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Two New Thymol Derivatives from the Roots of *Ageratina adenophora*

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Abstract: Two new thymol derivatives, 7,9-diisobutyryloxy-8-ethoxythymol (**1**) and 7-acetoxy-8-methoxy-9-isobutyryloxythymol (**2**), were isolated from fresh roots of *Ageratina adenophora*, together with four known compounds, 7,9-di-isobutyryloxy-8-methoxythymol (**3**), 9-oxoageraphorone (**4**), (−)-isochaminic acid (**5**) and (1α,6α)-10-hydroxycar-3-ene-2-one (**6**). Their structures were established on the basis of detailed spectroscopic analysis, and they were all isolated from the roots of *A. adenophora* for the first time. All the compounds were tested for their in vitro antibacterial activity toward three Gram-positive and two Gram-negative bacterial strains. Thymol derivatives **1–3** only selectively showed slight in vitro bacteriostatic activity toward three Gram-positive bacteria. The two known carene-type monoterpenes **5** and **6** were found to show moderate in vitro antibacterial activity against all five tested bacterial strains, with MIC values from 15.6 to 62.5 μg/mL. In addition, compounds **5** and **6** were further revealed to show in vitro cytotoxicity against human tumor A549, HeLa and HepG2 cell lines, with IC_{50} values ranging from 18.36 to 41.87 μM. However, their cytotoxic activities were inferior to those of reference compound adriamycin.

Keywords: *Ageratina adenophora*; thymol derivatives; monoterpenes; antibacterial; cytotoxicity

1. Introduction

Ageratina adenophora (Spreng.) King and Robinson is a perennial, herbaceous invasive plant, native to Mexico and Costa Rica, which has invaded around 30 countries in tropical and subtropical zones of the world [1,2]. This plant was first introduced to Yunnan Province of China in the 1940s, and by now it has rapidly spread 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 damaged the ecology and environment of China's native habitat [4,5].

A. adenophora is seldom attacked by microorganisms (including bacteria and fungi) and insects, suggesting that rich bioactive secondary metabolites that might be defense-related, would exist in this plant. Previous phytochemical studies have revealed structurally diverse chemicals including (mono-, sesqui-, di-, and tri-) terpenoids, phenylpropanoids, flavonoids, coumarins, sterols and alkaloids were reported from this species [6–8], some of which were shown to possess allelopathic [9,10], phytotoxic [11] and antifeedant [12] activities. Our recent study also revealed some bioactive natural products, including bioactive quinic acid derivatives and monoterpenes from the aerial parts of *A. adenophora* and some phenolic compounds with allelopathic potential from the roots of this species [13–15]. In continuation of our work on searching for bioactive natural compounds of *A. adenophora*, a further study on the roots of this invasive plant was conducted, which led to the isolation of two new thymol derivatives (**1** and **2**) along with four known compounds (**3**–**6**) (Figure 1). Herein, we report the isolation and structural elucidation of these compounds, as well as their antibacterial and cytotoxic activities.
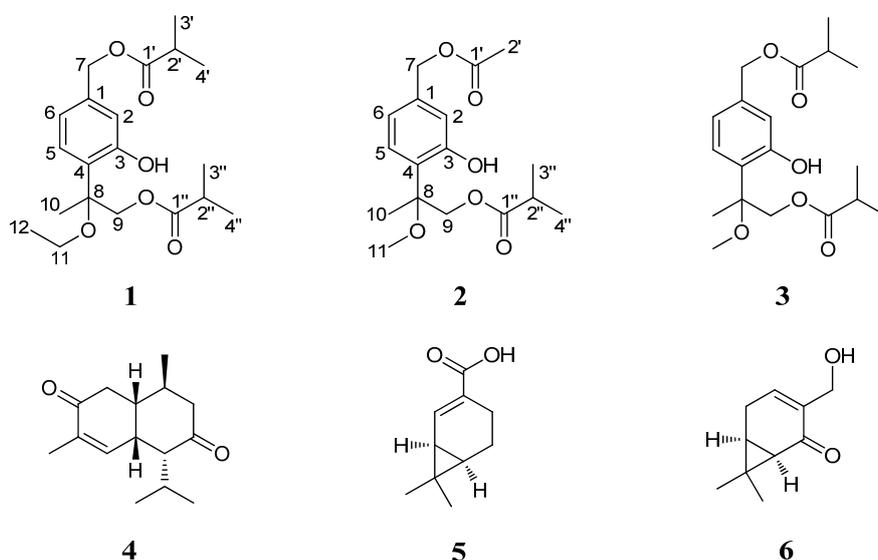

Figure 1. Chemical structures of compounds **1**−**6**.

2. Results and Discussion

The EtOH extract of the fresh roots of *A. adenophora* was initially partitioned between water and EtOAc. The EtOAc soluble fraction was then subjected to silica gel column chromatography (CC) followed by CCs of silica gel, reverse phase silica gel and Sephadex LH-20, to yield new thymols **1** and **2** along with other four known metabolites, 7,9-di-isobutyryloxy-8-methoxythymol (**3**) [16], 9-oxoageraphorone (**4**) [17], (−)-isochaminic acid (**5**) [18] and (1α,6α)-10-hydroxy-3-carene-2-one (**6**) [14]. The structures of the known compounds were determined by interpretation of their spectroscopic data, as well as by comparison with reported values.

Compound **1** was isolated as a yellowish oil. Its molecular formula $C_{20}H_{30}O_6$ was determined by HR-ESI-MS at *m/z* 389.1942 [M + Na]$^+$ (calcd. for $C_{20}H_{30}O_6Na$, 389.1940) (see the supplementary), corresponding to 6 degrees of unsaturation. The IR spectrum displayed absorptions at 3434 and 1737 cm^{-1} indicative of the existence of hydroxyl and carbonyl groups. In the ^{1}H-NMR spectrum, signals for an oxymethylene at δ_H 5.05 (s, 2H), one tertiary methyl at δ_H 1.67 (s, 3H), four secondary methyls at δ_H 1.20 (d, 6H) and 1.13 (d, 6H), a primary methyl at δ_H 1.25 (t, 3H), and a 1,3,4-trisubstituted phenyl group were readily recognized. In the ^{13}C-NMR and DEPT spectra (Table 1), 20 carbons including six methyls, three oxygenated methylenes, two methines, one quaternary carbon, two carboxyl carbons and six aromatic carbons (3 × C and 3 × CH) were displayed. These above data and literature precedents supported **1** to be a thymol derivative

with one ethoxy group and two isobutyryloxy groups in the molecule [16,19]. Careful comparison showed that the ^{1}H- and ^{13}C-NMR spectroscopic data (see Table 1) of **1** were very close to those of 7,9-di-isobutyryloxy-8-methoxythymol [16], a literature reported thymol derivative which was also obtained in the present study as compound **3**, except that the resonances for the methoxy group in **3** were replaced by signals (δ_H 3.54 (H-11a), 3.39 (H-11b), 1.25 (H$_3$-12); δ_C 59.1 (C-11), 15.4 (C-12)) for an ethoxy group in **1**. These findings suggested **1** to be a thymol derivative close to **3**, with only difference of the methoxy group at C-8 in **3** being replaced by an ethoxy group (Figure 1). This proposed structure was well supported by 2D NMR analyses including ^{1}H-^{1}H COSY and HMBC experiments (Figure 2). In the ^{1}H-^{1}H COSY spectrum, signals correlated to the four H-atom coupling systems, i.e., C-5 through C-6, C-11 through C-12, C-2′ through C-4′ and C-2″ through C-4″ were all exhibited. The observation of [1]H-^{13}C long-range correlation signals in the HMBC spectrum of H-9 and H-3″(4″) with C-1″ (δ_C 176.9) (Figure 2) evidenced the location of an isobutyryloxy group at C-9 (δ_C 68.3). The location of the ethoxy group at C-8 was assigned by significant HMBC correlations of H-11 with C-8 (δ_C 80.8). The ester bond linkage of C-7 with the other isobutyryloxy group at C-1′ was supported by the observation of HMBC correlations of H-7 (δ_H 5.04) and H-3′(4′) (δ_H 1.20) with C-1′ (δ_C 176.6). Therefore, compound **1** was determined as 7,9-diisobutyryloxy-8-ethoxythymol.

Compound **2** was also obtained as a yellowish oil. Its molecular formula $C_{17}H_{24}O_6$ was deduced from the HR-ESI-MS *m/z* 347.1479 [M + Na]$^+$ (calcd. for $C_{17}H_{24}O_6Na$, 347.1471). Its spectral features and physicochemical properties suggested **2** to be also a thymol derivative. Careful comparison indicated that the ^{1}H and ^{13}C (DEPT) NMR data (Table 1) of **2** were very similar to those of **3**, except that the resonances for the isobutyryloxy group that located at C-7 in **3** were replaced by the signals (δ_H 2.10 (3H, s, H$_3$-2′); δ_C 170.8 (C-1′), 20.9 (C-2′)) for an acetoxy group in **2** (Figure 1). This deduction was supported by its molecular formula and the observation of significant [1]H-^{13}C long-range correlation signals in the HMBC spectrum of H-7 (δ_H 5.03) and H-2′ (δ_H 2.10) with C-1′ (δ_C 170.8) (Figure 2). By further detailed analysis of 2D NMR (^{1}H-^{1}H COSY, HSQC, and HMBC) spectra (Figure 2), the ^{1}H- and ^{13}C spectroscopic NMR data were unambiguously assigned as shown in Table 1, and they were fully supported by the structure of **2** as shown in Figure 1. Accordingly, the structure of **2** was thus determined as 7-acetoxy-8-methoxy-9-isobutyryloxythymol.
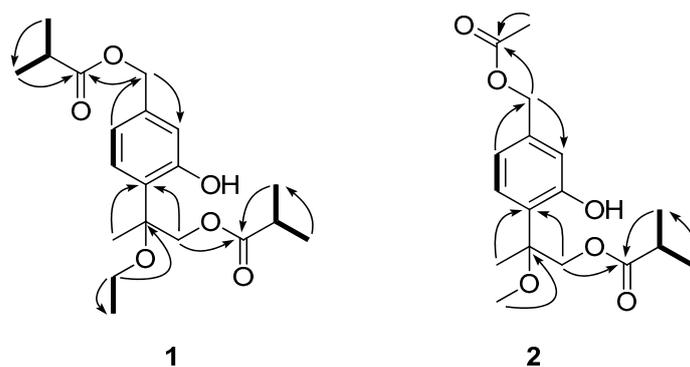

Figure 2. Selected HMBC (⟶) and COSY (▬) correlations of compounds **1** and **2**.

Among these isolated compounds, **1**–**3** were thymol derivatives. Thymols, first found in *Thymus* plants [20], were recently discovered to be in rich supply in *Eupatorium* species [19,21,22]. Due to the thymol derivatives usually being oily like compounds which were difficult to prepare single crystals for X-ray analysis, it is generally difficult to determine the stereochemistry of thymols at the stereogenic center C-8 [19,23]. Just like the thymol precedents in the literature, the stereochemistry of thymols **1**–**3** at the stereogenic center C-8 were also still an open question. Compounds **5** and **6** were two carene-type monoterpenes with potential anti-fungal activities that we have recently obtained from the aerial parts of *A. adenophora* [14]. To the best of our knowledge, this is the first time these compounds have been obtained from the roots of this plant.

Table 1. The ^{1}H- and ^{13}C-NMR data of compounds **1**, **2** and **3**.

H/C	1 [a]		2 [a]		3 [b]	
	δ_H (Mult., *J* in Hz)	δ_C (DEPT)	δ_H (Mult., *J* in Hz)	δ_C (DEPT)	δ_H (Mult., *J* in Hz)	δ_C (DEPT)
1		138.2 (s)		137.9 (s)		138.3 (s)
2	6.84 (d, 1.2)	116.3 (d)	6.85 (s)	116.6 (d)	6.86 (s)	116.4 (d)
3		156.5 (s)		156.4 (s)		156.4 (s)
4		124.4 (s)		123.8 (s)		123.6 (s)
5	7.01 (d, 8.0)	127.6 (d)	7.01 (d, 8.4)	127.8 (d)	7.02 (d, 8.4)	127.9 (d)
6	6.82 (dd, 8.0, 1.2)	118.8 (d)	6.82 (d, 8.4)	119.2 (d)	6.83 (d, 8.4)	119.0 (d)
7	5.04 (s)	65.3 (t)	5.03 (s)	65.5 (t)	5.05 (s)	65.3 (t)
8		80.8 (s)		81.3 (s)		81.4 (s)
9	4.36 (d, 11.6)	68.3 (t)	4.33 (d, 12.0)	68.2 (t)	4.34 (d, 11.6)	68.3 (t)
	4.23 (d, 11.6)		4.22 (d, 12.0)		4.23 (d, 11.6)	
10	1.66 (s)	21.0 (q)	1.64 (s)	20.2 (q)	1.65 (s)	20.2 (q)
11	3.54 (m), 3.39 (m)	59.1 (t)	3.28 (s)	50.9 (q)	3.29 (s)	51.0 (q)
12	1.25 (t, 7.2)	15.4 (q)				
1′		176.6 (s)		170.8 (s)		176.6 (s)
2′	2.56 (m)	34.0 (d)	2.10 (s)	20.9 (q)	2.60 (m)	33.9 (d)
3′	1.20 (d, 6.8)	19.0 (q)			1.20 (d, 6.8)	18.8 (q)
4′	1.20 (d, 6.8)	19.0 (q)			1.20 (d, 6.8)	18.9 (q)
1″		176.9 (s)		176.5 (s)		176.9 (s)
2″	2.62 (m)	34.0 (d)	2.55 (m)	33.9 (d)	2.60 (m)	34.0 (d)
3″	1.13 (d, 6.8)	18.9 (q)	1.12 (d, 6.6)	18.8 (q)	1.12 (d, 6.8)	18.9 (q)
4″	1.13 (d, 6.8)	18.9 (q)	1.13 (d, 6.6)	18.8 (q)	1.13 (d, 6.8)	20.2 (q)

[a] Recorded in CDCl$_3$; [b] Recorded in CD$_3$OD.

These six compounds were tested for their in vitro antibacterial activities against five bacterial strains, including three Gram-positive bacteria of *Staphylococcus aureus*, *Bacillus thuringiensis* and *Bacillus subtilis*, and two Gram-negative bacteria of *Escherichia coli* and *Shigella dysenteria*. The experimental results obtained from the bioassay (Table 2) showed that the two carene-type monoterpenes **5** and **6** were moderately active toward the five bacterial strains with MIC values from 15.6 to 62.5 μg/mL. The thymol compounds **1**–**3** only selectively showed weak bacteriastatic activity toward the three Gram-positive bacterial strains, and no antibacterial activity was detected for compound **4** in this bioactive assay.

Table 2. MIC values of compounds **1**–**6** in μg/mL against five bacterial strains.

Compounds	*Staphylococcus aureus*	*Bacillus thuringiensis*	*Bacillus subtilis*	*Escherichia coli*	*Shigella dysenteriae*
1	>200	>200	125	>200	>200
2	125	62.5	62.5	>200	>200
3	>200	125	125	>200	>200
4	>200	>200	>200	>200	>200
5	31.3	31.3	15.6	62.5	62.5
6	15.6	31.3	15.6	62.5	62.5
KS	3.9	3.9	3.9	3.9	3.9
C	>200	>200	>200	7.8	7.8

KS = Kanamycin sulfate, C = Cefradine.

Compounds **1**–**6** were further evaluated for their in vitro cytotoxicity against three human cancer cell lines (A549, HeLa and HepG2) using a MTT method as described. The resulting IC$_{50}$ values are displayed in Table 3, as compared to adriamycin as positive control. Compounds **5** and **6** showed moderate cytotoxicity against all the three tested cancer cell lines, with IC$_{50}$ values ranging from 18.36 to 41.87 μM. No obvious activity was detected for the other four isolates (**1**–**4**).

Table 3. Cytotoxicity of compounds **1–6** (IC$_{50}$, μM).

Compounds	A549	HeLa	HepG2
1–4	>100	>100	>100
5	32.37 ± 3.75	25.64 ± 2.34	41.87 ± 6.53
6	30.65 ± 3.87	18.36 ± 1.72	39.44 ± 3.61
Adriamycin	0.68 ± 0.06	0.46 ± 0.05	1.23 ± 0.02

Values represent mean ± SD (*n* = 3) based on three individual experiments.

A. adenophora is a well-known invasive plant which has spread rapidly in the southwest part of China. To date, phytochemical investigations have revealed diverse chemicals from this plant species. However, those studies were mainly focused on the aerial parts, and seldom concentrated on the roots. Our recent study revealed a series of phenolic compounds with allelopathic potential from the roots of this plant [15]. The present study further indicated that the roots of *A. adenophora* are rich in potential bioactive compounds worthy of further investigation.

3. Materials and Methods

3.1. General Information

Optical rotations were obtained on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Waltham, MA, USA) with MeOH as solvent. UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 35 UV-vis spectrophotometer. IR spectra (KBr) were recorded on a Bruker Tensor 27 spectrophotometer in cm^{-1}. ^{1}H (600 MHz and 400 MHz), ^{13}C (150 MHz and 100 MHz), and 2D NMR spectra were recorded in CDCl$_3$ and CD$_3$OD on a Bruker DRX-400 instrument and a Bruker AVANCE 600 instrument with TMS as an internal standard. HR-ESI-MS data were obtained on a Water Q-TOF Premier mass spectrometer and HR-EI-MS data were obtained on a Finigan MAT 95XP mass spectrometer. ESIMS were collected on an MDS SCIEX API 2000 LC/MS/MS instrument. Preparative HPLC was conducted using a CXTH P3000 HPLC pump and a UV3000 UV-Vis Detector with a Fuji-C18 column (10 μm–100A, ChuangXinTongHeng Science And Technology Co., Ltd., Beijing, China). For column chromatography (CC), silica gel (200–300 mesh, Qingdao Marine Chemical Inc.), YMC ODS-A (50 μm, YMC Co. Ltd., Kyoto, Japan) were used, and Sephadex LH-20 (Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden) were used. Analytical grade petroleum ether (b.p. 60–90 °C), methanol, ethyl acetate, chloroform, acetone were purchased from Tianjin Fuyu Fine Chemical Industry Co. (Tianjin, China); HPLC grade methanol was purchased from J&K Chemical Ltd. (Beijing, China); Fractions were monitored by pre-coated HSGF$_{254}$ TLC Yantai Jiangyou Silica Gel Co. Ltd. (Yantai, China), and spot detection was performed under fluorescent light (λ = 254 nm), and then spraying 10% H$_2$SO$_4$ in ethanol, followed by heating. RPMI-1640 medium and fetal calf serum were purchased from Gibco BRL (Gaithersburg, MD, USA). Kanamycin sulfate, Resazurin, Cefradine, CDCl$_3$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (Sigma-Aldrich, St. Louis, MO, USA). Adriamycin was obtained from Pfizer Italia SRL (Roma, Italy). *p*-Nitrophenyl-α-D-glucopyranoside (PNPG) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

3.2. Plant Materials

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.

3.3. Extraction and Isolation

The fresh root material (14.5 kg) of *A. adenophora* were cut into pieces and extracted three times with 95% EtOH (3 × 20 L) at room temperature. After removal of the EtOH in vacuo, the viscous concentrate was suspended in 10% ethanol in H_2O (2.5 L) and extracted with ethyl acetate (EtOAc) (3 × 2.5 L). The oily EtOAc extract (76 g) was then applied on silica gel (500 g) column chromatography and eluted with 2L petroleum ether/$CHCl_3$ (1:1, *v/v*) to yield an olily fraction (22.1 g) which was further subjected to silica gel column chromatography using a gradient of petroleum ether/$CHCl_3$ (90:10–0:100, *v/v*) and $CHCl_3$–MeOH (100:0–80:20, *v/v*) to give sub-fractions F_1–F_7. The fraction F_2 (4.0 g), obtained from the elution of petroleum ether/$CHCl_3$ of 1:1, was separated by RP-C_{18} MPLC eluted with MeOH/H_2O (from 4:6 to 7:3, *v/v*) and further purified by Sephadex LH-20 column chromatography with elution solvent of MeOH to give compound **5** (2.6 mg). The fraction F_4 (4.7 g), obtained from the elution of $CHCl_3$/MeOH of 95:5, was applied on silica gel (200–300 mesh) column chromatography and eluted with a gradient of *n*-hexane/ethyl acetate to afford fractions F_{4-1}–F_{4-8} on the basis of thin layer chromatography (TLC) profiles. Fraction F_{4-3} (1.3 g), obtained from the elution of *n*-hexane/ethyl acetate of 8.5:1.5, was separated by silica gel column chromatography eluted with petroleum ether/Me_2CO (from 9:1 to 7:3, *v/v*) to give subfractions F_{4-3-1}–F_{4-3-5}, of which subfraction F_{4-3-4} (0.4 g) was further purified by RP-C_{18} HPLC eluted with 70% MeOH in H_2O to afford compound **1** (t_R = 37.2 min, 2.8 mg). Fraction F_{4-4} (1.1 g), obtained from the elution of *n*-hexane/ethyl acetate of 7:3, was separated by RP-C_{18} HPLC eluted with a MeOH/H_2O gradient (from 50:50 to 80:20, *v/v*) to provide pure compound **4** (t_R = 68.1 min, 5.6 mg). Fraction F_{4-6} (0.7 g) was divided into subfractions F_{4-6-1}–F_{4-6-4}, of which subfraction F_{4-6-4} (39.2 mg) was purified repeatedly by Sephadex LH-20 columns eluted with $CHCl_3$/MeOH (20:80, *v/v*) to afford compound **2** (3.0 mg). Fraction F_{4-6-3} was separated by RP-C_{18} MPLC eluted with MeOH/H_2O (from 50:50–100:0, *v/v*) to attain subfractions $F_{4-6-3-1}$–$F_{4-6-3-4}$. Subfraction $F_{4-6-3-1}$ was then further purified by RP-C_{18} MPLC eluted with MeOH/H_2O (from 50:50–100:0, *v/v*) to attain pure compound **6** (t_R = 13.5 min, 2.4 mg), and subfraction $F_{4-6-3-3}$ was purified by Sephadex LH-20 column eluted with acetone to afford compound **3** (4.7 mg).

7,9-Diisobutyryloxy-8-ethoxythymol (**1**). yellowish oil; $[\alpha]_D^{20}$ −5.0 (*c* 0.06, $CHCl_3$); IR (KBr) ν_{max} 3434, 1737, 1265, 1187, 1155 cm^{-1}; UV (MeOH) λ_{max} (log ε) nm: 205 (4.16), 279 (3.31); ESI-MS *m/z* 755 $[2M + Na]^+$, 389 $[M + Na]^+$; HR-ESI-MS *m/z* 389.1942 $[M + Na]^+$ (calcd. for $C_{20}H_{30}O_6Na$, 389.1940); For ^1H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1.

7-Acetoxy-8-methoxy-9-isobutyryloxythymol (**2**). yellowish oil; $[\alpha]_D^{20}$ +3.8 (*c* 0.87, $CHCl_3$); IR (KBr) ν_{max} 3318, 1739, 1629, 1577 cm^{-1}; UV (MeOH) λ_{max} (log ε) nm: 204 (4.27), 219 (3.80), 279 (3.31); ESI-MS *m/z* 687 $[2M + K]^+$, 671 $[2M + Na]^+$, 363 $[M + K]^+$, 347 $[M + Na]^+$, 683 $[2M + Cl]^-$, 359 $[M + Cl]^-$, 323 $[M–H]^-$; HR-ESI-MS *m/z* 347.1479 $[M + Na]^+$ (calcd. for $C_{17}H_{24}O_6Na$, 347.1471). For ^1H ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data, see Table 1.

3.4. Antibacterial Assay

The antibacterial activities of **1**–**6** were tested by using a microdilution method as we described previously [24]. Three Gram-positive bacteria strains, *Staphylococcus aureus*, *Bacillus thuringiensis and Bacillus subtilis*, and two Gram-negative bacterial species, *Escherichia coli* and *Shigella dysenteria*, were used in the bioassay. In the test, indicator solution of resazurin (100 µg/mL, 100 µL) was first placed into each control wells (11th column) in 96-well microplates for the assay. Subsequently, indicator solution (100 µg/mL, 7.5 mL) was mixed with test organism (10^6 cfu/mL, 5 mL) followed by transferring (100 µL, each) to growth control wells (12th column) and all test wells (1–10th column) in the 96-well microplates. Then, each of the sample solutions (1.0 mg/mL of test compounds in methanol, 100 µL) and positive control solution (1.0 mg/mL of kanamycin sulfate or cefradine in methanol) as well as negative control sample (pure MeOH) were applied to the wells in the 1st column of the plates. In each test microplate, the six compound samples along with positive control and

negative control samples were applied. Once all controls and samples were properly applied to the 1st column of wells in the microplates, half of the homogenized content (100 µL) from these wells was then transferred parallel to the 2nd column of wells, and each subsequent column of wells was treated similarly (doubling dilution) up to the 10th column, followed by discarding the last 100 µL aliquot. Finally, the plates were incubated at 37 °C for 5–6 h until the color of growth control wells change to pink. The lowest concentration for each test compound at which color change occurred was recorded as its primary MIC value. The averages of the primary values from three individual tests were calculated and these were taken as the final MIC values for the test compounds. MIC values for test compounds were displayed in Table 2.

3.5. Cytotoxic Assay

Compounds **1**–**6** were evaluated for their cytotoxity against human lung adenocarcinoma (A549), human cervical carcinoma (HeLa) and human liver hepatocellular carcinoma (HepG2) cell lines. The three tumor cell lines were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences. The cytotoxic activities of the tested compounds were assayed according to an MTT method by using 96 well plates [25]. In brief, the cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37 °C. 100 µL adherent cells at the density of 5×10^4 cell/mL was seeded into each well of 96-well cell culture plates and incubated in 5% CO_2 at 37 °C for 24 h to form a monolayer on the flat bottoms. Then, the supernatant per well was removed and subsequently added with 100 µL fresh medium and 100 µL medium containing a test compound. The plate was then incubated in 5% CO_2 at 37 °C. After 72 h, 20 µL of 5 mg/mL MTT in DMSO was added into each well and incubated for 4 h. The supernatant per well was carefully removed and 150 µL DMSO was added. The plate was then vortex shaken for 15 min to dissolve blue formazan crystals. The optical density (OD) of each well was measured on a Genois microplate reader (Tecan GENios, Männedorf, Switzerland) at the wavelength of 570 nm. All assays were performed in triplicate and adriamycin was used as a positive control. In each experiment, each of the tumor cell lines was exposed to the test compound at concentrations 50, 25, 12.5, 6.25, 3.125, 1.5625 µg/mL. The inhibitory rate of cell growth was calculated according to the following formula: Inhibition rate (%) = $(OD_{control} - OD_{treated})/OD_{control} \times 100\%$. IC_{50} values were calculated by SPSS 16.0 statistic software. The values were based on three individual experiments and expressed as means $\pm$ standard deviation (SD).

4. Conclusions

Six natural products, including two new thymol derivatives **1** and **2**, were isolated from the roots of *A. adenophora*. Their structures were identified by extensive spectroscopic analysis, including NMR and HR-ESI-MS techniques. All the compounds were isolated from the roots of *A. adenophora* for the first time. Thymol compounds **1**−**3**, and monoterpenes **5** and **6** selectively showed in vitro antibacterial activity against five assayed bacterial strains. Compounds **5** and **6** were further revealed to show moderate in vitro cytotoxicity against human tumor A549, HeLa and HepG2 cell lines. The present study indicates that the roots of *A. adenophora* are also rich in potential bioactive compounds worthy of further investigation.

Author Contributions: L.-M. Dong, M. Zhang and Q.-L. Xu performed the isolation, structural elucidation of the chemicals and participated in the bioassay experiments. Q. Zhang, B. Luo, Q.-W. Luo and W.-B. Liu partially contributed the isolation of the chemicals and the bioactive assay. J.-W. Tan designed and organized the whole research of this study. All authors approved the final version manuscript.

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References

1. Qiang, S. The history and status of the study on crofton weed (*Eupatorium adenophorum* Spreng.), a worst worldwide weed. *J. Wuhan Bot. Res.* **1998**, *16*, 366–372.
2. Wang, J.J. The crofton weed, *Ageratina adenophora*. In *Biology and Management of Invasive Alien Species in Agriculture and Forestry*; Wan, F.H., Zheng, X.B., Guo, J.Y., Eds.; Science Press: Beijing, China, 2005; pp. 650–661.
3. Sun, X.Y.; Lu, Z.H.; Sang, W.G. Review on studies of *Eupatorium adenophorum*—An important invasive species in China. *J. For. Res.* **2004**, *15*, 319–322.
4. Wan, F.H.; Guo, J.Y.; Wang, D.H. Alien invasive species in China: Their damages and management strategies. *Biodivers. Sci.* **2002**, *10*, 119–125.
5. Wan, F.H.; Liu, W.X.; Guo, J.Y.; Qiang, S.; Li, B.P.; Wang, J.J.; Yang, G.Q.; Niu, H.B.; Gui, F.R.; Huang, W.K.; et al. Invasive mechanism and control strategy of *Ageratina adenophora* (Sprengel). *Sci. China Life Sci.* **2010**, *53*, 1291–1298. [CrossRef] [PubMed]
6. Yan, Q.S.; Yang, J.; Li, H.M.; Cao, A.C.; Chen, Q.H.; Wen, Y.Q.; He, L. Advances in the studies on the chemical components and bioactivity of *Eupatorium adenophorum* Spreng as a intruding species. *J. Beijing Norm. Univ.* **2006**, *42*, 70–73.
7. Li, Y.M.; Li, Z.Y.; Ye, M. The chemical compositions and their bioactivities in the different parts of *Eupatorium adenophorum* Spreng. *J. Yunnan Agric. Univ.* **2008**, *23*, 42–46.
8. He, L.; Hou, J.; Gan, M.L.; Shi, J.G.; Chantrapromma, S.; Fun, H.K.; Williams, I.D.; Sung, H.H.Y. Cadinane sesquiterpenes from the leaves of *Eupatorium adenophorum*. *J. Nat. Prod.* **2008**, *71*, 1485–1488. [CrossRef] [PubMed]
9. Yang, G.Q.; Wan, F.H.; Liu, W.X.; Zhang, X.W. Physiological effects of allelochemicals from leachates of *Ageratina adenophora* Spreng on rice seedlings. *Allelopathy J.* **2006**, *18*, 237–245.
10. Zhao, X.; Zheng, G.W.; Niu, X.M.; Li, W.Q.; Wang, F.S.; Li, S.H. Terpenes from *Eupatorium adenophorum* and their allelopathic effects on *Arabidopsis* seeds germination. *J. Agric. Food Chem.* **2009**, *57*, 478–482. [CrossRef] [PubMed]
11. Zheng, G.W.; Jia, Y.X.; Zhao, X.; Zhang, F.J.; Luo, S.H.; Li, S.H.; Li, W.Q. *o*-Coumaric acid from invasive *Eupatorium adenophorum* is a potent phytotoxin. *Chemoecology* **2012**, *22*, 131–138. [CrossRef]
12. Shi, W.; Luo, S.H.; Li, S.H. Defensive sesquiterpenoids from leaves of *Eupatorium adenophorum*. *Chin. J. Chem.* **2012**, *30*, 1331–1334. [CrossRef]
13. Zhang, M.; Liu, W.X.; Zheng, M.F.; Xu, Q.L.; Wan, F.H.; Wang, J.; Lei, T.; Zhou, Z.Y.; Tan, J.W. Bioactive quinic acid derivatives from *Ageratina adenophora*. *Molecules* **2013**, *18*, 14096–14104. [CrossRef] [PubMed]
14. Xu, Q.L.; Zhang, M.; Zhou, Z.Y.; Liu, W.X.; Wan, F.H.; Wang, H.F.; Tan, J.W. Two new carene-type monoterpenes from aerial parts of *Ageratina adenophora*. *Phytochem. Lett.* **2014**, *9*, 123–126. [CrossRef]
15. Zhou, Z.Y.; Liu, W.X.; Pei, G.; Ren, H.; Wang, J.; Xu, Q.L.; Xie, H.H.; Wan, F.H.; Tan, J.W. Phenolic compounds from *Ageratina adenophora* roots and their allelopathic effects on *Arabidopsis* seeds germination and seedling growth. *J. Agric. Food Chem.* **2013**, *61*, 111792–111799. [CrossRef] [PubMed]
16. Maldonado, E.; Marquez, C.L.; Ortega, A. A thymol derivative from *Calea nelsonii*. *Phytochemistry* **1992**, *31*, 2527–2528. [CrossRef]
17. Ding, Z.H.; Guo, Y.B.; Ding, J.K. Chemical constituents from the flowers of *Eupatorium adenophorum*. *Acta Bot. Yunnanica* **1999**, *21*, 505–511.
18. Jayasinghe, L.; Kumarihamy, B.; Jayarathna, K.; Udishani, N.; Bandara, B.; Hara, N.; Fujimoto, Y. Antifungal constituents of the stem bark of *Bridelia retusa*. *Phytochemistry* **2003**, *62*, 637–641. [CrossRef]
19. Tori, M.; Ohara, Y.; Nakashima, K.; Sono, M. Thymol derivatives from *Eupatorium fortunei*. *J. Nat. Prod.* **2001**, *64*, 1048–1051. [CrossRef] [PubMed]
20. Vernet, P. Composition of essential oil of *Thymus vulgaris* L. [Labiee]: Mode of hereditary transmission of three terpenes [thymol, carvacrol and linalol]. *C. R. Seances. Acad. Sci. Ser. D* **1977**, *284*, 1289–1292.
21. Zhang, K.M.; Shi, L.; Jiang, C.D.; Li, Z.Y. Inhibition of *Ageratina adenophora* on spore germination and gametophyte development of *Macrothelypteris torresiana*. *J. Integr. Plant. Biol.* **2008**, *50*, 559–564. [CrossRef] [PubMed]

22. Chen, J.J.; Tsai, Y.C.; Hwang, T.L.; Wang, T.C. Thymol, benzofuranoid, and phenylpropanoid derivatives: anti-inflammatory constituents from *Eupatorium cannabinum*. *J. Nat. Prod.* **2011**, *74*, 1021–1027. [CrossRef] [PubMed]

23. Talavera-Alemán, A.; Rodríguez-García, G.; López, Y.; García-Gutiérrez, H.A.; Torres-Valencia, J.M.; Río, R.E.D.; et al. Systematic evaluation of thymol derivatives possing stereogenic or prostereogenic centers. *Phytochem. Rev.* **2016**, *15*, 251–277. [CrossRef]

24. Wang, J.; Ren, H.; Xu, Q.L.; Zhou, Z.Y.; Wu, P.; Wei, X.Y.; Cao, Y.; Chen, X.X.; Tan, J.W. Antibacterial oleanane-type triterpenoids from pericarps of *Akebia trifoliata*. *Food Chem.* **2015**, *168*, 623–629. [CrossRef] [PubMed]

25. Wang, J.; Xu, Q.L.; Zheng, M.F.; Ren, H.; Lei, T.; Wu, P.; Zhou, Z.Y.; Wei, X.Y.; Tan, J.W. Bioactive 30-oroleanane riterpenes from the ericarps of *Akebia trifoliata*. *Molecules* **2014**, *19*, 4301–4312. [CrossRef] [PubMed]

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