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In vitro Antitubercular Screening and *in silico* Study of Germacradienolide from *Blainvillea latifolia*

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Bioassay-guided isolation from acetone extract of *Blainvillea latifolia* yielded one compound. The acetone extract, fractions and the compound **1** were investigated for antitubercular activity against *Mycobacterium tuberculosis* H37Ra. Compound **1** showed the activity with IC_{50} and MIC values at 8.9 and >100 μg/mL. However, the acetone extract of *Blainvillea latifolia* was inactive against two Gram negative (*E. coli*, *P. flurescence*) and two Gram-positive (*S. aureus*, *B. subtilis*) bacterial strains. Hence, it was concluded that the extract and the compound **1** are specifically active against MTB and not against bacterial strains. Molecular docking study was performed against crucial mycobacterial target MtInhA to gain an insight into the binding mode and the thermodynamic interactions governing the binding affinity of this molecule.

Keywords: *Blainvillea latifolia*, Asteraceae, Germacradienolide, Antitubercular activity, Molecular docking.

INTRODUCTION

Tuberculosis is the leading cause of infectious disease mortality in the world and which is caused by *Mycobacterium tuberculosis* (MTB). Around 32% of the world's population is infected with MTB, so there is urgent need of new and more efficient drugs [1] for the treatment of tuberculosis. One of the sources is nature which is considered to be safe and non-toxic.

Blainvillea latifolia is a weed occurring in some parts of Maharashtra and Andhra Pradesh states, India. A structural study on sesquiterpenoids and acetylenic derivatives from *Blainvillea acmella*, *Blainvillea latifolia* and *Eclipta erecta* were already carried out [2]. The isolation of oxygenated guaianolides along with several known guaianolides from *Blainvillea latifolia* (aerial parts) have also been reviewed [3]. Aromatic esters like benzyl 2,6-dimethoxy- and 2,3,5,6-tetramethoxybenzoate, along with known compounds were isolated from aerial parts of *Blainvillea latifolia* [4]. Two germacradienolides have been isolated from the aerial parts of *Blainvillea latifolia* along with a known sesquiterpene lactone. The structures of the compounds have been established as 8β-hydroxy-9β-acetoxy-14-(2-methylbutyryloxy)-6α-7-germacradienolide and 8β-hydroxy-9β-acetoxy-14-isobutyryloxy-6α,7-germacradienolide by spectral methods and chemical transformation [5]. A germacranolide has been isolated from the acetone extract of *Blainvillea latifolia* along with a known sesquiterpene lactone. The structure of the compound has been established as 10-acetoxymethyl-9β-hydroxy-8β-(2-methyl butyryloxy)-germacradien-6,7-olide by spectral methods and chemical correlation of this compound [6].

The aerial parts of *Blainvillea latifolia* afforded in addition to widespread compounds the heliangolides, zoapatanolide A and B, guaianolide, subacaulin, as well as a derivative of geranyl nerol and the structures were elucidated by high field ^{1}H NMR studies [7]. The germacrenolide, 9β-hydroxy-8β-isobutyryloxygermacradien-6,7-olide and other compounds were isolated

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from acetone extract of *Blainvillea latifolia* and its structure was detected by IR, ^{1}H & ^{13}C NMR and mass spectral analysis, which are previously isolated from *Blainvillea acmella* [8]. Stigmasterol was isolated from an oily triterpenoid fraction extracted from the whole plant. Alkaline hydrolysis of this fraction yielded α-amyrin acetate and β-amyrin acetate. Thus, the original triterpenoid was a mixture of α- and β-amyrin [9].

EXPERIMENTAL

Plant material: The plant *Blainvillea latifolia* was collected from Savitribai Phule Pune University, Pune, India, in the month of August 2021. The whole plant was authenticated by Department of Botany, Agharkar Research Institute, Pune, India. The voucher specimen of plant material was maintained under the reference number WP-093.

Extraction and fractionation: The whole plant (1.0 kg) was shade dried and grounded. The powdered material extracted by maceration using acetone (6.0 L × 3) at room temperature for 48 h × 3. The extract was filtered and concentrated on a rotary evaporator under reduced pressure at 40 ºC thereby providing crude acetone extract (19.14 g), which showed promising activity against *mycobacterium tuberculosis* H37Ra. The crude extract (15 g) was subjected to column chromatography over silica gel (100-200 mesh) by employing *n*-hexane-acetone gradient (100-0 %) as mobile phase. Four fractions (1-4) were collected by using 100% hexane (1), hexane:acetone 80:20 (2), hexane:acetone 60:40 (3) and 100% acetone (4). Fraction-2 showed promising antitubercular activity hence, it was further purified by column chromatography. Further, fraction-2 purified by repeated column chromatography with *n*-hexane: acetone mixture by increasing polarity of acetone gave four fractions. Fraction-2 yielded one impure compound **1**. Fraction-2 on repeated preparative TLC using acetone:hexane (20:80) as eluent and crystallization gave pure compound **1** (20 mg) and obtained as a white solid.

Antitubercular testing protocol

Mycobacterial cultures and growth conditions: At 470 nm, the activity against *M. tuberculosis* H37Ra (ATCC 25177) (MTB) was estimated with XTT reduction menadione assay (XRMA) through absorbance. Standard cultures of *M. bovis* BCG (ATCC 35743) and MTB were obtained from the American Type Culture Collection (Manassas, USA). *M. bovis* BCG and MTB were grown in 50 mM sodium nitrate containing Dubos medium (Difco, Detroit, USA) and a specified *Mycobacterium phlei* medium, respectively [10]. At -70 ºC , these were stored as glycerol stocks. Before inoculation, to acquire metabolically active mycobacteria, 50 µL of glycerol stock was pre-inoculated in a corresponding medium. For all experiments, cultures were grown at 150 rpm and 37 °C under aerobic conditions to log phase [optical density at 595 nm (OD_{595}) = 1]. Because mycobacteria grew as aggregated clumps in the culture medium, the cultures were sonicated for 2 min with a water-bath sonicator (Ultrasonic, Freeport, USA) to acquire viable dispersed cells for reproducible inoculation of *Mycobacterial bacilli* for the experiments in the fresh medium.

Primary screening: The inhibitory activity of the compound against dormant (12 days incubation) mycobacteria was analyzed for the concentrations of 10, 30 and 100 µg/mL. Its inhibitory activity against MTB was estimated using XRMA at 470 nm [11]. Percentage inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{CMPD})}{(\text{Control} - \text{Blank})} \times 100$$

where, control is the activity of mycobacteria without compounds; CMPD is the activity of mycobacteria in the presence of compound from *Blainvillea latifolia*; and 'blank' is the activity of the culture medium without mycobacteria.

The experiment was performed in triplicate and the quantitative value was expressed as the mean ± standard deviation (S.D.)

Determination of minimum inhibitory concentration (MIC) and 50% inhibitory concentration (IC_{50}): Based on primary screening, the MIC and IC_{50} of the active compound against mycobacteria was evaluated through dose-response assay at the concentration 0.78-100 µg/mL. Using Origin Pro software (Origin Lab Corp., Northampton, USA), the dose-response curve was obtained. The IC_{50} and MIC were considered as the minimum concentrations for compounds showing growth inhibition of 50% and ≥ 90%, respectively, in relation to the growth control without compounds. Standard antitubercular drug rifampicin, was considered the positive control. All the experiments were performed in triplicates.

***In vitro* assay**: *In vitro* activity against MTB at dormant (12 days) stages was performed using the XRMA assay [10].

Antimicrobial activity: Through antibacterial assay, 3, 10, 30 µg/mL compound was analyzed against four bacterial strains: two Gram-negative bacteria: *E. coli* (ATCC 25292) as well as *P. fluorescens* (ATCC 13525) and two Gram-positive bacteria: *S. aureus* (ATCC 29213) as well as *B. subtilis* (ATCC 23857).

To estimate specificity, compound concentrations of 3, 10, 30 µg/mL was analyzed against the aforementioned four bacteria. For antimicrobial assays, the culture with adjusted OD (OD_{620} = 1) was inoculated in the LB broth (1% v/v). Afterwards, in a 96-well microtitre plate, 2.5 and 247.5 µL of compound and culture, respectively, were dispensed and incubated at 37 ºC for 18 h before measuring the absorbance at 620 nm [12-14]. The positive control employed was ampicillin. Moreover, for sterility control and growth, wells were included. The IC_{50} and MIC values were calculated using the dose-response curve.

Molecular docking: To elucidate the thermodynamic interactions which governed binding affinity and understand the possible mechanism through which isolated compound **1** can provide the antitubercular activity, molecular docking was performed against the enoyl-acyl carrier protein reductase of *Mycobacterium tuberculosis* (mtInhA) by using the standard protocol provided in grid-based ligand docking with energetics (GLIDE) module of small drug discovery suite (Schrödinger, LLC, New York, USA) [15-17].

RESULTS AND DISCUSSION

The acetone extract of *Blainvillea latifolia* showed the promising antitubercular activity. Hence further subjected to fractionation by acetone:hexane gradient, which gave four fractions (1-4). Fraction-2 showed promising antitubercular activity (Table-1). This fraction afforded one pure compound by repeated column chromatography and preparative TLC. The structure of this compound **1** was established by IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

TABLE-1
in vitro ANTI-TUBERCULAR STUDY OF CRUDE EXTRACT, FRACTIONS AND COMPOUND **1** AGAINST MTB H37Ra

Purification stage	Fractions	Inhibition (%)
Crude extract	Acetone extract	85.20 ± 0.86% (at 25 µg/mL)
Sub-fractions	Fraction-1	59.20 ± 0.98% (at 100 µg/mL)
	Fraction-2	89.20 ± 0.87% (at 12.5 µg/mL)
	Fraction-3	33.90 ± 0.70% (at 100 µg/mL)
	Fraction-4	27.10 ± 0.29% (at 100 µg/mL)
Fraction-2	Compound **1**	86.40 ± 0.889 (at 100 µg/mL)

Compound **1** (Fig. 1) was obtained as a white solid having melting point 159-160 ºC, specific rotation was observed at $[\alpha]^{D}_{20}$ = -47.8º (*c* = 0.050, $CHCl_3$). The LC-MS showed the molecular ion peak at *m/z* = 392 $[M]^+$. The IR spectrum showed hydroxy group at 3580 cm^{-1}, α,β-unsaturated γ-lactone peak at 1760 cm^{-1} and acetate at 1740 & 1240 cm^{-1}. In ^{1}H NMR spectrum, the hydrogens on exomethylene group were observed at δ 5.86 and 6.37 supporting the presence of α,β-unsaturated γ-lactone. The vinylic methyl and acetyl group observed at 1.81 δ and 2.04 δ, respectively. An isopropyl group and two olefinic protons were observed at δ 2.58 (H-2′), 1.17 (H-3′), 1.19 (H-4′) and δ 4.76 (H-5), 5.36 (H-1), respectively. The methylene protons attached to oxygen showed peaks at δ 4.41 and 4.64. The methine protons at C-6, 7, 8, 9 were observed at 4.97, 2.95, 5.87 and 4.41 δ, respectively.

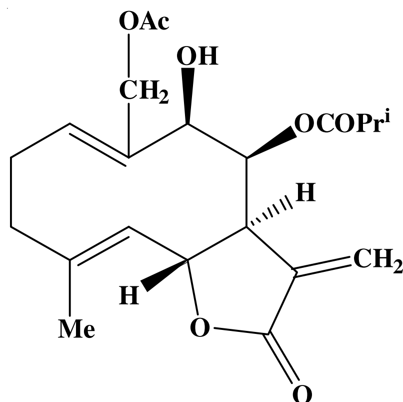

Fig. 1. Structure of compound **1**

The ^{13}C NMR spectrum showed 21 carbon signals. Two olefinic bonds observed at δ 138.62 (C-1), 135.70 (C-10) and 135.83(C-4), 127.38 (C-5), respectively. The lactone carbonyl (C-12), acetate carbonyl (-OAc) and carbonyl at C-1′ showed peaks at δ 169.43, 171.96 and 176.19, respectively which disappears in DEPT experiment. The exomethylene double bond present at C-11 and C-13 position showed peaks at δ 141.94 and 122.90. The methylene carbons at C-2, C-3 were observed at δ 39.40, 25.74 and methylene carbon at C-14 showed downfield shift near to acetate group at δ 59.85. The doublet carbons at C-6, 7, 8, 9 and 2′ were observed at δ 80.65, 50.95, 75.13, 74.93 and 34.38, respectively. The four methyl at C-15, 3′, 4′ and _-OAc) gave four signals at δ 17.29, 19.92, 18.92 and 21.12. In DEPT spectrum four methyl, four methylenes and seven methines were observed. The stereochemistry of lactone ring, olefinic bond and orientation of H-8 & H-9 was confirmed by single X-ray crystallography [7].

The crude acetone extract of the whole plant of *Blainvillea latifolia* exhibited promising antitubercular activity against MTB H37Ra with an efficiency of 85.20% inhibition at 25 µg/mL. Further, acetone extract was chromatographed to obtain four fractions (1-4). These fractions showed variable antitubercular activities with 59.20, 89.20, 33.90 and 27.10% inhibitions at 100, 12.5, 100 and 100 µg/mL, respectively (Table-1). Since fraction-2 showed promising activity, it was further purified to yield compound **1**. The antitubercular activity for compound **1** was determined by measuring inhibition of growth against non-virulent strain of *M. tuberculosis* (MTB H37Ra; ATCC 25177) in liquid medium. Compound **1** showed high inhibition with IC_{50} value of 8.9 µg/mL (Fig. 2).

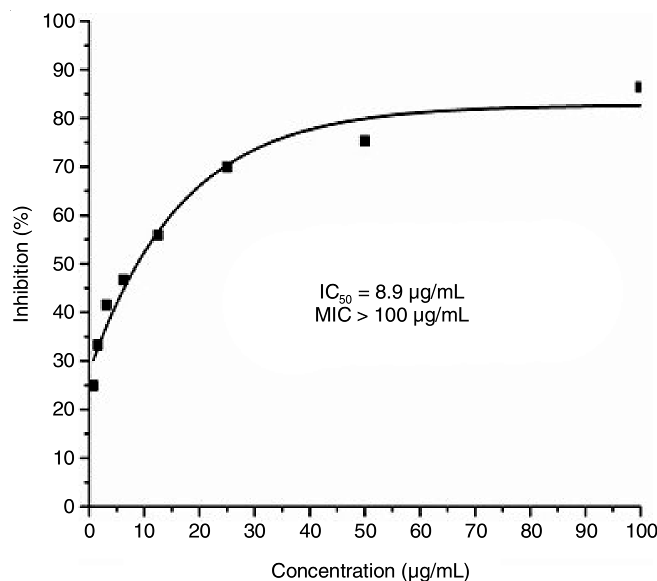

Fig. 2. Dose response curve of compound **1** against *M. Tuberculosis* H37Ra with IC_{50} and MIC values under *in vitro* condition

Acetone extract of *Blainvillea latifolia* was also tested for antibacterial activity against four bacterial strains (*E. coli*, *P. flurescence*, *S. aureus* and *B. subtilis*), but it was inactive at 30 µg/mL (Table-2).

MtInhA is one of the key enzymes involved in the type II fatty acid biosynthesis pathway, which controls mycobacterial cell wall construction and thus has been targeted in the development of antituberculosis drugs. Furthermore, in mammals, there is no homologue to InhA, which makes it an optimal target for selective antitubercular drugs.

Fig. 3. Binding mode of compound **1** into the active site of enoyl-acyl carrier protein reductase of *Mycobacterium tuberculosis* (mtInhA)

TABLE-2
ANTIBACTERIAL ACTIVITY OF ACETONE EXTRACT AGAINST FOUR BACTERIAL STRAINS

Bacterial strain	Concentration (µg/mL) ± SD			Activity +/–
	30 µg/mL	10 µg/mL	3 µg/mL	
S. aureus	1.5 ± 0.4	4.7 ± 6.7	-8.8 ± 1.2	–
B. subtilus	30.4 ± 0.5	22.1 ± 1.3	-3.2 ± 0.2	–
P. flurescence	65.1 ± 4.6	41.8 ± 0.3	-22.9 ± 2.4	–
E. coli	-0.2 ± 0.5	-1.9 ± 0.5	0.1 ± 2.7	–

The lowest energy docked conformation of compound **1** (Fig. 3) showed that the compound is nicely bound to the active site of MtInhA producing a Glide docking score of -8.434 and Glide binding energy: -41.577kcal/mol. The major driving force involved in the mechanical inter-locking of this molecule was observed to be a network of significant van der Waals interactions with Met199 (-4.352 kcal/mol), Ala198 (-1.155 kcal/mol), Thr196 (-1.051 kcal/mol), Ile194 (-3.166 kcal/mol), Pro193 (-2.446 kcal/mol), Gly192 (-1.478 kcal/mol), Ala191 (-1.419 kcal/mol), Lys165 (-2.308 kcal/mol), Met161 (-1.261 kcal/mol), Tyr158 (-2.22 kcal/mol), Phe149 (-5.123 kcal/mol), Asp148 (-2.024 kcal/mol), Met147 (-4.07 kcal/mol), Gly96 (-1.008 kcal/mol), Ile95 (-2.115 kcal/mol), Ser94 (-1.802 kcal/mol) and Ile21 (-2.153 kcal/mol) residues of MtInhA. The enhanced binding affinity of the compound is also attributed to favourable electrostatic interactions observed with Met199 (-1.296 kcal/mol), Thr196 (-2.114 kcal/mol), Lys165 (-2.244 kcal/mol) and Tyr158 (-1.473 kcal/mol) residues lining the active site. This balanced network of favourable non-bonded interactions observed with MtInhA were further supported by three very close hydrogen bonding interactions observed with Thr196 (2.081Å), Tyr158 (2.314Å) and Lys165 (1.994Å) residues, which serve as 3anchor3 to guide the orientation of molecule into the 3D space of the active site and further facilitates the steric and electrostatic interactions. This *in silico* binding affinity data suggest that this molecule could serve as a pertinent starting point for structure-based optimization for arriving at potent molecules with higher potency and selectivity towards this crucial target intervening antitubercular drug discovery.

Conclusion

Bioassay-guided isolation from the acetone extract of *Blainvillea latifolia* yielded one compound **1**. The acetone extract, fractions and the compound **1** were investigated for antitubercular activity against *Mycobacterium tuberculosis* H37Ra. Compound **1** showed the activity with IC_{50} and MIC values at 8.9 and >100 µg/mL. However, the acetone extract of *Blainvillea latifolia* was inactive against two Gram-negative (*E. coli* and *P. flurescence*) and two Gram-positive (*S. aureus* and *B. subtilis*) bacterial strains. Hence, it was concluded that the extract and the compound **1** are specifically active against *Mycobacterium tuberculosis* H37Ra and not against bacterial strains.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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