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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Potent in vitro and in vivo antitubercular activity of certain newly synthesized indophenazine 1,3,5-trisubstituted pyrazoline derivatives bearing benzofuran

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Abstract Fourteen newly synthesized derivatives of indophenazine 1,3,5-trisubstituted pyrazoline bearing benzofuran were prepared from benzofuran chalcones with indophenazine hydrazide through cycloaddition reaction. All the compounds were screened for their in vitro and in vivo antitubercular activity against drug resistant and multidrug-resistant *Mycobacterium tuberculosis* $H_{37}RV$. The MIC_{50} and MIC_{90} were estimated and compared with rifampicin and gatifloxacin standard drugs. Nitro group containing at *ortho* **5j**, *meta* **5e**, furan ring containing **5m** and *ortho* **5i**, *para* **5h** chloro containing compounds were exhibited significant in vitro, in vivo antitubercular activity against standard drugs.

Keywords In vitro and in vivo antitubercular activity · Multidrug-resistant *M. tuberculosis* · Indophenazine pyrazoline · Benzofuran

Introduction

A recent survey indicates the leading mortality cause by HIV/AIDS is closely associated with tuberculosis. Improved therapy for tuberculosis is recognized as a major need for the developing countries as well as developed countries (Albalak *et al.*, 2007). Current chemotherapy of tuberculosis is not likely to be successful in retroviral infected patients access to the world. Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) is another serious threat to treatment and control of tuberculosis. The drug resistance may be caused by mycobacterium unique cell wall structure, which is rich in long-chain fatty acids such as C60 to C90 mycolic acid, which covalently linked with peptidoglycan-associated polysaccharide arabinogalactan (Trias and Benz, 1993). The cell wall barrier alone is not sufficient to explain the intrinsic drug resistance. Resistance of *M. tuberculosis* strains by existing antitubercular agents is also increasing problem worldwide (Corbett *et al.*, 2003). Therefore, powerful novel antitubercular agents being new mechanism of action is needed to develop for the treatment of complex tuberculosis cases. In spite of drug resistance, complex tuberculosis cases severe and toxicity on repeated dosing of isoniazid (INH), thought to synthesize a novel series of indophenazine 1,3,5-trisubstituted pyrazoline containing benzofuran. According to the past researcher reports, pyrazoline derivatives are highly active against many mycobacteria (Ali *et al.*, 2007; Kini *et al.*, 2009). The incorporation of heterocyclic rings into the pyrazoline ring was reported as potent antibacterial agents (Kirilmis *et al.*, 2008), antiamoebic agents (Husain *et al.*, 2008), and antifungal agents (Prakash *et al.*, 2008). The present investigation focus on the synthesis of 1-[3-(5-hydroxybenzo[*b*]furan-2-yl)-5-substituted phenyl-4,5-dihydro-1*H*-1-pyrazolyl]-2-(5*H*-indolo[2,3-*b*]quinoxalin-5-yl)-1-ethanone derivatives **5a**–**n** from 2-(5,8-dihydroquinoxalino[2,3-*b*]indol-5-yl)acetohydrazide **4** with different benzofuran chalcones **3a**–**n** and tested for in vitro and in vivo antitubercular activity (Lenaerts *et al.*, 2005, 2008;

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Stover *et al.*, 2000; Tyagi *et al.*, 2005) against *M. tuberculosis*.

Chemistry

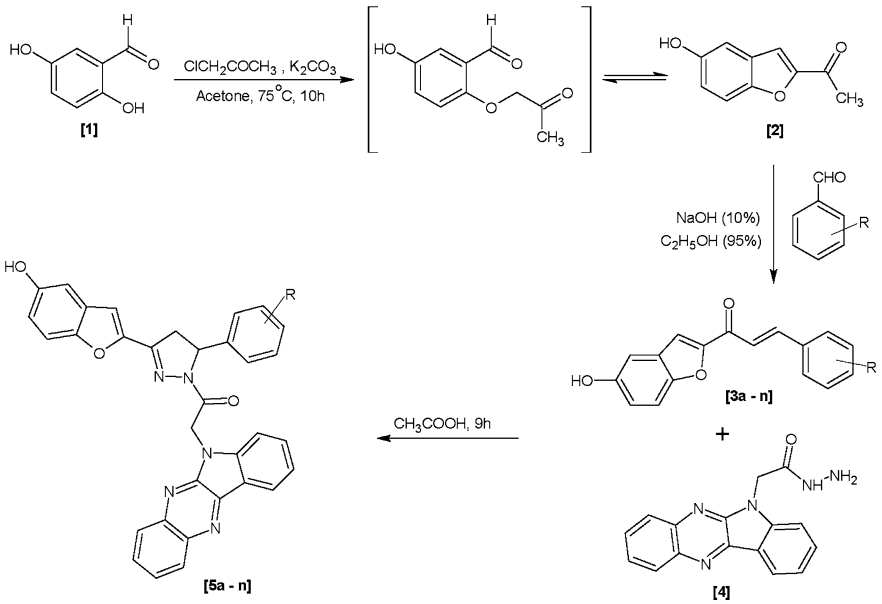
2,5-Dihydroxy benzaldehyde **1** was utilized for the synthesis of 5-hydroxy, 2-acetylbenzofuran **2** in presence of chloroacetone and K_2CO_3. A new series of (2*E*)-1-(5-hydroxy-1-benzofuran-2-yl)-3-phenylprop-2-en-1-one derivatives **3a–n** were prepared from 5-hydroxy, 2-acetylbenzofuran with different aromatic aldehydes in presence of strong alkaline medium. Fourteen new 1-[3-(5-hydroxybenzo[*b*]furan-2-yl)-5-substituted phenyl-4,5-dihydro-1*H*-1-pyrazolyl]-2-(5*H*-indolo[2,3-*b*]quinoxalin-5-yl)-1-ethanone derivatives **5a–n** were synthesized from 2-(5,8-dihydroquinoxalino[2,3-*b*]indol-5-yl)acetohydrazide **4** with different benzofuran chalcones **3a–n** in presence of glacial acetic acid (Scheme 1).

Biology

The in vitro and in vivo antitubercular activity was carried out with MTB and MDR-TB using $H_{37}Rv$ stains (Interleid, 1991). The clinically isolated stains were resistant to isoniazid (INH), rifampicin, ethambutol, and gatifloxacin were procured from Tuberculosis Research Centre, Chennai, India. Antitubercular activity was evaluated to determine the MIC_{50} and MIC_{90} using 10% oleic acid–albumin–dextrose–catalase-enriched 7H11 agar medium (Collins and Franzblau, 1997). The toxicity (IC_{50}) of the compounds was examined using mammalian VERO cell line.

Results and discussion

All the synthesized compounds were found satisfactory antitubercular agents against both MTB and MDR-TB with less cytotoxicity (IC_{50}) than standard drugs (Table 1). Out of fourteen compounds **5j**, **5e**, **5m**, **5i**, **5h**, **5l**, **5f** were found potent against both MTB and MDR-TB, MIC_{50} of 0.16, 0.62, 1.1, 1.75, 2.45, 3.8, 4.56 μg/ml, respectively. Among all the active compounds **5j** and **5e** were found more active than rifampicin and gatifloxacin (MIC_{50} = 0.5 μg/ml and MIC_{90} = 0.12 μg/ml) against MTB. The N–H, one proton in indophenazine ring was established by sharp ^{1}H NMR peak at 9.834 ppm (s, 1H) and 3402, 3169, 3130 cm^{-1} in FTIR. Oxazolidine ring of indophenazine was established by ^{1}H NMR peak at 8.42–7.65 ppm, by IR (cm^{-1}): 3402, 3169 m (NH), 1686 m (CH), 1617 m (C=N). 6-Carbethoxymethyl indophenazine structure was characterized by sharp ^{1}H NMR peak at 5.064–1.25 ppm (s, N–CH_2), where N–H peak was absent and additional 4.18 ppm (d, 2H, J = 7.12 Hz, O–CH_2–CH_3), 1.25 ppm (d, 3H, J = 9.32 Hz, O–CH_2–CH_3) peaks were obtained. The IR peaks 1738 and 1748 cm^{-1} (C=O), 1678 (CH), 1614 (C=C) and (C=N) were supported for the establishment of the structure. Indophenazine-6-acetic acid hydrazide structure was characterized by sharp ^{1}H NMR peak at 8.92 (s, 1H) $NHNH_2$ and 5.78 (s, 2H) $NHNH_2$. The IR peaks 1614 cm^{-1} (C=C) and (C=N), 1546 cm^{-1} (NH), 1488, 1467 cm^{-1} (C=N) and (C=C) were reconfirmed the structure. Compound **5a** structure was elucidated by ^{1}H NMR, IR, Mass, and elemental analysis data, where $NHNH_2$ peaks were found absent and a sharp ^{1}H NMR peak at 3.6 (s, 1H) was found for one proton in position 5 in pyrazoline ring and 2.3 (s, 2H) for two protons at position 4 in pyrazoline ring were



Scheme 1 General synthesis of **3a–n** and **5a–n**

Table 1 In vitro and in vivo antitubercular screening oft novel indophenazine 1,3,5-trisubstituted pyrazoline bearing benzofuran derivatives against *Mycobacterium tuberculosis* $H_{37}RV$

Name of comp.	−R	Results against MTB[a]		Results against MDR-TB[b]		IC_{50} (μM)[c]
		MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	
5a	–OH (*o*)	9.81	10.84	10.34	12.56	>226.8
5b	$–OCH_3$ (*o*)	11.80	14.20	10.67	13.64	>225.6
5c	$–N(CH_3)_2$ (*p*)	5.56	9.21	5.00	8.98	>216.2
5d	–COOH (*o*)	10.20	6.87	9.56	7.45	>220.5
5e	$–NO_2$ (*m*)	0.62	1.24	1.20	2.00	>180.2
5f	–OH (*o*), OCH_3 (*p*)	4.56	8.32	7.80	13.10	>206.5
5g	–OH (*p*)	10.53	7.52	9.86	11.21	>245.2
5h	–Cl (*p*)	2.45	4.20	5.23	8.66	>205.4
5i	–Cl (*o*)	1.75	2.56	4.68	8.61	>195.6
5j	$–NO_2$ (*o*)	0.16	0.42	3.24	7.2	>144.3
5k	$–OCH_3$ (*p*)	12.11	14.3	11.5	15.4	>225.5
5l	–H	3.8	1.5	10.56	6.12	>196.7
5m	Furan ring	1.1	3.12	6.40	9.78	>198.2
5n	–CH=CH–Ar	0.98	3.67	5.6	11.34	>206.5
Rifampicin		0.5	2.0	4.21	7.37	>77.4
Gatifloxacin		0.12	0.5	14.73	28.46	>159.5

[a] *Mycobacterium tuberculosis* $H_{37}RV$

[b] Multidrug-resistant *Mycobacterium tuberculosis*

[c] Cytotoxicity in VERO cell line

ensuring the formation of pyrazoline ring. In mass spectra, *m*/*z*: 553.75 (molecular ion peak) was recorded same with actual molecular weight of the compound and 100% base peak of the important fragment was also found at *m*/*z*: 145.20. The IR data, 3373 (O–H str.), 1345, 1169 (C–O str.) and the data of elemental analysis were reestablished the structure of **5a** by ±0.4% different in calculated C, H, N values with founded values. Similar way we interpreted and characterized all the synthesized final compounds.

Structure activity relationships

Structure activity relationship study recommended that substituted phenyl ring (**5a–k**) at 5 positions by –OH (*o*) and $–OCH_3$ (*m*) in 4,5-dihydro pyrazole posses a variable in vitro and in vivo antitubercular activity against MTB and MDR-TB listed in Table 1. The *ortho* and *meta* substituted phenyl ring with $–NO_2$ (**5e** and **5j**) produced very good antitubercular activity. Where, unsubstituted phenyl ring (**5l**) found moderate activity. When phenyl ring was replaced by five-membered ring (**5m**) at 5 position of pyrazole ring created good antitubercular activity. The single C–C bond between pyrazole ring and 5-phenyl ring can be replaced by ethylene bridge in compound **5n**, results potent inhibitor of *M. tuberculosis*. *Ortho* and *para* positions substituted with –Cl (**5i** and **5h**) in phenyl ring produced moderate activity. The other substitution in phenyl ring at position 5 in pyrazoline produced less active against *M. tuberculosis*.

Conclusion

It could be concluded that, the screening of antitubercular activity of the novel series of 1,3,5-trisubstituted indophenazine pyrazoline containing benzofuran derivatives as a new lead compounds endowed with high antitubercular activity toward MTB and MDR-TB, exhibiting MICs values between 0.16 and 29.0 μg/ml. The potency, selectivity, and low cytotoxicity of the synthesized compounds make them valid lead for synthesizing a new compound that can produce better activity.

Experimental protocols

Chemistry

All the chemicals were procured from Sigma-Aldrich (USA), E. Merck (Germany), and SD Fine Chemicals (India). Melting points were determined using one end open capillary tubes on a Buchi-530 melting point apparatus and are uncorrected. Infrared spectrophotometry

(FTIR) was recorded for all the compounds on Jasco-FTIR-6100 using KBr. ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) was recorded in Advance bruker (300 MHz). Chemical shift was recorded in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Parkin–Elmer 2400 Series II analyzer carried out the elemental analysis. The purity of the compounds was confirmed by thin layer chromatography (TLC) using silica gel G glass plates and solvent system of benzene:ethyl acetate. The spots were visualized in iodine vapor.

Synthesis of 5-hydroxy, 2-acetylbenzofuran ***2***

2,5-Dihydroxy benzaldehyde **1** (0.01 mol) and chloroacetone (0.01 mol) were dissolved in 150 ml of dry acetone into a 250 ml round bottom flask, to that added 30 g of anhydrous K_2CO_3 and refluxed for 10 h at 75°C on water bath. The solid was obtained after cooling and filtration. The solid was washed with cold acetone and recrystallized with petroleum ether (40–60°C). Yield was found 79% and m.p.: 85°C; IR (KBr) υ_{max} (cm^{-1}): 1173, 1768 (C–O–C), 1674 (C=O), 1614 (C=C), 1486, 1469 (C=C), 1318 (CH), 1228 (C–O), 1244 and 880 (C=C); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.826 (s, 3H, CH_3), 7.228–7.48 (s, 1H, CH), 7.26–7.40 (m, 4H, $J = 8.0$ Hz, Ph–H), 7.10–7.25 (m, 3H, $J = 8.3$ Hz) furan ring.

General procedure for the synthesis of (2E)-1-(5-hydroxy-1-benzofuran-2-yl)-3-phenylprop-2-en-1-one derivatives ***3a–n***

5-Hydroxy, 2-acetylbenzofuran **2** (0.05 mol) and different aromatic aldehydes (0.05 mol) were dissolved in 20 ml of ethanol (98%) in a 250 ml conical flask, the reaction was carried out according to previously published articles (Agrawal *et al.,* 2007; Manna *et al.*, 2004, 2008). 8 ml of KOH/NaOH solution (20%) was added to the reaction mixture. Then the flask was kept for stirring at room temperature for 20 h. After stirring the reaction mixture was poured into 100 ml ice-cold water, and acidified with conc. HCl. The obtained solid was filtered and washed with cold water and purified from ethanol to obtained different benzofuran chalcones **3a**–**n**.

Synthesis of 2-(5,8-dihydroquinoxalino[2,3-b]indol-5yl)acetohydrazide ***4***

2-(5,8-Dihydroquinoxalino[2,3-*b*]indol-5-yl)acetohydrazide **4** was synthesized according to published procedure (Manna and Agrawal, 2009) from indophenazine ester (0.1 mol) with hydrazine hydrate (99%, 0.4 mol) in presence of absolute ethanol.

General procedure for the synthesis of 1-[3-(5-hydroxybenzo[b]furan-2-yl)-5-substituted phenyl-4,5-dihydro-1H-1-pyrazolyl]-2-(5H-Indolo[2,3-b]quinoxalin-5-yl)-1-ethanone derivatives ***5a–n***

(2*E*)-1-(5-Hydroxy-1-benzofuran-2-yl)-3-phenylprop-2-en-1-one derivatives (0.01 mol) and 2-(5,8-dihydroquinoxalino[2,3-*b*]indol-5yl)acetohydrazide (0.02 mol) were dissolved in 20 ml of glacial acetic acid into a 100 ml of flat bottom flask and then refluxed for a period of 10 h at 175°C. Cool the reaction mixture at room temperature and removed excess solvent under reduce pressure. The reaction mixture was poured into the 250 ml of ice-cold water with stirring. The solid product was obtained by filtration and washed with cold water. Similar way we synthesized other compounds in this series.

5a: White solid was recrystallized from 95% of ethanol; yield: 48%, m.p.: 155–157°C, $R_f = 0.88$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 236.2 nm; IR (KBr) υ_{max} (cm^{-1}): 3373 (O–H str.), 1454 (Aromatic), 1345, 1169 (C–O str.); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.56 (dd, 4H, $J = 7.41$, 4.2 Hz, Ph–H), 7.92 (s, 2H, OH), 7.79 (dd, 2H, $J = 3.41$, 6.2 Hz, CH), 7.55 (s, 4H, CH), 7.39 (s, 6H, Ph–H), 3.6 (s, 1H, pyrazoline ring), 2.3 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 553.75 (m^+), 145.20 (100%); $C_{33}H_{23}N_5O_4$ (553.56): calcd. C 71.60%, H 4.19%, N 12.65%; found C 71.90%, H 4.18%, N 12.56%.

5b: Yellowish white solid was recrystallized from methanol; m.p.: 71–73°C, yield: 38%, $R_f = 0.79$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 306.6 nm; IR (KBr) υ_{max} (cm^{-1}): 1169–1026 (C–O–C str.), 2839 (CH_3 str.), 1456 (CH_3 def.); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.15 (m, 2H, $J = 3.44$, 6.32, 7.56 Hz, Ph–H), 7.87 (s, 2H, OH), 7.7 (dd, 2H, $J = 3.44$, 6.32 Hz, Ph–H), 7.55 (d, 4H, $J = 8.7$, 3.4 Hz, CH), 7.39 (s, 6H, Ph–H), 6.89 (d, 4H, $J = 8.73$, 2.65 Hz), 4.01 (s, 3H, OCH_3), 3.61 (s, 6H, Ph–H), 2.4 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 567.90 (m^+), 145.13 (100%); $C_{34}H_{25}N_5O_4$ (567.59): calcd. C 71.95%, H 4.44%, N 12.34%; found C 71.97%, H 4.48%, N 12.46%.

5c: White solid was recrystallized from methanol; m.p.: 103–105°C, yield: 72%, $R_f = 0.94$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 296.4 nm; IR (KBr) υ_{max} (cm^{-1}): 2925, 2804 (Ar–N–CH_3 str.), 1446 (CH_3 ban.), 1365, 1317 (C–N str.); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.05 (d, 1H, $J = 5.52$ Hz, Ph–H), 7.92 (s, 2H, OH), 7.58 (d, 1H, $J = 1.55$, 8.5 Hz), 7.48 (d, 6H, $J = 7.64$, 4.23 Hz), 6.9 (d, 4H, $J = 8.74$, 6.72 Hz), 5.85 (d, 1H, $J = 8.36$, 6.55 Hz, CH), 3.9 (s, 6H), 2.8 (s, 3H, CH_3), 2.3 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 580.12 (m^+), 145.20 (100%); $C_{35}H_{28}N_6O_3$ (580.64): calcd. C 72.4%, H 4.86%, N 14.47%; found C 72.96%, H 4.18%, N 14.51%.

5d: White solid was recrystallized from 95% of ethanol; m.p.: 198–200°C, yield: 56%, $R_f = 0.54$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 298.2 nm; IR (KBr) υ_{max} (cm^{-1}): 3402 (O–H str.), 1711, 1697 (C=O str.), 1173–1074 (C–O str.); ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 11.13 (s, 1H) COOH, 8.21 (dd, 2H, $J = 3.6$, 6.32 Hz), 8.10 (d, 1H, $J = 8.51$ Hz), 7.80 (dd, 2H, $J = 3.41$, 6.22 Hz), 7.57 (s, 1H, OH), 7.29 (s, 6H), 3.55 (s, 6H), 2.13 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 581.87 (m^+), 145.20 (100%); $C_{34}H_{23}N_5O_5$ (581.58): calcd. C 70.22%, H 3.99%, N 12.04%; found C 70.90%, H 3.88%, N 12.56%.

5e: White solid was recrystallized from methanol; m.p.: 97–99°C, yield: 48%, $R_f = 0.63$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 265.4 nm; IR (KBr) υ_{max} (cm^{-1}): 1527 (asy) (NO_2 str.), 1346 (sym) (NO_2 str.), 883 (C–N str.), 877; ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 9.2 (d, 1H, $J = 2.38$ Hz), 8.53 (dd, 1H, $J = 2.38$, 9.10 Hz), 8.39 (d, 1H, $J = 9.10$ Hz), 7.6 (s, 4H, OH), 7.42 (s, 6H), 3.61 (s, 6H), 2.32 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 582.35 (m^+), 145.14 (100%); $C_{33}H_{22}N_6O_5$ (582.56): calcd. C 68.04%, H 3.81%, N 14.43%; found C 67.98%, H 3.78%, N 14.36%.

5f: Light yellow solid was recrystallized from methanol; m.p.: 223–225°C, yield: 70%, $R_f = 0.54$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 267.6 nm; IR (KBr) υ_{max} (cm^{-1}): 3240 (O–H str.), 1383 (O–H ben.), 1170–1080 (C–O str.), 1020 (C–O–C str.), 2927 (CH_3 str.), 1450 (CH_3 def.); ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.25 (dd, 2H, $J = 3.34$, 6.34 Hz), 7.81 (dd, 2H, $J = 3.47$, 6.36 Hz), 7.35 (d, 4H, $J = 8.41$ Hz), 7.42 (s, 1H, OH), 7.31 (s, 1H, Ph–OH), 6.82 (d, 4H, $J = 8.73$ Hz), 3.20 (s, 3H, Ph–OCH_3), 2.4 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 583.62 (m^+), 145.22 (100%); $C_{34}H_{25}N_5O_5$ (583.59): calcd. C 69.97%, H 4.32%, N 12.00%; found C 70.23%, H 4.18%, N 13.56%.

5g: White solid was recrystallized from methanol; m.p.: 154–156°C, yield: 66%, $R_f = 0.78$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 220.3 nm; IR (KBr) υ_{max} (cm^{-1}): 3367 (O–H str.), 1367–1252 (O–H ben.), 1172–1012 (C–O str.); ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.32 (dd, 2H, $J = 3.45$, 6.32 Hz), 7.81 (dd, 2H, $J = 3.54$, 6.41 Hz), 7.24 (s, 4H), 7.42 (s, 6H), 6.27 (s, 1H, OH), 3.56 (s, 6H), 2.3 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 553.85 (m^+), 145.30 (100%); $C_{33}H_{23}N_5O_4$ (553.56): calcd. C 71.60%, H 4.19%, N 12.65%; found C 71.70%, H 4.38%, N 12.16%.

5h: White solid was recrystallized from 95% of ethanol; m.p.: 213–215°C, yield: 81%, $R_f = 0.95$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 197.6 nm; IR (KBr) υ_{max} (cm^{-1}): 3240 (C–Cl str.), 1035 (C–O–C str.), 2930 (CH_3 str.), 1447 (CH_3 def.), 1599, 786, 746 (C–Cl str.); ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.5 (dd, 2H, $J = 3.42$, 7.23 Hz), 7.82 (dd, 2H, $J = 3.40$, 8.40 Hz), 7.52 (dd, 4H, $J = 1.42$, 8.64 Hz), 7.15 (dd, 4H, $J = 2.63$, 8.64 Hz), 6.95 (s, 1H, OH), 4.96 (s, 2H), 3.56 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 572.84 (m^+), 144.97 (100%); $C_{33}H_{22}N_5O_3Cl$ (572.01): calcd. C 69.29%, H 3.88%, N 12.24%; found C 69.72%, H 3.14%, N 12.36%.

5i: White solid was recrystallized from 95% of ethanol; m.p.: 195–197°C, yield: 32%, $R_f = 0.87$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 216.4 nm; IR (KBr) υ_{max} (cm^{-1}): 3255 (C–Cl str.), 1042 (C–O–C str.), 2912 (CH_3 str.), 1433 (CH_3 def.), 1601, 790, 748 (C–Cl str.); ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.31 (dd, 2H, $J = 3.44$, 6.22 Hz), 7.82 (dd, 2H, $J = 3.44$, 6.40 Hz), 7.22 (s, 4H), 7.40 (s, 1H, OH), 3.55 (s, 6H), 2.3 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 571.94 (m^+), 144.97 (100%); $C_{33}H_{22}N_5O_3Cl$ (572.01): calcd. C 69.29%, H 3.88%, N 12.24%; found C 69.55%, H 3.76%, N 11.98%.

5j: Light yellow solid was recrystallized from methanol; m.p.: 105–107°C, yield: 54%, $R_f = 0.82$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 280.6 nm; IR (KBr) υ_{max} (cm^{-1}): 3089, 1599, 1552 (asy) (NO_2 str.), 1344 (sym) (NO_2 str.), 815 (C–N str.); ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 9.1 (d, 1H, $J = 2.41$ Hz), 8.56 (dd, 1H, $J = 2.32$, 9.20 Hz), 8.30 (d, 1H, $J = 9.17$ Hz), 7.6 (s, 1H, OH), 7.22 (s, 6H), 3.23 (s, 6H), 2.41 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 582.62 (m^+), 145.14 (100%); $C_{33}H_{22}N_6O_5$ (582.56): calcd. C 68.04%, H 3.81%, N 14.43%; found C 68.48%, H 3.70%, N 14.22%.

5k: Light brown solid was recrystallized from methanol; m.p.: 164–166°C, yield: 75%, $R_f = 0.64$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 240.2 nm; IR (KBr) υ_{max} (cm^{-1}): 3010, 1602, 1170–1026 (C–O–C str.), 2837 (CH_3 str.), 1446 (CH_3 def.); ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.12 (dd, 2H, $J = 3.42$, 6.30 Hz), 7.69 (dd, 2H, $J = 3.39$, 6.30 Hz), 7.57 (d, 4H, $J = 8.72$ Hz), 7.41 (s, 1H, OH), 6.90 (d, 4H, $J = 8.63$ Hz), 3.60 (s, 6H), 2.35 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 567.33 (m^+), 145.13 (100%); $C_{34}H_{25}N_5O_4$ (567.59): calcd. C 71.95%, H 4.44%, N 12.34%; found C 72.11%, H 4.31%, N 11.97%.

5l: Light yellowish solid was recrystallized from methanol; m.p.: 215–217°C, yield: 68%, $R_f = 0.86$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 203.2 nm; IR (KBr) υ_{max} (cm^{-1}): 3063, 1660, 1592; ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.10 (d, 1H, $J = 8.52$ Hz), 7.91 (s, 1H, OH), 7.63 (dd, 1H, $J = 1.72$, 8.52 Hz), 7.22 (s, 4H), 7.40 (s, 6H), 3.55 (s, 6H), 2.41 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 537.44 (m^+), 145.00 (100%); $C_{33}H_{23}N_5O_3$ (537.57): calcd. C 73.73%, H 4.31%, N 13.03%; found C 73.12%, H 4.68%, N 13.59%.

5m: Yellowish white solid was recrystallized from methanol; m.p.: 185–187°C, yield: 54%, $R_f = 0.95$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 358.0 nm; IR (KBr) υ_{max} (cm^{-1}): 3134 (C–H str.), 1550 (furan), 742

(C–H ban.); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.14 (d, 1H, $J = 8.53$ Hz), 7.89 (s, 1H), 7.62 (dd, 1H, $J = 1.70$, 8.56 Hz), 7.24 (s, 4H), 7.42 (s, 1H, OH), 5.73 (s, 1H), 5.82 (d, 2H, $J = 5.23$ Hz), 3.58 (s, 4H), 2.17 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 527.81 (m^+), 145.00 (100%); $C_{31}H_{21}N_5O_3$ (527.53): calcd. C 70.58%, H 4.01%, N 13.28%; found C 70.40%, H 4.18%, N 13.36%.

5n: Light brown solid was recrystallized from methanol; m.p.: 173–175°C, yield: 72%, $R_f = 0.89$ [benzene and ethylacetoacetate (3:1 ratio)], λ_{max}: 348.7 nm; IR (KBr) υ_{max} (cm^{-1}): 2933, 1610, 978 (R–CH=CHR); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.44 (d, 1H, $J = 8.60$ Hz), 7.78 (s, 1H), 7.55 (dd, 1H, $J = 2.70$, 8.20 Hz), 7.32 (s, 4H), 7.60 (s, 1H, OH), 3.52 (s, 4H), 2.35 (s, 2H, pyrazoline ring); MS (FAB) *m/z*: 563.60 (m^+), 145.10 (100%); $C_{35}H_{25}N_5O_3$ (563.60): calcd. C 74.59%, H 4.47%, N 12.43%; found C 76.90%, H 4.13%, N 12.56%.

Biology

In vitro antitubercular activity

The in vitro antitubercular activity was screened against *M. tuberculosis* using egg base *Löwenstein–Jensen* and *Ogawa* media (Tanoue *et al.*, 2002), by measuring the growth of *M. tuberculosis* ($H_{37}RV$) after 3 and 6 weeks. Cultures were incubated at 37°C in ambient air for up to 6 weeks. Bacterial counts were measured and compared with standard drugs (rifampicin, gatifloxacin) and controls (vehicle-treated).

In vivo antitubercular studies

The in vivo animal model was used for evaluating antitubercular activity against MTB at a dose of 25 mg/kg body weight in 6-week-old female CD-1 mice, six per group. The mice were infected intravenously through the caudal vein with 10^6 to 10^7 viable *M. tuberculosis* ATCC 35801 (Sriram *et al.*, 2005). Test drug treatment was carried out by the intra-peritoneal route began after 10 days of inoculation of the animal with the microorganism and continued for 10 days. Thirty-five days post infection, the spleen and right lung were aseptically removed and grounded using tissue homogenizer, and the numbers of viable organism were determined by serial tenfold dilutions. Viable cell counts were converted to logarithms, which were then evaluated by one or two-variable analyses of variance. Statistically significant effects from the analyses of variance were further evaluated by Tukey's honestly significant difference tests to make pair-wise comparisons among means.

MICs determination

The MDR-TB clinical isolate was obtained from the Tuberculosis Research Centre (Chennai, India) and was resistant to isoniazid, rifampicin, and gatifloxacin. Minimum inhibitory concentration is defined as the minimum concentration of compounds required to give complete inhibition of bacterial growth, the method was perfumed accordingly to Bryson and Szybalski (1952) published method. Fresh colonies of *M. tuberculosis* $H_{37}RV$ (MTB) were collected and suspended in distilled water; the turbidity of the resulting suspensions was then adjusted with distilled water to match that of a standard 1 mg/ml suspension of *M. bovis* BCG (containing approximately 10^8 CFU/ml), after which the suspensions were further diluted to 10^{-1} and 10^{-2} mg/ml. The MICs were determined on 10% oleic acid–albumin–dextrose–catalase-enriched 7H11 agar medium. All the synthesized final compounds and standard drugs were dissolved in dimethyl formamide (DMF). One volume of drug solution was added to 99 volume of culture medium, and serial twofold dilutions were carried out; final synthesized compounds concentrations ranged from 4 to 0.12 μg/ml and rifampicin, gatifloxacin from 4 to 0.12 μg/ml bacterial suspensions. The bacterial suspensions were plated, in duplicate, on both drug-free and drug-containing medias. MIC_{50} was defined as the lowest drug concentration that inhibited 50% of the bacterial growth, and the MIC_{90} was 90% of the bacterial growth, compared to that on drug-free medium after incubation at 30°C for 60 days. The MICs was determined against *M. tuberculosis* ($H_{37}Rv$) on 7H11 agar medium. The MICs of all the synthesized compounds and standard drugs were reported in Table 1.

Cytotoxicity

All the synthesized compounds were examined for in vitro cytotoxicity (Gundersen *et al.*, 2002; Sriram *et al.*, 2007; Mustafa *et al.*, 2005) using a mammalian VERO cell (CCL-81, American Type Culture Collection) by exposing monolayers in 96-well plates to threefold dilutions of test compounds for 72 h at concentration of 58.7 mg/l. Cell viability was measured using the CellTiter96 aqueous non-radioactive cell proliferation assay (Promega Corp, Madison, WI), which determines the extent of reduction of a tetrazolium dye by measuring the absorbance of the product at 490 nm. Untreated cells and cells lysed with sodium dodecyl sulfate were used to determine 0 and 100% inhibition, respectively.

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