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Research Paper

Preparation and effects of 2,3-dehydrosilymarin, a promising and potent antioxidant and free radical scavenger

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Abstract

Objectives Silymarin or silybin has been effectively used for treating liver diseases and acute liver injury partly due to its antioxidant activity. In this study, 2,3-dehydrosilymarin, a compound exhibiting remarkable antiradical/antioxidant activity, was prepared from silymarin for the first time. The solubility, radical scavenging capacity and liver protecting activity of 2,3-dehydrosilymarin were studied and compared with silybin, dehydrosilybin and silymarin.

Methods The structures of its main components were verified by ultra-performance liquid chromatography/mass spectrometry (UPLC-MS) and other spectral analysis. In addition, a rapid screening method, online high-performance liquid chromatography/1,1-dipheny1-2-picrylhydrazyl (HPLC-DPPH) system, was developed for identifying the individual antioxidants in 2,3-dehydrosilymarin.

Key findings Both in-vitro and in-vivo results markedly proved that dehydrosilymarin has decent aqueous solubility and remarkable antiradical/antioxidation capacity. Moreover, 2,3-dehydrosilybin and 2,3-dehydrosilychristin were identified to be the two major active compounds contained in 2,3-dehydrosilymarin.

Conclusions Our results suggest that 2,3-dehydrosilymarin may be a promising and potent alternative for inhibition of free radical and prevention of oxidation.

Keywords antioxidant; dehydrosilymarin; free radical scavenger; online HPLC-DPPH

Introduction

The flavonolignan silybin is the major bioactive component of the extract from the seeds of the milk thistle (*Silybum arianum* (L.) Gaertn.). Silybin is often used in the prevention and treatment of various liver diseases.[1,2] It acts mainly as an effective radical scavenger (anti-lipoperoxidant), and also as an antioxidant. Nevertheless, the bioavailability of silybin is rather limited by its low solubility in water.[1] An oxidized form of silybin (so-called 2,3-dehydrosilybin) was found to exhibit significantly greater antioxidant and anti-cancer activity than silybin.[3] Unfortunately, dehydrogenization of silybin led to an impaired water solubility, which considerably compromised the therapeutic efficacy of 2,3-dehydrosilybin.[2,4]

Silymarin consists of 70–80% of flavanolignans, including silybin, isosilybin, silydianin and silychristin; the remaining ingredients are mainly polyphenolic compounds.[1] It has been used in Europe since the 16th century and continues to be used there in the treatment of liver disease.[5] The therapeutic effects of silymarin suggest that it possesses potent antiradical and antioxidant activity.

Silybin, 2,3-dehydrosilybin and silymarin have been extensively studied in recent years. Based on the comparison between silybin and 2,3-dehydrosilybin, we inferred that 2,3-dehydrosilymarin might have a better antioxidant activity than silymarin. To the best of our knowledge, few studies involve 2,3-dehydrosilymarin and no reports on the drug effects of 2,3-dehydrosilymarin were found. Therefore, we prepared 2,3-dehydrosilymarin, and then studied its antioxidant activity and radical scavenging capacity. 1,1-Dipheny1-2-picrylhydrazyl free radical (DPPH) is often used for the evaluation of the general radical scavenging ability of antioxidants.[6,7] The O.D.517 nm absorbance decreases as the radical is scavenged by antioxidants to form the stable DPPH-H molecule. However, DPPH assay alone is not able to identify those active ingredients in a mixture of compounds such as silymarin or dehydrosilymarin. An improved online HPLC-DPPH method combining

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separation and activity evaluation of antioxidants was firstly reported by Koleva *et al*. [8] This method has subsequently been successfully used to detect radical scavenging compounds by another two groups.[9–11]

In this study, we investigated the antioxidant activity and the radical scavenging capacity of 2,3-dehydrosilymarin. We also identified its active ingredients with the online HPLC-DPPH method. The method, which combines separation of antioxidants and activity evaluation, presents a major advantage for such investigations. Our results suggested that 2,3-dehydrosilymarin had better antiradical and antioxidative capacity than silybin, 2,3-dehydrosilybin and silymarin. More interestingly, the solubility of 2,3-dehydrosilymarin was significantly improved compared with 2,3-dehydrosilybin, and could lead to a better bioavailability and therapeutic efficacy. Hepato-protective effects against CCl_4 were observed when mice were pre-treated with 2,3-dehydrosilymarin (doses 28.4 mg/kg, 142 mg/kg, 284 mg/kg), while no protection was afforded when mice were pretreated with silybin, 2,3-dehydrosilybin or silymarin at the same doses. Therefore, 2,3-dehydrosilymarin might be a good candidate for further development as an antioxidant remedy.

Materials and Methods

Chemicals

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH.) was purchased from Sigma-Aldrich (St Louis, MO, USA). Silybin and silymarin were kindly provided by Zhongxing Pharmaceutical Co. Ltd (Jiangsu, China). Silycristin was from Tauto Biotech Co. Ltd (Shanghai, China). All other solvents/chemicals were purchased from Guoyao Chemical Regent Co., Ltd (Shanghai, China) except specifically mentioned. HPLC-grade solvents were used in this study.

Animals

All the mice used were adult males, 17–22 g, purchased from the animal distribution center of Yangzhou University (Jiangsu, China). The mice were kept on an artificial 12-h light–dark cycle and given free access to standard laboratory diet and water according to the regulations for the administration of affairs concerning experimental animal care (State Council of the China, 1988). All the research protocols were approved by the Research Ethics Committee of Jiangsu University.

Preparation of 2,3-dehydrosilymarin, 2,3-dehydrosilybin and 2,3-dehydrosilycristin

Silymarin(6 g) was dissolved in 400 ml pyridine and heated to 90°C under reflux for 77 h with stirring. After reaction, pyridine was removed using a rotary evaporator under 45 mbar at 60°C. To remove the residual pyridine, 50 ml toluene was added and evaporated in vacuum at 80°C. The remaining pellet was dissolved in ethyl acetate, loaded onto a silica gel column, and then eluted with hot acetone. After these procedures, acetone was removed from the products by distillation. The leftover pellet was re-dissolved in hot ethanol and filtered through a Double-ring #102 filter paper (Xinhua Paper Industry Co. Ltd, Hangzhou, China). The pass-through was air dried until ~4 g brown 2,3-dehydrosilymarin pellet was obtained.

Table 1 Composition of the mobile phase used in the UPLC-MS analysis

Time (min)	Flow rate (ml/min)	%A	%B
Initial	0.300	30.0	70.0
0.10	0.300	30.0	70.0
10.00	0.300	100.0	0.0
12.00	0.300	100.0	0.0
12.10	0.300	30.0	70.0

A: Methanol; B: 0.2% formic acid aqueous solution.

2,3-Dehydrosilybin and 2,3-dehydrosilycristin were prepared in accordance with the methods stated above, expect silybin and silycristin were used as initial materials. Their structures, verified by IR and NMR, were consistent with the results reported in literature.[12]

UPLC-MS analysis

UPLC/MS/MS were performed on Waters MALDI Synapt Q-TOF MS (Milford, MA, USA) using an ESI source with positive ion mode. Chromatography was performed on an ACQUITY UPLC BEH C-18 column (100×2.1 mm, i.d. 1.7 μm particle size; Waters, Milford, MA, USA). The binary gradient employed methanol (A) and 0.2% formic acid aqueous solution (B) according to Table 1. The flow rate was 0.3 ml/min. The column temperature was kept at 40°C. UV spectra were recorded over the range of 200–750 nm. The injection volume was 5 μl. The ESI source was operated at 100°C in positive mode to produce MH+ ions. The desolvation temperature was set at 250°C, extract voltage was 3.0 V, desolvation gas and cone gas was set at 500 and 50 l/hr, respectively. The full-scan mass spectra were acquired over the range of 50–1000 amu. Capillary voltages were 3.5 kV in ESI+ and cone voltages were 30 V.

Solubility studies

The experiments were carried out essentially according to the reported method[13] with slight modifications. To prepare saturated solutions, excess amount (20 mg) of silybin, 2,3-dehydrosilybin, silymarin, 2,3-dehydrosilymarin were dissolved in 10 ml double-distilled H_2O and sonicated for 1 h by a sonicator (KQ-500DE, Kunshan, China). After sonication, those samples were shaken in a vibrator with homothermal air bath (25°C) for 60 h, and then centrifuged at 15 000*g* for 10 min. The supernatants were filtered through 0.45 μm cellulose acetate membrane filters to remove undissolved compounds. Once saturated solutions were prepared, they were diluted for UV absorbance analysis. The OD 288 nm absorbances of silybin and silymarin solutions, and the OD 255 nm absorbances of 2,3-dehydrosilybin and 2,3-dehydrosilymarin solutions were determined by a UV–Vis spectrophotometer (UV-2401PC, Shimadzu, Japan).

To quantify the concentrations of the saturated solutions, serial ethanol solutions of silybin, 2,3-dehydrosilybin, silymarin, and 2,3-dehydrosilymarin were prepared. The standard curves were plotted according to their OD 255 nm or 288 nm

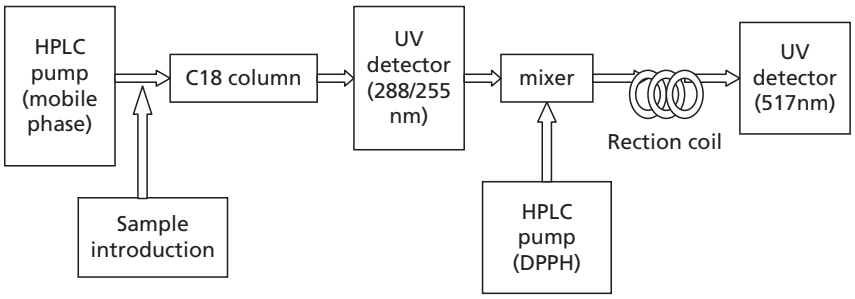

Figure 1 Instrumental setup for the online RP-HPLC-DPPH measurement of radical scavenging compounds.

Table 2 The malondialdehyde values of fresh liver tissue samples from 10 tested groups

Group	Treatment	MDA (nmol/mg protein)
Group I	Blank	1.62 ± 1.86
Group II	CCl_4	5.5912 ± 1.9471[a]
Group III	Silybin (142 mg/kg) + CCl_4	5.0742 ± 1.5732[a]
Group IV	2,3-Dehydrosilybin (28.4 mg/kg) + CCl_4	4.0071 ± 2.0734
Group V	2,3-Dehydrosilybin (142 mg/kg) + CCl_4	4.5482 ± 0.8705[a]
Group VI	2,3-Dehydrosilybin (284 mg/kg) + CCl_4	4.0259 ± 2.2688
Group VII	Silymarin (142 mg/kg) + CCl_4	4.6483 ± 1.1161[a]
Group VIII	2,3-Dehydrosilymarin (28.4 mg/kg) + CCl_4	2.8854 ± 0.8841[b]
Group IX	2,3-Dehydrosilymarin (142 mg/kg) + CCl_4	2.5416 ± 0.6359[b]
Group X	2,3-Dehydrosilymarin (284 mg/kg) + CCl_4	2.7666 ± 0.8815[b]

MDA, malondialdehyde. The experiments were repeated 10 times for each sample ($n = 10$). Data are shown as mean ± SD. The differences across all 10 groups were significant (Kruskal–Wallis test, H = 43.433, d.f. = 9, $P < 0.001$). [a]Significantly higher than the MDA values in Group I (Dunn's test subsequent to the Kruskal–Wallis test, $P < 0.05$). [b]Significantly lower than the MDA values in Group II (Dunn's test subsequent to the Kruskal–Wallis test, $P < 0.05$).

absorbances. The concentrations of those samples were calculated from the corresponding standard curves.

Online HPLC-DPPH analysis

The samples were dissolved in methanol at 100 mg/l concentration for on-line HPLC-DPPH analysis. The instrumental setup was applied according to the reported method (Figure 1).[8,9,11] The separation of antioxidative components was performed on a 150 × 4.6 mm i.d., 5 μm particle size, shimpak RP-18 column (Shimadzu, Japan). The mobile phase was methanol and water (6 : 4) and the flow rate was 0.3 ml/min. The signals were detected with a shimadzu SPD-10Avp photo diode array at 288 nm wavelength for silymarin and 255 nm for 2,3-dehydrosilymarin. For online DPPH radical-scavenging analysis, the flow reagent (2 mg/l DPPH in methanol) was set to be 0.3 ml/min, and the induced bleaching was detected photometrically as a negative peak at 517 nm. The length of the capillary used for the postcolumn reaction was adjusted to 465 cm (to achieve a reaction time of 90 s).

Quatification of DPPH radical scavenging activity

The superoxide radical scavenging capacity of 2,3-dehydrosilymarin was examined by free radical scavenging (DPPH, superoxide) assay.[6] A 1-ml volume of ethanol solution of DPPH (19.8×10^{-4} mM) was added into 3 ml of 2,3-dehydrosilymarin ethanol solutions at serial concentrations (1.8, 3.6, 5.4, 9, 27, 54 and 200 mg/l). After 30 min, the absorbance changes of mixed samples were measured with a UV–Vis spectrophotometer at 517 nm. The radical inhibition efficacy was calculated from the absorbance change. The radical scavenging activity of silybin, 2,3-dehydrosilybin, silymarin and 2,3-dehydrosilycristin was also measured and compared with that of 2,3-dehydrosilymarin. Their DPPH scavenging efficacies were compared by IC50 values (the concentration of tested compound which inhibited 50% radicals), calculated from the mean values of triplicates at different concentrations.

In-vivo study on the liver protection effects of 2,3-dehydrosilymarin

One-hundred mice were obtained and randomly divided into 10 groups ($n = 10$ in each group). Group I (blank group) mice were orally administered with a 0.5% aqueous solution of sodium carboxymethylcellulose (CMC-Na, 20 ml/kg dose) daily for 15 days, and sesame oil (5 ml/kg dose) on day 16. Group II (positive control group) were orally treated with 0.5% CMC-Na daily (20 ml/kg dose) for 15 days and then challenged with CCl_4 in sesame oil (0.3%, dose = 5 ml/kg) on day 16. The protective effects of silybin, 2,3-dehydrosilybin, silymarin and 2,3-dehydrosilymarin were tested in Groups III–X (Table 2). Group III were pre-treated orally with silybin (7.1 mg/ml in 0.5% CMC-Na, 20 ml/kg dose) daily for 15 days and CCl_4 in sesame oil (0.3%, 5 ml/kg body weight) on day 16. Group IV–VI mice were orally administered with 2,3-dehydrosilybin (1.42 mg/ml, 7.1 mg/ml or 14.2 mg/ml in 0.5% CMC-Na, 20 ml/kg dose, respectively) daily for 15 days

and CCl_4 in sesame oil (0.3%, 5 ml/kg) on day 16. Group VII mice were given silymarin (7.1 mg/ml in 0.5% CMC-Na, 20 ml/kg dose) orally for 15 days and then CCl_4 in sesame oil (0.3%, 5 ml/kg body weight) on day 16. Group VIII–X mice were given 2,3-dehydrosilymarin orally at different concentrations (1.42 mg/ml, 7.1 mg/ml or 14.2 mg/ml in 0.5% CMC-Na, 20 ml/kg dose, respectively) for 15 days and treated with CCl_4 in sesame oil (0.3%, 5 ml/kg) on day 16. All the mice were sacrificed 24 h after the CCl_4 challenge. Fresh liver samples were then dissected and the acute toxicity was tested with malondialdehyde (MDA) studies.

Liver malondialdehyde determination

Fresh liver samples were immediately washed with ice-cold saline. 0.3 g liver tissue was added into 3.0 ml ice-cold saline, homogenized with a Fluker homogenizer (Fluker, Gemany) for 15 s, and then centrifuged at 785*g* for 10 min to remove debris. MDA concentrations in the supernatants were determined with a kit from Jiancheng Co. Ltd (Nanjing, China) according to its instructions. The protein concentrations of the supernatants were determined with Coomassie Brilliant Blue assay. The final liver MDA values were normalized by the protein concentrations.

Statistical analysis

Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn's test was used for the solubility analysis and the MDA assay. Analysis was made using SigmaStat Software (Version 3.5, Jandel Scientific Software, Corte Madern, CA, USA)

Results and Discussion

Preparation and composition analysis of 2,3-dehydrosilymarin

2,3-Dehydrosilymarin was prepared essentially according to a reported method, which was originally designed for the preparation of 2,3-dehydrosilybin.[3] Before the composition analysis of the 2,3-dehydrosilymarin, we prepared pure standards of 2,3-dehydrosilybin and 2,3-dehydrosilycristin. The infrared spectra of 2,3-dehydrosilybin and 2,3-dehydrosilycristin were recorded using KBr pellets on a NICOLET AVATAR-370 spectrometer. The ^{1}H NMR spectra were obtained on Bruker AVANCE DPX 200 and AMX 400 WB spectrometers. DMSO-d6 was used as solvent, in which the observation of OH signals is possible. The solid-state infrared spectrum and ^{1}H NMR (400.13 MHz, 296 K) data of 2,3-dehydrosilybin and 2,3-dehydrosilycristin were consistent with the results reported in literature.[12]

The composition of our 2,3-dehydrosilymarin was studied using UPLC-MS analysis. A typical profile of 2,3-dehydrosilymarin is shown in Figure 2. The retention time of peak 1 was 4.567 min with λmax at 257 nm and 373 nm, while the retention time of peak 2 was 6.088 min with λmax at 254 nm and 369 nm. The mass spectrum of both peaks showed correct mass: m/z 481.1 (MH)+. Peaks 1 and 2 were identified as 2,3-dehydrosilychristin and 2,3-dehydrosilybin (Figure 3), respectively, by comparing their t_R values, absorbances from 200 nm to 600 nm, and mass spectra with those of corresponding pure standards. Interestingly, the $t_R = 6.37$ min peak (Figure 2) had similar prominent fragments to those of 2,3-dehydrosilybin and 2,3-dehydroisosilybin in m/z 400 to 200 region. This finding suggested that this peak could be a 2,3-dehydro-derivative of isosilybin.

Solubility of 2,3-dehydrosilymarin

The aqueous solubility of 2,3-dehydrosilymarin was studied on the room temperature (25°C). The solubilities of silybin, 2,3-dehydrosilybin and silymarin were also tested for comparison. The results are shown in Table 3. Among all samples, the solubility of 2,3-dehydrosilybin was the lowest, though 2,3-dehydrosilybin has been frequently reported to have significant antioxidant activity. The poor solubility of 2,3-dehydrosilybin in water was probably due to the dehydrogenation between C_2-C_3. A flavonoid plane structure, which led to a substantial decrease of solubility, was formed along with the conjugated double bond at C_2-C_3 in 2,3-dehydrosilybin. The application of 2,3-dehydrosilybin has been substantially restricted by its poor solubility.[3] However, in our results, the solubility of 2,3-dehydrosilymarin was distinctly higher than that of silybin and 2,3-dehydrosilybin. Therefore, 2,3-dehydrosilymarin, with better aqueous solubility, could be a more promising antioxidant drug, having improved bioavailability.

DPPH radical scavenging activity of 2,3-dehydrosilymarin

The ability of various antioxidants – both pure compounds and plant extracts – to terminate radical chain processes was indirectly evaluated by different methods using various substrates.[14] Conventional procedures for the screening and identification of antioxidants in complex matrices require the separation and purification of chemical compounds, which is tedious and time-consuming. On-line HPLC radical scavenging activity measurement makes it possible to directly identify active constituents in complex matrices.[15,16] In this study, an online DPPH-HPLC assay was used to identify antioxidant constituents in silymarin and 2,3-dehydrosilymarin separately. Combined UV (positive signals) and DPPH quenching (negative signals) chromatograms, fractions from 2,3-dehydrosilymarin are presented in Figure 4. With online HPLC-DPPH analysis of silymarin, negative (DPPH quenching) peaks were hardly observed in any of the fractions. But for 2,3-dehydrosilymarin, two fractions were detected as positive peaks on the UV detector (255 nm), which showed hydrogen-donating ability (negative peaks, with 90 s lag time for reaction) toward the DPPH radicals. These two fractions ($t_R = 16.0$ min and $t_R = 50.7$ min) were identified as 2,3-dehydrosilychristin and 2,3-dehydrosilybin by comparing their retention time with standard compounds.

Our results showed that the online HPLC-DPPH method could be applied for a quick screening of antioxidant compounds in silymarin and 2,3-dehydrosilymarin. With the on-line HPLC-DPPH assay, we demonstrated that 2,3-dehydrosilybin and 2,3-dehydrosilychristin were the two major active antioxidants in 2,3-dehydrosilymarin. DPPH free radical was also used to further quantify the free

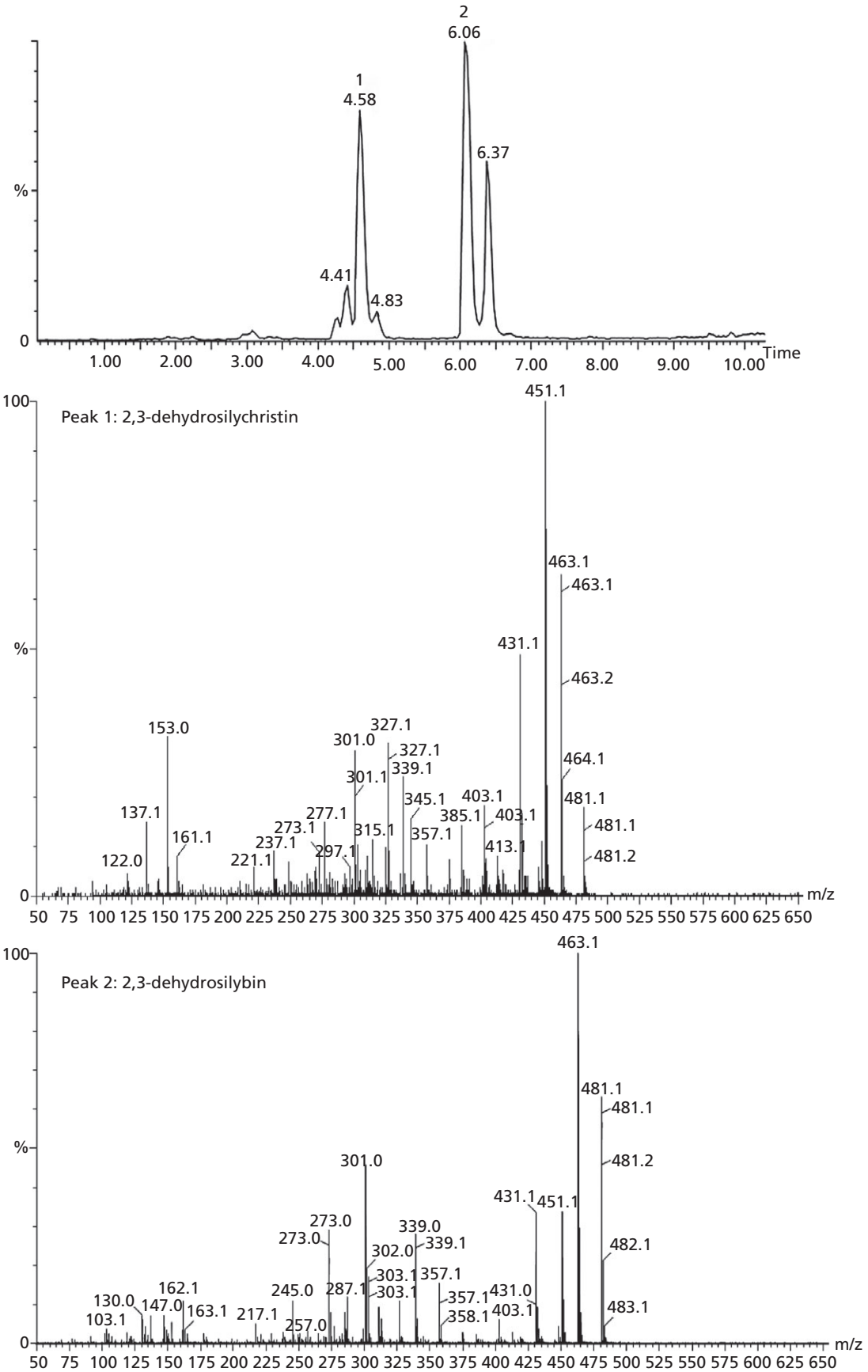

Figure 2 LC/MS analysis of 2,3-dehydrosilymarin, 1 = 2,3-dehydrosilychristin (4.58), 2 = 2,3-dehydrosilybin (6.06).

Table 3 The tested aqueous solubilities of silybin, 2,3-dehydrosilybin, silymarin and 2,3-dehydrosilymarin samples at 25°C

	Silybin	2,3-Dehydrosilybin	Silymarin	2,3-Dehydrosilymarin
Solubility (μg/ml)	55.28 ± 0.23	3.45 ± 0.39	452.12 ± 13.74[a,b]	406.39 ± 10.79[a,b]

The experiments were repeated 6 times for each sample. Data are shown as mean ± SD. The differences across all four groups were significant (Kruskal–Wallis test, H = 21.609, d.f. = 3, $P < 0.001$). [a]Significantly higher than the solubility values of silybin (Dunn's test subsequent to the Kruskal–Wallis test, $P < 0.05$). [b]Significantly higher than the solubility values of 2,3-dehydrosilybin (Dunn's test subsequent to the Kruskal–Wallis test, $P < 0.05$).

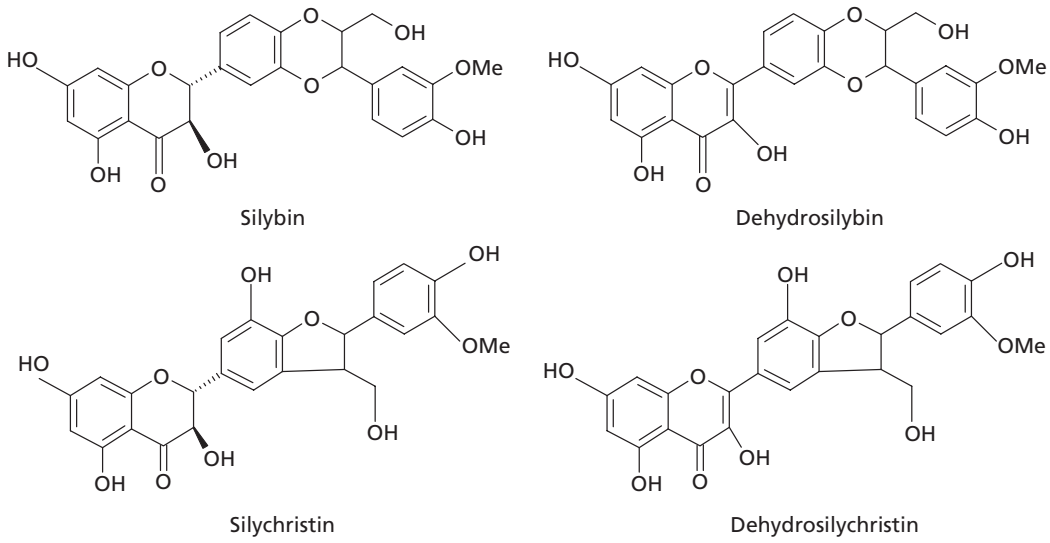

Figure 3 Structures of silybin, 2,3-dehydrosilybin, silychristin and 2,3-dehydrosilychristin.

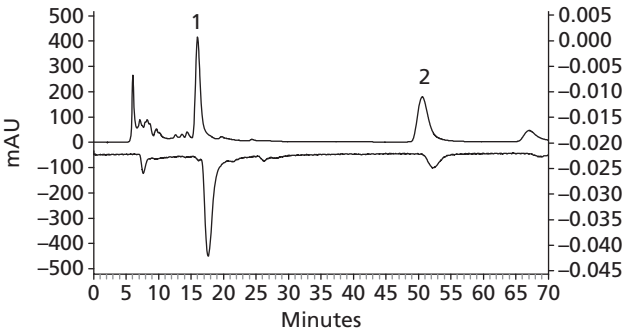

Figure 4 UV and DPPH radical quenching chromatograms of 2,3-dehydrosilymarin, 1 = 2,3-dehydrosilychristin, 2 = 2,3-dehydrosilybin.

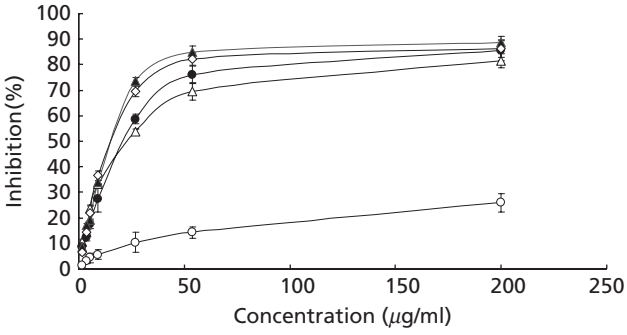

Figure 5 Scavenging of the DPPH radical by silybin (○), 2,3-dehydrosilybin (●), silymarin (△), 2,3-dehydrosilymarin (▲) and 2,3-dehydrosilycristin (◇). Results are means ± SD ($n = 3$).

radical-scavenging activity of 2,3-dehydrosilymarin, silymarin, silybin, 2,3-dehydrosilybin and 2,3-dehydrosilycristin. Their DPPH radical scavenging capacities were shown to be dose-dependent (Figure 5); the IC50 values were calculated for each sample. The tested IC50 values of silybin, 2,3-dehydrosilybin, silymarin, 2,3-dehydrosilymarin and 2,3-dehydrosilycristin were 803.93 μg/ml, 23.28 μg/ml, 24.67 μg/ml, 16.30 μg/ml and 18.43 μg/ml, respectively. These results indicated that 2,3-dehydrosilybin, silymarin, 2,3-dehydrosilymarin and 2,3-dehydrosilycristin had comparable DPPH radical scavenging activity, greatly higher than that of silybin. The DPPH radical scavenging activity of 2,3-dehydrosilymarin was apparently better than that of silymarin, as indicated by its lower IC50 value (16.30 μg/ml vs 24.67 μg/ml).

Effect of 2,3-dehydrosilymarin in carbon tetrachloride-induced hepatotoxicity

Before the studies on their liver protecting effect, the acute toxicity of silybin, 2,3-dehydrosilybin, silymarin and 2,3-dehydrosilymarin was studied in mice. No acute toxicity was observed in the mice given any of the drugs at oral doses up to 320 mg/kg. The liver protecting effects of those drugs were

studied with a CCl_4-intoxicated mouse model. MDA analysis was used to characterize the extent of liver injury in CCl_4-intoxicated mice. The liver protecting effects of silybin, 2,3-dehydrosilybin, silymarin and 2,3-dehydrosilymarin in mice are presented in Table 2. The results suggest that silybin, 2,3-dehydrosilybin or silymarin have little or no significant effects against liver damage at tested doses. However, 2,3-dehydrosilymarin showed clear protective effects against CCl_4-induced liver injury. The liver protection of 2,3-dehydrosilymarin was observed in the mice treated with a oral dose as low as 28.4 mg/kg. More interestingly, the liver protecting effects of 2,3-dehydrosilymarin seemed to be independent of dose (Group VIII–X).

When compared with silymarin, 2,3-dehydrosilymarin had slightly improved IC50 and comparable solubility. This fact suggested that the remarkable in-vivo liver protecting effects of 2,3-dehydrosilymarin could partly arise from its good radical scavenging activity and solubility. From online DPPH-HPLC analysis, we found that 2,3-dehydrosilychristin was also a potent antioxidant. It is plausible that 2,3-dehydrosilychristin played a significant role in the liver protection. Detailed study on 2,3-dehydrosilychristin is still under way in our group.

Conclusions

Based on this study, we conclude that our prepared 2,3-dehydrosilymarin has decent aqueous solubility, potent antiradical/antioxidation capacity and better in-vivo liver protecting effects when compared with silybin, 2,3-dehydrosilybin and silymarin. Our findings suggest that 2,3-dehydrosilymarin is a promising and potent antioxidant and free radical scavenger, which may have better efficacy than the widely used silymarin or silybin. We also found that 2,3-dehydrosilychristin is a potent bioactive ingredient in 2,3-dehydrosilymarin. This compound probably plays a significant role in the liver protection.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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