



**To the Esteemed Members of the Scientific Jury,**

Appointed by Order No. 218-OB/20.12.2024 of the Director of IMB-BAS, regarding the defense of the doctoral dissertation of Alexander Nikolaev Dushkov, a full-time doctoral student in the Structure and Function of Chromatin section at IMB-BAS, under the supervision of Prof. Iva Ugrinova, PhD

## REVIEW

by Prof. Margarita Apostolova, PhD

Head of Medical and Biological Research Laboratory, Rumen Tsanev Institute of Molecular Biology – BAS

on the dissertation for the awarding of the educational and scientific degree "Doctor" in the doctoral program "Molecular Biology" within the professional field 4.3. Biological Sciences, under the category 4. Natural Sciences, Mathematics, and Informatics.

**Dissertation 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".

I hereby declare that I have no conflict of interest as defined in § 1, item 2a, nor any restrictions related to affiliated parties as per § 1, item 5 of the additional provisions of the Act on the Development of the Academic Staff in the Republic of Bulgaria. I have no co-authorship with the doctoral student in the submitted publications. Furthermore, my review found no evidence of plagiarism.

### **I. Overview of the Procedure and the Doctoral Student.**

The doctoral student has been granted the right to defend his thesis within the deadline. The submitted set of materials on electronic media (dissertation and abstract) complies with the Regulations for the Implementation of the Law on the Development of the Academic Staff of the Republic of Bulgaria (Promulgated by the State Gazette No. 75 of September 24, 2010; amended by the State Gazette No. 19 of March 8, 2011; amended by the State Gazette No. 9 of January 31, 2012; amended by the State Gazette No. 62 of July 12, 2013; supplemented by the State Gazette No. 60 of July 22, 2014; amended by the State Gazette No. 57 of July 28, 2015; amended and

supplemented by the State Gazette No. 56 of July 6, 2018; amended and supplemented by the State Gazette No. 15 of February 19, 2019; amended by SG No. 76 of September 5, 2023; and amended by SG No. 23 of March 18, 2025) and the Regulations for the Development of Academic Staff at IMB-BAS. The minimum national requirements according to the Law on the Development of the Academic Staff in the Republic of Bulgaria have been met (Promulgated in the Official Gazette No. 38 of May 21, 2010; amended in the Official Gazette No. 81 of October 15, 2010; amended in the Official Gazette No. 101 of December 28, 2010; amended in the Official Gazette No. 68 of August 2, 2013; amended and supplemented in the Official Gazette No. 30 of April 3, 2018; amended in the Official Gazette No. 17 of February 26, 2019; amended in the Official Gazette No. 17 of February 25, 2020).

Alexander Dushkov has provided a list of 2 publications related to his dissertation work, with a total impact factor of 4.2 (JCR-IF, Web of Science). The articles were published in journals classified in quartiles 1 and 4, receiving 11 citations (Scopus). The doctoral student has participated in 7 scientific forums focused on the dissertation's theme, presenting at 6 of them.

## **II. Relevance of the Topic, Understanding the Problem, and Suitability of the Goals and Achieved Results**

The dissertation presented for defense addresses a significant issue related to studying the antitumor effects of various pharmacologically relevant substances found in mushrooms native to the Republic of Bulgaria. Mushrooms represent a vast yet underexplored and underutilized source of potential new pharmaceutical products. Many species contain bioactive polysaccharides with proven antitumor and immunostimulatory properties, making the subject of the dissertation particularly valuable.

The relevance of this topic is undeniable, as cancer-related diseases are the second leading cause of mortality, following cardiovascular diseases, with a concerning upward trend in morbidity in recent years. Given their profound social impact and the limited research on Bulgarian mushrooms, the subject of this dissertation is both timely and significant.

The dissertation by Alexander Dushkov consists of 117 pages, structured according to the requirements for obtaining the educational and scientific degree of "Doctor. " It includes four tables and 47 figures. The sections are as follows: Introduction (2 pages), Literature Review (38 pages), Goals and Objectives (1 page), Materials and Methods (20 pages), Results and Discussion



(38 pages), Conclusions (1 page), and Contributions (1 page). A total of 248 literary sources were cited.

The introduction effectively establishes the topic's relevance, while the literature review comprehensively examines its core problem. The goals and objectives are clearly outlined and relevant. Cellular, chemical, and molecular biological methods are modern and precise and produce reliable results.

The research scope is sufficient to produce both qualitative and statistically reliable results. However, no chapter outlines the statistical methods employed to analyze the findings.

To perform the tasks, a set of 8 human cell lines was used - one from normal diploid cells (MPC-5) and 7 of tumor origin - A549, NCL-H1299, PC-3, MDA-MB231, A375, A375-KRAS, HS 895 T.

The complex experiments conducted to achieve the dissertation's goals demonstrate that the doctoral student has mastered a range of modern, informative methods for conducting scientific research. These methods include cell line cultivation, cell viability assessment using the MTT method, FACS analysis, bacterial transformation, human cell transfection, fluorescence microscopy, image analysis, analysis of newly synthesized RNA via a click reaction, and composition analysis of extracts using HPLC and CZE.

Regarding the methodology, it is essential to note that ergosterol is susceptible to oxidation when exposed to air, resulting in degradation over time, particularly during the 21-day extraction process described in the dissertation. Oxygen dissolved in ethanol may contribute to this process. While ethanol helps preserve sterols, a 50% ethanol-water mixture introduces water, which may also promote hydrolysis. High drying temperatures (e.g., above 60–70°C) can cause thermal degradation of ergosterol, resulting in losses. The methodological description does not indicate whether the extraction was conducted in the dark and drying was carried out in a dehydrator at 73 °C overnight. For the reasons described above and for the proper use of the reference standard, it would be beneficial to follow the same preparatory procedures. Before conducting future research, it is necessary to obtain detailed information about the method of drying used to prepare fragmented mushroom bodies, as ergosterol is sensitive to oxygen and can degrade into oxidized derivatives when exposed to air, especially during prolonged drying. Exposure to UV and visible light can degrade ergosterol or convert it into vitamin D<sub>2</sub> (ergocalciferol), thereby reducing its overall content.

The dissertation presents the results in detail and a logical manner, supported by appropriate figures and tables. The findings clearly demonstrate the different levels of cytotoxic effects of ethanol extracts on various cell lines. The impact of *Fomitopsis betulina* is most pronounced, with the aqueous extract also demonstrating effectiveness. *A. muscaria* shows significant cytotoxicity against lung and prostate cancer cell cultures. An analysis of the neuroactive alkaloids that may potentially be responsible for the cytotoxic effect was also conducted: ibotenic acid, muscimol, and ergosterol. Notably, the finding is that ergosterol is absent in quantities that would lead to cytotoxicity. However, the absence of ergosterol suggests that other compounds with potential antitumor effects may be present in the extracts. Have there been efforts to identify them?

An analysis of the molecular and cellular changes that occur after treatment with *A. muscaria* extract on the proliferative activity of lung cancer cell lines A549 and H1299, as well as prostate cancer cell line PC-3, was also performed. It was concluded that the reduction of cells in the S-phase, established after the incorporation of EdU in A549, is relatively more prevalent than in H1299. The statement is relatively accurate; however, the fluorescent photographs indicate that the treatment was conducted with a concentration of 0.9%, whereas the statistical analyses reflect a concentration of 0.45% (Figures 39, 40, 41, and 42). The same inaccuracy was made in Figures 43, 44, 45, and 46.

Studies related to FACS analysis of the cell cycle of A549 cells treated with *A. muscaria* for 24 hours at concentrations close to and greater than the  $IC_{50}$  confirm a decrease in cells in the S-phase. Since the different experiments were performed with concentrations close to  $IC_{25}$ ,  $IC_{50}$ , and  $IC_{90}$  for varying treatment durations, it would be beneficial to include a summary table with all these values in the dissertation.

Importantly, the presence of stress granules (SGs) in the cytoplasm of A549 and H1299 cells was detected using G3BP1 covalently linked to a fluorescent FLAG tag after treatment with the ethanolic extract of *A. muscaria*. Since SGs promote therapy resistance, metastasis, and survival, targeting them could be a promising anti-cancer strategy.

Based on the experiments conducted, six key conclusions have been drawn. Six contributions have also been formulated, precisely and specifically. All of them are supported by the experimental data, with which I agree.

The dissertation and its abstract are fully aligned.

I have the following questions for the dissertation candidate:

1. Could the antitumor effect of *A. muscaria* be attributed to its polysaccharides?
2. Is the formation of SGs favorable after treatment with the ethanol extract of *A. muscaria*?

**III. Conclusion:** Based on extensive scientific research, I conclude that **Alexander Nikolaev Dushkov** possesses the skills to plan and execute experiments aligned with established goals, as well as the ability to discuss the obtained results. The dissertation is contemporary, utilizing modern methodologies with clear objectives and presenting significant results and conclusions. It provides undeniable scientific and applied contributions. The dissertation by **Alexander Nikolaev Dushkov** fully meets the requirements outlined in the Law on the State of the Republic of Bulgaria concerning the Promotion of Scientific Research, as well as the Regulations regarding the Conditions and Procedures for acquiring scientific degrees and holding academic positions at the Institute of Molecular Biology. I provide a **positive assessment** of the dissertation and recommend that the esteemed Scientific Jury award **Alexander Nikolaev Dushkov** the educational and scientific "Doctor" degree in the doctoral program in Molecular Biology "

Sofia, 19.03.2025

Prof. Margarita Apostolova,