Standardization and Identification of Minor Components of Silymarin (MK-001)

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Abstract: **Objective** To develop a highly effective HPLC method for standardization of silymarin and for characterization of minor components, which has not been reported previously. **Methods** An HPLC fingerprinting method and ^{1}H-NMR spectroscopy were employed to provide assurance for a standardized silymarin product (MK-001). **Results** A total of 18 marker compounds were identified and characterized including ten flavonolignans and eight small molecules including adenine, adenosine, uridine, and 3,5,7-trihydroxychromone which were first isolated and characterized from silymarin. The HPLC fingerprinting method and ^{1}H-NMR had been established for complete assignments of HPLC peaks with intensity over 1.0%. **Conclusion** The successfully established HPLC fingerprinting method and ^{1}H-NMR could be applied for the standardization of commercial silymarin products. MK-001 represents the first standardized silymarin with the highest content of flavonolignans (> 90%).

Key words: adenine; flavonolignans; ^{1}H-NMR; HPLC fingerprinting; silymarin
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Introduction

With increasing public demands for the medicine from plants based on dietary supplements, it is important to evaluate the efficacy of these products. Unfortunately, the standardization method for many botanical products is not currently available, which significantly hampers the assessment of their efficacy and safety in basic and/or clinical studies (Fong, 2002; Barrett, 2004; Tyler, 2004).

Silymarin is the extract from the fruit of *Silybum marianum* (L.) Gaertn (milk thistle), an ancient herbal remedy used to treat a range of liver and gallbladder disorders, including hepatitis and cirrhosis, and to protect the liver against poisoning from wild mushroom, alcohol, chemical, and environmental toxins (Flora *et al*, 1998; Foster, 1999). The hepato-protective property of *S. marianum* against a variety of toxins is substantiated by experimental data. In our recent study in mice, silymarin (MK-001) supplementation significantly attenuated the liver injury and prevented acute EtOH-induced enhancement of hepatic tumor necrosis factor (TNF) production (Song *et al*, 2006). Another study showed that MK-001 inhibited the expression of TNF-α in anti-CD3 stimulated human peripheral blood mononuclear cells and nuclear factor kappa B (NF-κB)-dependent transcription in human hepatoma Huh7 cells; MK-001 dose-dependently inhibited the infection of Huh7 and Huh7.5.1 cells by JFH-1 virus; And MK-001 displayed both prophylactic and therapeutic effects against HCV infection, and when combined with interferon-alpha, inhibited hepatitis C virus (HCV) replication more than interferon-alpha alone. Commercial preparations of silymarin also displayed antiviral activity, but the effects were not as potent as MK-001 (Polyak *et al*, 2007). However, its clinical benefits have not been consistently proved because several randomized trials had produced inconsistent results. This is primarily due to the unavailability of standardized product for testing. Rigorous clinical trials require a standardized product that will assure data

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quality and serve as a reference reagent for comparison with other remedies and for gaining insight into drug-herbal interactions and potential adverse effects.

Recent examples of compromised safety and adverse reactions as a result of adulteration, contamination, and substitution of raw herbal materials could be found in the literature (Fong, 2002a; 2002b; Awang, 1991; Slifman *et al*, 1998; Ernst, 2002; Béjar, Betz, and Barrett, 2004). While most botanical products are traditionally identified on the basis of morphological or experiential characterization and microscopic examination of the cross-sections and/or powders, the application of modern DNA analysis and chromatographic techniques such as TLC, GC, HPLC, etc has begun to show their power and practical value. By using modern analytical HPLC, the establishment of chemical profiles or fingerprints of botanical product is becoming a standard operation practice. In our case, the source material based on *Good Agricultural Practice* (GAP) was procured and authenticated by Dr. CHEN Shi-lin, a botanist at Institute of Medicinal Plant Development (IMPLAD) in Beijing, China.

The next step is to identify the marker compounds which are chemically defined constituents specific to a herb. The marker compounds play critical roles in the standardization of botanical products. Ideally, the marker compounds are also biologically active, which would ensure that each batch of plant material has essentially the same pharmacological potency. However, the identity of the bioactive constituent(s) may not be available for many commonly-used herbs. Fortunately in the case of silymarin, we isolated and characterized a group of flavonolignans: silybin A, silybin B, isosilybin A, isosilybin B, silydianin, and silychristin, which not only serve as marker compounds but also are biologically active (Lee and Liu, 2003a).

The development of modern analytical methods such as HPLC and NMR with various improved techniques enables the establishment of a comprehensive standardization method. Tittel and Wagner (1978) first reported the HPLC separation of silymarin and identified seven compounds: taxifolin, silychristin, silydianin, silybins (two isomers), and dehydrosilybins (two isomers). The dehydrosilybins were later proved to be isosilybins A and B (Lee and Liu, 2003a). Later, Ding *et al* (2000) using the micellar electrokinetic capillary chromatography method identified silychristin, silydianin, diastereoisomeric silybin A, silybin B, isosilybin A, and isosilybin B, and quantitatively determined these peaks. Lee *et al* (2006) reported the separation and characterization of six marker compounds: silybin, isosilybin, silydianin, and silychristin extracted from *S. marianum* by liquid chromatography-electrospray tandem mass spectrometry. Surprisingly in their continuous study of commercial products, the incorrect stereochemistry for isosilybins A and B was again used (Lee, Narayan, and Barrett, 2007) which has been corrected by us based on X-ray crystallography study (Lee and Liu, 2003a). Shibano *et al* (2007) reported the separation and characterization of seven active flavonolignans in *S. marianum* by using LC-MS. Unfortunately the structures of isosilybins A and B were incorrectly assigned. It is commonly known that the stereochemistry of isomeric silybin A, silybin B, isosilybin A, and isosilybin B could not be assigned by LC-MS (Ding *et al*, 2001). Recently we came cross another paper (Cai *et al*, 2009) using LC-MS to determine diastereoisomeric silybins A and B as well as diastereoisomeric isosilybins A and B. In fact, we reported the absolute stereochemistry of diastereo-isomeric silybin A (2*R*,3*R*,7′*R*,8′*R*), silybin B (2*R*,3*R*,7′*S*,8′*S*), isosilybin A (2*R*,3*R*,7′*R*,8′*R*), and isosilybin B (2*R*,3*R*,7′*S*,8′*S*), based on X-ray crystallography of isosilybin B in combination with optical rotation measurement (Lee and Liu, 2003a). In addition, we reported three new minor flavonolignans named as 2,3-*cis*-silybin A, 2,3-*cis*-silybin B, and neusilychristin, and a known one taxifolin (Lee and Liu, 2003b). Interestingly, under optimized HPLC condition, we found that there were still many minor peaks that have not yet been identified. These peaks also presented in EtOAc fraction from MeOH extract of *S. marianum* reported by Kim *et al* (2003). As part of our objective in the standardization of silymarin, we report here the isolation and characterization of these minor compounds which have not yet been reported before.

^{1}H-NMR spectroscopy has been successfully applied to providing ^{1}H-NMR fingerprint for crude extract of medicinal herbs (Qin *et al*, 2001). Since ^{1}H-NMR could provide characteristic signals for each individual flavonolignan together with the fact that flavonolignans account for more than 80% of silymarin,

we believe that ^{1}H-NMR fingerprint could serve as a secondary quality control method in the standardization of silymarin.

Material and methods

Botanical material

The fruits of *Silybum marianum* (L.) Gaertn were collected at the agricultural station in northern China (Heilongjiang Province) and were identified by Prof. CHEN Shi-lin, IMPLAD, Chinese Academy of Medical Science (Beijing, China). The voucher specimen was deposited in the herbarium collection of IMPLAD. Two commercial silymarin products called Brand A and Brand B were purchased from Vitamin Shop in Boston, USA.

Instruments and conditions

HPLC analyses were carried out with a Waters 1525 Binary HPLC System with Waters 2487 Dual Wavelength Detector and an ODS-A column (150 mm × 4.6 mm, 3 μm) using a solvent system of methanol-water (1:1) and a flow rate of 1.0 mL/min at room temperature. Gradient elution with increased amount of methanol to the water was used to achieve better resolution of polar components. The detection was monitored at 254 nm. Each individual peak was assigned by comparing the retention time value of individual compound isolated or by co-injection. Relative content of total flavonolignans was calculated based on the ratio of each known flavonolignan peak identified as marker compounds. ^{1}H-NMR spectra were recorded on a Varian VXR 300 Spectrometer operating at 300 MHz. Acetone-d_6 was chosen as solvent after comparing the spectra with those taken by methanol-d_4, DMSO-d_6, THF-d_4, and pyridine-d_5. Silica gel for column chromatography and all solvents with analytical grade were purchased from Fisher Chemicals and the Toyoperal HW-40 was obtained from Tosoh Chemicals (Japan).

Preparation of typical commercial silymarin

Although the efficiency of solvent extraction including boiling water (Duan, Carrier, and Clausen, 2004; Wallace, Carrier, and Clausen, 2005; Barreto *et al*, 2003) and the effect of defatted and undefatted fruits had been investigated (Wallace, Carrier, and Clausen, 2005), the advantages of organic solvent from the view of efficiency are still clearly shown. Defatted pulp of *S. marianum* was extracted with 90% aqueous acetone. The extract was concentrated to remove acetone, and then washed by hexane to eliminate hydrophobic impurities. The remaining concentrates were treated with aqueous NaCl and ethanol solution to remove water-soluble impurities. The precipitate and solid obtained through spray drying were combined together to provide silymarin.

Standardized silymarin (MK-001) preparation

Silymarin obtained from above procedure or from commercial product was washed for three times with eight times of aqueous ethanol (5%). The washed silymarin was dried over the oven at 80 ℃ and then the MK-001 was obtained.

Separation of small molecular components from silymarin

Aqueous ethanol (5%) was added to silymarin powder and stirred thoroughly at room temperature to dissolve the polar components which was treated as impurities of commercial silymarin. Aqueous ethanol solution after filtration was concentrated to dry and the polar extract from silymarin was obtained. The extract was isolated repeatedly by chromatography on silica gel and Toyopearl HW-40 columns. Total eight small molecules, including adenine, adenosine, uridine, and 3, 5,7-trihydroxychromone, were isolated.

Results and discussion

For the standardization of silymarin we investigated 16 brand name silymarin products on the US market using HPLC analysis. As examples, some of them are shown in Fig. 1. From Fig.1, it could be seen that the main components such as silybin A, silybin B, and silychristin in different brands were readily identifiable, however, the ratio between major components and the content of impurity peaks especially in the polar region was quite different.

To prepare further purified MK-001, silymarin prepared with above-mentioned procedure was purified by washing with 5% ethanol for three times. The solid after filtration was dried completely and milled to powder and then the purified silymarin called MK-001 was obtained. This procedure could be applied for the most of typical commercial silymarin products. MK-001 was used for the separation of various flavonolignans, such as silybins A and B, isosilybins A and B, etc. The

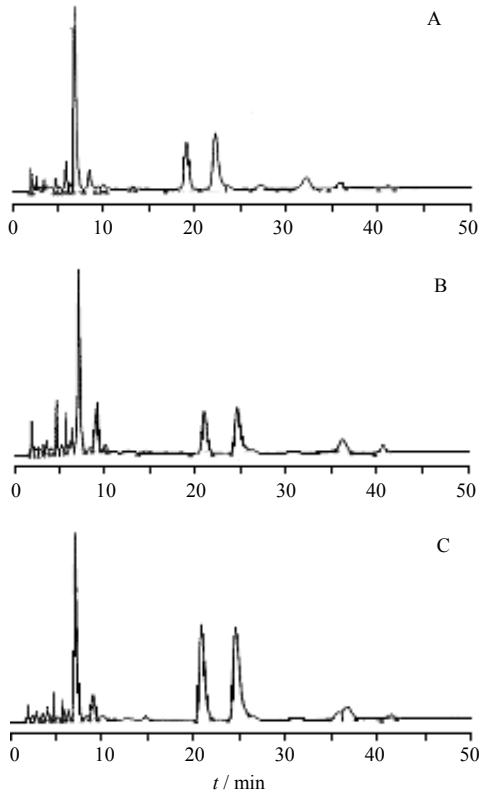


Fig. 1 HPLC comparison of commercial silymarin
A: MK-001 B: Brand A C: Brand B

polar part washed by 5% ethanol was used for the separation of minor compounds. As shown in Fig. 2, a total of ten flavonolignans including silybins A and B, isosilybins A and B, silychristin, isosilychristin, neusilychristin, silydianin, 2,3-*cis*-silybin A, and 2,3-*cis*-silybin B have been isolated from MK-001. These flavonolignans comprise up to 92% of the total flavonolignans in MK-001. Fig. 3 shows eight small molecules isolated from silymarin whereas sugar and mannitol could not be displayed in Fig. 4.

In the past, many efforts were focused on the isolation and identification of flavonolignans, especially in the HPLC region where t_R value is larger than 7.26 (silychristin). In order to clarify those minor components of silymarin, we investigated the polar fraction of MK-001. As shown in Fig. 4, a total of eight additional compounds, i.e. adenine, uracil, uridine, adenosine, 3,5,7-trihydroxychromone taxifolin (t_R 33.7), chromone (t_R 30.3), and phenol glycoside (t_R 49.6), have been separated and identified by silica gel and Toyopearl HW-40 column chromatography in combination with preparative HPLC. These 18 isolated marker compounds make us establish a complete HPLC fingerprint (Fig. 5) of silymarin for our primary quality control with a capability of complete HPLC peak assignments of >1.0% peak intensity.

HPLC fingerprint

Fig. 5 shows the complete assignment of HPLC peaks of MK-001.

^{1}H-NMR fingerprint

Flavonolignans in standardized silymarin have very similar ^{1}H-NMR spectra, making it difficult to assign individual isomers such as silybins A and B, isosilybins A and B by ^{1}H-NMR spectroscopy. However, the benefit from the similarity of the signals of total flavonolignans mixture simplifies the complex patterns of silymarin, so as to make it possible to use ^{1}H-NMR fingerprint as a secondary quality assurance (QA) method for the standardization of silymarin. We investigated five different NMR solvents along with D_2O exchange and found that the ^{1}H-NMR spectrum in acetone-d_6 + D_2O displayed very specific signals of total phenolic flavonolignans contained in the standardized silymarin. As evidenced, the ^{1}H-NMR spectrum could be used effectively as ^{1}H-NMR fingerprint for quality control of silymarin as shown in Fig. 6.

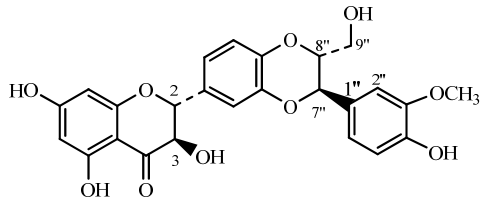
The ^{1}H-NMR fingerprint of MK-001 derived from flavonolignans could be divided into eight areas (A—H). Each area clearly represents the certain structural features of known flavonolignans. The assignment of areas A—H is summarized in Table 1.

Among these eight areas, area C represents the unique signal of H-7″ in silychristin, one of the main components in MK-001; Area F was only contributed by H-8″ of silybin A, silybin B, isosilybin A, and isosilybin B which were compounds found only from silymarin and its origin plant *S. marianum*. While we clearly understood that the flavonolignans were composed of flavanonol unit and phenyl propanol called lignan, either part or their mixture could not give the same or similar ^{1}H-NMR fingerprint with those in silymarin, especially in areas C and F. Therefore, it is safe to conclude that ^{1}H-NMR fingerprint of MK-001 certainly could be used as a secondary QA method.

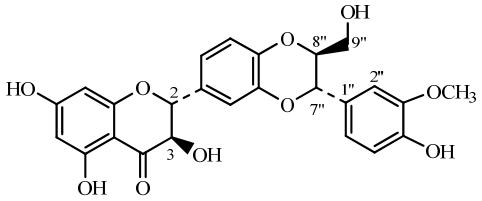
Identification of minor components from silymarin

Flavonolignans represented by silybins A and B, isosilybins A and B, and their other isomers were poorly dissolved in methanol. So the properties could

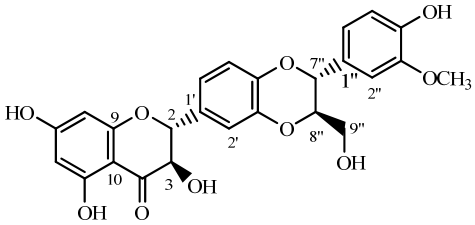
be used for the separation of minor polar components from silybins. Ethanol (5%) soluble part was isolated repeatedly over silica gel column chromatography. Total eight small molecules, including adenine, adenosine, uridine, and 3,5,7-trihydroxychromone, were isolated and characterized for the first time from silymarin. Because of the poor availability of the spectral data of the following small molecules isolated from *S. marianum*, their identification and necessary spectral data are listed as following.



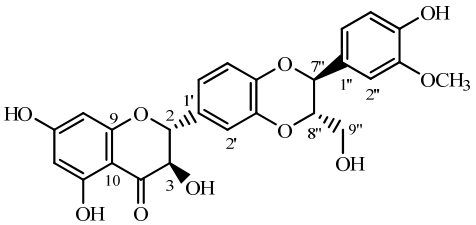
silybin A



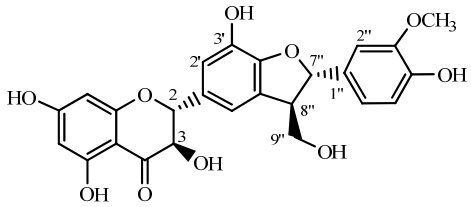
silybin B



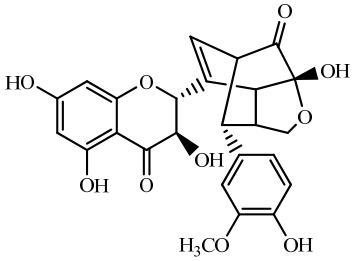
isosilybin A



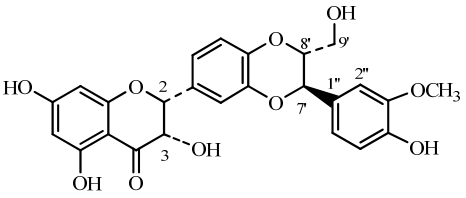
isosilybin B



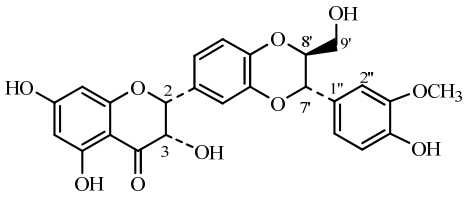
silychrisitin



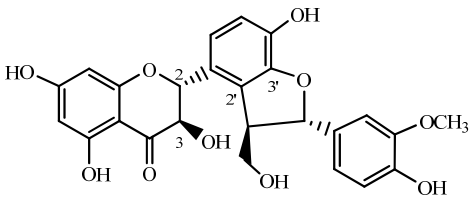
silydianin



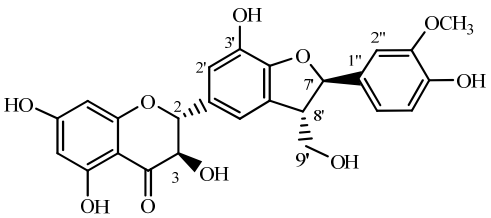
2,3-*cis*-silybin A



2,3-*cis*-silybin B



isosilychristin



neusilychristin

Fig. 2 Flavonolignans isolated from silymarin

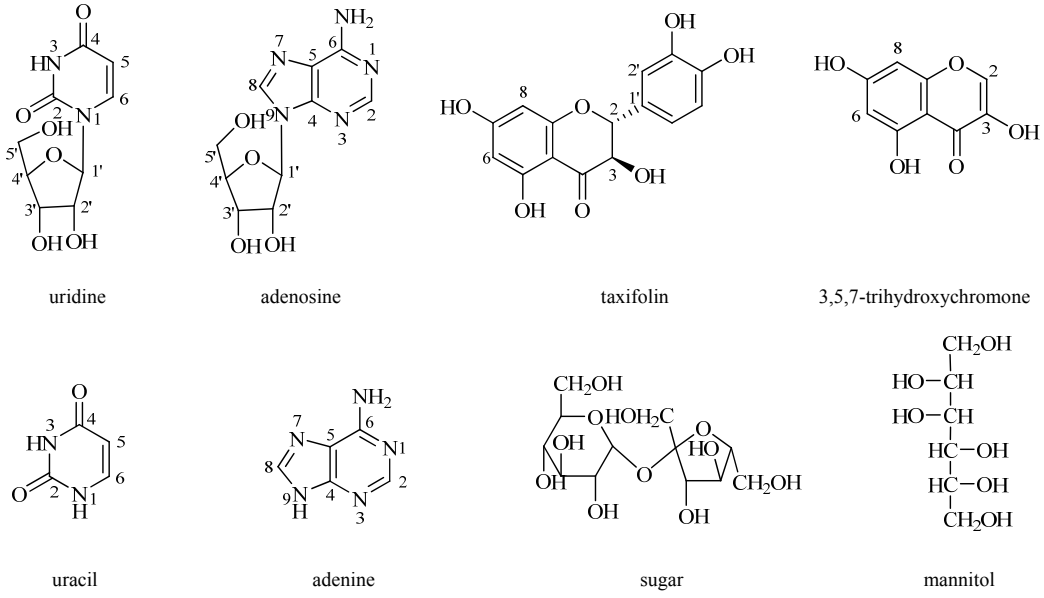


Fig. 3 Polar components and small molecular components isolated from silymarin

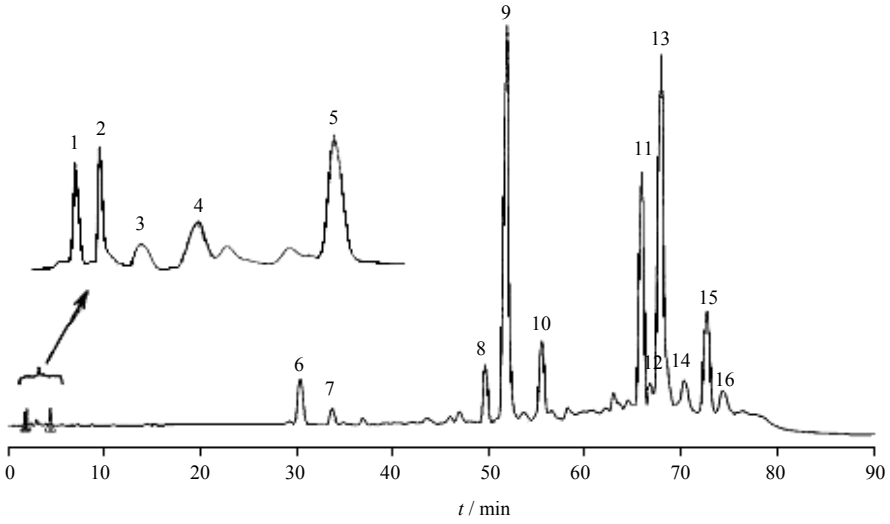


Fig. 4 HPLC fingerprint of MK-001 showing polar components by improved HPLC condition

1: uridine analog 2: adenine 3: uracil 4: uridine 5: adenosine 6: 3,5,7-trihydroxy-chronmone 7: taxifolin 8: phenol glycoside 9: silychristin 10: silydianin neusilychristin 11: silybin A 12: 2,3-*cis*-silybin B 13: silybin B 14: 2,3-*cis*-silybin A 15: isosilybin A 16: isosilybin B

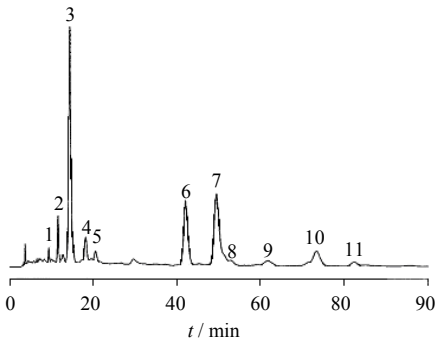


Fig. 5 HPLC fingerprint of MK-001

1: taxifoin 2: 3,5,7-trihydroxy-chromone 3: silychristin 4: silydianin neusilychristin 5: phenol glycoside 6: silybin A 7: silybin B 8: 2,3-*cis*-silybin B 9: 2,3-*cis*-silybin A 10: isosilybin A 11: isosilybin B

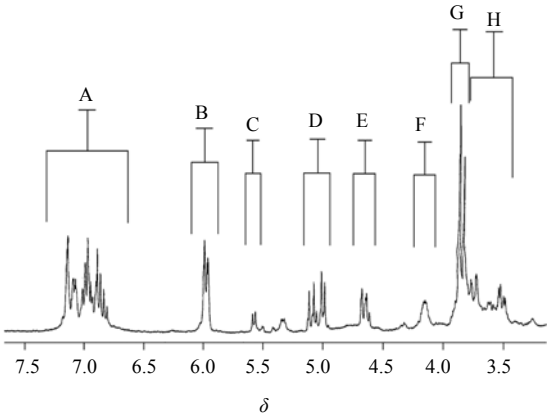


Fig. 6 ^{1}H-NMR fingerprint of MK-001

Table 1 Assignment of areas A—H in ^{1}H-NMR fingerprint of MK-001

Areas	Protons	Assignment
A (δ 6.7~7.3)	H-2′, 5′, 6′	silybin A, B, isosilybin A, B, silychristin, silydianin
B (δ 5.9~6.1)	H-6, 8	silybin A, B, isosilybin A, B, silychristin, silydianin
C (δ 5.5~5.7)	H-7″	silychristin
D (δ 4.9~5.2)	H-2	silybin A, B, isosilybin A, B, silychristin
	H-7″	silybin A, B, isosilybin A, B
E (δ 4.5~4.8)	H-3	silybin A, B, isosilybin A, B, silychristin
F (δ 4.1~4.3)	H-8″	silybin A, B, isosilybin A, B
G (δ 3.7~3.9)	OCH_3	silybin A, B, isosilybin A, B, silychristin
H (δ 3.4~3.7)	2 H-9″	silybin A, B, isosilybin A, B
	H-8″	silychristin

Adenine: $C_5H_5N_5$, molecular weight 135; ^{1}H-NMR (CD_3OD) δ: 8.14 (1H, s, H-8), 8.06 (1H, s, H-2); ^{13}C-NMR (CD_3OD) δ: 156.27 (C-6), 153.77 (C-4), 152.75 (C-2), 143.76 (C-8), 118.93 (C-5); TOF-MS-ES$^-$: 134 [M—1], 180 [M + Cl$^-$]. It is identical with Spectral Database for Organic Compounds recorded (SDBS 2004).

Adenosine: $C_{10}H_{13}N_5O_4$, molecular weight 267; ^{1}H-NMR (CD_3OD) δ: 8.32 (1H, s, H-8), 8.19 (1H, s, H-2), 5.98 (1H, d, J = 6.6 Hz, H-1′), 4.73 (1H, dd, J = 6.3, 5.1 Hz, H-2′), 4.34 (1H, dd, J = 5.1, 2.4 Hz, H-3′), 4.20 (1H, dd, J = 4.8, 2.7 Hz, H-4′), 3.89 (1H, dd, J = 12.6, 2.1 Hz, H-5′), 3.76 (1H, dd, J = 12.6, 2.7 Hz, H-5′); ^{13}C-NMR (THF-d_8) δ: 156.89 (C-6), 152.90 (C-2), 149.29 (C-4), 141.33 (C-8), 120.40 (C-5), 90.51 (C-1′), 87.53 (C-4′), 74.93 (C-2′), 71.98 (C-3′), 62.78 (C-5′); TOF-MS-ES$^-$: 302 [M + Cl$^-$], 312 [M + HCOO$^-$], 266 [M—1], 134 [adenine part]; TOF MS ES$^+$: 309 [M + 1 + CH_3CN], 268 [M + 1]; TOF MS ES$^-$: 312 [M + HCOO$^-$], 302 [M + Cl$^-$], 266 [M—1], 134 [M—sugar]. It is identical with Spectral Database for Organic Compounds recorded (SDBS, 2004).

Uridine: Off-white amorphous powder, $C_9H_{12}N_2O_6$, molecular weight 244; ^{1}H-NMR (CD_3OD) δ: 8.00 (1H, d, J = 8.1 Hz, H-5), 5.89 (1H, d, J = 4.2 Hz, H-1′), 5.69 (1H, d, J = 8.1 Hz, H-6), 4.17 (1H, t, J = 5.1 Hz, H-3′), 4.14 (1H, t, J = 5.1 Hz, H-2′), 3.99 (1H, m, H-4′), 3.83 (1H, dd, J = 12.0, 2.7 Hz, H-5′), 3.72 (1H, dd, J = 12.0, 2.7 Hz, H-5′); ^{13}C-NMR (CD_3OD) δ: 166.21 (C-4), 152.47 (C-2), 142.75 (C-6), 102.69 (C-5), 90.66 (C-1′), 86.36 (C-4′), 75.73 (C-2′), 71.30 (C-3′), 62.26 (C-5′). It is identical with Spectral Database for Organic Compounds recorded (SDBS 2004).

Uracil: $C_4H_4N_2O_2$, molecular weight 112; ^{1}H-NMR (CD_3OD) δ: 7.45 (1H, d, J = 7.5 Hz, H-5), 5.68 (1H, d, J = 7.8 Hz, H-6); ^{13}C-NMR (CD_3OD) δ: 167.69 (C-4), 153.66 (C-2), 143.97 (C-6), 101.80 (C-5). It is identical with Spectral Database for Organic Compounds recorded (SDBS 2004).

Taxifolin: Yellowish amorphous powder, $C_{15}H_{12}O_7$, molecular weight 304; ^{1}H-NMR (CD_3COCD_3 + D_2O) δ: 6.99 (1H, s, H-2′), 6.82 (2H, s, H-5′, 6′), 5.93 (1H, d, J = 2.1 Hz, H-8), 5.88 (1H, d, J = 2.1 Hz, H-6), 4.95 (1H, d, J = 11.4 Hz, H-2), 4.55 (1H, d, J = 11.4 Hz, H-3). It is identical with literature reported (Kim *et al*, 2003).

3,5,7-Trihydroxychromone: Yellow amorphous powder, $C_9H_6O_5$, molecular weight 194; ^{1}H-NMR (CD_3OD) δ: 7.86 (1H, s, H-2), 6.27 (1H, d, J = 1.5 Hz, H-8), 6.16 (1H, d, J = 1.5 Hz, H-6); ^{13}C-NMR (CD_3OD) δ: 178.23 (C-4), 165.49 (C-7), 162.61 (C-9), 159.34 (C-5), 141.79 (C-2), 141.25 (C-3), 105.83 (C-10), 99.61 (C-6), 94.92 (C-8); GC-MS: 194 [M]$^+$. It is identical with literature reported (Yang *et al*, 2003). It is confirmed by comparing its ^{1}H-NMR and ^{13}C-NMR data with those of its 3-*O*-α-*L*-rhamanopyranoside (Li *et al*, 1996) and its 3-hydroxy glycosidation effect for ^{13}C-NMR spectra (Xu, 1993).

Other two compounds identified are sucrose and mannitol.

In conclusion, a total of 18 marker compounds have been identified and characterized which allows the establishment of a complete HPLC fingerprint of MK-001. Eight polar components have been isolated and characterized from silymarin for the first time. HPLC fingerprint showed a complete assignment of all components with peak intensity over 1.0%.

In addition, ^{1}H-NMR fingerprinting method was established to serve as a secondary QA method for standardization of silymarin. As evidenced, MK-001 is the first fully standardized product from *S. marianum* currently available with the highest content (92%) of flavonolignans and with every single component (> 1%) being completely identified and characterized.

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