SUPPLEMENTARY MATERIAL

***In vitro* and *ex vivo* antitubercular activity of diarylheptanoids from the rhizomes of *Alpinia officinarum* Hance**

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Abstract

Phytochemical investigation of methanol extract of the rhizomes of *Alpinia officinarum* Hance afforded four known diarylheptanoids 1,7-diphenylhept-4-en-3-one (**1**), 5-hydroxy-1,7-diphenyl-3-heptanone (**2**), 5-hydroxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone (**3**), 7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl heptan-3-one (**4**). The acetate derivative of (**4**), 7-(4"-acetate-3"-methoxy phenyl)-1-phenyl heptan-3-one (**5**) was prepared. These diarylheptanoids exhibited promising *in vitro* and *ex vivo* antitubercular activity for the first time against dormant *Mycobacterium tuberculosis* H37Ra with the IC₅₀ values between 0.34 to 47.69 and 0.13 to 22.91 μ M respectively. All compounds showed comparable activity against *M. bovis* BCG (Dormant Phage), and did not show any activity against two gram +ve and two gram -ve bacterial strains. These compounds were also weakly cytotoxic up to 300 μ M against three human cancer cell lines THP-1, Panc-1 and A549.

Keywords: *Alpinia officinarum* Hance; zingiberaceae; diarylheptanoids; antitubercular

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1. Experimental

1.1 General Experimental Procedures

All solvents used were of LR grade. Extracts were concentrated using rotary evaporator (Buchi R-200, Switzerland). Precoated TLC plates of silica gel 60 F₂₅₄ (Merck, Germany) were used for TLC analysis. Silica gel 100-200 mesh (Merck) was used for column chromatography. IR spectra were recorded on Perkin-Elmer FT-IR spectrometer. ^1H and ^{13}C -NMR spectra were recorded on 400 MHz, 500 MHz and 100 MHz, 125MHz spectrometers, respectively (Bruker, Germany). Deuterated chloroform (CDCl_3) and methanol (CD_3OD) (Aldrich, USA) were used for recording NMR spectra and tetramethylsilane (TMS) was used as an internal standard. ESI-MS spectra were recorded with an API-QSTAR-PULSAR spectrometer. GC-MS was carried out on 6890 GC with 5973 mass selective detector. Optical rotations were recorded with a JASCO P-1020 polarimeter (Waltham, MA, USA). Human acute monocytic leukemia (Thp-1), pancreas carcinoma (Panc-1) and lung adenocarcinoma (A549) cell lines were purchased from the European Collection of Cell Cultures (ECACC). Isoniazid and Rifampicin (standard drugs), tetrazolium salt (MTT), isopropanol, XTT sodium salt powder, Menadione and DMSO were procured from Sigma. The optical density was read on a micro plate reader Spectramax plus384 plate reader, Molecular Devices Inc at 490 nm filter.

1.2 Plant material

Rhizomes of *A. officinarum* Hance, zingiberaceae, were purchased from Pathanamthitta in Kerala, India, and were authenticated by the Department of Botany, Agharkar Research Institute, Pune 411004, India. The voucher specimen of plant material was maintained under the reference number R-138.

1.3 Extraction and bioassay-guided isolation of compounds

The rhizomes (1.5 kg) were ground and extracted by maceration using methanol (6.0 L x 3) at room temperature (48 h x 3). The viscous extract was filtered and concentrated on a rotary evaporator under reduced pressure at 40°C thereby providing crude methanol extract (92.34 g) which was subsequently screened and found to be effective against MTB. It was therefore subjected for fractionation using different solvents and further purification.

The methanol extract (87 g) was redissolved in methanol: water (80:20, 100 ml) and partitioned with n-Hexane thrice. After removal of hexane layer, aqueous layer was further diluted with about 20% water and extracted thrice with chloroform and similarly extracted with ethyl acetate. The results of fractionation and antitubercular screening are collectively represented in Table S1. From these results it was evident that the chloroform and ethyl acetate fractions exhibited prominent inhibitory action against MTB endorsing further purification leading to marker compounds. The chloroform and ethyl acetate fraction (22 g) was collectively subjected to column chromatography over silica gel (100-200 mesh) by employing hexane-ethyl acetate gradient (0 -100%) as mobile phase. Similar fractions in TLC were pooled together to obtain 10 fractions and studied their antitubercular activity. Among those three fractions showing antitubercular activity were further purified and analyses to identify the four antitubercular isolates and also acetate derivative of compound (**4**). Fraction AO-1 on chromatographic separation gave compounds (**1**, 15 mg) and (**2**, 26 mg). Fractions AO-2 on purification gave compound (**3**, 643 mg) and AO-3 on further purification yielded compound (**4**, 8 mg). Acetylation of compound (**4**, 6 mg) gave compound (**5**, 5 mg). These compounds (**1-5**) were characterized by 1D, 2D spectral analysis and tested for antitubercular activity (Table S2).

1.4 Spectral data

7-(4"-Hydroxy-3"-methoxyphenyl)-1-phenyl heptan-3-one (**4**): Pale yellow liquid (Kiuchi et al. 1992), FT-IR (CHCl₃) cm⁻¹: 3600 (bs), 1707 (s), 1514 (s), 1446 (s), 1261(s), 1061(s). ¹H-NMR (400 MHz), in CDCl₃: δ (ppm) 2.90 (2H, t, J=7.6Hz, H-1), 2.73 (2H, t, J=7.6Hz, H-2), 2.39 (2H, t, J=7 Hz, H-4), 1.54-1.60 (2H, m, H-5, 6), 2.56 (2H, t, 7Hz, H-7), 7.24-7.27 (2H, m, H-2',6'), 7.15-7.18 (3H, m, H-3',4',5'), 6.74 (1H, d, J=2Hz, H-2''),6.90 (1H, d, J=8Hz, H-5''), 6.71 (1H, dd, J=2 and 8Hz, H-6''), 3.90 (OCH₃, s). ¹³C-NMR (100 MHz), in CDCl₃: δ (ppm) 29.79 (C-1), 44.29 (C-2), 210.13 (C-3), 42.85 (C-4), 23.37 (C-5), 31.46 (C-6), 35.42 (C-7), 141.12 (C-1'),128.49 (C-2', 6'),128.32 (C-3',5'),126.10 (C-4'),134.17 (C-1''),110.92 (C-2''),146.32, (C-3'')143.63 (C-

4''), 114.15 (C-5''), 120.88 (C-6''), 55.87 (OCH₃). GC-MS: m/z = 312 (M⁺), 210, 195(100), 180, 152, 137, 69, 43, 789.

7-(4''-Acetate-3''-methoxyphenyl)-1-phenyl heptan-3-one (**5**): yield 5 mg, yellow liquid, FT-IR (CHCl₃) cm⁻¹: 1762(s), 1711(s), 1508 (s), 1267 (s), 1032 (s). ¹H-NMR (400 MHz), in CDCl₃: δ (ppm) 2.92 (2H, t, J=7.6Hz, H-1), 2.74 (2H, t, J=7.6Hz, H-2), 2.40 (2H, t, J=7Hz, H-4), 1.58 (2H, m, H-5,6), 2.56 (2H, t, J=7Hz, H-7), 7.24-7.30 (2H, m, H-2',6'), 7.15-7.22 (3H, m, H-3',4',5'), 6.65 (1H, d, J=2Hz, H-2''), 6.82 (1H, d, J=8Hz, H-5''), 6.64 (1H, dd, J=2 and 8Hz, H-6''), 3.85 (OCH₃, s), 2.35 (COCH₃, s). ¹³C-NMR (100 MHz), in CDCl₃: δ (ppm) 29.79 (C-1), 44.32 (C-2), 210 (C-3), 42.79 (C-4), 23.41 (C-5), 30.91 (C-6), 35.72 (C-7), 141.20 (C-1'), 128.49 (C-2',6'), 128.32 (C-3',5'), 126.1 (C-4'), 141.11 (C-1''), 112.56 (C-2''), 150.76 (C-3''), 137.74 (C-4''), 120.43 (C-5''), 122.41 (C-6''), 55.83 (OCH₃), 20.70 (COCH₃). GC-MS: m/z = 354 (M⁺), 280, 197, 180, 167, 153, 126, 111, 97, 83, 69, 55, 41(100), 28.

1.5 Antitubercular activity

Crude extracts, sub-fractions and pure compounds (**1-5**) were tested for their *in vitro* and *ex vivo* effects against dormant and active stages MTB using XRMA protocol (Singh et al. 2011, Sarkar Sampa and Sarkar Dhiman 2012). MTB (ATCC No. 25177) were grown to logarithmic phase (O. D. 1.0) in a M. pheli medium. The stock culture was maintained at -70°C and sub-cultured once in M. pheli medium before inoculation into the experimental culture. All experiments were performed in triplicates and IC₅₀ and IC₉₀ values were calculated from their dose–response curves (Singh et al. 2011, Dzoyem et al. 2013). *M. bovis* BCG assay was carried out according to NR assay protocol (Khan Arshad and Sarkar Dhiman 2008, Sarkar Sampa and Sarkar Dhiman 2012).

1.6 Antimicrobial activity

To determine specificity, compounds (**1-5**) at concentrations of 0.1, 0.3, 1, 3, 10, 30 and 100 µg/mL were tested against bacteria. Bacterial strains, namely *Escherichia coli* (NCIM 2931), *Pseudomonas fluorescens* (ATCC 13525), *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 23857) and *Mycobacterium bovis* BCG (ATCC 35743), were obtained from the National Collection of Industrial Microorganisms (NCIM) (Pune, India) and American Type Culture Collection (ATCC) (USA). These cultures were grown in Luria–Bertani (LB) medium (HiMedia) at 37°C and 150 rpm. For the antibacterial assay, bacterial cultures were incubated for

18 h in the presence of (**1-5**) before reading the absorbance at 620 nm (Kang et al. 2014, Dzoyem et al. 2013).

1.7 Cytotoxicity assay

The cytotoxicity of the pure compounds (**1-5**) was determined using MTT assay against three different human cancer cell lines in duplicate (Ciapetti et al. 1993, Mosmann et al. 1983, Dzoyem et al. 2013). Leukaemia THP-1, lung A549 adenocarcinoma, pancreatic PANC-1 adenocarcinoma cell lines were obtained from the European Collection of Cell Cultures (ECCC), Salisbury, UK. Cell lines were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humidified environment.

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Table S1. Yield and antitubercular activity of *A. officinarum* rhizome extracts

Purification stage	Fractions	Quantity	Activity % Inhibition (at 25 µg/mL)
Crude extract	Methanol extract	92.34 g	85.20 ± 0.86
Sub-fractions	Hexane	31.03 g	33.09 ± 2.43
	Chloroform	10.65 g	98.70 ± 0.23
	Ethyl acetate	15.50 g	94.57 ± 1.11
	Aq. Methanol	30.70 g	17.02 ± 0.09
Chloroform + Ethyl acetate	AO- 1	1.43 g	95.18 ± 2.45
	AO- 2	3.97 g	98.67 ± 0.14
	AO- 3	2.40 g	95.66 ± 4.87

Table S2. *In vitro* anti-tubercular activity of compounds against *Mycobacterium tuberculosis* H37Ra

Comp.	% Inhibition of <i>M. tuberculosis</i> H37Ra growth in presence of compounds with SD values			Activity against MTB H37Ra
	30 µg/mL	10 µg/mL	3 µg/mL	
1	92.34 ± 0.30	90.28 ± 0.22	95.78 ± 0.45	+
2	92.08 ± 0.52	77.33 ± 0.98	57.05 ± 0.28	+
3	91.72 ± 0.85	88.78 ± 0.72	78.28 ± 0.24	+
4	96.17 ± 0.53	38.80 ± 0.98	31.16 ± 0.57	+
5	98.78 ± 1.42	40.38 ± 0.23	22.46 ± 0.36	+

+ : Compounds showing > 90% inhibition at 30 µg/mL ; - : Compounds showing <90% inhibition at 30 µg/mL

Table S3. ¹H and ¹³C NMR spectroscopic data (500 and 125 MHz, CDCl₃) for compounds (4) and (5)

4				5	
Position	δ _c , type	δ _H (J in Hz)	δ _c , type	δ _H (J in Hz)	HMBC
1	29.79, CH ₂	2.90, t (7.6)	29.79	2.92, t (7.6)	2, 3, 1', 2', 6'
2	44.29, CH ₂	2.73, t (7.6)	44.32	2.74, t (7.6)	1, 3, 1'
3	210.13, C	-	210	-	-
4	42.85, CH ₂	2.39, t (7)	42.79	2.40, t (7)	3, 5, 6
5	23.37, CH ₂	1.54-1.60, m	23.41	1.58, m	4, 7
6	31.46, CH ₂	1.54-1.60, m	30.91	1.58, m	4, 7
7	35.42, CH ₂	2.56, t (7)	35.72	2.56, t (7)	5, 6, 1'', 2'', 6''
1'	141.12, C	-	141.20	-	-
2'	128.49, CH	7.24-7.27, m	128.49	7.24-7.30, m	1', 3', 4', 5'
6'	128.49, CH	7.24-7.27, m	128.49	7.24-7.30, m	1', 3', 4', 5'
3'	128.32, CH	7.15-7.18, m	128.32	7.15-7.22, m	2', 6'
4'	126.10, CH	7.15-7.18	126.1	7.15-7.22, m	2', 6'
5'	128.32, CH	7.15-7.18	128.32	7.15-7.22, m	2', 6'
1''	134.17, C	-	141.11	-	-
2''	110.92, CH	6.65, d (2)	112.56	6.74, d (2)	7, 1'', 3'', 5''
3''	146.32, C	-	150.76	-	-
4''	143.63, C	-	137.74	-	-
5''	114.15, CH	6.82, d (8)	122.41	6.90, d (8)	7, 2''
6''	120.88, CH	6.64, dd (2,8)	120.43	6.71, dd (2,8)	1'', 2'', 3'', 4''
	55.87, OCH ₃	3.90, s	55.83	3.85, s	3''
			20.70, COCH ₃	2.35, s	

Table S4. Antimicrobial activity of compounds from the rhizomes of *Alpinia officinarum* Hance

Comp.	% Inhibition at 100 µg/mL			
	SA	BS	PF	EC
1	9.8	15.1	9.6	10.0
2	24.0	21.7	15.4	15.8
3	25.1	19.1	13.4	6.5
4	11.2	16.6	10.3	3.9
5	15.1	21.1	19.3	7.3
SA- <i>Staphylococcus aureus</i> ; BS- <i>Bacillus subtilis</i> ; PF <i>Pseudomonas fluorescens</i> ; EC- <i>Escherichia coli</i> ; Cell viability 80% at the highest (100 µg/mL) concentration; considered as not active against microorganisms				

Table S5. Cytotoxic activity of compounds (**1-5**) for 3 human cancer cell lines after 48 h of exposure ^a

Comp.	% Inhibition at 100 µg/mL		
	THP-1	PANC-1	A549
1	11.4	8.6	10.8
2	18.1	13.0	10.5
3	19.1	17.3	7.9
4	5.7	23.5	27.3
5	11.7	21.6	19.6
THP-1: Acute monocytic leukemia; PANC-1: Pancreas carcinoma; A549: Lung adenocarcinoma.			

Spectral data and structure of compound (**4**)

IR of compound (**4**)

¹H-NMR of compound (4)

¹³C-NMR of compound (4)

GC-MS of compound (4)

Spectral data and structure of compound (5)

IR of compound (**5**)

^1H -NMR of compound (**5**)

^{13}C -NMR of compound (**5**)

GC-MS of compound (5)

COSY of compound (5)

HSQC of compound (5)

HMBC of compound (5)

Figure S1. HMBC and ¹H-¹H COSY correlations of compound (5)