

Full Paper

## Antibacterial Thymol Derivatives Isolated from *Centipeda minima*

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**Abstract:** Two new monoterpenoids, 8,10-dihydroxy-9(2)-methylbutyryloxythymol (**1**) and 10-hydroxy-8,9-dioxyisopropylidene-thymol (**2**), together with five known thymol derivatives: 8,9,10-trihydroxythymol (**3**), thymol- $\beta$ -glucopyranoside (**4**), 9-hydroxythymol (**5**), 8,10-dihydroxy-9-isobutyryloxythymol (**6**), and 8-hydroxy-9,10-diisobutyryloxythymol (**7**), were isolated from *Centipeda minima*. Their structures were identified by means of spectroscopic analyses. Interestingly, compound **2** is not an extraction artifact according to a close HPLC examination of material after extraction by analytical MeOH at ambient temperature. The antibacterial activities of compounds **1-7** were evaluated against eight microbial strains by the agar dilution method.

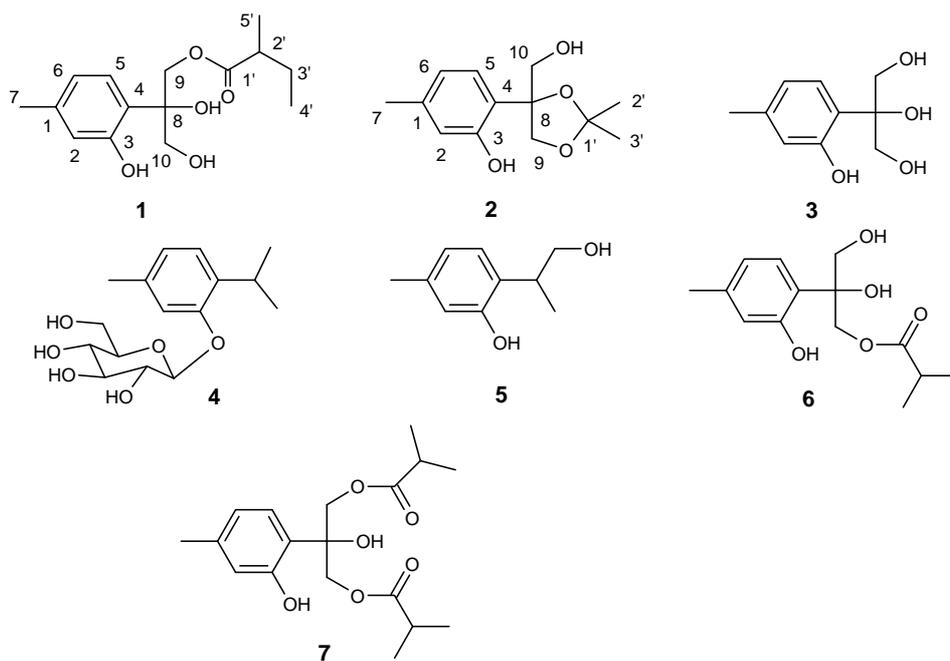
**Keywords:** *Centipeda minima*, Compositae, thymol derivatives, antibacterial activity

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## Introduction

The loss of quality and safety of food largely results from microorganisms. The use of antimicrobials and antiseptics is a common alternative to control bacteria in food [1]. However, the widespread use of antibiotics in human medicine and agriculture has caused serious problem of bacterial resistance [2]. Therefore, plant derived antimicrobial agents with high potency and low mammalian toxicity, useful for food preservation and human health, have gained special interest in recent decades [3-5]. China is a country well known for its utilization of traditional medicine; for centuries, indigenous people have been using herbal medicines to treat various diseases, including a wide range of infectious ones. Interestingly, there is not much documented data on microbial resistance. This indicates that traditional Chinese medicine is a potential source for discovering promising antibacterial substances. *Centipeda minima* (L.) (Compositae) is found spread throughout China, Japan, Korea, India, Malaysia, and Oceania [6], and is a commonly used Chinese folk medicine for colds, nasal allergy, diarrhea, malaria and asthma [7]. Previous studies on *C. minima* revealed that its flavonoids, sesquiterpene lactones and amides could block histamine release, and therefore contribute to its anti-allergy effects [8,9]. Sesquiterpenoids were also found to have antagonistic activities for platelet activating factor and antibacterial activities [10,11]. During our search for new antimicrobial components from traditional Chinese medicine, two new monoterpenoids, 8,10-dihydroxy-9(2)-methylbutyryloxythymol (**1**), 10-hydroxy-8,9-dioxisopropylidenethymol (**2**), along with five known thymol derivatives: 8,9,10-trihydroxythymol (**3**), thymol- $\beta$ -glucopyranoside (**4**), 9-hydroxythymol (**5**), 8,10-dihydroxy-9-isobutyryloxythymol (**6**), and 8-hydroxy-9,10-diisobutyryloxythymol (**7**) were isolated from the whole plants of *C. minima* (Figure 1). In this paper, we report the isolation and structural identification of the new compounds, and the antibacterial properties of compounds **1-7**.

**Figure 1.** Structures of compounds **1-7**.



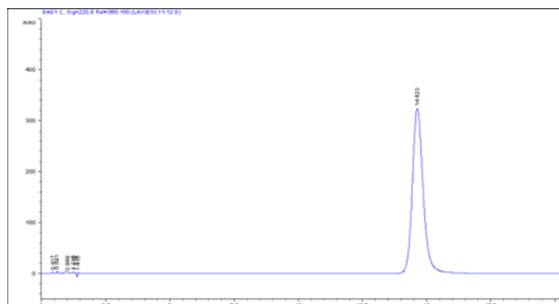
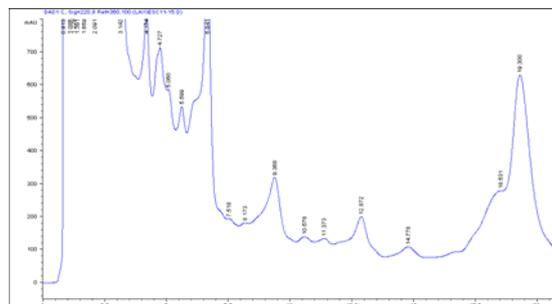
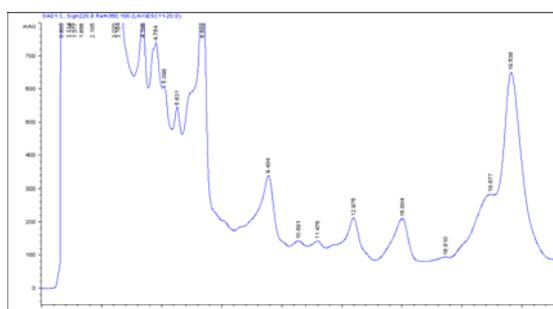
## Results and Discussion

Compound **1** was obtained as a colorless oil. Its molecular formula  $C_{15}H_{22}O_5$  was deduced on the basis of HRESIMS showing a peak at  $m/z$  305.1363  $[M+Na]^+$  (calcd.  $C_{15}H_{22}O_5Na$ , 305.1364). The  $^1H$ -NMR spectrum exhibited a set of signals characteristic of a 1,3,4-trisubstituted phenyl moiety. The  $^{13}C$ -NMR data (Table 1) were very similar to those of **6**, except for two methyls at  $\delta_C$  11.4 and 16.4, one methylene at  $\delta_C$  26.6, and one methine at  $\delta_C$  41.0 present in **1**, instead of two symmetrical methyl groups at  $\delta_C$  19.1, and one methine at  $\delta_C$  34.4 seen in **6**, which in conjunction with the  $^1H$ - $^1H$  COSY interactions of aliphatic protons, suggesting the existence of a  $CH_3-CH_2-CH-CH_3$  segment in **1**. The HMBC correlations of H-2', H-3', H-5' and H-9 all correlated with a carbonyl carbon at  $\delta_C$  177.9 established the linkage of C-1' and C-9 ( $\delta_C$  67.3). The absolute stereochemistry at C-8 and C-2' still remained obscure. Thus, the structure of **1** was determined to be 8,10-dihydroxy-9(2)-methylbutyryloxythymol.

The molecular composition of **2** was assigned as  $C_{13}H_{18}O_4$  by HRESIMS (peak at  $m/z$  261.1108  $[M+Na]^+$  (calcd.  $C_{13}H_{18}O_4Na$  261.1102), featuring five degrees of unsaturation. The resemblance of  $^{13}C$ -NMR spectra between **2** and **3** suggest they are analogues. However, two additional methyl signals ( $\delta_C$  25.9, 27.2) and one  $sp^3$  quaternary carbon ( $\delta_C$  110.6) were observed in the  $^{13}C$ -NMR spectrum of **2**, in conjunction with one more unsaturation and the 40 Da larger mw of **2** than **3**, disclosing the presence of an isopropyl moiety. This was further confirmed by the observed HMBC interactions of H-9, H-2', and H-3' with C-1'. The cross peaks of  $H_a-9$  with  $H_a-10$  and  $H_b-10$ , and  $H_b-9$  with H-2' observed in the ROESY spectrum indicated that hydroxymethyl group,  $H_a-9$ , and H-3' were at the same face of the five-membered ring. The structure of **2** suggests an extraction artifact, however, the unambiguous detection of **2** by a close HPLC analysis in the raw material after extracting with analytical methanol and concentrating at ambient temperature suggested that **2** is indeed a new natural product (Figure 2). Therefore, the structure of **2** was assigned as 10-hydroxy-8,9-dioxyisopropylidenethymol.

The five known monoterpenoids were identified as 8,9,10-trihydroxythymol (**3**) [12], thymol- $\beta$ -glucopyranoside (**4**) [13], 9-hydroxythymol (**5**) [14], 8,10-dihydroxy-9-isobutyryloxythymol (**6**) [15] and 8-hydroxy-9,10-diisobutyryloxythymol (**7**) [15], respectively, by comparison of their spectroscopic data ( $^1H$ -,  $^{13}C$ -NMR and MS) with those reported in the literature. Thymol derivatives have been isolated from other Compositae species [16-20], with compounds **3-7** being thymol derivatives were isolated from *C. minima* for the first time.

Antibacterial tests revealed that all the agents tested exhibited antibacterial effects against all the bacteria investigated (Table 2). At the MIC of 6.25  $\mu g/mL$ , compound **2** was found to be most effective against *B. subtilis*, compound **3** against *S. typhimurium*, and compound **7** against *S. aureus*, *S. flexneri*, and *S. paratyphi-B*. The MIC value of **2** against *S. aureus* is 12.5  $\mu g/mL$  comparable with that of cefradine. All the tested bacteria were less sensitive to compound **1** with the MIC larger than 100  $\mu g/mL$ . Thymol, as a component of volatile oil in many plants, has been proved to possess antimicrobial activities [21], the antibacterial activities of compounds **2-7** were probably resulted from their structural similarities with thymol. The results implied that *C. minima* could be a potential source for searching natural antibacterial substances applied for food preservation.

**Figure 2.** HPLC check of compound **2** in the plant <sup>a</sup>**A:** Compound **2****B:** The crude extracts**C:** Compound **2** and the crude extracts

<sup>a</sup> HPLC conditions: column: LiChrospher 100 RP-18e (125 x 4 mm, 5  $\mu$ m), mobile phase: 22% acetonitrile aqueous, flow rate: 1 mL/min, detection: 220 nm; A peak at  $t_R$ 14.8 min of the chromatogram of the crude extracts bears the same UV/DAD absorption and retention time as the reference compound; The proportion of peaks at 13.0 and 14.8 min is reversal when mixing reference with crude extracts.

## Experimental

### General

Melting points were obtained on an XRC-1 micromelting apparatus. Optical rotations were determined on a JASCO-20C digital polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. IR spectra were obtained with a Bruker Tensor 27 FT-IR spectrophotometer with KBr pellets. <sup>1</sup>H-NMR (400 MHz) and <sup>13</sup>C-NMR (100 MHz) spectra were recorded on a Bruker AM-400 spectrometer with TMS as an internal reference. 2D NMR spectra were measured with a DRX-500 spectrometer. EIMS (70 eV) were recorded on a VG Auto Spec-3000 spectrometer. ESIMS and HRESIMS were carried out with an API QSTAR Pulsar 1 spectrometer. Silica gel (200-300 mesh and 10-40  $\mu$ m) for column chromatography and GF<sub>254</sub> for TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, People's Republic of China. Sephadex LH-20 was obtained from Amersham Pharmacia Biotech, Sweden. RP-18 silica gel (40-63  $\mu$

column chromatography was purchased from Daiso Co., Japan. Diaion HP20 and MCI gel CHP 20P (75-150  $\mu\text{m}$ ) were obtained from Mitsubishi Kasei, Tokyo, Japan. Fractions were monitored by TLC and spots were visualized after spraying with 10%  $\text{H}_2\text{SO}_4$  in ethanol or anisaldehyde reagent followed by heating.

### Plant Material

The whole plants of *C. minima* were purchased from Yunnan Corporation of Materia Medica, Yunnan Province, P. R. China, and identified by Mr. H. Y. Sun at Yunnan Corporation of Materia Medica. A voucher specimen (No. CHYX0159) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, P. R. China.

### Extraction and Isolation

Dried and powdered plant materials of *C. minima* (10 kg) were extracted three times with 95% EtOH under reflux and the combined solvent was evaporated *in vacuo*. The extracts were suspended in water and successively partitioned with petroleum ether, EtOAc and *n*-BuOH, respectively. The EtOAc extracts (170 g) were subjected to column chromatography (CC) over silica gel (200-300 mesh) and eluted with  $\text{CHCl}_2$ -MeOH (8:1) to give fractions 1-4. Fraction 3 (70 g) was submitted to CC on silica gel, eluting with  $\text{CHCl}_3$ -MeOH (11:1) to give fractions 3.1-3.4. Fraction 3.3 (13 g) was chromatographed on MCI gel CHP 20P eluted with MeOH- $\text{H}_2\text{O}$  (9:1) followed by gel filtration on Sephadex LH-20 (MeOH) and repeated *vacuo* liquid chromatography (VLC) to yield **3** (22 mg) and **4** (52 mg). Fraction 2 (30 g) was chromatographed on Diaion HP20 (95% EtOH) to decolor, the eluents were then subjected to CC on silica gel, eluting with  $\text{CHCl}_3$ -MeOH (1:0.5:1) to give fractions 2.1-2.8. Fraction 2.2 (10 g) was passed through MCI gel CHP 20P eluted with MeOH- $\text{H}_2\text{O}$  (9:1) to decolor, and then subjected to Sephadex LH-20 (MeOH), RP-18 (MeOH- $\text{H}_2\text{O}$ , 50:50-100:0) and repeated VLC to yield **1** (44 mg), **2** (13 mg), **5** (28 mg), **6** (66 mg), and **7** (90 mg).

*8,10-Dihydroxy-9(2)-methylbutyryloxythymol (1)*. Colorless oil;  $[\alpha]_{\text{D}}^{20.8} +13.46^\circ$  (c 0.52,  $\text{CHCl}_3$ ); UV ( $\text{CHCl}_3$ )  $\lambda_{\text{max}}$  (log  $\epsilon$ ) nm : 278 (3.34), 238(3.08); IR (KBr)  $\nu_{\text{max}}$ : 3417, 1717, 1578, 1462  $\text{cm}^{-1}$ . ESIMS  $m/z$ : 305  $[\text{M}+\text{Na}]^+$ ; HRESIMS  $m/z$ : 305.1363  $[\text{M}+\text{Na}]^+$  (calcd.  $\text{C}_{15}\text{H}_{22}\text{O}_5\text{Na}$  305.1364, error = -0.635 ppm);  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see Table 1.

*10-Hydroxy-8,9-dioxyisopropylidene-thymol (2)*. Colorless crystal; mp 156-157 $^\circ\text{C}$ ;  $[\alpha]_{\text{D}}^{20.5} +9.36^\circ$  (c 0.29, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) nm: 282 (3.32), 275(3.33), 219 (3.87), 203 (4.36); IR (KBr)  $\nu_{\text{max}}$ : 3405, 1617, 1588, 1458  $\text{cm}^{-1}$ ; ESIMS  $m/z$ : 261  $[\text{M}+\text{Na}]^+$ ; HRESIMS  $m/z$ : 261.1108  $[\text{M}+\text{Na}]^+$  (calcd for  $\text{C}_{13}\text{H}_{18}\text{O}_4\text{Na}$  261.1102, error = 1.9953 ppm);  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data, see Table 1.

**Table 1.**  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra data for compounds **1** and **2**<sup>a</sup>.

position	1		2	
	$\delta_{\text{H}}$ (J=Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J=Hz)	$\delta_{\text{C}}$
1		139.9		139.5
2	6.72 br. s	118.5	6.57 br. s	117.1
3		156.4		154.6
4		119.6		127.3
5	6.94 d (7.9)	126.3	7.32 d (7.8)	128.5
6	6.67 br. d (7.9)	120.5	6.62 br. d (7.8)	120.9
7	2.29 s	20.9	2.23 s	21.1
8		78.9		86.5
9 <sub>a</sub>	4.54 d (12.0)	67.3	4.40 d (9.0)	72.3
9 <sub>b</sub>	4.46 d (12.0)		4.16 d (9.0)	
10 <sub>a</sub>	3.90 d (12.0)	65.9	3.73 br. d (11.5)	67.3
10 <sub>b</sub>	3.81 d (12.0)		3.60 br. d (11.5)	
1'		177.9		110.6
2'	2.40 m	41.0	1.27 s	25.9
3' <sub>a</sub>	1.62 m	26.6	1.52 s	27.2
3' <sub>b</sub>	1.51 m			
4'	0.84 m	11.4		
5'	1.11 d (7.7)	16.4		

<sup>a</sup>The spectra were recorded in  $\text{CDCl}_3$  (400 MHz for  $^1\text{H}$ , 100 MHz for  $^{13}\text{C}$ ).

### Antibacterial Assay

Antibacterial properties were tested by agar dilution method [22]. The bacterial strains employed were *Staphylococcus aureus* CMCC26001 (CMCC, National Center for Medical Culture Collections, Beijing, China), *Escherichia coli* CMCC44103, *Salmonella typhimurium* CMCC80087, and *Shigella flexneri* CMCC51335, and the following clinically isolated strains: *Staphylococcus epidermidis*, *Bacillus subtilis*, *Salmonella paratyphi-A*, *Salmonella paratyphi-B*. Cefradine and gentamycin were used as reference standards, plates containing only MHA medium and 1% DMSO in MHA medium served as negative and solvent controls. The tests were performed in triplicate and repeated once.

**Table 2.** Antibacterial activity of compounds **1-7** (MIC<sup>a</sup> values,  $\mu\text{g/mL}$ ).

Pathogen	1	2	3	4	5	6	7	standards <sup>b</sup>	
Reference strains									
<i>Staphylococcus aureus</i> CMCC26001	>100	12.5	>100	>100	>100	100	6.25	15	7.5
<i>Escherichia coli</i> CMCC44103	>100	>100	>100	>100	>100	>100	>100	7.5	7.5
<i>Salmonella typhimurium</i> CMCC80087	>100	>100	6.25	25	50	25	>100	7.5	7.5
<i>Shigella flexneri</i> CMCC51335	>100	12.5	>100	>100	>100	>100	6.25	3.25	3.25

Table 2. Cont.

Clinically isolated strains									
<i>Staphylococcus epidermidis</i>	>100	25	>100	50	>100	100	50	3.25	7.5
<i>Bacillus subtilis</i>	>100	6.25	>100	>100	>100	50	>100	3.25	3.25
<i>Salmonella paratyphi-A</i>	>100	>100	>100	>100	>100	>100	>100	3.25	3.25
<i>Salmonella paratyphi-B</i>	>100	>100	>100	>100	>100	>100	6.25	3.25	3.25

<sup>a</sup> Minimum inhibition concentration; <sup>b</sup> Left column is for cefradine and the right is for gentamycin.

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*Sample Availability:* Samples of compounds **4** and **6** are available from the authors.