



OPINION

On: Dissertation for awarding the educational and scientific degree of "Doctor" in the scientific specialty 4.3 Biological Sciences, Molecular Biology, on the topic "Natural low-toxic biologically active extracts of Bulgarian mushrooms with a focus on *Amanita muscaria* – analysis of composition, antitumor activity and mechanism of action" prepared and presented by full-time PhD student Alexander Nikolaev Dushkov, with scientific supervisor Prof. Dr. Iva Ugrinova

By Prof. Dr. Roumiana Petrova Stateva, Institute of Chemical Engineering - BAS

According to Order No. 218-OB of the Director of the Institute of Molecular Biology "Acad. Rumen Tsanev" (IMB-BAS) from 20.12.2024 I was appointed to act as a member of a scientific jury, and subsequently selected to prepare and present an opinion on the dissertation of A. Dushkov. After examining the documents, dissertation and abstract submitted by the doctoral student, I found that they fully comply with the requirements set out in the Rules of Procedure for Acquiring Scientific Degrees and Occupying Academic Positions at the Bulgarian Academy of Sciences (amended from 20.05.2024). The dissertation was presented and discussed at an extended seminar of the Department of Chromatin Structure and Function at IMB-BAS on 16.12.2024. During his doctoral studies, Mag. Dushkov has fully completed his doctoral program by collecting 518 credit points, which is reflected in his attestation card.

Brief biographical information about A. Dushkov:

Alexander Dushkov was born in 1981 in the town of Sofia. Sofia. He graduated from NPMG "Acad. Lyubomir Chakalov", profile Biology in 2013. The same year he was admitted as a full-time student at the Faculty of Biology at Sofia University "St. Kliment Ohridski". Kliment Ohridski", where in 2019 he received a bachelor's degree, and in 2021 - a master's degree. Since 2022 he has been a full-time PhD student at IMB-BAS. During his doctoral studies, he completed a one-month internship at the Faculty of Chemistry of Charles University in Prague, Czech Republic under the CEEPUS program. From 2018 until now, he has been working at IMB-BAS, first as a biologist, and later as an assistant.

Overview and characterization of the dissertation:

The dissertation is written on 111 pages, contains 47 figures, 4 tables, and includes the following sections: Introduction, Literature Review, Purpose and Objectives, Materials and Methods, Results and Discussion, Conclusions and Contributions. The list of References is impressive - 248 sources in Latin, which undoubtedly is a testament to the extremely good knowledge of the PhD student of the current state-of-the-art.

The detailed literature analysis presented in Section 2 allowed Alexander to clearly formulate the main goal of the dissertation: "Study of the antitumor effect of extracts obtained from different types of mushrooms distributed on the territory of Bulgaria".

I would especially like to note the relevance of the topic of the dissertation, aimed at:

- Study of the possibilities for transforming (valorizing) a sustainable, relatively easily accessible and available in sufficient quantities on the territory of Bulgaria plant biomass into bioactive substances and secondary metabolites with high added value;
- Clarification of the mechanism of their action and analysis of the possibilities and prospects for their application in various areas, mostly related to health and improving the quality of human life, and in particular aimed at the treatment of socially significant diseases.

The dissertation topic is consonant with the principles of the biocircular economy and follows the new trends, characterized by a change in the emphasis of the research paradigm – from biomass-to-energy/biofuels to biomass-to-biochemicals. The PhD student and his supervisor Prof. Ugrinova should be congratulated for this appropriate choice of a research topic.

The thus formulated aim of the dissertation predetermines the interdisciplinary nature of the research, aimed mainly at using the instrumentarium of molecular biology and biotechnology, but also analytical and to some extent chemical engineering, especially related to obtaining low-toxic biologically active extracts from the objects examined and analyzing their composition.

Section 2 convincingly demonstrates not only the very good knowledge of the specialized literature by the PhD student, but also his ability to conduct analytical, in-depth and critical analysis. This, without a doubt, was important in the formulation of five tasks (Section 3), clearly and specifically defined, for

the fulfillment of the ambitious goal, each of which requires a significant amount of experimental work. The only remark to this part of the dissertation is that the inscriptions in the Figures, and the Tables, are in English. Similarly, with a few exceptions, is the presentation of figures and tables in the other sections of the dissertation.

Section 4 is devoted to Materials and Methods. In certain subsections (4.1.1 – 4.1.5; 4.2.2 – 4.2.11) specialized terminology is used as well as particular methods discussed that are not within expertise in order thus, I am not in the position to comment with the necessary confidence.

As for the preparation of extracts from fungal fruiting bodies (subsection 4.2.1), I would like to note that if the generally accepted in chemical engineering (and not only) approach is followed, it would be necessary to analyze the influence of the operating parameters (e.g. temperature, nature of solvents, etc.) both on the effectiveness of the techniques used and on the component composition of the extracts obtained.

Each of the extracts obtained, as noted quite correctly, is a very complex mixture, the composition of which may vary depending on the type of solvent applied and its polarity – e.g. strongly or less polar (water vs ethanol). However, I believe that a brief analysis of the influence not only of the nature of the solvents, but also of the temperature regime as well as extraction time, would be very useful in obtaining important new information which can subsequently make it possible to identify the extraction method and/or the combination of conditions that allow the production of low-toxic biologically active extracts with the desired composition.

Regarding subsection 4.2.9, the following terminological clarification should be made: In the specialized literature, the process used by the PhD student and called "fractionation", is more correctly to be referred to as "sequential/multistep liquid-liquid extraction". In liquid-liquid extraction, a process of exchange of certain compounds between two solvents that are either partially or completely immiscible with each other is realized, and in the multi-step extraction, a new solvent is added at each subsequent step.

In other words, the term 'sequential extraction' should be used when the chemical differences between the extracts obtained are due to different solubility of their components in different solvents. While the term 'fractionation' should be used when the chemical differences between the resulting fractions are due to a different distribution of the individual components between two phases. These clarifications refer to the terminology, and in no way affect the reliability of either the results obtained by the PhD student or their interpretation. In other words, the term 'sequential extraction' should be used when the chemical differences between the extracts obtained are due to different solubility of their components in different solvents. While the term 'fractionation' should be used when the chemical differences between the resulting fractions are due to a different distribution of the individual components between two phases. These clarifications clarify the terminology, but in no way affect the reliability of the results obtained by the PhD student and their interpretation.

Section 5 is devoted to the analysis of *in vitro* studies of the cytotoxicity of the obtained extracts against different cell lines, and the PhD student correctly noted that it is necessary to control very carefully "location of collection, presence or absence of stumps in the biomass, clearly defined biomass/solvent ratios during extraction, etc.", I would also add biomass preparation, etc. (p. 70). Controlling these "important details" is a prerequisite for obtaining reliable and reproducible results, and their correct subsequent comparison and interpretation.

It is worth noting in particular the fact that Alexander, in the course of his training, not only got acquainted with, but mastered highly effective analytical methods for the analysis of the obtained extracts - HPLC, CZE/CCD and UHPLC-MS/MS, and proposed optimized procedures for the analysis of the content of the neuroactive alkaloids ibotenic acid (IBO), muscimol (MUS), as well as ergosterol, a substance with proven anticarcinogenic activity.

As already mentioned, the research carried out by A. Dushkov are a combination of precise experimental work and the use of modern methods for analysis and methods for processing the results obtained. The data is credible, interpreted creatively and competently. Their significance is systematized and summarized concisely as Conclusions (Section 6).

This, in turn, has allowed six Contributions to be formulated (Section 7), with specificity and accuracy of expression, which is rare (at least in my review practice, where I have seen descriptions of contributions within pages). I would, however, note that, in my opinion, if the order of importance is followed, contributions 4, 5, 6 should be placed respectively in places 1, 2 and 3, firstly, because they directly relate to the topic of the dissertation and are in the field of the scientific specialty molecular biology, and secondly, because in this way they will be in sync with those made in section 6 of the Conclusions.

There is a complete correspondence between the dissertation and its Abstract/summary.

Publications and contributions of the PhD student:

The scientific achievements of Assistant Dushkov on the topic of the dissertation are summarized in two publications in specialized international journals IF, quartiles Q1 and Q4 respectively. In both

publications, Alexander is the lead author, which is an undoubted confirmation of his personal participation in the elaboration, interpretation of the results and the formulation of conclusions.

Particularly impressive are the indicators of the article published in *Molecules* (Q1). According to the journal's statistics, it has been "accessed" so far over 6400 times and has 9 citations - unfortunately, a fact that has not been noted by the dissertation. The high citation rate of this scientific work, published in 2023, is a confirmation of the serious interest of the international academic community, and is direct evidence of the relevance of the topic and the significance of the results obtained. The second publication in *Bulg Chem Commun* (Q4) also has 2 citations, according to SCOPUS.

In connection with the publication activity of the PhD student, it should also be noted that according to the above-mentioned Regulations, the minimum required points for groups of indicators, in this case for the scientific degree of Doctor, are 30 points from indicators from 5 to 10. Alexander not only fully covers them - but exceeds them (25 points for an article published in a Q1 journal, and 12 points for publication in Q4 journal).

Alexander Dushkov has 4 participations in international scientific events and another three - in national ones, however the type of presentation - respectively a poster or an oral presentation - is not specified.

Since the results of the study "Analysis of the Ibotenic Acid, Muscimol, and Ergosterol Content of an *Amanita Muscaria* Hydroalcoholic Extract with an Evaluation of Its Cytotoxic Effect against a Panel of Lung Cell Lines In Vitro" were presented at two scientific forums, I believe that the following notation should be used: **Title, Type** (oral/poster), **Presented at:** 1. Name of the scientific event, place, date; 2. (analogous sequence), except in the case if at one of the events it was an oral presentation, while at the other – a poster.

Personal impressions:

I have known Alexander for over two years and I have excellent impressions of his knowledge, thoroughness, competence and attitude to scientific challenges. I especially note the very good knowledge of English.

I would also like to say that reading his dissertation gave me pleasure, not only because it is excellently structured, elegantly written and in very good Bulgarian, but also because it pointed out new and extremely relevant topics for possible future cooperation. If I have to extrapolate (with a wink), after *Amanita Muscaria* that inspired the creative process of Alexander, I am confident that we will be able to identify many other plants (even those considered and treated as weeds), forgotten herbs, etc., of which the flora of Bulgaria is so rich, as objects of an integral approach, focused on the prospects for transforming their biomass into valuable bioactive components with a wide range of applications.

CONCLUSION

The presented dissertation is an up-to-date, complex and completed research with serious scientific contributions, which fully meets all the requirements outlined in the corresponding Law and Procedures on its implementation.

All this gives me grounds to confidently vote positively and to recommend to the honorable Scientific Jury to award to Alexander Nikolaev Dushkov the educational and scientific degree of "Doctor" in the professional field 4.3. "Biological Sciences", Scientific specialty "Molecular Biology".

11.03.2025 r.

Sofia

Member of the Jury

Prof. Roumiana P. Stateva, PhD