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Preparation of C-23 esterified silybin derivatives and evaluation of their lipid peroxidation inhibitory and DNA protective properties

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ABSTRACT

A diverse series of C-23 esterified silybin derivatives (**1a**–**n**) were designed and synthesized. The antioxidative properties of these compounds were evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radical scavenging, ferrous ion chelation, and inhibition of rat liver homogenate lipid peroxidation. Their protective effects on the prevention of hydrogen peroxide induced DNA damage were also investigated. Most of the synthesized compounds exhibited more effective antioxidant activities than silybin. The esterified silybin analogues displayed satisfactory performance especially on iron chelation and antiperoxidative activity. Compound **1n** in particular exhibited remarkable antiperoxidative effect with an IC_{50} value of 0.2 ± 0.1 μM, which was stronger than that of quercetin (IC_{50} = 1.8 ± 0.6 μM). Compounds **1c**, **1e**, **1g**, **1h** and **1k** displayed potent, dose-dependent protective properties against DNA cleavage. The results of the bioassays support the antioxidative and DNA protective effects of these synthesized silybin derivatives.

1. Introduction

Excessive production of free radicals, a by-product of cellular redox reactions, causes oxidative damage to lipids, proteins, and DNA. The cumulative effects of oxidative damage have been implicated in a variety of diseases, such as neurodegenerative disorders,[1,2] cancer,[3] cardiovascular diseases,[4] and liver damage.[5] Antioxidants, including vitamins C, E and flavonoids, have positive effects on preventing or attenuating these diseases.[6] Thus, there is considerable interest in the discovery and development of efficient synthetic or natural antioxidants to prevent and treat diseases related to free radicals.

The natural flavonolignan silybin (**1**), isolated from the seeds of milk thistle *Silybum marianum*, is one of the main active principles of Legalon® (Fig. 1). It is an important hepatoprotective agent applied to the treatment of liver damage of various etiologies.[7] Recent studies indicated that the liver-protecting properties of silybin arise from its antioxidative activity.[8–10] Silybin may act as a radical scavenger to remove the reactive radicals resulting from liver detoxification of xenobiotics by monooxygenase systems, and an inhibitor of lipid peroxidant to stimulate the metabolism of phospholipids, thereby modulating the fluidity of cell membranes of hepatocytes.[11] Previous bioavailability studies showed that silybin derivatives such as glycosides and C-23 phosphoric esters exhibited improved water solubility, together with significant antioxidant and cytoprotective activities.[12,13] However, oxidation of the C-23 hydroxymethyl moiety of silybin to a carboxylic acid group caused a 50% decrease in the antiperoxidative capacity (Fig. 1).[14] The results suggest that the C-23 substituent of silybin could play an important role in the bioactivities of silybin analogues. Previous modification work exploring the antioxidant properties of B- and E-ring substituted versions of silybin showed that a silybin analogue possessing a methoxy group in the B-ring is a superior superoxide scavenger compared to quercetin (Fig. 1).[15] In an attempt to further improve the antioxidative properties of

Abbreviations: DEAD, diethyl azodicarboxylate; linDNA, linear deoxyribonucleic acid; ocDNA, open circular deoxyribonucleic acid; scDNA, supercoiled circular deoxyribonucleic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTA-2Na, disodium salt of ethylene diamine tetraacetic acid; ESIMS, electro spray ionization mass spectrum; LPO, lipid peroxidation; MDA, malondialdehyde; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; SAR, structure–activity relationship; TBA, thiobarbituric acid; TPP, triphenylphosphine.

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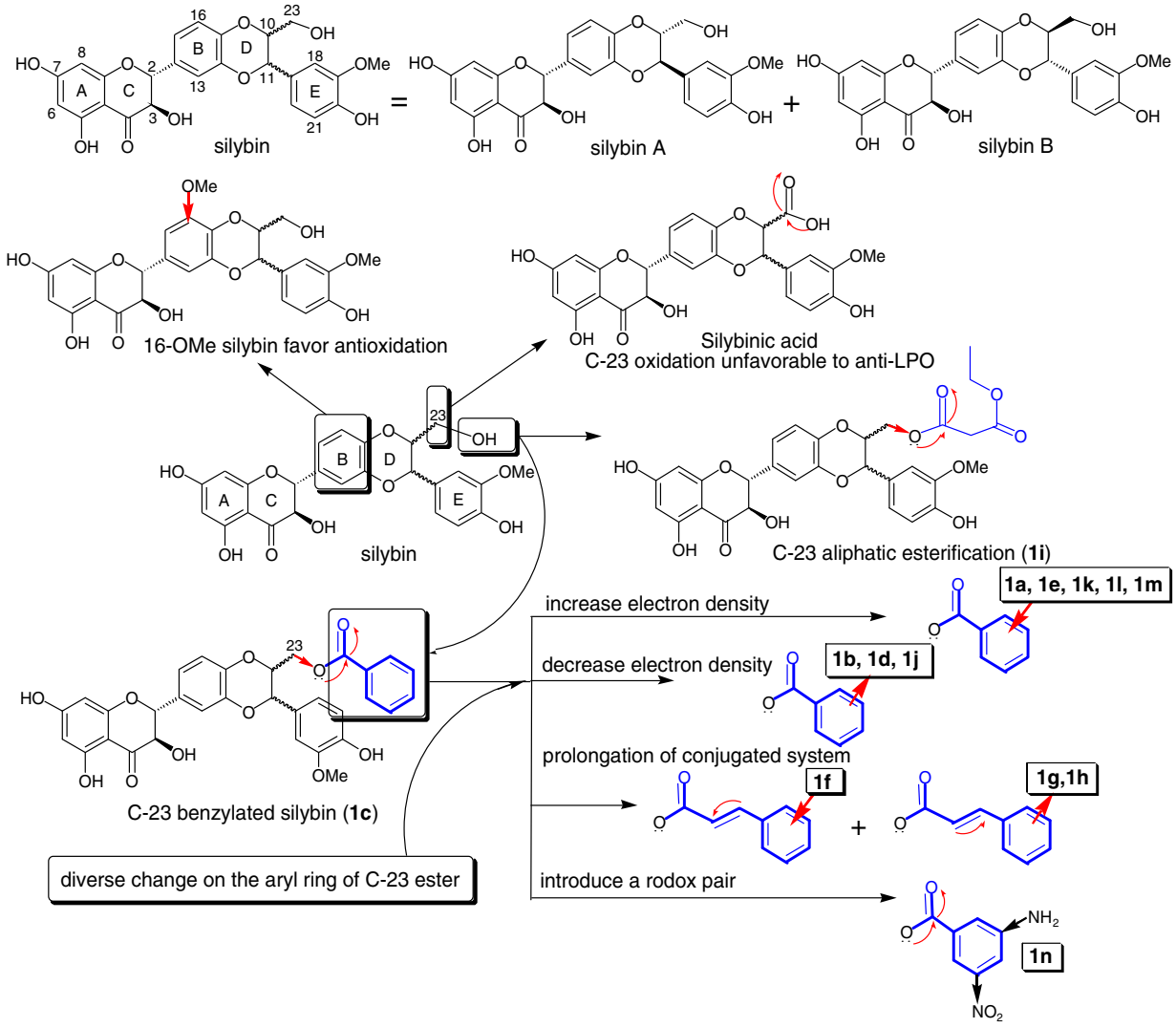


Figure 1. Rational design of C-23 esterified silybin analogues (**1a**–**1n**). The structure of silybin is composed of a pair of diastereomers: silybin A and silybin B. Newly designed modification in the present study concentrated on the C-23 aryl moiety is demonstrated by blue scaffolds. The direction of electron flow was illustrated by red arrows.

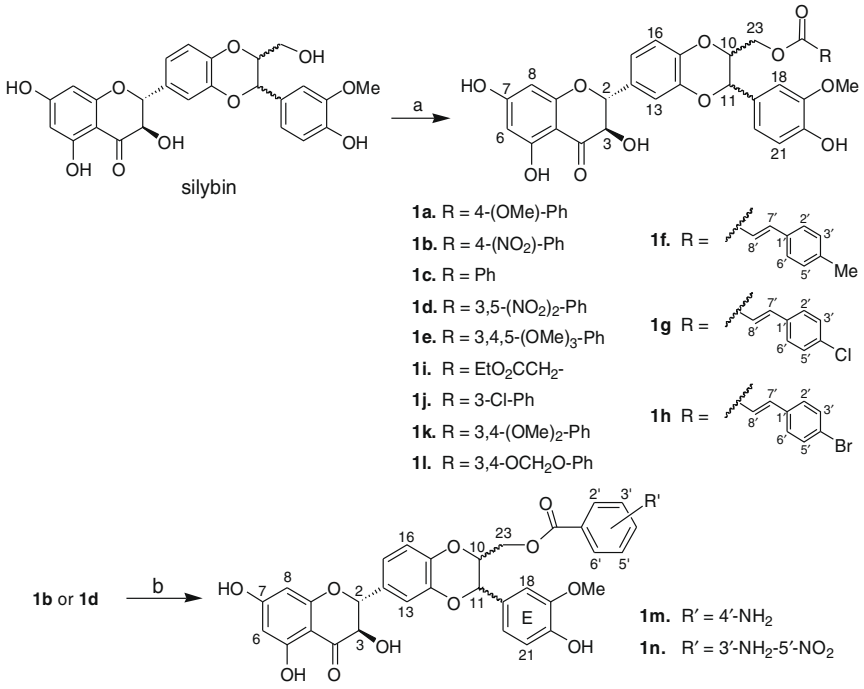
silybin, we have designed and synthesized a novel set of silybin derivatives, possessing diverse substituents, such as aromatic and aliphatic groups at C-23, selected to change the chain length by prolongation of the conjugated system, and modulate the electron density of the lead structure (Fig. 1).

The aim of this study was to elucidate the antioxidant properties of these new silybin derivatives by way of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radical scavenging, ferrous ion chelation, and rat liver homogenate lipid peroxidation inhibition. As a first step towards elucidating their bioactivity on the cell level, their protective properties against hydrogen peroxide induced DNA damage were also assayed. The present study may help to better understand the mechanisms underlying the antioxidative activities of silybin analogues and to further optimize their pharmaceutical applications, especially in neuroprotective efficacies against oxidative stress induced cell damage.

2. Results and discussion

2.1. Chemistry

Scheme 1 illustrates the chemoselective esterification of silybin. The fact that silybin contains five hydroxy groups of three different types, means that selective esterification is somewhat problematic. According to Appendino et al.,[16] esterification of silybin with various acids under Mitsunobu reaction conditions results in the selective 3,23-diester products with the three phenol hydroxyls remaining intact. However, slight modification of the conditions, [condensation of silybin with various substituted acids in the presence of triphenylphosphine (Ph_3P) and diethyl azodicarboxylate (DEAD)] led to the successful synthesis of the monoesters **1a**–**l**, based on the mass spectra obtained. Comparisons of the ^{1}H NMR spectra of **1a**–**l** with that of silybin suggest that the chemical shifts of hydroxymethyl proton (H-23) were downfield shifted (from ca. δ 3.10–3.70 to ca. δ 4.10–4.50), while the relative C-10/C-11 configurations were still *trans*, based on the coupling constants of the H-11 doublets (J ca. 8.0 Hz). Therefore, ester formation occurred regioselectively at C-23. The C-3 hydroxy group in silybin was not esterified under our reaction conditions, probably due to its steric hindrance. Despite modest yields, the procedure described here involved mild conditions, a one-step reaction and no selective protection of hydroxy groups. Moreover, the nitro groups in compounds **1b** and **1d** were further reduced under atmospheric pressure of hydrogen in the presence of 10% palladium charcoal to afford the amines **1m** and **1n**, respectively (Scheme 1). The structures of the synthesized compounds were elucidated by their



Scheme 1. Preparation of the 23-esterified silybin analogues **1a**–**n**. Reagents and conditions: (a) RCOOH, TPP, DEAD, THF, rt, 10 h; (b) H_2, 10% Pd–C, EtOAc, rt.

NMR, ESI-MS and HRESI-MS data. The starting material utilized was commercially available silybin composed of silybins A and B (Fig. 1), both of which possess *trans*-configuration at C-10 and C-11. Therefore, the purified products **1a**–**n** should be either 10*R*,11*R*-*trans* or 10*S*,11*S*-*trans* diastereomers. The absolute configurations of the chromatographic pure compounds **1a**–**n** have not been determined yet.

2.2. Biological evaluation and discussion

2.2.1. The free radical scavenging activities of the test compounds and structure–activity relationship (SAR)

The in vitro model of DPPH scavenging provided information pertaining to the capacity of the test compounds **1a**–**n** to scavenge free radicals independent from any enzymatic activity. Among the flavonolignans synthesized, the 3,4,5-trimethoxy benzoic acid ester **1e** and amino/nitro benzoic acid ester **1n** showed definite DPPH scavenging effects, with EC_{50} values of 145.2 ± 10.3 μM and 127.4 ± 19.8 μM, respectively (Table 1). As presented in Table 1, most of the esterified silybin derivatives exhibited comparable or better DPPH quenching activity than silybin. However, the overall relative weakness in DPPH radical scavenging activity compared to quercetin (EC_{50} = 5.5 ± 0.8 μM) shown by these flavonolignans confirmed the results of related research.[14]

The results of experiments on superoxide anion scavenging showed that most of the modified flavonolignans failed to enhance their scavenging effects against $O_2^{\cdot-}$ radicals when compared to silybin (Table 1). This suggests that the size of the free radicals may have influence on the free radical scavenging capacity of the synthesized silybin C-23 esters.[17]

From a SAR investigation point of view, it could be found that, as for DPPH radicals, introduction of a benzoyl moiety at C-23 (case **1c** in Fig. 1) may promote the scavenging ability of DPPH radicals when compared to the parent silybin (Table 1). Thereafter, the following SAR could be neatly obtained by comparing the bioactivity of compounds with **1c** as a reference. Enhancement of the electron density of the benzoyl ring of **1c** by piling up the electron-donating functionalities (e.g., **1a**, **1k**, and **1l**, see Fig. 1) resulted in a decreased quenching ability of the compounds towards DPPH radicals when compared to that of **1c**, though all of them possessed enhanced efficacy compared to silybin (Table 1). Furthermore, introduction of electron-withdrawing substituents into the benzene ring of **1c** (cases **1b**, **1d**, and **1j** in Fig. 1) did not elevate the scavenging capacity of the analogues (Table 1). Interestingly, the analogue possessing a 3,4,5-trimethoxy moiety (case **1e** in Fig. 1) exhibited a stronger DPPH scavenging effect than **1c**. In addition, the results also showed that the replacement of the aromatic moiety of **1c** with an aliphatic substituent (case **1i** in Fig. 1) moderated the quenching ability towards DPPH radicals. Furthermore, com-

Table 1
DPPH and superoxide anion radicals scavenging activities of **1a**–**n**[a]

Compound	DPPH scavenging activity (%)	Superoxide scavenging activity
1a	37.1 ± 4.3	6.5 ± 2.0%
1b	38.6 ± 3.6	—[c]
1c	40.4 ± 5.1	—[c]
1d	22.1 ± 3.0	32.0 ± 4.4%
1e	52.6 ± 6.4[b]	36.1 ± 3.8%
1f	33.3 ± 4.1	7.3 ± 1.7%
1g	18.4 ± 2.2	9.1 ± 2.1%
1h	19.8 ± 2.5	6.5 ± 1.8%
1i	16.2 ± 2.0	60.1 ± 7.9%[b]
1j	5.6 ± 1.6	7.0 ± 1.1%
1k	34.2 ± 6.3	37.2 ± 3.7%
1l	31.7 ± 4.7	7.3 ± 1.0%
1m	16.4 ± 3.0	43.3 ± 5.5%
1n	59.9 ± 6.2[b]	54.3 ± 4.9%[b]
Silybin	16.6 ± 2.5	38.2 ± 3.2%
Quercetin	96.8 ± 8.9[b]	93.8 ± 9.3%[b]

[a] The rate of radicals being scavenged at the concentration of 100 μg/mL. Data are expressed as the mean ± SD, n = 3.
[b] EC_{50} values were determined to be the effective concentrations at which radicals were scavenged by 50%. As for DPPH radical scavenging test, EC_{50} values for **1e**, **1n** and quercetin are 145.2 ± 10.3 μM, 127.4 ± 19.8 μM, and 5.5 ± 0.8 μM, respectively. For superoxide anion radical scavenging, EC_{50} values for **1i**, **1n** and quercetin are 113.4 ± 28.8 μM, 128.8 ± 21.6 μM, and 31.8 ± 3.2 μM, respectively.
[c] No detectable activity.

pounds **1g**, **1h**, and **1f**, the three silybin analogues possessing the prolonged C-23 side chains exhibited inferior DPPH scavenging when compared to **1c** (see Fig. 1 and Table 1). Nevertheless, the results suggest that introduction of an electron-donating methyl group into the cinnamic benzene ring of C-23 esterified silybin (case **1f**) may elevate the scavenging ability towards DPPH radicals, when compared with those cases introduced the electron-withdrawing substituents (**1g** and **1h** in Fig. 1) (Table 1). Furthermore, assembling of a redox pair (packing a nitro and an amino group simultaneously in the aromatic ring) moiety into a C-23 benzoyl ester of silybin (case **1n** in Fig. 1) significantly improved the scavenging ability of the compound, which is far superior to **1c** and the amino analogue **1m** (Table 1).

As for the SAR concept of $O_2^{\cdot-}$ scavenging, the analogues containing electron-donating substituents (e.g., **1a**, **1k**, and **1l** in Fig. 1) showed a decreased $O_2^{\cdot-}$ quenching ability compared to silybin (Table 1). Nevertheless, **1k** and **1e**, two compounds possessing 3,4-dimethoxy groups on the benzoyl ring, still retain effective quenching ability to $O_2^{\cdot-}$ radicals compared to silybin (Table 1). However, three conjugated cinnamic acid analogues **1g**, **1h**, and **1f** owning prolongated C-23 esterified side chains exhibited decreased $O_2^{\cdot-}$ radical scavenging ability compared to that of silybin. In contrast to the result of the DPPH test, replacement of the aromatic moiety with an aliphatic substitute (case **1i** in Fig. 1) improved the scavenging capability towards $O_2^{\cdot-}$ radicals (Table 1). This implied that the aromatic ring involved in the C-23 esterified moiety is not crucial to facilitate compounds' $O_2^{\cdot-}$ radicals scavenging. However, **1m** exhibited a stronger inhibitory effect on $O_2^{\cdot-}$ but a similar inhibition against DPPH radicals to silybin (Table 1), thereby differentiating itself from other electron-donating analogues **1a**, **1k**, **1l**, and **1e** (Fig. 1) which exhibited inferior $O_2^{\cdot-}$ radicals scavenging but clearly superior DPPH radicals quenching ability to silybin. This suggests that the amino group on the aromatic ring displays more than a simple electron-donating functionality; it may in fact possess an unusual antioxidant mechanism distinct from those of **1a**, **1k**, **1l**, and **1e**. This assumption is at least partially supported by the unique antioxidative properties of **1n**, which possesses both an electron-withdrawing nitro group and an amino group at the aromatic ring (Fig. 1). This compound exhibited certain scavenging abilities toward DPPH and $O_2^{\cdot-}$ radicals (Table 1). In a mechanism point of view, it might be proposed that the proton of the amino group can be subtracted during the free radical transition procedure, thus forming a free radical which may be successively delocalized by the adjacent aromatic π electrons of the nitrobenzoyl group (Fig. 2). This may constitute the background for **1n** possessing the apparent scavenging ability towards free radicals.

2.2.2. Fe^{2+} chelation and preliminary SAR concept of 1a–n

Ferrum plays an essential catalytic role in the formation of hydroxyl free radicals from hydrogen peroxides and superoxide anions. In the Fenton reaction, in the presence of ferrous ions, H_2O_2 can be converted to hydroxyl radicals. The ability to chelate Fe^{2+} is a practical indicator of antioxidant activity. The Fe^{2+} chelating activity of compounds **1a–n** was assessed by differences in absorbance at 562 nm, with the appearance of an absorption peak upon reaction of Fe^{2+} with ferrozine. The results showed that almost all of the C-23 esterified silybin derivatives except for **1b**, **1d** and **1n** displayed their chelating effects on Fe^{2+} (EC_{50} values ranged from 73 to 136 μM) superior both to quercetin (EC_{50} = 236.1 ± 36.7 μM) and to silybin (EC_{50} = 196.5 ± 23.5 μM). However, none of the analogues exhibited a chelating capacity comparable to the positive control, EDTA-2Na (EC_{50} = 16.2 ± 1.9 μM) (Table 2).

In the SAR investigation on **1a–n** upon Fe^{2+} chelation, it was seen that most of the synthesized electron-donating style analogues (cases **1a**, **1k**, and **1e**, see Fig. 1) demonstrated elevated che-

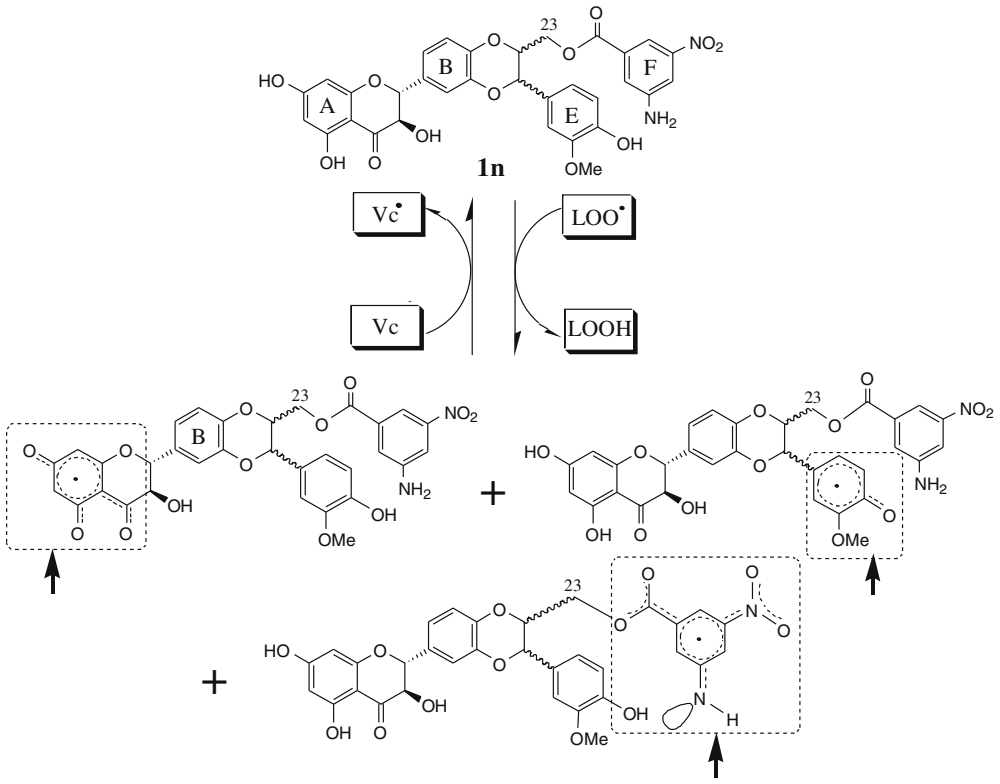


Figure 2. Possible mechanism of action of **1n** in the free radical quenching process associated with LPO inhibition. Three dotted rectangles represent the three hypothesized conjugated delocalization moieties.

Table 2
Fe^{2+} chelating activities of **1a–n**[a]

Compound	EC_{50} (μM) of Fe^{2+} chelating activities
1a	73.3 ± 8.9
1b	>300
1c	105.4 ± 8.6
1d	204.9 ± 16.7
1e	94.8 ± 7.8
1f	135.8 ± 13.1
1g	87.9 ± 7.7
1h	88.9 ± 8.6
1i	100.8 ± 9.5
1j	106.3 ± 9.6
1k	77.0 ± 6.4
1l	108.2 ± 9.1
1m	128.2 ± 10.9
1n	>300
Silybin	196.5 ± 23.5
Quercetin	236.1 ± 36.7
EDTA-2Na	16.2 ± 1.9

[a] EC_{50} value was determined to be the effective concentration at which ferrous ions were chelated by 50%. Data are expressed as the mean ± SD, $n = 3$.

Table 3
Inhibitory effects of **1a–n** on rat liver homogenates lipid peroxidation[a]

Compounds	IC_{50} (μM) of lipid peroxidation inhibition
1a	21.0 ± 6.3
1b	12.6 ± 2.2
1c	12.7 ± 2.1
1d	14.6 ± 1.6
1e	25.7 ± 5.3
1f	14.7 ± 1.3
1g	12.0 ± 0.8
1h	16.3 ± 2.5
1i	33.4 ± 4.7
1j	14.2 ± 1.8
1k	16.0 ± 1.7
1l	20.8 ± 2.9
1m	14.3 ± 2.5
1n	0.2 ± 0.1
Silybin	22.7 ± 2.6
Quercetin	1.8 ± 0.6

[a] Data are expressed as the mean ± SD, $n = 3$.

lating efficiency against ferrous ion compared to that of **1c** owning a bare aryl moiety (Table 2). This suggests that introduction of electron-donating substituents to the aromatic ring of silybin derivatives may raise the Fe^{2+} chelation ability, albeit **1l** which possesses a 3,4-methylene dioxy group showed slightly distinct characteristics (Table 2). Conversely, except for **1j**, compounds with electron-withdrawing substituents (cases **1b** and **1d**, see Fig. 1) exhibited weaker Fe^{2+} chelating ability than both **1c** and silybin. Moreover, both **1b** and **1n** owning nitro groups in the aryl moiety of the molecules showed indistinct chelating ability to Fe^{2+}, with IC_{50} values over 300 μM (Table 2). This suggests that introduction of one or more nitro groups into the aromatic ring of C-23 esterified analogues of silybin may result in a decrease in the efficiency of Fe^{2+} chelation.

In addition, for those analogues containing conjugated C-23 side chain (see Fig. 1), it was assumed that the electron-withdrawing substituents on the conjugated chain, **1g** and **1h** as examples, was a positive factor contributing to the enhancement of Fe^{2+} chelation. On the contrary, the analogue possessing an electron-donating methyl group (**1f**) demonstrated inferior chelating ability to the non-prolonged C-23 ester **1c** (Table 2). Furthermore, removal of the aromatic moiety and replacement by an aliphatic group at C-23 hydroxyl (case **1i**, see Fig. 1) did not alter the Fe^{2+} chelation ability when compared to **1c** (Table 2). This suggests that the 3,5-dioxygen-4-oxo moiety plays a more important role than the aliphatic substituent to chelation of ferrous ions.[18] Interestingly, though the analogue containing a redox pair moiety (case **1n**, see Fig. 1) lost the ability to chelate Fe^{2+}, the amino-containing analogue **1m** demonstrated better Fe^{2+} chelation than both silybin and quercetin (Table 2).

2.2.3. Inhibition of lipid peroxidation of rat liver homogenates and preliminary SAR

Free radicals, such as hydroxyl and hydroperoxyl radicals, can attack unsaturated fatty acids located in the cell membrane and initiate lipid peroxidation chain reactions (LPO), ultimately leading to functional disruption of membrane localized enzymes. Malondialdehyde (MDA) has long been used as a specific biomarker for oxidative stress, and the increase of MDA reflects an increase in lipid peroxidation. In the present study, investigation into the antiperoxidative effects of silybin derivatives **1a–n** in rat liver homogenates was carried out through application of lipid-derived MDA. As shown in Table 3, all of the test compounds exhibited positive activities toward LPO inhibition; among which the aliphatic ester **1i** showed the weakest inhibitory efficiency (IC_{50} = 33.4 ± 4.7 μM) against LPO. Furthermore, with the exception of compounds **1e** and **1i**, all the other derivatives (compounds **1a–d**, **1f–h**, and **1j–n**) possessed relatively higher antiperoxidative activities (IC_{50} values ranged from 0.2 to 21 μM) than that of silybin, with IC_{50} values of 22.7 ± 2.6 μM (Table 3).

The preliminary SAR study of **1a–n** was accomplished based on the obtained results. First of all, it was surmised that drawing electron-donating substituents to the aromatic ring of C-23 ester moiety did not ensure the strengthening of LPO inhibition, due to the finding that all of four derivatives **1a**, **1k**, **1l**, and **1e** owning increased electron density of the aryl moiety failed to exhibit better inhibitory ability against LPO than that of **1c** (see Fig. 1 and Table 3). Furthermore, the inhibitory efficacy of **1b**, **1d** and **1j** against LPO indicated that the three analogues possessing electron-withdrawing substituents (see Fig. 1) showed virtually unchanged IC_{50} values when compared to **1c**, though they did exhibit stronger inhibition against LPO than silybin (Table 3). The results imply that decrease of electron density of the C-23 aryl moiety by introduction of electron-withdrawing substituents, such as one or more nitro groups or a halide atom into the aromatic ring of C-23 esterified silybin analogues, could not significantly improve their inhibitory capability against LPO. Furthermore, the sequence (**1g** > **1f** > **1h**) of LPO inhibitory ability for the three chain-prolonged C-23 esters (Fig. 1) suggests that the chlorine substituent in the cinnamic aryl ring may elevate the ability of LPO suppression (Table 3). However, it is not facile to assign a hard and fast SAR rule to the chain extension analogues with the limited compounds available. Furthermore, the inhibitory efficacy of the aliphatic analogue **1i** against LPO was less effective compared to that of **1c**; this might be attributable to the lack of an aromatic functionality resulting in a weakness in reinforcing delocalization during the quenching process associated with LPO inhibition (the possible mechanism is illustrated in Fig. 2).

In addition, it should be noticed that, among the test compounds, although silybin analogue **1n** showed the weakest chelation towards Fe^{2+}, it demonstrated the strongest inhibition against LPO with an IC_{50} of 0.2 ± 0.1 μM, tenfold and sixfold superior to silybin and quercetin, respectively (Tables 2 and 3). Further investigation on the inhibitory activity of **1n** against LPO indicated that the compound inhibited lipid oxidation in a concentration-dependent manner (Fig. 3). More impressively, **1n** displayed its inhibitory capacity superior to the positive control, quercetin, in all of the six test concentrations (Fig. 3). This intensely suggested that this redox pair-bearing compound **1n** possess strong affinities for lipid peroxide radical (LOO·). Figure 2 describes the hypothetical mechanism of

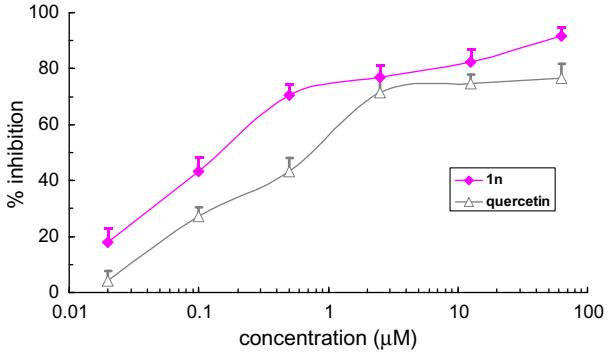


Figure 3. Concentration-dependent inhibitory activities of **1n** and quercetin against Fe^{2+}-mediated peroxidation of rat liver homogenates: ♦ = **1n** and △ = quercetin. Values are means ± SD (n = 3).

1n upon inhibition of LPO. It is proposed that the free radicals produced during lipid peroxidation can be trapped in three delocalized aromatic rings, based on the attacking probabilities of the free radicals occurring on rings A, E and F (Fig. 2). The multiple possibilities confer **1n** with a potent inhibitory capacity against rat homogenate lipid peroxidation. This assumption is also substantiated by the results of the clear anti-LPO effects of all the C-23 esters owning an aromatic substituent, and of the weakest anti-LPO effect of **1i** possessing an aliphatic C-23 side chain (Table 3).

2.2.4. Protection of DNA against H_2O_2-induced damage and SAR of the test compounds

The effects of the test compounds (**1a**–**n**) on H_2O_2-induced DNA damage were also investigated in this study. The pcDNA3.1 (+) plasmid showed 3 bands on agarose gel electrophoresis (lane 1, Fig. 4A–C): the faster moving band corresponds to the native form of supercoiled circular DNA (scDNA), the second band is linear DNA (linDNA) and the third is composed of the open circular form (ocDNA). It was found that cleavage of DNA damaged by a high concentration of H_2O_2 (final concentration = 25 mM) were suppressed by the presence of compounds **1c**, **1e**, **1f**, **1g**, **1h**, **1i**, **1j**, and **1k** at the highest test concentration of 100 μg/mL, in which compounds **1c**, **1e**, **1g**, **1h**, and **1k** showed positive protective effects in dose-dependent manners at the concentrations tested (Fig. 4A–D). Other compounds, including **1a**, **1b**, **1d**, and **1l**–**n**, failed to exhibit detectable protective efficacy at the highest test concentration, and were thus excluded from further investigations.

Analysis of the results pertaining to the DNA protection experiment led to the following conclusions. The introduction of electron-withdrawing substituents into the aromatic ring of C-23 ester moiety (e.g., **1b** and **1j** in Fig. 1) may have a negative influence on the protective ability; whilst adoption of an electron-donating functionality does not ensure the promotion of protective efficacy either: the 3,4-dimethoxy analogue **1k** exhibited a stronger protective effect than the 3,4,5-trimethoxy compound **1e**, whereas **1k** was less effective than the bare benzene analogue **1c** (see Fig. 1) at the highest test concentration (Fig. 4D). However, the extension of the C-23 side chain by introduction of a conjugated cinnamic acid aromatic moiety (see Fig. 1) can noticeably enhance the protective capacity against DNA damage: **1f** possessing an electron-donating methyl along with **1g** and **1h** owing halide atoms (see Fig. 1) showed apparent protective ability against DNA damage, though **1f** did not exhibit dose-dependent protection at higher concentrations. The above information led to the suggestion that extension of the C-23 side chain with a cinnamic moiety or introduction of a 3,4-dimethoxy pattern into the C-23 aryl ring, may enhance the DNA protection ability of the C-23 esterified silybin analogues against H_2O_2-induced damage. In addition, the

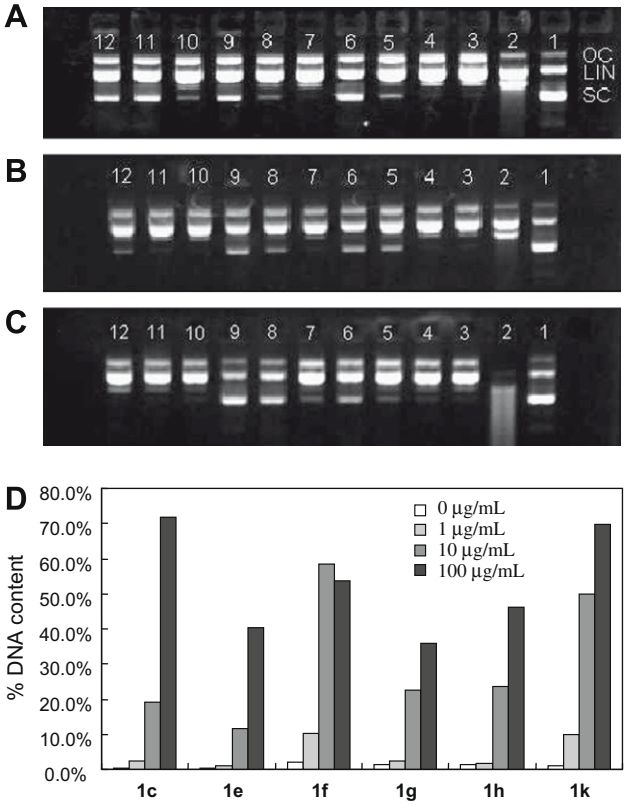


Figure 4. Protective effects of silybin derivatives on supercoiled DNA against high concentration of H_2O_2. For A–C, lane 1 represents untreated DNA, lane 2 for treated DNA (added 25 mM of H_2O_2), lane 3 for solvent control (25 mM of H_2O_2 in DMSO). Lane (4A: 4–6) displaying that added compound **1c** at 1 μg/mL, 10 μg/mL and 100 μg/mL concentrations, respectively into treated DNA. Lane (4A: 7–9) for compound **1e**, lane (4A: 10–12) for **1f**, lane (4B: 4–6) for **1g**, lane (4B: 7–9) for **1h**, lane (4B: 10–12) for **1i**; lane (4C: 4–6) for **1j**, lane (4C: 7–9) for **1k**, lane (4C: 10–12) for **1m**, respectively added into treated DNA with the concentration orders the same as **1c**.

aliphatic-substituted **1i** as well as the amino-containing analogues **1m** and **1n** did not exhibit noticeable protection against H_2O_2-induced DNA scission.

Compared to the assay of Fe^{2+} chelating activity, it was observed that, except for **1a** and **1l**, the synthetic silybin 23-esters exhibited similar tendency towards Fe^{2+} chelation and DNA protection against H_2O_2-induced damage: compounds **1b**, **1d** and **1n** with weak Fe^{2+} chelating activities showed no significant protective effect toward H_2O_2-induced damage, even at the highest test concentration, whereas compounds **1k**, **1g**, and **1h** possessed clear antioxidant activities of both kinds. The fact that **1n** unexpectedly failed to exhibit positive results on above-mentioned two assays suggests that **1n** may display a different mechanism of action towards DNA damage protection and Fe^{2+} chelation, compared to LPO inhibition and free radical scavenging. This might be due to the concurrence of amino and nitro groups consequently forming complex electronic or spatial effects. These findings may provide valuable insights into the SAR concept to inform future design and preparation of silybin analogues.

3. Conclusions

In summary, 14 monoester silybin derivatives with different patterns of substituents on C-23 were designed and synthesized by condensing silybin with various substituted acids in the presence of Ph_3P and DEAD. Most of the synthesized compounds supported the study design with overall superior antioxidative

activity to that of silybin. The effects of aliphatic versus aromatic substitutes, electron-donating groups versus electron-withdrawing ones and conjugated chain length of silybin derivatives on the antioxidant activities were also analyzed. Negative correlation between Fe^{2+} chelation and anti-LPO efficacy of the compounds was revealed. Furthermore, the redox pair-bearing silybin analogue **1n** might display various mechanisms of action for the respective antioxidative properties of LPO suppression, free radical scavenging, Fe^{2+} chelation and DNA damage protection. The results of the diverse bioassays implied that the C-23 esterified analogues could modify the properties of silybin, and influence their antioxidative capability.

4. Experimental

4.1. General experimental procedures

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), hydrogen peroxide (H_2O_2), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ferrozine, EDTA-2Na and silybin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tris base and RPMI 1640 medium were obtained from Gibco (Grand Island, NY, USA). Reduced form disodium salt (NADH) was supplied from Amresco (Solon, OH, USA). Quercetin was prepared in our laboratory (HPLC purity of 99%). Plasmid mini preparation kit was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). The pcDNA3.1 (+) was a gift from the Institute of Materia Medica of Zhejiang University. All other reagents were of the highest purity commercially available. Sprague-Dawley rats were obtained from the Zhejiang Center of Laboratory Animals, China. The use of animals was in accordance with Guideline for the Care and Use of Laboratory Animals of Zhejiang University.

4.2. Chemistry and synthesis

^{1}H NMR spectra were obtained at 400 MHz on a Bruker AM-400 spectrometer; chemical shifts δ in ppm with Me_4Si as internal standard (s, singlet; d, doublet; t, triplet; m, multiplet), coupling constants J in hertz. EIMS data were measured on a Varian MAT-95 mass spectrometer, direct inlet 70 eV and ESIMS were recorded on a Bruker Esquire 3000 Plus instrument. HRESI-MS data were recorded on a Micromass Q-Tof MS spectrometer. TLC was carried out with glass precoated Si gel GF_{254} plates; detection by UV light (254 nm). Column chromatography was performed with Si gel (200–300 mesh; Qingdao Ocean Chemical Plant, Qingdao, Shandong, China). The starting material, silybin (purity: 98%, HPLC), was supplied by Panjin Tianyuan Pharmaceutical Co., Ltd (Panjin, Liaoning, China). All reactions were carried out in oven or flame-dried glassware with magnetic stirring. Solvents were purified by standard procedures. Yields refer to chromatographically and spectroscopically homogeneous materials unless otherwise stated.

4.2.1. General procedure for the synthesis of compounds 1a–l

To a solution of silybin (1.00 g, 2.08 mmol), TPP (1.60 g, 5.71 mmol) and DEAD (1.00 g, 5.75 mmol) in absolute THF (15 mL) was added the appropriate acid (4.00 mmol) in absolute THF (5 mL) under argon. The reaction mixture was stirred at room temperature for 10 h. The mixture was then concentrated in vacuo. The residue was poured into water and extracted with $CHCl_3$ (3×20 mL). The organic phase was separated and washed with brine (40 mL), dried over Na_2SO_4, and evaporated under reduced pressure to yield yellow oil. The oil was chromatographed on silica gel to afford **1a–l**.

4.2.1.1. 4-Methoxybenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxy phenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-oxo-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1a). This compound was prepared by the general method with silybin and 4-methoxybenzoic acid (0.61 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CHCl_3$/MeOH (50:1) as eluent. Yellow solid, yield: 0.73 g (57%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.21 (s, 1H, OH-5), 6.85–7.95 (m, 10H, Ar-H), 6.05 (s, 1H, H-8), 5.99 (s, 1H, H-6), 5.00 (d, 1H, J = 8.0 Hz, H-11), 4.98 (d, 1H, J = 12.0 Hz, H-2), 4.53 (m, 1H, H-3), 4.48 (m, 1H, H-23a), 4.34 (m, 1H, H-10), 4.20 (m, 1H, H-23b), 3.86 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 195.5 (C, C-4), 166.0 (C, C-7), 165.8 (C, ArCOO), 163.7 (C, C-5), 163.6 (C, C-4′), 163.0 (C, C-8a), 146.9 (C, C-19), 146.5 (C, C-20), 144.0 (C, C-16a), 143.7 (C, C-12a), 131.6 (CH, C-3′, 5′), 129.3 (C, C-14), 127.4 (C, C-17), 121.7 (CH, C-15), 121.1 (C, C-1′), 120.6 (CH, C-22), 117.4 (CH, C-16), 116.4 (CH, C-13), 114.0 (CH, C-21), 113.7 (CH, C-2′, 6′), 109.3 (CH, C-18), 100.6 (C, C-4a), 97.0 (CH, C-6), 95.9 (CH, C-8), 82.9 (CH, C-2), 76.0 (CH, C-10), 72.3 (CH, C-11), 72.2 (CH, C-3), 63.1 (CH_2, C-23), 55.8 (OCH_3), 55.4 (OCH_3); ESI-MS m/z 615 $[M-H]^+$; HRESI-MS m/z: 615.1514 $[M-H]^+$ (calcd for $C_{33}H_{27}O_{12}$: 615.1503).

4.2.1.2. 4-Nitrobenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-oxo-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1b). This compound was prepared by the general method with silybin and 4-nitrobenzoic acid (0.67 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CHCl_3$/EtOAc (50:1) as eluent. Yellow solid, yield: 0.92 g (70%); ^{1}H NMR ($CDCl_3$, 400 MHz,) δ 11.17 (s, 1H, OH-5), 6.90–8.16 (m, 10H, Ar-H), 6.11 (s, 1H, H-8), 6.04 (s, 1H, H-6), 4.99 (d, 1H, J = 12.0 Hz, H-2), 4.97 (d, 1H, J = 6.8 Hz, H-11), 4.56 (m, 2H, H-3, 23a), 4.52 (m, 1H, H-10), 4.34 (m, 1H, H-23b), 3.87 (s, 3H, OCH_3); ESI-MS m/z 630 $[M-H]^+$; HRESI-MS m/z: 630.1253 $[M-H]^+$ (calcd for $C_{32}H_{24}NO_{13}$: 630.1248).

4.2.1.3. Benzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1c). This compound was prepared by the general method with silybin and benzoic acid (0.49 g, 4.00 mmol) as starting materials, and purified on silica gel chromatography using $CHCl_3$/EtOAc (50:1) as eluent. Yellow solid, yield: 0.67 g (55%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.20 (s, 1H, OH-5), 6.92–8.06 (m, 11H, Ar-H), 6.06 (s, 1H, H-8), 6.02 (s, 1H, H-6), 5.02 (d, 1H, J = 8.0 Hz, H-11), 4.96 (d, 1H, J = 12.0 Hz, H-2), 4.52 (m, 2H, H-3, 23a), 4.28 (m, 1H, H-10), 4.21 (m, 1H, H-23b), 3.85 (s, 3H, OCH_3); ESI-MS m/z 585 $[M-H]^+$; HRESI-MS m/z: 585.1401 $[M-H]^+$ (calcd for $C_{32}H_{25}O_{11}$: 585.1397).

4.2.1.4. 3,5-Dinitrobenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxy phenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1d). This compound was prepared by the general method with silybin and 3,5-dinitrobenzoic acid (0.85 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CHCl_3$/EtOAc (50:1) as eluent. Yellow solid, yield: 1.19 g (85%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.24 (s, 1H, OH-5), 6.92–8.24 (m, 9H, Ar-H), 6.12 (s, 1H, H-8), 6.07 (s, 1H, H-6), 5.01 (d, 1H, J = 12.0 Hz, H-2), 4.97 (d, 1H, J = 6.8 Hz, H-11), 4.58 (m, 2H, H-3, 23a), 4.53 (m, 1H, H-10), 4.34 (m, 1H, H-23b), 3.89 (s, 3H, OCH_3); ESI-MS m/z 675 $[M-H]^+$; HRESI-MS m/z: 675.1108 $[M-H]^+$ (calcd for $C_{32}H_{23}N_2O_{15}$: 675.1098).

4.2.1.5. 3,4,5-Trimethoxybenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1e). This compound was prepared by the general method with silybin and 3,4,5-trimethoxybenzoic acid (0.85 g, 4.00 mmol) as

starting materials and purified on silica gel chromatography using $CHCl_3$/EtOAc (50:1) as eluent. Yellow solid, yield: 0.63 g (45%); ^1H NMR ($CDCl_3$, 400 MHz) δ 11.19 (s, 1H, OH-5), 6.86–7.26 (m, 8H, Ar-H), 6.06 (s, 1H, H-8), 5.99 (s, 1H, H-6), 4.99 (d, 1H, J = 12.0 Hz, H-2), 4.98 (d, 1H, J = 8.0 Hz, H-11), 4.52 (m, 2H, H-3, 23a), 4.37 (m, 1H, H-10), 4.26 (m, 1H, H-23b), 3.91 (s, 3H, OCH_3), 3.90 (s, 6H, OCH_3), 3.83 (s, 3H, OCH_3); ESI-MS m/z 675 $[M-H]^+$; HRESI-MS m/z: 675.1725 $[M-H]^+$ (calcd for $C_{35}H_{31}O_{14}$: 675.1714).

4.2.1.6. 3-(4-Methylphenyl)-2-propenoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1f). This compound was prepared by the general method with silybin and 3-(4-methylphenyl)-2-propenoic acid (0.65 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using petroleum ether/EtOAc (2:1) as eluent. Yellow solid, yield: 0.43 g (33%); ^1H NMR ($CDCl_3$, 400 MHz) δ 11.20 (s, 1H, OH-5), 7.64 (d, 1H, J = 16.0 Hz, H-7′), 6.87–7.44 (m, 10H, Ar-H), 6.40 (d, 1H, J = 16.0 Hz, H-8′), 6.04 (s, 1H, H-8), 5.97 (s, 1H, H-6), 4.98 (d, 1H, J = 11.6 Hz, H-2), 4.93 (d, 1H, J = 8.0 Hz, H-11), 4.52 (dd, 1H, J = 11.6, 4.0 Hz, H-3), 4.38 (m, 1H, H-23a), 4.29 (m, 1H, H-10), 4.12 (m, 1H, H-23b), 3.87 (s, 3H, OCH_3), 2.38 (s, 3H, 4′-CH_3); ESI-MS m/z 625 $[M-H]^+$; HRESI-MS m/z: 625.1717 $[M-H]^+$ (calcd for $C_{35}H_{29}O_{11}$: 625.1710).

4.2.1.7. 3-(4-Chlorophenyl)-2-propenoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1g). This compound was prepared by the general method with silybin and 3-(4-chlorophenyl)-2-propenoic acid (0.73 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CHCl_3$/EtOAc (50:1) as eluent. Yellow solid, yield: 0.40 g (30%); ^1H NMR ($CDCl_3$, 400 MHz) δ 11.20 (s, 1H, OH-5), 7.63 (d, 1H, J = 16.0 Hz, H-7′), 6.87–7.20 (m, 10H, Ar-H), 6.42 (d, 1H, J = 16.0 Hz, H-8′), 5.96 (s, 1H, H-8), 5.95 (s, 1H, H-6), 4.99 (d, 1H, J = 11.6 Hz, H-2), 4.93 (d, 1H, J = 8.0 Hz, H-11), 4.53 (dd, 1H, J = 11.6, 4.4 Hz, H-3), 4.40 (m, 1H, H-23a), 4.30 (m, 1H, H-10), 4.14 (m, 1H, H-23b), 3.89 (s, 3H, OCH_3); ESI-MS m/z 645 $[M-H]^+$; HRESI-MS m/z: 645.1174 $[M-H]^+$ (calcd for $C_{34}H_{26}ClO_{11}$: 645.1164).

4.2.1.8. 3-(4-Bromophenyl)-2-propenoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1h). This compound was prepared by the general method with silybin and 3-(4-bromophenyl)-2-propenoic acid (0.91 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CHCl_3$/EtOAc (50:1) as eluent. Yellow solid, yield: 0.50 g (35%); ^1H NMR ($CDCl_3$, 400 MHz) δ 11.20 (s, 1H, OH-5), 7.59 (d, 1H, J = 16.0 Hz, H-7′), 6.87–7.54 (m, 10H, Ar-H), 6.43 (d, 1H, J = 16.0 Hz, H-8′), 5.98 (d, 1H, J = 0.8 Hz, H-8), 5.96 (d, 1H, J = 0.8 Hz, H-6), 4.99 (d, 1H, J = 12.0 Hz, H-2), 4.93 (d, 1H, J = 8.0 Hz, H-11), 4.54 (dd, 1H, J = 12.0, 4.8 Hz, H-3), 4.40 (m, 1H, H-23a), 4.29 (m, 1H, H-10), 4.16 (m, 1H, H-23b), 3.87 (s, 3H, OCH_3); ESI-MS m/z 689 $[M-H]^+$; HRESI-MS m/z: 689.0667 $[M-H]^+$ (calcd for $C_{34}H_{26}BrO_{11}$: 689.0658).

4.2.1.9. Propanedioic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ethyl ester (1i). This compound was prepared by the general method with silybin and propanedioic acid monoethyl ester (0.53 g, 4 mmol) as starting materials and purified on silica gel chromatography using $CHCl_3$/EtOAc (50:1) as eluent. Yellow solid, yield: 0.45 g (36%); ^1H NMR ($CDCl_3$, 400 MHz) δ 11.19 (s, 1H, OH-5), 6.82–8.25 (m, 6H, Ar-H), 6.07 (s, 1H, H-8), 5.99 (s, 1H, H-6), 4.98 (d, 1H, J = 10.8 Hz, H-2), 4.90 (d, 1H, J = 7.6 Hz, H-11), 4.36 (m, 1H, H-23a), 4.25 (q, 2H, J = 7.2 Hz, OCH_2CH_3), 4.22 (m, 2H, H-3, 10), 4.08 (m, 1H, H-23b), 3.76 (s, 3H, OCH_3), 3.41 (s, 2H, $COCH_2CO$), 1.28 (t, 3H, J = 7.2 Hz, OCH_2CH_3); ESI-MS m/z 595 $[M-H]^+$; HRESI-MS m/z: 595.1459 $[M-H]^+$ (calcd for $C_{30}H_{27}O_{13}$: 595.1452).

4.2.1.10. 3-Chlorobenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1j). This compound was prepared by the general method with silybin and 3-chlorobenzoic acid (0.62 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CDCl_3$/EtOAc (50:1) as eluent. Yield: 0.64 g (50%), yellow solid; ^1H NMR ($CDCl_3$, 400 MHz) δ 11.20 (s, 1H, OH-5), 6.87–7.87 (m, 10H, Ar-H), 6.07 (s, 1H, H-8), 6.00 (s, 1H, H-6), 5.00 (d, 1H, J = 8.0 Hz, H-11), 4.97 (d, 1H, J = 12.0 Hz, H-2), 4.53 (m, 2H, H-3, 23a), 4.38 (m, 1H, H-10), 4.26 (m, 1H, H-23b), 3.86 (s, 3H, OCH_3); ESI-MS m/z 619 $[M-H]^+$; HRESI-MS m/z: 619.1015 $[M-H]^+$ (calcd for $C_{32}H_{24}ClO_{11}$: 619.1007).

4.2.1.11. 3,4-Dimethoxybenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxy phenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1k). This compound was prepared by the general method with silybin and 3,4-dimethoxybenzoic acid (0.73 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CDCl_3$/EtOAc (50:1) as eluent. Yield: 0.70 g (52%), yellow solid; ^1H NMR ($CDCl_3$, 400 MHz) δ 11.20 (s, 1H, OH-5), 6.86–7.65 (m, 9H, Ar-H), 6.00 (s, 1H, H-8), 5.98 (s, 1H, H-6), 5.00 (d, 1H, J = 8.0 Hz, H-11), 4.99 (d, 1H, J = 12.0 Hz, H-2), 4.55 (m, 2H, H-3, 23a), 4.36 (m, 1H, H-10), 4.25 (m, 1H, H-23b), 3.97 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.81 (s, 3H, OMe); ESI-MS m/z 645 $[M-H]^+$; HRESI-MS m/z: 645.1617 $[M-H]^+$ (calcd for $C_{34}H_{29}O_{13}$: 645.1608).

4.2.1.12. 3,4-Methylene-dioxy-benzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1l). This compound was prepared by the general method with silybin and 1,3-benzodioxole-5-carboxylic acid (0.66 g, 4.00 mmol) as starting materials and purified on silica gel chromatography using $CDCl_3$/EtOAc (40:1) as eluent. Yield: 0.59 g (45%), yellow solid; ^1H NMR ($CDCl_3$, 400 MHz) δ 11.20 (1H, s, OH-5), 6.84–7.67 (9H, m, Ar-H), 6.05 (2H, s, OCH_2O), 6.03 (s, 1H, H-8), 5.98 (s, 1H, H-6), 4.99 (d, 1H, J = 12.0 Hz, H-2), 4.98 (d, 1H, J = 7.2 Hz, H-11), 4.54 (m, 2H, H-3, 23a), 4.33 (m, 1H, H-10), 4.24 (m, 1H, H-23b), 3.83 (s, 3H, OCH_3); ESI-MS m/z 629 $[M-H]^+$; HRESI-MS m/z: 629.1303 $[M-H]^+$ (calcd for $C_{33}H_{25}O_{13}$: 629.1295).

4.2.2. General procedure for the synthesis of compounds 1m–n

A solution of **1b** or **1d** (1 mM) in EtOAc (10 mL) was subjected to catalytic reduction over 10% Pd–C (2.00 g). After absorption of hydrogen had ceased, the catalyst was filtered off and the filtrate was evaporated under reduced pressure to give oil. The oil was chromatographed on silica gel to afford **1m** and **1n**, respectively.

4.2.2.1. 4-Aminobenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1m). This compound was prepared by the general method with **1b** (0.63 g, 1.00 mmol) as a starting material, and purified on silica gel chromatography using $CHCl_3$/EtOAc (30:1) as eluent. Yellow solid, yield: 0.51 g (85%); ^1H NMR ($CDCl_3$, 400 MHz) δ 11.53 (s, 1H, OH-5), 7.74 (d, 2H, J = 8.4 Hz, H-2′, 6′), 6.82–7.29 (m, 6H, Ar-H), 6.63 (d, 2H, J = 8.4 Hz, H-3′, 5′), 5.94 (s, 1H, H-8), 5.92 (s, 1H, H-6), 5.04 (d, 1H, J = 7.6 Hz, H-11), 5.02 (d, 1H, J = 10.8 Hz, H-2), 4.56 (m, 1H, H-23a), 4.41 (m, 2H, H-3, 10), 4.12 (m, 1H, H-23b),

3.73 (s, 3H, OCH_3); ESI-MS *m/z* 600 $[M-H]^+$; HRESI-MS *m/z*: 600.1509 $[M-H]^+$ (calcd for $C_{32}H_{26}NO_{11}$: 600.1506).

4.2.2.2. 3-Amino-5-nitrobenzoic acid, mono[[2,3-dihydro-3-(4-hydroxy-3-methoxy phenyl)-6-(2,3-dihydro-3,5,7-trihydroxy-4-*oxo*-4*H*-1-benzopyran-2-yl)-1,4-benzodioxin-2-yl] methyl] ester (1n). This compound was prepared by the general method with **1d** (0.68 g, 1.00 mmol) as a starting material and purified on silica gel chromatography using $CHCl_3$/EtOAc (30:1) as eluent. Yellow solid, yield: 0.52 g (80%); ^1H NMR ($CDCl_3$, 400 MHz) δ 11.60 (s, 1H, OH-5), 6.82–8.25 (m, 9H, Ar-H), 5.94 (d, 1H, *J* = 2.0 Hz, H-8), 5.92 (d, 1H, *J* = 2.0 Hz, H-6), 5.03 (d, 1H, *J* = 7.6 Hz, H-11), 5.01 (d, 1H, *J* = 10.8 Hz, H-2), 4.56 (m, 1H, H-23a), 4.50 (m, 2H, H-3, 10), 4.25 (m, 1H, H-23b), 3.73 (s, 3H, OCH_3); ESI-MS *m/z* 645 $[M-H]^+$; HRES-I-MS *m/z*: 645.1368 $[M-H]^+$ (calcd for $C_{32}H_{25}N_2O_{13}$: 645.1357).

4.3. DPPH radicals scavenging assay

The free radical scavenging activity of different concentrations of the test compounds were evaluated by their abilities to quench the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in vitro as reported by Shimada et al.[19,20] The reaction mixture contained in a total volume of 0.25 mL, 25 μL of various concentrations of the test compounds which were dissolved in dimethyl sulfoxide (DMSO) and 40 μL of DPPH (0.4 mg/mL) dissolved in methanol. The methanolic solution of DPPH served as a control; both of silybin and quercetin were applied as the reference. The absorbance was measured at 517 nm after incubating the mixture at 37 °C for 30 min.

4.4. Superoxide anion radicals scavenging assay

Superoxide anion was generated in vitro as described by Robak et al.[21] with a slight modification.[22] Briefly, superoxide anion was produced in a non-enzymic PMS-NADH system by following the reduction of NBT. The assay mixture contained in a total volume of 0.25 mL, 16 mM of Tris–HCl buffer (pH 8.0), 78 μM of NADH, 50 μM of NBT, 5 μM of PMS and the test samples with gradient concentrations. Optical density (OD) values were monitored at 560 nm after 5 min of the incubation at room temperature. The wells without PMS were taken as the blank control. Quercetin and silybin were employed as the reference compounds.

4.5. Ferrous ion chelating assay

Ferrous ion chelation by the test compounds was estimated by the method of Dinis et al.[23] Briefly, the samples with final concentrations ranging from 50 to 200 μg/mL were added to a solution of 2 mM $FeSO_4$ (5 μL) and 80% DMSO (200 μL). The reaction was initiated by addition of 5 mM ferrozine (10 μL), and the mixture shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. EDTA-2Na was taken as the positive control, while silybin and quercetin were also introduced as the reference compounds.

4.6. Measurement of rat liver homogenates lipid peroxidation

MDA was used as an indicator of lipid peroxidation inhibition and was determined by the thiobarbituric acid (TBA) assay[22] on freshly prepared Sprague-Dawley rat liver homogenates using colorimetric analysis.[24] The reaction mixtures which were composed of 200 μL of solution contained aqueous $FeSO_4$ (4 μM), vitamin C (50 μM), 50 μL of rat liver homogenates and 5 μL of the test compounds (concentrations from 2.5 to 100 μg/mL), were incubated at 37 °C in capped tubes for 1 h before 100 μL of trichloroacetic acid (20%, v/v) was added. The mixture reacted at room temperature for 30 min. Finally, 200 μL of HCl (0.1 M) and 100 μL of TBA (1%, v/v) were added into each tube and the mixture was incubated at 100 °C for another 1 h. Centrifugation was then carried out at 5000 rpm for 5 min, and the absorbance of the supernatant was measured at 532 nm, both quercetin and silybin were served as the positive standards.

4.7. Protection of 1a–n on DNA scission H_2O_2-induced

The experiments were performed in a volume of 10 μL, containing 0.3 μg of pcDNA3.1 (+) plasmid DNA, which was prepared by the plasmid mini preparation kit, 25 mM of H_2O_2 and 2 μL of the test compounds with final concentrations from 1 to 10 μg/mL. After incubating at 37 °C for 30 min, the samples mixed with 2 μL of 0.25% bromophenol blue, were then analyzed by electrophoresis on a 1% agarose gel containing 1 mg/mL of ethidium bromide in TAE buffer (40 mM Tris, 1 mM EDTA-Na_2-salt, 20 mM acetic acid). The pcDNA3.1 (+) plasmid treated with DMSO was included as a solvent control in each run of gel electrophoresis, which was carried out at voltage-stabilizing (100 V) for 45 min and photographed on gel formatter (Gel Doc2000, Bio-Rad, Hercules, CA, USA). The experiment was repeated three times.

4.8. Statistical analysis

All experimental data are expressed as means ± SD. The IC_{50} value was obtained by regression analysis.

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