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Introduction

Ageratina adenophora (Sprengel) King & Robinson (synonym: *Eupatorium adenophorum* Sprengel) is a perennial, herbaceous invasive plant, native to Mexico and Costa Rica.¹ As a well-known invasive species, this plant has successfully invaded more than thirty countries and regions in tropical and temperate zones of the world, including America, Australia, Europe, India, South Africa, Southeast Asia, and the southwest part of China.^{2,3} At its invasion places, the rapid spread of *A. adenophora* has caused serious economic losses to agriculture, forestry and livestock, and intensely damaged the local ecosystem and the original biodiversity.^{4,5}

Thymol derivatives with antibacterial and cytotoxic activity from the aerial parts of Ageratina adenophora[†]

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Three new thymol derivatives, 7-formyl-9-isobutyryloxy-8-hydroxythymol (1), 7,9-di-isobutyryloxy-8,10-dehydrothymol (2) and 2α -methoxyl-3 β -methyl-6-methylol-2,3-dihydrobenzofuran (3), along with five known ones (4–8), were isolated from the aerial parts of the invasive plant *Ageratina adenophora*. Their structures were elucidated by extensive spectroscopic analysis and they were all isolated from the aerial part of *A. adenophora* for the first time. These compounds, except 8, selectively showed *in vitro* antimicrobial activity against three Gram-(+) and two Gram-(-) bacterial strains. In particular, compounds 1 and 5 showed notable *in vitro* antimicrobial activity against all five bacterial strains with IC₅₀ values ranging from 3.9 to 15.6 µg mL⁻¹, as compared to reference compound kanamycin sulfate with a MIC value 1.9–3.9 µg mL⁻¹. Compounds 1 and 5 were further revealed to show *in vitro* cytotoxic activity against three tested human tumor (MCF-7, NCI-H460 and HeLa) cell lines, with IC₅₀ values ranging from 7.45 to 28.63 µM. Compounds 7 and 8 selectively showed slight but detectable *in vitro* cytotoxic effects were detected in the bioassay of the other four thymol derivatives. The present results provide new data to support that the aerial parts of *A. adenophora* are a rich source of bioactive chemicals valuable in medicinal applications.

In nature, A. adenophora is seldom attacked by microorganisms and insects. This suggests a rich defense related to bioactive chemicals, that possibly might also be pharmaceutically valuable, would exist in this plant. Previously, some literature reported that this plant was used in Nigeria and India as traditional or folk herb medicine for treatment of many human diseases, like fever, diabetes, inflammation, etc.67 To date, some terpenoids, flavonoids, phenylpropanoids, coumarins, sterols and alkaloids have been reported from this plant,⁸⁻¹⁰ with part of them exhibiting phytotoxic,11 allelopathic,12,13 antifungal14 and antifeedant15 activities. In our recent study, some bioactive compounds, including phytotoxic phenolics,16 antifungal monoterpenes17 and antibacterial quinic acid derivatives,18 were also revealed from the roots or aerial parts of this plant. In continuation of the work to clarify those potentially new and bioactive chemicals in the aerial parts of A. adenophora, eight thymol derivatives including three new (1-3) and five known (4-8) ones are here further obtained (Fig. 1). We herein report the isolation and structural elucidation of these compounds, as well as describe their in vitro antimicrobial activity against five bacteria and cytotoxicity toward three human cancer cell lines.

Results and discussion

The petroleum ether- and EtOAc-soluble fractions of the ethanol extract of the aerial parts of *Ageratina adenophora* (Spreng.) were

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isolated and purified by repeated column chromatography (CC) and HPLC to afford the three new (1–3) and five known (4–8) thymol derivatives. By comparing their NMR and MS data with those reported in literatures, the five known compounds were identified as 2α -methoxyl-3 β -methyl-6-(acetyl-*O*-methyl)-2,3-dihydrobenzofuran (4),¹⁹ 7,8,9-trihydroxythymol (5),²⁰ 7,9-di-isobutyryloxy-8-ethoxythymol (6),²¹ 7-acetoxy-9-isobutyryloxy-8-methoxythymol (7)²¹ and 7,9-di-isobutyryloxy-8-methoxythymol (8).²¹

Compound 1 was isolated as a yellow oil and determined to have a molecular formula C14H18O5 on the basis of HR-ESI-MS data (m/z 289.1056 [M + Na]⁺, calcd. for C₁₄H₁₈O₅Na, 289.1052). The IR absorptions at 3424 and 1695 cm⁻¹ revealed the presence of hydroxyl and carbonyl groups in the molecule. The existence of three methyl groups was revealed by ¹H NMR spectrum which provided methyl signals at $\delta_{\rm H}$ 1.66 (3H, s), 1.12 (3H, s) and 1.13 (3H, s). The three aromatic proton signals at $\delta_{\rm H}$ 7.34 (1H, d, J = 1.2 Hz), 7.18 (1H, d, J = 8.4 Hz), and 7.35 (1H, dd, J = 8.4, 1.2 Hz) were indicative of a typical pattern of 1,3,4trisubstituted phenyl group.²² The ¹³C NMR (DEPT) spectra (Table 2), coupled with HSQC spectral analysis, revealed the presence of a formyl group at $\delta_{\rm C}$ 191.9 (C-7), a carbonyl group at $\delta_{\rm C}$ 177.7 (C-1"), an oxymethylene group at $\delta_{\rm C}$ 70.4 (C-9), a methine group at $\delta_{\rm C}$ 33.9 (C-2"), and an oxygenated quaternary carbon at $\delta_{\rm C}$ 77.7 (C-8). Taken together these spectral data and the molecular formula into consideration, the existence of two hydroxyl groups could further be deduced. In the ¹H-¹H COSY spectrum, significant correlation signals for two proton systems were displayed, one at C-5 through C-6 and the other at C-3" through C-4" (Fig. 2), corresponding to structure fragments of -CH(5)-CH(6) and $CH_3(3'')-CH(2'')-CH_3(4'')$, respectively. In the HMBC spectrum, the exhibition of correlation signals of H-7 $(\delta_{\rm H}$ 9.92) with C-2 and C-6, and of H-2, H-6 with C-7 $(\delta_{\rm C}$ 191.9)

evidenced the connection of C-7 with C-1. The HMBC correlations of Me-10, H-9 with C-4 supported the connection of C-8 with C-4. The HMBC correlations of Me-3", Me-4" with C-1" supported the connection of C-1" with C-2". The HMBC correlations of H-9 with C-1" supported the ester bond linkage of C-1" with C-9. Further consideration of the substitution pattern of the aromatic ring and the other NMR data led to the assignment of the two hydroxyl groups at C-3 and C-8, respectively. Based on these above spectroscopic analyses, we can unambiguously establish the planner structure of **1** as depicted in Fig. 1. However, it is presently difficult to determine the configuration of C-8. Thus, the structure of **1** was elucidated as 7-formyl-9isobutyryloxy-8-hydroxythymol.

Compound 2 was assigned the molecular formula $C_{18}H_{24}O_5$, as deduced from ESI-MS and HR-ESI-MS data. Comparative analysis of the spectroscopic data and literature precedents^{21,23,24} supported 2 to be also a thymol derivative. Careful comparison of ¹H and ¹³C NMR data (Tables 1 and 2) of 2 with those of known compound 8 revealed the major differences of the two compounds that the resonances for Me-10, Me-11 and



Fig. 2 Key HMBC (\rightarrow) and ¹H–¹H COSY (\rightarrow) correlations of 1, 2 and 3.

3

Table 1 The ¹H NMR spectral data (δ in ppm, J in Hz) for compounds 1, 2 and 3

Position	1 ^{<i>a</i>}	2^a	3^b
2	7.34 (1H, d, 1.2)	6.92 (1H, d, 1.2)	5.21 (1H, d, 2.0)
3	_	_	3.15 (1H, brq, 7.2)
4	—	—	7.12 (1H, d, 7.6)
5	7.18 (1H, d, 8.4)	7.07 (1H, d, 7.6)	6.87 (1H, brd, 7.6)
6	7.35 (1H, dd, 8.4, 1.2)	6.84 (1H, dd, 7.6, 1.2)	_ *
7	9.92 (1H, s)	5.06 (2H, s)	6.79 (1H, brs)
9	4.47 (1H, d, 12.0)	4.73 (2H, s)	_
	4.33 (1H, d, 12.0)		
10	1.66 (3H, s)	5.47 (1H, d, 1.2)	1.23 (3H, d, 7.2)
		5.28 (1H, d, 1.2)	
11	_	_	4.53 (2H, s)
12			3.47 (3H, s)
2'	_	2.62 (1H, sept, 7.2)	_
3′	_	1.20 (3H, d, 7.2)	_
4'	_	1.20 (3H, d, 7.2)	_
2″	2.57 (1H, sept, 7.2)	2.62 (1H, sept, 7.2)	_
3″	1.12 (3H, d, 7.2)	1.20 (3H, d, 7.2)	
4''	1.13 (3H, d, 7.2)	1.20 (3H, d, 7.2)	_

the sp³ quaternary carbon C-8 in compound **8** were replaced by signals [$\delta_{\rm H}$ 5.28 (1H, H_a-10), 5.47 (1H, H_b-10); $\delta_{\rm C}$ 141.6 (C-8), 116.8 (C-10)] for a terminal double bond in 2. In the HMBC spectrum, significant correlation signals of H₂-10 to C-4 and C-9 were observed, which confirmed the location of the terminal double bond between C-8 and C-10. The ¹H–¹H COSY spectrum and HMBC correlations (Fig. 2) of Me-3' and Me-4' with C-1', of Me-3" and Me-4" with C-1", of H-7 with C-1', and of H-9 with C-1", evidenced the ester bond linkages of C-7 and C-9 with an individual isobutyryl group, respectively. The presence of

Table 2 The ^{13}C (DEPT) NMR spectral data (δ in ppm) for compounds 1, 2 and 3

Position	1 ^{<i>a</i>}	2 ^{<i>a</i>}	3 ^{<i>b</i>}
1	137.4 (C)	138.2 (C)	_
2	118.9 (CH)	115.5 (CH)	115.5 (CH)
3	157.1 (C)	153.7 (C)	44.4 (CH)
4	132.5 (C)	125.1 (C)	124.7 (CH)
5	127.0 (CH)	129.6 (CH)	120.8 (CH)
6	120.5 (CH)	119.3 (CH)	143.3 (C)
7	191.9 (CH)	$65.5 (CH_2)$	109.3 (CH)
8	77.7 (C)	141.9 (C)	159.3 (C)
9	70.4 (CH ₂)	$65.5 (CH_2)$	131.8 (C)
10	25.8 (CH ₃)	116.8 (CH ₂)	18.9 (CH ₃)
11		_ ` `	65.2 (CH ₂)
12	_	_	56.2 (CH ₃)
1′	_	176.9 (C)	
2'	_	34.0 (CH)	_
3'	_	18.8 (CH ₃)	_
4'	_	18.8 (CH ₃)	_
1″	177.7 (C)	177.8 (C)	_
2″	33.9 (CH)	34.0 (CH)	_
3″	18.8 (CH ₃)	18.9 (CH ₃)	_
4″	18.8 (CH ₃)	$18.8 (CH_3)$	_

^a Recorded at 150 MHz in CDCl₃. ^b Recorded at 100 MHz in CD₃OD.

diagnostic proton signals at $\delta_{\rm H}$ 6.92 (1H, d, J = 1.2 Hz), 7.07 (1H, d, J = 7.6 Hz), and 6.84 (1H, dd, J = 7.6, 1.2 Hz) supported the appearance of the core 1,3,4-trisubstituted phenyl moiety. The HMBC correlations of H-2 and H-6 with C-7, of H-10 and H-9 with C-4, and of H-5 with C-8 confirmed the connections of C7 with C-1, C-4 with C-8, and the location of a hydroxyl group at C-3. Therefore, compound **2** was determined as 7,9-di-iso-butyryloxy-8,10-dehydrothymol.

The molecular formula of compound 3 was determined as C11H14O3 by the HR-EI-MS, due to a quasi-molecular ion peak at m/z 194.0938 (calcd. for C₁₁H₁₄O₃, 194.0937). The IR absorption at 3448 cm⁻¹ revealed the presence of hydroxyl group in the molecule. The ¹H and ¹³C (DEPT) NMR spectra displayed closely related signals with those of 2α-methoxyl-3β-methyl-6-(acetyl-Omethyl)-2,3-dihydrobenzofuran,19 a known thymol compound which was also obtained as compound 4 in the present study. Careful comparison of their NMR spectral data indicated the major difference that the spectroscopic resonances for the acetoxy group connected to C-11 in 4 were absent in 3, suggesting that the acetoxy group located at C-11 in 4 was replaced by a free hydroxyl group in 3. Accordingly, we can preliminarily establish the structure of compound 3 as shown in Fig. 1, and this deduction was further well supported by ¹H-¹H COSY and HMBC analysis (Fig. 2). Furthermore, the presented coupling constant of H-2 ($J_{2,3} = 2.0$ Hz) and the NOE correlation between H-2 and H₃-10 in the NOESY spectrum supported the β-orientation of Me-10 and the α-orientation of the methoxy group at C-2.19,25 Therefore, compound 3 was assigned as 2α-methoxyl-3βmethyl-6-methylol-2,3-dihydrobenzofuran.

Among these thymol derivatives, **1–3** are new compounds that are here reported from nature for the first time. Compound **3** is structurally characterized with a dihydrobenzofuran skeleton and this type of monoterpene is rare in natural products.²⁵ Compound **5** was previously only reported from plant *Eupatorium fortunei* and this is the first time for it being isolated from

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Compounds	Staphylococcus aureus	Bacillus cereus	Bacillus thuringiensis	Escherichia coli	Salmonella enterica
1	7.8	3.9	7.8	15.6	15.6
2	>100	62.5	>100	>100	>100
3	31.3	31.3	62.5	>100	>100
4	31.3	62.5	62.5	>100	>100
5	7.8	7.8	15.6	15.6	15.6
6	62.5	62.5	62.5	>100	>100
7	>100	62.5	62.5	>100	>100
8	>100	>100	>100	>100	>100
KS	1.9	3.9	3.9	3.9	3.9

A. adenophora.²⁰ The other four known compounds **4**, **6**, **7** and **8** are here also isolated and identified from the aerial parts of *A. adenophora* for the first time.

Aimed to explore their potential and undiscovered pharmacological activity of these isolated thymol derivatives, compounds 1-8 were evaluated for their in vitro antimicrobial activity by testing their MIC values, using a bioassay method as previously we used and described.^{26,27} Table 3 lists the results obtained for these compounds on the viability of five tested bacterial strains, compared to kanamycin sulfate (KS) as a reference compound. Among them, compounds 1 and 5 were found to be strongly active against all the five assayed microorganisms with MIC values ranging from 3.9 to 15.6 µg mL⁻¹, which were comparable to the positive control KS (MICs = 1.9 to 3.9 μ g mL⁻¹). Compounds 2, 3, 4, 6 and 7 only selectively showed antibacterial activity (MIC 31.3-62.5 µg mL^{-1}) against three tested Gram-(+) bacteria, *i.e. S. aureus*, *B.* cereus and B. thuringiensis. While no antibacterial activity was detected for compound 8 toward all the five tested bacterial strains. From the result, it seems to show that the existence of both the hydroxyl group at C-3 and C-8 would be important for this group of thymol compounds to display their antibacterial potentials.

Compounds 1-8 were further tested for their in vitro cytotoxicity against human cancer MCF-7, HeLa and NCI-H460 cell lines, using a microdilution titre technique as recently we described.²⁸ The resulting IC₅₀ values are displayed in Table 4, compared to Adriamycin as positive control. Compounds 1 and 5 were found to show strong or moderate cytotoxicity against all the three tested cancer cell lines, with IC₅₀ values ranging from 7.45 to 28.63 $\mu M.$ Compounds 7 and 8 showed slight but detectable cytotoxicity toward MCF-7 and NCI-H460 cell lines, with IC₅₀ values ranging from 44.65 to 83.19 µM. While, no obvious cytotoxic activity was detected for the other compounds in this bioassay. Comparison of the chemical structures and the cytotoxic activity of these compounds indicated that the existence of both the hydroxyl group at C-3 and C-8 would be important for this group of thymol derivatives to fully display their cytotoxic potentials.

As a well-known invasive plant, *A. adenophora* has attracted much attention of scientists to investigate its invasion

mechanisms. Since that this plant is accumulating a huge biomass at its invasion areas, to explore the potential utilization of A. Adenophora is gradually also concentrated and emphasized by some researchers. Up to date, phytochemical studies have indicated that structurally diverse chemicals exist in this invasive species. Our present findings further support that the aerial part tissue of this plant is rich in bioactive natural products valuable to be explored for medicinal usage. It is interesting to note that compound 5 was reported as a natural product capable of strongly inhibiting the growth of Microcystis aeruginosa,20 suggesting that these thymol derivatives, at least for compound 5, might have some allelopathic potential to contribute the invasion success of A. adenophora. Noteworthily, for all these thymol derivatives identified from A. adenophora, the carbon C-7 (or C-11 in compounds 3 and 4) is generally appeared as an oxygenated carbon. While for those thymol compounds reported from some other Eupatorium plants, such as thymol compounds from E. fortunei and E. cannabinum,22-24 usually exhibited at C-7 is a methyl group (an unoxygenated carbon). Thus, the general oxygenation extent of thymol compounds at C-7 might have some chemotaxonomic significance for plants in Adenophora and (or) Eupatorium genus. Furthermore, it is evident that compounds 3 and 4 contain a typical dihydrobenzofuran skeleton. Dihydrobenzofuran type monoterpene is rare in nature and the discovery of dihydrobenzofuran type new compound 3 suggests that some so far undiscovered rare monoterpenes would still exist in the aerial parts of A. adenophora worthy of further investigation.

Table 4Cytotoxic activity of compounds $1-8 (IC_{50}, \mu M)^a$					
Compound	ds MCF-7	NCI-H460	HeLa		
1	7.45 ± 0.22	8.32 ± 0.21	9.45 ± 0.46		
2-4	>100	>100	>100		
5	11.54 ± 0.8	$6 15.67 \pm 1.03$	28.63 ± 1.93		
6	>100	>100	>100		
7	44.65 ± 4.0	8 52.74 \pm 5.16	>100		
8	83.19 ± 6.5	5 77.42 \pm 5.06	>100		
Adriamyci	n 0.78 ± 0.06	1.12 ± 0.05	0.54 ± 0.04		

^{*a*} Values represent mean \pm SD (n = 3) based on three individual experiments.

Materials and methods

General experimental procedures

UV spectra were recorded in MeOH on a PerkinElmer Lambda 35 UV-vis spectrophotometer. IR spectra (KBr) were recorded on a Bruker Tensor 27 spectrophotometer in cm⁻¹. ¹H (600 MHz and 400 MHz), ¹³C (150 MHz and 100 MHz), and 2D NMR spectra were recorded in CDCl₃ and CD₃OD 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 P3000 HPLC pump and a UV3000 UV-VIS Detector with a Fuji-C18 column (10 µm-100A). For column chromatography (CC), silica gel (200-300 mesh, Qingdao Marine Chemical Inc., Peoples Republic of China), YMC ODS-A (50 µm, YMC Co. Ltd., Japan) were used, and Sephadex LH-20 (Pharmacia Fine Chemical Co. Ltd.) were used. Fractions were monitored by TLC, and spots were visualized by heating the silica gel plates sprayed with 10% H₂SO₄ in ethanol.

Plant materials

The aerial part material of Ageratina adenophora (Spreng.) were collected in a suburb of Kunming, Yunnan province, P. R. China, in July 2009, and authenticated by Prof. Fu-Wu Xing, South China Botanical Garden, Chinese Academy of Sciences. A voucher specimen (no. 20090702) was deposited at the Laboratory of Phytochemistry at the South China Botanical Garden, Chinese Academy of Sciences.

Extraction and isolation

The air-dried and powdered aerial part material of A. adenophora (10 kg) were extracted with 95% aqueous ethanol at room temperature for three times (3 \times 20 L, each for 24 h). The ethanol extracts were next combined and concentrated in vacuo, with the resulting residue suspended in water and sequentially extracted with petroleum ether $(3 \times 3 L)$ and EtOAc $(3 \times 3 L)$. The petroleum ether and EtOAc layers were evaporated in vacuo to yield petroleum-soluble fraction (93.1 g) and EtOAc-soluble fraction (80.0 g), respectively.

The petroleum ether fraction (93.1 g) was subjected to silica gel CC (1000 mm \times 120 mm i.d.), eluting with a gradient of CHCl₃-MeOH (100 : 0 to 90 : 10, v/v) to give twelve fractions (P_1 - P_{12}) after pooled according to their TLC profiles. Fraction P_6 (20.6 g), obtained by elution with $CHCl_3$ -MeOH (98 : 2, v/v), was applied to silica gel CC (800 \times 75 mm i.d.) eluted with a gradient of petroleum ether-acetone (100:0 to 80:20, v/v) to obtain nine fractions ($P_{6-1}-P_{6-9}$). Fraction P_{6-4} (7.4 g) was subjected to silica gel CC (800 \times 50 mm i.d.) eluted with petroleum-acetone (300:1 to 100:5) in a gradient to obtain subfractions $P_{6-4-4}-P_{6-4-9}$, of which sub-fraction P_{6-4-4} (600.0 mg) was separated by preparative HPLC (flow rate 8 mL min⁻¹) using MeOH-H₂O (70 : 30, v/v) to afford compound 6 ($t_{\rm R}$ = 37.5 min, 2.4 mg). Fraction P_{6-7} (6.2 g) was applied to an ODS CC using

MeOH-H₂O (30 : 70 to 90 : 10) to obtain sub-fractions P_{6-7-1} -P₆₋ 7-12. Subsequently sub-fraction P₆₋₇₋₈ (19.2 mg) was purified by Sephadex LH-20 CC (1500 mm \times 25 mm i.d.) eluted with CHCl₃-MeOH (20: 80, v/v) to afford compounds 2 (2.1 mg) and 8 (2.5 mg), and sub-fraction P_{6-7-3} (930.0 mg) was purified by a Sephadex LH-20 CC using acetone to afford compound 7 (25.1 mg). Fraction P₆₋₈ (340.0 mg) was subjected to Sephadex LH-20 CC and preparative HPLC (flow rate 8 mL min⁻¹) using MeOH- $H_2O(45:55 \text{ to } 50:50, v/v)$ as mobile phase to afford compound $1 (t_{\rm R} = 48.0 \text{ min}, 6.7 \text{ mg})$. Fraction P₆₋₉ (600.0 mg) was subjected to silica gel CC eluted with petroleum-acetone (100:1 to 90 : 10, v/v) in gradient to obtain sub-fractions P_{6-8-1} and P_{6-8-2} , and sub-fraction P_{6-8-2} (45.5 mg) was further purified by an ODS CC using MeOH-H₂O (65 : 35, v/v) as eluent to afford compound 4 (3.0 mg).

The EtOAc-soluble fraction (80.0 g) was subjected to silica gel CC (1000 mm \times 120 mm i.d.) using a gradient of CHCl₃-MeOH (95:5 to 60:40, v/v) to give ten fractions (E_1-E_{10}) . Fraction E_6 (12.0 g), obtained by elution with $CHCl_3$ -MeOH (85 : 15, v/v), was subjected to silica gel CC eluted with CHCl3-MeOH (40:1 to 9:1, v/v) in gradient to obtain sub-fractions $E_{6-1}-E_{6-8}$. Subfraction E_{6-2} (213.1 mg) was further applied to ODS CC eluted with MeOH-H₂O (20:80 to 90:10, v/v) to afford compound 3 (7.0 mg). Fraction E₆₋₅ (2.7 g) was subjected to ODS CC using MeOH-H₂O (10 : 90 to 70 : 30, v/v) to afford five subfractions $(E_{6-5-1}-E_{6-5-5})$, of which sub-fraction E_{6-5-1} (312.0 mg) was further purified by Sephadex LH-20 CC using MeOH to afford compound 5 (30.0 mg).

7-Formyl-9-isobutyryloxy-8-hydroxythymol (1). Yellowness oil; $[\alpha]_{D}^{20}$ -2.0 (c 0.65, CHCl₃); IR (KBr) ν_{max} 3424, 1695, 1612, 1579 cm⁻¹; UV (MeOH) λ_{max} (log ε) nm: 222 (4.11), 260 (3.88), 317 (3.36); ¹H (CDCl₃, 600 MHz) and ¹³C NMR (CDCl₃, 150 MHz) data: see Tables 1 and 3; ESI-MS m/z 571 $[2M + K]^+$, 555 [2M + $Na^{+}_{, 567} [2M + Cl]^{-}_{, 289} [M + Na^{+}_{, 301} [M + Cl]^{-}_{, 265} [M - H]^{-}_{; 7}$ HR-ESI-MS m/z 289.1056 $[M + Na]^+$ (calcd. for $C_{14}H_{18}O_5Na$, 289.1052).

7,9-Di-isobutyryloxy-8,10-dehydrothymol (2). Yellowness oil; $[\alpha]_{D}^{20}$ 0.0 (c 0.20, CH₃OH); IR (KBr) ν_{max} 3438, 1733, 1677 cm⁻¹; UV (MeOH) λ_{max} (log ε) nm: 205 (4.34), 285 (3.42); ¹H (CDCl₃, 600 MHz) and ¹³C NMR (CDCl₃, 100 MHz) data: see Tables 1 and 3; ESI-MS m/z 343 $[M + Na]^+$, 359 $[M + K]^+$; HR-ESI-MS m/z $343.1526 [M + Na]^+$ (calcd for C₁₈H₂₄O₅Na, 343.1521).

2α-Methoxyl-3β-methyl-6-methylol-2,3-dihydrobenzofuran (3). Yellowness oil; $[\alpha]_{D}^{20}$ 2.8 (*c* 0.16, CH₃OH); IR (KBr) ν_{max} 3448, 1596, 1496, 1455 cm⁻¹; UV (MeOH) λ_{max} (log ε) nm: 204 (4.09), 279 (3.32); ¹H (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) data: see Tables 1 and 3; ESI-MS m/z 195 $[M + H]^+$, 217 [M + Na^{+} , 289 $[M + Na^{+}]$, 423 $[2M + Cl^{-}]$; HR-EI-MS m/z 194.0938 (calcd. for C₁₁H₁₄O₃, 194.0937).

Antimicrobial activity

Antimicrobial activity of these eight thymol compounds were evaluated, using 96-well plates, by a method based on a microdilution titre technique with modification in determination of the minimum inhibitory concentration (MIC) values.²⁶ Before the assay, proper amounts of the tested compounds and

kanamycin sulfate (positive control) were dissolved in methanol to prepare their 1.0 mg mL⁻¹ sample solutions and the positive control solution, respectively, and resazurin (indicator) was dissolved in distilled water to prepare 100 μ g mL⁻¹ indicator solution for the assay. In the test, 100 µL indicator solution (resazurin, 100 μ g mL⁻¹) was first placed into each of the sterility control wells (11th column) on the 96 well plates, and about 7.5 mL indicator solution was mixed with 5 mL test organism (10^6 cfu mL⁻¹) followed by transferring (100μ L, each) to growth control wells (12th column) and all test wells (1–10 $^{\rm th}$ column). Then, each of 100 µL of the sample solutions (1.0 mg mL⁻¹ of tested compounds in methanol) and the positive control solution (1.0 mg mL⁻¹ of kanamycin sulfate in methanol) as well as the negative control sample (pure MeOH) were allied to the wells in the 1st column of the plates. In each plate, up to six samples along with a positive control and a negative control samples were applied. Once all samples and controls were properly applied to the 1st column of wells on the plate, half of the homogenized content (100 µL) from these wells was then parallel transferred to the 2nd column of wells, and each subsequent well 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 change to pink. The lowest concentration for each tested compound at which color change occurred was recorded as its primary MIC value. The average of primary values from three individual tests were calculated and that was taken as the final MIC value for each of the test compounds.27 A total of five microorganisms including three Gram-(+) bacteria (Staphylococcus aureus, Bacillus cereus and Bacillus thuringiensis) and two Gram-(-) bacterial species (Escherichia coli and Salmonella enterica) were used in the bioassay. The resulting MIC values of the tested compounds were listed in Table 3.

Cytotoxic assay

The cytotoxic activity of compounds 1-8 against three human tumor (MCF-7, NCI-H460 and Hela) cell lines were assayed by using 96 well plates according to a MTT method as we used previously.28 In brief, the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO₂ at 37 °C. Each well of 96well cell culture plates was seeded with 100 µL adherent cells (5 \times 10⁴ cell per mL) and placed in an atmosphere with 5% CO₂ at 37 °C for 24 h to form a monolayer on the flat bottoms. Subsequently, in each well, the supernatant was removed and 100 μ L fresh medium and 100 μ L medium containing one of the test compounds was added. Then the plate was incubated in 5% CO2 atmosphere at 37 °C. After 3 days, 20 µL MTT at concentration 5 mg mL⁻¹ in DMSO was added into each well and incubated for 4 h. Carefully, the supernatant in each well was removed and 150 µL DMSO was added. Then the plate was vortex shaken for 15 min to dissolve blue formazan crystals. The OD (optical density) value of each well was tested on a Genois microplate reader (Tecan GENios, Männedorf, Switzerland) at 570 nm. All the tests were conducted by three individual experiments and adriamycin was applied as a positive control.

In a test, for each of the tumor cell lines, each of the test compounds was set at concentrations 50, 25, 12.5, 6.25, 3.125, 1.5625 µg mL⁻¹. The inhibitory rate of tumor cell growth was calculated by the formula: inhibition rate (%) = (OD_{control} – OD_{treated})/OD_{control} × 100%, and the IC₅₀ values were calculated by SPSS 16.0 statistic software. The three tumor cell lines were purchased from the Kunming Institute of Zoology, CAS. The resulting IC₅₀ values listed in Table 4 were based on three individual experiments and represented as means \pm standard deviation (SD).

Conclusions

Eight thymol derivatives, including three new (1-3) and five known ones (4-8), were obtained from the aerial parts of the invasive plant Ageratina adenophora. Their structures were elucidated by extensive spectroscopic analysis and they were all isolated from the aerial parts of A. Adenophora for the first time. Compounds 1 and 5 were found to show obviously in vitro antimicrobial activity against all the five tested bacterial strains with IC_{50} values ranging from 7.8 to 15.6 µg mL⁻¹. Other thymol compounds, except 9, only selectively showed detectable in vitro antimicrobial activity toward three tested Gram-(+) bacteria. Compounds 1 and 5 were further revealed to show in vitro cytotoxic activity against human tumor MCF-7, NCI-H460 and HeLa cell lines, with IC50 values ranging from 7.45 to 28.63 µM. Compounds 7 and 8 selectively showed slight in vitro cytotoxicity toward MCF-7 and NCI-H460 cell lines. These data supported that the aerial parts of A. Adenophora are rich in potential bioactive natural products valuable to be developed in medicinal field.

Conflicts of interest

There are no conflicts to declare.

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