SUPPLEMENTARY MATERIAL

Phenolics from *Lagotis brevituba* Maxim

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**Abstract:** A phytochemical investigation on *Lagotis brevituba* 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. brevituba* for the first time. Compound 4 and 11 displayed remarkable antioxidant activities against DPPH radical (IC\(_{50}\)=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 (IC\(_{50}\)=2.04±0.13mg/L).

**Keywords:** *Lagotis brevituba*; phenolics; spectroscopic methods

1. **Experimental**

1.1 **General**

NMR spectra was measured on a Bruker 500MHz NMR spectrometer at 500 MHz for \(^1\)H and 125 MHz for \(^{13}\)C 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% H\(_2\)SO\(_4\) 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 powered _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 CH$_2$Cl$_2$-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 CH$_2$Cl$_2$-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 (CH$_2$Cl$_2$-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-CH$_3$CN: 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 (CH$_2$Cl$_2$-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-CH$_3$CN: 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-CH$_3$CN: 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]. \(^1\)H-NMR (500MHz, CD_3OD) δ: 7.89 (2H, d, J=8.5Hz, H-2, 6), 6.84 (2H, d, J=8.5Hz, H-3, 5); \(^{13}\)C-NMR (125MHz, CD_3OD) 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]. \(^1\)H-NMR (500MHz, CD_3OD) δ: 7.40 (1H, dd, J=7.9, 1.7Hz, H-2), 7.38 (1H, d, J=1.7Hz, H-5), 6.79 (1H, d, J=7.9Hz, H-5), 3.76 (3H, s, -OCH_3); \(^{13}\)C-NMR (125MHz, CD_3OD) 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 (-OCH_3).

Vanillic acid (3)
White amorphous powder, C_8H_8O_4, Negative ESI-MS m/z: 167.2[M-H]. \(^1\)H-NMR (500MHz, DMSO-d_6) δ: 7.44 (1H, d, J=8.1Hz, H-6), 7.43 (1H, s, H-2), 6.84 (1H, d, J=8.1Hz, H-5), 3.80 (3H, s, -OCH_3); \(^{13}\)C-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]. \(^1\)H-NMR (500MHz, CD_3OD) δ: 7.42 (1H, brs, H-2), 7.40 (1H, d, J=8.2Hz, H-6), 6.78 (1H, d, J=8.2Hz, H-5). \(^{13}\)C-NMR (125MHz, CD_3OD) 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), C9H8O4, 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, C16H20O9, 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); 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), C9H8O3, 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), C7H6O3, Negative ESI-MS m/z: 139.0[M-H]⁺, ¹H-NMR (500MHz, DMSO-d6) 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-d6) 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, C22H22O11, 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=2.2Hz, H-2'), 6.90 (1H, d, J=8.4Hz, H-5'), 6.79 (1H, d, J=2.1Hz, H-6), 6.75 (1H, s, H-3), 6.44 (1H, d, J=2.1Hz, H-8), 5.08 (1H, d, J=7.5Hz, H-1''), 3.20-3.70 (sugar protons), 3.17 (3H, s, -OCH$_3$); $^{13}$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 (-OCH$_3$).

Chrysoeriol (10)

Yellow powder, C$_{12}$H$_{12}$O$_6$, Negative ESI-MS m/z: 299.2[M-H$^-$], $^1$H-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=8.8Hz, H-5'), 6.89 (1H, s, H-3), 6.49 (1H, d, J=1.5Hz, H-8), 6.18 (1H, d, J=1.5Hz, H-6), 3.89 (3H, s, -OCH$_3$); $^{13}$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 (-OCH$_3$).

Luteolin (11)

Yellow powder, C$_{15}$H$_{10}$O$_6$, Negative ESI-MS m/z: 287.1[M-H$^-$], $^1$H-NMR (500MHz, DMSO-d$_6$) δ: 7.41 (1H, dd, J=8.4, 2.4Hz, H-6'), 7.39 (1H, d, J=2.4Hz, H-2''), 6.89 (1H, d, J=8.4Hz, H-5'), 6.67 (1H, s, H-3), 6.44 (1H, d, J=2.4Hz, H-6), 6.18 (1H, d, J=2.4Hz, 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).
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 Na$_2$CO$_3$ (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.

Total flavonoid contents of the extract was examined by the AlCl$_3$ method. The reaction mixture contains 150 μl of sample solution, and 150 μl of AlCl$_3$ 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 ± 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, 150μL 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:

\[ \text{Inhibition percentage (\%)} = \left[ 1 - \frac{(A_i-A_j)}{A_c} \right] \times 100\% \]

\( A_i \) 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 2μL 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. 198μL 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:

\[ \text{Inhibition percentage (\%)} = \left[ 1 - \frac{(A_i-A_j)}{(A_i-A_c)} \right] \times 100\% \]

\( A_i \) 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).