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Phenolics from *Ageratina adenophora* Roots and Their Phytotoxic Effects on *Arabidopsis thaliana* Seed Germination and Seedling Growth

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ABSTRACT: A bioassay-directed phytochemical study was conducted to investigate potential allelochemicals in the roots of the invasive plant *Ageratina adenophora*. Eleven phenolic compounds, including seven new ones, 7-hydroxy-8,9-dehydrothymol 9-*O*-*trans*-ferulate (**1**), 7-hydroxythymol 9-*O*-*trans*-ferulate (**2**), 7,8-dihydroxythymol 9-*O*-*trans*-ferulate (**3**), 7,8-dihydroxythymol 9-*O*-*cis*-ferulate (**4**), methyl (7*R*)-3-deoxy-4,5-epoxy-D-*manno*-2-octulosonate 8-*O*-*trans*-*p*-coumarate (**5**), methyl (7*R*)-3-deoxy-4,5-epoxy-D-*manno*-2-octulosonate 8-*O*-*cis*-*p*-coumarate (**6**), and 3-(2-hydroxyphenyl)propyl methyl malonate (**7**), were isolated from a bioactive subfraction of the ethanol extract of the roots of *A. adenophora*. The new structures were established on the basis of detailed spectroscopic analysis. The potential phytotoxic effects of these compounds on the germination of *Arabidopsis thaliana* seeds were tested by a filter paper assay. Compound **7** and known compounds 3-(2-hydroxyphenyl)-1-propanol (**8**) and *o*-coumaric acid (**9**) remarkably showed inhibition activity against *Arabidopsis* seed germination at a concentration of 1.0 mM. Compounds **1**, **2**, **5**, **6**, and **10** showed slight inhibitory activity at the test concentration after treatment for 3 days, while the other compounds showed no obvious inhibitory effects. Moreover, **7**−**9** were further found to show obvious inhibitory activity on retarding the seedling growth of *Ar. thaliana* cultured in soil medium.

KEYWORDS: *Ageratina adenophora, phenolic compounds, phytotoxic, allelochemical, Arabidopsis*

INTRODUCTION

Ageratina adenophora (Spreng.) King & Robinson (also known as *Eupatorium adenophorum* Spreng.), belonging to the family Compositae and indigenous to Mexico and Costa Rica, is a perennial, herbaceous invasive plant that has invaded more than 30 countries and regions in tropical and subtropical zones of the world.[1,2] Since *A. adenophora* was first introduced to Yunnan province of China in the 1940s, it has spread rapidly across a large area of southwest China, including Yunnan, Guizhou, Guangxi, Sichuan, Chongqing, and Xizang provinces.[3] The rapid spread of *A. adenophora* in China has caused serious economic losses to agriculture, forestry, and livestock and severely damaged the ecology and environment of China's native habitat.[4,5]

A. adenophora is a plant rich in terpenes (mono-, sesqui-, di-, and triterpenes) and phenolic compounds (flavonoids, phenylpropanoids, and coumarins), and some sterols as well as a few nitrogen-containing natural products have also been reported from this species.[6−8] Since Tripathi et al. suggested that *A. adenophora* might adversely affect neighboring plants by releasing inhibitors,[9] additional research has supported the view that allelopathy could be an important strategy for this plant to achieve its invasive success.[10−12] In recent years, studies aimed to address potential allelochemicals of *A. adenophora* have revealed several cadinine sesquiterpenoids with phytotoxic activities.[13−16] However, because of their low polarity and possible short half-life in the soil, Zhao et al. suggested that cadinine-type sesquiterpenes might represent only part of the allelochemicals of this plant.[16] Very recently, Zheng et al. reported the existence of a particular phenolic compound, *o*-coumaric acid, in this plant and revealed that this compound was capable of exhibiting a wide range of phytotoxic effects on *Arabidopsis* and crop plants,[17] suggesting that nonterpene allelochemicals would play an even more important role in the allelopathy of *A. adenophora* and are worthy of further investigation.

Generally, the roots have been considered by some as the most important tissue for invasive plants for storing and releasing their potential allelochemicals.[18,19] Previous studies have also revealed that root extracts and leachates of *A. adenophora* showed obvious phytotoxic effects.[10,12] However, studies of allelochemicals of *A. adenophora* have so far mainly concentrated on the aerial parts of the plant, and few reports have ever focused on the root tissues. As one part of our study

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Table 1. ^{1}H NMR Data of Compounds 1−7

	1[a]	2[b]	3[a]	4[a]	5[b]	6[b]	7[c]
2	7.24, s	7.11, d (1.8)	7.15, d (1.8)	7.73, d (1.9)	7.45, br d (8.7)	7.63, br d (8.7)	
3					6.79, d (8.7)	6.73, d (8.7)	7.09, dd (7.2, 2.0)
4							6.85, ddd (7.3, 7.2, 1.8)
5	6.82, d (7.4)	6.77, d (8.2)[d]	6.80, d (8.2)[e]	6.74, d (8.3)	6.79, d (8.7)	6.73, d (8.7)	7.07, ddd (7.5, 7.3, 2.0)
6	7.11, br d (7.4)	7.00, dd (8.2, 1.8)	7.04, dd (8.2, 1.8)	7.07, dd (8.3, 1.9)	7.45, br d (8.7)	7.63, br d (8.7)	6.76, dd (7.5, 1.8)
7	7.73, d (15.8)	7.53, d (15.9)	7.58, d (15.9)	6.82, d (12.9)[e]	7.63, d (16.0)	6.85, d (12.8)	2.70, t (7.5)
8	6.48, d (15.8)	6.28, d (15.9)	6.31, d (15.9)	5.76, d (12.9)	6.34, d (16.0)	5.78, d (12.8)	1.99, m
9							4.19, t (6.4)
10	3.90, s	3.84, s	3.88, s	3.85, s			
11 (1′)							3.42, s
12 (2′)	6.83, s	6.81, s	6.80, s[e]	6.80, s[e]			
13 (3′)					2.26, m	2.26, m	3.77, s
4′					4.14, m	4.13, m	
5′	7.07, d (7.4)	7.11, d (7.2)	7.30, d (7.9)	7.24, d (8.4)	4.02, dd (4.8, 4.8)	4.00, dd (4.8, 4.8)	
6′	6.79, br d (7.4)	6.78, br d (7.2)[d]	6.83, br d (7.9)[e]	6.79, br d (8.4)[e]	4.48, dd (4.8, 4.3)	4.44, dd (4.8, 4.3)	
7′	4.52, s	4.49, s	4.52, s	4.51, s	4.40, m	4.35, m	
8′		3.52, m			4.97, dd (12.2, 8.3)	4.94, dd (12.2, 8.4)	
					4.76, dd (12.2, 3.2)	4.69, dd (12.2, 3.2)	
9′	7.47, s	4.31, dd (10.5, 6.4)	4.47, d (11.1)	4.43, d (11.2) 4.35,	3.78, s	3.78, s	
		4.24, dd (10.5, 7.4)	4.39, d (11.1)	d (11.2)			
10′	2.11, s	1.29, d (7.0)	1.66, s	1.61, s			

[a]Recorded in CD_3OD at 400 MHz. [b]Recorded in CD_3OD at 600 MHz. [c]Recorded in $CDCl_3$ at 400 MHz. [d]Overlapped signals. [e]Assignments were made by two-dimensional NMR.

of the invasion mechanisms of *A. adenophora*, we recently conducted a bioassay-directed phytochemical study of potential allelochemicals in the roots of this plant, which led to the isolation of 11 phenolic compounds, including seven new ones. Here we report the isolation and structure elucidation of these compounds and describe their phytotoxic effects on *Arabidopsis* seed germination and seedling growth.

MATERIALS AND METHODS

General Methods. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter (Perkin-Elmer, Inc., Waltham, MA). UV spectra were recorded on a Perkin-Elmer Lambda 650 UV−vis spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-400 NMR spectrometer (Bruker Biospin Gmbh, Rheistetten, Germany) and a Bruker AVANCE 600 instrument with tetramethylsilane as the internal standard. Electrospray ionization mass spectrometry (ESI-MS) was conducted on a MDS SCIEX API 2000 liquid chromatography−tandem mass spectrometry (LC−MS/MS) instrument (Applied Biosystems, Inc., Foster City, CA), and high-resolution (HR) ESI-MS spectra were recorded on an API QSTAR Pulsar 1 spectrometer (Advanced Biomics, Los Angeles, CA). HRESI-MS spectra were recorded on a Waters Auto Premier P776 spectrometer. Silica gel (200−300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), Lichroprep reversed-phase C18 silica gel (40−63 um, Merck, Darmstadt, Germany), and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Preparative middle-performance liquid chromatography (MPLC) was conducted on a CXTH P3000 instrument (Beijing ChuangXinTongHeng Science and Technology Co., Ltd., Beijing, China) equipped with a UV3000 UV−vis detector and a Fuji-C18 column (10 μm, 30 mm × 250 mm). Thin-layer chromatography (TLC) was conducted on precoated silica gel plates (HSGF254, Yantai Jiangyou Silica Gel Development Co., Ltd., Yantai, China), and spot detection was performed by spraying 10% H_2SO_4 in ethanol, followed by heating.

Plant Material. The root material of *A. adenophora* was collected in a suburb of Kunming, Yunnan province, P. R. China, in September 2010, identified by one of the authors (J.-W.T.). A voucher specimen (No. 20100902) was deposited at the Laboratory of Phytochemistry at the South China Botanical Garden, Chinese Academy of Sciences. Seeds of the *Arabidopsis thaliana* Columbia ecotype were from the laboratory of K. Wu at the South China Botanic Garden, Chinese Academy of Sciences.

Extraction and Isolation. The fresh root material of *A. adenophora* (14.5 kg) was cut into pieces and extracted three times with 95% EtOH (3 × 20 L) at room temperature. After removal of ethanol (EtOH) *in vacuo*, the viscous concentrate was suspended in 10% ethanol in H_2O (2.5 L) and then successively extracted with ethyl acetate (EtOAc) (3 × 2.5 L). The oily EtOAc extract (76 g) was subjected to silica gel (500 g) column chromatography and first eluted with 2 L of a petroleum ether/chloroform mixture [1:1 (v/v)] to remove most of the fatty acids. Then the silica gel column was further eluted with a chloroform/methanol ($CHCl_3$/MeOH) system from 9:1, 8:2, and 7:3 to 1:1 (v/v, each 2.5 L) to afford fractions Fr.1−Fr.4. As a result of the bioassay analysis (see Figure 1), the most active fraction, i.e., Fr.1, was further investigated. Fraction Fr.1 (21 g), obtained from the elution of a 9:1 $CHCl_3$/MeOH mixture, was further subjected to silica gel column chromatography and eluted with a petroleum ether/acetone gradient [from 1:0 to 0:1 (v/v)] to afford fractions A−F on the basis of TLC profiles. Fraction C, obtained from the elution of a 9:1 petroleum ether/acetone mixture, was separated by Sephadex LH-20 column chromatography and eluted with MeOH to give subfractions C1−C3; subfraction C2 was further purified by RP-C_{18} MPLC and eluted with a MeOH/H_2O mixture [from 4:6 to 7:3 (v/v)] to yield compound **7** (26 mg). After repeated silica gel [$CHCl_3$/MeOH, from 9.5:0.5 to 9:1 (v/v)] and Sephadex LH-20 column chromatography [1:4 (v/v) $CHCl_3$/MeOH], compound **8** (41 mg) was obtained from subfraction C3. Fraction D, obtained from the elution of an 8:2 petroleum ether/acetone mixture, was subjected to preparative RP-C_{18} MPLC and eluted with a MeOH/H_2O mixture [from 3:7 to 6:4 (v/v)], and the needle crystals of compound **10** (1045 mg) were precipitated from the elution with a 6:4 MeOH/H_2O mixture. Fraction E, obtained from the elution of a 7:3 petroleum ether/acetone mixture, was divided into subfractions E1−E3 by being passed over the RP-C_{18} MPLC system and eluted with a MeOH/H_2O mixture [from 2:8 to 9:1 (v/v)]. Subfractions E1−E3 were each purified repeatedly by silica gel chromatography and Sephadex LH-20 columns. Finally, compound **11** (63 mg) was obtained from subfraction E2, and **1** (11 mg) and **2** (41 mg) were obtained from

Table 2. ^{13}C NMR Data of Compounds 1−7

	1[a]	2[b]	3[a]	4[a]	5[a]	6[a]	7[c]
1	127.6, s	127.7, s	127.7, s	128.1, s	127.2, s	127.6, s	153.8, s
2	111.8, d	111.5, d	111.6, d	114.9, d	131.2, d	133.7, d	127.0, s
3	149.4, s	149.3, s	149.4, s	148.3, s	116.8, d	115.8, d	130.2, d
4	150.9, s	150.4, s	150.6, s	149.5, s	161.2, s	160.0, s	120.6, d
5	116.5, d	116.4, d	116.5, d	115.6, d	116.8, d	115.8, d	127.3, d
6	124.5, d	124.1, d	124.2, d	126.7, d	131.2, d	133.7, d	115.4, d
7	148.1, d	146.6, d	147.1, d	145.5, d	146.7, d	145.2, d	26.1, t
8	114.4, d	115.5, d	115.3, d	116.5, d	115.0, d	116.4, d	28.5, t
9	166.1, s	169.4, s	169.2, s	168.1, s	169.1, s	168.1, s	65.1, t
10	56.5, q	56.4, q	56.4, q	56.4, q			166.7, s
11 (1′)	143.2, s	141.9, s	143.6, s	143.6, s	169.6, s	169.6, s	41.3, t
12 (2′)	115.1, d	114.8, d	116.0, d	116.0, d	104.8, s	104.8, s	167.5, s
13 (3′)	156.3, s	156.2, s	156.7, s	156.7, s	40.3, t	40.3, t	52.6, q
4′	126.8, s	129.5, s	129.0, s	128.9, s	65.8, d	65.8, d	
5′	130.5, d	128.5, d	128.0, d	128.0, d	69.5, d	69.5, d	
6′	119.0, d	119.2, d	119.0, d	119.0, d	78.4, d	78.4, d	
7′	64.9, t	65.0, t	64.8, t	64.8, t	81.1, d	81.0, d	
8′	121.7, s	33.6, d	76.0, s	75.9, s	64.5, t	64.2, t	
9′	134.9, d	69.5, t	71.4, t	71.0, t	53.3, q	53.3, q	
10′	15.0, q	17.3, q	25.1, q	25.0, q			

[a]Recorded in CD_3OD at 100 MHz. [b]Recorded in CD_3OD at 150 MHz. [c]Recorded in $CDCl_3$ at 100 MHz.

subfraction E3. Fraction F, obtained from the elution of a 6:4 petroleum ether/acetone mixture, was separated by RP-C_{18} MPLC and eluted with a MeOH/H_2O mixture [from 2:8 to 6:4 (v/v)], yielding subfractions F1 and F2; subfraction F1 was purified by Sephadex LH-20 [1:4 (v/v) $CHCl_3$/MeOH] column chromatography to afford compound **9** (156 mg) and a mixture of **5** and **6** (17 mg). Subfraction F2 was first separated by Sephadex LH-20 column chromatography and eluted with MeOH and then purified by RP-C_{18} MPLC and eluted with a MeOH/H_2O mixture [6:4 (v/v)] to afford a mixture of **3** and **4** (9 mg).

*7-Hydroxy-8,9-dehydrothymol 9-O-trans-Ferulate (**1**).* Colorless oil: UV (MeOH) λ_{max} (log ε) 206 (4.55), 236 (4.22), 334 (4.37) nm; ^1H and ^{13}C NMR data in Tables 1 and 2, respectively; ESI-MS (+) *m/z* 379 $[M + Na]^+$; ESI-MS (−) *m/z* 355 $[M - H]^-$, 391 $[M + Cl]^-$; HRESI-MS (+) *m/z* 357.1338 $[M + H]^+$ (calcd for $C_{20}H_{21}O_6$, 357.1338).

*7-Hydroxythymol 9-O-trans-Ferulate (**2**).* Colorless oil: $[\alpha]^{26}_D$ 0 (*c* 0.21, CH_3OH); UV (MeOH) λ_{max} (log ε) 206 (4.44), 286 (4.07), 324 (4.21) nm; ^1H and ^{13}C NMR data in Tables 1 and 2, respectively; ESI-MS (+) *m/z* 381 $[M + Na]^+$; HRESI-MS (+) *m/z* 381.1321 $[M + Na]^+$ (calcd for $C_{20}H_{22}O_6Na$, 381.1314).

*7,8-Dihydroxythymol 9-O-trans-Ferulate (**3**) and 7,8-Dihydroxythymol 9-O-cis-Ferulate (**4**).* Amorphous powder: $[\alpha]^{26}_D$ 0 (*c* 0.17, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (4.49), 286 (4.05), 324 (4.20) nm; ^1H and ^{13}C NMR data in Tables 1 and 2, respectively; ESI-MS (+) *m/z* 397 $[M + Na]^+$; ESI-MS (−) *m/z* 373 $[M - H]^-$, 409 $[M + Cl]^-$; HRESI-MS (+) *m/z* 397.1265 $[M + Na]^+$ (calcd for $C_{20}H_{22}O_7Na$, 397.1263).

*Methyl (7R)-3-Deoxy-4,5-epoxy-D-manno-2-octulosonate 8-O-trans-p-Coumarate (**5**) and Methyl (7R)-3-Deoxy-4,5-epoxy-D-manno-2-octulosonate 8-O-cis-p-Coumarate (**6**).* Viscid solid: $[\alpha]^{26}_D$ 0.011 (*c* 0.24, CH_3OH); UV (MeOH) λ_{max} (log ε) 210 (4.00), 226 (3.91), 312 (4.23) nm; ^1H and ^{13}C NMR data in Tables 1 and 2, respectively; ESI-MS (+) *m/z* 381 $[M + H]^+$, 403 $[M + Na]^+$; ESI-MS (−) *m/z* 379 $[M - H]^-$, 415 $[M + Cl]^-$; HREIMS *m/z* 380.1102 $[M]^+$ (calcd for $C_{18}H_{20}O_9$, 380.1107).

*3-(2-Hydroxyphenyl)propyl Methyl Malonate (**7**).* Colorless oil: UV (MeOH) λ_{max} (log ε) 203 (3.99), 214 (3.83), 274 (3.32) nm; ^1H and ^{13}C NMR data in Tables 1 and 2, respectively; ESI-MS (+) *m/z* 253 $[M + H]^+$, 275 $[M + Na]^+$; ESI-MS (−) *m/z* 251 $[M - H]^-$, 287 $[M + Cl]^-$; HRESI-MS (+) *m/z* 275.0892 $[M + Na]^+$ (calcd for $C_{13}H_{16}O_5Na$, 275.0895).

*(2S,3R)-7-Hydroxytoxol (**11**).* Colorless oil: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.49 (1H, s, H-4), 7.34 (1H, s, H-6), 5.12 (1H, d, *J* = 3.4 Hz, H-3), 5.01 (1H, s, H-2′a), 4.94 (1H, d, *J* = 3.4 Hz, H-2), 4.84 (1H, s, H-2′b), 2.40 (1H, s, H-9), 1.65 (1H, s, H-3′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 95.1 (s, C-2), 76.3 (d, C-3), 119.1 (d, C-4), 129.1 (s, C-4a), 140.6 (s, C-5), 117.7 (d, C-6), 131.3 (s, C-7), 152.4 (s, C-7a), 198.6 (s, C-8), 26.3 (q, C-9), 140.7 (s, C-1′), 113.0 (t, C-2′), 17.2 (q, C-3′); ESI-MS (+) *m/z* 235 $[M + H]^+$, 257 $[M + Na]^+$, 491 $[2M + Na]^+$; ESI-MS (−) *m/z* 233 $[M - H]^-$, 269 $[M + Cl]^-$.

Seed Germination Bioassay. Seeds of *Ar. thaliana* were washed with ethanol [70% (v/v)] for 2 min and surface sterilized using sodium hypochlorite [0.5% (v/v)] for 2 min, followed by three washes with sterile distilled water. After surface sterilization, seeds were stored in a refrigerator at 4 °C for 3 days before being used. Three layers of filter paper were put in each 8.5 cm diameter glass Petri dish, and the filter papers were impregnated with subfractions or compounds dissolved in methanol (3 mL). Concentrations of subfractions were set at 3 and 5 mg/mL (Figure 1), while for compounds, the concentration was set at 1.0 mM (Figure 4). Two controls (filter paper treated with 3 mL of methanol or without any treatment) were set. To avoid potential effects of the organic solvent, the filter paper treated with the methanol solution was placed in a fume hood for complete solvent evaporation (for ∼1 h at 35 °C). Subsequently, 3 mL of Hoagland solution was added to each piece of filter paper in each Petri dish.[16,20] Thirty *Arabidopsis* seeds were evenly placed on the moist filter paper in each Petri dish. Each treatment had three duplicates. Seeds were allowed to germinate under a 12 h light−dark cycle at 25 °C (day) and 20 °C (night). The light intensity in the growth chamber was 100 μmol m^{-2} s^{-1}. The number of germinated seeds was recorded after treatment for 3 days, at which time most seeds (≥80%) in the control Petri dishes had germinated.[16] Data are presented as means ± the standard deviation (SD) in Figures 1 and 4. The significance of inhibitory effects of subfractions and compounds on the seed germination of *Arabidopsis* was examined by analysis of variance (ANOVA) at the $p < 0.01$ level.

Seedling Growth Bioassay. After surface sterilization and vernalization, the vigorously germinating seeds of *Ar. thaliana* were selected and evenly and carefully planted on the surface of moist soil in 12 plant cultivation pots (inside diameter of 6.5 and height of 5.5 cm); each pot contained four germinating seeds. Subsequently, the cultivation pots were placed into a growth chamber, set as the same temperature and light conditions as described in Seed Germination

Bioassay. After 4 days when the first two leaves of each seedling were observed, the 12 pots of seedlings were then divided into six groups (two pots per group). One pot of seedlings in each group were treated (cultured) with a water solution of compound **7**, **8**, **9**, **10**, or **11** or subfraction Fr.1 as a treatment, and the other pot of seedlings in the group was comparably cultured with pure water as a control. Different compounds (or Fr.1) were tested with different groups of seedlings. Each of the water solutions of test compounds and Fr.1 (for the treatments) or pure water (for the controls) was used to irrigate the individual groups of seedlings during the next 24 days. In total, the seedlings were irrigated four times with an interval of 4 days between each irrigation, and each time dropped with 1 mL the solution (or pure water) for a seedling. Concentrations of compounds **7**−**11** were set at 1.0 mM, while for Fr.1, the concentration was set at 2 mg/mL. The total fresh weights of the aerial parts of four seedlings for each treatment specialized for Fr.1 or compounds **7**−**11** or control were recorded after treatment (cultured) for 25 days. Three independent experiments for the tests described above were conducted. Data are presented as means ± SD in Figure 5. The significance was examined by ANOVA at the $p < 0.01$ level.

RESULTS AND DISCUSSION

The EtOH extract of *A. adenophora* roots was partitioned with EtOAc and 10% ethanol in H_2O, and the obtained EtOAc extract was subjected to silica gel column chromatography to remove less polar fatty acids and afford subfractions Fr.1−Fr.4. The phytotoxic activities of the four subfractions against *Arabidopsis* seed germination were tested. The results showed that Fr.1 was the most active subfraction capable of nearly completely inhibiting germination at a concentration of 3.0 mg/mL (Figure 1). This suggested that potential allelochemicals in the EtOAc extract would have been mainly partitioned and concentrated into Fr.1. Therefore, subfraction Fr.1 was further phytochemically analyzed, which eventually led to the isolation of eleven phenolic compounds, **1**−**11**.

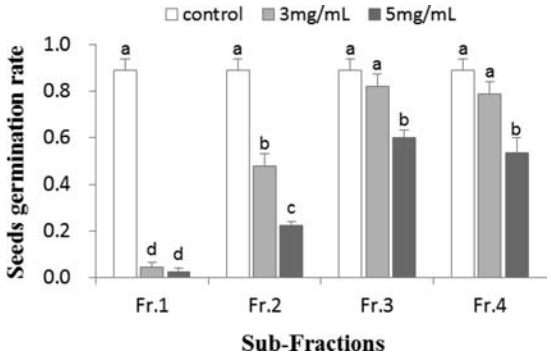

Figure 1. *Arabidopsis* seed germination rates recorded after 3 days in Hoagland medium containing subfractions (Fr.1−Fr.4) from the EtOH extract of the roots of *A. adenophora* at the indicated concentrations. Values are means ± the standard deviation from three independent experiments (30 seeds per treatment). The seed germination rate = (number of germinated seeds/30) × 100%. Significant differences are marked with different letters at the $p < 0.01$ level.

Compound **1**, obtained as a colorless oil, was deduced to have the molecular formula $C_{20}H_{20}O_6$ from pseudomolecular ions at m/z 379 $[M + Na]^+$ in positive ESI-MS and m/z 391 $[M + Cl]^-$ and 355 $[M - H]^-$ in negative ESI-MS as well as m/z 357.1338 $[M + H]^+$ in HRESI-MS (calcd for $C_{20}H_{21}O_6$, 357.1338). In the ^1H NMR spectrum (Table 1), signals for a tertiary methyl group at δ 2.11 (3H, Me-10′), a methoxy group at δ 3.90 (3H, Me-10), three olefinic protons at δ 7.73 (1H, H-7), δ 6.48 (1H, H-8), and δ 7.47 (1H, H-9′), one oxygenated methylene at δ 4.52 (2H, H_2-7′), and two sets of aromatic protons belonging to two 1,3,4-trisubsituted phenyl groups at δ 7.24 (1H, H-2), δ 6.82 (1H, H-5), and δ 7.11 (1H, H-6) and δ 6.83 (1H, H-2′), δ 7.07 (1H, H-5′), and δ 6.79 (1H, H-6′), respectively, were all observed. In the ^{13}C NMR and DEPT spectra (Table 2), 20 carbons, including two methyls (one oxygenated), one oxygenated methylene, 12 aromatic carbons (6 × C and 6 × CH), and four olefinic carbons (1 × C and 3 × CH), were displayed. These data support a preliminary construct as a planar structure of compound **1** as shown in Figure 2, in which a typical feruloyl moiety was included.[21] This proposed structure was further evidenced by detailed one-dimensional and two-dimensional (2D) NMR analysis. In the ^1H NMR spectrum, the identity of the coupling constants of signal δ_H 6.82 (H-5, d, J = 7.4 Hz) with 7.11 (H-6, br d, J = 7.4 Hz), δ_H 7.73 (H-7, d, J = 15.8 Hz) with 6.48 (H-8, d, J = 15.8 Hz), and δ_H 7.07 (H-5′, d, J = 7.4 Hz) with 6.79 (H-6′, br d, J = 7.4 Hz) supported the connectivity of C-5 with C-6, C-7 with C-8, and C-5′ with C-6′. The observation of $^1H-^{13}C$ long-range correlations of C$\underline{H_3}$-10 (δ_H 3.90), H-2 and H-5 with quaternary C-3 (δ_C 149.4), and H-2, H-5, and H-6 with quaternary C-4 (δ_C 150.9) in the HMBC spectrum (Figure 3) revealed the location of the methoxy group at C-3 (δ_C 149.4). The location of Me-10′ at C-8′ and the linkage of C-4′ with C-8′ were assigned by significant HMBC correlations of C$\underline{H_3}$-10′ (δ_H 2.11) with C-4′ (δ_C 126.8) and C-9′ (δ_C 134.9), H-9′ (δ_H 7.47) with C-4′ (δ_C 126.8), and H-5′ (δ_H 7.07) with C-8′ (δ_C 121.7). The direct linkage of C-7′ with C-1′ was supported by the observation of HMBC correlations of H-7′ (δ_H 4.52) with C-1′ (δ_C 143.2), C-2′ (δ_C 115.1), and C-6′ (δ_C 119.0). The coupling constants between H-7 and H-8 ($J_{7,8}$ = 15.8 Hz) further indicated the *trans* configuration of the −C-7-H═C-8-H− bond in the feruloyl moiety. The HMBC correlation from H-9′ (δ 7.47) to C-9 (δ 166.1) indicated the ester linkage between C-9 and C-9′. All the spectral data supported **1** as shown in Figure 2. Thus, compound **1** was identified as 7-hydroxy-8,9-dehydrothymol 9-*O*-*trans*-ferulate.

Compound **2** was obtained as a colorless oil. Its HRESI-MS spectrum exhibited a pseudomolecular ion peak at m/z 381.1321 $[M + Na]^+$ corresponding to a molecular formula of $C_{20}H_{22}O_6$ (calcd for $C_{20}H_{22}O_6Na$, 381.1314). By comparison, it was found that the ^1H and ^{13}C NMR spectroscopic data of **2** were closely related to those of **1** except that the signals for the trisubstituted −C-8′═C-9′-H− in **1** were absent in **2** (Tables 1 and 2). Instead, additional signals for an oxygenated methylene group at δ_H 4.31 (1H), δ_H 4.24 (1H), and δ_C 69.5 and a methine group at δ_H 3.52 (1H) and δ_C 33.6 were present. These findings led us to establish **2** as a molecule close to **1**, the only difference being that the double bond between C-8′ and C-9′ in **1** was replaced by a single bond. This deduction was in accordance with further HMBC analyses (Figure 3) and accounted well for the chemical shift change of the adjacent methyl group (Me-10′) from δ_H 2.11 (3H) in **1** to δ_H 1.29 (3H) in **2**. Therefore, compound **2** was identified as 7-hydroxythymol 9-*O*-*trans*-ferulate.

Compounds **3** and **4** were obtained as a mixture of isomers in an approximately 3:2 ratio, which was estimated from the intensity of signals in the ^1H NMR spectrum. The two compounds were determined to have the same molecular formula, $C_{20}H_{22}O_7$, with one more oxygen atom than **2**. The ^1H and ^{13}C NMR spectra, coupled with 2D NMR spectra, showed two closely related sets of 20 carbon signals that could be

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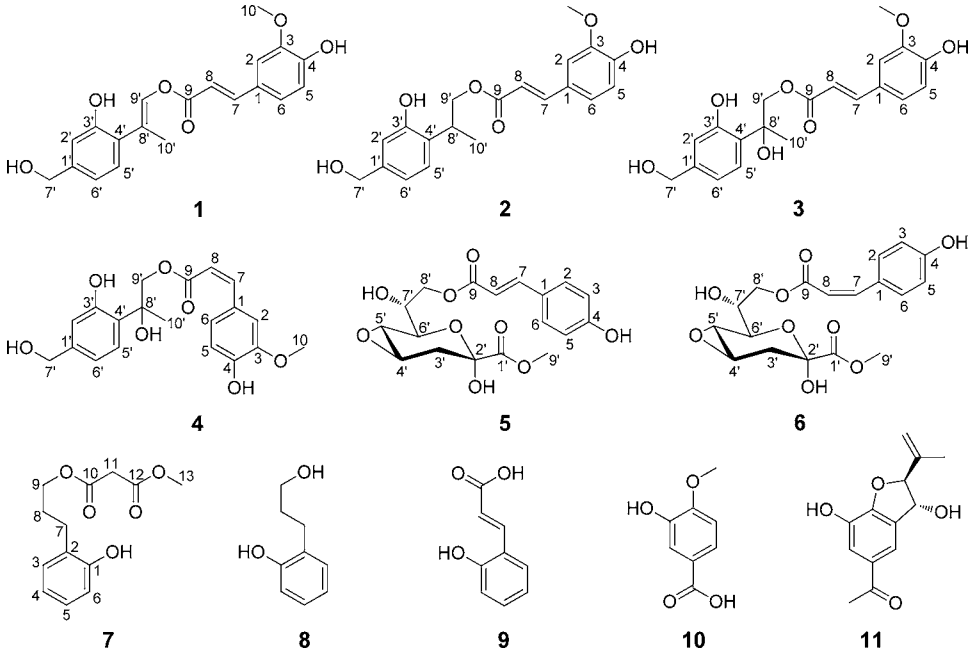

Figure 2. Structures of compounds **1**−**11**.

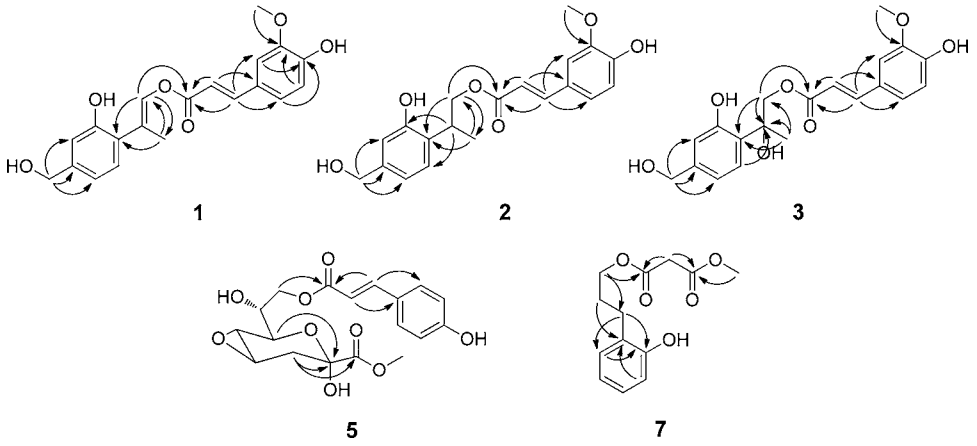

Figure 3. Significant HMBC correlations of compounds **1**−**3**, **5**, and **7**.

differentiated for assignment to compounds **3** and **4**, respectively (Tables 1 and 2). By comparison of the NMR data of **3** with those of **2**, it was found that the ^{1}H and ^{13}C NMR spectroscopic data of **3** were closely related to those of **2** except that the proton and carbon signals for the methine C-8′-H group in **2** were absent in **3**. Instead, an additional signal for an oxygenated quaternary carbon at δ_C 76.0 was present. These findings indicated that the C-8′-H group in **2** was oxygenated to a C-8′-OH group in **3**, which was in accord with the molecular formula and further supported by the observation of obvious changes in the chemical shift values from C-8′ through C-10′ (Tables 1 and 2). Thus, the structure of **3** was established as shown in Figure 2, in which the −C-7-H═C-8-H− bond in the feruloyl moiety was in the *trans* configuration. By further comparison, it was found that the major difference of the NMR spectroscopic data between **4** and **3** was the coupling constants between the two olefinic protons, H-7 and H-8 ($J_{7,8}$ = 15.9 Hz in **3**; $J_{7,8}$ = 12.9 Hz in **4**), from which the stereochemistry of the −C-7-═C-8-H− bond in **4** could be established as *cis*. Consequently, **3** and **4** were identified as 7,8-dihydroxythymol 9-*O*-*trans*-ferulate and 7,8-dihydroxythymol 9-*O*-*cis*-ferulate, respectively.

Compounds **5** and **6** were obtained as an inseparable mixture of isomers in a ratio of approximately 3:1, as estimated from the intensity of signals in the ^{1}H NMR spectrum. The molecular formula, $C_{18}H_{20}O_9$, was determined on the basis of HRESI-MS analysis (m/z 380.1102 $[M]^+$; calcd, 380.1107). The ^{1}H and ^{13}C NMR spectra, coupled with 2D NMR analysis, showed two sets of closely related resonances, which could be assigned to compounds **5** and **6**, respectively (Tables 1 and 2). Careful comparison indicated that the resonances of the NMR data from C-1′ through C-9′ of **5** and **6** were in accordance with those of methyl 2-keto-3-deoxyoctulosonate (KDO),[22] suggesting that compounds **5** and **6** each contain a KDO moiety in their molecules. Further comparison of the NMR data indicated that the resonances from C-1 through C-9 of **5** and **6** were consistent with a *trans*-*p*-coumaroyl moiety and a *cis*-*p*-coumaroyl moiety, respectively.[23] These data led us to preliminarily establish the planar structures of **5** and **6** as shown in Figure 2, which were further confirmed by the

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following spectroscopic data. In the $^1H-^1H$ COSY spectrum, signals for a H atom spin system from C-3′ through C-8′ in **5** and **6** were revealed, which supported the existence of the KDO moiety in the molecules. The hemiketal carbon in each of the KDO units was displayed in the ^{13}C NMR spectrum at δ 104.8 (s) as ascribed to C-2′. HMBC correlations (Figure 3) from H-3′ (δ_H 2.26), H-4′ (δ_H 4.14), and H-6′ (δ_H 4.48) to C-2′ (δ_C 104.8) and from H-3′ and H-9′ (δ_H 3.78) to C-1′ (δ_C 169.6) evidenced the C-1′−C-2′−C-3′ linkages and the O-bridged linkage of C-2′ with C-6′ as well as the ester bond linkage between C-1′ and C-9′. The stereochemistry of the KDO moiety was determined to be the same as that reported in the literature for authentic compound methyl (7*R*)-3-deoxy-D-*manno*-2-octulosonate,[24,25] which was supported by the small coupling constants between H-5′ and H-4′ ($J_{H5',4'}$ = 4.8 Hz) and between H-5′ and H-6′ ($J_{H5',6'}$ = 4.8 Hz) and further supported by the ROESY experiment in which key ROESY correlations of H-6′ with H-4′ and H-5′, of H-4′ with H_2-8′, and of H-5′ with H_2-8′ were displayed. The observation of the HMBC correlation of H-8′ with C-9 revealed the ester linkage between C-8′ and C-9. Therefore, **5** and **6** were identified as methyl (7*R*)-3-deoxy-4,5-epoxy-D-*manno*-2-octulosonate 8-*O*-*trans*-*p*-coumarate and methyl (7*R*)-3-deoxy-4,5-epoxy-D-*manno*-2-octulosonate 8-*O*-*cis*-*p*-coumarate, respectively.

Compound **7** was obtained as a colorless oil. Its HRESI-MS (positive) spectrum showed a $[M + Na]^+$ ion at *m*/*z* 275.0892, corresponding to the molecular formula $C_{13}H_{16}O_5$. In the ^1H NMR spectrum (Table 1), four aromatic proton signals at δ_H 7.09 (1H, H-3), δ_H 6.85 (1H, H-4), δ_H 7.07 (1H, H-5), and δ_H 6.76 (1H, H-6) and proton resonances for a methoxy group at δ_H 3.77 (s, 3H) were observed. In the ^{13}C NMR (DEPT) spectra (Table 2), 13 signals were recognized, viz., 4 × C, 4 × CH, 4 × CH_2, and 1 × CH_3, including two carbonyl group, four methylenes (one oxygenated), one methoxy group, and six sp^2 C atoms (Table 2), which suggested that an aromatic ring and one free hydroxyl group would be present in the structure. The $^1H-^1H$ COSY spectrum, coupled with HSQC analysis, allowed the establishment of two H atom spin systems, corresponding to the C-3−C-4−C-5−C-6 and C-7−C-8−C-9 structural fragments (Figure 3). The HMBC correlations of H-7 (δ_H 2.70) with C-1 (δ_C 153.8) and C-3 (δ_C 130.2) and of H-8 (δ_H 1.99) with C-2 (δ_C 127.0) indicated the linkage of C-7 with C-2. The HMBC correlations of H-11 (δ_H 3.42) with C-10 (δ_C 166.7) and C-12 (δ_C 167.5) confirmed the linkage from C-10 through C-12. Furthermore, the HMBC correlations of H-9 (δ_H 4.19) with C-10 (δ_C 166.7) and of Me-13 (δ_H 3.77) with C-12 (δ_C 167.5) revealed the two ester bond linkages of C-10 with C-9 and C-12 with Me-13, respectively. The location of the hydroxyl group at C-2 was supported by HMBC correlations of H-3 (δ_H 7.09), H-5 (δ_H 7.07), and H-6 (δ_H 6.76) with C-2 (δ_C 127). Thus, **7** was identified as 3-(2-hydroxyphenyl)propyl methyl malonate.

The four known compounds were identified as 3-(2-hydroxyphenyl)-1-propanol (**8**),[26] *o*-coumaric acid (**9**),[17] isovanillic acid (**10**),[27] and (2*S*,3*R*)-7-hydroxytoxol (**11**)[28−30] by comparison of their spectral data with literature values. In addition, the ^{13}C NMR data of **11** were assigned in this study for the first time.

All 11 phenolic compounds were tested for their phytotoxic effects on the germination of *Arabidopsis* seeds using a filter paper assay, in which the seed germination rates 3 and 6 days post-treatment were recorded. Results showed that **7**−**9** strongly inhibited seed germination at a concentration 1.0 mM, especially **7** and **9**, which were capable of nearly completely inhibiting seed germination after treatment for 3 days. Compounds **1**, **2**, **5**, **6**, and **10** showed slight inhibitory activity against *Arabidopsis* seed germination at this concentration after treatment for 3 days, but no inhibitory activities were exhibited after treatment for 6 days. No obvious inhibitory effects were displayed by the other compounds in this bioassay (see Figure 4).

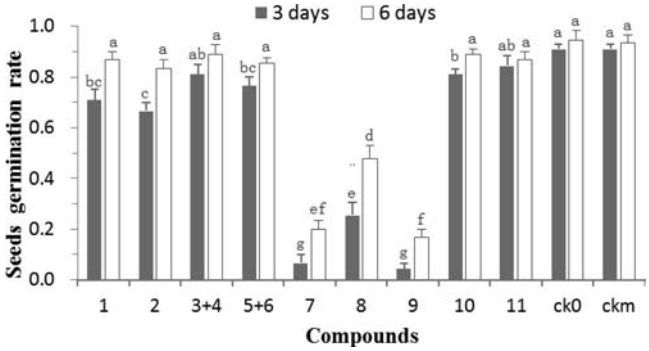

Figure 4. *Arabidopsis* seed germination rates recorded after 3 and 6 days in Hoagland medium containing one of the isolated compounds, **1**−**11** (1.0 mM), or nothing (ck0, control 1, filter paper without treatment; ckm, control 2, filter paper treated with 3 mL of methanol). Bars indicate maximal deviations from mean values derived from three independent measurements (30 seeds per treatment). The seed germination rate = (number of germinated seeds/30) × 100%. Significant differences were marked with different letters at the $p < 0.01$ level.

Limited by the quantity of the isolates, we further tested only compounds **7**−**11** together with subfraction Fr.1 for their inhibitory activity on the seedling growth of *Arabidopsis* cultivated in the soil, using a method described in Materials and Methods. The total fresh weight of the aerial parts of a fixed number of seedlings for each treatment was recorded to evaluate the inhibitory activity of Fr.1 and the tested compounds. Results displayed in Figure 5 showed that Fr.1 was active on retarding the seedling growth of *Arabidopsis* cultured in potted soil at a concentration of 2 mg/mL. Compounds **7** and **8** were also obviously active in inhibiting the

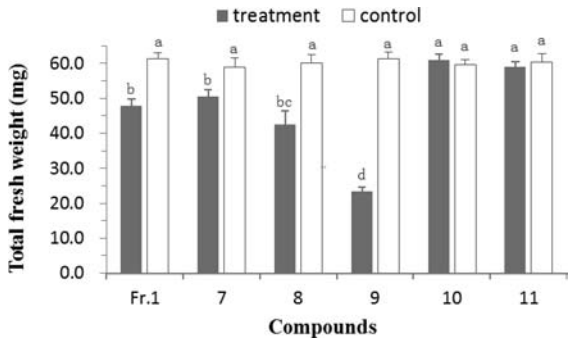

Figure 5. Total fresh weights of the aerial parts of *Ar. thaliana* seedlings (four seedlings for each treatment) growing in potted soil, cultured with water solutions of Fr.1 (2 mg/mL) or compounds **7**−**11** (1.0 mM) for the treatments, or with pure water for controls. Data were recorded after treatment for 25 days. Bars indicate maximal deviations from mean values derived from three independent measurements. Significant differences were marked with different letters at the $p < 0.01$ level.

seedling growth of *Arabidopsis* in soil at a concentration of 1.0 mM, but they were inferior to compound **9**, which showed the strongest inhibitory activity against the seedling growth of *Arabidopsis* in soil at the concentration tested. No inhibitory activities were exhibited by compounds **10** and **11** in this seedling growth bioassay (see Figure 5).

From a structural point of view, it is reasonable to deduce that compound **7** would derive from **9** via **8**. These three compounds were structurally related, and each contained a 2-hydroxyphenylpropanoid unit. It is interesting to see that **7**−**9** were the most active compounds with respect to not only inhibiting the germination of *Arabidopsis* seeds but also retarding the seedling growth of *Arabidopsis* in soil, especially for compound **9**, which showed the strongest activity in both assays. These results suggest that the 2-hydroxyphenylpropanoid unit might be necessary for compounds **7**−**9** to "maintain" their strong phytotoxic potentials.

Compounds **1**−**4** are four previously unreported thymol derivatives. Thymols, first found in *Thymus* plants,[31] were recently discovered to also be rich in *Eupatorium* species.[32−34] In the bioassay, these compounds had weak or no effects. However, it is worth noting that **1**−**4** represent a new subclass of thymol derivatives with an aromatic unit ester bond linked at C-9. **5** and **6** make up another group of phenolic compounds, with each having one 3-deoxy-D-*manno*-2-octulosonic acid (KDO) unit in the molecules. KDO was a unique natural product traditionally considered to have originated from Gram-negative bacteria.[35] It was also revealed as a component of the rhamnogalacturonan II pectin fraction of the primary cell walls of higher plants.[36,37] In our bioassay, these two compounds showed no obvious inhibitory activities against *Arabidopsis* seed germination. However, to the best of our knowledge, **5** and **6** were the first two examples of KDO present in the form of esters.

Interference of invasive plants with the growth and establishment of native plants via allelopathy has been indicated in a number of recent studies as an important contributing factor for invasive plants to achieve their invasion success. Recently, several cadinene sesquiterpenoids and a phenolic compound, *o*-coumaric acid, were revealed from *A. adenophora* and showed phytotoxic effects.[13−17] However, those studies of potential allelochemicals of *A. adenophora* have so far only concentrated on the aerial parts of the plant, and few reports have ever focused on the roots. In this study, 11 structurally diverse phenolic compounds were isolated from the roots of *A. adenophora*, of which seven were new compounds. Bioassays showed that three of them were not only remarkably active against *Arabidopsis* seed germination but also obviously active in retarding the seedling growth of *Arabidopsis* in the soil, especially for compound **9**, which showed the best bioactivity. These findings, to some extent, further explain the strong phytotoxic effects reported for the root extracts and leachates of *A. adenophora*.[10,12] The question of whether these compounds are directly involved in the allelopathy as functional allelochemicals and contribute to the invasive success of this plant still needs to be answered.

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Notes

The authors declare no competing financial interest.

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