

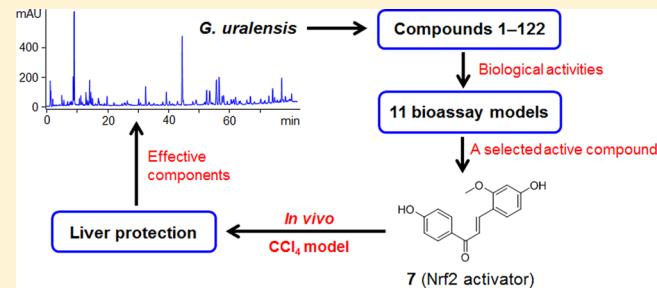
Bioactive Constituents of *Glycyrrhiza uralensis* (Licorice): Discovery of the Effective Components of a Traditional Herbal Medicine

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Supporting Information

ABSTRACT: Traditional herbal medicines have been reported to possess significant bioactivities. In this investigation, a combined strategy using both phytochemical and biological approaches was conducted to discern the effective components of licorice, a widely used herbal medicine. Altogether, 122 compounds (**1–122**), including six new structures (**1–6**), were isolated and identified from the roots and rhizomes of *Glycyrrhiza uralensis* (licorice). These compounds were then screened using 11 cell- and enzyme-based bioassay methods, including Nrf2 activation, NO inhibition, NF- κ B inhibition, H1N1 virus inhibition, cytotoxicity for cancer cells (HepG2, SW480, A549, MCF7), PTP1B inhibition, tyrosinase inhibition, and AChE inhibition. A number of bioactive compounds, particularly isoprenylated phenolics, were found for the first time. **Echinatin** (**7**), a potent Nrf2 activator, was selected as an example for further biological work. It attenuated CCl₄-induced liver damage in mice (5 or 10 mg/kg, ip) and thus is responsible, at least in part, for the hepatoprotective activity of licorice.



Natural products have continued to play an important role in drug discovery. Over the past 30 years, up to 60% of the new small-molecule drugs approved by the U.S. Food and Drug Administration are derived from or related to natural products.¹ Traditional herbal medicines have been used in the orient for a long time, and many of them have been shown to be effective by laboratory and clinical studies. Therefore, it may be hypothesized that there is a higher possibility of finding bioactive natural products from traditional herbal medicines based on their therapeutic effects, rather than from other natural sources through random screening. On the other hand, although the phytochemistry and biological activity of herbal medicines have been studied extensively, these studies have tended to be conducted separately by different research groups. Thus, a comprehensive correlation of chemical constituents and biological activities of a given herbal drug is usually lacking. Furthermore, the majority of prior biological studies may have been focused on a few major compounds that are obtained readily, and little is known on the bioactivities of the minor compounds present.²

Licorice is one of the most widely used herbal medicines and is recorded in the pharmacopoeia of the People's Republic of China and other countries.³ It has a history of use of at least 2000 years.⁴ Licorice is derived from the roots and rhizomes of *Glycyrrhiza uralensis* Fisch. (Leguminosae) and related species. Thus far, at least 400 compounds have been isolated from *Glycyrrhiza* species, including triterpenoid saponins, different classes of phenolic compounds such as flavanones, chalcones,

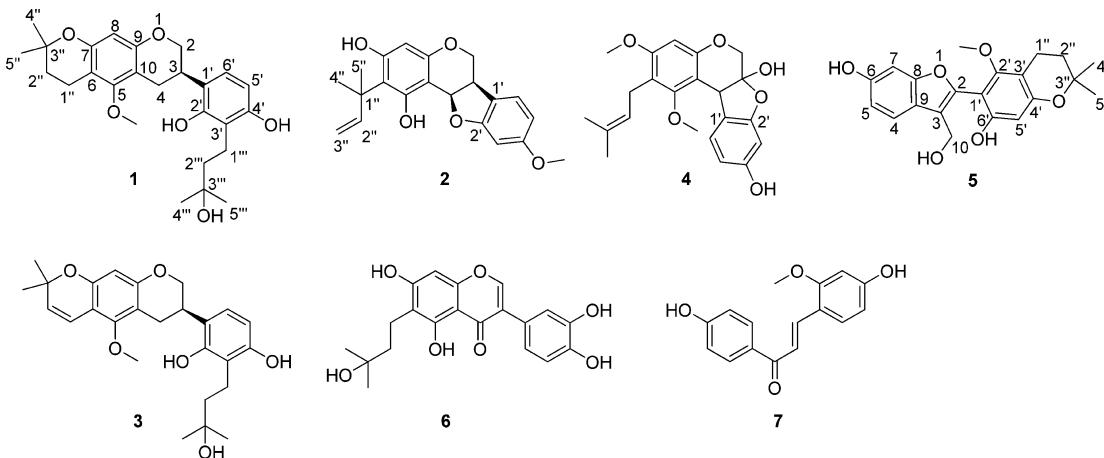
and coumarins, and their glycosides.⁵ In traditional medicine and oriental clinical practice, licorice is used mainly to treat coughs, influenza, and liver damage and for detoxification. Biological studies have revealed that licorice extracts show antioxidant, anti-inflammatory, antiviral, cytotoxic, antidiabetic, skin-whitening, and cholinergic activities, although the major component glycyrrhizin has toxic adrenocorticotropic effects.^{6–8} However, the effective components responsible for these bioactivities are usually unknown. The biological studies are limited to a few major compounds such as glycyrrhizin, liquiritin, liquiritigenin, and isoliquiritigenin. Little is known on the biological activities of the minor compounds that have been isolated from licorice.

In this report, a comprehensive and efficient strategy was conducted to determine the biologically active constituents of licorice. This strategy is depicted in Scheme S1 (Supporting Information). The chemical constituents of *G. uralensis* were isolated systematically to establish a compound library (**1–122**) having six new compounds (**1–6**). Then, all compounds isolated were subjected to testing in 11 in vitro bioassays selected according to the known biological activities of licorice to establish a chemical–bioactivity correlation. A number of compounds were found to be active for the first time. Finally, the bioactivity of a selected Nrf2 activator (**echinatin**, **7**) was

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Chart 1. Structures of Compounds 1–7



confirmed by in vivo and mechanistic studies and was shown to be able to protect against CCl_4 -induced liver damage in mice.

RESULTS AND DISCUSSION

Compound Structure Elucidation. In order to discern the potentially effective components of licorice, its chemical constituents were isolated systematically. The crude drug materials (35 kg) were extracted by 95% and 70% EtOH, and the combined extract was partitioned successively with EtOAc and *n*-BuOH. The extracts were repeatedly separated by column chromatography over macroporous resin, silica gel, polyamide, MCI gel, Sephadex LH-20, and ODS C₁₈ supports and were then purified by semipreparative RP-HPLC. Finally, 122 pure compounds (1–122) were obtained. These substances represented almost all of the observable peaks in the HPLC fingerprint of licorice. The structures of the known compounds (7–122) were identified by NMR and MS spectroscopic data measurement and by comparing with literature values (Chart S1, *Supporting Information*). They included 67 phenolic compounds (7–73), 20 flavonoid glycosides (74–93), and 29 triterpenoid saponins (94–122). Several new triterpenoid saponins and coumarins have been recently reported.⁹ In addition, the structural characterization and absolute configuration determination of six new compounds (1–6) were carried out, as described in the paragraphs below.

The HRESIMS data of 1 showed a $[\text{M} - \text{H}]^-$ ion at m/z 441.2265 (calcd for $\text{C}_{26}\text{H}_{33}\text{O}_6$, 441.2272), corresponding to a molecular formula of $\text{C}_{26}\text{H}_{34}\text{O}_6$. The 1D and 2D NMR spectra of 1 were very similar to those of kanzonol H¹⁰ (Table 1). The isoprenyl group in kanzonol H was replaced by a 3-hydroxy-3-methylbutyl group, which was confirmed by the HMBC correlations of H-1'' (δ_{H} 2.58) with C-2' (δ_{C} 153.5), C-4' (δ_{C} 154.5), and C-3' (δ_{C} 117.2) and of H-2'' (δ_{H} 1.51) with C-3' (Figure 1). The absolute configuration of C-3 in 1 was determined by comparing the experimentally obtained and calculated electronic circular dichroism (ECD) spectra. The calculated ECD spectra were obtained by time-dependent density functional theory (TDDFT) calculations at the B3LYP/6-31G* level in acetonitrile (MeCN) solution with the IEFPCM model.¹¹ As illustrated in Figure S1 (*Supporting Information*), the calculated ECD spectrum for (3*R*)-1, but not (3*S*)-1, agreed well with the recorded spectrum for 1. Therefore, the absolute configuration of 1 was established as

3*R*, and its structure was established as (3*R*)-2',4'-dihydroxy-5-methoxy-3'-(3-hydroxy-3-methylbutyl)-[6,6-dimethyl-4,5-dihydroprano(2,3;7,6)]isoflavan. This compound has been named glycyuralin A.

The HRESIMS data of 2 exhibited a $[\text{M} - \text{H}]^-$ ion at m/z 353.1374 (calcd for $\text{C}_{21}\text{H}_{21}\text{O}_5$, 353.1383), corresponding to a molecular formula of $\text{C}_{21}\text{H}_{22}\text{O}_5$. The ¹H NMR spectra showed four aliphatic proton resonances at δ_{H} 4.13 (1H, dd, $J = 4.8, 10.8$ Hz), 3.52 (1H, t, $J = 10.8$ Hz), 3.42 (1H, m), and 5.63 (1H, d, $J = 7.2$ Hz, H-4). They were correlated with carbon resonances at δ_{C} 67.2 (CH₂), 39.7 (CH), and 76.5 (CH) in the HSQC spectrum. Such resonances are characteristic for ring B of a pterocarpan skeleton.¹² The ¹H NMR resonances at δ_{H} 6.28 (1H, dd, $J = 10.4$ Hz), 4.89 (1H, d, $J = 17.6$ Hz), 4.81 (1H, d, $J = 10.4$ Hz), and 1.50 (6H, s), together with the ¹³C NMR resonances at δ_{C} 41.6 (C), 152.2 (CH), 107.9 (CH₂), and 29.6 (2 × CH₃), indicated the presence of a 1,1-dimethyl-2-propenyl group.¹³ This group was located at C-6, according to the HMBC correlations of H-8 (δ_{H} 6.17) with C-7 (δ_{C} 159.3) and C-9 (δ_{C} 157.8), of H-2 (δ_{H} 4.13 and 3.52) with C-9, and of H-2'' (δ_{H} 6.28), H-4'' (δ_{H} 1.50), and H-5'' (δ_{H} 1.50) with C-6 (δ_{C} 114.3). The H-3 and H-4 signals each showed a coupling constant of 7.2 Hz, indicating a *cis* configuration (cf. a *trans* configuration, $J = 14.0$ Hz).¹⁴ All currently known natural pterocarpans have a *cis* configuration. By ECD spectroscopic calculations, the absolute configuration of 2 was determined as 3*R*, 4*R* (Figure S1, *Supporting Information*). On the basis of the above evidence, the structure of 2 (glycyuralin B) was determined as (3*R*,4*R*)-5,7-dihydroxy-4'-methoxy-6-(1,1-dimethyl-2-propenyl)pterocarpan.

The molecular formula of 3 was determined to be $\text{C}_{26}\text{H}_{32}\text{O}_6$ by HRESIMS analysis ($[\text{M} - \text{H}]^-$ m/z 439.2102, calcd for $\text{C}_{26}\text{H}_{31}\text{O}_6$, 439.2115) and is two hydrogen atoms less than that of 1. The NMR spectra of 3 were very similar to those of 1. The two sp³ methylene resonances at δ_{C} 16.5 (C-1'') and 31.9 (C-2'') of 1 were absent, and two sp² methine resonances were apparent at δ_{C} 116.6 and 128.0 for 3. In accordance, the proton resonances appeared at lower fields of δ_{H} 6.46 (1H, d, $J = 10.0$ Hz) and 5.61 (1H, d, $J = 10.0$ Hz). This indicated 3 contains a 6,6-dimethylbenzopyran ring, which was confirmed by the HMBC correlations of H-1'' (δ_{H} 6.46) with C-2'' (δ_{C} 128.0), C-3'' (δ_{C} 75.6), C-5 (δ_{C} 155.1), C-7 (δ_{C} 153.0), and C-6 (δ_{C} 107.0) and of H-2'' (δ_{H} 5.61) with C-1'' (δ_{C} 116.6), C-3'', C-4'' (δ_{C} 27.4), C-5'' (δ_{C} 27.3), and C-6. The absolute configuration of C-3 was determined to be R by ECD spectral calculation

Table 1. NMR Spectroscopic Data (400 MHz, DMSO-*d*₆) for Compounds 1–6

^aRecorded in acetone-*d*₆.

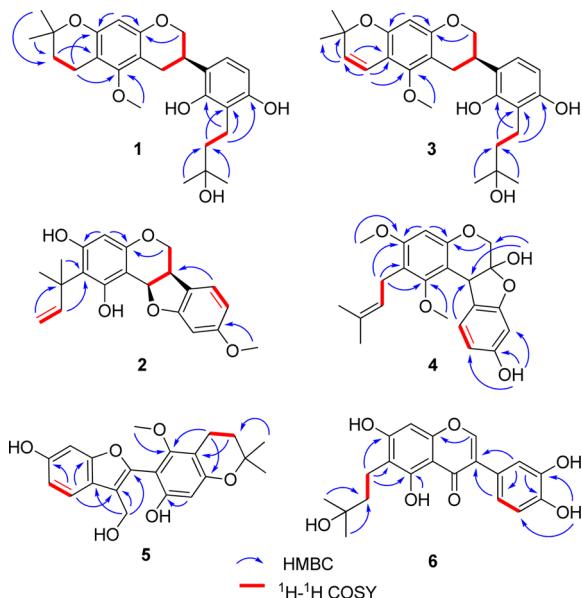


Figure 1. Key HMBC and ^1H - ^1H COSY correlations for compounds 1–6.

(Figure S1, Supporting Information). Thus, the structure of 3 (glycyuralin C) was established as (3*R*)-2',4'-dihydroxy-5-methoxy-3'-(3-hydroxy-3-methylbutyl)-[6,6-dimethylpyrano(2,3:7,6)]isoflavan.

The HRESIMS data of compound 4 exhibited a $[\text{M} - \text{H}]^-$ ion at m/z 383.1479 (calcd for $\text{C}_{22}\text{H}_{23}\text{O}_6$, 383.1489), consistent with a molecular formula of $\text{C}_{22}\text{H}_{24}\text{O}_6$. The NMR spectra showed proton resonances at δ_{H} 1.62 (3H, s), 1.71 (3H, s), 3.10 (1H, dd, $J = 6.0, 14.4$ Hz), 3.27 (1H, dd, $J = 8.0, 14.4$ Hz), and 5.08 (1H, m), indicating the presence of an isoprenyl group.¹⁵ Apart from two methoxy group resonances at δ_{C} 60.9 and 55.6, another 15 ^{13}C NMR resonances were observed, including five oxygenated aromatic quaternary carbons, four aromatic quaternary carbons, four aromatic methines, and two aliphatic carbons. Thus, 4 was found to include a $\text{C}_6-\text{C}_3-\text{C}_6$ flavonoid skeleton with two aliphatic carbons in ring C. In accordance, an aliphatic methylene at δ_{H} 3.71 (1H, d, $J = 11.6$ Hz) and 4.18 (1H, d, $J = 11.6$ Hz), together with an aliphatic methine at δ_{H} 4.27 (1H, s), were observed and were assigned to H-2 and H-4, respectively. The hydroxy group resonance at δ_{H} 7.19 (OH-3) showed HMBC correlations with C-2 (δ_{C} 69.1), C-4 (δ_{C} 43.8), and C-3 (δ_{C} 108.0), indicating C-3 as a hemiacetal carbon. On the basis of the molecular formula and degree of unsaturation of 4, it could be deduced that an ether linkage formed between C-3 and C-2'. In addition, the isoprenyl group was shown to be located at C-6, as evidenced by the HMBC correlations of H-1" (δ_{H} 3.10, 3.27) with C-7 (δ_{C} 157.0), C-6 (δ_{C} 116.6), and C-5 (δ_{C} 156.8). Therefore, the structure of 4 (glycyuralin D) was established as 3,4'-dihydroxy-5,7-dimethoxy-6-(3-methyl-2-butenyl)-4-aryl-3,2'-epoxy-2-deoxy-3,4-dihydrocoumarin.

Compound 5 gave a molecular formula of $\text{C}_{21}\text{H}_{22}\text{O}_6$, according to its HRESIMS data ($[\text{M} - \text{H}]^-$ m/z 369.1333, calcd for $\text{C}_{21}\text{H}_{21}\text{O}_6$, 369.1333). The UV maximum absorptions at 261 and 301 nm were characteristic for a 2-phenyl-3-methylcoumarone skeleton.¹⁶ The 1D and 2D NMR spectra of 5 were very similar to those of glycybenzofuran, which has been reported from *G. uralensis* previously.¹⁶ The methyl group of C-10 in glycybenzofuran was replaced by a hydroxymethyl group,

indicating that an additional hydroxy group occurred at C-10 (δ_{C} 54.8) of 5. This could be confirmed by the HMBC correlations of H-10 (δ_{H} 4.39) with C-2 (δ_{C} 144.8), C-3 (δ_{C} 118.0), and C-9 (δ_{C} 121.0). In addition, the isoprenyl group and OH-4' formed a new dihydropyran ring, as evidenced by the HMBC correlations of H-1" (δ_{H} 2.58) with C-2' (δ_{C} 158.3), C-3' (δ_{C} 105.3), and C-4' (δ_{C} 155.9) and of H-2" (δ_{H} 1.73) with C-3'. Therefore, the structure of 5 (glycyuralin E) was determined as 6,6'-dihydroxy-2'-methoxy-3-hydroxymethyl-[6,6-dimethyl-4,5-dihydropyrano(2,3:4',3')]-2-arylcoumarone.

The molecular formula of 6 was established as $\text{C}_{20}\text{H}_{20}\text{O}_7$ based on its HRESIMS data ($[\text{M} - \text{H}]^-$ m/z 371.1127, calcd for $\text{C}_{20}\text{H}_{19}\text{O}_7$, 371.1125). The UV (λ_{max} 291 sh and 265 nm), ^1H NMR (δ_{H} 8.26, s, H-2), and ^{13}C NMR [δ_{C} 153.6 (C-2), 122.3 (C-3), and 180.4 (C-4)] spectra were consistent with an isoflavone skeleton.¹⁷ The ^1H NMR resonances at δ_{H} 1.14 (6H, s), 1.50 (2H, m), 2.58 (2H, m), and 4.17 (1H, br s) indicated the presence of a 3-hydroxy-3-methylbutyl group.¹⁰ This could be located at C-6, according to the HMBC correlations of H-1" (δ_{H} 2.58) with C-5 (δ_{C} 158.9), C-6 (δ_{C} 109.5), and C-7 (δ_{C} 162.0). Thus, the structure of 6 (glycyuralin F) was established as 5,7,3',4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl)-isoflavone.

Biological Activity. The isolation of 122 chemical constituents from *G. uralensis* allowed a licorice compound library to be established. This is the most extensive compound library for licorice that has been reported to date and covered almost all the detectable peaks in the HPLC fingerprint of licorice (Figure 2A).

Next, a systematic biological activity screening study was conducted for all 122 pure compounds except for two triterpenoid saponins (108 and 113) due to their limited amounts available. Licorice extract has been reported to possess antioxidant, anti-inflammatory, antiviral, anticancer, antidiabetic, skin-whitening, and cholinergic activities.^{6,7} According to the literature, these biological effects might be attributed partly to Nrf2 activation, NO and NF- κ B inhibition, virus inhibition, cytotoxicity for cancer cells, PTP1B inhibition, tyrosinase inhibition, and AChE inhibition activities, respectively. Therefore, the 120 compounds were evaluated using these cell- or enzyme-based screening models to find out the potential effective components.

Nrf2 Activation Activity. Oxidative stress can lead to cancer, cardiovascular disease, chronic inflammation, and neurodegenerative diseases.¹⁸ Previous research has indicated that licorice shows potential hepatoprotective and anticancer activities, and antioxidation may play an important role.¹⁹ Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor, which binds to antioxidant response elements (ARE) located in the promoter region of genes encoding antioxidant enzymes such as heme oxygenase-1 (HO-1) and glutathione (GSH) peroxidase (GPx).²⁰ Therefore, the Nrf2 transcription activation activities of the licorice compounds were evaluated at $10\text{ }\mu\text{M}$, using a luciferase reporter assay in HepG2 human hepatocellular carcinoma cells stably transfected with Nrf2 luciferase reporter (HepG2C8 cells).²¹

Among the 120 compounds, 49 compounds were found to activate Nrf2 transcription to above 1.5-fold of the control (Table S1, Supporting Information). Among them, 7 (echinatin, 5.46-fold), 42 (isoliquiritigenin, 5.88-fold), 46 (luteone, 6.68-fold), and 53 (dehydroglasperin C, 4.70-fold) showed the most potent activities. The positive control used

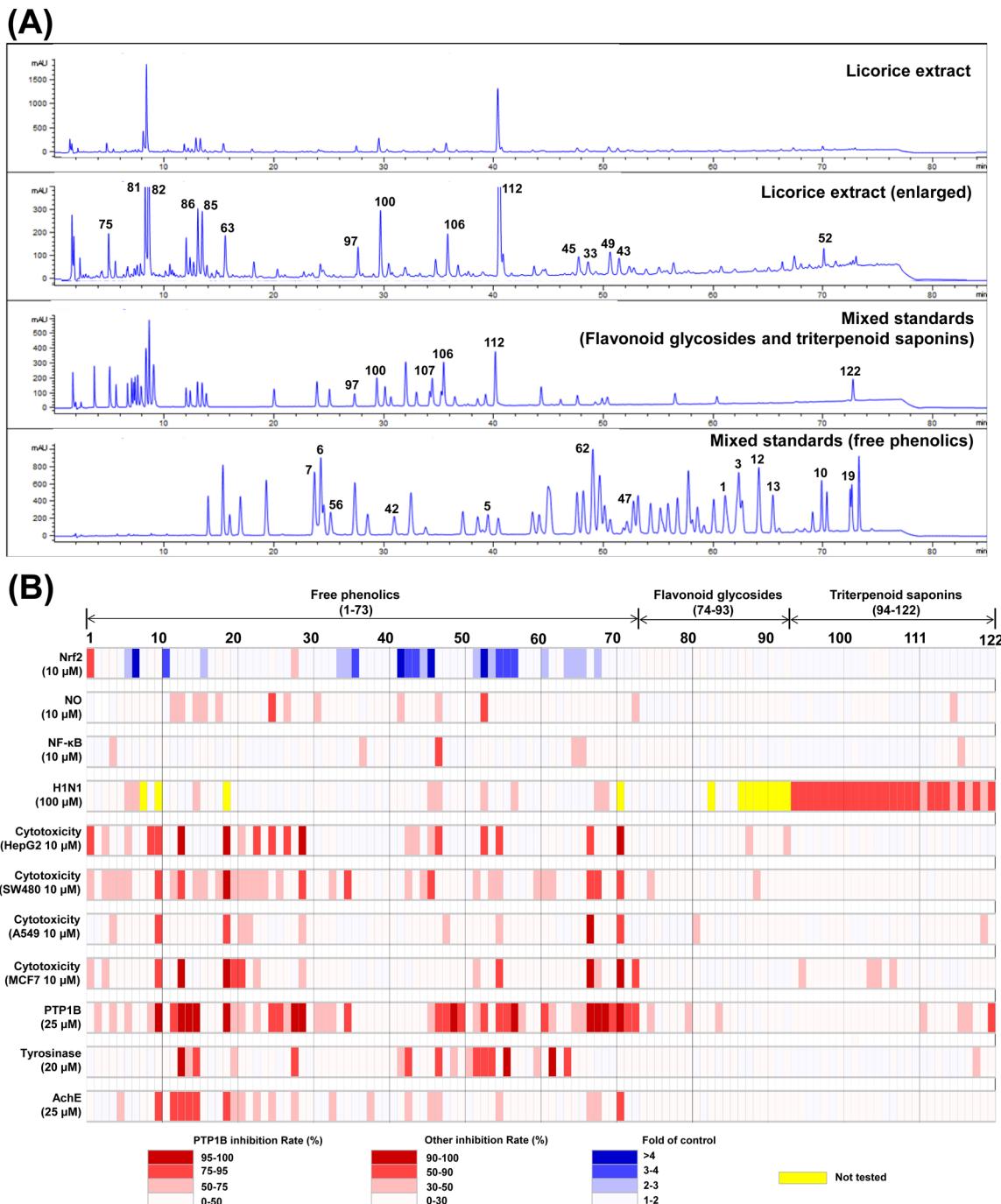


Figure 2. 2D plot for chemical–bioactivity correlation of licorice. (A) UHPLC-UV chromatograms of licorice and mixed standards at 254 nm. (B) Screening results of licorice compounds for their bioactivities, including Nrf2 activation (10 μ M), NO inhibition (10 μ M), NF- κ B inhibition (10 μ M), anti-H1N1 virus (100 μ M), HepG2, SW480, A549, and MCF7 cytotoxicity (10 μ M), PTP1B inhibition (25 μ M), tyrosinase inhibition (20 μ M), and AChE inhibition (25 μ M).

was tBHQ, which showed 1.51-fold of the control value at 10 μ M and 4.34-fold at 100 μ M. The activities for 42 and 53 have been reported in previous literature, whereas 7 and 46 were reported here for the first time.²² The dose-dependent assay indicated that 7 and 46 showed the maximum activities at the concentrations of 20 and 10 μ M, respectively, due to their cytotoxic activity at higher concentrations (Figure S2, Supporting Information). The majority of the positive compounds found using this assay were free phenolics (including prenylated phenolics), except for 83 (a flavone C-glycoside, 1.50-fold) and 96 (a saponin, 1.53-fold). In contrast,

the flavonoid glycosides and triterpenoid saponins showed weak activities on Nrf2 activation. These data were consistent with the preliminary screening results on licorice extracts. The EtOH crude extract or ethyl acetate extract, which contained free phenolic compounds (Figure S3, Supporting Information), activated Nrf2 transcription by 4.04-fold and 7.16-fold at 25 μ g/mL, respectively, whereas the aqueous crude extract or *n*-butanol extract, which contained mainly flavonoid glycosides and triterpenoid saponins, resulted in activation levels of only 1.38-fold and 1.24-fold, respectively.

Table 2. IC₅₀ Values (μ M) of Selected Active Compounds for Inhibitory Activities against NO, NF- κ B, PTP1B, Tyrosinase, and AChE, as Well as Cytotoxicity against HepG2, SW480, A549, and MCF7 Cells

compound	NO	NF- κ B	PTP1B	tyrosinase	AChE	HepG2	SW480	A549	MCF7
glycyrinal D (4)		24.6							
isoangustone A (10)			3.0			4.4	6.5	6.4	6.6
7-O-methylluteone (12)					9.9				
glyasperin D (13)				0.15		3.8	7.2	12.9	6.7
angustone A (19)				0.4		1.1	6.4	7.1	6.0
topazolin (21)						10.8	16.1	23.1	7.3
5,7,4'-trihydroxy-3'-(3-methylbut-2-enyl)-3-methoxyflavone (25)	12.1								
11b-hydroxy-11b,1-dihydromedicarpin (37)		16.9							
licocoumarone (47)	8.5		5.1						
dehydroglyasperin C (53)		8.1							
6-C-prenylorobol (55)						6.0	12.0	20.3	7.0
kaempferol (56)					1.0				
glyasperin C (62)					0.2				
1-methoxyphaseollin (65)		25.8							
glabridin (66)		21.4							
licoricidin (71)						15.5	0.3	7.1	6.9
curcumin ^a	19.1								
MG132 ^a		29.5			5.2				
ursolic acid ^a						0.3			
galanthamine ^a							0.9	1.4	0.8
irinotecan ^a								0.5	

^aPositive control.

Nine coumarins (8, 9, 11, 27, 32, 33, 43, 48, 50) showed varied activities (0.88- to 3.49-fold). It was found that the Nrf2 activation activities increased when the isoprenyl chain at C-6 formed a dihydropyran ring with OH-7. As a result, isoglycyrol (11, 3.32-fold) and isoglycoumarin (43, 3.49-fold) showed more potent activities than their isomers glycyrol (9, 0.88-fold) and glycoumarin (33, 1.72-fold), respectively. Interestingly, the new compound 1, an isoflavan containing the same dihydropyran ring, was found to be an Nrf2 inhibitor (76% inhibition at 10 μ M). Nrf2 inhibitors are rare among natural products and may enhance efficacy against cancer.²³

While several chalcones [e.g., echinatin (7) and isoliquiritigenin (42)] showed potent Nrf2 activation activities, homobutein (54) contains an additional OCH₃-3' substituent when compared to 42, and its activity decreased to 2.49-fold. Compound 60 (liquiritigenin) is the flavanone isomer of 42, and its activity decreased to 1.80-fold. These results were consistent with previous reports that chalcones are potent Nrf2 activators.²⁴

LPS-Induced NO Inhibition and NF- κ B Inhibition Activities.

Licorice is used for the treatment of inflammation in traditional Chinese medicine.¹⁹ When inflammation occurs, macrophages produce inflammatory cytokines and nitric oxide (NO), which, in turn, activate unprimed macrophages and other nearby cells.²⁵ In this study, the effects of licorice compounds (10 μ M) on LPS-induced NO production in the RAW 264.7 macrophage cell line were evaluated to find potential anti-inflammatory compounds (Table S1, Supporting Information). A total of 13 compounds could significantly inhibit LPS-induced NO production with an inhibition rate of >30%. The positive control used was curcumin, which gave inhibition rates of 12% at 10 μ M and 53% at 20 μ M, respectively. The active compounds found were mainly free phenolics except for 117 (a triterpenoid saponin). Consistent with this result, the licorice ethyl acetate extract (52% inhibition) showed stronger activities than the n-butanol extract (40% inhibition). Among the active

compounds, 25 (5,7,4'-trihydroxy-3'-(3-methylbut-2-enyl)-3-methoxyflavone) and 53 (dehydroglyasperin C) were shown to decrease NO production by >50% and gave IC₅₀ values of 12.1 and 8.1 μ M, respectively (Table 2). The activities of 53 have been reported previously, and the present results were consistent with these literature data.²⁶

NF- κ B is a pivotal regulator of pro-inflammatory gene expression.²⁷ To check for anti-inflammatory compounds that act on the NF- κ B pathway, the licorice compounds (10 μ M) were evaluated using a luciferase reporter assay in SW480 human colon carcinoma cells stably transfected with NF- κ B luciferase reporter. As shown in Table S1 (Supporting Information), only five free phenolics (4, 37, 47, 65, 66) and one triterpenoid saponin (118) inhibited NF- κ B transcription by >30% (the positive control used was MG132, with 23% inhibition at 10 μ M). A further dose-dependent assay provided the IC₅₀ values of 4, 37, 47, 65, and 66 (24.6, 16.9, 5.1, 25.8, and 21.4 μ M, respectively). Among these, the activity of glabridin (66), the major constituent of *G. glabra*, has been reported in this assay before.²⁸ Licocoumarone (47) was the most potent substance obtained, and it also inhibited NO production with an IC₅₀ value of 8.5 μ M. Its potential anti-inflammatory activity is reported here for the first time. Interestingly, its OH-7 methylated derivative 15 showed only very weak activity in this assay.

H1N1 Virus Inhibition Activity. Licorice has long been used for the treatment of influenza, and triterpenoid saponins are considered as the major bioactive constituents.²⁹ In this work, the inhibitory activities of licorice compounds (100 μ M) against influenza virus A/WSN/33 (H1N1) were evaluated in MDCK cells. The activities of several triterpenoid saponins (94–121) have been reported in previous publications.⁹ All the saponins showed anti-H1N1 virus activities, with inhibition rates varying from 47% to 82% at 100 μ M. Some of them showed IC₅₀ values from 39.6 to 49.1 μ M, which were comparable to the positive control substance oseltamivir

phosphate (IC_{50} value, 45.6 μM). According to their amounts present in licorice roots and rhizomes (data not shown), glycyrrhizin (112), 22 β -acetoxyglycyrrhizin (100), and licorice saponins A3 (97), E2 (107), and G2 (106) could be the major anti-H1N1 components of licorice. Glycyrrhetic acid (122) also showed obvious inhibitory activity (inhibition rate at 100 μM , 58%). This compound is a major *in vivo* metabolite of a number of licorice saponins and is found at considerable blood concentrations on oral administration of licorice.³⁰

The flavonoid glycosides showed no or very weak inhibitory activities against H1N1 virus. Only a few free phenolics showed discernible activities, and these were much weaker than for the saponins. Nine of them (6, 7, 15, 46, 47, 53, 57, 68, 69) showed inhibition rates of >30%, with these being mainly prenylated isoflavones and coumarones. The anti-H1N1 activities of 10, 19, and 71 were not evaluated due to their strong cytotoxicity against MDCK cells.

Cytotoxic Activities (SW480, HepG2, A549, and MCF7 Cells). Licorice has been investigated extensively as a potential anticancer or cancer chemopreventive natural agent.³¹ A dozen of prenylated flavonoids have been found to remarkably inhibit SW480 cancer cells.³² In this work, cytotoxic activities of licorice compounds (10 μM) against four human cancer cell lines were evaluated. These cell lines included HepG2 (liver cancer), SW480 (colorectal cancer), A549 (lung cancer), and MCF7 (breast cancer) cells. As shown in Table S1 (Supporting Information), a number of free phenolic compounds, particularly those that are prenylated, showed cytotoxic activities, whereas the flavonoid glycosides and triterpenoid saponins showed only weak activities. Consistent with this result, the licorice ethyl acetate extract showed more potent cytotoxic effects (76–99% inhibition against all four cell lines at 25 $\mu\text{g}/\text{mL}$) than the *n*-butanol extract.

Among the isoflavones, 10 (isoangustone A) and 19 (angustone A), a pair of isomers, showed the most potent activities, with IC_{50} values ranging from 1.1 to 7.1 μM against the four cell lines. Previously, 10 was found to induce apoptosis in SW480 cells by disrupting mitochondrial functions,³² in SW480 cells by disrupting mitochondrial functions. Licoricidin (71) was the most potent compound among the isoflavans, with IC_{50} values in the range 0.3–7.1 μM . It was noted that its IC_{50} value against HepG2 cells was at a submicromolar level, with potent cytotoxic activity being rarely observed for natural flavonoids. The flavones and coumarins showed low to moderate cytotoxic activities. The most potent compound was topazolin (21), which displayed IC_{50} values of 10.8, 16.1, 23.1, and 7.3 μM against HepG2, SW480, A549, and MCF7 cells, respectively.

Some compounds showed obvious selectivity among different cell lines. For instance, glyasperin D (13) showed inhibition rates of 99%, 78%, 25%, and 98% for HepG2, SW480, A549, and MCF7 cells, respectively. Likewise, isolupalbigenin (67) inhibited the cells by 68%, 59%, 93%, and 91%, respectively. The IC_{50} values for 6-C-prenylorobol (55) were 6.0, 12.0, 20.3, and 7.0 μM , respectively.

PTP1B Inhibition Activity. Licorice extracts have been reported to have potential antidiabetic activities.³³ Protein tyrosine phosphatase 1B (PTP1B) plays a critical role in the negative regulation of insulin and leptin signaling pathways and has been considered as a potential therapeutic target against type 2 diabetes.³⁴ In this work, the PTP1B inhibitory activities of licorice compounds (25 μM) were evaluated. A total of 51 compounds inhibited PTP1B with inhibitory rates of >50%

(Table S1, Supporting Information), including 44 free phenolics, three flavonoid glycosides, and four triterpenoid saponins (the positive control ursolic acid, 45% at 5 μM).

All the prenylated flavones and flavonols (20, 21, 25, 28, 49, 61) showed PTP1B inhibitory activities. The activity of licoflavonol (20) has been reported previously.¹⁶ Their inhibitory activities would increase when the isoprenyl group occurred in ring B rather than ring A, as evidenced by 20 (51%)/28 (98%) and 21 (52%)/25 (85%). The isoflavones and isoflavans showed varied activities. All 17 compounds with an isoprenyl group in ring A displayed positive activities, including the new compound 6. Among them, the six diprenyl compounds (10, 19, 67, 68, 69, 71) showed the most potent activities, with inhibition rates of >95%, suggesting that isoprenyl groups were critical for these effects. A further dose-dependent assay led to a determination of the IC_{50} values of 10 and 19 as 3.0 and 0.4 μM , respectively. In contrast, isoflavones without an isoprenyl group (such as 30, 36, and 37) usually showed moderate or poor activities. Similarly, the isoprenylated coumarones 15, 29, and 47 also showed strong inhibitory activities (>90% inhibition rate). The activities of 29 have been reported in recent publications.⁹ The chalcones and flavanone (60) showed low PTP1B inhibitory activities, which was consistent with the literature report.³⁵ It is worth noting that glycyrrhetic acid (122) could inhibit PTP1B by 81%. Since 122 is a major metabolite of saponins, the saponins may also contribute to the potential antidiabetic activity of licorice.

Tyrosinase Inhibition Activity. Licorice is used popularly in cosmetic products as a skin-whitening agent.³⁶ Tyrosinase catalyzes key reactions in the biosynthesis of melanin and has been used as a target for the discovery of new skin-whitening agents.³⁷ In this study, tyrosinase inhibitory activities of licorice compounds (20 μM) were screened. The saponins and flavonoid glycosides showed poor activities. In contrast, a number of free phenolic compounds showed potent activities, particularly those bearing an isoprenyl group.

Compounds 13, 56, and 62 showed the most potent activities (>95% inhibition rate). Compound 13 (glyasperin D) was discovered as a tyrosinase inhibitor for the first time, whereas this type of activity for 56 (kaempferol) and 62 (glyasperin C) has been reported.^{36,38} The two isoflavans 13 and 62 are close analogues. They showed IC_{50} values of 154 and 177 nM, respectively. Their C-3–C-4 dehydrogenated analogues 14 and 53 showed decreased activities. Three chalcones (7, 42, 54) showed moderate activities. Both 42 and 54 contain a resorcinol unit and showed more potent activities than 7. These results are consistent with a previous report.³⁹

AChE Inhibition Activity. Licorice extracts have been reported to show neuroprotective and cholinergic effects, since they can inhibit acetylcholinesterase (AChE) activity and thus potentially enhance acetylcholine levels in the brain.^{40,41} In this work, the licorice compounds (25 μM) were screened for their capabilities to inhibit AChE. As shown in Table S1 (Supporting Information), 21 compounds, all of which are free phenolics, were found to inhibit AChE by >30% (the positive control galanthamine, 90% inhibition at 5 μM), although the majority showed only moderate activities. The most potent compounds were 12 (7-O-methyluteone), 19 (angustone A), and 71 (licoricidin), with inhibition rates of 83%, 71%, and 82%, respectively. Among them, 12 and 71 showed IC_{50} values of 9.9 and 15.5 μM , respectively, and their activities in this assay are reported for the first time.

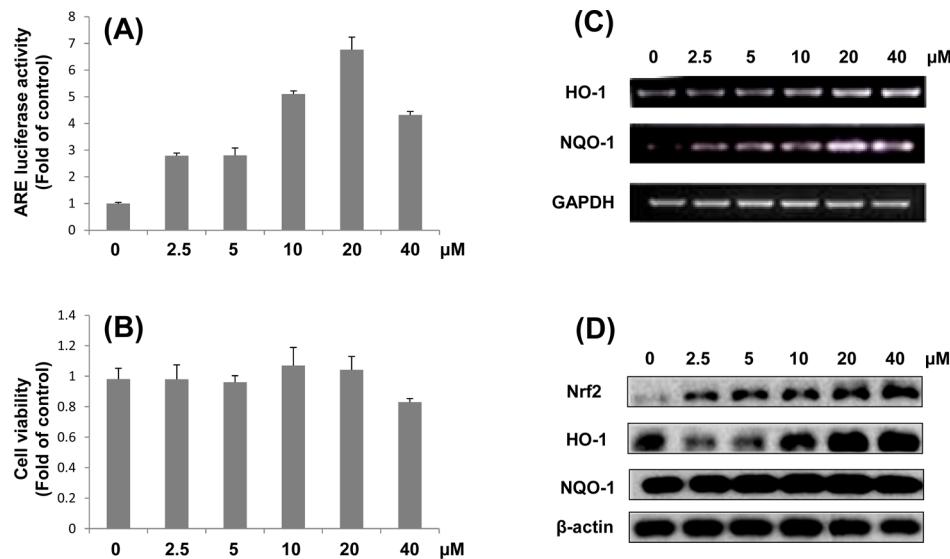


Figure 3. Nrf2 activation effects of echinatin (7) in HepG2 cells. (A) Dose-dependent activation effects of 7 on luciferase activity of HepG2 cells stably transfected with Nrf2 luciferase reporter. (B) Cytotoxicity of 7 against HepG2 cells at different concentrations. (C) Effect of 7 on the transcription of HO-1 and NQO1 in HepG2 cells by RT-PCR analysis. (D) Effect of 7 on the protein levels of Nrf2, HO-1, and NQO-1 in HepG2 cells by Western blotting analysis.

Chemical–Bioactivity Correlation Analysis of Licorice. Based on the above screening tests, a chemical–bioactivity correlation of licorice was established, which is illustrated as a two-dimensional plot (Figure 2B). Free phenolic compounds (particularly prenylated phenolics) were responsible for most of the bioactivities except for anti-H1N1. The bioactivities of the majority of these compounds were found for the first time, and these compounds may be important effective components of licorice roots and rhizomes. Triterpenoid saponins were mainly responsible for the anti-H1N1 virus activities. Although flavonoid glycosides only showed weak to moderate activities for most of the in vitro screening models, they may be metabolized into bioactive free flavonoids such as liquiritigenin or isoliquiritigenin after oral administration, and thus may play an important role in enhancing the therapeutic effects observed for licorice.³⁰ The active compounds are summarized as shown in Figures S4 and S5 (Supporting Information).

A number of these bioactive compounds show potential as drug candidates, although their drug ability needs to be further evaluated. For instance, although licoricidin (71) showed promising cytotoxic activities and inhibitory activities against PTP1B and AChE enzymes, previous work has indicated it has poor oral absorption.⁴² Thus, structural modification or improvements in dosage form need to be considered to improve its bioavailability. It is also noteworthy that some common natural products, such as genistein (44) and kaempferol (56), were found to show potent bioactivities in the present investigation.

Confirmation of the in Vitro Nrf2 Activation Activities of 7 in HepG2 Cells. According to the screening results obtained, compound 7 (echinatin) was found to activate Nrf2 to 5.5-fold of the control value at 10 μM . Its activities were confirmed using HepG2C8 cells with a luciferase reporter assay. The results showed that 7 activated Nrf2 in a dose-dependent manner, within the concentration range 2.5–40 μM (Figure 3). The most pronounced effect (6.8-fold) was observed at 20 μM , but the effect decreased to 4.3-fold at 40 μM , due to the cytotoxicity of 7. RT-PCR analysis indicated that 7 dose-dependently induced the transcription of HO-1 and NQO1,

two well-characterized transcriptional targets of Nrf2. Next, the protein levels of Nrf2, HO-1, and NQO-1 were measured in HepG2 cells treated with or without 7 by Western blotting analysis. As a result, 7 was found to increase the protein levels of Nrf2 and HO-1 in a concentration-dependent manner. The above results confirmed the Nrf2 activation activity of 7.

In Vivo Nrf2 Activation Activities of 7 in Mice. For the next step, the potency of 7 to activate the Nrf2 pathway in mice was evaluated. Due to the limited amount of 7 available, it was given to C57BL/6J mice by intraperitoneal injection. The drug was dissolved in corn oil and was administered at a dose of 5 or 10 mg/kg for three consecutive days. The liver tissues were harvested 3 h after the final treatment and were analyzed by qRT-PCR. As shown in Figure 4, 7 increased liver transcription of both HO-1 and NQO-1 in a dose-dependent manner. Western blotting analysis indicated the protein levels of Nrf2 and HO-1 were also increased.

Protective Effect of 7 against CCl_4 -Induced Liver Damage in Mice. Carbon tetrachloride (CCl_4) may cause acute hepatic injury partly due to the formation of trichloromethyl radicals ($\text{CCl}_3\cdot$) and reactive oxygen species.⁴³ In turn, Nrf2 activators have been shown to attenuate CCl_4 -induced hepatotoxicity through inhibition of oxidative damage.⁴⁴ In this study, the protective effect of the licorice EtOAc extract was tested initially against CCl_4 -induced oxidative hepatic damage in mice after oral administration. It was found that pretreatment with this extract (300 mg/kg) prior to administration of CCl_4 prevented the increases of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum. In addition, the licorice EtOAc extract prevented CCl_4 -induced apoptosis and necrosis, as indicated by liver histopathology analysis (Figure S6, Supporting Information). Next, the protective effect of 7 against CCl_4 -induced oxidative hepatic damage was evaluated. Mice were administered with 7 (dissolved in corn oil, 5 or 10 mg/kg, ip) once daily for three consecutive days and were then treated with CCl_4 [200 $\mu\text{L}/\text{kg}$, ip, dissolved in corn oil (2%, v/v)]. Twenty four hours after the treatment, the mice were sacrificed, and the serum levels of ALT and AST were determined. The results

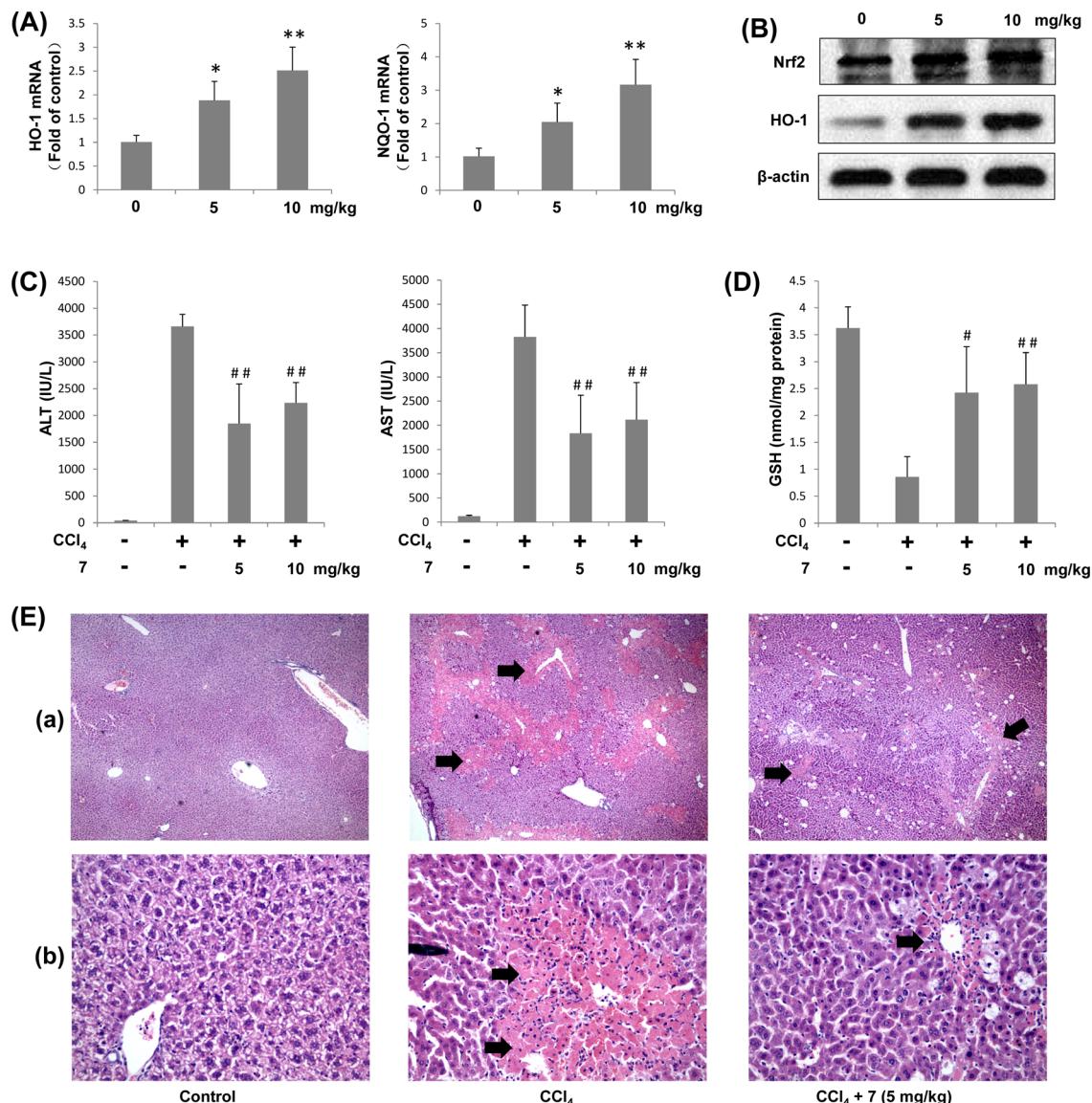


Figure 4. Nrf2 activation effects of echinatin (7) in the mouse liver and its protective effects against CCl₄-induced mouse liver damage. (A) Effects of 7 on the transcription of HO-1 and NQO1 in mouse liver by qRT-PCR analysis. (B) Effects of 7 on the protein levels of Nrf2 and HO-1 in the mouse liver by Western blotting analysis. (C) Effects of 7 on mouse serum ALT and AST levels after CCl₄ treatment. (D) Effects of 7 on mouse hepatic GSH level after CCl₄ treatment. (E) Effects of 7 on histological changes of the mouse liver after CCl₄ treatment. The thick arrows indicate necrotic areas. H&E-stained slices were observed under 4× (a) and 20× (b) magnification. **p* < 0.05, ***p* < 0.01, compared with the control group. #*p* < 0.05, ##*p* < 0.01, compared with the CCl₄ group.

demonstrated that a single dose of CCl₄ caused significant hepatotoxicity in mice, indicated by the increases of ALT and AST levels observed. Pretreatment with 7 prevented the CCl₄-induced elevations in ALT (*p* < 0.01) and AST (*p* < 0.01) levels at either 5 or 10 mg/kg, although the dose-dependent relationship was not significant (Figure 4). It was important to note that CCl₄-induced liver damage may be attributed to several possible mechanisms including covalent metabolite binding, lipid peroxidation, and the generation of inflammatory cytokines. Although echinatin (7) attenuated CCl₄-induced hepatotoxicity through inhibition of lipid peroxidation, it is not known whether it acts by other mechanisms. Histopathological analysis (hematoxylin-eosin stained slices) also showed that CCl₄ induced hepatic cord degeneration and focal necrosis in mice livers, and pretreatment with 7 ameliorated CCl₄-induced severe hepatic lesions.

Previous studies have shown that glutathione, a major antioxidant system against free-radical damage, plays a key role in eliminating the reactive toxic metabolites of CCl₄.⁴⁵ Therefore, reduced GSH levels were measured in this study. According to the results obtained, the administration of CCl₄ alone significantly depleted GSH levels from 3.62 ± 0.40 nmol/kg protein to 0.86 ± 0.38 nmol/kg protein, and pretreatment with 7 attenuated the depletion to 2.42 ± 0.85 nmol/kg protein (5 mg/kg) and 2.58 ± 0.59 nmol/kg protein (10 mg/kg), respectively (Figure 4).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. UV spectra were measured on a Cary 300 Bio UV-visible spectrophotometer. ECD spectra were recorded on a JASCO J-810 CD spectrophotometer. IR spectra were recorded as KBr disks on a

Nicolet Nexus-470 FT-IR spectrometer. NMR spectra were recorded at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR on a Bruker AVANCE III-400 spectrometer in DMSO-d₆ with TMS used as a reference (unless otherwise stated). HRESIMS data were obtained on a Bruker Apex IV FT-MS spectrometer. TLC was performed on precoated silica gel GF₂₅₄ plates (Qingdao Marine Chemical Inc., Qingdao, People's Republic of China), and the spots were visualized under UV light (365 nm). Open column chromatography was carried out using AB-8 macroporous resin (Cangzhou Bao'en Chemical Factory, Cangzhou, People's Republic of China), silica gel (200–300 mesh, Qingdao Marine Chemical Inc.), SBC MCI gel (75–150 μm, Sci-BioChem Co. Ltd., Chengdu, People's Republic of China), ODS C₁₈ (DAISO Company, Hiroshima, Japan), polyamide (80–120 mesh, Taizhou Lujiaosijia Plastic Factory, Taizhou, People's Republic of China), and Sephadex LH-20 (GE Healthcare Bio-Science AB, Uppsala, Sweden) as packing materials. Semipreparative HPLC was performed on an Agilent 1200 instrument equipped with a Zorbax SB C₁₈ column (250 mm × 9.4 mm i.d., 5 μm).

Plant Material. The roots and rhizomes of licorice (*Glycyrrhiza uralensis*) were collected when three years old in September 2012 in Chifeng City, Inner Mongolia Autonomous Region, People's Republic of China. It was identified by one of us (M.Y.). A voucher specimen (no. GC-201209) has been deposited at the School of Pharmaceutical Sciences, Peking University, Beijing, People's Republic of China.

Extraction and Isolation. The dried plant material (35 kg) was powdered and extracted with 95% (90 L × 2 h × 2) and 70% EtOH (90 L × 2 h × 1) under reflux. After concentration in vacuo, the extract (10 L) was dispersed in H₂O and successively extracted with EtOAc and n-BuOH. The EtOAc extract (1280 g) was separated by repeated column chromatography and preparative liquid chromatography to obtain the free phenolic compounds 1–73 (Figure S8, Supporting Information). The n-BuOH extract (1540 g) was separated on an AB-8 macroporous resin column eluted with EtOH/H₂O (10–95%, v/v) to obtain fractions A–E. Fractions B and D were further separated to yield triterpenoid saponins 94–122, as previously reported.⁹ Fraction A (200 g) was separated to obtain the flavonoid glycosides 74–76, 79–86, and 89–93 (Figure S7, Supporting Information). The detailed isolation procedure is described in the Supporting Information. In addition, schaftoside (77), isoschaftoside (78), neoliquiritin (87), and neoisoliquiritin (88) were purchased from Mansite Bio-Technology Co., Ltd. (Chengdu, People's Republic of China). The purity of all the compounds when tested was above 95% by HPLC/UV analysis.

Glycyuralin A (1): white powder; [α]_D²⁵ −17.1 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 228 (4.71), 282 (2.38) nm; IR (KBr) ν_{max} 3411, 2972, 2936, 1620, 1585, 1478, 1454, 1155, 1132 cm^{−1}; ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 441.2265 [M − H][−] (calcd for C₂₆H₃₃O₆, 441.2272).

Glycyuralin B (2): white powder; [α]_D²⁵ −57.2 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 231 (3.85), 281 (2.68) nm; IR (KBr) ν_{max} 3433, 2929, 2849, 1610, 1498, 1461, 1125, 1087 cm^{−1}; ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 353.1374 [M − H][−] (calcd for C₂₁H₂₁O₅, 353.1383).

Glycyuralin C (3): white powder; [α]_D²⁵ −9.2 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 225 (4.53), 286 (2.64) nm; IR (KBr) ν_{max} 3433, 2971, 2929, 1616, 1457, 1367, 1135, 1042 cm^{−1}; ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 439.2102 [M − H][−] (calcd for C₂₆H₃₁O₆, 439.2115).

Glycyuralin D (4): white powder; [α]_D²⁵ −30.4 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 228 (3.98), 284 (2.57) nm; IR (KBr) ν_{max} 3433, 2922, 2854, 1610, 1457, 1286, 1201, 1109, 1042 cm^{−1}; ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 383.1479 [M − H][−] (calcd for C₂₂H₂₃O₆, 383.1489).

Glycyuralin E (5): white powder; [α]_D²⁵ −0.8 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 261 (3.81), 301 (4.02) nm; IR (KBr) ν_{max} 3342, 2952, 1631, 1491, 1419, 1154, 1116, 975, 811 cm^{−1}; ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 369.1333 [M − H][−] (calcd for C₂₁H₂₁O₆, 369.1333).

Glycyuralin F (6): white powder; [α]_D²⁵ +0.2 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 265 (2.73) nm; IR (KBr) ν_{max} 3435, 2971, 2927, 1647, 1621, 1524, 1464, 1295, 1220, 1060, 823 cm^{−1}; ¹H and ¹³C

NMR data, see Table 1; HRESIMS m/z 371.1127 [M − H][−] (calcd for C₂₀H₁₉O₇, 371.1125).

Nrf2 Activation Assay. HepG2 human hepatocellular liver carcinoma cells were obtained from American Type Culture Collection (ATCC; Manassas, MD, USA) and were grown in MEM medium. The assay was conducted as recently reported.⁴⁶ tert-Butylhydroquinone (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control.

NO Inhibition Assay. RAW264.7 murine monocyte-macrophage cells were obtained from ATCC and were grown in RPMI1640 medium. Cells were plated at 1.2 × 10⁵ cells/well in 96-well plates and then treated with test compounds and 1 μg/mL LPS (Sigma-Aldrich). After 24 h of incubation, the level of nitrite (an indicator of NO synthesis) was measured using the Griess reagent (Beyotime Institute of Biotechnologies, Jiangsu, People's Republic of China). Briefly, 50 μL of cell culture medium was mixed with an equal volume of reagent I (1% sulfanilamide in 0.1% naphthylethylenediamine dihydrochloride) and reagent II (5% phosphoric acid in water). After being incubated at 37 °C for 15 min, the absorbance was measured at 540 nm. Curcumin was used as the positive control.⁴⁷

NF-κB Inhibition Assay. SW480 human colorectal adenocarcinoma cells were obtained from ATCC and were grown in RPMI1640 medium. The assay was conducted as previously reported.⁴⁸ MG132 was used as the positive control.

Anti-H1N1 Virus Assay. MDCK Madin-Darby canine kidney cells were obtained from ATCC and were grown in DMEM medium. The assay was conducted as previously reported.⁹ Oseltamivir phosphate (Osv-P, Tamiflu) was used as the positive control.

Cytotoxicity Assay. A549 human alveolar adenocarcinoma cells and MCF7 human breast carcinoma cells were obtained from ATCC and were grown in DMEM and MEM medium, respectively. The cytotoxic effects of test compounds against SW480, HepG2, A549, and MCF7 cells were determined using the MTS assay. Briefly, the cells were seeded at 5 × 10³ cells/well in 96-well plates and cultured overnight. Compounds at different concentrations were then added into the culture and incubated for another 24 h. Cell viability was measured by the MTS assay following the manufacturer's protocol (Promega, Madison, WI, USA). Irinotecan was used as the positive control.

PTP1B Inhibition Assay. The assay was conducted as previously reported.⁹ Ursolic acid was used as the positive control.

Tyrosinase Inhibition Assay. The ability of test compounds to inhibit tyrosinase (Sigma-Aldrich) was evaluated by a previously described method with slight modifications.³⁹ Briefly, compounds at different concentrations were added to a reaction mixture containing 30 μL of tyrosinase (333 U/mL), 69 μL of sodium phosphate buffer (45 mM, pH 6.6), and 100 μL of L-tyrosine (2 mM). The reaction mixture (200 μL) was incubated at 37 °C for 15 min, and the oxidation of L-tyrosine was determined by measuring the absorbance at 492 nm. Glyasperin C was used as the positive control.³⁶

AChE Inhibition Assay. The assay was conducted by a previously described method with slight modifications.⁴⁹ Briefly, compounds at different concentrations were added to a reaction mixture containing 2.5 μL of AChE (0.025 U/mL, Sigma-Aldrich), 100 μL of Tris-HCl buffer (30 mM, pH = 8.0), 76.5 μL of DTNB (0.26 mM), and 20 μL of ATCI (0.2 mM). The reaction mixture (200 μL) was incubated at room temperature, and the hydrolysis of ATCI was monitored by measuring the absorbance at 405 nm every 1 min for 10 times. The increase of absorbance measured was linear, and the slope represented the reaction rate. Galanthamine was used as the positive control.

Experimental Animals. The animal facilities and protocols were approved by Beijing Municipal Science and Technology Commission (SYXK-2011-0039). All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.⁵⁰ Seven-week-old male C57BL/6J mice were provided by the Experimental Animal Center of Peking University Health Science Center (Beijing, People's Republic of China). The mice had free access to water and a standard diet. They were maintained in a controlled environment at 21 ± 2 °C and 50 ± 5% relative humidity under a 12 h dark/light cycle and were acclimatized for at least 1 week prior to use.

Nrf2 Activation Activity of Echinatin (7) in the Mouse Liver. The mice used were divided randomly into three groups ($n = 5$ for each group). Compound 7 (5 or 10 mg/kg body weight, groups 2 and 3) in corn oil (0.5 or 1 mg/mL) was administered intraperitoneally (ip) once daily for three consecutive days. Control animals (group 1) were given an equal amount of corn oil ip. Three hours after the final treatment, mice were anesthetized with diethyl ether. Blood was then collected from the retro-orbital venous sinus, and the liver tissue was isolated and stored at -80°C for RT-PCR and Western blotting analysis.

Protective Effects of Echinatin (7) on CCl_4 -Induced Mice Liver Damage. The mice used were divided randomly into four groups ($n = 10$ for each group). Compound 7 (5 or 10 mg/kg body weight, groups 3 and 4) in corn oil (0.5 or 1 mg/mL) was administered intraperitoneally once daily for three consecutive days. Three hours after the final treatment, the mice were treated with CCl_4 [200 $\mu\text{L}/\text{kg}$, ip, dissolved in corn oil (2%, v/v)]. Control animals were given appropriate corn oil ip. Twenty-four hours after administration of CCl_4 , mice were anesthetized with diethyl ether. Blood was then collected from the retro-orbital venous sinus to determine serum ALT, AST, and LDH activities, and the liver tissue was isolated and stored at -80°C for GSH and MDA analysis, except for the left lobe, which was used for histological studies. A detailed experimental procedure used to investigate the protective effects of the EtOAc extract of *G. uralensis* on CCl_4 -induced mouse liver damage is described in the Supporting Information.

ALT and AST Levels. After coagulation, the collected mouse blood samples were centrifuged at 8000 rpm (4°C) for 10 min to obtain serum, which was sent to Peking University Third Hospital (Beijing, People's Republic of China) to test the ALT, AST, and LDH activities.

Histological Analysis. The liver tissues were fixed by immersion in 10% neutral-buffered formalin, which was sent to Peking University Third Hospital for hematoxylin-eosin (H&E) staining and blind histological assessment. Histological changes were evaluated in nonconsecutive, randomly chosen 4 \times and 20 \times histological fields.

Determination of Hepatic GSH Levels. The GSH levels in liver homogenates were measured following the manufacturer's protocol using a GSH/GSSG assay kit (Beyotime). The protein content in liver homogenates was determined using a BCA protein assay kit (Beyotime).

RT-PCR Analysis. Total RNA of HepG2 cells or liver tissue was extracted using the method described by Chomczynski and Sacchi,⁵¹ and the reverse transcription of total RNA was carried out following the manufacturer's protocol using a RevertAid First Strand cDNA synthesis kit (Thermo, Waltham, MA, USA). The PCR reaction was performed with a diluted cDNA sample and amplified in each 10 μL reaction volume. Primers (mice and human) for gene amplification of HO-1, NQO-1, and β -actin were listed as follows: mice HO-1, 5'-CAAGCCGAGAAATGCTGAGTTCATG-3' (forward) and 5'-GCAAGGGATTTCCCTGCCAG-3' (reverse); mice NQO-1, 5'-GCGAGAAGAGGCCCTGATTGTACTG-3' (forward) and 5'-TCTCAAACCAGCCTTCAGAATGG-3' (reverse); mice β -actin, 5'-CTGTCCCCTGTATGCCCTCTG-3' (forward) and 5'-ATGTCAC-GCACGATTTC-3' (reverse); human HO-1, 5'-GCAGAG-GGTGATAGAAGAGG-3' (forward) and 5'-AAGGAAGCCAGCC-AAGAG-3' (reverse); human NQO-1, 5'-GGAGTCGGA-CCTCTATG-3' (forward) and 5'-GGCAGCGTAAGTGTAAAG-3' (reverse); human β -actin, 5'-GTGGACATCCGCAAAGAC-3' (forward) and 5'-AAAGGGTGTAAACGCAACTAA-3' (reverse). Quantitative PCR was performed using a Stratagene MX3000P qPCR System (Agilent Technologies, Palo Alto, CA, USA).

Western Blotting. The total protein was extracted using RIPA cell lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 3 mM sodium pyrophosphate, 1 mM EGTA, 1 mM Na_2EDTA , 50 mM NaF, 1% NP-40, 1% sodium deoxycholate, 1 mM glycerol phosphate, 1× protease/phosphatase inhibitor cocktail, 1 mM PMSF, and 1 mM Na_3VO_4). The protein content was determined using a BCA protein assay kit (Beyotime). A 20 μg aliquot of protein was separated by 10% SDS-polyacrylamide gel electrophoresis, and proteins in SDS-polyacrylamide gel were electrotransferred to a polyvinylidene difluoride

membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h at room temperature with 5% skim milk in phosphate-buffered saline–0.1% Tween 20 (PBST) and then probed with specified primary antibodies (1:1000–2000) in 5% bovine serum albumin in PBST overnight at 4°C . Then the blots were incubated with the corresponding horse radish peroxidase-conjugated second antibodies (1:5000) at room temperature for 1 h. Protein expression was visualized by ECL Prime Western Blotting detection reagent (Amersham-Pharmacia, Jersey, NJ, USA) and scanned using a ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). The antibodies against β -actin, Nrf2, HO-1, and NQO-1 were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Statistical Analysis. The results are expressed as the means \pm SD of three independent experiments. Statistical analysis was performed by the two-tailed Student's *t* test for unpaired data, and differences were considered significant at $p \leq 0.05$.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jnatprod.5b00877](https://doi.org/10.1021/acs.jnatprod.5b00877).

Detailed experimental procedures for the isolation of 1–122, protective effects of the *G. uralensis* EtOAc extract on CCl_4 -induced mouse liver damage, ECD spectroscopic calculation, UHPLC analysis, and spectroscopic data (NMR, MS, UV, and IR) for compounds 1–6 (PDF)

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Notes

The authors declare no competing financial interest.

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