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Flavonol glycosides from the leaves of *Elaeagnus pungens*

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ABSTRACT

The leaves of *Elaeagnus pungens* were extracted and successively purified by a set chromatographic techniques. The structures of the obtained constituents were elucidated on the basis of spectroscopic methods including 1D/2D NMR and high resolution-MS. One new flavonoid glycosides, kaempferol 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl(1 → 2)]-β-D-galactopyranoside-7-*O*-β-D-glucopyranoside (**1**), along with five known compounds (**2**–**6**) were isolated and identified. The MTT assay *in vitro* showed that the isolated flavonol glycosides have no proliferating activity, suggesting no aggravating impact on the increased airway smooth muscle cells.

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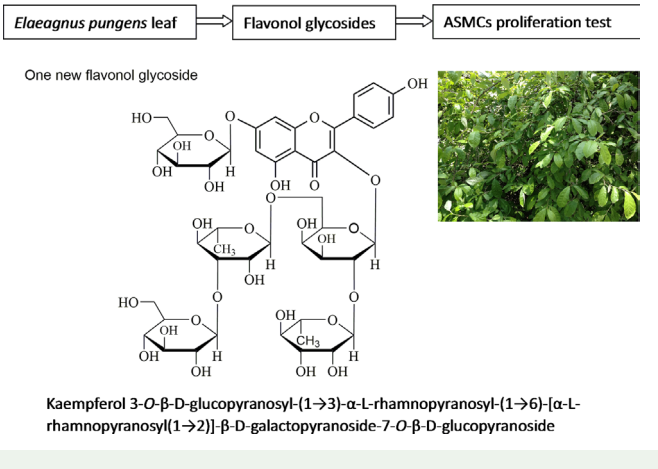

Kaempferol 3-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-[α-L-rhamnopyranosyl(1→2)]-β-D-galactopyranoside-7-*O*-β-D-glucopyranoside

1. Introduction

Family Elaeagnaceae comprises three genera and about eighty species, many of which are widely distributed in the east and southeast of Asia. The genus *Elaeagnus*, encompasses 55 species in China. The leaves of *Elaeagnus pungens* Thunb. have been used as Chinese folk medicine for the treatment of bronchitis, cough, asthma and other diseases (Chinese

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Pharmacopoeia Commission 1977; Fang et al. 2006). Previous phytochemical studies on *E. pungens* and other *Elaeagnus* plants have presented the isolation and identification of flavonoids (Bekker & Glushenkova 2001; Cao et al. 2001a, 2001b; Ge, Li et al. 2013; Li et al. 2012), terpenoids (Zhao et al. 2006), phenylpropanoids (Guo et al. 2008), along with other components (Zhao, Zhu et al. 2006).

Our preliminary studies have evaluated the anti-asthmatic, antitussive and expectorant activities of the ethanolic extract and fractions from *E. pungens in vivo* (Ge, Dai et al. 2009, 2013, 2015). Also, we have reported some research on chemical components and relaxant effect on guinea pig tracheal smooth muscle, which is related to the anti-asthmatic activity (Ge, Li et al. 2013; Li et al. 2012). Given the importance of its effect on respiratory diseases, the chemical compositions of *E. pungens* leaf were further studied and presented here along with the structures of the newly obtained flavonol glycosides. The effect of isolated compounds was evaluated on the proliferation of airway smooth muscle cells (ASMCs), in order to search for the active components of *E. pungens* leaf and provide further experimental evidence for the use and development of *E. pungens*.

2. Results and discussion

Compound **1** was isolated as yellow amorphous powder. The molecular formula, $C_{45}H_{59}O_{29}$, was determined by HR-ESI-MS (*m/z*: 1063.3133 $[M-H]^-$, Calcd for $C_{45}H_{59}O_{29}$: 1063.3142), as well as its ^{13}C and DEPT NMR data. Its UV spectrum exhibited absorption bands characteristic for flavonols at 266 and 348 nm. The ^1H NMR spectrum of **1** showed a 57-di-hydroxylated pattern for ring A (two meta-aromatic protons at δ_H 6.43 and 6.75, $J = 2.0$ Hz) and a 4′-hydroxylated pattern for ring B (AA′BB′ system signals at δ_H 8.06 and 6.86, each d, $J = 9.0$ Hz), which indicated the presence of kaempferol as aglycone by comparison of its NMR spectral data with literature (Feng et al. 2014).

The ^1H NMR of **1** exhibited five anomeric protons at δ_H 5.58 (1H, d, $J = 7.5$ Hz), 4.42 (1H, s), 4.28 (1H, d, $J = 7.5$ Hz), 5.05 (1H, s) and 5.06 (1H, d, $J = 7.0$ Hz), which gave correlations in the HSQC spectrum with the five anomeric carbon signals at δc 99.0, 99.8, 104.4, 100.6 and 99.9, respectively, confirming that compound **1** contains five sugar units. The sugars obtained by acid hydrolysis of **1** were identified as D-glucose, L-rhamnose and D-galactose by GC analysis of their respective derivatives.

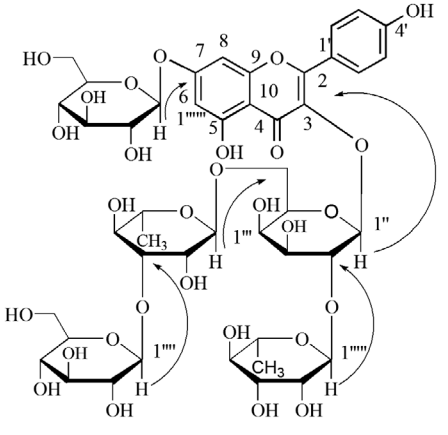
The *J* values of five anomeric protons at δ_H 5.58 (1H, d, $J = 7.5$ Hz, H-1″), 4.42 (1H, s, H-1‴), 4.28 (1H, d, $J = 7.5$ Hz, H-1⁗), 5.05 (1H, s, H-1⁗′), and 5.06 (1H, d, $J = 7.0$ Hz, H-1⁗″), proved sugar units as one β-galactose, two α-rhamnose, and two β-glucose, respectively. From five anomeric protons starting points, NMR signals of each sugar unit were assigned by ^1H–^1H COSY, HSQC and HSQC–TOCSY spectra and sugar moieties were two β-D-glucopyranosyl, two α-L-rhamnosyl and one β-D-galactosyl, and further connected based on long-range correlations in the HMBC spectrum.

In the HMBC spectrum, the glycosidic linkage positions were unambiguously determined from H-1″ (δ_H 5.58, Gal) to C-3 (δc 133.0) and from H-1⁗″ (δ_H 5.06, Glu) to C-7 (δc 162.8), which confirmed that the β-galactose and β-glucose units were attached to C-3 and C-7 of kaempferol, respectively. ROE connectivities were detected between the anomeric proton of the 7-*O*-linked sugar residue H-1⁗″ (δ_H 5.06, Glu-I) and both H-6 and H-8. Other HMBC correlations of C-2″ (δc 74.8) and C-6″ (δc 64.8) with anomeric protons at H-1‴ (δ_H 4.42, Rha-I) and H-1⁗′ (δ_H 5.05, Rha-II), respectively, and the reverse correlations, identified positions 2″

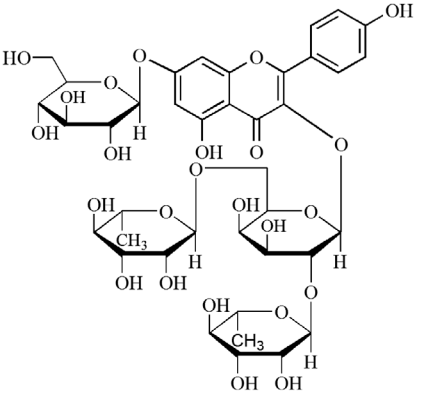
and 6″ of the galactose unit as glycosylation sites. Moreover, the correlations of the anomeric proton at δ_H 4.28 (1H, d, J = 7.5 Hz, H-1‴ Glu-II) with C-3‴ at δc 81.0 revealed that the glucose was linked to C-3‴ of Rha-I. The HMBC correlations are shown in Figure 1. This deduction was further supported by ROESY correlations (See Figure S1). Based on the above evidence, the structure of **1** was assigned as kaempferol 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranoside-7-*O*-β-D-glucopyranoside, and the structure is shown in Figure 1.

Five known compounds, kaempferol3-*O*-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranoside-7-*O*-β-D-glucopyranoside (**2**) (Kijima et al. 1995), (*6S*, *9S*)-6, 9-dihydroxymegastiman-4-megastigmen-3-one-9-*O*-β-D-glucopyranoside (**3**) (Wang et al. 2009), (*6S*, *9S*)-6, 9-dihydroxymegastiman-4-megastigmen-3-one-9-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (**4**) (Wang et al. 2009), blumenol C9-*O*-β-D-glucopy ranoside (**5**) (Song et al. 2010) and blumenol C9-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-lucopyranoside (**6**) (De Tommasi et al. 1992) were also isolated and identified by spectral analysis and comparison of data with those reported in the literature.

The proliferation effects of compounds **1**–**6** were evaluated on the ASMCs in the asthma rats. Compounds **1**–**6** exhibited no effects on the over-proliferation activity in the ASMCs, compared with the control group. It is reported that improving ASMCs proliferation is tightly related to increased airway smooth muscle mass, which could generate more shortening, leading to increased airway narrowing and airflow obstruction in asthma (Bentley &



1



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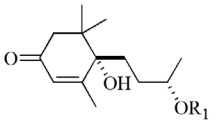

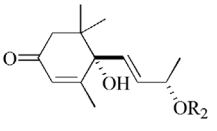

3 R_1=9-*O*-β-D-glucoside
4 R_1=9-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucoside
5 R_2=9-*O*-β-D-glucoside
6 R_2=9-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucoside

Figure 1. Chemical structures of compounds **1**–**6** and HMBC correlations of compound **1**.

Hershenson 2008; Zhao et al. 2013). That suggested the isolated flavonol glycosides maybe have no aggravating effect for the increased ASMCs proliferation in *E. pungens* leaf.

3. Experimental

3.1. General procedures

Optical rotations were measured on a JASCO P-1020 automatic digital polarimeter (JASCO, Tokyo, Japan). UV spectra were obtained on Shimadzu UV260 spectrometer (Shimadzu Co, Kyoto, Japan). HR-ESI-MS data were recorded on *Agilent* G6230 TOF MS. 1D- (^{1}H at 500 MHz and ^{13}C at 125 MHz) and 2D-NMR spectra were run on a BRUKER DRX-500 spectrometer (Ettlingen, Germany), using TMS as internal standard. The preparative HPLC were performed on Ultimate 3000 HPLC system (Dionex Co, Sunnyvale, CA, USA), and a semi-preparative column (10 mm × 250 mm, Nacalaitesque Co, Ltd, Kyoto) was used for separations. GC-MS data were recorded on an on Agilent Technologies 5973 Network Mass Selective Detector-6890N Network GC System (Santa Clara, CA, USA). Precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co, Ltd, Qingdao, China) were employed for TLC. For column chromatography (CC), silica gel (200–300 mesh, Qingdao Haiyang Chemical Group Co, Qingdao, China), reversed phase C18 silica gel (50 mm, YMC Co, Ltd, Kyoto, Japan), D-101 macroporous absorbent resin (Anhui Sanxing Resin Technology Co, Ltd, Bangbu, China), and Sephadex LH-20 (Shanghai Ai-yan Biological Co, Ltd, Shanghai, China) were used. All of the solvents and materials were analytical reagent grade except for Chromatography methanol.

3.2. Plant material

The fresh leaves of *E. pungens* were collected in May 2014 at GuiFeng Hill, Macheng city, Hubei province, China, and authenticated by Dr Dingrong Wan, Professor in Pharmacognosy at School of Pharmacy, South-central University for Nationalities, where a voucher specimen (SCUN08015) was deposited.

3.3. Extraction and isolation

The dried and powdered leaves of *E. pungens* (4.6 kg) were extracted with 70% EtOH under reflux for three times, and the EtOH extract was evaporated *in vacuo* to give a gummy residue (886 g). The residue was suspended in distilled water and then successively partitioned with petroleum ether (3 × 2.0 L), AcOEt (3 × 2.0 L), and BuOH (3 × 2.0 L). Each part was concentrated to get petroleum ether (fraction A, 39.8 g), AcOEt (fraction B, 161.5 g), BuOH (fraction C, 65.0 g) and H_2O fraction (fraction D, 420.0 g). 50.0 g of fraction D was subjected to D-101 macroporous absorbent resin CC and eluted with gradient solvent system of EtOH–H_2O (0:1–9:1) to afford four fractions (Fr. D1–4). Fr. D2 was further separated into three fractions (Fr. D2.1–2.3) by reversed-phase C_{18} silica gel CC using a gradient of CH_2Cl_2–MeOH (19:1–3:7). Separation of Fr. D2.1 with Sephadex LH-20 CC (MeOH) gave Fr. D2.1.1–2.1.2. After further purification by semi-preparative HPLC (particle size 5 μm, MeOH–H_2O; 40:60, 3 mL/min), compound **1** (20.0 mg, t_R = 16.1 min), compound **2** (7.0 mg), compound **6** (4.0 mg) was obtained from Fr. D2.1.1. 62.0 g of fraction C was separated into nine fractions (Fr. C1–9) by silica gel CC with gradient elution of CH_2Cl_2–MeOH (15:1–3:7). Fr. C1 and Fr. C3 were

repeatedly purified by silica gel CC and semi-preparative HPLC to afford compound **3** (8.5 mg), compound **4** (5.0 mg), compound **5** (4.7 mg).

3.4. Acidic hydrolysis

Compounds **1** (3.0 mg) were hydrolyzed with 4 N trifluoroacetic acid (6 mL) for 3 h at 90 °C. After cooling, the hydrolytical solution was extracted with chloroform (3 × 6.0 mL). The water layer obtained on acid hydrolysis gave the sugar residue after drying. The residue and reference substances of L-rhamnose, D-glucose and D-galactose were derived by reacting with 1.5 mg hydroxylamine hydrochloride and 0.5 mL pyridine for 1 h at 90 °C. After cooled to room temperature, 0.9 mL Ac_2O was added with heating at 90 °C continued for another 1 h. The reaction mixtures were concentrated under reduced pressure, and the resulting aldononitrile peracetates were subjected to GC-MS analysis. The samples (10 µL) were injected into a HP-1 chromatographic column (30 m × 0.2 mm, Agilent).Temperature of the injector was 280 °C. Flow of the helium carrier gas was maintained at 1 mL/min. A temperature gradient system was used for the oven: starting at 100 °C for 2 min, 20 °C/min to 200 °C (remaining at 200 °C for 2 min), then 10 °C/min to 260 °C (remaining at 260 °C for 3 min) – the total run time was 18 min. The range of MS was *m/z* 30–500. Peaks of the hydrolysate were detected by comparison with retention times of the standard L-rhamnose (8.86 min), D-glucose (11.05 min) and D-galactose (11.26 min) prepared in a similar manner.

3.4.1. *Kaempferol 3-O-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranoside-7-O-β-D-glucopyranoside* (1)

Yellow amorphous powder; $[\alpha]_D$ −80.09 (*c* = 0.216, MeOH); UV (MeOH) λ_{max} (log ε) nm: 266 (4.20), 348 (4.09); ^{1}H NMR (500 MHz, MeOH-d_4): δ 6.43 (1H, d, *J* = 2.0 Hz, H-6), 6.75 (1H, d, *J* = 2.0 Hz, H-8), 8.06 (2H, d, *J* = 9.0 Hz, H-2′ and H-6′), 6.86 (2H, d, *J* = 9.0 Hz, H-3′ and H-5′), 5.58 (1H, d, *J* = 7.5 Hz, H-1″), 3.79 (1H, t, *J* = 8.8 Hz, H-2″) 3.11 (1H, m, H-3″) 3.59 (2H, m, H-4″ and H-5″), 3.21 and 3.56 (1H, m, H-6″), 4.42 (1H, s, H-1‴), 3.59 (1H, m, H-2‴), 3.28 (2H, m, H-3‴ and H-4‴), 3.41 (1H, m, H-5‴), 1.04 (1H, d, *J* = 6.0 Hz, H-6‴), 4.28 (1H, d, *J* = 7.5 Hz, H-1⁗), 2.99 (1H, t, *J* = 8.5 Hz, H-2⁗), 3.13 (1H, m, H-3⁗), 3.04 (1H, m, H-4⁗), 3.28 (1H, m, H-5⁗), 3.56 and 3.42 (1H, m, H-6⁗), 5.05 (1H, s, H-1⁗′), 3.65 (1H, m, H-2⁗′), 3.46 (1H, m, H-3⁗′), 3.71 (1H, m, H-4⁗′), 3.73 (1H, m, H-5⁗′), 0.76 (1H, d, *J* = 6.0 Hz, H-6⁗′), 5.06 (1H, d, *J* = 6.0 Hz, H-1⁗″), 3.59 (1H, m, H-2⁗″), 3.04 (1H, m, H-3⁗″), 3.14 (1H, m, H-4⁗″), 3.42 (1H, m, H-5⁗″), 3.67 and 3.42 (1H, m, H-6⁗″), and ^{13}C NMR (125 MHz, MeOH-d_4): δ 156.8 (C-2), 133.0 (C-3), 177.3 (C-4), 160.8 (C-5), 99.3 (C-6), 162.8 (C-7), 94.6 (C-8), 155.9 (C-9), 105.6 (C-10), 120.6 (C-1′), 130.9 (C-2′ and C-6′), 115.2 (C-3′ and C-5′), 160.1 (C-4′), 99.0 (C-1″), 74.8 (C-2″), 71.8 (C-3″), 68.3 (C-4″), 73.1 (C-5″), 64.8 (C-6″), 99.8 (C-1‴), 73.9 (C-2‴), 81.7 (C-3‴), 70.6 (C-4‴), 67.8 (C-5‴), 17.9 (C-6‴), 104.4 (C-1⁗), 73.8 (C-2⁗), 76.0 (C-3⁗), 69.6 (C-4⁗), 76.3 (C-5⁗), 60.8 (C-6⁗), 100.6 (C-1⁗′), 69.3 (C-2⁗′), 70.5 (C-3⁗′), 70.7 (C-4⁗′), 68.2 (C-5⁗′), 17.3 (C-6⁗′), 99.9 (C-1⁗″), 72.9 (C-2⁗″), 76.6 (C-3⁗″), 69.5 (C-4⁗″), 77.1 (C-5⁗″), 60.6 (C-6⁗″). Negative HR-ESI-MS *m/z*: 1063.3133[M − H]$^-$ (Calcd, $C_{45}H_{59}O_{29}$: 1063.3142).

3.5. Proliferation experiment

Male Sprague Dawley rats weighing 180–200 g (Hubei Province Center for Disease Control and Prevention, Wuhan, China) were used to make the asthma model according to the

literature (Salmon et al. 1999). In brief, rats were actively sensitized on days 1 and 8 using 50 mg.kg^{-1} subcutaneous injection of ovalbumin in 0.9% sterile saline containing 200 mg of aluminium hydroxide. Challenges were performed every third day after sensitization, being exposed to a 2% ovalbumin aerosol mist produced by a Ultrasonic nebulizer (YuYue Medical Device Ltd, Jiangsu, China) for 30 min. After asthma symptoms took place, rats were killed on day 29 using an overdose of sodium pentobarbitone (500 mg kg^{-1}; i.p.). Primary ASMCs were culture by enzymatic method (Hirst 2006) and the proliferation of ASMCs was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu-mbromide) assay. In brief, the airway smooth muscles from the asthma rats were cut into small pieces and digested by D-Hanks solution containing 1 mg/mL type I collagenase, 2 mg/mL papain (Invitrogen, Grand Island, NY, USA) and 2 mg/mL BSA at 37 °C for 40 min. The collected pellets by centrifuge were cultured with DMEM (Dulbecco's modified eagle medium) supplemented with 20% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin and subcultured with 0.25% trypsin-0.02% EDTA (Ethylenediaminetetraacetic acid disodium salt) solution. The cells between 3 and 8 passages were used for the experiment.

After compounds **1**–**6** at the concentrations of 10, 30 and 100 μM were cultured with the ASMCs for 48 h, MTT (5 mg/mL) was added and incubated for 4 h, then the absorbance at 490 nm was measured on a microplate reader (Thermo Electron Co, Waltham, MA, USA).

4. Conclusion

One new flavonoid glycosides, kaempferol 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranoside-7-*O*-β-D-glucopyranoside (**1**), along with five known compounds (**2**–**6**) were isolated and identified from the leaves of *E. pungens*. The MTT assay *in vitro* showed that the isolated flavonol glycosides showed no proliferating activity in the asthma ASMCs, implying no aggravating impact on the increased airway smooth muscle within airway wall of asthmatic disease.

Disclosure statement

No potential conflict of interest was reported by the authors.

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