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In silico assessment of skin sensitization potential of polyphenolic metabolites from *Sideritis syriaca* using QSAR toolbox

G. Rusev*, V. Dzhogov, L. Gonsalvesh

[1]*Department of Chemistry, Burgas State University "Prof. Assen Zlatarov", 1 Prof. Yakimov Str., 8010 Burgas, Bulgaria*

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Accurate prediction of skin sensitization potential and potency is essential for the safety of consumers and workers across multiple industries, including cosmetics, pharmaceuticals, and nutraceuticals. Although animal testing has traditionally served as the gold standard for hazard assessment, the development and adoption of non-animal and *in silico* approaches are rapidly advancing in response to ethical and regulatory demands. In this study, the skin sensitization risk of ten predominant polyphenolic compounds isolated from *Sideritis syriaca* was evaluated using the QSAR toolbox (version 4.7.1). Available *in vivo* data were limited, with only one compound having published results. Protein-binding alerts, as identified by the OASIS profiler, were detected in two of the parent compounds. Further simulation of abiotic and metabolic transformation pathways generated 89 predicted metabolites, uncovering mechanistic alerts including Michael addition, nucleophilic addition, Schiff base formation, acylation, and radical-mediated reactions. Among the compounds studied, verbascoside exhibited the highest predicted reactivity. While most parent compounds displayed a low sensitization hazard, simulation of their metabolites indicated potential for downstream risk. These findings underscore the utility of *in silico* tools for robust and early safety evaluation of plant-derived secondary metabolites and support the ongoing transition toward animal-free toxicological assessments.

Keywords: Skin sensitization, *in silico*, metabolic simulation, polyphenols, QSAR toolbox

INTRODUCTION

Skin sensitization testing is a regulatory cornerstone for the safety assessment of chemical ingredients, particularly in the cosmetics industry, prior to their market release. Historically, skin sensitization hazard identification relied predominantly on animal-based models. The guinea pig maximization test, established by Magnusson and Kligman [1], was one of the earliest standardized assays for evaluating a chemical's potential to induce human skin sensitization. This was subsequently superseded by the murine local lymph node assay (LLNA), recognized for its higher sensitivity, improved quantification of allergic potency, and suitability for dose–response assessment [2]. The landscape of skin sensitization testing has, however, evolved dramatically in response to regulatory, ethical, and scientific developments. The implementation of the European Union Cosmetics Directive (Regulation (EC) No 1223/2009), notably Article 18, and the REACH regulation (EC No 1907/2006), strictly prohibit animal testing for cosmetic ingredients and finished products, setting a precedent for similar restrictions worldwide. In parallel, increased societal concern for animal welfare and strengthened international consensus have catalyzed the development and validation of alternative test methods, in alignment with the principles of the 3Rs (Replacement, Reduction, Refinement).

Concurrently, our understanding of the immunological mechanisms underlying skin sensitization has deepened. Skin sensitization is now recognized as an adaptive immune reaction, tightly orchestrated through a cascade of molecular and cellular events occurring across two primary phases: induction and elicitation. Advances in toxicological research have culminated in the adverse outcome pathway (AOP) framework for skin sensitization [3], codified in the OECD Test Guideline No. 497. Briefly, this AOP encompasses four well-defined key events: (*i*) covalent binding of electrophilic chemicals (haptens) to skin proteins, (*ii*) keratinocyte activation and release of pro-inflammatory signals, (*iii*) activation and maturation of dendritic cells and their migration to lymph nodes, and (*iv*) proliferation of antigen-specific T-cells, ultimately leading to allergic contact dermatitis upon re-exposure. Given the complexity of these biochemical interactions and their relevance to public health, the accurate assessment of skin sensitization potential has become critically important, especially as a wider array of natural

* To whom all correspondence should be sent:
E-mail: *georgi-rusev@uniburgas.bg*

substances—including botanicals, nutraceuticals, and phytopharmaceuticals—enters consumer product markets [4].

Computational (*in silico*) approaches such as quantitative structure–activity relationship (QSAR) modeling and expert rule-based systems have emerged as essential tools for predicting sensitization hazards, capable of integrating vast datasets, minimizing animal use, and supporting regulatory compliance [5]. These models, often calibrated to LLNA and human data, are especially valuable for screening large chemical libraries and structurally diverse secondary metabolites, including those from medicinal plants such as *Sideritis syriaca*. A skin sensitizer, as defined by the Globally Harmonized System (GHS) and REACH, is any substance that induces an allergic response following repeated dermal exposure. The present study aims to predict the skin sensitization potential of the ten selected secondary metabolites from *Sideritis syriaca* by applying the OECD QSAR toolbox (v.4.7.1), evaluating both the parent compounds and their putative metabolites generated *via* simulation of abiotic and skin metabolic pathways. This approach provides a comprehensive, animal-free evaluation framework that aligns with current scientific and regulatory standards.

METHODOLOGY

Using QSAR toolbox (version 4.7.1), the ten plant secondary metabolites, named as parent compounds (Supplementary Table), with the highest concentrations (ranging from 11 to 95 $mg \cdot g^{-1}$ extract), identified *via* HPLC-MS/MS analysis of methanolic extracts from both cultivated and wild *Sideritis syriaca*, were selected for evaluation. HPLC-MS/MS is a powerful analytical technique widely used for the separation, identification, and quantification of polyphenols in complex matrices such as plant extracts, foods, and biological samples. The method combines the high-resolution separation capability of high-performance liquid chromatography (HPLC) with the specificity and sensitivity of tandem mass spectrometry (MS/MS), enabling the detection and structural elucidation of a broad range of polyphenolic compounds, including phenolic acids, flavonoids, and their derivatives [6]. MS/MS fragmentation patterns are crucial for identifying polyphenol classes, as each class displays a characteristic cleavage behavior that reflects their core structure and substituents. For flavonoids, common fragmentation pathways include Retro-Diels-Alder (RDA) reactions in the C-ring, which generate diagnostic ions, and alpha-cleavage or loss of sugar moieties for glycosides, producing aglycone fragments. Phenolic acids typically show losses of CO_2, CO, and H_2O, with methoxy groups cleaving at lower energies, aiding in their differentiation. Glycosylated polyphenols exhibit neutral losses corresponding to the sugar unit (e.g., 162 Da for hexoses, 120 Da for pentoses), and the fragmentation pattern can distinguish between O-glycosides and C-glycosides, as C-glycosides often show more complex sugar fragmentation and water loss. These reproducible fragmentation trees enable confident identification and classification of polyphenols in complex samples [7].

The compounds comprised three phenolic acids (syringic, salicylic, gentisic), three cinnamic acid derivatives (caffeic, ferulic, chlorogenic) [8], the phenylethanoid glycoside verbascoside, the flavone apigenin, and two flavanols – kaempferol and isorhamnetin [9]. Where available, *in vivo* skin sensitization data (LLNA and GPMT) were retrieved and analyzed. Further profiling of the selected parent structures was conducted using the OASIS protein-binding profiler. Additionally, two simulation modules were applied to predict the formation and reactivity of potential metabolites: the abiotic autoxidation simulator and the skin sensitization metabolism simulator [3, 10].

Autoxidation (AO) refers to the spontaneous oxidation of organic molecules triggered by exposure to air [11, 12]. This process involves a free-radical chain reaction between the compound and molecular oxygen, leading to the production of various oxidation products. Among these, organic hydroperoxides are considered especially significant due to their potential to cause adverse effects such as contact allergy. To investigate and predict AO pathways, an AO model was designed. A training set comprising 140 chemicals—including terpenes, simple aliphatic and polyethyleneglycol ethers, aldehydes, and aminophenols—with published data on AO pathways was assembled. To ensure data consistency, experimental conditions were standardized: exposure to air or oxygen at room temperature and atmospheric pressure, in bulk or with different solvents, and under nearly neutral (pH 7–7.5) or slightly alkaline (pH 8–9) conditions. The duration of AU ranged from a few hours to several months. The AU simulator is based on a collection of molecular transformations extracted from these documented AO pathways.

The skin metabolism simulator (SMS) is designed to replicate how chemicals are metabolized within the skin compartment. Due to the scarcity of experimental data on skin metabolism and the prevailing assumption that skin enzymes process xenobiotics in ways similar to the liver, the simulator

is modeled as a streamlined version of a mammalian liver metabolism simulator. The SMS employs a structured sequence of primary transformations, categorized into two types:

- Rate-determining transformations: These include Phase I and Phase II metabolic reactions such as C-hydroxylation, ester hydrolysis, oxidation, glutathione conjugation, glucuronidation, and sulfonation.
- Non-rate-determining transformations: These involve molecular changes of highly reactive intermediates that occur following the primary metabolic steps.

Simulation process begins by comparing the parent compound to a set of reaction fragments, focusing on the transformation with the highest likelihood of occurring. This yields a group of first-level metabolites. Each new metabolite formed is evaluated using the same sequence of hierarchical transformations, generating a second tier of metabolites. The simulation continues iteratively, processing each metabolite, until it meets specific propagation constraints—for example, when the likelihood of forming additional metabolites becomes low or when a Phase II reaction has taken place.

RESULTS AND DISCUSSION

Protein-binding alerts associated with skin sensitization were identified in only two of the ten evaluated parent compounds – verbascoside and chlorogenic acid. Both compounds possess aromatic rings linked to side chains with conjugated double bonds (alternating single and double bonds), often adjacent to electron-withdrawing groups like carbonyls (C=O). This arrangement increases the electrophilicity (electron deficiency) of certain carbon atoms, making them reactive toward nucleophilic groups (e.g., amino acids in skin proteins). The presence of a carbonyl group (C=O) at the β-position (two atoms away from a reactive center) polarizes the double bond, further enhancing its reactivity. This is a hallmark of structures prone to Michael-type addition (Fig. 1).

This reaction involves the addition of a nucleophile (such as the amino group of a skin protein) to an α, β-unsaturated carbonyl compound. The conjugated system with a polarized double bond acts as an electrophilic trap for nucleophiles, facilitating covalent protein binding – a recognized mechanism in skin sensitization.

Upon application of the abiotic autoxidation simulator, 26 metabolites were generated from six parent structures. These metabolites exhibited protein-binding alerts across four mechanistic domains: Michael addition, Acylation, Radical-based reactions and Schiff base formation. Autoxidation typically generates reactive oxygen species (ROS) and may modify certain chemicals to more reactive forms. The detailed results show that more metabolites have been generated for isorhamnetin and kaempferol (10 for each compound). Three of them, which are similar for both chemicals, have no structural alerts.

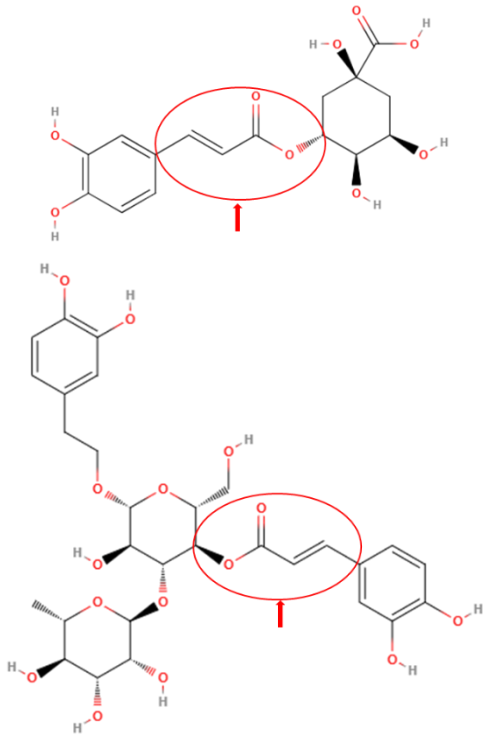

Fig. 1. Reactive centers for two of the parent structures – chlorogenic acid (up) and verbascoside (down).

Both molecules possess several free hydroxyl groups on their aromatic rings, especially at positions that are highly susceptible to oxidation (e.g., 3, 5, 7, and 4′ for kaempferol; 3, 5, 7, and 4′ for isorhamnetin, with the 3′ position methylated in isorhamnetin – Fig. 2).

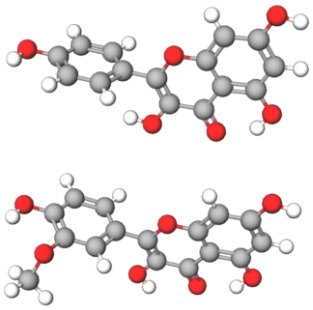

Fig. 2. 3D molecular models of kaempferol (up) and isorhamnetin (down). C atoms are gray, H atoms are white and O atoms are red.

These hydroxyl groups facilitate the formation of phenoxyl radicals, which can undergo further transformation, leading to a cascade of chemical reactions and metabolite diversity.

The conjugated double bonds in the flavanol structure allow for electron delocalization, making the molecules more reactive toward oxygen and free radicals. This delocalization promotes the formation of quinones, ring-opened products, and other oxidized derivatives [13, 14]. The presence of multiple hydroxyl groups, especially at the 3-position, gives these compounds a high hydrogen-donor capacity, which is crucial for initiating autoxidation and subsequent metabolite formation (Fig. 3).

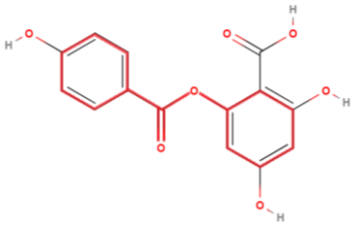

Fig. 3. One of the isorhamnetin-generated metabolites with possible active sites (centers).

For the caffeic and chlorogenic acids two metabolites have been generated. The two chemicals are highly prone to autoxidation due to the catechol structure, leading to rapid formation of o-quinones, dimers, and polymeric products [15]. The primary pathway involves hydrogen atom transfer and radical adduct formation, resulting in cyclic metabolites and oxidized dimers. With two generated metabolites for each compound after simulated abiotic autoxidation apigenin and verbascoside show moderate diversity.

When using an autoxidation metabolic simulator on phenolic acids such as syringic, salicylic, gentisic and ferulic acids, several factors could explain the absence of predicted metabolites – lack of highly reactive functional groups and methoxy group stabilization [16]. These phenolic acids possess relatively simple structures without highly reactive sites (such as ortho-dihydroxy or α, β-unsaturated carbonyl groups) that are more prone to autoxidation. For example, syringic acid has methoxy substituents that stabilize the aromatic ring, while salicylic and gentisic acids have hydroxyl groups that do not favor easy radical formation or further transformation under mild oxidative conditions. In syringic acid, the methoxy groups at the 3- and 5- positions increase the bond dissociation enthalpy of the phenolic O–H bond, making radical formation less favorable and thus reducing the likelihood of autoxidative transformation. These phenolic acids are known for their antioxidant properties, meaning that they can efficiently neutralize free radicals rather than propagate radical chain reactions [17, 18]. This property can limit their own autoxidation and the subsequent formation of detectable metabolites. In some cases, phenolic acids can form dimers or quinones upon oxidation, but these reactions may require specific conditions (e.g., high pH, presence of metal ions, or enzymatic catalysis) that are not replicated in the simulator's standard autoxidation model. Studies have shown that benzoic acid derivatives (which include syringic, salicylic, and gentisic acids) are less prone to autoxidation compared to cinnamic acid derivatives, often taking part in side reactions rather than producing distinct oxidation products under standard conditions [19,20]. The antioxidant and radical-scavenging nature of ferulic acid also limits further oxidation under mild conditions. It possesses a methoxy group and a conjugated side chain, which can participate in radical reactions, but under standard autoxidation, the main products are often dimers or oligomers rather than distinct small-molecule metabolites.

The skin sensitization metabolism simulator yielded 63 metabolites derived from nine of the ten parent compounds [21–23]. The only one compound with no metabolites generated is salicylic acid. Its structure lacks electrophilic centers or functional groups that could readily undergo autoxidation to form reactive metabolites under physiological conditions. In autoxidation, salicylic acid can act as a free radical scavenger rather than a substrate that forms reactive intermediates. The main reaction with ROS (such as hydroxyl radicals) leads to hydroxylation, producing minor metabolites like 2,3- and 2,5-dihydroxybenzoic acid, but not highly reactive or protein-binding metabolites. These metabolites are not considered electrophilic enough to form stable protein adducts, which are crucial for skin sensitization. Salicylic acid does not generate metabolites with structural alerts for skin sensitization because its metabolic products (mainly through hydroxylation and conjugation) are not electrophilic and do not form covalent bonds with proteins. The other two cinnamic acid derivatives (caffeic actd and ferulic acid) generated 1 and 4 metabolites, respectively. For two of the ferulic acid metabolites there is no alert. The rest 3 show mechanistic alert Michael addition on quinoid type compounds and Schiff base formation with carbonyl compounds (Fig. 4).

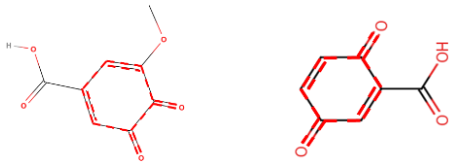

Fig. 4. Structural formulas of two of the generated metabolites for syringic and gentisic acids (red dashed lines show possible reactive sites)

Ortho-quinones and para-quinones can react with proteins through 1,4-addition, in which the amino group (NH_2) of lysine or the thiol group (SH) of cysteine adds across the quinone ring system [24]. These quinones are typically generated by oxidation of para- and ortho-dihydroxy aromatic compounds (pro-Michael acceptors) which, through well-established metabolic or abiotic transformations, become Michael acceptors—for example, the oxidation of hydroquinone to benzoquinone. This Michael-type addition allows covalent bond formation at an electrophilic center without the need for a leaving group [25]. The reactive groups of the Michael acceptor can be present either in open-chain form or as part of a ring structure, such as a quinone (Fig. 4).

Similarly, ortho- and para-aminophenols, after undergoing metabolic or abiotic transformation, can form quinone imines which also react with proteins *via* Michael-type addition. Nitrophenols, following metabolic or abiotic processes such as keto-aci tautomerism, can form nitroquinones that react with proteins in the same manner. For instance, 2,4-dinitrophenol acts as a cellular metabolic toxin, and exposure may provoke allergic reactions including hives, blisters, or unexplained rashes [26]. Additionally, aromatic primary or secondary amines situated ortho to a nitro group may induce sensitization through the formation of aci-tautomers. However, for Michael adducts to form by 1,4-addition, at least one position on the aromatic ring must remain unsubstituted.

A comprehensive assessment of the *in vivo* and *in vitro* data available for all compounds generated by the two simulation models (AO and SMS) demonstrated that such information exists for only a limited subset of the 89 predicted metabolites. For the three cinnamic acid derivatives investigated (caffeic, ferulic, and chlorogenic acids), *in vivo* and *in vitro* data were identified solely for two metabolites of ferulic acid generated by AO and SMS, namely formaldehyde and formic acid. Evidence of potential skin sensitization, including positive test outcomes, was observed only for formaldehyde, whereas the *in vivo* and *in vitro* data for formic acid were negative. For the three phenolic acids examined – syringic, salicylic, and gentisic acids – experimental data were available exclusively for two metabolites of syringic acid, again identified as formaldehyde and formic acid [27]. A systematic review of *in* vivo and *in vitro* data for metabolites generated from the phenylalanine glycoside verbascoside by the SMS simulator revealed that *in vivo* information is available for only one out of the 28 predicted metabolites – hydroxytyrosol, which has been characterized as a possible skin sensitizer [28]. *In vitro* data exist solely for D-(+)-glucose, which has been found to yield negative results. No experimental data were found for the flavone apigenin. For the two flavanols, isorhamnetin and kaempferol, data are available for two distinct compounds: *in vivo* data for vanillic acid indicate no evidence of skin sensitization [29], while *in vitro* data for para-salicylic acid are also negative [30].

CONCLUSIONS

In silico methods using the QSAR toolbox can effectively predict the skin sensitization potential of polyphenolic compounds and their metabolites, supporting animal-free toxicological assessment approaches. Among the ten polyphenolic parent compounds examined, most showed low skin sensitization hazard; however, two parent compounds – verbascoside and chlorogenic acid – displayed protein-binding alerts indicative of potential sensitization mechanisms such as Michael addition. Simulation of abiotic autoxidation and skin metabolism pathways generated 89 predicted metabolites, many with mechanistic alerts including Michael addition, Schiff base formation, acylation, and radical-mediated reactions, revealing a higher risk of sensitization from metabolites than from parent compounds alone. Verbascoside exhibited the highest predicted reactivity and number of reactive metabolites, highlighting molecular complexity as a factor that increases potential sensitization risk. Certain compounds like syringic, salicylic, gentisic, and ferulic acids were less prone to autoxidation and did not generate metabolites with sensitization alerts due to structural features that confer antioxidant stability. The findings emphasize the critical role of metabolic transformation in modulating skin sensitization risk and the utility of combining abiotic and metabolic simulation to comprehensively evaluate hazards of natural product secondary metabolites. The overall results support the use of computational models aligned with regulatory frameworks such as the OECD adverse outcome pathway for skin sensitization as robust tools for early safety evaluation, aiding industry compliance with animal testing bans and promoting ethical research. The paucity of available experimental data

underscores the necessity for robust physicochemical and mathematical models to enable accurate forecasting and prediction of the potential metabolic activities of the compounds under investigation.

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