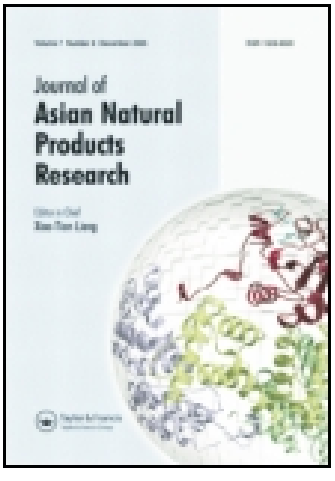

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Two new flavonol glycosides from the leaves of Elaeagnus pungens

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Two new flavonol glycosides from the leaves of *Elaeagnus pungens*

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The leaves of *Elaeagnus pungens* were extracted with 70% ethanol and successively purified by column chromatography. Seven constituents were obtained and characterized, all of which belong to the class of flavonol glycosides. Their structures were elucidated on the basis of spectroscopic methods including 1D/2D NMR and MS analysis techniques. The seven flavonol glycosides were determined as kaempferol 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl(1 → 2)]-β-D-galactopyranoside (**1**), isorhamnetin 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside (**2**), together with five known compounds, respectively. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu-m bromide assay *in vitro* showed that the isolated flavonol glycosides showed no proliferation activity in the asthma airway smooth muscle cells, comparing with solvent as the control group.

Keywords: Elaeagnaceae; *Elaeagnus pungens*; flavonol glycoside; spectroscopic analysis

1. Introduction

The *Elaeagnus* is a member of the family Elaeagnaceae, which comprises three genera and about 80 species that are widespread in subtropical and temperate areas of East and Southeast Asia [1,2]. Fifty-five species, including *Elaeagnus pungens* Thunb., are found in China. In traditional Chinese medicine, *E. pungens* leaf has been documented as an anti-asthmatic remedy to treat the severe asthma, chronic bronchitis, or other respiratory disorders in the early material medica 'Bencao Gangmu' (Ming dynasty, about 430 years ago). It is also generally used by Tujia nationality people in Hubei province to treat shortness of breath, cough, or bronchitis [3]. It is nontoxic under oral administration for a long time in adult without definite IC_{50} value in mice [4].

Experimentally, the chemical constituents of *E. pungens* have been investigated in the past years, including flavonoids [5], lignanoids [6], organic acids [7], and terpenoids [8]. Our preliminary study has evaluated the anti-asthmatic, antitussive, and expectorant activities *in vivo* of the ethanolic extract and fractions from the plant [9]. In general, despite itslong-time use in human health, literatures about *E. pungens* are limited, especially on the research on chemical components and effect on airway smooth muscle cells (ASMCs), the change of which is related to asthmatic development [10]. Given the importance of its effect on respiratory diseases, the chemical composition of *E. pungens* leaf was studied in detail and presented here along with the structures of the newly obtained flavonol glycosides. The effect of isolated compounds was evaluated on the proliferation of ASMCs, in order to search for the active components of *E. pungens* leaf.

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2. Results and discussion

Compound **1** was obtained as a yellow powder. Its molecular formula of $C_{39}H_{50}O_{24}$ was deduced from the HR-ESI-MS at *m/z* 925.2584 $[M + Na]^+$. Acidic hydrolysis of **1** showed the presence of glucose, rhamnose, and galactose. The ^1H NMR spectrum of aglycon showed a set of AA′BB′ systems at δ_H 8.08 (2H, d, $J = 9.0$ Hz) and 6.91 (2H, d, $J = 9.0$ Hz), as well as two *meta*-aromatic protons at δ_H 6.17 (s, 1H) and 6.35 (s, 1H). The ^{13}C NMR spectral data of the aglycone spectra of **1** revealed the presence of nine quaternary C-atoms (including one C=O group at δ_C 179.3) and six CH groups. Detailed analysis of HSQC and HMBC spectra indicated compound **1** was characterized as being kaempferol 3-*O*-glycoside with four sugar moieties.

The ^1H and ^{13}C NMR data also showed four glycosyl signals by four anomeric protons at δ_H 5.58 (1H, d, $J = 7.5$ Hz), 4.58 (1H, s), 4.39 (1H, d, $J = 8.0$ Hz), and 5.24 (1H, s), and the related anomeric C-atom signals at δ_C 101.0, 101.7, 105.7, and 102.6. Signals of each sugar unit were assigned by HSQC, HMBC, TOCSY, and ^1H–^1H COSY analyses and sugar moieties were one β-D-glucose, one β-D-galactose, and two α-L-rhamnoses. The configurations of glucopyranosyl and galactopyranosyl assigned as β were further supported by the coupling constants of the anomeric protons at δ_H 4.39 (1H, d, $J = 8.0$ Hz) and 5.58 (1H, d, $J = 7.5$ Hz). The sequence of the sugar moieties was determined by HMBC correlations between H-1″ at δ 5.58 and C-3 at δ 134.4; H-1‴ at δ 4.58 and C-6″ at δ 67.5; H-1^{IV} at δ 4.39 and C-3‴ at δ 83.0; H-1^{V} at δ 5.24 and C-2″ at δ 77.4. Thus, compound **1** was deduced as kaempferol 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl(1 → 2)]-β-D-galactopyranoside, and the structure is shown in Figure 1.

Compound **2** was isolated as a yellow powder. The molecular formula was established to be $C_{34}H_{42}O_{21}$ by HR-ESI-MS at *m/z* 809.2111 $[M + Na]^+$. Acidic hydrolysis also showed the presence of glucose, rhamnose, and galactose in compound **2**. By comparing the NMR data of **2** with those of **1**, it was found that

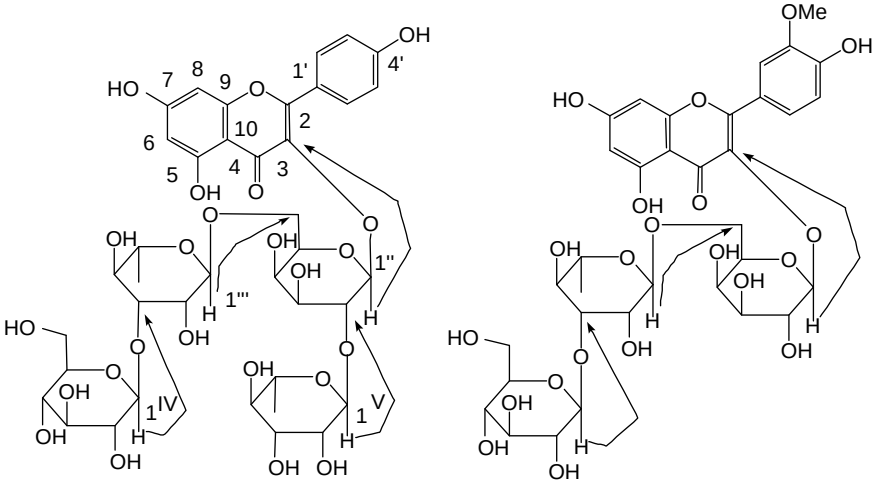

Figure 1. Key HMBC correlations of compounds **1** and **2**.

the structure of the sugar moiety in **2** corresponded to that in **1** except for the absence of one rhamnosyl group in **2**, and the structure of aglycon of **2** had one more methoxyl group. The MeO group was located at C-3′ based on the HMBC correlation of δ_H 3.89 (3H, s)/C-3′ (δ_C 148.5). Therefore, the aglycon of **2** was identified as isorhamnetin. The ^{1}H and ^{13}C NMR data also showed three glycosyl signals by three anomeric protons at δ_H 5.12 (1H, d, $J = 8.0$ Hz), 4.48 (s, 1H), and 4.25 (1H, d, $J = 8.0$ Hz), and the related anomeric C-atom signals at δ_C 105.2, 102.0, and 105.8. In the same way as that of **1**, the sequence of the sugar moieties was determined by HMBC correlations (see Figure 1). The HMBC correlation of H-2″ [δ_H 5.12 (1H, d, $J = 8.0$ Hz)] with C-3 (δ_C 135.6) revealed the sugar chain was connected to C-3. Therefore, compound **2** was deduced as isorhamnetin 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside, and the structure is shown in Figure 1.

Five known compounds were identified as kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl(1 → 2)]-β-D-galactopyranoside (**3**) [11], kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl(1 → 2)]-β-D-galactopyranoside-7-*O*-β-D-glucopyranoside (**4**) [12], kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**5**) [12], kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**6**) [13], and kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside (**7**) [14].

The proliferation effects of compounds **1**–**7** were evaluated on the ASMCs in the asthma rats. It exhibited that there were no effects of compounds **1**–**7** on the proliferation activity in the ASMCs, compared with the control group. That suggested the isolated flavonol glycosides may be not the effective components for the ASMCs proliferation in *E. pungens* leaf.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured using a P-1020 digital polarimeter in MeOH solution from JASCO, Tokyo, Japan. UV spectra were measured on *Shimadzu UV-260* spectrometer from Shimadzu Co., Kyoto, Japan. ^{1}H, ^{13}C, and 2D NMR spectra were run on a Bruker DRX-500 spectrometer with tetramethylsilane as internal standard from Ettlingen, Germany. HR-ESI-MS data were obtained on *Bruker FT-MS Apex III* mass spectrometer. GC–MS data were obtained on *Agilent Technologies 5973* Network Mass Selective Detector-6890N Network GC System from Santa Clara, CA, USA. High performance liquid chromatography (HPLC) was carried out on Ultimate 3000 HPLC system, Ultimate 3000 pump, Ultimate 3000 Variable Wavelength from Dionex Co., Sunnyvale, CA, USA and 10 mm × 250 mm semi-preparative column from Nacalai tesque Co., Ltd, Kyoto, Japan. Thin-layer chromatography was carried out on silica gel 60 GF254 (Qingdao Haiyang Chemical Co., Ltd, Qingdao, China), and column chromatography (CC) was carried out using silica gel (200–300 mesh) from Qingdao Haiyang Chemical Group Co., macroporous resin D-101 from Anhui Sanxing Resin Technology Co., Ltd, Bangbu, China; Sephadex LH-20 from Shanghai Ai-yan Biological Co., Ltd, Shanghai, China and C18 reverse-phase silica gel from YMC Co., Ltd, Kyoto, Japan. All of the solvents and materials were reagent grade and purified as required.

3.2 Plant material

The fresh leaves of *E. pungens* were collected in June 2010 at She Hill, Wuhan

city, Hubei province, China. The plant was authenticated by Dr Dingrong Wan, Professor in Pharmacognosy at School of Pharmacy, South-central University for Nationalities (SCUN). The collected *E. pungens* leaf is also consistent with the standardized herbal specimen (No. 42046) in Hubei Provincial Institute of Drug Control. The voucher specimen (SCUN08010) has been deposited in the Herbarium of School of Pharmacy, SCUN.

3.3 Extraction and isolation

The collected leaves were dried in shade and reduced to coarse powder using a mortar and pestle. The powdered leaves of *E. pungens* (4.2 kg) were extracted with 70% EtOH and then successively partitioned with petroleum ether (3 × 2.0 liters), AcOEt (3 × 2.0 liters), and BuOH (2.0 liters). The residual water layer was concentrated (400 g) and subjected to CC [macroporous resin; EtOH–H_2O; 0:1, 3:7, 6:4, 9:1, 1:0 (v/v)] to give five fractions (Frs 1–5). Fr. 2 (40.0 g) was subjected to CC (SiO_2; CH_2Cl_2–MeOH; 15:1 → 0:1 gradient system) to give five fractions (Frs 2.1–2.5). Fr. 2.4 (2.6 g) was successively separated by CC (SiO_2; CH_2Cl_2–MeOH; 10:1 → 0:1) to yield Frs 2.4.1–2.4.6. Fr. 2.4.4 (0.3 g) was further purified by CC (Sephadex; MeOH) and followed by semi-preparative HPLC (MeOH–H_2O; 35:65, 3 ml/min) to afford compound **1** (10 mg; t_R 4.4 min). Fr. 2.5 (13.7 g) was purified by CC (Sephadex; MeOH) to give Frs 2.5.1–2.5.10. Fr. 2.5.2 (9.1 g) was subjected to CC (SiO_2; CH_2Cl_2–MeOH; 9.3:0.7 → 1:1) to afford Frs 2.5.2.1–2.5.2.10. Fr. 2.5.2.8 (1.4 g) was subjected to CC (*RP-18*; MeOH–H_2O; 1:10 → 3:2) to provide Frs 2.5.2.8.1–2.5.2.8.13. Fr. 2.5.2.8.8 (57 mg) was further purified by CC (Sephadex; MeOH) to give compound **2** (3.5 mg) and compound **3** (9.0 mg). Fr. 2.5.2.8.4 (0.25 g) was further purified by CC (Sephadex; MeOH) and followed by semi-preparative HPLC (MeOH–H_2O; 24:76, 3 ml/min) to afford compound **4** (9.3 mg; t_R 11.4 min). Fr. 2.5.2.8.11 (135 mg) was further purified by CC (Sephadex; MeOH) to get compound **5** (5.1 mg).

The BuOH fraction (62.0 g) was subjected to CC (SiO_2; CH_2Cl_2–MeOH; 15:1 → 3:7 gradient system), to give Frs 1–10. Fr. 5 (12 g) was successively separated by CC (SiO_2; CH_2Cl_2–MeOH; 1:0 → 4:6) to provide Frs 5.1–5.12. Fr. 5.9 (1.3 g) was further subjected to CC (Sephadex; MeOH) to afford Frs 5.9.1–5.9.4. Fr. 5.9.2 (0.2 g) was further purified by semi-preparative HPLC (MeOH–H_2O; 49:51, 3 ml/min) to afford compound **6** (21.9 mg; t_R 9.9 min). Fr. 5.9.3 (120 mg) was further purified by semi-preparative HPLC (MeOH–H_2O; 40:60, 3 ml/min) to afford compound **7** (11.5 mg; t_R 10.6 min).

3.4 Acidic hydrolysis

Compounds **1** and **2** (3 mg) were, respectively, added to trifluoroacetic acid (4 N) solution (6 ml), then heated for 3 h under 90°C. After cooled to room temperature, the hydrolytical solution was extracted with chloroform (3 × 6.0 ml). The water layer was concentrated to 0.5 ml. The completely concentrated water layer and reference substances of β-D-glucose, β-D-galactose, and α-L-rhamnose were derived by reacting with 1.5 mg hydroxylamine hydrochloride and 0.5 ml pyridine for 1 h at 90°C. After cooling, 0.9 ml Ac_2O was added and the mixture was heated at 90°C for 1 h. The reaction mixtures were evaporated under reduced pressure, and the resulting aldononitrile peracetates were analyzed by GC–MS. The samples (10 μl) were injected into a HP-1 30 m × 0.2 mm chromatographic column (Agilent). The injector temperature was 280°C. The helium carrier gas flow was maintained at 1 ml/min. The oven temperature program was as follows: 100°C for 2 min, 200°C at 20°C/min (remaining

Table 1. ^{13}C and ^{1}H NMR spectral data of compounds **1** and **2**.

	^{13}C NMR		^{1}H NMR	
No.	**1**	**2**	**1**	**2**
2	158.6	158.9		
3	134.4	135.6		
4	179.3	179.5		
5	163.1	163.0		
6	100.6	100.1	6.17 (1H, s)	6.13 (1H, s)
7	167.7	166.3		
8	95.3	95.0	6.35 (1H, s)	6.34 (1H, s)
9	158.5	158.6		
10	105.4	105.7		
1′	123.4	123.0		
2′	132.2	114.8	8.08 (1H, d, $J = 9.0$ Hz)	7.99 (1H, s)
3′	116.2	148.5	6.91 (1H, d, $J = 9.0$ Hz)	
4′	161.3	150.9		
5′	116.2	116.2	6.91 (1H, d, $J = 9.0$ Hz)	6.82 (1H, d, $J = 8.0$ Hz)
6′	132.2	123.9	8.08 (1H, d, $J = 9.0$ Hz)	7.53 (1H, d, $J = 8.0$ Hz)
3′-OMe		57.0		3.89 (3H, s)
1″	101.0	105.2	5.58 (1H, d, $J = 7.5$ Hz)	5.12 (1H, d, $J = 8.0$ Hz)
2″	77.4	73.1	3.85–3.90 (m)	3.72–3.74 (m)
3″	75.2	75.1	3.72–3.74 (m)	3.47–3.48 (m)
4″	70.8	70.1	3.98–4.01 (m)	3.68–3.72 (m)
5″	75.7	75.6	3.51–3.54 (m)	3.57–3.62 (m)
6″	67.5	67.9	3.73–3.76 (m); 3.40–3.50 (m)	3.62–3.67 (m); 3.44–3.48 (m)
1‴	101.7	102.0	4.58 (1H, s)	4.48 (1H, s)
2‴	71.3	71.4	3.72–3.74 (m)	3.80–3.82 (m)
3‴	83.0	83.2	3.50–3.70 (m)	3.45–3.50 (m)
4‴	72.6	72.6	3.94–3.96 (m)	3.32–3.37 (m)
5‴	69.5	69.5	3.39–3.42 (m)	3.47–3.52 (m)
6‴	18.0	18.1	1.20 (3H, d, $J = 6.0$ Hz)	1.05 (3H, d, $J = 6.0$ Hz)
1^{IV}	105.7	105.8	4.39 (1H, d, $J = 8.0$ Hz)	4.25 (1H, d, $J = 8.0$ Hz)
2^{IV}	75.4	75.4	3.68–3.72 (m)	3.12–3.17 (m)
3^{IV}	77.6	77.6	3.30–3.41 (m)	3.27–3.28 (m)
4^{IV}	71.0	70.9	3.27–3.34 (m)	3.26–3.28 (m)
5^{IV}	77.7	77.7	3.10–3.20 (m)	3.06–3.09 (m)
6^{IV}	62.1	62.1	3.66–3.80 (m); 3.58–3.64 (m)	3.57–3.65 (m)
1^{V}	102.6		5.24 (1H, s)	
2^{V}	72.4		3.39–3.41 (m)	
3^{V}	72.5		3.65–3.69 (m)	
4^{V}	74.2		3.38–3.42 (m)	
5^{V}	69.9		3.35–3.38 (m)	
6^{V}	17.7		1.01 (3H, d, $J = 6.0$ Hz)	

at 200°C for 2 min), then to 260°C at 10°C/min (remaining at 300°C for 3 min) – the total run time was 18 min. The range of MS was *m/z* 30–500. The t_R values of β-D-glucose, β-D-galactose, and α-L-rhamnose derivatives were 11.05, 11.26, and 8.86 min, respectively.

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

Yellow powder; $[\alpha]_D$ −78.3 ($c = 0.121$, MeOH); UV(MeOH) λ_{max} 266, 349; ^{13}C

NMR (125 MHz, MeOH-d_4) and ^{1}H NMR (500 MHz, MeOH-d_4) spectral data are shown in Table 1. HR-ESI-MS: *m/z* 925.2584 $[M + Na]^+$(calcd for $C_{39}H_{50}O_{24}Na$, 925.2571).

*3.4.2 Isorhamnetin 3-O-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside (**2**)*

Yellow powder; $[\alpha]_D$ −33.8 ($c = 0.106$, MeOH); UV (MeOH) λ_{max} 255, 354 nm; ^{13}C NMR (125 MHz, MeOH-d_4) and ^{1}H NMR (500 MHz, MeOH-d_4) spectral data are shown in Table 1. HR-ESI-MS: *m/z* 809.2111 $[M + Na]^+$ (calcd for $C_{34}H_{42}O_{21}Na$, 809.2108).

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 [15]. 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 aluminum hydroxide. Challenges were carried out every third day after sensitization, being exposed to a 2% ovalbumin aerosol mist produced by an Ultrasonic nebulizer (YuYue Medical Device Ltd, Jiangsu, China) for 30 min. After asthma symptoms appear, 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 [16] and the proliferation of ASMCs was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu-m bromide) 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 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% ethylenediaminetetraacetic acid disodium salt solution. The cells between 3 and 8 passages were used for the experiment.

After compounds **1**–**7** 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).

Acknowledgments

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References

[1] N.P. Bekker and A.I. Glushenkova, *Chem. Nat. Compd.* **37**, 97 (2001).
[2] M. Ayaz, M. Riaz, A. Malik, E. Ahmad, I. Fatima, M. Arif Lodhi, and M.I. Chaudhary, *Nat. Prod. Res.* **23**, 409 (2009).
[3] Z.X. Fang, H. Zhao, and J.H. Zhao, *Yaocai Zhi of Tujia Nationality* (China Science and Technology of Medicine Press, Beijing, 2006), p. 466.
[4] P.A. Shen, *Pharmacological and Clinical Application of Chinese Material Medica* (People's Medical Publishing House, Beijing, 2006), p. 721.
[5] K. Tagahara, J. Koyama, and T. Sugita, *Shoyakugaku Zasshi* **35**, 340 (1981).
[6] M.J. Guo, H.B. Jiang, X.Q. Tian, N. Huang, and J. Huang, *West China J. Pharm. Sci.* **23**, 381 (2008).
[7] X. Zhao, H. Huang, and R.L. Zhu, *Chin. Tradit. Patent Med.* **28**, 403 (2006).
[8] X. Zhao, R.L. Zhu, B. Jiang, and H. Huang, *China J. Chin. Mater. Med.* **31**, 472 (2006).
[9] Y.B. Ge, J.Q. Liu, and D.F. Su, *J. Ethnophamacol.* **126**, 538 (2009).
[10] J.K. Bentley and M.B. Hershenson, *Proc. Am. Thorac. Soc.* **5**, 89 (2008).

[11] G.C. Kite, C.A. Stoneham, and N.C. Veitch, *Phytochemistry* **68**, 1407 (2007).
[12] B. Schwarz and T. Hofmann, *J. Agric. Food Chem.* **55**, 1394 (2007).
[13] H. Wu, S. Dushenkov, C.T. Ho, and S.M. Sang, *Food Chem.* **115**, 592 (2009).
[14] H.M. Ho, R.Y. Chen, L.K. Leung, F.L. Chan, Y. Huang, and Z.Y. Chen, *Biomed. Pharmacother.* **56**, 289 (2002).
[15] M. Salmon, D.A. Walsh, H. Koto, P.J. Barnes, and K.F. Chung, *Eur. Respir. J.* **14**, 633 (1999).
[16] S.J. Hirst, *Eur. Respir. J.* **9**, 808 (2006).