

REVIEW

by assoc. prof. **Maria Delcheva Zhelyazkova-Savova, MD, PhD**
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Regarding the dissertation work on the topic:

PHARMACOLOGICAL STUDY OF BEHAVIORAL EFFECTS OF BIOLOGICALLY ACTIVE SUBSTANCES OF PLANT ORIGIN IN EXPERIMENTAL ANIMAL MODELS OF DEPRESSION

for awarding the educational and scientific degree "DOCTOR"
to the PhD student in an independent form of study

Milena Todorova Salbashyan

In the area of Higher Education 7. Healthcare and sports, Professional direction 7.1. Medicine, Doctoral program "Pharmacology (incl. Pharmacokinetics and Chemotherapy)"

By order No. R-109-441/11.11.2022 of the Rector of MU-Varna and with the decision of the Faculty Council meeting of Faculty of Medicine at MU-Varna (protocol No. 75/02.11.2022), I have been appointed as a member of the Scientific Jury for the above procedure. As such, I submit my review of **Milena Todorova Salbashyan's** dissertation work on the topic "**Pharmacological study of behavioral effects of biologically active substances of plant origin in models of depression in experimental animals**"

The set of electronic/paper materials presented to me by the applicant includes all the necessary documents in accordance with the requirements of the Law on the Development of the Academic Staff and the Rules for the Development of the Academic Staff of MU-Varna.

Education and professional development of the PhD student

Milena Todorova graduated from the Medical College of Medical University Varna in 1987 as a nurse. She received her higher medical education in the MU-Varna in 1999.

Her work experience includes 4 years of work as a nurse in a cardiology department, and after her graduation as a medical doctor she has worked in the city of Dobrich, successively in the Epidemiological department and in an outpatient individual practice for primary care. During the period 2005-2013, she resided in the USA, where she held positions of a medical assistant and a phlebotomist in various health care facilities. Since 2015, she has been working as an assistant at the Department of Pharmacology and Clinical Pharmacology and Therapeutics of the Medical University of Varna.

Her present teaching activity is related to conducting seminars on pharmacology and clinical pharmacology for medical students, including those from the English language program.

She has participated in three research projects dedicated to pharmacological studies of biologically active substances of plant origin, one of which is closely connected to her dissertation work.

She is proficient in English and speaks Russian. She works with basic functions of the office package and the statistical/graphic software GraphPad Prizm, as well as Corel Draw, and Insta.

Relevance of the problem

The dissertation of Milena Todorova deals with important current problems of modern medicine, related to the treatment of neuropsychiatric conditions. Anxiety-depressive disorders and neurodegenerative diseases with their high rate and social importance are emerging as leading debilitating conditions along with cardiovascular and metabolic diseases. The therapeutic results from the use of conventional antidepressants, benzodiazepines and other specific pharmacological agents are often unsatisfactory, and accompanied by side effects limiting their use. This opens the doors to the scientific search of new possibilities for the treatment of affective and cognitive disorders, including based on newer aspects in the understanding of their pathogenic mechanisms. Given the accumulated data on the involvement of inflammation and oxidative stress in the genesis of these diseases, along with the classical role of neurotransmitter, hormonal and neurotrophic imbalance, natural biologically active substances of plant origin, rich in polyphenols, are emerging as potentially useful and relatively harmless, with their pronounced anti-inflammatory and antioxidant properties. The focus in the present dissertation is put on the psychopharmacological effects of *Aronia Melanocarpa* fruit juice (AMFJ) and the phenolic acids found in it, in experimental models of anxiety, depression, pain and memory deficits.

A review of the PhD thesis

Dissertation structure

The dissertation is written on 177 pages. It is constructed in accordance with the adopted national standards and includes the following sections: Introduction – 2 pages, Literature review – 53 pages; Aim and tasks – 2 pages; Materials and methods – 12 pages; Personal results with discussion – 49 pages; Conclusions – 4 pages; Contributions – 1 page; List of publications and participations related to the dissertation work – 2 pages; Literature (631 references, including 3 in Bulgarian) – 38 pages; Acknowledgments – 1 page. The work is well balanced structurally. Summary discussion as an essential part of the exposition is missing.

Literature awareness

The review of the literature is written concisely and follows a logical sequence. The author shows good awareness of the issues of interest. Contemporary data has been included, with 36% of the cited literature (227 sources) from the last 10 years, and 8% (51 sources) from the last 5 years.

The literature review includes extensive information on polyphenols from a biochemical point of view, with an emphasis on the most abundant polyphenols in AMFJ. The pharmacokinetics of these biologically active substances is thoroughly discussed, especially the factors responsible for their relatively low bioavailability. Logically, special attention has been paid to the possibility of polyphenols to pass through the blood-brain barrier. A detailed presentation of the data concerning the central effects of polyphenols is given, and specifically, the numerous studies with *Aronia melanocarpa*, including those conducted over the years at the Department of Pharmacology in Varna. The discussion concerning the effects on the CNS is organized in the same directions as those in the dissertation itself, namely: anxiolytic, pro-cognitive, antidepressant and analgesic. For each of these effects, possible mechanisms through which they can be realized, are commented.

The summary of the literature review ends by formulating the yet unexplored psychopharmacological aspects of AMFJ action, particularly in experimental rat models of ovariectomy and olfactory bulbectomy.

On this basis, the aim and tasks of the study have been derived. The aim is given as: "Pharmacological study of the effects of biologically active substances of plant origin (fruit juice of *Aronia melanocarpa* and phenolic acids) in experimental models of behavioral disorders in experimental animals induced by ovariectomy and bulbectomy". The aim, as one might expect, accurately reflects the content of the dissertation, while the title is somewhat misleading, emphasizing only the depression modeled by the experimental settings used.

The tasks to achieve the aim can be regarded in two groups, depending on the model used – in fact, this is how the results have been reported in the dissertation itself (although 4 separate tasks have been formulated):

- Modeling of behavioral changes by ovariectomy (OVE) and, on this background, studying the effects of AMFJ and chlorogenic acid (CGA) as related to the locomotor activity, anxiety, depressive-like behavior and pain sensitivity.
- Modeling of behavioral changes by means of bilateral olfactory bulbectomy (OBE) and, on this background, investigating the effects of the phenolic acids – chlorogenic, ferulic and gallic, in terms of anxiety, learning and memory.

Materials, methods and design of experiments

30 male and 70 female white Wistar rats, weighing 200-240 g, bred at an ambient temperature of 20-25°C and a 12-hour light/dark cycle, with unlimited access to food and water, have been used.

All experimental procedures were carried out in accordance with national and international requirements for the protection and humane treatment of experimental animals (European Directive 2010/63/EU) and in accordance with the rules of the Ethics Committee at the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 from the US Department of Health and Human Resources).

The methods used have been adequately selected according to the tasks. These include established behavioral tests to assess locomotor activity (open field), anxiety (social interaction test and elevated plus maze), depressive-like behavior (forced swim test), memory and learning (active two-way avoidance learning and passive avoidance training), pain sensitivity (hot plate). These behavioral tests were administered on the background of a certain duration of treatment of the experimental rats (according to the setting), after the animals had recovered from the applied surgical intervention.

Surgical procedures include bilateral ovariectomy in female rats and bilateral olfactory bulbectomy in male rats. The operative interventions are briefly and clearly described, with the prophylactic antibacterial and antihemorrhagic measures taken and the recovery period provided (14-15 days) being indicated. Bilateral olfactory bulbectomy has been verified macroscopically after completion of behavioral tests. For each of the settings, two control groups were included – sham operated rats and untreated rats with OVE or OBE.

AMFJ doses were 5 and 10 ml/kg and are probably based on previous experiments conducted in the Department. A juice prepared from fresh fruits of *Aronia melanocarpa* by grinding, crushing, squeezing, filtering and preserving with potassium sorbate (1.0 g/l) was used, stored at 0°C until use. The composition of the used juice is given according to literature data.

By design, the described settings are chronic experiments. In ovariectomized animals, treatment with AMFJ or CGA has begun 14 days after surgery and continued for a minimum of 30 or 75 days, after which the various behavioral tests have been performed within 4 days in a specific sequence. Bulbectomized rats have been treated for 14 days with the phenolic acids, starting on the 15th day after the intervention, in doses of 20 mg/kg, the reason for using this dose not being stated.

For statistical processing of the results, the use of one-way and two-way analysis of variance with Dunnett's post-test is indicated, as well as Student's t-test when comparing two independent groups. Results are presented as mean±SEM and statistical significance is set at $p < 0.05$. It is not clear, however, which results have been evaluated with the two-way analysis of variance and which with the Student's t-test, as this information is missing from the presentation of the results.

Analysis and evaluation of results

The results are organized into two main parts according to the models used. They are illustrated with 17 figures and 9 tables. Each part includes a presentation of the results of the experiments performed in the corresponding model – ovariectomy or bulbectomy, together with a discussion. The discussion of the current results allows a focused analysis of the specific work done, highlighting the ability of the PhD student to consider the obtained results in the context of the already existing information and to search and find out literature data for comparison and for explanation of potential mechanisms.

The results of the conducted experiments are confirmatory by their nature, insofar as the anxiolytic, antidepressant, analgesic and anti-amnesic activity of AMFJ and phenolic acids

have already been tested and established over the years in the Department of Pharmacology of the University of Varna. What is new in this case are the models in which these effects were tested. The formulation of the tasks implies a separate description of these models as inducing the expected behavioral changes. However, the observed changes have been included in the general presentation of the results of the effects of the applied active substances.

In the discussion of the results from the application of AMFJ and phenolic acids in the various behavioral tests, the mechanisms by which the obtained beneficial effects can be explained, are commented. I appreciate the fact that much of the discussion is focused on the specific pathogenic mechanisms that distinguish the particular experimental models used as generators of behavioral changes, distinct from other common triggers of anxiety, depression, or cognitive problems, and, accordingly, on the involvement of appropriate mechanisms of action of the test substances in the observed effects.

The dissertation is easy to read, the presentation is for the most part smooth, logical and understandable, regardless of the mistakes and repetitions (see below).

Analysis and assessment of conclusions and contributions

The **conclusions** are organized in two main directions, corresponding to the experimental models, and reflect correctly and in a condensed form the obtained results. They include 4 points each for ovariectomy and for olfactory bulbectomy, summarizing the data obtained for the studied behavioral indicators in each model, as well as details regarding doses and duration of treatment. The established sedative, anxiolytic, antidepressant, and analgesic effects of PSAM and chlorogenic acid in ovariectomized rats are highlighted, as well as the antagonizing effects of chlorogenic, ferulic, and gallic acids on the hyperactivity, anxiety, and memory deficits induced by olfactory bulbectomy.

I accept the **contributions** in the way they are formulated, with emphasis on the original nature of what was established in the experimental work of the present dissertation work.

Publication activity

In connection with her dissertation, Milena Todorova has published four full-text articles, one of which in an international scientific journal with an impact factor. In two of the publications, she is the first author. Related to the dissertation are nine participations in national and international scientific forums, of which five were published as abstracts in prestigious international scientific publications with an impact factor.

Notes, omissions and recommendations

The notes and recommendations that I address to the author do not change my overall positive assessment and do not undervalue the work as a whole, but rather have the character of constructive criticism aimed at gaining useful experience and improving.

- Structure and content
 - ✓ There is a discrepancy between the wording of the title and the aim, with the aim being more closely related to the content of the dissertation work.

- ✓ My personal view is that every PhD thesis should end with a short general discussion that unites the individual parts, shapes the work as a whole and gives it a finished look. The presence of such a final discussion would have increased the scientific value of the dissertation work.
- ✓ Most of the results are presented in text, figure and table, which is redundant as it does not provide additional information.
- ✓ The statistical analysis used must be specified when presenting individual results.
- ✓ Some references are found which appear in the list but are not mentioned in the text, e.g. Hozumi S et al, 2003; Jancsar SM and Leonard BE, 1984; Moriguchi S et al, 2009; Slotkin et al, 1999; Imwalle et al, 2005; Ito et al., 2008; Mar A et al, 2000; Mazzucco et al, 2006.
- Language and style
 - ✓ Literal repetitions of sentences, groups of sentences or individual paragraphs of the literature review are allowed in discussions or between different discussions in separate parts of the dissertation.
 - ✓ Spelling errors are detected.

Evaluation of the abstract

The abstract is written on 83 pages and meets the requirements of the regulatory framework, correctly reflecting the content of the dissertation work.

Personal impressions of the candidate

I have known Milena Todorova since she joined the Department of Pharmacology at MU-Varna. She is quiet and modest, diligent and precise in her work, responsible in fulfilling her duties as a researcher and a teacher. Friendly and smiling, she enjoys the respect of her colleagues.

In conclusion, it is my believe that Milena Todorova's dissertation can be qualified as an up-to-date scientific work with interesting results and valuable contributions. They contribute to expanding the psychopharmacological characteristics of AMFJ and individual phenolic acids, as well as to the possibility of their clinical utilization. The PhD student demonstrates good scientific and theoretical training, ability to independently conduct experimental research and analyze the results. The scientific markers satisfy the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the Regulations for the Development of the Academic Staff at the MU-Varna for awarding the scientific and educational degree "DOCTOR". This all gives me the reason to vote with conviction in favor of awarding the scientific and educational degree "**DOCTOR**" to **Milena Todorova Salbashyan**.

Varna

09.01.2023

Reviewer:



/assoc. prof. Maria D. Zhelyazkova-Savova, MD, PhD/