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Antioxidant and anti-tubercular activity of some 3-substituted-1,3-dihydro-2H-indole-2-one derivatives.

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ABSTRACT

A series of 3-substituted-1,3-dihydro-2H-indol-2-one derivatives were synthesized, characterized and evaluated for antioxidant activity and anti-tubercular activity. Antioxidant activity was determined in vitro using DPPH (2,2-diphenyl-1-picrylhydrazyl), butylated hydroxy toluene (BHT) and butylated hydroxy anisol (BHA). Compounds **E9**, **E11**, **E12**, **E14** and **E15** exhibited good antioxidant activity in comparison to positive controls ascorbic acid (AA), butylated hydroxyl toluene (BHT) and butylated hydroxyl anisol (BHA). Preliminary anti-tubercular activity was performed against Mtb H_{37}Rv. Compounds **E5**, **E6**, **E9**, **E10** and **E14** displayed mild anti-tubercular activity in the range of 12.50-50 µM (MIC) on comparison to standards Amikacin, Cycloserine, Ethambutol, Isoniazide, Pyrimethamine and Rifampin.

Keywords: 1,3-dihydro-2H-indol-2-one derivatives, antioxidant, antitubercular, DPPH

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INTRODUCTION

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, and they are known as mediators of intracellular signaling cascades [1]. Excessive ROS produced in most living organisms are metabolized through normal physiological processes. Accumulation of excessive ROS leads to oxidative stress and is also responsible for damaging lipids, proteins and the cells containing DNA [2]. In an effort to reduce the damaging effect of ROS, antioxidants are capable of scavenging excess ROS [3]. The important classes of antioxidants are represented from phenolic compounds [4]. Some of the examples include resveratrol, epigallocatachin and curcumin possessing anticancer and antioxidant properties.

One third of the world's population is thought to have been infected with *M. tuberculosis* [5], with new infections occurring at a rate of about one per second [5]. In 2007, there were an estimated 13.7 million chronic active cases globally [6], while in 2010, there were an estimated 8.8 million new cases and 1.5 million associated deaths, mostly occurring in developing countries [7]. The absolute number of tuberculosis cases has been decreasing since 2006, and new cases have decreased since 2002 [7]. The distribution of tuberculosis is not uniform across the globe; about 80% of the population in many Asian and African countries test positive in tuberculin tests, while only 5–10% of the United States population tests positive [8]. More people in the developing world contract tuberculosis because of compromised immunity, largely due to high rates of HIV infection and the corresponding development of AIDS [9].

A large number of imidazo[2,1-b][1,3,4]thiadiazole have been reported to possess diverse pharmacological properties such as anticancer [10,11], antitubercular [12,13], antibacterial [14,15], antifungal [16,17], anticonvulsant, analgesic [18], antisecretory [19], anti-inflammatory [20-22], cardiotonic [23] diuretic [24], and herbicidal activities [25,26].

Considering the potential effect of imidazo[2,1-b][1,3,4]thiadiazole, we have condensed this scaffold with 1,3-dihydro-2H-indol-2-one with the aim of generating antioxidant and anti-tubercular analogues.

Chemistry

5-amino-2-aralkyl-1,3,4-thiadiazole (**A**), [11] and intermediate compounds 2,6-diphenylimidazo[2,1-*b*][1,3,4]thiadiazole (**C21**) [27], 2-(4-methoxyphenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazole (**C22**) [28], 6-phenyl-2-(3,4,5-trimethoxyphenyl)imidazo [2,1-*b*] [1,3,4] Thiadiazole (**C23**) [29], 2-(4-nitro-phenyl)-6-phenylimidazo [2,1-*b*] [1,3,4]thiadiazole (**C24**) [30], 2-(4-chlorophenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazole (**C25**) [31], 2-(4-chlorophenyl)-6-(4-methoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**C27**), 2-(4-chlorophenyl)-6-(4-methylphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**C28**), 6-phenyl-2-(3,4,5-trimethoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde (**D23**), 2-(4-chlorophenyl)-6-(4-methoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde (**D27**), 2-(4-chlorophenyl)-6-(4-methylphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde (**D28**) [27] were prepared as per literature. Compounds **C1-20**, **D1-20** and **E1-20** were prepared as per literature. [32]

General procedure for the preparation of 2,6-disubstitutedimidazo[2,1-b][1,3,4]thiadiazole (C25 and C29)

The appropriate 2-amino-5-substituted-1,3,4-thiadiazole (**A**) (30 mmol) was treated with the appropriate phenacyl bromide (**B**) (30 mmol), in ethanol (150 mL). The mixture was refluxed for 10-12 h. Excess of solvent was removed under reduced pressure and the solid hydrobromide was separated by filtration, washed with cold ethanol and dried. Neutralization of hydrobromide salts with cold aqueous solution of sodium carbonate yielded the corresponding free base which was filtered with a yield of 52-62 %.

2-(Naphthalen-1-yl-methyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazole (C25*)*

Yield 48 %, IR (KBr cm^{-1}) 3068 (C-H), 2929-2872(C-H), 1621 (C=C), 1460 (C=N). ^{1}H-NMR (δ): 8.61 (s, 1H), 8.14-8.11 (m, 1H), 7.99-7.93 (m, 2H), 7.81-7.79 (m, 2H), 7.66 (d, 2H, J=6), 7.60-7.52 (m, 3H), 7.37 (t, 2H, J=16), 7.26-7.24 (m, 1H), 4.93 (s, 2H, -CH2-).

6-(4-Methylphenyl)-2-(naphthalen-1-yl-methyl)imidazo[2,1-*b*][1,3,4]thiadiazole *(C29)*

Yield 52 %, IR (KBr cm^{-1}) 3080 (C-H), 2924-2871(C-H), 1613 (C=C), 1459 (C=N). ^{1}H-NMR (δ): 8.54 (s, 1H), 8.13-8.12 (m, 1H), 7.99-7.92 (m, 2H), 7.70 (d, 2H, J=8), 7.65-7.63 (m, 1H), 7.58-7.52 (m, 3H), 7.19 (d, 2H, J=8), 4.92 (s, 2H, -CH2-), 2.28 s, 3H, -CH3).

General procedure for the preparation of [2-(4-substitutedbenzyl-6-(4'-substitutedphenylimidazo[2,1-b][1,3,4]thiadiazole-5-carbaldehyde] (D)

The Vilsmeier Haack reagent was prepared at 0-5 °C by dropping $POCl_3$ (2.3 g, 15 mmol) into a stirred solution of DMF (10 mL). The appropriate imidazo[2,1-b][1,3,4]thiadiazole (**C)** (4 mmol) was added slowly to the Vilsmeier reagent while maintaining stirring and cooling for 2 h. Further stirring was continued for 6 h at 80-90°C. The resulting reaction mixture was poured into 100 mL of water; the precipitate was filtered, pressed, suspended in water and neutralized to pH 7 with cold aqueous solution of sodium carbonate. The solid was separated by filtration, washed with water, dried and crystallized from EtOH with a yield of 45-55 %.

2,6-Diphenylimidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde (D21)

Yield 48 %, IR (KBr cm^{-1}) 3051(C-H), 2924-2871(C-H), 1698 (C=O), 1619 (C=C), 1460 (C=N). ^{1}H-NMR (δ): 10.05 (s, 1H, -CHO), 8.00-7.98 (m, 2H), 7.93 (d, 2H, J = 7.6 Hz), 7.56-7.49 (m, 4H), 7.46 (d, 2H, J = 8).

2-(4-Methoxyphenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde (D22)

Yield IR (KBr cm^{-1}) 3100 (C-H), 2951-2801(C-H), 1701 (C=O), 1613 (C=C), 1459 (C=N). ^{1}H-NMR (δ): 10.05 (s, 1H, -CHO), 8.00-7.95 (m, 5H), 7.54 (d, 2H, J = 8 Hz), 7.19 (d, 2H, J = 8), 3.87 (s, 3H, OCH_3).

2-(4-Nitrophenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde (D24)

Yield IR (KBr cm^{-1}) 3071 (C-H), 2906-2899(C-H), 1679 (C=O), 1621 (C=C), 1448 (C=N). ^{1}H-NMR (δ): 10.08 (s, 1H, -CHO), 8.47 (d, 2H, J = 8), 8.32 (d, 2H, J = 8 Hz), 8.02-7.99 (m, 2H), 7.58-7.53 (m, 3H).

2-(Naphthalen-1-ylmethyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde *(*D25*)*

Yield IR (KBr cm^{-1}) 3096 (C-H), 2920-2899(C-H), 1700 (C=O), 1654 (C=C), 1455 (C=N). ^{1}H-NMR (δ): 10.05 (s, 1H, -CHO), 8.07-8.00 (m, 1H), 7.94-7.90 (m, 2H), 7.82-7.77 (m, 2H), 7.58-7.47 (m, 8H), 4.91 (s, 2H, -CH2-).

2-(4-Chlorophenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde *(D26)*

Yield IR (KBr cm^{-1}) 3079 (C-H), 2920-2877 (C-H), 1692 (C=O), 1625 (C=C), 1455 (C=N). ^{1}H-NMR (δ): 10.06 (s, 1H, -CHO), 8.04-7.99 (m, 5H), 7.66 (d, 2H, J = 8 Hz), 7.55 (d, 2H).

6-(4-Methylphenyl)-2-(naphthalen-1-ylmethyl)imidazo[2,1-*b*][1,3,4]thiadiazole-5-carbaldehyde *(D29)*

Yield IR (KBr cm^{-1}) 3092 (C-H), 2936-2857(C-H), 1705 (C=O), 1631 (C=C), 1449 (C=N). ^{1}H-NMR (δ): 9.94 (s, 1H, -CHO), 8.15-8.12 (m, 1H), 8.00-7.94 (m, 2H), 7.80 (d, 2H, J=8), 7.67-7.65 (m, 1H), 7.61-7.55 (m, 3H), 7.30 (d, 2H, J=8), 5.02 (s, 2H, -CH2-), 2.35 (s, 3H, $-CH_3$).

General procedure for the preparation of 3-((2-(4-substitutedbenzyl-6-(4-substitutedphenyl)imidazo[2,1-b][1,3,4]thiadiazol-5-yl)methylidene)-1,3-dihydro-2H-indol-2-ones (E).

The 2-indolinone (10 mmol) was dissolved in methanol (100 ml) and treated with appropriate carbaldehyde **(D)** (10 mmol) and piperidine (1 ml). The reaction mixture was refluxed for 1-5 h (according to a TLC test), cooled and concentrated under reduced pressure. The resulting precipitate was collected by filtration with a yield of 70-85% and it was purified by recrystallisation from ethanol.

3-[(2,6-Diphenylimidazo[2,1-*b*][1,3,4]thiadiazol-5-yl)methylidene]-1,3-dihydro-2*H*-indol-2-one (E21)

Yield 77 %, IR (KBr cm^{-1}) 3208 (N-H), 3055 (C-H), 2924-2875 (C-H), 1708 (C=O), 1613 (C=C), 1459 (C=N). ^{1}H-NMR (δ): 10.66 (s, 1H), 7.89-7.75 (m, 3H), 7.63-7.52 (m, 4H), 7.46 (t, 2H, J=16), 7.38-7.35 (m, 1H), 7.18 (t, 1H, J=16), 6.88 (d, 1H, J=8), 6.82 (d, 1H, J=8), 6.69 (t. 1H, J=16). MS (ESI) m/z: 421.10 (420.49).

3-{[2-(4-Methoxyphenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazol-5-yl]methylidene}-1,3-dihydro-2*H*-indol-2-one (E22)

Yield 70 %, IR (KBr cm^{-1}) 3125 (N-H), 3022 (C-H), 2923-2866 (C-H), 1700 (C=O), 1597 (C=C), 1413 (C=N). ^{1}H-NMR (δ): 10.65 (s, 1H), 7.76-7.73 (m, 4H), 7.62 (s, 1H), 7.45 (t, 2H, J=16), 7.37-7.34 (m, 1H), 7.17 (t, 1H, J=16), 7.10 (d, 2H, J=8), 6.87 (d, 1H, J=8), 6.81 (d, 1H, J=8), 6.69 (t, 1H, J=16), 3.28 (s, 3H, -OCH_3). MS (ESI) m/z: 451.30 (450.51).

3-{[2-(3,4,5-Trimethoxyphenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazol-5-yl]met- hylidene}-1,3-dihydro-2*H*-indol-2-one (E23)

Yield 75%, IR (KBr cm^{-1}) 3140 (N-H), 3011 (C-H), 2936-2731 (C-H) 1701 (C=O), 1603 (C=C), 1477 (C=N). ^{1}H-NMR (δ): 10.66 (s, 1H), 7.78 (d, 2H, J=8), 7.59 (s, 1H), 7.49 (t, 2H, J=16), 7.41-7.38 (m, 1H), 7.19 (t, 1H, J=16), 7.13 (s, 1H), 6.98 (s, 2H), 6.89-6.86 (m, 2H), 6.79 (t, 1H, J=16), 3.74 (s, 6H, 2-OCH_3), 3.72 (s, 3H, OCH_3). MS (ESI) m/z: 511.20 (510.56).

3-{[2-(4-Nitrophenyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazol-5-yl]methylidene}-1,3-dihydro-2*H*-indol-2-one (E24)

Yield 71 %, IR (KBr cm^{-1}) 3180 (N-H), 3092 (C-H), 2935 (C-H), 1670 (C=O), 1599 (C=C), 1520 (-NO_2), 1343(C=N). MS (ESI) m/z: 466.10 (465.48).

3-{[2-(Naphthalen-1-ylmethyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazol-5-yl]meth- ylidene}-1,3-dihydro-2*H*-indol-2-one (E25)

Yield 77 %, IR (KBr cm^{-1}) 3139 (N-H), 3081-3028 (C-H), 2972-2863 (C-H), 1676 (C=O), 1602 (C=C), 1469 (C=N). ^{1}H-NMR (δ): 10.63 (s, 1H), 8.05-7.92 (m, 3H), 7.69 (d, 2H, J=8), 7.60-7.50 (m, 4H), 7.40 (t, 2H, J=16), 7.33-7.29 (m, 1H), 7.19-7.15 (m, 1H), 6.85 (d, 1H, J=8), 6.67-6.64 (m, 2H), 4.89 (s. 2H, -CH_2-). MS (ESI) m/z: 485.2 (484.57).

3-{[6-(4-Chlorophenyl)-2-phenylimidazo[2,1-*b*][1,3,4]thiadiazol-5-yl]methylidene}-1,3 -dihydro-2*H*-indol-2-one (E26)

Yield 79 %, IR (KBr cm^{-1}) 3180 (N-H), 3070-3030 (C-H), 2916-2831 (C-H), 1695 (C=O), 1599 (C=C), 1407 (C=N). ^{1}H-NMR (δ): 10.68 (s, 1H), 7.82 (d, 2H, J=8), 7.78 (d, 2H, J=8), 7.63-7.49 (m, 6H), 7.18 (t, 1H, J=16), 6.87 (d, 1H, J=8), 6.79 (d, 1H, J=8), 6.69 (t, 1H, J=16). MS (ESI) m/z: 455.3 (454.93).

3-{[6-(4-Chlorophenyl)-2-(4-methoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazol-5-yl] methylidene}-1,3-dihydro-2*H*-indol-2-one (E27)

Yield 80 %, IR (KBr cm^{-1}) 3100 (N-H), 3081-3058 (C-H), 2995 (C-H), 1701 (C=O), 1601 (C=C), 1525 (C=N). MS (ESI) m/z: 485.2 (484.96).

3-{[6-(4-Chlorophenyl)-2-(4-methylphenyl)imidazo[2,1-*b*][1,3,4]thiadiazol-5-yl]methylidene}-1,3-dihydro-2*H*-indol-2-one (E28)

Yield 77 %, IR (KBr cm^{-1}) 3100 (N-H), 3079 (C-H), 2952-2859 (C-H), 1698 (C=O), 1601 (C=C), 1480 (C=N). ^{1}H-NMR (δ): 10.65 (s, 1H), 7.72 (d, 2H, J=8), 7.58 (s, 1H), 7.47 (d, 2H, J=8), 7.23-7.15 (m, 5H), 6.85 (d, 1H, J=8), 6.67 (t, 1H, J=16), 6.61 (d, 1H, J=8), 2.28 (s, 3H, -CH_3).

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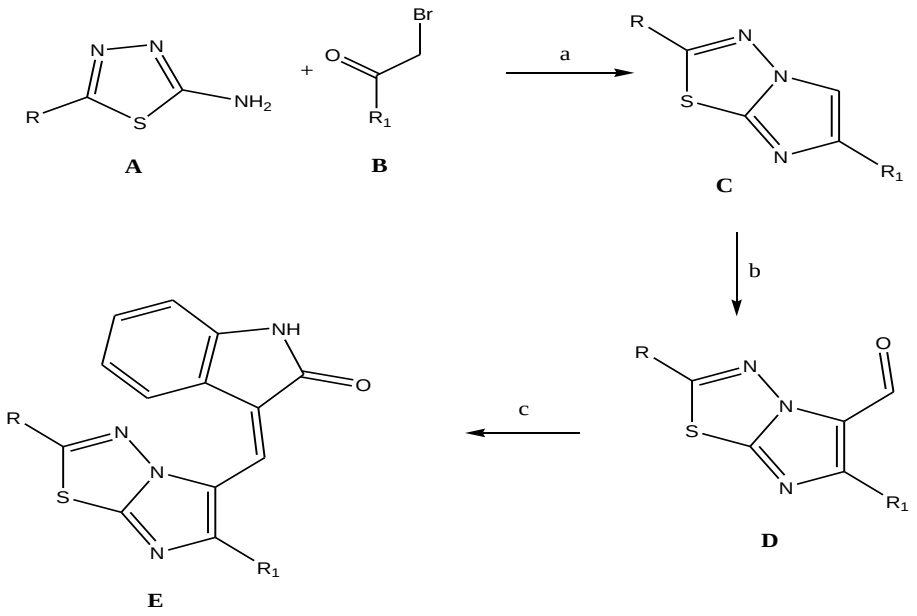
3-{[2-(Naphthalen-1-ylmethyl)-6-(4-methylphenyl)imidazo[2,1-*b*][1,3,4]thiadiazol-5-yl]methylidene}-1,3-dihydro-2*H*-indol-2-one (E29)

Yield 69 %, IR (KBr cm^{-1}) 3140 (N-H), 30-3028 (C-H), 2970-2861 (C-H), 1676 (C=O), 1602 (C=C), 1469 (C=N). ^{1}H-NMR (δ): 10.62 (s, 1H), 8.04-7.92 (m, 3H), 7.59-7.49 (m, 6H), 7.22-7.15 (m, 3H), 6.85 (d, 1H, J=8), 6.67 (d, 2H, J=4), 4.88 (s. 2H, -CH_2-), 2.29 (3H, s, -CH_3). MS (ESI) m/z: 485.2 (484.57).

Scheme



Reagente and conditions: a) alcohol, 12 hrs reflux, Na_2CO_3; **b)** $POCl_3$ + DMF, 80-90 ^{0}C, 3-4 hrs
c) 2-oxindole, CH_3OH, piperidine.

Table: 1

No	R	R_1	No	R	R_1	No	R	R_1
E1	Benzyl	4-Br-ph	**E12**	4-Cl-benzyl	4-NO_2-ph	**E23**	3,4,5-tri-methoxy-ph	ph
E2	Benzyl	4-Cl-ph	**E13**	4-Cl-benzyl	4-Br-ph	**E24**	4-NO_2-ph	ph
E3	Benzyl	4-OCH_3-ph	**E14**	4-Cl-benzyl	4-F-ph	**E25**	Naphthalene-1-yl-CH_2-	ph
E4	Benzyl	4-CH_3-ph	**E15**	4-Cl-benzyl	2*H*-chromen-2-one	**E26**	4-Cl-ph	ph
E5	4-Cl-benzyl	ph	**E16**	CH_3	4-Cl-ph	**E27**	4-Cl-ph	4-OCH_3-ph
E6	4-Cl-benzyl	4-Cl-ph	**E17**	CH_3	4-Br-ph	**E28**	4-Cl-ph	4-CH_3-ph
E7	4-Cl-benzyl	4-CH_3-ph	**E18**	CH_3	4-NO_2-ph	**E29**	Naphthalene-1-yl-CH_2-	4-CH_3-ph
E8	4-Cl-benzyl	4-OCH_3-ph	**E19**	CH_3	4-CH_3-ph			
E9	Benzyl	4-F-ph	**E20**	CH_3	4-OCH_3-ph			
E10	Benzyl	ph	**E21**	Ph	ph			
E11	Benzyl	4-NO_2-ph	**E22**	4-OCH_3-ph	ph			

BIOEVALUATIONS

ANTIOXIDANT ACTIVITY

DPPH radical scavenging assay [33]

1ml (0.1mM) of DPPH (2,2-diphenyl-1-picrylhydrazyl) solution ethanol was added to 3mL of test and reference compound (50µg/mL). The reaction mixture was shaken vigorously and kept in dark at room temperature for 1hr. Absorbance was measured at 517nm using UV-visible spectrophotometer (Shimadzu-Uv-1800). The % scavenging activity was calculated using the following formula:

$$\% SA = (A_0 - A_1) / A_0 X 100,$$

Where, % SA = Percentage scavenging activity.
A_0 = Absorbance of control, A = Absorbance of sample.

Table 2: DPPH scavenging activity of test and reference compounds.

Compound (50µg/mL)	% Scavenging Activity	Compound (50µg/mL)	% Scavenging Activity
E1	35.91±0.236	E19	35.08±2.103
E2	34.53±0.622	E20	35.72±1.504
E3	20.68±0.433	E21	25.11±0.651
E4	36.10±1.018	E22	32.12±0.753
E5	30.04±0.222	E23	36.75±0.912
E6	29.54±0.482	E24	26.37±1.115
E7	36.14±1.103	E25	29.12±0.912
E8	28.09±0.666	E26	22.35±0.835
E9	**48.40±0.458**	E27	33.14±1.125
E10	22.50±0.761	E28	29.15±0.750
E11	**45.12±0.215**	E29	28.15±0.759
E12	**44.06±0.256**	AA	38.67± 0.437
E13	33.47±0.501	BHT	44.16±0.733
E14	**44.16±0.118**	BHA	51.07±0.216
E15	**55.50±0.871**		
E16	33.60±0.590		
E17	21.22±1.116		
E18	30.62±0.806		

All values are expressed in Mean ± SEM of triplicate reading, n=3
AA= Ascorbic acid, BHT= Butylated hydroxy toluene, BHA= Butylated hydroxy anisol

Anti-tubercular activity

Bacteria. MIC screening was conducted for *Mtb* H37Rv (SRI 1345), isoniazid (INH)-resistant Mtb (SRI 1369), rifampin (RMP)-resistant *Mtb* (SRI 1367), and ofloxacin (OFX)-resistant Mtb (SRI 4000). MBC, LORA and intracellular drug screening assays were conducted using only *Mtb* H37Rv (SRI 1345). Subsequent to initial dose response testing, the test compound was subjected to secondary assays for evaluation of anti-mycobacterial activity in a low throughput format. These assays included MIC, MBC, LORA, intracellular (macrophage) drug screening, and MTT cell proliferation.

Minimal Inhibitory Concentration (MIC)

The broth microdilution assay format following guidelines established by the *Clinical and Laboratory Standards Institute* (CLSI) was routinely utilized for MIC testing. Briefly, testing was conducted using 96-well, U-

bottom microplates with an assay volume of 0.2 mL / well. First, the test media, Middlebrook 7H9 broth supplemented with OADC Enrichment (BD BioSciences; Sparks, MD), was added (0.1 mL/well) to each well. The test compounds, solubilized in appropriate solvent and subsequently diluted in test media, were subsequently added (0.1 mL/well) to appropriate wells at twice the intended starting concentration and serially diluted two-fold across the plate. The plates were then inoculated (0.1 mL/well) with a targeted concentration of 1.0×10^6 CFU/mL *M. tuberculosis* and incubated at 37ºC for 7 days in approximately 90% humidity. Following incubation, the plates were read visually and individual wells scored for turbidity, partial clearing or complete clearing. Testing was conducted in duplicate and the following controls were included in each test plate: i) medium only (sterility control); ii) organism in medium (negative control); and iii) rifampin or isoniazid (positive control). The MIC is reported as the lowest concentration (µg/mL) of drug that visually inhibits growth of the organism.

Minimal Bactericidal Concentration (MBC)

The MBC was determined subsequent to MIC testing by sub-culturing diluted aliquots from wells that fail to exhibit macroscopic growth. The sample aliquots were inoculated onto Middlebrook 7H10 agar plates and subsequently incubated for 16-21 days at 37°C. Once growth was readily apparent, the bacterial colonies were enumerated. The MBC is defined as the lowest concentration (µg/mL) of compound exhibiting 99.9% kill over the same time period used to determine the MIC (18-24 h). MBC values greater than 16 times the MIC typically indicate antimicrobial tolerance.

***Low-Oxygen Recovery Assay* (LORA)**

Traditional screening of drugs against *M. tuberculosis* only addresses or targets the organism in an active replicating state. It is well documented that *Mtb* can reside in a state of non-replicating persistence (NRP) which has not been adequately assessed in the development of new antimicrobials. Briefly, microplates were prepared in the same manner as the MIC testing format. Instead of incubating aerobically, the plates are placed under anaerobic conditions using a MACS MIC automated jar gassing system and incubated for 7 days at 37°C. The plates were subsequently transferred to an ambient gaseous condition (5% CO_2) for 7 days after which the plates are read visually and individual wells scored for turbidity, partial clearing or complete clearing. Testing was conducted in duplicate and the following controls were included in each test plate: i) medium only (sterility control); ii) organism in medium (negative control); and iii) rifampin or isoniazid (positive control). Results are reported as the lowest concentration (µg/mL) of drug that visually inhibits growth of the organism.

Intracellular Drug Activity

Briefly, the murine J774 cell line was propagated in RPMI 1640 supplemented with L-glutamine and fetal bovine serum (FBS). Cells were maintained in tissue culture flasks at 37°C in the presence of 5% CO_2. For infection studies, J774 cells were transferred to 12-well tissue culture chambers in 1 mL volumes at a density of 2.0×10^5 in the presence of 10% FBS. After overnight incubation, the medium was replaced with fresh medium containing 1% FBS to stop macrophage division while maintaining cell viability. Twenty-four hours later, the macrophage monolayer was enumerated with an ocular micrometer for total number of cells per well to determine the infection ratio. The medium was removed and replaced with 1 mL of fresh medium with 1% FBS containing *Mtb* at a multiplicity of infection (MOI) of 5 Mycobacteria/macrophage. The cells are infected for 4 h after which time nonphagocytosed Mycobacteria were washed from the monolayers and fresh medium added. Drugs were then added, using 3 concentrations, and infection allowed to proceed for 7 days. At 0 and 7 days, the macrophages were lysed with sodium dodecyl sulfate, treated with DNAase, diluted and plated onto 7H10 agar to determine the cell number or colony forming units (CFU). Each drug concentration wastested in duplicate and rifampin was used as the positive control drug. A drug cytotoxicity control plate assay (MTT proliferation) was also conducted in parallel using uninfected macrophages to confirm that concentrations utilized for testing were not toxic to the macrophages.

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Table 3: Antitubercular activity of test and reference compounds

Compound code	MIC (µM)
E1	NA
E3	NA
E4	NA
E5	12.50
E6	25.00
E8	NA
E9	50.00
E10	25.00
E11	NA
E12	NA
E13	NA
E14	25.00
E15	NA
Amikacin	0.16
Cycloserine	50
Ethambutol	6.25
INH	0.31
Pyrimethamine	50
Rifampin	0.04

Table 4: MIC and MBC results

Compound	MIC	% inhibition[a]	MBC $H_{37}Rv$ (µg/mL)	MIC INH-R[b] (µg/mL)	% inhibition	MIC RMP-R[c] (µg/mL)	% inhibition	MIC RMP-R[d] (µg/mL)	% inhibition
E5	1.56	46	NA	>50	--	>50	--	>50	--
E6	1.56-3.125	44-51	NA	>50	--	>50	--	>50	--
E14	3.125	54	NA	>50	--	>50	--	>50	--
Rifampin (positive control)	0.098	74	1.56	0.05	75	NA	NA	NA	NA
Isoniazid (positive control)	NA	NA	NA	NA	NA	0.02	70	0.05	86

[a]Percent inhibition at the MIC concentration [b]INH-R= Isoniazid Resistance [c]RMP-R= Rifampin Resistance
[d]OFX-R= Ofloxacin Resistance [e]NA= Not Applicable:Conony Counts above the established rejection value of ≥40
fNA=Not Applicable: Compound not used in assay

Table 5: MBC results

Compound	MBC (µg/mL)	Colony Count (CFU)
E5	--[a]	TNTC[b]
E6	--	TNTC
E14	--	TNTC
Rifampin[c]	1.56	15

[a]—MBC above the established rejection value of ≥ 40 colonies [b]TNTC = Too Numerous To Count

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Table 6: LORA, Macrophage and MTT Results

Compound	LORA µg/mL	Macrophage log reduction (low conc.)	Macrophage log reduction (mid conc.)	Macrophage log reduction (high conc.)	MTT % viability (low conc.)	MTT % viability (mid conc.)	MTT % viability (high conc.)
E5	>50	0.69	0.53	0.29	84	79	52
E6	>50	0.71	0.38	0.38	59	24	7
E14	>50	0.53	0.38	0.64	78	47	13
Rifampin (Positive Control)	0.78	0.46	1.72	2.32	90	89	85

RESULTS AND DISCUSSION

Chemistry

Thirty two compounds (**E1-E29**) were prepared as per the methodologies outlined in **Scheme** and **Table 1.** by reacting formyl compound (**D**) with indolin-2-one in methanol with catalytic amount of piperidine. The required α-bromoketones were prepared according to the literature by using bromine in acetic acid. Compounds 2-alkyl or 2-aralkyl-6-arylimidazo[2,1-*b*][1,3,4] thiadiazoles (**C**) were prepared by reacting 2-amino-1,3,4-thiadiazole (**A**) with phenacyl bromides (**B**) in anhydrous alcohol. Formyl compounds (**D**) were prepared by reacting 2-alkyl or 2-aralkyl-6-arylimidazo[2,1-*b*][1,3,4] thiadiazoles (**C**) with $POCl_3$ and DMF by Vilsmeier-Haak reaction.

Compounds namely **E21-E29** exhibited absorption bands for N-H stretching from 3208 to 3125 cm^{-1} and 1708-1678 cm^{-1} for C=O functional group. All the compounds showed vibration bands between 3100-3011 for C-H aromatic stretching and for aliphatic C-H stretching from 2978-2731. Compounds D21 to D32 showed vibration bands at 1705-1679 cm^{-1} for CHO in their respective IR spectra.

In 1H NMR spectrum of compounds C25 and C32, the presence of singlet between δ 8.61 and 8.54 ppm for the imidazole proton (C5-H) confirmed the cyclization of 2-amino-1,3,4-thiadiazole **A** with respective phenacyl bromide (**B)**. All the 5-formyl substituted derivatives (**D21-29**) showed the presence of singlet peak between δ 10.08-9.94 ppm in their respective spectra. All the compounds showed prominent signals for aromatic protons around 8.61-6.59 ppm. Bridge headed methylene proton at C2 appeared between δ 5.02 to 4.45 ppm for all derivatives. The compounds **D22, E22** and **E23** showed OCH3 proton between δ 3.87 - 3.28 ppm. Compounds **C29, D29,** and **E28** showed a methyl proton between δ 2.35-2.28 ppm as singlet. The structures of all the compounds were finally ascertained by mass spectra.

ANTIOXIDANT ACTIVITIES

All of the synthesized derivatives were evaluated for their free radical scavenging ability and compared with positive controls—ascorbic acid (AA) and butylated hydroxy toluene (BHT), butylated hydroxyl anisol (BHA) and the results are shown in **Table 2**. Compound **E15** showed the highest radical scavenging activities on comparing to all standards. Whereas derivatives **E9, E11, E12** and **E14** demonstrated greater radical scavenging activity than the ascorbic acid.

ANTI-TUBERCULAR ACTIVITY

MIC. The MIC for each compound was determined by testing ten, two-fold dilutions. The MIC is reported as the lowest concentration (µg/mL) of drug that visually inhibited growth of the organism. In addition, the percentage of inhibition at the MIC is provided. Compounds **E5**, **E6**, **E9**, **E10** and **E14** displayed mild anti-tubercular activity in the range of 12.50-50 µM (MIC) on comparison to standards Amikacin, Cycloserine, Ethambutol, Isoniazide, Pyrimethamine and Rifampin (**Table 3**).

MBC. The established rejection value of >40 colonies for the MBC assay was based on the calculated concentration of *Mtb* in the MIC plates. Results, reported as µg/mL concentration, are determined based on

Colony Forming Units (CFUs) enumerated from agar plates. Only agar plates with countable colonies have reportable counts. If a compound lacks bactericidal activity, many times the CFUs are too numerous to count (TNTC) and are thus reported as such. Results are provided in **Tables 4** & **5** and none of the compounds exhibited minimal bactericidal concentration on comparison to standard Rifampin.

***LORA*.** Results for the LORA assay are reported as the lowest concentration (μg/mL) of drug that visually inhibited growth of the organism. The data is presented in **Table 6**. Compounds failed to display any LORA activity on comparison to positive control Rifampin.

***Intracellular Drug Activity*.** Intracellular drug activity is reported as log reduction values calculated as reduction in *Mtb* concentration from zero hour to 7 days post-infection. The three concentrations chosen were based on the MIC data generated in the HTS primary screen. The mid concentration bracketed the reported MIC with the lower concentration ten-fold below the mid and the higher concentration ten-fold above the mid. The data is presented in **Table 6**. Drug cytotoxicity is reported as cell proliferation, or percentage of viability. This data is also presented in **Table 6**.

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