

OPINION

From: Prof. Evdokia Pasheva, D.Sci.

Subject: PhD thesis entitled: "Natural low-toxic biologically active extracts of Bulgarian mushrooms with a focus on *Amanita muscaria* – analysis of composition, antitumor activity and mechanism of action" by PhD student Alexander Dushkov.

The dissertation submitted to me for an opinion contains 117 pages and is richly illustrated, which contributes to the easy reading and analysis of the results. The bibliographic reference includes 248 titles. The literature review is entirely related to the topic, highlighting the growing trend of searching for anti-cancer agents among plant and fungal species as a valuable resource in choosing therapy. The wide variety of plant and fungal species on the one hand and the specific modes of action related to antioxidant, anti-inflammatory and antimicrobial properties of many of them motivate the scientific community to study new species and establish antitumor potential. There is still not enough information in the literature about the antitumor potential of mushrooms, which motivates the dissertation to focus on this topic. The goals and objectives set are clearly formulated and logically follow the conclusions made in the literature review. To achieve the results in the dissertation, modern methods are used, which the PhD student has mastered. It is noteworthy that the analysis of the results is based entirely on the evidence obtained in the laboratory. Various types of tree mushroom extracts were used to determine the cytotoxic effect, and different cell cultures were used to determine possible selectivity. Specific substances that would be responsible for the cytotoxic effect have also been analyzed, such as the neuroactive alkaloids ibotenic acid (IBO) and muscimol (MUS), as well as ergosterol (ERG). After optimizing the chromatographic method for determining the content of the three components, the results obtained showed that the ERG did not present in quantities sufficient to induce the cytotoxic effect observed in MTT tests. The changes in the cell under the action of *A. muscaria* extract were also analyzed. The presence of aggregates of newly synthesized RNA in the cytoplasm of cells has been found, which is atypical for untreated cancer cells. It has been proven that these structures are the so-called stress granules by coincidence of their localization with the signal of the FLAG-labeled protein G3BP1. These observations suggest that cells of the A549 and H1299 lines form cytoplasmic SGs when treated with amounts of ethanol extract from the red fly agaric close to its IC25 value. When studying the effect of *A.*

Muscaria extract on the proliferative activity of cancer lung cell lines A549 and H1299 and prostate cancer PC3, interesting results were obtained that suggest a certain selectivity. The reduction of S-phase cells in A549 is comparatively more prevalent compared to in H1299. One explanation offered by the thesis for this difference is related to the presence of more mesenchymal characteristics in H1299, the absence of active p53 and a pronounced higher proliferative activity, which suggests a weaker effect of the extract. The result for PC3 is intriguing. Analysis of the number of S-phase cells in the presence of A. muscaria extract at doses close to the established IC50 values in the medium of PC3 line cells showed that the treatment did not lead to visible or a statistically significant decline, suggesting that the extraction will not favor the therapy of patients suffering from prostate cancer. The conclusions and contributions in the dissertation are specific and are based on the original results obtained.

Recommendation: I believe that the dissertation would benefit if at the end of it there was a summarized short discussion in which to analyze and summarize the original results obtained. It would be useful to mark what potential is revealed for possible future developments, since the topic has not been studied in detail so far and there is not much accumulated literature data.

Conclusion: In the scientific literature, there is an increasing interest in studying the properties of plant extracts as possible antitumor agents. That is why I consider that the chosen topic of the PhD thesis is relevant and up-to-date. For its development, modern methodologies have been applied, which the candidate has mastered and optimized. The conclusions from the original results obtained have been analyzed on the basis of the experimental data combined with the conclusions of current publications on the topic. Two articles related to the dissertation have been published in Q1 (25 points), Q4 (12 points), in which Dushkov is the first author, which unequivocally shows his significant participation in the development of the problem. All stated above gives me the reason to most confidently recommend to the respected jury to award the PhD student Alexander Dushkov the educational and scientific degree of "Doctor".