REVIEW
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of a dissertation for the award of the educational and scientific degree "Doctor"
in the field of higher education 4. Natural Sciences, Mathematics and Informatics; Professional field: 4.3. Biological Sciences; Doctoral program: Botany

Author: Donika Petrova Gyuzeleva

Topic: Biological and phytochemical study of plants from the Bulgarian flora with potential for biotechnological application

Scientific supervisors:

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1. General description of the procedure and the submitted materials

By order No. RD-21-2058 of 15.11.2024 of the Rector of Plovdiv University "Paisii Hilendarski" (PU), I have been appointed as a member of the scientific jury for the defense of a dissertation on the topic "Biological and phytochemical study of plants from the Bulgarian flora with potential for biotechnological application" for the acquisition of the educational and scientific degree "doctor" (PhD). Author of the dissertation is Donika Petrova Gyuzeleva - a full-time doctoral student at the Department of Botany and Biological Education. At the first meeting of the scientific jury, held on 19.11.2024, I was assigned to prepare a review of the dissertation. I received all the materials necessary for the preparation of this review in electronic format. They are in accordance with Art. 36 (1) of the Regulations for the Development of the Academic Staff of the University of Plovdiv and include the following documents:

– a request to the Rector of the University of Plovdiv for opening of the procedure for defense of the dissertation;
– a CV in European format;
– a report from the departmental council, related to a preliminary discussion of the dissertation and reporting the readiness to open the procedure to defense;
– a dissertation;
– an abstract;
– a list of scientific publications on the topic of the dissertation;
– copies of the scientific publications;
– a declaration of originality and authenticity of the presented documents.

2. Brief biographical data

PhD student Donika Gyuzeleva is an alumnus of the Paisii Hilendarski University, where in 2015 she graduated as a bachelor with a degree in "Medical Biology", and in 2016 she received a master's degree in "Microbiological Control and Food Safety". Over the past two academic years, she has conducted practical exercises with students in plant anatomy and morphology in the Department of Botany and Biological Education of the Faculty of Biology of the Paisii Hilendarski University. She was enrolled as a full-time doctoral student in the Botany doctoral program on 01.08.2019 and was discharged on 01.08.2022 with the right to defend her dissertation.

3. Relevance of the topic and appropriateness of the set aim and tasks

The dissertation, presented by the doctoral student Donika Gyuzeleva, aims to establish the possibility of medical and pharmaceutical application of three species of the genus *Marrubium* (*Marrubium friwaldskyanum*, *Marrubium peregrinum*, *Marrubium vulgare*) and *Centaurea thracica* through their biological and phytochemical study. The basis for this work is the insufficient information on the phytochemical composition of plants found in different regions of Bulgaria, one of which (*Marrubium friwaldskyanum*) is endemic to Bulgaria, as well as the lack of detailed studies of the individual morphological parts (leaves, flowers, stems, fruits) in terms of their individual phytochemical content and biological activity. The topic of the dissertation is in line with the recommendations of the World Health Organization, which encourages the use of the unique strengths of natural sources to improve people's health and well-being, while also promoting more detailed scientific research to understand the benefits and risks of traditional medicine (WHO global report on traditional and complementary medicine 2019).

4. Knowledge of the problem

The doctoral student introduces the current knowledge about the studied plants through a literature review on the morphological characteristics of the plants, their application in traditional medicine, and the reported phytochemical constituents. Some known biological properties of extracts obtained from these plants are also reviewed: antioxidant, antimicrobial and antitumor, as well as the plausible mechanisms associated with these effects. It would be good to summarize the literature review with a conclusion that would better justify the following aim and tasks of the dissertation.

5. Research methodology

The methods used by the doctoral student are adequate to the set aim and tasks. Elucidating the phytochemical composition of such a complex mixture of chemical constituents as plant extracts, identifying individual compounds and their reliable quantification is a very difficult task due to the chemical complexity and dynamic concentration range of the metabolome and it is unthinkable without application of modern chromatographic methods with mass detection. The doctoral student uses GC-MS for the analysis of moderately polar analytes after preliminary derivatization and liquid chromatography with tandem mass detection. The annotation of metabolites was done using either an internal database with standard compounds or available database libraries. The gas and liquid chromatography data were subjected to multivariate analysis

to outline the similarities and differences in the phytochemical composition of individual members of the genus *Marrubium*.

Biological tests of total extracts or extracts from individual plant parts include studies on their cytotoxicity and antitumor properties, antimicrobial potential and immunomodulatory effect. The antitumor properties of extracts from leaves, flowers and stems of the endemic Bulgarian species were studied against three types of tumor cells in a monolayer, and one of the tumor lines as cell spheroids, which are a closer model of solid tumors. The vitality of the cells was monitored by two standard methods – by the tetrazolium dye MTT and the test with neutral red dye (NR). The antibacterial properties were tested on two strains of bacteria - one Gram (+) and one Gram (-), and for each extract the minimum inhibitory concentration and the minimum bactericidal concentration were determined.

To clarify the chemical composition of the seeds and fruits of *Centaurea thracica*, standardized methods according to BDS, ISO or other international organizations were used.

Appropriate statistical methods were applied to process the obtained results.

The description of some of the methods used is incomplete, for example, whether the conditions of the chromatographic analyses are original, or methods developed by other authors were used (only the derivatization method is cited). It is not clear which extracts in terms of the solvent used were used in the biological tests (the article in Heliyon refers to the article in the International Journal of Molecular Sciences, and there are several extracts obtained and reported there).

6. Characteristics and evaluation of the dissertation work

The dissertation covers 128 pages with a good balance between the individual parts. The literature review, written on 15 pages, analyses the known data on the botanical and morphological characteristics of the plants that are objects of study, data on their phytochemical composition and therapeutic properties. The chapter “Materials and Methods” (23 pages) describes in a relatively detailed manner the methods used. It is quite appropriate that the doctoral student combines the results and their discussion in one chapter, since in this way the diversity of results is logically bonded to their meaning and significance.

The bibliography includes 257 literary sources, of which 7 are in Cyrillic.

The experimental studies were conducted in the scientific laboratories of the Department of Botany and Biological Education, the Department of Developmental Biology at the Faculty of Biology, the Department of Chemical Technology at the Faculty of Chemistry at the Paisii Hilendarski University; the Department of Medical Biochemistry at the Faculty of Pharmacy at the Medical University – Plovdiv, and the Center for Plant and Systems Biology and Biotechnology – Plovdiv. These are established scientific centers where proven specialists in their fields of work, which does not cast doubt on the reliability of the results obtained and their discussion.

The dissertation is a good basis for further research, enabling the practical application of plants or their extracts as food supplements, phytotherapeutics, etc.

7. Contributions and significance of the work for science and practice

The formulated aim and tasks of the dissertation work show that the doctoral student's intention is not only to obtain new data for the scientific literature about the studied plants, but also to outline possible practical applications of their extracts. In this sense, the dissertation has not only scientific, but also scientific and applied contributions. I would outline the principal ones:

- A comparative anatomical and morphological description of the three members of the genus *Marrubium* has been made with an emphasis on the characteristics of the endemic for Bulgaria species, which may be useful for future taxonomic studies within the genus *Marrubium*;
- Based on the data from gas chromatography analysis, a hierarchical cluster analysis of the primary metabolites (mainly amino acids) contained in extracts of *Marrubium peregrinum* L. and *Marrubium friwaldskyanum* has been carried out, which makes it possible to determine the main compounds essential for the development of plants;
- Metabolic profiling, performed using liquid chromatography data and the selection of 20 target compounds with the highest concentration, showed significant differences in the type and quantity of secondary metabolites synthesized by *M. friwaldskyanum* and *M. Peregrinum*, which indicates significant differences in the biochemical networks of the species leading to the synthesis of secondary metabolites. This is best highlighted in the phytochemical composition of the flowers, whose biological role is different from that of the other plant parts;
- Two compounds were identified – the dipeptide phenylalanylmethionine and the conjugate acid of taurine with deoxycholic acid, which are found only in the endemic species;
- The ability of extracts from different parts of *M. Friwaldskyanum* to selectively inhibit the growth, activity and viability of different types of tumor cells has been demonstrated for the first time, while a similar cytotoxic effect was not found in normal fibroblast cells. Based on the results of the two cytotoxicity tests, a hypothesis was proposed about the mechanism of the possible effect of the tested extracts on tumor cells;
- A detailed chemical analysis of unripe and ripe seeds of Thracian cornflower (*Centaurea thracica*) was performed, comparing the dynamics in the synthesis of basic compounds: proteins, carbohydrates, lipids, content of insoluble fiber and minerals during the ripening process. Special attention was paid to the lipid composition of the fruits and seeds. The calculated values of the atherogenicity and thrombogenicity indices show good antiatherogenic and antithrombogenic properties of the studied oils, and the values of the polyunsaturated/saturated fatty acid ratio confirm the high nutritional value of the oil, which makes it promising for use in food supplements and pharmaceutical products for the prevention of some chronic diseases.

8. Assessment of publications on the dissertation work

The dissertation work is based on the content of 4 scientific articles, all of which have been published in international journals, referenced in international databases for scientific literature WoS and/or Scopus. The doctoral student is the first author of three of them. It should be pointed

the interest of other Bulgarian and foreign researchers in them, as evidenced by the fact that the articles, although published in the last 2 years, have already been cited 9 times (as of the date of preparation of the review).

Regarding the points specified in the Regulations for the Implementation of the Development of the Academic Staff in the Republic of Bulgaria Act (DASRBA), the doctoral student fully covers the 30 points of publications required by it, with the published articles on the dissertation work scoring 70 points:

Article	Impact-factor WoS	Scopus Journal Rank	Points	Citations
Gyuzeleva, D., Batsalova, T., Dzhambazov, B., Teneva, I., Mladenova, T., Mladenov, R., ... & Bivolarska, A. (2024). Assessment of the biological activity of *Marrubium friwaldskyanum* Boiss.(Lamiaceae). Heliyon.	3.4, Q1	0.62, Q1	25	1
Gyuzeleva, D. P., Stoyanov, P. S., Bivolarska, A. V., Mladenov, R. D., Mladenova, T. R., Petkov, V. H., & Todorov, K. T. (2022). Anatomical Investigation of *Marrubium friwaldskyanum* Boiss. and *Marrubium peregrinum* L.(Lamiaceae) from Bulgaria. Ecologia Balkanica, 14(1).	-	0.202, Q4	10	6
Gyuzeleva, D., Benina, M., Ivanova, V., Vatov, E., Alseekh, S., Mladenova, T., ... & Stoyanov, P. (2023). Metabolome Profiling of *Marrubium peregrinum* L. and *Marrubium friwaldskyanum* Boiss Reveals Their Potential as Sources of Plant-Based Pharmaceuticals. International journal of molecular sciences, 24(23), 17035.	4.9, Q1	1.18, Q1	25	2
Teneva, O., Petkova, Z., Antova, G., Angelova-Romova, M., Stoyanov, P., Todorov, K., ... & **Gyuzeleva, D**. (2024). Chemical Composition and Lipid Bioactive Components of *Centaurea thracica* Dwelling in Bulgaria. Molecules, 29(14), 3282.	4.2, Q2	0.74, Q1	25	1

The results included in the dissertation have received public promotion to the scientific community not only through publication in international journals, but also through presentation at 3 scientific forums.

9. Personal participation of the doctoral student

I could not assess the personal participation of the doctoral student in the conducted dissertation research, and to what extent the formulated contributions and the results obtained are her personal merit, since most of the research was done by large multidisciplinary scientific teams. It is noteworthy that she is the first author of 3 of the publications. As I have already emphasized, the experiments were conducted in established scientific centers and the participation of the doctoral student in the experimental work and the discussion of the results has undoubtedly contributed to her professional enhancement and growth.

I believe that the dissertation is the doctoral student's own work and that no data or texts have been plagiarized from publications by other authors.

10. Abstract

The abstract covers 47 pages and includes the content of the dissertation in a condensed but comprehensive form. A large part of the figures that support the conclusions and main contributions of the work are included. A bibliography of publications on the dissertation is also attached.

11. Critical notes and recommendations for future use of the dissertation contributions and results

The dissertation is written in very good scientific language, follows the logical sequence of the experiments and their interpretation, and this makes it very convincing. It impresses with the variety of methods used and a significant amount of data, graphically presented in 39 figures and 12 tables. Sometime there are small terminological inaccuracies, for example, UV radiation is not light; on p. 62, 74 and some figures hydroxygallic acid is mentioned, but it refers probably to hydroxy derivatives (glycosides) of gallic acid, since it itself is trihydroxybenzoic acid; "stock solution", etc.

My notes on the content of the dissertation do not reflect on my overall positive assessment, but I think they would be useful in the further research and publication activities of the doctoral student.

- To me, the title of the dissertation sounds a bit peculiar. Indeed, in a broad sense, "biotechnology" is the use of biology to make useful products, but in a narrower sense, the doctoral student's research is not so much on biotechnological production as on possible biomedical or pharmaceutical applications; moreover, this is the sense of the discussions of the results obtained;
- The list of abbreviations used is quite incomplete;
- The literature review pays considerable attention to the antioxidant properties of plant extracts and especially to the group of polyphenols. The dissertation lacks such data. Some of the standard antioxidant methods could be applied and how they correlate with the phytochemical composition could be discussed;
- The chapter "Materials and Methods" states that the calibration plots for ICP-MS were made in the intervals 0.01 to 30 ppm, while much of the data in Table 4 exceed 1000 ppm. How was this difference overcome? It is worth commenting on the high content of K, which is nearly 4 orders of magnitude higher than that of Na, considering the importance of the K^+/Na^+ ratio for the vital functions of cells, for regulating blood pressure and reducing the risks of cardiovascular diseases;
- Some of the conclusions are generally accepted and do not need citations (pp. 6 and 15). However, on p. 59, the penultimate paragraph needs a citation;
- Some of the dissertation conclusions formulated in this way sound more like contributions and vice versa – some of the contributions are essentially conclusions.

CONCLUSION

According to the DASRBA, “Doctor” is an educational and scientific degree. The content of the dissertation convincingly shows that the doctoral student Donika Gyuzeleva has not only built on the knowledge obtained in the master's degree and has covered the educational component of the “Doctor” degree but has also mastered several instrumental and biological methods. She can critically discuss the results obtained and present them in writing. The dissertation submitted for review, in terms of its scientific value, meets the requirements of the DASRBA, the Regulations for the implementation of the DASRBA and the relevant Regulations of the “Paisiy Hilendarski” University.

Based on the analysis of the significance of the research conducted, its presentation in the dissertation and the published articles, I confidently give my positive assessment and recommend to the esteemed members of the scientific jury to award the educational and scientific degree “Doctor” to Donika Petrova Gyuzeleva in the scientific field 4. Natural sciences, mathematics and informatics, professional field 4.3. Biological Sciences under the Botany PhD program.

January 8, 2025
Plovdiv

Reviewer: .....