HHS Public Access

Author manuscript

Fitoterapia. Author manuscript; available in PMC 2018 June 01.

Published in final edited form as:

Fitoterapia. 2017 June ; 119: 175–184. doi:10.1016/j.fitote.2017.04.002.

Chemotaxonomic and biosynthetic relationships between flavonolignans produced by *Silybum marianum* populations

Sameh F. AbouZid[a,*], **Hayam S. Ahmed**[a], **Abeer S. Moawad**[a], **Asmaa I. Owis**[a], **Shao-Nong Chen**[b,c], **Amandine Nachtergael**[c], **James B. McAlpine**[c], **J. Brent Friesen**[c,d], and **Guido F. Pauli**[b,c]

[a]Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62111, Egypt

[b]UIC/NIH Center for Botanical Dietary Supplements Research, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St., M/C 781, Chicago, IL 60612, United States

[c]Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St., M/C 781, Chicago, IL 60612, United States

[d]Physical Sciences Department, Rosary College of Arts and Sciences, Dominican University, 7900 West Division Street, River Forest, IL 60305, United States

Abstract

Flavonolignans constitute an important class of plant secondary metabolites formed by oxidative coupling of one flavonoid and one phenylpropanoid moiety. The standardized flavonolignan-rich extract prepared from the fruits of *Silybum marianum* is known as silymarin and has long been used medicinally, prominently as an antihepatotoxic and as a chemopreventive agent. Principal component analysis of the variation in flavonolignan content in *S. marianum* samples collected from different locations in Egypt revealed biosynthetic relationships between the flavonolignans. Silybin A, silybin B, and silychristin are positively correlated as are silydianin, isosilychristin, and isosilybin B. The detection of silyamandin in the extracts of *S. marianum* correlates with isosilychristin and silydianin content. The positive correlation between silydianin, isosilychristin, and silyamandin was demonstrated using quantitative ^{1}H nuclear magnetic resonance spectroscopy (qHNMR). These correlations can be interpreted as evidence for the involvement of a flavonoid radical in the biosynthesis of the flavonolignans in *S. marianum*. The predominance of silybins A & B over isosilybin A & B in the silybin-rich samples is discussed in light of the relative stabilities of their respective radical flavonoid biosynthetic intermediates.

Keywords

Silybum marianum; Flavonolignans; Biosynthesis; Principal component analysis; qHNMR

[*]Corresponding author. sameh.zaid@pharm.bsu.edu.eg (S.F. AbouZid).

1. Introduction

Flavonolignans are an important class of plant natural products. These compounds are a special class of lignans which are basically defined as dimers of substituted cinnamic alcohols. Biosynthetically flavonolignans result from oxidative coupling between a flavonoid moiety and a phenylpropanoid part. The flavonoid moiety can be taxifolin, eriodictyol, naringenin, chrysoeriol, tricin, luteolin, apigenin, or quercetin. Coniferyl alcohol, a monolignol, constitutes the phenyl-propanoid part in most flavonolignans [1]. The direct precursors of the flavonolignans, the flavonoids and monolignols, are synthesized via the phenylpropanoid pathway transforming phenylalanine into 4-coumaroyl-CoA [2]. Cloning and characterization of a full-length chalcone synthase cDNA and one partial gene from *S. marianum* that is probably involved in flavonolignan biosynthesis have been reported recently [3].

The silybins A & B, isosilybins A & B, silychristin A, isosilychristin, and silydianin are the major flavonolignans produced by the fruits of *Silybum marianum* (L.) Gaertn. var. *purple* (Asteraceae). They are formed by oxidative coupling of a taxifolin flavanonol precursor and a coniferyl alcohol [4–5]. The standardized extract of *S. marianum* fruits is known as silymarin and has long been used for the treatment of chronic inflammatory liver diseases [6] and more recently for prostate cancer chemoprevention [7]. Silandrin, isosilandrin, silyhermin, and silymonin have been isolated from the fruits of white-flowered *S. marianum*, var. *albiflorum*, growing in Hungary [8–10] and identified as 3-deoxy-isosilybin, 3-deoxy-silybin, 3-deoxy-silychristin and 3-deoxy-silydianin, respectively. Accordingly, eriodictyol constitutes the flavonoid moiety of these 3-deoxy flavonolignans.

The oxidative coupling reaction between the flavonoid precursor and coniferyl alcohol is probably catalyzed by a peroxidase enzyme. These enzymes are known to be radical generators. According to the most accepted hypothesis, flavonolignans are likely biosynthesized by oxidative radicalization of their flavonoid precursors and coniferyl alcohol, followed by coupling of the two radicals. The enzyme catalyzing the oxidative coupling of flavonolignans has not yet been characterized. However, in vitro synthesis of flavonolignans (silybinins) was achieved by peroxidase (EC 1.11.1.7) of *S. marianum* cell suspension culture [11]. This reaction is initiated by one-electron oxidation of (+)-taxifolin to a phenoxy radical that couples with a quinone methide radical, generated from coniferyl alcohol. Subsequent intramolecular nucleophilic attack of the hydroxyl group of ring B of the flavonoid part on the quinone methide ring produces the silybins and isosilybins. Silychristin A, isosilychristin, and silydianin are derived from mesomeric forms of the taxifolin-derived free radical (Fig. 1). This reaction pathway is neither regionor enantiospecific [12], which would explain the large structural diversity of isomers produced and points to simple radical thermodynamics as being the determining factor of their relative distribution.

We reported previously on the principal component analysis (PCA) of the variation of the flavonolignan content in *S. marianum* fruits collected from different locations in Egypt [13]. The focus of the current report is the analysis of correlations between variables from the previous PCA analysis and variables related to the contents of all major flavonolignans

detectable by HPLC. This data is subsequently used to establish potential biosynthetic links between the flavonolignan as indicators of biosynthetic pathways.

2. Materials and methods

2.1. Plant material

S. marianum fruits were collected from wild populations growing in nine different locations in Egypt including Assiut, Beni-Suef, Qaliubiya, Menofia, El-Beheira, Alexandria, and the Cairo-Alexandria desert road. The exact latitudes and longitudes of the locations were previously reported [13]. This work analyzed most, but not all, of the extracts from these samples.

2.2. Extraction and analysis

Flavonolignans were extracted from *S. marianum* fruits collected from different locations using Accelerated Solvent Extraction on a Dionex ASE350 instrument (Dionex Corporation, Sunnyvale, CA, USA) as previously described [13]. A validated HPLC method carried out on a Waters Alliance 2695 equipped with a PDA and an Agilent ZORBAX SB-C18 column were used to determine the content of flavonolignans as previously described [13]. A gradient of MeOH and H_2O/0.1% formic acid was used as mobile phase, starting at 30:70 and increasing linearly to 60:40 over 32 min. The flow rate was 1.0 ml/min, and ambient temperature was used. The extracts were reconstituted in 5 ml methanol, 10 μl were injected and quantified at 288 nm. The method yielded the following retention times (minutes) for the major silymarin compounds (Fig. 2A): taxifolin (R_t = 13.2), isosilychristin (R_t = 17.8), silychristin A (R_t = 19.7), silydianin (R_t = 21.5), silybin A (R_t = 27.1), silybin B (R_t = 28.1), isosilybin A (R_t = 30.2), and isosilybin B (R_t = 30.8).

2.3. qHNMR analysis of S. marianum samples

An absolute qHNMR method was used to quantify silydianin and isosilychristin content in the extracts prepared from *S. marianum* samples. A Bruker Avance NMR spectrophotometer AV400 (9.4 T/400 MHz, Oxford magnets) equipped with a 5 mm Z-gradient probe BBO (broadband observe) and a computer with XWIN-NMR for acquisition and processing of the spectra was used for acquiring the ^{1}H NMR spectra (400 MHz). Samples are weighed to 0.01 mg accuracy, dissolved in 600 μl of DMSO-d_6 (Dimethylsulfoxide-d_6, Lot no. 10E-645, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) using a Pressure-Lok gas syringe (VICI Precision Sampling Inc., Baton Rouge, LA, USA), and transferred into 5 mm standard NMR tubes (NORELL Inc., Landisville, NJ, USA). The qHNMR spectrum was measured using quantitative acquisition and processing parameters as follows: single 90° ^{1}H excitation pulse with GARP carbon decoupling; relaxation delay 60 s, acquisition time 4 s, number of scans 64, non-spinning mode, sample temperature 25 °C, data points 64 K, dummy scans 4, and preacquisition delay 59.57 μs. Probe tuning and matching were performed on each sample. ^{1}HNMR data was analyzed by ACD NMR Manager Version 12.01. Interactive Fourier Transform allowed finding the most suitable FT parameters and spectrum phase adjusted correctly. Basic spectral manipulations such as zero filling, Fourier transform, phase correction, baseline correction, calibration, integration, and peak

assignment were carried out. Zero filling was carried out to 262,144 (256 k) data points for digital resolution enhancement.

2.4. Silyamandin isolation

Silyamandin was isolated from pericarp that was separated from the fruits of *S. marianum* as previously reported [14]. The isolation scheme included a two-step column chromatography using silica gel and Sephadex LH20 as stationary phases. The ^{1}H NMR spectra of silyamandin, isosilychristin, and silydianin were compared using ACD NMR manager version 12.01. The latter two flavonolignans were isolated in a previous study [13] and analyzed using the quantitative, acquisition and processing parameters mentioned earlier. The ^{1}H NMR spectrum was measured on a Bruker Avance III 400 MHz for ^{1}H (Bruker AG, Switzerland) equipped with a BBFO Smart Probe on a Bruker 400 AEON Nitrogen-Free Magnet NMR spectrometer. The following conditions were used: 30° pulse experiment; 4.1 second acquisition time; 1.0 second relaxation delay; 15.1 ppm (8012 Hz) sweep width; 2997.5464 Hz spectrum Offset; 65,536 data points and 2 dummy scans. The data were processed using line broadening 0.1 Hz. For each sample, 16 scans were acquired.

2.5. Statistical analysis

Multivariate data analysis was carried out using XLSTAT2016. PCA was used to visualize the correlations between the content of the major flavonolignans produced by the fruits of *S. marianum*. The Pearson test was used to calculate the linear correlation coefficients between the variables.

3. Results and discussion

3.1. PCA analysis of S. marianum samples

We previously reported the PCA of *S. marianum* samples collected from different locations in Egypt [13]. The samples (observations) were grouped into three categories (samples with an average silymarin content of < 18.8 mg/g, samples enriched in silymarin (> 18.8 mg/g), and silydianin-rich samples) and visualized into 2-D space according to the content of the major flavonolignans (variables). In the first two categories, silybin A, silybin B, and silychristin A were the predominant flavonolignans. Silydianin, isosilychristin, and the isosilybins were predominant in the third category. Fig. 2 shows HPLC chromatograms for samples of these categories, the silybin-rich (Fig. 2B) vs. the silydianin-rich (Fig. 2C) samples. The current report studies the correlations between the flavonolignans content (variables) and how these correlations can be explained according to their biosynthetic pathways. In the PCA analysis, the cumulative variability showed eight factors (F1–F8), with F1 (80.98%) and F2 (14.19%) representing 95.18% of the total variability.

PCA, as a method of exploratory data analysis, allowed formulating a number of hypotheses based on the correlations between variables. For example, in the current study, silybin A, silybin B, and silychristin showed a correlation in their relative abundance among the analyzed samples. This is evident from the acute angle between the vectors representing these variables/flavonolignans content in Fig. 3. Silydianin, isosilychristin, and isosilybin B are correlated to a lesser extent. Taxifolin had a positive correlation to silybins A and B,

isosilybins A, and silychristin A. At the same time, taxifolin exhibited negative correlations to silydianin and isosilychristin. It can be seen that vectors representing the variables measured in this study are almost reaching the circumference of the circle in Fig. 3. This indicates that these variables are highly represented in the F1 and F2 space. The hypotheses extracted from the PCA variable analysis were statistically tested to either validate or reject the null hypothesis. A correlation test based on Pearson coefficient was undertaken. The alpha risk threshold was set at 0.05. The p-values resulting from the statistical test are shown in Table 1.

The Pearson correlation coefficient (ranging from −1 to +1) was used to measure the degree of linear correlation between the variations in content for each pair of flavonolignans. The correlation matrix (Table 1) confirmed the observed correlations with numerical values. For example, the Pearson coefficient for silybin A and silybin B was 1. This can be at least partially explained by the fact that the two diastereomers are formed from the same flavonoid radical and share the same biosynthetic pathway, except for the final non-stereoselective step (Fig. 1). However, a common observation in all of the analyzed samples was that the content of silybin B was higher than that of silybin A, and that the abundance of isosilybin A was elevated relative to that of isosilybin B.

A near unity positive correlation (0.999) was also found between silychristin A and silybins A and B. This can also be explained by the conception that silychristin A is formed from a mesomer of the 4′-*O*-taxifolin radical, which is considered to be responsible for formation of the silybins A & B. On the other hand, silydianin showed a strong positive correlation with isosilychristin and isosilybin B (Table 1, Fig. 3), with correlation coefficients of 0.868 and 0.887, respectively. Fig. 1 shows that silydianin and isosilychristin are formed from the same flavonoid radical, whereas isosilybin B can be formed from the mesomeric 3′-*O*-taxifolin radical.

The positive correlation between silydianin and isosilychristin can also demonstrated using quantitative ^{1}H nuclear magnetic resonance spectroscopy (qHNMR). Quantitative comparison between these two flavonolignans may be made by careful analysis of the spectra of crude extracts of eight *S. marianum* samples collected from different locations in Egypt. In these eight samples, silydianin and isosilychristin can be quantified using resonances corresponding to H-6′ of silydianin and H-α of isosilychristin. The ^{1}H NMR spectra of extracts of the eight populations are shown in Fig. 4. The doublet at 6.01 ppm corresponds to the H-6′ of silydianin, and the multiplet at 5.57 ppm corresponds to the H-α of isosilychristin [13,15]. These two resonances are well isolated from others in the spectra of *S. marianum* extracts. The integral values of the two resonances were compared after correcting for the weights of the extract samples (Table 2). The residual protonated solvent signal (DMSO-d_5) was set to an arbitrary value of 100. The correlation between the integral values corresponding to the content of the two flavonolignans was tested using the Spearman correlation test, a non-parametric statistical test used to measure correlation between quantitative variables of independent samples. The correlation coefficient between the two flavonolignans was 0.905 with a p-value equal to 0.005 at alpha risk threshold equal to 0.05.

3.2. Silyamandin hypothetical biosynthetic pathway

Quantitative analyses of the qHNMR spectra of the crude extracts of *S. marianum* populations revealed the presence of silyamandin. This flavonolignan was recently isolated from a tincture of *S. marianum* that had been incubated at 40 °C for 3 months [16] and postulated to have been formed via oxidative degradation of silydianin. The broad singlet at 6.11 ppm corresponding to the H-6′ of silyamandin, well-separated from other signals in the spectra of *S. marianum* extracts, was used to determine the silyamandin content. Table 2 shows the integral values of this resonance in spectra of *S. marianum* extracts. The same statistical test as mentioned earlier was used to demonstrate the correlation between silyamandin, silydianin, and isosilychristin. The correlation coefficient between silyamandin with the other two flavonolignans was 0.857 with a p-value equals to 0.011 at an alpha risk threshold of 0.05. This correlation shows the possible biosynthetic relationship between the three flavonolignans. Fig. 5 shows a possible biosynthetic scheme in which silyamandin, silydianin, and isosilychristin share the same precursor. This hypothetical biosynthetic pathway is at variance with the postulate that silyamandin is (exclusively) an artifact formed from oxidative coupling of silydianin. However, analysis of the ^{1}H NMR spectra of isosilychristin and silydianin, previously isolated from *S. marianum* extract [13], showed the presence of 10.8% silyamandin as an impurity of isosilychristin (Fig. 6). An absolute qHNMR method was used to determine the silyamandin content in the isosilychristin sample using external calibration via the residual protonated solvent signal of DMSO-d_5 as internal calibrant [17].

Identification of the silyamandin was carried out by a detailed interpretation of its 1D ^{1}H NMR spectrum, using a manual form of ^{1}H iterative Full Spin Analysis (HiFSA). Utilizing the spin simulation tool of NUTS NMR software, the spin parameters (δ, *J*, half width) were extracted from the spectrum under first order assumptions and then refined by manual adjustment until the lines and intensities of the simulated matched those of the experimental spectrum. The observed characteristic *J*-patterns included several non-first order resonances and were fully consistent with the structure of silyamandin. While the data align well with those first reported by MacKinnon et al. [16], several of the previously reported coupling values and multiplicities require revision. Table 3 provides all chemical shifts and a comprehensive *J*-correlation map of silyamandin is represented in the form of a Quantum Interaction and Linkage Table (QuILT) [18].

The negative correlations of isosilybin A with isosilybin B, silydianin, and isosilychristin also challenge the alleged biosynthetically relationships of these flavonolignans according to the hypothetical scheme shown in Fig. 1. The p-values for the correlations between isosilybin A with isosilychristin and isosilybin B were 0.092 and 0.196, respectively. Notably, these values are higher than the alpha risk threshold taken when carrying out the statistical testing. This means that the null hypothesis is accepted in the two cases. Interestingly, isosilybin A was detected along with the silybins A and B from a fungal endophyte, *Aspergillus iizukae*, which had been isolated from the surface-sterilized leaves of *S. marianum* [19].

3.3. Taxifolin radical stability and flavonolignan biosynthesis

In silybin-rich samples (Fig. 2B), the content of the silybins A and B is usually higher than their corresponding regioisomers isosilybins A and B. The situation is reversed in silydianin-rich samples (Fig. 2C). A literature survey assessed the ratio between silybins and isosilybins in *S. marianum* grown in different countries and in tissue culture. Table 4 shows the difference in content between the silybins and the isosilybins in cultivars of *S. marianum* growing in Egypt, Iran, and Poland [13,20–24]. It also shows the difference in plant tissue culture systems established from the same plant. The silybins A & B consistently show a higher content than the isosilybins A and B, across different cultivars and tissue culture systems. It is plausible that both pairs of diastereomers share the same biosynthetic pathway except that the silybins A and B are formed from a 4′-*O*-taxifolin radical, while the isosilybin A & B are formed from a 3′-*O*-taxifolin radical.

Flavonolignan biosynthesis (Fig. 1) shares a correlation with the well-studied antioxidant activity of its flavonoid moieties. Both processes involve the formation of a flavonoid free radical. This is in line with the observed correlations in light of the hypothetical biosynthetic scheme of flavonolignans and quantum chemistry studies on taxifolin radical formation. Two main mechanisms have been described in the literature for the free radical formation: H-atom abstraction (direct O—H bond breaking), and a one electron transfer (indirect H-atom abstraction) mechanism [25]. In the H-atom abstraction mechanism, a hydrogen atom is removed from a flavonoid hydroxyl group to form a radical, ArȮ.

$$\mathrm{ArOH} \rightarrow \mathrm{Ar\dot{O}} + \dot{\mathrm{H}}$$

Two important quantum parameters correlate with this reaction: O—H bond breaking and the stability of the formed radical ArȮ. The former is measured by the bond dissociation enthalpy (BDE) of the O—H bond, which is defined as the difference in heat of formation between the flavonoid and the corresponding radical. The weaker the O—H bond (low BDE), the faster the radical formation [26]. The ArȮ stability is determined by the number of hydrogen bonds, conjugation, and resonance effects.

In the one-electron transfer mechanism, an electron is removed from an oxygen atom of the hydroxyl group in the flavonoid to form a radical cation. The stability of this radical cation can be measured by the Ionization Potential (IP). It has been postulated that the formed cation is then rapidly deprotonated [26].

$$\mathrm{ArOH} \rightarrow \mathrm{ArO\dot{H}^{+}} + \dot{\mathrm{e}} \rightarrow \mathrm{Ar\dot{O}} + \mathrm{H^{+}} + \dot{\mathrm{e}}$$

In both mechanisms, the formed radical/radical cation must be relatively stable so that their forming reactions are thermodynamically favorable. In the case of the H-atom abstraction mechanism, the B-ring is the most important site for H-transfer, especially when it is a catechol moiety. The C-ring OH at C-3 is important as a site for H-atom abstraction when a 2,3-double bond exists. The presence of this unsaturation stabilizes the ArȮ radical after H-abstraction by delocalization between the B and C rings [27]. Neohydnocarpin isolated from the fruits of *Hydnocarpus wightiana* [28] represents a typical example of a flavonolignan

biosynthesized by radical formation on the C-ring OH at C-3. Some flavonolignans possess a 2,3-double bond similar to that in hydnocarpin and its congeners, salcolins A & B, calquiquelignans D & E, and sinaiticin [1]. None of these flavonolignans possess an OH group at C-3. However, flavonolignans possessing a 2,3-double bond as well as an OH group at C-3 have been isolated from *S. marianum* fruits [29–30]. Little is known about H-atom abstraction in the A-ring. However, rhodiolin isolated from the rhizomes of *Rhodiola rosea* [31] represents a flavonolignan that is considered to be formed by radical formation on the A-ring OH at C-8. Fig. 7 shows examples of flavonolignans with different coupling positions on the three flavonoid rings.

Taxifolin is the flavonoid involved in the biosynthesis of flavonolignans in *S. marianum* variety *purple*. Four stereoisomers of this dihydroflavonol exist due to the presence of two chiral centers in the C-ring: 2*S*3*S*, 2*R*3*R*, 2*R*3*S*, and 2*S*3*R* (Fig. 8) [26]. The first two isomers are *trans*, and the latter two are *cis* configured. The *trans*-2*R*3*R* taxifolin isomer is the predominant flavonoid in flavonolignans, especially those isolated from the *S. marianum* variety *purple*. Quantum mechanical calculations have shown that the *trans*-dihydroflavonols are more stable than the *cis*-compounds, explaining the predominance of *trans*-compounds. BDE calculated as the difference in total enthalpy between 2*S*,3*S*-taxifolin and the radical formed after H-atom abstraction from each of the 3, 5, 7, 3′, 4′ positions has been reported [27]. In this earlier study, all calculations correspond to systems in a vacuum. The effect of solvent was reported to be below 2 kcal/mol. The electronic structures for the most stable conformations of the four isomers of taxifolin were studied [26]. Predominant H-abstraction should occur from 4′- or 3′-OH groups, compared to 3-, 5-, or 7-OH groups based on BDE calculations. Moreover, the electronic density distribution in the highest occupied molecular orbitals (HOMO) level, which is directly related to the electron transfer capacity, was found to be delocalized on the B-ring and not extended to the 2,3-bond and 4-carbonyl groups. This confirms the minor role of the 3-OH group in taxifolin radical formation. In addition, the authors also studied the electronic density distribution using the singly-occupied molecular orbital (SOMO) level for the radicals obtained after H-atom abstraction. The SOMOs of the 4′- and 3′-OH group radicals were found to be delocalized, in contrast to that of 3-OH radical which was determined to be localized on the O-atom. This explains the predominant formation of flavonolignans from the 4′- and 3′-*O*-taxifolin radical in *S. marianum* variety *purple* (Fig. 1).

The difference in heat of formation ($\Delta_f H$) between the radical and the parent taxifolin molecule for the 2*S*3*S* and 2*R*3*R* isomers has been reported [26]. The lowest value for $\Delta_f H$ was found for the 4′-OH radical, followed by 3′-OH radical. This confirms that the B-ring is the most energetically favorable site for H-transfer and radical formation. Stabilization by H-bond formation between the remaining OH group and the neighboring oxygen atom provides further support to this point. A small difference in $\Delta_f H$ was found between 4′-OH and 3′-OH, which was attributed to steric hindrance between 3′-OH and the rest of the molecule. Nevertheless, this small difference in $\Delta_f H$ can explain, at least in part, the higher content of silybins A and B, derived from the 4′-*O*-taxifolin radical, over isosilybins A and B, derived from the 3′-*O*-taxifolin radical.

4. Conclusions

PCA of the flavonolignans content in the investigated *S. marianum* populations provided important clues for the biosynthesis of these pharmaceutically important secondary metabolites. The outcome supports the depicted hypothetical biosynthetic pathway of flavonolignans, involving oxidative coupling between the flavonoid taxifolin and the phenylpropanoid coniferyl alcohol with certain site preferences (Figs. 1 and 5). These plausible pathways can be extended to other flavonolignans produced by different plant species. The usefulness of the multivariate statistical analysis and the 2D model that resulted from the analysis of the *S. marianum* samples may serve to explain the biosynthetic relations between known flavonolignans. In addition, these rationales might be suitable to predict the biosynthetic routes for yet unknown analogues. Open questions exist regarding the presence of silybin-rich and silydianin-rich cultivars growing under different environmental conditions. More detailed studies on the enzymatic and genetic levels are required for better understanding of the biosynthesis of flavonolignans in *S. marianum*.

Acknowledgments

Conflict of interest

The authors appreciate the support by the Science and Technology Development Fund (STDF-STF), Egypt, project ID 6081 (PI: SAZ), as well as through grant P50 AT000155 from NCCIH and ODS/NIH.

References

1. Chambers SC, Valentová K, Křen V. “Non-Taxifolin” derived flavonolignans: phytochemistry and biology. Curr. Pharm. Des. 2015; 21:5489–5500. [PubMed: 26429716]
2. Dewick, P. Medicinal Natural Products: A Biosynthetic Approach. John Wiley & Sons; England: 2002. p. 151-154.
3. Sanjari S, Shobbar ZS, Ebrahimi M, Hasanloo T, Sadat-Noori SA, Tirnaz S. Chalcone synthase genes from milk thistle (*Silybum marianum*): isolation and expression analysis. J. Genet. 2015; 94:611–617. [PubMed: 26690515]
4. Bernards MA. Plant natural products: a primer. Can. J. Zool. 2010; 88:601–614.
5. Althagafy HS, Meza-Aviña ME, Oberlies NH, Croatt MP. Mechanistic study of the biomimetic synthesis of flavonolignan diastereoisomers in milk thistle. J. Org. Chem. 2013; 78:7594–7600. [PubMed: 23876147]
6. Flora K, Hahn M, Rosen H, Benner K. Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am. J. Gastroenterol. 1998; 93:139–143. [PubMed: 9468229]
7. Deep G, Agarwal R. Antimetastatic efficacy of silibinin: molecular mechanisms and therapeutic potential against cancer. Cancer Metastasis Rev. 2010; 29:447–463. [PubMed: 20714788]
8. Steiber G, Szilágyi I, Tétényi P. Differences in active agent content and composition of two species of the *Silybum* genus (*Silybum marianum* (L.) Gaertn. and *Silybum eburneum* Coss. et Durr.). Herb. Hung. 1977; 16:55–75.
9. Szilágyi I, Tétényi P, Antus S, Seligmann O, Chari M, Seitz M, Wagner H. Structure of silandrin and silymonin, two new flavanolignans from a white blooming *Silybum marianum* variety. Planta Med. 1981; 43:121–127. [PubMed: 17402024]
10. Samu Z, Nyiredy S, Baitz-Gács E, Varga Z, Kurtán T, Dinya Z, Antus S. Structure elucidation and antioxidant activity of (−)-isosilandrin isolated from *Silybum marianum* L. Chem. Biodivers. 2004; 1:1668–1677. [PubMed: 17191807]
11. Sánchez-Sampedro MA, Fernández-Tárrago J, Corchete P. Silymarin synthesis and degradation by peroxidases of cell suspension cultures of *Silybum marianum*. J. Plant Physiol. 2007; 164:669–674. [PubMed: 16901583]

12. Nyiredy S, Samu Z, Szücs Z, Gulácsi K, Kurtán T, Antus S. New insight into the biosynthesis of flavanolignans in the white-flowered variant of *Silybum marianum*. J. Chromatogr. Sci. 2008; 46:93–96. [PubMed: 18366865]
13. AbouZid S, Chen SN, Pauli GF. Silymarin content in *Silybum marianum* populations growing in Egypt. Ind. Crop. Prod. 2016; 83:729–737.
14. AbouZid S, Chen SN, McAlpine JB, Friesen JB, Pauli GF. *Silybum marianum* pericarp yields enhanced silymarin products. Fitoterapia. 2016; 112:136–143. [PubMed: 27233988]
15. Kim NC, Graf TN, Sparacino CM, Wani MC, Wall ME. Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). Org. Biomol. Chem. 2003; 1:1684–1689. [PubMed: 12926355]
16. MacKinnon SL, Hodder M, Craft C, Simmons-Boyce J. Silyamandin, a new flavonolignan isolated from milk thistle tinctures. Planta Med. 2007; 73:1214–1216. [PubMed: 17823870]
17. Pauli GF, Chen SN, Simmler C, Lankin DC, Gödecke T, Jaki BU, Friesen JB, McAlpine JB, Napolitano JG. Importance of purity evaluation and the potential of quantitative ^{1}H NMR as a purity assay. J. Med. Chem. 2014; 57:9220–9231. [PubMed: 25295852]
18. Pauli GF, Niemitz M, Bisson J, Lodewyk MW, Soldi C, Shaw JT, Tantillo DJ, Saya JM, Vos K, Kleinnijenhuis RA, Hiemstra H. Toward structural correctness: aquatolide and the importance of 1D proton NMR FID archiving. J. Org. Chem. 2016; 81:878–889. [PubMed: 26812443]
19. El-Elimat T, Raja HA, Graf TN, Faeth SH, Cech NB, Oberlies N. Flavonolignans from *Aspergillus iizukae*, a fungal endophyte of milk thistle (*Silybum marianum*). J. Nat. Prod. 2014; 77:193–199. [PubMed: 24456525]
20. Radjabian T, Rezazadeh S, Fallah Huseini H. Analysis of silymarin components in the seed extracts of some milk thistle ecotypes from Iran by HPLC. Iran. J. Sci. Technol. Trans. A. 2008; 32:141–146.
21. Andrzejewska J, Sadowska K, Mielcarek S. Effect of sowing date and rate on the yield and flavonolignan content of the fruits of milk thistle (*Silybum marianum* L. Gaertn.) grown on light soil in a moderate climate. Ind. Crop. Prod. 2011; 33:462–468.
22. El Sherif F, Khattab S, Ibrahim A, Ahmed S. Improved silymarin content in elicited multiple shoot cultures of *Silybum marianum* L. Physiol. Mol. Biol. Plants. 2013; 19:127–136. [PubMed: 24381444]
23. Rahimi S, Hasanloo T, Najafi F, Khavari-Nejad RA. Enhancement of silymarin accumulation using precursor feeding in *Silybum marianum* hairy root cultures. Plant Omics J. 2011; 4:34–39.
24. Alikaridis F, Papadakis D, Pantelia K, Kephalas T. Flavonolignan production from *Silybum marianum* transformed and untransformed root cultures. Fitoterapia. 2000; 71:379–384. [PubMed: 10925007]
25. Leopoldini M, Pitarch IP, Russo N, Toscano M. Structure, conformation, and electronic properties of apigenin, luteolin, and taxifolin antioxidants. A first principle theoretical study. J. Phys. Chem. 2004; 108:92–96.
26. Trouillas P, Fagnere C, Lazzaroni R, Calliste C, Marfak A, Duroux J. A theoretical study of the conformational behavior and electronic structure of taxifolin correlated with the free radical-scavenging activity. Food Chem. 2004; 88:571–582.
27. Trouillas P, Marsal P, Siri D, Lazzaroni R, Duroux JL. A DFT study of the reactivity of OH groups in quercetin and taxifolin antioxidants: the specificity of the 3-OH site. Food Chem. 2006; 97:679–688.
28. Ranganathan KR, Seshadri TR. Constitution of isohydnocarpin isolated from the seed hulls of *Hydnocarpus wightiana*. Indian J. Chem. 1974; 12:888–889.
29. Bandopadhyaya M, Pardeshi NP, Seshadri TR. Compounds of *Silybum marianum*. Indian J. Chem. 1972; 10:808–809.
30. Takemoto T, Ikegawa S, Nomoto K. Studies on constituents of *Silybum marianum* (L.) Gaertn. I. New flavonolignans named 2,3-dehydrosilymarin and 2,3-dehydro-silychristin. Yakugaku Zasshi. 1975; 95:1017–1021. [PubMed: 1237601]
31. Zapesochnaya GG, Kurkin VA. The flavonoids of the rhizomes *Rhodiola rosea*. II. A flavonolignan and glycosides of herbacetin. Khimiya Prirodnykh Soedinenii. 1983; 19:23–321.

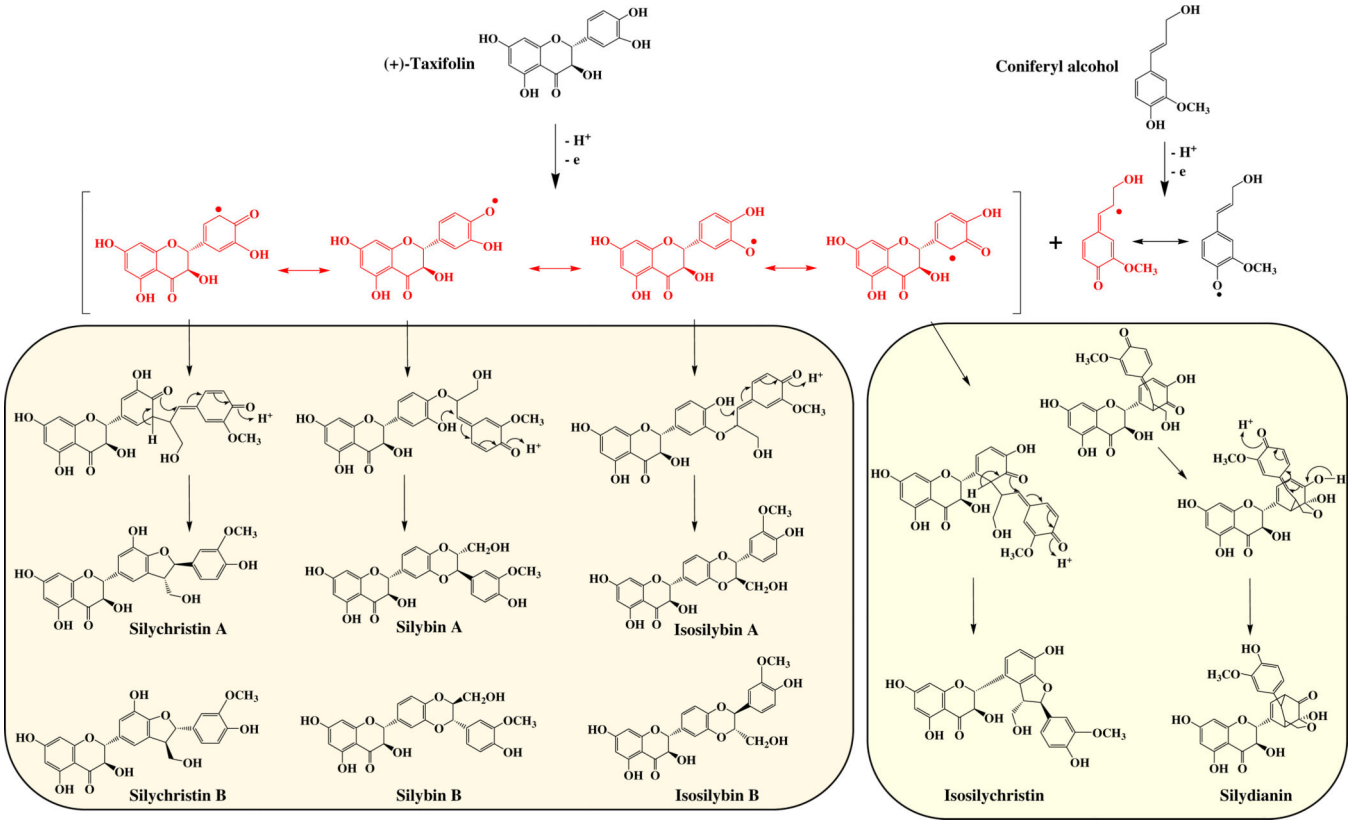

Fig. 1.
Biosynthetic pathways for *Silybum marianum* flavonolignans.

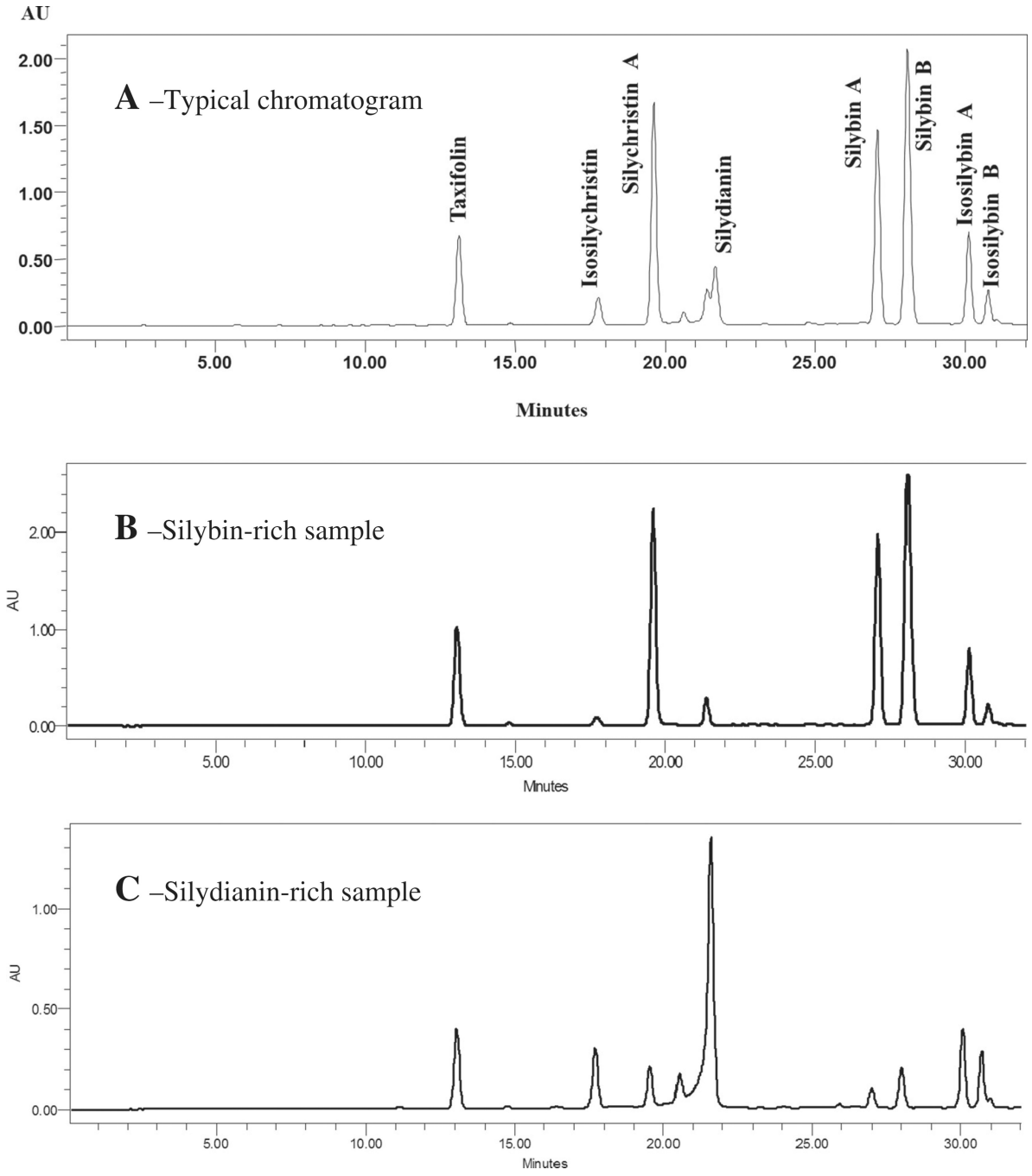

Fig. 2.

HPLC chromatograms for analysis of flavonolignans in *Silybum marianum* samples collected in Egypt. A: typical chromatogram, B: chromatogram for silybin-rich sample, and C: chromatogram for silydianin-rich sample.

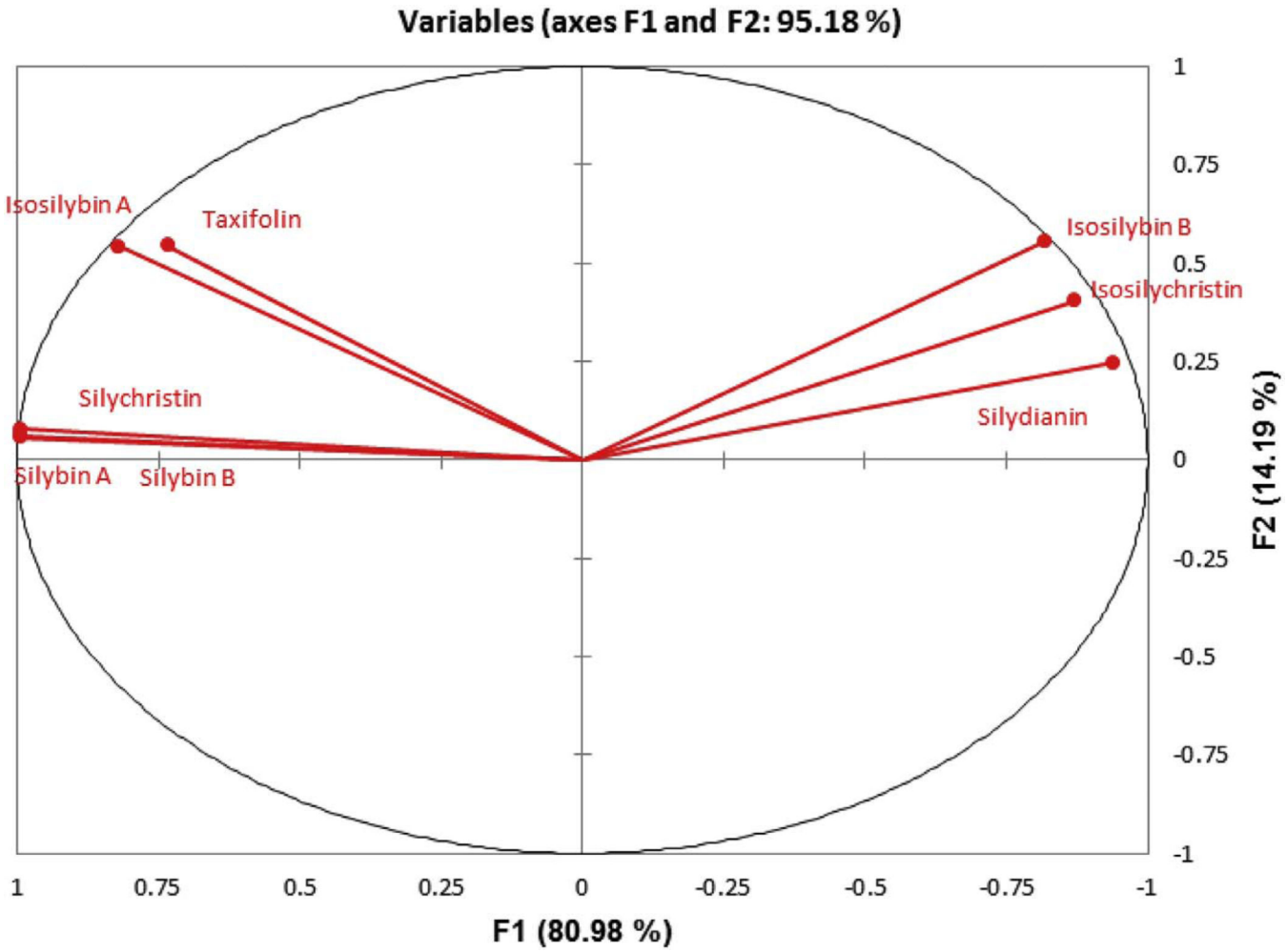

Fig. 3.

Correlation map showing the variables (flavonolignan contents) in the space of F1 and F2 representing 95.18% of variation among the analyzed *Silybum marianum* samples.

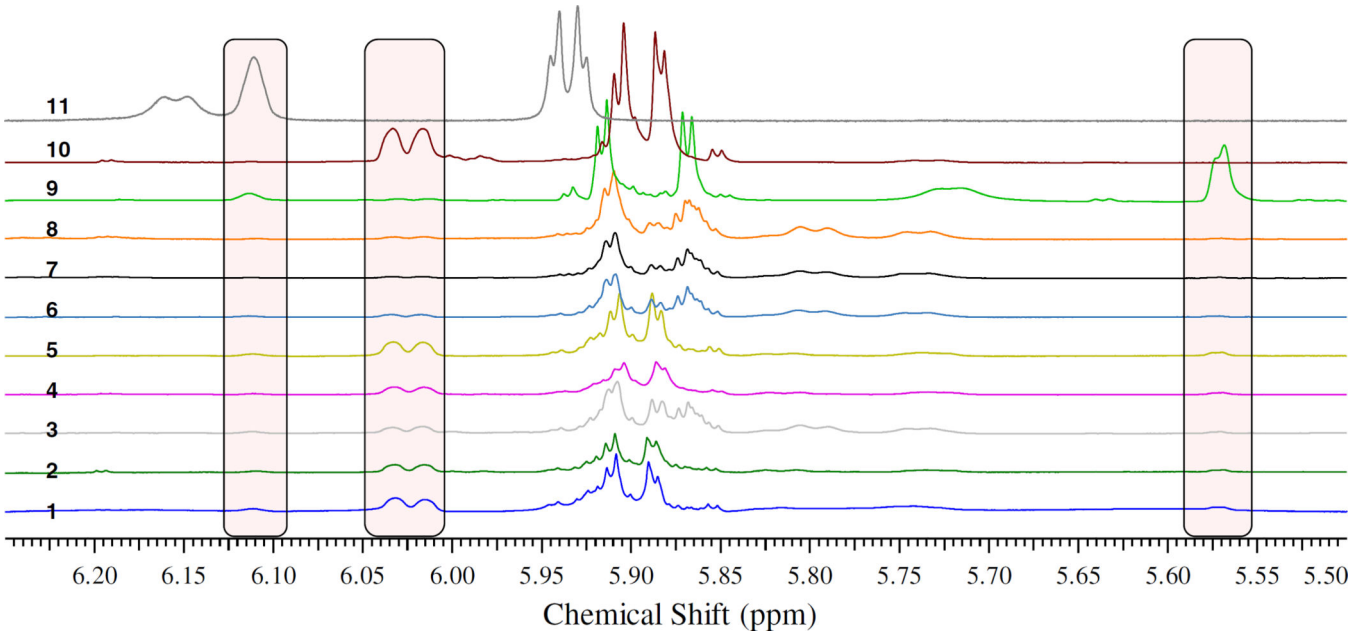

Fig. 4.

Expanded regions of the spectra (5.50–6.20 ppm) measured for extracts prepared from eight samples of *Silybum marianum* collected from seven locations in Egypt. Numbers above each spectrum represent location from which the plant was collected; 1 = Qaliubiya, 2 = Cairo Alexandria desert road – white flowered variety, 3 = Alexandria, 4 = Beni-Suef, 5 & 6 = Cairo Alexandria desert road – purple flowered variety – black and brown fruit, respectively, 7 = Assiut, 8 = Cairo Alexandria desert road – purple flowered variety – creamy white fruits, 9 = isosilychristin, 10 = silydianin, and 11 = silyamandin. The residual protonated solvent signal (DMSO-*d*5) was used as a calibrant to compare the quantities of individual flavonolignans in the extracts.

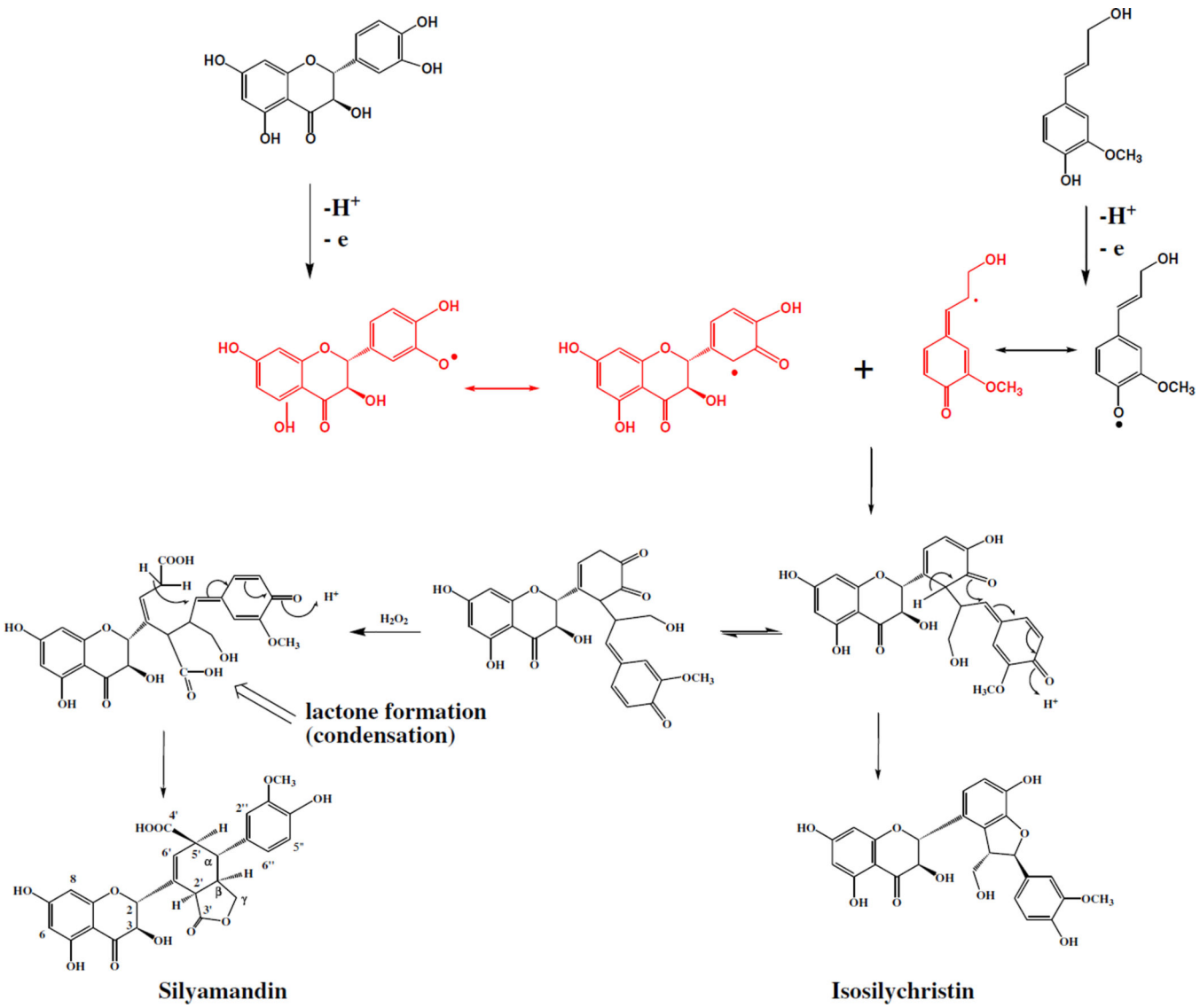

Fig. 5.
Hypothetical biosynthetic pathway of silyamandin.

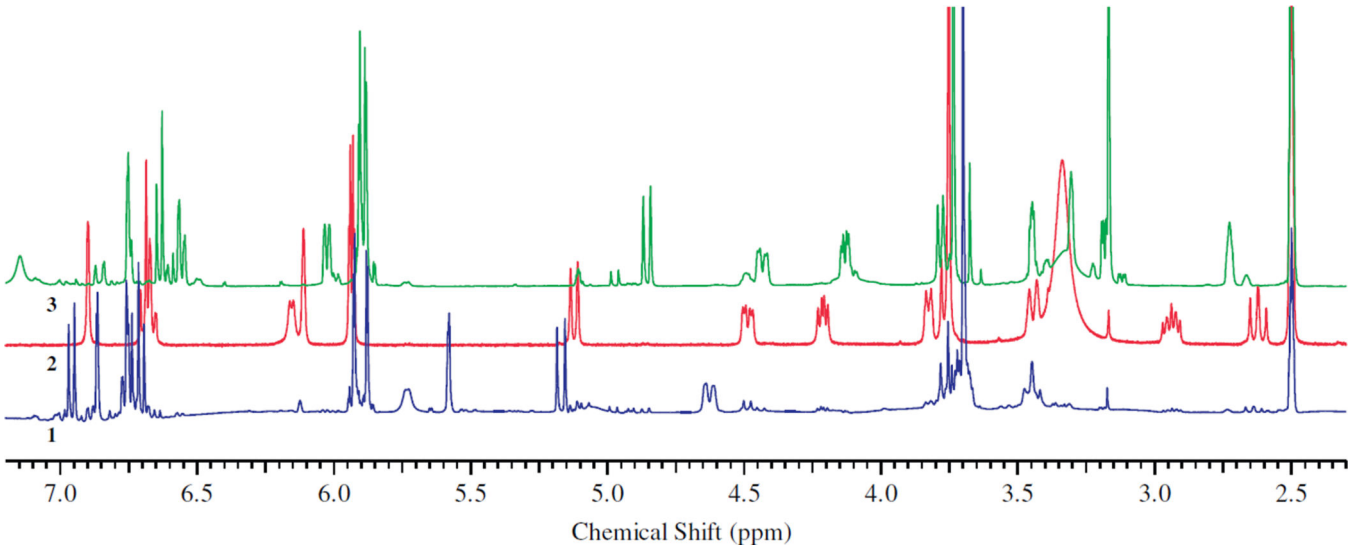

Fig. 6.

^{1}H NMR spectra (an expanded region 2.30–7.20 ppm) of isosilychristin (1, blue), silyamandin (2, red), and silydianin (3, green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

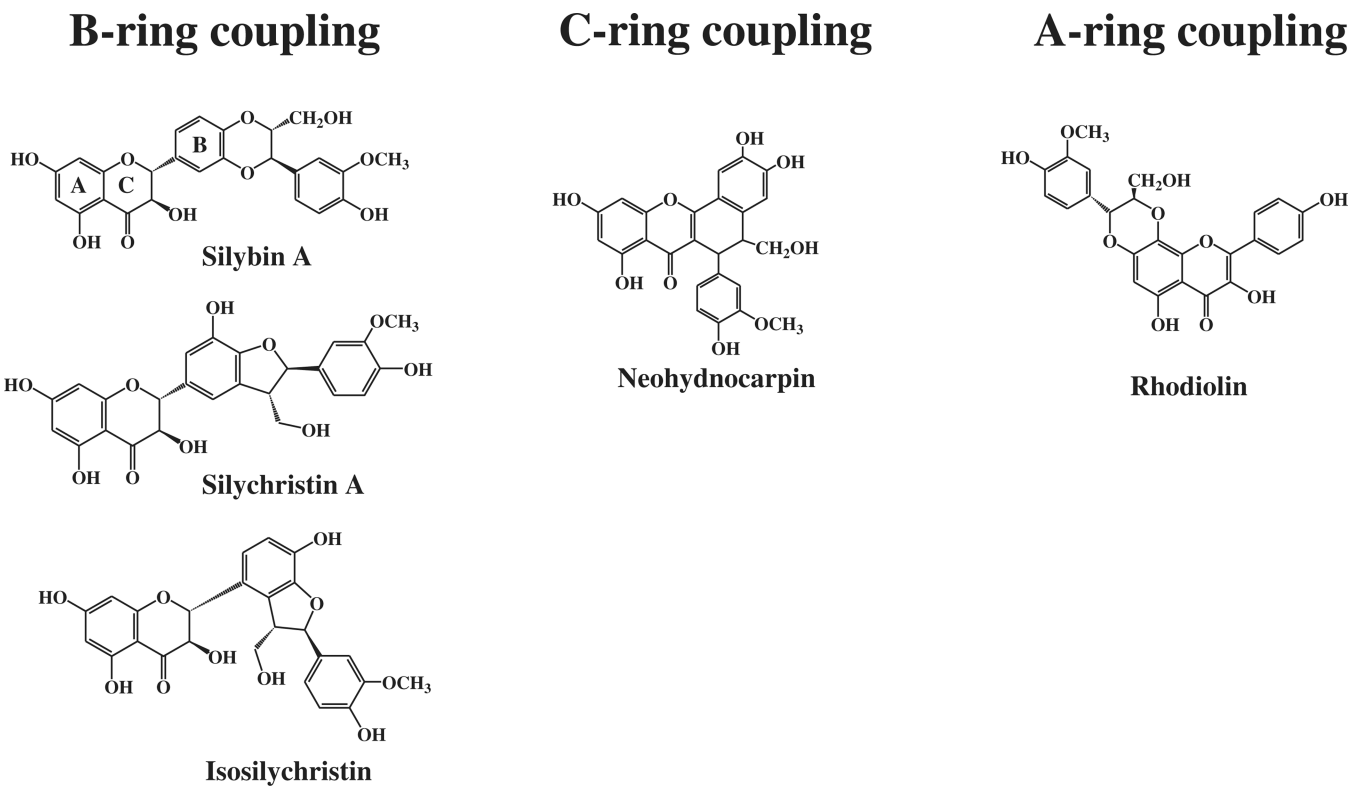

Fig. 7.
Examples of flavonolignans with different coupling positions on the three flavonoid rings.

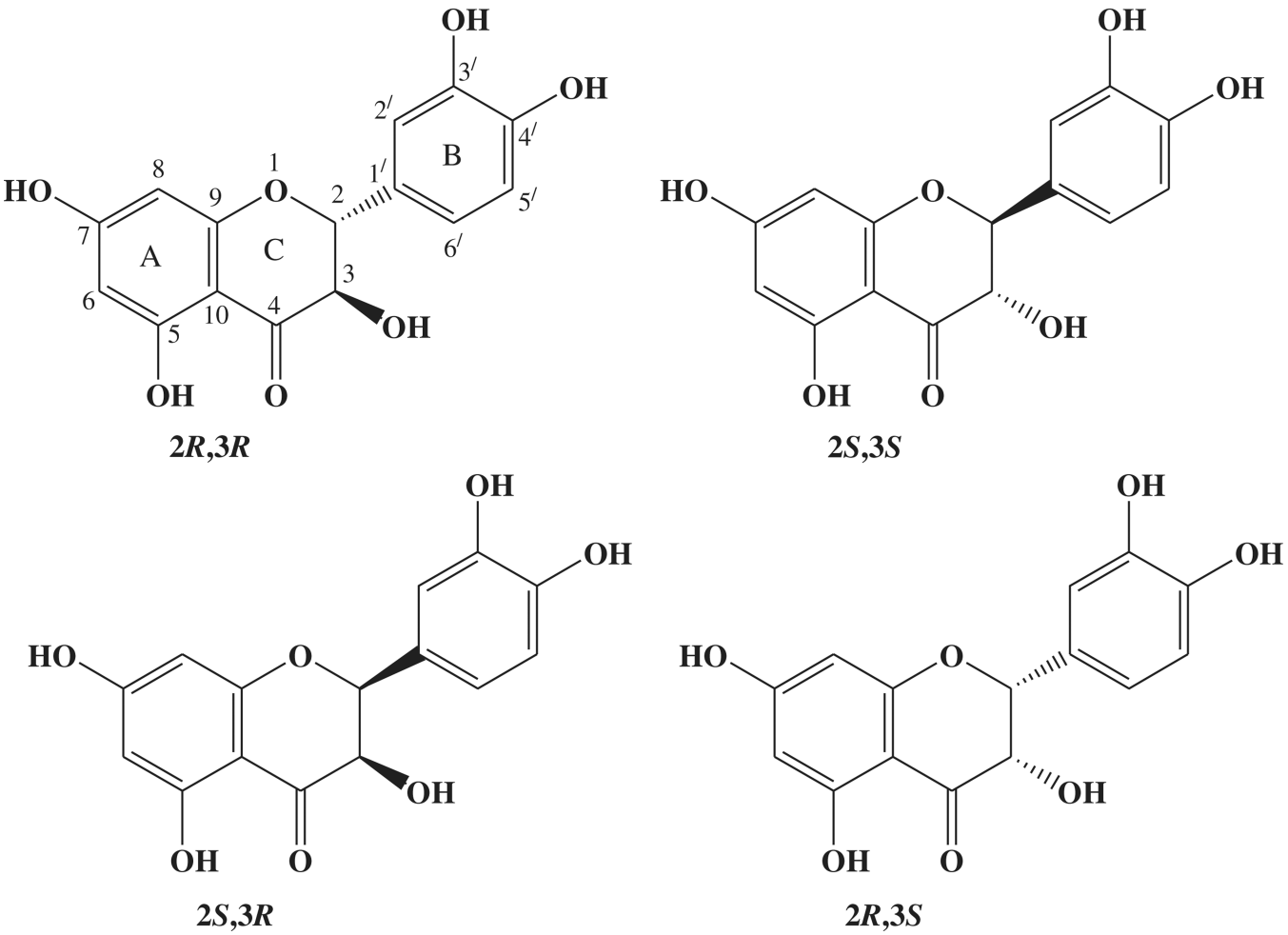

Fig. 8.

The four stereoisomers of taxifolin 2*R*3*R*, 2*S*3*S*, 2*S*3*R*, and 2*R*3*S*.

Table 1

Correlation matrix and p-value tables showing the correlations between the variables (flavonolignan content). Values in bold are different from 0 with a significance level alpha = 0.05. Red > 0.99, orange > 0.82, yellow > 0.71.

Variables	Silydianin	Isosilychristin	Isosilybin B	Taxifolin	Isosilybin A	Silybin B	Silychristin A	Silybin A
Correlation matrix								
Silydianin	**1**							
Isosilychristin	**0.868**	**1**						
Isosilybin B	**0.887**	**0.945**	**1**					
Taxifolin	–0.489	**–0.516**	–0.334	**1**				
Isosilybin A	**–0.666**	–0.451	–0.354	**0.821**	**1**			
Silybin B	**–0.932**	**–0.811**	**–0.772**	**0.718**	**0.865**	**1**		
Silychristin A	**–0.921**	**–0.815**	**–0.768**	**0.742**	**0.868**	**0.999**	**1**	
Silybin A	**–0.933**	**–0.817**	**–0.776**	**0.721**	**0.862**	**1.000**	**0.999**	**1**
p–Values								
Silydianin	**0**	**0.000**	**0.000**	0.064	**0.007**	**0.000**	**0.000**	**0.000**
Isosilychristin	**<0.0001**	**0**	**<0.0001**	**0.049**	0.092	**0.000**	**0.000**	**0.000**
Isosilybin B	**<0.0001**	**<0.0001**	**0**	0.224	0.196	**0.001**	**0.001**	**0.001**
Taxifolin	0.064	**0.049**	0.224	**0**	**0.000**	**0.003**	**0.002**	**0.002**
Isosilybin A	**0.007**	0.092	0.196	**0.000**	**0**	**<0.0001**	**<0.0001**	**<0.0001**
Silybin B	**<0.0001**	**0.000**	**0.001**	**0.003**	**<0.0001**	**0**	**<0.0001**	**<0.0001**
Silychristin A	**<0.0001**	**0.000**	**0.001**	**0.002**	**<0.0001**	**<0.0001**	**0**	**<0.0001**
Silybin A	**<0.0001**	**0.000**	**0.001**	**0.002**	**<0.0001**	**<0.0001**	**<0.0001**	**0**

Table 2

qHNMR integrals corresponding to silydianin, isosilychristin, and silyamandin in extracts prepared from eight different samples of *Silybum marianum* growing in Egypt.

Sample	Silydianin	Isosilychristin	Silyamandin
1	0.774	0.082	0.083
2	0.617	0.078	0.062
3	0.325	0.036	0.033
4	0.318	0.043	0.024
5	0.260	0.042	0.043
6	0.126	0.029	0.029
7	0.101	0.022	0.022
8	0.108	0.018	0.013

Table 3

The Quantum Interaction Linkage Table (QuILT) resulting from manual iterative full spin analysis (HiFSA) of the non-first order400 MHz 1D ^{1}H NMR spectrum of silyamandin. Couplings less than an absolute value of 1.0 Hz are given as Φ. Cells in the upper right represent the number of bonds separating the two hydrogens. In the lower left are the observed coupling constants in Hz. The numbers of bonds separating two coupled nuclei are color-coded: violet = ^{2}J, blue = ^{3}J, yellow = ^{4}J, green = ^{5}J, and pink = ^{6}J.

ppm	mult.	H	H–2	H–3	OH–3	H–6	H–8	H–2′	H–5′	H–6′	H–α	H–β	H–γa	H–γb	H–2″	H–5″	H–6″	OCH_3	COOH
5.111	dddd	H–2		3	4	7	5	4	5	4	6	5	6	6	**8**	9	8	11	7
4.477	ddddd	H–3	10.4		3	6	6	5	6	5	7	6	7	7	9	10	9	12	8
6.144	d(dd)	OH–3	Φ	4.8		**7**	7	6	7	6	8	7	8	8	10	11	10	13	9
5.9176	d	H–6	Φ	Φ	Φ		4	9	10	9	11	10	11	11	13	14	13	16	12
5.9318	d	H–8	Φ	Φ	Φ	2.1		7	8	7	9	8	9	9	11	12	11	14	10
3.816	ddd(d)	H–2′	1.2	Φ	Φ	Φ	Φ		5	4	4	3	4	4	6	7	6	9	7
3.434	dddd(d)	H–5′	Φ	Φ	Φ	Φ	Φ	Φ		3	3	4	5	5	5	6	5	8	4
6.101	dddd	H–6′	Φ	Φ	Φ	Φ	Φ	1.9	Φ		4	5	6	6	6	7	6	9	5
2.612	ddd	H–α	Φ	Φ	Φ	Φ	Φ	Φ	10.7	Φ		3	4	4	4	5	4	7	5
2.928	dddd	H–β	Φ	Φ	Φ	Φ	Φ	7.2	Φ	Φ	12.5		3	3	5	6	5	8	6
4.202	dddd	H–γa	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	5.4		2	6	7	6	9	7
3.758	dddd	H–γb	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	9.0		6	7	6	9	7
6.8876	d	H–2″	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	d	5	4	5	7
6.682	d	H–5″	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ		3	6	8
6.654	dd	H–6″	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	1.9	8.0		7	7
3.850	s	OCH_3	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ		10
3.330	brs	COOH	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	Φ	

Table 4

Silybins/isosilybins ratios in different cultivars and tissue culture systems of *Silybum marianum*.

Cultivar/tissue culture	Silybins/Isosilybins Ratio	Reference
S. marianum var. *purple*, Nile delta, Egypt	5.88	[13]
S. marianum var. *purple*, Marzan Abad, Iran	3.25	[20]
S. marianum var. *purple*, Mockelek, Poland	3.27	[21]
Shoot tip culture of *S. marianum*	3.38	[22]
Hairy root culture of *S. marianum*	1.15	[23]
Untransformed root culture of *S. marianum*	2.52	[24]