration of *Aronia melanocarpa* fruit juice and phenolic acids during the research. The passage of polyphenols through the blood-brain barrier and their accumulation in certain brain structures is a necessary prerequisite for producing effects in the central nervous system. The last section of the literature review is about the effects of polyphenols on the central nervous system. Data on the effects of these compounds on anxiety, memory, neuronal signaling and synaptic functions, neuroinflammation and neurodegeneration, cerebral blood flow, neurogenesis, acetylcholinesterase activity, glutamate-induced excitotoxicity, depressive symptomatology, and pain are discussed. The doctoral student presents detailed information on the mechanisms involved in these effects. This information is important for both the design of the experimental study and the discussion of the results obtained by the doctoral student.

The presented literature overview shows that Dr. Salbashian has excellent knowledge about the data on the problem being developed, knows how to analyze and use them in formulating the tasks and interpreting the obtained results.

At the end of the literature review, Dr. Salbashian summarizes the presented literature data regarding the biological effects of *Aronia melanocarpa* fruit juice and the polyphenols found in it. Based on this, she justifies the relevance of the dissertation and the choice of experimental models in which the pharmacological effects of *Aronia melanocarpa* fruit juice and/or the phenolic acids found in it (chlorogenic, ferulic and gallic) have not been studied yet. The current PhD thesis is a continuation of the scientific investigations of the research team at the Department of Pharmacology and Clinical Pharmacology and Therapeutics of MU-Varna. In this aspect, very important is the role of the scientific supervisor of the doctoral student - Prof. Dr. S. Valcheva-Kuzmanova.

Research methodology

The study design is described in-depth. The chosen methods are contemporary, reliable and provide the achievement of the aim of the study. They are described in detail and precisely, which guarantees reproducibility of the obtained results. A wide range of *in vivo* methods have been used. This is indicative for the good practical training of Dr. Salbashian.

The experiments were carried out on male and female white Wistar rats. Two contemporary experimental models of depression are used – bilateral ovariectomy and bilateral olfactory bulbectomy. The PhD student has used a large number of behavioral methods for recording the studied pharmacological effects – open field test, two-way active avoidance test, one-way passive avoidance test, social interaction test, elevated plus maze, forced swimming test and hot plate test. This allows the investigation of a large number of effects of the studied substances on the CNS - motor activity, anxiolytic, antidepressant, analgesic effect and impact on working memory.

Competent and appropriate statistical processing of the obtained results was performed using the statistical package GraphPad Prism (Version 5.00, GraphPad Software, Inc.).

Characterization and evaluation of the PhD thesis and contributions

The PhD thesis is structured according to the requirements for the educational and scientific degree "Doctor". It is laid out on 177 standard typewritten pages and is well balanced between its individual parts - introduction (2 pages), literature review (54 pages), objective and tasks (2 pages), material and methods (13 pages), results and discussion (50 pages), conclusion (4 pages), scientific contributions (1 page). The bibliographic reference is written on 39 pages and includes 631 authors, of which 3 are in Cyrillic and 628 are in Latin. The dissertation is illustrated with 12 tables and 41 figures.

The aim is clearly and precisely formulated. The doctoral student planned 4 tasks for the achievement of the aim of the study.

The conducted experiments are very precisely and correctly planned and carried out. The results are competently processed statistically, described accurately and in-depth and illustrated with 18 figures and 10 tables. The presentation of the results follows in a logical sequence the tasks and is divided into two sections. The first section is about the effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on the behavior of ovariectomized rats. The pharmacological effects are studied after 30-day and 75-day treatment of the experimental animals. This section includes 4 subsections in which the doctoral student describes the results from the first two tasks - effect of *Aronia melanocarpa* fruit juice and chlorogenic acid on motor activity, anxiety, depressive behavior and pain sensitivity. The second section of results is about the effects of chlorogenic, ferulic and gallic acids on the behavior of bilateral bulbectomized rats. The effects are studied after 14-day treatment of the experimental animals. It includes 2

subsections in which are described the results from the third and fourth task – effect of chlorogenic, ferulic and gallic acids on anxiety and memory functions. Each of the subsections is followed by a detailed discussion of the results obtained.

Dr. Salbashian competently analyzes and interprets the results obtained, as well as compares them with those of other authors. The doctoral student intelligently discusses the possible mechanisms of the registered pharmacological effects. This is based on her good knowledge of the literature data and the information obtained from the performed behavioral tests.

The doctoral student has formulated 2 conclusions that correspond to the obtained experimental results and fully meet the set tasks.

Dr. Salbashian has formulated 4 contributions. They are original and important for the development of the science in the researched field and its practical application. For the first time, the effects of *Aronia melanocarpa* fruit juice are investigated in ovariectomized rats, and it was found that it suppresses motor activity, exhibits a certain anxiolytic effect; has an antidepressant-like effect and increases threshold sensitivity for thermal pain. It is also demonstrated for the first time that chlorogenic acid in ovariectomized rats causes a decrease in locomotor activity; prevents the development of anxiety; has no effect on depressive symptoms and increases threshold sensitivity for thermal pain. It is found for the first time that chlorogenic, ferulic, and gallic acids in bulbectomized rats prevent bulbectomy-induced hyperactivity; exhibit an anxiolytic-like effect and improve memory and learning processes. The conducted experiments with *Aronia melanocarpa* fruit juice and chlorogenic, ferulic, and gallic acids contribute to their complete pharmacological characterization and reveal future perspectives in the treatment of anxiety and depressive disorders, neurodegenerative and other diseases of the nervous system. I accept the contributions of the dissertation.

Author's abstract (thesis summary)

The author's abstract of the PhD thesis is structured according to the requirements and contains 83 pages. It is illustrated with 18 figures and 11 tables, and sufficiently reflects the main content of the dissertation - the methods used, the results obtained, discussion, formulated conclusions and scientific contributions.

Evaluation of the publications of the PhD student

Dr. Salbashian has submitted 4 full-text articles and 9 participations in scientific forums on the topic of the dissertation. All full-text publications are in English, and in two of them the doctoral student is the first author. Three of the full-text articles are published in scientific journals refereed in international scientific databases - two publications in Bulgarian journals refereed in Scopus ((Folia Medica, Bulgarian Chemical Communications)) and one in a foreign journal with IF (Farmacia, IF=1,48). The doctoral student participates in six scientific forums abroad and three in Bulgaria. Dr. Salbashian also has 1 participation in a scientific project on the topic of the dissertation.

The PhD student's publication activity indicates her ability to analyze, summarize and present the data from experimental studies in publications and scientific forums. The number and type of publications meet the requirements for the acquisition of 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-Varna.

I have no critical remarks and recommendations.

Conclusion

The PhD thesis of Dr. Milena Todorova Salbashian, entitled "Pharmacological study of behavioral effects of biologically active substances of plant origin in experimental animal models of depression", is dedicated to a relevant and important topic. The PhD student shows an excellent knowledge of the researched problem and uses a wide range of contemporary *in vivo* research methods for achieving the formulated tasks of the research. The obtained results are a consequence of precisely planned and conducted scientific research. They are documented precisely and in-depth. The contributions of the PhD thesis are original and important for the development of the science in the researched field and its practical application.

Milena Todorova Salbashian is an established specialist in pharmacology, who possesses in-depth theoretical knowledge and practical training, and demonstrates qualities for independent planning and conducting scientific research.

The presented dissertation fully meets the requirements of 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 „Prof. Dr. Paraskev Stoyanov" – Varna.

In conclusion, I confidently give my positive assessment of the research, presented by the above peer-reviewed dissertation, author's abstract, obtained results and scientific contributions, and would recommend to the honorable members of the scientific jury to award the educational and scientific degree "Doctor" to Milena Todorova Salbashian in a doctoral program "Pharmacology (including Pharmacokinetics and Chemotherapy)".

04.01.2023

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