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Flavonoids from *Taxillus theifer*

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ABSTRACT

A new catechin derivative, catechin-5-*O*-(6-*O*-galloyl-*β*-glucopyranoside) (**2**), together with ten known flavonoids, was isolated from the leaves and stems of *Taxillus theifer* (Hayata) H. S. Kiu. *T. theifer* is a shrub parasitizing on different species of plants. Three samples of *T. theifer* on various host plants were collected and their chemical constituent variations were identified and estimated by HPLC/UV methods. A comparison of the constituents from *T. theifer* parasitizing on different host trees demonstrated that catechin (**1**), quercetin-3-*O*-(6-*O*-galloyl-*β*-glucopyranoside) (**3**), quercetin-3-*O*-*β*-glucopyranoside (**5**), and quercetin-3-*O*-*β*-glucuronide (**6**) were the major components in the title plant irrespective of the host tree. However, the content of some components varies for each sample.

Key words: *Taxillus theifer, Scurrula ritozanensis*, Loranthaceae, catechin-5-*O*-(6-*O*-galloyl-*β*-glucopyranoside), flavonoid

INTRODUCTION

Loranthaceae plants have the special characteristics of hemi-parasitic. They often hemi-parasitize in shrubby form on the branches of diverse host plants(1). Some species of Loranthaceae from China have been used as medicinal materials for the treatment of cancer, bacteria infection, hypertension and rheumatics(2). Various compounds have been found in Loranthaceaeous plants and some of them have been identified with antimicrobial and hypotensive properties(3,4).

Taxillus theifer (Hayata) H. S. Kiu (*Scurrula ritozanensis*), a Loranthaceaeous plant endemic to Taiwan(1), has been used as an anti-hypertensive agent in Formosan folk medicine. Our preliminary bioassay also showed that the ethanolic extract of *T. theifer* has a significant vaso-relaxing effect to reduce vascular tone induced by vaso-constrictor in rat aortic ring preparation. Parasitic plant *T. theifer* may go after many different species of plants as hosts. Three samples of *T. theifer* parasitized on different host-plants were collected from the mountain area of Nantou, Taiwan. It is generally considered that different host plants offer different nutrients which may in turns effect the composition of parasite metabolites. The variation of chemical composition correlates closely with the performance of biological activity. To our knowledge, the chemical constituents of this title plant have not been studied. Here, we reported the isolation of flavonoids from *T. theifer* and further analysis of the content variations of the chemicals in all collected samples.

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MATERIALS AND METHODS

I. *Plant Materials*

The leaves and stems of *T. theifer* parasite on *Zelkva serrata*, *Lagerstroemia subcostata* and *Pyrus serotina* were collected in March 2007, in Nantou, Taiwan, and called TT-ZS, TT-LS and TT-PS, respectively. These samples were verified by Dr. Cheng-Jen Chou, research fellow of National Research Institute of Chinese Medicine, Taipei, Taiwan. Voucher specimens were deposited in the same Institute.

II. *Extraction and Isolation*

The leaves and stems of *T. theifer* parasite on *Zelkva serrata* (5.0 kg) were crushed and extracted with ethanol (40 L × 3) under reflux. The ethanolic extract was evaporated to dryness and partitioned successively between H_2O and *n*-hexane, followed by *n*-BuOH (each 1 L × 3). The *n*-BuOH fraction (103 g) was subjected to column chromatography on Sephadex LH-20 (10 × 120 cm; GE Healthcare

Biosciences AB, Sweden), with a gradient elution of MeOH in H_2O from 50 to 100%, and 12 fractions (Fr.1-12) were collected. Fractions 4, 5, 6, 8, 9 and 10 were further purified over Sephadex LH-20 by eluting with MeOH or acetone to get compounds **1** (19.8 mg), **2** (48.2 mg), **3** (0.84 g), **4** (21.7 mg), **5** (0.81 g), **6** (1.18 g), **7** (5.2 mg), **8** (7.6 mg), **9** (159.3 mg), **10** (46.7 mg), and **11** (17.9 mg).

III. *Spectroscopic Analysis*

IR spectra were acquired as KBr pellets on a Nicolet Avatar 320 IR spectrometer. UV spectra were carried on a Hitachi U-3200 spectrophotometer in MeOH. ^{1}H-, ^{13}C- and 2D-NMR spectra were measured on a Varian Inova-500 spectrometer using deuterated solvents (methanol-d_4 or DMSO- d_6, CIL, MA, USA) as internal standards. ESIMS and HR-ESI-MS were recorded on Finnigan LCQ and Finnigan MAT 95S MS spectrometers, respectively.

IV. *Preparation of Stock Solutions and Standard Curves*

After spectroscopic analysis and purity assessment by HPLC, the identified compounds **1-11** (purity > 99.0%) served as standards in the following analytic experiment. Stock solutions were prepared by dissolving 10 mg of standard substances [catechin (**1**), quercetin-3-*O*-(6-*O*-galloyl-β-glucopyranoside) (**3**), quercetin-3-*O*-β-glucopyranoside (**5**) or quercetin-3-*O*-β-glucuronide (**6**)] in 10 mL of methanol. Two hundred microliters of aliquot of each standard was mixed and diluted in methanol at the concentration of 0.2 mg/mL each. Calibration standards of 100, 25, 20, 10, and 5 μg/mL were prepared by serial dilution of the mixed solution. The internal standard (IS) was mangiferin (50 μg/mL).

V. *Plant Sample Preparation*

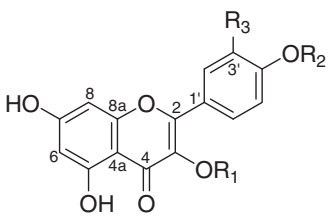
About 10 g of leaves and stems of *T. theifer* from three different host plants were extracted with 100 mL of ethanol twice under reflux, and the ethanolic extracts were dried under vacuum. The ethanolic extracts of TT-ZS (sample 1), TT-LS (sample 2) and TT-PS (sample 3) were prepared in concentration of 5 mg/mL (sample 1) and 3 mg/mL (samples 2 and 3) with MeOH, respectively. After filtering using a 0.45-μm PTFE filter, the filtrates were analyzed by HPLC.

VI. *Chromatographic System*

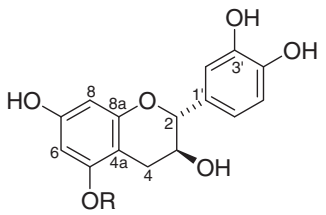
HPLC analysis was carried out using a Hitachi system (Tokyo, Japan) consisting of an L-7100 HPLC pump, an L-7200 autosampler equipped with a 100-μL sample loop, an L-7450A photodiode array detector, and a D-7000 HPLC Multi-System Manager chromatographic data system. Separation of samples was performed on a Cosmosil® 5C18-AR-II column (4.6 × 250 mm, 5 μm, Nacalai Tesque, Kyoto, Japan). Mobile phases consisted of solvent A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). A linear gradient program was performed from 10 to 30% of solvent B over 100 min, at flow rate 1.0 mL/min. An aliquot (20 μL) of sample was injected onto analysis and the profile was recorded at UV 280 nm.

VII. *Evaluation of Precision and Accuracy for HPLC Analysis*

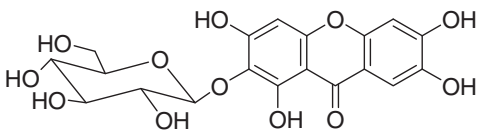
The precision and accuracy were determined by carrying out six independent assays of test compounds **1**, **3**, **5** and **6** at five concentrations of 5, 10, 15, 25 and 100 μg/mL. The precision of the HPLC method was determined as the coefficient of variation (CV%, 100 × mean standard derivation/average %) of intra- and inter-day assays. The accuracy of the HPLC method was demonstrated as the percentage derivation [(true value-measured value) × 100/true value %)].



3 R_1 = glucosyl(6"-O-galloyl) R_2 = H R_3 = OH
4 R_1 = glucosyl(6"-O-rhamnosyl) R_2 = H R_3 = OH
5 R_1 = glucosyl R_2 = H R_3 = OH
6 R_1 = glucuronic acid R_2 = H R_3 = OH
7 R_1 = glucosyl R_2 = H R_3 = H
8 R_1 = H R_2 = glucosyl R_3 = OH
9 R_1 = glucuronic acid methyl ester R_2 = H R_3 = OH
10 R_1 = R_2 = H R_3 = OH
11 R_1 = glucuronic acid butyl ester R_2 = H R_3 = OH



1 R = H
2 R = glucosyl(6"-O-galloyl)



Internal standard: Mangiferin

Figure 1. Chemical structures of isolated compounds **1-11** and internal standard, mangiferin.

RESULTS AND DISCUSSION

Phytochemical investigation of the ethanolic extract of *T. theifer* yielded one new and ten known compounds (Figure 1). The chemical structures of these known compounds have been identified as catechin (**1**), quercetin-3-*O*-(6-*O*-galloyl-β-glucopyranoside) (**3**)[5], quercetin-3-*O*-rutinoside (**4**)[6], quercetin-3-*O*-β-glucopyranoside (**5**)[7], quercetin-3-*O*-β-glucuronide (**6**)[8], kaempferol-3-*O*-β-glucopyranoside (**7**)[7], quercetin-4'-*O*-β-glucopyranoside (**8**)[7], quercetin-3-*O*-β-glucuronic acid methyl ester (**9**), quercetin (**10**)[7] and quercetin-3-*O*-β-glucuronic acid butyl ester (**11**) on the basis of spectral analyses (1D, 2D NMR and MS) and comparison with reported data.

Compound **2** was obtained as an amorphous solid from MeOH. Its molecular formula $C_{28}H_{28}O_{15}$ was deduced from ^{13}C NMR, DEPT, and ESIMS, and further confirmed by HR ESIMS *m/z* 603.1407 $[M-H]^-$ (calcd. for $C_{28}H_{27}O_{15}$ 603.1350). IR (ν_{max} 3360, 2913, 1699, 1607, 1531, 1505, 1455, 1352, 1247, 1073, 1025 /cm) and UV [λ_{max} (log ε) 279 (4.27), 223 sh. (4.70) nm] spectra showed hydroxyl, benzene, carbonyl, and glycosyl systems. The ^1H-NMR and COSY spectra (Table 1) of compound **2** showed a pair of aromatic doublets at δ 6.05/6.28 (each *d*,

Table 1. ^1H- and ^{13}C-NMR data of compound **2**[a]

	no.	δ ^{13}C	δ ^1H	HMBC (^2J and ^3J)
catechin	2	81.6	4.60 (d, 7.5)	C-3, C-4, C-1', C-2', C-6', C-8a
	3	67.4	3.98 (ddd, 5.5, 7.5, 8.0)	
	4	27.2	2.59 (dd, 16.0, 8.0)/3.01 (dd, 16.0, 5.5)	C-2, C-3, C-5, C-4a, C-8a
	4a	102.6	---	
	5	156.7	---	
	6	95.9	6.28 (d, 2.0)	C-4a, C-5, C-7, C-8
	7	156.5	---	
	8	97.3	6.05 (d, 2.0)	C-4a, C-6, C-7, C-8a
	8a	155.5	---	
	1'	130.9	---	
	2'	114.0	6.82 (d, 1.5)	C-2, C-4', C-6'
	3'	145.1	---	
	4'	145.1	---	
	5'	115.0	6.75 (d, 8.0)	C-1', C-3'
	6'	118.8	6.70 (dd, 8.0, 1.5)	C-2, C-2', C-4'
glucosyl	1"	101.3	4.89 (d, 7.5)	C-5
	2"	73.7	3.50 [b]	
	3"	76.8	3.50 [b]	
	4"	70.2	3.54 [b]	
	5"	74.4	3.71 (br. t, 6.5)	
	6"	63.5	4.41 (dd, 12.0, 5.5)/4.57 (d, 12.0)	C-7"
galloyl	1"'	120.1	---	
	2"'/6"'	109.1	7.10 (s)	C-1", C-3", C-4", C-5", C-7"
	3"'/5"'	145.3	---	
	4"'	138.7	---	
	7"'	167.2	---	

[a] measured in MeOH-d_4; multiplicity and coupling constant (*J* in Hz) assigned in parentheses.
[b] Signal patterns are unclear due to overlapping.

Table 2. Precision and accuracy of the method

	Nominal conc. (μg/mL)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)
			1			**3**			**5**			**6**	
Inter-day	5	4.35 ± 0.26	6.06	-14.89	5.18 ± 0.59	11.40	3.54	5.03 ± 0.74	14.64	0.63	4.82 ± 0.72	14.88	-3.78
	10	9.61 ± 0.76	7.88	-4.02	9.77 ± 0.84	8.61	-2.38	9.54 ± 0.55	5.81	-4.83	9.41 ± 0.55	5.80	-6.32
	15	15.33 ± 0.86	5.59	2.16	14.77 ± 1.11	7.54	-1.55	15.28 ± 1.30	8.50	1.81	15.17 ± 0.39	2.59	1.15
	25	25.95 ± 0.67	2.60	3.67	25.50 ± 2.03	7.97	1.97	25.25 ± 1.19	4.71	0.98	25.67 ± 1.08	4.23	2.60
	100	99.75 ± 0.15	0.15	-0.25	99.87 ± 0.37	0.37	-0.13	99.90 ± 0.25	0.26	-0.10	99.84 ± 0.22	0.22	-0.16
			1			**3**			**5**			**6**	
Intra-day	5	4.59 ± 0.63	13.64	-8.89	4.40 ± 0.34	7.70	-13.69	4.49 ± 0.64	14.28	-11.29	5.09 ± 0.59	11.68	1.80
	10	9.62 ± 0.95	9.83	-3.91	9.44 ± 1.21	12.85	-5.90	9.82 ± 0.67	6.84	-1.84	9.24 ± 1.29	14.00	-8.17
	15	15.52 ± 0.96	6.17	3.34	14.60 ± 0.68	4.62	-2.71	15.22 ± 0.76	4.96	1.44	15.70 ± 1.19	7.60	4.47
	25	25.64 ± 2.51	9.78	2.48	27.00 ± 2.22	8.23	7.40	25.66 ± 1.43	5.57	2.56	25.20 ± 2.81	11.13	0.81
	100	99.74 ± 0.49	0.49	-0.26	99.53 ± 0.58	0.58	-0.47	99.81 ± 0.30	0.30	-1.19	99.81 ± 0.48	0.48	-0.19

[D]ata are expressed as means ± S.D. (n = 6).

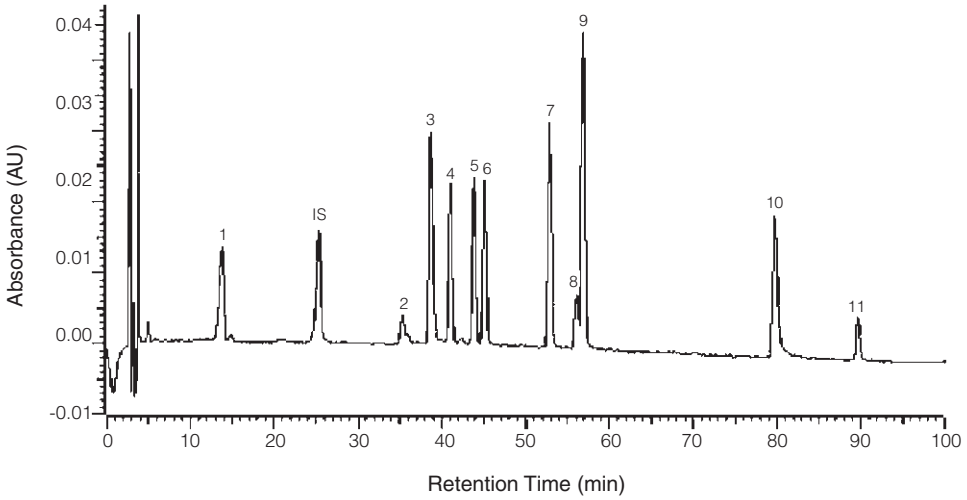


Figure 2. HPLC Chromatograms of flavonoids mixture with internal standard (IS, mangiferin) at UV 280 nm detection. **1**: catechin, **2**: catechin-5-*O*-(6-*O*-galloyl-β-glucopyranoside), **3**: quercetin-3-*O*-(6-*O*-galloyl-β-glucopyranoside), **4**: quercetin-3-*O*-rutinoside, **5**: quercetin-3-*O*-β-glucopyranoside, **6**: quercetin-3-*O*-β-glucuronide, **7**: kaempferol-3-*O*-β-glucopyranoside, **8**: quercetin-4'-*O*- β-glucopyranoside, **9**: quercetin-3-*O*-β-glucuronic acid methyl ester, **10**: quercetin, and **11**: quercetin-3-*O*-β-glucuronic acid butyl ester.

J = 2.0 Hz), two hydroxyl methine signals at δ 3.98/4.60, a benzylic methylene signal at δ 3.01/2.59, and a set of ABC-type signals at δ 6.75/6.70/6.82, suggesting a flavan skeleton with a 3,5,7,3',4'-pentahydroxy substitution. The appearance of a doublet at δ 4.60 (*J* = 7.5 Hz) due to flavan H-2 suggested that this unit had catechin (2, 3-*trans*) stereochemistry[9]. An anomeric proton at δ 4.89 (*d*, *J* = 7.5 Hz) and a two-proton singlet at δ 7.10 suggested β-glucosyl and galloyl units. The signals of H-6 and H-8 were determined by ROESY experiment. The signal at δ 6.05 displayed cross-peaks with H-2' and H-6' and is thus identified as H-8[10]. Therefore, H-6 is at δ 6.28. The locations of oxygenated quaternary carbons C-4a, C-8a, and C-7 were deduced by HMBC correlations from H-4, H-6 and H-8 to C-4a, from H-2, 4 and H-8 to C-8a, and from H-6 and H-8 to C-7. Significant HMBC correlations of compound **2** from H-4 (δ 2.59/3.01) to C-5 (δ 156.7), and H-1" (δ 4.89) to C-5, indicated the position of the glucosyl group on C-5. The cross-peak in the ROESY experiment between H-6/H-1" (δ 4.89) further confirmed the location of β-glucose on C-5[10]. The low-field shift of H-6" (δ_H 4.41/4.57) clarified the location of the galloyl group on C-6". HMBC correlations of H-6", H-2''' and H-6''' with C-7''' reconfirmed the above deduction. According to the above data, the structure of compound **2** was elucidated as catechin-5-*O*-(6-*O*-galloyl-β-glucopyranoside). The UV spectrum of **compound 2** is similar to that of catechin, indicating that 5-*O*-glycosyl linkage did not affect the UV absorbance.

The major flavonoids **1**, **3**, **5** and **6** in *T. theifer* were isolated from the crude drug and served as pure standards for quantitative analysis. Satisfactory linearity for the analysis of each compound was obtained. Linearity was examined with a series of standard solutions in the concentration range of 4.59-99.74 μg/mL for catechin (**1**), 4.40-99.53 μg/mL for quercetin-3-*O*-(6-*O*-galloyl-β-glucopyranoside) (**3**), 4.49-99.81 μg/mL for quercetin-3-*O*-β-glucopyranoside (**5**) and 4.09-99.81 μg/mL for quercetin-3-*O*-β-glucuronide (**6**). The linear regression equation of the calibration curves of catechin was calculated to be y = 0.0184x + 0.0115 with a correlation coefficient of r^2 = 0.9997 (n = 5), for quercetin-3-*O*- (6-*O*-galloyl-β-glucopyranoside): y = 0.0416x - 0.1294 with a correlation coefficient of r^2 = 0.9982, for quercetin-3-*O*-β-glucopyranoside: y = 0.0311x + 0.0224 with a correlation coefficient of r^2 = 0.9992, and for quercetin-3-*O*-β-glucuronide: y =0.032x - 0.0589 with a correlation coefficient of r^2 = 0.9996. Method precision was investigated by repeated analysis of standard solutions; the values of the CV of intra-day and inter-day were less than 15% (n = 6) for compounds **1**, **3**, **5** and **6** (Table 2). Percentage derivation was performed to confirm the accuracy of method. The percentage derivations were in the range of -0.08-11.29% (Table 2).

As shown in Figure 2, a reversed-phase C18 column chromatography with the linear gradient elution of mobile phase acetonitrile-H_2O (0.1% formic acid added) from 10 to 30% acetonitrile over 100 min provided good separation of the 11 flavonoids mixture and internal standard. This HPLC condition also fulfilled the requirements for resolution in crude extract analysis.

The ethanolic extracts of the samples were quantitatively analyzed by HPLC without any solvent partition process or other column chromatography. A comparison of HPLC of the extracts from *T. theifer* parasite on different host trees, *Zelkva serrata* (sample 1), *Lagerstroemia subcostata* (sample 2) and *Pyrus serotina* (sample 3), showed that compounds **1**, **3**, **5** and **6** were the four

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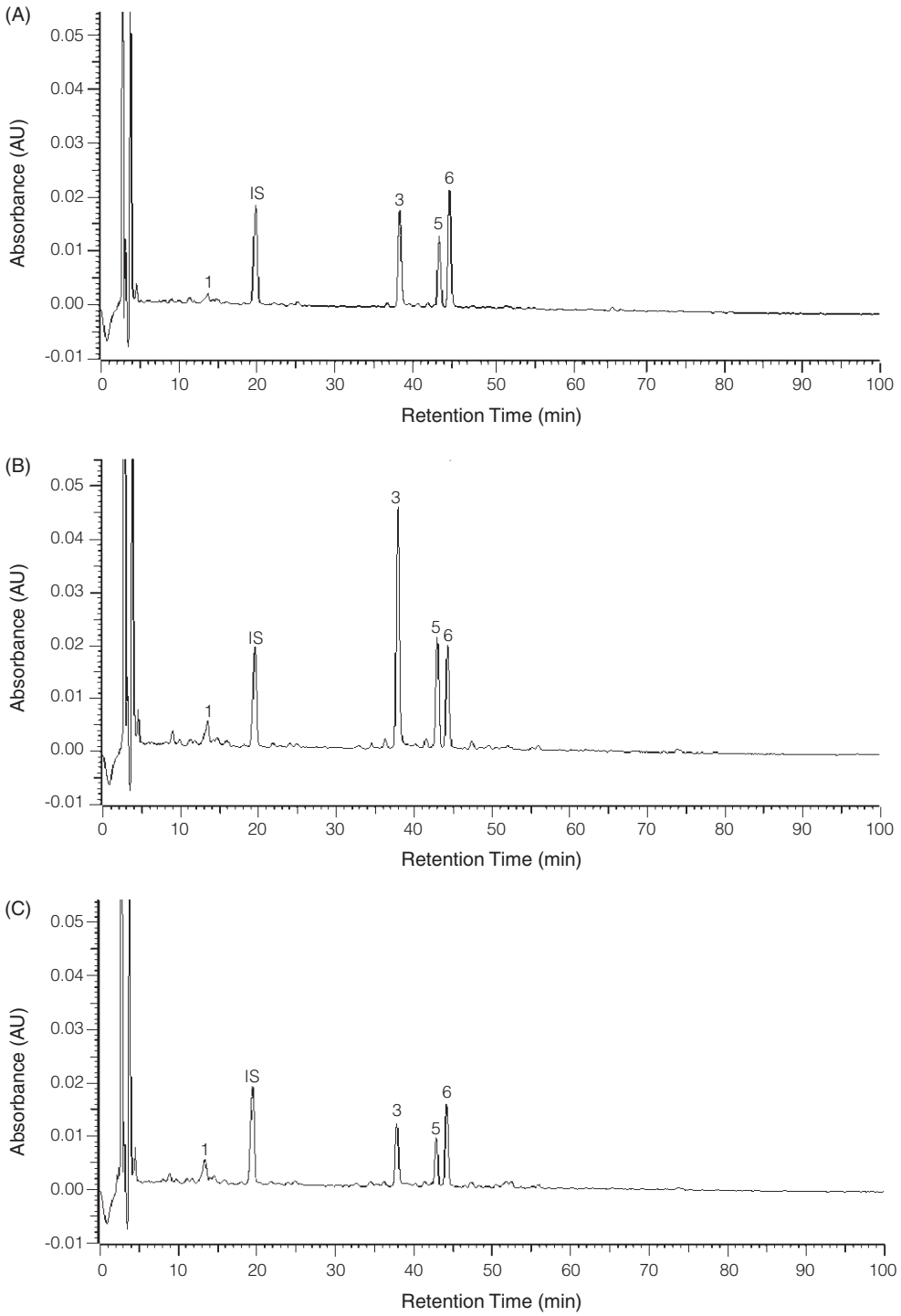

Figure 3. HPLC Chromatograms of EtOH extracts of (A) TT-ZS (sample 1), (B) TT-LS (sample 2) and (C) TT-PS (sample 3) with internal standard (IS, mangiferin) at UV 280 nm detection. **1**: catechin, **3**: quercetin-3-*O*-(6-*O*-galloyl-β-glucopyranoside), **5**: quercetin-3-*O*-β-glucopyranoside, **6**: quercetin-3-*O*-β-glucuronide.

major compounds contained in the title plant irrespective of the host trees (Figure 3A-3C). Compounds **2**, **4**, **7**-**11** were not detected in *T. theifer* extract, indicating that some of them are fairly low yield or lacking. The contents of compounds **1**, **3**, **5**, **6** varied greatly in samples 1-3 and the results were showed in Table 3.

Compounds **9** and **11** could be artifacts arising from esterification of compound **6** in the column purification process and the *n*-BuOH partition process. Compound **1** has an advantage of content in the HPLC analysis but not in the phytochemical investigation. This variation may derive from a different experimental process.

Table 3. Major flavonoids (**1**, **3**, **5** and **6**) content of *T. theifer* parasite on different host trees, *Zelkva serrata* (sample 1), *Lagerstroemia subcostata* (sample 2) and *Pyrus serotina* (sample 3)

	Content in EtOH extract (%, w/w)[a]			
	1	**3**	**5**	**6**
Sample 1	0.09 ± 0.01	1.15 ± 0.10	0.80 ± 0.09	1.37 ± 0.06
Sample 2	0.19 ± 0.01	2.52 ± 0.16	0.97 ± 0.06	0.96 ± 0.07
Sample 3	0.22 ± 0.03	0.66 ± 0.03	0.52 ± 0.04	0.96 ± 0.06

[a]Samples were analysed in triplicate and results are expressed as means ± S.D.

Compound **1** is more hydrophilic than compounds **2**, **4**, **7**, **8**, and **10** so that compound **1** is expected to distribute in both water and *n*-BuOH layer. This fact caused only a small amount of compound **1** isolated from the *n*-BuOH layer. The total contents of major flavonoids of samples 1-3 were in the order of sample 2 > sample 1 > sample 3. Sample 2 had major flavonoids content which was about twice quantity of sample 1.

CONCLUSIONS

In summary, this study indicated that *T. theifer* had similar secondary metabolite patterns but different contents depending on the host plant. Their corresponding pharmacological activity requires further explanation.

ACKNOWLEDGMENTS

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