



REVIEW

for the dissertation work for the acquisition of the scientific and educational degree "doctor" (PhD)

on the topic "Natural low-toxic biologically active extracts from Bulgarian mushrooms with a focus on *Amanita muscaria* - analysis of the composition, antitumor activity and mechanism of action",

of Alexander Nikolaev Dushkov, full-time doctoral student at the Laboratory of Chromatin Structure and Function, Institute of Molecular Biology "Acad. Rumen Tsanev", Bulgarian Academy of Sciences, in the scientific direction 4.3. Biological Sciences (Molecular Biology)

with scientific supervisor Prof. Dr. Iva Ugrinova

By Prof. Dr. Tanya Ivanova Topuzova-Hristova, Department of Cell and Developmental Biology, Faculty of Biology, Sofia University "St. Kliment Ohridski"

Data about the doctoral student and the doctoral program.

Alexander Nikolaev Dushkov graduated with a Bachelor degree in Molecular Biology from the Faculty of Biology at Sofia University "St. Kliment Ohridski" in 2019 and a Master's degree in Cell Biology and Pathology from the same faculty at Sofia University "St. Kliment Ohridski" in 2021 with excellent results. Since 2018, he has been working as a technician, laboratory assistant, biologist and later as an assistant in the "Chromatin Structure and Function" section at the Institute of Molecular Biology, BAS. Alexander Dushkov was enrolled as a full-time doctoral student in 2021 at the the Laboratory of Chromatin Structure and Function of the Institute of Molecular Biology at the Bulgarian Academy of Sciences, in the scientific field 4.3. Biological Sciences (Molecular Biology) with scientific supervisor Prof. Dr. Iva Ugrinova. The doctoral student was dismissed with the right to defend his/her thesis on time, and all requirements according to the Regulations for the Implementation of the Law on the Development of the Academic Staff of the Republic of Bulgaria and the Regulations for the Development of the Academic Staff of the Institute of Molecular Biology "Acad. Rumen Tsanev" at the Bulgarian Academy of Sciences, Section 4, have been met and there are no violations. The minimum national requirements according to the Law on the Development of the Academic Staff in the Republic of Bulgaria, Promulgated by the State Gazette No. 38 of May 21, 2010, amended by the State Gazette No. 81 of October 15, 2010, amended by the State Gazette No. 101 of December 28, 2010, amended by the State Gazette No. 68 of August 2, 2013, amended and supplemented

by the State Gazette No. 30 of April 3, 2018, amended by the State Gazette No. No. 17 of February 26, 2019, amended by SG No. 17 of February 25, 2020.

Dissertation data.

The topic of the dissertation "Natural low-toxic biologically active extracts from Bulgarian mushrooms with a focus on *Amanita muscaria* – analysis of the composition, antitumor activity and mechanism of action" accurately reflects its content. The dissertation covers 114 pages, contains 47 figures and 4 tables. The topic of the dissertation is actual and of a scientific-applied nature. The discovery of new biologically active substances with potential antitumor activity, as well as the clarification of their mechanisms of action are of great interest for biomedical and pharmacological research in search of new drugs.

The main parts of the dissertation follow the generally accepted plan for such work and include: Literature review – 31 pages, Aims and objectives – 1 page, Materials and methods – 20 pages, Results and Discussion – 28 pages and Conclusions - 1 and contributions – 1 page.

The literature review provides an overview of data on the main tumor diseases in humans, the pharmacological applications of biologically active metabolites from 5 species of mushrooms included in the study, DNA damage and cellular stress and their role in tumorigenesis. This part of the dissertation is written in understandable language, concisely and clearly, sufficiently motivating the choice of a scientific problem for further research.

The formulated goal and objectives logically follow from the review, and the materials and methods used are described correctly and clearly, in sufficient detail to be repeated. The aim of the dissertation is to study the antitumor effect of extracts obtained from different species of mushrooms distributed on the territory of Bulgaria. 8 human cell lines were used - one from normal diploid cells and 7 with tumor origin, which were tested with water and alcohol extracts from a total of 5 species of mushrooms (wood mushrooms and fly agaric).

The set of methods includes determination of cytotoxicity by MTT, transformation of bacteria and transfection of human cells with fluorescently tagged proteins to determine the formation of stress granules, immunofluorescence analysis, analysis of cell cycle and synthetic phase, analysis of newly synthesized RNA by click reaction, fractionation and analysis of the composition of the extracts by High Performance Liquid Chromatography and Capillary Electrophoresis with a non-contact capacitively coupled conductivity detector. Basic theoretical principles that justify their use are explained for the applied methods, accompanied by the necessary schemes. The experiments were carried out precisely, and the obtained results were interpreted logically. A discussion was made to the presented results, which could be expanded in view of more data on the chemical composition and possible candidates for the observed cytotoxic effect and selective effect. This issue is particularly important,

considering that the studied alcoholic and aqueous extracts of *Amanita muscaria* do not contain ergosterol, which has proven antitumor activity. Is there any data on another potential candidate with such an action?

Most of the figures included in this section are microphotographs from immunofluorescence microscopy studies and graphical quantification of cell viability and chemical composition analyses and give an idea of the large amount of work that has been done. The results are well illustrated and explained, allowing the drawing of 6 conclusions regarding the action of the extracts on the studied cell lines, as well as the composition of the red fly agaric extracts. Some of the conclusions are formulated as a summary of the results obtained and need better precision. In addition to the conclusions, 6 contributions of the dissertation work are also formulated, three of which are related to the optimization of the methods for chemical composition analysis.

Scientific apparatus

248 sources were cited, which allow for a comprehensive review of scientific achievements on the topic. Some of the sources related to the incidence of cancer (over 20) are from medical data sites such as Medical News Today, American Cancer Society, HWO, which, although based on scientific data, are not essentially scientific publications with an explained methodology and experimental or analytical approach and should not be cited as sources for a strictly scientific work such as a doctoral dissertation. The same applies to the botanical description of mushrooms, where Wikipedia is cited as a source (a total of 5 items) and the pages cited as separate sources with information about cell lines from the ATCC, Cyton and Cellosaurus websites (a total of 15). I recommend that the doctoral student refrain from such citations in his future scientific works, and to orient himself towards strictly scientific sources that have undergone at least double scientific review. When using photos or graphics from the Internet in complex or illustrative figures and diagrams, the source could be cited under the figure itself, and commercial sites are cited only in the Materials and Methods section, and not as references at the end of the paper. Another note is that two of the cited sources were published in Japanese, two in German and one in French, and one of them is cited twice – in Bulgarian and in translation. These sources were probably cited by someone later, and were not used in the original. It is good to avoid such a practice in subsequent works. The remaining citations are made in compliance with the established standards for citing scientific literature.

Abstract of the thesis

The abstract contains 50 pages and fully reflects the content of the dissertation work. The main results are correctly presented, illustrated with a total of 19 figures, which include graphs, color microphotographs and diagrams. The conclusions and contributions are presented correctly and correspond to those in the dissertation work. Publications on the topic of the dissertation are also included, as well as a shortened list of cited literature used mainly in the discussion of the results.

Publications

The main results of the dissertation work have been published in two articles in scientific journals with an impact factor and with quartiles Q1 and Q4. In both publications, the doctoral student is the first author, which reflects the significant contribution to their development. According to the requirements of the Academic Staff Development Act, the two published articles carry a total of 32 points, which exceeds the required minimum of 30 points. In addition to these publications, Alexander Dushkov has attached a list of 7 participations in national and international conferences, at which he has popularized the results obtained in his dissertation research. This scientific and research activity characterizes Alexander Dushkov as a well-established and active young scientist with excellent prospects for future development.

Scientific and applied scientific contributions

The following contributions from the dissertation are indicated: optimization of a new chromatographic method for analyzing the content of the alkaloids ibotenic acid and muscimol, as well as the substance ergosterol in a complex mixture of substances; optimization of a capillary-electrophoretic method with non-contact conductivity detection for analyzing the content of the alkaloids ibotenic acid and muscimol in a complex mixture of substances; and optimization of a new UHPLC/MS-MS method for analyzing the content of the alkaloids ibotenic acid and muscimol in a complex mixture of substances. Since these are not essentially new protocols, but optimization of existing ones, they are rather part of the tasks completed in connection with the dissertation, and not independent applied scientific contributions. The remaining contributions are related to the assessment of the cytotoxic effect of a panel of different types of extracts of wood mushrooms, distributed on the territory of Bulgaria, on cancer cell lines; evaluation of the cytotoxic and cytostatic effect of *Amanita muscaria* tincture on cell cultures in vitro and the first information on the accumulation of stress granules in cell cultures in vitro treated with *A. muscaria* extract. The latter contribution is personally the most intriguing for me and has the potential to develop into an interesting independent topic for research.

Conclusion

In conclusion, a comprehensive study of the pharmacological potential of extracts of biologically active substances from mushrooms from Bulgaria is presented. An in vitro assessment of the cytotoxic effect of a panel of different types of tree mushroom extracts and of *Amanita muscaria* tincture on seven human cell lines of tumor and non-tumor origin was performed and the first data on the accumulation of stress granules in cells treated in vitro with the extract were obtained. An analysis of alkaloids and certain biologically active substances such as ergosterol and psychotropic substances in the used *A. muscaria* extracts was performed. The obtained results provide a basis for the development of a promising topic on the mechanisms of action of plant and mushroom components on tumor cells in vitro.

Based on the presented materials, I believe that the doctoral student fully meets the requirements of the ZRASRB for awarding the scientific and educational degree "doctor" and I recommend that Alexander Dushkov be awarded the scientific and educational degree "doctor" in scientific direction 4.3. Biological Sciences, scientific specialty Molecular Biology.

Reviewer:

City of Sofia

/Prof. Dr. Tanya Topuzova-Hristova/