Full Paper

Thienopyrimidines as Novel Inhibitors of *Mycobacterium tuberclosis*: Synthesis and *In-vitro* Studies

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A series of novel 5,6-unsubstituted thieno-[2,3-*d*]-pyrimidines has been synthesized and tested for growth inhibition of *Mycobacterium tuberculosis* H37Rv. Of twelve compounds synthesized eleven have shown antimycobacterial activity that differs in potency. Compounds **7b**, **7c**, **7d**, **7e**, **7f**, and **7g** exhibited good antimycobacterial activity. MIC values of the compounds tested were comparable with pyrazinamide. Six compounds which have shown good antimycobacterial activity were also subjected for cytotoxicity studies and were found to possess poor cytotoxicity. The study indicates the definite need for focusing attention on thienopyrimidines for further lead optimization.

Keywords: Antimycobacterials / *Mycobacterium tuberculosis* / Thienopyrimidines

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Introduction

The World Health Organization has warned that the incidence of drug resistant tuberculosis has reached a record level, becoming a serious threat to global health [1]. HIV and other immune suppressing conditions have dramatically increased the risk of developing active tuberculosis and HIV co-infection makes tuberculosis more difficult to diagnose and treat. Multi-drug resistant strains of tuberculosis (TB) could become dominant forms of the disease in the next few decades, adding heavy financial and medical burdens to already struggling health systems.

In a series of studies into TB, scientists said ''superbug'' strains of the disease were have been gaining ground in some countries and called for greater investment into research and development of new drugs and possible vaccines. Scientists pointed out that the combined impact of new drugs, vaccines, and diagnostic tests could cut worldwide incidence of TB by 94 percent by 2050, but the investment needed to bring this about is falling way too short of the requirement.

This has created the need for developing simpler, economically viable molecules for tuberculosis. Priority for the discovery of new anti-tuberculosis drugs interest has led to a keen focus on a family of proteins which are involved in modifications of mycolic acids, very long chain lipids and these proteins have been demonstrated to be important in the persistent phase of infection, many of which contribute to its pathogenicity. Several studies indicate that functional groups in the acyl chain of mycolic acids are important for pathogenesis and persistence [2].

Some reports have already indicated the usefulness of thienopyrimidine as antitubercular drug [1, 2]. This prompted us to continue our studies with the intention of designing and synthesizing thienopyrimidines **7**(**a–l**) and evaluating them for their antitubercular activity.

Results and discussion

Compound **3** was formed (Scheme 1) by the condensation of ethylcyanoactate with 2,5-dihydroxy-1,4-dithiane in presence of triethylamine. Compound **4** was prepared through condensation reaction between formamide and compound (**3**) followed by cyclization. IR and NMR spectra confirmed the formation of compound **4**. Compound **4** as it exists in two tautomeric forms IR spectra showed the presence of ketone at 1660 cm^{-1} and NMR spectra showed a broad peak which was not prominent due to tautomerism. Absence of doublet due to NH_2 in IR spectra, absence of quartet and triplet due to $-CH_2CH_3$ at 2-4 ppm in NMR spectra confirms the cyclization. The 4-hydroxythieno[2,3-*d*] pyrimidine **4** treated with potassium carbonate in dry acetone to form potassium salt to make the compound to exist in lactim form (lactam–lactim

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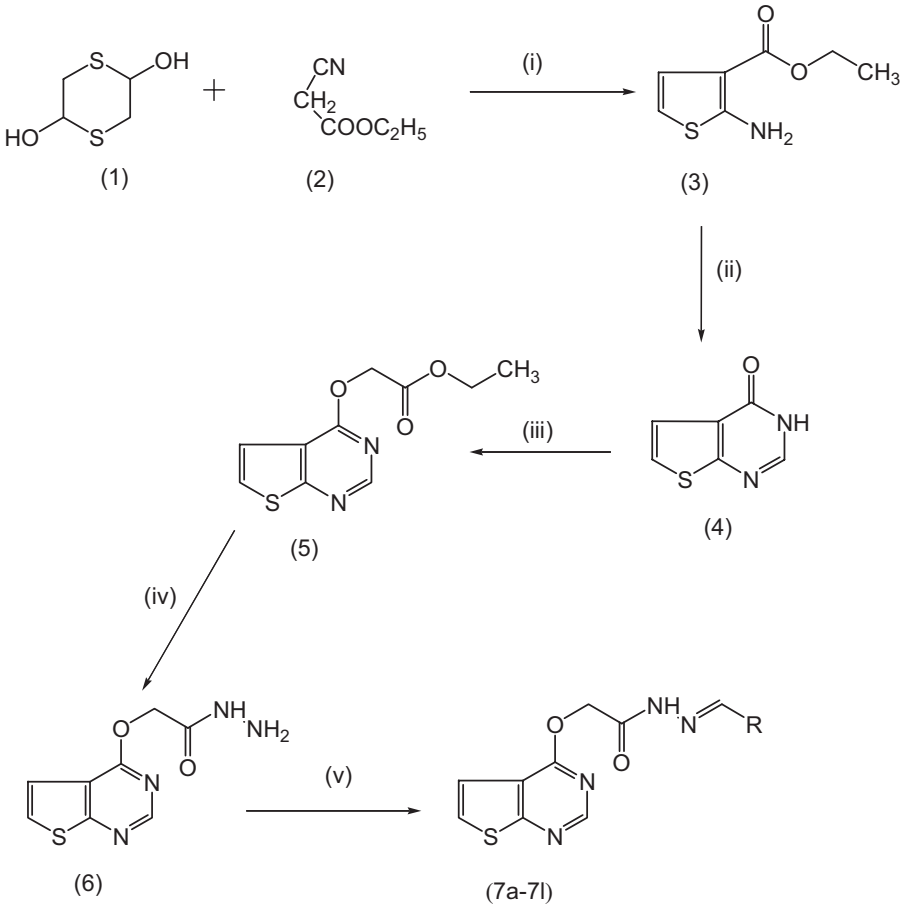


Scheme 1. (i) DMF, triethylamine, (ii) $HCONH_2$, (iii) $ClCH_2COOC_2H_5$, CH_3ONa, dry acetone, (iv) $NH_2NH_2 \cdot H_2O$, ethanol, (v) R-CHO, glacial acetic acid, ethanol.

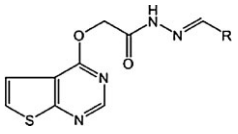
tautomerism) which was then allowed to react with ethylchloroacetate to form **5**. Disappearance of –NH peak in IR spectra and appearance of quartet at 4.16–4.21 ppm and triplet at 1.21–1.25 confirms the formation of the compound.

Peak at 3165.29 cm^{-1} due to –NH and doublet at 3290.67 cm^{-1} and 3178.79 cm^{-1} due to $-NH_2$ in IR spectra as well as singlet at 4.2 ppm due to $-NH_2$ and singlet at 9.1 ppm due to NH in NMR confirms the formation of compound (**6**) obtained by treating compound **5** with hydrazine hydrate confirms the structure. Compound **6** condensed with different aromatic aldehydes in alcohol in presence of glacial acetic acid to form Schiff bases **7(a–l)**. Disappearance of doublet peak ($-NH_2$) in IR spectra and singlet at 4.2 ppm due to $-NH_2$, appearance of =CH peak at 7.7 ppm in NMR proved the structure of these compounds.

Table 1 shows the physicochemical properties and MIC values against *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and Table 2 shows spectral data.

The compounds (**7a-l**) were evaluated against *M. tuberculosis* H37Rv (ATCC 27294) using Middlebrook 7H9 broth. The ability of compounds to inhibit the growth of *Mycobacterium* species was determined by Ziehl-Neelsen staining. Pyrazinamide was used as standard. Eleven of twelve synthesized compounds showed antimycobacterial activity differing in potency. Compounds **7b**, **7c**, **7d**, **7e**, **7f**, and **7g** exhibited good antimycobacterial activity. Compounds with deactivating groups at *o*- and *p*-positions (**7b**, **7c**, **7d**, and **7e**) were found to be more potent antimycobacterials. Compounds **7j** and **7l** with –OH (activating) group at *p*- and *m*-position exhibited less potent antimycobacterial activity compared to compounds with deactivating groups. The compounds **7f** and **7g** showed good antimycobacterial activity even though they have activating groups. The compound **7h** exhibited less potent antimycobacterial activity, in spite of having two $-OCH_3$ groups. **7i** having –OH and $-OCH_3$ groups has shown weak antimycobacterial activity. Compound **7k** has not exhibited antimycobacterial activity.

Table 1. Physical constants and *in vitro* antimycobacterial activities of compounds **3**, **4**, **5**, **6**, **7(a-l)**.



Sl. No	R	% Yield	Melting point, °C	*M. tuberculosis* MIC[a], μM
7a	C_6H_5	43.69	288	320
7b	C_6H_4-4-$N(CH_3)_2$	94.57	272	70
7c	C_6H_4-6-Br	54.56	154	64
7d	C_6H_4-6-NO_2	74.23	240	70
7e	C_6H_4-4-Cl	80.12	285	71
7f	C_6H_4-4-OCH_3	78.1	225	66
7g	C_6H_4-3,4,5- OCH_3	86.24	264	62
7h	C_6H_4-3,4- OCH_3	89.13	279	266
7i	C_6H_4-3- OCH_3-4-OH	89.44	281	293
7j	C_6H_4-4-OH	84.25	317	137
7k	C_6H_4-5-OH	81.56	305	–
7l	C_6H_4-3-OH	58.1	236	274
	Pyrazinamide	–	–	60.97

[a] MIC: minimum inhibitory concentration.

The six compounds **7b**, **7c**, **7d**, **7e**, **7f**, and **7g** were tested for cytotoxicity (IC_{50}) using THP-1 (human, monocytes) cell line. All six exhibited poor cytotoxicity to THP-1 cells. Table 3 shows the cytotoxicity to THP-1 cells and selective index (SI).

Conclusion

Though the MIC values obtained for H37Rv are large, the compounds' cytotoxicity concentration is thirty times more than the MIC. This study proves that the class of thienopyrimidines can potentially serve as lead structure for further optimization. In depth studies are underway and we hope to develop therapeutic agents for TB which are of low cost and were useful in developing countries.

Experimental

General

The melting points were determined and are uncorrected. Infrared spectra (KBr disc) were performed on FTIR-8300 Shimadzu and the frequencies were expressed in cm^{-1}. ^{1}H-NMR spectra were recorded on Bruker-Avance 400 MHz instrument with TMS (0 ppm) as an internal standard; the chemical shifts (δ) are reported in ppm and coupling constants (J) are given in Hertz (Hz). Signal multiplicities are represented by s (singlet), d (doublet), t (triplet), m (multiplet), and br s (broad singlet). Completion of the reaction and the purity of the compounds were checked on Merck precoated silica gel 60 F-254. Yields were not optimized. All the solvents and reagents were used without further purification.

Synthesis

*Synthesis of ethyl 2-aminothiophene-3-carboxylate (**3**) [3, 4]*

Triethylamine (50 m mol) was added dropwise over 10 min to a mixture of 2,5-dihydroxy-1,4-dithiane (50 m mol), ethylcyanoacetate (100 m mol), and dimethylformamide(40 mL). The mixture was stirred for at 45°C for 30 min, diluted with 0.4 M acetic acid, extracted with ether. The ethereal layer was dried over sodium sulphate. The solvent removed and residue cooled to get the product.

Yield 66.5%, mp 44°C, white crystals.

*Synthesis of 4-hydroxythieno[2,3-d]pyrimidine (**4**) [5]*

A mixture of ethyl-2-aminothiophene-3-carboxylate **3** (0.05 mol) and formamide (20 mL) was refluxed for 8 h and allowed to cool overnight and added water. The crystals filtered dried and recrystallized with hot alcohol.

Yield 76.6%, mp 260°C, light brownish yellow colored crystals.

*Procedure for the synthesis of 4-(ethylacetoxy)thieno [2,3-d]pyrimidine (**5**) [6–10]*

A mixture of 4-hydroxy thieno[2,3-*d*]pyrimidine **4** (0.05 mol) and anhydrous K_2CO_3 (0.1 mol) in excess of dry acetone (100 mL) was stirred at reflux temperature for 4 h. To the stirred suspension, a mixture of ethylchloroacetate (0.05 mol) in dry acetone was added in dropwise over a period of 30 min at reflux temperature, and the reflux continued for further 6 h. After keeping the reaction mixture overnight, the excess of solvent was removed to get the solid. The solid was recrystallized from acetone.

Yield 74.64%, mp 140°C, buff color crystalline solid.

Table 2. Spectral data of compounds **3**, **4**, **5**, **6**, **7(a-l)**.

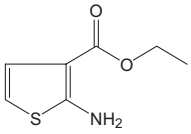
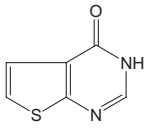
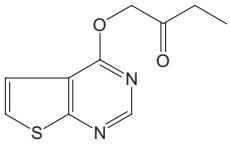
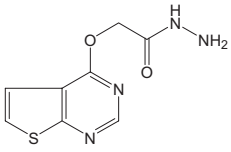
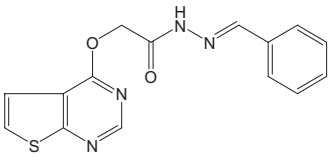
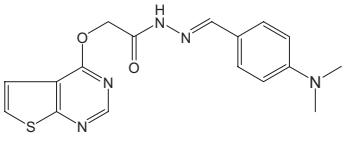
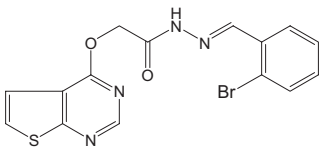
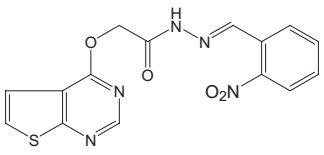
Sl. No.	Structure	IR ν_{max}, cm^{-1}, KBr	NMR ($CDCl_3$) δ ppm
3		3412.19, 3304.17(NH_2), 1654.98 (C=O), 1521.98, 1490 (C=C)	7.06–7.08 (d, H, CH (H1)), 7.25–7.26 (d, H, CH (H2)), 7.32 (s, 2H, NH_2), 4.42–4.48 (q, 2H, CH_2), 1.33–1.36 (t, 3H, CH_3)
4		3285 (N–H), 1660 (C=O), 1590, 1520 (C=C)	7.26–7.27 (d, H, CH (H1)), 7.44–7.46 (d, H, CH (H2)), 7.99 (s, H, CH (H3)), 5–6 (brs, H, OH)
5		1746 (C=O), 3081.98–3097.42 (Ar–CH), 1568, 1579.51, 1551.49 (C=C), 1667.29 (C=N)	7.21–7.22 (d, H, SCH=), 7.41–7.42 (d, H, =CH–), 7.90 (s, H, NCHN), 4.66 (s, 2H, OCH_2), 4.16–4.21 (q, 2H, CH_2), 1.21–1.25 (t, 3H, CH_3)
6		3290.67, 3178.79 (NH_2), 3165.29 (NH), 1685.8 (C=O), 3047.63–3109.35 (Ar–CH), 1566, 1535.39, (C=C), 1660.77 (C=N)	7.30–7.31 (d, H SCH=), 7.36–7.37 (d, H =CH–), 8.17 (s, H, NCHN), 4.63 (s, 2H, OCH_2), 7.7 (s, H, NH), 2.5 (s, 2H, NH_2)
7a		3223.16 (NH), 1697.41 (C=O), 1651. 12 (C=N)	7.63–7.68 (d, H, SCH=, J = 5.8), 7.40–7.42 (d, H, =CH–, J = 5.8), 8.41 (s, H, NCHN), 5.23 (s, 2H, OCH_2), 8.05 (s, H, CONH), 7.75 (s, H, N=CH) 7.4–7.73 (m, 5H, ArH)
7b		3238.59 (NH), 1678.13 (C=O), 1612.54 (C=N)	7.60–7.62 (d, H, SCH=, J = 5.78), 7.4–7.43 (d, H, =CH–, J = 5.78), 8.39 (s, H, NCHN), 5.18 (s, 2H, OCH_2), 7.9 (s, H, CONH), 7.90 (s, H, NNCH), 6.73 (d, 2H, ArH, J = 7.89), 7.52 (d, 2H, ArH, J = 7.89), 2.49 (s, 6H, N–CH_3)
7c		3238.59 (NH), 1678.13 (C=O), 1612.54 (C=N), 582.52 (C–Br)	7.60–7.61 (d, H, SCH=, J = 5.8), 7.39–7.40 (d, H, =CH–, J = 5.8), 8.35 (s, H, NCHN), 5.06 (s, 2H, OCH_2), 10.58 (s, H, CONH), 7.7 (s, H, N=CH), 7.42–7.70 (m, 4H, ArH)
7d		3228.39 (NH), 1678.13 (C=O), 1645.24 (C=N)	7.66–7.68 (d, H, SCH=, J = 5.8), 7.41–7.42 (d, H, =CH–, J = 5.8), 8.44 (s, H, NCHN), 5.2 (s, 2H, OCH_2), 8.44 (s, H, CONH), 8.06 (s, H, N=CH), 7.69–8.13 (m, 4H, ArH)

Table 2. (*continued*)

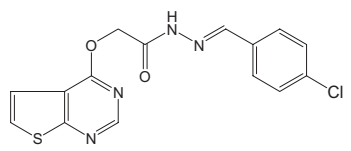
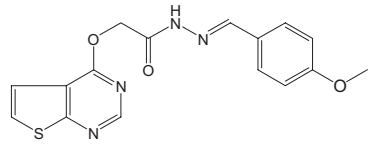
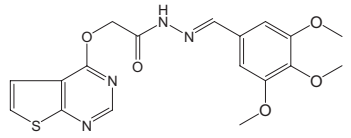
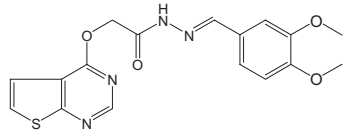
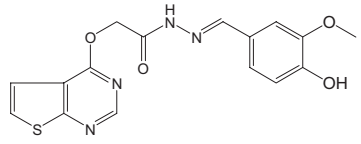
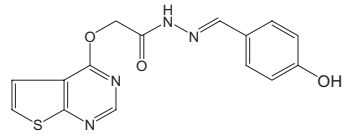
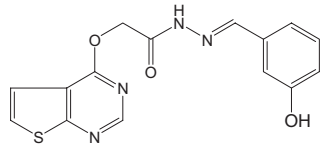
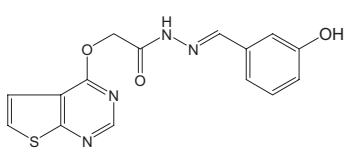
Sl. No.	Structure	IR ν_{max}, cm^{-1}, KBr	NMR ($CDCl_3$) δ ppm
7e		3172.1 (NH), 1732.11 (C=O), 1639.47 (C=N)	7.62–7.63 (d, H, SCH=, J = 5.8), 7.4–7.48 (d, H, =CH–, J = 5.8), 8.4 (s, H, NCHN), 5.23 (s, 2H, OCH_2), 8.04 (s, H, CONH), 11.85 (s, H, NNCH), 7.50–7.52 (d, 2H, ArH, J = 8.55), 7.75–7.77 (d, 2H, ArH, J = 8.55)
7f		3259.09 (NH), 1671.83 (C=O), 1656.65 (C=N)	7.63–7.65 (d, H, SCH=, J = 5.8), 7.4–7.42 (d, H, =CH–, J = 5.8), 8.4 (s, H, NCHN), 5.2 (s, 2H, OCH_2), 8.14 (s, H, CONH), 7.96 (s, H, NNCH), 6.99–7.02 (d, H, ArH, J = 8.84), 7.65–7.68 (m, 2H, ArH), 3.79 (s, 3H, OCH_3)
7g		3189.09 (NH), 1670.33 (C=O), 1583.65 (C=N)	7.61–7.63 (d, H, SCH=, J = 5.79), 7.41–7.42 (d, H, =CH–, J = 5.79), 8.42 (s, H, NCHN), 5.24 (s, 2H, OCH_2), 9.6 (s, H, CONH), 7.96 (s, H, NNCH), 7.04 (s, 2H, ArH), 3.8 (s, 6H, OCH_3), 3.6 (s, 3H, OCH_3)
7h		3186.51 (NH), 1674.27 (C=O), 1597.11 (C=N)	7.61–7.63 (d, H, SCH=, J = 5.8), 7.40–7.42 (d, H, =CH–, J = 5.8), 8.41 (s, H, NCHN), 5.23 (s, 2H, OCH_2), 9.70 (s, H, CONH), 7.97 (s, H, NNCH), 7.22–7.34 (d, H, ArH, J = 8.6), 7.00–7.22 (m, 2H, ArH), 3.79 (d, 6H, OCH_3)
7i		3292.60 (OH), 3198.08 (NH), 1687.77 (C=O), 1666.55 (C=N)	7.61–7.62 (d, H, SCH=, J = 5.8), 7.4–7.42 (d, H, =CH–, J = 5.8), 8.41 (s, H, NCHN), 5.25 (s, 2H, OCH_2), 9.4 (s, H, CONH), 7.09 (s, H, NNCH), 6.81–6.83 (d, H, ArH, J = 8.13), 7.098–7.119 (m, 2H, ArH), 3.82 (s, 3H, OCH_3), 9.4 (s, H, OH)
7j		3209.66 (OH), 3146 (NH), 1701.27 (C=O), 1648.48 (C=N)	7.61–7.62 (d, H, SCH=, J = 5.8), 7.4–7.41 (d, H, =CH–, J = 5.8), 8.4 (s, H, NCHN), 5.18 (s, 2H, OCH_2), 7.94 (s, H, CONH), 7.99 (s, H, NNCH), 6.81–6.83 (d, 2H, ArH, J = 8.64), 7.54–7.56 (d, 2H, ArH, J = 8.64), 9.89 (s, H, OH)
7k		3248.69 (OH), 3159 (NH), 1711.17 (C=O), 1668.48 (C=N)	7.61–7.62 (d, H, SCH=, J = 5.8), 7.4–7.41 (d, H, =CH–, J = 5.8), 8.4 (s, H, NCHN), 5.20 (s, 2H, OCH_2), 8.41 (s, H, CONH), 7.21 (s, H, NNCH), 6.84–6.9 (m, 3H, ArH), 7.73–7.75 (d, 2H, ArH, J = 7.8), 10.04 (s, H, OH)
7l		3253.69 (OH), 3172.1 (NH), 1732.11 (C=O), 1639.47 (C=N)	7.61–7.63 (d, H, SCH=, J = 5.8), 7.4–7.42 (d, H, =CH–, J = 5.8), 8.4 (s, H, NCHN), 5.20 (s, 2H, OCH_2), 7.96 (s, H, CONH), 7.94 (s, H, NNCH), 6.83–6.9 (d, 2H, ArH, J = 8.64), 7.09–7.24 (d, 2H, ArH, J = 8.64), 9.60 (s, H, OH)

Table 3. Cytotoxicity and selective index.

Compound	MIC[a], μM	IC_{50}[b], μM	SI[c], μM
7b	70	2492.90	35.61
7c	64	2387.80	37.31
7d	70	2415.90	34.51
7e	71	2264.80	31.89
7f	66	2454.70	37.19
7g	62	2019.90	32.58

[a] MIC: Minimum inhibitory concentration. [b] IC_{50}: Concentration of test drug needed to inhibit cell growth by 50%. [c] SI: Selectivity index calculated by dividing the IC_{50} by the MIC.

*Procedure for synthesis of thieno[2,3-d]pyrimidin-4-acetoxyhydrazide (**6**) [6]*

To a suspension of **5** (0.01 mol) in 40 ml ethanol 0.015 mol of hydrazine hydrate was added and the reaction mixture was refluxed for 4 h. The resulting mixture was allowed to cool and filtered. The solid obtained dried and recrystallized with hot water.

Yield 64.2%; 257–260°C; yellowish crystalline solid.

*General procedure for synthesis of N-substituted-2-(thieno[2,3-d]pyrimidine-4-yloxy)acetohydrazides (**7a–7l**) [11]*

Equimolar (0.03 mol) quantity of **4** and different aldehydes was refluxed in 25 mL alcohol for 2–3 h in the presence of a few drops of glacial acetic acid. Solvent was evaporated and the product was poured to water, filtered and dried. The crude solid was recrystallized in appropriate solvent systems to give the products.

Antitubercular activity

The ability of compounds to inhibit the growth of *Mycobacterium* species grown in Middlebrook 7H9 broth and standard strain of *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was determined by Ziehl-Neelsen stain. The basal medium is prepared according to manufacturer's instructions (Hi-Media) and sterilized by autoclaving. 4.5 mL of broth is poured into each one of the sterile bottles. To this, 0.5 mL of ADC supplement is added. This supplement contains catalase, dextrose and bovine serum albumin fraction. Then a stock solution of the compound is prepared (10 mg/mL). From this appropriate amount of solution is transferred to media bottles to achieve final concentrations of 25, 50, and 100 μg/mL. Finally, 10-μL suspension of *Mycobacterium tuberculosis* strain (100 000 organisms/mL, adjusted by McFarland's turbidity standard) is transferred to each of the tubes and incubated at 37°C. Along with this, one growth control without compound and standard drug controls were also set up. The bottles are inspected for growth twice a week for a period of three weeks. The appearance of turbidity is considered as growth and indicates resistance to the compound. The growth is confirmed by making a smear from each bottle and performing a Ziehl-Neelsen stain.

In-vitro cytotoxicity evaluation [12]

The compounds were tested for cytotoxicity using THP-1(human, monocytes) cell line.

Procedure with THP-1 culture

The cell suspension from the confluent culture flask was transferred to sterile tubes, centrifuged at 2000 rpm for 10 min and cell pellet was separated. Known volume of media was added to the pellet and cells were resuspended and cell count was adjusted to 1.0×10^5 cells/mL using RPMI medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10 000 cells) was added. After 2 h, 100 mL of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, 10 mL of MTT (5mg/mL) in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. Microtitre plates were centrifuged at 2000 rpm for 15 min and supernatant was removed. 100 mL of propanol was added and the plates were gently shaken to solublise the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and inhibit cell concentration of test drug needed to growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

$$\%\ \text{Growth Inhibition} = \frac{\text{Mean OD of individual tested group}}{\text{Mean OD of control group}} \times 100$$

Selectivity index

A selectivity index can be calculated by dividing the IC_{50} by the MIC: If the SI is >10, the compound is then evaluated further [13].

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