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Phenolic Constituents from the Roots of *Mikania micrantha* and Their Allelopathic Effects

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ABSTRACT: Four new thymol derivatives, 8,10-dihydroxy-9-benzoyloxythymol (**1**), 9-isobutyryloxy-10-hydroxythymol (**2**), 7,8,9,10-tetrahydroxythymol (**3**), and 7,8,10-trihydroxy-9-*E*-feruloyloxythymol (**4**), were isolated from the fresh roots of *Mikania micrantha*, along with 8,9,10-trihydroxythymol (**5**), 8,10-dihydroxy-9-acetoxythymol (**6**), 8,10-dihydroxy-9-isobutyryloxythymol (**7**), 8,10-dihydroxy-9-(2-methylbutyryloxy)thymol (**8**), 8,9-dehydro-10-hydroxythymol (**9**), 8-methoxy-9-hydroxythymol (**10**), ethyl caffeate (**11**), ethyl ferulate (**12**), 3,5-di-*O*-caffeoylquinic acid (**13**), and mikanin (**14**). Their structures were determined by spectroscopic methods. The known thymol derivatives (**5**−**10**) were obtained from the genus *Mikania* for the first time. Allelopathic effects of these compounds on *Arabidopsis thaliana* seeds were evaluated by a filter paper assay. After the treatment at 0.1 mM for 4 days, the seed germination rate with compound **8** was 48% and the inhibitory rates of shoot growth with compounds **1**, **2**, **7**−**10**, and **12** were over 40%. The IC_{50} values of compounds **1** and **8** on shoot growth were 342.5 and 625 μM, respectively.

KEYWORDS: *Mikania micrantha, root, thymols, phenolic acids, allelopathy, Arabidopsis thaliana seeds*

INTRODUCTION

Mikania micrantha Kunth (Asteraceae), a perennial creeping vine native to tropical Central and South America, has been spread to the Pacific islands and southeast Asia, including southern China, since the early 20th century. Known by the common name of "mile-a-minute", it grows fast in non-native areas away from its natural enemies and becomes an invasive pest to plantation crops and commercial forests.[1]

Allelopathy has been considered to be an important invasive strategy of exotic plants.[2] It was reported that the seed germination percentage and radicle length of Chinese cabbage and tomato decreased progressively when their seeds were exposed to increasing concentrations of the aqueous extract of *M. micrantha* leaves.[3] From the aerial parts of the plant, four sesquiterpene dilactones and a flavonol glucoside were obtained, and their allelopathic effects on certain plants were reported.[4,5] On the other hand, *M. micrantha* has well-developed roots. The aqueous extract of the roots was reported to exhibit inhibitory activity against the seed germination and seedling growth of *Coix lacryma-jobi*.[6] Field experiments also showed that the average shoot lengths of *Panicum* grown in *Mikania* rhizosphere soil were shorter by 21% when compared to those grown in non-*Mikania* soil, and a higher level of water-soluble phenolics and lower microbial activity were observed in *Mikania* rhizosphere soil.[7] However, chemicals involved in the root allelopathy have not yet been discovered.

The objective of this research was to isolate and identify chemical compounds in the fresh roots of *M. micrantha* and to evaluate their allelopathic activity.

MATERIALS AND METHODS

General Experimental Procedures. Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for column chromatography. Medium-pressure liquid chromatography (MPLC) was performed on an EZ Purifier (Lisure Science, Suzhou, China), and the columns used were 400 × 40 mm inner diameter and 400 × 25 mm inner diameter, 20−45 μm, Chromatorex RP-18 SMB100 (Shanghai Lisui E-Tech Co., Shanghai, China). Preparative high-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6AD pump (Shimadzu, Kyoto, Japan) connected to a Shimadzu RID-10A refractive index detector (Shimadzu), and the column used was 250 × 20 mm inner diameter, 5 μm, YMC-Pack ODS-A with a 23 × 4 mm inner diameter guard column of the same material (YMC Co., Kyoto, Japan). Nuclear magnetic resonance (NMR) spectra, including ^{1}H and ^{13}C NMR, $^{13}C-^{1}H$ correlation spectroscopy (COSY), and heteronuclear multiple-bond correlation (HMBC), were recorded on a Bruker DRX-400 instrument (Bruker BioSpin Gmbh, Rheinstetten, Germany) in deuterated methanol (CD_3OD) with the solvent residual peaks of δ_H 3.31 and δ_C 49.0 ppm as reference. Electrospray ionization mass spectrometry (ESI−MS) was acquired on a MDS SCIEX API 2000 LC/MS/MS apparatus (Applied Biosystems, Inc., Forster, CA). High-resolution (HR)-ESI−MS was measured on a Bruker Bio TOF IIIQ mass spectrometer (Bruker Daltonics, Inc., Billerica, MA). The zoom-stereo microscope SMZ-T4OP (Optec, Chongqing, China) and OPTPro 2008 digital microscopic image processing system (Optec) were used for the counting of seeds and measurement of shoot lengths. The commercial

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herbicide, Harness, was purchased from Monsanto Co., (St. Louis, MO). The others were the same as in a previous paper.[8]

Plant Material. Fresh roots of *M. micrantha* were collected from the countryside near Guangzhou in November 2011 and botanically authenticated by Prof. Huagu Ye, South China Botanical Garden, Chinese Academy of Sciences. A voucher specimen (21751) was deposited at the Herbarium of South China Botanical Garden, Chinese Academy of Science. *Arabidopsis thaliana* seeds (Columbia ecotype) were obtained from Max Planck Institute for Chemical Ecology, Jena, Germany.

Extraction and Isolation. The fresh roots were rinsed and manually cut to pieces (4050 g) and then immediately immersed in 55% aqueous ethanol twice (20 and 16 L) at room temperature (16−25 °C) for 3 days each time. Filtration and condensation of the solution under vacuum gave an extract (266.1 g). The extract (264.5 g) was subjected to silica gel (2600 g) column (602 × 116 mm inner diameter) chromatography using chloroform ($CHCl_3$)/methanol (MeOH) [1:9, 2:8, 3:7, 4:6, and 5:5 (v/v) in 26.4, 26.4, 26.4, 26.4, and 20.0 L, respectively] as an eluent to afford fractions 1−14 after being pooled according to their thin-layer chromatography (TLC) profiles. Fraction 1 (5.98 g) was separated by MPLC and 400 × 40 mm inner diameter column using MeOH/H_2O [2:8, 3:7, 4:6, 5:5, 6:4, and 7:3 (v/v) in 2000 mL each] as an eluent to furnish fractions 1-1−1-20. Fraction 1-5 was further separated by Sephadex LH-20 column (1280 × 23 mm inner diameter) chromatography using MeOH as an eluent, and the resultant two fractions were individually purified by preparative HPLC using 50% aqueous MeOH (v/v, the same below) as the mobile phase at the flow rate of 5 mL/min to afford compound **6** [retention time (t_R) of 30.5 min, 162.5 mg] and 43% aqueous MeOH as the mobile phase at the flow rate of 5 mL/min to yield compound **4** (t_R of 52.0 min, 5.6 mg). Fraction 1-7 was further separated by LH-20 column (1280 × 23 mm inner diameter) chromatography and purified by preparative HPLC using aqueous MeOH as the mobile phase at the flow rate of 5 mL/min to give compounds **7** (50% MeOH, t_R of 70.2 min, 235.6 mg), **9** (41% MeOH, t_R of 74.3 min, 12.9 mg), **10** (50% MeOH, t_R of 57.6 min, 4.1 mg), and **11** (48% MeOH, t_R of 56.0 min, 34.2 mg). Fraction 1-10 was further separated by LH-20 column (1215 × 14 mm inner diameter) chromatography using MeOH as an eluent to yield compound **12** (3.2 mg). Fraction 1-11 was further separated by Sephadex LH-20 column (1215 × 14 mm inner diameter) chromatography and purified by preparative HPLC using 58% aqueous MeOH as the mobile phase at the flow rate of 5 mL/min to furnish compounds **2** (t_R of 44.9 min, 4.4 mg) and **8** (t_R of 48.7 min, 27.6 mg). Fraction 1-14 was successively separated by Sepahdex LH-20 (1280 × 23 mm inner diameter) and silica gel (520 × 10 mm inner diameter) column chromatography to yield compound **1** (16.5 mg). Fraction 3 (4.39 g) was separated by MPLC and 400 × 40 mm inner diameter column using MeOH/H_2O [1:9, 2:8, 3:7, 4:6, 5:5, 65:35, 75:25, and 9:1 (v/v) in 2000 mL each] as an eluent to give fractions 3-1−3-17. Fraction 3-1 was further separated by LH-20 column (1215 × 14 mm inner diameter) chromatography and purified by preparative HPLC using 20% aqueous MeOH as the mobile phase at the flow rate of 5 mL/min to yield compound **3** (t_R of 16.1 min, 1.4 mg). Fraction 3-3 was further separated by LH-20 column (1280 × 23 mm inner diameter) chromatography and purified by preparative HPLC using 18% aqueous MeOH as the mobile phase at the flow rate of 5 mL/min to furnish compound **5** (t_R of 55.6 min, 485.1 mg). Fraction 3-6 was further separated by LH-20 column (1215 × 14 mm inner diameter) chromatography using MeOH as an eluent to give compound **14** (12.1 mg). Fraction 9 (9.22 g) was subjected to silica gel (450 g) column (950 × 52 mm inner diameter) chromatography using $CHCl_3$/MeOH [10:0, 9:1, 85:15, 8:2, 75:25, 7:3, 6:4, and 5:5 (v/v) in 5.5 L each] as an eluent to yield fractions 9-1−9-43. Fraction 9-21 (2.5 g) was separated by MPLC and 400 × 25 mm inner diameter column using MeOH/H_2O [1:9, 2:8, 3:7, 4:6, and 5:5 (v/v) in 1000 mL each] as an eluent to give fractions 9-21-1−9-21-29. Fraction 9-21-18 was further separated by LH-20 column (1215 × 14 mm inner diameter) chromatography using MeOH as an eluent to afford compound **13** (19.3 mg).

8,10-Dihydroxy-9-benzoyloxythymol (1). Colorless oil. $[\alpha]_D^{20}$ +1.4 (*c* 0.145, MeOH). UV (MeOH) λ_{max} nm (log ε): 225 (4.05), 282 (3.32). HR-ESI−MS positive *m/z*: 325.1033 $[M + Na]^+$ (calcd for $C_{17}H_{18}O_5Na^+$, 325.1046; error, 4.0 ppm). ESI−MS positive *m/z*: 325 $[M + Na]^+$. ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) in Table 1.

Table 1. ^1H and ^{13}C NMR Data of Compounds 1 and 2 in CD_3OD

	1		2	
C/H	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1		140.0		138.7
2	6.62 (d, 1.7)	118.1	6.60 (br s)	116.9
3		156.6		156.5
4		124.0		123.8
5	7.25 (d, 8.0)	128.5	6.97 (d, 7.6)	129.7
6	6.66 (dd, 8.0, 1.7)	121.3	6.59 (br d, 7.6)	121.1
7	2.23 (3H, s)	21.0	2.22 (3H, s)	21.1
8		78.7	3.46 (pentet, 6.4)	42.5
9	4.75 (d, 11.3)	69.0	4.40 (dd, 11.0, 6.4)	65.5
	4.68 (d, 11.3)		4.36 (dd, 11.0, 6.4)	
10	4.02 (d, 11.4)	66.7	3.81 (2H, d, 6.4)	63.3
	3.94 (d, 11.4)			
1′		131.4		178.9
2′	7.93 (dd, 7.8, 1.5)	130.6	2.48 (heptet, 7.0)	35.2
3′	7.41 (br t, 7.8)	129.4	1.07 (3H, d, 7.0)	19.3
4′	7.55 (tt, 7.8, 1.5)	134.1	1.06 (3H, d, 7.0)	19.3
5′	7.41 (br t, 7.8)	129.4		
6′	7.93 (dd, 7.8, 1.5)	130.6		
7′		168.1		

9-Isobutyloxy-10-hydroxythymol (2). Colorless oil. $[\alpha]_D^{20}$ −1.2 (*c* 0.167, MeOH). UV (MeOH) λ_{max} nm (log ε): 218 (3.75), 282 (3.06). HR-ESI−MS positive *m/z*: 275.1255 $[M + Na]^+$ (calcd for $C_{14}H_{20}O_4Na^+$, 275.1254; error, −0.3 ppm). ESI−MS positive *m/z*: 275 $[M + Na]^+$. ESI−MS negative *m/z*: 251 $[M - H]^-$. ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) in Table 1.

7,8,9,10-Tetrahydroxythymol (3). Colorless oil. UV (MeOH) λ_{max} nm (log ε): 218 (3.87), 278 (3.47). HR-ESI−MS negative *m/z*: 213.0764 $[M - H]^-$ (calcd for $C_{10}H_{13}O_5^-$, 213.0768; error, 2.0 ppm). ESI−MS positive *m/z*: 237 $[M + Na]^+$. ESI−MS negative *m/z*: 213 $[M - H]^-$. ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) in Table 2.

7,8,10-Trihydroxy-9-*E*-feruloyloxythymol (4). Colorless oil. $[\alpha]_D^{20}$ +0.6 (*c* 0.310, MeOH). UV (MeOH) λ_{max} nm (log ε): 218 (4.01), 285 (3.74), 327 (3.84). HR-ESI−MS positive *m/z*: 413.1208 $[M + Na]^+$ (calcd for $C_{20}H_{22}O_8Na^+$, 413.1207; error, 0.2 ppm). ESI−MS positive *m/z*: 413 $[M + Na]^+$. ESI−MS negative *m/z*: 389 $[M - H]^-$, 425 $[M + Cl]^-$. ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) in Table 2.

8,9,10-Trihydroxythymol (5). Colorless oil. ESI−MS positive *m/z*: 221 $[M + Na]^+$. ESI−MS negative *m/z*: 197 $[M - H]^-$, 233 $[M + Cl]^-$. ^1H NMR (400 MHz, CD_3OD) δ: 6.59 (1H, d, *J* = 1.7 Hz, H-2), 7.17 (1H, d, *J* = 7.8 Hz, H-5), 6.64 (1H, dd, *J* = 7.8 and 1.7 Hz, H-6), 2.20 (3H, s, H_3-7), 3.88 (2H, d, *J* = 12.0 Hz, H_2-9), 3.85 (2H, d, *J* = 12.0 Hz, H_2-10). ^{13}C NMR (100 MHz, CD_3OD) δ: 139.6 (C-1), 118.2 (C-2), 157.0 (C-3), 124.4 (C-4), 128.6 (C-5), 121.2 (C-6), 21.0 (C-7), 79.9 (C-8), 66.5 (C-9), 66.5 (C-10).

8,10-Dihydroxy-9-acetoxythymol (6). Colorless oil. $[\alpha]_D^{20}$ 0 (*c* 0.310, $CHCl_3$). ESI−MS positive *m/z*: 263 $[M + Na]^+$. ESI−MS negative *m/z*: 239 $[M - H]^-$, 275 $[M + Cl]^-$. ^1H NMR (400 MHz, CD_3OD) δ: 6.61 (1H, d, *J* = 1.7 Hz, H-2), 7.17 (1H, d, *J* = 8.0 Hz, H-5), 6.64 (1H, dd, *J* = 8.0 and 1.7 Hz, H-6), 4.53 (1H, d, *J* = 11.4 Hz, H-9), 4.46 (1H, d, *J* = 11.4 Hz, H-9), 3.90 (1H, d, *J* = 11.4 Hz, H-10), 3.85 (1H, d, *J* = 11.4 Hz, H-10), 2.23 (3H, s, H_3-7), 1.98 (3H, s, H_3-2′). ^{13}C NMR (100 MHz, CD_3OD) δ: 139.9 (C-1), 118.1 (C-2), 156.5

Table 2. ^{1}H and ^{13}C NMR Data of Compounds 3 and 4 in CD_3OD

	3		4	
C/H	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C
1		143.6		143.7
2	6.77 (d, 1.8)	116.1	6.81 (d, 1.8)	116.0
3		157.3		156.8
4		126.5		126.0
5	7.29 (d, 7.8)	128.9	7.35 (d, 7.9)	128.9
6	6.80 (dd, 7.8, 1.8)	118.8	6.84 (dd, 7.9, 1.8)	118.9
7	4.51 (2H, s)	64.8	4.48 (2H, s)	64.8
8		80.0		78.7
9	3.89 (1H, d, 11.8)	66.5	4.69 (d, 11.4)	68.4
	3.87 (1H, d, 11.8)		4.56 (d, 11.4)	
10	3.89 (1H, d, 11.8)	66.5	3.98 (d, 11.4)	66.7
	3.87 (1H, d, 11.8)		3.89 (d, 11.4)	
1′				127.7
2′			7.14 (d, 2.0)	111.6
3′				149.4
4′				150.6
5′			6.80 (d, 8.1)	116.4
6′			7.02 (dd, 8.1, 2.0)	124.2
7′			7.55 (d, 15.9)	147.0
8′			6.29 (d, 15.9)	115.3
9′				169.2
OCH_3			3.87 (3H, s)	56.4

(C-3), 123.7 (C-4), 128.5 (C-5), 121.2 (C-6), 21.0 (C-7), 78.4 (C-8), 68.2 (C-9), 66.5 (C-10), 172.9 (C-1′), 20.7 (C-2′).

8,10-Dihydroxy-9-isobutyryloxythymol (7). Colorless oil. $[\alpha]_D^{20}$ −0.1 (*c* 0.104, $CHCl_3$). ESI−MS positive *m*/*z*: 291 $[M + Na]^+$. ESI−MS negative *m*/*z*: 267 $[M - H]^-$, 303 $[M + Cl]^-$. ^{1}H NMR (400 MHz, CD_3OD) δ: 6.59 (1H, d, *J* = 1.8 Hz, H-2), 7.16 (1H, d, *J* = 7.8 Hz, H-5), 6.63 (1H, dd, *J* = 7.8 and 1.8 Hz, H-6), 2.23 (3H, s, H_3-7), 4.57 (1H, d, *J* = 11.2 Hz, H-9), 4.40 (1H, d, *J* = 11.2 Hz, H-9), 3.91 (1H, d, *J* = 11.4 Hz, H-10), 3.84 (1H, d, *J* = 11.4 Hz, H-10), 2.47 (1H, heptet, *J* = 7.0 Hz, H-2′), 1.06 (3H, d, *J* = 7.0 Hz, H_3-3′), 1.03 (3H, d, *J* = 7.0 Hz, H_3-4′). ^{13}C NMR (100 MHz, CD_3OD) δ: 139.9 (C-1), 118.0 (C-2), 156.6 (C-3), 123.9 (C-4), 128.6 (C-5), 121.1 (C-6), 21.0 (C-7), 78.7 (C-8), 68.2 (C-9), 66.6 (C-10), 178.8 (C-1′), 35.1 (C-2′), 19.2 (C-3′), 19.1 (C-4′).

8,10-Dihydroxy-9-(2-methylbutyryloxy)thymol (8). Colorless oil. $[\alpha]_D^{20}$ 0 (*c* 0.128, $CHCl_3$). ESI−MS positive *m*/*z*: 305 $[M + Na]^+$. ESI−MS negative *m*/*z*: 281 $[M - H]^-$, 317 $[M + Cl]^-$. ^{1}H NMR (400 MHz, CD_3OD) δ: 6.59 (1H, br s, H-2), 7.16 (1H, d, *J* = 7.9 Hz, H-5), 6.63 (1H, br d, *J* = 7.9 Hz, H-6), 4.58 (1H, d, *J* = 11.4 Hz, H-9), 4.39 (1H, d, *J* = 11.4 Hz, H-9), 3.91 (1H, d, *J* = 11.4 Hz, H-10), 3.83 (1H, dd, *J* = 11.4 Hz, H-10), 2.23 (3H, s, H_3-7), 2.31 (1H, m, H-2′), 1.54 (1H, m, H-3′), 1.37 (1H, m, H-3′), 0.78 (3H, t, *J* = 7.5 Hz, H_3-4′), 1.02 (3H, d, *J* = 7.3 Hz, H_3-5′). ^{13}C NMR (100 MHz, CD_3OD) δ: 139.9 (C-1), 118.0 (C-2), 156.6 (C-3), 123.9 (C-4), 128.6 (C-5), 121.1 (C-6), 21.0 (C-7), 78.6 (C-8), 68.2 (C-9), 66.7 (C-10), 178.4 (C-1′), 42.2 (C-2′), 27.8 (C-3′), 11.8 (C-4′), 17.0 (C-5′).

8,9-Dehydro-10-hydroxythymol (9). Colorless oil. ESI−MS positive *m*/*z*: 165 $[M + H]^+$, 187 $[M + Na]^+$. ESI−MS negative *m*/*z*: 163 $[M - H]^-$, 199 $[M + Cl]^-$. ^{1}H NMR (400 MHz, CD_3OD) δ: 6.60 (1H, br s, H-2), 7.00 (1H, d, *J* = 8.2 Hz, H-5), 6.61 (1H, br d, *J* = 8.2 Hz, H-6), 2.24 (3H, s, H_3-7), 5.36 (1H, d, *J* = 2.1 Hz, H-9), 5.16 (1H, d, *J* = 2.1 Hz, H-9), 4.36 (2H, br s, H_2-10). ^{13}C NMR (100 MHz, CD_3OD) δ: 139.9 (C-1), 117.2 (C-2), 155.4 (C-3), 125.9 (C-4), 130.9 (C-5), 121.3 (C-6), 21.1 (C-7), 149.3 (C-8), 65.9 (C-9), 114.5 (C-10).

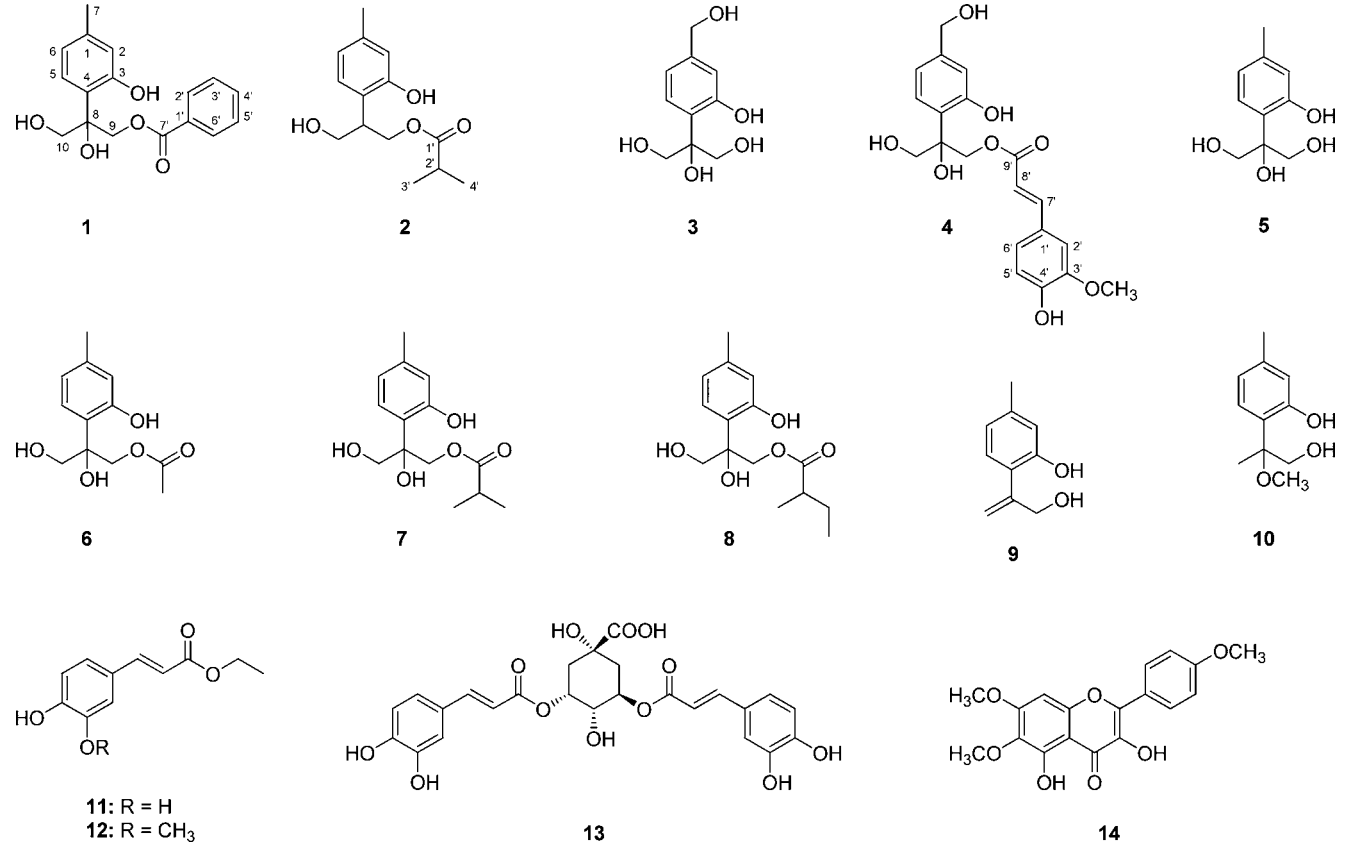

Figure 1. Structures of compounds **1**−**14** from the roots of *M. micrantha*.

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8-Methoxy-9-hydroxythymol (10). Colorless oil. $[\alpha]_D^{20}$ 0 (*c* 0.142, $CHCl_3$). ESI−MS positive *m/z*: 219 $[M + Na]^+$. ESI−MS negative *m/z*: 195 $[M - H]^-$. ^1H NMR (400 MHz, CD_3OD) δ: 6.61 (1H, d, *J* = 1.7 Hz, H-2), 7.01 (1H, d, *J* = 8.0 Hz, H-5), 6.66 (1H, dd, *J* = 8.0 and 1.7 Hz, H-6), 2.25 (3H, s, H_3-7), 3.74 (1H, d, *J* = 11.5 Hz, H-9), 3.62 (1H, d, *J* = 11.5 Hz, H-9), 1.60 (3H, s, H_3-10), 3.23 (3H, s, OCH_3). ^{13}C NMR (100 MHz, CD_3OD) δ: 140.4 (C-1), 118.2 (C-2), 157.1 (C-3), 123.7 (C-4), 129.2 (C-5), 121.5 (C-6), 21.0 (C-7), 83.5 (C-8), 69.0 (C-9), 20.1 (C-10), 50.9 (OCH_3).

Allelopathy Bioassay. Allelopathic effects of the obtained compounds on the seed germination and shoot growth of *A. thaliana* were assessed by a filter paper assay as previously described,[9] with slight modification. In brief, the compounds were dissolved in MeOH to the concentration of 100 mM and then diluted with distilled water to 1.0 mM. For each test solution, 1.65 mL was added to sterile three layers of filter paper in a glass Petri dish (60 mm inner diameter), and 1.65 mL of distilled water containing 1% aqueous MeOH (v/v) was used as a solvent control. Three replicates were set for each treatment. A total of 40 surface-sterilized seeds of *A. thaliana* were evenly placed into each Petri dish and allowed to germinate at room temperature (15−20 °C) without extra light treatment. The numbers of germinated seeds were counted after 4 days when over 90% seeds of the control were germinated. The shoot lengths were measured after 4 days using the zoom-stereo microscope SMZ-T4 and OPTPro 2008 digital microscopic image processing system. Compounds **1** and **8** were diluted 2-fold to 500, 250, 125, and 62.5 μM for a dose-dependent experiment. A commercial herbicide, Harness (common name Acetochlor), was used as a positive control. Three replicates, 20 seeds each, were set for each treatment, and shoot lengths were measured after 5 days.

Values were expressed as the mean ± standard deviation (SD). For statistical analysis, a normality test ($p > 0.05$) was evaluated before one-way analysis of variance (ANOVA) and Tukey's test was used to determine the differences among these means. Half-maximal inhibitory concentration (IC_{50}) values were calculated by the SPSS 16.0 statistic software.

RESULTS AND DISCUSSION

The aqueous ethanolic extract of the fresh roots of *M. micrantha* was successively separated by column chromatography, MPLC, and preparative HPLC to yield 14 phenolic compounds, including four new thymol derivatives (**1**−**4**).

Compound **1** was obtained as a colorless oil and deduced to have the molecular formula $C_{17}H_{18}O_5$ from the HR-ESI−MS molecular ion and NMR data. The ^1H NMR spectrum (Table 1) showed signals composed of three ABX-type aromatic protons at δ 6.62 (1H, d, *J* = 1.7 Hz, H-2), 7.25 (1H, d, *J* = 8.0 Hz, H-5), and 6.66 (1H, dd, *J* = 8.0 and 1.7 Hz, H-6) attributable to a 1,3,4-trisubstituted benzene ring, four methylene protons at δ 4.75 and 4.68 (1H each, d, *J* = 11.3 Hz, H_2-9) and 4.02 and 3.94 (1H each, d, *J* = 11.4 Hz, H_2-10), and methyl protons at δ 2.23 (3H, s, H_3-7) linked to an aromatic ring. Moreover, signals of five aromatic protons at δ 7.93 (2H, dd, *J* = 7.8 and 1.5 Hz), 7.55 (1H, tt, *J* = 7.8 and 1.5 Hz), and 7.41 (2H, br t, *J* = 7.8 Hz) suggested the presence of a monosubstituted aromatic ring. The ^{13}C NMR spectrum (Table 1) exhibited a total of 17 carbon signals composed of 12 aromatic carbons, one carboxylic carbon at δ 168.1 (C-7′), three oxygenated alkyl carbons at δ 78.7 (C-8), 69.0 (C-9), and 66.7 (C-10), and a methyl carbon at δ 21.0 (C-7). These data were characteristic of a thymol nucleus and a benzoyl moiety.[10] In the HMBC spectrum, the long-range correlations from H_2-9 and H_2-10 to δ 124.0 (C-4), H-5 to δ 140.0 (C-1), 156.6 (C-3), and 78.7 (C-8), and H_3-7 to δ 118.1 (C-2) and 121.3 (C-6) confirmed the thymol nucleus. Furthermore, the HMBC correlations from H_2-9 to C-7′ revealed the connection of the benzoyl moiety to C-9. Therefore, compound **1** was determined as 8,10-dihydroxy-9-benzoyloxythymol, as shown in Figure 1.

Compound **2** was deduced to have the molecular formula $C_{14}H_{20}O_4$ from the molecular ion in the HR-ESI−MS spectrum and NMR data. The ^1H NMR spectrum (Table 1) exhibited signals readily recognized for three ABX-type aromatic protons at δ 6.60 (1H, br s, H-2), 6.97 (1H, d, *J* = 7.6 Hz, H-5), and 6.59 (1H, br d, *J* = 7.6 Hz, H-6), two oxygenated methylenes at δ 4.40 and 4.36 (1H each, dd, *J* = 11.0 and 6.4 Hz, H_2-9) and 3.81 (2H, d, *J* = 6.4 Hz, H_2-10), and a tertiary methyl at δ 2.22 (3H, s, H_3-7). The ^{13}C NMR spectrum (Table 1) displayed a total of 14 carbon signals, including six aromatic carbons ranging from δ 156.5 to 116.9 and two oxygenated methylene carbons at δ 65.5 (C-9) and 63.3 (C-10), which were also typical of a thymol nucleus. The HMBC correlations from δ 1.07 and 1.06 (3H each, d, *J* = 7.0 Hz) to δ 178.9 (C-1′) and 35.2 (C-2′) revealed the presence of an isobutyryl moiety.[11] Furthermore, the HMBC correlation from H_2-9 to C-1′ clarified the connection of the acyl moiety to C-9. Hence, compound **2** was identified as 9-isobutyryloxy-10-hydroxythymol.

Compound **3** was assigned the molecular formula $C_{10}H_{14}O_5$ on the basis of the HR-ESI−MS molecular ion and NMR data. The ^1H NMR spectrum (Table 2) displayed three ABX-type aromatic protons, four doublets at δ 3.89 and 3.87 (2H each, d, *J* = 11.8 Hz) assignable for two oxygenated methylenes, and two singlet protons for an oxygenated methylene at δ 4.51 (2H, H_2-7). The ^{13}C NMR spectrum (Table 2) showed a total of 10 carbon signals, which were similar to 8,9,10-trihydroxythymol (**5**), except for the significant downfield shift at C-7 (+43.8 ppm) and slight changes at C-1 (+4.0 ppm), C-2 (−1.9 ppm), and C-6 (−2.4 ppm), suggesting the connection of a hydroxyl group to C-7. Consequently, compound **3** was established as 7,8,9,10-tetrahydroxythymol.

Compound **4** was deduced the molecular formula $C_{20}H_{22}O_8$ from the HR-ESI−MS molecular ion and NMR data. The ^1H and ^{13}C NMR spectra (Table 2) showed signals of three ABX-type aromatic protons, six oxygenated methylene protons, and 10 carbons, which were very close to those of compound **3**. Moreover, the ^1H and ^{13}C NMR spectra exhibited signals of a pair of olefinic protons at δ 7.55 and 6.29 (1H each, d, *J* = 15.9 Hz), three additional ABX-type aromatic protons at δ 7.14 (1H, d, *J* = 2.0 Hz), 6.80 (1H, d, *J* = 8.1 Hz), and 7.02 (1H, dd, *J* = 8.1 and 2.0 Hz), methoxy protons at δ 3.87 (3H, s), and nine carbons, including a carboxyl at δ 169.2 (C-9′), which suggested the presence of an *E*-feruloyl moiety.[12] Furthermore, the long-range HMBC correlations from δ 4.69 and 4.56 (1H each, d, *J* = 11.4 Hz, H_2-9) to C-9′ and δ 4.48 (2H, s, H_2-7) to δ 116.0 (C-2) and 118.9 (C-6) revealed the connections of the acyl moiety to C-9 and a hydroxymethyl group to C-1. According, compound **4** was clarified as 7,8,10-trihydroxy-9-*E*-feruloyloxythymol.

The known phenolic compounds were identified as 8,9,10-trihydroxythymol (**5**),[13] 8,10-dihydroxy-9-acetoxythymol (**6**),[14] 8,10-dihydroxy-9-isobutyryloxythymol (**7**),[15] 8,10-dihydroxy-9-(2-methylbutyryloxy)thymol (**8**),[16] 8,9-dehydro-10-hydroxythymol (**9**),[17] 8-methoxy-9-hydroxythymol (**10**),[10] ethyl caffeate (**11**),[18] ethyl ferulate (**12**),[19] 3,5-di-*O*-caffeoylquinic acid (**13**),[20] and mikanin (**14**)[21] by analysis of their ^1H and ^{13}C NMR and ESI−MS data and a comparison of the data to the literature.

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Compounds **1**−**10**, **12**, and **13** were evaluated for their allelopathic effects on the seed germination and shoot growth of *A. thaliana* using a filter paper assay.[9] As shown in Table 3,

Table 3. Allelopathic Effects of the Isolated Compounds at the Concentration of 1.0 mM on the Seed Germination and Shoot Growth of *A. thaliana*[a]

compound	seed germination rate (%)	inhibitory rate of shoot length (%)
1	86.0 ± 4.0 a	96.4 ± 0.7 c
2	78.7 ± 3.2 a	45.0 ± 13.6 b
3	94.5 ± 4.8 a	22.1 ± 15.7 ab
4	81.6 ± 7.2 a	26.4 ± 15.7 ab
5	89.3 ± 2.5 a	12.9 ± 17.1 ab
6	94.2 ± 2.9 a	37.8 ± 12.1 b
7	86.5 ± 6.2 a	41.4 ± 10.7 b
8	48.0 ± 8.5 b	85.7 ± 14.3 c
9	87.1 ± 7.5 a	45.0 ± 11.4 b
10	86.9 ± 1.3 a	49.3 ± 8.6 bc
12	88.2 ± 5.9 a	47.8 ± 8.6 b
13	79.4 ± 9.6 a	12.9 ± 14.3 ab
control	94.3 ± 2.2 a	0 ± 11.4 a

[a]Each value represents the mean ± SD (n = 3). For each treatment, the means within the column followed by different letters were significantly different at the $p < 0.05$ level.

the seed germination rates of *A. thaliana* treated with test compounds at the concentration of 1.0 mM for 4 days were in the range of 78.7−94.5%, except for compound **8**, with which the germination rate was only 48.0%. On the other hand, the inhibitory rates of shoot length treated with compounds **1**, **2**, **7**−**10**, and **12** were over 40%, especially compounds **1** and **8**, with which the inhibitory rates were over 85%. As a result, compounds **1** and **8** were chosen for a dose-dependent experiment, and their IC_{50} values were calculated to be 342.5 and 625.0 μM, respectively, which were comparable to the commercial herbicide, Harness (Figure 2).

With respect to the phenolic constituents from the aerial parts of *M. micrantha*, seven flavonoids, including mikanin (**14**), and two di-*O*-caffeoylquinic acid *n*-butyl esters were reported, among which 3,4′,5,7-tetrahydoxy-6-methoxyflavone 3-*O*-β-D-glucopyranoside was evaluated for its inhibitory effect on the root growth of *Brassica parachinensis*.[5,21,22] The known thymol

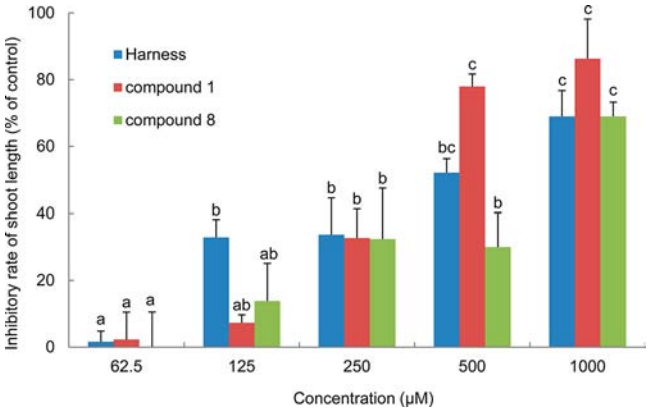

Figure 2. Inhibitory effects of compounds **1** and **8** on the shoot growth of *A. thaliana*. For each treatment, the means within the column followed by different letters were significantly different at the $p < 0.05$ level.

derivatives (**5**−**10**) were previously obtained from some species of other genera, such as *Eupatorium*, *Centipeda*, and *Inula*, in the family Asteraceae.[15,16,23] This is the first report of their existence in the genus *Mikania* and their allelopathic effects.

In consideration of extraction of the roots with ethanol and condensation of the ethanolic solution at 45−50 °C, compounds **11** and **12** are more likely to be the esters of ethanol and caffeic and ferulic acids; that is to say, compounds **11** and **12** are more likely to exist in the roots as their corresponding acids. Caffeic and ferulic acids have been isolated from a variety of crops, weed residues, and other plants. They are important allelochemicals that release into the soil from plants through various mechanisms, such as root exudation, leachation, and residue decomposition. Upon release, they play a multitude of ecological and physiological roles, such as the inhibition of seed germination and root growth.[24,25]

This research reveals that the fresh roots of *M. micrantha* contain diverse phenolic constituents, including thymol derivatives and phenolic acids, and some of these phenolic compounds possess variable allelopathic properties.

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Notes

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