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Two new flavonoids from *Artemisia argyi* with their anticoagulation activities

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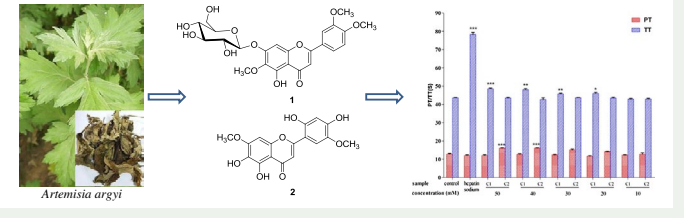
ABSTRACT

A new flavone glycoside, eupatilin 7-*O*-*β*-D-glucopyranoside (**1**) and a new flavone, 5,6,2′,4′-tetrahydroxy-7,5′-dimethoxyflavone (**2**), were isolated from *Artemisia argyi*. Their structures were unambiguously elucidated by extensive spectroscopic analysis. Both flavonoids were evaluated for *in vitro* anticoagulation activities. Compound **1** significantly extended thrombin time. Compound **2** had obvious effect in increasing prothrombin time.



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KEYWORDS
Artemisia argyi; Compositae; flavone; anticoagulation activity

1. Introduction

Thromboembolic diseases continue to be the leading cause of death throughout the world (Wu et al. 2015). As is well-known, thrombosis is closely related to activating platelet adhesion, aggregation, secretion functions and activation of intrinsic and extrinsic coagulation systems, which cause blood coagulation and fibrin formation (Xin et al. 2011). Therefore, anticoagulants play a pivotal role in the prevention and treatment of thrombotic disorders (Xu et al. 2016).

The genus *Artemisia* (Compositae) consists of about 500 species and subspecies mainly distributed in the temperate zones of North America, Europe and Asia. Sesquiterpenoids and flavonoids were the most abundant secondary metabolites in *Artemisia* species (Ornano et al. 2016; Allison et al. 2017; Peron et al. 2017). *Artemisia argyi* is one of the most widely distributed *Artemisia* species in China. The dried leaves of *A. argyi*, known as 'Aiye', was firstly recorded as medicine in 'Ming Yi Bie Lu' in the Liang Dynasty (A.D. 502–560). *A. argyi* leaf

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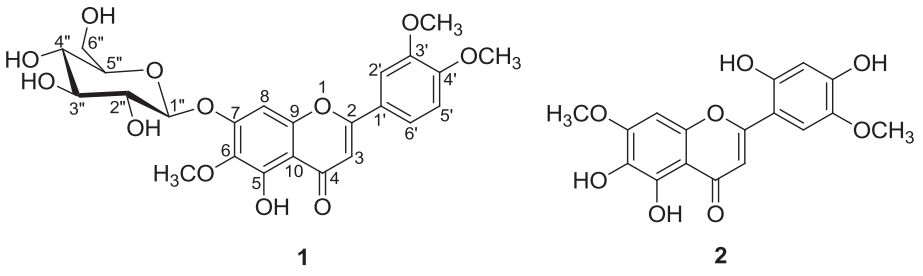

Figure 1. Structures of compounds **1** and **2**.

from Qichun, Hubei province (Qi Zhou, in ancient times), is traditionally regarded as the genuine Aiye (Lv et al. 2013; Ge et al. 2016). It has been used as a traditional Chinese herb for moxibustion and for curing homeostasis, menstruation-related symptoms, eczema, diarrhoea and tuberculosis (Wang et al. 2013). Phytochemical researches on this species have led to the isolation of sesquiterpenoids, triterpenoids, steroids, coumarins and flavonoids, showing anti-inflammatory, immunomodulatory, antitumour, antimutagen and antimicrobial activities (Nakasugi et al. 2000; Lee et al. 2002, 2005; Liu et al. 2006; Ji et al. 2010; Wang et al. 2013; Zhang et al. 2013; Wang et al. 2014; Zeng et al. 2014). Particularly, the essential oil from *A. argyi* has obvious effect on improving haemorheology in the rat acute blood stasis model (Ge et al. 2016). 5,7-Dihydroxy-6,3′,4′-trimethoxy-flavone from the leaves of *A. argyi* exhibited antiplatelet aggregation activity *in vitro* (Zhong & Cui 1992). In further research on the anticoagulation-active constituents of *A. argyi*, two new flavonoids (**1** and **2**) (Figure 1) were isolated and characterised, respectively. Herein, the isolation and structural elucidation of the new compounds and their anticoagulation activities are described.

2. Results and discussion

2.1. Structure elucidation of two new flavonoids

Compound **1** was isolated as a yellowish amorphous powder. Its molecular formula was established as $C_{24}H_{26}O_{12}$ on the basis of the HRESI-MS ion at *m/z* 529.1313 $[M + Na]^+$ (Calcd for $C_{24}H_{26}O_{12}Na$, 529.1322), indicating 12° of unsaturation. The UV maximum absorptions at 275 and 338 nm of **1** were characteristic of a flavone skeleton (Ngankeu Pagning et al. 2016; Xiao et al. 2016), which was corroborated by the absorption bands at 3380 (hydroxyl), 1662 (conjugated carbonyl) and 1614 and 1465 (aromatic ring) cm^{-1} in its IR spectrum. In the ^{13}C NMR and HSQC spectra, 24 carbon signals were exhibited, corresponding to a flavone skeleton with one glucosyl unit and three methoxy groups.

In the ^1H NMR spectrum of **1**, the appearance of a typical ABX system at δ_H 7.71 (1H, dd, J = 8.6, 1.4 Hz, H-6′), 7.59 (1H, d, J = 1.4 Hz, H-2′) and 7.15 (1H, d, J = 8.6 Hz, H-5′) corresponded to a 1′,3′,4′-trisubstituted phenyl moiety of ring B, which was further confirmed through HMBC correlations (Figure S11) from H-2′ to C-2, C-3′, C-4′ and C-6′, from H-5′ to C-1′, C-3′ and C-4′ and from H-6′ to C-2, C-2′ and C-4′. In addition, the other two aromatic protons were observed on this spectrum, assignable to H-8 (δ_H 7.08, 1H, s) and H-3 (δ_H 7.07, 1H, s) by analyses of the HMBC correlations from H-8 to C-6, C-7, C-9 and C-10 and from H-3 to C-2, C-4, C-10 and C-1′. The ^1H NMR spectrum of **1** also exhibited a hydrogen-bonded hydroxyl proton signal at δ_H 12.92 (1H, s, OH-5) that can be assigned to the OH-5 group, as further

confirmed on the basis of the correlations between δ_H 12.92 (OH-5) and δ_C 152.3 (C-5), 132.6 (C-6) and 105.8 (C-10) in the HMBC spectrum. Moreover, three methoxy singlets at δ_H 3.78, 3.86 and 3.88 (each 3H, s) were observed. The locations of the three methoxy groups at C-6, C-4′ and C-3′ were inferred from the HMBC correlations between the methoxy protons and C-6/C-4′/C-3′, respectively. This ^{1}H-NMR spectrum also showed the presence of glycosyl unit with the anomeric proton at δ_H 5.13 (1H, overlapped, H-1″), a methylene group at δ_H 3.74 (1H, m, H_a-6″) and 3.50 (1H, overlapped, H_b-6″). Other protons (H-2″-H-5″) of the sugar moiety were observed between 3.19 and 3.47 ppm. The signals at δ_H 5.48 (1H, d, J = 4.9 Hz), 5.19 (1H, d, J = 4.3 Hz), 5.13 (1H, overlapped) and 4.68 (1H, t, J = 5.2 Hz) were assigned to OH-2″, OH-3″, OH-4″ and OH-6″, respectively, as supported by the HMBC correlations of OH-2″/C-2″, C-1″, C-3″; OH-3″/C-3″, C-2″, C-4″; OH-4″/C-4″, C-3″, C-5″ and OH-6″/C-6″, C-5″.

Acid hydrolysis of **1** followed by TLC analysis and optical rotation of the hydrolysate and direct comparison with authentic sugars further indicated the presence of a D-glucose unit. Simultaneously, the anomeric configuration of glucosyl moiety was assigned as β on the basis of the chemical shift of its anomeric carbon (δ_C 100.4). The glucosyl residue was located at C-7 of the aglycon flavone by the appearance of HMBC cross peak of H-1″ (δ_H 5.13) with C-7 (δ_C 156.6). Therefore, the structure of compound **1** was elucidated as 5-hydroxy-6,3′,4′-trimethoxyflavone 7-*O*-β-D-glucopyranoside, named eupatilin 7-*O*-β-D-glucopyranoside.

Compound **2** was obtained as a yellow powder. The molecular formula of compound **2** was determined to be $C_{17}H_{14}O_8$ by HRESI-MS, consistent with the molecular ion peak at *m/z* 369.0584 $[M + Na]^+$ (Calcd for $C_{17}H_{14}O_8Na$, 369.0586). The UV spectrum of **2** showed absorption bands at λ_{max} 283 and 367 nm, which were characteristic of a flavone skeleton. The IR signals at 3375, 1663, 1603 and 1428 cm^{-1} suggested the presence of hydroxyl, carbonyl and aromatic functionalities. The ^{13}C NMR and HSQC spectra exhibited 17 carbon signals, comprising a flavone skeleton with two methoxy groups.

The ^{1}H NMR spectrum of **2** exhibited a characteristic proton signal at δ_H 12.78 corresponding to a chelated hydroxyl group at C-5, which was confirmed by the HMBC correlations (Figure S11) from OH-5 to C-5, C-6 and C-10. Moreover, four aromatic protons were observed on this spectrum, assignable to H-3 (δ_H 7.08, 1H, s), H-8 (δ_H 6.94, 1H, s), H-3′ (δ_H 6.57, 1H, s) and H-6′ (δ_H 7.45, 1H, s) by analyses of the HMBC correlations from H-3 to C-2, C-4, C-10 and C-1′, from H-8 to C-6, C-7, C-9 and C-10, from H-3′ to C-1′, C-2′, C-4′ and C-5′ and from H-6′ to C-2, C-1′, C-2′, C-4′ and C-5′. Additionally, the other ^{1}H NMR spectral information include two methoxy groups (δ_H 3.93 and 3.82), three hydroxyl groups at δ_H 8.65 (1H, br s, OH-6), 10.42 (1H, br s, OH-2′) and 10.03 (1H, br s, OH-4′). The long-range HMBC correlations from one methoxy group at δ_H 3.93 (OCH_3-7) to C-7 (δ_C 154.2) and from another one at δ_H 3.82 (OCH_3-5′) to C-5′ (δ_C 141.6) revealed that they were located at C-7 and C-5′, respectively. Furthermore, the HMBC correlations from OH-6 to C-6/C-5/C-7, from OH-2′ to C-2′/C-1′/C-3′ and from OH-4′ to C-4′/C-3′/C-5′ confirmed that the three hydroxyl groups were attached to C-6, C-2′ and C-4′, respectively. Therefore, the structure of compound **2** was determined as 5,6,2′,4′-tetrahydroxy-7,5′-dimethoxyflavone.

2.2. Anticoagulation activities

Compounds **1** and **2** were evaluated for their *in vitro* anticoagulation activities on the thrombin time (TT) and prothrombin time (PT). As shown in Figure 2, compound **1** remarkably prolonged the TT with a good dose–effect relationship at the concentration from 20 to

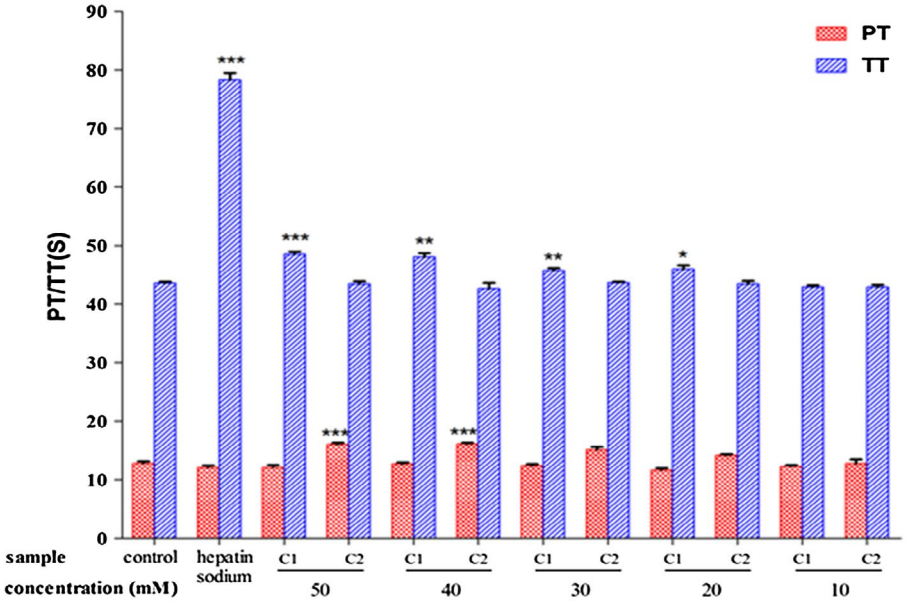

Figure 2. Anticoagulant activities of compound **1** (C1) and compound **2** (C2) at different concentrations with respect to TT and PT *in vitro*. The activity is expressed as the clotting time in seconds (s). DMSO was used as the control. Heparin sodium (0.5 μM) was used as the positive control. Values are expressed as means ± SD ($n = 3$) and $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the control group.

50 mM, compared with the DMSO control. However, **2** had significant effect in increasing PT at the concentration from 40 to 50 mM.

PT value reflects the activity of the extrinsic pathways of coagulation. TT is related to the third coagulation phase in plasma, and the prolongation of TT indicates inhibition of thrombin-mediated fibrin formation (Dang et al. 2015; Zhang et al. 2016). In this study, the results implied that compounds **1** and **2** exhibited potential anticoagulant effects by acting on different coagulation pathways.

3. Experimental

3.1. General experimental procedure

NMR spectra were recorded on a Bruker AVANCE-III HD 400 spectrometer. HRESI-MS was carried out in the positive ion mode with a Bruker microOTOF-Q III spectrometer. Optical rotations were measured on a polAAr 31 polarimeter. UV spectra were run on a Shimadzu UV-2700 spectrophotometer. IR spectra were recorded on a PerkinElmer Spectrum Two spectrophotometer in a KBr disc. Column chromatography (CC) was performed on silica gel (200–300 mesh, Yantai Institute of Chemical Technology, Yantai, P. R. China), Sephadex LH-20 gel (GE Healthcare Amersham Biosciences, Uppsala, Sweden) and Diaion HP-20 resin (Mitsubishi Chemical Corporation, Tokyo, Japan). TLC analysis was done on pre-coated silica gel GF_{254} plates (10–40 μm, Yantai Institute of Chemical Technology, Yantai, P. R. China). The anticoagulant activity was measured on platelet aggregation and blood coagulation factors analyser (LG-PABER-I, Steellex, Beijing, P. R. China). TT (No. 121167) and PT (No. 105A278) kits

were obtained from Sun Biochemical Co. Ltd. (Shanghai, P. R. China). Heparin sodium was purchased from Macklin Biochemical Co. Ltd. (Shanghai, P. R. China).

3.2. Material

The leaves of *A. argyi* were collected in May 2014 from Qichun, Hubei province, People's Republic of China. The sample was identified by Prof. Sui-Qing Chen from School of Pharmacy, Henan University of Traditional Chinese Medicine. A voucher specimen (2014-Y0524) was deposited at the herbarium of School of Pharmacy, Xinxiang Medical University.

3.3. Extraction and isolation

The air-dried and milled leaves of *A. argyi* (12.5 kg) were percolated with 95% EtOH (125 L) at room temperature. The filtrate was evaporated in vacuum to obtain a residue (2.64 kg), which was suspended in H_2O (5 L) and extracted with petroleum ether (6 × 1 L), CH_2Cl_2 (6 × 1 L) and EtOAc (6 × 1 L), respectively. The EtOAc extract (525 g) was subjected to Diaion HP-20 CC, eluted with EtOH/H_2O (gradient 80% and 90%) to obtain 80% and 90% EtOH fractions. About 80% EtOH fraction (385 g) was chromatographed over silica gel CC eluting with petroleum ether–acetone (gradient 8:1, 4:1, 2:1 and 1:1) and petroleum ether–acetone–MeOH (1:1:0.5) to yield 14 fractions 1–14. Fraction 14 (249.3 g) was subjected to Diaion HP-20 column, eluting with EtOH/H_2O (gradient 40, 60, 80 and 90%) to obtain subfractions 14.1–14.4. Subfraction 14.1 (40% EtOH fraction, 197.9 g) was separated by CC over silica gel eluted with $CHCl_3$–MeOH (30:1, 20:1, 15:1, 10:1, 5:1, 2:1, 1:1 and 0:1) to yield 13 subfractions, 14.1.1–14.1.13. Compound **1** (17.5 mg) was afforded from subfraction 14.1.11 by repeating CC on silica gel. Subfraction 14.1.8 was repeatedly chromatographed over silica gel and purified by Sephadex LH-20 CC (CH_2Cl_2/MeOH, 1:1) to afford compound **2** (4.6 mg).

3.3.1. Eupatilin 7-O-β-D-glucopyranoside (1)

Yellowish amorphous powder. $[\alpha]_D^{20}$ − 64 (*c* 0.05, MeOH). UV (MeOH) λ_{max} (log ε): 214 (4.99), 241 (sh) (4.56), 250 (4.57), 275 (4.62), 338 (4.77). IR (KBr) ν_{max} cm^{-1}: 3380, 2936, 1662, 1614, 1465, 1367, 1268, 1072, 814, 686. HRESI-MS: *m/z* 529.1313 $[M + Na]^+$ (Calcd for $C_{24}H_{26}O_{12}Na$, 529.1322). ^1H NMR (DMSO-d_6, 400 MHz): δ_H 7.07 (1H, s, H-3), 7.08 (1H, s, H-8), 7.59 (1H, d, *J* = 1.4 Hz, H-2′), 7.15 (H, d, *J* = 8.6 Hz, H-5′), 7.71 (H, dd, *J* = 8.6, 1.4 Hz, H-6′), 5.13 (1H, overlapped, H-1″), 3.35 (1H, overlapped, H-2″), 3.30 (1H, overlapped, H-3″), 3.19 (1H, m, H-4″), 3.47 (1H, overlapped, H-5″), 3.74 (1H, m, H_a-6″), 3.50 (1H, overlapped, H_b-6″), 3.78 (3H, s, OCH_3-6), 3.88 (3H, s, OCH_3-3′), 3.86 (3H, s, OCH_3-4′), 12.92 (1H, s, OH-5), 5.48 (1H, d, *J* = 4.9 Hz, OH-2″), 5.19 (1H, d, *J* = 4.3 Hz, OH-3″), 5.13 (1H, overlapped, OH-4″), 4.68 (1H, t, *J* = 5.2 Hz, OH-6″). ^{13}C NMR (DMSO-d_6, 100 MHz): δ_C 163.9 (C-2), 103.7 (C-3), 182.5 (C-4), 152.3 (C-5), 132.6 (C-6), 156.6 (C-7), 94.7 (C-8), 152.2 (C-9), 105.8 (C-10), 122.8 (C-1′), 109.5 (C-2′), 149.1 (C-3′), 152.5 (C-4′), 117.7 (C-5′), 120.2 (C-6′), 100.4 (C-1″), 73.2 (C-2″), 76.8 (C-3″), 69.7 (C-4″), 77.5 (C-5″), 60.7 (C-6″), 60.3 (OCH_3-6), 55.9 (OCH_3-3′), 55.8 (OCH_3-4′).

3.3.2. 5,6,2′,4′-tetrahydroxy-7,5′-dimethoxyflavone (2)

Yellow amorphous powder. UV (MeOH) λ_{max} (log ε): 211 (4.81), 242 (sh) (4.31), 262 (sh) (4.40), 283(4.47), 367(4.61) nm. IR (KBr) ν_{max} cm^{-1}: 3375, 2925, 1663, 1603, 1428, 1360, 1267, 1203, 1118, 860, 797, 565. HRESI-MS: *m/z* 369.0584 $[M + Na]^+$ (Calcd for $C_{17}H_{14}O_8Na$, 369.0586). ^1H

NMR (DMSO-d_6, 400 MHz): δ_H 7.08 (1H, s, H-3), 6.94 (1H, s, H-8), 6.57 (1H, s, H-3′), 7.45 (H, s, H-6′), 3.93 (3H, s, OC$\underline{H}_3$-7), 3.82 (3H, s, OC$\underline{H}_3$-5′), 12.78 (1H, s, OH-5), 8.65 (1H, br s, OH-6), 10.42 (1H, br s, OH-2′), 10.03 (1H, br s, OH-4′). ^{13}C NMR (DMSO-d_6, 100 MHz): δ_C 161.7 (C-2), 106.7 (C-3), 182.2 (C-4), 146.1 (C-5), 129.7 (C-6), 154.2 (C-7), 91.2 (C-8), 149.6 (C-9), 104.8 (C-10), 107.1 (C-1′), 152.9 (C-2′), 104.4 (C-3′), 151.7 (C-4′), 141.6 (C-5′), 111.9 (C-6′), 56.3 (O$\underline{C}H_3$-7), 56.7 (O$\underline{C}H_3$-5′).

3.4. Acid hydrolysis of compound 1

A solution of **1** (1.9 mg) in MeOH (3.0 mL) was treated with 2.0 M HCl (5.0 mL) and refluxed for 4 h at 100°C. After removal of the solution under vacuum, 5 mL of distilled water were added, and then partitioned with 5 mL of ethyl acetate (EtOAc) thrice. The aqueous layer was neutralised with $NaHCO_3$ and condensed to dryness. The residue was dissolved in 1 mL of distilled water and was analysed by co-TLC using the solvent system, $CHCl_3$–MeOH–H_2O (2:1:0.1). The TLC plate was sprayed with 10% H_2SO_4 for detection. Comparison of the retention factor value (R_f) in the TLC and optical rotation of the sugar in aqueous layer with those of the authentic sugar confirmed that the sugar in **1** was D-glucose (Xiao et al. 2016).

3.5. Anticoagulation activities assay

Male New Zealand white rabbits (Animal Experimental Center of Zhengzhou University, Zhengzhou, China) weighing 2.0 ± 0.2 kg were used. The animal experimental procedures were performed in accordance with the permission of the animal ethical committee of Xinxiang Medical University. The fresh blood samples were acquired from the common carotid artery and mixed with 3.8% sodium citrate (blood/citrate: 9/1, v/v), then the mixture was centrifuged at 3000 rpm for 10 min to obtain plasma (Zhang et al. 2016).

The samples were dissolved in DMSO, and DMSO was used as a control group. Heparin sodium was used as positive control. The LG-PABER-I coagulation analysis instrument was used to estimate TT and PT. TT was measured by incubating 50 µL of plasma with 10 µL of compound for 3 min at 37°C. Then 50 µL of thrombin was added, and the clotting time was recorded. PT was measured by incubating 50 µL of plasma with 10 µL of compound for 5 min at 37°C. Then 100 µL of warmed thromboplastin agent was added, and the clotting time was recorded. All the results were expressed as mean ± standard deviation, three times in four channels. Statistical significance was determined with t-test. The *P*-values of less than 0.05 ($p < 0.05$) were considered significant.

4. Conclusion

Our present work on the leaves of *A. argyi* yielded two new flavonoids (**1** and **2**). Their structures were elucidated on the basis of extensive spectroscopic analysis. Compound **1** significantly extended TT. Compound **2** had obvious effect in increasing PT. The both new compounds exhibited potential anticoagulation activities through different pathways.

Supplementary material

HRMS and NMR spectra of compounds **1** and **2** are available online.

Disclosure statement

No potential conflict of interest was reported by the authors.

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