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Anti-inflammatory flavonol acylglycosides from the aerial part of *Lindera akoensis* Hayata†

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Five new flavonol acylglycosides, linderakosides A–E (1–5), together with 30 known compounds were isolated from the aerial part of *Lindera akoensis* Hayata. The structures were established using extensive spectroscopic analysis and comparison of NMR data with those of known compounds. The flavonol acylglycosides 1, 2, and 5 showed *in vitro* anti-inflammatory activity, which decreased LPS-stimulated nitrite production in RAW 264.7 cells. The structure activity relationships (SAR) of the flavonol acylglycoside compounds were also established to research for potential lead compounds as anti-inflammatory drugs.

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Introduction

Lindera akoensis Hayata (Lauraceae) is an endemic large evergreen shrub that is widely distributed in Taiwan broad-leaved forests. This plant is a synonym of *Benzoïn akoense* (Hayata) Kamik.¹ *L. akoensis* has been used as a folk medicine for treatment of inflammation and trauma.² Previous phytochemical studies revealed that the genus *Lindera* contains alkaloids,³ anthraquinone,⁴ aporphines, butanolides,⁵ essential oil,⁶ flavonoids, furanoids, chalconoids,⁵ phenolic compounds,^{7,8} and sesquiterpenoids.^{9,10} Earlier chemical and pharmacological studies on this endemic species of *L. akoensis* suggested that its butanolides account for its anti-inflammatory activities and antimycobacterial activities against *Mycobacterium tuberculosis* H37Rv.^{5,11,12} Previous studies on the aerial part of *L. akoensis* resulted in isolation of 10 butanolides, five lignans, and five flavonols.^{5,11} In the present study, further detailed chemical investigation of the same 95% ethanol extract of *L. akoensis* has

resulted in isolation of five new flavonol acylglycosides (1–5) (Fig. 1), along with known compounds including three amides, eight apocarotenoids, 10 phenolic compounds, and nine porphyrinoids, which were isolated from this plant for the first time. Potential anti-inflammatory activity of isolates 1, 2, 4 and 5 was investigated *via* examining the inhibitory activity toward nitric oxide (NO) production induced by lipopolysaccharide in mouse macrophage RAW 264.7 cells tested *in vitro* (Table 1).

Results and discussion

The aerial part of *L. akoensis* was collected in Taiwan and extracted with 95% ethanol. The extract was partitioned into EtOAc and H₂O layers. The EtOAc layer was purified using conventional chromatographic techniques yielding 35 compounds. Structures of the compounds were elucidated using spectroscopic techniques and these structures were compared with data from the literature.

Compound 1 was isolated as a pale yellow solid. The molecular formula C₃₁H₂₈O₁₂ was determined on the basis of its HR-ESI-MS (calcd for C₃₁H₂₈O₁₂Na, 615.1473), which showed a pseudo-molecular ion peak at *m/z* 615.1479, corresponding to 18 degrees of unsaturation. The IR spectrum indicated the existence of a hydroxyl group (3426 cm^{−1}), conjugated carbonyl group (1651 cm^{−1}), and aromatic ring (1605 and 1513 cm^{−1}). The UV absorption bands indicated λ_{max} at 267 nm (log ε 4.56) and 313 (log ε 4.69) nm. The NMR spectra showed that 1 has a structure to similar to that of 4'-O-methylkaempferol-3-O-α-L-(4''-E-p-coumaroyl)rhamnoside, which has been isolated from this plant previously.⁵ This evidence suggests that 1 is a flavonoid glycoside derivative from its characteristic yellow color and spectral properties. The ¹H, DEPT, and HSQC spectra of 1 indicated the presence of a 5,7-dihydroxy A ring system [δ_H 6.20

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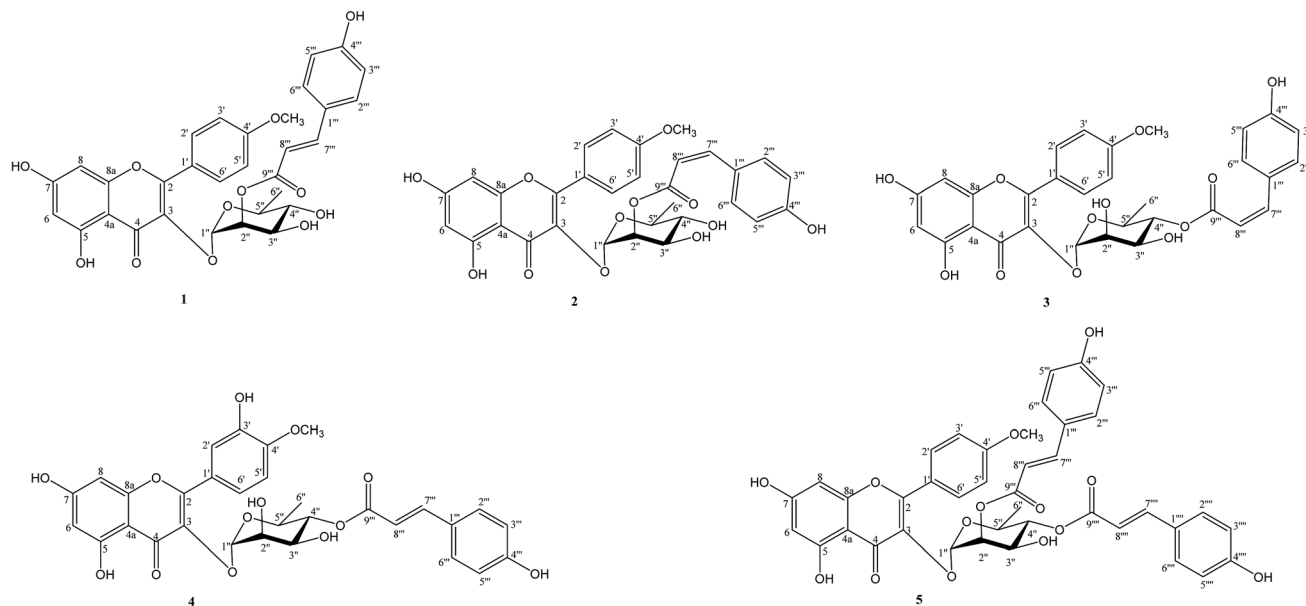


Fig. 1 Chemical structures of new compounds 1–5.

(d, $J = 2.0$ Hz, H-6) and 6.38 (d, $J = 2.0$ Hz, H-8)] and a 1,4-disubstituted B ring [δ_{H} 7.09 (d, $J = 8.8$ Hz, H-3', 5') and 7.88 (d, $J = 8.8$ Hz, H-2', 6')] structure in flavonol, one sugar resonance [δ_{H} 5.48 (d, $J = 1.4$ Hz, H-1'')/ δ_{C} 100.8], and a methoxy group [δ_{H} 3.89/ δ_{C} 56.2]. These data suggest that 1 contains a flavonol glycoside derivative.¹³ In the ^{13}C NMR spectrum of 1, significant flavonol signals were observed at δ_{C} 159.0 (C-2), 136.0 (C-3), and

176.5 (C-4). The NOESY correlations between the methoxy singlet resonance with δ_{H} 7.09 (H-3', 5') suggested location of a methoxy at C-4'. HMBC correlations from δ_{H} 7.88 (2H, H-2' and H-6') to δ_{C} 159.0 (C-2), as well as δ_{H} 3.89 (OCH₃) to 163.7 (C-4'), assumed that the aglycone of 1 was a kaempferide skeleton.¹⁴ For the glycone moiety, the carbon signal at δ_{C} 100.8 showed correlation with the anomeric proton at δ_{H} 5.48 in the

Table 1 ^1H NMR spectroscopic data of compounds 1–5 (in CDCl₃, 500 MHz)^a

No.	1	2	3	4	5
6	6.20, d (2.0)	6.21, d (2.1)	6.21, d (2.1)	6.21, d (2.0)	6.22, d (2.0)
8	6.38, d (2.0)	6.38, d (2.1)	6.38, d (2.1)	6.39, d (2.0)	6.39, d (2.0)
2'	7.88, d (8.8)	7.88, d (8.8)	7.81, d (8.5)	7.37, br s	7.90, d (8.9)
3'	7.09, d (8.8)	7.10, d (8.8)	7.06, d (8.5)		7.18, d (8.9)
5'	7.09, d (8.8)	7.10, d (8.8)	7.06, d (8.5)	7.12, d (8.2)	7.18, d (8.9)
6'	7.87, d (8.8)	7.88, d (8.8)	7.81, d (8.5)	7.38, d (8.2)	7.90, d (8.9)
1''	5.48, d (1.4)	5.40, d (1.0)	5.51, d (1.0)	5.60, br s	5.72, d (1.4)
2''	5.52, dd (3.0, 1.4)	5.49, dd (3.5, 1.0)	4.23, dd (3.0, 1.0)	4.23, br s	5.54, dd (2.5, 1.4)
3''	3.93, dd (9.5, 3.0)	3.94, dd (9.5, 3.5)	3.89, dd (9.7, 3.0)	3.94, dd (9.7, 3.2)	4.17, dd (9.8, 2.5)
4''	3.39, dd (9.5, 6.7)	3.28, t (9.5)	4.90, t (9.7)	4.91, t (9.7)	4.97, t (9.8)
5''	3.39, qd (5.5, 6.7)	3.49, qd (6.2, 9.5)	3.28, qd (6.3, 9.7)	3.23, qd (6.3, 9.7)	3.31, qd (6.2, 9.8)
6''	0.99, d (5.5)	0.96, d (6.2)	0.76, d (6.3)	0.78, d (6.3)	0.85, d (6.2)
2'''	7.45, d (8.7)	7.61, d (8.6)	7.66, d (8.6)	7.53, d (8.6)	7.50, d (8.6)
3'''	6.79, d (8.7)	6.75, d (8.6)	6.74, d (8.6)	6.83, d (8.6)	6.82, d (8.6)
5'''	6.79, d (8.7)	6.75, d (8.6)	6.74, d (8.6)	6.83, d (8.6)	6.82, d (8.6)
6'''	7.45, d (8.7)	7.61, d (8.6)	7.66, d (8.6)	7.53, d (8.6)	7.50, d (8.6)
7'''	7.53, d (15.8)	6.85, d (12.8)	6.87, d (12.8)	7.59, d (16.0)	7.55, d (16.0)
8'''	6.33, d (15.8)	5.78, d (12.8)	5.72, d (12.8)	6.29, d (16.0)	6.27, d (16.0)
OCH ₃	3.89, s	3.88, s	3.89, s	3.89, s	3.87, s
2''''					7.50, d (8.6)
3''''					6.85, d (8.6)
5''''					6.85, d (8.6)
6''''					7.50, d (8.6)
7''''					7.68, d (16.0)
8''''					6.42, d (16.0)

^a The chemical shifts are expressed in δ ppm. The coupling constants (J) are expressed in Hz.



HSQC. The distinct methyl proton of Rha H-6 (δ_{H} 0.99, 3H, d, J = 5.5 Hz) and small coupling constant (J = 1.4 Hz) of the anomeric proton were assigned as a α -L-rhamnopyranoside moiety using the characteristic ^1H NMR signals. The α -L-rhamnopyranoside moiety was linked at C-3 of the flavone, from a cross-peak between H-1'' (δ_{H} 5.48) of rhamnose and C-3 (δ_{C} 136.0) of the aglycon. In addition, a 1,4-disubstituted aromatic ring [δ_{H} 6.79 (d, J = 8.7 Hz, H-3''', 5''') and 7.45 (d, J = 8.7 Hz, H-2''', 6''') as well as *trans*-olefinic signals [δ_{H} 7.53 and 6.33 (each 1H, d, J = 15.8 Hz)] were observed in the presence of an (*E*)-*p*-coumaroyl moiety. A detailed comparison of the ^{13}C -NMR data between **1** and the afzelin literature data,¹⁵ the downfield shifts for C-2'' of Rha ($\Delta\delta$ + 1.6 ppm) and upfield shifts for C-1'' ($\Delta\delta$ - 2.7 ppm) and C-3'' ($\Delta\delta$ - 1.9 ppm) of Rha, suggested that **1** was esterified at C-2''. Furthermore, the HMBC correlation between the H-2'' and C-9''' indicated that an (*E*)-*p*-coumaroyl moiety was located at the C-2'' position. Accordingly, the structure of **1** was elucidated as 4'-*O*-methyl-2''-(*E*)-*p*-coumaroylafzelin, and named linderakoside A.

Compound **2** was isolated as a pale yellow solid, with molecular formula obtained as $\text{C}_{31}\text{H}_{28}\text{O}_{12}$ from HR-ESI-MS (m/z 615.1477 [$\text{M} + \text{Na}$]⁺, calcd 615.1473) analyses with 18 degrees of unsaturation. IR and UV spectra were nearly the same as those of **1**. 1D and 2D NMR spectra analyses established a kaempferide glycoside skeleton, which was also closely related to that of **1**. Compound **2** was identified as the *Z*-isomer of **1**, according to *cis*-olefinic protons at δ_{H} 6.85 and 5.78 (each 1H, d, J = 12.8 Hz) in **1**. Moreover, the (*Z*)-*p*-coumaroyl moiety position was determined to be C-2'' using the HMBC correlations between H-2'' (δ_{H} 5.47) and C-9''' (δ_{C} 167.3). Based on the obtained data, compound **2** was determined as 4'-*O*-methyl-2''-(*Z*)-*p*-coumaroylafzelin, and named linderakoside B.

The molecular formula of compound **3** was given as $\text{C}_{31}\text{H}_{28}\text{O}_{12}$ with 18 degrees of unsaturation from HR-ESI-MS at m/z 615.1476 [$\text{M} + \text{Na}$]⁺ (calcd 615.1473), and compound **3** exhibited the same molecular weight as **2**. The NMR, UV, and IR data showed signal patterns similar to those of **2**; however, the rhamnopyranose moiety substitution patterns differed. The ^1H -NMR of rhamnose signals were easily assigned using the characteristic doublet signal of methyl. The ^1H -NMR signal for Rha-6'' (δ_{H} 0.76, d, J = 6.3 Hz) shifted upfield ($\Delta\delta$ - 0.20 ppm) compared with that of **2**, which was shielded by the flavone C-ring, and the ^1H -NMR signal of Rha-4'' (δ_{H} 4.90, t, J = 9.7 Hz) appeared relatively downfield ($\Delta\delta$ + 1.51 ppm) using the esterified *p*-coumaroyl moiety.¹⁶ From ^{13}C NMR spectra comparison of **3** and **2** in the L-rhamnose moiety, the downfield shifts for Rha C-1'' ($\Delta\delta$ + 2.1 ppm) and Rha C-4'' ($\Delta\delta$ + 2.4 ppm) and upfield shifts of Rha C-2'' (-1.5) and C-5'' (-3.3) in **3**, implied that the (*Z*)-*p*-coumaroyl moiety was located at Rha C-4'' in **3**, instead of Rha C-2'' as in **2**. The HMBC correlation between δ_{H} 4.90 (H-4'') and δ_{C} 167.9 (C-9''') of **3** indicated that the (*Z*)-*p*-coumaroyl moiety was located at the C-4 position. Thus, the structure of **3** was elucidated as 4'-*O*-methyl-4''-(*Z*)-*p*-coumaroylafzelin, and named linderakoside C.

The molecular formula of compound **4** was obtained as $\text{C}_{31}\text{H}_{28}\text{O}_{13}$ from HR-ESI-MS (m/z 631.1423 [$\text{M} + \text{Na}$]⁺, calcd 631.1422) with 18 degrees of unsaturation, and thus contains

one more oxygen atom than **1**. The spectroscopic features of **4** were closely related to the spectroscopic features of **1**, except for the presence of aromatic ABX-coupling signals [δ_{H} 7.37 (1H, br s), 7.12 (1H, d, J = 8.2 Hz), and 7.38 (1H, d, J = 8.2 Hz)] rather than a 1,4-disubstituted B ring structure in flavonol, and the rhamnopyranoside unit substitution patterns differed. The ^1H -NMR data for **4** showed that an aromatic ABX-coupling system was ascribed to the presence of hydroxyl and methoxy substituents. The 4'-OMe was deduced according to a NOESY correlation between a methoxy proton (δ_{H} 3.89) and H-5' (δ_{H} 7.12) and HMBC correlation between δ_{H} 3.89 (OCH₃) and δ_{C} 152.0 (C-4'), and thus the hydroxyl group was located at C-3' (Fig. 2). From the HMBC spectrum, the correlation between δ_{H} 7.37 (H-2') and δ_{C} 159.4 (C-2), 152.0 (C-4'), 148.0 (C-3'), and 124.5 (C-1'), δ_{H} 7.12 (H-5') and δ_{C} 148.0 (C-3'), 152.0 (C-4'), and 124.5 (C-1'), and δ_{H} 7.39 (H-6') and δ_{C} 159.4 (C-2), 112.7 (C-5'), 131.4 (C-2'), and 152.0 (C-4'), assumed that the flavone moiety of **4** was tamarixetin.¹⁷ The characteristic doublet methyl signal (CH₃-6'') of rhamnose upfield (δ_{H} 0.78, d, J = 6.3 Hz) and the triplet of H-4'' downfield (δ_{H} 4.91, t, J = 9.7 Hz) in **4** was the same as in **3**, which suggested that the (*E*)-*p*-coumaroyl moiety was located at Rha C-4'' in **4**. HMBC spectrum inspection showed correlations between δ_{H} 5.60 (Rha-1'') and δ_{C} 135.7 (C-3) and between δ_{H} 4.91 (Rha-4'') and δ_{C} 168.9 (C-9'''), indicating Rha-C-1'' linkage to C-3 of the flavone and of Rha-C-4'' to (*E*)-*p*-coumaroyl-C-9''', respectively. The above evidence was used to identify **4** as a 2''-(*E*)-*p*-coumaroyltamarixetin, and the compound was named linderakoside D.

With molecular formula calculated as $\text{C}_{40}\text{H}_{34}\text{O}_{14}$ by HR-ESI-MS (m/z 761.1846 [$\text{M} + \text{Na}$]⁺ calcd 761.1841), further combined with the observation of ^{13}C and DEPT spectra, compound **5** was suggested to have a similar kaempferide glycoside skeleton to **1**. Comparing **5** with **1**, there were similarities in both the UV and IR data and the ^1H NMR spectra, but a difference appeared in the HR-ESI-MS analysis of one more (*E*)-*p*-coumaroyl moiety (C₉H₇O₂). In the ^1H NMR spectrum, *ortho*-coupled proton signals at δ_{H} 7.50, 6.85 (each 2H, d, J = 8.6 Hz, H-2''', H-6''') and *trans*-olefinic protons at δ_{H} 7.68, 6.42 (each 1H, d, J = 16.0 Hz, H-7''', H-8''') indicated that **5** possessed an *E*-olefinic functionality. NMR data for **5** compared with those of **1** revealed the downfield shifts for Rha C-4'' ($\Delta\delta$ + 2.9 ppm) and upfield shifts of Rha C-3'' (-1.9) and C-5'' (-3.2) in **5**, suggesting an additional *p*-coumaroyl moiety at C-4''. This conclusion was supported by the HMBC correlation between H-4'' (δ_{H} 4.97) of the L-rhamnose and C-9''' (δ_{C} 168.4). Based on the above evidence, **5** was determined to be 4'-*O*-methyl-2'',4''-di-(*E*)-*p*-coumaroylafzelin, and named linderakoside E.

The 30 known compounds including three amides, moupinamide (**6**),¹⁸ *N*-*p*-coumaroyltyramine (**7**),¹⁹ and *N*-*trans*-sinapoyltyramine (**8**),¹⁹ eight apocarotenoids, 4,5-dihydroblumenol A (**9**),²⁰ epiloliolide (**10**),²¹ (7*E*)-3 β -hydroxy-5 α ,6 α -epoxy-magastigmen-9-one (**11**),²² 2 α ,4 β -dihydroxy-2,6,6-trimethylcyclohexanone (**12**),²³ (3*S*,4*S*,5*S*,6*S*,9*R*)-3,4-dihydroxy-5,6-dihydro- β -ionone (**13**),²⁴ boscialin (**14**),²⁵ grasshopper ketone (**15**),²⁶ and loliolide (**16**),²¹ 10 phenolic compounds, 2-hydroxymethyl-4-nitrophenol (**17**),²⁷ 4-hydroxy-3,5-dimethoxybenzaldehyde (**18**),²⁸ isovanillin (**19**),²⁸ *p*-hydroxybenzaldehyde (**20**),²⁹ vanillin (**21**),³⁰ *p*-hydroxybenzoic acid (**22**),³¹ 4-hydroxy-3-methoxynitrobenzene (**23**),³² *trans*-ferulic



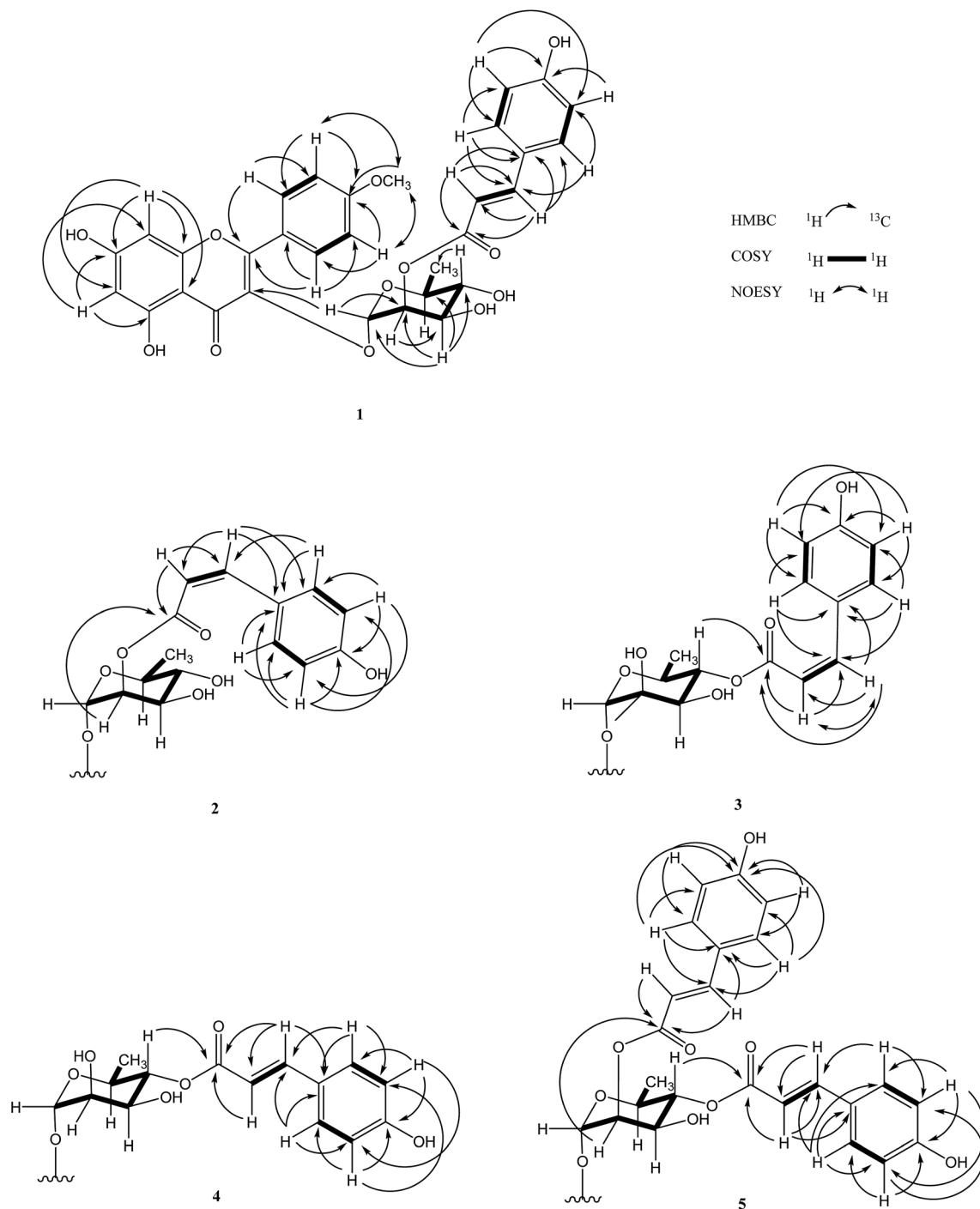


Fig. 2 Selected key HMBC, COSY, and NOESY correlations of compounds 1–5.

ester (24),³³ 2-methyl-4-nitrophenol (25),³⁴ and 3-hydroxy-4-methoxybenzoic acid (26),³⁵ and nine porphyrinoids, (13²*R*)-13²-hydroxypheophytin a (27),³⁶ (13²*S*)-13²-hydroxypheophytin a (28),³⁶ pheophytin a (29),³⁷ (10*S*)-pheophytin a (30),³⁸ pheophytin b (31),³⁹ aristophyll-C (32),³⁶ 7'-oxoaristophyll-C (33),³⁶ (13²*S*)-13²-hydroxypheophytin b (34),³⁶ and methyl rel-(15¹*R*)-3¹,3²-didehydro-15¹-hydroxy-7¹-oxo-17³-*O*-phytyl-rhodochlorin 15-acetate δ -lactone (35),⁴⁰ were identified by comparison of their physical and reported spectroscopic data (Table 2).

The potential anti-inflammatory activities of compounds 1, 2, 4, and 5 from *L. akoensis* were tested *in vitro*, by examining any decrease in LPS-stimulated nitrite production in RAW 264.7 cells (Table 3). Compounds 1, 2, and 5 exhibited significant inhibitory activities against nitric oxide production with IC₅₀ values of 19.1, 25.1, and 9.4 μM , respectively. There was no significant change in cell viability among these active compounds (Table 3). These results are consistent with data from the literature on the kaempferide glycoside skeleton, in



Table 2 ^{13}C NMR spectroscopic data of compounds 1–5 (in CDCl_3 , 125 MHz)

No.	1	2	3	4	5
2	159.0	159.1	158.8	159.4	159.1
3	136.0	136.1	136.0	135.7	135.2
4	179.5	179.7	179.6	179.7	179.4
4a	106.1	106.1	106.1	106.1	106.1
5	158.7	158.7	158.8	158.8	158.8
6	100.1	100.1	100.1	100.2	100.2
7	166.1	166.1	166.3	166.3	166.2
8	95.0	95.0	95.0	95.0	95.0
8a	163.6	163.4	163.4	163.4	163.4
1'	123.8	123.8	124.0	124.5	124.0
2'	131.8	131.8	132.1	131.4	132.0
3'	115.5	115.5	116.6	148.0	115.5
4'	163.3	163.7	163.4	152.0	163.7
5'	115.4	115.5	116.6	112.7	115.5
6'	131.9	131.8	132.0	131.4	132.0
1''	100.8	100.8	102.9	102.5	99.5
2''	73.5	73.5	72.0	71.9	73.3
3''	70.8	70.7	70.2	70.3	68.6
4''	72.3	72.3	74.7	75.0	74.9
5''	73.7	73.2	69.9	69.9	69.9
6''	17.9	17.9	17.8	17.8	17.9
1'''	127.3	127.7	127.7	127.4	127.3
2'''	131.4	133.9	134.0	131.5	131.4
3'''	117.0	116.1	116.0	117.0	117.0
4'''	161.4	160.2	160.3	161.5	161.6
5'''	117.0	116.1	116.0	117.0	117.0
6'''	131.4	133.9	134.0	131.5	131.4
7'''	147.3	145.8	145.5	146.9	147.1
8'''	115.0	116.3	116.0	115.4	114.8
9'''	168.4	167.3	167.8	168.9	168.6
OCH_3	56.2	56.2	56.3	56.7	56.3
1''''					127.3
2''''					131.4
3''''					117.0
4''''					161.6
5''''					117.0
6''''					131.4
7''''					147.6
8''''					115.1
9''''					168.4

which the (*E*) or (*Z*)-*p*-coumaroyl moiety was located at Rha C-4'' showing weak activity ($\text{IC}_{50} > 50 \mu\text{M}$).⁵ However, the (*E*) or (*Z*)-*p*-coumaroyl moiety was located at Rha C-2'' in compounds 1 and 2 rather than at Rha C-4'' in compound 4, and showed the highest inhibitory effects ($\text{IC}_{50} < 25 \mu\text{M}$). The activities of Rha C-

2'' with (*E*)-*p*-coumaroyl moiety (1) are better than Rha C-2'' with (*Z*)-*p*-coumaroyl moiety (2). The disubstituted (*E*)-*p*-coumaroyl moieties of compound 5 were located at Rha C-4'' and Rha C-2'', and showed the strongest activity ($\text{IC}_{50} < 10 \mu\text{M}$). The mono-substituted (*E*)-*p*-coumaroyl group at Rha C-2'' of compound 1 decreased activity. The observed structure–activity relationships (SAR) imply that the presence of the disubstituted (*E*)-*p*-coumaroyl groups at C-4 and C-2 of rhamnose moiety have an important role in enhancing the anti-inflammatory potential of kaempferide glycoside.

Conclusions

This study investigated chemically the aerial part of *L. akoensis* and isolated five new flavonol acylglycosides, linderakosides A–E (1–5) along with 30 known compounds, including three amides (6–8), eight apocarotenoids (9–16), 10 phenolic compounds (17–26), and eight porphyrinoids (27–35). These compounds were isolated from this plant for the first time. Compounds 1, 2, and 5 displayed potential anti-inflammatory activity with IC_{50} values of 9.4–25.1 μM , and exhibited no cytotoxic activity. These results provide a basis for evaluating the structure–activity relationships of flavonol acylglycosides, as well as for developing compound 5 as an anti-inflammatory drug.

Experimental section

General experimental procedures

Optical rotations were obtained in MeOH using a JASCO P-1020 digital polarimeter. IR and UV spectra were recorded on a Shimadzu IR Prestige-21 Fourier transform infrared and a Shimadzu Pharmaspec-1700 UV-Visible spectrophotometer, respectively. 1D and 2D NMR spectra were measured in CDCl_3 and referenced to δ_{H} 7.26 and δ_{C} 77.0, and were recorded on a Bruker AVANCE III-500 MHz spectrometer. The HRESIMS data were recorded on a Finnigan LCQ ion-trap mass spectrometer. Column chromatography was performed using silica gel (Merck, 30–65 μm), and TLC analysis was performed using aluminum pre-coated silica gel plates (Merck, Kieselgel 60 F₂₅₄). HPLC was obtained with Shimadzu LC-6A apparatus equipped with an IOTA-2RI-detector. Phenomenex luna silica (Φ 250 \times 10 column) were used for preparative purposes.

Plant material

The aerial part of *L. akoensis* was collected in Taichung, Taiwan, in July, 2008. This material was identified by Prof. Yen-Hsueh Tseng, Department of Forestry, National Chung Hsing University, Taichung, Taiwan. A voucher specimen (CMU2008-06-LA) was deposited in the School of Pharmacy, China Medical University.

Extraction and isolation

The dried aerial part of *L. akoensis* (5.9 kg) was extracted with 95% ethanol for 7 days (20 L, three times). The dried extract (337.8 g) was suspended in H_2O and partitioned successively

Table 3 Cell viability and *in vitro* decrease of nitrite of LPS-stimulated production in RAW 264.7 cell activities of compounds 1, 2, 4, and 5^a

Compound	Cytotoxicity IC_{50} (μM)	Inhibition of NO production IC_{50} (μM)
1	92.6 \pm 0.51	19.1
2	92.2 \pm 0.47	25.1
4	92.5 \pm 0.13	>50
5	90.9 \pm 0.40	9.4
Indomethacine		182.9 \pm 5.5

^a Values are expressed as mean \pm SD of three replicates.



with ethyl acetate (EtOAc) and *n*-BuOH. The EtOAc layer was evaporated *in vacuo* to yield a residue (127.8 g) that was subjected to silica gel column chromatography (particle size 0.063–0.200 mm; Φ 250 \times 15 column) and eluted with a gradient of increasing polarity with solvent of *n*-hexane/EtOAc solvent (99 : 1 \rightarrow 0 : 100) to give 21 fractions. Fraction 3 (10.0 g) was separated using semi-preparative HPLC ($\text{CH}_2\text{Cl}_2/\text{EtOAc}$, v/v 4 : 1) to afford pure, **25** (14.1 mg), **26** (23.5 mg), **27** (16.2 mg), and **35** (4.5 mg). Fraction 11 (5.08 g) was fractionated after repeated chromatography over silica gel (*n*-hexane/acetone, v/v 50 : 1 \rightarrow 0 : 100) to afford Fr. 11-1–11-10. Fr. 11-6 (109.5 mg) was chromatographed on semi-preparative HPLC ($\text{CH}_2\text{Cl}_2/\text{EtOAc}$, v/v 7 : 3) to afford **10** (16.5 mg), **11** (2.1 mg), **12** (3.3 mg), **17** (6.2 mg), **18** (3.6 mg), and **21** (2.2 mg). Fr. 11-7 (98.7 mg) was further purified using semi-preparative HPLC ($\text{CH}_2\text{Cl}_2/\text{EtOAc}$, v/v 2 : 1) to produce **24** (3.4 mg), **30** (2.9 mg), **31** (4.1 mg), **32** (4.4 mg), and **33** (2.3 mg). Fr. 11-8 (97.3 mg) was chromatographed on semi-preparative HPLC ($\text{CH}_2\text{Cl}_2/\text{EtOAc}$, v/v 2 : 1) to afford **19** (19.1 mg), **20** (12.3 mg), **22** (9.6 mg), **28** (8.3 mg), and **29** (12.4 mg). Fraction 15 (6.82 g) was re-separated by chromatography and semi-preparative HPLC (EtOAc/*n*-hexane, v/v 1 : 1) to afford pure **6** (6.6 mg), **9** (9.8 mg), **23** (8.3 mg), and **34** (3.4 mg). Fraction 16 (7.15 g) was re-separated by chromatography and semi-preparative HPLC (EtOAc/*n*-hexane, v/v 2 : 3) to afford **1** (6.5 mg), **2** (4.6 mg), **3** (0.8 mg), **4** (5.5 mg), **5** (6.5 mg), **7** (9.1 mg), and **8** (5.9 mg). Fraction 17 (1.25 g) was further passed over Sephadex LH-20 column and then purified by semi-preparative HPLC (EtOAc/*n*-hexane, v/v 2 : 3) to yield **13** (7.7 mg), **14** (6.5 mg), **15** (3.7 mg), and **16** (3.5 mg).

4'-O-methyl-2''-E-p-coumaroylazfelin (1). Pale yellow solid; mp: 160–163 °C; $[\alpha]_{\text{D}}^{24}$ –20.6 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ϵ) 313.0 (4.69), 277.0 (4.50), 267.0 (4.56), 247.0 (4.31), 211 (4.71) nm; IR ν_{max} 3426, 1651, 1605, 1513, 1173 cm^{-1} ; HR-ESI-MS *m/z*: 615.1479 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{31}\text{H}_{28}\text{O}_{12}\text{Na}$, 615.1473).

4'-O-methyl-2''-Z-p-coumaroylazfelin (2). Pale yellow solid; mp: 169–170 °C; $[\alpha]_{\text{D}}^{24}$ –29.3 (*c* 0.98, MeOH); UV (MeOH) λ_{max} (log ϵ) 313.0 (4.60), 278.0 (4.48), 267.0 (4.57), 247.0 (4.34) nm, 211.0 (4.72); IR ν_{max} 3426, 1651, 1605, 1512, 1173 cm^{-1} ; HR-ESI-MS *m/z*: 615.1477 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{31}\text{H}_{28}\text{O}_{12}\text{Na}$, 615.1473).

4'-O-methyl-4''-Z-p-coumaroylazfelin (3). Pale yellow solid; mp: 162–164 °C; $[\alpha]_{\text{D}}^{24}$ –24.5 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ϵ) 312.0 (4.75), 247.0 (4.44), 211.0 (3.94) nm; IR ν_{max} 3442, 2936, 1655, 1607, 1510, 1177 cm^{-1} ; HR-ESI-MS *m/z*: 615.1476 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{31}\text{H}_{28}\text{O}_{12}$, 615.1473).

4''-E-p-coumaroyltamarixetin (4). Pale yellow solid; mp: 200–203 °C; $[\alpha]_{\text{D}}^{24}$ –183.2 (*c* 1.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 313.0 (4.42), 276.0 (4.27), 267.0 (4.33), 246.0 (4.24), 208.0 (4.61) nm; IR ν_{max} 3426, 2932, 1651, 1604, 1512, 1173 cm^{-1} ; HR-ESI-MS *m/z*: 631.1423 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{31}\text{H}_{28}\text{O}_{13}\text{Na}$, 631.1422).

4'-O-methyl-2'',4''-di-E-p-coumaroylazfelin (5). Pale yellow solid; mp: 201–205 °C; $[\alpha]_{\text{D}}^{24}$ –88.8 (*c* 1.18, MeOH); UV (MeOH) λ_{max} (log ϵ) 314.0 (4.97), 248.0 (4.44) nm, 212 (4.85) nm; IR ν_{max} 3402, 2936, 1651, 1605, 1512, 1173 cm^{-1} ; HR-ESI-MS *m/z*: 761.1846 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{40}\text{H}_{34}\text{O}_{14}\text{Na}$, 761.1841).

Cell culture

RAW264.7 (BCRC no. 60001) was purchased from the Bio-resources Collection and Research Center (BCRC) of the Food

Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA, USA) with 5% CO_2 incubator at 37 °C and subcultured every 3 days at a dilution of 1 : 5 using 0.05% trypsin–0.02% EDTA in Ca^{2+} -, Mg^{2+} -free phosphate-buffered saline (DPBS).

Nitric oxide (NO) production assay⁴¹

RAW 264.7 cells were incubated in a 96-well plate for 24 h and then pretreated with LPS (100 ng mL^{-1}) with compounds (0, 3.125, 6.25, 12.5, 25 and 50 $\mu\text{g mL}^{-1}$). Then, the supernatant (100 μL) was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 10 min; the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices Orleans Drive, Sunnyvale, CA, USA). Using sodium nitrite to generate a standard curve, the concentration of nitrite was measured from absorbance at 540 nm.

Cell viability assay⁴¹

RAW 264.7 cells (2×10^5 cells per well) were seeded in a 96-well plate containing DMEM medium with 10% FBS for 24 h. Then cells were treated with various concentrations of compounds **1**, **2**, **4**, and **5** in the presence of 100 ng mL^{-1} LPS (lipopolysaccharide) and incubated for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg mL^{-1} MTT for 2 h at 37 °C testing for cell viability. The medium was then discarded, and 100 μL dimethyl sulfoxide (DMSO) was added. The absorbance with cell viability was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

Statistical analysis

IC_{50} values were estimated using a non-linear regression algorithm (SigmaPlot 8.0; SPSS Inc., Chicago, IL, USA, 2002). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests).

Conflicts of interest

There are no conflicts to declare.

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