SUPPLEMENTARY MATERIAL

Phenolics from *Lagotis brevītuba* Maxim

Xiang Yuan^{a,b†}, Huaixiu Wen^{a,b†}, Yulei Cui^{a,b}, Minxia Fan^{a,b}, Zenggen Liu^a, Lijuan Mei^a, Yun Shao^a, Yiping Wang^c and Yanduo Tao^a

^aKey Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, 810001, P. R. China

^bCollege of life science, University of Chinese Academy of Sciences, Beijing, 100000, P. R. China

^cShanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, 201203, P. R. China

Abstract: A phytochemical investigation on *Lagotis brevītuba* led to the isolation and characterization of eleven phenolic compounds: p-hydroxy-benzoic acid **1**, methyl 3,4-dihydroxybenzoate **2**, vanillic acid **3**, protocatechuic acid **4**, caffeic acid **5**, glucose ester of (E)-ferulic acid **6**, p-coumaric acid **7**, vanillin **8**, diosmetin-7-O-β-D-glucoside **9**, chrysoeriol **10**, and luteolin **11**. Their structures were elucidated using spectroscopic methods and by comparison with data in the literature. Compounds **1–6** were firstly obtained from the genus *Lagotis*, and compounds **1–9** were isolated from *L. brevītuba* for the first time. Compound **4** and **11** displayed remarkable antioxidant activities against DPPH radical (IC₅₀=5.60±0.09mg/L, 27.5±0.06mg/L, respectively), which were superior to positive control rutin. And compound **11** was also superior to rutin in ABTS assay (IC₅₀=2.04±0.13mg/L).

Keywords: *Lagotis brevītuba*; phenolics; spectroscopic methods

1. Experimental

1.1 General

NMR spectra was measured on a Bruker 500MHz NMR spectrometer at 500 MHz for ¹H and 125 MHz for ¹³C NMR (internal standard: TMS). MS spectra was obtained on LTQ Orbitrap XL mass spectra (ESI, positive-ion mode). Column chromatography (CC) was carried out on silica gel (100-200 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). Thin-layer chromatography was performed with silica gel G-precoated plates (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). The spots were visualized under UV light (254 and 365nm) and further by spraying with 10% H₂SO₄ in EtOH followed by heating. Preparative high performance liquid chromatography was performed on Hanbon liquid chromatography with column HCE

C18 (20mm × 250mm).

1.2 Plant material

The plants of *Lagotis brevituba* were collected from Qilian mountains, Qinghai Province, China, in September 2015, and identified by Prof. Mei Lijuan, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, China. A voucher specimen (2015090101) was preserved in herbarium of Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, China.

1.3 Extraction and isolation

The air-dried and powdered *Lagotis brevituba* (1.5kg) were extracted three times using 95% ethanol under reflux for 3h (70°C). The combined ethanol extract was then concentrated under reduced pressure to obtain a dark crude extract. The residue was suspended in water, and partitioned sequentially with petroleum (PE, boiling point 60~90), EtOAc and *n*-BuOH.

The EtOAc extraction (10g) was conducted by column chromatography (CC) on silica gel eluted with CH₂Cl₂-MeOH (10:0~1:1, v/v) to yield four fractions (Fr.1~4). Fr.1 (2.1g) was subjected to CC on silica gel eluted with CH₂Cl₂-MeOH (9:1~7:3, v/v) to give two fractions (Fr.5, 6). Fr.5 (163mg) was re-chromatographed over CC on silica gel (CH₂Cl₂-MeOH, 8:2, v/v) to afford compound **1** (51mg), **2** (26mg). Fr.6 (50mg) was purified by preparative HPLC (column: HC RP-C18, 10μm, 250×20mm; Water-CH₃CN: 90-10; flow rate 15mL/min; detection 210nm) to obtain compound **3** (19mg). Fr.2 (1.5g) was further separated by CC on silica gel to afford two fractions (Fr.7, 8). Fr.7 (90mg) was purified by Sephadex LH-20 (CH₂Cl₂-MeOH, 1:1, v/v) to yield compound **4** (12mg). Fr.8 (155mg) was submitted to semi-preparative HPLC (column: HC RP-C18, 10μm, 250×20mm; Water-CH₃CN: 85-15; flow rate 15mL/min; detection 210nm) to achieve compounds **5** (8mg), **10** (33mg), and **11** (16mg). Fr.3 (0.7g) was repeatedly chromatographed on silica gel CC to yield compounds **6** (31mg), **7** (51mg), **8** (26mg). Fr.4 (0.6g) was directly applied on semi-preparative HPLC (column: HC RP-C18, 10μm, 250×20mm; Water-CH₃CN: 87-13; flow rate 15mL/min; detection 210nm) to afford compound **9** (35mg), **10** (106mg) and **11** (81mg).

The structures of the isolated compounds were elucidated according to their spectroscopic data and compared with those previously reported in the literature. They were identified as p-hydroxy-benzoic acid **1**, methyl-3,4-dihydroxybenzoate **2**, vanillic acid **3**, protocatechuic acid **4**, caffeic acid **5**, glucose ester of (E)-Ferulic Acid **6**, p-coumaric acid **7**, vanillin **8**, diosmetin-7-O- β -D-glucoside **9**, chrysoeriol **10**, luteolin **11**.

1.4 Identification of compounds

p-Hydroxy-benzoic acid (1)

White powder, $C_7H_6O_3$, Negative ESI-MS m/z : 137.1[M-H]⁻. ¹H-NMR (500MHz, CD₃OD) δ : 7.89 (2H, d, $J=8.5$ Hz, H-2, 6), 6.84 (2H, d, $J=8.5$ Hz, H-3, 5); ¹³C-NMR (125MHz, CD₃OD) δ : 170.3 (C-7), 163.3 (C-4), 133.0 (C-2, 6), 122.9 (C-1), 116.0 (C-3, 5).

Methyl-3,4-dihydroxybenzoate (2)

Colorless needle crystal (MeOH), $C_8H_8O_4$, Negative ESI-MS m/z : 167.0[M-H]⁻, ¹H-NMR (500MHz, CD₃OD) δ : 7.40 (1H, dd, $J=7.9, 1.7$ Hz, H-6), 7.38 (1H, d, $J=1.7$ Hz, H-2), 6.79 (1H, d, $J=7.9$ Hz, H-5), 3.76 (3H, s, -OCH₃); ¹³C-NMR (125MHz, CD₃OD) δ : 167.2 (C-7), 151.1 (C-4), 147.2 (C-3), 123.5 (C-1), 121.6 (C-6), 115.0 (C-5), 112.7 (C-2), 55.6 (-OCH₃).

Vanillic acid (3)

White amorphous powder, $C_8H_8O_4$, Negative ESI-MS m/z : 167.2[M-H]⁻, ¹H-NMR (500MHz, DMSO- d_6) δ : 7.44 (1H, d, $J=8.1$ Hz, H-6), 7.43 (1H, s, H-2), 6.84 (1H, d, $J=8.1$ Hz, H-5), 3.80 (3H, s, -OCH₃); ¹³C-NMR (125MHz, DMSO- d_6) δ : 166.5 (C-7), 151.1 (C-2), 147.2 (C-3), 123.9 (C-6), 121.9 (C-1), 114.9 (C-5), 112.5 (C-2), 55.3 (OMe).

Protocatechuic acid (4)

Colorless needle crystal (MeOH), $C_7H_6O_4$, Negative ESI-MS m/z : 153.6[M-H]⁻, ¹H-NMR (500MHz, CD₃OD) δ : 7.42 (1H, brs, H-2), 7.40 (1H, d, $J=8.2$ Hz, H-6), 6.78 (1H, d, $J=8.2$ Hz, H-5). ¹³C-NMR (125MHz, CD₃OD) δ : 170.8 (C-7), 151.3 (C-4),

146.0 (C-3), 123.8 (C-6), 123.7 (C-1), 117.7 (C-5), 115.7 (C-2).

Caffeic acid (5)

Yellow crystal (EtOAc), $C_9H_8O_4$, Negative ESI-MS m/z : 179.4[M-H]⁻, ¹H-NMR (500MHz, CD₃OD) δ : 7.39 (1H, d, J =15.8Hz, H-7), 6.90 (1H, s, H-2), 6.80 (1H, d, J =8.0Hz, H-6), 6.64 (1H, d, J =8.0Hz, H-5), 6.09 (1H, d, J =15.8Hz, H-8); ¹³C-NMR (125MHz, CD₃OD) δ : 171.2 (C=O), 149.4 (C-4), 146.9 (C-7), 146.7 (C-3), 127.8 (C-1), 122.8 (C-6), 116.5 (C-8), 115.7 (C-2), 115.1 (C-5).

Glucose ester of (E)-Ferulic Acid (6)

Amorphous powder, $C_{16}H_{20}O_9$, Negative ESI-MS m/z : 355.0[M-H]⁻, ¹H-NMR (500MHz, CD₃OD) δ : 7.70 (1H, d, J =15.8Hz, H-7), 7.18 (1H, s, H-2), 7.16 (1H, d, J =8.3Hz, H-6), 6.95 (1H, d, J =8.3Hz, H-5), 6.42 (1H, d, J =15.8Hz, H-8), 5.55 (1H, d, J =7.8Hz, H-1'), 3.83 (3H, s, OCH₃), 3.66 (1H, dd, J =12.1, 5.0Hz, H-6'a), 3.33-3.45 (4H, m, H-2', 3', 4', 5'); ¹³C-NMR (125MHz, CD₃OD) δ : 167.5 (C=O), 153.0 (C-4), 150.8 (C-7), 147.8 (C-3), 128.6 (C-1), 124.3 (C-6), 115.7 (C-8), 114.8 (C-5), 111.6 (C-2), 95.8 (C-1'), 78.8 (C-5'), 78.0 (C-3'), 74.0 (C-2'), 71.1 (C-4'), 62.3 (C-6'), 56.5 (OCH₃).

p-Coumaric acid (7)

White needles (MeOH), $C_9H_8O_3$, Negative ESI-MS m/z : 163.2[M-H]⁻, ¹H-NMR (500MHz, CD₃OD) δ : 7.48 (1H, d, J =15.7Hz, H-7), 7.39 (2H, d, J =8.5Hz, H-3, 5), 7.76 (2H, d, J =8.5Hz, H-2, 6), 6.28 (1H, d, J =15.6Hz, H-8). ¹³C-NMR (125MHz, CD₃OD) δ : 168.6 (C-9), 160.7 (C-4), 144.7 (C-7), 130.7 (C-2, 6), 127.8 (C-1), 116.7 (C-3, 5), 115.8 (C-8).

Vanillin (8)

Colorless needle crystal (MeOH), $C_7H_8O_3$, Negative ESI-MS m/z : 139.0[M-H]⁻, ¹H-NMR (500MHz, DMSO-*d*₆) δ : 9.76 (1H, s, -CHO), 7.42 (1H, d, J =8.0Hz, H-6), 7.38 (1H, brs, H-2), 6.96 (1H, d, J =8.0Hz, H-5), 3.83 (3H, s, OCH₃); ¹³C-NMR (125MHz, DMSO-*d*₆) δ : 191.1 (C-7), 153.3 (C-4), 148.2 (C-3), 128.7 (C-1), 126.2 (C-6), 115.4 (C-5), 110.7 (C-2), 55.6 (C-8).

Diosmetin-7-O- β -D-glucoside (9)

Amorphous powder, $C_{22}H_{22}O_{11}$, Negative ESI-MS m/z : 461.3[M-H]⁻, ¹H-NMR

(500MHz, DMSO- d_6) δ : 7.45 (1H, dd, $J=8.4, 2.2$ Hz, H-6'), 7.42 (1H, d, $J=2.2$ Hz, H-2'), 6.90 (1H, d, $J=8.4$ Hz, H-5'), 6.79 (1H, d, $J=2.1$ Hz, H-6), 6.75 (1H, s, H-3), 6.44 (1H, d, $J=2.1$ Hz, H-8), 5.08 (1H, d, $J=7.5$ Hz, H-1''), 3.20-3.70 (sugar protons), 3.17 (3H, s, -OCH₃); ^{13}C -NMR (125MHz, DMSO- d_6) δ : 181.9 (C-4), 164.5 (C-7), 163.0 (C-5), 161.2 (C-2), 157.0 (C-9), 149.9 (C-4'), 145.8 (C-3'), 121.4 (C-1'), 119.2 (C-6'), 116.0 (C-5'), 113.6 (C-2'), 105.3 (C-10), 103.2 (C-3), 99.9 (C-1''), 99.5 (C-6), 94.7 (C-8), 77.2 (C-5''), 76.4 (C-3''), 73.1 (C-2''), 69.6 (C-4''), 60.6 (C-6''), 48.6 (-OCH₃).

Chrysoeriol (10)

Yellow powder, C₁₂H₁₂O₆, Negative ESI-MS m/z : 299.2[M-H]⁻, ^1H -NMR (500MHz, DMSO- d_6) δ : 12.97 (1H, s, OH-5), 7.56 (1H, brs, H-6'), 7.55 (1H, brs, H-2'), 6.93 (1H, d, $J=8.8$ Hz, H-5'), 6.89 (1H, s, H-3), 6.49 (1H, d, $J=1.5$ Hz, H-8), 6.18 (1H, d, $J=1.5$ Hz, H-6), 3.89 (3H, s, -OCH₃); ^{13}C -NMR (125MHz, DMSO- d_6) δ : 181.8 (C-4), 164.4 (C-7), 163.6 (C-2), 161.4 (C-5), 157.3 (C-9), 150.7 (C-4'), 148.0 (C-3'), 121.5 (C-1'), 120.4 (C-6'), 115.8 (C-5'), 110.2 (C-2'), 103.6 (C-10), 103.2 (C-3), 98.9 (C-6), 94.1 (C-8), 56.0 (-OCH₃).

Luteolin (11)

Yellow powder, C₁₅H₁₀O₆, Negative ESI-MS m/z : 287.1[M-H]⁻, ^1H -NMR (500MHz, DMSO- d_6) δ : 7.41 (1H, dd, $J=8.4, 2.4$ Hz, H-6'), 7.39 (1H, d, $J=2.4$ Hz, H-2'), 6.89 (1H, d, $J=8.4$ Hz, H-5'), 6.67 (1H, s, H-3), 6.44 (1H, d, $J=2.4$ Hz, H-6), 6.18 (1H, d, $J=2.4$ Hz, H-8), 12.97 (1H, s, 5-OH); ^{13}C -NMR (125MHz, DMSO- d_6) δ : 181.6 (C-4), 164.2 (C-2), 163.9 (C-7), 161.5 (C-5), 157.3 (C-9), 149.7 (C-4'), 145.7 (C-3'), 121.5 (C-1'), 119.0 (C-6'), 116.0 (C-5'), 113.3 (C-2'), 103.7 (C-10), 102.9 (C-3), 98.8 (C-6), 93.9 (C-8).

Figure S1: Structures of compounds from *Lagotis brevītuba*.

1.5 Total phenolic contents (TPC)

Total phenolic contents of the extract was evaluated by the Folin–Ciocalteu method. The standard calibration curve was prepared with gallic acid solutions over ranges of 5~80μg/ml. All test samples were appropriately diluted for adjusting absorbance within the working range of standard calibration curve. A 400 μl of test samples was mixed with 400 μl of 1 mol/l Folin–Ciocalteu reagent and 800μl of Na₂CO₃(20%, w/v) at room temperature for 10 min. The mixture was centrifuged at 12,000×g for 10 min, and the absorbance was measured by an UV-visible spectrophotometer at 765nm. Blanks were prepared similarly but containing 50% ethanol instead of Folin–Ciocalteu reagent. The total phenolic contents were expressed as gallic acidequivalents (GAE) in milligrams per gram of sample (mg GAE/g). Data were reported as mean ± SD for at least triplicates

1.6 Total flavonoid contents (TFC)

Total flavonoid contents of the extract was examined by the AlCl₃ method. The reaction mixture contains 150 μl of sample solution, and 150 μl of AlCl₃ solution (2%, w/v). After 10 min incubation at ambient temperature, the absorbance of mixture was measured at 435 nm. The calibration curve was prepared with rutin (as a standard)

solutions. The total flavonoid contents were expressed in milligrams of rutin equivalents (RE) per gram of samples. Data were reported as mean $\pm$ SD for at least triplicates.

1.7 Antioxidant activities of these compounds in DPPH radical scavenging assay

The antioxidant activities of these compounds were evaluated in the DPPH radical scavenging assay. In brief, 150 μ L of 0.2 mM DPPH prepared in Ethanol were added to 96-well plates with compounds at different concentrations. The samples were incubated for 30 min in the dark at room temperatures, and the decrease of absorbance at 517 nm was measured against Ethanol using a Microplate reader. All determinations were carried out more than triplicate. The radical scavenging activity of the tested samples was calculated as 'inhibition percentage' according to the equation:

$$\text{Inhibition percentage (\%)} = [1 - (A_i - A_j)/A_c] \times 100\%$$

A_i is an absorbance of test samples at 30 min; A_j is an absorbance of ethanol at 30min; A_c is an absorbance of control DPPH at 30 min.

1.8 Antioxidant activities of these compounds in ABTS radical scavenging assay

Antioxidant assessment of the compounds was conducted using the ABTS assay. A series of 2 μ L samples were placed in individual wells of a 96-well microplate. The same amounts of Rutin and 50% methanol were added to columns designated as positive and negative controls, respectively. 198 μ L of ABTS⁺ were added to each well. The absorbance was measured by the UV-visible spectrophotometer at 730 nm after 6 min reaction in spectrophotometer set at 30 °C . The percent inhibition values for each sample were then calculated using the equation:

$$\text{Inhibition percentage (\%)} = [1 - (A_i - A_j)/(A_i - A_c)] \times 100\%$$

A_i is an absorbance of test samples at 6 min; A_j is an absorbance of ethanol at 6 min; A_c is an absorbance of control DPPH at 6 min.

Table 1. Total phenolic contents and total flavonoid contents of ethanolic extract

Sample	Total phenolic content (mg GAE/g) ^a	Total flavonoid content (mg RE/g) ^a
Crude extract	133.1±0.12	16.3±0.16

^a Each value is expressed as mean ± SD (n=3).

Table 2. Free radical scavenging activities of compounds

Compound	IC ₅₀ (DPPH)/(mg/L) ^a	IC ₅₀ (ABTS)/(mg/L) ^a
1	41.80±0.16	5.85±0.12
2	35.12±0.03	3.56±0.11
3	62.25±0.15	18.23±0.3
4	5.60±0.09	41.60±0.25
5	35.72±0.08	3.97±0.09
6	281.51±0.11	47.80±0.10
7	45.56±0.12	6.62±0.08
8	34.26±0.15	7.04±0.15
9	155.74±0.13	35.75±0.12
10	56.23±0.03	6.96±0.09
11	27.5±0.06	2.04±0.13
Rutin	30.86±0.03	2.85±0.12

^a Each value is expressed as mean ± SD (n=3).