Naturally Occurring Anti-TB Agents: Isolation, Chemical Transformations and *In Vitro* Antitubercular Activities of Secondary Metabolites of Rhizomes of *Alpinia galanga*

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Abstract

A bioactivity-guided chemical examination of the acetone extract of the rhizomes of *Alpinia galanga* led to the isolation of six secondary metabolites, eucalyptol derivative (**1**) and phenylpropanoids (**2**–**6**). The structures of all of the isolated compounds (**1**–**6**) were elucidated on the basis of their spectral data. The isolated compounds (**1**–**6**) were *in vitro* assayed against active and dormant phenotypes of *Mycobacterium tuberculosis* H37Ra, respectively. Interestingly, 1′*S*-1′-acetoxychavicol acetate (**2**) showed good antitubercular activities against both active and dormant phenotypes of *M. tuberculosis* with IC_{50} values of 1.04 µM and 2.69 µM, respectively. Tsuji-Trost and homodimerization reactions of the active compound (**2**) respectively resulted in the formation of two analogues, **7** and **8**. Both of these synthesized analogues were also found to be active *in vitro* against active [IC_{50}s of 3.24 and 3.87 µM, respectively, for compounds **7** and **8**] and dormant [IC_{50}s of 8.33 and 2.41 µM, respectively, for compounds **7** and **8**] phenotypes of *M. tuberculosis* H37Ra, respectively.

Key words

Alpinia galanga · Zingiberaceae · phenyl propanoids · natural products · antitubercular activity

Supporting information available online at http://www.thieme-connect.de/products

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (MTB), is a leading cause of death worldwide. The World Health Organization (WHO) reported [1] that approximately 9 million people were infected with TB globally in the year 2013 alone, which resulted in 1.5 million deaths, out of which an estimated 360 000 were infected with both human immunodeficiency virus (HIV) as well as tuberculosis. It is estimated that more than half of the TB-infected population is from Southeast Asia and Western Pacific Regions with China and India alone accounting for 11% and 24% of total cases, respectively. The treatment requires long spells due to which several patients discontinue the treatment in between, which results in the development of multidrug resistance (MDR) and extensively drug-resistant (XDR) TB. Both of these forms of TB are highly fatal, and the treatment is both expensive and complicated, thereby further complicating the prevention, control, and treatment of TB [2–4]. Although, at present, isoniazid, ethambutol, pyrazinamide, and rifampicin are available as effective anti-TB drugs, the threat posed by the development of multidrug resistance tuberculosis (MDR-TB) against the first-line as well as the second-line drugs is a serious issue [5,6]. Hence, the need for the development of new naturally occurring molecules to effectively treat TB and also address MDR and XDR assumes significance.

The Zingiberaceae plant, *Alpinia galanga* (L.) Willd., is commonly known as galangal and is widely cultivated in China, India, and Southeast Asian countries such as Thailand, Indonesia, and the Philippines [7,8]. The rhizomes of this plant are extensively used as a spice or ginger substitute for flavoring foods. The rhizome has found several uses in the traditional system of medicine such as stomachic in China, or for carminative, antiflatulent, antifungal, and anti-itching in Thailand. In India, it has been traditionally used as a nervine tonic and for a stimulant effect [9]. Also, the use of the extract of the rhizome as an aphrodisiac, anti-inflammatory, revulsive, antiproliferative activity, antioxidant, anticholinergic, immunostimulating activity, hypoglycemic, and antimicrobial has been reported [8–17]. The chemical examination of *A. galanga* has resulted in the isolation of several bioactive molecules [18–35]. The pungent principal compound 1′*S*-1′-acetoxychavicol acetate (**2**) of *A. galanga* has been reported to possess various biological activities, such as antioxidative [36], antitumor [37–41], anti-inflammatory [42], xanthine oxidase inhibitory activity [43], and antifungal [44].

In continuation of our work on naturally occurring bioactive secondary metabolites [45–49], we initiated a systematic chemical examination of *A. galanga* for its antitubercular secondary metabolites. The dried rhizomes of *A. galanga* were successively extracted with acetone and MeOH to furnish acetone and MeOH extracts, respectively, which were assayed against both active and dormant phenotypes of *M. tuberculosis*. The acetone extract showed antitubercular activity (**Table 1**) against both the active and dormant phenotypes of *M. tuberculosis* with MIC_{90} (IC_{50}) values of 17.80 (10.44) and 18.27 (10.87) µM, respectively, however, the MeOH extract was found to be totally inactive. Bioactive crude acetone was taken up for the isolation of the bioactive secondary metabolites and was fractionated over SiO_2 column (100–200 mesh) into nine fractions (A–I). Fraction B was flash chromatographed using a RediSep® column (SiO_2, 12 g) to furnish a pale yellow viscous oil that was identified as 2-acetoxy-1,8-cineole (**1**) by comparison with its reported spectral data [20,22]. Silica gel column chromatography of fraction C resulted in 12 subfractions (C1 to C12). These subfractions were further flash chromatographed and resulted in the isolation of four compounds that were identified on the basis of their spectral data as 1′*S*-1′-acetoxychavicol acetate (**2**) [24–25,29,34], *trans*-*p*-coumaryl diacetate (**3**) [24,29], 1′*S*-1′-acetoxyeugenol acetate (**4**) [24,29,34,43], and *trans*-coniferyl diacetate (**5**) [43]. Further, fraction D on flash chromatography furnished a viscous liquid that was identified as 1′*S*-1′-hydroxychavicol acetate (**6**) by comparison with its reported spectral data [24,29].

The isolated compounds **1**–**6** (**Fig. 1**) were assayed *in vitro* against active and dormant phenotypes of *M. tuberculosis* H37Ra, respectively, using an established XTT reduction menadione assay (XRMA) antitubercular screening protocol [50–52]. The first-line antitubercular drug rifampicin (Sigma) was used as a reference standard and data obtained are presented in **Table 1**. Interestingly, 1′*S*-1′-acetoxychavicol acetate (**2**) was found to be the most active amongst all of the isolated metabolites against both active and dormant phenotypes of *M. tuberculosis*, having IC_{50} values of 1.04 µM and 2.69 µM, respectively. However, out of

Table 1 *In vitro* antitubercular activity of pure isolated compounds (**1**–**6**) and their synthetic analogues (**7** and **8**).

Extract/Compound	Antitubercular activity against *M. tuberculosis* H37Ra in µM with SD values			
	Active state		Dormant state	
	MIC_{90}	IC_{50} (µM)	MIC_{90}	IC_{50} (µM)
Acetone ext.	17.80 ± 0.17	10.44 ± 0.37	18.27 ± 1.02	10.87 ± 0.57
MeOH ext.	NA	–	NA	–
1	NA	–	NA	–
2	3.27 ± 0.08	1.04 ± 0.04	4.73 ± 0.58	2.69 ± 0.14
3	40.95 ± 1.05	25.04 ± 1.36	60.11 ± 1.20	27.12 ± 1.10
4	9.18 ± 1.29	5.04 ± 0.23	8.53 ± 0.20	5.40 ± 0.40
5	23.16 ± 1.19	17.38 ± 0.65	15.07 ± 0.78	7.80 ± 1.44
6	21.98 ± 0.09	7.98 ± 1.13	21.03 ± 0.20	9.33 ± 0.20
7	14.53 ± 1.88	3.24 ± 0.71	11.98 ± 1.10	8.33 ± 0.20
8	5.01 ± 0.41	3.87 ± 0.61	4.04 ± 0.30	2.41 ± 0.30
Rifampicin	0.048	0.0018	0.043	0.0014

NA: not active; both IC_{50} and MIC_{90} are > 100 µg/mL; SD (±): standard deviation

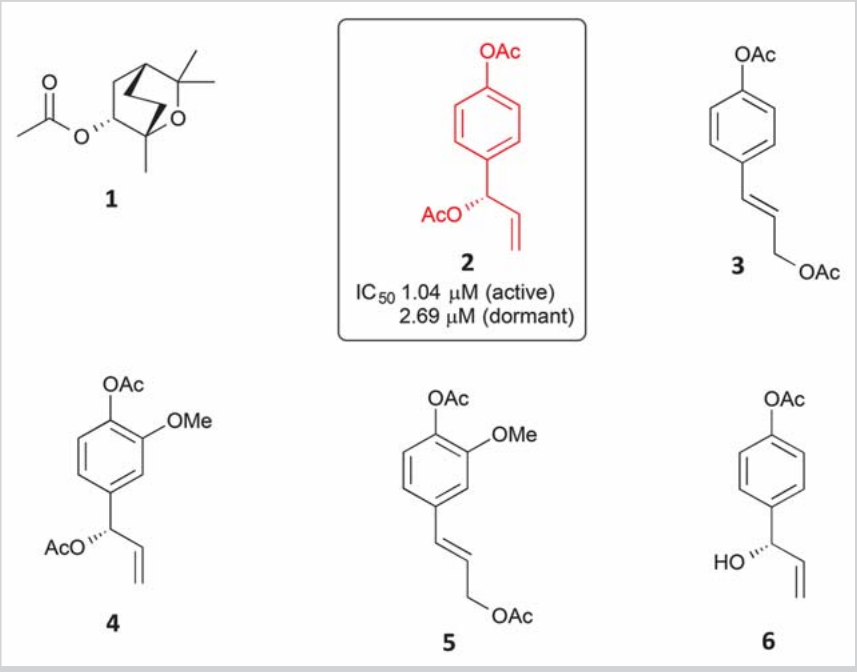

Fig. 1 Isolated compounds (**1**–**6**) from the acetone extract of the rhizomes of *A. galanga*.

all of the isolated secondary metabolites *viz. trans-p*-coumaryl diacetate (**3**), 1′*S*-1′-acetoxyeugenol acetate (**4**), *trans*-coniferyl diacetate (**5**), and 1′*S*-1′-hydroxychavicol acetate (**6**), only compound **4** showed moderate activities with IC_{50} values of 5.04 µM and 5.40 µM against active and dormant phenotypes of *M. tuberculosis*, respectively.

Since compound **2**, 1′*S*-1′-acetoxychavicol acetate (yield 562 mg), showed antitubercular activities compared to other isolated secondary metabolites against both active and dormant phenotypes of *M. tuberculosis*, we thought of carrying out synthesis of the analogues of 1′*S*-1′-acetoxychavicol acetate (**2**) and evaluate their *in vitro* antitubercular activities in order to further improve activities. The presence of allylic acetate's functionality in compound **2** prompted us to attempt a palladium-catalyzed Tsuji-Trost reaction [53] to synthesize its analogue (**7**) via a C–C bond-forming reaction (**Fig. 2**). Reaction of 1′*S*-1′-acetoxychavicol acetate (**2**) with cyclohexanone in DMSO catalyzed by $Pd(OAc)_2$ at room temperature furnished a reaction mixture that was flash chromatographed using RediSep® column (SiO_2, 12 g) and eluted with petroleum ether : ethyl acetate (0 → 10%) to furnish compound **7** as a viscous liquid (37%). Homodimerization [54] of compound **2** was carried out using Grubb's Ist generation catalyst to furnish homodimer **8** (91%) as a colorless solid [m.p. 83–85 °C; $[\alpha]_D^{25}$ − 36.6 (*c* 1, $CHCl_3$)].

The synthesized analogues **7** and **8** were assayed *in vitro* against both active and dormant phenotypes of *M. tuberculosis* for their antitubercular activities (**Table 1**). Both compounds showed *in vitro* antitubercular activities. Compound **7** was found to possess IC_{50} values of 3.24 µM and 8.33 µM, whereas compound **8** had IC_{50} values of 3.87 µM and 2.41 µM against active and dormant phenotypes of *M. tuberculosis*, respectively.

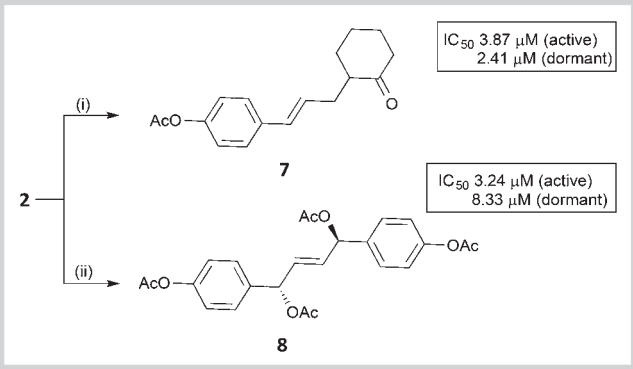

Fig. 2 Scheme of the preparation of derivatives of **2**. Reagents and conditions: (i) $Pd(OAc)_2$, PPh_3, DMSO, cyclohexanone, pyrrolidine, rt, 3 h, 37%; (ii) Grubb's Ist generation catalyst, CH_2Cl_2, rt, 16 h, 91%.

Materials and Methods

Plant material

The rhizomes of *A. galanga* were collected and identified by Prof. Kornkanok Ingkainan, Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Thailand from Phitsanulok, Thailand in May 2008. A herbarium specimen (003566) is being maintained at the Department of Biology, Faculty of Pharmaceutical Sciences, Naresuan University, Thailand.

Synthesis of compound 7

A mixture of 1′*S*-1′-acetoxychavicol acetate (**2**; 40 mg, 0.5 mmol), $Pd(OAc)_2$ (10 mol%), and ligand PPh_3 (25 mg) in DMSO (2 mL) was stirred at room temperature for 5 min. Next, cyclohexanone (1.5 mmol, 3 equiv.) and pyrrolidine (30 mol%) were added and the reaction mixture was further stirred at room temperature for 3 h. After completion of the reaction (TLC), the reaction mixture was quenched with H_2O (5 mL) and was extracted with EtOAc (3 × 25 mL). The organic layers were pooled together and washed with brine solution (1 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude reaction mixture was flash chromatographed using a RediSep® column (SiO_2, 12 g) and eluted with petroleum ether : ethyl acetate (0 → 10%) to furnish pure compound **7** as a viscous liquid (17 mg, 37%); R_f 0.30 (EtOAc-petroleum ether, 1 : 4); $[\alpha]_D^{25}$ +0.23 (*c* 1, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 7.33 (d, *J* = 8.6 Hz, 2 H), 7.00 (d, *J* = 8.6 Hz, 2 H), 6.44–6.29 (m, 1 H), 6.24–6.05 (m, 1 H), 2.75–2.57 (m, 1 H), 2.52–2.32 (m, 3 H), 2.29 (s, 3 H), 2.24–2.01 (m, 4 H), 1.88 (dd, *J* = 3.4, 8.3 Hz, 1 H), 1.74–1.59 (m, 2 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 212.5, 169.6, 149.6, 135.4, 130.7, 128.7, 126.9, 121.6, 77.7, 77.0, 76.4, 50.7, 42.2, 33.6, 33.0, 27.9, 25.1, 21.2; ESI-MS: *m/z* 295.1 [M + Na]$^+$; HRMS (ESI): calcd. for $C_{17}H_{20}O_3Na$ [M + Na]$^+$ 295.1305, found 295.1298.

Synthesis of compound 8

A stirred solution of 1′*S*-1′-acetoxychavicol acetate (**2**; 40 mg) was dissolved in dry CH_2Cl_2 (2 mL) and degassed for 15 min. Then Grubb's Ist generation catalyst (15 mol%) was added to the reaction mixture and stirring was continued for a further 16 h at room temperature under an argon atmosphere. After the completion of reaction (TLC), the solvent was removed under reduced pressure. The crude reaction mixture was flash chromatographed using RediSep® column (SiO_2, 12 g) and eluted with petroleum ether : ethyl acetate (0 → 20%) to furnish pure homodimer **8** as a colorless solid (68 mg, 91%); R_f 0.30 (EtOAc-petroleum ether, 3 : 7); m. p. 83–85 °C; $[\alpha]_D^{25}$ −36.6 (*c* 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 7.34 (d, *J* = 8.7 Hz, 4 H), 7.07 (d, *J* = 8.2 Hz, 4 H), 6.27–6.32 (m, 2 H), 5.90 (dd, *J* = 2.7, 1.4 Hz, 2 H), 2.29 (s, 6 H), 2.09 (s, 6 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 169.9, 169.5, 150.6, 136.3, 130.6, 130.6, 128.6, 121.9, 74.4, 21.3, 21.2; ESI-MS: *m/z* 463.1 [M + Na]$^+$; HRMS (ESI): calcd. for $C_{24}H_{24}O_8Na$ [M + Na]$^+$ 463.1363, found 463.1351.

Antitubercular assay using the XTT reduction menadione assay protocol

Crude extracts and pure compounds **1**–**8** were evaluated for their *in vitro* effects against the active and dormant phase of *M. tuberculosis* H37Ra (MTB) using the XRMA protocol [51]. *M. tuberculosis* H37Ra (ATCC 25177) was obtained from MTCC. MTB (ATCC No. 25177) were grown to the logarithmic phase (O.D. 1.0) in a *Mycobacterium phlei* medium. The stock culture was maintained at −70 °C and subcultured once in *M. phlei* medium before inoculation into the experimental culture. All experiments were performed in triplicate, and IC_{50} and MIC values were calculated from their dose-response curves.

$$\%\text{Inhibition} = 100 - (A_1 - \text{blank})/(A_2 - \text{blank}) \times 100$$

where A_1 is the culture absorbance at 470 nm in the presence of the compound after the addition of menadione, A_2 is the culture absorbance at 470 nm (DMSO solvent control) after the addition of menadione, and blank is the culture absorbance at 470 nm of the respective data points before the addition of XTT/menadione [51].

XTT reduction menadione assay protocol

Activity against MTB was determined through the XRMA, reading absorbance at 470 nm, as per the protocol [51]. A compound solution (2.5 µL) was added in a total volume of 250 µL of *M. pheli* medium consisting of the MTB, sealed with plate sealers and allowed to incubate for 8 (active phase) and 12 (dormant phase) days at 37 °C. The XRMA was then carried out to estimate the viable cells present in different wells of the assay plate. To all wells, 200 µM of XTT were added and incubated at 37 °C for another 20 min. It was followed by the addition of 60 µM of menadione and incubated at 37 °C for 40 min. The optical density was measured using a microplate reader (SpectraMax Plus 384 plate reader, Molecular Devices, Inc.) at 470 nm filter against a blank prepared from a well free of cells. Absorbance obtained from the cells treated with 1% DMSO alone was considered 100% cell growth. The %inhibition in the presence of test material is calculated by using formula,

$$\%\text{Inhibition} = (\text{average of control} - \text{average of compound})/(\text{average of control} - \text{average of blank}) \times 100$$

where control is culture medium with cells and DMSO and blank are culture medium without cells. For all samples, each compound concentration was tested in triplicate in a single experiment and the quantitative value is expressed as the mean ± standard deviation (S.D.).

Supporting information

The general experimental procedures, extractions of the plant material, isolation of compounds, and antitubercular assay protocol as well as copies of their ^{1}H, ^{13}C, DEPT, LCMS, and HRMS spectra are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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