

OPINION

Subject: evaluation of dissertation work for the acquisition of an educational and scientific Doctor of Philosophy degree (PhD)

Author of the dissertation: Antonia Georgieva Diukendjieva-Todorova, MSc

Dissertation topic: "*In silico* and *in vitro* studies of ADME/TOX properties and molecular interactions of flavonolignans from *Silybum marianum* L. (milk thistle)"

Scientific organization: Institute of Biophysics and Biomedical Engineering at the Bulgarian Academy of Sciences (IBPBMI-BAS), "QSAR and Molecular Modeling" section

Scientific supervisors: cor.-mem. Prof. Ilza Pajeva, DSc and Prof. Ivanka Tsakovska, PhD

Member of the scientific jury: Assoc. Prof. Yordan Nikolaev Georgiev, PhD, Eng., Department of Physiology, Pathophysiology, Chemistry and Biochemistry at the Faculty of Medicine of the Prof. Dr. Assen Zlatarov University - Burgas, E-mail address: yordan.georgiev@uniburgas.bg

1. General preparation of the materials under the procedure

By Order №708/12.09.2023 of the Director of IBPBMI-BAS, I was elected as a member of the scientific jury and appointed to prepare an opinion on the present procedure. The candidate for the defense of the PhD degree, a full-time doctoral student Antonia Georgieva Diukendjieva-Todorova, MSc, has submitted a set of documents for the procedure in an electronic format, which contains: an application for admission to the defense procedure, protocols and certificates for passed exams, a short professional biography, a copy of the diploma for the educational and qualification Master's degree, dissertation in English, summary of the dissertation in Bulgarian and English, list of scientific publications on the dissertation, copies of scientific publications and list of noted citations on the dissertation. The documents for the procedure have been carefully prepared and are in accordance with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Rules for the Implementation of the LDASRB and the IBPBMI-BAS Regulations for the Implementation of the LDASRB from 12.03.2021.

2. Relevance of the problem developed in the dissertation in scientific and scientific-applied terms

The search and discovery of valuable bioactive molecules of therapeutic importance from medicinal plants for the preparation of phytotherapeutics, their (semi)synthetic analogues or nutraceuticals for the needs of dietary and/or functional nutrition will never subside. Silymarin, which is extracted from the seeds of milk thistle (*Silybum marianum* L. Gaertn., Asteraceae), through various extraction approaches with water, aqueous-alcohol mixtures and other solvents, is a typical example of a commercially available extract with enormous therapeutic potential, included in a number of clinical trials. It has long been known mainly for its hepatoprotective properties, which are associated with its antioxidant, immunomodulatory, anti-inflammatory effects, and regulation of carbohydrate and lipid metabolism. Silymarin also exhibits antimicrobial, antitumor, antidiabetic, neuro- and cardioprotective activities, etc.. Its main active molecules are several flavonolignans called silybin A and B, isosilybin A and B, silydianin, silychristin, the flavonoid dihydroquercetin,

etc.. The anticancer effects of silymarin deserve special attention and are associated with modulation of MAPK, PI3K/Akt, Wnt/ β -Catenin, STAT3, and NF- κ B signaling pathways, leading to cell cycle suppression, tumor growth, angiogenesis and metastasis, and activation of apoptosis. In the search for the molecular basis for the manifestation of an anticancer activity by some of the flavonolignans of silymarin, in the present thesis it is shown that they can affect the action of the estrogen receptor alpha (ER α), the B-raf serine/threonine protein kinase, involved in MAPK pathway, and the Smoothed receptor (SMO) of the Hedgehog signaling pathway. The potential effects of silybins as agonists/antagonists of ER α may prove important for the treatment of oncological and cardiovascular diseases. It is possible that mutations in the BRAF gene are associated with uncontrolled cell growth and tumorigenesis (e.g., in melanoma), and therefore finding inhibitors of this kinase is of therapeutic importance. Similarly, mutations in the SMO gene are also associated with oncogenesis and flavonolignans can be considered as potential therapeutic inhibitors. That is why it can be summarized that the scientific problems involved in the developed dissertation work are relevant and lead to the achievement of meaningful and significant scientific and scientific-applied goals for the protection of human health.

3. Degree of knowledge of the state of the problem in the literature review

The literature review of the dissertation work is placed on 45 pages, and it is written in a scientifically sound style and fully corresponds to the research goals of the dissertation. Antonia Diukendjieva-Todorova has conducted a thorough analysis of the available literature in the research area, including literature sources published in the period from 1960 (including 1 article from 1868) to 2023. The review is divided into sub-chapters, in which are discussed as follows: 1) the role of natural compounds in the development of drugs for the treatment of socially significant diseases, with a brief historical overview; 2) the composition and structural features of the silymarin extract isolated from milk thistle (*S. marianum* L.) and some of its pharmacological activities, with an idea of the metabolism of some of the active ingredients, toxicity and future prospects for the application of silymarin; 3) application of integrated *in vitro/in silico* experimental approaches to study pharmacological effects of natural compounds, with an emphasis on the advantages of some *in silico* methods (QSAR, Molecular docking, Molecular similarity), such as the possibility to study the biological effects of optical isomers of the active compounds and *in vitro* artificial models for the study of absorption of biomolecules in the gastrointestinal tract. Evidence of the in-depth critical analysis of literary data is the discovery of unexplored aspects of scientific problems and the suggestion of new research niches. This suggests that Antonia Diukendjieva-Todorova has conducted an adequate literature review of part of the scientific literature devoted to the scientific problems studied in the dissertation work. Essentially, the literature review is well structured, organized, and concise.

4. Correspondence of the chosen research methodology with the set goal and tasks of the dissertation work

The purpose of the dissertation work is precisely and clearly formulated, and is logically related to the scientific problems addressed in the literature review. Namely, the study of absorption, distribution, metabolism, excretion and toxicity, and unraveling the molecular mechanisms of anticancer action of flavonolignans from milk thistle-derived silymarin. The presented scientific tasks are current and aim at the search for solutions to real

problems in the field of Theoretical Chemistry regarding molecular interactions, molecular modeling and prediction of the structure-activity relationship, with application in Experimental Biology and Medicine. The methodology of the research is in accordance with the set goals and tasks of the dissertation work. PhD student Diukendjieva-Todorova has used a wide range of *in silico* and *in vitro* methods to predict membrane permeability, gastrointestinal absorption of flavonolignans, metabolism, receptor recognition (human ER α) and structure-activity relationship. She has applied interesting modern *in vitro* molecular biological and biochemical techniques for an integrated evaluation of the potential anticancer properties of flavonolignans in silymarin as cytotoxic compounds against human tumor cell lines, interaction of biomolecules with B-raf kinase, and SMO receptor of the Hedgehog signaling pathway.

From the attached experimentarium, it can be seen that the doctoral student has mastered a set of mathematical, chemical, biochemical and biological methods.

5. Results and contributions of the dissertation work

The thesis investigated various flavonolignans (e.g. silybin A and B, dehydrosilybin A and B) contained in silymarin as potential new anticancer agents through a combination of *in silico* theoretical simulation approaches. Subsequently, *in vitro* experimental molecular biological analyzes were also performed by treating some target proteins in the development of antitumor therapies, such as B-raf V600E kinase and SMO receptor, in both tumor (A-375 and A-431) and normal cell lines (HaCaT). The main results of the *in silico* analyzes were also confirmed by the *in vitro* biological experiments. Very good membrane permeability was observed for the dehydrogenated silybins in the *in silico* and *in vitro* assays. It has been suggested that the most likely metabolization of flavonolignans from silymarin is via glucuronidation and oxidative *O*-demethylation. Molecular docking analyzes demonstrate that silybin B can bind to ER α . The most potent *in vitro* inhibitory effect on B-raf V600E kinase activity of all tested flavonolignans was shown by dehydrosilybin B with IC₅₀= 24.9 μ M. Dehydrosilybin A (IC₅₀= 83 μ M) and B (IC₅₀= 86 μ M) expressed higher inhibitory activities on the development of A-375 human malignant melanoma cell line, compared to silybin A (IC₅₀= 97 μ M) and B (IC₅₀= 120 μ M), and the first two substances also exhibited lower cytotoxic effects on normal human keratinocytes (cell line HaCaT). It is interesting to note that dehydrosilybin A and the racemic mixture of dehydrosilybin A and B possessed potent inhibitory activities on the Hedgehog signaling pathway, playing the role of potential inhibitors of this pathway. At the same time, a mixture of silybin A and B, and dehydrosilybin B alone did not lead to a similar activity. In addition, dehydrosilybin A and dehydrosilybin AB were able to attach as antagonists for the G-protein-coupled receptor SMO, the mutated form of which is involved in the development of tumours.

The conclusions are clearly and correctly formulated, entirely based on the experimental data obtained, without entering into uncertain hypotheses. I fully support and welcome scientific contributions. Essentially, they can be summarized as follows: 1) A new QSAR model was developed to predict membrane permeability and gastrointestinal absorption of bioactive molecules after oral administration; 2) It has been found that milk thistle flavonolignans can be absorbed in the gastrointestinal tract to achieve adequate bioavailability; 3) The silybin diastereoisomers of silymarin can interact with ER α , suppress the action of B-raf kinase and the SMO receptor, thus explaining their *in vitro* anticancer

effects on human malignant melanoma and epidermoid carcinoma; 4) The most active antitumor agents of the flavonolignans studied from silymarin are the optical isomers dehydrosilybin A and B, which are interesting from a pharmacological point of view.

6. Evaluation of the publications and presentation of the dissertation work

The results of the dissertation have been published in 4 articles in scientific journals that fall into the leading first (Q1, 1 paper.) and second quartiles (Q2, 3 papers). The scientific publications have a high overall impact factor of 11.39, with the doctoral student being the first author in all four articles. They have been cited a total of 43 times as of the date of submission of the dissertation according to the Web of Science/Scopus data basis, which is a testament to the relevance and quality of the research. In addition, two reports from participation in conferences have been published in full text, in which the doctoral student Diukendjieva-Todorova is also the first author. The research on the dissertation was presented by the doctoral student at 5 international scientific forums and three national conferences. The development of the dissertation is related to the implementation of two young scientist projects within the BAS, as well as participation in an international and accompanying national project under the COST program. The candidate conducted part of the *in vitro* experiments in the dissertation during specialization at the University of Siena in Italy. The publication activity on the dissertation fully meets and exceeds the requirements in the IBPBMI-BAS Regulations for the implementation of the LDASRB from 12.03.2021.

The summary of the PhD thesis is well-formed and reflects the overall results obtained in the dissertation work.

7. Opinions, recommendations, notes and questions

Regarding the literature review, I have the following remarks:

The introduction of abbreviations should be either in a list at the beginning of the dissertation or just straight into the text where they first appear. In the literature review, it would be useful to consider the immunomodulatory effects of silymarin in the treatment of the socially significant diseases considered, in more detail the structure-activity relationship and receptor recognition of the active ingredients, where possible, as well as to present the doses, animal models (as some cell lines were included in the *in vitro* studies presented) and duration of treatment for the observed positive effects.

Regarding the design of the Materials and Methods section, I have the following recommendations:

The cell concentrations of the cell lines investigated and the dosage of B-raf kinase can be added in the section.

The remarks or recommendations made only partially affect the technical layout, not the scientific content of the dissertation work, and in no way can underestimate the seriousness of the doctoral student, her hard work, as well as the high professionalism of the scientific supervisors in the development and writing of the dissertation work. Rather, they should be useful into the future work of the doctoral student as a researcher and author of scientific content.

The decision to write the dissertation in English is commendable, making the full text of the work easily accessible in the international scientific space.

I have the following questions for the PhD student:

1. Do you have literature or other information on the molecular mechanisms of absorption of flavonolignans from silymarin in the gastrointestinal tract?
2. Can you predict what the metabolic fate of unabsorbed flavonolignans from silymarin will be in the large intestine under the action of gut microflora?
3. What explanation can you give for the higher suppressive activity of the racemic dehydrosilybin AB mixture than that of silybin A and B, and the lack of activity of the silybin AB mixture on the Hedgehog signaling pathway?

8. Conclusion with a clear positive or negative evaluation of the dissertation work

The presented dissertation work is up-to-date and represents a comprehensive and complete scientific study in the field of Theoretical Chemistry, Biochemistry and Experimental Biology, according to the set goals and objectives. A prerequisite for that is the qualification of the doctoral student and adequate guidance from the scientific supervisors. During her studies, Antonia Diukendjieva-Todorova from secondary education, through the Bachelor's course in Biotechnology, the Master's course in Biochemistry and doctoral studies at the IBPBMI-BAS, developed on the border between Chemistry and Biology. During this entire period, she has accumulated the necessary basic knowledge and experience to work as a qualitative researcher.

Due to all of the above, I convincingly **give a positive assessment** of the dissertation work and **propose to the honorable jury to vote on awarding the educational and scientific PhD degree to Antonia Georgieva Diukendjieva-Todorova, MSc**, in the field of Higher Education 4. Natural Sciences, Mathematics and Informatics, by Professional direction: 4.3. Biological Sciences and scientific specialty: "Application of the principles and methods of Cybernetics in the field of structure-activity dependencies of biologically active compounds".

22.11.2023
Burgas

Member of the jury:.....
/Assoc. Prof. Yordan Georgiev, PhD, Eng./