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Acylated flavonol monorhamnosides, α-glucosidase inhibitors, from *Machilus philippinensis*

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ABSTRACT

Bioassay-guided fractionation and isolation of the active constituents from the leaf extract of *Machilus philippinense* Merr. yielded two active compounds, kaempferol-3-*O*-α-L-rhamnopyranoside 3″,4″-di-*E*-*p*-coumaroic acid ester (**1**) and 3″-*E*,4″-*Z*-di-*p*-coumaroic acid ester (**2**) when tested against a *Bacillus stearothermophilus*, a α-glucosidase type IV. The IC_{50} values of **1** and **2** were 6.10 and 1.00 μM, respectively. Further application of the HPLC–SPE–NMR hyphenated technique in the on-line characterization of other active ingredients present in the CH_2Cl_2 – soluble fraction led to identification of luteolin (**3**) and seven additional 3-*O*-(coumaroyl-rhamnopyranosyl)-flavonols (**4–10**). Their structures were determined mainly by ^1H NMR spectroscopic analyses. Among the compounds identified, compounds **2**, **4**, **5**, and **7** were hitherto unknown natural products.

1. Introduction

One of the strategies to monitor blood glucose for type II diabetes mellitus is to either inhibit or reduce production of glucose from the small intestine. α-Glucosidase inhibitors (AGH) target enzymes in the small intestine brush border and interfere with the digestion of carbohydrates, achieving better glycemic control (Cheng and Fantus, 2005). Therefore, the development of such inhibitors will be beneficial for therapy of type II diabetes. Several natural α-glucosidase inhibitors including acarbose, voglibose, and miglitol (Scott and Spencer, 2000) are clinically used. Thus, natural products of great structural diversity are still a good source for searching for such inhibitors, thereby motivating us to explore biologically active compounds from the highly diverse Formosan plants. We had screened the extracts of nine Formosan *Machilus* plants and found that the CH_2Cl_2 – soluble fraction of the leaves of *M. philippinensis* Merr. (Lauraceae) (Huang, 1996) was active against α-glucosidase type IV from *Bacillus stearothermophilus* (93.7% inhibition at 100 μg/ml). Following a bioassay guided approach, two active compounds (**1–2**) were isolated by general chromatographic methods. The constituents in some active subfractions were more complicated mixtures, as observed from HPLC analysis, and could not be separated readily by the general chromatographic methods used. However, the high performance liquid chromatography–solid phase extraction–nuclear magnetic resonance (HPLC–SPE–NMR) hyphenated technique which combines the strength of analytical resolution (HPLC), multiple peak trapping and single deuteriated solvent used for NMR sampling (SPE), gave definitive information for structural elucidation (NMR, 1D and 2D). This recently developed hyphenated technique had been demonstrated to be very powerful and efficient to analyze and identify the ingredients in complicated mixtures (Seger et al., 2005; Lee et al., 2007), especially for known compounds. Accordingly, we applied the HPLC–SPE–NMR technique in this study to facilitate the separation and structural characterization of the compounds in the mixture. This article describes the outcome of our effort in identifying the α-glucosidase inhibitors from the leaves of *M. philippinense* via the approaches indicated above.

2. Results and discussion

The MeOH extract of the leaves of *M. philippinense* Merr. (Lauraceae) was divided into fractions soluble in hexane, CH_2Cl_2, EtOAc, *n*-BuOH and H_2O. Of them, the CH_2Cl_2 – soluble fraction was active against a (*B. stearothermophilus*) α-glucosidase type IV from showing 93.7% inhibition at 100 μg/ml. This fraction was further fractionated on a Sephadex LH-20 column to give two active fractions (Fractions II and III). Fraction III was separated by the general chromatographic methods to give two active compounds (**1** and **2**) (Fig. 1).

The ^1H NMR spectrum of **1** (CD_3OD) displayed signals for a kaempferol moiety, an AA'XX' system for H-2′ (H-6′) (δ 7.84) and H-3′ (H-5′) (δ 7.03) (J = 8.8 Hz), and AX system for H-6 (δ 6.21) and H-8 (δ 6.39) (J = 2.0 Hz), a rhamnopyranosyl (rha) moiety

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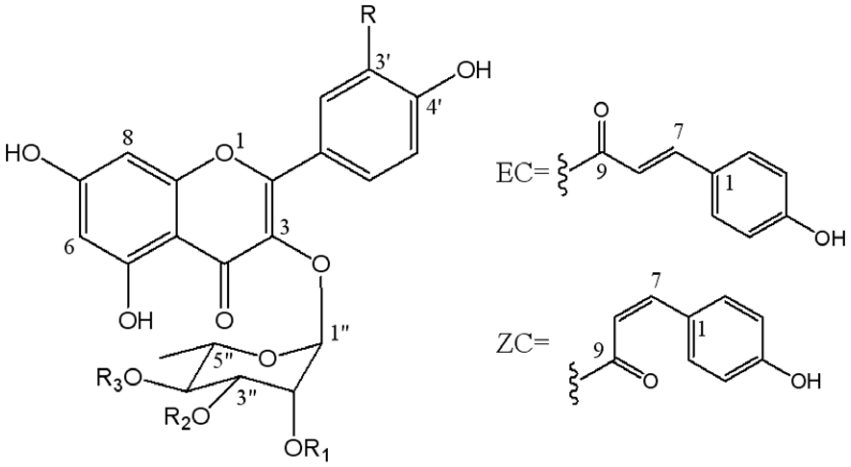

Compd	1	2	4	5	6	7	8	9	10
R	H	H	OH	OH	H	H	H	H	H
R_1	H	H	H	H	H	H	EC	ZC	ZC
R_2	EC	EC	ZC	ZC	ZC	ZC	H	H	H
R_3	EC	ZC	EC	ZC	EC	ZC	EC	ZC	EC

Fig. 1. Structures of compounds **1–2**, and **4–10**.

($\delta_{H\text{-}1}$ 5.78, *d*, *J* = 1.7 Hz; $\delta_{Me\text{-}6}$ 0.85, *d*, *J* = 6.2 Hz), and two *E*-coumaroyl (*E*C) moieties ($J_{H\text{-}7/H\text{-}8}$ = 15.9 Hz). The signals of rha-H_3 and rha-H_4, assigned by analyzing a COSY spectrum, were downfield shifted relative to those in the non-substituted rhamnose. Therefore, both C-3 and C-4 of the glycone were ester linked to *E*C. These data together with the ESI-MS, which displayed the $[M+H]^+$ at *m/z* 725, established **1** as the known kaempferol-3-*O*-α-L-(3″,4″-di-*E*-*p*-coumaroyl)-rhamnopyranoside (Fiorini et al., 1998). The HR-FAB-MS of **2** showed a $[M+H]^+$ at *m/z* 725.1870, in agreement with the molecular formula of $C_{39}H_{32}O_{14}$, the same as that of **1**. The ^1H NMR spectroscopic data of **2** (CD_3OD) were also very similar to those of **1**, except that proton signals of an *E*C moiety in **1** were replaced by those of a *Z*-coumaroyl (*Z*C) moiety. Since signals of both rha-H_3 and rha-H_4 appeared at relatively downfield positions, as confirmed from the COSY spectroscopic analysis, these two coumaroyl groups were also ester linked to the rha-C_3 and rha-C_4 positions. The HMBC spectrum of **2** showed shift correlations of both rha-H_3 (δ 5.28) and *E*-coum-H_7 (δ 7.61) to *E*C-C_9 (δ 168.4), and both rha-H_4 (δ 5.18) and *Z*C-H_7 (δ 6.83) to *Z*C-C_9 (δ 167.0) via three-bond coupling (Fig. 2), establishing C-4 of the glycone to be ester linked to the *Z*-coumaric acid. Accordingly, compound **2** was established as kaempferol-3-*O*-α-L-(3″-*E*,4″-*Z*-di-*p*-coumaroyl)-rhamnopyranoside, a new natural product.

To assist in the structural characterization of their geometric isomers, the full ^1H NMR and ^{13}C NMR assignments of **1** and **2** (CD_3OD) (Table 1 and see Section 4) were made by analysis of the HMQC and HMBC spectra.

The reversed phase C-18 HPLC profile of Fraction II (Fig. 3), an active subfraction possessing 72.2% inhibition against α-glucosidase at 10 μg/ml, established the presence of at least ten additional compounds. Application of HPLC–SPE–NMR under such HPLC conditions as indicated in the experimental was employed to analyze the components in this complicated mixture. This led to characterization of ten compounds (**1–10**) (Fig. 1), using 2.7 mg of Fraction II, theoretically equivalent to ca. 3.6 g of the dry leaves.

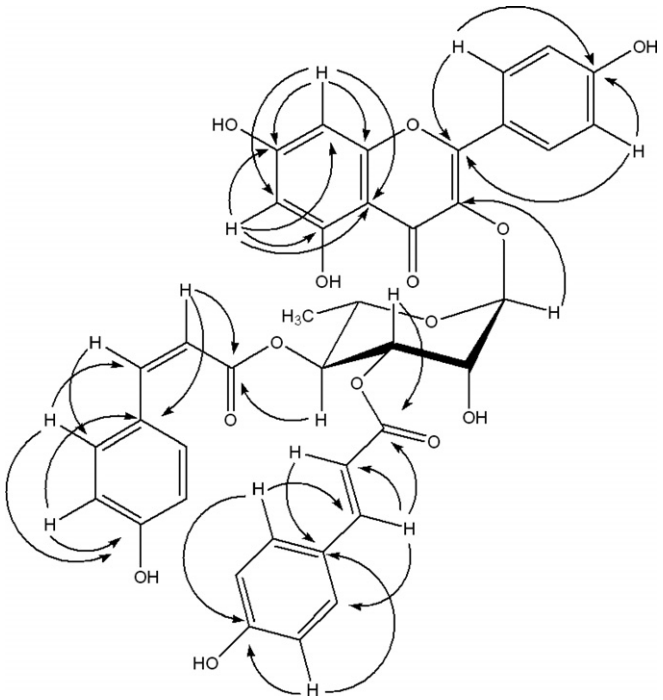

Fig. 2. HMBC correlations of compound **2** (CD_3OD, 400 MHz).

Analysis of the on-line ^1H NMR and ESI-MS spectra of **3** identified it as luteolin (Owen et al., 2003). Compounds **4–10** showed similar UV absorption maxima around 265 and 310 nm, obtained from the on-line photodiode array detection, to **1** and **2**. The HR-ESI-MS of **4** and **5** showed $[M-H]^-$ at *m/z* 739.1680 and *m/z* 739.1685, respectively, in agreement with the same molecular

Table 1
^{1}H NMR spectroscopic Data (δ/ppm, *m*, *J*[a]) of compounds **1–2** and **4–10** (400 MHz)

Proton	**1**[b]	**1**[c]	**2**[b]	**2**[c]	**4**[b]	**5**[b]	**6**[b]	**7**[b]	**8**[b]	**9**[b]	**10**[b]
6	06.26 *d*	6.21 *d*	6.25 *d*	6.20 *d*	6.25 *d*	6.25 *d*	6.26 *d*	6.25 *d*	6.25 *d*	6.25 *d*	6.25 *d*
8	6.44 *d*	6.39 *d*	6.44 *d*	6.39 *d*	6.44 *d*	6.44 *d*	6.44 *d*	6.43 *d*	6.44 *d*	6.43 *d*	6.43 *d*
2′, 6′	7.84 *d*	7.84 *d*	7.81 *d*	7.80 *d*	7.44 *d* (2′)	7.41 *d* (2′)	7.83 *d*	7.80 *d*	7.83 *d*	7.80 *d*	7.83 *d*
3′, 5′	7.03 *d*	7.05 *d*	6.97 *d*	6.97 *d*	7.03 *d* (5′)	6.97 *d* (5′)	7.04 *d*	6.97 *d*	7.04 *d*	6.98 *d*	7.04 *d*
6′					7.38 *dd*	7.35 *dd*					
Rha											
1″	5.56 *brs*	5.78 *brs*	5.46 *brs*	5.63 *d*	5.51 *brs*	5.41 *brs*	5.55 *brs*	5.44 *brs*	5.70 *brs*	5.55 *brs*	5.66 *brs*
2″	4.39 *brs*	4.42 *brs*	4.40 *brs*	4.46 *dd*	4.37 *brs*	4.39 *brs*	4.36 *brs*	4.38 *brs*	5.47 *brs*	5.46 *brs*	5.45 *brs*
3″	5.27 *dd*	5.37 *dd*	5.20 *dd*	5.28 *dd*	5.30 *dd*	5.23 *dd*	5.27 *dd*	5.20 *dd*	4.06 *m*	4.02 *m*	4.05 *m*
4″	5.10 *t*	5.19 *t*	5.08 *t*	5.18 *t*	5.06 *t*	5.04 *t*	5.04 *t*	5.03 *t*	4.87 *t*	4.74 *t*	4.75 *t*
5″	3.40 *m*	3.25 *m*	3.45 *m*	3.36 *m*	3.45 *m*	3.44 *m*	3.39 *m*	3.44 *m*	ND[d]	3.35 *m*	ND[d]
6″	0.82 *d*	0.85 *d*	0.82 *d*	0.85 *d*	0.86 *d*	0.87 *d*	0.81 *d*	0.80 *d*	0.83 *d*	0.80 *d*	0.80 *d*
Coum-1	3″-EC	3″-EC	3″-EC	3″-EC	3″-ZC	3″-ZC	3″-ZC	3″-ZC	2″-EC	2″-ZC	2″-ZC
2‴, 6‴	7.45 *d*	7.40 *d*	7.41 *d*	7.33 *d*	7.62 *d*	7.65 *d*	7.63 *d*	7.66 *d*	7.52 *d*	7.64 *d*	7.64 *d*
3‴, 5‴	6.80 *d*	6.76 *d*	6.79 *d*	6.74 *d*	6.75 *d*	6.81 *d*	6.76 *d*	6.81 *d*	6.84 *d*	6.80 *d*	6.80 *d*
7‴	7.59 *d*	7.62 *d*	7.60 *d*	7.61 *d*	6.86 *d*	6.87 *d*	6.84 *d*	6.86 *d*	7.67 *d*	6.92 *d*	6.93 *d*
8‴	6.28 *d*	6.28 *d*	6.30 *d*	6.27 *d*	5.75 *d*	5.77 *d*	5.74 *d*	5.76 *d*	6.40 *d*	5.84 *d*	5.85 *d*
Coum-2	4-EC	4″-EC	4″-ZC	4″-ZC	4″-EC	4″-ZC	4″-EC	4″-ZC	4″-EC	4″-ZC	4″-EC
2⁗, 6⁗	7.47 *d*	7.46 *d*	7.59 *d*	7.62 *d*	7.48 *d*	7.57 *d*	7.47 *d*	7.56 *d*	7.54 *d*	7.65 *d*	7.54 *d*
3⁗, 5⁗	6.82 *d*	6.78 *d*	6.74 *d*	6.70 *d*	6.83 *d*	6.77 *d*	6.83 *d*	6.77 *d*	6.87 *d*	6.79 *d*	6.86 *d*
7⁗	7.51 *d*	7.49 *d*	6.85 *d*	6.83 *d*	7.53 *d*	6.86 *d*	7.51 *d*	6.86 *d*	7.57 *d*	6.91 *d*	7.56 *d*
8⁗	6.20 *d*	6.19 *d*	5.66 *d*	5.65 *d*	6.21 *d*	5.68 *d*	6.20 *d*	5.67 *d*	6.29 *d*	5.75 *d*	6.28 *d*

[a] Coupling constants (Hz) $J_{6,8}$ 2.0, $J_{2',3'}$ ($J_{5',6'}$) 8.9 (kaempferol), $J_{5',6'}$ 8.4 and $J_{2',6'}$ 1.9 (quercetin); rha part $J_{1,2}$ 1.4, $J_{2,3}$ 3.0, $J_{3,4}$ 10.0, $J_{4,5}$ *10.0*, $J_{5,6}$ 6.2; coumaroyl part $J_{7,8}$ 12.8 (*ZC*), 16.0 (*EC*), $J_{2,3}$ ($J_{5,6}$) 8.6.
[b] Data obtained from analyzing the on-line ^{1}H NMR spectra of the HPLC–SPE–NMR (CD_3CN, 400 MHz).
[c] Data obtained from analyzing the general ^{1}H NMR spectra (CD_3OD, 400 MHz).
[d] Not detected since the signals could be buried by the signal of H_2O, existing in CD_3CN, and was suppressed during data acquisition.

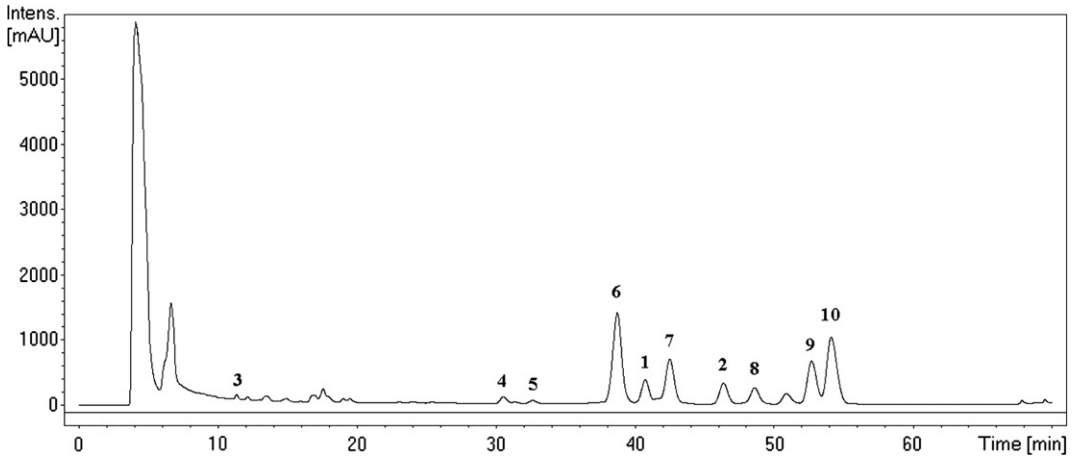

Fig. 3. HPLC chromatogram of Fraction II; for conditions see Section 4.

formula of $C_{39}H_{32}O_{15}$, one more oxygen atom than that of **1**. The ^{1}H NMR spectroscopic data suggested compounds **4–5** to possess quercetin as the aglycone, different from those of kaempferol in **1** and **2** by showing an ABX system instead of an AA'XX' pattern for the B ring protons. The proton signals for the 3,4-diesterified rhamnosyl part were similar except for the difference caused by the isomeric coumaroyl moieties. The ^{1}H NMR spectrum of **5** showed signals for two *ZC* groups and that of **4** showed resonances for a *ZC* and an *EC* moiety, as evidenced by the coupling constants of H-7 and H-8 of these residues (Table 1). Accordingly, compound **5** was established as quercetin-3-*O*-α-L-(3″,4″-di-*Z*-*p*-coumaroyl)-rhamnopyranoside. Comparison of the proton chemical shifts for these geometrical isomers and the linked rhamnosyl part (CD_3CN) would draw a consistent correlation as observed from the data listed in Table 1. That is, the chemical shifts of H-7 and H-8 in the di-coumaroyls linked to the rha-C_3 (C-3″) and rha-C_4 (C-4″) will appear around 7.59 and 6.28 ppm for rha-3-*EC* (3″-*EC*) (**1** and **2**) vs. 7.51 and 6.20 ppm for rha-4-*EC* (4″-*EC*) (**1**); around 6.87 and 5.77 ppm for rha-3-*ZC* (3″-*ZC*) (**5**) vs. around 6.85 and 5.66 ppm for rha-4-*ZC* (4″-*ZC*) (**2** and **5**). In addition, the signal of the rha-H_3 in the presence of 4″-*EC* substitution displayed a meaningful shift relative to that in 4″-*ZC* substitution, e.g. δ 5.27 in **1** vs. δ 5.20 in **2**. Based on these correlations, compound **4** was elucidated as quercetin-3-*O*-α-L-(3″-*Z*,4″-*E*-di-*p*-coumaroyl)-rhamnopyranoside.

The ESI-MS of compounds **6–10** showed the same quasi molecular ion as those of **1** and **2**. Their ^{1}H NMR spectra (Fig. 4) were also similar to those of **1** and **2**, except for resonances arising from the isomeric coumaric acids and the substituted position of these esters in the rhamnosyl group (Table 1). Of these, compounds **8–10** were identified as the known kaempferol-3-*O*-α-L-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnopyranoside (**8**) (Rao et al., 2002),

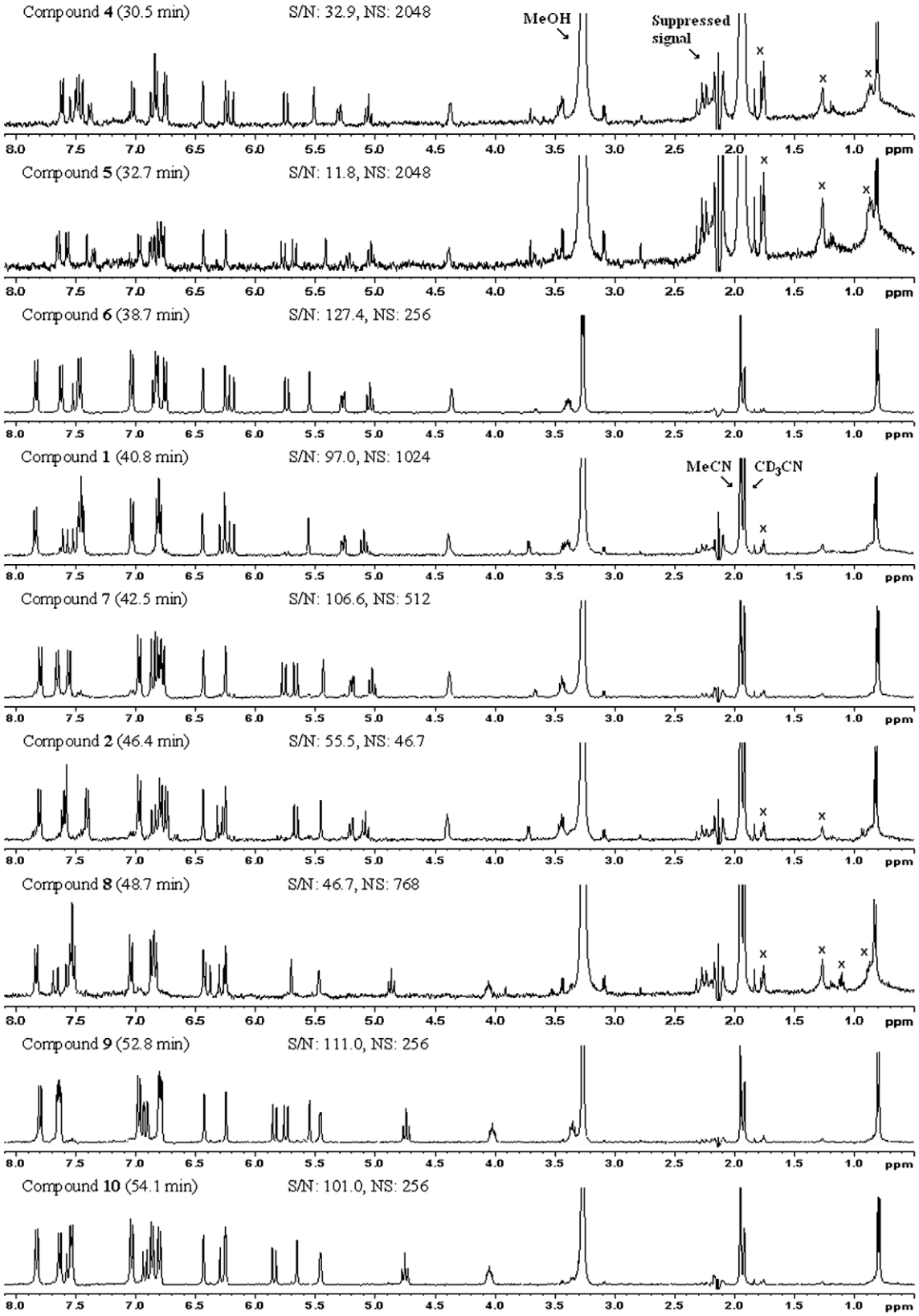

Fig. 4. On-line ^{1}H NMR spectra of compounds **1–2,** and **4–10** obtained from HPLC–SPE–NMR (400 MHz) (CD_3CN).

kaempferol-3-*O*-α-L-(2″,4″-di-*Z*-*p*-coumaroyl)-rhamnopyranoside (**9**) (Fiorini et al., 1998), and kaempferol-3-*O*-α-L-(2″-*Z*,4″-*E*-di-*p*-coumaroyl)-rhamnopyranoside (**10**) (Kuo et al., 2005), respectively. The ^{1}H NMR assignments for compound **10** (CD_3CN) were confirmed by analysis of 2D NMR spectroscopic data (HMBC, HMQC, COSY and NOESY). In addition to on-line measured ^{1}H

NMR spectra, the homo-COSY spectra of **1**, **2**, **6–9** in CD_3CN were also acquired on-line. Analysis of the ^1H NMR and COSY spectra of these compounds allowed unambiguous assignment of the protons in the rhamnosyl moiety as listed in Table 1. Since the rha-H_3 and rha-H_4 in compounds **6** and **7** were downfield shifted, both rha-C_3 and rha-C_4 in these two compounds were ester linked. The coupling constants of H-7 and H-8 in the *p*-coumaroyl groups indicated compound **6** contained one *Z*C (J = 12.8 Hz) and one *E*C (J = 16.0 Hz) moiety, and compound **7** contained two *Z*C groups. Hence, compound **7** was elucidated as kaempferol-3-*O*-α-L-(3″,4″-di-*Z*-*p*-coumaroyl)-rhamnopyranoside. The substitution effect caused by the geometric isomers of the rha-C_4 esters on the chemical shift of rha-H_1 was also observed. In summary, 4″-*E*C caused a downfield shift to rha-H_1 around 0.10 ppm relative to 4″-*Z*C as exemplified in **1** vs. **2**, and **9** vs. **10** (CD_3CN). This effect was little influenced by the substitution at rha-C_2 and rha-C_3 as shown in Table 1 (e.g. **8** vs. **10**). Based on this correlation, compound **6** [$\delta_{\text{rha-1}}$ 5.55 vs. 5.44 in **7** (4″-*Z*C)] should contain 4″-*E*C. Therefore, compound **6** was established as kaempferol-3-*O*-α-L-(3″-*Z*,4″-*E*-di-*p*-coumaroyl)-rhamnopyranoside (Wang et al., 2007).

The ^1H NMR assignments of compounds **4–9** (CD_3CN) were made by analysis of the ^1H NMR and COSY spectra, and by correlation with those of well-assigned data for **1**, **2** and **10**. For instance, the proton chemical shifts in rha-4-*E*C of **4** and rha-4-*Z*C of **5** were assigned by correlation with the corresponding signals in those of **1** and **2**. The proton signals of the rha-3-*Z*C in **4** were thus easily designated by elimination of the rha-4-*E*C resonances. By comparison of the ^1H NMR spectroscopic data of **4**, the ^1H NMR signal assignments for **6** were readily made since both compounds contained identical substitutions at the C-3 and C-4 positions of the rhamnosyl group. Likewise, those of rha-3-*Z*C and rha-4-*Z*C in **7** were assigned by correlation with those of **5,** together with analysis of the COSY spectrum. As for the ^1H NMR assignments of the C-2 and C-4 esterified rhamnosyl compounds (**8** and **9**), they were made by correlation with the ^1H NMR spectroscopic data of **10** (CD_3CN) (Table 1), which were assigned by analysis of COSY and NOESY spectra. The on-line measured NOESY spectrum of **10** showed a key NOE between H-7 (δ 6.93) and H-2/6 (δ 7.64) of the rha-2-*Z*C unit, which allowed the distinct of the chemical shifts of H-2/6 in both rha-2-*Z*C and rha-4-*E*C.

Since these isomers were well-resolved in the analytical RP C-18 HPLC, separation via semi-preparative RP-18 HPLC was attempted to provide enough materials for bioassay. Each separated compound, however, became a mixture after storage overnight in the delivery system (MeCN–H_2O). For instance, compound **6** became the mixture of **1**, **2**, **6**, **7**, **9** and **10** after such late workup in an integration ratio of 32.8:11.5:100.0:34.7:2.9:6.1, detected at 254 nm, in an HPLC analysis using the same conditions for HPLC–SPE–NMR. Based on such observation, these structural isomers could be yielded via isomerization (**6** to **1**, **2** and **7**) and *trans*-esterification (**6–9** and **10**). This study demonstrated that through HPLC–SPE–NMR technique such structural isomers could be characterized and thus this technique is also useful to disclose the artifacts of labile natural products during processing.

Under the RP-HPLC conditions used in the HPLC–SPE–NMR experiment, the elution order of the components in Fraction II was as follows: **4**, **5**, **6**, **1**, **7**, **2**, **8**, **9** and **10** (Fig. 3). Of these, compounds **4** and **5** contain the 3′-hydroxykaempferol moiety, i.e. quercetin, as the aglycons part, while the rest contained the kaempferol moiety as the aglycons part and thus were less polar. The rhamnosyl residue in compounds **1**, **2, 6**, and **7** was 3,4-disubstituted, while that in **8–10** is 2,4-disubstituted. The elution order indicated that the axially oriented rha–C_2–OH, as in **1**, **2, 6**, and **7**, was more polar than the equatorially oriented rha–C_3–OH, as in **8–10**. As for the relationship of the geometric isomer (*Z* or *E*) and the polarity, no conclusion could be drawn from the elution order of both groups (**1–2, 6–7**; **8–10**). Other factors such as steric effects might play some role in this.

Table 2
Inhibitory effect of compounds **1**, **2** and related compounds against α-glucosidase

Compounds	IC_{50}[a]
1	6.10 ± 0.28 μM
2	1.00 ± 0.01 μM
Quercetin-3-*O*-rhamnopyranoside	33.05 ± 2.68 μM
Kaempferol-3-*O*-rhamnopyranoside	228.11 ± 9.50 μM
Mixture of 1-2, 4-10	2.67 ± 0.14 μg/ml
Acarbose	0.046 ± 0.01 μM

[a] The IC_{50} values were calculated from the dose-response curve of six concentrations of each test sample in triplicate.

The inhibitory effect of the isolated compounds and two non-acylated parent flavonol rhamnosides against α-glucosidase type IV from *B. stearothermophilus* was evaluated and the results are summarized in Table 2. The data indicated that compounds **1** and **2** possessed high potency with the IC_{50} value of 6.10 and 1.00 μM, respectively. The mixture of nine flavonol rhamnoside dicoumaric acid esters, composed of **1**, **2**, and **4–10**, obtained from a semi-preparative HPLC of Fraction II, in an area integration of 1.6:1.0:30.1:20.1:16.7:11.0:12.2:10.5:21.1 detection at 254 nm, was found to have an IC_{50} value of 2.67 μg/ml against α-glucosidase. Kaempferol rhamnoside, IC_{50} 228.11 μM, however, was much less active than its acylated derivative **1** or **2**. It is noted that quercetrin, having one additional phenolic group at C-3′, is much more active than kaempferol rhamnoside. The simple flavonoids (Gao et al., 2004; Gao and Kawabata, 2005), flavonoid glycosides (Hanamura et al., 2005; Matsui et al., 2007) and *trans*-cinnamic acid derivatives (Adisakwattana et al., 2004) against α-glucosidase have been reported. However, they displayed weak activity against α-glucosidase. Our study demonstrates for the first time that coumaroylated flavanol mono-rhamnosides possesses potent activity against α-glucosidase and are much more potent than those reported for the individual moiety as indicated above. This result suggests that acylated moiety plays an important role in exerting inhibitory activity, in agreement with those concluded from the studies of acylated anthocyanins and caffeoylquinic acids against α-glucosidase (Matsui et al., 2001, 2004). This study also indicated that the geometric orientation could play a certain role against this specific target, since compound **2** with 3″-*E*, 4″-*Z*-di-*p*-coumaroyl groups was more potent than compound **1** with 3″, 4″-di-*E*-*p*-coumaroyl groups.

3. Conclusion

This study led to isolation of two active acylated flavonol monoglycosides against α-glucosidase type IV from *Bacillus stearothermophilus* from *M. philippinense* Merr. and identified seven additional isomers using HPLC–SPE–NMR. The combination of these nine isomeric flavonol monorhamnoside dicoumaroisc acid esters also showed high potency against α-glucosidase. This type of compounds might serve as leads to develop drugs for treatment of type 2 diabetes. In addition, this study demonstrates the powerfulness of HPLC–SPE–NMR in thorough exploration of either complicated or mixtures bioactive labile natural products.

4. Experimental section

4.1. General experimental procedures

UV spectra were measured in MeOH using an Hitachi 150-20 Double Beam spectrophotometer, whereas optical rotations were recorded using a JASCO DIP-370 polarimeter. ^1H and ^{13}C NMR spectra and 2D NMR were obtained on a Bruker AV400 spectrometer

(CD_3OD, δ_H 3.30 and δ_C 49.0 ppm; CD_3CN, δ_H 1.93 ppm) using standard pulse programs. HR-FAB mass data was measured using a JEOL JMS-700 (Japan) mass spectrometer. TLC analyses were carried out on silica gel plates (KG60-F_{254}, Merck). The HPLC–SPE–NMR instrument consisted of an Agilent (Waldbronn, Germany) model 1100 liquid chromatograph with photodiode array detector, a Knauer (Berlin, Germany) model K120 HPLC makeup pump, a Prospekt 2 (Spark Holland) solid-phase extraction (SPE) unit containing 192 Hyphere Resin GP cartridges (10 × 2 mm, 10–12 μm) for analyte trapping, a Bruker (Rheinstetten, Germany) nitrogen synthesizer for flushing cartridge, and a Bruker AV400 MHz NMR spectrometer equipped with an LC SEI ^{13}C–^{1}H probehead with an active volume of 30 μl. All cartridges were conditioned automatically and equilibrated twice CH_3CN(2 × 0.5 ml) and H_2O in sequence at a flow rate of 1 ml/min before use. HPLC separation was performed on a RP-18 column (Phenomenex® Prodigy ODS-3, 250 × 4.6 mm, 5 μm). The HPLC-MS consisted of Agilent 1100 system and Esquire 2000 ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany). The HR-ESI-MS data were measured on a micrOTOF orthogonal ESI-TOF mass spectrometer (Bruker, Daltonik, Bremen, Germany) coupled to an HPLC system from Agilent (Waldbronn, Germany). The microplate spectrophotometer was SPECTRAmax® PLUS (Molecular Devices, USA).

4.2. Chemicals and reagents

CH_3CN (HPLC grade) and MeOH were purchased from Mallinckrodt Baker Inc., (USA) and deionized H_2O was prepared from a Barnstead water purification system (Dubuque, IA, USA). CD_3CN (99.8%) and CD_3OD (99.8%) were purchased from Cambridge Isotope Lab. Inc., (USA) α-Glucosidase type IV from *B. stearothermophilus*, *p*-nitrophenyl α-D-glucopyranoside, K_2HPO_4 and KH_2PO_4 were purchased from Sigma–Aldrich Co., Germany. Authentic quercetin-3-*O*-rhamnopyranoside and kaempferol-3-*O*-rhamnopyranoside were obtained from our natural product collection (Lee et al., 2006).

4.3. Plant Material

Leaves of *M. philippinensis* Merr. were collected in September 2005 at the Fu-shan Research Center, Taiwan Forestry Research Institute, Yilan county, Taiwan. The specimen (NTUSP200509MP) was authenticated by Mr. Jer-Tone Lin, Associate Researcher, Taiwan Forestry Research Institute.

4.4. Extraction and Isolation

The dried leaf of *M. philippinensis* (774 g) was percolated by 95% EtOH (6 l × 4) at room temperature to give the EtOH extract (160 g) after evaporation under reduced pressure. The extract was divided into fractions soluble in CH_2Cl_2 (38.30 g), EtOAc (10.51 g), *n*-BuOH (31.02 g) and H_2O (74.12 g), respectively, by partitioning the suspension of the extract in H_2O (1.2 l) against the corresponding solvent, each 1.2 l × 3, in sequence. Part of the CH_2Cl_2 soluble part (23.5 g), which showed 93.7% inhibition against α-glucosidase at 100 μg/ml) was fractionated on a Sephadex LH-20 column (2.6 l, MeOH) to give 3 fractions (Fractions I–III). Fraction III (890 mg), which showed an 82.5% inhibition against α-glucosidase at 10 μg/ml, was further separated by RP-18 HPLC (LiChroprep RP-18, size B, 310 × 25 mm; 40–63 μm, Merck) using a stepwise gradient of MeOH–H_2O from 10:90 to 100:0, followed by preparative HPLC (Lichrospher RP-18e, 250 × 25 mm, 5 μm, Merck), eluted with MeOH–H_2O (72:28, v/v) with flow rate of 8 ml/min, to give compound **1** (5.8 mg, t_R = 29.3 min) and **2** (2.6 mg, t_R = 34.6 min).

4.5. HPLC–SPE–NMR experiments

Fraction II, showing 72.2% inhibition against α-glucosidase at 10 μg/ml, was analyzed by the HPLC–SPE–NMR hyphenated technique. The optimal HPLC conditions, which gave base-line separation for Fraction II (Fig. 3), were found as follows: RP-18 column (Phenomenex® Prodigy ODS-3, 250 × 4.6 mm, 5 μm), MeOH–H_2O (40:60–44:56, v/v) in 20 min, to 47% in a 40 min linear gradient; flow rate 0.5 ml/min, injection volume 20 μl (10 mg/0.2 ml), detection at 280 nm. The post-column eluent was mixed with H_2O, supplied by the make-up pump (1.0 ml/min), before peak trapping on the HySphere-Resin GP cartridges, using the Prospekt II SPE system. This HPLC–SPE procedure was repeated three times. The loaded cartridges were flushed with dry N_2 gas for 30 min, and the analyte in each dried cartridge was washed by CD_3CN into a 30 μl inverted NMR probe, where on-line NMR spectra were measured. The ^{1}H NMR spectra were recorded using multiple solvent suppression pulse program for residual proton and H_2O signals in CD_3CN. Shaped low-power rf pulse and CW decoupling on the F2 channel for decoupling of the ^{13}C satellites were utilized. All spectra were measured at 300 K and the ^{1}H chemical shift was referenced to a residual signal of CD_2HCN at δ 1.93. A total of 256–2048 scans for each measurement were accumulated into 16 k data points with a sweep width of 8000 Hz. 2D NMR spectra were recorded using standard pulse programs (COSY and NOESY) and the correlation maps consisted of 2048 × 256 data points per spectrum.

4.6. Concentration of compounds ***1,2, 4-10*** *from Fraction II*

Fraction II was fractionated via a semi-preparative RP-18 column (Phenomenex® Prodigy ODS-3, 250 × 10 mm, 5 μm), using the same conditions indicated above for HPLC–SPE–NMR, except that the flow rate was changed to 2.6 ml/min and the injection volume was 70 μl (100 mg/ml), to give a fraction (0.9 mg) containing the acylated flavonol rhamnosides **1**, **2**, **4–10** in an area integration of 1.6:1.0:30.1:20.1:16.7:11.0:12.2:10.5:21.1 detected at 254 nm.

4.7. HPLC–MS and HPLC–HR-ESI-MS experiments

Fr. II was analyzed using the same HPLC program as described for HPLC-SPE-NMR. ESI-MS data were acquired using the positive mode with the following settings: nebulizer pressure 15 psi, drying gas 5 l/min at 300 °C. The HR-ESI-MS data were measured in the negative ionization mode with nebulizer pressure 34.8 psi, drying gas 6 l/min at 200 °C.

4.8. Assay for α-glucosidase activity

The inhibitory activity against α-glucosidase was measured with a slight modification of the reported method (Pistia-Brueggeman and Hollingsworth, 2001). To each well of 96-well microtiter plate was added 20 μl of α-glucosidase [3 U/ml in phosphate buffer (PBS), pH 6.5], 40 μl of PBS (pH 6.5), 10 μl of the sample (concentrations: 1000 μg/ml, 100 μg/ml, 10 μg/ml in 1% MeOH) and H_2O (20 μl). The mixture was incubated at 37 °C for 10 min, then *p*-nitrophenyl α-D-glucopyranoside (10 μl, 20 mM in PBS) was added, and the mixture was incubated for additional 35 min. The absorbance (A) of each well was measured at 405 nm with microplate spectrophotometer (SPECTRAmax® PLUS, Molecular Devices). The inhibition activity was calculated by the equation: Inhibition (%) = $[1-(A_{sample}/A_{control})] \times 100\%$. The positive control was Acarbose (Bayer) which was found to have the IC_{50} value of 0.046 μM against the same enzyme.

4.9. Kaempferol-3-O-α-L-(3″,4″-di-E-p-coumaroyl)-rhamnopyranoside (**1**)

$[\alpha]^{2}7_{D}$-144.0 (MeOH, *c* 0.20); t_R: 40.8 min (Fig. 3).

4.10. Kaempferol-3-O-α-L-(3″-E,4″-Z-di-p-coumaroyl)-rhamnopyranoside (**2**)

Yellow amorphous powder; $[\alpha]_D^{27}$ −224.0 (MeOH, *c* 0.10); UV (MeOH) λ_{max} nm (log ε): 313 (4.69), 266 (4.41); for ^{1}H NMR spectrum, see Table 1; for HMBC correlations: see Fig. 2; ^{13}C NMR (CD_3OD, 100 MHz) δ 179.4 (*s*, C-4), 168.4 (*s*, C-9‴), 167.0 (*s*, C-9⁗), 166.4 (*s*, C-7), 163.3 (*s*, C-5), 161.8 (*s*, C-4′), 161.3 (*s*, C-4‴), 160.3 (*s*, C-4⁗), 159.4 (*s*, C-2), 158.7 (*s*, C-9), 147.4 (*d*, C-7‴), 146.5 (*d*, C-7⁗), 135.3 (*s*, C-3), 134.0 (*d*, C-2⁗/6⁗), 132.0 (*d*, C-2′/6′), 131.3 (*d*, C-2‴/6‴), 127.4 (*s*, C-1⁗), 127.1 (*s*, C-1‴), 122.5 (*s*, C-1′), 116.8 (*d*, C-3′/5′), 116.7 (*d*, C-3‴/5‴), 115.9 (*d*, C-3⁗/5⁗), 115.6 (*d*, C-8⁗), 105.9 (*s*, C-10), 114.7 (*d*, C-8‴), 102.1 (*d*, C-1″), 100.1 (*d*, C-6), 94.9 (*d*, C-8), 73.0 (*d*, C-3″), 71.3 (*d*, C-4″), 69.9 (*d*, C-5″), 69.7 (*d*, C-2″), 17.6 (*q*, C-6″); ESI-MS (positive mode) *m/z* 724.7 $[M+H]^+$; HR-FAB-MS (positive mode) *m/z* 725.1870 $[M+H]^+$ (calc. for $C_{39}H_{32}O_{14}$, 725.1870). t_R: 46.4 min (Fig. 3).

4.11. Quercetin-3-O-α-L-(3″-Z,4″-E-di-p-coumaroyl)-rhamnopyranoside (**4**)

UV (MeCN–H_2O) λ_{max} nm: 310, 265; for ^{1}H NMR spectrum, see Table 1; ESI-MS (positive mode) *m/z* 739.1 $[M+H]^+$; HR-ESI-MS (negative mode) *m/z* 739.1680 $[M-H]^-$ (calc. for $C_{39}H_{31}O_{15}$, 739.1663); t_R: 30.5 min (Fig. 3).

4.12. Quercetin-3-O-α-L-(3″,4″-di-Z-p-coumaroyl)-rhamnopyranoside (**5**)

UV (MeCN–H_2O) λ_{max} nm: 310, 265; for ^{1}H NMR spectrum, see Table 1; ESI-MS (positive mode) *m/z* 739.1 $[M+H]^+$; HR-ESI-MS (negative mode) *m/z* 739.1685 $[M-H]^-$ (calc. for $C_{39}H_{31}O_{15}$, 739.1663); t_R: 32.7 min (Fig. 3).

4.13. Kaempferol-3-O-α-L-(3″,4″-di-Z-p-coumaroyl)-rhamnopyranoside (**7**)

UV (MeCN–H_2O) λ_{max} nm: 310, 265; for ^{1}H NMR spectrum, see Table 1; ESI-MS (positive mode) *m/z* 724.7 $[M+H]^+$; HR-ESI-MS (negative mode) *m/z* 723.1710 $[M-H]^-$ (calc. for $C_{39}H_{31}O_{14}$, 723.1714); t_R: 42.5 min (Fig. 3).

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