

STATEMENT

from

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of a dissertation for the award of the educational and scientific degree “Doctor of Philosophy” in the

scientific field 7. Health and sports, scientific direction

7.3 Pharmacy, doctoral program Pharmaceutical chemistry

Doctoral candidate: MPharm Velislava Dimitrova Todorova

Department: Pharmacognosy and pharmaceutical chemistry

Dissertation title: Pharmacanalytical control of substances with adaptogenic properties from *Rhaponticum carthamoides* Willd.

Supervisor: Assoc. Prof. Kalin Valentinov Ivanov MPharm, PhD, Medical University - Plovdiv

1. Overview of the procedure and the doctoral candidate

After preliminary discussion by the extended Department Council (pr. No. 8/14.11.2024) and acceptance by the Faculty Council (pr. No. 10/27.11.2024) of the Faculty of Pharmacy, I have been appointed by order No. P-2121/23.12.2024 of Prof. Dr. Maria Tokmakova - Vice Rector for Research of MU-Plovdiv as a member of the scientific jury in the procedure for the defense of a dissertation on the topic "Pharmacanalytical control of substances with adaptogenic properties from *Rhaponticum carthamoides* Willd" for the acquisition of the educational and scientific degree "doctor" in the field of higher education 7. Health and Sports, professional field 7.3 Pharmacy, doctoral program Pharmaceutical Chemistry.

The presented set of materials on paper/electronic media is in accordance with the requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADSRB), the Regulations for the Implementation of the ADSRB, as well as with Art. 70 (1) of Section I. Acquisition of the educational and scientific degree "DOCTOR OF PHILOSOPHY " and the scientific degree "DOCTOR OF SCIENCES" at MU-Plovdiv; Regulations of MU-Plovdiv dated 28.01.2021 and includes the following documents:

- Application to the Rector of MU-Plovdiv for the opening of the procedure for the defense of a dissertation
- CV in European format with the signature of the doctoral student
- Notarized copy of a higher education diploma

- Orders for enrollment in doctoral studies and for withdrawal with the right to defense
- Order for conducting an exam from the individual plan and a corresponding protocol for a passed exam or doctoral minimum in the specialty
- Protocol of the department council for preliminary discussion of the doctoral thesis and the decisions made to open a procedure and for the composition of a scientific jury
- Dissertation work
- Abstract
- List of scientific publications on the topic of the dissertation
- Copies of scientific publications
- List of participation in scientific forums
- List of noted citations
- Declaration of originality and authenticity of the attached documents
- Other documents related to the course of the procedure

The doctoral student has applied 3 scientific publications with a total impact factor of 14.306 (IF), with a total number of citations of 58 excluding self-citations. The doctoral student is the first author of all submitted publications.

Velislava Dimitrova Todorova was born on September 7, 1995 and completed her secondary education with a humanitarian profile in the city of Plovdiv. In 2020, she graduated with honors as a Master of Pharmacy at the Faculty of Pharmacy of the Medical University - Plovdiv. The same year, she won a competition for an assistant professor in Pharmaceutical Chemistry and Pharmaceutical Analysis at the Department of Pharmacognosy and Pharmaceutical Chemistry of the Faculty of Pharmacy at the Medical University - Plovdiv, where she has been teaching to this day. Since 2021, Velislava has been enrolled as a full-time doctoral student at the same department under the supervision of Assoc. Prof. Kalin Ivanov. The doctoral student's professional growth is accompanied by the acquisition of the specialty "Analysis of Medicinal Products" in 2024. Her intensive and impressive scientific work, which is characterized by her dedication, was awarded the "Pythagoras" award for a young scientist in the field of life sciences and medicine by the Ministry of Education and Science in 2024.

The opinion was drawn up in accordance with the requirements of the Regulations for the Implementation of the ADSRB.

2. Relevance of the dissertation topic

Chronic exposure to stress in everyday life is among the leading risk factors for premature aging of the body as well as the development of a number of chronic diseases, such as obesity, non-insulin-dependent diabetes and cardiovascular diseases. This also determines the sustained interest in the biological activity and phytochemical characteristics of adaptogenic plants. Adaptogens have the potential to normalize the biological functions of the body and strengthen it in stress conditions.

Among the best-studied plants that are classified as adaptogens are: *Panax ginseng* C.A.Mey. (Araliaceae), *Eleutherococcus senticosus* Max. (Araliaceae), *Rhodiola rosea* L. (Crassulaceae), *Schisandra chinensis* (Turcz.) Bail, as well as *Rhaponticum carthamoides* (Wild.) Iljin (Asteraceae). *Leuzea*, as the plant *R. carthamoides* is popularly known, stands out as a valuable source of active compounds with proven adaptogenic effects.

Epidemiological data on the prevalence of overweight and obesity indicate that by 2035 It is expected that every second adult will be overweight and every fourth adult will be obese. This forecast requires global efforts to reduce, prevent and treat obesity. The modern approach to obesity includes lifestyle changes with diet and physical activity, drug therapy and metabolic surgery. In Europe, 4 drugs are registered for the treatment of obesity – orlistat, naltrexone/bupropion, liraglutide at a dose of 3.0 mg daily and semaglutide at a dose of 2.4 mg weekly.

It should be taken into account that less than 40% of people with obesity have a diagnosis, with less than 20% undergoing treatment, and despite the available options for treating obesity, only about 1% of them are on drug therapy.

A significant percentage of people with obesity do not achieve a significant effect on their body weight with modern therapeutic agents. Maintaining the achieved body weight reduction over time also proves to be a serious problem. It is necessary to know the modern possibilities for treating obesity in order to improve the prognosis of people with this disease. The complex nature of obesity as a disease necessitates the search for therapeutic options aimed at various mechanisms involved in its pathogenesis. That is why extremely intensive work is being done to find new means of controlling body weight. Plant extracts and their secondary biologically active metabolites have the potential to influence the signaling pathways and mechanisms that underlie the function and physiology of fat cells, as the main structural units of adipose tissue, and accordingly to influence the development of obesity.

The unresolved issues surrounding the pharmacoanalytical control of the quality composition and effectiveness of the applied adaptogens determine the relevance of the topic of Velislava Todorova's dissertation work, which aims to evaluate the therapeutic potential of *R. carthamoides* and the ecdysteroids contained in it in obesity, overweight and aging. To achieve the main goal of this dissertation, 7 main tasks have been formulated precisely and clearly, which relate to plant selection and determination of macroscopic histochemical analysis of roots; characterization of the essential oils isolated in a cultivated population of *R. carthamoides* ; development and validation of analytical methods for quantitative determination of selected phytoecdysteroids in the extract of *R. carthamoides* by means of HPTLC; HPLC-UV and LC-PDA-MS; assessment of the influence of *R. carthamoides* extract and the pure compounds ecdysterone, turkesterone and ponasterone A on the processes of adipogenesis and lipolysis in an in vitro model of human adipocytes; as well as assessment of the influence of *Leuzea* extract and ecdysterone on the lifespan of the model organism *Caenorhabditis elegans*. The well-formulated tasks fully correspond to the goal of the

dissertation and include modern pharmacanalytical methods and adequate model systems for assessing biological activity.

3. Awareness of the subject matter

The literature review is very well structured and presents up-to-date information on the phytochemical characteristics and available data on biological activity of adaptogenic plants. The role of phytoecdysteroids in the effect of adaptogenic plants is analyzed in depth: *Panax ginseng* C.A.Mey. (Araliaceae), *Eleutherococcus senticosus* Max. (Araliaceae), *Rhodiola rosea* L. (Crassulaceae), *Schisandra chinensis* (Turcz.) Bail, as well as *Rhaponticum carthamoides* (Wild.) Iljin (Asteraceae). Special emphasis is placed on the use of adaptogenic plants and phytoecdysteroids in the form of extracts, food supplements and so-called "superfoods" to increase resistance to stress and improve overall homeostasis in the body, assessment of the quality and composition of adaptogenic plant products. The advantages and disadvantages of chromatographic methods for the analysis of ecdysteroids, which are very well described, are presented. Plants rich in 20-hydroxyecdysterone, which are edible and often recommended in healthy diets, namely fresh spinach leaves, asparagus stems and quinoa seeds, were analyzed in order to ensure their safe use. General information, phytochemical composition, biological activity are presented in detail and secondary metabolites in extracts of the indicated plants, grouped according to their chemical structure, have been identified.

387 literary sources are cited, with over 40% of them from the last 10 years; they are from international journals, and authors from the country with their publications in refereed scientific journals are also cited, regulatory documents of the European Medicines Agency (EMA) and the World Anti-Doping Agency (WADA) are also cited.

The doctoral student knows the state of the problem very well and has presented a thorough creative assessment of the analyzed literary material, having well identified the need to expand scientific knowledge in the field.

4. Research methodology

The dissertation used a strategy that includes several stages: characterization of the plant material from *R. carthamoides* ; isolation and analysis of the essential oil profile of Leuzea extract by gas chromatography in tandem with mass spectrometry (GC-MS); development and validation of pharmacanalytical methods for quantification by HPTLC, HPLC-UV; LC-PDA-MS chromatographic techniques; modern and reliable methods for determining biological activity - assessment of the anti-adipogenic potential of the plant extract, as well as selected secondary metabolites in human adipocytes, and assessment of the effect of Leuzea extract and ecdysterone in vivo in *C. elegans*.

The collection of plant material and the extraction processes are described in detail. The process of developing and validating the analytical methods is clearly and in detail described, with precisely derived formulas used to determine linearity, accuracy, precision and limits of detection and quantification. An *in vitro* assessment of anti-adipogenic potential in human adipocytes was conducted, describing in detail the cell culture and treatment, the assessment of cell viability, the analysis of intracellular lipid accumulation. An *in vivo* model of aging in *C. elegans* was used.

Standard methods of pharmacognostic and pharmacochemical analysis were used, including modern methods of high-performance liquid chromatography, precise methods for determining cell viability, lipid accumulation and lipolysis in human fat cells and a modern approach for assessing the potential for increased resistance to stress stimuli of different origins and prolonging lifespan in an *in vivo* model system of *C. elegans*. Statistical analysis of the data was performed using SigmaPlot v11.0 from Systat Software GmbH (Erkrath, Germany).

The chosen research methodology allows achieving the set goal and obtaining an adequate answer to the tasks solved in the dissertation.

5. Characteristic and evaluation of the dissertation, its impact and contributions

The dissertation is written on 178 pages, and is well structured in the following main sections: Title page (1 page), Table of contents (3 pages), Abbreviations used (2 pages), Introduction (2 pages), Literature review (49 pages), Aim and objectives (1 page), Material and methods (16 pages), Results and discussion illustrated in 24 tables and 39 figures (58 pages), Discussion of the results illustrated with 3 figures (19 pages), Conclusions (1 page), Contributions (1 page), List of publications related to the dissertation (2 pages), Bibliography with 387 literature sources (38 pages), of which 114 from the last 5 years.

In fulfilling the tasks of the dissertation, the effects of an extract of *R. carthamoides*, 20-XE, TC and PS on adipogenesis in an *in vitro* model of human adipocytes and the aging processes in *C. elegans* were studied. It was found that extracts of *R. carthamoides* and 20-XE reduced adipogenesis and stimulated lipolysis, TC affected only adipogenesis, and PS had no such effects. In addition, extract of *R. carthamoides* affected lipid metabolism in glucose-fed *C. elegans*. Extract of *R. carthamoides* was found to extend lifespan and delay physiological aging in *C. elegans*, and also increased resistance to heat stress and survival under oxidative stress. In contrast, 20-XE increased survival in *C. elegans* exposed to oxidative stress and improved resistance to heat stress.

The results of the conducted research lay new foundations for further studies on the molecular mechanisms of action of 20-XE, TC and *R. carthamoides* and their potential application as a means of improving the quality of life

In this dissertation work, data were obtained from a comparative analysis of essential oil from wild and cultivated *R. carthamoides* samples, which showed differences in the component composition, probably due to the different climatic conditions of growth. Of contribution is the

introduction for the first time in Bulgaria of a method for extracting and characterizing essential oil from *R. carthamoides* cultivated in Bulgaria and for the first time histochemical localization of secretory channels and lipid accumulations in the roots and rhizome of *R. carthamoides* was carried out.

The results of the experiments conducted in this work for the development and validation of HPTLC, HPLC-UV and LC-PDA-MS methods for the identification and quantification of 20-hydroxyecdysterone, turkesterone and ponasterone in plant extracts, "superfoods" and food supplements are of serious scientific and applied contribution. By means of the validated methods, the quantitative content of the indicated phytoecdysterones in an extract of *R. carthamoides*, from a plant cultivated in Bulgaria, was determined for the first time.

As a contribution of a scientific and fundamental nature, the data on the biological activity of the identified 20-hydroxyecdysterone, turkesterone and ponasterone, as participating in the mechanism of anti-adipogenic action of *Leuzea* extract in human adipocytes, are presented. The results support the conclusions that the extract of *R. carthamoides* reduces adipogenesis and stimulates lipolysis in an in vitro model of human adipocytes, and among its pure compounds studied, 20-hydroxyecdysone reduces adipogenesis and stimulates lipolysis, TC affects only adipogenesis, and PS does not affect either adipogenesis or lipolysis in an in vitro model of human adipocytes. In addition, data from the in vivo model system show the potential of the extract of *R. carthamoides* to reduce lipid accumulation in *C. elegans* fed with glucose. The extract of the adaptogenic plant *Leuzea* also has the potential to extend lifespan, delay physiological aging, and increase resistance to heat stress and oxidative stress in *C. elegans*. Of the studied secondary metabolites, the biological activity of 20-hydroxyecdysone stands out in terms of increasing survival in *C. elegans* exposed to oxidative stress and heat stress. The established antiadipogenic activity of both *R. carthamoides* extract and 20-XE in SGBS cells and the positive influence of *R. carthamoides* on aging and lifespan in *C. elegans* are evidence for the potential use of adaptogenic plants and their secondary metabolites for body weight control and reduction of signs of aging.

6. Assessment of the publications and personal contribution of the doctoral candidate

In relation to the topic of the dissertation, MPharm Todorova has presented 3 publications in international scientific journals, with an impact factor - total IF 14.306, with two of them located in the first quartile for the relevant scientific field (Q1) and one in Q3, and all three are published with open access, which is in accordance with the principles of open science. In all publications, Velislava Todorova is the first author, which is evidence of her leading role in the research conducted. Scientific publications on the topic of Velislava Todorova's dissertation have 58 citations (excluding auto-citations in Scopus). Separate fragments of Velislava Todorova's dissertation have been presented at 3 international scientific conferences, 1 as a report and 3 as posters, with her being the lead author in 3 of her participations. The participation of MPharm

Todorova as a leading researcher in a project with number DPDP 06/2022: Pharmacanalytical control of adaptogens of plant origin - characterization of an extract of *Rhaponticum carthamoides*, related to the topic of the dissertation, which is indicative of her personal development as an independent scientist. The participation of scientists from several teams outside MU-Plovdiv - one from the country and one abroad in the publications included in the dissertation is also impressive, which shows skills for working in a multidisciplinary team.

The provided documentation shows the personal participation of Velislava Todorova in the conducted dissertation research. The results obtained and the formulated contributions are her personal merit.

I have no critical remarks about the conducted research and the presented materials. I recommend that Velislava Todorova continue her in-depth research into pharmacanalytical approaches in the same direction by further expanding her knowledge and experience in metabolomics of plant extracts, for example by adding nuclear magnetic resonance spectroscopy.

7. Summary

The abstract for the dissertation work of Velislava Todorova is presented in 53 pages and reflects the main results achieved in the dissertation work and meets all generally accepted requirements in the Regulations for the Implementation of the ADSRB for its preparation.

CONCLUSION

In conclusion, I believe that the dissertation work of Velislava Dimitrova Todorova is completely complete, modern, in-depth, well-structured and designed, with clear and precisely formulated and implemented tasks, with certain conclusions and solid scientific-fundamental and scientific-applied results, which represent an original contribution to science in an interesting area - pharmacanalytical approaches for phytochemical characterization of plant extracts.

The dissertation work meets all the requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADSRB), the Regulations for the Implementation of ADSRB and the Regulations for the Implementation of ZRASRB of the Bulgarian Academy of Sciences. The presented materials and dissertation results fully comply with and even exceed the specific requirements of the Regulations for the Conditions and Procedure for Acquiring Scientific Degrees and Holding Academic Positions at the Medical University - Plovdiv.

The dissertation work shows that the doctoral student MPharm Velislava Dimitrova Todorova possesses in-depth theoretical knowledge and professional skills in the scientific specialty of Pharmaceutical Chemistry, demonstrating qualities and skills for independent conduct of scientific research.

Due to the above, I confidently give my positive assessment of the conducted research, presented by the above-reviewed dissertation, abstract, achieved results and contributions, and I

propose to the members of the esteemed scientific jury to award the educational and scientific degree of 'doctor' to Assoc. Prof. Velislava Dimitrova Todorova in the field of higher education 7. Health and Sports 7.3. Pharmacy in the doctoral program in Pharmaceutical Chemistry.

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

15.01.2025

Prepared by:.....

/Assoc. Prof. Liliya V. Mihaylova, MPharm PhD/