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PHENYLETHANOID GLYCOSIDES FROM *Cistanche tubulosa*

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Cistanche tubulosa (Schrenk) Wight (*Rou Cong Rong* in Chinese) is a kind of perennial phanerogamic parasitic plant (Orobanchaeceae) attached underground to the roots of *Tamarix talamakanensis*, *T. vamosissima*, *T. hohenackeri, T. arceuthoide*, etc., which is widely cultivated in desert areas such as the southern edge of the Tarim Basin in Xinjiang of China, and which grows by absorbing nutrients from the host plant [1–3]. The dried succulent stem of *C. tubulosa* is a precious traditional Chinese herb and has been recorded in the Chinese Pharmacopoeia (2010).

C. tubulosa is used to nourish the kidney, supplement essence and blood, and relieve constipation as a laxative [4]. A great number of investigations have proved its medicinal activities in scavenging free oxygen radicals [5–7]. Its stem extracts are used as an anti-decrepitude [8], to improve learning-memory ability [9], for neuroprotection [10], and for immunoregulation [11]. The major active compounds in *Cistanche* species are phenylethanoid glycosides such as echinacoside, acteoside, isoacteoside, and tubuloside A, which are usually chosen as marker compounds to assess the quality of *C. tubulosa*, and species sources of *C. tubulos*a var *C. deserticola*, *C. salsa,* and *C. sinensis* are distinguished based only on these compounds [12, 13].

In the course of further studies on active compounds from *C. tubulosa*, we have sitimultaneous isolated and purified echinacoside (**1**), cistanoside A (**2**), cistantubuloside A (**3**), acteoside (**4**), isoacteoside (**5**), 2′-acetylacteoside (**6**), and tubuloside A (**7**) from *C. tubulosa* by prep-HPLC, each at over 96.3% purity as determined by HPLC. The compounds were identified by their retention time, and confirmed by ESI-MS, and NMR experiments.

In *in vitro* assays, the mouse skin melanoma action of phenylethanoid glycoside compounds on cell line KML was studied. Antitumor activities of one extract and five compounds were found for KML, such as Fr. 2 (31%), echinacoside (75%), cistanoside A (33%), cistantubuloside A (83%), acteoside (81%), and 2′-acetylacteoside (93%), expressed as percent inhibition of tumor growth.

Plant Material. The stems of *Cistanche tubulosa* were collected from Keriya country, Xinjiang of China in September 2007 and was identified by Chief Pharmacist Sulayman Khalik at the Xinjiang Uyghur Autonomous Regional Institute for Food and Drug Control. A voucher specimen was deposited in the Xinjiang Technical Institute of Physics and Chemistry.

Sample Preparation. The dried, powdered fresh stems (3.0 kg) of *Cistanche tubulosa* were soaked twice with 80% aqueous methanol (1.5 L, 1.2 L) for 78 h at room temperature, twice filtered, combined, and concentrated under reduced pressure before being suspended in water, then partitioned consecutively with petroleum-ether, CH_2Cl_2, and *n*-butanol. The *n*-butanol fraction (17.0 g) was separated by solid phase-extraction (SPE), and the stationary phase of the normal-phase silica gel was eluted by a $CHCl_3$–CH_3OH gradient (100:0; 75:25) and aqueous methanol (95% to 80%) to give three fractions [Fr. 1 (1.2 g), Fr. 2 (12.0 g), and Fr. 3 (2.2 g)]. Fraction 2 (6.0 g) was separated by reversed-phase silica gel column chromatography. The prep-HPLC conditions were as follows: The binary mobile phase consisted of methanol (solvent A) and water (solvent B). The flow-rate was kept constant at 5.0 mL/min for a total run time of 145 min. The system was run with a gradient program: 15.0% A in 0–120 min and 35% A in 25 min. The sample injection volume was from 500 to 1000 μL, which afforded eleven fractions [Fr. 2-1 (251 mg), Fr. 2-2 (586 mg), Fr. 2-3 (74 mg), Fr. 2-4 (92 mg), Fr. 2-5 (92 mg), Fr. 2-6 (420 mg), Fr. 2-7 (78 mg),

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Fr. 2-8 (75 mg), Fr. 2-9 (103 mg), Fr. 2-10 (64 mg), and Fr. 2-11 (261 mg)]. Then each fraction, such as Fr. 2-2, Fr. 2-4, Fr. 2-5, Fr. 2-6, Fr. 2-9, and Fr. 2-10, was further purified by prep-HPLC [CH_3OH–H_2O (20:80→30:70, v/v)] to give seven compounds (**1**, 508 mg), (**2**, 48 mg), (**3**, 42 mg), (**4**, 395 mg), (**5**, 53 mg), (**6**, 87 mg), and (**7**, 103 mg), and then identified by comparison of their physical data (^{1}H NMR, ^{13}C NMR, ESI-MS) with reported values [14–18].

Echinacoside (**1**). White powder, mp 202–204°C; ESI-MS *m/z*: 786 $[M]^+$, 623 $[M - C_6H_5O_5]^+$. ^{1}H NMR (600 MHz, CD_3OD, δ, ppm, J/Hz): 7.60 (1H, d, J = 16, H-7′), 6.56–7.07 (6H, aromatic H), 6.28 (1H, d, J = 16, H-8′), 5.18 (1H, s, H-1 of rhamnosyl), 4.39, 4.30 (each 1H, d, J = 7.8, H-1 of glucosyl), 3.56 (2H, q, J = 6.0, H-8), 2.79 (2H, d, J = 6.0, H-7), 1.08 (3H, d, J = 6.0, H-6 of rhamnosyl). The ^{13}C NMR spectrum data of compound **1** were in accord with the reported values in the literature [14, 15, 17].

Cistanoside A (**2**). White powder, mp 207–209°C, ESI-MS *m/z*: 823 $[M + Na]^+$, 800 $[M]^+$, 637 $[M - C_6H_5O_5]^+$. ^{1}H NMR (600 MHz, CD_3OD, δ, ppm, J/Hz): 7.61 (1H, d, J = 16, H-7′), 6.56–7.14 (6H, aromatic H), 6.27 (1H, d, J = 16, H-8′), 5.17 (1H, s, H-1 of rhamnosyl), 4.38 and 4.29 (each 1H, d, J = 7.8, H-1 of glucosyl), 3.83 (3H, s, OCH_3), 3.55 (2H, q, J =6.0, H-8), 2.80 (2H, d, J = 6.0, H-7), 1.09 (3H, d, J = 6.0, H-6 of rhamnosyl). The ^{13}C NMR spectrum data of compound **2** were in accord with the reported values in the literature [14].

Cistantubuloside A (**3**). Primrose yellow powder, mp 210–212°C. ^{1}H NMR (600 MHz, CD_3OD, δ, ppm, J/Hz): 7.54 (1H, d, J = 16, H-7′), 7.03 (2H, dd, J = 7.8, 2.4, H-2, H-6), 7.00 (1H, d, J = 1.5, H-2′), 6.91 (1H, dd, J = 7.8, 1.8, H-6′), 6.72 (1H, d, J = 7.8, H-5′), 6.64 (2H, dd, J = 7.8, 2.4, H-3, H-5), 6.22 (1H, d, J = 16, H-8′), 5.12 (1H, d, J = 2.4, H-1 of rhamnosyl), 4.31, 4.22 (each 1H, d, J = 7.8, H-1 of glucosyl), 3.59 (2H, m, H-8), 2.79 (2H, m, H-7), 1.02 (3H, d, J = 6.0, H-6 of rhamnosyl). The ^{13}C NMR spectrum data of compound **3** were in accord with the reported values in the literature [18].

Acteoside (**4**). Primrose yellow powder, mp 143–146°C; ESI-MS *m/z*: 625 $[M + H]^+$; 624 $[M]^+$, 461 $[M - C_6H_5O_5]^+$. ^{1}H NMR (600 MHz, CD_3OD, δ, ppm, J/Hz): 7.60 (1H, d, J = 16, H-7′), 6.56–7.07 (6H, aromatic H), 6.28 (1H, d, J = 16, H-8′), 5.20 (1H, s, H-1 of rhamnosyl), 4.37 (1H, d, J = 7.8, H-1 of glucosyl), 3.54 (2H, q, J = 6.0, H-8), 2.79 (2H, d, J = 6.0, H-7), 1.10 (3H, d, J = 6.0, H-6 of rhamnosyl). The ^{13}C NMR spectrum data of compound **4** were in accord with the reported values in the literature [14, 15, 17].

Isoacteoside (5). Primrose yellow powder, mp 145–147°C; ESI-MS *m/z*: 624 $[M]^+$, 461 $[M - C_6H_5O_5]^+$. ^{1}H NMR (600 MHz, CD_3OD, δ, ppm, J/Hz): 7.60 (1H, d, J =16, H-7′), 6.56–7.07 (6H, aromatic H), 6.28 (1H, d, J = 16, H-8′), 5.20 (1H, s, H-1 of rhamnosyl), 4.37 (1H, d, J =7.8, H-1 of glucosyl), 3.54 (2H, q, J = 6.0, H-8), 2.79 (2H, d, J = 6.0, H-7), 1.10 (3H, d, J = 6.0, H-6 of rhamnosyl). The ^{13}C NMR spectrum data of compound **5** were in accord with the reported values of isoacteoside in the literature [14, 15, 17].

2′-Acetylacteoside (**6**). White powder, mp 151–153°C, ESI-MS *m/z*: 689 $[M + Na]^+$, 665 $[M - H]^+$, 623 $[M - COCH_3]^+$, 503 $[M - C_6H_5O_5]^+$. ^{1}H NMR (600 MHz, CD_3OD, δ, ppm, J/Hz): 7.60 (1H, d, J =15.6, H-7′), 6.54–7.05 (6H, aromatic H), 6.27 (1H, d, J = 16, H-8′), 5.00 (1H, s, H-1 of rhamnosyl), 4.53 (1H, d, J = 8.0, H-1 of glucosyl), 3.61 (2H, q, J = 8.8, H-8), 2.70 (2H, t, J =6.8, H-7), 1.98 (3H, s, -CO$\underline{CH_3}$), 1.07 (3H, d, J = 6.0, H-6 of rhamnosyl). The ^{13}C NMR spectrum data of compound **6** were in accord with the reported values of 2′-acetyl-acteoside in the literature [14, 15, 17].

Tubuloside A (**7**). White powder, mp 213–215°C, ESI-MS *m/z*: 828 $[M]^+$, 785 $[M - COCH_3]^+$, 665 $[M - C_6H_5O_5]^+$. ^{1}H NMR (600 MHz, CD_3OD, δ, ppm, J/Hz): 7.54 (1H, d, J = 16.2, H-7″), 6.54–7.05 (6H, aromatic H), 6.22 (1H, d, J = 16, H-8″), 5.03 (1H, s, H-1 of rhamnosyl), 4.48 and 4.24 (each 1H, d, J = 8.4, H-1 of glucosyl), 3.60 (2H, m, J = 8.8, H-8), 2.64 (2H, m, J = 6.8, H-7), 1.92 (3H, s, -CO$\underline{CH_3}$), 1.01 (3H, d, J = 6.0, H-6 of rhamnosyl). The ^{13}C NMR spectrum data of compound **7** were in accord with the reported values in the literature [14, 15, 17].

Cytotoxic Potential against Mouse Skin Melanoma. The *in vitro* cytotoxic potential of compounds **1**–**7** and Fr. 2 against mouse skin melanoma cancer cell line was determined from the reduction of the yellow color in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the common method [19].

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