Antimycobacterial activity of two natural alkaloids, vasicine acetate and 2-acetyl benzylamine, isolated from Indian shrub *Adhatoda vasica* Ness. leaves

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In folk medicine, *Adhatoda vasica* Ness. (Acanthaceae) is used to treat asthma and cough. The leaves of *A.vasica* were powdered and extracted with hexane, ethyl acetate and methanol. The hexane extract showed 97% reduction in colony-forming units (CFU) at 100 μg/ml. The hexane extract was subjected to column chromatography. Two natural compounds, vasicine acetate and 2-acetyl benzylamine, were isolated from it. They were bioassayed against *Mycobacterium tuberculosis*. The two compounds showed strong antimycobacterial activity. Vasicine acetate and 2-acetyl benzylamine isolated from hexane extract of *A.vasica* leaves, significantly inhibited *M. tuberculosis* and one multi-drug-resistant (MDR) strain and one sensitive strain at 200 and 50 μg/ml, respectively. Our study demonstrated that both the compounds, vasicine acetate and 2-acetyl benzylamine, could be evaluated further for developing a drug to control *M. tuberculosis*.

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1. Introduction

Tuberculosis (TB) is a disease that has affected mankind from very ancient times. It is caused by *Mycobacterium tuberculosis.* Tuberculosis is a reemerging infectious disease causing considerable mortality. India accounts for nearly one-third of the global burden of tuberculosis and the disease is one of India's most significant public health problems. In India approximately 2 million people acquire TB every year (Young *et al.* 2009). Indian medicinal plants are used in different Ayurvedic formulations to treat TB (Kirtikar and Basu 1935).

The World Health Organization (WHO 2000) has estimated that between the years 2000 and 2020 nearly one billion people will be infected and more than 200 million will develop the disease. *M. tuberculosis* has developed resistance to many antibiotics. Plants are an important source to find useful compounds.

The Indian shrub *Adhatoda vasica* Ness. (Malabar nut) is an evergreen indigenous medicinal plant used to treat cold, cough, asthma and tuberculosis (Chopra and Ghosh 1925). It is a highly valuable Ayurvedic medicinal plant. The importance of *A. vasica* plant in the treatment of respiratory disorders can be understood from the ancient Indian saying, 'No man suffering from phthisis need despair as long as the vasaka plant (*A. vasica*) exists' (Dymock *et al.* 1893). The antimicrobial activity of *A. vasica* leaf extracts against *Staphylococcus aureus, S. epidermidis, Bacillus subtilis, Proteus vulgaris* and *Candida albicans* had been established (Karthikeyan *et al.* 2009). However, studies on antimycobacterial activity using *A. vasica* compounds are very few. The bronchodilatory activity of *A. vasica* compounds have been well studied by Dorch and Wagner (1991). Grange and Snell (1996) have studied the antimycobacterial activity of semi-synthetic derivatives of compounds of *A. vasica* against *M. tuberculosis* by conventional method.

Keywords. 2-acetyl benzylamine; *Adhatoda vasica*; antimycobacterial activity; *Mycobacterium tuberculosis*; vasicine acetate

Abbreviations used: CFU, colony-forming units; LRP, luciferase reporter phage; MIC, minimum inhibitory concentrations; MDR, multi-drug-resistant; OADC, oleic acid–albumin-dextrose-catalase, RLU, relative light unit; TB, tuberculosis

The most studied chemical component in *A. vasica* is a bitter quinazoline alkaloid, vasicine, which is present in the leaves, roots and flowers. Apart from vasicine, the leaves contain several alkaloids (vasicinone, vasicinol, adhatodine, adhatonine, adhavasinone, anisotine and peganine), betaine, steroids and alkanes (Lahiri and Prahdan 1964; Bhat *et al*. 1978; Atal 1980; Chowdhury and Bhattacharyya 1987). This paper describes the antimycobacterial effects of two new compounds, vasicine acetate and 2-acetyl benzylamine, isolated from *A. vasica.*

2. Materials and methods

2.1 *Plant material*

Leaves from *A. vasica* were collected from Padappai, near Chennai, India, and shade-dried. The plant was identified and authenticated by a plant taxonomist at the Department of Botany, Loyola College, Chennai, India. The voucher specimen (ERIH:292) is deposited at the Herbarium of Entomology Research Institute, Loyola College, Chennai, India.

2.2 *Chemicals and phage*

Middlebrook 7H11 agar (Difco, USA), Middlebrook 7H9 broth (Difco, USA)**,** Rifampicin and Isoniazid were obtained from Himedia, Mumbai. D-luciferin was purchased from R&D System, Minneapolis, USA, and phAE 129 was obtained from Tuberculosis Research Centre, Chennai.

2.3 *Preparation of plant extracts*

Dried plant powder (1.5 kg) was exhaustively extracted sequentially with 3 l of hexane, ethyl acetate and methanol (at room temperature) after 48 h of soaking with intermittent shaking. The extracts were collected and concentrated using rotary evaporator under reduced pressure at less than 40°C and then further concentrated in vacuum rotary evaporator at 40°C. The residues were stored at 4°C until use.

2.4 *Bioassay guided fractionation*

The hexane extract (7.5 g) was subjected to column chromatography on a silica gel column (Merck 70–230 mesh size 2.5 × 60 cm) and eluted with stepwise gradient to *n*-hexane:ethyl acetate ratios 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9. Fractions 5 and 7 eluted with 8:2 hexane: ethyl acetate showed single spots on thin-layer chromatography. The identification and structural elucidation of the active compounds were carried out using ^{1}H NMR, ^{13}C NMR and ^{13}C DEPT-NMR. The NMR spectra were taken in $CDCL_3$ on a BRUKER instrument at 300 MHz (^{1}H) and 75 MHz (^{13}C), and IR spectra were taken on a Perkin Elmer FT-IR spectrophotometer. The EIMS was taken on a JEOL instrument at 70 eV by direct inlet method. The isolated compounds were assayed for their antimycobacterial activity.

2.5 *Test organisms and test extracts and compounds*

Clinical isolates of reference strains for *M. tuberculosis* (H37Rv ATCC 27294) and one MDR (Rifampicin and Isoniazid) strain (Rf 10385) and one sensitive strain (Rf 10321) were obtained from Bacteriology Department, Tuberculosis Research Centre (TRC), Chennai, India.

Stock solution of the crude extracts (10 mg/ml) was prepared using 1% DMSO. It was verified that DMSO did not suppress or delay the growth of *M. tuberculosis* strains (Grange and Snell 1996). Four working concentrations at 12.5, 25, 50 and 100 μg/ml were prepared from the stock solutions of the crude extracts. Stock solution of the isolated compound (10 mg) was also prepared by dissolving in 1% DMSO. Four working concentrations of 12.5, 25, 50, 100, 200 and 400 μg/ml of the compound and reference drug controls with Rifampicin (2.0 μg/ml) and Isoniazid (0.2 μg/ml) were also prepared by dissolving in 1% DMSO and mixed with Middlebrook medium.

2.6 *Antitubercular activity assay*

The antimycobacterial activity of the crude extracts was assessed by the conventional method of Grange and Snell (1996). Cultures of *M. tuberculosis* (H37 Rv), MDR and sensitive strains were grown on LJ medium and maintained at −70°C. The culture revived from −70°C were sub cultured on Middlebrook 7H9 broth supplemented with oleic acid–albumin-dextrose-catalase (OADC) enrichment for 10 days and stored at 4°C until used. A standard suspension of 10^7 CFU/ml (equivalent to # 1 McFarland standard) was used in the inoculation process. Media were prepared as 10 ml slopes in glass bottles inoculated with 0.1 ml of suspension with and incubated at 37°C. The percentage inhibition of CFU was determined after 3 weeks of inoculation.

The antitubercular activity of the compounds was evaluated following the method of Kamal *et al.* (2006). Minimum inhibitory concentrations (MIC in μg/ml) of compounds against strains of *M. tuberculosis* were determined by a reference agar dilution method as per the NCCLS- M24-T2 recommendations (NCCLS, 2000). The compounds and reference drugs were dissolved in DMSO as described above. Appropriate volumes of compounds

were incorporated into duplicate plates of Middlebrook 7H10 agar medium supplemented with OADC enrichment at a concentration of 0.03–16 μg/ml. Test organisms (Mycobacterium strains) were grown in Middlebrook 7H9 broth containing 0.05% Tween80 and 10% ADC supplement. After 10 days of incubation at 37°C, the broths were adjusted to 10^5 CFU/ml (equivalent to 0.5 McFarland standard). The organisms were further diluted 10 fold in sterile water containing 0.10% Tween80. The resulting mycobacterial suspensions were spotted (3–5 μl/spot) onto drug supplemented 7H10 media plates. The plates were sealed and incubated at 37°C with 10% CO_2 for 3–4 weeks in an upright position. The MIC was recorded as the lowest concentration of the drug that completely inhibited the growth of mycobacterial cultures.

2.7 *Luciferase reporter phage assay*

Luciferase reporter phage (LRP) assay was carried out following the method of Sivakumar *et al.* (2007) using luciferase reporter phage assay. Hundred microliter bacterial suspension of 2×10^7 CFU /ml (equivalent to McFarland #2 standard) was added to 350 μl of G7H9 with and without the test compound. For each sample, two drug-free controls and four drug concentrations (12.5, 25, 50 and 100 μg/ml) were prepared, and this setup was incubated for 72 h at 37°C. After incubation, 50 μl of the high-titer LRP (phAE129) and 40 μl of 0.1 M $CaCl_2$ were added to all the vials and this setup was incubated at 37°C for 4 h. After incubation, 100 ml of the mixture was taken from each tube into a luminometer cuvette and equal amount of working D-luciferin (0.3 mM in 0.05 M sodium citrate buffer, pH 4.5) solution was added. The relative light unit (RLU) was measured after 10 s of integration in the luminometer (Monolight 2010). Duplicate readings were recorded for each sample and the mean was calculated. The percentage reduction in RLU was calculated for each test sample and compared with the control. The experiment was repeated when the mean RLU of the control was less than 1000.

3. Results

When the hexane extract of *A.vasica* was subjected to chromatography, it yielded nine fractions. Fraction 5 eluted with hexane:ethyl acetate (8:2 ratio) appeared to be an individual compound. It was white powder. It was subjected to spectroscopic analysis. The yield of the compound was 85 mg. The IR Spectrum gave the following data: Max cm^{-1} (400–4000) 1720 (acetate), 1629 (>C=N) 1579, 1498, 1406 (aromatic system), 1385, 1345, 1241 (acetate), 1162, 1129, 1105, 1072, 933, 903, 767, 722 (aromatic system). ^{1}H NMR data for the active molecule were the following: 7.14 (2H, m, H-5 and H-6), 6.96 (1H,brs, H-7) 6.84 (1H, bs, H-8), 4.72, 4.80 (each 1H, d, J= 14.5 HZ, H-9), 2.09 (3H, s, −OCOCH_3), 3.52, 3.70 (1H each, m, H-1), 2.25, 2.55 (1H each, m, H-2), 5.19 (1H, brt, H-3). ^{13}C NMR and DEPT-NMR spectra of the active molecule were the following: 50.7 (C-1), 28.0 (C-2), 70.2 (C-3), 163.9 (C-3a), 134.0 (C-4a), 129.5 (C-5), 126.6 (C-6), 126.2 (C-7), 120.3 (C-8), 116.3 (C-8a), 46.8 (C-9), 167.3 (−OCOCH$_3$), 23.3 (−OCOCH$_3$). MS: M+ M/Z 230, M/ f- C_{13} H_{14} N_2 O_2, m.p. 122°. The structure was elucidated using the above data (figure 1).

The compound tested positive for alkaloid in the Dragendroff test. The IR spectrum did not show the presence of hydroxyl or amino group. The presence of the ester carbonyl group was shown by peaks at 1720 and 1241 cm^{-1}. The peaks at 1629cm^{-1} showed the presence of >C=N group. The presence of aromatic system was shown by peaks at 1579, 1498, 1406, 933, 903, 767 and 722 cm^{-1}. The ^{1}H NMR spectrum showed the presence of an acetate group appearing as three proton singlet at δ 2.09. An ortho disubstituted benzene ring was also suggested by the peaks in the region δ 6.84–7.14 integrating for four protons.

The structure of vasicine acetate was suggested by the remaining peaks. The −CHOAC group was confirmed by the one proton triplet at δ 5.19. The two methylene proton at C-1 appeared as multiplets at δ 3.52 and 3.70. The methylene proton at C-2 appeared as multiplets at δ 2.25 and 2.55. H-9 appeared as two one proton doublets (J=14.5 H_2) at 4.72 and 4.80. The above spectral data confirmed the structure of the compound to be vasicine acetate. This is reported for the first time.

The compound from fraction 7 was eluted with *n*-hexane: ethyl acetate (8:2 ratio). It appeared to be an individual compound. It was white powder. It was subjected to spectroscopic analysis. The compound yield was 72 mg. The IR spectrum gave the following data: Max cm^{-1}= (500–4000) 3400 (−NH_2), 2928, 1685 (ArCO−), 1628, 1576, 1498, 1459 and 1406 (aromatic system), 1188, 775 (aromatic system). ^{1}H NMR data for the active molecule were the following: 7.91 (2H, m., H-1 and H-3), 7.52 (2H, m, H-2 and H-4), 4.42 (1H, brs, −CH_2NH_2), 2.6 (3H, s, COCH_3). The ^{13}C NMR spectrum of the active molecule was the following: 129.1 (C-1), 127.8 (C-2), 132.6 (C-3), 128.2 (C-4), 136.1 (C-5), 137.6 (C-6),

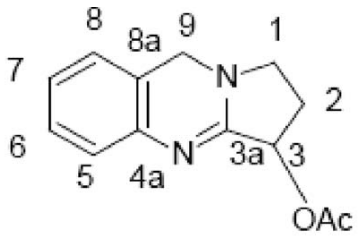

Figure 1. Vasicine acetate isolated from *A.vasica.*

54.3 (−$\underline{C}H_2NH_2$), 197.6 (−$\underline{C}OCH_3$), 26.6 (−CO$\underline{C}H_3$). MS: M+ M/Z 147, M/f- C_9 H_9 N O. The structure was elucidated using the above data (figure 2)

The compound 2-scetyl benzyl amine tested positive for primary amine in the dye test (diazotisation and coupling with β-naphthol in aqueous NaOH). The IR spectrum showed the presence of amine (3400 cm^{-1}) and aromatic ketone (1685 cm^{-1}). The presence of aromatic system was shown by peaks at 1628, 1576, 1498, 1459, 1406 and 775 cm^{-1}. In ^{1}H NMR the four aromatic protons appeared in the region δ 7.52 - 7.91. The presence of $-CH_2NH_2$ was shown by the benzyl methylene proton appearing as broad singlet at δ 4.42. The methyl proton of the acetyl group appeared as three proton singlet at δ 2.6. In the ^{13}C NMR spectrum there were four aromatic CH groups and two quaternary carbons. The methylene carbon of $-CH_2NH_2$ group appeared at δ 54.3. The presence of acetyl group was shown by peaks at δ 197.6 and 26.6. The above spectral data confirmed the structure of compound to be 2-acetyl benzylamine. This compound has previously been synthesized (Meindl e*t al*. 1984), but this is the first time that it has been isolated from a natural source.

The percentage inhibition of CFU by the three crude extracts is given in table 1. The hexane extract of *A.vasica* showed 98% reduction in CFU.

Table 2 shows the MIC values (100 μg/ml) of both the compounds against *M. tuberculosis* MDR strains using agar dilution assays. The growth of resistant strain RF-10385 was arrested by vasicine acetate and 2-acetyl benzylamine at 200 μg/ml. The growth of sensitive strain RF-10321 was controlled by vasicine acetate and 2- acetyl benzylamine at 50 μg/ml.

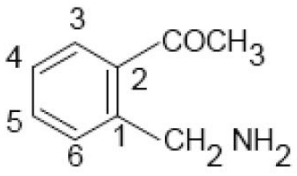

Figure 2. 2-Acetyl benzyl amine isolated from *A. vasica.*

Table 3 shows the percentage of reduction in RLU against *M. tuberculosis* and MDR strains using LRP assay. A compound is considered to be an antimycobacterial agent if 50% reduction in the RLU is observed as compared with the control when a luminometer is used. The percentage of RLU unit against *M. tuberculosis* by vasicine acetate was recorded as 99.96% in *M. tuberculosis,* 97.68 in the resistant strain and 99.85% in the sensitive strain. The percentage of reduction in RLU by 2- Acetyl benzylamine was recoded as 98.93% in *M. tuberculosis,* 95.55 in the resistant strain and 98.81% in the sensitive strain (table 4).

4. Discussion

The prevalence of MDR strains of *M. tuberculosis* is an important reason for the resurgence of TB as a major disease in many parts of the world. In many countries, medicinal plants are used by traditional medical practitioners to combat TB. *A. vasica* has been used in folk medicine to treat TB (Gupta and Chopra, 1954; Grange and Snell, 1996). The reduction in CFU of *M. tuberculosis* by the hexane crude extract of *A.vasica* leaves in our study clearly indicated the effectiveness of this plant to control tuberculosis *in vitro*.

The agar dilution assay specifically showed the percentage inhibition of CFU of the three strains of *M. tuberculosis*. These results were confirmed by the LRP assay of three strains at four different concentrations. Both compounds were highly effective against *M. tuberculosis* reference strain, MDR and sensitive strains. Vasicine acetate was more active than 2- acetyl benzylamine at all concentrations. Shawar *et al.* (1997) stated that luciferase assay had several advantages owing to its feasibility, amenability and high speed.

Cladwell *et al.* (2000) reported that 3-epioleanolic acid and oleanolic acid isolated from *Junellia tridens* had shown antimycobacterial activity with MIC values ranging from 16 to 128 μg/ml.

Grange and Snell (1996) tested the activity of bromhexine and ambroxol , semisynthetic derivatives of vasicine from

Table 1. Percentage inhibition of *M. tuberculosis* by the three extracts of *A.vasica* leaves

		Percentage inhibition of CFU			
Name of the plant	Extracts	Concentrations μg/ml			
Adhatoda vasica		12.5	25	50	100
	He	44.76±3.43	64.25±1.03	82.53±1.32	96.76±3.74
	Ea	20.83±3.48	38.25±4.06	59.62±3.21	70.16±2.40
	Me	24.75±3.06	37.63±2.46	49.70±4.07	61.41±0.63
Negative control		03.24±4.62			
Rifampicin 2.0 μg/ml		99.78±6.95			

Mean values represent mean CFU±SD of three replicates.
He, hexane; Ea, ethyl acetate; Me, methanol.
Negative control, medium and culture alone. Rifampicin, standard.

Table 2. MIC values for inhibition by vasicine acetate and 2-acetyl benzylamine against *M. tuberculosis*-resistant and *M. tuberculosis*-sensitive cultures using absolute concentration method

Strains	Lab code: No	A μg/ml	Percentage inhibition of CFU	B μg/ml	Percentage inhibition of CFU	RIF control 2 μg/ml	INH control 0.2 μg/ml	Positive control
M. tuberculosis H37Rv (HR-Sen) ATCC 27294	H37Rv	100	99.46	100	98.76	NG	NG	4.74
Resistant (HR-Res) Rf- 10385	RF-10385	200	97.3	200	95.37	NG	NG	4.27
Sensitive (HR-Sen) Rf- 10321	RF-10321	50	99.59	50	98.12	NG	NG	4.96

A, vasicine acetate; B, 2-acetyl benzylamine; RIF, Rifampicin; INH, Isoniazid; NG, no growth; Control (log value/ml); HR Res, Isoniazid- and Rifampin-resistant ; HR Sen: Isoniazid- and Rifampin-sensitive.

Table 3. Percentage reductions in relative light units (RLU) by different concentrations of vasicine acetate against clinical isolates of *M. tuberculosis*-resistant and *M. tuberculosis*-sensitive cultures

Strains	% reduction in RLU at concentration of μg/ml						
	Control	RIF 2.0 μg/ml	INH 0.2 μg/ml	12.5	25	50	100
M. tuberculosis H37Rv(HR-Sen) ATCC 27294	5.46	100	100	76.24	88.75	98.79	99.96
MDR Resistant (HR-Res) Rf- 10385	6.39	100	100	68.70	80.13	94.57	97.68
Sensitive (HR-Sen) Rf 10321	5.73	100	100	71.53	86.65	96.82	99.85

RIF, Rifampicin; INH, Isoniazid; control (log value/ml); HR Res, Isoniazid- and Rifampin-resistant; HR Sen, Isoniazid- and rifampin-sensitive.

Table 4. Percentage reductions in relative light units (RLU) by different concentrations of 2- acetyl benzylamine against clinical isolates of *M. tuberculosis*-resistant and *M. tuberculosis*-sensitive cultures

Strains	% of reduction in RLU at concentration of μg/ml						
	Control	RIF 2.0 μg/ml	INH 0.2 μg/ml	12.5	25	50	100
M.tuberculosis H37Rv (HR-Sen) ATCC 27294	5.46	100	100	70.40	81. 75	95.52	98.93
MDR Resistant (HR-Res) Rf 10385	6.39	100	100	65.52	72. 43	92.77	95.55
Sensitive (HR-Sen) Rf 10321	5.73	100	100	71.85	84. 75	94.87	98.81

RIF, Rifampicin; INH, Isoniazid; control (Log value/ml); HR Res, Isoniazid- and Rifampin-resistant; HR Sen, Isoniazid- and Rifampin-sensitive.

A. vasica against *M. tuberculosis* and found that the MIC for ambroxol was 50 to 100 mg/l and for bromhexine, the MIC was 6 to 12 mg/l at pH 6.5 after dissolving the compounds in DMSO. Our study indicated that the MIC values for vasicine acetate and 2-acetyl benzylamine ranged from 50 to 200 μg/ml when they were dissolved in DMSO. This clearly showed that the natural compounds also possessed equally good mycobacterial activity.

Gordien *et al.* (2009) found MIC values ranging from 21.1 to 92.4 μg/ml for terpenoids isolated from *Juniperous communis*. Murillo *et al.* (2003) reported MIC values of 100 μg/ml for the flavones isolated from *Haplopappus sonorensis*. Our results are similar to the activity of these natural compounds. Okunade *et al.* (2004) described 88 naturally occurring compounds, and in some cases synthetic analogues, largely from plants, fungi and marine organisms that demonstrated significant activity in the *in vitro* bioassays against *Mycobacterium tuberculosis* and other Mycobacterial species. We believe that vasicine acetate and 2-acetyl benzyl amine can also play an important role in the management of tuberculosis.

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