Large-Scale Isolation of Flavonolignans from *Silybum marianum* Extract Affords New Minor Constituents and Preliminary Structure-Activity Relationships

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Abstract:

The gram-scale isolation of the major flavonolignan diastereoisomers from milk thistle (*Silybum marianum*) extract provided an entré into the isolation of two related analogs that are present in extremely minute quantities. The isolation and structure elucidation of these two new compounds, which we have termed isosilybin C and isosilybin D due to their structural similarities to isosilybin A and isosilybin B, respectively, afforded a preliminary analysis of structure-activity relationships toward prostate cancer growth, survival, and apoptotic endpoints.

Keywords: *Silybum marianum* | Asteraceae | milk thistle | silymarin | silybin | isosilybin

Article:

In previously reported studies [1], our team performed the gram-scale isolation of four key flavonolignan diastereoisomers (silybin A, silybin B, isosilybin A, and isosilybin B) from milk thistle [*Silybum marianum*(L.) Gaertn. (Asteraceae)]. Presently, these materials are undergoing evaluation, both *in vitro* and *in vivo*, for their prostate cancer chemopreventive, antiproliferative, and pro-apoptotic properties [2-5]. In that earlier work, over 250 g of milk thistle extract [termed ‘silymarin’; see [6] for a review on the nomenclature of milk thistle components] were processed through several rounds of chromatography to yield 3 to 4 grams of each diastereoisomer [1]. Based on the distinctive UV profile of this class of flavonolignans, and retention times that were longer than the most retained compound, isosilybin B, in reverse phase HPLC, we hypothesized that some of the side fractions contained constituents of a similar structural type present in low yields. As isosilybin B and, to a lesser extent, isosilybin A seem to be the most potent in various prostate cancer chemopreventive and antiproliferative activity assays [2-5, 7] the evaluation of these new compounds *in vitro*could expand our knowledge of the structure-activity relationships of flavonolignans against this type of neoplasm.

Compound **1** was isolated as a yellow film. The molecular formula, determined by HRESIMS, was $C_{25}H_{22}O_{10}$, giving an index of hydrogen deficiency of 15. This is the same molecular formula of flavonolignan diastereoisomers from milk thistle [8, 9]. The 1D NMR data of **1** showed signals typical of a*trans*-3-hydroxyflavanone moiety with hydroxyl substitutions at positions C-5 and C-7 (Table 1). Additional aromatic proton and carbon signals, as well as two oxygenated methines, an oxymethylene, and a methoxy signal also indicated a flavonolignan structural type [8, 9]. However, the lignan-derived third aromatic ring in**1** displayed a 1,3,5-substitution pattern, as evidenced by coupling constants ($J_{2''}=J_{4''}=J_{6''}$=2 Hz), positioning a hydroxyl group at C-5″ and a methoxy group at C-3″. Alternatively, the major flavonolignans, silybin A (**3**), silybin B (**4**), isosilybin A (**5**), and isosilybin B (**6**) (Figure 1), have a 1,3,4-substitution pattern. HMBC data revealed that the regiochemistry of **1** (Figure 2) was similar to that of compounds **5** and **6**.

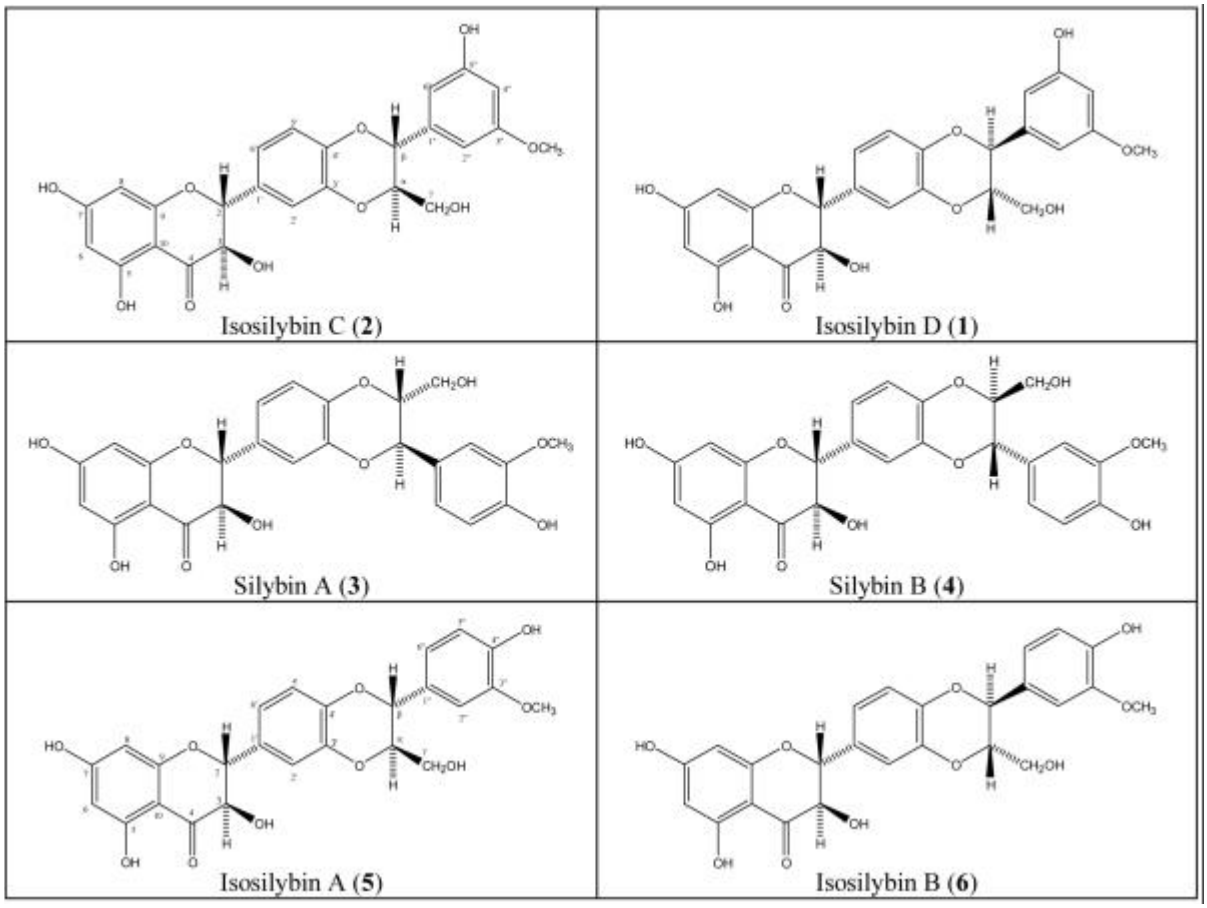

Figure 1. Structures of flavonolignans from milk thistle.

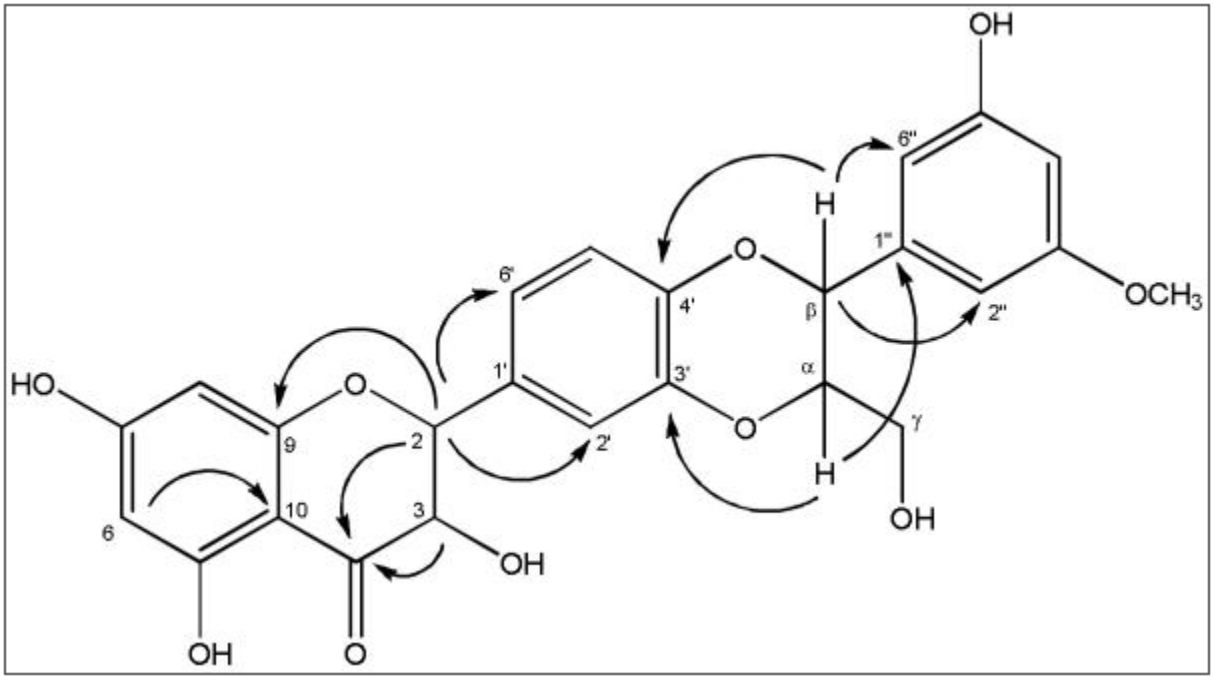

Figure 2. Key HMBC correlations in **1**

	1		2	
	δ_C	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)
1	-	-	-	-
2	82.5	5.10 d (11)	82.4	5.05 d (11)
3	71.4	4.62 d (11)	71.4	4.57 dd (11, 4)
4	197.4	-	197.0	-
5	162.5	-	162.3	-
6	95.3	5.87 d (1)	95.6	5.79 d (1)
7	167.7	-	164.1	-
8	96.3	5.90 d (1)	96.6	5.81 d (1)
9	163.3	-	163.4	-
10	100.2	-	99.6	-
1′	130.6	-	130.7	-
2′	116.9	7.15 d (2)	117.2	7.11 d (2)
3′	141.8	-	141.7	-
4′	143.4	-	143.3	-
5′	116.5	6.97 d (8)	116.6	6.97 d (8)
6′	121.5	7.02 dd (8, 2)	121.2	7.04 dd (8, 2)
α	77.3	4.46 ddd (3, 4, 8)	77.3	4.46 ddd (3, 4, 8)
β	75.2	5.27 d (3)	75.2	5.27 d (3)
γ	58.0	3.39 dd (8, 12); 3.34 dd (4, 12)	58.1	3.30 - 3.40 br. m
1″	127.2	-	127.2	-
2″	118.9	6.79 d (2)	118.9	6.77 d (2)
3″	146.4	-	146.4	-
4″	115.4	6.79 d (2)	115.4	6.77 d (2)
5″	147.4	-	147.4	-
6″	111.0	6.96 d (2)	111.1	6.97 d (2)
3″-OCH_3	55.7	3.74 s	55.7	3.73 s

Table 1. NMR data of compounds **1** and **2** in DMSO-d_6

The spectroscopic and spectrometric data of **2** were nearly identical to those of **1.** The major differences were discernable in the CD (Figure 3) and optical rotation data, where **1** and **2** had values of equal magnitude but opposite sign. The trivial names isosilybin C (**2**) and isosilybin D (**1**) were assigned to these new compounds, given their stereochemical similarities to isosilybin A (**5**) and isosilybin B (**6**), respectively.

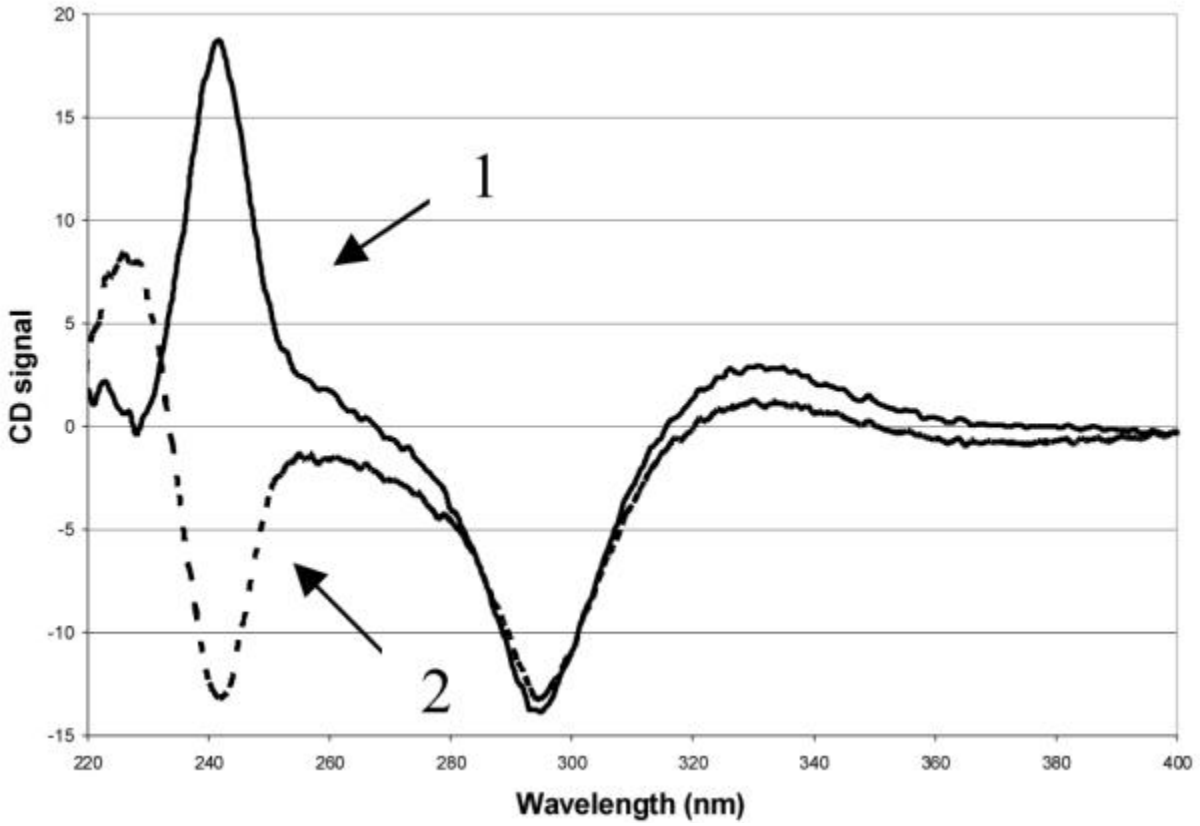

Figure 3. CD data of compounds **1** and **2**; the CD data for compounds **3**-**6** has been published previously [8].

Compounds **1**-**6** were evaluated for antiproliferative/cytotoxic activity against a panel of three prostate cancer cell lines. Compounds **1** and **2** had decreased activity, relative to the well studied compounds **3**-**6** [2-4, 8]. This suggested that an *ortho* relationship for the hydroxyl and methoxy substituents (in **3**-**6**) was more favorable than the *meta* relationship for the same substituents (in **1** and **2**), although other structural features may also be important. The configuration that was most similar to isosilybin B (**6**), being compound **1**, was more potent, as we predicted. Activity for the multicomponent mixture from which these compounds were isolated, silymarin, was also examined as a positive control. Medicinal chemistry studies are ongoing to probe the structure-activity relationships in the flavonolignan series further.

MATERIALS AND METHODS

Optical rotations, IR spectra, UV spectra, and CD spectra were recorded on a Rudolph Research Autopol III automatic polarimeter, a Nicolet Avatar 360 FT-IR, a Varian Cary 3 UV-Vis

spectrophotometer, and an Aviv Circular Dichroism Stopped Flow Model 202, respectively. NMR experiments were performed on a Varian-500 spectrometer with TMS as an internal standard. High resolution mass spectrometry data were acquired via an Agilent G1969A MSD-TOF instrument (Santa Clara, CA). Internal standards were infused via the reference nebulizer simultaneous with sample analysis, and data were acquired with reference mass correction enabled. Data were processed and lists of possible molecular formulae were generated using Analyst QS (Applied Biosystems; Foster City, CA). HPLC-grade solvents were purchased from Burdick & Jackson (Muskegon, MI, USA). Reverse Phase-HPLC was carried out on Varian Prostar HPLC systems (Walnut Creek, CA, USA) using YMC ODS-A (5 μm; 250 × 25 mm; Waters; Milford, MA, USA) columns, as described previously [1].

Powdered extract (1 kg; Product No. 345066, Lot No. 37501) of the seeds (achenes) of *Silybum marianum*(L.) Gaertn. was obtained from Euromed, S.A. (Barcelona, Spain), which is a part of the Madaus Group (Cologne, Germany) [1]. This extract (silymarin) was used also as a positive control for the antiproliferative/cytotoxic activity experiments, since it has been evaluated extensively in biological assays [4,5, 7, 10, 11]. Our team published the chemical profile of this extract previously [7]. Compounds **3**-**6** were isolated from this same extract and their purities were as described [1].

The processing of milk thistle extract to yield gram quantities of flavonolignans was described in detail previously [1]. In the process of isolating gram quantities of isosilybin B (**6**), which retained the longest on RP-HPLC of the seven major flavonolignan diastereoisomers [1], numerous fractions were obtained that contained minor compounds that eluted after **6**. Compounds **1** and **2** were isolated from a pool of these fractions. Briefly, a pool (1029 mg) of later eluting fractions were purified using the reverse phase column and equipment described above via an isocratic system of 50:50 MeOH:H_2O over 90 minutes. Two fractions that were enriched in **1** and **2** were pooled (204 mg; pool B), and Figure 4 (Supporting Information) displays the chromatogram of pool B via analytical HPLC; the elution profile of isosilybin A (**5**), isosilybin B (**6**), isosilybin D (**2**), and isosilybin C (**1**), respectfully, is discernable, showing the longer retention time of the later two. An isocratic system of 50:50 MeOH:H_2O over 100 minutes was used to purify this pool. From this, four fractions were pooled (95 mg) and rechromatographed via the same solvent system over 115 minutes. Compound **1** (21 mg) was isolated in >99% purity. A single fraction (14 mg) was rechromatographed via the same system over 120 minutes to isolate compound **2** (3 mg) in >99% purity.

Isosilybin D (**1**): Yellow film; $[\alpha]_D$: +11 (c 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 288 (0.87), 230 (0.95), 212 (1.24); CD (MeOH) λ_{ext} (Δε) 326 (+2.9), 295 (−13.8), 242 (+18.7); HRESIMS *m/z* = 483.1290 $[M+H]^+$ (calcd for $C_{25}H_{23}O_{10}$, 483.1285). ^{1}H and ^{13}C NMR data, see Table 1; HMBC: H-2 → C-3, 4, 9, 1′, 2′, 6′; H-3 → C-2, 4, 1′; H-6→ C-5, 7, 8, 10; H-8→ C-6, 7, 9, 10; H-2′→ C-2, 1′, 3′, 4′, 6′; H-5′→ C-1′, 3′, 4′, 6′; H-6′→ C-2, 1′, 2′, 4′, 5′; H-α→ C-3′, β, γ, 1″; H-β → C-4′, α, γ, 1″, 2″, 6″; H-γ → C- α , β.

Isosilybin C (**2**). Yellow film; $[\alpha]_D$ = −13 (c 0.003, MeOH); UV (MeOH) λ_{max} (log ε) 288 (1.14), 230 (1.33), 212 (1.60); CD (MeOH) λ_{ext} (Δε) 326 (+0.9), 294 (−13.2), 242 (−13.2); HRESIMS *m/z* 483.1293 $[M+H]^+$ (calcd for $C_{25}H_{23}O_{10}$, 483.1285). H and ^{13}C NMR data, see Table 1; HMBC:H-2 → C-3, 4, 9, 1′, 2′, 6′; H-3 → C-2, 4, 1′; H-6→ C-5, 7, 8, 10; H-8→ C-

6, 7, 9, 10; H-2′→ C-2, 1′, 3′, 4′, 6′; H-5′→ C-1′, 3′, 4′, 6′; H-6′→ C-2, 1′, 2′, 4′, 5′; H-α→ C-3′, β, γ, 1″; H-β → C-4′, α, γ, 1″, 2″, 6″; H-γ → C- α , β.

Compounds were tested for antiproliferative/cytotoxic activity using modifications of a published procedure [7]. Prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA): DU145 (HTB-81, an androgen-independent line derived from a central nervous system metastasis of prostate adenocarcinoma), PC-3 (CRL-1435, an androgen-independent line derived from a bone metastasis of prostate adenocarcinoma), and LNCaP (CRL-1740, an androgen-dependent line derived from a lymph node metastasis of prostate adenocarcinoma). All cell lines were cultured and maintained exactly as described previously [7], except for LNCaP cells, which were maintained at an initial density of 2×10^6 cells per 75 cm^2flask in 30 mL medium.

For cytotoxicity experiments, logarithmically growing cells were seeded at either 1500 cells per well (DU145) or 3000 cells per well (PC-3 and LNCaP) in 96-well plates in 50 µL of medium and allowed to attach to plate overnight. The following day, stock solutions of pure compounds were prepared in DMSO at 100 mM and diluted into RPMI 1640 (Life Technologies/Invitrogen, Carlsbad, CA) at twice the intended final concentration of 5, 10, 15, 30, 60, 90, or 120 µM. A 50 µL aliquot of each compound in medium was added to each well (in triplicate for each final concentration). After incubating for five days, 10 µL of the mitochondrial dehydrogenase substrate WST-8/PMS [Cell Counting Kit-8 (CCK-8), Dojindo, Gaithersburg, MD] was added to each well and incubated 2 h further. Cell-free wells of medium were incubated similarly to correct for nonenzymatic hydrolysis of the substrate to its formazan product. Mitochondrial respiratory activity was quantified by spectrophotometry as described previously [7] and cellular survival expressed as a percentage relative to the corrected control values.

IC_{50} values of compounds **1-6** against three human prostate cancer cell lines

Compounds	DU-145	PC-3	LNCaP
silybin A (**3**)	99.6 ± 24.3	69.5 ± 25.5	106 .1 ± 15.1
silybin B (**4**)	70.9 ± 12.6	43.8 ± 14.5	89.9 ± 5.1
isosilybin A (**5**)	72.5 ± 11.8	53.4 ± 28.0	102 ± 17.9
isosilybin B (**6**)	48.4 ± 19.2	42.2 ± 4.1	67.4 ± 23.6
isosilybin C (**2**)	>120(85)	>120(65)	>120(60)
isosilybin D (**1**)	109.3 ± 14.1	74.1 ± 9.4	86.4 ± 26.5
silymarin	113.4 ± 8.3	75.0 ± 15.3	107.8 ± 15.8

Table 2. IC_{50} values in µM reported as the average ± SD from three dose-response experiments. Within each experiment, compounds were assessed in triplicate. IC_{50} values were calculated by linear regression.Parentheses indicate percent survival at 120 µM. Data for silymarin are provided as a positive control, given its extensive investigation for activity against human prostate cancer *in vitro* and *in vivo*; the molarity calculation for this multicomponent mixture was as described [7].

SUPPLEMENTARY MATERIAL

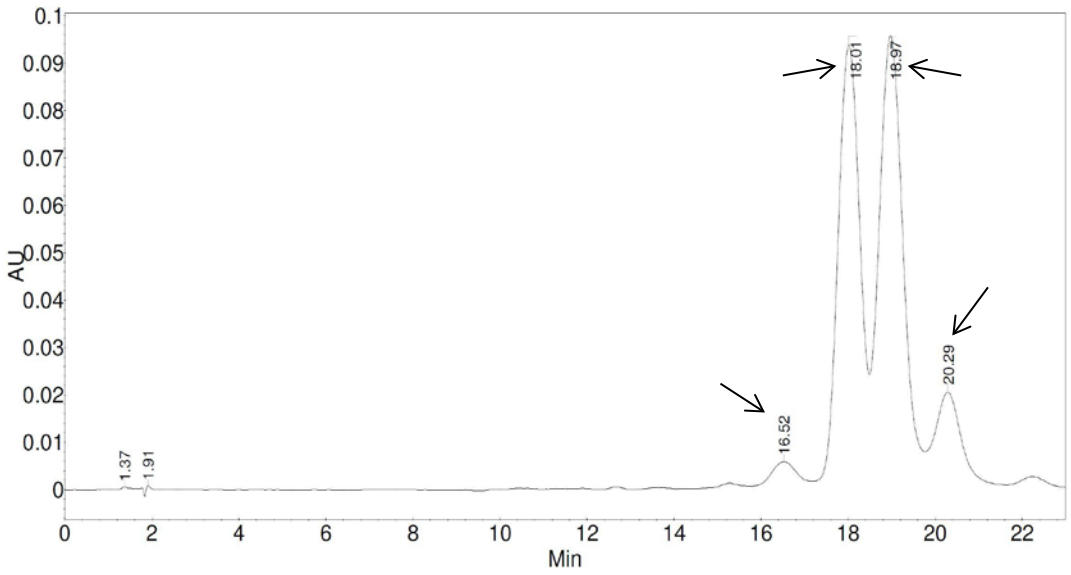

Figure 4 (Supporting Information). Chromatogram (284 nm) of pool B showing the elution order of isosilybin A (**5**), isosilybin B (**6**), isosilybin D (**1**), and isosilybin C (**2**). The sample (~1 mg/mL dissolved in MeOH, 5µL injected) was analyzed using a YMC ODS-A (5 µm; 250 × 4.6 mm; Waters; Milford, MA, USA) column eluted with 50:50 MeOH:H_2O isocratically for 23 min using the instrument described in the Materials and Methods. These methods were used for all other analytical experiments performed for this study.

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