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Original article

Synthesis and antimycobacterial activity of *N*′-[(*E*)-(monosubstituted-benzylidene)]-2-pyrazinecarbohydrazide derivatives

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ABSTRACT

The present article describes a series of twenty-six *N*′-[(*E*)-(monosubstituted-benzylidene)]-2-pyrazinecarbohydrazide (**4**–**29**), which were synthesized and evaluated for their cell viabilities in non infected and infected macrophages with *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG). Afterwards, the non-cytotoxic compounds (**4**, **6**, **8**, **15**, **21**, **23**, **24**, **27** and **28**) were assessed against *Mycobacterium tuberculosis* ATCC 27294 using the micro plate Alamar Blue assay (MABA) and the activity expressed as the minimum inhibitory concentration (MIC) in μg/mL. The compounds **6**, **23**, **27** and **28** exhibited a significant activity (50–100 μg/mL) when compared with first line drugs such as pyrazinamide and were not cytotoxic in their respective MIC values.

1. Introduction

Tuberculosis (TB) is a serious public health problem and it is estimated that one-third of the world's population is currently infected with TB bacillus and there are 2.0 million annual deaths [1]. Some facts contribute to the alarming global scenery of TB such as, the low financial investment and the advent of strains that cannot be cured by standard anti-tuberculosis drug treatment. For example, it has been nearly 40 years since the introduction of a new class of compounds for the treatment of TB. In this context, there is an urgent need for new drugs to fight against this disease.

Furthermore, it is particularly alarming the advent of strains, which are resistant to at least two major anti-tuberculosis drugs, isoniazid and rifampicin. This kind of resistance has been called multidrug-resistant tuberculosis (MDR-TB). The spread of MDR-TB could cost between 100 and 1400 times the available treatment costs and further threatens to make TB incurable. Exact data are hard to estimate, but at least 4% of all world-wide TB patients are resistant to at least one of the current first line drugs [2].

Another serious problem is the XDR-TB (extensively drug-resistant tuberculosis, which is strains resistant to first and second line anti-TB drugs) that raises concerns of a future TB epidemic which, restricted treatment options. The true scale of XDR-TB is unknown due to many countries lack the necessary equipment and capacity to accurately diagnose it. However, it is estimated that there are around 40,000 cases per year [3]. Due to the importance of TB nowadays, there is an urgent need for new drugs to treat MDR-TB and now XDR-TB. In this context, the pyrazine nucleus is an important heteroaromatic class of compounds, which is present in many flavorings [4], natural [5,6] and synthetic products with a wide range of pharmacological activities, as anti-inflammatory, anticancer, anti-diabetic, sedative and antibacterial [7].

Another important application of this nucleus is the PZA (pyrazinamide) (Fig. 1), a first-line drug used in TB treatment [8,9]. PZA is a nicotinamide analog and a pro-drug with an excellent sterilizing effect on semidormant tubercle bacilli. This effect is responsible to decrease the time of treatment from twelve to six months. Into the bacillus, PZA is converted by the mycobacterial enzyme pyrazinamidase to its active form pyrazinoic acid (POA).

Experimental evidences suggest that PZA diffuse into *Mycobacterium tuberculosis* through passive conduction and after that it is converted in POA by pyramidase, which promotes an accumulation of this metabolite in the mycobacterial cytoplasm. This accumulation is possible because *M. tuberculosis* has an inefficient efflux system [10] thereby occurs a decrease of the intracellular pH. The inhibition of the growth of bacteria is caused due to the

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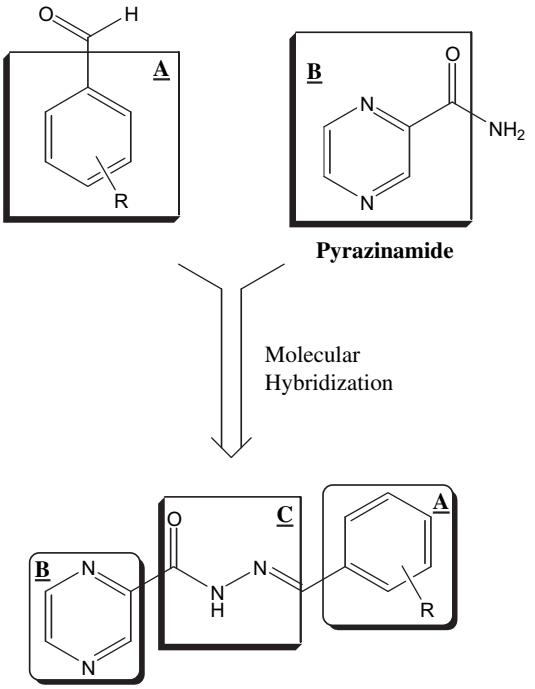


Fig. 1. Design concept of *N*-acylhidrazones pyrazine derivatives.

deactivation of fatty acid synthetase enzyme [11]. Alterations in the gene responsible for the synthesis of pyramidase are described in the literature as the principal mechanism of resistance against pyrazinamide [12,13].

In our continuous program in the search of new candidates to antitubercular agents, we proposed the synthesis of some *N*-acylhydrazones contained the pyrazine nucleus that was designed by molecular hybridization (Fig. 1). According to the literature, many *N*-acylhydrazones are described with a wide range of pharmacological activities, such as antibacterial agents [14,15]. For example, the isoniazid derivatives prepared by our group, exhibits minimum inhibitory concentrations (MIC) comparable with the first line anti-TB drugs as isoniazid and rifampicin [16–19].

The design concept of these compounds explores the introduction of monosubstituted benzaldehydes moieties (**A**) into pyrazine core (**B**) to afford *N*-acylhydrazones groups (**C**). This modification aims to evaluate the cytotoxic effects of these compounds, their selectivity against *M. tuberculosis* and their mechanism of action. Furthermore, we investigated the influence of some substituents in phenyl ring (**B**) on cytotoxicity and biological activity of these compounds in health and infected macrophages.

2. Results and discussion

2.1. Chemistry

The synthetic route for preparation of (monosubstituted-benzylidene)-2-pyrazinecarbohydrazide derivatives **4**–**29** are summarized in Scheme 1. The compound **2** was obtained from 2-pyrazinecarboxylic acid **1** using $SOCl_2$ and MeOH. This compound was confirmed by ^{1}H NMR spectrum due to the presence of a signal at 3.93 ppm as a singlet relative to methyl hydrogens. In addition, the compound **3** was prepared by reaction of compound **2** with hydrazine hydrate under reflux. In ^{1}H NMR spectrum we observed the disappearance of that singlet relative to methyl group and appear a singlet at 4.70 ppm relative to CONH proton. After that, the compounds **4**–**29** were obtained through reaction between the compound **3** and appropriated benzaldehydes as it is described in experimental section.

In general, ^{1}H NMR spectra **4**–**29** compounds showed the signals of the respective protons of the synthesized compounds, which were verified on the basis of their chemicals shifts, multiplicities and coupling constants. These spectra showed two characteristic signals about N=CH proton at 8.60–9.10 ppm and CONH protons at 11.90–12.70 ppm. The ^{13}C NMR spectra showed the C=O and C=N signals at 162.1–159.6 and 143.4–142.7 ppm, respectively (Table 1).

2.2. Cell viability assay

The cellular viability in the presence and absence of test compound (**4**–**29**) was determined by Mosmans's MTT (3-(4,5-demethylthylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide; Merck) microcultured tetrazolium assay [26,27]. The results were represented as percentage cell viability (Table 2). This table shows that the compounds **4**, **6**, **8**, **11**, **12**, **14**, **15**, **20**, **21**, **27** and **28** did not kill more than 10 percent of the host cells in the minimum concentration tested. These compounds were selected to be tested in macrophages infected with BCG (Table 3).

The purpose of this test is to evaluate the action of these compounds against macrophages that show their metabolism changed after infection. Therefore, the derivatives **4**, **6**, **8**, **15**, **18**, **23**, **24**, **27** and **28** were not cytotoxic, due to they did not kill more than 10% of the cells at the minimum concentration tested.

2.3. Antimycobacterial activity

After to evaluate the cell viability in non infected or infected macrophages with *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) in the presence and absence of test compounds (**4**–**29**), only the derivatives **4**, **6**, **8**, **15**, **18**, **23**, **24**, **27**, and **28** were submitted to antimycobacterial evaluation.

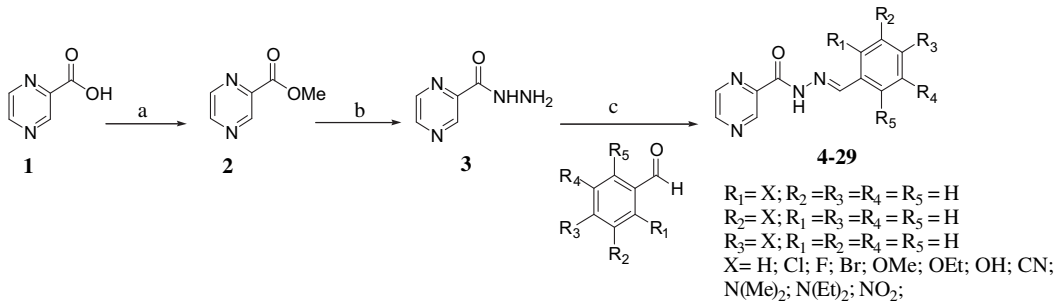
The antimycobacterial activities of these compounds were assessed against *M. tuberculosis* ATCC 27294[28] using the micro plate Alamar Blue assay (MABA) [29] (Table 4). This methodology is nontoxic, uses a thermally-stable reagent and shows good correlation with proportional and BACTEC radiometric methods [30,31].

These results showed that only compounds **6**, **8**, **23**, **27** and **28** exhibited antimycobacterial activity between 50 and 100 μg/mL. Nevertheless, if we analyze Tables 3 and 4, we could verify that the compound **8** was cytotoxic (80% cell viability) in its respective MIC (100 μg/mL).

Moreover, we can observe that the active compounds (**6**, **23**, **27** and **28**) have electron withdrawing groups in ortho or meta positions that indicates the position of the group in the ring is important for the biological activity in this series of compounds. Unfortunately, *c* log *P* values not show a linear correlation with the biological activity in this series.

3. Conclusion

The synthesis of twenty-six *N*′-[(*E*)-(monosubstituted-benzylidene)]-2-pyrazinecarbohydrazide derivatives (**4**–**29**) was performed in good yields (50–90%), among them fifteen are new compounds (**8**, **9**, **11**, **12**, **13**, **14**, **15**, **17**, **18**, **20**, **22**, **23**, **24**, **27** and **28**). All these compounds were submitted to cell viability evaluation, but only eighth derivatives (**4**, **6**, **8**, **15**, **21**, **23**, **24**, **27** and **28**) were not cytotoxic in non infected or infected macrophages with *M. bovis* Bacillus Calmette–Guerin (BCG). In relation to the antimycobacterial activity, it was found that the compounds **6**, **8**, **23**, **27** and **28** (50–100 μg/mL) exhibited activities better than PZA (>100 μg/mL), when used Alamar Blue assay. These results suggest promising perspectives for MDR/XDR-TB, therefore it is necessary



Scheme 1. Reagents and conditions: (a) MeOH, $SOCl_2$, r.t, 1 h, 78%; (b) $N_2H_4.H_2O$ (55%), EtOH, 80 °C, 2 h, 77%; (c) EtOH/H_2O, r.t, 4–24 h, 50–90%.

to examine the mechanism of action in detail to ascertain the reason for the increased activity of the *N*-acylhydrazones.

Currently, we are performing specific tests to determine the probable mechanism of action of these compounds and if exists a possible synergism amongst pyrazine core and *N*-acylhydrazone group in the biological activity of this series.

4. Experimental

4.1. General procedures

Melting points were determined on a Buchi apparatus and are uncorrected. Infrared spectra were recorded on a Thermo Nicolet Nexus 670 spectrometer as potassium bromide pellets and frequencies are expressed in cm^{-1}. Mass spectra (ESI assay in solution of ammonium chloride) were recorded on Micromass ZQ Waters mass spectrometer. NMR spectra were recorded on a Bruker Avance 400 operating at 400.00 MHz (^1H) and 100.0 MHz (^{13}C) and Bruker Avance 500 spectrometer operating at 500.00 MHz (^1H) and 125.0 MHz (^{13}C), in deuterated dimethylsulfoxide. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane and *J*-coupling in Hertz (Hz). Proton and carbon spectra were typically obtained at room temperature. For TLC plates coated with silica gel were run in chloroform/methanol mixture and spots were developed in ultraviolet and solution of ninhidrine (0.2% p/v in ethanol).

4.1.1. Synthesis of methyl 2-pyrazinecarboxylate (**2**)

The methyl 2-pyrazinecarboxylate **2** was prepared by slow addition (during 15 min) of $SOCl_2$ (3.0 equiv., 24.3 mmol) in MeOH (20 mL) under stirring at 0 °C, followed by the introduction of the compound **1** (1.0 g, 8.1 mmol). The temperature of the reaction mixture was then, increased from 0 °C to the room temperature and after 1 h, the excess of $SOCl_2$ was removed under reduced pressure. The crude product was neutralized with saturated aqueous solution of $NaHCO_3$ (60 mL) and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic phases were dried over Na_2SO_4 and concentrated under reduced pressure to afford only the desired product **2** as a solid. This compound was used as such in the synthesis of 2-pyrazinehidrazide without further purification. Yield: (0.89 g, 80%) mp: 59–60 °C (62 °C) [32].

Table 1
Melting points and yields of *N*′-[(*E*)-(monosubstituted-benzylidene)]-2-pyrazinecarbohydrazide (**4**–**29**).

Compound	Substituents					Yield (%)	mp (°C)
	R_1	R_2	R_3	R_4	R_5		
4	H	H	H	H	H	50	204[20]
5	Cl	H	H	H	H	58	175–176[21]
6	H	Cl	H	H	H	90	203[22]
7	H	H	Cl	H	H	79	215–216[22]
8	F	H	H	H	H	73	218–219
9	H	F	H	H	H	75	216–217
10	H	H	F	H	H	58	195–197[20]
11	Br	H	H	H	H	52	175–176
12	H	Br	H	H	H	75	178–179
13	H	H	Br	H	H	71	221–223
14	OMe	H	H	H	H	52	180–181
15	H	OMe	H	H	H	50	160–161
16	H	H	OMe	H	H	65	190–191[23]
17	H	OEt	H	H	H	76	168–169
18	H	H	OEt	H	H	79	224–225
19	OH	H	H	H	H	65	175–176[20]
20	H	OH	H	H	H	73	230–231
21	H	H	OH	H	H	89	252–253[20]
22	CN	H	H	H	H	80	210–211
23	H	CN	H	H	H	88	200–201
24	H	H	CN	H	H	80	247–248
25	H	H	N $(Me)_2$	H	H	72	244–245[24]
26	H	H	N $(Et)_2$	H	H	73	141–142[25]
27	NO_2	H	H	H	H	72	182–183
28	H	NO_2	H	H	H	62	230–231
29	H	H	NO_2	H	H	67	262–263[20]

Table 2
Data of the cellular viability for a macrophage cell line J774 (ATCC TIB-67™) by Mosmans's assay.

Compound	% Cell viability/dose (μg/mL)		
	50	100	150
4	100	75	100
5	70	66	69
6	100	100	100
7	83	64	56
8	100	100	81
9	49	46	40
10	78	69	71
11	98	100	91
12	100	100	93
13	100	100	94
14	97	100	97
15	100	100	97
16	74	55	55
17	74	71	69
18	100	98	100
19	1	0.3	0
20	100	100	88
21	100	81	80
22	92	75	70
23	100	100	100
24	100	100	100
25	93	87	89
26	97	92	93
27	90	59	46
28	100	85	76
29	50	44	44
Pyrazinamide	100	100	100

Table 3
Data of the cellular viability for a macrophage cell line J774 infected (ATCC TIB-67™) with BCG by Mosmanśs assay.

Compound	% Cell viability/dose (μg/mL)		
	50	100	150
4	100	88	80
6	100	96	94
8	100	80	88
11	73	64	52
12	87	96	56
13	52	56	54
14	87	54	56
15	100	98	87
18	90	85	88
20	73	64	57
21	100	72	91
22	80	73	75
23	100	98	89
24	100	98	100
25	80	73	71
26	85	84	78
27	100	94	88
28	92	92	72
Pyrazinamide	92	87	67

*4.1.2. Synthesis of 2-pyrazinehidrazide (**3**)*

The 2-pyrazinehidrazide **3** were prepared by addition of $N_2H_4 \cdot H_2O$ (25%, 20 equiv., 144 mmol) and 36 mL ethanolic solution of compound **2** (1.0 g, 7.2 mmol) and refluxed for 2 h. The solvent was removed under reduce pressure and the residue was purified by washing cold Et_2O (30 mL) to afford the compound **3** as solid. Yield: (0.81 g, 80%), mp: 158 °C (158–159 °C) [33].

4.1.3. General procedures of synthesis of N′*-[(*E*) -(monosubstituted-benzylidene)]-2-pyrazinecarbohydrazide **4–29***

The synthesis of *N′-(E)*-(monosubstituted-benzylidene)-2-pyrazinecarbohydrazide **4**–**29** were prepared by reaction between compound **3** (1.0 equiv.) and the appropriate benzaldehyde (1.2 equiv.) in mixture of ethanol and water distillate (1:1, 10 mL). Initially dissolving **3** in water distillate (5 mL) and adding the respective benzaldehyde in ethanol (5 mL). After stirring for 4–24 h at room temperature, the resulting mixture was concentrated under reduced pressure and the residue purified by washing with cold Et_2O (3 × 10 mL), leading the pure derivatives **4**–**29** as a solid in 50–90% yields.

4.1.3.1. N′*-[(*E*)-phenylmethylidene]-2-pyrazinecarbohydrazide (**4**).* Yield: 50%; mp: 204 °C; (228–230 °C) [20].

4.1.3.2. N′*-[(*E*)-(2-chlorophenyl)methylidene]-2-pyrazinecarbohydrazide (**5**).* Yield: 58%; mp: 175–176 °C; (180–182 °C) [21].

Table 4
The *in vitro* activity of compounds **4**, **6**, **8**, **15**, **18**, **23**, **24**, **27**, and **28** against *M. tuberculosis* $H_{37}R_v$ strain (ATCC 27294), susceptible both to rifampicin and isoniazid.

Compounds	MIC[a]	*C* Log *P*[b]
4	>100	1.63
6	50	2.29
8	100	1.75
15	>100	1.66
18	>100	2.06
23	50	1.36
24	>100	1.39
27	50	1.54
28	100	1.57
Pyrazinamide	>100	−0.71

[a] Minimum inhibitory concentration.
[b] Calculated using online www.molinspiration.com site.

4.1.3.3. N′*-[(*E*)-(3-chlorophenyl)methylidene]-2-pyrazinecarbohydrazide (**6**).* Yield: 90%; mp: 203 °C; (209–211 °C)[22].

4.1.3.4. N′*-[(*E*)-(4-chlorophenyl)methylidene]-2-pyrazinecarbohydrazide (**7**).* Yield: 79%; mp: 215–216 °C; (240–242 °C) [22].

4.1.3.5. N′*-[(*E*)-(2-fluorophenyl)methylidene]-2-pyrazinecarbohydrazide (**8**).* Yield: 73%; mp: 218–219 °C.

^{1}H NMR (400MHz, DMSO-d_6) δ: 12.52(1H; s; NH); 9.29(1H; d; J = 1.3 Hz; H_3); 8.94(1H; d; J = 2.4 Hz; H_6); 8.92(1H; s; N=CH); 8.81(1H; dd; J = 2.4 and 1.3 Hz; H_5); 8.01–7.96(1H; m; $H_{3'}$); 7.54–7.49(1H; m; $H_{6'}$); 7.34–7.29(2H; m; $H_{4'}$ and $H_{5'}$) ppm.

^{13}C NMR (100MHz, DMSO-d_6) δ: 162.1; 159.7; 147.9; 144.5; 144.1; 143.3; 142.7; 132.3; 126.9; 124.9; 121.8; 116.1 ppm.

MS/ESI: [M-H]:243.

IR ν_{max} (cm^{-1}; KBr pellets): 3308 (N–H); 1683 (C=O).

4.1.3.6. N′*-[(*E*)-(3-fluorophenyl)methylidene]-2-pyrazinecarbohydrazide (**9**).* Yield: 75%; mp: 216–217 °C.

^{1}H NMR (400MHz, DMSO-d_6) δ: 12.41(1H; s; NH); 9.27(1H; d; J = 1.2 Hz; H_3); 8.94(1H; d; J = 2.4 Hz; H_6); 8.80(1H; dd; J = 2.4 and 1.2 Hz; H_5); 8.66(1H; s; N=CH); 7.58–7.50(3H; m; $H_{4'}$; $H_{5'}$ and $H_{6'}$); 7.33–7.28(1H; m; $H_{2'}$)ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 159.7; 148.1; 147.9; 144.5; 144.2; 143.4; 136.6; 132.9; 131.1; 129.2; 126.5; 122.2 ppm; MS/ESI: [M-H]: 243. IR ν_{max} (cm^{-1}; KBr pellets): 3308 (N–H); 1683 (C=O).

4.1.3.7. N′*-[(*E*)-(4-fluorophenyl)methylidene]-2-pyrazinecarbohydrazide (**10**).* Yield: 58%; mp: 195–197 °C; (210–212 °C) [20].

4.1.3.8. N′*-[(*E*)-(2-bromophenyl)methylidene]-2-pyrazinecarbohydrazide (**11**).* Yield: 52%; mp: 175–176 °C.

^{1}H NMR (400MHz, DMSO-d_6) δ: 12.68(1H; s; NH); 9.28(1H; d; J = 1.0 Hz; H_3); 9.05(1H; s; N=CH); 8.94(1H; d; J = 2.4 Hz; H_6); 8.81(1H; s; H_5); 8.04(1H; dd; J = 7.8 and 1.4 Hz; $H_{3'}$); 7.72(1H; d; J = 7.8 Hz; $H_{6'}$); 7.50(1H; t; J = 7.4 Hz; $H_{5'}$); 7.40(1H; ddd; J = 7.8; 7.4 and 1.4 Hz; $H_{4'}$) ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 160.3; 148.8; 148.2; 144.7; 144.4; 143.7; 133.5; 133.2; 132.4; 128.5; 127.8; 124.1 ppm; MS/ESI: [M-H]: 303. IR ν_{max} (cm^{-1}; KBr pellets): 3307 (N–H); 1703 (C=O).

4.1.3.9. N′*-[(*E*)-(3-bromophenyl)methylidene]-2-pyrazinecarbohydrazide (**12**).* Yield: 75%; mp: 178–179 °C.

^{1}H NMR (400MHz, DMSO-d_6) δ: 12.44(1H; s; NH); 9.28(1H; d; J = 1.4 Hz; H_3); 8.94(1H; d; J = 2.4 Hz; H_6); 8.80(1H; dd; J = 2.4 and 1.4 Hz; H_5); 8.62(1H; s; N=CH); 7.92(1H; d; J = 1.0 Hz; $H_{2'}$); 7.73(1H; d; J = 7.9 Hz; $H_{4'}$); 7.66(1H; dd; J = 8.0 and 1.0 Hz; $H_{6'}$); 7.45(1H; t; J = 7.8 Hz; $H_{5'}$) ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 159.7; 148.1; 147.9; 144.5; 144.2; 143.4; 136.6; 132.9; 131.1; 129.2; 126.5; 122.2 ppm; MS/ESI: [M-H]:303. IR ν_{max} (cm^{-1}; KBr pellets): 3297 (N–H); 1698 (C=O).

4.1.3.10. N′*-[(*E*)-(4-bromophenyl)methylidene]-2-pyrazinecarbohydrazide (**13**).* Yield: 71%; mp: 221–223 °C.

^{1}H NMR (400MHz, DMSO-d_6) δ: 12.37(1H; s; NH); 9.27(1H; d; J = 1.4 Hz; H_3); 8.93(1H; d; J = 2.5 Hz; H_6); 8.79(1H; dd; J = 2.5 and 1.4 Hz; H_5); 8.62(1H; s; N=CH); 7.68(4H; m; $H_{2'}$; $H_{3'}$; $H_{5'}$ and $H_{6'}$)ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 159.6; 148.6; 147.9; 144.6; 144.1; 143.3; 133.4; 132.0; 130.2; 123.6 ppm; MS/ESI: [M-H]: 303. IR ν_{max} (cm^{-1}; KBr pellets): 3302 (N–H); 1690 (C=O).

4.1.3.11. N′*-[(*E*)-(2-methoxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**14**).* Yield: 52%; mp: 180–181 °C.

^{1}H NMR (400MHz, DMSO-d_6) δ: 12.35(1H; s; NH); 9.26(1H; s; H_3); 9.00(1H; s; H_6); 8.92(1H; s; H_5); 8.80(1H; s; N=CH); 7.91(1H;

d; $J = 7.5$ Hz; $H_{6'}$); 7.45(1H; t; $J = 7.5$; $H_{4'}$); 7.12(1H; d; $J = 7.5$ Hz; $H_{3'}$); 7.04(1H; t; $J = 7.5$ Hz; $H_{5'}$); 3.87(3H; s; OCH_3) ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 160.5; 148.8; 148.2; 144.7; 144.4; 143.7; 133.5; 133.2; 132.2; 128.4; 127.8; 124.1; 55.4 ppm; MS/ESI: [M-H]: 255. IR ν_{max} (cm^{-1}; KBr pellets): 3300 (N–H); 1680 (C=O).

4.1.3.12. N′-*[(E)-(3-methoxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**15**).* Yield: 50%; mp: 160–161 °C.

^1H NMR (400MHz, DMSO-d_6) δ: 12.29(1H; s; NH); 9.28(1H; d; $J = 1.4$ Hz; H_3); 8.93(1H; d; $J = 2.4$ Hz; H_6); 8.80(1H; dd; $J = 2.4$ and 1.4 Hz; H_5); 8.63(1H; s; N=CH); 7.40(1H; t; $J = 8.0$ Hz; $H_{5'}$); 7.30–7.28(2H; m; $H_{2'}$ and $H_{4'}$); 7.04(1H; dd; $J = 8.0$ and 2.0 Hz; $H_{6'}$); 3.82(3H; s; OCH_3) ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 159.5; 149.7; 147.8; 144.6; 144.1; 143.3; 135.5; 130.0; 120.2; 116.5; 111.3; 55.2 ppm; MS/ESI: [M-H]: 255. IR ν_{max} (cm^{-1}; KBr pellets): 3298 (N–H); 1674 (C=O).

4.1.3.13. N′-*[(E)-(4-methoxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**16**).* Yield: 65%; mp: 190–191 °C; (223–224 °C) [24].

4.1.3.14. N′-*[(E)-(3-ethoxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**17**).* Yield: 76%; mp: 168–169 °C.

^1H NMR (400MHz, DMSO-d_6): δ: 12.30(1H; s; NH); 9.27 (1H; d; $J = 1.3$ Hz; H_3); 8.93 (1H; d; $J = 2.4$ Hz; H_6); 8.39 (1H; dd; $J = 2.4$ and 1.3 Hz; H_5); 8.61(1H; s; N=CH); 7.37 (1H; t; $J = 7.7$ Hz; $H_{5'}$); 7.30–7.27 (2H, m; $H_{2'}$ and $H_{6'}$); 7.02 (1H; dd; $J = 7.4$ and 1.8 Hz; $H_{4'}$); 4.08 (2H; q; $J = 13.9$ and 6.9 Hz; OCH_2CH_3); 1.35 (3H; t; $J = 6.9$ Hz; OCH_2CH_3) ppm.

^{13}C NMR (100MHz, DMSO-d_6) δ: 159.6; 158.8; 149.8; 147.9; 144.7; 144.1; 143.3; 135.6; 130.0; 120.1; 116.9; 111.9; 63.2; 14.6 ppm; MS/ESI: [M-H]: 269. IR ν_{max} (cm^{-1}; KBr pellets): 3302 (N–H); 1686 (C=O).

4.1.3.15. N′-*[(E)-(4-ethoxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**18**).* Yield: 79%; mp: 224–225 °C.

^1H NMR (500MHz, DMSO-d_6): δ: 12.15 (1H; s; NH); 9.25 (1H; d; $J = 1.3$ Hz; H_3); 8.92 (1H; d; $J = 2.4$ Hz; H_6); 8.79 (1H; m; H_5); 8.58 (1H; s; N=CH); 7.66 (2H; d; $J = 8.7$ Hz; $H_{2'}$ and $H_{6'}$); 7.01 (2H, d; $J = 8.7$ Hz; $H_{3'}$ and $H_{5'}$); 4.09 (2H; q; $J = 13.7$ and 7.0 Hz; OCH_2CH_3); 1.35 (3H; t; $J = 7.0$ Hz; OCH_2CH_3) ppm.

^{13}C NMR (125MHz, DMSO-d_6) δ: 159.1; 149.6; 147.6; 144.7; 143.9; 143.2; 129.9; 128.8; 126.4; 114.7; 63.2; 14.5 ppm.

MS/ESI: [M-H]: 269.

IR ν_{max} (cm^{-1}; KBr pellets): 3305 (N–H); 1688 (C=O).

4.1.3.16. N′-*[(E)-(2-hydroxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**19**).* Yield: 65%; mp: 175–176 °C; (198–200 °C) [20].

4.1.3.17. N′-*[(E)-(3-hydroxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**20**).* Yield: 73%; mp: 230–231 °C.

^1H NMR (400MHz, DMSO-d_6) δ: 12.21(1H; s; NH); 9.64(1H; s; OH); 9.27(1H; d; $J = 1.4$ Hz; H_3); 8.93(1H; d; $J = 2.4$ Hz; H_6); 8.79(1H; dd; $J = 2.4$ and 1.4 Hz; H_5); 8.57(1H; s; N=CH); 7.27(1H; t; $J = 7.80$ Hz; $H_{5'}$); 7.22(1H; s; $H_{2'}$); 7.11(1H; d; $J = 7.8$ Hz; $H_{4'}$); 6.86(1H; dd; $J = 8.0$ and 1.6 Hz; $H_{6'}$)ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 159.4; 157.7; 149.9; 147.8; 144.6; 144.1; 143.3; 135.4; 129.9; 118.9; 117.7; 112.8 ppm; MS/ESI: [M-H]:241. IR ν_{max} (cm^{-1}; KBr pellets): 3460 (O–H); 3259 (N–H); 1680 (C=O).

4.1.3.18. N′-*[(E)-(4-hydroxyphenyl)methylidene]-2-pyrazinecarbohydrazide (**21**).* Yield: 89%; m.p: 252–253 °C; (290–292 °C) [20].

4.1.3.19. N′-*[(E)-(2-cyanophenyl)methylidene]-2-pyrazinecarbohydrazide (**22**).* Yield: 80%; mp: 210–211 °C.

^1H NMR (400MHz, DMSO-d_6): δ: 12.82 (1H; s; NH); 9.28 (1H; s; H_3); 9.08 (1H; s; H_6); 8.94 (1H; s; H_5); 8.82 (1H; s; N=CH); 8.16 (1H; d; $J = 7.8$ Hz; $H_{6'}$); 7.94 (1H; d; $J = 7.6$ Hz; $H_{3'}$); 7.82 (1H; t; $J = 7.6$ Hz; $H_{5'}$); 7.65 (1H; t; $J = 7.6$ Hz; $H_{4'}$) ppm.

^{13}C NMR (100MHz, DMSO-d_6) δ: 160.7; 148.4; 146.0; 144.7; 144.5; 143.9; 137.0; 134.1; 133.9; 131.3; 126.5; 117.4; 111.5 ppm.

MS/ESI: [M-H]: 250.

IR ν_{max} (cm^{-1}; KBr pellets): 3261 (N–H); 2228 (CN); 1700 (C=O).

4.1.3.20. N′-*[(E)-(3-cyanophenyl)methylidene]-2-pyrazinecarbohydrazide (**23**).* Yield: 88%; mp: 200–201 °C.

^1H NMR (400MHz, DMSO-d_6): δ: 12.50 (1H; s; NH); 9.27 (1H; s; H_3); 8.94 (1H; s; H_6); 8.80 (1H; s; H_5); 8.68 (1H; s; N=CH); 8.12 (1H; s; $H_{2'}$); 8.07 (1H; d; $J = 7.8$ Hz; $H_{4'}$); 7.91 (1H; t; $J = 7.6$ Hz; $H_{5'}$); 7.68 (1H; t; 7.8 Hz; $H_{6'}$) ppm.

^{13}C NMR (100MHz, DMSO-d_6) δ: 159.7; 147.9; 147.5; 144.5; 144.2; 143.3; 135.5; 133.5; 131.2; 130.2; 118.3; 112.1 ppm.

MS/ESI: [M-H]: 250.

IR ν_{max} (cm^{-1}; KBr pellets): 3306 (N–H); 2233 (CN); 1688 (C=O).

4.1.3.21. N′-*[(E)-(4-cyanophenyl)methylidene]-2-pyrazinecarbohydrazide (**24**).* Yield: 80%; mp: 247–248 °C.

^1H NMR (500MHz, DMSO-d_6): δ: 12.56 (1H; s; NH); 9.28 (1H; d; $J = 1.3$ Hz; H_3); 8.95 (1H; d; $J = 2.4$ Hz; H_6); 8.81 (1H; dd; $J = 2.4$ and 1.3 Hz; H_5); 8.71 (1H; s; N=CH); 7.94 (2H; d; $J = 8.6$ Hz; $H_{3'}$ and $H_{5'}$); 7.91 (2H; d; $J = 8.6$ Hz; $H_{2'}$ and $H_{6'}$) ppm.

^{13}C NMR (125MHz, DMSO-d_6) δ: 161.4; 159.7; 147.9; 147.1; 144.8; 143.4; 138.5; 132.7; 127.7; 118.5; 112.1 ppm.

MS/ESI: [M-H]: 250.

IR ν_{max} (cm^{-1}; KBr pellets): 3301 (N–H); 2232 (CN); 1683 (C=O).

4.1.3.22. N′-*[(E)-(4-(dimethylamino)phenyl)methylidene]-2-pyrazinecarbohydrazide (**25**).* Yield: 65%; mp: 244–245 °C; (250–252 °C) [25].

4.1.3.23. N′-*[(E)-(4- diethylamino)methylidene]-2-pyrazinecarbohydrazide (**26**).* Yield: 65%; mp: 141–142 °C; (162–164 °C)[26].

4.1.3.24. N′-*[(E)-(2-nitrophenyl)methylidene]-2-pyrazinecarbohydrazide (**27**).* Yield: 72%; mp: 182–183 °C.

^1H NMR (400MHz, DMSO-d_6) δ: 12.71 (1H; s; NH); 9.28 (1H; d; $J = 1.0$ Hz; H_3); 9.08 (1H; s; N=CH); 8.95 (1H; d; $J = 2.4$ Hz; H_6); 8.81 (1H; dd; $J = 2.4$ and 1.0 Hz; H_5); 8.14 (1H; d; $J = 7.2$ Hz; $H_{3'}$); 8.10 (1H; d; $J = 7.8$ Hz; $H_{6'}$); 7.85 (1H; t; $J = 7.4$ Hz; $H_{4'}$); 7.71 (1H; t; $J = 7.4$ Hz; $H_{5'}$)ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 160.0; 148.4; 147.9; 145.0; 144.5; 144.2; 143.3; 133.7; 130.9; 128.5; 128.1; 124.6 ppm; MS/ESI: [M-H]: 270. IR: (KBr pellets, cm^{-1}): 3400 (NH); 1700 (CO).

4.1.3.25. N′-*[(E)-(3-nitrophenyl)methylidene]-2-pyrazinecarbohydrazide (**28**).* Yield: 62%; mp: 230–231 °C.

^1H NMR (400MHz, DMSO-d_6) δ: 12.55(1H; s; NH); 9.29(1H; s;H_3); 8.95(1H; d; $J = 2.1$ Hz; H_6); 8.81(1H; s; N=CH); 8.77(1H; s; H_5); 8.53(1H; s; $H_{2'}$); 8.29(1H; d; $J = 8.0$ Hz: $H_{4'}$); 8.16(1H; d; $J = 8.0$ Hz; $H_{6'}$); 7.78(1H; t; $J = 8.0$ Hz; $H_{5'}$)ppm; ^{13}C NMR (100MHz, DMSO-d_6) δ: 159.8; 148.2; 147.9; 147.4; 144.4; 144.2; 143.3; 135.9; 133.5; 130.5; 124.52; 121.1 ppm; MS/ESI: [M-H]: 270. IR ν_{max} (cm^{-1}; KBr pellets): 3443 (N–H); 1660 (C=O).

4.1.3.26. N′-*[(E)-(4-nitrophenyl)methylidene]-2-pyrazinecarbohydrazide (**29**).* Yield: 67%; mp: 262–263 °C; (278–280 °C) [20].

4.2. General procedures for biological tests

4.2.1. Cell viability assay

The cellular viability for a macrophage cell line J774 (ATCC TIB-67™) was determined by Mosmans's MTT (3-(4,5-dimethylthylthiazol-2yl)-2,5-dimethyl tetrazolium bromide; Merck) microcultured tetrazolium assay. We evaluated non infected or infected macrophages with *M. bovis* Bacillus Calmette–Guerin (BCG) in the presence and absence of test compounds (**4**–**29**). The cells were plated in flat bottom 96 well plates (2.5×10^6 cells/well/100 μL) cultured for 24 h in a controlled atmosphere (CO_2 5% at 37 °C), and non-adherent cells were washed by gentle flushing with RPMI 1640 supplemented with fetal bovine serum (10%) and gentamicin (25 μg/mL). Adherent cells were infected or not with BCG (2.5×10^6 UFC/well/100 μL) cultured in the presence of medium alone, tween 20 (3%) (live and dead controls, respectively) or different concentrations of compounds (1.0, 10.0 and 100 μg/mL) in a triplicate assay. After 48 h, stock MTT solution (5 mg/mL of saline; 20 mL/well) was added to the culture and 4 h later, the plate was centrifuged for 2 min at 2800 rpm, supernatant was discharged and Dimethyl sulfoxide (DMSO) (100 μL/well) was added for formazan crystals solubilization and the absorbance was read at 540 nm in a plate reader (Biorad – 450).

4.2.2. Antimycobacterial activity

Briefly, 200 μL of sterile deionized water was added to all outer-perimeter wells of sterile 96 well plates (falcon, 3072: Becton Dickinson, Lincoln Park, NJ) to minimize evaporation of the medium in the test wells during incubation. The 96 plates received 100 μL of the Middlebrook 7H9 broth (Difco laboratories, Detroit, MI, USA) and a serial dilution of the compounds **4**–**29** was made directly on the plate. The final drug concentrations tests were 0.01–100 μg/mL. Plates were covered and sealed with parafilm and incubated at 37 °C for five days. After this time, 25 μL of a freshly prepared 1:1 mixture of Alamar Blue (Accumed International, Westlake, Ohio) reagent and 10% tween 80 was added to plate and incubated for 24 h. A blue color in the well was interpreted as no bacterial growth, and a pink color was scored as growth. The MIC (minimal inhibition concentration) was defined as the lowest drug concentration, which prevented a color change from blue to pink.

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