Chromones and Tannins from the Fruit of *Euscaphis japonica* var. *wupingensis*

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Euscaphis japonica var. *wupingensis* is a variety of *E. japonica* in the family Staphyleaceae. The biological characteristics of *E. japonica* var. *wupingensis* have been well described, but few studies have been conducted on its constituents. After repeated separation using column chromatography and preparative high performance liquid chromatography (HPLC), ten compounds were isolated from the methanol extract of fruit of *E. japonica* var. *wupingensis*. The structure of the components was characterized on the basis of spectroscopic data, including nuclear magnetic resonance (NMR), and mass spectra (MS). These compounds included five chromone derivatives: isobiflorin (**1**), biflorin (**2**), 2-methyl-5,7-dihydroxy-chromone-7-*O*-ß-D-glucopyranoside (**5**), 5,7-dihydroxy-2-methyl-4H-chromen-4-one (**6**), and quercetin-3-*O*-D-arabinoside (**7**), and four tannins: eugeniin (**3**), ellagic acid (**8**), 3, 3'-di-*O*-methylellagic acid 4-(5''-acetyl) - α -L-arabinofuranoside (**9**), 3,3'-di-*O*-methylellagic acid (**10**), and methyl 3,4,5-trihydroxybenzoate (**4**). The antioxidant activity of the isolated compounds were tested. Ellagic acid (**8**) exhibited potent 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity with an SC_{50} value of 4.05 μM.

Keywords: Euscaphis japonica var. *wupingensis; Tannins; Chromones; DPPH scavenging activity*

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INTRODUCTION

Euscaphis japonica var. *wupingensis* (Cai *et al*. 2018) is a variety in the bladdernut family Staphyleaceae. It is a deciduous shrub or small tree, glabrous, with leaves opposite, 5-7-pinnate, and it is generally found in the forests and mountains of Fujian, China. Its pericarp is yellow, and it can be distinguished easily from *E. japonica*.

E. japonica is a traditional Chinese medicinal material (Luo *et al*. 2012). Its roots, root bark, fruit, and flowers can be used as medicines because of a variety of effects (Li *et al*. 2016, 2018), and the pesticide effects are very different in the plant (Dong *et al.* 2004; Lee *et al*. 2009; Zhang *et al*. 2012). The fruits have the effect of treating headache, dizziness, and cold. The study of plant secondary metabolites is a crucial step in the scientific and rational use of plants (Yu *et al*. 2017). At present, 84 kinds of compounds have been isolated from *Euscaphis* Sieb. et Zucc., and the main active substances are triterpenoids, flavonoids, and phenolic acids (Tekeda *et al*. 2000; Hajime *et al*. 2009; Huang *et al*. 2014; Liang *et al*. 2018).

As a new variety, studies up to this point on *E. japonica* var. *wupingensis* have focused mainly on the morphological and biological characteristics, while its chemical properties have not been reported yet. In this paper, the chemical constituents from the fruits of this new variety were studied, which provides a theoretical basis for its rational utilization and functional development.

EXPERIMENTAL

Materials

Fruits of *E. japonica* var. *wupingensis* were collected from Huangtu hill (Wuping country, Fujian province, China) in October 2017. This site is located at 25°11'30″ N, 116°02'37″ E, altitude 400 to 520 m. The samples were oven-dried at 80 °C for 24 h and crushed before extraction.

The analytically pure solvents used in the extract and open-column separation process, including ethanol, methanol, and acetonitrile, were purchased from China National Pharmaceutical Corporation, Limited. The HPLC grade acetonitrile and methanol were obtained from Tedia (Fairfield, CT, USA). Ascorbic acid (Vc) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from J&K Scientific Co. Ltd. (Beijing, China).

General Equipment

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were taken using TMS as the internal standard on a Bruker Avance III 600 FT NMR spectrometer (Bruker Corporation, Billerica, MA, USA). Chemical shifts were recorded as δ (ppm) values with dimethyl sulfoxide-deuterium (Sigma-Aldrich, St. Louis, MO, USA) as the solvent. Coupling constants (*J*) were recorded in Hz, and multiplicities were abbreviated as follows: s = singlet, d = doublet, t = triplet, br = broad, and m = multiplet.

The ESI-MS analysis was detected on a Thermo Q-Exactive Orbitrap Mass spectrometer (Thermo Fisher Scientific Corporation, Waltham, MA, USA), which was equipped with electrospray ionization source (ESI).

Column chromatography was performed on a Cosmosil 75 C18-OPN Column (75 μm, Nakalai Tesque Co. Ltd., Kyoto, Japan). The preparative HPLC was performed with a Varian binary gradient LC system (Varian Inc. Corporate, Santa Clara, CA, USA) containing two solvent deliver modules (PrepStar 218), a photodiode array detector (ProStar 335), using an the preparative Cosmosil ODS column (250 mm × 20.0 mm i.d., 5 μm, Cosmosil, Nakalai Tesque Co. Ltd., Kyoto, Japan). A Sephadex LH-20 column (GE Healthcare, Sweden) was used for column chromatography with a glass column (120 cm × 1.5 cm inner diameter); methanol was used as the eluent.

Extraction and Isolation

The fruits (100 g) were ultrasonically extracted with methanol for one hour (×3) under room temperature and evaporated until dry before weighing. The methanol extraction yielded 5.8 g of solid material having a brown color. For separation, 5.0 g of extract was chromatographed into a reversed-phase column, resulting in 5 fractions (abbreviated Fr. 1 – Fr. 5) with eluent of CH_3OH-water (20:80, 40:60, 60:40, 80:20, 100, *v/v*). Fr. 2 (2.5 g) was subjected to ODS column (25 g) and gradiently eluted with acetonitrile/H_2O (10% to 25%, 2L) to give 12 subfractions Fr. 2-1 – Fr. 2-12. Compound **1** was isolated from Fr. 2-6 (500 mg), through a preparative reversed-phase HPLC column,

and gradient-eluted with CH_3OH-water from 10:90 to 50:50 (*v/v*) at a flow of 8 mL/min. Fr. 3 (120 mg) was subjected to a Sephadex LH-20 column and eluted with methanol to afford compounds **6** and **7**. Further separation on Fr. 2-7 (800 mg), Fr. 2-9 (300 mg), and Fr. 2-12 (400 mg) by preparative HPLC resulted in the isolation of compounds **2**, **3**, and **5**, respectively. A preparative reversed-phase HPLC isocratic gradient eluted with CH_3OH-H_2O (0 – 60 min; 5% – 100 % CH_3OH, *v/v*) at a flow of 8 mL/min was used to isolate compounds **8**, **9**, and **10** from Fr. 4. The isolation scheme of these compounds is shown in Fig. 1.

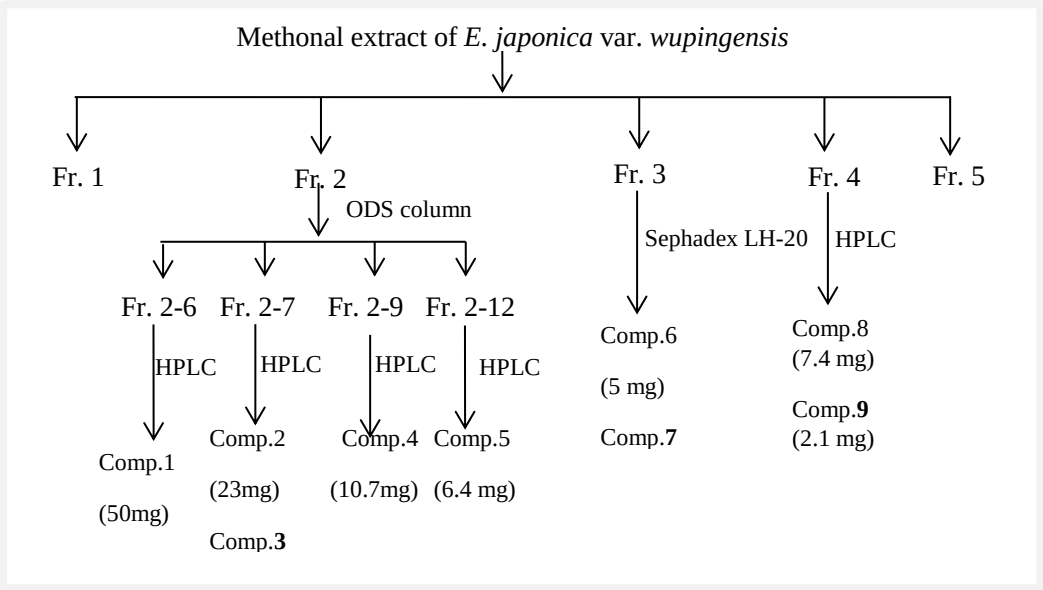

Fig. 1. Diagram of the extract and isolation of *E. japonica* var. *wupingensis*

DPPH Radical Scavenging Activity

The compound solutions of 50, 25, 12.5, 6.25, and 3.125 μM were prepared with anhydrous ethanol, using vitamin C (Vc) as a positive control. DPPH radical scavenging activities of the isolated compounds were investigated according to the reported literature (Qiu *et al.* 2002). Briefly, a solution of the test compound in EtOH (0.1 mL), and 80 mM DPPH radical in EtOH (0.1 mL) was incubated at room temperature for 30 min. Reduction of the DPPH radical was measured at 517 nm. Measurements were performed in duplicate, and the concentration required for a 50% reduction (50% scavenging concentration, SC_{50}) of 40 mM DPPH radical was determined graphically, as shown in Table 2.

*Isobiflorin, $C_{16}H_{18}O_9$ (**1**)*

Compound **1** was obtained as colorless needles. ESI-MS: *m/z* 353 $[M-H]^-$. The ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 13.03 (1H, br.s, 5-OH), 6.25 (1H, s, H-6), 6.20 (1H, s, H-3), 4.63 (1H, d, J = 9.5 Hz, H-1'), 2.35 (3H, s, 2-CH_3); The ^{13}C-NMR (DMSO-d_6, 151 MHz) spectral data are listed in Table 1.

*Biflorin, $C_{16}H_{18}O_9$ (**2**)*

Compound **2** was isolated as colorless needles. ESI-MS: *m/z* 353 $[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 13.40 (1H, s, 5-OH), 6.39 (1H, s, H-8), 6.17 (1H, s, H-3), 4.56 (1H, d, J = 9.7 Hz, H-1'), 2.35 (3H, s, 2-CH_3); The ^{13}C-NMR (DMSO-d_6, 151 MHz) spectral data are shown in Table 1.

*Eugeniin, $C_{41}H_{30}O_{26}$ (**3**)*

Compound **3** was obtained as a puce amorphous powder. ESI-MS: *m/z* 939 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 6.88 (2H, s, galloyl-H), 6.87 (2H, s, galloyl-H), 6.81 (2H, s, galloyl-H), 6.76 (1H, s, HHDP-H), 6.28 (1H, s, HHDP-H), 5.68 (1H, t, J = 9.9 Hz, H-1), 5.53 (1H, t, *J* = 9.7 Hz, H-3), 5.37 (1H, br. d, *J* = 8.5 Hz, H-2), 5.12 (1H, dt, *J* = 14.3, 7.0 Hz, H-6), 4.55 (1H, br. dd, *J* = 9.9, 7.2 Hz, H-5), 3.83 (1H, br. d, *J* = 13.0 Hz, H-6). The ^{13}C-NMR (DMSO-d_6, 151 MHz) spectral data were summarized as follows: δ 168.1 (C-7’’’’), 168.1 (C-7’’’’’), 167.4 (C-7’), 165.9 (C-7’’), 165.7 (C-7’’’), 146.1 (C-3, 5’), 146.0 (C-3’’, 5’’), 145.9 (C-3’’’, 5’’’), 145.7 (C-4’’’’, 6’’’’), 145.7 (C-4’’’’’, 6’’’’’), 139.4 (C-4’), 139.2 (C-4’’), 139.2 (C-4’’’), 135.9 (C-5’’’’), 135.7 (C-5’’’’’), 124.8 (C-2’’’’), 124.2 (C-2’’’’’), 119.2 (C-1’), 118.9 (C-1’’), 118.8 (C-1’’’), 115.8 (C-3’’’’), 115.7 (C-3’’’’’), 109.3 (C-2’, 2’’, 2’’’), 109.2 (C-6’, 6’’, 6’’’), 106.2 (C-1’’’’), 105.8 (C-1’’’’’), 98.8 (C-1), 73.5 (C-3), 72.7 (C-5), 72.1 (C-2), 70.6 (C-4), 63.0 (C-6).

*Methyl 3,4,5-trihydroxybenzoate , $C_8H_8O_5$ (**4**)*

Compound **4** was obtained as a colorless needles. ESI-MS: *m/z* 185 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 6.94 (2H, s, H-2, 6), 3.74 (3H, s, CH_3). ^{13}C-NMR (DMSO-d_6, 151 MHz): δ_C 166.8 (CO), 146.0 (C-3, 5), 138.9 (C-4), 119.7 (C-1), 108.9 (C-2, 6), 52.1 (CH_3).

*2-Methyl-5,7-dihydroxy-chromone-7-O-ß-D-glucopyranoside, $C_{16}H_{18}O_9$ (**5**)*

Compound **5** was a light yellow powder. ESI-MS: *m/z* 355 $[M+H]^+$, 353$[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 12.83 (1H, br s, 5-OH), 6.66 (1H, d, *J* = 2.0 Hz, H-8), 6.42 (1H, d, *J* = 2.2 Hz, H-6), 6.26 (1H, s, H-3), 5.04 (1H, d, *J* = 7.5 Hz, H-1’), 2.39 (3H, s, 2-CH_3); The ^{13}C-NMR (DMSO-d_6, 151 MHz) spectral data are shown in Table 1.

*5,7-Dihydroxy-2-methyl-4H-chromen-4-one, $C_{10}H_8O_4$ (**6**)*

Compound **6** was a light yellow crystal. ESI-MS: *m/z* 193$[M+H]^+$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 6.33 (1H, d, *J* = 2.0 Hz, H-3), 6.17 (2H, s, H-6, 8), 2.35 (3H, s, 2-CH_3). The ^{13}C-NMR (DMSO-d_6, 151 MHz) spectral data are shown in Table 1.

*Quercetin-3-O-ß-D-arabinoside, $C_{20}H_{18}O_{11}$ (**7**)*

Compound **7** was a yellow powder. ESI-MS: *m/z* 435 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 12.63 (1H, s, 5-OH), 7.65 (1H, dd, *J* = 2.0, 8.4Hz, H-6’), 7.53 (1H, d, *J* = 2.2 Hz, H-2’), 6.85 (1H, d, *J* = 8.4 Hz, H-5’), 6.39 (1H, d, *J* = 2.0 Hz, H-8), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 5.26 (1H, d, *J* = 5.3 Hz, H-1"); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ_C 177.9 (C-4), 164.9 (C-7), 161.6 (C-5), 156.7 (C-2), 156.7 (C-9), 149.1 (C-4’), 145.5 (C-3’, 5’), 134.1 (C-3), 122.4 (C-2’, 6’), 121.3 (C-1’), 116.2 (C-3’, 5’), 115.9 (C-2’, 6’), 104.3 (C-10), 101.9 (C-1’’), 99.2 (C-6), 94.0 (C-8), 72.1 (C-3’’), 71.2 (C-2’’), 66.6 (C-4’’), 64.8 (C-5’’).

*Ellagic acid, $C_{14}H_6O_8$ (**8**)*

Compound **8** was a grey powder. ESI-MS: *m/z* 301 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 7.45 (2H, s, H-5,5'); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ_C 159.9 (C-7,7'), 148.9 (C-4,4'), 136.8 (C-3,3'), 130.1 (C-2,2'), 113.1 (C-6,6'), 110.1 (C-5,5'), 106.7 (C-1,1').

*3,3'-Di-O-methylellagic acid 4-(5''-acetyl)-a-L-arabinofuranoside, $C_{23}H_{20}O_{13}$ (**9**)*

Compound **9** was obtained as white amorphous powder. ESI-MS: *m/z* 503 $[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 7.73 (1H, s, H-5), 7.52 (1H, s, H-5'), 5.68 (1H, d, H-1''), 4.08 (3H, s, 3-OCH_3), 4.05 (3H, s, 3'-OCH_3), 3.87 (1H, dd, J=6 and 4 Hz, H-3''), 2.04 (3H, s, 6''-CH_3); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ_C 170.7 (C-6''), 159.1 (C-7), 142.5 (C-3, 2), 142.2 (C-3', 2'), 113.3 (C-5, 5'), 112.4 (C-6, 6'), 108.0 (C-1''), 83.1 (C-4''), 82.2 (C-2''), 77.4 (C-3''), 64.1 (C-5''), 62.0 (3-OCH_3), 61.3 (3'-OCH_3), 21.1 (6''-CH_3)。

*3,3'-Di-O-methylellagic acid ,$C_{16}H_{10}O_8$ (**10**)*

Compound **10** was obtained as yellow powder. ESI-MS: *m/z* 329 $[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ_H 7.51 (2H, s, H-5, 5'), 4.04 (6H, s, 3, 3'-OCH_3); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ_C 159.0 (C-7,7'), 152.9 (C-4,4'), 140.8 (C-3,3'), 141.7 (C-2,2'), 112.6 (C-6, 6'), 112.0 (C-5, 5', 1, 1'), 61.4 (3,3'-OCH_3).

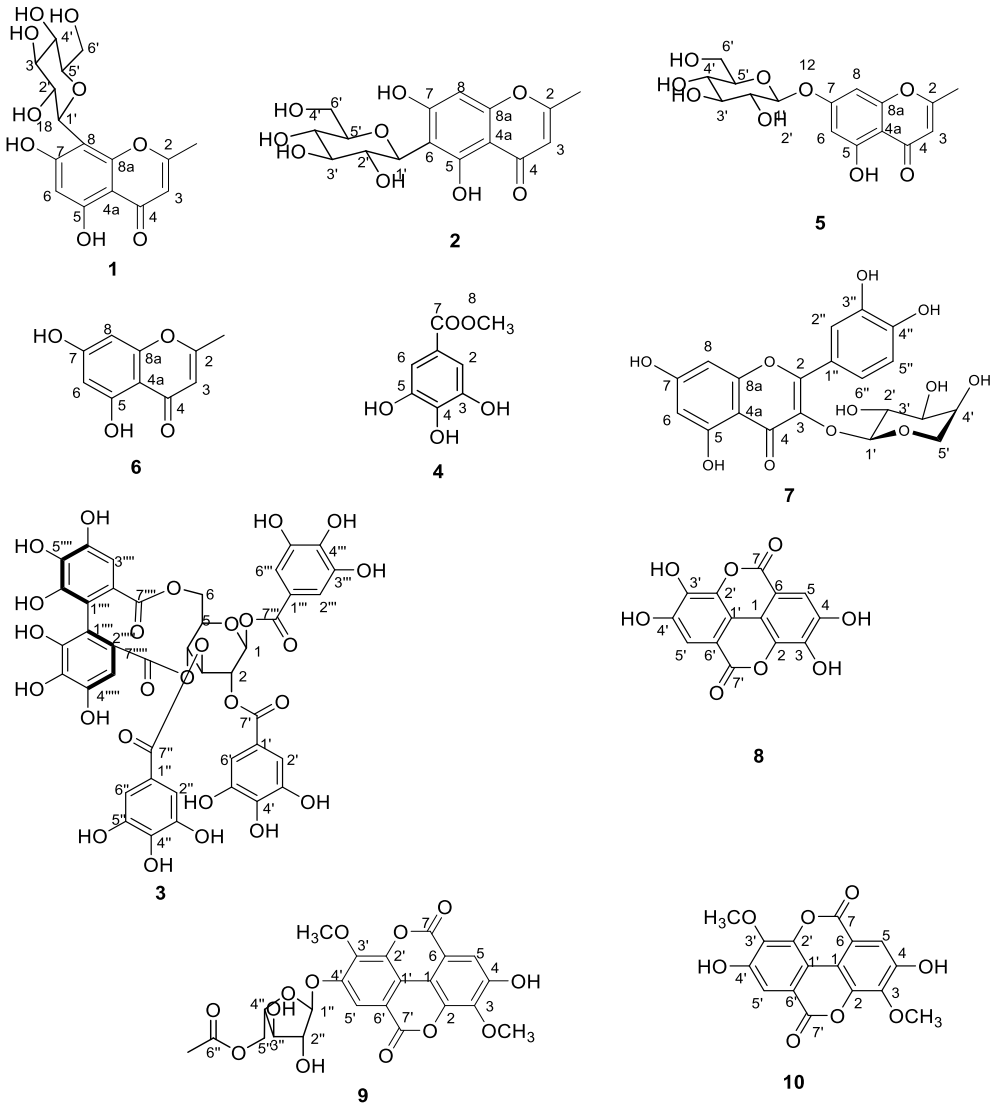

Fig. 2. Chromones and tannins from the fruit of *Euscaphis japonica* var. *wupingensis*

Table 1. ^{13}C-NMR (DMSO-d_6, 151 MHz) data of compounds **1**, **2**, **5**, and **6**

Number	**1**	**2**	**5**	**6**
2	167.9	167.9	161.6	168.1
3	108.4	108.1	108.8	108.4
4	182.4	182.6	182.5	182.2
5	161.2	160.9	157.9	164.6
6	109.2	98.7	100.3	99.2
7	163.9	163.2	168.9	162.0
8	93.9	104.95	95.0	94.2
9	157.2	156.87	163.4	158.3
10	103.5	104.1	105.5	103.9
2-CH_3	20.3	20.3	20.5	20.4
1'	73.4	73.58	99.9	
2'	71.1	71.4	73.5	
3'	79.4	79	77.6	
4'	70.6	70.9	70.0	
5'	82.1	81.8	76.8	
6'	62.0	61.9	61.1	

RESULTS AND DISCUSSION

Phytochemistry of the Fruit of *E. japonica* var. *wupingensis*

Isolation of the fruit of *E. japonica* var. *wupingensis* yielded five chromones, four tannins, and methyl 3,4,5-trihydroxybenzoate. Their chemical structures were determined by their ESI-MS, ^{1}H-NMR, and ^{13}C-NMR spectra. Two chromones, 2-methyl-5,7-dihydroxy-chromone-7-*O*-ß-D-glucopyranoside (**5**) and quercetin-3-*O*-ß-D-arabinoside (**7**), and two tannin, eugeniin (**3**) and 3,3'-di-O-methylellagic acid 4-(5"-acetyl)-α-L-arabinofuranoside (**9**), were isolated from the family of Staphyleaceae for the first time. The structures of the isolated compounds are shown in Fig. 2.

Compound **3** was isolated as a puce amorphous powder. The dark color, high polarity, as well as the high molecular weight, induced by the ESI-MS qusio-molecular ion peak at *m/z* 939 $[M+H]^+$, indicated that compound **3** is a tannin derivate. By comparing it ^{13}C-NMR data with those reported in literature (Lian *et al.* 2017), the structure of **3** was determined as eugeniin.

Compound **5** was obtained as a light yellow powder. In low field of ^{1}H-NMR (DMSO-d_6, 600 MHz) of **5**, the signal of δ_H 12.83 (1H, br.s) belongs to the C-5 hydroxyl, forming intramolecular hydrogen bond with C=O. A pair of AM spin system aromatic proton signals were found at δ_H 6.66 (1H, d, J = 2.0 Hz) and 6.42 (1H, d, J = 2.2 Hz), belonging to the H-8 and H-6 of the chromone's ring. The signal at δ_H 6.26 (1H, s) was assigned to H-3. The high field of ^{1}H-NMR shown the presence of methyl at δ_H 2.39 (3H, s, 2-CH_3). The ^{13}C-NMR revealed an oxy-glycosylated glucose, based on the signals at δ_c 95.0 (C-1'), 77.6 (C-3'), 76.8 (C-5'), 73.5 (C-2'), 70.0 (C-4') and 61.1 (C-6'). According to the presence of 5-OH and absence of 7-OH ^{1}H-NMR signal, the glucosyl was considered to be attached to C-7. Thus, compound **5** was 2-mehyl-5,7-dihydroxychromanone glucoside. The above data is consistent with the reported NMR data (Yang *et al.* 2006). Therefore, the compound was identified as 5,7-dihydroxy-2-methylchromanone-7-O-β-D-glucoside.

Compound **7** was isolated as yellow powder. Both the hydrochloric acid-magnesium powder (HCl-Mg) reaction and the Molish reaction were positive, indicating that the **7** is a flavonoid glycoside. In the ^{1}H-NMR spectrum, the signal of 5-OH was shown at δ_H 12.63 (1H, s, 5-OH). The AMX spin system signals at δ_H 7.65 (1H, d, J = 2.0 Hz), 7.64 (1H, d, J = 2.2 Hz), 7.53 (1H, d, J = 2.2 Hz) suggests the 3',4'-disubstituted B flavonoid ring; The meta-coupled benzene ring signals at δ_H 6.39 (1H, d, J = 2.0 Hz) and 6.21 (1H, d, J = 2.0 Hz) belonged to H-8 and H-6 in the A ring, respectively. The signal at δ_H 5.26 (1H, d, J = 5.3 Hz, H-1") was attributed to a terminal proton of the sugar. By comparing the ^{13}C-NMR data with those in the literature (Jiang *et al*. 2009), compound **7** was identified as quercetin 3-O-α-L-arabinopyranoside.

Compound **9** was obtained as white amorphous powder. The ^{1}H-NMR spectrum showed 9 proton signals, including two benzene ring signals at δ_H 7.73, 7.52; two methoxy signals at δ_H 4.08, 4.05; an acetyl signal at δ_H 2.04, and a set of glycoside proton signal ranging from δ_H 5.68 to 3.87. The ^{13}C-NMR shows the unsaturated lactone signal at δ 159.1; an arabinofuranose signal δc 108.1, 83.1, 82.2, 77.4, and 64.1. ^{1}H-NMR and ^{13}C-NMR indicate that the compound is a steroidal compound linked to a glycoside; the ^{13}C-NMR signals at δ_C 62.0 and δ 61.3 suggest methoxy groups. The NMR data were similar to the reported results (Tanaka *et al*. 2001) (Jules *et al*. 2007). Therefore, compound **9** is identified as 3,3'-di-*O*-methylellagic acid 4-(5''-acetyl)-α-L-arabinofuranoside.

By comparing NMR data with those reported in literature, other known compounds were identified as: isobiflorin (**1**) (Takashi *et al*. 1993) (Lee *et al*. 2016), biflorin (**2**) (Li *et al*. 2009) , methyl 3,4,5-trihydroxybenzoate (**4**) (Yang *et al*. 1998), 5, 7-dihydroxy-2-methyl-4*H*-chromen-4-one (**6**) (Cao *et al*. 2005), ellagic acid (**8**) (Xiao *et al*. 2017), and 3,3'-di-*O*-methylellagic acid (**10**) (Nono, *et al*. 2014).

Antioxidant Activity

Table 2 shows that the chromones and quinones in the extract of *E. japonica* var. *wupingensis* have a good scavenging effect on DPPH free radicals. Higher concentrations resulted in a stronger ability to scavenge free radicals. Ellagic acid (**8**, SC_{50} of 4.05 μM) has the strongest effect on scavenging DPPH freeness, which is twice as high as that of vitamin C (SC_{50} of 9.85 μM).

Table 2. Antioxidant Activity of Compounds Isolated from *E. japonica* var. *wupingensis*

Compounds	SC_{50} (μM)
Isobiflorin (**1**)	14.20
biflorin (**2**)	22.06
2-methyl-5,7-dihydroxy-chromone-7-O-ß-D-glucopyranoside (**5**)	19.48
Quercetin-3-O-β-D-arabinoside (**7**)	14.88
Ellagic acid (**8**)	4.05
3,3'-di-O-methylellagic acid (**10**)	29.57
Vc	9.85

CONCLUSIONS

1. *E. japonica* var. *wupingensis* was extracted with methanol, and the extract components were isolated using column chromatography. Five chromones, four tannins, and methyl 3,4,5-trihydroxybenzoate were identified as isobiflorin (**1**), biflorin (**2**), 2-methyl-5,7-

dihydroxy-chromone-7-O-*β*-D-glucopyranoside (**5**), 5,7-dihydroxy-2-methyl-4H-chromen-4-one (**6**), quercetin-3-O-D-arabinoside (**7**), eugeniin (**3**), ellagic acid (**8**), 3,3'-di-*O*-methylellagic acid 4-(5"-acetyl)-α-L-arabinofuranoside (**9**), and 3,3'-di-*O*-methylellagic acid (**10**).

2. For the first time, the chemical composition isolated from the *Euscaphis japonica* var. *wupingensis* is reported. Two chromones (**5** and **7**) and two tannins (**3** and **9**) were first isolated from the genus of *Euscaphis.*
3. Ellagic acid (**8**) has stronger antioxidant activity than vitamin C, and the specific efficacy needs to be further studied.

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