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Antitubercular potential of some semisynthetic analogues of phytol

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ABSTRACT

Phytol, a diterpene alcohol was modified to several semisynthetic analogues. Some of the modifications were done logically to enhance lipophilicity of the molecule. Analogues **14**, **16** and **18** exhibited antitubercular activity (MIC 15.6–50 μg/mL) better than phytol (100 μg/mL). The most potent analogue **18** was evaluated for in vivo toxicity in Swiss albino mice and was well tolerated by the experimental animals up to 300 mg/kg body weight as a single oral acute dose.

According to the latest report of World Health Organisation on tuberculosis (TB), globally there was an increase in incidence of TB in 2007.[1] Most of the estimated number of cases in 2007 were in Asia (55%) and Africa (31%), with small proportions of cases in the Eastern Mediterranean Region (6%), the European Region (5%), and the Americas (3%). In terms of maximum number of cases, the top five countries were India (2.0 million), China (1.3 million), Indonesia (0.53 million), Nigeria (0.46%) and South Africa (0.46%) in 2007. Although the prevalence and mortality rates are falling globally and in all six WHO regions, the number of new cases are still increasing. So, WHO has set an immediate target that TB prevalence and death rates should be halved by 2015 compared with their level in 1990. Increase in the multidrug resistance tuberculosis (MDR-TB) and extensive drug resistance tuberculosis (XDR-TB) cases make it imperative to explore for new anti-TB agents. Many natural products and their semisynthetic analogues have been playing an important role in the chemotherapy of tuberculosis.[2] Streptomycin (**1**) and its semisynthetic analogues amikacin (**2**), Rifamycin (**3**, **4**) and its semisynthetic analogues rifampicin (**5**), rifabutin (**6**), rifapentine (**7**), etc. are used in combination with other antitubercular agents as either frontline or second line drugs (see Fig. 1).[3]

The present study was intended to explore possibility of modification of phytol (**8**) to better antitubercular agent. Several analogues were synthesized and evaluated against *Mycobacterium tuberculosis* $H_{37}Rv$ strain radiorespirometrically. The most active derivative thus obtained was then logically modified to get better antitubercular analogues of phytol. The most potent antitubercular analogue of phytol was further evaluated for in vivo oral acute toxicity in Swiss albino mice.

In our experiments, phytol (**8**) exhibited antitubercular activity against *M. tuberculosis* $H_{37}Rv$ strain at 100 μg/mL (MIC). To get better analogues for antitubercular activity, we modified it to various derivatives (Scheme 1) at hydroxyl and double bond positions. Phytol (**8**) was treated with acetic anhydride in dry pyridine to get an acetyl derivative (**9**). Epoxidation of phytol with *m*-chloroperbenzoic acid (*m*-CPBA) in dichloromethane afforded compound **10**.[4] Formylation of phytol was done using Vilsmeier reagent (DMF–$POCl_3$) to get a formate ester (**11**).[5] The alcoholic hydroxyl of phytol was converted into a bromo derivative (**12**) using phosphorus tribromide in dry benzene.[6] Phytyl bromide (**12**) was further treated with sodium azide in DMF to get an azide derivative (**13**).[7] The primary alcoholic group of phytol was oxidised to an aldehyde (**14**) by treating it with pyridinium chlorochromate (PCC) in dry dichloromethane.[8] Phytol was dimerised at alcoholic position to get an ether derivative (**15**) on dehydrating with conc. sulfuric acid in dry benzene.[8]

Further, the aldehyde derivative of phytol (**8**) was modified to some more lipophilic analogues (Scheme 2). Phytal (**14**) was refluxed with hydroxylamine hydrochloride in ethanol to get corresponding oxime derivative (**16**) of aldehyde.[8] Two different fatty acid ester chains were hooked up at hydroxyl position of oxime derivative to enhance lipophilicity of the molecule. It was first treated with ethyl bromoacetate in presence of anhydrous potassium carbonate in dry acetone to get **17**. Similarly, **18** was obtained by

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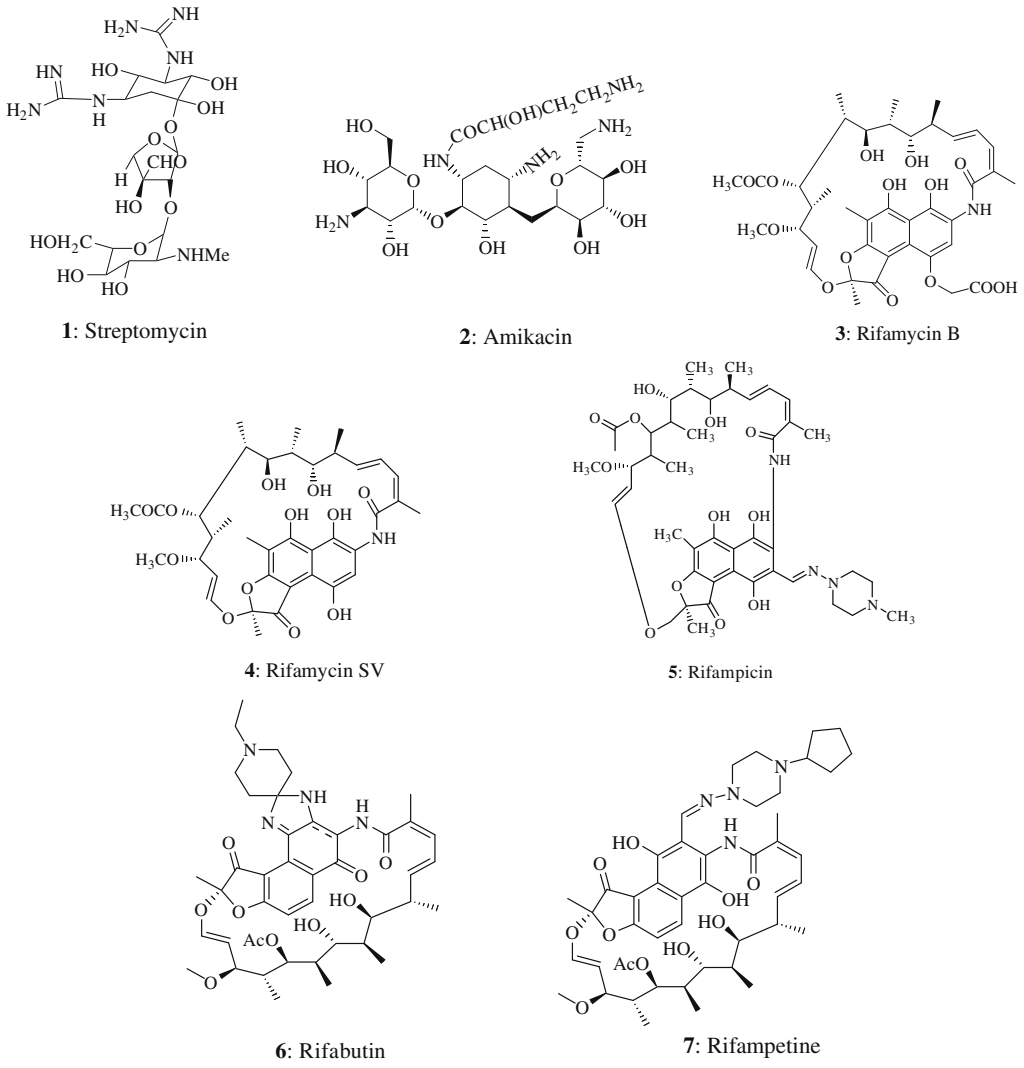


Figure 1. Some of the natural and semisynthetic antitubercular drugs.

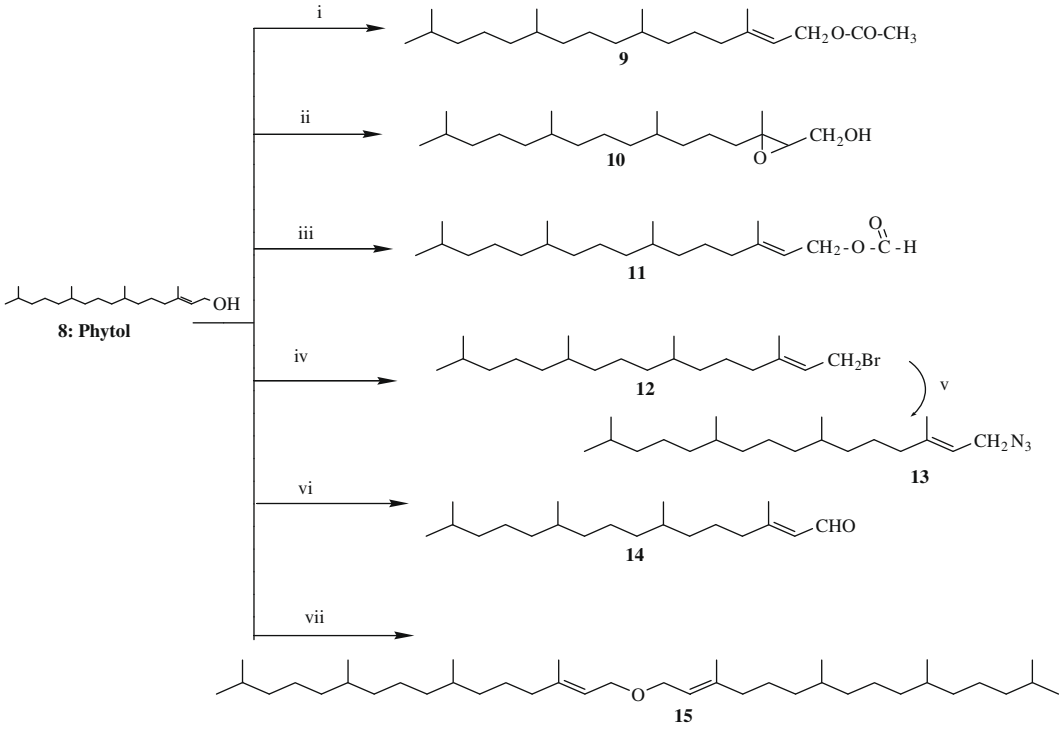
treating **16** with ethyl bromocrotonate by the same procedure. Another lipophilic derivative was obtained on treating phytyl bromide with demethoxycurcumin to get a derivative (**19**) possessing two phytyl chains attached to both the phenolic positions of demethoxycurcumin. Demethoxycurcumin has been reported to exhibit antitubercular activity at MIC 200 μg/mL.[9] All the analogues **9**–**19** were confirmed by spectroscopy.[10]

Compounds **8**–**19** were evaluated for in vitro antitubercular activity with BACTEC 460 Radiometric Susceptibility Assay against M. tuberculosis $H_{37}Rv$ (ATCC 27294).[11] Rifampicin (Sigma Biochemicals) and streptomycin (Sigma Biochemicals), were used as positive controls. Analogues **14**, **16** and **18** exhibited potent antitubercular activity, **11** and **17** possessed lower activity than the phytol while rest of the analogues were found to be inactive (Table 1).

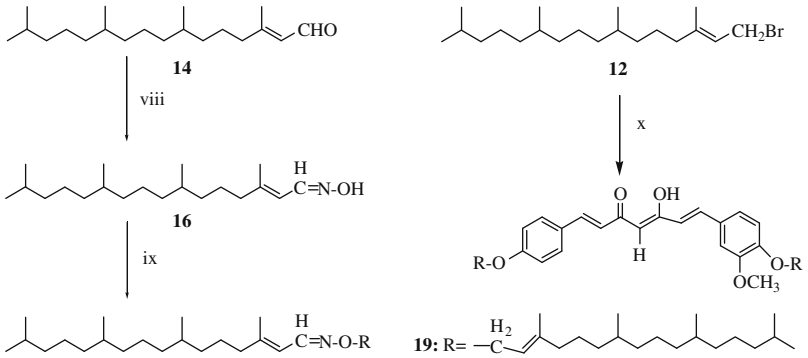
Rajab et al. reported antitubercular activity of phytol at MIC 2 μg/mL against *M. tuberculosis* $H_{37}Rv$ strain radiorespirometrically.[12] They synthesized several analogues of phytol and reported structure–activity relationship. Saludes et al. reported antitubercular activity of phytol at 32 μg/mL against *M. tuberculosis* $H_{37}Rv$ strain radiorespirometrically.[13] However, in our experiments phytol which was a mixture of cis- and trans-isomers (1:1) exhibited antitubercular activity MIC at 100 μg/mL against *M. tuberculosis* $H_{37}Rv$ strain radiorespirometrically. The antitubercular activity reported earlier was for trans-isomer of phytol by other workers.[12,13] The aldehyde derivative of Phytol (Phytal, **14**) was further modified to three other derivatives having enhanced lipophilicity. A moderate to high lipophilicity of the compounds exhibit better antitubercular activity due to the lipophilic nature of the Mycobacterium cell wall.[14–16] Over 60% of its cell wall is lipid due to which it poses difficulty to permeability to drug molecules, which ultimately retards the transport of polar compounds through outer lipid layer of mycobacterium and thus, the pathogen is resistant to many antibiotics, acidic and alkaline compounds, resistant to osmotic lysis via complement deposition, resistant to lethal oxidations and survival inside of macrophages.[17] So, lipophilicity was enhanced by modifying the aldehydic position of **14**.

The most potent analogue of phytol that is, **18** was evaluated for in vivo acute oral toxicity in Swiss albino mice in accordance with the 'Organisation for Economic Co-operation and Development (OECD)' test guideline No. 423 (1987).[18]

Three different doses 5, 50 and 300 mg/kg body weight of the test compound were given to mice after making suspension of **18** in distilled water using traces of ethanol as co-solvent. No observational changes, morbidity and mortality were observed throughout the experimental period (7 days). Blood and serum samples upon analysis showed non-significant changes in all the parameters



Scheme 1. Reagents and conditions: (i) dry pyridine, acetic anhydride, rt, overnight (16–18 h), 94%; (ii) dry DCM, *m*-CPBA, 10 °C for 1 h, then rt overnight (16–18 h), 88%; (iii) dry DMF, $POCl_3$, 0 °C for 1 h, then rt 3 h, 91%; (iv) benzene, PBr_3, 10 °C for 30 min., then rt for 2 h, 89%; (v) DMF, NaN_3, 70 °C, 73%; (vi) dry DCM, pyridinium chlorochromate, refluxed 5 h, 78%; (vii) dry benzene, 1 drop concd H_2SO_4, reflux for 5 h, 73%.



Scheme 2. Reagents and conditions: (viii) $NH_2OH{\cdot}HCl$, ethanol, pyridine, refluxed 2 h, 91%; (ix) **10:** dry acetone, anhydrous K_2CO_3, ethyl bromoacetate, refluxed, 4 h, 79%; and **11:** dry acetone, anhydrous K_2CO_3, ethyl bromocrotonate, refluxed, 4 h, 61%; (x) dry acetone, anhydrous K_2CO_3, demethoxycurcumin, rt, 3 h, 48%.

Table 1
Antimycobacterial activity of phytol and its analogues against *M. tuberculosis* $H_{37}Rv$ strain by BACTEC assay

S. No.	Compound No.	MIC (μg/mL)
1	**8**	100
2	**9**	n.a[a]
3	**10**	n.a
4	**11**	500
5	**12**	n.a
6	**13**	n.a
7	**14**	50
8	**15**	n.a
9	**16**	25
10	**17**	500
11	**18**	15.6
12	**19**	n.a
13	Rifampicin	2.0
14	Streptomycin	2.0

[a] n.a. = not active at 500 μg/mL concentration.

studied like total RBC, WBC count, differential leukocyte count, haemoglobin, serum total cholesterol, triglycerides, creatinine level, SGPT and SGOT activity (Table 2). Similarly, animals on gross pathological study showed no changes in any of the organs studied including their absolute and relative weight (see Figs. 2 and 3). Therefore, the experiment showed that analogue **18** was well tolerated by the Swiss albino mice up to the dose level 300 mg/kg body weight as a single acute oral dose. However, sub-acute and chronic experiments with this analogue need to be carried out to look for adverse effect if any on repeated exposure to compound **18** for its future development.

In conclusion, the present study provides three better antitubercular analogues (**14**, **16** and, **18**) of phytol against *M. tuberculosis* $H_{37}Rv$ strain. Analogue **18**, exhibited most potent antitubercular activity and was well tolerable at 300 mg/kg body weight as single acute oral dose.

Table 2
Effect of phytyl derivative **18**, as a single acute oral dose @ 5, 50 and 300 mg/kg body weight on body weight, haemogram and serum biochemical parameters in Swiss albino mice

Parameters	Dose of phytyl derivative, **18**, in mg/kg body weight as a single oral dose			
	Control	5 mg/kg	50 mg/kg	300 mg/kg
Body weight (g)	23.56 ± 1.547	25.41 ± 2.31	25.39 ± 2.43	20.56 ± 1.53
Total RBC count (millions/mm^3)	6.24 ± 0.51	5.98 ± 0.21	6.58 ± 0.19	5.14 ± 0.78
Total WBC count (thousands/mm^3)	10.71 ± 2.47	14.45 ± 1.16	13.46 ± 0.99	14.24 ± 1.55
Haemoglobin (g/dL)	7.54 ± 1.05	6.41 ± 0.61	10.09 ± 2.36	5.55 ± 0.07
Serum total cholesterol (mg/dL)	43.25 ± 4.04	57.67 ± 7.95	54.91 ± 4.95	62.27 ± 8.28
Serum triglycerides (mg/dL)	72.43 ± 8.40	84.31 ± 14.76	71.90 ± 3.46	68.95 ± 7.80
Serum creatinine (mg/dL)	0.71 ± 0.14	0.61 ± 0.12	0.59 ± 0.08	1.20 ± 0.32
SGPT (U/L)	8.13 ± 0.79	11.15 ± 4.50	10.06 ± 1.19	11.36 ± 1.00
SGOT (U/L)	28.34 ± 3.37	28.03 ± 3.52	35.44 ± 7.64	41.73 ± 9.44

n = 4, non significant changes were found in all the parameters studied compared to control.

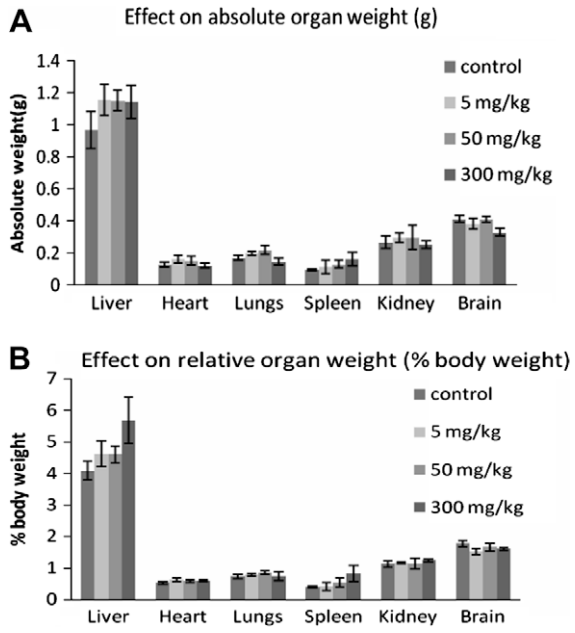


Figure 2. (A and B) Effect of phytyl derivative **18,** as a single acute oral dose at 5, 50 and 300 mg/kg body weight on absolute and relative organ weight in Swiss albino mice. (n = 4, Non significant changes were found compared to control).

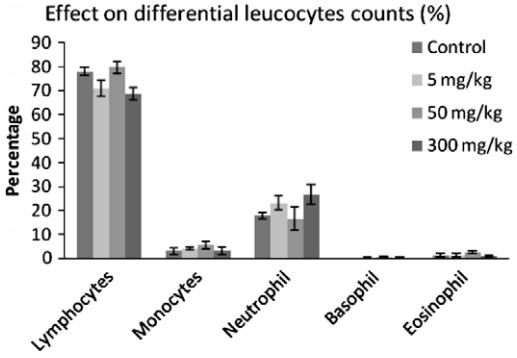


Figure 3. Effect of phytyl derivative **18**, as a single acute oral dose at 5, 50 and 300 mg/kg body weight on differential leucocytes counts in Swiss albino mice. (n = 4, Non significant changes were found compared to control).

Acknowledgements

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10. *Selected physical data*: Compound **11**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.76–0.87 (m, 12H, 4 × CH_3 of phytyl chain), 1.07–2.05 (m, 24H, 1 × CH_3, 9 × CH_2 and 3 × CH of phytyl chain), 2.95 (s, 3H, CH_3–C=), 4.09 (d, 2H, OCH_2), 5.45 (t, 1H, =CH–C–C–O–, J = 7.95 Hz), 8.01 (s, 1H, O–CHO formate); ESI-MS (MeOH): 325 $[M+H]^+$, 347 $[M+Na]^+$; IR (CCl_4, cm^{-1}): 2928, 1719, 1654; **12**: ^{1}H NMR ($CDCl_3$): δ 0.79–0.92 (m, 12H, 4 × CH_3 of phytyl chain), 1.09–2.10 (m, 24H, 1 × CH_3, 9 × CH_2 and 3 × CH of phytyl chain), 1.76 (s, 3H, CH_3–C=), 4.04 (d, 2H, OCH_2), 5.52 (t, 1H, =CH–C–C–O–); ESI-MS (MeOH): 359 $[M+H]^+$, 381 $[M+Na]^+$; IR (CCl_4, cm^{-1}): 2928, 1460, 1201; **14**: ^{1}H NMR ($CDCl_3$): δ 0.83–0.94 (m, 12H, 4 × CH_3 of phytyl chain), 2.18 (s, 3H, CH_3–C=), 1.09–2.54 (m, 21H, 9 × CH_2 and 3 × CH of phytyl chain), 5.89 (d, 1H, CH=C, J = 8.1 Hz), 9.97 (d, 1H, –CHO, J = 8.1 Hz); ESI-MS (MeOH): 295 $[M+H]^+$, 333 $[M+K]^+$; IR (CCl_4, cm^{-1}): 2927, 2370, 1719, 1654; **16**: ^{1}H NMR ($CDCl_3$): δ 0.81–0.92 (m, 12H, 4 × CH_3 of phytyl chain), 1.34 (s, 3H, CH_3–C=), 1.04–2.36 (m, 21H, 9 × CH_2 and 3 × CH of phytyl chain), 5.91 (d, 1H, CH=C, J = 5.1 Hz), 6.52 (d, 1H, CH=NO–, J = 4.8 Hz), 8.04 (d, 1H, N–OH, J = 4.8 Hz); ESI-MS (MeOH): 310 $[M+H]^+$, 332 $[M+Na]^+$; IR (CCl_4, cm^{-1}): 3312, 2928, 1653; **18**: ^{1}H NMR ($CDCl_3$): δ 0.77–0.81 (m, 12H, 4 × CH_3 of phytyl chain), 1.05 (t, 3H, COO–C–CH_3), 1.02–2.10 (m, 21H, 9 × CH_2 and 3 × CH of phytyl chain), 2.11 (s, 3H, CH_3–C=), 3.93 (d, 2H, OCH_2–C=, J = 7.5 Hz), 4.11 (q, 2H, OCH_2–C, J = 7.2 Hz), 5.93–5.99 (m, 2H, C=CH, –CH=N), 6.89–6.99 (m, 2H, CH= and =CH–C=N); ESI-MS (MeOH): 445 $[M+Na]^+$; IR (CCl_4, cm^{-1}): 2928, 1724, 1654, 975.
11. In vitro antimycobacterial assay by BACTEC Radiometric Susceptibility Assay.*Mycobacterium tuberculosis* H_{37}Rv (ATCC 27294) used in this screening was obtained from National JALMA Institute for Leprosy and other Mycobacterial Diseases (ICMR), Agra, India and maintained on Löwenstein-Jansen media slant at 37 °C. After 21 days of incubation bacterial cells were scraped from slants and transferred in 1.0 mL of BACTEC diluting fluid and made complete homogenized suspension by vortexing with glass beads (2 mm diameter). The suspension was allowed to stand for a few minutes to permit sedimentation of the bacterial clumps if any. The turbidity of the homogenous suspension was adjusted to McFarland standard 1.0 with diluting fluid. A BACTEC 12B vial (Becton-Dickinson) was injected with 0.1 mL of this suspension. This vial was used as primary inoculum after the growth index (GI) reached a value of about 500 (approximately 1 × 10^6 cfu/mL).
Briefly, 0.1 mL of bacterial suspension from the primary inoculum culture vial (GI 500) was injected into test compound-containing vials using 1.0 mL insulin syringe. To comply with 1% proportion method, 0.1 mL of primary inoculum was added to 9.9 mL BACTEC diluting fluid to obtain 1:100 dilutions. From this 0.1 mL was injected into two 12B vials for two test compounds containing 4.0 ml medium along with 40 μL of DMSO. Vials were incubated at 37 °C, and the GI was recorded every 24 h in a BACTEC 460TB instrument (Becton-

Dickinson). Once the GI of the control vial (1:100) reached 30 then the GI values of the test (compound-containing) vials were compared with that of control vials based on difference in growth (ΔGI). The result was interpreted as follows: If the difference (called as ΔGI) of current GI from previous day GI in the case of drug containing vials is lower than the ΔGI of 1:100 control vial for the same period then the test compound is termed as active against MTB or otherwise inactive.

Twofold serial dilution technique was used to assess the minimum inhibitory concentration (MIC) of a test compounds. Only broth culture was used as a positive control and media as a negative control.

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18. In vivo acute oral toxicity evaluation.Sixteen mice (8 male and 8 female) were taken and divided into four groups comprising 2 male and 2 female in each group weighing between 20 and 25 g. The animals were maintained at 22 ± 5 °C with humidity control and also on an automatic dark and light cycle of 12 h. The animals were fed with the standard rat feed and provided ad libitum drinking water. Mice of group 1 were kept as control and rest were kept as experimental. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. The test compound was suspended in distilled water using traces of ethanol as co-solvent and was given at 5, 50 and 300 mg/kg body weight to animals of groups 2, 3 and 4, respectively. Control animals received only vehicle. All the animals were sacrificed on 7th day after the experimentation.

The animals were checked for mortality and any signs of ill health at hourly interval on the day of administration of drug and there after a daily general case side clinical examination was carried out including changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, pilo-erection respiratory patterns, etc. Also changes in gait, posture and response to handling were also recorded. In addition to observational study, body weights were recorded and blood and serum samples were collected from all the animals on 7th day after experiment and were analysed for total RBC, WBC, differential leukocyte count, haemoglobin percentage and biochemical parameters like total cholesterol, triglycerides, creatinine, SGPT and SGOT activity. The animals were then sacrificed and necropsed for any gross pathological changes. Weights of vital organs like liver, heart, kidney, etc. were recorded.