Bulgarian Academy of Sciences

Institute of Biophysics and Biomedical Engineering

Department of QSAR and Molecular Modelling

IN SILICO AND *IN VITRO* STUDIES OF ADME/TOX PROPERTIES AND MOLECULAR INTERACTIONS OF FLAVONOLIGNANS FROM *SILYBUM MARIANUM* L. (MILK THISTLE)

AUTHOR'S SUMMARY OF

A DISSERTATION THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

ANTONIA GEORGIEVA DIUKENDJIEVA-TODOROVA

SCIENTIFIC FIELD: 4. "NATURAL SCIENCES, MATHEMATICS AND INFORMATICS"
PROFESSIONAL FIELD: 4.3. "BIOLOGICAL SCIENCES"
SCIENTIFIC SPECIALTY: "APPLICATION OF THE PRINCIPLES AND METHODS OF
CYBERNETICS IN THE FIELD OF STRUCTURE – ACTIVITY RELATIONSHIPS
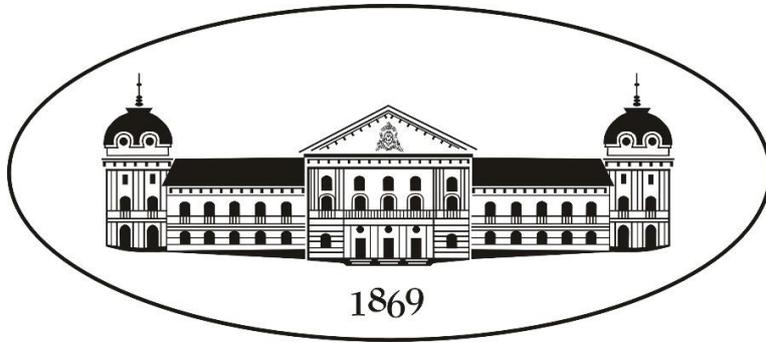OF BIOLOGICALLY ACTIVE SUBSTANCES"

SUPERVISORS:

PROF. IVANKA TSAKOVSKA, PhD

and

CORR. MEMBER PROF. ILZA PAJEVA, DSc

Sofia, 2023

1869

The defense of the dissertation will be on ……….…….. at ….……………… in ……………….…...

The dissertation was discussed at an extended scientific seminar of Department of QSAR and Molecular Modelling, Institute of Biophysics and Biomedical Engineering on 28 July 2023.

The dissertation consists of introduction, aims and objectives, three chapters, contributions,

list of cited literature (202 literary sources), a list of publications and participation in scientific forums, 2 appendices and its volume is 144 pages.

Author: Antonia Georgieva Diukendjieva-Todorova

Title: *In Silico* and *In Vitro* Studies of ADME/Tox Properties and Molecular Interactions of Flavonolignans from *Silybum Marianum* L. (Milk Thistle)

TABLE OF CONTENTS

ABBREVIATIONS

ABL - aqueous boundary layer

ADME/Tox - absorption, distribution, metabolism, excretion, and toxicity

BC - BODIPY-Cyclopamine

DS - Double-Sink

F - variance ratio

GIA - gastrointestinal absorption

HH - Hedgehog

ERα - human estrogen receptor alpha

logP - octanol-water partition coefficient

logD - octanol-water distribution coefficient

LOO - leave-one-out

MW – molecular weight

NP – natural product

PAMPA - parallel artificial membrane permeation assay

pKa - acid dissociation constant

PSA - polar surface area

Q^2 - cross-validated multiple correlation coefficient

QSAR - quantitative structure–activity relationship

R^2 - multiple correlation coefficient (determination coefficient)

SEE - standard error of estimate

SMO – Smoothened

TPSA - topological polar surface area

TSA – total surface area

INTRODUCTION

For millennia, humans have used naturally occurring substances for various medical purposes. Nowadays natural products (NP) continue to be recognized as a primary resource for drug discovery with particular effectiveness in cancerous and infectious diseases. They possess multiple beneficial characteristics enabling them to interact with pharmacological targets more efficiently than synthetic compounds. It is envisaged that the technological advances in biosynthetic engineering, genomics and computational tools will reinforce NP-based drug discovery in both established and newly introduced fields.

An example for a well-known NP that is used since antiquity, but only recently was shown to exert many new effects, is silymarin, the active extract derived from the medicinal plant *Silybum marianum* (L.) Gaertn (milk thistle). Silymarin's main constituents (flavonolignans) are identified as multi-target compounds with potential for use in the treatment of oncological, neurological, cardiovascular, infectious and metabolic diseases. They are under especially intense research in the clinical therapy of cancer for chemoprevention, treatment, and amelioration of chemotherapy-associated side effects. However, many of the mechanisms of action as well as the relevant targets of flavonolignans in the context of human pathologies are not well understood. Additionally, the pharmacokinetic properties of all individual components in silymarin are understudied which hinders the evaluation of possible variations in the features of different silymarin formulations. In this context, the integration of *in silico* studies to silymarin research may be considered as especially useful in providing reliable cost- and time-saving strategies to better understand their pharmacological properties.

Therefore, the aim of this study is to evaluate properties and to elucidate mechanisms of action, related to potential pharmacological effects of flavonolignans from *Silybum marianum,* using both *in silico* and *in vitro* investigations. An interdisciplinary approach based on *in silico* quantitative structure–activity relationship (QSAR) predictions and *in vitro* parallel artificial membrane permeation assay (PAMPA) measurements was used for evaluation of gastrointestinal absorption (GIA) of all major components of the milk thistle. Additionally, potential toxic effects and metabolic transformations were predicted *in silico* and possible interactions with the estrogenic receptor alpha were further studied as a potential mechanism of toxicity. A combined *in silico / in vitro* analysis clarified mechanisms of anticancer activities of flavonolignans and identified BRAF kinase and Smoothened receptor (SMO) as their novel pharmacological targets.

CHAPTER 1.   LITERATURE REVIEW

1. Role of natural products in drug development.

The NPs have been used by humans since antiquity for various medical purposes and they continue to provide inspiration for modern drug design notably due to the vast diversity of their chemical structures and biological activities (Li et al. 2019).

Recent reports, assessing the past 30 years of developments in drug discovery worldwide, show that despite the research focus was shifted away from NPs, they are a major source of novel lead compounds or pharmacophores for medicinal chemistry. An analysis of NPs as sources of new drugs from 01/1981 to 09/2019 demonstrate that at the end of the third quarter of 2019 the NP field is still highly productive, with 441 of the 1394 small molecules (32%) falling into this overall category over the complete time period (Newman and Cragg 2020).

NPs possess special characteristics in comparison with conventional synthetic molecules, which result in both advantages and challenges for the drug discovery process. The reduced NP drug discovery efforts by the pharmaceutical industry in the past few years may be largely associated with the multifaceted difficulties associated with NPs research: the need for harvesting adequate amounts of biological material for the production of pharmaceutical preparations, variability of the biological material, complications in the isolation and/or purification procedures, toxicity of some active compounds. Despite all difficulties, the modern approaches in biosynthesis, biochemistry and engineering of natural compounds has witnessed an unprecedented series of breakthroughs (Wright 2019). Firstly, the processes that govern the production of NPs from various sources are becoming clearer thus enabling informed modification and manipulation. Secondly, the rapid and cost-effective sequencing of whole genome of NP producing organisms has revealed various genes involved in NP biosynthesis and shown that frequently these genes are assembled in clusters within the genomes that facilitate identification (Wright 2017).

Earlier comparative studies of NPs with chemically synthesized molecules have already shown that NPs possess more favourable characteristics associated with active target engagement including increased numbers of sp3-hybridized carbons and of chiral centres, fewer aromatic rings, larger macrocyclic aliphatic rings, lower nitrogen content and increased oxygen content, all contributing to more complex three-dimensional structures. These properties enable NPs to interact with biological targets more effectively rather than the more planar and less

stereochemically complex features that are predominant in synthetic compound libraries (Rodrigues et al. 2016). Indeed, results from some large screening collections suggest that diversity within the biologically relevant 'chemical space' is probably more important than the library size (Harvey, Edrada-Ebel, and Quinn 2015). The beneficial chemical characteristics of NPs can be further enhanced through medical chemical strategies to produce compounds with improved drug-like properties. These include stability to host biochemical processes and targeting specific tissues, and extension of the receptors range. Moreover, NP-derived substructures appear to be particularly suited for fragment growth into ligand-efficient and selective new chemical entities (Grabowski, Baringhaus, and Schneider 2008).

2. Flavonolignans – a small subgroup of natural compounds with big potential as drug leads.

Silybum marianum (L.) Gaertn, commonly known as milk thistle, is an annual or biennial plant of the *Asteraceae* family, native to the Mediterranean and North African regions (Khan, Blackshaw, and Marwat 2009), that is well known since ancient times for its various therapeutic effects. In the past milk thistle has been used mainly to treat kidney, spleen, liver, and gallbladder diseases (Abenavoli et al. 2018). Its beneficial health-related activities have been proven by many researchers over the years and in the 1970s the extract of milk thistle fruits, silymarin, was classified by the World Health Organization as an official medicine with hepatoprotective properties (Bijak 2017). Nowadays milk thistle has retained its popularity with silymarin herbal supplements being among the top-selling ones in the USA in 2015 (Andrew and Izzo 2017). In the recent decades studies addressing silymarin's antioxidant and anti-inflammatory capacities and its ability to modulate cellular signalling pathways have also gained the interest of pharmacologists. The results indicate silymarin as a multi-target substance with potential for use in the treatment of oncological, neurological, cardiovascular, infectious and metabolic diseases as well as a skin protector in dermatology and cosmetics (X. Wang, Zhang, and Wu, 2020).

The proven ability of silymarin to act on key stages of carcinogenesis, namely initiation, promotion and tumour progression, makes it a promising agent for chemopreventive strategies and as an adjuvant in chemotherapy. The potential of these applications should be further studied in well-planned clinical trials to determine precisely to the adequate doses and the actual efficacy of this extract in different types of cancers. Additionally, to improve the efficacy and

avoid possible side effects of silymarin, its mechanism needs to be elucidated in relation to its chemical compounds and their action on disease pathogenesis.

In the past decades extensive studies were conducted to explore the metabolism and transport of silymarin as well as the impact of its consumption on the pharmacokinetics of other clinical drugs. However, it should be noted that pharmacokinetic studies were mainly performed on silybin, the major component of silymarin. Therefore, possible variations in oral content, dissolution, and oral bioavailability of other flavonolignans in silymarin should be considered, since they are shown to be responsible for the distinct beneficial effects apart from silybin (Camini and Costa 2020).

Studies of acute, subacute and chronic toxicity have indicated favourable safety profiles of silymarin in humans and only transient side effects like gastrointestinal upsets were seen in some studies, however again, most toxicity studies did not discuss absorption and formulation of silymarin (Soleimani et al. 2019). As silymarin contains several flavonolignans the investigation of these substances separately could provide a more thorough safety profile of various silymarin preparations and explain better possible interactions when co-administered with other drugs.

Silymarin is a complex extract from milk thistle flowers, roots, and mainly fruits containing flavonolignans (about 70%–80% *w*/w) as well as polymeric and oxidized polyphenolic compounds consisting of a mixture of flavonoids. Flavonolignans are a relatively small subclass of compounds, whose common name suggests that the molecular skeleton could be divided in two moieties (flavonol and lignan), but either hybrid or non-conventional lignans can be considered more appropriate for silymarin compounds group (Csupor, Csorba, and Hohmann 2016). Due to the presence of chiral centers in the common skeleton, flavonolignans occur as stereoisomers in natural sources, most commonly denoted as A and B. The main silymarin flavonolignans are silybins A and B, isosilybins A and B, silychristins A and B, isosilychristin and silydianin (Kvasnička et al. 2003) (Figure 1). The main component of silymarin - silybin is present as a quasi-equimolar mixture of diastereoisomers A and B. Silybin is small, but highly functionalised molecule with alternating carbocycles and heterocycles, and, due to its structure, it is quite resistant to reduction but oxidizes easily to 2,3-dehydrosilybin (Abenavoli et al. 2018).
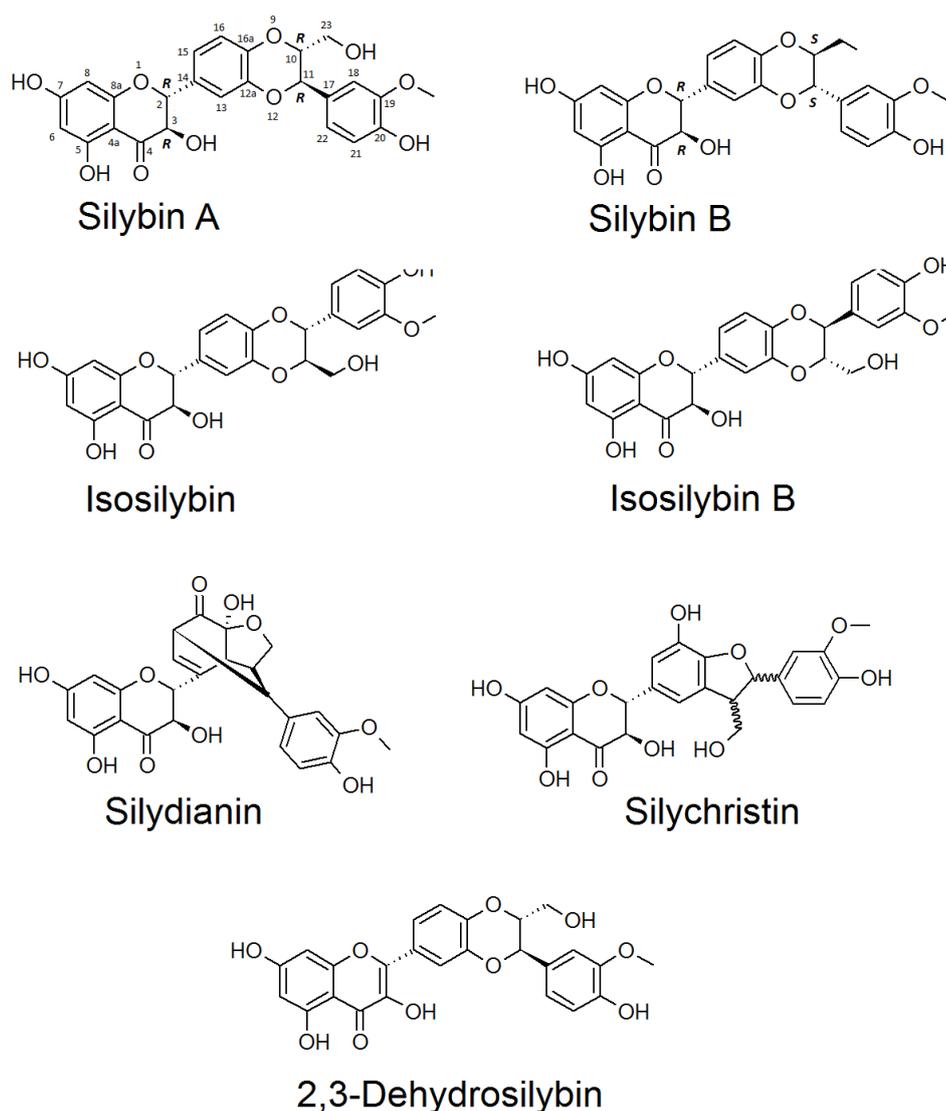
Figure 1. Major flavonolignans in silymarin. The structure of silybin A is numbered according to a proprietary numbering system used in most chemical papers. For simplicity, the chiral centers are shown for silybins only. Adapted from Biedermann et al. 2014 and Lorenzo et al. 2020.

Being the main flavonolignan in silymarin and thanks to its easy isolation, silybin is often assigned as the sole contributor of biological activity of the whole extract. However, there is evidence that other individual components contained in silymarin are selectively or synergistically responsible for various bioactivities. Therefore, the precise analytical determination of silymarin components is essential prior to any biological study to keep reproducibility and validity of the results (Petrásková et al. 2020). Additionally, it is indicated that stereochemistry plays an important role for multiple biological activities of flavonolignans and there is a need for more focused research on the pure forms of the compounds that are otherwise therapeutically used as mixtures (Křen 2021). However, determination,

quantification and separation of optically pure compounds is often considered as difficult to conduct in a single run and at reasonable time due to the complex nature of silymarin containing structurally related compounds. In this context, the integration of *in silico* studies to silymarin research before or in parallel with experimental validation may be especially useful as these allow for working with individual chemical structures providing reliable cost- and time-saving strategies to better understanding their pharmacological properties.

3. Integration of *in silico/in vitro* approaches to pharmacological studies of bioactive NP compounds.

In recent years, the integration of *in silico* approaches to NP research has made significant contributions to the understanding of chemistry and biology of NPs including expanding the drug relevant chemical space and unveiling more putative macromolecular targets. *In silico* modelling has become a necessary tool for drug discovery and is referred as major accelerator of the reawakening interest in NP-based drug discovery efforts. Integrated computer-assisted strategies are indispensable in processing huge amount of available structural and biological information in a reasonably short time and may have a substantial impact on the discovery success rate. As a rule, NPs are characterized with high structural diversity and complexity and very often engage high number of targets. Bearing in mind that high throughput *in vitro/vivo* experiments for studying the pharmacology of NPs are cost- and time-consuming, highly efficient *in silico* predictions serve as rapid and economical strategies to decipher NP-target associations, prior to experimental validation (Moumbock et al. 2019). Additionally, *in silico* predictions of ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) properties play an important role in facilitating the appropriate selection of NPs as candidate drugs since poor pharmacokinetic properties and toxicity issues are considered the main reasons for terminating the development process for drug candidates. The significance of *in silico* modelling has become more apparent as ADME/Tox properties are increasingly being evaluated at an earlier stage of the drug development process (Alqahtani 2017), especially in the research of unmodified NPs which may display unfavourable ADME/tox profiles (International Natural Product Sciences Taskforce et al. 2021).

In the sections below the main *in silico* approaches and tools to study molecular interactions and ADME/Tox properties of NPs that have been used in this PhD thesis are presented.

3.1. QSAR analyses.

The fundamentals of QSAR modelling that attempts to predict biological activities of chemical structures by analysis of characteristics of structure features dates back to the nineteenth century. In 1868, Crum-Brown and Fraser published Eq (1), which is considered to be the founding formula for the development of QSAR concept: the physiological activity, Φ, is expressed as a function of the chemical structure C (Brown and Fraser 1868).

$$\Phi = f(C) \tag{1}$$

Various QSAR studies have been carried out to understand biological effects at different levels e.g. interactions with macromolecules, cellular responses, antimicrobial, antifungal, antioxidant, anticancer, anti-inflammatory activities, etc. (Peter et al. 2019). As regarded to ADME/Tox properties numerous QSAR and related approaches have successfully made their way in the form of software and new methods to predict ADME/Tox profile are continuously introduced to the drug discovery community. The main purpose of these continuously evolving predictive ADME/Tox approaches is to reduce late-stage drug development failures by focusing on the most promising drug lead(s) with desired ADME/Tox properties (Silva and Trossini 2014).

Classical QSAR analysis (Hansch analysis) may be defined as the application of multivariate statistical analysis approaches, namely regression and classification methods, on the pursuing of quantitative relationships between the biological activity of a set of congener compounds and physicochemical properties. This knowledge field assumes that the behaviour of a substance in the biological environment depends on its structural characteristics, which affect its overall properties (Rudrapal and Egbuna 2022).

3.2. *In silico* approaches and tools to study molecular interactions and ADME/Tox properties of NPs.

In this work the following approaches have been applied to study:
(1) molecular interactions of NPs: molecular docking and similarity search;
(2) ADME/Tox properties of NPs: expert systems for ADME/Tox assessment.

Molecular docking

Molecular docking is the most common computational structure-based drug design approach that has been widely used ever since the early 1980s to simulate molecular interactions

between two molecules (most commonly a protein and a small molecule) and may provide a prediction about the binding mode and affinity between them (Stanzione, Giangreco, and Cole 2021). It is a useful tool in drug discovery programs when the 3D structure of the protein target is available and allows for virtual screening of large numbers of small molecules to facilitate target and hit identification. The application of molecular docking in NP-based drug discovery programs can help to explain some traditional uses and potentially identify new uses for medicinal plants (Asiamah et al. 2023).

The molecular docking process involves prediction of the ligand conformation as well as its position and orientation within the protein binding site (usually referred to as a pose) and assessment of the quality of the pose using a scoring function to evaluate the binding energy between the ligand and the receptor. For a molecular docking procedure, the basic requirements are the availability of structures of receptor and ligand (most usually a small molecule), conformational search procedures for sampling the receptor and ligand conformations, and ranking or scoring scheme to find out the best docking orientation. The 3D structure of receptor can be obtained by experimental methods such as X-ray crystallography and accessed through sources of crystallographic structure of proteins and protein-ligands complexes such as Protein Data Bank (PDB) (https://www.rcsb.org/). The structure of ligands can be obtained from chemical databases, or the structures can be drawn using software. These structures have to be sampled for their minimum energy, physiologically stable conformations using search algorithms.

Molecular similarity searching

Molecular similarity is a key concept that has been routinely used in the discovery and design of new molecules. It is based on the notion that two molecules often share similar physical properties and biological function if they are structurally similar (Kumar and Zhang 2018). Similarity methods for searching databases of chemical structures are usually applied early in drug discovery programmes when little is known about the biological target of interest.

Molecular similarity analysis comprises two main components - structural representations and quantitative measurements of similarity between two molecular structures. Many types of structural representations have been suggested to measure the similarity between two molecules. These include physiochemical properties, topological indices, molecular graphs, pharmacophore features, molecular shapes, molecular fields, etc. Additionally, there are various methods to quantify the similarity between two structural representations, e.g., Tanimoto

coefficient, Tversky index, Dice index, cosine coefficient, Euclidean distance, etc. Among these, Tanimoto coefficient (Rogers and Tanimoto 1960) is the most popular and widely used similarity measure. Based on the structural representation, molecular similarity approaches can be classified into 2D or 3D (shape) similarity methods.

Shape similarity methods have been successfully utilized as a virtual screening tool in drug discovery to identify molecules similar to a given query from the library of chemicals with both retrospective and prospective studies utilizing this approach being published in the literature. It is also frequently combined with structure-based methods and several groups adopted it to improve the performance of molecular docking (Kumar and Zhang 2018). One application of shape similarity methods is to hop from one chemical scaffold to another in order to improve the potency, selectivity, physicochemical properties of lead compounds in drug discovery. Scaffold hopping may be highly effective in rescuing problematic leads that cannot be pursued further due to problems in selectivity, pharmacology and pharmacokinetics. Another application is *in silico* target fishing or the identification of new protein targets of chemical compounds.

Expert systems for ADME/Tox assessment of bioactive compounds.

In silico methods used for the prediction of various properties including ADME/Tox properties in the drug discovery belong to two classes – 1) expert systems and 2) data-driven (statistical) systems. The expert systems utilize the knowledge of human experts while data-driven systems rely on the experimental data (Silva and Trossini 2014). Expert systems employ a reasoning engine that solves problems (or makes predictions) by applying rules from a knowledge base in response to single or multiple queries (or hypotheses). Advantages of expert systems include the transparency of the predictions made and the capacity for the human expert developing the rules to assimilate diverse information types in a way that cannot be easily replicated by automated analysis (Marchant, Briggs, and Long 2008). The last decades have seen a steady rise in the use of expert systems that aid the drug discovery process, particularly in the area of toxicity. Both commercial and open source systems are available such as Derek Nexus, among others like ToxTree, CASE Ultra Expert Rules, and Leadscope Expert Alert system (Brigo, Naga, and Muster 2022). The accessibility of modern modelling techniques, powerful computational resources and good-quality data have made it possible to generate reliable predictions for new chemical entities, impurities, chemicals, NPs and a lot of other substances (Machhar et al. 2019).

QSAR PAMPA permeability models.

GIA is a key ADME property when the biological effects of substances are evaluated. PAMPA has gained popularity as a screening method of choice for determining passive transcellular permeability, which is the main GIA mechanism for many drugs. Recently the PAMPA assay has attracted increasing interest from various other industrial sectors, including cosmetics, where non-animal models may provide a crucial source of information for *in vitro - in vivo* extrapolation.

PAMPA is an experimental model introduced by Kansy et al. (Kansy, Senner, and Gubernator 1998) to predict the oral absorption of new therapeutic agents in a simple, reproducible and high throughput manner. The assay measures effective/apparent permeability and/or the fraction of the permeated test compound. A limitation of PAMPA is that active and efflux transporters are not modelled by the PAMPA membrane. Despite this, it has been shown that PAMPA permeability correlates well with Caco-2 cell permeability and human intestinal absorption *in vivo*, this undoubtedly being a result of the fact that most of the known drugs are absorbed via passive diffusion. The correlations were confirmed to be statistically reliable in studies utilising linear regression reported by Ano et al. (Ano et al. 2004), Fujikawa et al. (Fujikawa et al. 2005; 2007), Verma et al. (Verma, Hansch, and Selassie 2007).

The common experimental set-up of the PAMPA consists of: 1) donor and acceptor compartments, containing an aqueous solution of the test molecule and aqueous buffer initially free of the test molecule respectively; (2) an artificial membrane, which is composed of a variety of organic solvents or phospholipid mixtures, and used to separate the donor and acceptor compartments; and (3) a filter, used for immobilisation and stabilisation of the membrane. A number of PAMPA variants have been developed which differ in membrane composition, presence of specific ingredients in the acceptor chamber, and permeation models used for calculation of the permeability coefficients.

A number of datasets with PAMPA permeability values are published in the literature where the permeability parameter estimated, literature sources and the main experimental conditions are presented. The largest publicly available dataset has been collected by Avdeef and coworkers and summarised in Avdeef 2012. It comprises Double-Sink (DS) PAMPA intrinsic and effective permeability coefficients determined for nearly 300 compounds (mostly commercial drugs).

The usability of QSAR models for PAMPA relies on the fact that permeability (particularly

when combined with aqueous solubility and pKa) can be applied as a predictor of GIA of orally administered drugs (Avdeef 2012; Avdeef et al. 2007). The models reported in the literature follow the classical QSAR approach employing experimental and/or theoretical structural descriptors to formulate a relationship with permeability. Most models are based on multiple linear regression or partial least squares statistical methods. Only a small number of models apply artificial neural networks to incorporate nonlinear dependencies. Generally, simple models based on few descriptors are easier to interpret than models based on many descriptors relating permeability in a complex way (Stenberg et al. 2002). In addition, the main requirements for good QSAR practice have to be followed, including thorough model validation and applicability domain analysis (Worth et al. 2005).

QSAR models for PAMPA relate the relevant permeability parameter, e.g. apparent permeability coefficient (P_a), or flux, with physico-chemical properties such as the octanol-water partition coefficient (logP), the acid dissociation constant (pKa) and the octanol-water distribution coefficient (logD) or structural descriptors including the polar surface area (PSA), the surface area of hydrogen bond donors or acceptors; Abraham solute descriptors; indicator variables for specific functional groups; VolSurf parameters, etc.

The QSAR models of PAMPA permeability may be a valuable tool, particularly in the initial steps of drug design. They may assist drug designers in the estimation of the passive intestinal permeability of drugs and other bioactive compounds thus saving time and reducing costs as by the potential elimination of *in vivo* and /or *in vitro* experiments. However, only a few large PAMPA permeability datasets are available in the public domain and a relatively small number of QSAR models of PAMPA permeability are reported compared to some other endpoints. Therefore, there is need for construction of large diverse compounds datasets that would be useful for development of reliable and highly predictable models with a broader applicability domain. Another potential enabler of the broader use of QSAR models for prediction of PAMPA permeability and estimation of GIA is the introduction of reliable models employing calculated parameters, especially when that can be easily obtained, especially from free software resources.

4. Conclusions.

NPs have been used since antiquity for various medical purposes and they continue to provide inspiration for modern drug design notably due to the vast diversity of their chemical structures and biological activities. In the recent years a renewed interest in NPs as drug leads is recorded mainly due to their favourable characteristics associated with active target

engagement and it is envisaged that that NP research will be reinforced by advances in analytical techniques, biosynthetic engineering, genomics and computational tools.

Silymarin, an extract from the well-known medicinal plant *Silybum marianum*, is a NP that offers multiple health benefits for individuals and has retained the interest of pharmacologists on account of its antioxidant and anti-inflammatory capacities and its ability to modulate cellular signalling pathways. Various studies indicate silymarin as a multi-target substance with potential for use in the treatment of oncological, neurological, cardiovascular, infectious and metabolic diseases as well as a skin protector in dermatology and cosmetics. Silymarin's constituents, mainly flavonolignans, are under especially intense research in the clinical therapy of cancer for chemoprevention, treatment, and amelioration of chemotherapy-associated side effects. The broad spectrum of biological activities of silymarin components suggests their potential as lead compounds in the context of multifaceted pathologies and offers an attractive possibility to further enhance the therapeutic potential of these molecules through suitable chemical modifications of their structure. In line with this prospect, there is need for more focused efforts on elucidating the mechanisms of action and the relevant targets of flavonolignans in the context of human pathologies. Additionally, more precise evaluation of silymarin's ADME/Tox properties should be performed - possible variations in bioavailability, metabolic transformations and safety profile of individual flavonolignans in silymarin should be considered, since they are shown to be responsible for distinct effects apart from the main component, silybin. Furthermore, it is indicated that stereochemistry plays an important role for multiple biological activities of flavonolignans and there is a need for more focused research on the pure forms of the compounds that are otherwise therapeutically used as mixtures (Křen 2021). In this context, the integration of *in silico* studies to silymarin research may be especially useful as these allow for working with individual chemical structures providing reliable cost- and time-saving strategies to better understanding their pharmacological properties. Indeed, *in silico* predictions have proven their potential to aid in deciphering NP-target associations and in evaluation of ADME/Tox properties in multiple studies. The vast variety of computational approaches may be used at different stages of NP research prior or in parallel with *in vitro* and *in vivo* methods. *In silico* methods are notably valuable in providing mechanistic explanations of anticipated and observed effects at molecular and even atomic level. The diversity of available *in silico* methods can be applied in combinations with experimental techniques to extract new knowledge about the properties and the mechanisms of action of NPs more efficiently and to facilitate the rational design of novel NP-based drugs.

AIMS AND TASKS OF THE PHD THESIS

AIMS

The aim of this PhD thesis is to evaluate ADME/Tox properties and to elucidate mechanisms of action, related to potential pharmacological effects of flavonolignans from *Silybum marianum*.

TASKS

1. **Studies of ADME/Tox properties of flavonolignans from *Silybum marianum*.**

    1.1. *In vitro* and *in silico* evaluation of gastrointestinal absorption.

    1.2. *In silico* prediction of toxicity and metabolism.

    1.3. Molecular modeling of interactions with the human estrogen receptor alpha (ERα).

2. **Studies of molecular interactions of flavonolignans from *Silybum marianum* with novel target proteins.**

    2.1. Evaluation of similarity between selected flavonolignans and drug molecules with known mechanism of action.

    2.2. Selection of potential common target proteins for the studied flavonolignans and drug molecules.

    2.3. Molecular modeling of flavonolignans' interactions with selected target proteins.

    2.4. *In vitro* studies of the flavonolignans' effects on mechanisms involving the selected target proteins.

CHAPTER 2.   MATERIALS AND METHODS USED IN THE STUDIES

1. METHODS

1.1.   PAMPA.

DS-PAMPA (Avdeef 2012) measurements were performed in the PAMPA Explorer Test System from Pion Inc. Measurements at pH 5.0, 6.2, and 7.4 in the donor compartment are conducted in order to mimic the *in vivo* pH conditions on the surface of the gastrointestinal tract.

1.2.   QSAR modelling to predict PAMPA permeability.

The structural information for the compounds used in the QSAR model development was collected from the NCI/CADD Chemical Identifier Resolver service and from the NCBI PubChem project. Octanol-water distribution-related molecular descriptors (logD at pH 7.4) were calculated by ACD/Percepta or by the calculator plugins of ChemAxon Marvin v. 14.8.25 (http://chemaxon.com). Molecular size-related descriptors were calculated by the KNIME-integrated Chemistry Development Kit (CDK, v. 1.5.1) and Indigo (v. 1.1.4) nodes. The multiple linear regression models were derived and refined in the KNIME Analytics Platform v. 2.12.2 (http://www.knime.com).

1.3.   Knowledge-based expert systems for prediction of toxicity and metabolism.

Predictions of toxicity and metabolism of the studied flavonolignans were performed using Derek Nexus v.5.0.1 and Meteor Nexus v.3.0.0 knowledge-based expert systems (Lhasa Ltd.).

1.4.   Molecular modelling of flavonolignans.

1.4.1.   Molecular modelling of flavonolignans' interactions with the human estrogen receptor alpha (ERα).

MOE 2015.10 software (Montreal, Canada) and GOLD v. 5.1 (Cambridge Crystallographic Data Centre Ltd.) software were used for docking studies, analysis and comparison of protein-ligand interactions and identification of important protein residues.

1.4.2.   Molecular modelling of flavonolignans' interactions with novel

target proteins.

Flexible Alignment and Surfaces and Maps tools in MOE 2016.08 software were used for initial assessment of similarity between the bioactive conformations of the selected drugs and silybins using atom-based properties and molecular surfaces analysis.

The chemical similarity between silybin and dehydrosilybin stereomers and approved drugs from the DrugBank database (Wishart 2006) was evaluated by the ROCS (Rapid Overlay of Chemical Structures) software (OpenEye 2019.05, Santa Fe, NM, USA).

Molecular Operating Environment (MOE 2019.0102) software (Montreal, Canada) was used for docking studies in the binding pockets of SMO (PDB ID: 5L7I, Structure of human Smoothened in complex with vismodegib, chain A) and BRAF kinase (PDB ID: 4RZV, Crystal structure of the BRAF (R509H) kinase domain monomer bound to vemurafenib, chain A).

1.5. *In vitro* studies on flavonolignans' potential to interact with selected anticancer drug targets.

The following *in vitro* methods were used to study the flavonolignans' potential to interact with selected anticancer drug targets:

- BRAF Kinase Assay.
- Cytotoxicity Assay.
- Hedgehog (HH) dependent luciferase reporter assay.
- BODIPY-Cyclopamine Binding Assay.

These *in vitro* studies were performed in collaboration with Department of Infectious Microbiology, Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences and Department of Biotechnology, chemistry and pharmacy, University of Siena.

2. MATERIALS AND DATA

2.1. PAMPA.

Seven compounds, provided by Laboratory of Biotransformation, Institute of Microbiology, Czech Academy of Sciences were investigated *in vitro*: silybin AB (Biedermann et al. 2014), isosilybin A (Gažák, Fuksová, et al. 2013), silychristin A, silydianin (Křenek et al. 2014), 2-3-dehydrosilybin AB (Gažák et al. 2011), taxifolin and quercetin. This set of compounds was

purposefully selected empirically to allow analysis of the structural features and physico-chemical properties that can influence permeability. Purity of the flavonolignans was above 96% (HPLC/PDA) and of taxifolin and quercetin above 99% (Sigma-Aldrich).

2.2. QSAR modelling to predict PAMPA permeability.

The QSAR model used DS-PAMPA data of 269 compounds collected from "Database of Double-Sink PAMPA log P_0, log $Pm^{6.5}$, and log $Pm^{7.4}$" (Avdeef 2012) and expressed as effective membrane permeability values (logP_e).

The structures and SMILES codes of 31 silybin derivatives, whose PAMPA permeability was predicted *in silico* (Džubák et al. 2006; Gažák et al. 2009; 2011; Kosina et al. 2002) are given in Appendix 2.

2.3. *In vitro* studies on flavonolignans' potential to interact with selected anticancer drug targets.

Four compounds, provided by Laboratory of Biotransformation, Institute of Microbiology of the Czech Academy of Sciences, Prague, were investigated *in vitro*: silybin A, silybin B (Biedermann et al. 2014), 2,3-dehydrosilybin A, and 2,3-dehydrosilybin B (Gažák, Trouillas, et al. 2013). Optically pure diastereoisomers were studied as it had already been shown that stereochemistry was pivotal for the biological activities of flavonolignans (Gazak, Walterova, and Kren 2007; Diukendjieva et al. 2017). The purity of flavonolignans was above 96% (HPLC/PDA).

CHAPTER 3.   RESULTS AND DISCUSSION

1. Studies of ADME/Tox properties of flavonolignans from *Silybum marianum*.

Despite the frequent therapeutic use of silybin and its congeners, many of their ADME/Tox properties have not been well investigated. Therefore, the aim of the studies in this section is to address this paucity of information by combining *in vitro* and *in silico* methods to evaluate the GIA, toxic effects and metabolism of NPs from *Silybum marianum* and their derivatives. The GIA of all major components of silymarin as well as of some structurally similar flavonoids was estimated using the parallel artificial membrane permeability assay (PAMPA). Additionally, an *in silico* evaluation of GIA for a broader set of silybin congeners using a QSAR model for the prediction of PAMPA permeability was reported. Predictions of toxicity and metabolism were performed using knowledge-based expert systems, and molecular modelling studies were further applied to reveal the differences in the interactions of the stereoisomeric forms of silybin with the ligand-binding domain of the human estrogen receptor alpha (ERα).

1.1.   PAMPA permeability.

1.1.1.   Measurement of PAMPA permeability.

The compounds subjected to PAMPA permeability measurements were selected intentionally based on their plant distribution and structural relations: silybin AB (Biedermann et al. 2014), isosilybin A (Gažák, Fuksová, et al. 2013), silychristin A and silydianin (Křenek et al. 2014) are the main components of *Silybum marianum*; 2-3-dehydrosilybin AB (Gažák, Trouillas, et al. 2013) is an NP derivative but also occurs in silymarin as a minor component – up to 1–2% (Chambers et al. 2017); taxifolin and quercetin are structurally identical to the flavonoid part of silybin and dehydrosilybin, respectively, and can be found in many fruits, vegetables, leaves, and grains.

The logarithms of the effective membrane permeability values (logPe) of the compounds studied are reported in Table 1. Measurements at pH 5.0, 6.2, and 7.4 in the donor compartment are conducted in order to mimic the *in vivo* pH conditions on the surface of the gastrointestinal tract (Waterbeemd, Lennernäs, and Artursson 2003). Good agreement is observed between the logPe values of silybin and quercetin reported by Avdeef (2012) and those measured in the present study: −5.08 vs. –5.25±0.05 for silybin, and –4.77 vs. –5.02±0.07 for quercetin (pH=7.4).

Table 1. Effective membrane permeability logPe ± SD of the compounds studied. The SD values have been calculated based on 3 parallel experiments.

pH Compound	5.0	6.2	7.4
Silybin AB	−4.11 ± 0.03	−4.14 ± 0.03	−5.25 ± 0.05
2,3-Dehydrosilybin AB	−4.11 ± 0.06	−4.17 ± 0.03	−4.06 ± 0.03
Isosilybin A	−4.32 ± 0.09	−4.31 ±0.06	−5.19 ± 0.02
Silychristin A	−6.14 ± 0.08	−6.09 ± 0.05	−6.75 ± 0.11
Silydianin	−5.76 ± 0.05	−5.79 ± 0.04	−6.64 ± 0.09
Taxifolin	−5.95 ± 0.10	−5.93 ± 0.02	−5.23 ± 0.01
Quercetin	−5.14 ± 0.42	−5.10 ± 0.17	−5.02 ± 0.07

According to the high/low-to-moderate logPe classification threshold of −6 (explained in Section 1.1.2.) and the analysis of the measured logPe values, the main active component of *Silybum marianum*, silybin, its 2,3-dehydro-derivative and isosilybin A can be considered to be highly permeable in the gastrointestinal tract. The flavonoids taxifolin and quercetin demonstrate a similar permeability profile. Silydianin and silychristin A, the second most abundant flavonolignans (after silybin) have lower logPe values, suggesting lower absorption in the gastrointestinal tract.

The results demonstrate variation in the permeability profiles of the compounds depending on their structure that is affected additionally by pH. There is a difference of more than one log unit in logPe at pH 7.4 between silybin and dehydrosilybin; however, there is no significant variation at pH 5.0 and/or 6.2. Conversely, the difference in the permeability values between taxifolin and quercetin is higher at the lower pH (6.2 and 5.0). It may be assumed that dehydrogenation in the flavonoid core increases permeability of the flavonolignans at pH 7.4, but does not affect the permeability of the related flavonoids (quercetin and taxifolin), possibly related to the lignan part that is absent in taxifolin and quercetin. Regarding the influence of isomerism, comparison of the permeability values for silybin and isosilybin shows no significant difference with pH.

Analysis of the pH dependence of permeability of individual compounds shows other significant variations. For silybin, isosilybin A, silychristin A and taxifolin there is a difference of ca. one log unit between log Pe values measured at pH 6.2 and 7.4 (Table 1). However, such

a difference was not observed for dehydrosilybin and quercetin. It was assumed that these variations may be related to the ionization states of the compounds influencing the ratio between their neutral and ionized forms and thus their permeability. As an indicator of relative ionization, which would affect passive diffusion, the ACD/Percepta pKa values of the compounds were calculated. The lowest calculated acidic pKa values vary between 6.3 and 7.4, implying that at pH 7.4 the portion of their ionized forms is higher compared to that at pH 6.2 and that should result in a lower permeability of the compounds. However, such a tendency has not been observed. Thus, the calculated pKa values alone are unlikely to explain the pH-dependent log Pe of the studied compounds.

1.1.2. QSAR model for prediction of PAMPA permeability.

To predict the PAMPA permeability of the tested compounds in addition to 31 experimentally untested flavonolignans, a QSAR model for prediction of PAMPA permeability was used. The model was developed within the SEURAT-1 initiative (https://www.seurat-1.eu/) and in particular the COSMOS project (COSMetics to Optimise Safety, https://www.seurat-1.eu/pages/cluster-projects/cosmos.php – last access on 17 July 2023), where *in silico* studies were conducted to GIA of bioactive substances. The model was intentionally developed using descriptors calculated from open-source or free software tools or obtainable from free online resources (Cronin et al., 2012) and has also been included in the DataBase service on Alternative Methods of the European Union Reference Laboratory for alternatives to animal testing (https://jeodpp.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/DBALM_docs/176_M_PAMPA.pdf - last access on 17 July 2023).

The model was developed using DS-PAMPA data (Avdeef, 2012) obtained under experimental conditions equivalent to the PAMPA measurements performed in this study. The dataset of 269 compounds was characterized by a broad distribution of the Pe values. The sink conditions of DS-PAMPA (lowering the active concentration of free permeant in the acceptor compartment) together with the aqueous boundary layer (ABL) control (40-60 μm ABL achieved by in-well stirring) allowed for elimination of non-linearity of the Pe data across a broad range of lipophilicity.

Molecular descriptors similar to those suggested by Kansy et al. 2001 – the logarithm of the apparent octanol/water distribution coefficient (log D), and the ratio of polar to total molecular surface area (PSA/TSA) – were utilized in the QSAR. LogD estimates are readily available

from http://www.chemspider.com (calculated by ACD/Percepta for compounds already included in the ChemSpider database) or from http://chemicalize.com (calculated by ChemAxon tools for any submitted compound). Substitution of the PSA/TSA ratio was considered to allow for the calculation of all descriptors with freely available software tools. As such PSA was substituted by TPSA (topological polar surface area) (Ertl, Rohde, and Selzer 2000). Considering that molecular weight (MW) is the most fundamental descriptor of the molecular size, and that the statistical parameters of the models using it were among the best, MW was selected to substitute for TSA. The two implementations of the model based on logD at pH 7.4 as estimated by the ACD/Percepta or ChemAxon tools are presented in the following equations, respectively:

$$logPe = -2.20(\pm 0.21) + 0.49(\pm 0.04)logD - 10.14(\pm 0.74)TPSA/MW$$

$$n = 251, R^2 = 0.75, SEE = 1.10, F = 371.3,$$

$$LOO\ Q^2 = 0.74,\ external\ validation\ Q^2 = 0.79\ (200/51)$$

$$logPe = -2.11(\pm 0.22) + 0.47(\pm 0.05)logD - 10.71(\pm 0.78)TPSA/MW$$

$$n = 248, R^2 = 0.74, SEE = 1.11, F = 345.1,$$

$$LOO\ Q^2 = 0.73,\ external\ validation\ Q^2 = 0.77\ (198/50)$$

The ability of these models to predict GIA was assessed using an external dataset (accessible at http://biomed.bas.bg/qsarmm/Human_intestinal_absorption_and_bioavalability/) of 783 compounds (1227 distinct values) with reported GIA collected from the literature, 167 of them (383 distinct GIA values) with DS-PAMPA Pe in the training set of the model developed. The data collected did not distinguish low and medium GIA, due to the low percentage of compounds with low and medium GIA (Figure 2A). However, a rapid decrease in the percentage of observations belonging to the highest GIA class (>80%) is evident for compounds with PAMPA log Pe lower than –6 (Figure 2B), which confirms the recommendation in Avdeef (2012) to use logPe < –6 as an indication for possible low GIA. The model classified the remaining 616 compounds into high or medium-to-low GIA classes with reasonable accuracy, sensitivity and specificity of the classification calculated (Table 2).
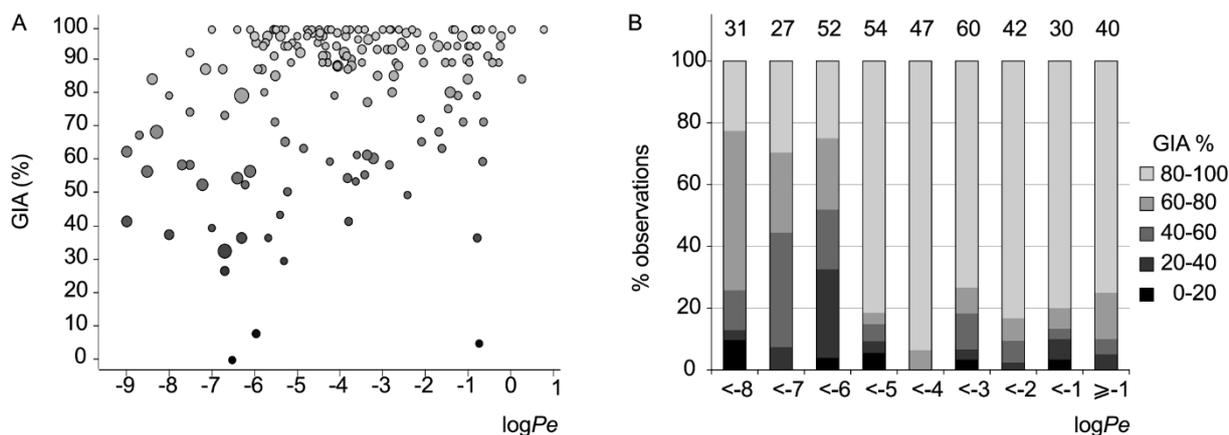
Figure 2. Correspondence between logPe and GIA (%) for 167 compounds present in both PAMPA Pe and GIA datasets: A – mean GIA values vs. PAMPA logPe; size of the circles corresponds to the number of averaged GIA values for the compound. B – distribution of GIA classes among PAMPA Pe classes (numbers on top of the columns correspond to the number of distinct GIA values in each PAMPA Pe class).

Table 2. Statistical parameters for the classification power of the PAMPA Pe, predicted by TPSA/MW-based models, with respect to GIA.

Model implementation	accuracy	sensitivity	specificity	% outliers
ACD/Percepta-calculated logD	76.1	83.9	58.3	11.6
ChemAxon tools-calculated logD	77.1	84.4	60.0	14.6

1.1.3. *In silico* prediction of PAMPA permeability.

The QSAR model was used to predict PAMPA permeability of the tested compounds in addition to 31 experimentally untested flavonolignans. The results from the *in silico* prediction of PAMPA permeability for the compounds studied *in vitro* using the QSAR model are reported in Table 3. Figure 3 represents their positions within the space defined by the physico-chemical parameters used for the development of the model for the compounds in the training set. The figure demonstrates that the compounds fall into the applicability domain of the model thus confirming the reliability of the predictions.

Table 3. Calculated molecular descriptors and logPe values predicted by the QSAR model for the flavonoids studied.

Compound	log D at pH 7.4	TPSA/MW	Predicted log Pe
Silybin AB	1.77	0.322	–4.60
2,3-Dehydrosilybin AB	1.03	0.331	–5.06
Isosilybin A	1.82	0.322	–4.57
Silychristin A	1.70	0.345	–4.86
Silydianin	1.03	0.338	–5.12
Taxifolin	1.15	0.419	–5.89
Quercetin	0.59	0.435	–6.32
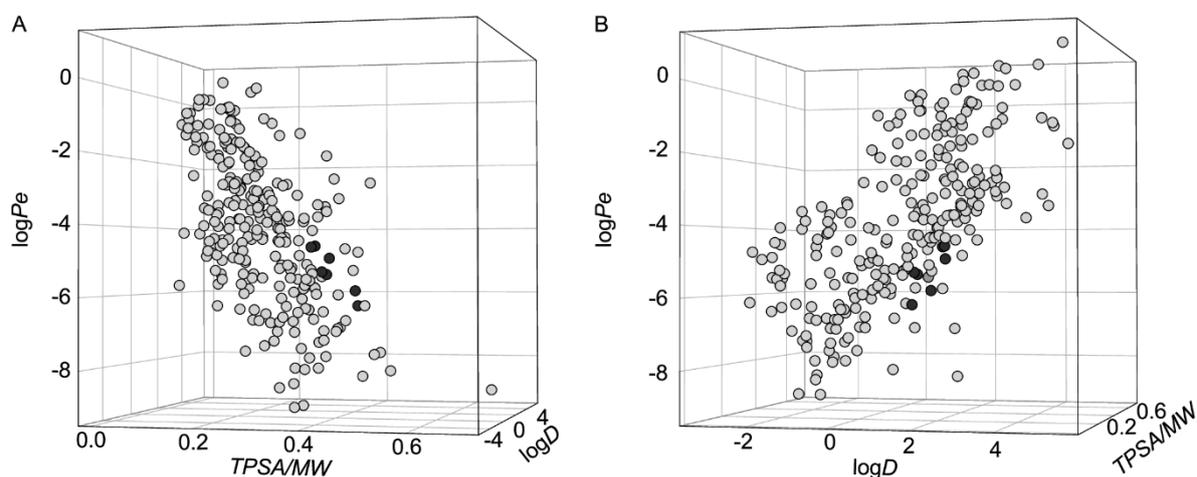
Figure 3. 3-D plots of experimental log Pe vs. calculated structural descriptors TPSA/MW (A) and logD at pH 7.4 (B) obtained by the ACD/Percepta model as the x-axis respectively for the training set of compounds (○) and the predicted flavonoids (•). The parameters' intervals are: – 9÷0.78 for log Pe; 0.011÷0.695 for TPSA/MW and –3.16÷5.51 for log D (pH 7.4).

Based on the good correspondence between the observed and calculated permeability of the silybin congeners (silybin AB, dehydrosilybin AB and isosilyibn A), the permeability of a further 31 silybin derivatives, with structural skeleton similar to those of the studied silybins and unknown permeability, was also predicted. The majority of the compounds have log Pe values between –4 and –5, which classifies them as highly permeable.

1.2. Biotransformations and toxic effects.

1.2.1. *In silico* prediction of toxicity and metabolism.

Predictions of toxicity and metabolism of selected flavonolignans (silybin AB, isosilybin A, silychristin A, and silydianin, being the main components of *Silybum marianum* and 2-3-dehydrosilybin AB that occurs in silymarin as a minor component – up to 1–2%, but reported to account for specific bioactivities) were performed using knowledge-based expert systems Derek Nexus and Meteor Nexus (Marchant, Briggs, and Long 2008), respectively.

Based on the alerts detected in the structures of silybin congeners, three potential toxic effects in mammals (chromosome damage *in vitro*, ERα modulation and skin sensitization) and one potential toxic effect in bacteria (mutagenicity *in vitro*) were outlined as plausible. Further studies of the estrogen receptor alpha modulation by flavonolignans were performed and reported in Section 1.2.2. This effect is selected for more detailed examination because it is described by a well-defined endpoint including a single protein target, making it particularly suitable for docking studies. Additionally, these allow for studying possible stereospecific effects that are not taken into account by Derek Nexus workflow.

With respect to metabolism Meteor Nexus was used as a tool that predicts the metabolic fate of a chemical from its structure (Langowski and Long 2002). The two most probable metabolic transformations for the studied compounds predicted by Meteor that use experimental data for compounds that match the same biotransformation, have similar molecular weights and are chemically similar around the site of metabolism to the query compound, are glucuronidation and oxidative *O*-demethylation.

1.2.2. Molecular modelling of interactions with the human estrogen receptor alpha (ERα).

In order to investigate the interactions of stereoisomeric forms of the main component of *Silybum marianum*, silybin, with the ligand-binding domain (LBD) of the ERα a docking study of each of the isomers, silybin A and silybin B, was further performed. It is known that agonists and antagonists stabilize differently the helix 12 (H12) in the LBD C-terminus which plays a crucial role in determining ERα interactions with coactivators and corepressors (Ascenzi, Bocedi, and Marino 2006). The antagonist-bound LBD conformation (with H12 position in green, Figure 4) was selected for docking of silybins as the agonist conformation (H12 position in magenta, Figure 4) was not large enough to accommodate the silybins (poses were generated

with the compounds in unrealistic folded conformations, data not shown).
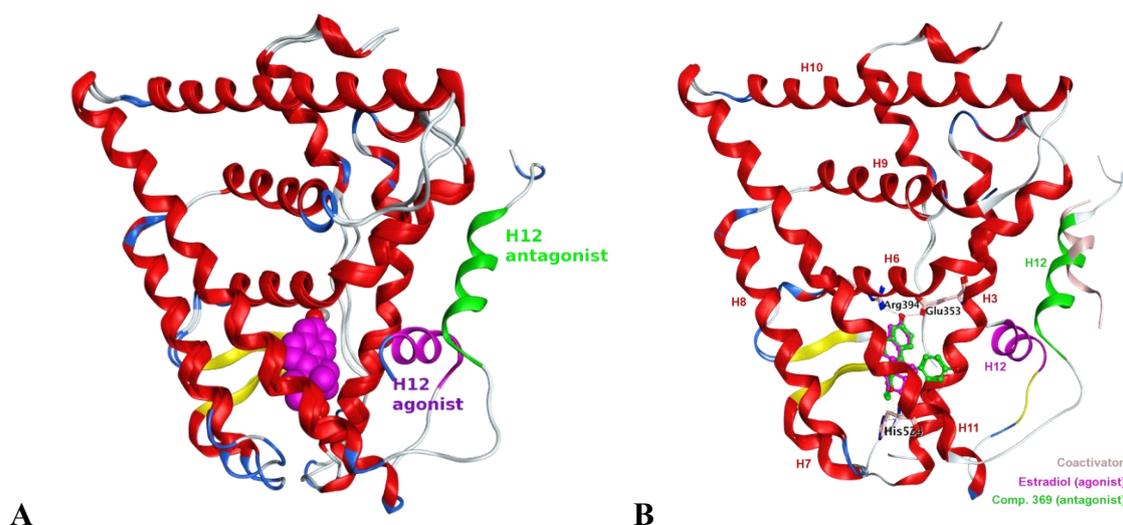
Figure 4. The LBD of ERα with (**A)** the full agonist estradiol in the active site shown in space-filled rendering and colored in magenta; (**B)** the full agonist estradiol (magenta) and an antagonist (green). Positions of the activation helix H12: agonist conformation (magenta); antagonist conformation (green).

This selection was additionally justified by the analysis of ERα X-ray complexes of partial agonists in the Protein Data Bank (PDB) (Berman 2000) that showed some partial agonists bound in the antagonist conformations of ERα. Our docking results demonstrate that both silybin A and silybin B can be accommodated into the ERα active site, but the stereoisomers showed different poses and interactions in the receptor active site. The results obtained from docking with MOE software reveal no specific interactions of silybin A with amino acids in the active site of ERα (Figure 5). In contrast, silybin B forms hydrogen bond (HB) interactions with Leu525and Asp 351 (Figure 5). Leu525 is located adjacent to His524, which is one of the amino acids that are implicated in the interactions of the agonist of ERα, estradiol.
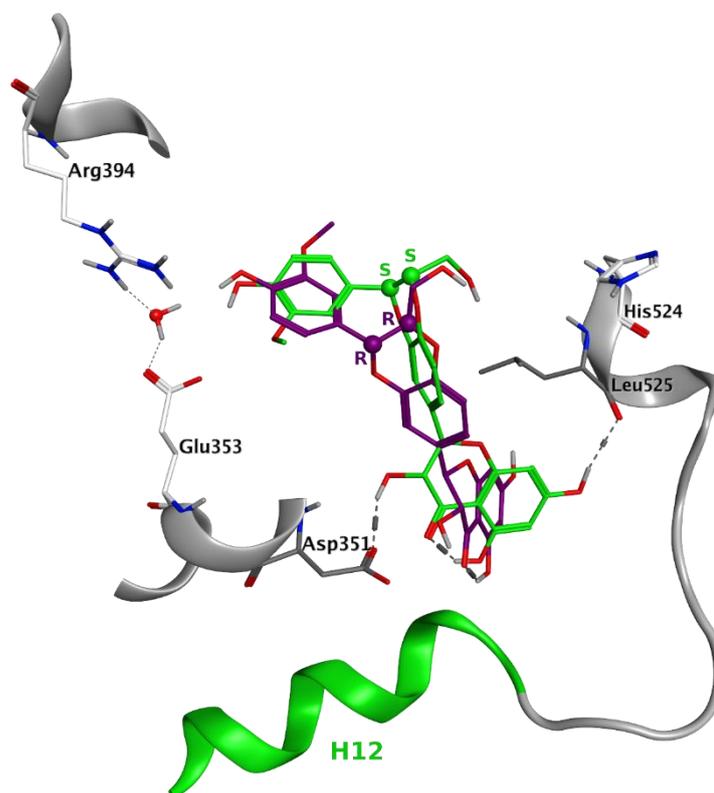
Figure 5. Poses of silybin A (purple) and silybin B (green) and HB interactions of silybin B in the ERα active site. The R- and S-stereo C-atoms of the compounds are shown as balls; the O atom of the water molecule in the active site is shown as a red ball.

Unlike the agonist estradiol and the antagonist 4-hydroxytamoxifen (Figure 6), silybin B does not interact directly with Glu353 and Arg394, but remains close to them and the active water molecule (distances not shown). Similarly to the antagonist 4-hydroxytamoxifen, silybin B interacts with Asp351 (Figure 6), but this interaction is through HB.
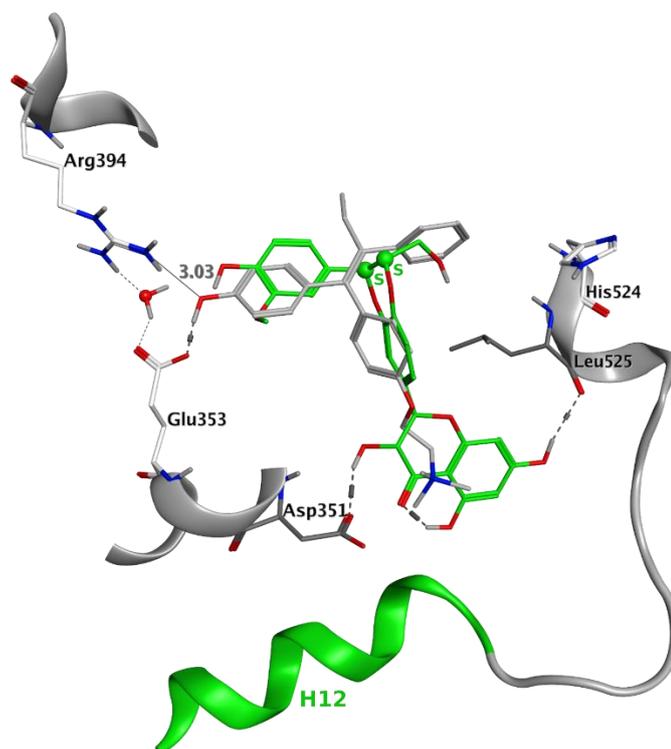
Figure 6. Poses of 4-hydroxytamoxifen (atom type colored) and silybin B (green) and their HB interactions in the ERα active site.

The docking results clearly show stereospecific interactions of silybin A and silybin B in the ERα active site, independently of the orientation of the stereoatoms $10R/11R$ and $10S/11S$ in the active site (towards H12 or opposite to it). The recorded specific interactions of silybin B in the best docking poses reproduce some of the interactions observed for the agonists and some of the interactions of the antagonists.

The docking results shed light on differences in the interactions of both stereoisomers with ERα and on the experimental observations of their ERα effects showing that silybin B and not silybin A is probably responsible for the partial ERα-mediated activity of silymarin (Plíšková et al. 2005).

1.3. Conclusions.

In the present studies the PAMPA methodology has been applied to estimate the membrane permeability of all major components of *Silybum marianum* (L.) Gaertn. A QSAR model for PAMPA has been developed and combined with the *in vitro* results to predict the GIA of all major components of the milk thistle and their derivatives. The QSAR model uses descriptors calculated by open-source or free software tools or those obtainable from free online resources that makes it appropriate for a broader application. The model is freely-available through the

30

Database Service on Alternative Methods of the European Union Reference Laboratory for Alternatives to Animal Testing.

According to both *in vitro* and *in silico* methods most flavonolignans are highly permeable in the gastrointestinal tract, which is a good prerequisite for sufficient bioavailability. The estimated permeability of the studied flavonoids makes them appropriate lead structures for drug development purposes. The results confirm that the combined interdisciplinary approach based on *in silico* QSAR predictions and *in vitro* PAMPA measurements can be used for preliminary evaluation of GIA and can guide further laboratory experiments for characterization of bioactive compounds, including NPs.

With respect to toxicity and metabolism, four potential toxic effects and two most probable metabolic transformations of the major components of *Silybum marianum* were outlined, as well as possible orientations and interactions of silybin stereoisomeric forms in the ERα active site were revealed that agree with and explain stereospecific effects studied in experimental settings.

The reported ADME/tox data for flavonolignans may be useful for the rational modification and design of new NP derivatives with potential positive effects for the human health.

2. Identification of novel pharmacological target proteins of flavonolignans from *Silybum marianum* and studies of their molecular interactions.
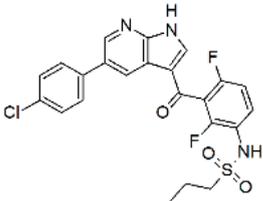
The broad spectrum of biological activities of silymarin components suggests their potential as lead compounds in the context of multifaceted pathologies with silybins and their 2,3-dehydro derivatives, being shown to exert anticancer activities, yet with unclear mechanisms. Addressing this issue, the studies in the current section combine *in silico* and *in vitro* methods to give insights into the possible interactions of flavonolignans from *Silybum marianum* with target proteins endowed with therapeutic implications in cancer.

2.1. Selection of potential novel protein targets of flavonolignans based on chemical similarity with approved drugs.

The chemical similarity between silybin and dehydrosilybin stereoisomers and approved drugs from the DrugBank database was assessed using the ROCS software (OpenEye) (Hawkins, Skillman, and Nicholls 2007). The TanimotoCombo index (TCI) was used as a

similarity metrics as it combines the shape and chemical features based on the alignment between known drugs and silybin and dehydrosilybin queries. Given the known anticancer effect of the flavonolignans, the structural and chemical similarity of these molecules with respect to drugs exerting clinical antitumor activity becomes of particular interest to predict the mechanism of action of these compounds. Therefore, we filtered and analysed anticancer drugs for which similarity with silybin and dehydrosilybin isomers was scored with TCI ≥ 0.9 (Table 4). Vemurafenib is approved for the treatment of metastatic melanoma as a competitive inhibitor of BRAF kinase bearing a substitution of glutamic acid for valine (V600E mutation) (Luke and Hodi 2012). Vismodegib selectively binds to and inhibits the transmembrane receptor SMO, i.e., the upstream regulator of the HH signaling pathway, and it is indicated by the FDA for the treatment of metastatic or locally advanced basal cell carcinoma (Sandhiya et al. 2013). ROCS results showed slightly higher TCI values for dehydrosilybins compared to silybins with respect to both vemurafenib and vismodegib.

Table 4. TanimotoCombo indices for antitumor drugs whose similarity with silybin and dehydrosilybin stereomers is scored higher than 0.9.

	Vemurafenib	Vismodegib
	Tanimoto-Combo Index	
Silybin A	0.963	0.962
Silybin B	0.943	0.962
Dehydrosilybin A	0.976	0.979
Dehydrosilybin B	0.963	0.979
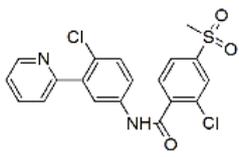
Based on the observed similarity, we can assume that the studied flavonolignans may interact with the same targets that these antitumor drugs do. To examine this hypothesis, the potential interactions with targets of vemurafenib and vismodegib, SMO, and BRAF kinase, respectively, were further studied with *in silico* methods.

2.2.   Molecular modelling studies.

2.2.1.   Analysis of shape and molecular surface properties.

Analysis of the similarity in the shape and molecular surface properties generated by flexible alignment separately for silybin A and B and vemurafenib, and silybin A and B and vismodegib revealed that both silybins produce similar scores in their flexible alignment on the bioactive conformations of vemurafenib and vismodegib. Interestingly, the best scored alignments show stereospecific orientation of superimposed silybins on vemurafenib, but not on vismodegib. Additionally, the correspondence between the surface properties of silybins is better expressed for vemurafenib; the shape correspondence is better expressed for vismodegib.

The flexible alignment and similarity between molecular properties of silybin isomers and the ligands of BRAF kinase (vemurafenib) and SMO (vismodegib) suggest the possibility for silybins to interact with these targets.

2.2.2.   Docking studies in SMO and BRAF kinase.

Molecular docking simulations were carried out using high-resolution X-ray structures of the protein targets of vismodegib (SMO) and vemurafenib (BRAF kinase) available in the Protein Data Bank (PDB ID 5L7I and 4RZV, respectively). The docking results demonstrate that silybins and dehydrosilybins can be accommodated into the binding sites of the studied targets. Docking poses were further analyzed in relation to the best correspondence of flavonolignans to the X-ray ligands in the protein pocket, and their interaction energies with the receptor. The results are summarized in Table 5. The most reasonable docking pose of dehydrosilybins yielded better docking scores than silybins in both receptors. For BRAF kinase, the docking score of dehydrosilybin B was the lowest (-8.354), compared to the other flavonolignans, although it was higher than the redocked score of vemurafenib. Regarding SMO, dehydrosilybin A and dehydrosilybin B have the lowest docking scores, comparable to that of vismodegib.

Table 5. Docking scores (kcal/mol) of silybins, dehydrosilybins, vemurafenib, and vismodegib (docking in BRAF kinase and SMO; the lower scores suggest higher binding affinities).

Compound	BRAF kinase	SMO
Silybin A	−5.787	−7.928
Silybin B	−6.158	−5.545
Dehydrosilybin A	−7.696	−8.090
Dehydrosilybin B	−8.354	−8.490
Vemurafenib	−10.196	N.A.
Vismodegib	N.A.	−8.429

In all docking simulations, binding site residues involved in specific interactions with flavonolignans were identified, including those involved in interactions with vemurafenib (Lys483, Cys532) and vismodegib (Ser387) (Figures 9 and 10). Multiple interactions that differed from those specified for the approved anticancer drugs were also observed, which were particularly evident for dehydrosilybins in both receptors. Specifically, dehydrosilybin A performs an aromatic interaction with Phe583, while dehydrosilybin B interacts through a hydrogen bond with Thr529 and Ile527, as well as through an aromatic interaction with Phe595 in the BRAF kinase. Notably, despite the comparable docking scores, only dehydrosilybin A forms two hydrogen bonds with the residues Asn219 and Met301 in the binding site of SMO (Figure 10A). Inverted (mirror-like) poses were observed for all diastereoisomer pairs in both proteins, with the exception of silybin A and silybin B in SMO. Such stereospecific orientation of the compounds in the binding sites of the studied proteins is not surprising, considering the already demonstrated ability of these compounds to interact in a stereospecific manner with ERα. This also clearly demonstrates the utmost importance of experimental testing of the pure stereomers of these flavonolignans.
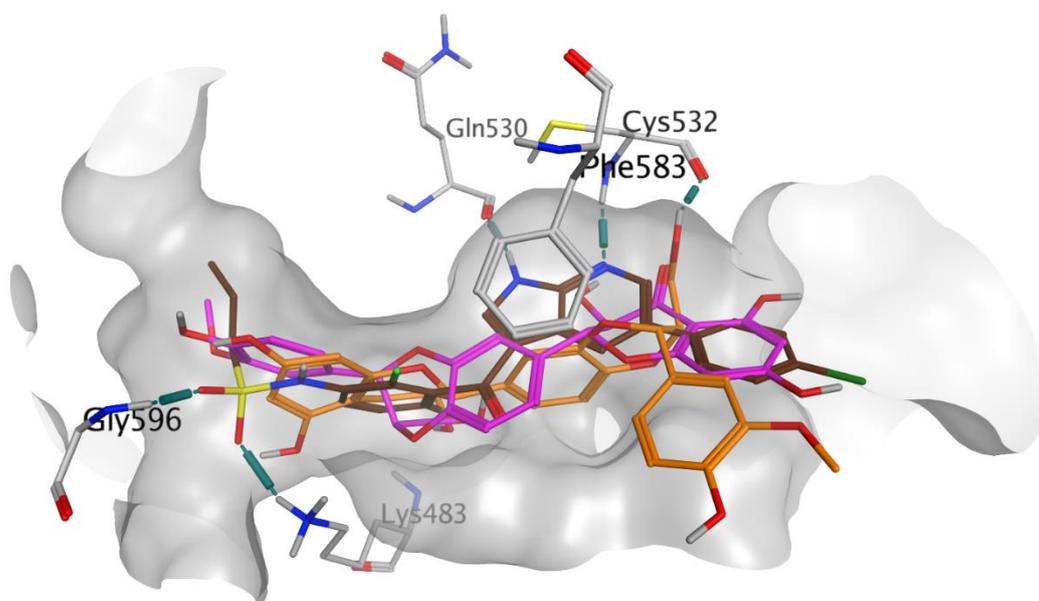
A
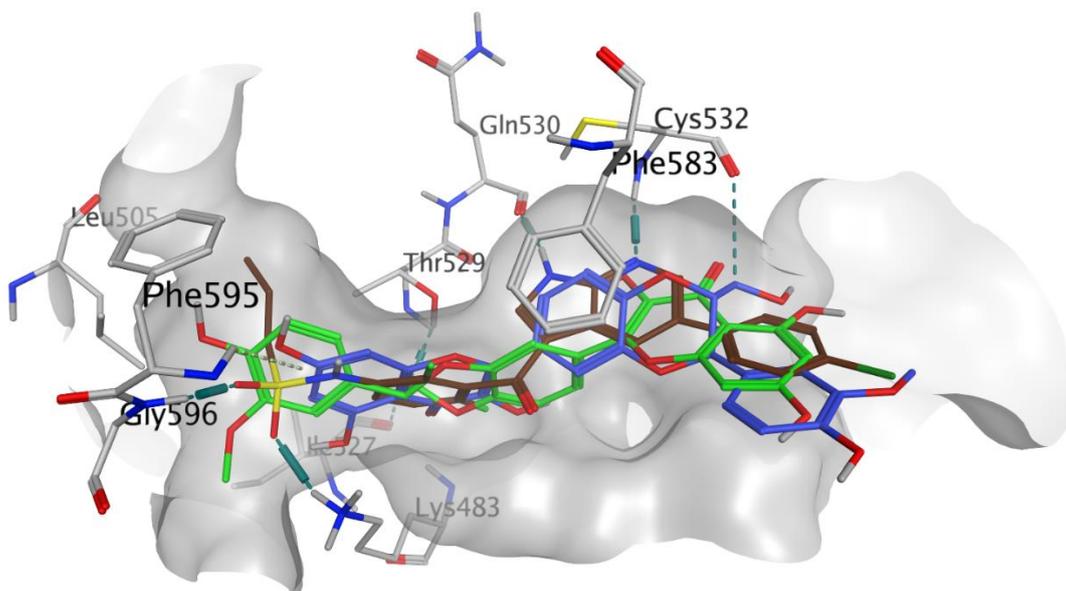
B

Figure 9. Poses and interactions of the studied compounds in the BRAF kinase binding pocket: (**A**) Silybin A (magenta) and silybin B (orange); (**B**) Dehydrosilybin A (blue) and dehydrosilybin B (green). In both panels, vemurafenib is shown in brown, the pocket is shown in grey, and the interacting protein residues are coloured in atom types.
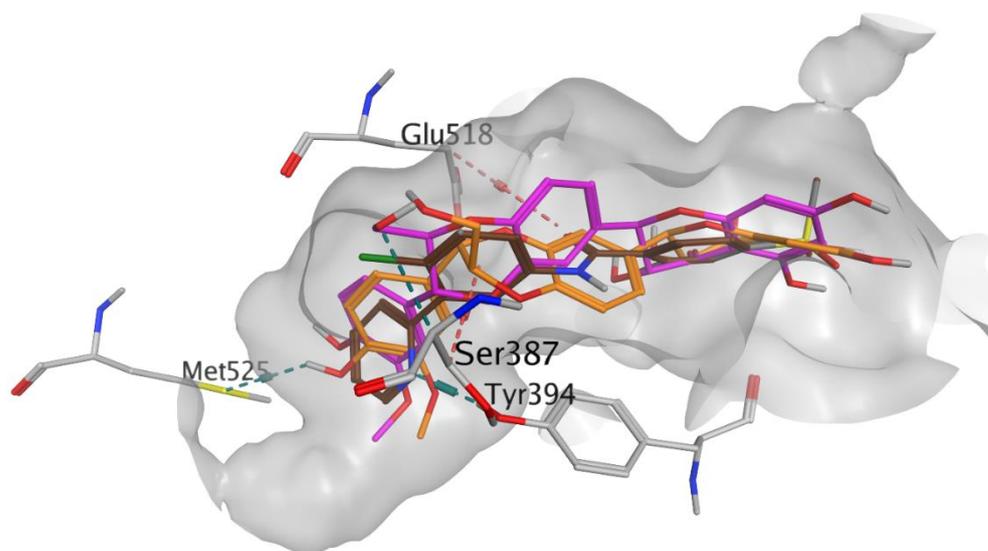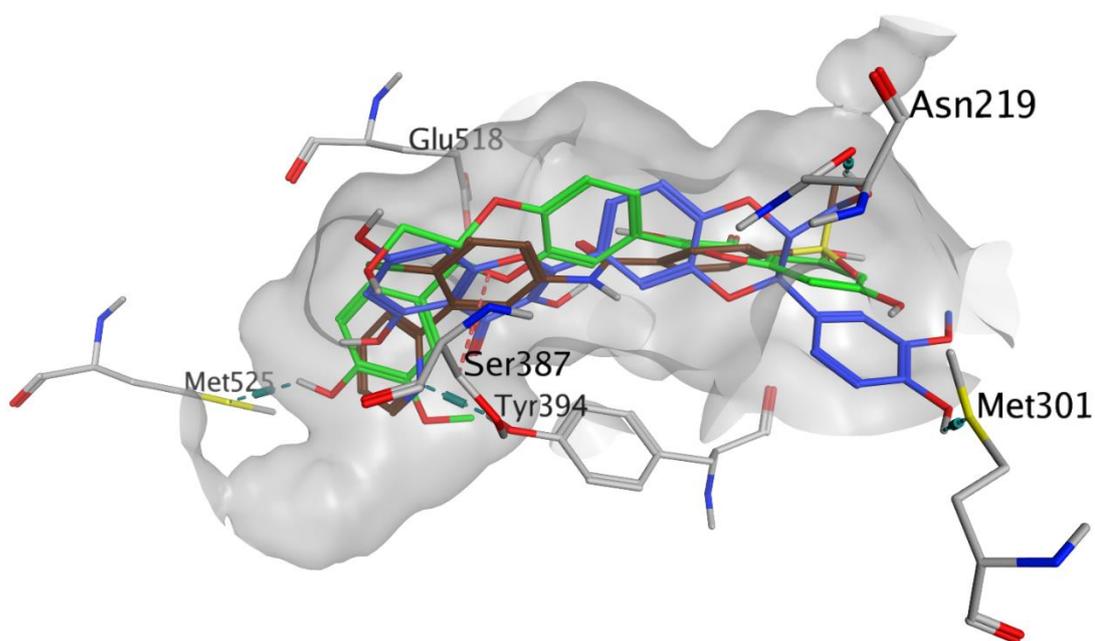
Figure 10. Poses and interactions of the studied compounds in the SMO binding pocket: (**A**) Silybin A (magenta) and silybin B (orange); (**B**) Dehydrosilybin A (blue) and dehydrosilybin B (green). In both panels, vismodegib is shown in brown, the pocket is shown in grey, and the interacting protein residues are coloured in atom types.

2.3. *In vitro* studies on flavonolignans' potential to interact with selected anticancer drug targets.

The results of the *in silico* study pointed to the flavonolignans' potential to interact with the identified anticancer drug targets. Therefore, *in vitro* experiments were further conducted to examine their possible effects on these proteins and cancer cell lines, and to possibly corroborate computational predictions. With respect to BRAF kinase, studies were performed on the effects of silybins on BRAF V600E kinase activity as well as on the viability of human malignant melanoma cells expressing BRAF V600E kinase and non-tumorigenic skin cells (keratinocytes). As for SMO, the effects on the HH pathway were investigated together with the potential interaction with the SMO receptor. Since it is anticipated that flavonolignans may act as inhibitors of the target proteins, like chemically similar drugs do, assays for testing inhibitory activity were selected for this purpose.

2.3.1. Effects on the BRAF V600E kinase activity.

Studies of the effects of silybins on the activity of BRAF V600E kinase activity demonstrate that dehydrosilybin B exhibits the highest inhibitory activity, with an IC_{50} of 24.9 µM, followed by dehydrosilybin A, silybin B, and silybin A (Figure 11). As for comparison, the reported IC_{50} value of vemurafenib is 32.4 nM. These results are in good agreement with the reported *in silico* results that demonstrate better docking scores for dehydrosilybins in the BRAF kinase as well as a larger number of interactions of the dehydro- derivatives in its binding site compared to silybins. These observations along with the stereospecific orientations of the diastereoisomer pairs provide mechanistic explanation of the distinct effects of the flavonolignans on the BRAF V600E kinase activity.
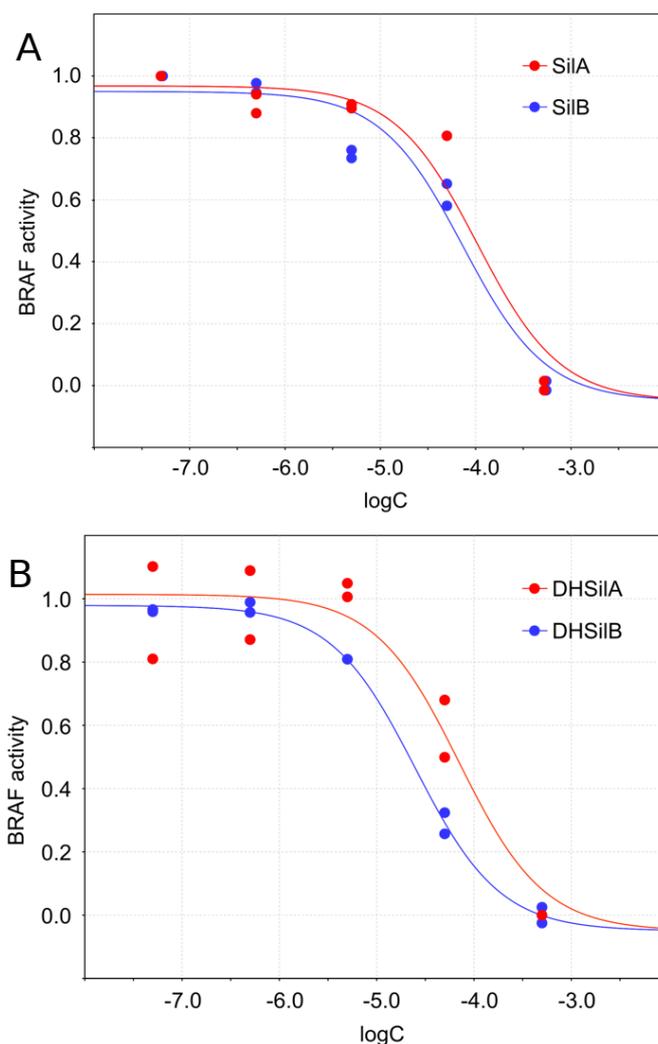
Figure 11. Inhibitory effects of the studied compounds on BRAF V600E kinase activity: (**A**) Silybin A (IC$_{50}$ = 104.0 μM, 95%, confidence interval, CI = 34.7 ÷ 204.2 μM) and silybin B (IC$_{50}$=73.9 μM, CI = 32.4 ÷ 112.2 μM); (**B**) dehydrosilybin A (IC$_{50}$ = 70.6 μM, CI = 30.9 ÷ 131.8 μM) and dehydrosilybin B (IC$_{50}$ = 24.9 μM, CI = 17.8 ÷ 26.3 μM).

2.3.2. Cytotoxicity on malignant skin cell lines.

Regarding cell viability, all tested flavonolignans exhibited higher cytotoxicity in the A-375 cell line, which is representative of malignant melanoma expressing the BRAF V600E mutation, compared to the non-melanoma tumor cell line A-431 and the non-tumorigenic cell line HaCaT. The IC$_{50}$ values obtained for the three tested cell lines are reported in Table 6. Dehydrosilybin A and dehydrosilybin B exhibit higher cytotoxicity toward the A-375 cell line and appear to be less toxic in A-431 and HaCaT cell lines compared to silybin A and silybin B. This trend is especially pronounced for the cell line HaCaT, in which dehydrosilybins exhibit the lowest toxicity.

Table 6. *In vitro* cytotoxicity of silybins and dehydrosilybins on malignant skin cell lines and nontumorigenic keratinocytes.

Compound	IC$_{50}$, µM (95% Confidence Interval)		
	Cell lines		
	A-375	**A-431**	**HaCaT**
Silybin A	97.0	126.0	120.0
	(38.0 ÷ 245.5)	(51.3 ÷ 288.4)	(56.2 ÷ 245.5)
Silybin B	120.0	1	150.0
	(53.7 ÷ 257.0)	66.0	(57.5 ÷ 426.6)
		(52.5 ÷ n.d.)	
Dehydrosilybin A	83.0	97.0	231.0
	(44.7 ÷ 158.5)	(55.0 ÷ 169.8)	(91.2 ÷ 457.1)
Dehydrosilybin B	86.0	130.0	164.0
	(64.6 ÷ 120.2)	(79.4 ÷ 213.8)	(75.9 ÷ 309.0)

2.3.3. Hedgehog inhibitory activity.

With respect to the SMO receptor, the HH inhibitory activity of the compounds, alone and in a racemate, was investigated in a luciferase reporter assay, which is widely used for characterizing HH inhibitors. The HH inhibitory activity of synthesized molecules was evaluated in NIH3T3 Shh Light II cells, stably incorporating a Gli-responsive firefly luciferase (Alfonsi et al. 2017) treated with the synthetic SMO agonist SAG (Chen 2002), alone and in combination with the tested compounds. At the maximum concentration of 30 µM, silybin A and silybin B (Figure 12A) showed mild activity as HH inhibitors, while dehydrosilybin A and the racemate of dehydrosilybin A and dehydrosilybin B (namely, dehydrosilybin AB) showed high activity in this assay, having an IC$_{50}$ of 5–10 µM (Figure 12B). The remaining silybin AB and dehydrosilybin B proved to be inactive.
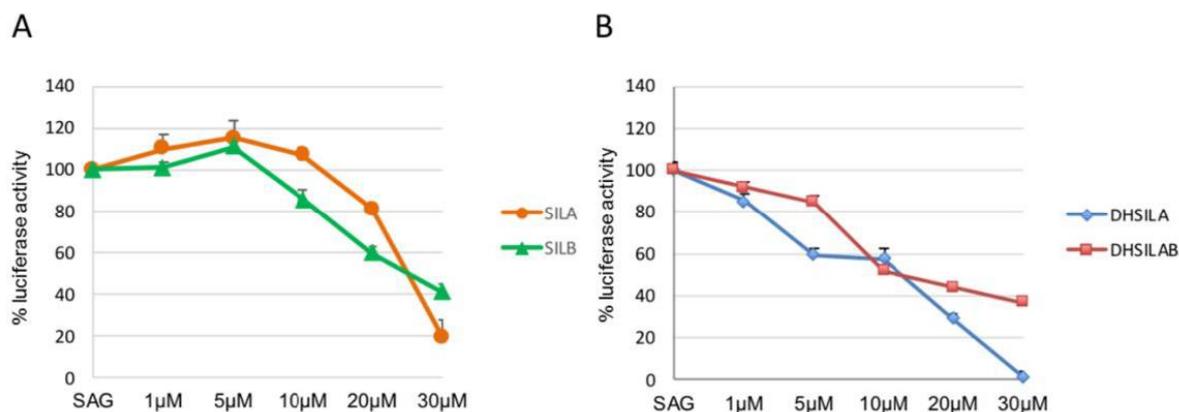
Figure 12. Hedgehog signaling pathway inhibitory activity of: (**A**) Silybin A and silybin B; (**B**) Dehydrosilybin A and dehydrosilybin AB.

2.3.4.   SMO binding properties.

To further investigate the binding properties of the selected compounds to the SMO receptor, we performed a displacement assay based on the use of BODIPY-Cyclopamine (BC) (Alfonsi et al. 2017; Infante et al. 2016), which is known to interact with the binding site of SMO antagonists located within the heptahelical bundle of the receptor. To this aim, HEK293T cells were transiently transfected for expression of SMO and then incubated with BC in the presence or absence of increased amounts of dehydrosilybin A or dehydrosilybin AB. These compounds inhibited BC binding to cells expressing SMO in a dose-dependent manner (Figure 13).
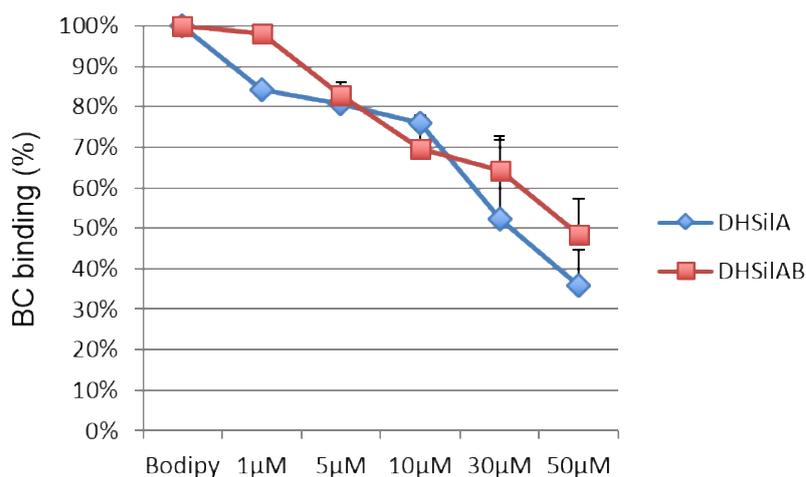
Figure 13. Inhibition of BODIPY-Cyclopamine binding by dehydrosilybin A and dehydrosilybin AB.

Overall, the findings have shown that dehydrosilybin A and dehydrosilybin AB bind the SMO receptor at the level of its heptahelical bundle, which is supportive of the SMO antagonism effect, as previously observed for other natural product chemotypes (Berardozzi et al. 2018; Lospinoso Severini et al. 2019).

The results from the *in vitro* analyses on the activity of the flavonolignans on SMO may be explained by the observations obtained *in silico,* similarly to those for the BRAF kinase. Dehydrosilybins have lower docking scores than silybins with values close to those of vismodegib. Additionally, the analysis of flavonolignans' interactions with SMO indicated that only dehydrosilybin A forms hydrogen bonds with amino acid residues in the binding site of the protein, implying its specific activity against the receptor that is confirmed by the *in vitro* experiments.

2.4.   Conclusions.

The combined *in silico* and *in vitro* approach applied in the studies of this section points to novel anticancer targets for flavonolignans from *Silybum marianum* (L.) Gaertn. The *in silico* simulations helped in the identification of novel potential protein targets relying on: (i) the chemical similarity of the studied compounds to the well-known antitumor drugs vemurafenib and vismodegib, and (ii) the ability of flavonolignans to interact with the targets of these drugs, proved by molecular docking simulations.

The *in silico* results suggest that the active components of silymarin may act as dual inhibitors of BRAF kinase and SMO, two major targets in current anticancer therapy. *In vitro* assays were further performed on the proteins and cell lines, outlining dose-dependent profiles of the studied compounds and suggesting their possible effects on these targets. As a whole, a good consistency between the results from the *in silico* simulations and the *in vitro* experiments was observed. The docking scores as a measure of the ligand-receptor interaction energy illustrate a good correspondence with IC_{50} values determined in the *in vitro* assays. Clearly, dehydrosilybins yield better docking values compared to silybins, and they are the more active compounds in the BRAF kinase and HH pathway assays. This observation is in agreement with other *in vitro* studies confirming the higher activity of dehydrosilybins in comparison with silybins (Agarwal et al. 2013). The cytotoxicity experiments additionally corroborate the cytotoxic properties of the compounds in malignant skin cell lines demonstrating higher activity in A-375 cells than in A-431 and HaCaT.

CONTRIBUTIONS

1. A new predictive QSAR model has been developed that allows for reliable prediction of membrane permeability and gastrointestinal absorption of orally applied bioactive compounds. The model is freely-available through the Database Service on Alternative Methods of the European Union Reference Laboratory for Alternatives to Animal Testing.

2. The applied combined *in silico / in vitro* analysis has explained molecular mechanisms of action of flavonolignans from *Silybum marianum* and has outlined them as appropriate lead structures for design of new natural product derivatives with potential positive effects on human health:

   - It has been found that the main components of *Silybum marianum* as well as their derivatives may be considered as highly permeable in the gastrointestinal tract.

   - It has been shown that silybin diastereomers perform stereospecific interactions with the nuclear estrogen receptor alpha thus explaining at a molecular level the experimentally observed differences in their toxic effects.

   - The enzyme BRAF kinase and Smoothened receptor have been shown as novel pharmacological targets implicated in the mechanisms of anticancer activities of flavonolignans from *Silybum marianum*. Dehydrosilybins have been outlined as promising lead structures for development of anticancer drugs.

REFERENCES

Abenavoli, Ludovico, Angelo A. Izzo, Natasa Milić, Carla Cicala, Antonello Santini, and Raffaele Capasso. 2018. "Milk Thistle (*Silybum Marianum*): A Concise Overview on Its Chemistry, Pharmacological, and Nutraceutical Uses in Liver Diseases: Milk Thistle and Liver Diseases." *Phytotherapy Research* 32 (11): 2202–13. https://doi.org/10.1002/ptr.6171.

Agarwal, Chapla, Ritambhara Wadhwa, Gagan Deep, David Biedermann, Radek Gažák, Vladimír Křen, and Rajesh Agarwal. 2013. "Anti-Cancer Efficacy of Silybin Derivatives - A Structure-Activity Relationship." Edited by Stephen J. Polyak. *PLoS ONE* 8 (3): e60074. https://doi.org/10.1371/journal.pone.0060074.

Alfonsi, Romina, Bruno Botta, Sandro Cacchi, Lucia Di Marcotullio, Giancarlo Fabrizi, Roberta Faedda, Antonella Goggiamani, Antonia Iazzetti, and Mattia Mori. 2017. "Design, Palladium-Catalyzed Synthesis, and Biological Investigation of 2-Substituted 3-Aroylquinolin-4(1 *H*)-Ones as Inhibitors of the Hedgehog Signaling Pathway." *Journal of Medicinal Chemistry* 60 (4): 1469–77. https://doi.org/10.1021/acs.jmedchem.6b01135.

Alqahtani, Saeed. 2017. "*In Silico* ADME-Tox Modeling: Progress and Prospects." *Expert Opinion on Drug Metabolism & Toxicology* 13 (11): 1147–58. https://doi.org/10.1080/17425255.2017.1389897.

Andrew, Ruth, and Angelo A Izzo. 2017. "Principles of Pharmacological Research of Nutraceuticals: Editorial." *British Journal of Pharmacology* 174 (11): 1177–94. https://doi.org/10.1111/bph.13779.

Ano, Rieko, Yukitaka Kimura, Motohiro Shima, Ryuichi Matsuno, Tamio Ueno, and Miki Akamatsu. 2004. "Relationships between Structure and High-Throughput Screening Permeability of Peptide Derivatives and Related Compounds with Artificial Membranes: Application to Prediction of Caco-2 Cell Permeability." *Bioorganic & Medicinal Chemistry* 12 (1): 257–64. https://doi.org/10.1016/j.bmc.2003.10.002.

Andrew, Worth, Bassan, A., Gallegos Saliner, A., Netzeva, T., Pavan, M., Tsakovska, I., Vracko Grobelsek, M. and Tier, G., authors. The Characterisation of (Quantitative) Structure-Activity Relationships - Preliminary Guidance. EUR 21866 EN. European Commission; 2005.

Ascenzi, Paolo, Alessio Bocedi, and Maria Marino. 2006. "Structure–Function Relationship of Estrogen Receptor α and β: Impact on Human Health." *Molecular Aspects of Medicine* 27 (4): 299–402. https://doi.org/10.1016/j.mam.2006.07.001.

Asiamah, Isaac, Samuel Asiamah Obiri, Woasiedem Tamekloe, Francis Ackah Armah, and Lawrence Sheringham Borquaye. 2023. "Applications of Molecular Docking in Natural Products-Based Drug Discovery." *Scientific African* 20 (July): e01593. https://doi.org/10.1016/j.sciaf.2023.e01593.

Avdeef, Alex. 2012. *Absorption and Drug Development: Solubility, Permeability, and Charge State*. Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/9781118286067.

Avdeef, Alex, Stefanie Bendels, L.i. Di, Bernard Faller, Manfred Kansy, Kiyohiko Sugano, and Yukinori Yamauchi. 2007. "PAMPA—Critical Factors for Better Predictions of Absorption." *Journal of Pharmaceutical Sciences* 96 (11): 2893–2909. https://doi.org/10.1002/jps.21068.

Berardozzi, Simone, Flavia Bernardi, Paola Infante, Cinzia Ingallina, Sara Toscano, Elisa De Paolis, Romina Alfonsi, et al. 2018. "Synergistic Inhibition of the Hedgehog Pathway by Newly Designed Smo and Gli Antagonists Bearing the Isoflavone Scaffold." *European Journal of Medicinal Chemistry* 156 (August): 554–62.

https://doi.org/10.1016/j.ejmech.2018.07.017.

Berman, H. M. 2000. "The Protein Data Bank." *Nucleic Acids Research* 28 (1): 235–42. https://doi.org/10.1093/nar/28.1.235.

Biedermann, D., E. Vavříková, L. Cvak, and V. Křen. 2014. "Chemistry of Silybin." *Nat. Prod. Rep.* 31 (9): 1138–57. https://doi.org/10.1039/C3NP70122K.

Bijak, Michal. 2017. "Silybin, a Major Bioactive Component of Milk Thistle (Silybum Marianum L. Gaernt.)—Chemistry, Bioavailability, and Metabolism." *Molecules* 22 (11): 1942. https://doi.org/10.3390/molecules22111942.

Brigo, Alessandro, Doha Naga, and Wolfgang Muster. 2022. "Increasing the Value of Data Within a Large Pharmaceutical Company Through In Silico Models." In *In Silico Methods for Predicting Drug Toxicity*, edited by Emilio Benfenati, 2425:637–74. Methods in Molecular Biology. New York, NY: Springer US. https://doi.org/10.1007/978-1-0716-1960-5_24.

Brown, A. C., and T. R. Fraser. 1868. "On the Connection between Chemical Constitution and Physiological Action; with Special Reference to the Physiological Action of the Salts of the Ammonium Bases Derived from Strychnia, Brucia, Thebaia, Codeia, Morphia, and Nicotia." *Journal of Anatomy and Physiology* 2 (2): 224–42.

Butina, Darko, Matthew D Segall, and Katrina Frankcombe. 2002. "Predicting ADME Properties in Silico: Methods and Models." *Drug Discovery Today* 7 (11): S83–88. https://doi.org/10.1016/S1359-6446(02)02288-2.

Camini, Fernanda Caetano, and Daniela Caldeira Costa. 2020. "Silymarin: Not Just Another Antioxidant." *Journal of Basic and Clinical Physiology and Pharmacology* 0 (0). https://doi.org/10.1515/jbcpp-2019-0206.

Chambers, Christopher Steven, Veronika Holečková, Lucie Petrásková, David Biedermann, Kateřina Valentová, Martin Buchta, and Vladimír Křen. 2017. "The Silymarin Composition… and Why Does It Matter???" *Food Research International* 100 (October): 339–53. https://doi.org/10.1016/j.foodres.2017.07.017.

Chen, J. K. 2002. "Inhibition of Hedgehog Signaling by Direct Binding of Cyclopamine to Smoothened." *Genes & Development* 16 (21): 2743–48. https://doi.org/10.1101/gad.1025302.

Csupor, Dezső, Attila Csorba, and Judit Hohmann. 2016. "Recent Advances in the Analysis of Flavonolignans of Silybum Marianum." *Journal of Pharmaceutical and Biomedical Analysis* 130 (October): 301–17. https://doi.org/10.1016/j.jpba.2016.05.034.

Diukendjieva, Antonia, Merilin Al Sharif, Petko Alov, Tania Pencheva, Ivanka Tsakovska, and Ilza Pajeva. 2017. "ADME/Tox Properties and Biochemical Interactions of Silybin Congeners: *In Silico* Study." *Natural Product Communications* 12 (2): 1934578X1701200. https://doi.org/10.1177/1934578X1701200208.

Džubák, Petr, Marián Hajdúch, Radek Gažák, Alena Svobodová, Jitka Psotová, Daniela Walterová, Petr Sedmera, and Vladimír Křen. 2006. "New Derivatives of Silybin and 2,3-Dehydrosilybin and Their Cytotoxic and P-Glycoprotein Modulatory Activity." *Bioorganic & Medicinal Chemistry* 14 (11): 3793–3810. https://doi.org/10.1016/j.bmc.2006.01.035.

Ertl, Peter, Bernhard Rohde, and Paul Selzer. 2000. "Fast Calculation of Molecular Polar Surface Area as a Sum of Fragment-Based Contributions and Its Application to the Prediction of Drug Transport Properties." *Journal of Medicinal Chemistry* 43 (20): 3714–17. https://doi.org/10.1021/jm000942e.

Fujikawa, Masaaki, Rieko Ano, Kazuya Nakao, Ryo Shimizu, and Miki Akamatsu. 2005. "Relationships between Structure and High-Throughput Screening Permeability of Diverse Drugs with Artificial Membranes: Application to Prediction of Caco-2 Cell Permeability." *Bioorganic & Medicinal Chemistry* 13 (15): 4721–32.

https://doi.org/10.1016/j.bmc.2005.04.076.

Fujikawa, Masaaki, Kazuya Nakao, Ryo Shimizu, and Miki Akamatsu. 2007. "QSAR Study on Permeability of Hydrophobic Compounds with Artificial Membranes." *Bioorganic & Medicinal Chemistry* 15 (11): 3756–67. https://doi.org/10.1016/j.bmc.2007.03.040.

Gažák, Radek, Kateřina Fuksová, Petr Marhol, Marek Kuzma, Rajesh Agarwal, and Vladimír Křen. 2013. "Preparative Method for Isosilybin Isolation Based on Enzymatic Kinetic Resolution of Silymarin Mixture." *Process Biochemistry* 48 (1): 184–89. https://doi.org/10.1016/j.procbio.2012.11.006.

Gažák, Radek, Petr Sedmera, Marek Vrbacký, Jitka Vostálová, Zdeněk Drahota, Petr Marhol, Daniela Walterová, and Vladimír Křen. 2009. "Molecular Mechanisms of Silybin and 2,3-Dehydrosilybin Antiradical Activity—Role of Individual Hydroxyl Groups." *Free Radical Biology and Medicine* 46 (6): 745–58. https://doi.org/10.1016/j.freeradbiomed.2008.11.016.

Gažák, Radek, Patrick Trouillas, David Biedermann, Kateřina Fuksová, Petr Marhol, Marek Kuzma, and Vladimír Křen. 2013. "Base-Catalyzed Oxidation of Silybin and Isosilybin into 2,3-Dehydro Derivatives." *Tetrahedron Letters* 54 (4): 315–17. https://doi.org/10.1016/j.tetlet.2012.11.049.

Gažák, Radek, Kateřina Valentová, Kateřina Fuksová, Petr Marhol, Marek Kuzma, Miguel Ángel Medina, Ivana Oborná, Jitka Ulrichová, and Vladimír Křen. 2011. "Synthesis and Antiangiogenic Activity of New Silybin Galloyl Esters." *Journal of Medicinal Chemistry* 54 (20): 7397–7407. https://doi.org/10.1021/jm201034h.

Gazak, Radek, Daniela Walterova, and Vladimir Kren. 2007. "Silybin and Silymarin - New and Emerging Applications in Medicine." *Current Medicinal Chemistry* 14 (3): 315–38. https://doi.org/10.2174/092986707779941159.

Grabowski, Kristina, Karl-Heinz Baringhaus, and Gisbert Schneider. 2008. "Scaffold Diversity of Natural Products: Inspiration for Combinatorial Library Design." *Natural Product Reports* 25 (5): 892. https://doi.org/10.1039/b715668p.

Harvey, Alan L., RuAngelie Edrada-Ebel, and Ronald J. Quinn. 2015. "The Re-Emergence of Natural Products for Drug Discovery in the Genomics Era." *Nature Reviews Drug Discovery* 14 (2): 111–29. https://doi.org/10.1038/nrd4510.

Hawkins, Paul C. D., A. Geoffrey Skillman, and Anthony Nicholls. 2007. "Comparison of Shape-Matching and Docking as Virtual Screening Tools." *Journal of Medicinal Chemistry* 50 (1): 74–82. https://doi.org/10.1021/jm0603365.

Infante, Paola, Romina Alfonsi, Cinzia Ingallina, Deborah Quaglio, Francesca Ghirga, Ilaria D'Acquarica, Flavia Bernardi, et al. 2016. "Inhibition of Hedgehog-Dependent Tumors and Cancer Stem Cells by a Newly Identified Naturally Occurring Chemotype." *Cell Death & Disease* 7 (9): e2376–e2376. https://doi.org/10.1038/cddis.2016.195.

Kansy, Manfred, Holger Fischer, Krystyna Kratzat, Frank Senner, Björn Wagner, and Isabelle Parrilla. 2001. "High-Throughput Artificial Membrane Permeability Studies in Early Lead Discovery and Development." In *Pharmacokinetic Optimization in Drug Research*, edited by Bernard Testa, Han van de Waterbeemd, Gerd Folkers, and Richard Guy, 447–64. Zürich: Verlag Helvetica Chimica Acta. https://doi.org/10.1002/9783906390437.ch24.

Kansy, Manfred, Frank Senner, and Klaus Gubernator. 1998. "Physicochemical High Throughput Screening: Parallel Artificial Membrane Permeation Assay in the Description of Passive Absorption Processes." *Journal of Medicinal Chemistry* 41 (7): 1007–10. https://doi.org/10.1021/jm970530e.

Khan, Muhammad Azim, Robert E. Blackshaw, and Khan B. Marwat. 2009. "Biology of Milk Thistle (*Silybum Marianum*) and the Management Options for Growers in North-

Western Pakistan." *Weed Biology and Management* 9 (2): 99–105. https://doi.org/10.1111/j.1445-6664.2009.00326.x.

Kosina, Pavel, Vladimír Kren, Rolf Gebhardt, Franti?ek Grambal, Jitka Ulrichová, and Daniela Walterová. 2002. "Antioxidant Properties of Silybin Glycosides." *Phytotherapy Research* 16 (S1): 33–39. https://doi.org/10.1002/ptr.796.

Křen, Vladimír. 2021. "Chirality Matters: Biological Activity of Optically Pure Silybin and Its Congeners." *International Journal of Molecular Sciences* 22 (15): 7885. https://doi.org/10.3390/ijms22157885.

Křenek, K., P. Marhol, Ž. Peikerová, V. Křen, and D. Biedermann. 2014. "Preparatory Separation of the Silymarin Flavonolignans by Sephadex LH-20 Gel." *Food Research International* 65 (November): 115–20. https://doi.org/10.1016/j.foodres.2014.02.001.

Kumar, Ashutosh, and Kam Y. J. Zhang. 2018. "Advances in the Development of Shape Similarity Methods and Their Application in Drug Discovery." *Frontiers in Chemistry* 6 (July): 315. https://doi.org/10.3389/fchem.2018.00315.

Kvasnička, F, B Bíba, R Ševčík, M Voldřich, and J Krátká. 2003. "Analysis of the Active Components of Silymarin." *Journal of Chromatography A* 990 (1–2): 239–45. https://doi.org/10.1016/S0021-9673(02)01971-4.

Langowski, Jan, and Anthony Long. 2002. "Computer Systems for the Prediction of Xenobiotic Metabolism." *Advanced Drug Delivery Reviews* 54 (3): 407–15. https://doi.org/10.1016/S0169-409X(02)00011-X.

Li, Feng, Yongli Wang, Dapeng Li, Yilun Chen, and Q. Ping Dou. 2019. "Are We Seeing a Resurgence in the Use of Natural Products for New Drug Discovery?" *Expert Opinion on Drug Discovery* 14 (5): 417–20. https://doi.org/10.1080/17460441.2019.1582639.

Lorenzo, José M., Predrag Putnik, Danijela Bursać Kovačević, Marinko Petrović, Paulo E. Munekata, Belén Gómez, Krystian Marszałek, Shahin Roohinejad, and Francisco J. Barba. 2020. "Silymarin Compounds: Chemistry, Innovative Extraction Techniques and Synthesis." In *Studies in Natural Products Chemistry*, 64:111–30. Elsevier. https://doi.org/10.1016/B978-0-12-817903-1.00004-8.

Lospinoso Severini, Quaglio, Basili, Ghirga, Bufalieri, Caimano, Balducci, et al. 2019. "A Smo/Gli Multitarget Hedgehog Pathway Inhibitor Impairs Tumor Growth." *Cancers* 11 (10): 1518. https://doi.org/10.3390/cancers11101518.

Luke, Jason J., and F. Stephen Hodi. 2012. "Vemurafenib and BRAF Inhibition: A New Class of Treatment for Metastatic Melanoma." *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 18 (1): 9–14. https://doi.org/10.1158/1078-0432.CCR-11-2197.

Machhar, Janvhi, Ansh Mittal, Surendra Agrawal, Anil M. Pethe, and Prashant S. Kharkar. 2019. "Computational Prediction of Toxicity of Small Organic Molecules: State-of-the-Art." *Physical Sciences Reviews* 4 (10). https://doi.org/10.1515/psr-2019-0009.

Marchant, Carol A., Katharine A. Briggs, and Anthony Long. 2008. "In Silico Tools for Sharing Data and Knowledge on Toxicity and Metabolism: Derek for Windows, Meteor, and Vitic." *Toxicology Mechanisms and Methods* 18 (2–3): 177–87. https://doi.org/10.1080/15376510701857320.

Moumbock, Aurélien F.A., Jianyu Li, Pankaj Mishra, Mingjie Gao, and Stefan Günther. 2019. "Current Computational Methods for Predicting Protein Interactions of Natural Products." *Computational and Structural Biotechnology Journal* 17: 1367–76. https://doi.org/10.1016/j.csbj.2019.08.008.

Newman, David J., and Gordon M. Cragg. 2020. "Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019." *Journal of Natural Products* 83 (3): 770–803. https://doi.org/10.1021/acs.jnatprod.9b01285.

Peter, Swathik Clarancia, Jaspreet Kaur Dhanjal, Vidhi Malik, Navaneethan Radhakrishnan,

Mannu Jayakanthan, and Durai Sundar. 2019. "Quantitative Structure-Activity Relationship (QSAR): Modeling Approaches to Biological Applications." In *Encyclopedia of Bioinformatics and Computational Biology*, 661–76. Elsevier. https://doi.org/10.1016/B978-0-12-809633-8.20197-0.

Petrásková, Káňová, Biedermann, Křen, and Valentová. 2020. "Simple and Rapid HPLC Separation and Quantification of Flavonoid, Flavonolignans, and 2,3-Dehydroflavonolignans in Silymarin." *Foods* 9 (2): 116. https://doi.org/10.3390/foods9020116.

Plíšková, Martina, Jan Vondráček, Vladimír Křen, Radek Gažák, Petr Sedmera, Daniela Walterová, Jitka Psotová, Vilím Šimánek, and Miroslav Machala. 2005. "Effects of Silymarin Flavonolignans and Synthetic Silybin Derivatives on Estrogen and Aryl Hydrocarbon Receptor Activation." *Toxicology* 215 (1–2): 80–89. https://doi.org/10.1016/j.tox.2005.06.020.

Rodrigues, Tiago, Daniel Reker, Petra Schneider, and Gisbert Schneider. 2016. "Counting on Natural Products for Drug Design." *Nature Chemistry* 8 (6): 531–41. https://doi.org/10.1038/nchem.2479.

Rogers, David J., and Taffee T. Tanimoto. 1960. "A Computer Program for Classifying Plants: The Computer Is Programmed to Simulate the Taxonomic Process of Comparing Each Case with Every Other Case." *Science* 132 (3434): 1115–18. https://doi.org/10.1126/science.132.3434.1115.

Rudrapal, Mithun, and Chukwuebuka Egbuna. 2022. *Computer Aided Drug Design (CADD): From Ligand-Based Methods to Structure-Based Approaches*. San Diego: Elsevier.

Sandhiya, Selvarajan, George Melvin, Srinivasamurthy Suresh Kumar, and Steven Aibor Dkhar. 2013. "The Dawn of Hedgehog Inhibitors: Vismodegib." *Journal of Pharmacology & Pharmacotherapeutics* 4 (1): 4–7. https://doi.org/10.4103/0976-500X.107628.

Silva, Fredson, and Gustavo Trossini. 2014. "The Survey of the Use of QSAR Methods to Determine Intestinal Absorption and Oral Bioavailability During Drug Design." *Medicinal Chemistry* 10 (5): 441–48. https://doi.org/10.2174/1573406410666140415122115.

Soleimani, Vahid, Parisa Sadat Delghandi, Seyed Adel Moallem, and Gholamreza Karimi. 2019. "Safety and Toxicity of Silymarin, the Major Constituent of Milk Thistle Extract: An Updated Review." *Phytotherapy Research*, May. https://doi.org/10.1002/ptr.6361.

Stanzione, Francesca, Ilenia Giangreco, and Jason C. Cole. 2021. "Use of Molecular Docking Computational Tools in Drug Discovery." In *Progress in Medicinal Chemistry*, 60:273–343. Elsevier. https://doi.org/10.1016/bs.pmch.2021.01.004.

Stenberg, Patric, Christel A.S. Bergstr??m, Kristina Luthman, and Per Artursson. 2002. "Theoretical Predictions of Drug Absorption in Drug Discovery and Development:" *Clinical Pharmacokinetics* 41 (11): 877–99. https://doi.org/10.2165/00003088-200241110-00005.

the International Natural Product Sciences Taskforce, Atanas G. Atanasov, Sergey B. Zotchev, Verena M. Dirsch, and Claudiu T. Supuran. 2021. "Natural Products in Drug Discovery: Advances and Opportunities." *Nature Reviews Drug Discovery* 20 (3): 200–216. https://doi.org/10.1038/s41573-020-00114-z.

Verma, Rajeshwar P., Corwin Hansch, and Cynthia D. Selassie. 2007. "Comparative QSAR Studies on PAMPA/Modified PAMPA for High Throughput Profiling of Drug Absorption Potential with Respect to Caco-2 Cells and Human Intestinal Absorption." *Journal of Computer-Aided Molecular Design* 21 (1–3): 3–22. https://doi.org/10.1007/s10822-006-9101-z.

Wang, Junmei, and Tingjun Hou. 2009. "Chapter 5 Recent Advances on in Silico ADME Modeling." In *Annual Reports in Computational Chemistry*, 5:101–27. Elsevier. https://doi.org/10.1016/S1574-1400(09)00505-2.

Wang, Xin, Zhen Zhang, and Shuai-Cheng Wu. 2020 "Health Benefits of Silybum Marianum: Phytochemistry, Pharmacology, and Applications." *Journal of Agricultural and Food Chemistry*, 21.

Waterbeemd, Han van de, Hans Lennernäs, and Per Artursson, eds. 2003. *Drug Bioavailability: Estimation of Solubility, Permeability, Absorption, and Bioavailability*. Methods and Principles in Medicinal Chemistry, v. 18. Weinheim: Wiley-VCH.

Wishart, D. S. 2006. "DrugBank: A Comprehensive Resource for in Silico Drug Discovery and Exploration." *Nucleic Acids Research* 34 (90001): D668–72. https://doi.org/10.1093/nar/gkj067.

Wright, Gerard D. 2017. "Opportunities for Natural Products in 21 [st] Century Antibiotic Discovery." *Natural Product Reports* 34 (7): 694–701. https://doi.org/10.1039/C7NP00019G.

Wright. 2019. "Unlocking the Potential of Natural Products in Drug Discovery." *Microbial Biotechnology* 12 (1): 55–57. https://doi.org/10.1111/1751-7915.13351.

PUBLICATIONS RELATED TO THE PHD THESIS

PUBLICATIONS IN SCIENTIFIC JOURNALS

1. **Diukendjieva A.**, M. Al Sharif, P. Alov, T. Pencheva, I. Tsakovska, I. Pajeva. ADME/Tox Properties and Biochemical interactions of Silybin Congeners: *In Silico* Study. *Natural Product Communications*, 2017, 12(2), 175-178. **IF=0.809 (2017), Q2. Citations: 6 (WoS), 5 (Scopus)**

2. **Diukendjieva A.**, P. Alov, I. Tsakovska, T. Pencheva, A. Richarz,V. Kren,M.T.D. Cronin, I. Pajeva. *In vitro* and *in silico* studies of the membrane permeability of natural flavonoids from *Silybum marianum(*L.) Gaertn. and their derivatives. *Phytomedicine*, 2018, 53. pp. 79-85. ISSN 0944-7113 . **IF = 4.268 (2019), Q1. Citations: 11 (WoS), 11 (Scopus)**

3. **Diukendjieva A.**, I. Tsakovska, P. Alov, T. Pencheva, I. Pajeva, A. Worth, M.T.D. Cronin. Advances in the prediction of gastrointestinal absorption: QSAR PAMPA modelling. *Computational Toxicology* (Special Issue *"In Silico* Approaches for the Safety Assessment of Cosmetic-Related Substances"), 2019, 51–59. **SJR = 0.579(2019), Q2. Citations: 12 (Scopus)**

4. **Diukendjieva A**., M. Zaharieva, M. Mori, P. Alov, I. Tsakovska, T. Pencheva, H. Najdenski. Dual SMO/BRAF Inhibition by Flavonolignans from *Silybum Marianum*. *Antioxidants,* 2020, 9 (5): 384. **IF=6.313 (2020), Q2. Citations: 14 (WoS); 9 (Scopus)**

REPORTS IN PROCEEDINGS

1. **Diukendjieva A.**, L. Marinov, P. Alov, I. Tsakovska, I. Pajeva. *In silico* modelling to predict transcellular permeability of bioactive compounds. *Series G. Medicine, Pharmacy and Dental medicine, Union of Scientists in Bulgaria – Plovdiv*, Vol. XVII, p. 187-190, 2015. ISSN 1311-9427

2. **Diukendjieva, A.**, M. Al Sharif, I. Tsakovska, T. Pencheva, P. Alov, I. Pajeva. *In silico* study of natural compounds: prediction of metabolism, toxicity and biochemical interactions. In: *Proceedings of the Humboldt Kolleg,16-18 Nov 2017, Sofia*, Faber, 2018, 24-33.

CONTRIBUTIONS TO SCIENTIFIC FORUMS

INTERNATIONAL SCIENTIFIC EVENTS

1. Diukendjieva A., Mori M., Alov P., Tsakovska I., Botta M., Pajeva I**.** *In silico* evaluation of new potential target proteins of flavonolignans from *Silybum marianum*. *6*[th] *Edition of International Conference on Pharmacognosy and Medicinal Plants,* 16-17 April, 2018, Amsterdam, Netherlands.

2. Diukendjieva A., Al Sharif M., Tsakovska I., Pencheva T., Alov P., Pajeva I. *In silico* study of natural compounds: prediction of metabolism, toxicity and biochemical interactions. *Humboldt Kolleg, Humboldtians and scientific progress in the Central and East European countries,* 16–18 November, 2017, Sofia, Bulgaria.

3. Diukendjieva A., Alov P., Tsakovska I., Pencheva T., Cronin M., Madden J., Yang C., Pajeva I. *In Silico* Model of PAMPA Permeability as an Estimator of Gastrointestinal Absorption of Bioactive Compounds. *21st European Symposium On Quantitative Structure-Activity Relationship*, 4-8 September 2016, Verona, Italy.

4. Diukendjieva A., Fratev F. Alov P. Al Sharif M., Pencheva T., Kren V., Tsakovska I., Pajeva I. *In Silico* Study of ADME/Tox Properties of Selected Flavonolignans. 2nd Meeting of Cost Action CM1407 "*Challenging Organic Syntheses Inspired By Nature - From Natural Products Chemistry To Drug Discovery*", 4-5 April 2016, Madrid, Spain.

5. Diukendjieva A., Al Sharif M., Alov P., Pencheva T., Kren V., Tsakovska I., Pajeva I. Adme/Tox Properties and Biochemical Interactions of Silybin Congeners: *In Silico* Study. *9th Conference on Medicinal and Aromatic Plants of Southeast European Countries – 9th CMAPSEEC*, 11 – 12 June 2015, Plovdiv, Bulgaria.

NATIONAL SCIENTIFIC EVENTS

1. Diukendjieva A., Al Sharif M,, Alov P., Pencheva T., Tsakovska I., Pajeva I. Natural flavonoids from *Silybum marianum L.* (milk thistle)*: in vitro* and *in silico* studies of pharmacokinetic properties and toxicity. *Scientific session "Biomedicine and quality of life - young people in science",* 26 - 27 June 2017, Sofia, Bulgaria.

2. Diukendjieva A., Alov P., Pencheva T., Tsakovska I., Fratev F., Al Sharif M., Pajeva I. *In Vitro and In Silico* Studies of the Membrane Transport of Selected Flavonoids. *Third National Congress On Physical Sciences*, 29 September – 2 October, 2016, Sofia, Bulgaria.

3. Diukendjieva A, Alov P, Tsakovska I, Pajeva I. QSAR Models For Predicting Transcellular Permeability of Bioactive Compounds,. *9th Workshop "Biological Activity of Metals, Synthetic Compounds and Natural Products*, 26 – 28 November, 2014, Sofia, Bulgaria.

1869