

## REVIEW

**by Assoc. Prof. Yulichka Sabeva Sabeva, PhD,  
scientific specialty "Toxicology",**

member of the Scientific Jury appointed by Order № 406/09.07.2020 of the Director of the Institute of Neurobiology at the Bulgarian Academy of Sciences – Sofia and Decision of the Scientific Council of INB – BAS, Protocol № 03/09.07.2020

**In reference with:** Dissertation thesis for award of a scientific and educational degree “PhD” in the field of higher education 7. Healthcare and sports, Professional field 7.1. Medicine, Scientific specialty "Pharmacology"

### **Data about the procedure**

The review was prepared on the basis of Order № 408/16.07.2020 of the Director of the Institute of Neurobiology at the Bulgarian Academy of Sciences – Sofia, and Protocol of the first meeting of the Scientific Jury on 16.07.2020.

**Doctoral candidate:** Master of Pharmacy Stela Toshkova Dragomanova, Assistant Professor at the Department of Pharmacology, Toxicology and Pharmacotherapy, Faculty of Pharmacy at Medical University – Varna, PhD student at the Institute of Neurobiology, Research Department of Behavioral Neurobiology – Bulgarian Academy of Sciences, Sofia.

**Topic of the dissertation thesis:** "Pharmacological, toxicological and neurobiological studies of Myrtenal – a bicyclic monoterpenoid of natural origin"

**Scientific supervisor of the doctoral student:** Prof. Dr. Lyubka Tancheva, Institute of Neurobiology – Bulgarian Academy of Sciences, Sofia

All necessary documents for obtaining the Educational-Scientific Degree "Doctor" are presented, according to the requirements of the Law for the development of the academic staff in the Republic of Bulgaria, the Regulations for its application and the Regulations for the conditions for acquiring scientific degrees and for holding academic positions at the Institute of Neurobiology at the Bulgarian Academy of Sciences.

**Brief biographical data and career development of the candidate.** In 1998 Stela Toshkova Dragomanova graduated as an assistant pharmacist at the Medical College – Varna, and in 2004 she graduated at the Medical University – Sofia, with a master's degree in pharmacy. From 2005 to 2010 she was a manager of a pharmacy, and her total experience in the pharmacy network is 13 years. In 2010, after winning a competition, she was appointed as a full-time assistant in the Department of Pharmacology and Clinical Pharmacology and Therapy, Faculty of Medicine, Medical University – Varna. During the period 2014 - 2015 she was an assistant in the newly established Educational and Scientific Sector "Pharmacology and Toxicology" at the Department of Preclinical and Clinical Sciences, Faculty of Pharmacy. Due to a change in the status of the Educational and Scientific Sector, from 2015 to the present, she is an assistant in the Department of Pharmacology, Toxicology and Pharmacotherapy at the Faculty of Pharmacy, MU – Varna, and from 2016 to 2020 she is an administrative assistant. Dragomanova conducted exercises in the main disciplines "Pharmacology" and "Toxicology" for the specialty "Master of Pharmacy" and lectures on the main disciplines "Pharmacology" and "Toxicology" for the specialty "Pharmacist Assistant", and on "Pharmacology" for the specialty "Medical Laboratory Assistant". In 2014 she was enrolled as a doctoral student in pharmacology in part-time study at INB – BAS, where after respective education she receives the required number of credits (Reference Ex. № 331 / 08.07.2020). She was the coordinator of a project funded by the Science Fund at the Medical University "Prof. Dr. P. Stoyanov" – Varna (2014 – 2018) on the topic: "Preventive effects of natural monoterpenes on memory disorders of experimental rodents". The doctoral student also participated as a researcher in Project DN 03/8/2016 at the Research Fund, on the topic: "Galantamine and 4-aminopyridine derivatives containing a peptide motif with an expected effect on Alzheimer's disease and multiple sclerosis". She graduated from the Qualification Course "Protection and welfare of experimental animals used for scientific and educational purposes", Faculty of Veterinary Medicine at the Thracian University, Stara Zagora, in 2015. In 2017, Dragomanova acquired the specialty "Clinical Pharmacist". She is a co-author of a university manual for pharmacology exercises, 2 collections of test questions in pharmacology and other textbooks for students of pharmacy, medicine and dentistry. The candidate has 13 publications (total IF 2.568) and 29 participation in scientific forums with published abstract (total IF 17.610). She has been a member of the Bulgarian Pharmaceutical Union since 2004. Speaks written and spoken English and Russian and has the necessary computer skills.

By Order of the Director of INB – BAS (№ 265 / 01.06.2018) Stela Dragomanova, master of Pharmacy, was expelled from the doctoral program with the right to defense. After the internal defense of the Advanced Scientific Seminar of Scientific Behavioral Neurobiology and Synaptic Signaling and Communications at INB - BAS, held on 30.06.2020, according to the Rules of INB, a procedure for official defense of the dissertation was started.

**Relevance and significance of the topic.** The dissertation that was submitted to me is dedicated to neurodegenerative diseases, in particular Alzheimer's disease (AD), an extremely actual

medical and social problem of particular clinical importance. Although since the identification of the disease in 1906, many scientific teams have been actively working to find a reliable treatment, at this stage the available and used drugs are to some extent effective in the symptomatic response to neurodegenerative changes. The most important reason for this is the complex nature of the disease process, which includes various mechanisms contributing to its progression or the so-called multifactorial pathoethology. Therefore, the pharmacological profile research of little known and / or insufficiently studied so far biologically active substances with potential effects for therapy and prevention of neurodegenerative disorders, including AD, is a step towards the introduction of new and more effective drugs in clinical practice and is an important direction in modern experimental science. Terpenes (terpenoids, isoprenoids) are the largest group of natural products present in all classes of living organisms. Some of them are used as medicinal products (taxanes, artemisin), and others (iridoid glycosides, oleanolic acid, ursolic acid, etc.) are in the preclinical stage of research (*in vitro* and *in vivo*). An interesting and promising object for more detailed research is the classic representative of low molecular weight monoterpenoids – Myrtenal, a secondary metabolite of  $\alpha$ -pinene, contained in the essential oils of many plants common in Bulgaria (myrtle, wormwood, marjoram, oregano, rosemary, thyme, sage, lavender), as well as in bee glue (propolis). It is important to note that the published studies on the biological properties of Myrtenal and its' related effects are aimed primarily at influencing experimental models of various diseases such as diabetes, tumors, psoriasis. Bronchodilator, anti-inflammatory, antiplatelet and antihaemolytic, hypotonic, antibacterial, as well as antitumor, anti-acetylcholinesterase and antimalarial pharmacological activities have been found in experimental conditions. Despite new isolated reports in recent years of positive effects of Myrtenal-containing essential oils on the memory of experimental rodents, the monoterpene itself has been relatively little studied in the field of neuroscience. Information on the neurobiological properties of Myrtenal is scarce and at present no data are available on its effect in experimental animal models of neurodegeneration, which justifies the aim of the present study.

**Structure and content of the dissertation.** The dissertation on scientific-metric indicators fully meets the requirements of the Law for development of the academic staff in the Republic of Bulgaria, the Regulations for its application and the Regulations for the conditions for acquiring scientific degrees and for holding academic positions at the Institute of Neurobiology at the Bulgarian Academy of Sciences. Written on 255 standard typewritten pages, the text is very well illustrated with 9 tables and 118 precisely made figures, which not only facilitates the analysis of the results and their summarization, but also further illustrates the scale of the study. It is structured according to the rules for forming a doctoral dissertation and contains all the basic elements. It has been developed on the basis of an *impressive Bibliographic reference* from 534 contemporary literary sources, of which 2 in Cyrillic and 532 in English (most of the last 15 years, and 121 of all or 23% - of the last 5 years). The 3-page **Introduction** briefly and very

precisely justifies the relevance of the problem. **The literature review** (62 pages) covers all aspects of the developed topic, and the data are supported by 30 informative figures and original diagrams. The pharmacological effects of natural terpenes and terpenoids of plant origin, the biological properties and action of monoterpenes and a number of plant Myrtenal-containing essential oils, as well as Myrtenal and in particular, its effect on the CNS are described and analyzed. The general mechanisms of neurodegeneration and oxidative stress as a pathogenic factor in neurodegenerative diseases are considered with special attention. Alzheimer's disease, including hypothesis-based etiology, is characterized in detail, with an emphasis on the understanding of the complex mechanism initiating the pathological process. The modern understanding of leading specialists is emphasized, according to which, despite the similar symptoms, in practice it is not just a disease, but a combination of pathological conditions. Modern scientific data have been thoroughly analyzed, which show that AD is a protective response of the brain, especially to 3 separate processes: inflammatory, deficiency of trophic substances and other molecules supporting synaptic function, as well as in contact with toxins. In this regard, the risk factors for the development of the disease are indicated. I believe that this part of the review is one of the most valuable contributions to the dissertation, which deserves admiration. Particular attention is paid to the pharmacotherapy of AD, whose main problem is the uncertain influence of the progression of the pathological process. The therapeutic benefits of the use of known medicinal products with different indications are considered. It is emphasized that the action of the applied drugs most often affects only one stage of the unclear and complex etiopathological picture, which changes in the course of the disease. Modern therapeutic strategies are outlined, an important part of which is the multitarget approach in the treatment of neurodegenerative diseases. At the heart of this approach is the concept of providing a wider spectrum of a particular substance to affect different molecular structures as pharmacological targets, as a way to reduce symptoms, slow progression and prevent further harm in an already started process. In this regard, the main directions in the search for therapeutic agents - targets for the treatment of the disease are indicated and the advantages of natural products with effects in AD are considered, in particular a number of monoterpenoids with promising neuroprotective activity. There is growing evidence to support the hypothesis that neurodegenerative disorders, such as AD, are mediated by disruption of synaptic function and increased oxidative stress, which justifies efforts to find antioxidant natural substances with a combined mechanism of action for prevention and / or treatment of AD, capable of strengthening the cholinergic neurotransmission and antioxidant protection capacity. The information available in the literature on the effects of Myrtenal on the levels of major brain neurotransmitters is very limited. *In vitro* inhibitory effects of the monoterpenoid on the activity of acetylcholinesterase, the enzyme responsible for regulating acetylcholine levels in the brain, have been identified. This suggests the presence of positive effects of Myrtenal on the memory of rodents with experimental dementia. At present, there are no studies on the effect of monoterpenoid on animal models of neurodegeneration, which is reason to consider the criteria for selection of adequate experimental models in the study of neurodegenerative disorders. The experimental model of scopolamine-induced dementia was analyzed in great detail. The data from the extremely in-depth and impressive literature review,

prepared with the necessary competence, define, on the one hand, the chosen topic as very important and significant, and on the other hand, show the excellent preparation and awareness of the doctoral candidate on a particularly topical issue that is the focus of a huge number of scientists around the world.

**The purpose** is clearly defined and fully justifies the need for such a study. To achieve it, 4 main tasks and 7 subtasks are formulated.

**The Materials and Methods** section is developed within 22 pages, and the relevant methods and techniques are described in detail and in a reproducible manner. A total number of 382 male sexually mature ICR mice and *Wistar* rats were used for the study. The studies were performed on the basis of approved behavioral experimental protocols and in accordance with all rules and requirements for work with laboratory animals.

**The dissertation work** was performed in three experimental stages: 1. Study of the effects of Myrtenal in healthy rodents; 2. Study of the preventive effects of Myrtenal in rodents with experimental dementia of the Alzheimer's type; 3. Study of the mechanisms of neuroprotective action of Myrtenal in rodents with scopolamine-induced dementia, by comparing its effects in healthy animals and those with model impairment. Complex behavioral, clinical-laboratory, software, histological and statistical methods are used. The affinity of the Myrtenal to the AChE and to the GABAA receptor was studied by specialized software. Biochemical, docking studies, as well as histopathological studies of brain samples (in the cortex and hippocampus) of experimental animals (conducted using a specially developed algorithm for software image processing) were performed with the assistance of experts in the relevant fields. This does not diminish the doctoral candidate's contributions, as it is this multidisciplinary that provides a different perspective on some of the tasks set. This approach demonstrates the qualities of Stela Dragomanova for attracting like-minded people in scientific research, as well as her skills for teamwork.

Appropriate modern methods for statistical calculations and mathematical models for analysis of the results have been selected for all stages of the research, ensuring the necessary reliability of the study.

**Results and discussion** (110 pages). The goals of the dissertation are achieved by performing a large volume and various types of research. During the first stage the pharmacological, toxicological, neurobiological and biochemical effects of Myrtenal in healthy laboratory rodents were studied. A mean lethal dose of LD<sub>50</sub> i.p. for mice (acute toxicity), prolonged toxicity study in mice after treatment with toxic doses; effects of Myrtenal when combined with CNS depressants model drugs, anxiolytic properties, analgesic properties in different models of pain; effects on memory and learning, neuromuscular coordination and research activity with behavioral tests; antioxidant activity, changes in AChE activity and study of the neuromodulatory

properties of the substance were determined. In the second stage, the preventive effects of Myrtenal in rodents with experimental Alzheimer's dementia were studied, and for this purpose, behavioral, biochemical and histopathological verification of the scopolamine-induced dementia model in laboratory mice and rats was performed. In the third stage, the mechanisms of neuroprotective action of Myrtenal were studied by comparing its effects in intact rodents and those with scopolamine-induced dementia (neuroprotective action in mice at increasing doses and in rats in various experimental protocols).

90 mice and 76 rats were used to study the effects of Myrtenal in healthy rodents and 138 mice and 78 rats for those with dementia, which also shows the scale of the study.

For the first time, the mean lethal dose of Myrtenal to mice after intraperitoneal administration was determined ( $LD_{50}$  i.p. = 191.5 mg / kg), as the study was provoked by the lack of such data. The observed central effects of Myrtenal, manifested by potentiation of the action of CNS depressants (barbiturates and benzodiazepines), as well as the established anxiolytic properties, confirm the results of docking studies showing its affinity for the GABAA receptor. Analgesic properties have been established in the induction of pain by central and peripheral mechanism, after acute and repeated administration. For the first time, the preventive effects of monoterpenoid in rodents with experimental dementia of Alzheimer's type were studied, and for this purpose a behavioral, biochemical and histopathological verification of the model of scopolamine-induced dementia in laboratory rodents was performed. Additionally, a special dose combination has been developed in experimental rats to mimic the nonlinear progression of scopolamine-induced damage. The mechanisms of neuroprotective action of Myrtenal in rodents with scopolamine-induced dementia have also been studied for the first time by comparing its effects in healthy laboratory animals. Properly planned and successfully implemented models with experimental animals, as well as precisely conducted numerous laboratory tests, allow the doctoral candidate to make a comparative analysis of the effects of the test substance in healthy and dementia rodents. For this purpose, the effects of Myrtenal on basic behavioral and biochemical parameters in healthy and rodents with scopolamine-induced dementia were compared. A parallel was drawn between the changes in healthy monoterpenoid-treated animals compared to controls, as well as in scopolamine-treated animals compared to those treated with scopolamine and Myrtenal simultaneously. For these reasons, I allow myself to draw attention to this small part of the section, which in my opinion is the quintessence of this work and deserves very high evaluation.

In healthy rodents, the test substance was found to have no adverse effects on memory, neuromuscular coordination and research behavior. In dementia animals, Myrtenal improves memory and learning abilities, with more pronounced effects in rats. Also in dementia animals, the monoterpenoid lowers the levels of LPO products and dose-dependently increases the content

of tGSH, significantly reduces the increased by scopolamine activity of superoxide dismutase (SOD) to levels close to those achieved by the antioxidant reference Lipoic acid. Myrtenal increases the levels of the brain neurotransmitter ACh in both healthy and dementia rodents, and its neuromodulatory effects are more pronounced in healthy ones. Elevated concentrations of ACh in the brain are accompanied by increased activity of the enzyme responsible for its degradation at the synapse.

It is noteworthy that the doctoral student competently and argumentatively analyzes her own results, comparing them with similar data of other researchers. The discussion of identified differences and probable reasons for them, as well as the hypotheses presented show, on the one hand, determination and competence to express personal position and on the other, scientific maturity, excellent knowledge and appropriate use of available information on the broad and particularly important topic of this thesis.

Based on the results of the study, I believe that the dissertation of Stela Dragomanova confirms the complex pathogenetic mechanism of neurodegenerative disorders, in particular AD, with an emphasis on impaired cholinergic transmission and oxidative stress, which proves the need for multi-target strategies involving natural substances such as Myrtenal.

**Conclusions and contributions.** The conclusions made by the doctoral candidate (10) are logical, precise and clearly formulated. The presented contributions of the dissertation are 8, which I evaluate as significant. A contribution of high scientific and theoretical value is an extremely rich and impressive literary review, with the value of a separate study on the problem, which demonstrates the author's ability to collect, analyze and summarize literary data, and which should be published. The experimental model for studying the effects of Myrtenal in healthy rodents (mice and rats), in order to monitor its safety in repeated use, was implemented for the first time in our country and is a scientifically applied contribution of high value and importance for prevention and prophylaxis, as at this time research to elucidate the mechanisms of action of the monoterpenoid worldwide is mainly on experimental disease models. The first-determined LD<sub>50</sub> i.p. of Myrtenal for mice is a significant contribution to the toxicological characteristics of the substance, which is incomplete at this time. An important scientific contribution to the elucidation of the main pharmacological properties of Myrtenal are the first proven CNS effects, potentiated by potentiation of the action of barbiturates and benzodiazepines, as well as the anxiolytic potential in self-administration, which confirm the results of docking studies and show greater its affinity for the GABAA-receptor, compared with Diazepam. A contribution to the elucidation of the pharmacological effects of Myrtenal is the established presence of analgesic properties with different duration of exposure. For the first time, its effects in an experimental model of neurodegenerative damage were studied, for which purpose behavioral, biochemical and histopathological verification of scopolamine-induced Alzheimer's dementia in laboratory rodents

was performed. Of great importance is the first established protective potential of the monoterpenoid in rodents with an experimental model of dementia associated with its antioxidant and neuromodulator properties. Of particular importance for future preclinical studies in the field of neurodegenerative diseases are the original histopathological data, which point to the localization of the neuroprotective effect of Myrtenal in the brain cortex. I highly appreciate the doctoral candidate's idea to recreate the nonlinear progression of the injury, successfully realized through behavioral, biochemical and histopathological verification of the proposed new dose modification of scopolamine-induced dementia. The first comparative analysis of the effects of Myrtenal in healthy and dementia rodents, which demonstrates more pronounced antioxidant properties in animals with impaired and pro-oxidant properties in intact ones, is one of the most significant contributions of the dissertation. I believe that the study of Stela Dragomanova, conducted for the first time with significant in volume and different types of research (pharmacological, toxicological, neurobiological, biochemical and histological), especially important for studying the effects of Myrtenal in healthy rodents and those with experimental model of dementia, is a particularly significant contribution, both to the verification and supplementation of data from existing studies, and to the discovery of new mechanisms of action of the monoterpenoid in neurodegenerative disorders and in particular in AD.

**Assessment of the publication activity related to the dissertation work and the personal contribution of the PhD student.** In connection with the dissertation, 3 articles have been published, in 2 of which the doctoral student is the first author. Mag. farm. St. Dragomanova has participated in 17 specialized scientific forums – 10 international conferences and 7 congresses with published abstracts, 5 of which, respectively, in the Netherlands (won international grant), Austria, Hungary and Serbia, and in 12 of them she is the first presenting author. Two of the published articles have been cited a total of 4 times (once by foreign and Bulgarian authors). The authoritative specialized publications (all with IF) and the citation period (2018 - 2020) are indicative of both the value of the study and the relevance of the issue. I evaluate the scientific activity as very good, and the contributions as a personal work of the doctoral candidate.

**Evaluation of the preparation of the doctoral student in the credit system,** according to the Law for the development of the academic staff of the Republic of Bulgaria and Regulations for training of doctoral students in INB - BAS. Stela Dragomanova covers and significantly exceeds, according to two of the criteria, the required credits in the three main areas: I. Implementation of the educational program (130 points); II. Approbation of the implementation of the scientific program (total number of points - 312, with a mandatory minimum of 40 points). III. Publications of scientific results on the topic of the dissertation (total number of points - 168, with a mandatory minimum of 80 points).



**The Autoreferat** contains 92 standard printed pages with 1 table and 44 figures included, it is properly structured, fully reproduces the content of the dissertation and is designed according to the requirements.

**Critical remarks and recommendations.** I have no remarks on the merits that would affect my overall very high assessment of the qualities of the developed dissertation thesis. In connection with all that I have said so far, I believe that this in-depth and comprehensive scientific study, including a particularly rich literary review, fully meet the requirements for a monograph, so I strongly recommend that in the foreseeable future, the dissertation be formed and published as a monograph.

**Conclusion.** The presented dissertation is a development of a current topic, with very important scientific-theoretical, original and significant scientific-practical contributions. Stela Dragomanova, master of Pharmacy, demonstrates qualities and skills for independent research, including analysis and interpretation of scientific data. I highly appreciate the mastery and appropriate application of various experimental models, techniques and procedures, including a large number of behavioral tests related to the use of experimental animals for specific scientific purposes. The results of the experimental studies, the conclusions made and the outlined contributions show that the doctoral candidate has the necessary theoretical knowledge and a high level of professional competence in the field of scientific interests.

**The complex positive evaluation of the dissertation, which fully complies with and even exceeds the specific requirements, as well as my personal opinion, based on professional contacts, allow me to confidently propose to the esteemed Scientific Jury to vote "pro" the doctoral candidate Stella Toshkova Dragomanova to obtain the educational and scientific degree "Doctor" of in the scientific specialty "Pharmacology", in the field of higher education 7. Health and sports, Professional field 7.1. Medicine.**

Sofia, August 24<sup>th</sup> 2020

Review drawn by:

Assoc. Prof. Yulichka Sabeva, PhD

