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RAPID IDENTIFICATION OF FLAVONOID GLYCOSIDES IN *Pasania kawakamii* AND *Cyclobalanopsis morii* VIA HPLC/MS AND HPLC-SPE-NMR[a]

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Pasania kawakamii (Hayata) Schottky and *Cyclobalanopsis morii* (Hayata) Schottky (Fagaceae) are broad-leaved evergreen trees, both endemic at medium-high altitude in Taiwan [1, 2]. Fagaceous plants have been reported to be rich in polyphenolic compounds, including both hydrolyzable and condensed tannins [3, 4]. However, few studies were related to the composition of flavonoids. Thus, this study was aimed at identifying flavonoids in the leaves of these two plants via an analytic approach. First, the flavonoids containing polyphenolic functions that showed strong UV absorption were focused by Sephadex LH-20 column chromatography from the *n*-BuOH-soluble fractions of the EtOH extract of the leaves of *P. kawakamii* and *C. morii* and detected by TLC under UV 254 nm. Second, the HPLC conditions for analysis of flavonoids from 12 *Litsea* and *Neolitsea* plants [5] and *Machilus philippinense* [6] developed in our laboratory were applied to identify the constituents in the UV-positive subfractions. This approach led to the identification of six known flavonoid glycosides **1**–**6** in total on the basis of the HPLC retention time and MS data (Table 1). The presence of **1**–**3** in subfraction 2 from *P. kawakamii* and **3**–**6** in the subfraction I–3 from *C. morii* was further confirmed by HPLC analysis *via* co-injection with authentic samples from our laboratory collection.

Compounds **1**–**6** were identified as rutin (**1**), kaempferol 3-*O*-(6″-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**2**), quercetin 3-*O*-α-L-rhamnopyranoside (**3**), quercetin 3-*O*-β-D-galactopyranoside (**4**), quercetin 3-*O*-β-D-glucopyranoside (**5**), and kaempferol 3-*O*-α-L-rhamnopyranoside (**6**).

As the retention time and MS data of most constituents in *C. morii* did not match those identified compounds from our collection, the flavonoid-rich fraction (subfraction II-2) was subjected to HPLC-SPE-NMR analysis. Using the same delivery system as that for LC-MS analysis for subfraction I-3, the application of HPLC-SPE-NMR in combination with the ESI-MS data led to the identification of 11 flavonoid glycosides **5**–**15** whose ESI-MS (Table 2) and characteristic PMR data are listed in Table 3.

Flavonoid glycosides **5**–**15** could be classified into two groups according to the skeletons of their aglycons, i.e., seven flavonols (**5**–**10**, **15**) and four flavones (**11**–**14**). The PMR spectral data of **6**, **7**, and **9** (600 MHz, CD_3CN) indicated an AA′XX′ system [δ 8.09 (H-2′/H-6′) and 6.92 (H-3′/5′), J = 8.8 Hz, **7**] and a *meta*-coupled AX system [δ 6.26 (H-6) and 6.47 (H-8), J = 1.9 Hz, **7**] for a kaempferol moiety, while those of **8**, **10**, and **15** showed signals for an isorhamnetin moiety, an AMX system for H-5′, H-6′, and H-2′ (δ 6.94, 7.66, and 7.92, J_m = 2.0 Hz and J_o = 8.4 Hz, **10**), an AX system for H-6 and H-8 (δ 6.26 and 6.49, J =1.8 Hz, **10**) and a methoxy singlet (δ 3.92, **10**). The NOESY spectrum of **10** showed the correlation of δ 3.92 (MeO-3′) to δ 7.92 (H-2′), supporting the designation for MeO-3′. The PMR spectrum of **11** showed typical signals for the flavone apigenin moiety, an aryl proton singlet at δ 6.63 for H-3 in addition to the AA′XX′ system for H-2′/H-6′ (δ 7.88) and H-3′/5′ (δ 6.97, J = 8.8 Hz), and an AX system for H-6 and H-8 (δ 6.44 and 6.74, J = 2.2 Hz). Compounds **12–14** possessed an *O*-methylluteolin moiety, as exemplified by the PMR spectral data (600 MHz, CD_3CN), all revealing a methoxy singlet (δ 3.95, **12**) in addition to the H-3 singlet (δ 6.69, **12**), an AMX system for H-5′, H-2′, and H-6′ (δ 6.98, 7.51, and 7.55, J_m = 1.9 Hz, and J_o = 8.4 Hz, **12**), and an AX system for H-6 and H-8 (δ 6.45 and 6.77, J = 2.2 Hz, **12**).

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TABLE 1. HPLC/MS Data for **1–3** from Subfraction 2 of *P. kawakamii* Leaf and **3–6** from Subfraction I-3 of *C. morii* Leaf

Compound	MW	t_R(min)[a]	Δt_R, min[c]	ESI-MS
1	610	31.2 (lit. 31.2 [5])[b1]	0	611.1 $[M + H]^+$, 465.0, 303.0 (lit. 611.1 $[M + H]^+$, 465.0, 303.0 [5])
2	594	34.5 (lit. 34.5 [5])[b1]	0	595.1 $[M + H]^+$, 287.1 (lit. 595.0 $[M + H]^+$, 449.0, 287.0 [5])
3	448	37.1 (lit. 37.2 [5])[b1]	–0.1	449.1 $[M + H]^+$, 303.1
		47.8 (lit. 48.5 [6])[b2]	–0.7	447.0 $[M – H]^–$, 300.9
4	464	31.7 (lit. 33.5 [6])[b2]	–1.8	463.0 $[M – H]^–$, 300.9
5	464	33.2 (lit. 35.1 [6])[b2]	–1.9	463.0 $[M – H]^–$, 300.9
6	432	68.5 (lit. 68.8 [6])[b2]	–0.3	431.0 $[M – H]^–$, 284.9

[a]HPLC conditions for subfraction 2 and subfraction I-3; [b]monitored at 365 nm (b1) and 254 nm (b2); [c]Δt_R, min = t_R (comp) – t_R (lit.).

TABLE 2. ESI-MS Data of Compounds **5–15**

Compound	ESI-MS, *m/z*	Compound	ESI-MS, *m/z*
5	463.0 $[M – H]^–$, 300.9	**11**	431.0 $[M – H]^–$, 268.9
6	431.0 $[M – H]^–$, 284.9	**12**	461.0 $[M – H]^–$, 283.9
7	447.0 $[M – H]^–$, 284.9	**13**	461.0 $[M – H]^–$, 299.0
8	477.0 $[M – H]^–$, 299.9	**14**	461.0 $[M – H]^–$, 298.9
9	447.0 $[M – H]^–$, 284.9	**15**	461.0 $[M – H]^–$, 314.9
10	477.0 $[M – H]^–$, 314.9		

The presence of an MeO at the C-3′ position in **12** was determined by the NOESY spectrum, which revealed the NOE correlation between δ 7.51 and δ 3.95. The glycon moiety in each compound was identified by the characteristic sugar proton signals, e.g., $\delta_{H\text{-}1''}$ 4.99 (d, J = 7.8 Hz) and $\delta_{H\text{-}4''}$ 3.72 (br.d, J = 3.2 Hz) in **7** for the *β*-D-galactopyranosyl moiety, $\delta_{H\text{-}1''}$ 5.06 (d, J = 7.0 Hz) in **8** for the *β*-D-glucopyranosyl moiety [6], and $\delta_{H\text{-}1''}$ 5.43 (br.s) and $\delta_{H\text{-}6''}$ 0.84 (d, J = 5.9 Hz) in **6** for the *α*-L-rhamnopyranosyl moiety [7]. The isomeric compounds such as **7** vs. **9** and **8** vs. **10**, the former being 3-*O*-galactopyranosylated and the latter being 3-*O*-glucopyranosylated, were further distinguished by co-injection of authentic samples, kaempferol 3-*O*-*β*-D-glucopyranoside and isorhamnetin 3-*O*-*β*-D-glucopyranoside, in HPLC analysis. The glycosidic linkage position in **11–14** was supported by the NOESY spectra, which showed the correlation of Glc H-1″ (δ 5.04, **11**) to H-6 (δ 6.44, **11**) and H-8 (δ 6.74, **11**) in **11**–**13** and Glc H-1″ (δ 5.02) to H-2′ (δ 7.73) in **14**, designating the presence of 7-*O*-Glc in **11–13** and 3′-*O*-Glc in **14**.

Based on these analyses, compounds **7–15** were identified as kampferol 3-*O*-*β*-D-galactopyranoside (**7**) [6], kaempferol 3-*O*-*β*-D-glucopyranoside (**9**) [6], isorhamnetin 3-*O*-*β*-D-galactopyranoside (**8**) [8], isorhamnetin 3-*O*-*β*-D-glucopyranoside (**10**) [9], apigenin 7-*O*-*β*-D-glucopyranoside (**11**) [10], chrysoeriol 7-*O*-*β*-D-glucopyranoside (**12**) [11], diosmetin 7-*O*-*β*-D-glucopyranoside (**13**) [12], diosmetin 3′-*O*-*β*-D-glucopyranoside (**14**) [13], and isorhamnetin 3-*O*-*α*-L-rhamnopyranoside (**15**) [14].

This study demonstrates the applicability of the established HPLC methods [5, 6] in analyzing the flavonoid composition of *Pasania kawakamii* and *Cyclobalanopsis morii*. With the assistance of HPLC-SPE-NMR, the minor or unidentified constituents could be clarified more comprehensively on an analytical scale. Thus, the combination of the established HPLC/MS method and HPLC-SPE-NMR not only serves as an efficient tool to explore flavonoid natural resources but is also good for natural reservation.

The physical data were obtained using the following instruments: column chromatography (CC): Sephadex LH-20 (Pharmacia); HPLC: Agilent 1100 system, Phenomenex Prodigy ODS-3 (anal: 250 × 4.6 mm, 5 μm); HPLC-SPE-NMR: Agilent (Waldbronn, Germany) model 1100 liquid chromatograph with photodiode array detector (Bruker DAD, Rheinstetten, Germany), HySphere-Resin GP cartridges (10 × 2 mm, 10 μm; Spark Holland) with a Prospekt 2 (Spark Holland) solid-phase extraction unit, a Knauer (Berlin, Germany) model K120 HPLC makeup pump, a Bruker (Rheinstetten, Germany) nitrogen synthesizer for flushing cartridge, and an Avance III 600 NMR spectrometer equipped with a 5 mm cryoprobe; HPLC-MS: chromatographic system as indicated above, coupled to a Bruker Doltinotics Esquire-2000 ion trap mass spectrometer with electrospray ion source.

TABLE 3. ^{1}H NMR Data of Compounds **5–15** (600 MHz, CD_3CN, δ, ppm, J/Hz)*

Compound	H-3	H-6	H-8	H-2′	H-3′	H-5′
5		6.27 (d, J = 1.8)	6.47 (d, J = 1.8)	7.93 br.s		6.93 (d, J = 8.4)
6		6.23 (d, J = 1.9)	6.42 (d, J = 1.9)	7.79 (d, J = 8.7)	6.95 (d, J = 8.7)	6.95 (d, J = 8.7)
7		6.26 (d, J = 1.9)	6.47 (d, J = 1.9)	8.09 (d, J = 8.8)	6.92 (d, J = 8.8)	6.92 (d, J = 8.8)
8		6.26 (d, J = 1.8)	6.49 (d, J = 1.8)	8.03 br.s		6.94 (d, J = 8.4)
9		6.26 (d, J = 1.3)	6.47 (d, J = 1.3)	8.07 (d, J = 8.6)	6.92 (d, J = 8.6)	6.92 (d, J = 8.6)
10		6.26 (d, J = 1.8)	6.49 (d, J = 1.8)	7.92 (d, J = 2.0)		6.94 (d, J = 8.4)
11	6.63 s	6.44 (d, J = 2.2)	6.74 (d, J = 2.2)	7.88 (d, J = 8.8)	6.97 (d, J = 8.8)	6.97 (d, J = 8.8)
12	6.69 s	6.45 (d, J = 2.2)	6.77 (d, J = 2.2)	7.51 (d, J = 1.9)		6.98 (d, J = 8.4)
13	6.65 s	6.44 (d, J = 2.0)	6.76 (d, J = 2.0)	7.46 (d, J = 2.2)		7.08 (d, J = 8.5)
14	6.64 s	6.22 (d, J = 1.9)	6.54 (d, J = 1.9)	7.73 (d, J = 1.9)		7.13 (d, J = 8.6)
15		6.24 (d, J = 1.9)	6.44 (d, J = 1.9)	7.46 (d, J = 1.8)		6.96 (d, J = 8.3)

Compound	H-6′	H-1″	H-4″	H-6″	MeO-3′	MeO-4′
5	7.50 (dd, J = 1.8, 8.4)	5.07 (d, J = 7.3)				
6	7.79 (d, J = 8.7)	5.43 br.s		0.84 (d, J = 5.9)		
7	8.09 (d, J = 8.8)	4.99 (d, J = 7.8)	3.72 (br.d, J = 3.2)			
8	7.64 (dd, J = 1.7, 8.4)	5.21 (d, J = 7.8)	3.76 (br.d, J = 3.3)		3.93 s	
9	8.07 (d, J = 8.6)	5.06 (d, J = 7.0)				
10	7.66 (dd, J = 2.0, 8.4)	5.19 (d, J = 7.4)			3.92 s	
11	7.88 (d, J = 8.8)	5.04 (d, J = 7.2)				
12	7.55 (dd, J = 1.9, 8.4)	5.04 (d, J = 7.4)			3.95 s	
13	7.54 (dd, J = 2.2, 8.5)	5.04 (d, J = 7.2)				3.93 s
14	7.69 (dd, J = 1.9, 8.6)	5.02 (d, J = 7.0)				3.91 s
15	7.44 (dd, J = 1.8, 8.3)	5.43 br.s		0.83 (d, J = 6.1)	3.92 s	

*^{1}H NMR data were analyzed based on adopted spectra from HPLC-SPE-NMR.

Chemicals and Reagents. MeCN (CAS and chromatographic grade), MeOH, CH_2Cl_2, *n*-BuOH, and trifluoroacetic acid (TFA) were purchased from Mallinckrodt Baker Inc. (KY, USA). Acetonitrile-d_3 (99.8%) was purchased from Cambridge Isotope Lab., Inc. (Andover, MA, USA), and deionized water was obtained from a Barnstead water purification system (Dubuque, IA, USA). Authentic rutin (**1**), kaempferol 3-*O*-(6″-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside (**2**), quercetin 3-*O*-α-L-rhamnopyranoside (**3**), quercetin 3-*O*-β-D-galactopyranoside (**4**), quercetin 3-*O*-β-D-glucopyranoside (**5**), kaempferol 3-*O*-α-L-rhamnopyranoside (**6**), kaempferol 3-*O*-β-D-glucopyranoside (**8**), and isorhamnetin 3-*O*-β-D-glucopyranoside (**10**) were obtained from our laboratory collection.

Plant Materials. The leaves of *C. morii* and *P. kawakamii* were collected in September 2009 at Highland Experimental Farm, National Taiwan University, Nantou County, Taiwan. The voucher specimens (SPNTU200909HEFA/B) were authenticated by Mr. Jer-Tone Lin, Fu-shan Research Center, Taiwan Forestry Research Institute (TFRI), Yilan County, Taiwan.

Extraction and Flavonoid Focusing. Dried leaves of *P. kawakamii* (733.8 g) were powdered and macerated in EtOH (95%) (3.5 L × 4). The EtOH extract (92.4 g), obtained after evaporation under reduced pressure, was suspended in H_2O (500 mL) and then partitioned against CH_2Cl_2 and *n*-BuOH (water saturated), each 500 mL × 3. A small portion of the *n*-BuOH-soluble fraction (105.7 mg out of 35.5 g) was chromatographed over a Sephadex LH-20 column (70 cm high, O.D. 1.0 cm; MeOH) to give three subfractions, combined on the basis of TLC examination (254 and 366 nm), of which subfraction 2 (11.1 mg) was found to be UV-positive. Dry powdered leaves of *C. morii* (401.5 g) were macerated in EtOH (95%) (2.3 L × 5) at room temperature. The EtOH extract (63.5 g) obtained after evaporation under reduced pressure was partitioned and chromatographed in the same manner as described above for *P. kawakamii*. From an aliquot of the *n*-BuOH-soluble fraction from *C. morii* (97.3 mg out of 19.8 g), subfraction I-3 (20.8 mg) was found to be UV-positive. The UV-positive subfractions of both plants were subjected to further analysis, including LC-DAD-MS and spike test. Another aliquot of the *n*-BuOH-soluble fraction from *C. morii* (541.6 mg out of 19.8 g) was chromatographed over a Sephadex LH-20 column (90 cm high, O.D. 2.0 cm; MeOH) to give subfraction II-2, which was found to contain most of the unidentified constituents in subfraction I-3 by HPLC analysis. Thus, this subfraction was subjected to HPLC-SPE-NMR analysis.

LC/MS Analysis of *P. kawakamii*. The UV-positive subfraction 2 (10 μL, 1 mg/mL) as described above was analyzed on an analytical RP-18 column, delivered with MeCN-0.1% TFA_{aq}, 7% to 33% in 44 min to 95% in 3 min, both linear

gradients, and 95% for 6 min; flow rate 0.5 mL/min [5]; UV detection 365 nm. A small proportion (5%) of the LC flow was directed to the MS via a splitter (1:20). The temperature of the ESI interface heated capillary was 250°C. The nebulizer gas (N_2) pressure was set to 15 psi, and a dry gas (N_2) flow of 8 L/min was used. MS data were acquired in the positive mode over a scan range of 100–1200 Da.

LC/MS Analysis of *C. morii*. The UV-positive subfraction I-3 (10 μL, 1 mg/mL) and subfraction II-2 (10 μL, 1 mg/mL) as described above were analyzed on an analytical RP-18 column, delivered with MeCN–H_2O, 15% to 18% in 20 min to 22% in 60 min, both linear gradients; flow rate 0.5 mL/min [6]; UV detection 254 nm. A small proportion (5%) of the LC flow was directed to the MS via a splitter (1:20). The parameter set for ESI-MS was the same as those for subfraction 2 described above, except that MS data were acquired in the negative mode over a scan range of 100–1500 Da.

HPLC-SPE-NMR Analysis. The UV-positive subfraction II-2 (20 μL, 50 mg/mL) of *C. morii* was analyzed using the same HPLC conditions as described for LC-DAD-MS. The post-column eluates were diluted with water, supplied by a makeup pump (flow rate 1.0 mL/min), then trapped by HySphere-Resin GP cartridges. Such LC-SPE procedure was repeated three times. Each compound-loaded cartridge was flushed with dry nitrogen for 30 min to remove the residual solvent, followed by elution with acetonitrile-d_3 into a 2.5 mm NMR tube. The PMR spectra were recorded using a multiple solvent suppression pulse program for residual protons and water signals in the D-solvent. All spectra were measured at 300 K, and the ^1H chemical shift was referenced to the residual proton signal of CD_3CN (99.8%) at δ_H 1.93 ppm.

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