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Isolation of an Antifungal Compound from *Anaphalis sinica* Hance

LINGYUN YANG[1], ZHIYI YU[3], XINGANG ZHANG[1], JIEYU FAN[1], MINGJING CHEN[2], HUI DENG[2], TAIPING HOU[2] and KE TAO[2,*]

[1]Key Laboratory of Bio-Resource and Eco-Environment of Ministry of Education, Sichuan University, Chengdu 610064, Sichuan, P.R. China
[2]College of Life Science, Sichuan University, Chengdu 610064, Sichuan Province, P.R. China
[3]West China School of Pharmacy, Sichuan University, Chengdu 610064, Sichuan Province, P.R. China

*Corresponding author: Fax: +86 28 85214048; Tel: +86 28 88846045; E-mail: taoke@scu.edu.cn

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The present investigation was in order to isolate antifungal constituents from *Anaphalis sinica* Hance, using activity against several phytopathogenic fungi as a lead. Bioactivity guided fractionation of the petroleum ether extract has led to the isolation of a tremetone derivative (compound **1**), whose structure had been elucidated as 4,6-dihydroxy-14,14-dimethyltremeton based on extensive 1D and 2D NMR and HR-ESI-MS spectral data. Compound **1** had exhibited excellent antifungal activity against *Valsa mali*, *Elsinoe ampelina* and *Gibberella saubinetii* and the values of their EC_{50} reached 2.27, 7.18 and 12.30 μg/mL respectively, that was comparable to the commercial fungicide carbendazim, whose EC_{50} values were 1.60, 33.54 and 6.60 μg/mL respectively.

Key Words: *Anaphalis sinica* hance, Antifungal activity.

INTRODUCTION

Anaphalis consists of about 80 species is one of the largest genera of the A*sian Gnaphalieae (Asteraceae)* and more than 50 species are distributed in China. *Anaphalis sinica* Hance, a species of *Anaphalis*, is widespread in northern, eastern and southern China[1]. In traditional Chinese medicine (TCM), *A. sinica* Hance has long been used by local inhabitants to treat tracheitis and enteritis[2]. Previous phytochemical investigation of *Anaphalis* revealed the presence of flavonoids[3-6], triterpenoids[5], furobenzopyranones[7,8], diterpenes, hydroxylactones[9], kaempferol, tiliroside and quercetin[6] and several species from the genus *Anaphalis* showed significant bioactivity[10]. However, according to existed literatures, little tremetone derivatives isolated from *Anaphalis* have been reported until now and the antifungal activity of these compounds from *A. sinica* Hance has rarely been studied. During the research in our laboratory to find new antifungal agents from plateau plants, we observed that the crude extract of *A. sinica* Hance displayed obvious antifungal activity against several phytopathogenic fungi. Under this circumstance, we aimed to find active constituents from this plant by bioactivity guided isolation and we had already obtained and identified the major antifungal constituent of this plant, which turned out to be a tremetone derivative (compound **1**, Fig. 1). Afterwards, we evaluated the effect of compound **1** on the mycelial growth of *Valsa mali, Elsinoe ampelina* and *Gibberella saubinetii* in solid media according to the standard operating procedure for new agrochemicals (SOP) method of China[11]. Further studies on *A. sinica* Hance will be summarized in our next study.

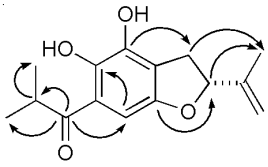

Fig. 1. Chemical structures and main HMBC correlations of compound **1**

EXPERIMENTAL

Melting points were obtained in an EXSTAR 6000 thermal analyzer from SEIKO. ^{1}H NMR, ^{13}C NMR and ^{2}D NMR (135°-DEPT NMR, HMBC NMR, HMQC NMR and ^{1}H-^{1}H COSY NMR) spectra were measured at 27 °C in $CDCl_3$ on Bruker AV II-400 MHz nuclear magnetic resonance apparatuses. IR spectra was recorded on KBr on Nicolet AVATAR 360 spectrophotometer. UV spectra was recorded on a Shimadzu SPD-M10AVP photodiode detector. HR-ESI-MS spectra was recorded on Bruker MS micrOTOF-QII. Medium pressure liquid chromatography (MPLC) was performed on Büchi's apparatus. Semi-preparative HPLC was performed on a Shimadzu LC-2010 with a Shim-pack PRC-SIL(H) column (20 mm × 250 mm I.D.).

Aerial parts of *A. sinica* Hance were collected from Ruoergai Plateau, Sichuan Province, P.R. China in August 2010 and the plant was identified by Dr. Ke Tao of Sichuan

TABLE-1
ANTIFUNGAL ACTIVITIES OF ALL THE FRACTIONS AND SUBFRACTIONS OF PETROLEUM ETHER EXTRACT AGAINST SEVERAL PHYTOPATHOGENIC FUNGI AT 200 μg/mL

Fractions	Inhibition of growth (%)					
	G. saubinetii	*V. mali*	*E. ampelina*	*P. anthracnose*	*C. gloeosporioides*	*B. maydis*
frs. 1	21	27	9	11	9	11
frs. 2	11	12	0	0	0	0
frs. 3	15	7	0	0	0	7
frs. 4	0	9	0	0	0	0
frs. 5	0	0	0	0	0	0
frs. 6	6	16	8	5	5	11
frs. 7	0	0	0	0	0	0
frs. 8	69	100	100	77	82	96
frs. 9	42	87	92	45	47	53
frs. 10	43	65	11	16	15	32
frs. 11	0	33	21	0	0	0
frs. 12	0	0	0	0	0	0
frs. 8.1	7	28	19	0	0	9
frs. 8.2	72	100	100	89	81	100
frs. 8.3	0	0	0	0	0	0

University. A voucher specimen (LY-201008-SA057) is deposited at the Institute for Pesticides and Crop Protection of Sichuan University.

Extraction and isolation: The extraction scheme followed the standard procedure[12]. Air dried, powdered plant material (500 g) of *A. sinica* Hance was extracted exhaustively with EtOH/H_2O (95:5, v/v) at 50 ºC (2L × 5) and the solvent was evaporated *in vacuo* yielding a dark brown viscous extract (45 g). The crude extract was suspended in twice weight of white diatomite and then partitioned at room temperature with petroleum ether (petroleum ether, 60-90 ºC), $CHCl_2$, EtOAc and MeOH successively, yielding petroleum ether extract (12.7 g), $CHCl_2$ extract (5.5 g), EtOAc extract (2.9 g) and MeOH extract (21.2 g) after removal of solvents under vacuum. Each extract was tested for antifungal activity against several phytopathogenic fungi with the plate growth rate method[13]. The petroleum ether extract (4 g) showed the best antifungal activity was subjected to Büchi's medium pressure liquid chromatography (MPLC) on silica gel (38-45 μm, 150 g) with the solvent gradient PE (60-90 ºC)/CH_2Cl_2 6:1, PE (60-90 ºC)/CH_2Cl_2 1:4, CH_2Cl_2, CH_2Cl_2/EtOAc 2:1, EtOAc/acetone 2:1, MeOH (each step 1000 mL). The MPLC yielded twelve fractions (frs.). (frs.1-12) and their antifungal activity was tested respectively (Table-1). The active frs. 8 (197 mg) with better antifungal activity was chromatographed on a silica gel column (38-45 μm, 50 g) and eluted with petroleum ether (60-90 ºC) in EtOAc (4/1, v/v, 1500 mL) to yield three subfractions. (frs. 8.1-8.3). Further separation of frs.8.2 (137 mg) was performed on a the Semi-preparative HPLC with a Shim-pack PRC-SIL(H) column (5 μm; 20 × 250 mm) using a isocratic elution (*n*-hexane/EtOAc 95:5, 4 mL/min; wavelength: 288 nm; room temperature) and yielded compound **1** (45 mg). The purity of compound **1** was determined by NP-HPLC with area normalization method on a Shim-pack CLC-SIL(M) column (5 μm; 4.6 × 150 mm; 290 nm; 30 ºC; in *n*-hexane/EtOAc). Fortunately, the purity of compound **1** was more than 99 % in the end.

Compound 1 (Fig. 1): Pale yellow needle crystal at room temperature; m.p. 129.7 ºC; UV_{max} (petroleum ether/EtOAc, 95/5, v/v): 287.80, 243.29, 226.21 nm; IR bands (KBr, ν_{max}, cm^{-1}): 3396.93, 2930.95, 1639.80, 1609.71, 1490-1100.47; HR-ESI-MS: *m/z* 285.1098 ($[M+Na]^+$; cald. for $C_{15}H_{18}O_4$; 262.1205); ^1H NMR (400.13 MHz, in $CDCl_3$) and ^{13}C NMR (100.62 MHz, in $CDCl_3$) Table-2.

TABLE-2
^1H AND ^{13}C NMR DATA OF COMPOUND **1** (δ in ppm)

Proton no.	^1H NMR signal	Carbon no.	^{13}C NMR signal
OH-4	11.94 (s, 1H)	C-13	210.72 (1C, C=O)
OH-6	8.65 (s, 1H)	C-8	166.21 (1C, C)
H-7	5.91 (s, 1H)	C-6	162.53 (1C, C)
H-2	5.26 (t, *J* = 8Hz, 1H)	C-4	159.62 (1C, C)
H-12	5.06 (s, 1H)	C-10	143.16 (1C, C)
H-12	4.92 (s, 1H)	C-12	112.68 (1C, CH_2)
H-14	3.90 (q, *J* = 6.4Hz, 1H)	C-9	104.72 (1C, C)
H-3	3.25 (dd, *J* = 9.6, 14.8Hz, 1H)	C-5	104.23 (1C, C)
H-3	2.90 (dd, *J* = 7.6, 14.8Hz, 1H)	C-7	90.49 (1C, CH)
H-11	1.75 (s, 3H)	C-2	88.02 (1C, CH)
H-15, H-16	1.19 (d, *J* = 6.8Hz, 6H)	C-14	39.15 (1C, CH)
		C-3	30.73 (1C, CH_2)
		C-15, C-16	19.31 (1C, CH_3)
		C-11	16.92 (1C, CH_3)

Antifungal bioassays: The antifungal activity of all the of samples was tested against several phytopathogenic fungi, *Gibberella saubinetii, Valsa mali Miyabe et Yamada, Elsinoe ampelina (de Bary) Shear, Pepper anthracnose, Colletotrichum gloeosporioides, Bipolaris maydis,* using the plate growth rate method[13,14], according to the SOP method[11]. Each sample was dissolved in acetone and mixed with sterile molten potato dextrose agar (PDA) to obtain final concentrations. The various fungi were incubated at 28 ºC in the dark. The zones of growth were measured when the control group had a growth cycle diameter of *ca*. 60 mm. The inhibition rate was calculated according to eqn. 1.

$$I = \frac{(\overline{D}_1 - \overline{D}_0)}{(D_1)} \times 100\ \% \quad (1)$$

where, I is the inhibition rate, D_1 is the average diameter of mycelia in the blank test and D_0 is the average diameter of mycelia in the presence of fractions.

The method given in ref.[14] was used to determine the median effect concentration (EC_{50}) of compound **1** against *Valsa mali, Elsinoe ampelina* and *Gibberella saubinetii*. Compound **1** and Carbendazim (used as the positive control) were tested at concentrations ranging from 100 to 0.78 μg/mL and from 100 to 0.39 μg/mL, respectively. The EC_{50} values were estimated using probit methods[15] and the results are given in Table -3. All assays were conducted in triplicate.

TABLE-3
ANTIFUNGAL ACTIVITY (EC_{50}, μg/mL) OF COMPOUND **1**

Phytopathogenic fungi	Median effective concentration	
	Compound **1**	Carbendazim
V. mali	2.27	1.60
E. ampelina	7.18	33.54
G. saubinetii	12.30	6.60

RESULTS AND DISCUSSION

Bioactivity guided fractionation of petroleum ether extract of *A. sinica* Hance ultimately led to the isolation of compound **1**, which was conclusively identified on the basis of extensive ^{1}D and ^{2}D NMR spectroscopic analysis (^{1}H, ^{13}C, HMQC, HMBC, DEPT, ^{1}H-^{1}H COSY). The HR-ESI-MS exhibited an ion at *m/z* 285.1098 $[M+Na]^+$, which was in accordance with the molecular formula of $C_{15}H_{18}O_4$. The IR absorptions indicated the presence of hydroxyl (3396 cm^{-1}), carbonyl (1639 cm^{-1}), olefin (1609 and 1100 cm^{-1}) and benzene (1444 cm^{-1} and 1490 cm^{-1}) functions.

Comprehensive analysis of the ^{1}H-^{1}H COSY, HMQC and HMBC spectra and comparison with the NMR data of 5-acetyl-4-hydroxy-2-isopropenyl-6-methoxy-2,3-dihydrobenzofuran[20] allowed to assign all the chemical shifts in the ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR data (Table-2) indicated the existence of 18 H-atoms and showed signals assignable to two phenolic hydroxyls at δ_H 11.94 (1H, s, OH-4) and 8.65 (1H, s, OH-6), an isopropyl group at δ_H 1.19 (6H, d, J = 6.8Hz, H-15 and H-16) and 3.90 (1H, q, J = 6.4Hz, H-14), a benzene ring's hydrogen at δ_H 5.91 (1H, s, H-7) and an alkylene at δ_H 4.92 (1H, s, H-12) and 5.06 (1H, s, H-12). Compound **1** contains 15 carbons, the ^{13}C NMR spectrum displayed 14 peaks, which were attributed by DEPT experiment to three methyl carbons (δ_C = 19.31, 16.92), two methylene carbons (δ_C = 112.68, 30.73), three methane carbons (δ_C = 90.49, 88.02, 39.15) and six quaternary carbons (δ_C = 166.21, 162.53, 159.62, 143.16, 104.72, 104.23). The ^{13}C NMR spectrum revealed characteristic signals for one carbonyl carbon at δ_C 210.72, one olefinic carbon at δ_C 112.68 and six benzene rings' carbons at δ_C 166.21, 162.53, 159.62, 104.72, 104.23 and 90.49. For the signal of C-7 was upfield to δ_C 90.49 whose chemical shifts should be influenced by substituent electronegativity of hydroxyl group at C-6, we could deduce that the acyl group and furane oxygen were in para. This conclusion was secured by further proton NOEDs experiments. In the NOEDs experiment, the correlation between the methylene (CH_2-3) proton and the hydroxyl (OH-4) proton, also the methylene (CH_2-7) proton and the hydroxyl (OH-6) proton were observed.

The substitution sites on the molecule were confirmed by the HMBC experiment (Fig. 1), displaying cross-peak correlations from H-11 to C-10, C-12 and C-2; from H-2 to C-3 and C-8; from H-3 to C-9, C-4 and C-8; from H-7 to C-6, C-4, C-5 and C-8; and from H-14 to C-15, C-16 and C-13.

The absolute configuration of 6-isopropyl-3-methyl-6,7-dihydro-5*H*-furo[3,2-g]-chromene-4,9-dione at C-6 was elucidated to be R as compared with the rotation of the lactone, obtained by exhaustive ozonolysis of (+) isopropyl succinic acid[21], similarly, we investigated the absolute configuration of compound **1** at C-2, which was also as R. Thus, the structure of compound **1** was established (Fig. 1).

Petroleum ether extract of *A. sinica* Hance exhibited potent antifungal activity against several phytopathogenic fungi at 1000 μg/mL. However, previous phytochemical studies of *A. sinica* Hance had not found the antifungal components. During our research, compound **1** which is partly responsible for the effectiveness of this plant was obtained by bioactivity guided isolation. compound **1** was isolated from *A. sinica* Hance for the first time, although this compound had already been found from *Helichrysum species*[15]. The bioactivity of this compound had never been reported. Compound 1 had exhibited excellent antifungal activity and cytotoxic activity in our research. The EC_{50} values of compound **1** against *Valsa mali, Elsinoe ampelina* and *Gibberella saubinetii* were 2.27, 7.18 and 12.30 μg/mL and that of the commercial fungicide carbendazim were 1.60, 33.54 and 6.60 μg/mL respectively. We can see that compound **1** had a better antifungal activity than carbendazim against *Elsinoe ampelina* and a comparable activity as carbendazim against *Valsa mali* and *Gibberella saubinetii*, which indicated that compound **1** had the potential to be developed as a new sort of environmentally-friendly antifungal drug in the agricultural chemistry field. Further research on the application of compound **1** and the isolation of bioactive constituents from *A. sinica* Hance are undergoing in our laboratory.

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