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Research Article



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# Inhibition of Mycobacterial Growth by Plumbagin Derivatives

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**Electron transport and respiratory pathways are active in both latent and rapidly growing mycobacteria and remain conserved in all mycobacterial species. In mycobacteria, menaquinone is the sole electron carrier responsible for electron transport. Menaquinone biosynthesis pathway is found to be essential for the growth of mycobacteria. Structural analogs of the substrate or product of this pathway are found to be inhibitory for the growth of *Mycobacterium smegmatis* and *M. tuberculosis*. Several plumbagin [5-hydroxy-2-methyl-1, 4-naphthaquinone] derivatives have been analyzed for their inhibitory effects of which butyrate plumbagin was found to be most effective on *M. smegmatis* mc$^2$155, whereas crotonate plumbagin showed greater activity on *M. tuberculosis* H37Rv. Effect on electron transport and respiration was demonstrated by butyrate plumbagin inhibiting oxygen consumption in *M. smegmatis*. Structural modifications of these molecules can further be improved upon to generate new molecules against mycobacteria.**

**Key words:** FIC, inhibition, *M. smegmatis*, Menaquinone, MIC, plumbagin

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Inhibition of Gram-positive bacteria by different chemical molecules, which are selective for specific metabolic pathways, is a subject of active investigation since last decade. These studies gain additional importance, keeping in mind the necessity of specific inhibitors toward the growth of *Mycobacterium tuberculosis,* the main causative agent for TB. With the sequencing of *M. tuberculosis* genome (1), a wide arena opened up for scientists working on eradicating this most successful pathogen. Later, most of the genes and essential pathways, showing details of structural and metabolic requirements for mycobacteria, were identified (2). These genes are found to be essential for the normal growth of this organism and provide useful information in the development of more specific antimicrobial inhibitors. Among these, the pathways for electron transport and respiration are involved in both latent and rapidly growing mycobacteria (3). The respiratory system of mycobacteria has both aerobic and anaerobic components (1), enabling them to survive in the unfavorable environment of the host. The recent study on the respiratory chain of mycobacteria as a rich source of antimycobacterial drug target (4–8) is the driving force behind this work.

Quinones shuttle electrons between the membrane-bound protein moieties in the electron transport chain. In mammalian cells, the membrane-soluble quinone is ubiquinone (coenzyme Q), whereas in prokaryotes, in addition to or instead of ubiquinone, bacteria utilize menaquinone (vitamin $K_2$) (9). Menaquinone is the sole quinone in mycobacteria (10–13). In humans, although vitamin $K_2$ plays an important role in the blood-clotting mechanism (14), the main source of it is diet or intestinal bacteria (15). Hence, menaquinone biosynthesis pathway is considered as an attractive target for developing novel antimycobacterial inhibitors (2,16–19) as it is considered as an essential pathway for the viability of mycobacteria (20).

Menaquinone biosynthetic pathway has been extensively studied in *E. coli* (10,21–24). The genome of *M. tuberculosis* contains homologs of most of the *E. coli men* genes (2), whereas some of the genes in ubiquinone pathway are absent. The proposed menaquinone pathway from *E. coli* (25) is shown in Figure 1, where chorismate is converted into menaquinone through a series of reactions catalyzed by several enzymes.

Structural analogs of the substrate or product of intermediate steps in the biosynthetic pathway are well known to function as inhibitors of the selected pathway (26). This approach motivated us to use naphthoquinone derivatives to analyze their effect on mycobacterial growth. In the menaquinone biosynthetic pathway, the 5th intermediate product DHNA (1,4-dihydroxy-2-naphthoate) has a naphthoquinone skeleton. Plumbagin (Figure 2A), which is already reported in the literature (27) as a naturally occurring naphthoquinone, is known to have antimicrobial activity (28–32).

The 1, 4-diketone quinone motif (Figure 3A) has strong inhibitory activity on microbial growth. 1,2-naphthoquinone (Figure 3B) is found to be eightfold less active than 1,4-naphthoquinone (Figure 3C) (31). Study on the antimycobacterial activity of naturally occurring phenolic compounds indicated that compounds without

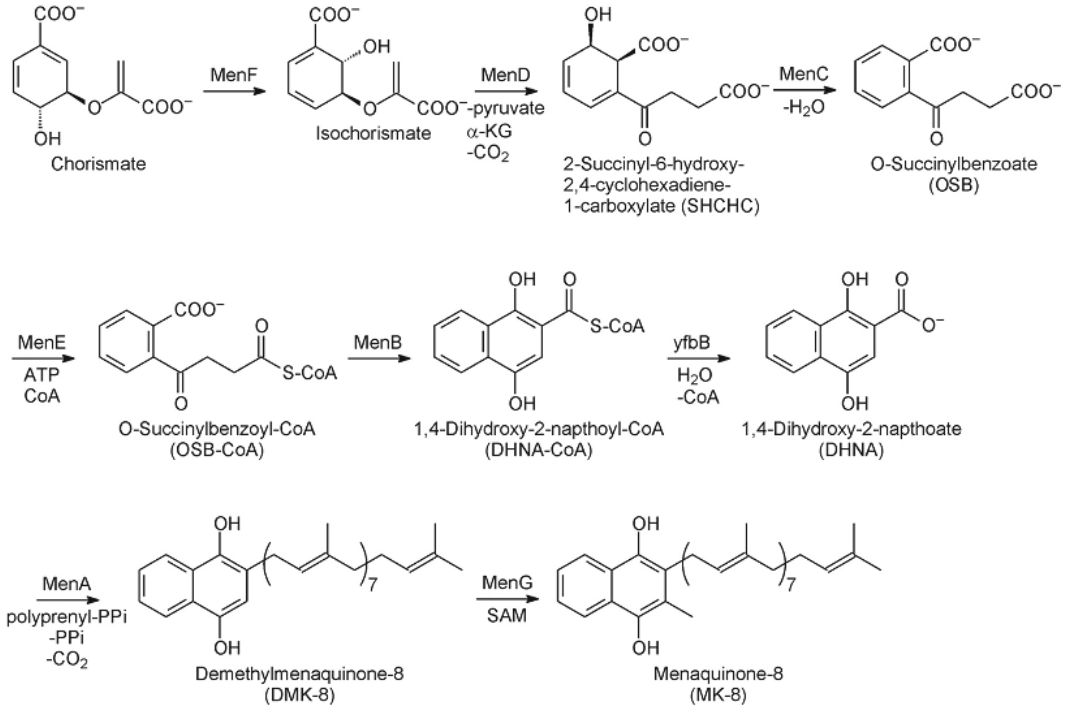



**Figure 1:** Schematic representation of menaquinone biosynthetic pathway in *E. coli,* adapted from the work of Truglio *et al.* (25).

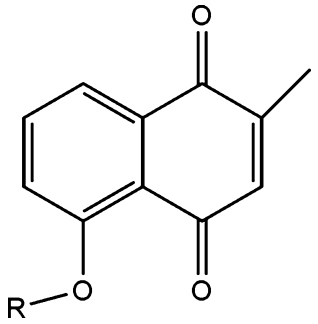


**Figure 2:** Plumbagin and its derivatives (A) plumbagin (HNQ); R=H, (B) acetyl plumbagin (AHNQ); R=CO-$CH_3$, (C) benzoate plumbagin (BHNQ); R=CO-Ph, (D) butyrate plumbagin (ByHNQ); R=CO-$CH_2$-$CH_2$-$CH_3$, (E) cinnamate plumbagin (CHNQ); R=CO-CH=CH-Ph, (F) crotonate plumbagin (CrHNQ); R=CO-CH=CH-$CH_3$, (G) iodobenzoate plumbagin (IHNQ); R=CO-Ph-I, (H) levulinoate plumbagin (LHNQ); R=CO-$CH_2$-$CH_2$-CO-$CH_3$, (I) propionate plumbagin (PHNQ); R=CO-$CH_2$-$CH_3$.

this quinone structure had much lower activity. Benzil (Figure 3D), where the diketone motif itself was retained yet moved off of the aromatic ring, shows a decrease in activity indicating that the ketone groups themselves are not toxic. Tran and coworkers found that adding a second conjugated ring mildly improved activity [in *M. smegmatis* minimal inhibitory concentration (MIC) of benzoquinone (Figure 3E) is 463 $\mu$M, MIC of naphthoquinone (Figure 3C) is 316 $\mu$M], adding a third ring strongly decreased activity (anthraquinone (Figure 3F) MIC is 30 700 $\mu$M). The presence of hydroxyl groups on the second ring had a major effect on potency. Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) (Figure 3G), which has two hydroxyl groups on the second ring (5,8-hydroxy), is found to be twofold more effective than its derivative, which has only one hydroxyl (5-hydroxy) (31).

It should be mentioned here that electron transport inhibitors have not been developed against *M. smegmatis*. As *M. smegmatis* is considered as a model organism for *M. tuberculosis* (33), the focus of this work is to analyze the effect of different plumbagin derivatives on their growth. However, we have also tested the effect of these molecules on the growth of *M. tuberculosis*, and the results show similar trend as that with *M. smegmatis*.

## Materials and Methods

### *Synthesis of plumbagin derivatives*

All starting chemicals, acetyl chloride, propionoyl chloride, plumbagin, cinnamic acid, butyric acid, levulinic acid, crotonoic acid, benzoic acid, and 2-iodobenzoic acid were purchased from Sigma Aldrich, USA/Merck, USA/Spectrochem, India. Reactions were monitored by thin-layer chromatography (Merck, Kieselgel 60 $PF_{254}$ TLC plates); TLC spots were visualized under ultraviolet light.

### *Method A: esterification using dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP)/pyridine (Scheme 1)*

#### General procedure

The solution of R-acid (2.0 eq) in 10 mL of dichloromethane (DCM) was cooled to 0 °C. DCC (77 mg, 1.5 eq) and pyridine (0.168 mL,

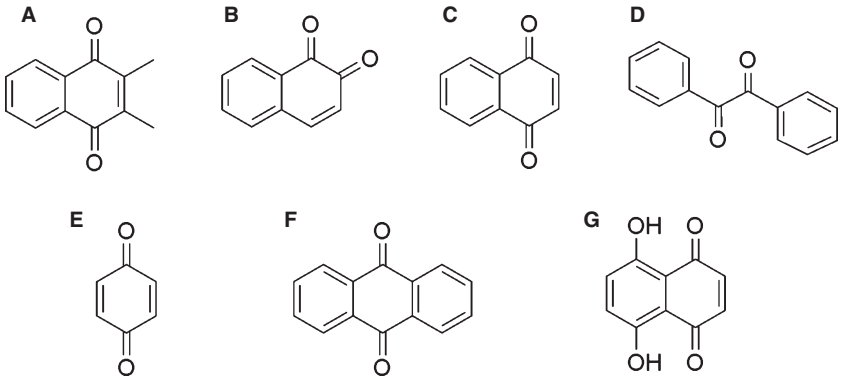
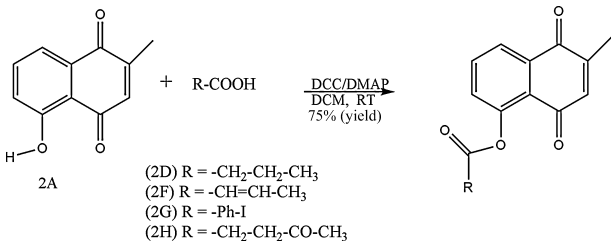



**Figure 3:** (A) 1,4-diketone quinone (B) 1,2-naphthoquinone (C) 1,4-naphthoquinone (D) benzil (E) benzoquinone (F) anthraquinone (G) naphthazarin.



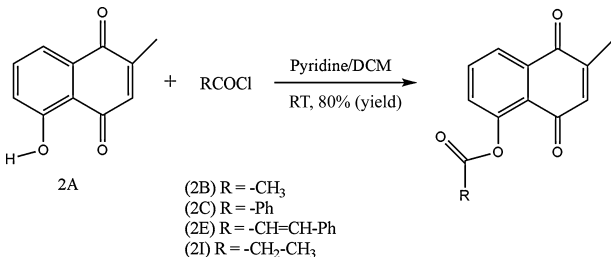

**Scheme 1:** Esterification using dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP)/pyridine (Method A).

2.0 eq) were added to this, followed by plumbagin (Figure 2A) (47 mg, 1.0 eq) and catalytic amount of DMAP (0.1 eq). The reaction mixture was stirred overnight at room temperature and was diluted with DCM and washed with water and brine solution. The dried organic layer on column chromatography yielded the desired product.

### *Method B: esterification using acid chloride/ pyridine (Scheme 2)*

#### General procedure: preparation of acid chloride

Acid (2.0 eq) in 10-mL DCM was cooled to 0 °C, and thionyl chloride (2.2 eq) was added to it. The solution was stirred from room temperature to 50 °C for 3 h. After conversion to acid chloride, reaction mixture was concentrated on rotavapour and dried on high vacuum. To the stirred solution of plumbagin (Figure 2A) (47 mg, 1.0 eq) in 10 mL of DCM, pyridine (0.252 mL, 3.0 eq) was added at




**Scheme 2:** Esterification using acid chloride/pyridine (Method B).

0 °C and stirred for 5 min at the same temperature. Freshly prepared acid chloride (2.0 eq) (described earlier) was added dropwise to this reaction mixture at 0 °C and was stirred for 3 h at room temperature. This was diluted with dichloromethane and washed with water and brine solution. The dried organic layer on column chromatography yielded the desired product.

### *Plumbagin (HNQ) [5-hydroxy 2-methyl 1,4 naphthoquinone] ($C_{11}H_8O_3$) (Figure 2A)*

Plumbagin was purchased from Sigma Aldrich. (400 MHz, $CDCl_3$), $\delta_H$ ppm: 12.0 (1H, OH), 8.09–8.05 (1H, m), 7.74–7.70 (1H, t, J = 8.0 Hz), 7.36–7.34 (1H, d, J = 8.0 Hz), 6.70 (1H, s), 2.16 (3H, s).

### *Acetyl plumbagin (AHNQ) [5-O-acetyl 2-methyl 1,4 naphthoquinone] ($C_{13}H_{10}O_4$) (Figure 2B)*

This was synthesized using Method B. $R_f$ 0.35 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$ 1765, 1735, 1655, 1630, 1590; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.09–8.05 (1H, m), 7.74–7.70 (1H, t, J = 8.0 Hz), 7.36–7.34 (1H, d, J = 8.0 Hz), 6.70 (1H, s), 2.438 (3H, s), 2.16 (3H, s); *m/z* 230 ($M^+$).

### *Benzoate plumbagin (BHNQ) [5-O-benzoyl 2-methyl 1,4 naphthoquinone] ($C_{18}H_{12}O_4$) (Figure 2C)*

This was synthesized using Method B. $R_f$ 0.40 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$ 1737, 1662, 1626, 1594; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.26–8.10 (3H, m), 7.80–7.47 (5H, m), 6.70 (1H, s), 2.16 (3H, s); *m/z* 292 ($M^+$).

### *Butyrate plumbagin (ByHNQ) [5-O-butyroyl 2-methyl 1,4 naphthoquinone] ($C_{15}H_{14}O_4$) (Figure 2D)*

This was synthesized using Method A. $R_f$ 0.370 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$ 1752, 1665, 1627, 1595; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.09-8.05 (1H, m), 7.74-7.70 (1H, t, J = 8.0 Hz), 7.36-7.34 (1H, d, J = 8.0 Hz), 6.70 (1H, s), 2.73–2.69 (2H, t, J = 7.6 Hz), 2.16 (3H, s), 1.89–1.80 (2H, m), 1.1-1.07 (3H, t, J = 7.6 Hz); *m/z* 258 ($M^+$).

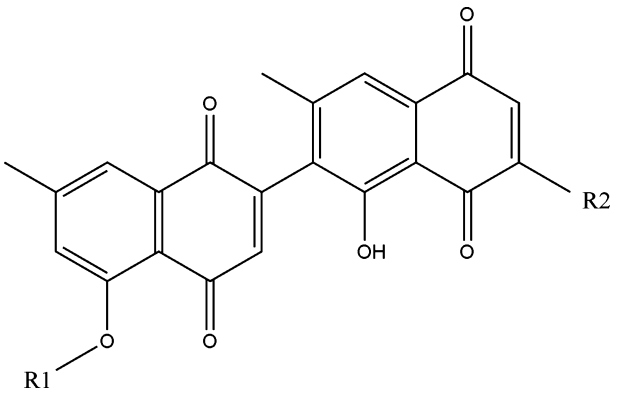



**Figure 4:** Diospyrin, R1 = $R_2$ = H; diospyrin derivatives:-D-1: R1 = $CH_3$, $R_2$ = H; D-2: R1 = $CH_2$-$CH_3$, $R_2$ = H; D-3: R1 = $CH_3$, $R_2$ = -NH-CO-$CH_3$.

### *Cinnamate plumbagin (CHNQ) [5-O-cinnamoyl 2-methyl 1,4 naphthoquinone] ($C_{20}H_{14}O_4$) (Figure 2E)*

This was synthesized using Method A. $R_f$ 0.45 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$ 3080, 2956, 1733, 1694, 1662, 1637; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.10-8.08 (1H, d, J = 6.4 Hz), 7.95-7.91 (1H, d, J = 16.0 Hz), 7.77–7.61 (3H, m) 7.45–7.20 (4H, m), 6.77–6.73 (1H, d, J = 12.4 Hz), 6.71 (1H, s), 2.16 (3H, s); m/z 318 ($M^+$).

### *Crotonate plumbagin (CrHNQ) [5-O-crotonoyl 2-methyl 1,4 naphthoquinone] ($C_{15}H_{12}O_4$) (Figure 2F)*

This was synthesized using Method B. $R_f$ 0.47 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$ 1736, 1726, 1660, 1627; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.09–8.05 (1H, m), 7.74–7.70 (1H, t, J = 8.0 Hz), 7.36–7.34 (1H, d, J = 8.0 Hz), 7.20 (1H, m), 6.70 (1H, s), 6.19–6.15 (1H, dd, J = 12.4 Hz & 1.6 Hz), 2.16 (3H, s), 2.03–2.01 (3H, dd, J = 5.2 Hz & 1.6 Hz); *m/z* 256 ($M^+$).

### *Iodobenzoate plumbagin (IHNQ) [5-O-(2-iodobenzoyl) 2-methyl 1,4 naphthoquinone] ($C_{18}H_{11}IO_4$) (Figure 2G)*

This was synthesized using Method A. $R_f$ 0.47 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$ 1740, 1668, 1629, 1590; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.36–8.34 (1H, dd, J = 6.4 Hz), 8.13-8.07 (2H, m), 7.81–7.77 (1H, t, J = 8.0 Hz), 7.57-7.52 (2H, m), 7.28 (1H, m) 6.72 (1H, s), 2.18 (3H, s); *m/z* 418 ($M^+$).

### *Levulinoate plumbagin (LHNQ) [5-O-levuloyl 2-methyl 1,4 naphthoquinone] ($C_{16}H_{14}O_5$) (Figure 2H)*

This was synthesized using Method A. $R_f$ 0.33 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$ 1758, 1717, 1668, 1657, 1627, 1594, 1577, 1536; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.07-8.05 (1H, m), 7.74–7.70 (1H, t, J = 8.0 Hz), 7.4–7.37 (1H, d, J = 8.0 Hz), 6.70 (1H, s), 3.03–2.932 (4H, m), 2.23 (3H, s) 2.16 (3H, s); *m/z* 286 ($M^+$).

### *Propionate plumbagin (PHNQ) [5-O-propionoyl 2-methyl 1,4 naphthoquinone] ($C_{14}H_{12}O_4$) (Figure 2I)*

This was synthesized using Method B. $R_f$ 0.33 ($SiO_2$, hexane: EtOAc, 100:20); υmax (KBr) $cm^{-1}$1765, 1739, 1659, 1630, 1592; (400 MHz, $CDCl_3$), $\delta_H$ ppm: 8.09–8.05(1H, m), 7.74–7.70 (1H, t, J = 8.0 Hz), 7.36-7.34 (1H, d, J = 8.0 Hz), 6.70 (1H, s), 2.79-2.741 (2H, q, J = 7.6 Hz), 2.16 (3H, s), 1.34–1.30 (3H, t, J = 7.6 Hz); *m/z* 244 ($M^+$).

### *Mycobacterial growth inhibitory assay*

The wild-type strain of *Mycobacterium smegmatis* $mc^2155$ was grown in Middlebrook 7H9 (MB7H9; Difco, USA) medium supplemented with 0.05% Tween 80 and glucose. Both 0.02% glucose (starvation) and 2% glucose (high concentration) concentrations were studied in this assay (34). The mycobacterial cultures were grown, and along with it, the synthetic derivative molecules of plumbagin were added into the medium (35). Different concentrations of these derivative molecules were dissolved in a final concentration of 1% dimethylsulfoxide (DMSO). Cultures without inhibitor molecules and DMSO were considered as positive control. Also, cultures were grown in the presence of DMSO alone to examine whether there is any inhibitory effect of DMSO. Optical densities were measured at 600 nm for more than 100 h, and growth rates were determined. Each experiment was performed in triplicates.

### *Determination of minimal inhibitory concentration (MIC)*

Minimal inhibitory concentration is the lowest concentration of an antimicrobial that will inhibit the visible growth of an organism. In this study, MIC for each plumbagin derivative was determined from the respective growth curves of *M. smegmatis* $mc^2155$.

*Mycobacterium tuberculosis* H37Rv were grown in MB7H9 medium supplemented with 0.2% glycerol, 0.25% Tween 80% and 10% albumin dextrose catalase. Minimal inhibitory concentrations in *M. tuberculosis* H37Rv were determined by resazurin microplate assay. Using a multichannel pipette, 200 μL of *M. tuberculosis* cultures was added into the wells along with different concentrations of plumbagin derivatives. Some wells served as inhibitor-free media or inoculum controls. The plates were packed in gas-permeable polythene bags and were incubated at 37 °C for 5 days. Thereafter, 40 μL of freshly prepared resazurin (20 mg/100 mL) reagent and 10% Tween 80 were added. The wells were observed after 24 and 48 h for a color change from blue to pink. A blue color in the well was interpreted as no growth, and pink color was scored as growth (36).

### *Biofilm inhibition assay*

Biofilms were grown as described by (37), and their quantification was performed according to O'Toole and coworkers (38). *M. smegmatis* cultures were grown in 96-well polystyrene plates in Sauton's medium, and at each time-point, samples (cultures with or without derivative molecules) were removed from the wells and incubated with 1% crystal violet for 45 min. Later the dye was

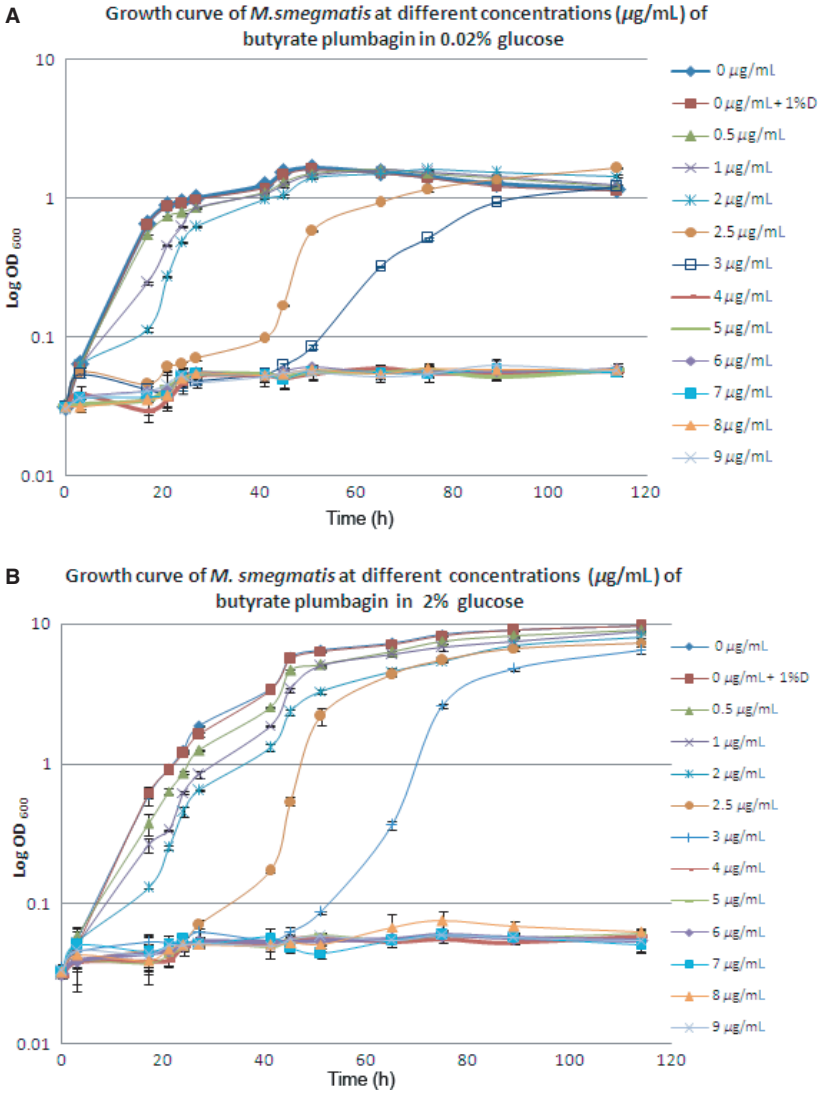



**Figure 5:** (A) Growth curve of *M. smegmatis* in 0 μg/ml, 0 μg/ml + 1% D (DMSO), 0.5, 1, 2, 2.5, 3, 4, 5, 6,7, 8 and 9 μg/mL of butyrate plumbagin (BHNQ) in 0.02% glucose. (B) Growth curve of *M. smegmatis* in 0 μg/mL, 0 μg/mL + 1% D (DMSO), 0.5, 1, 2, 2.5, 3, 4, 5, 6, 7, 8 and 9 μg/mL of butyrate plumbagin (BHNQ) in 2% glucose.

washed off with water, and 80% ethanol was added. Optical densities were measured at 590 nm using a microplate reader (39).

### *Fractional inhibitory concentration (FIC) assay*

FIC is the coefficient indicating whether the combined inhibitory effects of molecules are synergistic, additive, indifferent or antagonistic (40). This assay was carried out along with rifampicin, which is the most potent antitubercular drug in the market (41). Both plumbagin and butyrate plumbagin were incorporated separately along with rifampicin to the *M. smegmatis* cultures as mentioned earlier, and inhibitory concentrations were determined.

### *Oxygen-consumption assay*

*M. smegmatis* were grown as mentioned previously in the presence and absence of butyrate plumbagin. Cultures without butyrate plumbagin were considered as positive control. After incubation for more than 100 h, 0.01% methylene blue was added and was further incubated at 37 °C for 12 h. Rate of oxygen consumption was determined by decolorization of methylene blue as described by Boshoff *et al.* (42).

## Results and Discussions

### *Design and synthesis*

Plumbagin and its derivatives (Figure 2A–I) were chosen for our work because a naphthoquinone moiety is the substrate/product of the last three steps in the biosynthesis of menaquinone (Figure 1). Also, plumbagin, which is a naphthoquinone, has a basic structural scaffold similar to 1, 4-dihydroxy-2-naphthoate (DHNA) of this pathway.

In our attempt to understand the effect of derivatization of the naphthoquinone moiety on the bactericidal action, we started by

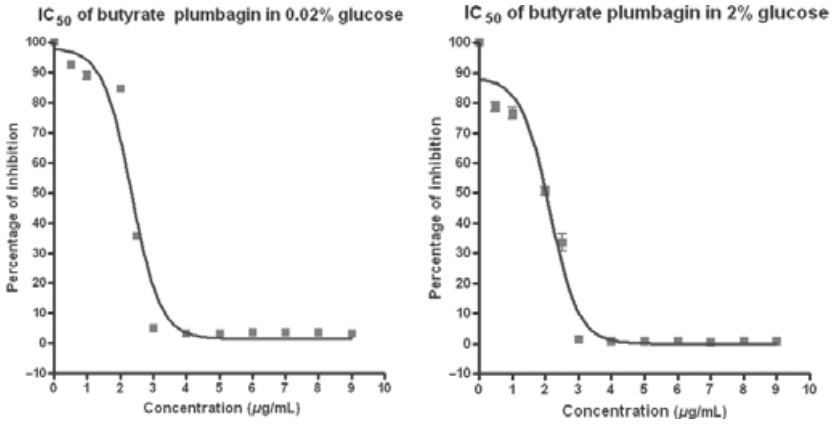



**Figure 6:** $IC_{50}$ of butyrate plumbagin in 0.02% and 2% glucose.

first derivatizing the phenolic group, because modification of phenolic functionality was found to affect the activity. For example, diospyrin (Figure 4), a known dinaphthoquinone isolated from *Euclea natalensis*, exhibits mild antitubercular activity with an MIC of 100 mg/L. Its semi-synthetic ether derivatives (D-1 & D-2) (Figure 4) were found to be less active than the methylether aminoacetate derivative D-3 (Figure 4) (MIC of 10–50 mg/L) against both wild-type *M. tuberculosis* H37Rv and a range of drug-resistant strains (43).

### *Effect of plumbagin derivatives on mycobacterial growth*

Figures 5A and B show a representative growth curve for the inhibition of *M. smegmatis* in 0.02% and 2% glucose-containing media at different concentrations of butyrate plumbagin. Growth of *M. smegmatis* in different butyrate plumbagin concentrations showed the maximum inhibition among all the derivatives synthesized. Although the nature of inhibition appears to be the same in both the glucose concentrations, certain interesting features, nonetheless, became apparent like cells showed no growth till 40 h at medium inhibitor concentrations, whereas cells grew well in lower concentrations as though there were no inhibitors at all. However, cell density increases at further time-points for the medium inhibitor concentrations and reaches saturation, similar to the positive control. This raises a possibility of bacterial adaptation to the inhibitors at medium inhibitor concentrations. However, no growth of *M. smegmatis* was observed when the concentration of the inhibitor was further increased to lethal levels.

From Figure 5A,B, one can derive the $IC_{50}$ value, which is the amount of inhibitor required for 50% inhibition on the growth of an organism and is depicted in Figure 6. Among the eight different derivatives of plumbagin (Figure 2B–I), only five of them had inhibitory effect on *M. smegmatis* $mc^2155$ (Table 1).

Interestingly, the growth curves showed adaptation of *M. smegmatis* to the inhibitors present in the media, so the growth was monitored for more than 100 h. It should be mentioned here that the first-line TB drug, rifampicin shows a monotonous inhibitory growth pattern (data not shown), while all the plumbagin derivatives showed adaptive response, although the time required to show this

**Table 1:** $IC_{50}$ data for plumbagin and its derivatives

| Sl. No. | Chemical molecules | 0.02% Glucose $IC_{50}$ ($\mu$M) | Standard deviation | 2% Glucose $IC_{50}$ ($\mu$M) | Standard deviation |
|---|---|---|---|---|---|
| 1. | Plumbagin | 9.13 | 0.074 | 9.19 | 0.007 |
| 2. | Acetyl Plumbagin | 19.39 | 0.083 | 18.53 | 0.052 |
| 3. | Benzoate Plumbagin | 14.44 | 0.041 | 10.54 | 0.015 |
| 4. | Butyrate Plumbagin | 9.06 | 0.003 | 8.26 | 0.064 |
| 5. | Cinnamate Plumbagin | – | – | – | – |
| 6. | Crotonate Plumbagin | 18.00 | 0.016 | 17.61 | 0.013 |
| 7. | Iodobenzoate Plumbagin | – | – | – | – |
| 8. | Levulinoate Plumbagin | – | – | – | – |
| 9. | Propionate Plumbagin | 13.58 | 0.009 | 13.79 | 0.017 |

– stands for no inhibition.

behavior and the concentration of the inhibitors were different in each case. Chromatographic analysis of the *M. smegmatis* culture filtrate, which showed inhibition even after 100 h, did not show any traces of plumbagin, indicating that the ester derivatives of plumbagin (Figure 2B–I) did not hydrolyze under the experimental conditions.

Unlike the growth curve observed in rifampicin, there was no significant difference between starvation (0.02%) and high (2%) glucose concentrations in these derivatives. Table 2 shows the MIC values of the different plumbagin derivatives for both *M. smegmatis* and *M. tuberculosis*. When the *M. smegmatis* cultures with these MIC values were further subjected to 2% glucose with 0.05% Tween 80, restoration of growth occurs. However, such was not the case when cultures were taken from the media with higher concentration (concentration above the MIC value: plumbagin – 15 $\mu$M, acetyl plumbagin – 35 $\mu$M, benzoate plumbagin – 24 $\mu$M, butyrate plumbagin – 19 $\mu$M, crotonate plumbagin – 23 $\mu$M, propionate plumbagin – 20 $\mu$M) of inhibitor. No growth was observed beyond this concentration even when grown for more than 200 h.

We observed that there were no inhibitory effects of these derivatives on biofilm formation by *M. smegmatis* (data not shown). Hence, these molecules appear to have effect only on planktonic cultures.

**Table 2:** Minimal inhibitory concentration ($\mu$M) data for plumbagin and its derivatives

| Compound | *M. smegmatis* mc$^2$155 0.02% Glucose | *M. smegmatis* mc$^2$155 2% Glucose | *M. tuberculosis* H37Rv |
|---|---|---|---|
| Plumbagin | 13.3 | 13.3 | 21.3 |
| Acetyl Plumbagin | 28.2 | 30.4 | 34.8 |
| Benzoate Plumbagin | 20.5 | 20.5 | 27.4 |
| Butyrate Plumbagin | 15.5 | 15.5 | 77.4 |
| Cinnamate Plumbagin | – | – | NT |
| Crotonate Plumbagin | 19.5 | 19.5 | 15.6 |
| Iodobenzoate Plumbagin | – | – | NT |
| Levulinoate Plumbagin | – | – | NT |
| Propionate Plumbagin | 16.4 | 16.4 | 16.4 |

– stands for no inhibition.
NT, not tested.

Fractional inhibitory concentration is the coefficient indicating whether the combined inhibitory effects of molecules are synergistic, additive, indifferent or antagonistic. FIC = A+B; where A = (MIC of combination X + Y)/(MIC of drug X alone) and B = (MIC of combination X + Y)/(MIC of drug Y alone). FIC is interpreted as synergistic when FIC $\leq$ 0.5; additive when FIC > 0.5–1.0; indifferent when FIC > 1.0 - $\leq$ 4.0 and antagonistic when FIC $\geq$ 4. When experimentally calculated, the FIC of rifampicin with plumbagin or butyrate plumbagin was indifferent in both starvation (0.02%) and high (2%) glucose concentrations for *M. smegmatis*.

Oxygen-consumption assay showed decolorization of methylene blue in the control and also in the concentrations below the MIC of butyrate plumbagin (Figure 7). These results demonstrated that treatment with butyrate plumbagin inhibited oxygen consumption in *M. smegmatis*. This may imply that there is effect of this inhibitor molecule on electron transport and respiration as decolorization of methylene blue which is a well-known redox dye has been reported to unambiguously demonstrate effects on respiration (42).

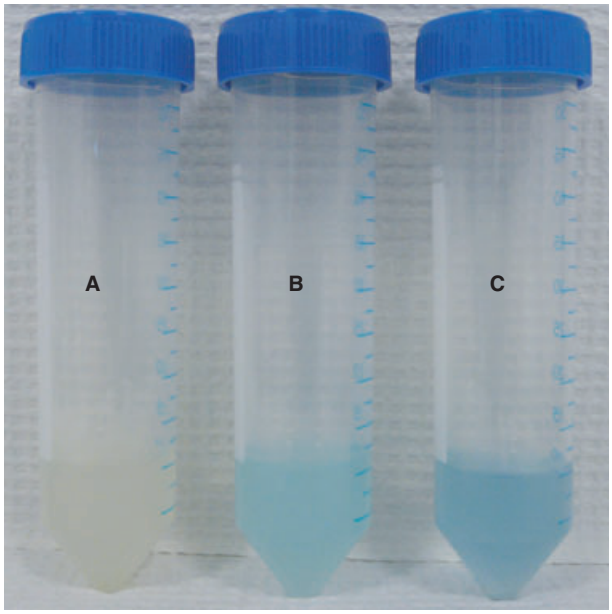



**Figure 7:** Oxygen consumption assay in *M. smegmatis* using butyrate plumbagin (A) control showing complete decolorization of methylene blue (B) culture with 3 $\mu$g/mL of butyrate plumbagin (C) culture with 6 $\mu$g/mL of butyrate plumbagin.

These studies were extensively carried out in *M. smegmatis*, as it allowed us to manipulate different growth conditions because of its faster growth rate and non-pathogenicity. These results were informative while testing these inhibitors on the pathogenic counterpart *M. tuberculosis*, as they share the major metabolic pathways and have related homologs with the virulence genes (33,44,45).

## Conclusion

To design an effective derivative of naphthaquinone moiety for antimycobacterial effect, we started by first derivatizing the phenolic group of plumbagin. Among the different derivatives of plumbagin, five of them had inhibitory effect on *M. smegmatis* mc$^2$155 of which butyrate plumbagin was the most effective one, whereas on *M. tuberculosis*, H37Rv crotonate plumbagin showed greater activity. Moreover, butyrate plumbagin demonstrated effect on electron transport and respiration by inhibiting oxygen consumption in *M. smegmatis*. Future work will concentrate on the diversity of oxygen containing functional groups on the aromatic ring and also derivatizing positions C-2 and C-3 of the quinonoid moiety. Structural modifications of these molecules can further be improved to generate better molecules against both rapidly multiplying and latent mycobacteria.

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