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REVIEW REPORT

from Prof. Irini Doytchinova, DSc.,

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On the PhD thesis of **Antonia Georgieva Diukendjieva-Todorova**, PhD student in the Institute of Biophysics and Biomedical Engineering at the Bulgarian Academy of Sciences, with title: IN SILICO AND IN VITRO STUDIES OF ADME/TOX PROPERTIES AND MOLECULAR INTERACTIONS OF FLAVONOLIGNANS FROM SILYBUM MARIANUM L. (MILK THISTLE) for awarding the educational and scientific degree "Philosophy Doctor" in AHE 4. Natural sciences, mathematics and informatics, PD 4.3 Biological sciences, DP "Application of the principles and methods of cybernetics in the field of structure-activity relationships of biologically active substances"

Antonia Diukenjieva's PhD thesis is in the field of drug design and investigates the ADME/Tox properties and modes of action of flavonolignans from *Silybum marianum* (milk thistle) using *in silico* and *in vitro* methods. Milk thistle is a medicinal plant known for centuries, primarily for its hepatoprotective effects attributed to silymarin. Silymarin is the total extract from the plant and includes silybin (racemate), isosilybin (racemate), silychristin, silydianin, and other flavolignans and flavonoids in smaller quantities. In recent years, interest in milk thistle has grown due to its proven protective properties on the cardiovascular, nervous, endocrine, and immune systems. The aim of the PhD thesis is to examine the pharmacokinetic and toxicological properties of flavonolignans from *Silybum marianum* and clarify the molecular mechanisms underlying some of their pharmacological effects.

The dissertation consists of 146 pages and includes 11 chapters: literature review, objectives and tasks, materials and methods, results and discussion, contributions, acknowledgments, a declaration of the originality of the results, references, publications related to the PhD thesis, contributions to scientific events, and two appendices. It is enriched with 16 tables and 25 figures. The bibliography comprises 425 titles.

In the literature review, Diukenjieva demonstrates a strong understanding of both the subjects under investigation and the methods for *in silico* and *in vitro* analyses of the properties and modes of action. The importance of natural products as sources of drug molecules from ancient times to the present is examined, along with their advantages over synthetic drugs. Special attention is given to the flavonolignans from *Silybum marianum*, their diverse therapeutic applications, ADME properties, and toxic effects. A comprehensive

overview is provided of the *in silico* and *in vitro* approaches used to study the pharmacological and ADME/Tox properties of natural products, with a closer look at the QSAR models in the literature on permeability determined through a parallel artificial membrane permeability assay (PAMBA). The literature analysis shows that flavonolignans are a promising group of natural compounds with potential in several therapeutic areas, and further *in vitro* and *in silico* studies on their intestinal permeability and specific modes of action are needed.

The aim of the study directly stems from the conclusions drawn in the literature review and determines seven clearly defined tasks, grouped into two directions: *in vitro* analysis and QSAR modeling of intestinal absorption and toxic effects, and *in silico* identification of novel target proteins for flavonolignans, followed by *in vitro* validation.

In the chapter "Materials and Methods," Diukenjieva thoroughly and comprehensibly describes the *in silico* and *in vitro* methods, software tools and expert systems she used in her PhD thesis.

The results are structured into two directions: ADME/Tox and new targets. Intestinal permeability was measured using the PAMBA test (logPe), and a pH-dependent relationship was found. As weak acids with pKa values around 7.4, the absorption of flavonolignans is pH-dependent and increases at pH values lower than their pKa, where non-ionized molecules dominate. Such a dependency is absent for compounds with an additional double bond in the flavone ring, such as 2,3-dehydrosilybin and quercetin, which further polarizes the O-H bond and lowers the pKa values. In the measured pH range, both molecules are ionized, and their absorption is pH-independent.

A QSAR model for intestinal permeability logPe of 269 compounds was derived, and it was found that the distribution coefficient at pH 7.4, $logD_{7.4}$, and the polar surface area per unit molecular weight determine the permeability of the compounds. The model is logical, statistically significant, and has good predictive ability, validated by an external test set of 783 compounds. It was used to assess the permeability of the tested flavonolignans, and the predicted values are close to the experimental values, except for silicristin and silidianin, where the chromone ring of the lignan is replaced with benzofuran. The model is included and freely accessible in the database of the European Union Reference Laboratory for Alternatives to Animal Testing.

The toxicity of the compounds was assessed by the expert systems Derek Nexus and Meteor Nexus and revealed three toxic hazards related to chromosomal damage, modulation of the estrogen receptor ERα, and skin sensitization. The interaction with ERα was modeled *in silico* by molecular docking, and stereoselective binding of silybin B, similar to the estradiol agonist, was found. This finding is a scientific novelty and sheds light on a putative xenoestrogenic toxicity of flavonolignans.

In the second direction of the PhD research, two new targets related to the antitumor activity of flavonolignans have been identified. These targets are the BRAF kinase and SMO protein. BRAF kinase with the V600E mutation is a proto-oncogene found in various malignancies such as colorectal cancer and melanoma, while the SMO protein is part of the

Hedgehog signaling pathway, and its inhibition has application in the treatment of basal cell carcinoma. The targets were identified by virtual screening of a database of antineoplastic drugs for structural similarity with the studied flavonolignans. Two molecules, vemurafenib and vismodegib, were found to be structurally similar to silybin A and B, and dehydrosilybin A and B. Vemurafenib is an inhibitor of BRAF kinase, while vismodegib inhibits the SMO receptor. Subsequent docking simulations on both proteins demonstrated that the binding of flavonolignans is stereoselective and driven by the formation of several hydrogen bonds and π-π interactions with amino acids from the protein binding site. Dehydrosilybins showed a stronger affinity for both proteins compared to silybins. The obtained *in silico* results were experimentally validated, which is a significant contribution to the work. The inhibitory effects of the tested compounds on the proteins were assessed *in vitro* as well as the anti-cancer activity on tumor and healthy cell lines. Unfortunately, in the *in vitro* studies, vemurafenib and/or vismodegib were not used as reference molecules, which would have provided information about the significance of the found activities. Despite the low selectivity indexes (not provided in the PhD thesis), ranging between 1.2 and 2.8, the studied compounds can be considered hit molecules whose structure can be further optimized into a lead molecule and even to a candidate drug.

Diukenjieva has formulated four contributions of her PhD thesis: the QSAR model for intestinal permeability, the experimentally determined permeability coefficients logPe of the five most common flavonolignans in *Silybum marianum*, the *in silico* hypothesis for interaction with the estrogen receptor ERα, and the newly identified and confirmed target proteins related to their anti-cancer activity of the tested compounds. I fully accept these contributions and consider them solid evidence for the quality of the PhD thesis. The PhD student has acquired a wide range of *in silico* methods for analyzing natural compounds and applied them in practice, formulating scientific hypotheses that she experimentally validated.

The results of the PhD thesis have been published in four articles, three of which are in journals with IF, one in a journal with SJR, and two as reports in proceedings. Two of the articles are in journals from Q1, and the other two are in journals from Q2. The publications have been cited 49 times (Scopus, October 25, 2023). Antonia Diukenjieva is first author in all six publications. The results have been presented at five international and three national scientific forums. The PhD research was funded by projects for young scientists from the Bulgarian Academy of Sciences, the National Science Fund, and a COST action. According to the Regulations of the Bulgarian Academy of Sciences for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the PhD candidate must have 30 points in a group of indicators G, formed by three publications related to the PhD thesis, of which at least one in an international journal with an IF according to WoS, and at least one article as first author. All three requirements have been exceeded. The four publications bring a total of 80 points to Diukendjieva (2 x 25 points for Q1 + 20 points for Q2 + 10 points for SJR without IF). Her first position in the authorship list of the publications is evidence that the PhD thesis is her personal achievement.

No plagiarism in the PhD thesis and the associated publications was found, using the application StrikePlagiarism (strikeplagiarism.com) approved by the Ministry of Education

and Science. The results are original and are obtained within Diukendjieva's own scientific research, as stated by herself in her PhD thesis.

I give a **positive assessment** of Antonia Diukenjieva's PhD thesis and, as a member of the scientific jury, confidently recommend to the Scientific Council of the Institute of Biophysics and Biomedical Engineering at the Bulgarian Academy of Sciences that she be awarded the educational and scientific degree "Doctor" in AHE 4. Natural Sciences, Mathematics, and Informatics, PD 4.3 Biological Sciences, DP "Application of the principles and methods of cybernetics in the field of structure-activity relationships of biologically active substances."

My reasons for this evaluation are as follows:

1. During her training in the doctoral program, Diukendjieva acquired new knowledge and practical skills for conducting thorough scientific research in the field of drug design.
2. The PhD student has applied the acquired knowledge and skills to the study of the ADME/Tox properties and modes of action of natural products from the species *Silybum marianum*. The results obtained have been published in two prestigious scientific journals, *Phytomedicine* and *Antioxidants*, which is recognition of their high scientific value.
3. The PhD student covers and exceeds the minimum requirements for the scientific activity of candidates for the acquisition of the educational and scientific degree of "Philosophy Doctor" in AHE 4. Natural Sciences, Mathematics, and Informatics, according to Appendix 1 of the Regulations for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria of the Bulgarian Academy of Sciences and the national Regulations for the Implementation of the Law on the Development of the Academic Staff in the Republic of Bulgaria. The total sum of points for Diukendjieva in the group of indicators G is 80, while the minimum requirement is 30 points.

Sofia, October 26, 2023 Reviewer:

(Prof. Irini Doytchinova)