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High-performance liquid chromatographic profile and ^{1}H quantitative nuclear magnetic resonance analyses for quality control of a Xinjiang licorice extract

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ABSTRACT

Various pharmacological properties of Xinjiang licorice flavonoids have been reported recently. We have investigated constituents corresponding to distinct peaks on the high-performance liquid chromatography (HPLC) profile of a flavonoid-rich extract from licorice, and identified 13 flavonoids, including licochalcone A (**1**), licochalcone B (**3**), glabrone (**4**), and echinatin (**5**), by isolating them and then performing high-resolution electrospray ionization mass spectrometry and ^{1}H nuclear magnetic resonance (NMR) spectral analyses. We then applied the ^{1}H quantitative NMR (qNMR) method for analysis of major flavonoids, **1** and **3–5** in the extract. The ^{1}H qNMR results were supported by ^{13}C NMR analysis. The results demonstrated the utility of the combination of HPLC profiling and qNMR analyses for quality control of Xinjiang licorice. Additionally, we observed a moderate inhibitory effect of the most abundant constituent, licochalcone A (**1**), on acetylcholine esterase activity, suggesting utility as a seed for drug development.

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Underground parts of *Glycyrrhiza* species (Fabaceae), called licorice roots, have been used widely for medicinal purposes in Asian traditional medicine and also as sweeteners in food industries [1]. Large differences in flavonoid constituents, especially in unglycosylated flavonoids, have been reported among the licorice roots derived from three different *Glycyrrhiza* species, i.e., *G. glabra, G. uralensis*, and *G. inflata*, all of which have been used medicinally [2–5]. Xinjiang licorice, which is based on *G. inflata* (i.e., licorice from the Xinjiang region of China), contains licochalcone A (**1**) as a characteristic constituent among flavonoid constituents. This compound has been found to have various pharmacological properties including antiviral, antibacterial, antitumor, and anti-inflammatory effects [6–11]. These studies, and more recent ones [12,13], prompted us to examine the flavonoid composition of Xinjiang licorice as a quality control measure, as a part of our study on licorice flavonoids [4].

Liquid chromatography-mass spectrometry (LC-MS) is an excellent method for identifying the many co-existing constituents in a plant extract. However, various compounds with isomeric structures have been identified in *Glycyrrhiza* species [3], and comparisons with standard constituents and combinations with other methods to differentiate these isomers are required for the precise identification of their respective high-performance liquid chromatography (HPLC) peaks. Our preliminary examination also suggested that identifying peaks corresponding to flavonoid constituents by LC-MS is difficult, especially in Xinjiang licorice, if only mass spectral data are available.

We therefore isolated the constituents corresponding to distinctive HPLC peaks of the flavonoid-rich ethyl acetate extract from Xinjiang licorice, followed by identification of 13 compounds via high-resolution electrospray ionization mass spectrometry (HR-ESIMS) and ^{1}H nuclear magnetic resonance (NMR) spectral analyses (referred to as **1**–**13**; see the following section).

^{1}H quantitative nuclear magnetic resonance (^{1}H qNMR) spectral measurement has been shown to be a useful method for quantitative analysis of major constituents in plant extracts when suitable NMR conditions with an appropriate internal standard (IS) are available. This method does not require the respective standard constituent samples and respective calibration lines of the samples that are required in HPLC [14–17]. Some previous reports have also mentioned using LC-MS and qNMR methods in conjunction with principal component analysis to determine the origins of licorice extracts [18,19]. Considering this background, we investigated the applicability of the

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qNMR method to quantitative analysis of major, flavonoid constituents of Xinjiang licorice (compounds **1**, and **3**–**5**).

Investigation of compounds with acetylcholine esterase (AChE) inhibitory activity from natural resources is important because these compounds, or plant extracts containing these compounds, are required for identifying candidate remedies for neurotropic diseases including dementia [17,20,21]. Some flavonoid constituents, and a triterpenoid glycyrrhizin (**14**), in licorice have been reported to have inhibitory effects on AChE [22,23]. We therefore compared the effects of major constituents of Xinjiang licorice (constituents **1** and **3**) on the activity of this enzyme, in comparison with the effect of constituent **14**.

Materials and methods

Instruments

Both ^{1}H NMR and ^{13}C NMR spectra were measured using a Varian PS600 NMR system (600 MHz for ^{1}H and 151 MHz for ^{13}C: Varian, Palo Alto, CA, USA) at room temperature (ca. 21°C). A Varian 400-MR ASW instrument was also used for ^{1}H NMR (400 MHz) measurements. VnmrJ 3.2 software incorporating qEstimate for qNMR was used for ordinary NMR and qNMR measurements. Chemical shifts were given in δ (ppm) values based on those of the solvent acetone-d_6 signals (δ 2.05 for ^{1}H and δ 29.8 for ^{13}C). Circular dichroism (CD) spectra were measured using a JASCO J720 W spectropolarimeter (Jasco, Tokyo, Japan). High-resolution electrospray ionization mass spectra (HR-ESIMS) were obtained using a Bruker micrOTOF II system (Bruker, Billerica, MA, US) by direct infusion. Liquid chromatography-mass spectrometry measurements were carried out using a liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF-MS) system (Agilent Technologies, Santa Clara, CA, US), which consisted of an Agilent 1200 series capillary LC system equipped with a Zorbax XDB-C18 column (0.5 × 150 mm) and Agilent G6520 Accurate-Mass Q-TOF system with a dual ESI source. The measurements described here were performed in negative mode using the following gradient profile of solvents: (A) H_2O containing 0.1% HCOOH and (B) CH_3CN containing 0.1% HCOOH: 0.5–15.5 min, 40–60% (B in A + B); 15.5–17.5 min, 60% (B in A + B); 17.5–20 min, 60–98% (B in A + B); 20.-0–25.0 min 98% (B in A + B). The flow rate was set at 10.0 μL/min. Gas temperature, gas flow, and nebulizer pressure were set at 350°C, 8 L/min, and 45 psig, respectively. The HPLC with photodiode-array UV detection was performed on a system composed of an L-2130 pump and L-2455 detector (Hitachi, Tokyo, Japan), equipped with a YMC Pro C18 column (6.0 × 150 mm) (YMC, Kyoto, Japan), unless specified otherwise. The solvents used in HPLC were combinations of: (A) H_2O–CH_3CN–CH_3COOH (60:40:1) and (B) H_2O–CH_3CN–CH_3COOH (40:60:1), with the following gradient profile: 0–45 min, 10–90% (B in A + B); 45–50 min, 90% (B in A + B). The flow rate was set at 1.5 mL/min. Reversed-phase (RP) preparative HPLC was performed using a YMC ODS A324 column (10 × 300 mm) or a YMC Pro C18 column (10 × 250 mm) in an oven set at 40°C, with combinations of H_2O–CH_3CN–CH_3COOH (70:30:1–50:50:1), or H_2O–CH_3OH–CH_3COOH (60:40:1) as solvents, with a flow rate of 2.0 mL/min. Normal-phase (NP) preparative HPLC on a YMC-Pack SIL column (10 × 300 mm) with *n*-hexane-ethyl acetate (9:1) (flow rate 3.0 mL/min, at room temperature) was also used for preparative HPLC. A model 680 microplate reader (Bio-Rad, Tokyo, Japan) was used together with Biolite 96-well plates (Thermo Fischer Scientific, Tokyo, Japan) for the AChE assay.

Column adsorbents used for constituent isolation

MCI-gel CHP-20P (Mitsubishi Chemical, Tokyo, Japan), Silica-gel PSQ100B (Fuji-Silysia, Kasugai, Japan), and Chromatorex ODS DM1020 T (Fuji-Silysia) were used for column chromatography (CC), with YMC Dispo-Pack AT ODS cartridge columns also used for purification.

Reagents

Glycyrrhizin and 1,4-bis(trimethylsilyl)benzene-d_4 (1,4-BTMSB-d_4) were purchased from Fujifilm Wako (Osaka, Japan). AChE (derived from *Electrophorus electricus*), acetylthiocholine iodide (ATCI), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), tacrine, and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Plant material

The Xinjiang licorice used in this study was purchased from Yarkand County Fuyuan Licorice Co., Ltd. (Yarkand, Xinjiang, China). The extract used for confirming the similarity of the HPLC profile to that previously reported for the *G. inflata* standard material [5] was prepared as follows. The Xinjiang licorice material (302 mg) was pulverized and homogenized in MeOH (40 mL × 2), and the filtered solution was then evaporated to give an extract (31.6 mg). This whole extract was analyzed by HPLC to assess the similarity of the profiles to that reported previously for *G. inflata* material, with licochalcone A (**1**) and licochalcone B (**3**) as the main peaks (see Figure S1 in Supporting information) [5].

Preparation of extracts for constituent isolation, NMR analysis, and AChE assay

Pulverized Xinjiang licorice (300 g) material was successively extracted with *n*-hexane (2 L × 3), ethyl acetate (2 L × 3), and MeOH (2 L × 3) to produce *n*-hexane (0.98 g), ethyl acetate (15.3 g), and MeOH (24.3 g) extracts. Ethyl acetate and MeOH extracts were used for the AChE assay. The ethyl acetate extract was also used for the subsequent purification of flavonoids, and for qNMR and HPLC analyses and AChE assay.

Isolation of the flavonoids in Xinjiang licorice

As indicated below, the ethyl acetate extract was subjected to countercurrent distribution (CCD), and chromatography of the CCD fractions on MCI-gel CHP-20P, Silica-gel PSQ100B, Chromatorex ODS DM1020 T, and/or YMC Dispo-Pack AT ODS cartridge columns in combination with preparative HPLC, to identify the 13 compounds.

A portion (2.06 g) of the ethyl acetate extract was subjected to CCD (n = 2, r = 2) with $CHCl_3$–MeOH–H_2O (7:13:8, by volume) to give four fractions [Upper Layer (UL)-I-1, 165.1 mg; UL-I-2, 140.9 mg; Lower Layer (LL)-I-2, 270.1 mg; LL-I-1, 1418.2 mg (polarity: most polar to least polar fractions)]. A large portion (1.38 g) of the LL-I-1 fraction was dissolved in water and the insoluble material (1.14 g) was removed by centrifugation. Next, the aqueous solution was chromatographed on an MCI-gel CHP-20P column (2.2 × 30 cm), with increasing concentrations of MeOH in H_2O (0–100%) [CC-M1]. A portion (10.0 mg) of the second fraction (110.5 mg) of the 100% MeOH eluate was purified by RP preparative HPLC to obtain licochalcone A (**1**) (4.2 mg). A portion (1.11 g) of the remaining water-insoluble material was chromatographed on a Silica-gel PSQ100B column (2.2 × 30 cm), with increasing concentrations of ethyl acetate in *n*-hexane (50–100%) (CC-S1). The eluate with 50% ethyl acetate in *n*-hexane (675 mg) from CC-S1 was chromatographed on a MCI-gel column (2.2 × 30 cm), with increasing concentrations of MeOH in H_2O (50–100%) (CC-M2). A large portion (202.4 mg) of the 100% MeOH eluate (213.3 mg) from CC-M2 was subjected to CC on Silica gel (1.1 × 30 cm), with increasing concentrations of ethyl acetate in *n*-hexane (40–100%) (CC-S2). A portion (37.0 mg) of the fifth fraction (59.2 mg) of the eluate with 40% ethyl acetate in *n*-hexane from CC-S2 was purified by RP preparative HPLC to obtain licochalcone A (**1**) (31.2 mg) and licochalcone E (**8**) (1.2 mg). A large portion (38.0 mg) of the second fraction of the 40% ethyl acetate in *n*-hexane eluate (43.6 mg) from CC-S2 was subjected to CC on Chromatorex ODS-DM-120 T (1.1 × 30 cm), with increasing concentrations of MeOH in water (80–100%) (CC-C1). The second fraction (6.4 mg) of the 80% MeOH eluate of CC-C1 was purified with NP preparative HPLC to obtain licoisoflavone B (**2**) (1.7 mg). A large portion (43.7 mg) of the third fraction (47.9 mg) of the eluate with 40% ethyl acetate in *n*-hexane from CC-S2 was chromatographed on a Chromatorex column (1.1 × 30 cm) with increasing concentrations of MeOH in water (80–100%) (CC-C2) to obtain glabrone (**4**) (19.1 mg) from the first fraction of the 80% MeOH eluate.

Separately, a portion (2.02 g) of the ethyl acetate extract was subjected to CCD (n = 2, r = 2) with $CHCl_3$–MeOH–*n*-propanol–H_2O (45:60:10:40, by volume), to give four fractions [UL-II-1, 91.0 mg; UL-II-2, 97.1 mg; LL-II-2, 289.2 mg; LL-II-1, 1531.2 mg (in order of polarity)]. The polar fractions UL-I-1, UL-I-2, UL-II-1, and UL-II-2 were combined (440.5 mg in total) and subjected to CC on MCI-gel (2.2 × 30 cm), with increasing concentrations of MeOH in water (0–100%) (CC-M3). A large portion (147.0 mg) of the 70% MeOH eluate (169.1 mg) from CC-M3 was chromatographed on a MCI-gel column (2.2 × 30 cm), with increasing concentrations of MeOH in water (60–100%) (CC-M4), and a portion (68.2 mg) of the eluate with 65% MeOH (109.6 mg) from CC-M4 was purified by RP preparative HPLC to obtain licochalcone B (**3**) (45.0 mg). A large portion (74.0 mg) of the 80% MeOH eluate (85.1 mg) from CC-M3 was further purified by CC on MCI-gel, with increasing concentrations of MeOH in water (65–100%) (CC-M5), and then by preparative HPLC of most (23.5 mg) of the 75% MeOH eluate (33.1 mg) from CC-M5 to obtain echinatin (**5**) (9.0 mg).

A portion (1.01 g) of the remaining ethyl acetate extract was subjected to CCD (n = 2, r = 2) $CHCl_3$–MeOH–H_2O (7:13:8, by volume) to give four fractions [UL-III-1, 70.4 mg; UL-III-2, 62.7 mg; LL-III-2, 141.7 mg; LL-III-1, 818.1 mg (in order of polarity)]. A large portion (779.7 mg) of the least polar LL-III-1 fraction was chromatographed on a Chromatorex column (2.2 × 30 cm), with increasing concentrations of MeOH in water (50–100%) (CC-C3). A large portion (32.1 mg) of the 60% MeOH eluate (35.8 mg) from CC-C3 was purified on a YMC Dispo-Pack AT ODS cartridge column, with increasing concentrations of MeOH in water (45–100%) (CC-Y1), and the eluate with 50% MeOH (11.6 mg) was subjected to preparative HPLC to obtain 4′,7-dihydroxyflavone (**10**) (1.4 mg). A large portion (263.0 mg) of the 80% MeOH eluate (284.1 mg) from CC-C3 was purified on a YMC cartridge column, with increasing concentrations of MeOH in water (72–100%) (CC-Y2), and a large portion (260.2 mg) of the 72% MeOH eluate (290.2 mg) from CC-Y2 was again chromatographed on a YMC cartridge column, with increasing concentrations of MeOH in water

(50–100%) (CC-Y3). A large portion (80.3 mg) of the 65% MeOH eluate (89.0 mg) from CC-Y3 was chromatographed on a YMC cartridge column, with increasing concentrations of MeOH in water (56–100%) (CC-Y4), and a large portion (16.0 mg) of the 62% MeOH eluate (17.8 mg) from CC-Y4 was further chromatographed on a YMC cartridge column, with increasing concentrations of MeOH in water (54–100%) (CC-Y5). A large portion (16.0 mg) of the 62% MeOH eluate (17.8 mg) from CC-Y5 was further purified by RP preparative HPLC to obtain licochalcone A (**1**) (3.7 mg), glabrone (**4**) (2.1 mg), licoflavone A (**6**) (1.8 mg), licochalcone E (**8**) (1.6 mg), 5-(1,1-dimethylallyl)-3,4,4′-trihydroxy-2-methoxychalcone (**11**) (2.1 mg), and licochalcone C (**12**) (1.9 mg). A large portion (71.1 mg) of the 70% MeOH eluate (79.6 mg) from CC-Y3 was chromatographed on a YMC cartridge column, with increasing concentrations of MeOH in water (60–100%) (CC-Y6), and a large portion (30.0 mg) of the 64% MeOH eluate (35.2 mg) from CC-Y6 was purified by RP preparative HPLC to obtain licochalcone A (**1**) (21.1 mg), licoisoflavone B (**2**) (2.1 mg), glabrone (**4**) (4.4 mg), and licoflavone A (**6**) (3.3 mg). A large portion (16.0 mg) of the 66% MeOH eluate (21.7 mg) from CC-Y6 was purified by RP preparative HPLC to obtain licoisoflavone B (**2**) (2.0 mg) and 8-(4-hydroxyphenyl)-2,2-dimethylpyrano[3,2-*g*]chromen-6-one (**13**) (1.1 mg). A large portion (46.7 mg) of the 75% eluate (51.5 mg) from CC-Y3 was chromatographed on a YMC cartridge column, with increasing concentrations of MeOH in water (66–100%) (CC-Y7), and a large portion (22.5 mg) of the 72% MeOH eluate (23.5 mg) was purified by RP preparative HPLC to obtain glabrol (**7**) (5.2 mg). A large portion (20.0 mg) of the 80% MeOH eluate (23.8 mg) from CC-Y3 was chromatographed on a YMC cartridge column, with increasing concentrations of MeOH in water (70–100%) (CC-Y8), and a large portion (7.8 mg) of the 76% MeOH eluate (9.4 mg) was purified by RP preparative HPLC to obtain licoflavone B (**9**) (2.4 mg).

Spectral properties of isolated constituents

Licochalcone A (**1**) [24,25]. HR-ESIMS, *m/z* 337.1438 $[M–H]^-$ (Calculated for $C_{21}H_{22}O_4$–H, 337.1445). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 8.02 (1 H, d, *J* = 15.6 Hz, H-β), 8.01 (2 H, d, *J* = 9.0 Hz, H-2′, H-6′), 7.67 (1 H, d, *J* = 15.6 Hz, H-α), 7.63 (1 H, s, H-6), 6.95 (2 H, d, *J* = 9.0 Hz, H-3′, H-5′), 6.58 (1 H, s, H-3), 6.30 (1 H, dd, *J* = 10.8, 18.0 Hz, H-2″), 5.02 (1 H, dd, *J* = 1.2, 18.0 Hz, H-3″), 4.99 (1 H, dd, *J* = 1.2, 10.8 Hz, H-3″), 3.87 (3 H, s, -OCH_3), 1.51 (6H, s, -CH_3 × 2). ^{13}C-NMR (151 MHz, acetone-d_6) δ: 188.4 (>C = O), 162.3 (C-4′), 160.0 (C-4), 159.7 (C-2), 148.6 (C-2″), 139.9 (C-β), 131.8 (C-1′), 131.5 (2 × C, C-2′, C-6′), 129.3 (C-6), 127.6 (C-5), 119.5 (C-α), 116.1 (2 C, C-3′, C-5′), 115.9 (C-1), 110.8 (C-3″), 101.0 (C-3), 55.9 (-OCH_3), 40.7 (C-1″), 27.5 (2 C, C-4″, C-5″).

Licoisoflavone B (**2**) [26]. HR-ESIMS, *m/z* 351.0869 $[M–H]^-$ (Calculated for $C_{20}H_{14}O_6$–H, 351.0874) ^{1}H-NMR [400 MHz, acetone-d_6 + D_2O (9:1)] δ: 8.16 (1 H, s, H-2), 7.00 (1 H, d, *J* = 8.0 Hz, H-6′), 6.72 (1 H, d, *J* = 10.0 Hz, H-4″), 6.43 (1 H, d, *J* = 2.0 Hz, H-8), 6.36 (1 H, d, *J* = 8.0 Hz, H-5′), 6.28 (1 H, d, *J* = 2.0 Hz, H-6), 5.67 (1 H, d, *J* = 10.0 Hz, H-3″), 1.53 (6H, s, -CH_3 × 2).

Licochalcone B (**3**) [24]. HR-ESIMS, *m/z* 285.0770 $[M–H]^-$ (Calculated for $C_{16}H_{14}O_5$–H, 285.0768). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 8.05 (2 H, d, *J* = 9.0 Hz, H-2′, H-6′), 7.97 (1 H, d, *J* = 15.6 Hz, H-β), 7.72 (1 H, d, *J* = 15.6 Hz, H-α), 7.33 (1 H, d, *J* = 8.4 Hz, H-6), 6.96 (2 H, d, *J* = 9.0 Hz, H-3′, H-5′), 6.71 (1 H, d, *J* = 8.4 Hz, H-5), 3.86 (3 H, s, -OCH_3). ^{13}C-NMR (151 MHz, acetone-d_6) δ: 188.2 (>C = O), 162.5 (C-4′), 149.8 (C-4), 149.2 (C-2), 139.2 (C-3), 139.1 (C-β), 131.7 (2 C, C-2′, C-6′), 131.6 (C-1′), 121.4 (C-1), 120.6 (C-α), 119.9 (C-6), 116.1 (2 C, C-3′, C-5′), 112.5 (C-5), 61.7 (-OCH_3).

Glabrone (**4**) [27,28]. HR-ESIMS, *m/z* 335.0919 $[M–H]^-$ (Calculated for $C_{20}H_{16}O_5$–H, 335.0925). ^{1}H-NMR [400 MHz, acetone-d_6 + D_2O (9:1)] δ: 8.31 (1 H, s, H-2), 8.09 (1 H, d, *J* = 8.8 Hz, H-5), 7.06 (1 H, dd, *J* = 2.4, 8.8 Hz, H-6), 7.04 (1 H, d, *J* = 8.4 Hz, H-6′), 6.96 (1 H, d, *J* = 2.4 Hz, H-8), 6.73 (1 H, d, *J* = 10.0 Hz, H-4″), 6.36 (1 H, d, *J* = 8.4 Hz, H-5′), 5.67 (1 H, d, *J* = 10.0 Hz, H-3″), 1.37 (6H, s, -CH_3 × 2).

Echinatin (**5**) [24]. HR-ESIMS, *m/z* 269.0820 $[M–H]^-$ (Calculated for $C_{16}H_{14}O_4$–H, 269.0819). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 8.05 (1 H, d, *J* = 15.6 Hz, H-β), 8.03 (2 H, d, *J* = 8.4 Hz, H-2′, H-6′), 7.70 (1 H, d, *J* = 8.4 Hz, H-6), 7.69 (1 H, d, *J* = 15.6 Hz, H-α), 6.95 (2 H, d, *J* = 8.4 Hz, H-3′, H-5′), 6.56 (1 H, d, *J* = 2.4 Hz, H-3), 6.52 (1 H, dd, *J* = 2.4, 8.4 Hz, H-5), 3.91 (3 H, s, -OCH_3). ^{13}C-NMR (151 MHz, acetone-d_6) δ: 188.3 (>C = O), 162.5 (C-4′), 162.3 (C-4), 161.4 (C-2), 139.2 (C-β), 131.7 (C-1′), 131.6 (2 C, C-2′, C-6′), 131.1 (C-6), 119.5 (C-α), 116.6 (C-1), 116.1 (2 C, C-3′, C-5′), 109.0 (C-5), 100.0 (C-3), 56.0 (-OCH_3)

Licoflavone A (**6**) [29]. HR-ESIMS, *m/z* 321.1132 $[M–H]^-$ (Calculated for $C_{20}H_{18}O_4$–H, 321.1132). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 7.94 (2 H, d, *J* = 9.0 Hz, H-2′, H-6′), 7.82 (1 H, s, H-5), 7.06 (1 H, s, H-8), 7.01 (2 H, d, *J* = 9.0 Hz, H-3′, H-5′), 6.59 (1 H, s, H-3), 5.40 (1 H, m, H-2″), 3.36 (2 H, d, *J* = 7.2 Hz, H-1′′), 1.76, 1.74 (each 3 H, s, -CH_3 × 2).

Glabrol (**7**) [30]. HR-ESIMS, *m/z* 391.1910 $[M–H]^-$ (Calculated for $C_{25}H_{28}O_4$–H, 391.1915). CD (MeOH) $[\theta]_{331} + 1.7 \times 10^4$, $[\theta]_{300} - 2.6 \times 10^4$, $[\theta]_{300} - 2.6 \times 10^4$, $[\theta]_{239} + 1.5 \times 10^4$, $[\theta]_{230} - 5.1 \times 10^3$, $[\theta]_{216} + 3.0 \times 10^4$. ^{1}H-NMR (600 MHz, acetone-d_6) δ: 7.58 (1 H, d, *J* = 8.4 Hz, H-6), 7.32 (1 H, d, *J* = 2.4 Hz, H-2′), 7.22 (1 H, dd, *J* = 2.4, 8.4 Hz, H-6′), 6.89 (1 H, d, *J* = 8.4 Hz,

H-5′), 7.62 (1 H, d, J = 8.4 Hz, H-5), 5.41 (1 H, dd, J = 3.0, 12.6 Hz, H-2), 5.37 (1 H, m, H-2″), 5.24 (1 H, m, H-2″), 3.36 (2 H, d, J = 7.8 Hz, H-1″), 3.33 (2 H, d, J = 7.8 Hz, H-1″), 2.99 (1 H, dd, J = 12.6, 16.8 Hz, H-3), 2.69 (1 H, dd, J = 3.0, 16.8 Hz, H-3), 1.72 (6 H, s, -CH_3 × 2), 1.63, 1.62 (each 3 H, s, -CH_3 × 2).

Licochalcone E (**8**) [31]. HR-ESIMS, m/z 337.1436 $[M–H]^-$ (Calculated for C_{21} $H_{22}O_4$–H, 337.1445). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 8.01 (1 H, d, J = 15.6 Hz, H-β), 7.98 (2 H, d, J = 9.0 Hz, H-2′, H-6′), 7.65 (1 H, d, J = 15.6 Hz, H-α), 7.51 (1 H, s, H-6), 6.94 (2 H, d, J = 9.0 Hz, H-3′, H-5′), 6.60 (1 H, s, H-3), 4.89, 4.88 (each 1 H, br s, H-3″ × 2), 3.81 (1 H, q, J = 7.2 Hz, H-1″), 3.78 (3 H, s, -OCH_3), 1.67 (3 H, s, -CH_3 at C-2′′), 1.35 (3 H, d, J = 7.2 Hz, -CH_3 at C-1″).

Licoflavone B (**9**) [29]. HR-ESIMS, m/z 389.1759 $[M–H]^-$ (Calculated for $C_{25}H_{24}O_4$–H, 389.1758). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 7.81 (1 H, s, H-5), 7.77 (1 H, d, J = 2.4 Hz, H-2′), 7.72 (1 H, dd, J = 2.4, 8.4 Hz, H-6′), 7.04 (1 H, s, H-8), 7.01 (1 H, d, J = 8.4 Hz, H-5′), 6.56 (1 H, s, H-3), 5.40 (2 H, m, H-2″), 3.36 (2 H, d, J = 7.8 Hz, H-1″), 1.76 (6 H, s, -CH_3 × 2), 1.75, 1.74 (each 3 H, s, -CH_3 × 2).

7,4′-Dihydroxyflavone (**10**) [32]. HR-ESIMS, m/z 253.0498 $[M–H]^-$ (Calculated for $C_{15}H_{10}O_4$–H, 253.0506). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 7.93 (1 H, d, J = 8.4 Hz, H-5), 7.89 (2 H, d, J = 9.0 Hz, H-2′, H-6′), 7.00 (1 H, d, J = 2.4 Hz, H-8), 6.98 (2 H, d, J = 9.0 Hz, H-3′, H-5′), 6.95 (1 H, dd, J = 2.4, 8.4 Hz, H-6), 6.67 (1 H, s, H-3).

5-(1,1-Dimethylallyl)-3,4,4′-trihydroxy-2-methoxy-chalcone (**11**) [33]. HR-ESIMS, m/z 353.1398 $[M–H]^-$ (Calculated for $C_{21}H_{22}O_5$–H, 353.1394). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 8.02 (2 H, d, J = 9.0 Hz, H-2′, H-6′), 7.93 (1 H, d, J = 15.6 Hz, H-β), 7.70 (1 H, d, J = 15.6 Hz, H-α), 7.29 (1 H, s, H-6), 6.96 (2 H, d, J = 9.0 Hz, H-3′, H-5′), 6.33 (1 H, dd, J = 10.2, 18.0 Hz, H-2″), 5.03 (1 H, dd, J = 1.8, 18.0 Hz, H-3″), 4.99 (1 H, dd, J = 6.6 Hz, H-3″), 3.80 (3 H, s, -OCH_3), 1.53 (6 H, s, -CH_3 × 2).

Licochalcone C (**12**) [24]. HR-ESIMS, m/z 337.1436 $[M–H]^-$ (Calculated for $C_{21}H_{22}O_4$–H, 337.1445). ^{1}H-NMR (600 MHz, acetone-d_6) δ: 8.05 (2 H, d, J = 9.0 Hz, H-2′, H-6′), 7.99 (1 H, d, J = 15.6 Hz, H-β), 7.69 (1 H, d, J = 15.6 Hz, H-α), 7.67 (1 H, d, J = 8.4 Hz, H-6), 6.96 (2 H, d, J = 9.0 Hz, H-3′, H-5′), 6.77 (1 H, d, J = 8.4 Hz, H-5), 5.27 (1 H, m, H-2″), 3.77 (3 H, s, -OCH_3), 3.39 (2 H, d, J = 6.6 Hz, H-1″), 1.79, 1.60 (each 3 H, s, -CH_3 × 2).

8-(4-Hydroxyphenyl)-2,2-dimethylpyrano[3,2-g]-chromen-6-one (**13**) [34]. HR-ESIMS, m/z 319.0976 $[M–H]^-$ (Calculated for $C_{20}H_{16}O_4$–H, 319.0976) ^{1}H-NMR (600 MHz, acetone-d_6) δ: 7.93 (1 H, d, J = 8.4 Hz, H-2′, H-6′), 7.73 (1 H, s, H-5), 7.02 (1 H, d, J = 8.4 Hz, H-3′, H-5′), 6.95 (1 H, s, H-8), 6.61 (1 H, s, H-3), 6.60 (1 H, d, J = 10.2 Hz, H-1″), 5.91 (1 H, d, J = 10.2 Hz, H-2″), 1.49 (6 H, s, -CH_3 × 2).

^{1}H qNMR measurements and ^{13}C NMR analysis

Acetone-d_6 was used as an NMR solvent. 1,4-BTMSB-d_4, which is soluble in this solvent, was selected as the IS because its 18 protons in the methyl signal at δ 0.25 did not overlap any of the licorice ethyl acetate extract signals. The parameters for qNMR measurements were as follows: pulse angle, 10.0 μs for 90°; relaxation delay, 40.0 s; acquisition time, 3.408 s; apodization, 0.3 Hz for exponential line broadening; spin, off. The total run time was 11.35 min for 16 scans for the measurement of the ethyl acetate extract sample (10.0 mg in 700 μL acetone-d_6). The molar concentrations of each of the constituents were determined using the following equation:

$$MC_x = \frac{I_x/H_x}{I_{std}/H_{std}} \times MC_{std}$$

where I_x, MC_x, and H_x represent the signal intensity (integral value), molar concentration, and number of protons in the target functional group of the constituent; I_{std}, MC_{std}, and H_{std} are the corresponding values for the IS (MC_{std}, 6.31 × 10^{-4} M). The ^{13}C NMR spectrum of the licorice ethyl acetate extract sample (20.0 mg in 700 μL acetone-d_6 with 0.2 mg 1,4-BTMSB-d_4) was measured with the same parameters as those used for the ordinary ^{13}C experiments: pulse width, 3.8 μs (for a pulse angle of 30°); relaxation delay, 1.000 s; acquisition time, 0.865 s; spectral width, 37,878.8 Hz; and total acquisition time 111 min for 3,584 repetitions. The WALTZ-16 pulse was used for proton decoupling during the ^{13}C measurement. A Fourier transformation was performed using 65,536 points, with line broadening at 3.0 Hz as the window function.

Estimation of AChE activity in the presence of Xinjiang licorice constituents and extracts

The effects of Xinjiang licorice constituents and extracts on AChE activity were estimated based on Ellman's method [35], with slight modifications [17]. AChE solution (0.5 U/mL,10 μL) and test compounds (or extract) solutions in DMSO (20 μL) were added to a solution of 0.1 M MOPS buffer (pH 7.5, 150 μL) in 96-well plates, and the mixture was preincubated for 10 min at 25°C. Next, ATCI (75 mM, 10 μL) and DTNB (10 mM, 10 μL) were added to the solution, which was incubated for a further 30 min at 25°C. The absorbance of the resulting solution at 415 nm was measured using a microplate reader, and the enzyme activity in the presence of the test compound was compared with that in the absence of the compound using the following equation:

$$\text{Enzyme activity}(\%) = \frac{S_E - S_B}{C_E - C_B} \times 100$$

where C_E and C_B represent the absorbance of the control solution (without the test compound) in the presence of AChE and in the absence of AChE, respectively; S_E and S_B are the absorbance of the solution with the test compound in the presence of AChE and in the absence of AChE, respectively. A statistical analysis was conducted based on an analysis of variance (ANOVA), and significant differences were evaluated using Dunnett's test [36,37]. When the P value was less than 0.05, the difference was regarded as being statistically significant.

Quantitative HPLC analyses of licochalcone A in the ethyl acetate extract and glycyrrhizin in the MeOH extract

For the quantitative analysis of licochalcone A (**1**) in the licorice ethyl acetate extract, a solution of the extract in MeOH (0.50 mg/mL) was subjected to a YMC Pro C18 column (6.0 × 150 mm) in an oven set at 40°C. The solvents used were combinations of (A) H_2O–CH_3CN–CH_3COOH (60:40:1) and (B) H_2O–CH_3CN–CH_3COOH (40:60:1), with the following gradient profile: 0–30 min, 50–90% (B in A + B); 30–40 min, 90% (B in A + B). The flow rate was set at 1.5 mL/min, and UV detection was set at 280 nm. The amount of licochalcone A in the ethyl acetate extract was estimated to be

$$23.4 \pm 0.94\text{w/w\%},$$

which corresponded to

$$1.19 \pm 0.05\text{w/w\%}$$

in the root material. The results were expressed as the means of triplicate experiments.

Analogously, a solution of the MeOH extract (1.00 mg/mL) was analyzed for the quantitation of glycyrrhizin (**14**) (see Figure S4 in Supporting information). The amount of glycyrrhizin in the MeOH extract was estimated to be

$$14.1 \pm 0.98\text{w/w\%}$$

which corresponded to

$$1.14 \pm 0.08\text{w/w\%}$$

in the root material.

Results and discussion

Isolation of flavonoids, corresponding to distinctive HPLC peaks in the Xinjiang licorice ethyl acetate extract

The HPLC profile of the extract is shown in Figure 1(a). Distinctive peaks corresponding to the thirteen constituents in the extract were identified using a UV detector and isolated as described in the Materials and methods section. The identity of each of the HPLC peaks was confirmed by LC-MS (Figure 1(b) and Table 1).

Twelve of the compounds were identified to be licochalcone A (**1**), licoisoflavone B (**2**), licochalcone B (**3**), glabrone (**4**), echinatin (**5**), licoflavone A (**6**), glabrol (**7**), licochalcone E (**8**), licoflavone B (**9**), 7,4′-dihydroxyflavone (**10**), 5-(1,1-dimethylallyl)-3,4,4′-trihydroxy-2-methoxychalcone (**11**), and licochalcone C (**12**) (Figure 2), based on the HR-ESIMS data and comparisons of the ^{1}H NMR data with reported records.

The structure of compound **13** was assigned as follows. The protons in the ^{1}H NMR spectrum of compound **13** formed a signal pattern [H-3 (δ 6.61, s), H-5 (δ 7.73, s), and H-8 (δ 6.95, s) on the flavone skeleton, and H-1″ (δ 6.60, d, J = 10.2 Hz), H-2″ (δ 5.91, d, J = 10.2 Hz), and two dimethyl groups at C-3″ (δ 1.49, 6 H, s) on the dimethylpyran moiety (**13**)], which was similar to that corresponding to the protons of licoflavone E (**13a**) [12] [H-3 (δ 6.70, s), H-5 (δ 7.71), and H-8 (δ 7.02) on the flavone skeleton; H-1″ (δ 6.59, d, J = 10.0 Hz), H-2″ (δ 5.89, d, J = 10.0 Hz), and

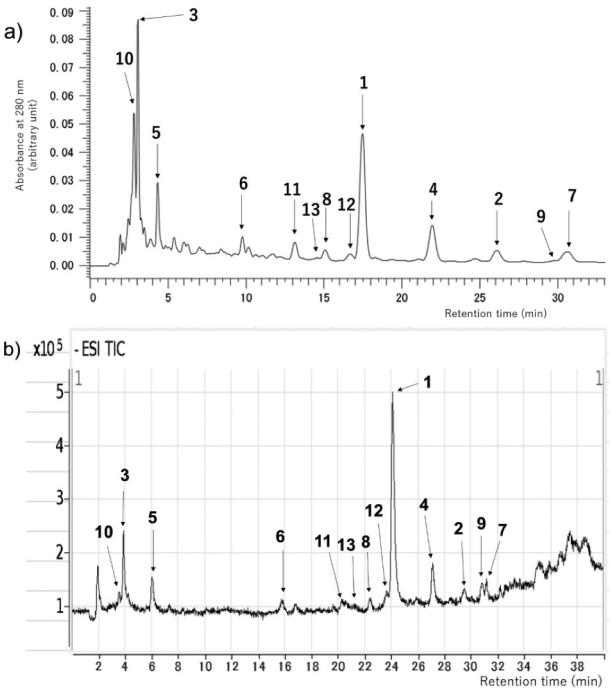

Figure 1. HPLC profiles of Xinjiang licorice ethyl acetate extract. Compound numbers were assigned to respective HPLC peaks. a) Profile with UV absorption at 280 nm. Column, YMC-Pack Pro C18 (6.0 mm i.d. × 150 mm) in an oven at 40°C; solvents (a) H_2O – CH_3CN – CH_3COOH (60:40:1) and (b) H_2O – CH_3CN – CH_3COOH with the gradient profile [0–45 min, 10 → 90% (B in A + B); 45–50 min, 90% (B in A + B)]; flow rate, 1.5 mL/min b) Total ion chromatogram on LC-MS. Zorbax XDB-C18 column (0.5 × 150 mm); solvents (A) H_2O containing 0.1% HCOOH and (B) CH_3CN containing 0.1% HCOOH with the gradient profile [0.5–15.5 min, 40 → 60% (B in A + B); 15.5–17.5 min, 60% (B in A + B); 17.5–20 min, 60 → 98% (B in A + B); 20.0–25.0 min 98% (B in A + B)]; flow rate, 10.0 μL/min.

Table 1. Retention times, compounds corresponding to the $[M–H]^-$ ion peaks in the ESIMS (negative-ion mode) in the LC-MS profile of Figure 1(b).

Retention time (min)	Compound	Molecular formula	$[M-H]^-$ ion peak (*m/z*)
3.54	7,4′ -Dihydroxyflavone (**10**)	$C_{15}H_{10}O_4$	253
3.88	Licochalcone B (**3**)	$C_{16}H_{14}O_5$	285
6.02	Echinatin (**5**)	$C_{16}H_{14}O_4$	269
15.80	Licoflavone A (**6**)	$C_{20}H_{18}O_4$	321
16.87	5-(1,1-Dimethylallyl)-3,4,4′ -trihydroxy-2-methoxychalcone (**11**)	$C_{21}H_{22}O_5$	353
20.29	8-(4-Hydroxyphenyl)-2,2-dimethylpyrano-[3,2-*g*]chromen-6-one (**13**)	$C_{20}H_{16}O_4$	353
22.42	Licochalcone E (**8**)	$C_{21}H_{22}O_4$	337
23.67	Licochalcone C (**12**)	$C_{21}H_{22}O_4$	337
24.13	Licochalcone A (**1**)	$C_{21}H_{22}O_4$	337
27.11	Glabrone (**4**)	$C_{20}H_{16}O_5$	335
29.50	Licoisoflavone B (**2**)	$C_{20}H_{16}O_6$	351
30.81	Licoflavone B (**9**)	$C_{25}H_{26}O_4$	389
31.17	Glabrol (**7**)	$C_{25}H_{28}O_4$	391

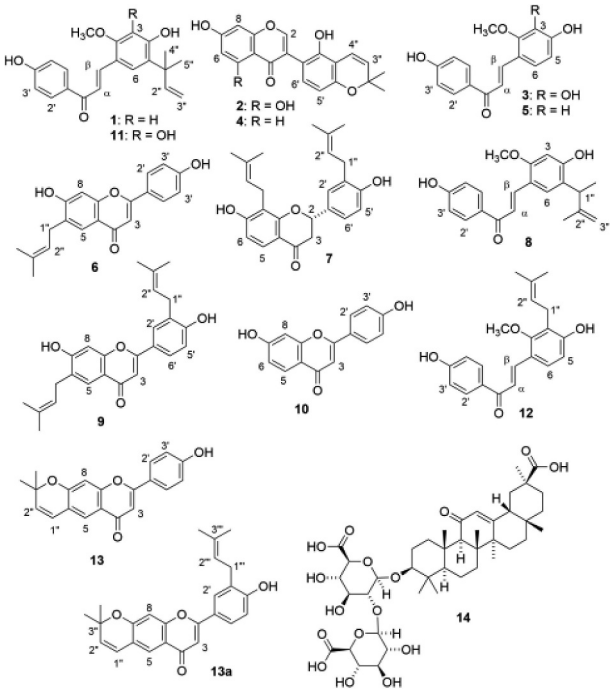

Figure 2. Structural formulae of the flavonoids isolated in the present study (1–13) and other licorice constituents (13a and 14).

two dimethyl groups at C-3″ (δ 1.44, 6 H, s) on the dimethylpyran moiety]. Differences in the spectra were observed for the A_2B_2 (AA′BB′) type signals of **13** [δ 7.93 (H-2′ and H-6′) and 7.02 (H-3′ and H-5′) (each 2 H, d, J = 10.2 Hz)], instead of the signals of the B-ring [δ 7.72 (H-2′), 6.92 (H-5′), and 7.74 (H-6′)] and dimethylallyl [δ 3.27 (2 H, H-1‴), 5.31 (1 H, H-2‴), and 1.71 (6 H, dimethyl at C-3‴)] protons in **13a**. Therefore, the structure of 8-(4-hydroxyphenyl)-2,2-dimethylpyrano[3,2-*g*]chromen-6-one (**13**) was assigned for this compound. As far as we know, the isolation of this compound from natural resources has not been reported previously, although the compound with structure **13** was previously reported as a synthetic compound [34]. Further data to substantiate the structure of the naturally occurring one is expected to confirm the identity.

Quantitation of major flavonoids in the ethyl acetate extract with ^{1}H qNMR

Among the isolated flavonoids, both licochalcone A (the main constituent in *G. inflata*) and accompanying flavonoids have been reported to have various pharmacological properties, as discussed in the Introduction section. Therefore, we examined the applicability of the ^{1}H qNMR method to the quantitative analysis of the main flavonoids, including licochalcone A (**1**).

The ^{1}H NMR spectrum of the ethyl acetate extract is shown in Figure 3, where the methyl signal of the 1,4-BTMSB-d_4 IS did not overlap with constituent signals. The signal patterns of compounds **1, 3, 4**, and **5** in their ^{1}H NMR spectra are shown in Figure S2 (see Supporting information). Based on these spectra, the respective amounts of these major constituents in the extract were estimated based on the peak area of their signals, A–D in Figure 3, relative to that of the IS signal. Table 2 summarizes the amounts of these compounds in the ethyl acetate extract, and those converted to the contents in the Xinjiang licorice root material (0.12–1.15 w/w %). The result for licochalcone A (**1**) based on the ^{1}H qNMR method

$(1.15 \pm 0.02\%)$

was also substantiated by HPLC

$$(1.19 \pm 0.05\%)$$

as discussed in the Materials and methods section, although some minor constituent peaks may have overlapped with the large HPLC peak of this compound. The composition profile obtained from the qNMR analysis could be utilized for quality control of Xinjiang licorice materials. Further investigation of Xinjiang licorice roots from various markets is required to confirm its applicability. Comparisons of the values for the other major constituents with the HPLC data were difficult because the profile suggested that the peaks partly overlapped those of many minor constituents.

^{13}C NMR analysis

^{1}H qNMR measurements of plant extracts are often affected by a severe overlapping of signals due to their co-existing constituents. Signal separation in the ^{13}C NMR spectrum has been widely recognized based on the wide range of chemical shifts (ca. 250 ppm), and quantitative ^{13}C NMR has also been applied for use in natural materials [38]. In contrast, in ordinary ^{13}C measurements, the intensities of respective

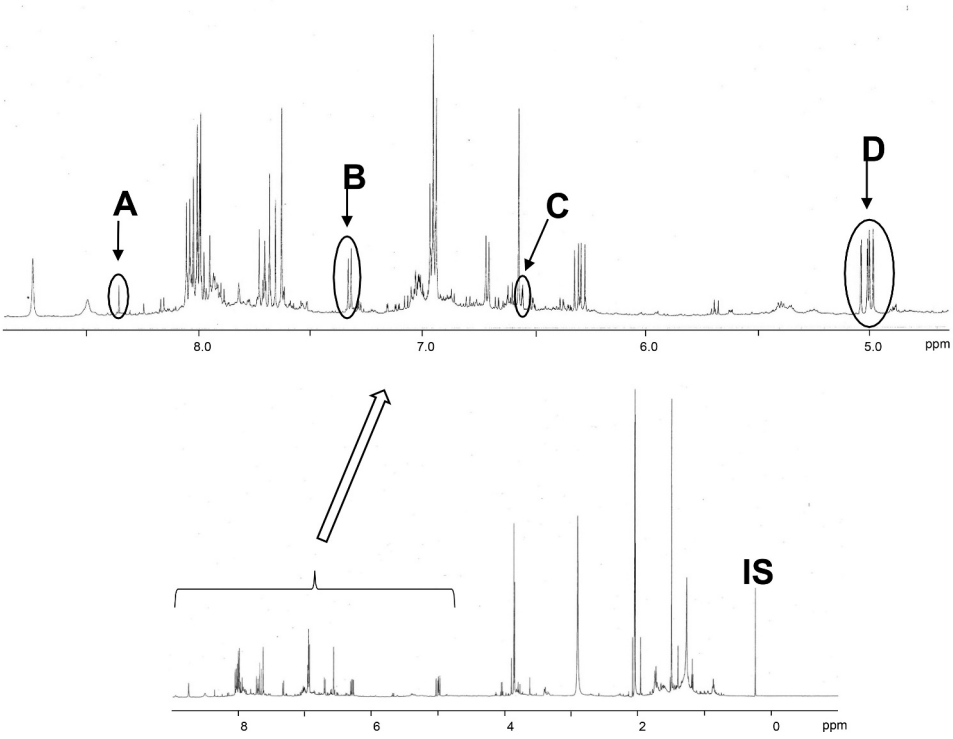

Figure 3. The ^{1}H qNMR spectrum of the ethyl acetate extract from Xinjiang licorice in the presence of 1,4-BTMSB-d_4 (IS). Signals A, B, C, and D on the expanded spectrum are attributed to H-2 of glabrone (**4**), H-6 of licochalcone B (**3**), H-3 of echinatin (**5**), and H-3″ (2 × H) of licochalcone A (**1**), respectively.

signals for a compound are not homogeneous because of the different nuclear Overhauser effects due to the irradiation of protons in the ^{13}C NMR measurements and different relaxation times of respective ^{13}C signals. However, the relative intensities of the ^{13}C signals of the same functional groups in an analogous environment are considered to be approximately proportional to the concentrations of the compounds in a mixture, unless some molecular interactions occur between the compounds. If signal intensities are indicative of the relative amounts of the constituents in these cases, ^{13}C NMR measurements may semi-quantitatively substantiate ^{1}H qNMR results when constituents with structures containing analogous functional groups are compared. In this context, we examined the applications of the ^{13}C NMR spectrum of the extract to compare the signal intensities of compounds with analogous structures.

The ^{13}C NMR spectrum of the Xinjiang licorice extract is shown in Figure 4, and the regions that are characteristic of the three chalcones, **1, 3**, and **5**, are expanded as indicated in the figure (See also Figure S3 in Supporting Information). The methoxy carbon signals of licochalcone A (**1**) and echinatin (**5**) at around δ 60 overlapped with each other, while the C-β position carbon signals of the α–β unsaturated ketone structures of all three chalcones at around δ 140 were well separated. Although the ketonic carbon signals in the region δ 180–190 were also separated, the signal-to-noise

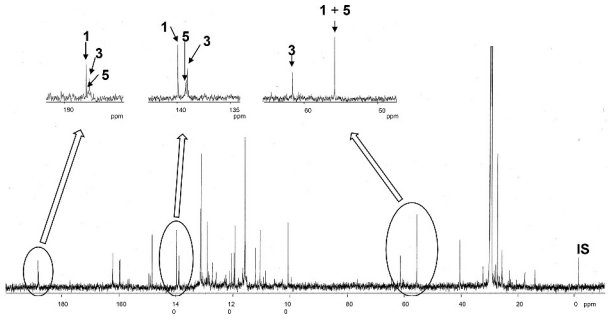

Figure 4. ^{13}C NMR spectrum of the Xinjiang licorice ethyl acetate extract. Ketone signals, C-β signals, and methoxyl signals appearing at around δ 180–190, δ 140, and δ 60, respectively, were observed. Compound numbers are given to the signals in the expanded spectrum.

(S/N) ratio of the echinatin (**5**) signal in this region (ca. 2–3) was too low to estimate its quantity. The ^{13}C signal of glabrone (**4**) was not used for estimation because of its low content, and also the large differences in its structural factors compared to the chalcones. Based on the signal intensities of the three regions, the molar ratios of the three chalcones were estimated as shown in Table 2. The relative molar ratio values from the ^{13}C NMR spectrum were similar to those estimated from the ^{1}H qNMR method. This suggests that ^{13}C NMR measurements can be applied for substantiating results from the ^{1}H qNMR method in a limited way and in a semi-quantitative manner.

Table 2. Licochalcone A (**1**), licochalcone B (**3**), glabrone (**4**), and echinatin (**5**) concentrations estimated by the ^{1}H qNMR and ^{13}C semiquantitative NMR analyses.

	Licochalcone A	Licochalcone B	Glabrone	Echinatin
Content in ethyl acetate extract (%) [a]	22.5 ± 0.39	12.4 ± 0.06	2.37 ± 0.39	4.48 ± 0.21
Content in licorice root (%) [a]	1.15 ± 0.020	0.634 ± 0.0033	0.121 ± 0.011	0.228 ± 0.020
Relative molar ratio based on ^{1}H qNMR (Licochalcone A = 1)	1.00	0.65	0.11	0.25
Relative molar ratio based on ^{13}C semiquantitative NMR (Licochalcone A = 1)	1.00	0.57	- [b]	0.29

[a]Mean value and SD based on triplicate measurements of ^{1}H qNMR.
[b]Signals attributable to this compound [28] were too small to be estimated or were overlapped by those of other constituents in the ^{13}C NMR spectrum of the extract.

Effects of Xinjiang licorice constituents on AChE activity

The inhibitory effects of the licorice flavonoids, 7-*O*-methyllutone and licoricidin, isolated from *G. uralensis*, on AChE activity have been reported previously [22]. The inhibitory effect of glycyrrhizin, which is the main constituent of *Glycyrrhiza* roots, on this enzyme has also been described [23]. We therefore examined the effects of two major chalcones from Xinjiang licorice on this enzyme activity, in comparison with that of glycyrrhizin (**14**).

The results are shown in Table 3. Licochalcone A (**1**), as well as glycyrrhizin (**14**), had inhibitory effects on AChE at concentrations of 10–1000 μM. Licochalcone B (**3**) had a weaker effect on the enzyme. However, the potentiating effect of glycyrrhizin at high concentrations was also observed.

The MeOH extract displayed weak inhibition at 10 μg/mL, but it did not inhibit enzyme activity at all at higher concentrations of the extract. The effect of the extract was not simply explainable by its main constituent of glycyrrhizin (14.1 w/w % in the MeOH extract). The ethyl acetate extract did not have an inhibitory effect on the enzyme at concentrations of 10–1000 μg/mL, despite the high licochalcone A (**1**) concentration in the extract (22.5 w/w % in the extract).

Table 3. Effects of Xinjiang licorice constituents and extracts on AChE activity.

Compound/Extract	Concentration	AChE activity (%) [a] ± SD
Tacrine [b]	50 μM	12.8 ± 0.94 [c]
Glycyrrhizin	10 μM	74.9 ± 3.00 [c]
	100	71.7 ± 7.17 [c]
	1000	68.0 ± 4.93 [c]
	10,000	114.8 ± 4.86 [c]
Licochalcone A	10 μM	85.2 ± 5.12 [c]
	100	89.9 ± 4.83 [c]
	1000	62.4 ± 0.36 [c]
Licochalcone B	10 μM	90.6 ± 1.92 [c]
	100	91.1 ± 1.77 [c]
	1000	94.9 ± 0.81 [c]
MeOH extract	10 μg/mL	77.0 ± 2.80 [c]
	100	99.7 ± 0.01
	1000	107.0 ± 1.41[c]
Ethyl acetate extract	10 μg/mL	99.3 ± 1.04
	100	112.8 ± 3.18 [c]
	1000	120.6 ± 2.92 [c]

[a]Means based on triplicate experiments.
[b]Used for the positive control.
[c]Significant difference from the control, $p < 0.05$.

We previously examined the effects of *Acorus* rhizome herbal drug extracts and the constituents on the AChE enzyme activity, and found the co-existence of constituents with inhibitory effects and a constituent with an enhancing effect in the extract. Analogously, some constituents with enhancing effects on enzyme activity might also be present in the Xinjiang licorice ethyl acetate extract, although we have not yet identified any such constituents. These results suggested the importance of tests using not only extracts but also isolated compounds. Further research is needed to identify constituents with different effects in licorice extracts.

Conclusions

The findings in the present study suggested the utility of the combination of HPLC profiling and qNMR analyses for quality control of Xinjiang licorice. Thirteen flavonoid constituents in an ethyl acetate extract of Xinjiang licorice, which is derived from *G. inflata*, were isolated and identified from spectral analyses. These compounds were assigned to distinctive HPLC peaks. Based on these results, we also examined the applicability of ^{1}H qNMR analysis for simultaneous estimations of the major constituents in the licorice extract, to demonstrate its utility for quality control of Xinjiang licorice, although future examinations of materials from a wider range of origins may be required. We also revealed a moderate inhibitory effect of a major functional constituent, licochalcone A (**1**), on AChE activity as an additional pharmacological effect, and demonstrated the importance of the plant material as a source of candidates for drug development.

Disclosure statement

Y. Lian is an employee of Chenguang Biotech Group Co., Ltd.

Author contributions

A.M. contributed isolation of constituents, structure elucidation, qNMR measurements, and AChE assay. Eerdunbayer and Y.L. performed preliminary experiments, sample preparations for the experiments, and also provided examined materials. T.S. and H. T. contributed mass spectral measurements including LC-MS. Eerdunbayer and S.T. searched the literature and designed the study. T.H. prepared and edited the

manuscript. All authors have read and approved the final manuscript.

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