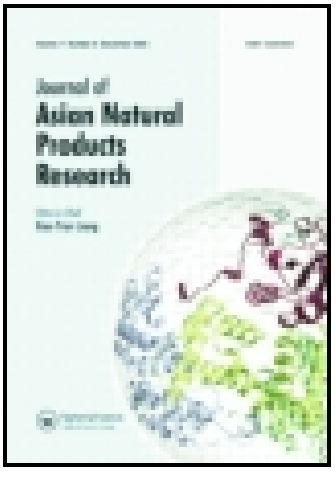

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Chemical constituents of *Euonymus fortunei*

Li-Hua Yan[ab], Xiao-Qian Liu[ab], Hui Zhu[ab], Qing-Rui Xu[c], Wei-Ming Wang[c], Shu-Ming Zhang[c], Qi-Wei Zhang[ab], Shan-Shan Zhang[ab] and Zhi-Min Wang[ab]*

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A new flavonol glycoside, kaempferol-3-*O*-β-D-(2-*O*-*E*-*p*-coumaroyl)-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**1**), along with eleven known compounds including five flavonol glycosides (**2**–**6**), one phenolic glycoside (**7**), two megastigmane glycosides (**8** and **9**), two triterpenoids (**10** and **11**) and one alditol (**12**), was isolated from the aerial parts of *Euonymus fortunei*. Their structures were determined on the basis of spectroscopic analysis and chemical evidence. Compounds **2**–**4**, **7**, **8,** and **10**–**12** were evaluated their antimicrobial activities against *Ureaplasma urealyticum in vitro*, but all tested compounds have no useful activities against *Ureaplasma urealyticum*.

Keywords: Celastraceae; *Euonymus fortunei;* flavonol glycosides; megastigmane glycosides; *Ureaplasma urealyticum*

1. Introduction

Euonymus fortunei (Turcz.) Hand.-Mazz (Celastraceae), known as "Fu-Fang-Teng" in Chinese, is an evergreen shrub with decumbent stems widely distributed in China. It has been used in Traditional Chinese Medicine for the treatment of rheumatism, metrorrhagia, traumatic injury, and so on [1]. Known constituents of *E. fortunei* include triterpenes, flavonoids, lignans, and sesquiterpene pyridine alkaloids [2–5]. Dihydro-β-agarofuran sesquiterpenoids are characteristic constituents of the family Celastraceae, and they have attracted a great deal of interest because of their cytotoxic, insect antifeedant, insecticidal, and anti-HIV activities. Some species of the family Celastraceae have been used for cancer, psoriasis, contraception and as an insecticide [6]. *Ureaplasma urealyticum* has been implicated in both genital disease and infertility in man. Drug tolerance may occur with continuing use of existing antibiotics, so an effective antibiotic treatment is still urgently required [7].

In this study, a phytochemical investigation is reported on a 70% ethanol extract from the aerial parts of *E. fortunei* and focuses on the isolation of higher polarity compounds. A new flavonol glycoside, kaempferol-3-*O*-β-D-(2-*O*-*E*-*p*-coumaroyl)-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**1**), along with 11 known compounds (**2**–**12**) were isolated and identified (Figure 1). Compounds **6**, **8,** and **9** were obtained from the genus *Euonymus* for the first time. The *in vitro* activities of compounds **2**–**4**, **7**, **8**, and **10**–**12** against *Ureaplasma urealyticum* were attemped to evaluate.

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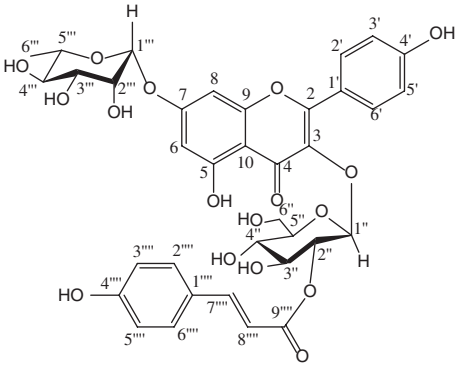

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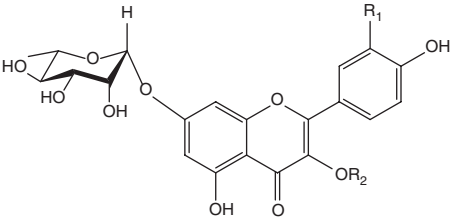
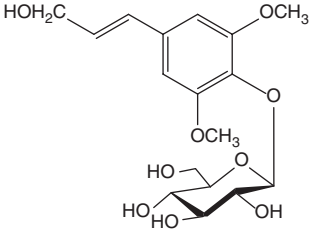


2 R_1 = H, R_2 = β-D-Glc
3 R_1 = H, R_2 = H
4 R_1 = H, R_2 = α-L-Rha
5 R_1 = H, R_2 = CH_3COO^4-α-L-Rha
6 R_1 = OH, R_2 = H



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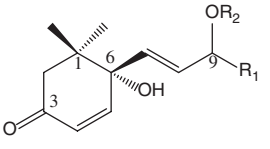
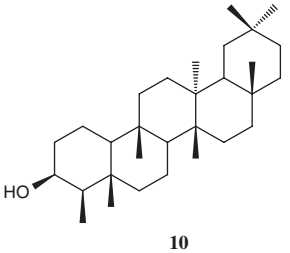


8 R_1 = CH_2OH, R_2 = β-D-Glc; 6*S*, 9*S*
9 R_1 =CH_3, R_2 = β-D-Glc; 6*S*, 9*R*



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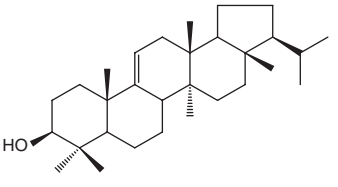

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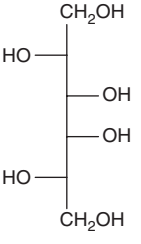

12

Figure 1. Structures of compounds **1**–**12**.

2. Results and discussion

Compound **1** was isolated as pale yellow amorphous powder. Its molecular formula was determined as $C_{36}H_{36}O_{17}$ by HR-ESI-MS (*m/z* 763.1866 $[M + Na]^+$). The UV absorption maxima (269, 317 nm) indicated the flavonoid skeleton. The IR spectrum showed absorption bands for hydroxyl groups (3364 cm^{-1}), chelated carbonyl (1655 cm^{-1}), and ester carbonyl groups (1702 cm^{-1}). The ^{1}H NMR spectrum of **1** (Table 1) showed a 5, 7-di-hydroxylated pattern for ring A (two *meta*-coupled doublets at δ_H 6.70 and 6.88, $J = 1.8$ Hz) and a 4′-hydroxylated pattern for ring B (AA′BB′ system signals at δ_H 8.46 and 7.30, each d, $J = 8.4$ Hz), which indicated the presence of kaempferol as aglycone. The ^{1}H NMR spectrum also exhibited two doublets at δ_H 8.02 and 6.68 with large coupling constants (15.6 Hz), which identified *trans* olefinic double bond protons, and *p*-hydroxyl phenyl ring showing another AA′BB′ pattern (δ_H 7.47 and 7.09, each d, $J = 8.4$ Hz), which suggested the presence of the *p*-coumaroyl moiety.

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **1** in C_5D_5N.

Positions	δ_H (J in Hz)	δ_C	HMBC(H-C)
2		157.1	
3		134.3	
4		178.3	
5		156.5	
6	6.70(1H, d, 1.8)	100.1	C-8, 10, 4, 7
7		162.4	
8	6.88(1H, d, 1.8)	94.4	C-6, 10, 5, 7,
9		162.1	
10		106.6	
1′		121.5	
2′, 6′	8.46(2H, d, 8.4)	131.7	C-2, 4′
3′, 5′	7.30(2H, d, 8.4)	116.1	C-1′, 4′
4′		161.7	
1″	6.69(1H, d, 7.8)	100.0	C-3, 3″, 5″
2″	6.04(1H, dd, 7.8, 9.6)	75.5	C-9‴′, 1″, 3″
3″	4.52(1H, t, 9.6)	76.0	C-2″, 4″
4″	4.28(1H, t, 9.6)	71.5	C-3″, 5″, 6″
5″	4.12(1H, m)	79.2	C-4″, 6″
6″	4.42(1H, dd, 12.0, 2.4)	62.1	C-4″
	4.24(1H, dd, 12.0, 5.4)		C-5″
1‴	6.18(1H, d, 1.2)	99.7	C-7, 5‴
2‴	4.67(1H, dd, 3.0, 1.2)	71.4	C-4‴, 3‴, 1‴
3‴	4.61(1H, dd, 9.0, 3.0)	72.1	C-4‴, 2‴
4‴	4.35(1H, t, 9.0)	73.3	C-3‴, 5‴
5‴	4.25(1H, dd, 9.0, 6.6)	71.2	C-4‴, 6‴
6‴	1.62(1H, d, 6.6)	18.4	C-4‴, 5‴
1⁗′		125.9	
2⁗′, 6⁗′	7.47(2H, d, 8.4)	130.4	C-4⁗′, 7⁗′
3⁗′, 5⁗′	7.09(2H, d, 8.4)	116.5	C-1⁗′, 4⁗′
4⁗′		161.1	
7⁗′	8.02(1H, d, 15.6)	145.3	C-9⁗′, 2⁗′, 6⁗′, 8⁗′
8⁗′	6.68(1H, d, 15.6)	115.2	C-1⁗′, 7⁗′, 9⁗′
9⁗′		166.9	
5-OH	13.25(1H, br s)		

The ^{1}H NMR spectrum also supported the presence of one glucose and one rhamnose with the glucose anomeric proton at δ_H 6.69 (d, $J = 7.8$ Hz) and the rhamnose H-1 at δ_H 6.18 (d, $J = 1.2$ Hz). The ^{13}C NMR spectrum displayed the corresponding signals attributable to kaempferol, *p*-coumaroyl, and two sugar units. The sugars obtained by acid hydrolysis of **1** were identified as D-glucose and L-rhamnose by GC analysis of their trimethylsilyl-L-cysteine derivatives of the hydrolysate of **1** and the authentic sugars. The J values of the anomeric proton signals indicated that the glycosidic linkage of glucose was β configuration, and those of rhamnose was α configuration. The *p*-coumaroyl subunit was confirmed by the HMBC correlations (Figure 2) from H-8⁗′ (δ_H 6.68) to C-1⁗′ (δ_C 125.9) and from H-7⁗′ (δ_H 8.02) to C-9⁗′ (δ_C 166.9). In addition, the glycosidic linkage positions were unambiguously determined by HMBC correlations from H-1″ (δ_H 6.69, Glc) to C-3 (δ_C 134.3) and from H-1‴ (δ_H 6.18, Rha) to C-7 (δ_C 162.4), which confirmed that the β-glucopyranose and α-rhamnopyranose units were attached to C-3 and C-7 of kaempferol, respectively. These spectral data suggested that the structure of **1** was

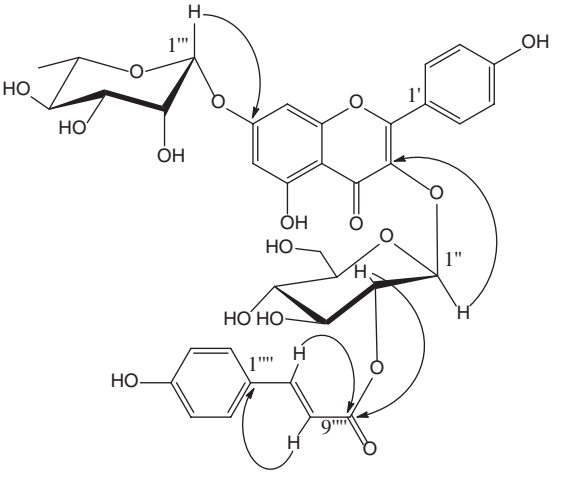

Figure 2. Key HMBC correlations of compound **1**.

similar to that of kaempferol-3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**2**) except for the presence of a *p*-coumaroyl group. The *p*-coumaroyl group was assigned to the C-2″ hydroxyl group via an ester bond, which was determined by the HMBC correlation between H-2″ (δ_H 6.04) and the ester carbonyl at δ_C 166.9 of the *p*-coumaroyl group. Therefore, the structure of compound **1** was characterized as kaempferol-3-*O*-β-D-(2-*O*-*E*-*p*-coumaroyl)-glucopyranosyl-7-*O*-α-L-rhamnopyranoside.

Other 11 known compounds (**2**-**12**) were identified as kaempferol-3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**2**) [3], kaempferol-7-*O*-α-L-rhamnopyranoside (**3**) [3], kaempferol-3, 7-*O*-α-L-dirhamnopyranoside (**4**) [3], kaempferol-3-(4-*O*-acetyl)-*O*-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (**5**) [8], quercitin-7-O-α-L-rhamnopyranoside (**6**) [9], syringin (**7**) [10], (6S, 7E, 9S)-6, 9, 10-trihydroxy-4, 7-megastigmadien-3-one-9-*O*-β-D-glucopyranoside (**8**) [11], (6S, 9R)-roseoside (**9**) [12], epifriedelinol (**10**) [13], isoarborinol (**11**) [14], and dulcitol (**12**) [15], respectively, by comparison of their physical and spectroscopic data with those values reported in the literature.

Compounds **2**–**4**, **7**, **8**, and **10**–**12** were evaluated for their antimicrobial activities against *Ureaplasma urealyticum in vitro*, but all tested compounds have no useful activities against *Ureaplasma urealyticum*. The minimum inhibitory concentration (MIC) range of compound **3** was 32–64 μg/ml. The MICs of other tested compounds were more than 128 μg/ml. Erythromycin served as a positive control, with the MIC range of 4–8 μg/ml.

3. Experimental

3.1 General experimental procedures

Melting points were determined using a SGW X-4 micromelting point apparatus (Shanghai Precision Instruments Co., Ltd, Shanghai, China). Optical rotations were measured on a PE Model 343 digital polarimeter (Perkin-Elmer, Waltham, MA, USA). IR spectra were obtained on a Nicolet 5700 FT-IR microscope instrument (Thermo Electron Corp., Madison, WI, USA). UV spectra were determined by a JASCO V-650 spectrophotometer (JASCO, Tokyo, Japan). HR-ESI-MS data were obtained on an Agilent 6520 Accurate-Mass LC/MS Q-TOF instrument (Agilent, Santa Clara, CA, USA). NMR

spectra were measured on a Bruker Avance 600 spectrometer (Bruker Biospin, Fallanden, Switzerland), using TMS as internal standard. GC data were recorded on an Agilent 7890A instrument (Agilent). Precoated silica gel GF_{254} plates (Qingdao Marine Chemical Factory, Qingdao, China) were employed for TLC. For column chromatography (CC), silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), reversed-phase C_{18} silica gel (50 μm, YMC Co. Ltd, Kyoto, Japan), HPD-100 macroporous absorbent resin (Hebei Bonherb Technology Corp., Cangzhou, China), and Sephadex LH-20 (GE healthcare, Uppsala, Sweden) were used. The preparative HPLC were performed on a Varian Pro Star HPLC system (Varian, Palo Alto, CA, USA), and a semi-preparative column (YMC-Pack ODS-A, 20 mm × 250 mm, 5 μm) was used for separations.

3.2 Plant material

The aerial parts of *Euonymus fortunei* were purchased from Nanning, Guangxi Zhuang Autonomous Region, China, in May 2011, and identified by Prof. Xue-Feng Feng, Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, where a voucher specimen (No. 90820110504) was deposited.

3.3 Extraction and isolation

Dried and powdered aerial parts of *E. fortunei* (20 kg) were extracted with 70% EtOH under reflux, and the EtOH extract was concentrated to dryness. The residue was suspended in H_2O and then extracted successively with petroleum ether (60–90°C) and EtOAc. The petroleum ether (60–90°C) extract (140 g) was subjected to silica gel CC and eluted with petroleum ether (60–90°C)-EtOAc (98:2–2:8) to yield 17 fractions (Fr. A1–Fr. A17). Compound **10** (1.65 g) and compound **11** (1.88 g) were recrystallized in petroleum ether (60–90°C)-acetone (1:1) from the Fr. A3 and Fr. A4, respectively. The EtOAc extract (35 g) was fractioned by Sephadex LH-20 CC and eluted with MeOH to obtain three fractions (Fr. B1–Fr. B3). Fr. B1 was further separated by reversed-phase C_{18} silica gel CC using a gradient of MeOH–H_2O (2:8–9:1) to yield 8 subfractions (Fr. B1-1–Fr. B1-8). After further purification by preparative HPLC (MeOH–H_2O, 45:55), compound **2** (35 mg, $t_R = 17.49$ min) was obtained from Fr. B1-5. Separation of Fr. B1-6 with Sephadex LH-20 CC (MeOH) gave compound **4** (210 mg). Compounds **1** (13 mg, $t_R = 25.63$ min), **5** (22 mg, $t_R = 28.66$ min), and **6** (6 mg, $t_R = 22.73$ min) were obtained from Fr. B1-8 by preparative HPLC (MeOH–H_2O, 1:1). Fr. B2 was applied to Sephadex LH-20 CC, with MeOH as eluent, to achieve compound **3** (150 mg). The aqueous phase was evaporated in vacuum to give a supernatant solution (1.8 L) and a precipitation. The precipitation was repeatedly recrystallized in MeOH–H_2O (1:1) to obtain compound **12** (11 g). The solution was subjected to HPD-100 macroporous absorbent resin CC using an EtOH/ H_2O gradient (0, 10, 30, 50, and 95% EtOH) to yield five fractions. The fraction (28 g) eluted by 30% EtOH was separated on a reversed-phase C_{18} silica gel column eluting with MeOH–H_2O (1:9–1:0), followed by preparative HPLC (MeOH–H_2O, 15: 85) to afford compounds **7** (37 mg, $t_R = 24.18$ min), **8** (56 mg, $t_R = 16.09$ min), and **9** (8 mg, $t_R = 8.92$ min).

3.3.1 Compound **1**

Pale yellow amorphous powder; mp 211–212°C; $[\alpha]_D^{20}$ − 168.0 (c 0.103, MeOH). UV (MeOH) λ_{max} nm (log ε): 203 (4.69), 269 (4.37), 317 (4.49). IR ν_{max}: 3364, 1702, 1655, 1600, 1513, 1492, 1451, 1172, 832, and 595 cm^{-1}. ^1H and ^{13}C NMR spectral data were listed in Table 1; HR-ESI-MS *m/z*: 763.1866 $[M + Na]^+$ (calcd for $C_{36}H_{36}O_{17}Na$, 763.1850).

3.4 Acid hydrolysis of compound 1

According to the reported method [16], compound **1** (2 mg) was hydrolyzed with 2 M $CF_3COOH-H_2O$ (2 ml) at 110°C for 3 h. After removal of CF_3COOH by evaporation and extraction with $CHCl_3$, the H_2O extract was evaporated and dried *in vacuo* to give the monosaccharide residue. The residue was dissolved in pyridine (100 μl), and then 0.06 M L-cysteine methyl ester hydrochloride-pyridine (100 μl) was added. The mixture was kept at 60°C for 1 h. Next, 150 μl trimethylsilylation reagent HMDS–TMCS (hexamethyldisilazane-trimethylchlorosilane, 1:1) was added, and the resultant reaction mixture was maintained at 60°C for 30 min. After centrifugation, the supernatant was analyzed by GC under the following conditions: capillary column, HP-5 (30 m × 0.32 mm × 0.25 μm); detector, FID (Flame Ionization Detector); detector temperature, 280°C; injection temperature, 250°C; initial temperature, 100°C for 2 min and subsequent increase to 280°C at a rate of 10°C/min; final temperature, 280°C for 5 min; carrier, N_2 gas. The absolute configurations of the sugars isolated from the hydrolysate of **1** were determined by comparing the retention times of their trimethylsilyl-L-cysteine derivatives with those of authentic sugars prepared by a similar procedure. The retention times of the trimethylsilyl-L-cysteine derivatives of the sugars were as follows: D-glucose, 19.79 min; L-rhamnose, 18.63 min.

3.5 Antimicrobial activity assay

Ureaplasma urealyticum serotypes 4 and 8 standard strains were purchased from the American Type Culture Collection. The assay was carried out adopting the agar dilution method [17,18]. Stock solutions of compounds **2**–**4**, **7**, **8,** and **10**–**12** were made up to 512 mg/ml using DMSO. Thereafter, stock solution (100 μl) of each compound was transferred into the first row of the micro-titer plate. Serial dilutions were performed, starting from 0.25 μg/ml to 128 μg/ml. Culture mediums (100 μl) were added to all wells of the micro-titer plate. Negative controls were included to ensure that the relevant broth was not contaminated. Erythromycin served as a positive control. The final MIC was determined to be the lowest concentration of compound inhibiting color change after incubation at 37°C for 48 h.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

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