Supplementary Files

Identification of key licorice constituents which interact with cytochrome P450: Evaluation by LC/MS/MS cocktail assay and metabolic profiling

Running head: Key licorice constituents to interact with CYP450.

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Experimental

LC/MS fingerprint analysis of licorice extracts. HPLC-DAD-MS^n analysis was performed on an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) coupled with an LCQ Advantage ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) *via* an electrospray ionization (ESI) interface. The HPLC eluent was introduced into ESI source of mass spectrometer in a post-column splitting ratio of 4:1. For HPLC analysis, samples were separated on an Atlantis T3 column (5 μm, ID 4.6 mm × 250 mm) equipped with an XTerra MS C_{18} guard column (5 μm, ID 3.9 mm × 20 mm) (Waters, MA, USA). The mobile phase consisted of acetonitrile (A) and water containing 0.1% (*v*/*v*) formic acid (B). A gradient program was used as follows: 0 min, 5% A; 8 min, 19% A; 15 min, 29% A; 23 min, 29% A; 23.1 min, 30% A; 25 min, 30% A; 30 min, 45% A; 45 min, 55% A; 55 min, 65% A; 70 min, 95% A; 80 min, 95% A. The flow rate was 0.7 mL/min. The column temperature was 40 °C. The sample injection volume was 10 μL. For MS detection, high purity nitrogen and ultra-high purity helium were used as the sheath/auxiliary gas and the collision gas. The ESI source was operated in the negative ion mode. The parameters were as follows: source voltage, 4.5 kV; sheath gas (N_2), 50 arbitrary units; auxiliary gas (N_2), 10 units; capillary temperature, 320 °C; capillary voltage, -13 V; tube lens offset voltage, -60 V. For full scan MS analysis, spectra were recorded in the range of *m/z* 130-1200. Data were analyzed using Xcalibur™ 1.4 software (Thermo Fisher).

LC/IT-TOF-MS analysis of licorice extracts. To confirm the elemental composition of licorice compounds with high-accurate mass, liquid chromatography coupled with ion trap-time of flight mass spectrometry (LC/IT-TOF-MS) was employed. The system consisted of an LC-20AD pump, an SIL-20AC autosampler, a CTO-20A column oven, an ESI source, and an IT-TOF mass spectrometer (Shimadzu, Tokyo, Japan). The HPLC conditions were the same as above. MS parameters were set as follows: collision and cooling gas, high purity argon (Ar); nebulizing gas, high purity nitrogen (N_2, 1.5 L/min); drying gas (N_2) pressure, 100 kPa; electrospray ionization, negative mode; curved desolvation line temperature, 200°C; interface voltage, -3.5 kV; detector voltage, 1.70 kV; endcap acceleration, 3.5 V; flight tube voltage, +7.0 kV negative mode); CID energy, 50%; CID gas, 50%. MS and MS^n scan range, *m/z* 220-1200; precursor ion isolation, 3.0 Th. Instrument calibration was performed with a methanol solution of vitamin B_{12} ($C_{63}H_{88}CoN_{14}O_{14}P$). The data were recorded and processed by the LC/MS solution V3.41 software, including a chemical formula predict program.

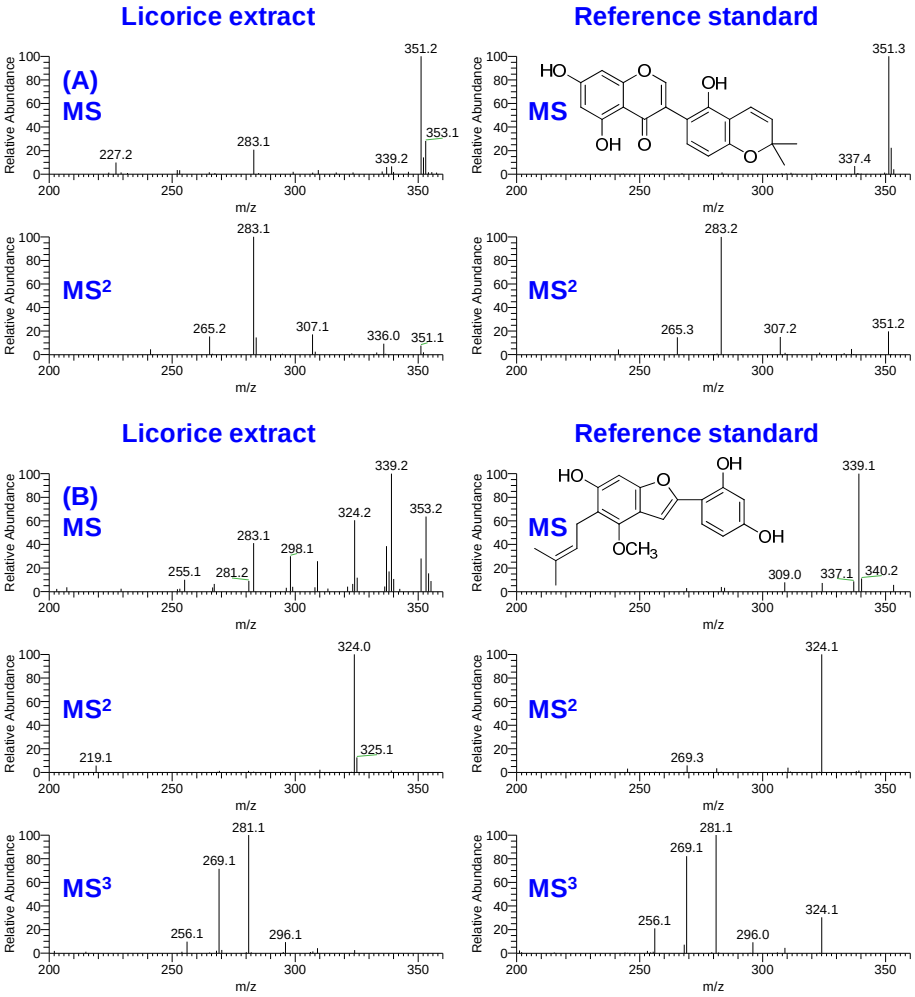


Figure 1S.Chemical structure and tandem mass spectrometry of **C15** (A) and **C17** (B), in comparison of authentic standards.

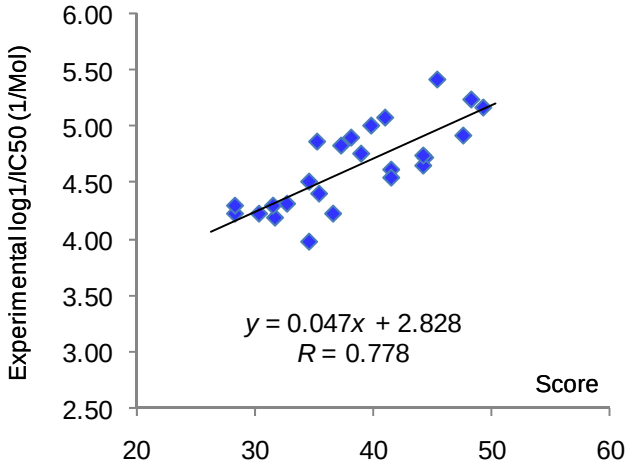


Figure 2S. Linear correlation of GOLD score and experimental log1/IC_{50} for CYP 2C19 inhibitors.

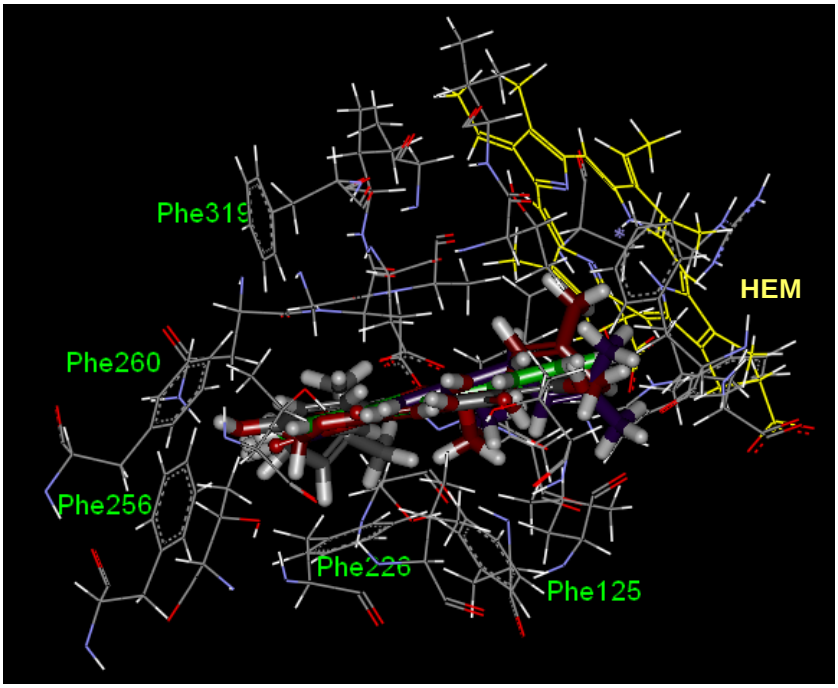


Figure 3S. Docking simulation of **28**, **30**, **31** and α-naphthoflavone (positive control) with CYP 1A2 protein.

Licorice compounds were marked in different colors: isolicoflavonol (**28**, gray), glycyrol (**30**, red), glyurallin A (**31**, purple), α-naphthoflavone (positive control, green). HEM stands for the heme group.

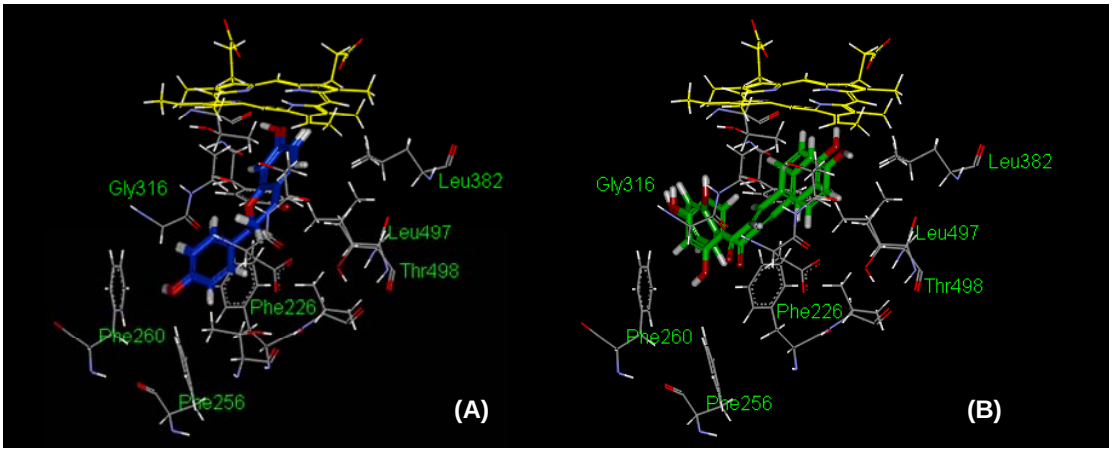


Figure 4S. Molecular docking of liquiritigenin (**9**, A) and isoliquiritigenin (**20**, B) with CYP 1A2.

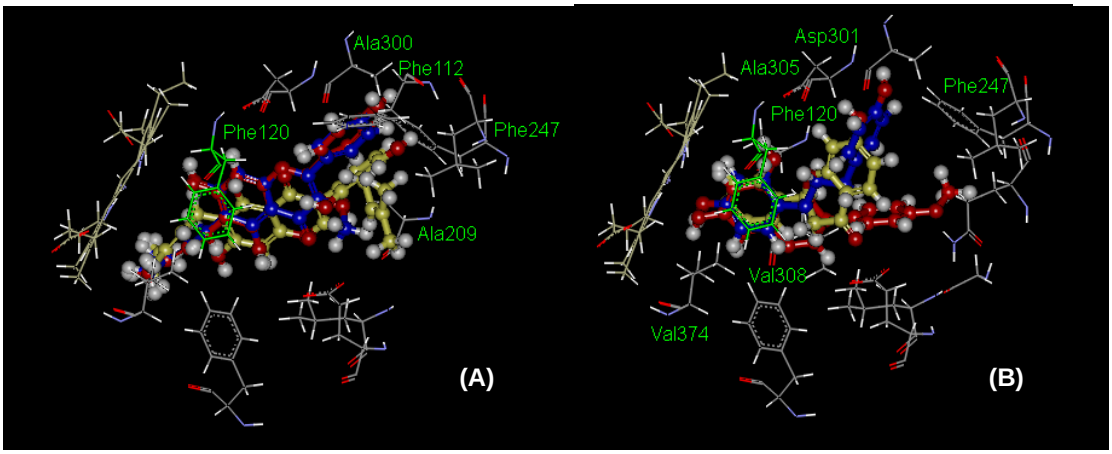


Figure 5S. Molecular docking of 2D6 inhibitors.

(A) prenylated flavonoids **32** (red), **33** (blue), and **36** (yellow); (B) unprenylated flavonoids **9** (yellow), **18** (blue), and **22** (red).

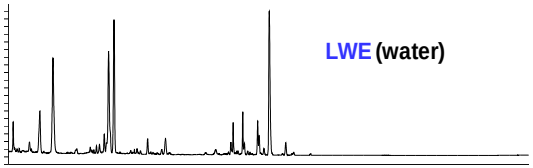
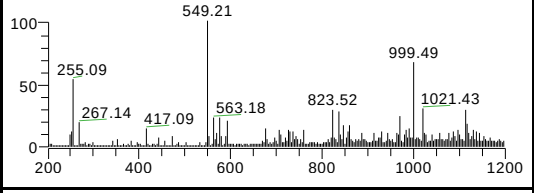
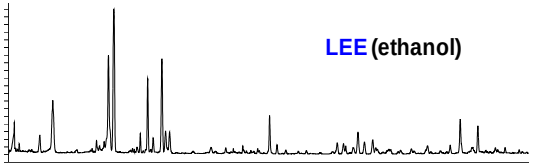
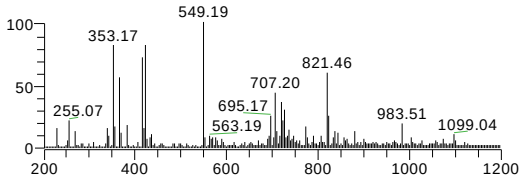
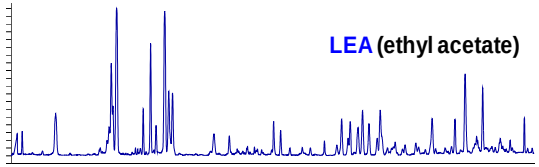
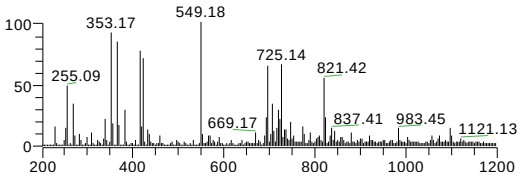
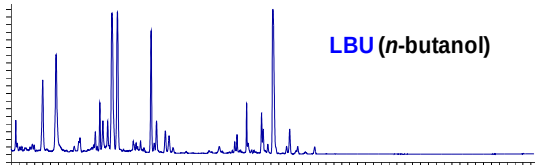
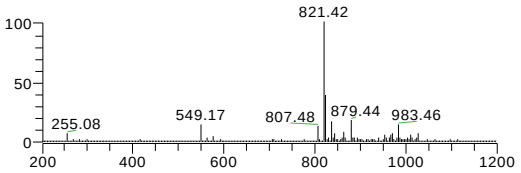
Extract	Constituent in LCD	LC/UV analysis (Figure 5)	LC/MS analysis (average 5-70 min)	MW used	Extraction yeild
LWE	170 agents reported from *G. uralensis*. Average MW was 453.			500	36.3%
LEE				500	22.8%
LEA				500	9.0%
LBU	40 saponins reported from *G. uralensis*; Average MW was 693.			700	12.2%

Figure 6S. LC/MS analysis of licorice extracts to estimate their mean molecular weights.

LCD, licorice chemical database (please refer to *Drug Metab Disp.* **2012**, *39*, 1597, and *Acta Pharmaceutica Sinia* **2012**, *47*, 1023); LWE, licorice water extract; LEE, licorice ethanol extract; LBU, licorice *n*-butanol fraction; LEA, licorice ethyl acetate fraction; The extraction yield refers to the total weight of constituents extracted from the crude drug, divided by the original weight of the drug before extraction.

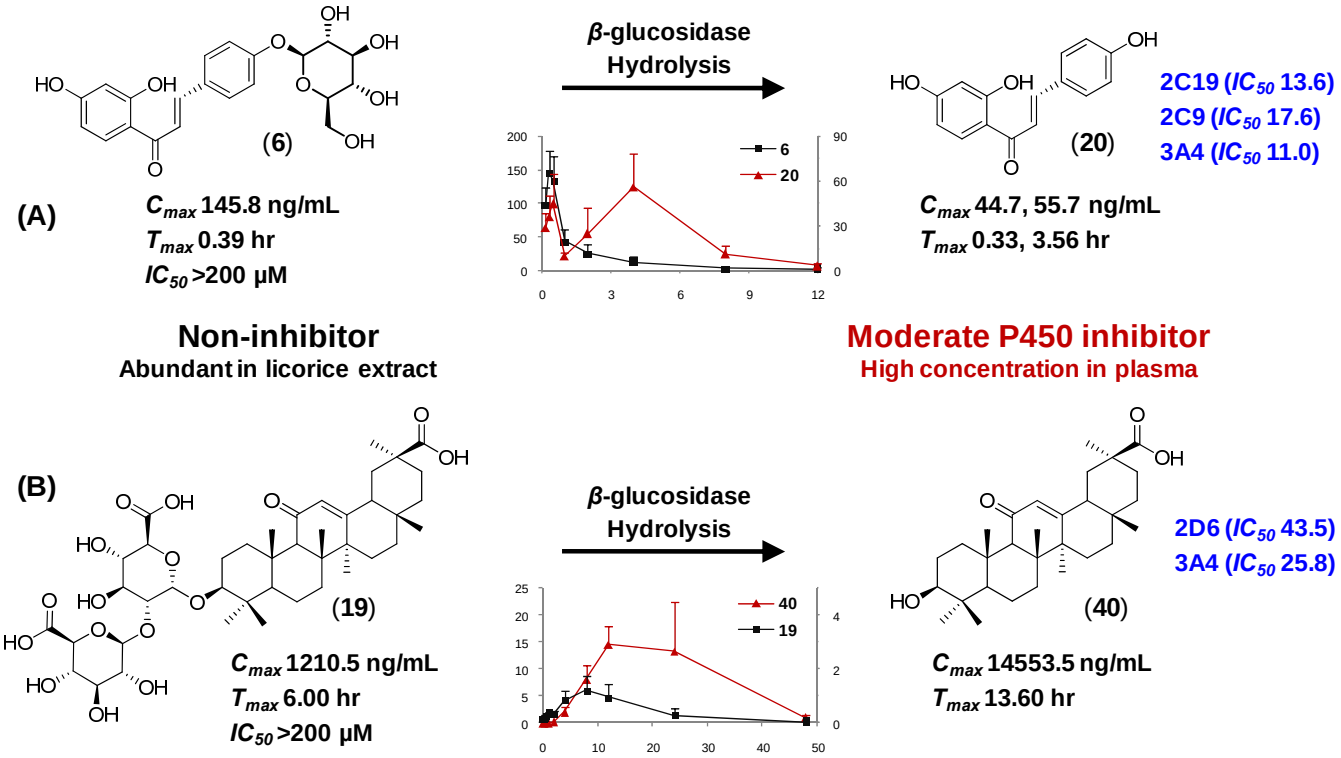


Figure 7S. Proposed *in vivo* P450 action for isoliquiritin (**6**, A) and glycyrrhizic acid (**19**, B).

PK parameters and curves were obtained in our previous study (20).

Table 1S. Interday and intraday variation of the analytical method.

	Analyte					
	dEtPHE	OHOME	OHTOL	dMeDEX	OHMID	OHTES
LLOQ (nM)						
NC	25.0	1.00	20.0	1.26	1.00	50.0
intraday MC	26.5	0.95	20.4	1.27	1.06	53.0
A	106	94	102	101	106	106
RSD%	9.0	6.8	8.8	7.8	6.8	3.6
interday MC	26.8	0.91	20.6	1.18	1.08	54.9
A	107	91	103	94	108	110
RSD%	8.3	8.8	8.7	8.1	10.4	6.3
MQC (nM)						
NC	125	5.00	100	6.32	5.00	250
intraday MC	127	4.90	101	6.08	5.13	258
A	102	98	101	96	103	103
RSD%	3.2	3.9	4.1	4.2	4.1	3.0
interday MC	136	5.03	102	6.23	5.21	263
A	109	101	102	99	104	105
RSD%	0.8	5.5	4.6	8.0	4.2	4.0
HQC (nM)						
NC	500	20.0	400	25.3	20.0	1000
intraday MC	480	18.6	415	25.0	18.3	1050
A	96	93	104	99	91	105
RSD%	9.6	9.5	4.7	4.8	7.0	4.3
interday MC	477	19.1	423	26.0	19.4	1081
A	95	96	106	103	97	108
RSD%	6.7	6.3	4.0	6.0	8.1	9.3

HQC, quality control sample in high concentration; MQC, quality control sample in middle concentration; LLOQ, quality control sample near lower limit of quantitation.

NC, nominated concentration; MC, measured concentration; A, accuracy in %.

Abbreviations for analytes were consistent in following tables, and in the manuscript. dEtPHE, acetaminophen (metabolite of phenacetin by 1A2); OHOME, 5-hydroxyomeprazole (metabolite of omeprazole by 2C19); OHTOL, hydroxytolbutamide (metabolite of tolbutamide by 2C9); dMeDEX, dextrorphan (metabolite of dextromethorphan by 2D6); OHMID, 1'-hydroxymidazolam (metabolite of midazolam by 3A4/5); OHTES, 6β-hydroxytestosterone (metabolite of testosterone by 3A4/5).

Table 2S. MS parameters to analyze the metabolites of probe substrates.

Compd	Parent	Product_1	CE_2	Product_2	CE_2	TLO	SID	Scan time
CIM	253.2	117.1	27	159.1	23	100	10	0.1
dEtPHE	152.1	110.2	15	65.2	30	100	5	0.1
dMeDEX	258.2	157.1	40	199.1	30	100	5	0.1
OHTOL	287.2	171.1	20	107.2	25	100	5	0.1
OHTES	305.3	269.1	15	287.2	15	100	15	0.1
OHMID	342.1	324.1	20	203.2	25	100	5	0.1
OHOME	362.2	214.1	10	152.1	35	80	5	0.2

Parent and product refers to ion pairs in SRM transition. “1”and “2” refers to quantitative and qualitative (backup) product ions. CE, collision energy (%); TLO, tube lens offset (V); Scan time (second). CIM refers to the internal standard Cimetidine. Abbreviation of probe metabolites please refer to Note of Table 1S.

Table 3S. Calibration curves for probe metabolites.

Compd	*y*-Intercept	Slope	Correlation coefficient (r^2)
dEtPHE	0.292	0.0115	0.991
OHOME	-0.00154	0.0732	0.992
dMeDEX	0.114	0.0746	0.994
OHMID	-0.0290	0.114	0.991
OHTOL	-0.00500	0.00175	0.990
OHTES	-0.00773	0.000360	0.992

Note: Abbreviation of probe metabolites please refer to Note of Table 1S.

Table 4S. Matrix effect and extraction efficiency of the method.

	Analyte					
	dEtPHE	OHOME	OHTOL	dMeDEX	OHMID	OHTES
LLOQ (nM)						
NC	25.0	1.00	20.0	1.26	1.00	50.0
Extract MC	24.5	1.01	21.1	1.28	0.95	54.4
Matrix MC	27.5	1.03	21.4	1.28	1.13	56.5
MeOH MC	28.7	1.17	20.9	1.33	1.12	58.4
REC%	98	100	106	102	95	109
EE%	89	98	99	100	84	96
ME%	96	88	102	96	100	97
MQC (nM)						
NC	125	5.00	100	6.32	5.00	250
Extract MC	118	4.86	101	5.96	5.13	262
Matrix MC	140	5.35	108	6.05	5.29	273
MeOH MC	148	5.80	112	6.37	5.51	281
REC%	94	97	100	94	103	105
EE%	84	91	94	98	97	96
ME%	94	92	96	95	96	97
HQC (nM)						
NC	500	20.0	400	25.3	20.0	1000
Extract MC	456	17.7	385	25.0	17.8	1040
Matrix MC	497	19.6	430	26.6	21.0	1078
MeOH MC	532	23.2	441	28.4	22.2	1146
REC%	91	88	96	99.1	89.2	104
EE%	92	90	90	94.3	84.9	96
ME%	94	85	97	93.7	94.9	94

HQC, quality control sample in high concentration; MQC, quality control sample in middle concentration; LLOQ, quality control sample near lower limit of quantitation.

Extract, standards were extracted following the preparation of QC samples; Matrix, standards were added to matrix; MeOH, standards were added into methanol. NC, nominated concentration; MC, measured concentration. EE%, extraction efficiency; ME%, matrix effect; REC%, recovery. Calculation of these rates please refer to the "*Instrument method validation*" section.

Abbreviation of probe metabolites please refer to Note of Table 1S.

Table 5S. Stability of the analytes.

	dEtPHE	OHOME	OHTOL	dMeDEX	OHMID	OHTES
L	0.34 ± 0.05	0.29 ± 0.00	0.12 ± 0.01	0.86 ± 0.11	2.28 ± 0.01	0.30 ± 0.01
FT	0.30 ± 0.03	0.28 ± 0.03	0.12 ± 0.01	0.87 ± 0.07	2.29 ± 0.21	0.30 ± 0.03
V %	-10.3	-3.6	-3.4	1.4	0.3	-2.1
LS	0.30 ± 0.01	0.28 ± 0.01	0.11 ± 0.01	0.88 ± 0.05	2.19 ± 0.15	0.29 ± 0.02
V %	-10.1	-6.1	-9.8	2.8	-3.7	-2.8
AT	0.30 ± 0.03	0.31 ± 0.01	0.11 ± 0.01	0.94 ± 0.02	2.55 ± 0.10	0.32 ± 0.02
V %	-10.6	6.0	-7.6	9.7	11.9	6.8
H	0.98 ± 0.13	0.82 ± 0.04	0.32 ± 0.02	2.82 ± 0.26	6.44 ± 0.29	0.80 ± 0.09
FT	0.94 ± 0.13	0.84 ± 0.02	0.36 ± 0.02	2.65 ± 0.22	6.52 ± 0.55	0.89 ± 0.05
V %	-3.4	2.7	11.9	-6.1	1.2	10.6
LS	0.91 ± 0.02	0.84 ± 0.02	0.32 ± 0.01	2.56 ± 0.14	6.36 ± 0.22	0.90 ± 0.11
V %	-6.7	2.9	1.4	-9.1	-1.2	11.6
AT	0.99 ± 0.05	0.85 ± 0.02	0.35 ± 0.01	2.63 ± 0.02	6.67 ± 0.14	0.91 ± 0.02
V %	1.4	3.8	8.5	-6.8	3.6	13.5

Note: data were presented in the form of relative peak area (analyte/internal standard) ± standard deviation; L, low concentration; H, high concentration; V, variation in %. FT, freeze-thaw; AT, ambient temperature; LS, long storage. Abbreviation of probe metabolites please refer to Note of Table 1S.

Table 6S. UV and MS spectra data of chemical constituents in *Glycyrrhiza uralensis*.

No.	UV	Tandem mass spectrometry (% of intensity)
C1	226, 276 (m)	MS^2[593]: 575(7), 503(30), 473(100), 383(19), 353(33)
		MS^3[593-->473]: 383(19), 353(100)
		MS^3[593-->353]: 353(20), 325(100), 297(11)
C2	226, 276 (m)	MS^2[695]: 485(100), 441(6)
		MS^3[695-->485]: 441(100), 397(48)
		MS^3[695-->441]: 397(100)
C3	224, 240	N/A
C4	230, 274 (m)	MS^2[433]: 313(9), 271(100)
		MS^3[433-->271]: 177(12), 169(6), 151(100)
		MS^4[433-->271-->151]: 107(100)
C5	226, 290	MS^2[433]: 271(100)
		MS^3[433-->271]: 177(31), 165(6), 151(100), 93(6)
		MS^4[433-->271-->151]: 107(100)
C6	242, 372	MS^2[549]: 429(72), 297(17), 255(100)
		MS^3[549-->255]: 153(100), 135(60), 119(10)
		MS^4[549-->255-->153]: 153(100)
C7	232, 288, 314 (m)	MS^2[695]: 549(100), 531(65), 255(7)
		MS^3[695-->549]: 429(16), 417(20), 297(16), 255(100)
		MS^4[695-->549-->255]: 153(100), 135(37), 119(6)
C8	200, 238, 370	MS^2[725]: 549(100), 531(64), 417(6), 399(9), 255(9)
		MS^3[725-->549]: 417(18), 297(15), 255(100)
		MS^4[725-->549-->255]: 153(100), 135(75)
C9	202, 232, 322 (m)	MS^2[695]: 549(75), 531(100), 255(6)
		MS^3[695-->531]: 399(33), 297(9), 255(100), 254(7)
		MS^4[695-->531-->255]: 153(100), 135(59), 119(7)
C10	202, 232, 322 (m)	MS^2[725]: 549(47), 531(100)
		MS^3[725-->531]: 399(17), 297(6), 255(100)
		MS^4[725-->531-->255]: 153(100), 135(95)
C11	242, 286	MS^2[367]: 367(18), 352(54), 335(20), 309(38), 298(100), 297(6), 283(15)
		MS^3[367-->298]: 283(100), 267(25), 255(7)
		MS^4[367-->298-->283]: 255(100), 239(14)
C12	232, 286	MS^2[369]: 229(100), 139(8)
		MS^3[369-->229]: 229(38), 174(100)
		MS^3[369-->139]: 124(100)
C13	204, 234, 286 (m)	MS^2[807]: 807(9), 764(10), 763(22), 598(7), 351(100)
		MS^3[807-->351]: 193(100), 175(17)
		MS^4[807-->351-->193]: 71(100)
C14	204, 234, 286 (m)	MS^2[367]: 367(46), 352(9), 337(81), 323(8), 298(100)
		MS^3[367-->298]: 297(9), 284(7), 270(100), 269(11), 268(10), 148(16)
		MS^4[367-->298-->270]: 255(100)
C15	232, 286	MS^2[351]: 351(9), 336(7), 307(16), 283(100), 265(13)
		MS^3[351-->283]: 283(68), 265(18), 255(22), 241(16), 239(100), 175(63)

		MS^4[351-->283-->239]: 240(39), 239(100), 209(74), 197(52)
C16	206, 230, 288	MS[Full ms]651(6), 547(15), 355(42), 229(100)
		MS^2[355]: 229(100), 125(16)
		MS^3[355-->229]: 229(45), 174(100)
		MS^2[229]: 229(37), 174(100)
		MS^3[229-->174]: 146(100), 136(10)
C17	228, 322, 334 (m)	MS^2[339]: 324(100), 310(8), 165(32)
		MS^3[339-->324]: 296(9), 281(100), 269(71), 256(10)
		MS^4[339-->324-->281]: 281(78), 253(100), 237(25)
C18	228, 322, 334 (m)	MS^2[353]: 353(72), 338(100), 337(12), 321(47), 310(9), 309(6), 298(21), 297(26), 295(11), 284(38), 283(13), 270(6)
		MS^3[353-->338]: 338(11), 323(31), 310(10), 295(100), 294(7), 283(28), 270(41), 241(10)
C19	266, 332	MS^2[337]: 337(100), 282(53)
C20	236, 276, 312	MS^2[261]: 246(100)
		MS^3[261-->246]: 231(100)
		MS^4[261-->246-->231]: 176(100)
C21	258	MS^2[335]: 335(100), 320(21), 317(7)
C22	202, 228, 286	MS^2[423]: 229(100), 193(46)
		MS^3[423-->229]: 229(45), 174(100)
		MS^4[423-->229-->174]: 146(100)
C23	204, 216, 230, 282	MS^2[437]: 405(100), 221(10), 215(24), 203(19), 177(7)
		MS^3[437-->405]: 405(100), 390(8), 347(47), 335(9)

Note, m refers to multiple UV absorption wavelengths due to overlapped peaks. The analytical method was described in Supplementary Notes.

Table 7S. Identification and predicted IC_{50} ranges for minor licorice compounds.

	Identification	1A2	2C9	2C19	2D6	3A4
C1	apigenin 6,8-di-*C*-glucopyranoside	>200	>200	>200	50-200	10-50
C2	licorice glycoside D1	>200	>200	>200	>200	10-50
C3	naringenin 5-glucoside	10-50	50-200	50-200	10-50	10-50
C4	naringenin 7-glucoside	<10	<10	<10	10-50	10-50
C5	naringenin 4'-glucoside	>200	>200	50-200	10-50	10-50
C6	liquiritigenin-7-*O*-*β*-*D*-apiofuranosyl-4'-*O*-*β*-*D*-glucopyranoside	>200	50-200	>200	50-200	10-50
C7	licorice glycoside D2	>200	>200	10-50	>200	10-50
C8	licorice glycoside C2	>200	>200	>200	>200	50-200
C9	licorice glycoside B	>200	>200	>200	>200	10-50
C10	licorice glycoside A	>200	>200	>200	50-200	10-50
C11	isoglycycoumarin	<10	<10	10-50	10-50	10-50
C12	glycyrrhiza isoflavone A	<10	<10	10-50	10-50	10-50
C13	licoricesaponin B2	>200	>200	>200	>200	>200
C14	glycyrrhisoflavanone	10-50	<10	<10	10-50	10-50
C15	licoisoflavone B	10-50	<10	10-50	<10	10-50
C16	glyasperin C	<10	<10	10-50	10-50	10-50
C17	licocoumarone	<10	<10	<10	<10	10-50
C18	licobenzofuran	<10	<10	<10	<10	10-50
C19	wighteone	<10	10-50	<10	10-50	10-50
C20	3-methoxy-2-(3-methylbut-2-enyl)-5-(2-pentyl)phenol	<10	<10	10-50	10-50	50-200
C21	glabrone	<10	<10	<10	<10	10-50
C22	glisoflavanone	>200	<10	50-200	10-50	10-50
C23	kanzonol T	>200	>200	10-50	10-50	10-50