Gallotannins and Related Polyphenols from *Pistacia weinmannifolia*

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Abstract: **Two new gallotannins, pistafolins A (1) and B (2), were isolated from the leaf extract of *Pistacia weinmannifolia*. Their structures were determined by spectral methods. Four known gallotannins (3–6), seven known flavonoid glycosides (7–13), along with 1-*O*-β-D-(6′-*O*-galloyl)-glucopyranosyl-3-methoxy-5-hydroxybenzene (14), gallic acid (15), methyl gallate (16), (+)-catechin (17), and (+)-gallocatechin (18), were also isolated. Some of these compounds were tested for their cytotoxicity toward K562 cells, and two small molecular phenolic compounds, 15 and 18, showed significant inhibitory effects with IC_{50} values less than 5 μg/ml.**

Key words: *Pistacia weinmannifolia*, Anacardiaceae, gallotannin, polyphenols, pistafolins A, B, cytotoxicity.

Introduction

Pistacia weinmannifolia J. Poisson ex Franch. (Anacardiaceae) is a shrub or an arbor widely found in Yunnan province of China. The leaves of this plant are used against dysentery, enteritis, influenza, traumatic bleeding, headache and lung cancer (1). However, its chemical constituents have never been studied. In a survey on them, we found that this medicinal plant was rich in tannins and polyphenols, and an aqueous acetone extract from the leaves showed potent cytotoxic activity against K562 cells.

Materials and Methods

General

Optical rotations were taken on a SEPA-300 polarimeter. IR spectral data were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV spectra were obtained on a UV 210A spectrometer. Mass spectra were recorded on a VG Auto Spec-300 spectrometer. 1D- and 2D-NMR spectra were run on a Bruker AM-400 and DRX-500 instruments with TMS as internal standard, respectively. Column chromatography was performed on Toyopearl HW-40 (coarse and fine grades) (Tosoh), Sephadex LH-20 (Merck), RP-18 (Merck) and MCI-gel CHP-20P (Mitsubishi Chemical Industry Co. Ltd).

Plant material

The leaves of *P. weinmannifolia* were collected in Lunan County, Yunnan Province, People's Republic of China, in April, 1997. The identity of plant material was verified by Prof. Zhong-Wen Lin, and a voucher specimen (No. KIB-97-04-20) was deposited in the Herbarium of the Department of Taxonomy, Kunming Institute of Botany, Academia Sinica.

Extraction and isolation

The dried and powdered leaves (3.2 kg) were extracted with 70% aqueous acetone at room temperature and filtered. The filtrate was concentrated and extracted with ether, EtOAc and *n*-BuOH successively. A part (50 g) of the EtOAc extract (169 g) was chromatographed over Toyopearl HW-40 (coarse, 250 g) with aqueous MeOH and Me_2CO (20% MeOH → 40 % → 60% → MeOH-Me_2CO-H_2O 8 : 1 : 1 → 70% Me_2CO) to give **15** (2.8 g) from 20% MeOH elute and fractions A–G. Fraction B from 20% MeOH elute was further purified by MCI-gel CHP-20P (100 g) with aqueous MeOH in stepwise gradient mode (H_2O → 10% MeOH → 20% → 30% → 40%) to afford **16** (13 mg), **17** (4 mg), and **18** (125 mg). Fraction C from 40% MeOH elute was rechromatographed over RP-18 (150 g) with 20% MeOH → 30% → 40% to yield **4** (140 mg) and **5** (134 mg). Fractions D and E from 60% MeOH and MeOH-Me_2CO-H_2O (8 : 1 : 1) elutes were subjected to repeated column chromatograph over MCI-gel CHP-20P (100 g) and RP-18 (150 g) developing with aqueous MeOH in the similar way, which led to the isolation of **2** (36 mg), **3** (243 mg), **6** (40 mg), **7** (612 mg), **8** (85 mg), **9** (70 mg), **12** (143 mg), **13** (25 mg), and **14** (99 mg). A part (50 g) of the *n*-BuOH extract (214 g) was applied to column chromatography over Sephadex LH-20 eluting with H_2O, aqueous MeOH and Me_2CO (20% MeOH → 30% → 40% → 60% → MeOH-Me_2CO-H_2O 8 : 1 : 1 → 70% Me_2CO) to yield fractions H-M. Fractions K and L from 60% MeOH and MeOH-Me_2CO-H_2O (8 : 1 : 1) elutes were similarly purified by a combination of rechromatography over Toyopearl HW-40 (fine, 80 g) and MCI-gel CHP-20P (100 g) to give **1** (198 mg), **10** (200 mg) and **11** (14 mg).

Pistafolin A (**1**): Off-white amorphous powder, $[\alpha]_D^{22}$: −9.9 (*c* 0.68, MeOH); UV: λ_{max}^{MeOH} (log ε) = 217.5 (4.79), 271.5 nm (4.43); IR (KBr): ν_{max} = 3382, 1715, 1611, 1534, 1446, 1323, 1195, 1086

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and 1025 cm^{-1}; FABMS: *m/z* (rel. intensity) = 647 $[M - H]^-$ (100); negative HRFABMS *m/z*: observed 647.0880 for $C_{28}H_{23}O_{18}$ calcd. 647.0884; ^{1}H-NMR (MeOH-d_4 + D_2O, 400 MHz): δ = 7.58 – 7.13 (galloyl H), 2.30 – 2.00 (4H, m, H-2, 6), 4.21 (1H, br s, $J_{wh/2}$ = 8 Hz, H-3), 3.82 (1H, br s, H-4), 5.41 (1H, br s, $J_{wh/2}$ = 20 Hz, H-5); ^{13}C-NMR data see Table **1**.

Pistafolin B (**2**): Off-white amorphous powder, $[\alpha]_D^{22}$: – 67.9° (*c* 0.24, MeOH): UV: λ_{max}^{MeOH} (log ε) = 217.5 (4.53), 278.0 nm (4.11); IR (KBr): ν_{max} = 3378, 2927, 2855, 1711, 1611, 1533, 1446, 1324, 1205, 1129, 1086 and 1032 cm^{-1}; FABMS: *m/z* (rel. intensity) = 495 $[M - H]^-$ (57); negative HRFABMS *m/z*: observed 495.0834 for $C_{21}H_{19}O_{14}$ calcd. 495.0775; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ = 7.30 – 6.99 (galloyl H), 2.10 – 1.18 (4H, m, H-2, 6), 3.96 (1H, br s, $J_{wh/2}$ = 8 Hz, H-3), 3.62 (1H, br s, H-4), 5.14 (1H, br s, $J_{wh/2}$ = 20 Hz, H-5); ^{13}C-NMR data see Table **1**.

Cytotoxicity assays

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was performed in 96-well plates. K562 cells at a log phase of their growth cycle (4×10^4 cell/ml) were added to each well (90 μl/well), then treated in four replicates at various concentrations of the drugs (10^{-4} – 10^{-8} mol/l) and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. After 48 h, 10 μl of MTT solution (5 mg/ml) per well were added to each cultured medium, which were incubated for a further 4 h. Then, 10% SDS-5% isobutanol-0.012 ml/l HCl was added to each well (100 μl/well). After 12 h at room temperature, the OD of each well was measured on an ELISA reader (Bioteck EL-340, U.S.A.) at two wavelengths (570 and 630 nm). In these experiments, the negative reference agents were isochoric normal saline, 1% DMSO or 0.1% DMSO, and mitoxantrone was used as the positive reference substance with concentrations of 10^{-5} – 10^{-8} mol/l.

Results and Discussion

Pistafolin A (**1**) was obtained as an off-white amorphous powder and gave a dark blue color with ferric chloride characteristic of gallotannins. The molecular formula was established as $C_{28}H_{24}O_{18}$ based on negative HRFABMS (obsd 647.0880, calcd 647.0884). The presence of galloyl groups was revealed by the analysis of the ^{1}H-NMR and ^{13}C-NMR spectra. The ^{13}C-NMR and DEPT spectra also showed seven carbon signals due to two methylenes (δ = 37.9, 38.6), three oxygen-bearing methines (δ = 71.3, 72.8, 73.3), a quaternary carbon (δ = 73.6), and a carboxyl signal (δ = 176.8), which corresponded to a quinic acid moiety.

In the ^{1}H-NMR spectrum, besides the signals of galloyl groups in the aromatic region and two methylenes at δ = 2.00 – 2.30, three broad 1H singlets were observed. One bearing the galloyl groups appeared in the lowfield (δ = 5.41), the other two free from them were shown in the upfield (δ = 3.82, 4.21) regions. So, when these three signals were assigned, the location of the galloyl groups on the quinic acid nucleus would be determined. Based on the ^{1}H-^{1}H COSY spectrum, the methine at δ = 3.82 was ascribable to H-4 of the quinic acid moiety. The assignment of H-3 and H-5 was achieved through the half-width values (2). Because H-3, H-4 and H-5 possess equatorial, axial and axial orientations respectively, H-5 should have a large half-width, while H-3 should have a small one. Accordingly, the lowfield proton (δ = 5.41, br s, $J_{wh/2}$ = 20 Hz) and the upfield signal (δ = 4.21, br s, $J_{wh/2}$ = 8 Hz) were assigned to H-5 and H-3 respectively, indicating the gallic acid ester at C-5. Simultaneously, the carbon signals of the quinic acid moiety were also assigned by the HMQC spectrum.

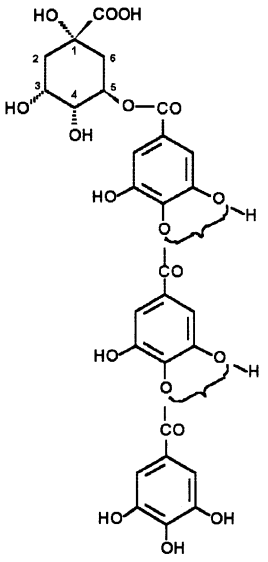
From the molecular formula, the occurrence of three galloyl groups linked through depside bonds could be deduced. Depsidically linked galloyl groups have been reported to occur in solution as an equilibrium mixture of *m*- and *p*-depside forms (3). The complication of the signals due to galloyl groups in the ^{1}H-NMR and ^{3}C-NMR spectra of **1** indicated that it actually existed as an equilibrium mixture of *m*- and *p*-isomers. Thus, pistafolin A (**1**) was characterized as 5-*O*-trigalloylquinic acid.

Pistafolin B (**2**), off-white amorphous powder, afforded a dark blue color with ferric chloride, indicating the presence of gal-

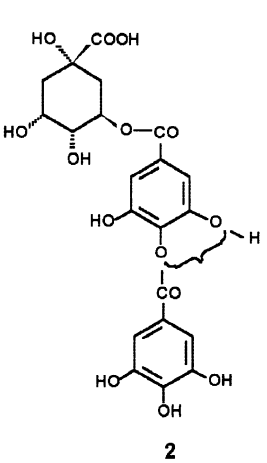
Table 1 ^{13}C-NMR data for compounds **1** and **2** (100 MHz, δ in ppm).

Carbon	1[a]	2[b]
Quinic acid moiety		
1	73.6 (s)	73.7 (s)
2	37.9 (t)	36.3 (t)
3	71.3 (d)	68.4 (d)
4	73.3 (d)	72.3 (d)
5	72.8 (d)	70.6 (d)
6	38.6 (t)	37.2 (t)
COOH	176.8 (s)	175.2 (s)
Galloyl moiety		
1′	121.8, 120.6, 120.5 (s)	120.1, 118.8 (s)
2′, 6′	118.2 – 110.1 (d)	116.1 – 108.6 (d)
3′, 5′	151.8 – 144.2 (s)	150.5 – 143.1 (s)
4′	140.4, 140.2, 140.0 (s)	139.3, 139.1 (s)
CO	167.1, 166.6, 165.9 (s)	165.2, 164.5, 164.0 (s)

[a] Recorded in MeOH-d_4.
[b] Recorded in DMSO-d_6.



1



2

loyl group. The signals in the ^{1}H-NMR and ^{13}C-NMR spectra were very similar to those of pistafolin A (**1**), suggesting the same structural skeleton as **1**. From ^{1}H-^{1}H COSY and the half-width values, the location of the galloyl groups was considered to be at C-5. The molecular formula, $C_{21}H_{20}O_{14}$ established by negative HRFABMS (obsd 495.0834, calcd 495.0775), determined that **2** was a quinic acid digallate, where two galloyls were linked by a depside bond. Accordingly, pistafolin B (**2**) was identified as 5-*O*-digalloylquinic acid.

Besides the two new gallotannins described above, four known gallotannins, octa-*O*-galloyl-*β*-D-glucose (**3**) (4), 3-*O*-galloylshikimic acid (**4**), $[\alpha]_D^{24}$: −111.7° (*c* 0.97, acetone), 5-*O*-galloylshikimic acid (**5**), $[\alpha]_D^{24}$: −142.0° (*c* 0.50, acetone) (5) and 4,5-di-*O*-galloylquinic acid $[\alpha]_D^{27}$: −35.5° (*c* 0.20, acetone) (**6**) (2), seven known flavonoid glycosides, myricetin 3-*O*-*α*-L-rhamnopyranoside (**7**) (6), quercetin 3-*O*-*α*-L-rhamnopyranoside (**8**) (7), myricetin 3-*O*-(3″-*O*-galloyl)-*α*-L-rhamnopyranoside (**9**) (6), myricetin 3-*O*-(6″-*O*-galloyl)-*β*-D-galactopyranoside (**10**) (8), myricetin 3-*O*-(6″-*O*-galloyl)-*β*-D-glucopyranoside (**11**) (9), myricetin 3-*O*-*β*-D-glucuronide (**12**), $[\alpha]_D^{24}$: −50.6° (*c* 0.30, MeOH) (10) and quercetin 3-*O*-*β*-D-glucuronide (**13**) (7), and five phenolic compounds, 1-*O*-*β*-D-(6′-*O*-galloyl)-glucopyranosyl-3-methoxy-5-hydroxybenzene (**14**) (11), gallic acid (**15**), methyl gallate (**16**), (+)-catechin (**17**) $[\alpha]_D^{24}$: +17.1° (*c* 0.45, acetone) (12) and (+)-gallocatechin (**18**) $[\alpha]_D^{24}$: +14.7° (*c* 0.30, acetone), were also isolated.

Compounds **1**, **3**, **5**, **6**, **7**, **8**, **9**, **10**, **12**, **15** and **18** were evaluated for their cytotoxicity against K562 cells. The IC_{50} values of them were 128.24, ND, ND, ND, 547.06, ND, ND, 147.57, 44.84, 2.22, and 4.16, respectively. Two small molecular phenolic compounds, gallic acid (**15**) and (+)-gallocatechin (**18**) showed significant inhibitory effect on K562 cells.

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