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Isolation, identification and antiviral activities of metabolites of calycosin-7-*O*-*β*-D-glucopyranoside

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ABSTRACT

In vivo and *in vitro* metabolites of calycosin-7-*O*-*β*-D-glucopyranoside in rats were identified using a specific and sensitive high performance liquid chromatography–tandem mass spectrometry (HPLC–MS^n) method. The parent compound and twelve metabolites were found in rat urine after oral administration of calycosin-7-*O*-*β*-D-glucopyranoside. The parent compound and six metabolites were detected in rat plasma. In heart, liver, spleen, lung and kidney samples, respectively, six, eight, seven, nine and nine metabolites were identified, in addition to the parent compound. Three metabolites, but no trace of parent drug, were found in the rat intestinal flora incubation mixture and feces, which demonstrated cleavage of the glycosidic bond of the parent compound in intestines. The main phase I metabolic pathways of calycosin-7-*O*-*β*-D-glucopyranoside in rats were deglycosylation, dehydroxylation and demethylation reactions; phase II metabolism included sulfation, methylation, glucuronidation and glycosylation (probably). Furthermore, two metabolites commonly found in rat urine, plasma and tissues were isolated from feces and characterized by NMR. The antiviral activities of the metabolite calycosin against coxsackie virus B_3 (CVB_3) and human immunodeficiency virus (HIV) were remarkably stronger than those of calycosin-7-*O*-*β*-D-glucopyranoside.

1. Introduction

Calycosin-7-*O*-*β*-D-glucopyranoside is the main isoflavonoid compound isolated from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao, which is a popular Chinese herbal compound long used in China to treat chronic diarrhea, prolapse of the rectum, hematochezia and abnormal uterine bleeding, edema, anemia, albuminuria in chronic nephritis, as well as diabetes [1]. Investigations on calycosin-7-*O*-*β*-D-glucopyranoside have been conducted in the fields of pharmacology [2–4], pharmacokinetics [5–7] and phytochemistry.

Our research group has focused attention on calycosin-7-*O*-*β*-D-glucopyranoside for several years. In our previous pharmacological research, calycosin-7-*O*-*β*-D-glucopyranoside was shown to display significant antiviral activities against coxsackie virus B_3 (CVB_3) both *in vivo* and *in vitro* [8]. This compound may also be a promising anti-HIV agent given its high therapeutic index with respect to inhibiting a virus-induced cytopathic effect [9]. According to our earlier study on its pharmacokinetics [6], the low cumulative urinary excretion and low concentration of calycosin-7-*O*-*β*-D-glucopyranoside detected in the plasma after oral administration to rats indicated that it might be metabolized by intestinal bacteria, its metabolic mechanisms were various and the hepatic first-pass effect existed. However, researchers still do not know the site of metabolism, the fate of the compound in the body, the amount of metabolites absorbed into blood after oral administration, or its effective metabolites. This limited knowledge about the metabolism of calycosin-7-*O*-*β*-D-glucopyranoside restricts deeper study of its pharmacological mechanism and its wider clinical application. Thus, the aim of this study is to thoroughly profile the *in vivo* metabolic pathways of calycosin-7-*O*-*β*-D-glucopyranoside

Abbreviations: HPLC–MS^n, high performance liquid chromatography–tandem mass spectrometry; CVB_3, coxsackie virus B_3; DMSO, dimethyl sulfoxide; AZT, zidovudine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; HRP, horseradish peroxidase; IgG, immunoglobulin G; Fc, fragment Fc; MAb, monoclonal antibody; DMF, dimethyl formamide; SDS, sodium dodecyl sulfate; SPE, solid phase extraction; ODS, octadecyl silane; MEM, minimum essential medium; CPE, cytopathic effects; $TCID_{50}$, median tissue culture infective dose; TC_0, maximal atoxic concentration; TC_{50}, median toxic concentration; ELISA, enzyme-linked immunosorbent assay; CC_{50}, median cytotoxic concentration; IC_{50}, half maximal inhibitory concentration; TI, therapeutic index; M.O.I., multiplicity of infection; ESI, electrospray ionization; EIC, extracted ion chromatogram.

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in rat urine, feces, plasma and organs; to ascertain the site of glycosidic bond cleavage by evaluating its incubation with intestinal flora *in vitro*; and to find out the actual compounds responsible for its antiviral activities.

2. Materials and methods

2.1. Chemicals and reagents

Calycosin-7-*O*-*β*-D-glucopyranoside was isolated from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by our laboratory. The structure was elucidated by ^{1}H and ^{13}C NMR using a Bruker DRX400 instrument (Bruker, USA), operating at 400 MHz for ^{1}H and ^{13}C spectra. Purity was higher than 99% as checked by HPLC–UV. HPLC grade acetonitrile, methanol and formic acid were purchased from Dikma Company (Dikma, USA). Water was deionized and double distilled. All other analytical grade reagents were from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Macroporous resin D101, silica gel H60, and Sephadex LH-20 were purchased from Anhui Sanxing Resin Technology Co., Ltd. (Anhui, China), Qingdao Haiyang Chemical Co., Ltd. (Shandong, China) and Amersham Biosciences (Amersham Biosciences, Sweden), respectively.

Zidovudine (AZT), N-2-(2-hydroxyothyl) piperazine-N′-(2-ethanesufonic acid) (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl formamide (DMF), sodium dodecyl sulfate (SDS), penicillin, streptomycin sulfate, horseradish peroxidase (HRP)-labeled goat anti-rabbit Immunoglobulin G (IgG) and Fragment Fc (Fc) specific anti-mouse IgG were purchased from Sigma (Sigma, USA). The monoclonal antibody (MAb) to HIV-1 p24 and rabbit polyclonal anti-HIV-1 serum were prepared by Dr. Zheng's laboratory [10].

2.2. Animals

All animal treatments were strictly in accordance with protocols approved by the Review Committee of Animal Care and Use at the Shanghai Institute of Materia Medica (Shanghai, China). Forty male Sprague-Dawley rats (200 ± 10 g; Shanghai SLAC Laboratory Animal Co., Shanghai, China) were acclimatized to the facilities for one week prior to experiments. All animals were fasted (but received water) for 12 h prior to administration of the dose, which was formulated in a mixture of calycosin-7-*O*-*β*-D-glucopyranoside suspended in an aqueous solution of 0.5% carboxymethyl cellulose sodium salt (CMC-Na) at a concentration of 10 mg/mL. The rats received the suspension by gavage administration with a single dosage of 120 mg/kg body weight.

The animals were divided into four groups at random: Group A ($n = 5$) was for post-dose blood collection; Group B ($n = 4$) was for post-dose urine and feces obtained; Group C ($n = 25$) was for post-dose organs withdrawn (with five rats at each time point); Group D ($n = 6$) was for pre-dose samples (i.e., blanks) collection.

2.3. Sample preparation

2.3.1. In vivo samples extraction

Blood samples were collected in heparinized tubes via caudal vein at 0.5, 1.5, 2.5, 3.5, 5, 6.5, 8 and 24 h after the dose, centrifuged at 4000 × *g* for 10 min to obtain the plasma. Samples of urine (0–2, 2–4, 4–6, 6–8, 8–10, 10–24, 24–48 h post-dose) and feces (0–10, 10–24, 24–48 h post-dose) were collected and combined separately. Organs including heart, lungs, liver, spleen and kidneys were removed 2, 4, 6, 8 and 24 h after dosing, washed with a 0.9% NaCl solution, dried with filter paper, weighed and homogenized with 0.1 M phosphate balanced solution (PBS) buffer (pH 7.4) in the ratio of 1 g:5 mL. After centrifugation at 10,000 × *g* for 20 min, the supernatants were separated out. All the samples were stored frozen at −80 °C until analysis.

Urine, plasma and tissue homogenates were pretreated according to the procedures as follows: 600 μL sample was loaded on to a SPE column (1 mL capacity, 100 mg ODS, Supelco, USA) that was preconditioned with 2 mL methanol and 2 mL water subsequently. The cartridge was then washed with 2 mL water and the analytes were eluted with 1 mL methanol. Fecal samples were dried for 24 h at 40 °C and crushed. An aliquot of 0.1 g feces was weighed and extracted by ultrasonication three times with 1 mL methanol for 30 min each time. Extracting solutions with different pre-treatment methods described above were all dried under nitrogen gas over a water bath of 37 °C. The residues were dissolved in 200 μL methanol and centrifuged twice at 12,000 × *g* for 10 min. And 20 μL of the supernatants was injected into the HPLC–MS^n for analysis. Blank samples as control were prepared with the same method as drug containing samples.

2.3.2. In vitro samples extraction

Anaerobic cultural solutions were prepared as literature described [11]. Fresh intestinal contents obtained from three male SD rats in Group D were immediately homogenized with anaerobic culture solution in the ratio of 1 g:4 mL under anaerobic environment. The homogenate was then filtered using gauze. A standard solution of calycosin-7-*O*-*β*-D-glucopyranoside was added into the intestinal flora culture solution described above to a final concentration of 80 μg/mL and cultured in anaerobic incubation bags over a shaking water-bath at 37 °C for 2, 4, 6, 8, 10 and 24 h then growth was terminated by cooling down to 4 °C. After centrifugation at 12,000 × *g* for 10 min, 600 μL of the supernatant was transferred into an SPE column and eluted as described above.

2.4. Instrumentation and conditions

HPLC–MS^n experiments were performed with an Agilent 1200 Series (Agilent, USA) coupled with a Bruker HCT ion trap mass spectrometer (Bruker, USA). The HyStar version 3.2 software was applied for system operation and data collection. Separations of the metabolites were achieved on a reversed-phase column (Apollo-C_{18}, 4.6 mm × 250 mm i.d., 5 μm, Grace, USA) connected to an EasyGuard Kit C_{18} (4 mm × 2 mm, Grace, USA) guard column. The column was maintained at 25 °C. Detection wavelengths were set at 254 nm and flow rate at 0.5 mL/min. The mobile phases consisting of 0.5% aqueous formic acid (A) and acetonitrile (B) were run according to the following profile: 10–40% B from 0 to 10 min, 40% B from 10 to 30 min, 40–70% B from 30 to 35 min, 70–100% B from 35 to 40 min, and 100% B from 40 to 45 min.

MS^n analyses were conducted in positive ion mode and the operating parameters were optimized as follows: collision gas, ultrahigh-purity helium (He); nebulizing gas, high-purity nitrogen (N_2); drying gas flow rate, 8 L/min; drying gas temperature, 350 °C; nebulizer gas pressure, 30 psi; capillary exit voltage, 117.3 V; skimmer voltage, 40.0 V and mass range of 50–1000 *m*/*z*.

Semi-preparative HPLC was performed on a Waters 1525 pump (Waters, USA) equipped with a Waters 2489 detector (254 nm) and an YMC-Pack ODS-A column (250 mm × 10 mm, 3–5 μm, 12 nm, YMC, Japan).

2.5. Isolation and purification of metabolites M11 and M12

In order to ascertain metabolite structure using NMR, larger amounts of metabolites were needed for analysis. Thirty normal SD rats were housed and administrated a dose of 120 mg/kg via gavage twice per day for 20 days. A total of 500 g feces was collected and stored at −80 °C. After drying for 24 h at 40 °C, samples were crushed and extracted with 1500 mL of methanol three times.

The eluent was evaporated to dryness, applied onto the macroporous resin D101, and eluted with H_2O/EtOH in a stepwise manner. The 50% EtOH eluate was subjected to Sephadex LH-20 using an EtOH system and collected in three fractions labeled A, B, and C. After A and B were subjected to silica gel chromatography with a CH_2Cl_2–MeOH solvent system (from 100:1 to 5:1, v/v), A was fractionated into A1, A2, and A3, and B was fractionated into B1, B2, B3, and B4. All eight fractions were further purified by semi-preparative HPLC.

2.6. Cells and viruses

Hep-2 cells were obtained from the Cell Center, Institute of Basic Medical Sciences, Peking Union Medical College (Beijing, China) and were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Tianjin Haoyang Biological Manufacture Co., Ltd., China), 2 mM glutamine, 10,000 units/mL penicillin, 50 μg/mL streptomycin and 2.5 μg/mL amphotericin B. A Nancy variant of CVB_3, was propagated in monolayers of Hep-2 cells, stored in small test tubes, and frozen at −80 °C. Human T-cell lines C8166 was maintained in RPMI-1640 supplemented with 10% heat-inactivated newborn calf serum (Gibco, USA). HIV-1_{IIIB} was obtained from the culture supernatant of H9/HIV-1_{IIIB} cells. CVB_3 titers were determined to be $10^{-6.5}$ in terms of the viral cytopathic effects (CPE), expressed as the median tissue culture infective dose ($TCID_{50}$). The $TCID_{50}$ in C8166 cells was calculated by the Reed–Muench method [12]. Virus stocks were stored in small aliquots at −80 °C.

2.7. Cytotoxicity assay

Hep-2 cells were cultured in 96-well plates and incubated for 72 h with different dilutions of compounds. The dead cells showed crimple and refractive brightness. The maximal atoxic concentration (TC_0) and median toxic concentration (TC_{50}) were defined by the Reed–Muench method [13].

The cytotoxicity of the compounds on C8166 cells was determined using the MTT colorimetric assay, as described previously [14]. Aliquots of 100 μL/well (4×10^5/mL) C8166 cell suspensions were seeded on a microtiter plate. Next, 100 μL/well of various concentrations of compounds were added, and the plates were incubated at 37 °C and 5% CO_2 for three days. The MTT reagent was added and incubated for 4 h, 100 μL of supernatant was discarded and then 100 μL of 50% DMF–20% SDS was added. After the dissolution of formazan, the plates were read on an enzyme-linked immunosorbent assay (ELISA) reader (Elx800, Bio-Tek, USA) at 570 nm and 630 nm. The median cytotoxic concentration (CC_{50}) was calculated from the dose response curve.

2.8. Antiviral activity

Hep-2 cells were cultured in 96-well plates, exposed to CVB_3 (100 $TCID_{50}$, 100 μL/well) for 1 h, and washed with PBS three times. Infected cells were then incubated for 72 h with different dilutions of chemicals. The infected cells showed obvious CPE characterized by crimple and refractive brightness and quantified by the high proportion of cells with CPE. Maximal viral CPE occurred at 72 h post-infection. Values for IC_{50} and therapeutic index (TI) were defined by the Reed–Muench method.

The inhibitory activities of compounds against the HIV-1_{IIIB} by CPE or p24 were determined as previously described [14–16]. Briefly, 4×10^4 C8166 cells were infected with HIV-1_{IIB} at a multiplicity of infection (M.O.I.) of 0.06 for CPE assay or M.O.I. of 0.1 for 2 h, resuspending the infected cells with 100 μL complete medium for p24 detection. The plates were then incubated in the presence or absence of 100 μL of various concentrations of compounds at 37 °C and 5% CO_2. AZT was used as a positive drug control. After three days of culture, either the percentage of inhibited syncytia formation was scored, or the level of p24 was measured by ELISA. From these measurements, the IC_{50} value was calculated.

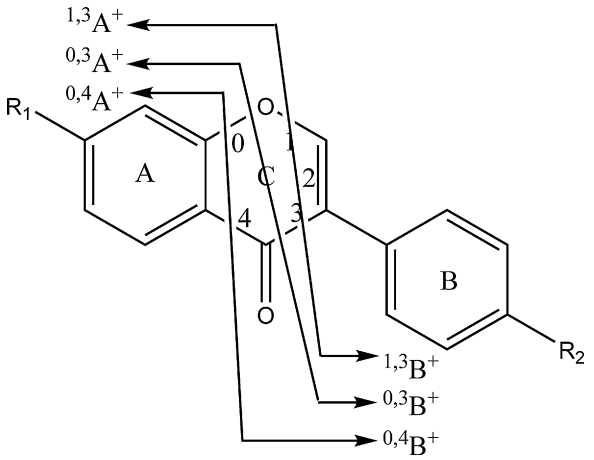

Fig. 1. Nomenclature adopted for cross-ring cleavages of isoflavones.

3. Results

3.1. LC–MS^n analysis of calycosin-7-O-β-D-glucopyranoside

Since the basic sub-structure of the parent drug could be retained in metabolites, the first step of this study focused on the characterization of the chromatographic and mass spectrometric properties of the parent drug. The resulting data provided a structural template for interpreting the structures of the metabolites.

Chromatographic and mass spectrometric conditions were optimized using a calycosin-7-*O*-β-D-glucopyranoside standard in positive-ion detection mode. A full-scan mass spectral analysis of calycosin-7-*O*-β-D-glucopyranoside showed protonated molecular ions at a value of *m/z* 447. The $[M+H]^+$ ion yielded the $[M+H-Glu]^+$ ion at *m/z* 285 due to the elimination of glycoside in the MS^2 spectrum. Successively, the $[M+H-Glu]^+$ ion was selected for MS^3 analysis and generated product ions at *m/z* 270 $[M+H-Glu-CH_3]^+$, 253 $[M+H-Glu-CH_3-OH]^+$, 225 $[M+H-Glu-CH_3-OH-CO]^+$, 197 $[M+H-Glu-CH_3-OH-2CO]^+$ and 137, a moderate $^{1,3}A^+$ (R_1 = OH) product ion attributed to the cross-ring cleavage (Fig. 1) [17–19]. The ions at *m/z* 285, 270, 253, 225, 197, and 137 were concluded to be characteristic product ions of calycosin-7-*O*-β-D-glucopyranoside. These characteristic product ions along with neutral losses provided a sound basis for identifying the metabolites of calycosin-7-*O*-β-D-glucopyranoside.

3.2. Identification of metabolites of calycosin-7-O-β-D-glucopyranoside

Compared drug-treated samples with corresponding control samples directly, a total of fourteen metabolites were detected and identified. Extracted ion chromatogram (EIC) of calycosin-7-*O*-β-D-glucopyranoside and its metabolites are presented in Fig. 2. The location, MS^2 data, biotransformation along with the retention time of calycosin-7-*O*-β-D-glucopyranoside and its metabolites are summarized in Table 1.

The MS^2 spectra of the molecular ion at *m/z* 285 (M12) exhibited $[M+H]^+$ signals at *m/z* 270, 253, 225, 197 and 137, which was the same as the MS^3 spectra of *m/z* 447 → 285. Therefore, M12 could be characterized as the deglycosylated product of calycosin-7-*O*-β-D-glucopyranoside, calycosin.

Table 1
Analysis of metabolites of calycosin-7-*O*-*β*-D-glucopyranoside in rat biological samples.

Analyte	Biotransformation	$[M+H]^+$ (m/z)	Rt (min)	Data-dependent MS^2 data (% base peak) (m/z)	Location								
					A	B	C	D	E	F	G	H	I
Calycosin-7-*O*-*β*-D-glucopyranoside (M0)		447	15.6	285 (100)	+	–	+	+	+	+	+	+	–
Calycosin-3′-*O*-glucuronide-7-*O*-glucoside (M1)	Glucuronidation	623	13.1	447 (66), 285 (100)	+	–	+	+	+	+	+	+	–
calycosin-3′,7-*O*-diglucuronide (M2)	Deglycosylation + glucuronidation	637	13.2	461 (100), 285 (53)	+	–	–	–	–	–	–	–	–
Calycosin-3′,7-*O*-disulfate (M3)	Deglycosylation + sulfation	445	14.0	365 (48), 285 (100)	+	–	–	+	+	+	+	+	–
Daidzein-7-*O*-glucuronide (M4)	Deglycosylation + dehydroxylation + demethylation + glucuronidation	431	14.8	255 (100)	+	–	–	–	–	–	+	+	–
Daidzein-7-*O*-glucoside (M5)	Dehydroxylation + demethylation	417	14.9	255 (100)	+	–	–	–	–	–	–	–	–
Calycosin-7-*O*-glucuronide (M6)	Deglycosylation + glucuronidation	461	16.0	285 (100)	+	–	+	+	+	+	+	+	–
Dehydroxylated calycosin glucuronide (M7)	Deglycosylation + dehydroxylation + glucuronidation	445	16.0	269 (100)	+	–	–	+	+	+	+	+	–
Daidzein sulfate (M8)	Deglycosylation + dehydroxylation + demethylation + sulfation	335	16.0	255 (100)	+	–	–	–	–	–	–	–	–
Calycosin sulfate (M9)	Deglycosylation + sulfation	365	16.2	285 (100)	+	–	+	+	+	+	+	+	–
3′,4′,7-trihydroxyisoflavone (M10)	Deglycosylation + demethylation	271	17.0	253 (69), 225 (56), 215 (76), 197 (52), 137 (100)	–	+	–	–	–	–	–	–	+
Daidzein (M11)	Deglycosylation + dehydroxylation + demethylation	255	21.3	237 (100), 219 (90), 199 (72), 137 (52), 123 (40)	+	+	+	–	+	–	+	+	+
Calycosin (M12)	Deglycosylation	285	22.8	270 (100), 253 (36), 225 (30), 197 (4), 137 (33)	+	+	+	+	+	+	+	+	+
Dehydroxylated calycosin (M13)	Deglycosylation + dehydroxylation	269	39.4	253 (100), 239 (57), 205 (61), 189 (20), 165 (66), 137 (15), 123 (33)	+	–	–	–	+	+	+	+	–
Mono-methylated calycosin (M14)	Deglycosylation + methylation	299	40.5	271 (15), 253 (27), 225 (12), 197 (39), 171 (22), 147 (28), 121 (100)	–	–	+	–	–	–	–	–	–

A, urine; B, feces; C, plasma; D, heart; E, liver; F, spleen; G, lung; H, kidney; I, intestinal incubation; +, found; –, not found.

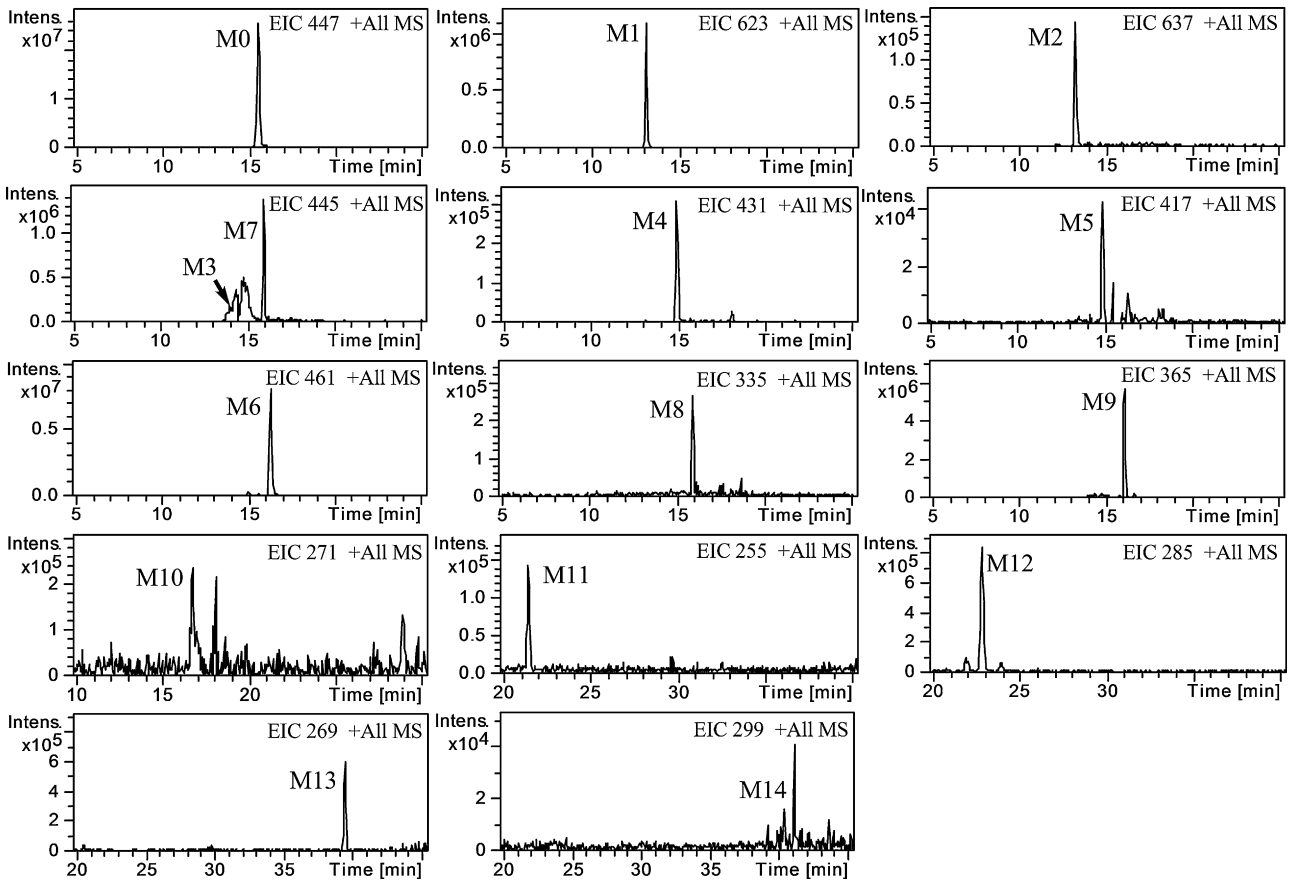

Fig. 2. Extract ion chromatography (EIC) of calycosin-7-*O*-*β*-D-glucopyranoside and its metabolites.

The molecular ion at *m/z* 271 (M10), 255 (M11) and 269 $[M+H]^+$ (M13) were all showed similar product ions to M12 and the base product ion of isoflavones at *m/z* 137 ($^{1,3}A^+$, R_1 = OH, Fig. 1). And molecular weights of them were 14, 30 and 16 Da smaller than that of calycosin respectively, it is plausible that M10, M11 and M13 were demethylated, demethylated/dehydroxylated and dehydroxylated products of M12, respectively.

In the MS^2 spectra of *m/z* values of 623 (M1), 431 (M4), 461 (M6) and 445 $[M+H]^+$ (M7), the parent ions lost a neutral fragment of 176 Da, giving product ions at *m/z* values of 447, 255, 285 and 269 The MS^3 spectra of *m/z* values of 623 → 447, 431 → 255, 461 → 285 and 445 → 269 were the same as the MS^2 spectra of M0, M11, M12 and M13. These results indicate that M1, M4, M6 and M7 should be the glucuronidated products of M0, M11, M12 and M13, respectively.

The MS^2 spectra of the molecular ions at *m/z* 335 (M8) and 365 $[M+H]^+$ (M9) showed abundant product ions at *m/z* 255 and 285, respectively; these changes were produced by the neutral loss of SO_3 (80 Da) from their molecular ions. Moreover, the MS^3 spectra of *m/z* 335 → 255 and 365 → 285 were the same as the MS^2 spectra of M11 and M12, respectively. Based on these data, M8 and M9 should be the sulfates of M11 and M12, respectively.

M3 also displayed the $[M+H]^+$ ion at a *m/z* 445 as M7, but its product ions at *m/z* 365 and 285 were produced by the sequential loss of 80 Da. Therefore, M3 should be the di-sulfate of M12. Similarly, the product ions of *m/z* 637 $[M+H]^+$, M2, at *m/z* 461 and 285 were produced by the sequential loss of 176 Da, Hence M2 should be the di-glucuronide of M12.

$[M+H]^+$ at *m/z* 417 given by M5 yielded no other product ions than *m/z* 255 in the MS^2 spectra and *m/z* 417 → 255 in the MS^3 spectra was the same as the MS^2 spectra of M11, indicating that M5 should be the glycosylated product of M11.

The molecular ion at *m/z* 299 $[M+H]^+$ (M14) showed almost the same product ions as M12. According to the fact that its molecular weight was 14 Da greater than that of calycosin, M14 was identified as the methylated metabolite of M12.

3.3. Characterization of metabolites M11 and M12

M11 and M12 were isolated from fractions C and B4 by semi-preparative HPLC, respectively.

M11 was obtained as pale yellow powder. The ESI–MS of M11 gave the molecular ion $[M+H]^+$ at *m/z* 255. The ^{13}C NMR data of M11 were as follows: (DMSO–d_6, δ ppm): 174.64 (C-4), 162.44 (C-7), 157.38 (C-9), 157.12 (C-4′), 152.72(C-2), 130.01 (C-2′), 130.01 (C-6′), 127.23 (C-5), 123.45 (C-1′), 122.50 (C-3), 116.61 (C-10), 115.05 (C-6), 114.90 (C-3′), 114.90 (C-5′), 102.04 (C-8). The structure was confirmed as daidzein by comparison with data from the existing literature [20].

M12 was obtained as pale yellow powder. The ESI–MS of M12 gave the molecular ion $[M+H]^+$ at *m/z* 285. The ^{13}C NMR data of M12 were as follows: (DMSO–d_6, δ ppm): 174.53 (C-4), 162.48 (C-7), 157.33 (C-9), 152.98 (C-2), 147.47 (C-4′), 146.00 (C-3′), 127.24 (C-5), 124.68 (C-1′), 123.33 (C-3), 119.65 (C-6′), 116.62 (C-10), 116.42 (C-2′), 111.95 (C-5′), 102.05 (C-8), 55.65 (OMe). The structure was confirmed as calycosin by comparison with data from the existing literature [21].

M0, M11 and M12 were dissolved in DMSO and diluted with complete medium for further use.

3.4. Cell viability

The effects of three chemicals on the viability of Hep-2 cells were as follows: The TC_0 values of M0, M11 and M12 were 250, 15.63 and

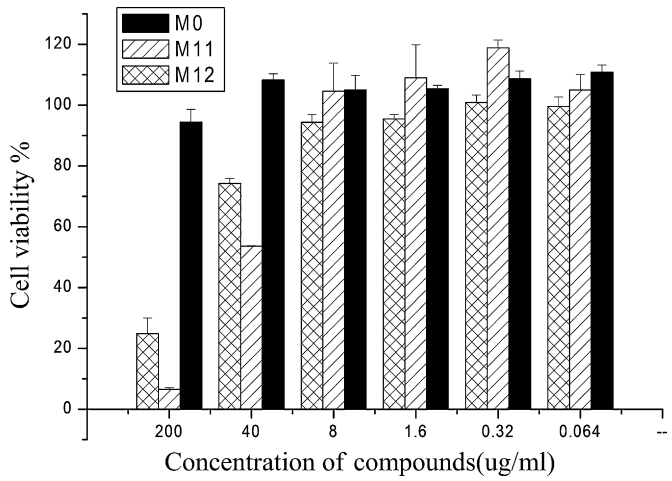

Fig. 3. Cytotoxicities of compounds in C8166 cells measured by MTT assay. Results were expressed as mean ± SEM (n = 6–9; SEM, standard error of the mean).

31.25 μg/mL, respectively. The TC_{50} values of them were 354.81, 22.39 and 44.67 μg/mL, respectively. The effect of compounds on C8166 cells were examined (Fig. 3). The CC_{50} values of M0, M11 and M12 were above 200, 42.80 and 87.13 μg/mL, respectively.

3.5. Antiviral activities

Both M0 and M12 have antiviral activities against CVB_3 *in vitro*. Their IC_{50} values were 251.19 and 7.94 μg/mL, respectively. Their TI values were 1.41 and 5.63, respectively. The higher TI implies that M12 exhibited more significant anti-CVB_3 activity than M0; M11 showed no antiviral activity.

M0, M11 and M12 inhibited HIV-1-induced syncytium formation. The median effective concentration (EC_{50}) values of these compounds were 176.66, 2.65 and 2.20 μg/mL, respectively, while their TI values were above 1.13, 16.15 and 39.60, respectively (Fig. 4A). To confirm the anti-HIV activity in different systems, p24 antigen production in HIV-1 acute infected C8166 cells was performed. M0, M11 and M12 inhibited the p24 antigen production with EC_{50} values above 200, 5.11 and 4.48 μg/mL, respectively. The TI values of M11 and M12 were 8.38 and 19.45, respectively (Fig. 4B); M0 showed no activity.

4. Discussion

This is the first comprehensive investigation of *in vivo* metabolites of calycosin-7-*O*-*β*-D-glucopyranoside in rats by HPLC–MS^n. Based on the previous investigations of credible MS^n fragmentation, the structures of most metabolites were deduced in the present paper, thereby clarifying the general metabolism and metabolic pathways of calycosin-7-*O*-*β*-D-glucopyranoside *in vivo* (Fig. 5). First, hydrolysis was considered as an initial step in biotransformation, which would allow for easier diffusion and absorption in the intestines. The hydrolysis metabolite, calycosin (M12), was then converted to twelve other metabolites (M2–M11, M13–M14) through glucuronidation, glycosylation, sulfation, dehydroxylation, demethylation and methylation reactions, whereas M1 were directly formed from the parent compound as a one-step metabolism reaction. Among these metabolites, the metabolic pathway of M5 was controversial. It might be transformed from M11 via glycosylation, or M0 via demethylation and dehydroxylation reactions. Except that calycosin has been reported, other thirteen metabolites were reported as metabolites of calycosin-7-*O*-*β*-D-glucopyranoside *in vivo* for the first time [6,7,19]. Besides, calycosin-7-*O*-*β*-D-glucuronic acid methyl ester determined by Yang et al. [7] was not detected in rat urine in our research; it is presumably by reason of matrix suppression in HPLC–MS^n.

Location of calycosin-7-*O*-*β*-D-glucopyranoside and its metabolites shown in Table 1 indicated that all compounds excluding M10 and M14 appeared in other samples could be detected in urine (M0–M9, M11–M13). M10 was detected in rat feces and incubation mixtures, metabolites in which (M10, M11, M12) were all phase I metabolites, but the parent compound was absent. *In vitro* incubation suggested intestinal bacteria involved in the demethylation, deglycosylation and dehydroxylation reaction. Hence, although the hydrolysis of certain flavonoid glycosides could reportedly be accomplished throughout the entire alimentary canal, including the oral cavity and stomach [22], combining the results with reference of hydrolysis of isoflavonoid glycosides [19,23], the intestine was believed to be the major site of hydrolysis of calycosin-7-*O*-*β*-D-glucopyranoside.

M14, the methylated metabolite, was detected in rat plasma, metabolites in which (M0, M1, M6, M9, M11, M12, M14) were not surprisingly less than those in urine [24]. The metabolites in rat plasma and organs were different by virtue of the difference between the dynamic circulation of plasma and slight enrichment

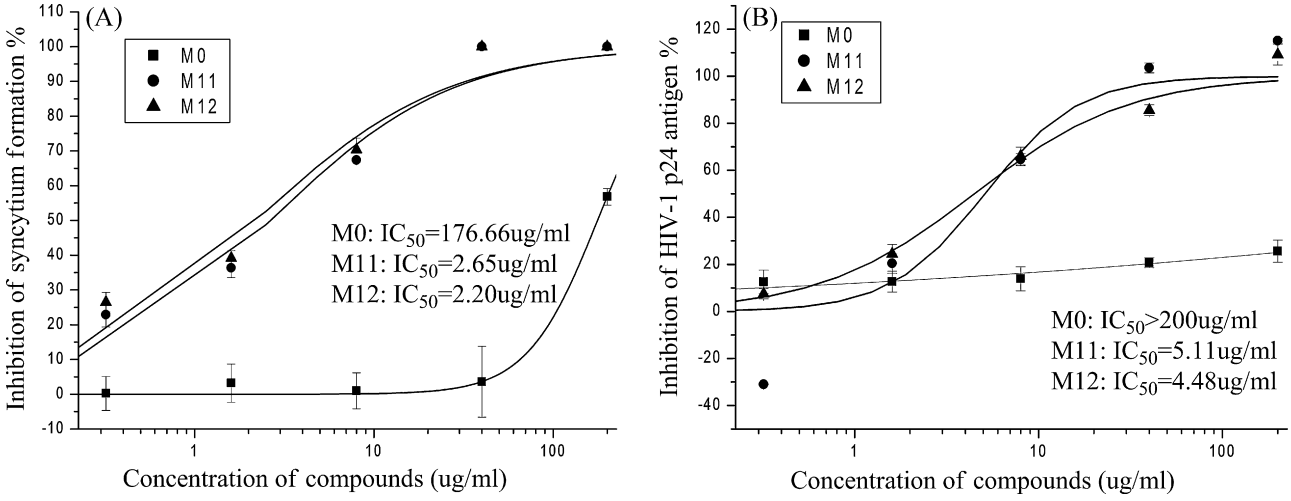

Fig. 4. The antiviral effects of compounds on HIV-1_{IIIB} in C8166 cells. Inhibition of HIV-1_{IIIB} replication was assessed by syncytium formation (A) and p24 antigen (B). Results were expressed as mean ± SEM (n = 6–9; SEM, standard error of the mean).

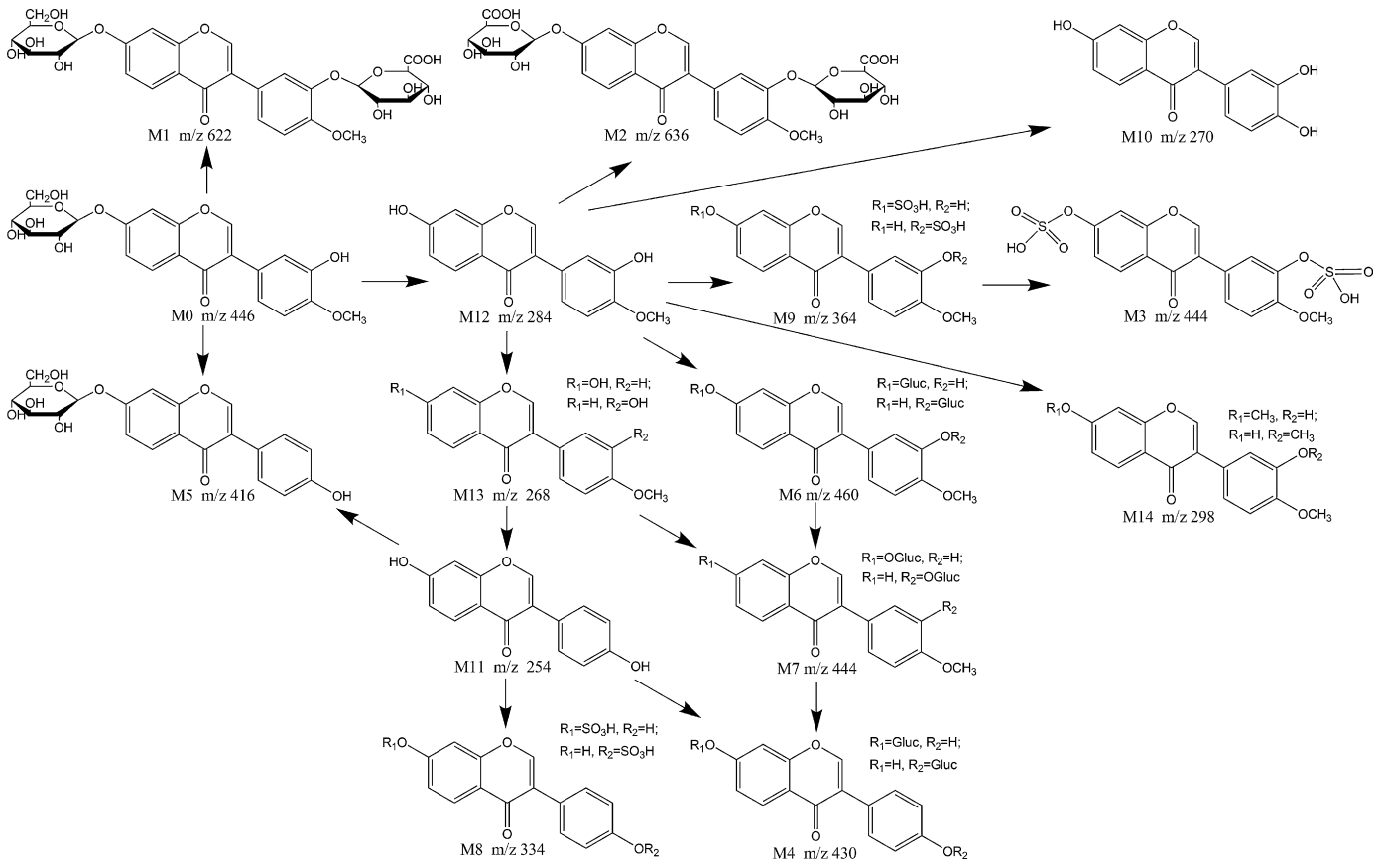

Fig. 5. Proposed major metabolic pathways of calycosin-7-*O*-*β*-D-glucopyranoside in rats.

of organs. The metabolites in organs were homologous to those in urine. Furthermore, M0, M1, M3, M6, M7, M9 and M12 were widely distributed in the body. Among them, M12, the aglycone of calycosin-7-*O*-*β*-D-glucopyranoside, the definitely major metabolite, is likely to have a greater biological effect than the parent compound [25]. We therefore isolated and compared the antiviral activity of M12 against coxsackie virus B_3 and human immunodeficiency virus with those of M0. In addition, several studies [26,27] have reported that *O*-glucuronidation or *O*-sulfation acts as a bioactivation step. So M6, M3 and M9, the *O*-glucuronidated and *O*-sulfated products of M12, also probably possessed antiviral effects. Still, the isolation of feces, but not urine, was performed for two reasons: One is the restriction on the parent drug supply. Calycosin-7-*O*-*β*-D-glucopyranoside showed content of no more than 0.04% in *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao. Thus, extracting a sufficient amount and purity of calycosin-7-*O*-*β*-D-glucopyranoside requires a very large amount of time and *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao. The other is that an earlier experiment showed that urine isolation is more difficult, because urine contains much more endogenesis compounds but less calycosin than feces. Thus, the isolation of feces was performed first. And our investigation of potential active metabolites is in progress.

The *in vitro* anti-HIV-1 and anti-CVB_3 activities of daidzein and calycosin were first evaluated and compared with those of calycosin-7-*O*-*β*-D-glucopyranoside in the present study. In the syncytium formation assay, daidzein and calycosin showed better anti-HIV-1 activities than calycosin-7-*O*-*β*-D-glucopyranoside. Especially calycosin has good activity for inhibiting HIV-1 replication. In the p24 antigen production assay, a similar result was observed. The anti-CVB_3 activity of calycosin was also better than that of calycosin-7-*O*-*β*-D-glucopyranoside, whereas daidzein showed non anti-CVB_3 activity. Therefore, calycosin shows good antiviral activities against HIV and CVB_3.

5. Conclusion

In summary, for the first time, the *in vivo* metabolism of calycosin-7-*O*-*β*-D-glucopyranoside was extensively studied and the antiviral activities of its metabolites were investigated and compared. Fourteen metabolites and the parent compound were found in rats. Twelve, six, six, eight, seven, nine and nine metabolites were identified in rat urine, plasma, heart, liver, spleen, lungs and kidneys, respectively. However, the parent compound was not detected in incubation mixtures of rat intestinal flora and feces; only three metabolites were found. The result showed that the intestine was the site of the deglycosylation reaction. Further, if more extensive trials are conducted on other animals or humans for the pre-clinical safety evaluation of calycosin-7-*O*-*β*-D-glucopyranoside from a metabolic perspective, the consistency and correlation of *in vitro* and *in vivo* findings could be used to predict *in vivo* metabolism from *in vitro* metabolism. The present study has demonstrated that calycosin-7-*O*-*β*-D-glucopyranoside has diverse and important metabolic pathways. This chemical was also widely distributed throughout the entire body with phase I metabolic reactions of deglycosylation, dehydroxylation and demethylation and phase II metabolic reactions of sulfation, methylation, glucuronidation and glycosylation (probably). Moreover, after isolation from feces and characterization by NMR, the anti-CVB_3 and anti-HIV effects of two metabolites were compared with the parent compound; those of calycosin, the aglycone, were tested and found to be stronger than those of calycosin-7-*O*-*β*-D-glucopyranoside. This study determines the actual compound responsible for the

antiviral activities of calycosin-7-*O*-*β*-D-glucopyranoside and lays the foundation for its further discovery and development research.

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