

# Comprehensive Analysis and Assessment of the Role of Major Bioactive Compounds Isolated from *Justicia Adhatoda* Leaves as Potent Antimycobacterial Agents

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## Research Article

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# Abstract

Tuberculosis (TB) continues to be one of the world's leading causes of death by the infectious pathogen *Mycobacterium tuberculosis*, which infects one-third of the global population. The emergence of the COVID-19 pandemic made its spread rapid and the treatment task more daunting. With the havoc of infectious disease expansion, traditional medicines have triggered tremendous interest worldwide. However, less availability of scientific evidence still hinders its practical use. In the present study, we evaluated the potential of the traditional medicinal plant, *Justicia adhatoda*, which has been used to treat respiratory ailments since ancient times. We have successfully isolated and characterized several bioactive compounds viz- Vasicoline, Vasicolinone, Adhatodine, Adhavasine, Aniflorine, and Vasicinone from *J. adhatoda* plant leaves, including Vasicine as the principal compound, and showed their anti-tubercular activity on nutrient-starved *Mycobacterium smegmatis* and *Mycobacterium bovis*. The study also directs their *in-vitro* and *ex-vivo* antimycobacterial potential on THP1 macrophages with internalized *Mycobacterium*. Our study is one of its first kind, where we assessed the synergistic antimycobacterial effect of the isolated compounds with the first-line drug Isoniazid (INH). Their potential role in promoting phagolysosome fusion and apoptosis of *M. bovis* infected THP1 macrophages is further evaluated.

## Introduction

Modern medicines mitigate chronic illnesses, correct disabling physical conditions, and cure molecular deficiencies, but their treatments are often marred by debilitating and severe side effects.<sup>1,2</sup> Tuberculosis (TB) is a chronic respiratory disease affecting more than one-fourth of the world population, causing around 2 million deaths every year, which becomes worrisome with the emergence of the global COVID-19 pandemic. International analysts predict a 26% increase in TB deaths in upcoming years, with additional 1.4 million TB deaths between 2020 and 2025 as a direct consequence of the pandemic.<sup>3,4</sup>

The bacteria responsible for the disease, *Mycobacterium tuberculosis*, is known for developing resistance against drugs rapidly, due to which the disease eradication is almost impossible, despite several anti-TB medicines at disposal. In the last four decades, two new TB drugs, bedaquiline, and delamanid have become available for MDR-TB and XDR-TB treatment, but resistant cases continue to surge.<sup>5,6</sup> Urgent need for new anti-TB drugs to improve cure rates for primary and drug-resistant TB has led to a renewed interest in plant-based bioactives as a possible medication. Natural and herbal products have caught the interest of global pharmaceutical companies and researchers worldwide to rediscover their potential as the source of safer drug candidates. According to the World Health Organization (WHO), approximately 75% of the world's population relies on plant extracts as traditional medicines and therapies for treatment.<sup>7</sup> However, due to a lack of scientific evidence about their pharmacological potential, mainstream therapies are often considered more reliable.<sup>8</sup> In recent years, natural products from plant sources are being relooked as they are abundant, readily available, cost-effective, have minimal or no side effects, and are beneficial in chronic disease treatment.<sup>8-10</sup>

*Justicia adhatoda* (L.) Nees *vasica* (family Acanthaceae) is a shrub widespread throughout Southeast Asia and the Indian subcontinent. It is commonly known as Malabar nut, Vasaka, or Adulsa (the greater 'neem').

The plant possesses anti-inflammatory, antispasmodic (bronchodilator), and expectorant properties.<sup>11</sup> Plant extracts are also traditionally used to treat cold, cough, asthma, and tuberculosis.<sup>12,13</sup> The ancient Indian saying, "No man suffering from phthisis need despair as long as the Vasaka plant exists," proves the importance of *J. adhatoda* plant in treating respiratory ailments.<sup>8</sup> WHO has recognized the properties of *J. adhatoda* in their manual "The Use of Traditional Medicine in Primary Health Care."<sup>14</sup> In North-East India, the "Meitei" community uses plant leaves and inflorescence to cure fever, cough, asthma, and dysentery. They also prepare a variety of cuisines for good digestion and health.<sup>15</sup> Recent studies on *J. adhatoda L.* plant leaf extracts showed that the plant has broad-spectrum antimicrobial and antifungal against several pathogens such as *E. coli*, *S. epidermidis*, *E. aerogenes*, *P. aeruginosa*, *B. subtilis*, *Rhizopus*, *Penicillium notatum*, *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus flavus*. Studies conducted on *J. adhatoda L.* leaf extracts showed anti-tubercular activity for *M. tuberculosis* MDR strains.<sup>16-19</sup>

*J. adhatoda L.* plant leaves contain alkaloids as major bioactive components, out of which study of vasicine is extensively carried out for drug development purposes.<sup>20,21</sup> Our previous study on the plant leaf extract of *J. adhatoda L.* proved the presence of several bioactive compounds besides vasicine, possessing antimycobacterial activity with low cytotoxic effects. We further found the components to exhibit a synergistic effect with Isoniazid (INH) on *M. smegmatis* and *M. bovis* (BCG).<sup>22</sup> In the present study, we have characterized the isolated active compounds and analyzed their role as antimycobacterial agents on nutrient-starved *Mycobacterium smegmatis* and *Mycobacterium bovis* (BCG). The synergistic antimycobacterial effect of the isolated components was further assessed with Isoniazid (INH) on the *Mycobacterium*. Additionally, we also studied the action of the isolated compounds on BCG-infected THP1 macrophages to analyze their activity on intracellular *Mycobacterium*.

## Results And Discussion

### Bioactive compound isolation and characterization

Alkaloids isolated from *J. adhatoda* majorly constituted quinazoline alkaloids, as depicted from our study of the <sup>1</sup>HNMR spectrum. We isolated ~99% pure Vasicine, a white-colored powder, and the highest content (~27%) amongst the total isolated alkaloids. The purity of Vasicine was confirmed via HPLC, HRMS, <sup>1</sup>HNMR, and FTIR results. (Table 1) Besides Vasicine, several other fractions of alkaloids were also isolated. They were all subsequently tested for their in-vitro antimycobacterial activity (Data already published). Six of the best fractions, labeled as 2A, 2B, 3A, 3B, 5A, and 5B, were selected and subjected to HPLC (Supplementary Figure 1), HRMS (Supplementary Figure 2), and <sup>1</sup>HNMR (Table 2) analysis for further characterization. The fractions were found to constitute Vasicoline (mw. 291.4) majorly, Vasicolinone (mw. 305.4), Adhatodine (mw. 335.4), Adhavasine (mw. 218.23), Aniflorine (mw. 349.4), and Vasicinone (mw. 202.4), respectively. (Table 1, Supplementary Figure 2) The isolated compounds were quinazoline compounds, confirmed by observing the <sup>1</sup>HNMR spectrum, having signature peaks in nitrogen, aromatic, and sugar region. Signature peaks in the <sup>1</sup>HNMR spectrum are identified and depicted in Table 2. Minor peaks observed in the spectrum were considered trace impurities often seen during isolation. Several functional groups like -OH, -N-H, C-H, C=O, and others were recognized from the FTIR results. The molecular

weight of the isolated fractions was obtained from HRMS in (M + H)<sup>+</sup> form, further confirming our findings. (Table 2)

Table 1  
Description of obtained isolated fractions HPLC peaks and HRMS details.

Fraction name	Compound	IUPAC Name	PubChem ID	Exact Mol. Wt.	Isolated Mol. Wt.	HPLC peak (2ml/min flow rate)
2A	Vasicoline	N,N-dimethyl-2-(1,2,3,9-tetrahydropyrrolo [2,1-b]quinazolin-3-yl)aniline	626005	291.4	291.5	1.604
2B	Vasicolinone	3-[2-(dimethylamino)phenyl]-2,3-dihydro-1H-pyrrolo[2,1-b]quinazolin-9-one	627712	305.4	305.5	1.077
3A	Adhatodine	methyl 2-(methylamino)-5-(1,2,3,9tetrahydropyrrolo[2,1-b]quinazolin-3-yl)benzoate	5316460	335.4	335.6	1.527
3B	Adhavasine	NA	NA	218.23	218.4	1.856
5A	Aniflorine	(3R)-3-[2-(dimethylamino)phenyl]-3-hydroxy-5-methoxy-1,2-dihydropyrrolo[2,1-b]quinazolin-9-one	442881	351.4	351.6	2.076
5B	Vasicinone	(3S)-3-hydroxy-2,3-dihydro1H-pyrrolo[2,1-b]quinazolin9-one	442935	202.21	202.4	1.389
BA	Vasicine	(3S)-1,2,3,9-tetrahydropyrrolo[2,1-b]quinazolin-3-ol	667496	188.23	188.4	1.137

Table 2  
FTIR and NMR peak details obtained of isolated fractions.

Fraction name	Compound Identified	FTIR peaks obtained	NMR peaks obtained
2A	Vasicoline	3300-3000 – NH, 2000-1650 – CH (aromatic ring), 1700-1650 – C=N	<sup>1</sup> H NMR: δ 2.15 (1H, ddt, <i>J</i> = 13.4, 8.0, 6.7 Hz), 2.37 (1H, dddd, <i>J</i> = 13.4, 7.5, 6.8, 1.7 Hz), 2.75 (6H, s), 3.42-3.61 (2H, 3.50 (ddd, <i>J</i> = 14.4, 6.7, 1.7 Hz), 3.53 (ddd, <i>J</i> = 14.4, 8.0, 6.8 Hz)), 4.18 (1H, dd, <i>J</i> = 7.5, 6.8 Hz), 4.61-4.83 (2H, 4.68 (d, <i>J</i> = 14.2 Hz), 4.76 (d, <i>J</i> = 14.2 Hz)), 6.52-6.70 (2H, 6.59 (ddd, <i>J</i> = 7.9, 7.6, 1.2 Hz), 6.64 (ddd, <i>J</i> = 8.1, 1.2, 0.5 Hz)), 6.78-6.93 (2H, 6.84 (ddd, <i>J</i> = 7.9, 1.3, 0.5 Hz), 6.86 (ddd, <i>J</i> = 7.9, 7.5, 1.3 Hz)), 6.95-7.19 (2H, 7.03 (ddd, <i>J</i> = 8.1, 7.6, 1.3 Hz), 7.13 (ddd, <i>J</i> = 8.1, 1.3, 0.5 Hz)), 7.20-7.34 (2H, 7.27 (ddd, <i>J</i> = 8.1, 7.5, 1.3 Hz), 7.28 (ddd, <i>J</i> = 7.9, 1.3, 0.5 Hz)).
2B	Vasicolinone	3300-3000 – NH, 2000-1650 – CH (aromatic ring), 1750 – C=O, 1700-1650 – C=N	<sup>1</sup> H NMR: δ 2.16-2.45 (2H, 2.24 (dtd, <i>J</i> = 13.5, 8.1, 6.5 Hz), 2.37 (dtd, <i>J</i> = 13.5, 6.7, 1.6 Hz)), 2.75 (6H, s), 3.78-3.97 (2H, 3.86 (ddd, <i>J</i> = 16.3, 6.5, 1.6 Hz), 3.88 (ddd, <i>J</i> = 16.3, 8.1, 6.7 Hz)), 4.30 (1H, dd, <i>J</i> = 8.0, 6.8 Hz), 6.52-6.70 (2H, 6.59 (ddd, <i>J</i> = 7.9, 7.6, 1.2 Hz), 6.64 (ddd, <i>J</i> = 8.1, 1.2, 0.5 Hz)), 6.84 (1H, ddd, <i>J</i> = 7.9, 1.3, 0.5 Hz), 7.03 (1H, ddd, <i>J</i> = 8.1, 7.6, 1.3 Hz), 7.22-7.53 (3H, 7.29 (ddd, <i>J</i> = 8.0, 7.4, 1.5 Hz), 7.39 (ddd, <i>J</i> = 7.8, 7.4, 1.4 Hz), 7.47 (ddd, <i>J</i> = 7.8, 1.5, 0.5 Hz)), 8.12 (1H, ddd, <i>J</i> = 8.0, 1.4, 0.5 Hz).
3A	Adhatodine	3300-3000 – NH, 2000-1650 – CH (aromatic ring), 1700-1650 – C=N, 1200-1000 – C-N, 3050 -OH, 1755-1700 – C=O	<sup>1</sup> H NMR: δ 2.05-2.36 (2H, 2.14 (ddt, <i>J</i> = 13.2, 8.0, 6.7 Hz), 2.28 (dddd, <i>J</i> = 13.2, 7.5, 6.8, 1.7 Hz)), 2.56 (3H, s), 3.42-3.61 (2H, 3.50 (ddd, <i>J</i> = 14.5, 6.7, 1.7 Hz), 3.52 (ddd, <i>J</i> = 14.5, 8.0, 6.8 Hz)), 3.81 (3H, s), 4.12 (1H, dd, <i>J</i> = 7.5, 6.8 Hz), 4.61-4.82 (2H, 4.68 (d, <i>J</i> = 14.2 Hz), 4.75 (d, <i>J</i> = 14.2 Hz)), 6.65 (1H, dd, <i>J</i> = 7.9, 2.6 Hz), 6.79-6.95 (2H, 6.86 (ddd, <i>J</i> = 7.9, 7.5, 1.3 Hz), 6.89 (dd, <i>J</i> = 7.9, 0.5 Hz)), 7.13 (1H, ddd, <i>J</i> = 8.1, 1.3, 0.5 Hz), 7.20-7.34 (2H, 7.27 (ddd, <i>J</i> = 8.1, 7.5, 1.3 Hz), 7.28 (ddd, <i>J</i> = 7.9, 1.3, 0.5 Hz)), 7.45 (1H, dd, <i>J</i> = 2.6, 0.5 Hz).
3B	Adhavasine	3300-3000 – NH, 2000-1650 – CH (aromatic ring), 1700-1650 – C=N, 3500-3150 – OH (intramolecular), 1259- Ar-O-CH <sub>3</sub>	<sup>1</sup> H NMR: δ 2.10-2.36 (2H, 2.17 (dddd, <i>J</i> = 11.7, 6.8, 2.2, 1.7 Hz), 2.27 (dddd, <i>J</i> = 11.7, 7.9, 7.4, 6.8 Hz)), 2.57 (1H, ddd, <i>J</i> = 13.6, 6.8, 1.7 Hz), 2.81 (1H, ddd, <i>J</i> = 13.6, 7.9, 6.8 Hz), 3.81 (3H, s), 4.51-4.72 (2H, 4.58 (d, <i>J</i> = 13.2 Hz), 4.65 (d, <i>J</i> = 13.2 Hz)), 5.82 (1H, dd, <i>J</i> = 7.4, 2.2 Hz), 6.46-6.60 (2H, 6.52 (dd, <i>J</i> = 7.6, 1.5 Hz), 6.54 (dd, <i>J</i> = 7.9, 1.5 Hz)), 7.34 (1H, dd, <i>J</i> = 7.9, 7.6 Hz).
5A	Aniflorine	3300-3000 – NH, 2000-1650 – CH (aromatic ring), 1754 – C=O, 1700-1650 – C=N, 3500-3150 – OH (intramolecular), 1259- Ar-O-CH <sub>3</sub>	<sup>1</sup> H NMR: δ 2.02 (1H, ddd, <i>J</i> = 13.9, 6.7, 1.6 Hz), 2.24 (1H, ddd, <i>J</i> = 13.9, 8.1, 6.5 Hz), 2.81 (6H, s), 3.73-4.00 (5H, 3.81 (ddd, <i>J</i> = 15.7, 6.5, 1.6 Hz), 3.91 (ddd, <i>J</i> = 15.7, 8.1, 6.7 Hz), 3.88 (s)), 6.61-6.82 (2H, 6.67 (ddd, <i>J</i> = 8.1, 1.2, 0.5 Hz), 6.75 (ddd, <i>J</i> = 7.8, 7.6, 1.2 Hz)), 6.88-7.10 (3H, 6.94 (dd, <i>J</i> = 8.5, 1.4 Hz), 7.01 (dd, <i>J</i> = 8.5, 7.8 Hz), 7.03 (ddd, <i>J</i> = 8.1, 7.6, 1.4 Hz)), 7.22 (1H, dd, <i>J</i> = 7.8, 1.4 Hz), 7.35 (1H, ddd, <i>J</i> = 7.8, 1.4, 0.5 Hz).

Fraction name	Compound Identified	FTIR peaks obtained	NMR peaks obtained
5B	Vasicinone	3300-3000 – NH, 2000-1650 – CH (aromatic ring), 1700-1650 – C=N, 3500-3150 – OH, 1754 – C=O	<sup>1</sup> H NMR: δ 2.15 (1H, dtd, <i>J</i> = 12.1, 8.1, 6.5 Hz), 2.33 (1H, dtd, <i>J</i> = 12.1, 6.7, 1.6 Hz), 3.71-3.89 (2H, 3.79 (ddd, <i>J</i> = 15.8, 6.5, 1.6 Hz), 3.81 (ddd, <i>J</i> = 15.8, 8.1, 6.7 Hz)), 5.27 (1H, dd, <i>J</i> = 8.0, 6.8 Hz), 7.22-7.54 (3H, 7.29 (ddd, <i>J</i> = 8.0, 7.4, 1.5 Hz), 7.39 (ddd, <i>J</i> = 7.8, 7.4, 1.4 Hz), 7.47 (ddd, <i>J</i> = 7.8, 1.5, 0.5 Hz)), 8.12 (1H, ddd, <i>J</i> = 8.0, 1.4, 0.5 Hz).
BA	Vasicine	3300-3000 – NH, 2000-1650 – CH (aromatic ring), 1700-1650 – C=N, 3500-3150 – OH	<sup>1</sup> H NMR: δ 2.02-2.19 (2H, 2.11 (ddt, <i>J</i> = 14.0, 7.4, 6.8 Hz), 2.11 (dddd, <i>J</i> = 14.0, 7.5, 2.2, 1.7 Hz)), 3.26-3.55 (2H, 3.34 (ddd, <i>J</i> = 14.0, 7.4, 2.2 Hz), 3.47 (ddd, <i>J</i> = 14.0, 7.5, 6.7 Hz)), 4.68-4.85 (2H, 4.75 (d, <i>J</i> = 14.0 Hz), 4.78 (d, <i>J</i> = 14.0 Hz)), 4.98 (1H, dd, <i>J</i> = 6.8, 1.7 Hz), 6.87 (1H, ddd, <i>J</i> = 7.9, 7.5, 1.3 Hz), 7.07-7.31 (3H, 7.13 (ddd, <i>J</i> = 8.2, 1.3, 0.6 Hz), 7.24 (ddd, <i>J</i> = 8.2, 7.5, 1.3 Hz), 7.24 (ddd, <i>J</i> = 7.9, 1.3, 0.6 Hz)).

**Time-kill kinetic antimycobacterial activity assay reveals synergy of the isolated fractions with INH on nutrient-starved *M. smegmatis* and *M. bovis* (BCG).**

Nutrient starved *M. smegmatis* and *M. bovis* (BCG) showed enhanced growth than the normal replicating bacteria when inoculated in 7H9 media after starvation. The nutrient-starved *M. smegmatis* and *M. bovis* (BCG) exhibited a shorter lag phase than the non-nutrient starved one. In the case of nutrient-starved *M. bovis* (BCG), we also observed biofilm formation upon a more extended incubation period. Both nutrient-starved bacilli developed resistance against INH. For normal *M. smegmatis*, percentage inhibition upon INH treatment was observed to be (38.70±0.023), the growth inhibition for nutrient-starved *M. smegmatis* was significantly decreased to (3.45±0.009). Similarly, for nutrient-starved BCG, the percentage growth inhibition in the presence of INH was recorded (6.50±0.004), which was significantly lower than that of the normal one (31.65±0.016). (Figure 1)

The isolated alkaloid fractions obtained from *J. adhatoda* plant leaves showed significant growth inhibition on nutrient-starved *M. smegmatis* and *M. bovis* (BCG). The fractions significantly increased the lag phase and decreased the log phase of the bacterial growth curve. In the standard growth curve of nutrient-starved *M. smegmatis*, the lag phase was observed for 6 hours, followed by the log phase, which continued beyond Day 2, subsequently transforming to the stationary phase. While, most of the fractions decrease the log phase to Day 1, making it less steep with time progress. Maximum inhibition was observed in 5A, followed by 5B, Vasicine, 2A, and 2B. 3A and 3B showed lower inhibition but the maximum synergy with INH. The bacterial CFU reached >10<sup>11</sup> CFU/mL for the assay at Day 2, from initial inoculum 1.5×10<sup>6</sup> CFU/mL. While total CFU counted for the bacteria treated with the fractions was significantly lower than the control, indicating the bactericidal activity of the fractions on the bacteria. 2A, 5B, and Vasicine showed bactericidal activity as log<sub>10</sub> CFU decreased to 4-5 for these fractions, even without INH. Whereas 3A, 3B, and 5A showed bacteriostatic effect having log<sub>10</sub> CFU more than 6, i.e., >10<sup>6</sup> CFU/mL.

However, they exhibited bactericidal activity when incubated with INH, as  $\log_{10}$  CFU was reduced to 3 ( $10^3$  CFU/mL). The cumulative effect of the fractions and INH showed synergy in 3A, 3B, 5A, and 2B, where 3A showed the best synergy and additive effect for Vasicine, 2A, and 5B. (Figure 2) For *M. bovis* (BCG), the lag phase was observed till Day 2, followed by the log phase from Day 2 to Day 7. The stationary phase started from Day 8 to Day 28, with little growth from Day 21. In *M. bovis* (BCG), the maximum growth inhibition was observed on Day 7, followed by Day 21, and total CFU was counted on Day 7. For BCG, the fractions showed the bactericidal property in general, except 3A and 3B. 5A and 5B showed the highest antimycobacterial activity, followed by Vasicine. Co-administration of the fractions with INH greatly enhanced the bactericidal property of the fractions. 3A and 3B showed bacteriostatic effect but exhibited bactericidal activity in the presence of INH. An additive effect was observed in the case of Vasicine, while 2A and 2B show no synergy with INH. Other fractions exhibited synergy with INH, with fraction 3A showing the best synergy. (Figure 3)

### **Antimycobacterial activity of the fractions on BCG infected THP1 macrophages**

Annexin V dead cell apoptosis assay revealed that the isolated fractions targeted the BCG-infected macrophages when added at  $IC_{50}$  concentration, leading to cell death via apoptosis and necrosis. There was an increase in the percentage of cell death with the increase in incubation time, i.e., the rate of cell death was observed higher after 48h incubation than 24h incubation (Figure 4). Vasicine was ineffective on macrophages having internalized BCG, showing negligible cell death at 24h, while ~20% death at 48h (Figure 4, G). Fractions 2A, 3A, and 5A showed insignificant death at 24h incubation, which increased approximately 40% at 48h. Fraction 3B showed ~40% cell death at 24h incubation, which reached 100% at 48h. A similar trend was observed for fraction 2B, exhibiting 40% death at 24h, while ~80% cell death at 48h incubation. Effect of time was not observed in the case of 5B, as percentage cell death (~20%) remained the same at 24h and 48h.

Synergy was observed in the case of fractions 3A, 3B, 5A, and 5B against BCG-infected macrophages when treated along with INH, where fractions 5A and 5B showed the best synergy. The antimycobacterial activity of these fractions increased more than 60% when treated with INH. Fraction 2A and 2B showed additive effects with INH on BCG-infected macrophages with a ~30% increase in antimycobacterial activity. In the case of Vasicine, there was no impact of INH, as no increase in the cell death of BCG-infected macrophages. While most of the fractions showed late apoptosis, 3A and 3B caused cell death due to necrosis. (Figure 4&5)

### **THP1 macrophages showed colocalization of *M. bovis* (BCG) and Lysosomes when treated with the isolated fractions.**

*Mycobacterium* bacilli, i.e., *M. bovis* (BCG) and *M. tuberculosis*, can prevent the host cell from forming lysosomes, making it their temporary resident. Upon treatment with the isolated fractions, the BCG-infected THP1 macrophage cells produce lysosomes targeted explicitly towards the bacilli. GFP-tagged BCG and LysoTracker Red-stained LAMP-1 protein, expressed on the lysosomes, were colocalized and analyzed by using the software. Yellow coloration indicates colocalization, obtained due to the superimposition of red

and green colors. Percent colocalization, measured by the software, was recorded highest in 2A, 2B, and 5B. 5A and Vasicine showed moderate, while no colocalization was found for 3A, 3B, and INH. For the fractions showing significant colocalization, the percent green overlap was significantly higher than the red overlap, except 5A and 5B.

The above result implies that other fractions promote lysosome production spread across the cytoplasm, while 5A and 5B direct lysosomes towards the internalized bacteria. In Vasicine and 2B, profound lysosome production was noted. (Figure 6) Macrophages expressed a significantly higher number of lysosomes directed towards the ingested bacilli when co-administered with the fractions and INH. All fractions except 3A showed an increase in the percent colocalization when co-administered with INH. Fractions 2B and 5B showed synergy with INH, while VAS, 2A, 3B, and 5A showed additive effect with INH for phagolysosome fusion. (Figure 7) The above result shows that the alkaloid fractions isolated from *J. adhatoda* leaf help macrophages kill internalized BCG by promoting phagolysosome fusion.

## Discussion

Plants are the inexhaustible resource of natural products used as folk medicines since humanity. Although the popularity of synthetic drugs increased due to their cost effectiveness, easy quality control, and immediate effects, the rise of resistant bacterial strains and severe side effects makes their use questionable. A vast number of natural product-derived compounds in the pipeline highlights the significance of plants as sources of new drug candidates.<sup>8,9,23</sup>

*Justicia adhatoda*, a well-known indigenous Indian subcontinental plant, is commonly used to treat respiratory ailments in folk medicine. Our study explored the antimycobacterial potential of the plant leaf extracts and successfully isolated and characterized bioactive compounds, besides Vasicine, responsible for the activity. The isolated fractions, labeled as 2A, 2B, 3A, 3B, 5A, and 5B, were identified as alkaloids, constituting Vasicoline, Vasicolinone, Adhatodine, Adhavasine, Aniflorine, and Vasicinone, respectively. Our previous study proved that the compounds showed excellent antimycobacterial activity against *M. smegmatis* and *M. bovis*, better than the first-line drugs Isoniazid (INH).<sup>22</sup> The fractions also showed synergy against both bacilli with INH. The present study analyzed the potential antimycobacterial activity of the isolated fractions on nutrient-starved *M. smegmatis* and *M. bovis* (BCG). We found that the fractions inhibit bacterial growth and possess a profound bactericidal effect when co-administered with INH. As the nutrient-starved bacilli act as a Latent TB model, the results are a significant milestone for further research for anti-TB drug development. The activity of Aniflorine, Vasicoline, and Vasicolinone was exceptional, as these compounds not only showed profound antimycobacterial inhibition on the nutrient-starved *M. smegmatis* and *M. bovis* (BCG) but also showed synergy with INH. Although Adhatodine and Adhavasine exhibited lower antimycobacterial activity than the other compounds, they showed maximum synergy with INH.

Dead cell apoptosis assay, performed on BCG-infected THP1 macrophages in the presence of the isolated compounds, gave more insight. The compounds were efficacious on the intracellular BCG, causing infected macrophages' death and showing synergy with INH. When co-administered with INH, compounds caused

cell death due to apoptosis, while Adhatodine and Adhavasine showed necrosis even with INH. Although Vasicine showed excellent antimycobacterial activity on *M. smegmatis* and *M. bovis* (BCG), it was ineffective on intracellular BCG. The efficacy of Vasicine remains unchanged even in the presence of INH. *Mycobacterium tuberculosis* is well known for its adaptability and quick resistant development. A significant paradigm in TB pathogenesis is that the internalized bacteria use the host cell mechanism to inhibit phagosome-lysosome fusion, making the condition favorable for latency. The extant drugs cannot target the internalized TB bacilli, which targets host cells, thus causing severe side effects. Our preliminary results suggest that the compounds isolated from *J. adhatoda* leaf promote phagosome-lysosome fusion. The lysosomes were directed towards the internalized bacilli. The combined effect of the compounds and INH showed an increase in phagolysosome fusion. However, Vasicine and Adhavasine showed no change in percentage colocalization of internalized BCG and lysosomes, even when administered with INH.

Vasicine is a well-known bronchodilator used for cough treatment. Several in-silico studies and a few in-vitro studies show its antimicrobial, antioxidant, and cytotoxic potential.<sup>24,25</sup> Vasicine is also known as a moderate anti-tubercular agent.<sup>26</sup> Some in-silico studies on the derivatives of Vasicine, such as Vasicinone, Vasicoline, and Vasicolinone, suggest the anti-TB activity along with anti-proliferative and hepatoprotective effects, where the activity of Adhatodine, Adhavasine, and Aniflorine has not yet been assigned.<sup>27,28</sup>

Our study is one of its first kind, where we assessed the in-vitro antimycobacterial activity of these derivatives on nutrient-starved *M. smegmatis* and *M. bovis* (BCG). We support our findings with the study conducted on BCG-infected macrophages. The compounds showed effective in-vitro antimycobacterial activity on both bacilli and showed synergy with the first-line drug INH. The alkaloids also showed anti-proliferative activity on BCG-infected macrophages and proved more effective than INH and Vasicine. They also promote phagolysosome fusion which shows their effect targeted towards the internalized bacilli. Aniflorine, Vasicoline, Vasicolinone, and Vasicinone showed excellent in-vitro antimycobacterial effects and good anti-proliferative activity. They also showed phagosome-lysosome fusion, better than the other compounds. Vasicoline and Vasicolinone showed additive effects, while Aniflorine and Vasicinone showed synergy with INH. Although Vasicine shows excellent in-vitro antimycobacterial activity, its impact on internalized *M. bovis* (BCG) was poor. Neither it showed the anti-proliferative effect nor showed phagolysosome fusion in BCG-infected THP1 macrophages. However, Vasicine showed synergy with INH for phagolysosome fusion.

Our study proves that *J. adhatoda* is rich in bioactive compounds responsible for the anti-tubercular activity, which should be pursued further for anti-TB drug development. The study also directs the in-vitro and ex-vivo antimycobacterial potential of quinazoline alkaloids in *J. adhatoda* and suggests their effectiveness more than vasicine, the principal alkaloid in the plant. The compounds need to be analyzed further on *M. tuberculosis* and also on the animal model. In-silico and in-vitro study of the isolated compounds, especially Aniflorine, Adhavasine, and Adhatodine, should be conducted in perspective of phagosome-lysosome fusion in *M. tuberculosis* internalized in macrophages to understand the mechanism of action of the compounds.

## Methods

# Bacterial samples, cell lines, and chemical procurement

Bacterial samples viz.- *Mycobacterium smegmatis* strain mc<sup>2</sup>155 and *Mycobacterium bovis* (BCG-Pasteur) were procured from the Department of Pathology, AIIMS, and Department of Biomedical Sciences, University of Delhi, North Campus, respectively. Procurement of GFP tagged *Mycobacterium bovis* (BCG) and THP1 cell lines from Department of Biomedical Sciences, University of Delhi, North Campus. Middlebrook 7H9 and 7H11, Oleic acid Albumin Dextrose Catalase (OADC), and Albumin Dextrose Catalase (ADC) supplements were purchased to maintain the bacterial cultures from Himedia.

For animal cell line propagation- Rosewell Park Memorial Institute 1640 (RPMI1640), Foetal Bovine Serum (FBS), 0.25% Trypsin-EDTA solution, Pen-Strep solution (10,000U/mL), 96 well microtiter plates, serological pipettes, and T<sub>25</sub> and T<sub>75</sub> Flasks (Gibco and Nunc) were used. THP1 differentiation into macrophages, cell stimulation cocktail containing phorbol 12-myristate 13-acetate (PMA) and ionomycin, Dead cell apoptosis kit, LysoTracker Red, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher. Solvents (both analytical and HPLC grade) were purchased from SRL chemicals and Merck. Deuterated Chloroform for NMR analysis, tyloxapol, and Isoniazid (INH) were purchased from Sigma Aldrich.

## Declaration Statement

*Justicia adhatoda* L. plant leaves were acquired from Kamala Nehru Ridge, New Delhi, located on the University of Delhi campus, provided by the garden officer of the University of Delhi. The plant material was procured legally, under the national guidelines and legislation of the National Biological Diversity Act, 2002, formulated by the Central Government vide notification S.O.1911(E) of the Government of India.<sup>29</sup> Submission of the plant for sample verification and authentication was made at NISCAIR, PUSA Road, Delhi. The sample authentication number - NISCAIR/RHMD/Consult/ 2015/2907/100. The herbarium was also created and deposited in the Department of Botany, University of Delhi. All experiments were performed by following the standard protocols approved by WHO biological reference standards.<sup>30</sup>

## Plant Material Collection, Extract Preparation, And Alkaloid Isolation

Plant leaves, acquired in October from Kamala Nehru Ridge, New Delhi, were washed, shade dried, and powdered. The methanol extract was prepared by dissolving powdered leaves in methanol (1:10 leaf: solvent ratio). The mixtures were stirred overnight on the magnetic stirrer at room temperature. The solution was filtered after 72 hours of incubation and concentrated by a rotary evaporator. Alkaloids were isolated via the acid-base extraction method by treating the extract with an excess of 1% citric acid solution. The mixture was stirred overnight, filtered, and suspended in chloroform. The aqueous and chloroform layer was separated and concentrated. The aqueous layer was basified to pH 9.5 using NH<sub>4</sub>OH and further extracted using chloroform and treated with Na<sub>2</sub>SO<sub>4</sub> (in excess), filtered, and again concentrated. The obtained compound mixture was measured with Acetone and Petroleum ether (1:1 ratio; 40ml each) and filtered to

get purified alkaloid – Vasicine. The qualitative tests for the first fraction confirmed the identity as an alkaloid.

## Alkaloid Fractionation And Isolation

The alkaloid fraction obtained was subjected to TLC analysis to standardize the mobile phase for further fractionation. After standardization, Prep TLC was performed using 45% ethyl acetate, 35% methanol, and 20% chloroform as mobile phase at pH 9.5 adjusted by adding  $\text{NH}_4\text{OH}$ . Different bands were obtained and re-fractionated to varying polarities of the mobile phase adjusted accordingly. Fractions were subjected to antimycobacterial activity assay, and out of several, the best fractions were selected for further analysis. (Antimycobacterial activity data has been published)

## Compound Characterization

**HPLC analysis:** Isolated fractions were first subjected to HPLC analysis, C18 column, 95% Methanol; 4% Acetonitrile; 1% Tetrahydrofuran as mobile phase isocratic solvent. The injection volume was 20 $\mu\text{l}$ , and the running time was 20 minutes. **Mass Spectrometry:** 5mg of the isolated fractions were submitted to the Q-ToF HRMS facility of SAIF CDRI Lucknow for analysis. **NMR analysis:** Isolated compounds were subjected to  $\text{H}^+$  NMR analysis. 7-8mg/ml of the samples were dissolved in 500  $\mu\text{l}$  of Deuterated Chloroform and subjected for NMR analysis at USIC, North Campus, University of Delhi. **FTIR spectrometry:** To determine the functional groups present, 5mg of the samples were dissolved in methanol (HPLC grade solvent) and submitted for FTIR analysis to AIRF, JNU.

### Preparation of nutrient-starved *M. smegmatis* and *M. bovis* (BCG)

The nutrient starvation model is designed by following the method standardized by Loebel et al.<sup>31</sup> Stocks of bacterial culture obtained were revived in 7H9 broth containing 10% OADC/ADC supplement. The *M. smegmatis* and *M. bovis* (BCG) cultures were incubated at 37°C for 24 hours and seven days. Afterward, the cultures were streaked on a 7H11 agar plate (containing 10% ADC and OADC growth supplement for *M. smegmatis* and BCG), and colonies were obtained after 3-5 days and 21 days for *M. smegmatis* and BCG, respectively. Cultures of *M. smegmatis* mc<sup>2</sup>155 and *M. bovis* (BCG) were grown till the log phase reached (24 hours for *M. smegmatis*, where the OD of culture, taken at 600 nm, was observed to be 0.6 and 7 days for *M. bovis* where the OD of culture, taken at 600 nm, was honored to be 0.48.) in Middlebrook 7H9 media supplemented with 10% OADC (for *M. bovis*) or ADC (for *M. smegmatis*) and 1% glycerol. Both cultures were centrifuged at 3000 rpm for 5 minutes. Media was discarded, and culture pellets were washed out three times with 1X PBS. The bacterial cultures were incubated at 37°C in PBS solution supplemented with Tyloxapol for single-cell suspension (non-degradable detergent). The incubation period for *M. smegmatis* was 15 days, whereas for *M. bovis*, its 4-6 weeks long. As nutrients were not provided, the OD of both cultures remained the same after the incubation period. After the completion of the incubation period, cultures were centrifuged at 3000 rpm for 5 minutes and resuspended in fresh 7H9 media supplemented

with 10% OADC/ADC and 0.5% glycerol. OD of the cultures was standardized at 0.1 (*M. smegmatis*) and 0.3 (*M. bovis*) for further experimentation.

### **Antimycobacterial activity assay and synergy with INH on nutrient-starved *M. smegmatis* and *M. bovis* (BCG) in a time-kill kinetic manner**

Cultures of *M. smegmatis* and *M. bovis* (BCG) ( $10^6$  CFU/ml) were incubated with the isolated fractions at their  $IC_{50}$  concentration determined previously (Data has been published). Another set containing the isolated fractions with the first-line drug INH was incubated with bacterial cultures ( $10^6$  CFU/ml). The culture tubes were incubated at 37°C in an incubator shaker. Before the incubation, 500µl of the milieu was taken out, and absorbance was recorded at 600nm as 0hour reading. For *M. smegmatis*, the absorbance of the treated cultures was recorded at 1, 2, ...8, 24hours, till day 7. For *M. bovis* (BCG), the readings were taken at 4, 8, 24hours, till day 28. Positive and negative controls were maintained to negate the errors. The graph between absorbance at 600nm and time was plotted to obtain the bacterial growth curve. The treated bacterial culture was centrifuged, washed by PBS, and suspended in fresh uninoculated 7H9 broth at the maximum inhibition. Dilutions were made for the suspended bacterial culture, spread on the 7H11 plates, and determined bacterial CFU.

## **Intracellular Antimycobacterial Assay**

### **Dead cell apoptosis assay**

THP-1 cell lines were maintained in RPMI 1640 with 10% FBS in the presence of Pen-strep solution. THP1 cells were transferred to 12 well plates ( $10^6$  cells per well) and stimulated to Macrophages using a Cell stimulation cocktail. The cell lines were left overnight, washed with 1XPBS, and replenished with RPMI 1640 media. Newly differentiated THP1 cells into macrophages were then infected with *M. bovis* (BCG), cultivated in 7H9 broth containing 10% OADC and 1% Glycerol. Bacterial culture was grown till log phase (0.6  $OD_{600}$ ) and then diluted to get 7.5 MoI (Multiplicity of Infection). The infected macrophages were treated with the isolated fractions at their  $IC_{50}$  concentration for 24 hours and 48 hours. Incubated cells were then harvested and washed with 1XPBS twice and pelleted out. Using Annexin V dead cell apoptosis kit by Thermo Fisher, the cells were treated with Annexin V binding buffer and subsequently tagged with Fluorescein isothiocyanate (FITC) and Propidium Iodide (PI) as per the instruction manual. The cells were subjected to FACS analysis.

### **Co-localization of *M. bovis* (BCG) and Lysosomes in THP1 Macrophage infected.**

In 12 well plates, sterile coverslips were inserted, and 1 ml RPMI 1640 media was added to each well. Wells were seeded with THP1 cells ( $10^6$  cells per well) and treated with a cell stimulation cocktail at 1µg/ml concentration to induce THP1 differentiation into macrophages. Treated cells were left for overnight incubation at 37°C. The media was removed, cells were washed gently with RPMI 1640 media after incubation, and fresh media was added for further use. On the other hand, GFP tagged *M. bovis* (BCG)

colony was cultured on 7H11 agar plates, containing 10% OADC and 1% Glycerol. Hygromycin was used to select the GFP-expressing colonies. The bacterial colony was grown in 7H9 broth under optimum temperature with 10% OADC and 1% Glycerol as growth supplement till log phase (0.6 OD<sub>600</sub>) and then diluted to get 7.5 MoI (Multiplicity of Infection). The cells were treated with isolated fractions at their IC<sub>50</sub> concentration, and GFP tagged BCG were added and incubated for 3 hours. After incubation completion, media was discarded, and cells were gently washed. Cells were then treated with LysoTracker Red (1ng/ml) for 1 hour and washed twice with PBS. Slides were prepared by putting the coverslips from the wells on the slide containing a drop of DAPI stain. Slides were observed under a confocal microscope at specific wavelengths and analyzed with the help of Just Another Colocalization Plugin (JACoP) plugin in ImageJ software.

## Statistical analysis

Graphpad Prism 5.0 was used to plot graphs and calculate p-value by performing a t-test for time-kill kinetic assay on nutrient-starved *Mycobacterium*. All Experiments were performed at least thrice, and results are represented as Mean and Standard Deviation (SD). Negative and positive controls were taken into consideration in all assays performed. INH was used as a standard anti-TB drug for comparison. Grammar and plagiarism have been checked using the software Grammarly Premium. FACS images were analyzed by using offline software Cyflgoic for calculating percentage apoptosis and necrosis. Co-localization in confocal images, Pearson's coefficient, and percentage overlap was calculated with the help of offline software ImageJ plugin JACoP.

## Declarations

## Acknowledgment

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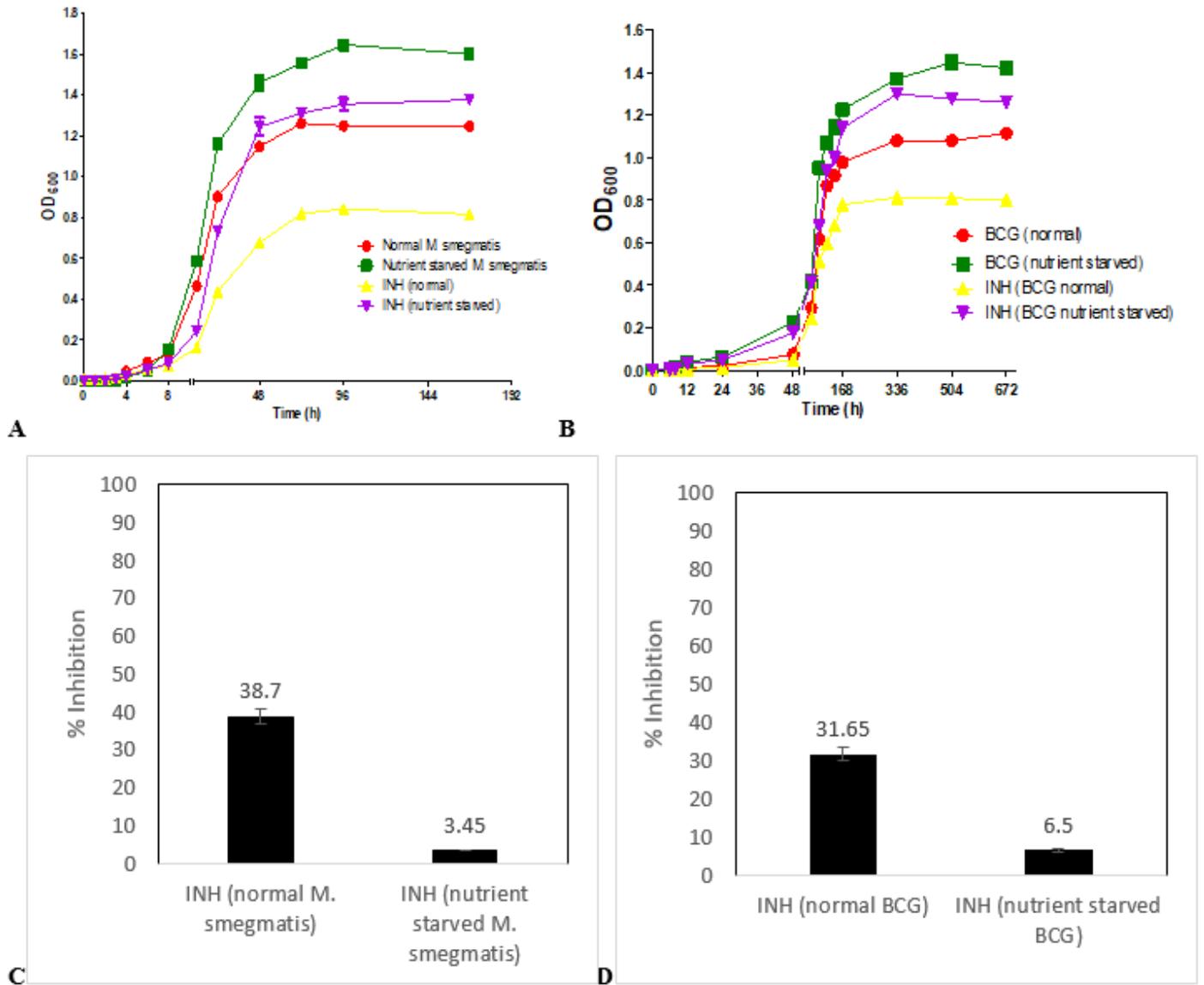
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## Figures



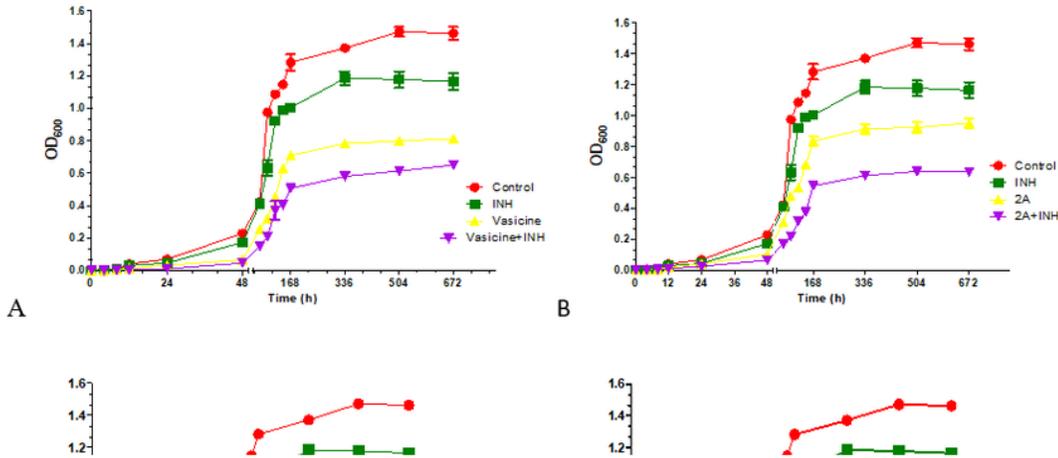
**Figure 1**

Comparison between normal and nutrient-starved bacterial growth for (A) *M. smegmatis* and (B) *M. bovis* (BCG) in the presence and absence of the first-line drug INH, by plotting the growth curve. Percentage inhibition by INH is shown in (C) for *M. smegmatis* at Day 2 and (D) for *M. bovis* (BCG) at Day 7. Values are plotted as Mean  $\pm$  SEM, n=3.

**Figure 2**

Graphical representation of time-kill kinetic assay on Nutrient starved *M. smegmatis* showing the change observed in the growth curve in the presence of (A) Vasicine, (B) 2A, (C) 2B, (D) 3A, (E) 3B, (F) 5A, and (G) 5B, with and without INH. (H) shows the bacterial CFU on Day 2 when treated with the isolated fractions with and without the co-treatment of INH to determine potential synergy. Values are plotted as Mean  $\pm$  SEM,

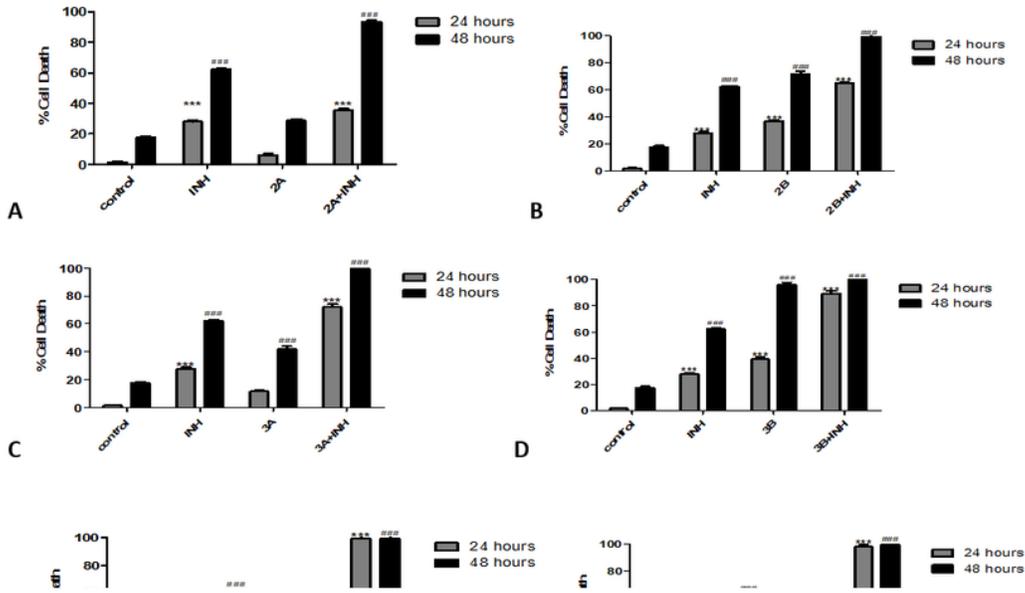
n=3. \*p-value < 0.05, \*\*p-value < 0.001, \*\*\* p-value < 0.001; t-test result compared to Control (bacterial growth without inhibitor) at Day 2.



**Figure 3**

Graphical representation of time-kill kinetic assay on Nutrient starved *M. bovis* (BCG) showing the change observed in the growth curve in the presence of (A) Vasicine, (B) 2A, (C) 2B, (D) 3A, (E) 3B, (F) 5A, and (G) 5B, with and without INH. (H) shows the bacterial CFU on Day 2 when treated with the isolated fractions with and without the co-treatment of INH to determine potential synergy. Values are plotted as Mean  $\pm$  SEM,

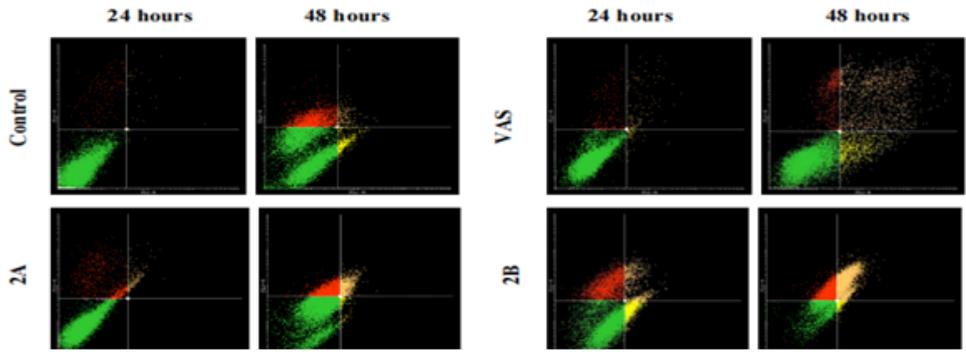
n=3. \*p-value < 0.05, \*\*p-value < 0.001, \*\*\* p-value < 0.001; t-test result compared to Control (bacterial growth without inhibitor) at Day 7.



**Figure 4**

Description of %Cell death of BCG infected macrophages upon treatment with (A) 2A, (B) 2B, (C) 3A, (D) 3B, (E) 5A, (F) 5B, and (G) Vasicine. Graphs (H) and (I) represented %Apoptosis and %Necrosis respectively, occurred upon treatment with isolated fractions for 24 and 48 hours. Values are plotted as Mean  $\pm$  SEM, n=3. \*p value < 0.05, \*\*p value < 0.001, \*\*\* p value < 0.001; t-test result compared to Control (bacterial

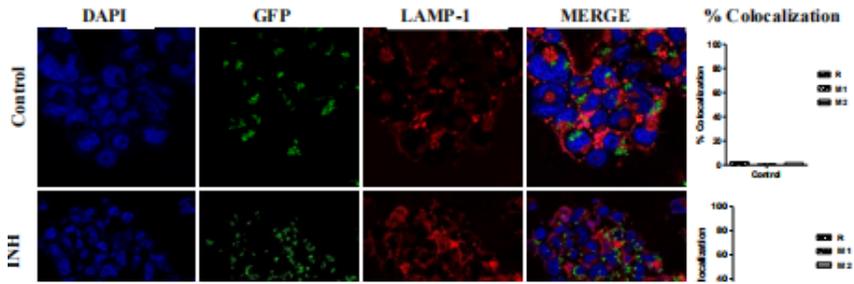
growth without inhibitor) at 24 hours. #p value < 0.05, ##p value < 0.001, ### p value < 0.001; t-test result compared to Control (bacterial growth without inhibitor) at 48 hours.



**Figure 5**

Figure representing the Dot plot, obtained upon subjecting the BCG infected THP1 macrophages upon treating with (A) isolated compounds and (B) compounds along with INH. PI (Propidium Iodide) and Annexin V FITC staining were done to evaluate apoptosis and necrosis. Lower Left quadrant represents live cells (green), Lower Right quadrant represents early apoptotic cells (Annexin V stained, yellow), Upper Right

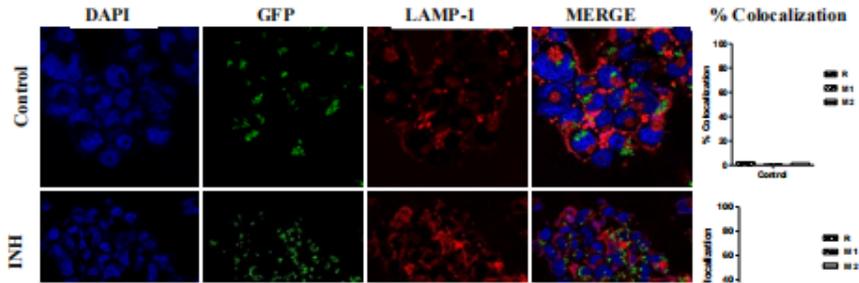
quadrant represents late apoptotic cells (stained with Annexin V and PI, orange). Upper Left quadrant represents necrotic cells (PI stained, red).



**Figure 6**

Confocal imaging of BCG-infected THP1 macrophages treated with fractions 2A, 2B, 3A, 3B, 5A, 5B, and Vasicine, compared with Control (untreated macrophages) and INH. DAPI stains THP1 nucleus in blue, GFP is expressed by internalized BCG, and LysoTracker Red is used to stain LAMP-1 proteins present on lysosomes. Merged image is used to identify and measure colocalization using ImageJ application. Percent

colocalization (R), red overlap (M1), and green overlap (M2) is showed in adjacent graphs. \*p value < 0.05, \*\*p value < 0.001, \*\*\*p value < 0.001; t-test result compared to Control for % colocalization. #p value < 0.05, ##p value < 0.001, ###p value < 0.001; t-test result compared to Control for % red overlap. \$p value < 0.05, \$p value < 0.001, \$\$\$p value < 0.001; t-test result compared to Control for % green overlap.



**Figure 7**

Confocal imaging of BCG-infected THP1 macrophages upon co-treatment with INH and fractions 2A, 2B, 3A, 3B, 5A, 5B, and Vasicine, compared with Control (untreated macrophages) and INH alone. DAPI stains THP1

nucleus in blue, GFP is expressed by internalized BCG, and LysoTracker Red is used to stain LAMP-1 proteins present on lysosomes. Merged image is used to identify and measure colocalization using ImageJ application. Percent colocalization (R), red overlap (M1), and green overlap (M2) is showed in adjacent graphs. \*p value < 0.05, \*\*p value < 0.001, \*\*\*p value < 0.001; t-test result compared to Control for % colocalization. #p value < 0.05, ##p value < 0.001, ###p value < 0.001; t-test result compared to Control for % red overlap. \$p value < 0.05, \$p value < 0.001, \$\$\$p value < 0.001; t-test result compared to Control for % green overlap.

## Supplementary Files

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