

Synthesis and evaluation of isonicotinoyl hydrazone derivatives as antimycobacterial and anticancer agents

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Abstract A new series of isonicotinoyl hydrazone derivatives (**3a–3o**) have been synthesized, characterized and evaluated for in vitro antimycobacterial activity against *M. tuberculosis* H37Rv and two clinical isolates using tetrazolium microplate assay (TEMA). Some of these compounds showed moderate to good antimycobacterial activity at micro molar concentrations. Among them, **3k** and **3m** were the most potent analogues with an inhibition concentration at 0.59 and 0.65 μM , respectively, against *M. tuberculosis* H37Rv compared to parent drug, isoniazid (0.57 μM). Additionally, all the synthesized compounds were subjected to in vitro anticancer activity against human colorectal cancer cell lines (HCT 116). Compounds **3b** and **3l** displayed antiproliferative activity at inhibitory concentration 3.1 and 0.29 μM , respectively, when compared to standard, 5-fluorouracil (5 μM). These results can be considered as an important start point for the rational design of new leads for antitubercular and anticancer drug discovery.

Keywords Isonicotinoyl hydrazone ·
Antimycobacterial activity · Anticancer activity ·
Tetrazolium microplate assay

Introduction

Tuberculosis (TB) is a contagious chronic granulomatous airborne disease ubiquitously caused by *Mycobacterium*

tuberculosis (*M.tb*) and mostly affects young adults in the developing world (WHO, 2011). The World Health Organization (WHO) reported (in year 2010) an estimate of 8.5–9.2 million cases and 1.2–1.5 million deaths (including deaths from TB among HIV-positive patients) (WHO). The emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of *M.tb*, particularly in settings where TB patients are co-infected with human immunodeficiency virus (HIV), poses a serious threat (Mital *et al.*, 2006). In spite of the increasing worldwide incidence of TB, no new drugs have been brought to the market over the past four decades. According to WHO's TB treatment guidelines, chemotherapy for TB-infected patient is based on the combined use of multiple chemotherapeutic agents: isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), streptomycin (SM) and ethambutol (EMB), over a period of 6–10 months (Whitney and Wainberg, 2002; Ellner *et al.*, 1991). Therefore, to combat the *M.tb* and its resistant strains there is an urgent need to develop novel anti-tubercular drugs, which are safe and effective.

Cancer is a disease characterized by disruption of controlling mechanisms that govern cell proliferation and differentiation. Malignancy is caused by abnormalities in cells, which might be either inherited or due to exposure to chemicals, radiation, or even infectious agents. The rapid proliferation of abnormal cells that grows beyond the usual boundaries, and then invades adjoining parts of the body and spread to other organs is one of the characteristic features of cancer. This process is referred to as metastasis, and it is the major cause of death from cancer. Lungs, stomach, liver, colon and breast are the most commonly affected organs, which lead to death every year (7.9 million deaths). It was projected that worldwide death toll from cancer is continuing to rise, with an estimated 12 million deaths by 2030 (WHO, 2012). The colon cancer is the third most

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common malignancy worldwide (Parkin *et al.*, 2005). The increase in the death toll year after year due to cancer, have made clear that there is an urgent need for development of newer and more powerful chemotherapeutic agents that can eradicate cancer cells without harming normal tissues.

Isoniazid (INH) showed wide interest because of their diverse biological and clinical applications. Recent years, hydrazide–hydrazone derivatives have attracted interest due to their wide range of applications in medicinal chemistry. Generally, presence of azometine (–NHN=CH–) moiety with these compounds contributes to its biological activity (Rollas and Küçükgüzel, 2007). Schwab and Tuschl (2003) conducted *in vitro* studies on the toxicity of INH in different cell lines showed that, INH induces cytotoxicity via apoptosis, and can lead to a significant disturbance of the cell cycle in mammalian cells.

Recently, several novel hydrazine and hydrazones derivatives showed strong *in vitro* anticancer activity on carcinoma cells (Demirbas *et al.*, 2004; Sztanke *et al.*, 2008; Vicini *et al.*, 2006). Apart from antitumor activity, several hydrazone derivatives also exhibited a broad spectrum of biological activity, such as antimicrobial, anticonvulsant, antidepressant, analgesic, anti-inflammatory (Kaymakç,ioğlu *et al.*, 2009) and antimycobacterial activity (Maccari *et al.*, 2005; Sinha *et al.*, 2005; Kaymakç,ioğlu *et al.*, 2006; Gurkok *et al.*, Gurkok *et al.* 2009; Pavan *et al.*, 2010).

Prompted by the above discussion, our rationale was to synthesize and characterise new isonicotinoyl hydrazone derivatives and examine their efficacy against *M.tb* H37Rv and two drug sensitive virulent clinical isolates strains (*MTB-1* and *MTB-2*). Simultaneously, we also present the structure activity relationship and *in vitro* antiproliferative activity against human colorectal cancer cell line (HCT 116).

Results and discussion

Chemistry

The synthesis of isonicotinoyl hydrazone derivatives involved the reaction (Fig. 1) between appropriately substituted benzaldehydes and isoniazid, by previously

reported procedure (Lourenaso *et al.*, 2008). Reaction mixtures were maintained at room temperature, leading to the desired compounds (**3a–3o**) in 60–79 % yields (Table 1). Progress of the reaction was monitored by thin layer chromatography, and spots were visualized under UV light as well as iodine chamber containing iodine vapours. All the compounds were identified by their UV, IR, NMR, Mass spectroscopy, X-ray crystallography as well as elemental analysis. The elemental analysis results were within ± 0.4 % of the theoretical values.

In general, IR spectra showed the C=O peak at 1634–1687 cm^{-1} , NH stretching vibrations at 3052–3290 cm^{-1} and N=C stretching at 1581–1630 cm^{-1} . In the nuclear magnetic resonance spectra (^1H NMR) the signals of the respective protons of the synthesized compounds were verified on the basis of their chemical shifts, multiplicities and coupling constants (*J*). The spectra showed the hydrazide (NH) proton as a singlet around δ 12.54–11.73 ppm and the imine proton (N=C–H) at δ 9.48–8.38 ppm. The ^{13}C NMR spectrum showed the C=O signals between δ 163.4–158.0 ppm and C=N signals δ 149.2–142.9 ppm. The crystals suitable for the X-ray crystallography analysis were grown using different solvents by slow evaporation. The X-ray crystallographic structures of most of the synthesized compounds were reported (Naveenkumar *et al.*, 2009; 2010a,b,c,d,e,f,g,h,i,j). The lipophilicities of the all synthesized compounds and the standard drug (INH) were expressed as *clog P* values, and were determined using ChemBioDraw ultra 11.0 software (Table 2). All the compounds exhibited higher lipophilicity compared to INH.

Pharmacology

Antimycobacterial activity

The antimycobacterial activities of compounds **3a–o** were assessed against *M.tb* H37Rv (ATCC-25618) and two clinical isolates (*MTB-1* and *MTB-2*), using calorimetric tetrazolium microplate assay method (TEMA) in triplicate with slight modification. This methodology is found to be reliable, faster and inexpensive method for the determination MICs (minimum inhibitory concentration) of *M.tb* strains (Mshana *et al.*, 1998; Caviedes *et al.*, 2002). The

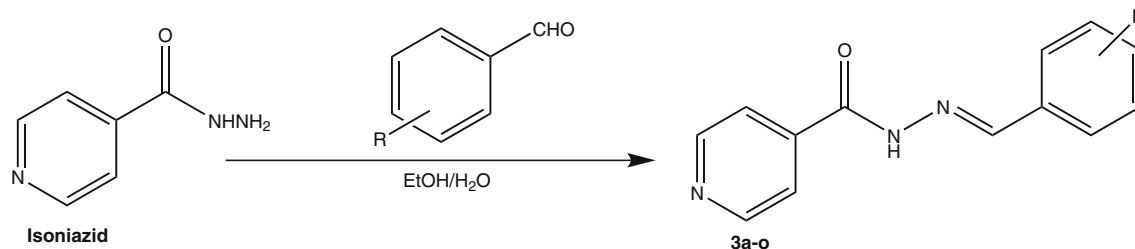


Fig. 1 Synthetic route used for the preparation of isonicotinoyl hydrazone derivatives (**3a–o**)

Table 1 Chemical structures and physical constants of isonicotinoyl hydrazone derivatives (**3a–o**)

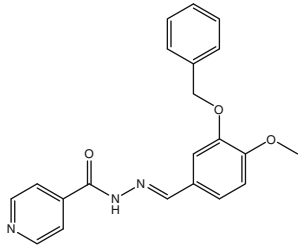
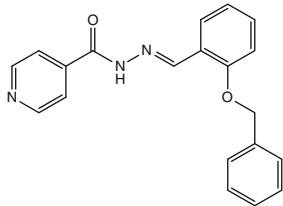
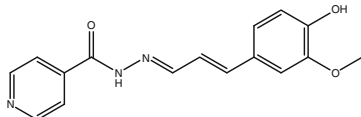
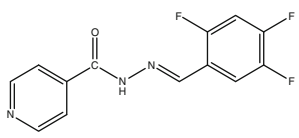
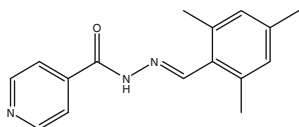
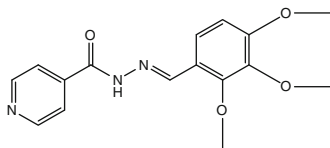
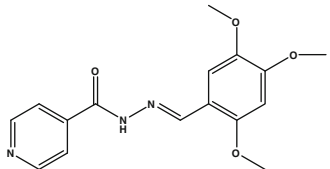
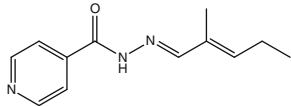
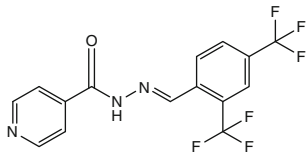
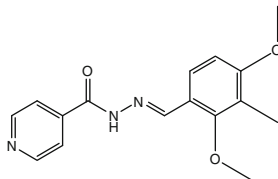
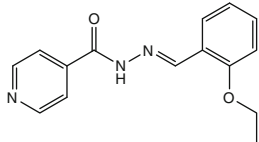
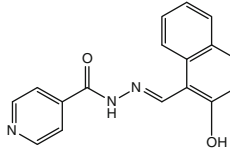
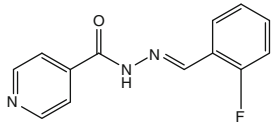
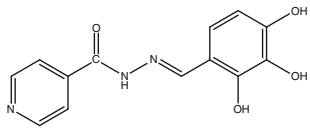
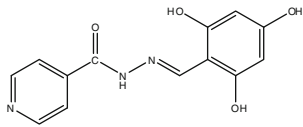
Sample code	Chemical structure	Melting point (°C)	Yield (%)
3a		238–239	79
3b		186–187	75
3c		215–217	69
3d		209–210	74
3e		196–197	68
3f		194–196	69
3g		206–208	67
3h		177–179	73

Table 1 continued

Sample code	Chemical structure	Melting point (°C)	Yield (%)
3i		176–178	78
3j		211–213	73
3k		201–203	76
3l		258–260	70
3m		191–193	72
3n		240–242	78
3o		238–240	72

MICs of all the individual compounds were reported in Table 2. The MIC was defined as the lowest drug concentration, required for the complete inhibition of bacterial growth and expressed in μM (micro molar).

The results showed that compounds (**3a–o**) exhibited antimycobacterial activity between 18.29 and 0.28 μM against all the tested stains. Synthesized derivatives [**3f**, **3g**, **3h**, **3j**, **3k** and **3m**] and INH were equipotent against the tested *M.tb* H37Rv and two clinical isolates. Compound **3k** was the most potent derivative with MIC 0.59 μM against *M.tb*, and 0.28 μM against *MTB-1&2*. Unfortunately, clog *P* values haven't shown a linear correlation with the

biological activity in this series of compounds. This result indicated that lipophilicity of the tested compounds is not sole criteria affecting their anti-tubercular activity.

From the results of in vitro antimycobacterial activity the following conclusions regarding structure activity relationship (SAR) can be drawn:

1. We observed that, in most of the active compounds (**3f**, **3g**, **3j**, and **3k**), the presence of electron donating groups ($-\text{OCH}_3$, $-\text{CH}_3$, $-\text{OCH}_2\text{CH}_3$) on the substituted aromatic ring increased the anti-tubercular activity. These results were in concordance with the findings of

Table 2 The in vitro activity of isonicotinoyl hydrazone derivatives (**3a–o**) against *M.tb* H37Rv strain (ATCC-25618, two clinical isolates) and anticancer activity (HCT 116 Cells)

Compound	MIC ^a <i>M.tb</i> H37Rv (μM)	MIC Clinical isolates (μM)		IC50 (μM) (HCT 116 cell lines)	clog <i>P</i> ^b
		<i>MTB-1</i>	<i>MTB-2</i>		
3a ^a	>13.83	13.83	13.83	17.2	3.13
3b ^a	3.77	1.9	0.93	3.1	3.02
3c	2.11	1.04	0.53	13.4	1.28
3d ^a	2.25	1.11	0.57	23.9	1.61
3e	4.67	1.15	1.15	17.1	2.51
3f ^a	0.98	0.5	0.24	22.3	0.54
3g ^a	0.98	0.5	0.24	25.9	0.89
3h ^a	1.42	0.35	0.35	15.5	1.52
3i ^a	13.84	3.46	0.21	11.6	2.58
3j ^a	1.03	0.53	0.26	16	1.78
3k ^a	0.59	0.28	0.28	13.7	1.78
3l	>17.16	17.16	>17.16	0.29	3.24
3m	0.65	0.65	0.32	26.8	1.4
3n ^a	>18.29	9.14	9.14	10.4	0.99
3o ^a	9.14	4.57	4.57	28.4	1.55
INH	0.57	0.28	0.28	–	–0.66
5-FU	–	–	–	5	–0.57

MIC minimum inhibitory concentration, IC50 median lethal dose against Human colorectal cancer cell lines (HCT 116), 5-FU 5-fluorouracil

^a X-ray crystal structures were published in Acta Crystallographica (section E)

^b clog *P* was calculated using Chem BioDraw Ultra 11.00

other researchers, who observed similar activity by introduction of –OCH₃ group (Emami *et al.*, 2008; Ferreira *et al.*, 2008).

- In contrast with above fact, the electron withdrawing group (–F) present at ortho position (compound **3m**) of phenyl ring showed an increase in antimycobacterial activity. The role of electron withdrawing group in improving activity is supported by the earlier studies (Lourenaso *et al.*, 2008; Junior *et al.*, 2005; Sharma *et al.*, 2004). On the other hand, additional fluoro groups at position 4th and 5th position of substitute ring (compound **3d**) had witnessed a decrease in the activity.
- The replacement of the phenyl ring with aliphatic chain (compound **3h**), gave the molecule with highly improved antimycobacterial activity. This is similar to one of a previous report by Narasimhan *et al.*, (2007), in which an increase in antimicrobial activity was observed with long aliphatic chain compounds.
- The replacement of –OH group from 3rd position of the phenyl ring (compound **3n**) to 6th position (compound **3o**) showed a significant increase in antimycobacterial activity. The presence of the –OH group at ortho position (compound **3l**, **3n** and **3o**) showed decreased in anti-tubercular activity of the compounds, which was in contrast with Tripathi *et al.* (2006), who stated that the –OH group at ortho position leads to a measurable change in the activity of the compounds.
- The presence of benzoyloxy group at ortho position of aromatic ring (compound **3b**) showed decreased

activity compared to ethoxy group (compound **3k**) at the same position.

- Slight decrease in the antitubercular activity was observed (compound **3j**), when the methyl group at 3rd position of aromatic ring was replaced with a methoxy group (compound **3f**).

Anticancer activity

All the synthesized isonicotinoyl hydrazone derivatives (**3a–o**) were evaluated for their in vitro anticancer effect via the standard MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Mosmann, 1983), against human colorectal cell lines (HCT 116). MTT assay is a standard colorimetric assay for measuring cell growth. Compound 5-fluorouracil (5FU) was used as positive control. IC50 values (in μM) were summarized in Table 2, to represent the concentration inducing a 50 % decrease of cell growth after 72 h incubation. HCT116 cells were treated with 5 μM of 5FU alone for 72 h. Similarly, HCT 116 cell lines were continuously exposed to test compounds **3a–3o** for 72 h, and their effects on cellular viability were calculated. The profiles of percentage inhibition of cell proliferation against the concentration of the test compound were established to calculate IC50 values of each derivative. Percentage inhibition of cell proliferation was determined at different concentrations of the test compounds ranging from 1.5625 to 100 μM. Figures 2a, b, c and 3a, b, c, depict the

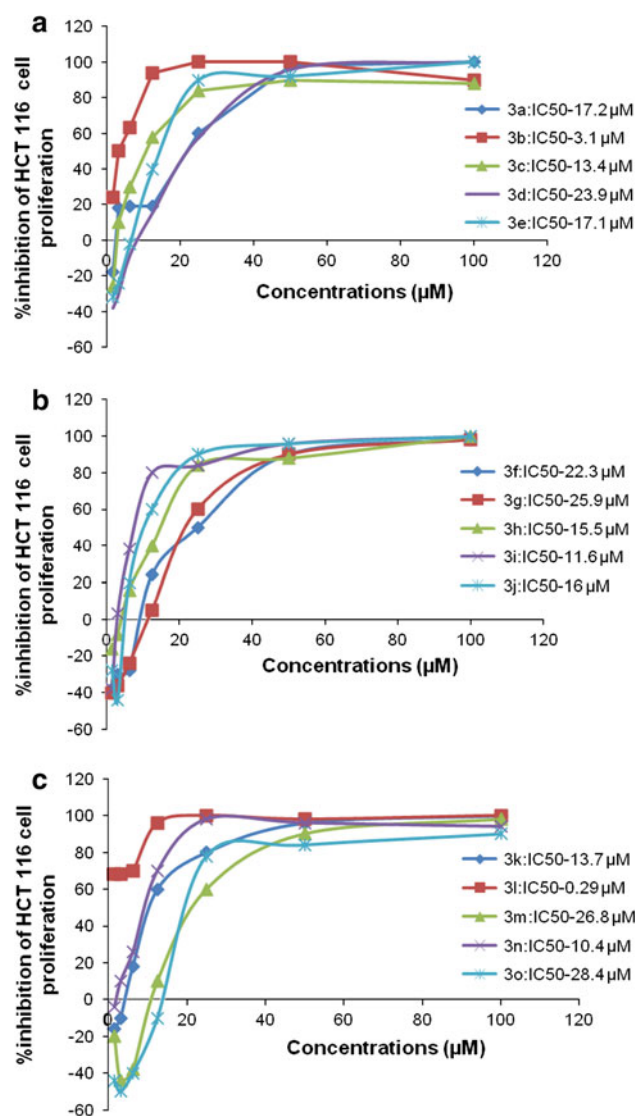


Fig. 2 MTT assay results of isonicotinoyl hydrazone derivatives against the HCT 116 cell line. **a** Compounds **3a–e**. **b** Compounds **3f–j**. **c** Compounds **3k–o**

effect of concentration of test compounds on HCT 116 cell lines after 72 h incubation time.

All the tested compounds displayed moderate to excellent antiproliferative effect against tested cell line, with IC₅₀ values in the range 0.29–28.4 μ M. Among them, compounds **3b** and **3l** exhibited excellent antitumor activity (IC₅₀ = 3.1 and 0.29 μ M, respectively). Interestingly, **3l** being 17 times more potent than standard 5FU could be easily used as a lead to be developed as a novel more potent anticancer agent. Overall, the results obtained from this study showed a limited correlation between the lipophilicity (*clog P*) and anticancer properties of the synthesized compounds.

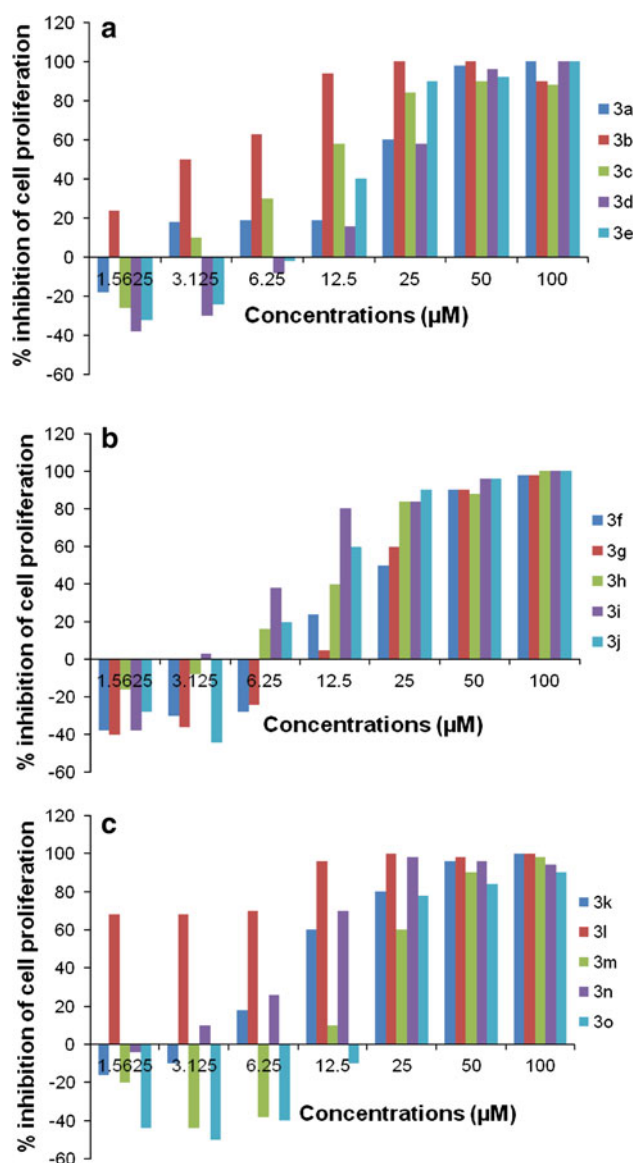


Fig. 3 The effects of increasing concentration of isonicotinoyl hydrazone derivatives on the percentage inhibition of cell proliferation. **a** Compounds **3a–e**. **b** Compounds **3f–j**. **c** Compounds **3k–o**

Conclusion

In the search for effective and selective antitubercular and anticancer agents, we synthesized a series of isonicotinoyl hydrazone derivatives. All the newly synthesized derivatives were characterized and evaluated for in vitro antimycobacterial and anticancer activity. The antimycobacterial results indicated that compounds **3k** and **3m** found to be most effective against both *M.tb* H37Rv and two clinical isolates compared to INH. The anticancer screening results of this series of compounds indicated that compounds **3b** and **3l** displayed superior antiproliferative activity in the low micro molar range as compared to 5FU against HCT116 cell line.

These compounds look like promising lead for further modification for the design and development of more superior antimycobacterial and anticancer agents with high selectivity and enhanced activity.

Experimental

Chemistry

All substituted benzaldehydes were obtained from Aldrich Chemical Company (USA). HPLC grade solvents were used for the reaction. Reagents and all solvents were analytically pure and were used without further purification. All the melting points (m.p. °C) were uncorrected and determined in open capillary tubes using digital melting point apparatus 9100 Electrothermal (UK). Pre-coated aluminium sheets silica gel 60 F₂₅₄ (Merck, Darmstadt Germany), TLC plates were used for monitoring the progress of the reaction, using dichloromethane/methanol as a mobile phase. UV spectra were taken dissolving compounds in methanol and recorded by using a Perkin-Elmer Lambda 45 UV spectrophotometer (USA). Infrared (FT-IR) spectra were recorded on Nicolet FT-IR Spectrometer (USA) apparatus, using the KBr pellets method and intensity of signals reported in wave numbers (cm⁻¹). Nuclear Magnetic resonance was recorded on Bruker Fourier transform instrument in dimethyl sulfoxide-*d*₆, recorded at 400 MHz (¹H NMR and ¹³C NMR). The chemical shifts are reported in parts per million delta (δ) using tetramethylsilane (TMS) as reference standard. Mass spectra were recorded with Agilent 1100 Series LC/MS (liquid chromatography/mass spectrometry) using electrospray ionization (ESI). The elemental analyses were recorded on Perkin-Elmer 2400 Series II CHN Elemental Analyzer. Names for all of the new compounds were given with the help of ChemBiodraw ultra 11.0.

General procedure for the synthesis of isonicotinoyl hydrazone derivatives (3a–3o)

Isoniazid (1.0 equiv.) was made to react with appropriate benzaldehyde **2a–2o** (1.0 equiv.) in ethanol/water (20 mL). Initially isoniazid was dissolved in water and a solution of respective benzaldehyde in ethanol (Fig. 1) solution was added. The reaction mixture was stirred for 1–5 h at room temperature. The reaction mixture was concentrated under reduced pressure, and given a cold ethanol wash followed by ethyl ether. It was further recrystallized with ethanol to obtain pure derivatives (**3a–3o**).

Analytical data for compounds 3a–3o

(*E*)-*N'*-(3-(benzyloxy-4-methoxybenzylidene) isonicotinohydrazide (**3a**) Pale yellow solid; UV (λ_{max}):328 nm. IR (cm⁻¹; KBr) 3289(NH), 1671(C=O), 1597(C=N). ¹H NMR (DMSO-*d*₆, 400 MHz) δ:11.96 (s, 1H, CONH), 8.79–8.77 [dd, 2H, *J* = 5.88 Hz, 2 Hz, (Pr-α-CH)], 8.38 (s, 1H, N=CH), 7.82–7.80 [dd, 2H, *J* = 5.92 Hz, 2.16 Hz, (Pr-β-CH)], 7.50–7.27 (m, 7H, Ar-H), 7.10–7.07 [d, 1H, *J* = 11.2 Hz, (Ar-H)], 5.15 (s, 2H, O-CH₂), 3.83 (s, 3H, CH₃). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 162.26, 152.22, 151.16, 150.06, 148.99, 141.50, 137.74, 129.27, 128.76, 128.72, 127.54, 123.13, 122.35, 112.35, 111.44, 70.84, 56.57. MS-ESI *m/z*(%)-[M+H]⁺ 362.20 (100). Anal. Calcd. for C₂₁H₁₉N₃O₃ (361.14) C, 69.79; H, 5.30; N, 11.63; found C, 69.71; H, 5.15; N, 11.70.

(*E*)-*N'*-(2-(benzyloxy) benzylidene) isonicotinohydrazide (**3b**) White solid; UV (λ_{max}): 328 nm. IR (cm⁻¹; KBr) 3228 (NH), 1670 (C=O), 1597(C=N). ¹H NMR (DMSO-*d*₆, 400 MHz) δ:12.11 (s, 1H, -CONH), 8.83 (s, 1H, N=CH), 8.77–8.75 [dd, 2H, *J* = 5.88 Hz, 2.20 Hz (Pr-α-CH)], 7.93–7.90 [dd, 1H, *J* = 10.32 Hz, 2.24 Hz, (Ar-H)], 7.82–7.80 [dd, 2H, *J* = 5.88 Hz, 2.20 Hz (Pr-β-CH)], 7.53–7.32(m, 6H, Ar-H), 7.24–7.21(d, 1H, *J* = 10.52, (Ar-H)), 7.07–7.02 [t, 1H, *J* = 9.82 Hz, (Ar-H)], 5.20 (s, 2H, O-CH₂). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ:158.02, 151.01,145.38, 137.66,132.47, 129.34, 128.78, 128.56, 126.80, 123.69, 122.38, 121.95,114.36, 70.99. MS-ESI *m/z* (%) -[M+Na]⁺ 354.10(100).Anal. Calcd for C₂₀H₁₇ N₃O₂ (331.13) C, 72.49; H, 5.17; N, 12.68; found C, 72.83; H, 4.91; N, 12.81.

(*E*)-*N'*-((*E*)-3-(4-hydroxy-3-methoxyphenyl) allylidene)isonicotinohydrazide(**3c**) Yellow solid; UV (λ_{max}): 353 nm. IR (cm⁻¹; KBr) 3228 (NH), 1650 (C=O), 1597 (C=N). ¹H NMR (DMSO-*d*₆, 400 MHz) δ: 11.86 (s, 1H, CONH), 9.41 (s, 1H, OH), 8.78–8.76 [dd, 2H, *J* = 4.56 Hz, 1.4 Hz, (Pr-α-CH)], 8.21–8.18 (dd, 1H, *J* = 6 Hz, 2.66 Hz, N=CH), 7.81–7.79 [dd, 2H, *J* = 4.46 Hz, 1.58 Hz, (Pr-β-CH)], 7.25 (s, 1H, Ar-H), 7.04–7.01 [dd, 1H, *J* = 8.18 Hz, 1.70 Hz, (Ar-CH)], 6.97–6.96 (m, 2H, =CH), 6.79–6.77 [d, 1H, *J* = 8.12 Hz, (Ar-H)], 3.82 (s, 3H, OCH₃). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ: 162.11,152.36, 151.13, 148.87, 148.78, 141.44, 141.35, 128.36,123.25,122.34, 122.30, 116.43, 111.18, 56.51. MS-ESI *m/z* (%) -[M+H]⁺ 298.2 (100). Anal. Calcd for C₁₆H₁₅N₃O₃ (297.11) C, 64.64; H, 5.09; N, 14.13; found C, 64.56; H, 4.86; N, 14.21.

(*E*)-*N'*-(2,4,5-trifluorobenzylidene) isonicotinohydrazide (**3d**) White solid; UV (λ_{max}): 309 nm. IR (cm^{-1} ; KBr) 3203 (NH), 1679 (C=O), 1622 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 12.25 (s, 1H, -CONH), 8.80–8.78 [dd, 2H, $J = 5.9$ Hz, 2.18 Hz, (Pr- α -CH)], 8.60 (s, 1H, N=CH), 7.82–7.80 [dd, 2H, $J = 5.9$ Hz, 2.14 Hz, (Pr- β -CH)], 7.78–7.65 (m, 2H, Ar-H). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 162.50, 158.37, 155.89, 151.25, 148.71, 146.29, 140.88, 122.34, 119.90, 114.52, 108.12. MS-ESI m/z (%) - [M + H] $^+$ 280.0 (100). Anal. Calcd for $\text{C}_{13}\text{H}_8\text{F}_3\text{N}_3\text{O}$ (279.06) C, 55.92; H, 2.89; N, 15.05; found C, 55.53; H, 2.49; N, 14.97.

(*E*)-*N'*-(2,4,6-trimethylbenzylidene) isonicotinohydrazide (**3e**) White solid; UV (λ_{max}): 302 nm. IR (cm^{-1} ; KBr) 3191 (NH), 1650 (C=O), 1601 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 11.92 (s, 1H, -CONH), 8.79–8.77 [dd, 2H, $J = 5.86$ Hz, 2.22 Hz, (Pr- α -CH)], 8.76 (s, 1H, N=CH), 7.83–7.81 [dd, 2H, $J = 5.86$ Hz, 2.2 Hz, (Pr- β -CH)], 6.93 (s, 2H, Ar-H), 2.42 (s, 3H, CH_3), 2.24 (s, 3H, CH_3), 2.18 (s, 3H, CH_3). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 162.14, 151.16, 150.04, 141.47, 139.44, 138.39, 130.35, 128.74, 123.36, 109.98, 21.91, 21.56. MS-ESI m/z (%) - [M + Na] $^+$ 290.10 (100). Anal. Calcd for $\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}$ (267.14) C, 71.89; H, 6.41; N, 15.72; found C, 71.86; H, 6.08; N, 15.76.

(*E*)-*N'*-(2,3,4-trimethoxybenzylidene) isonicotinohydrazide (**3f**) White solid; UV (λ_{max}): 305 nm. IR (cm^{-1} ; KBr) 3183 (NH), 1679 (C=O), 1614 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 12.01 (s, 1H, -CONH), 8.78–8.76 [d, 2H, $J = 7.96$ Hz, (Pr- α -CH)], 8.65 (s, 1H, N=CH), 7.84–7.82 [dd, 2H, $J = 6$ Hz, 2.08 Hz, (Pr- β -CH)], 7.65–7.62 (d, 1H, $J = 11.8$ Hz, Ar-H), 6.95–6.92 (d, 1H, $J = 11.92$ Hz, Ar-H), 3.85 (s, 3H, -OCH $_3$), 3.84 (s, 3H, OCH $_3$), 3.76 (s, 3H, OCH $_3$). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 162.26, 156.28, 153.65, 151.16, 145.44, 142.39, 141.50, 122.35, 121.54, 120.93, 109.60, 62.70, 61.32, 56.84. MS-ESI m/z (%) - [M + H] $^+$ 316.20 (100). Anal. Calcd for $\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}_4$ (315.12) C, 60.94; H, 5.43; N, 13.33; found C, 60.95; H, 5.13; N, 13.40.

(*E*)-*N'*-(2, 4, 5-trimethoxybenzylidene) isonicotinohydrazide (**3g**) White solid; UV (λ_{max}): 355 nm. IR (cm^{-1} ; KBr) 3290 (NH), 1654 (C=O), 1597 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 11.92 (s, 1H, -CONH), 8.77–8.76 [dd, 2H, $J = 4.5$ Hz, 1.52 Hz, (Pr- α -CH)], 8.75 (s, 1H, N=CH), 7.83–7.82 [dd, 2H, $J = 4.5$ Hz, 1.58 Hz, (Pr- β -CH)], 7.36 (s, 1H, Ar-H), 6.76 (s, 1H, Ar-H), 3.87–3.76 (m, 9H, -OCH $_3$). ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 161.98, 154.49, 153.24, 151.11, 145.53, 144.15, 141.43, 122.30, 114.12, 108.64, 98.76, 57.40, 56.79, 56.67. MS-ESI m/z (%) - [M + H] $^+$ 316.10 (100). Anal. Calcd for $\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}_4$ (315.12) C, 60.94; H, 5.43; N, 13.33; found C, 60.94; H, 5.74; N, 13.39.

(*E*)-*N'*-(*E*)-2-methylpent-2-enylidene isonicotinohydrazide (**3h**) White solid; UV (λ_{max}): 282 nm. IR (cm^{-1} ; KBr) 3052 (NH), 1667 (C=O), 1630 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 11.73 (s, 1H, CONH), 8.77–8.75 [dd, 2H, $J = 5.8$ Hz, 2.2 Hz, (Pr- α -CH)], 8.03 (s, 1H, N=CH), 7.78–7.76 [dd, 2H, $J = 5.8$ Hz, 2.2 Hz, (Pr- β -CH)], 5.94–5.91 $J = 4.98$ Hz, [t, 1H, (=CH)], 2.29–2.19 [p, 2H, $J = 19.84$ Hz (-CH $_2$)], 1.8 (s, 3H, -CH $_3$), 1.03–0.98 [t, 3H, $J = 10.02$ Hz, (CH $_3$)]. ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 162.22, 154.90, 151.12, 143.41, 141.57, 133.38, 122.32, 22.11, 14.24, 11.84. MS-ESI m/z (%) - [M + Na] $^+$ 240.10 (100). Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{N}_3\text{O}$ (217.12) C, 66.34; H, 6.96; N, 19.34; found C, 66.28; H, 6.86; N, 19.54.

(*E*)-*N'*-(2, 4-bis (trifluoromethyl) benzylidene) isonicotinohydrazide (**3i**) White solid; UV (λ_{max}): 305 nm. IR (cm^{-1} ; KBr) 3187 (NH), 1663 (C=O), 1556 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 12.53 (s, 1H, -CONH), 8.86 (s, 1H, N=CH), 8.82–8.80 [d, 2H, $J = 7.92$ Hz, (Pr- α -CH)], 8.46–8.43 [d, 1H, $J = 11.28$ Hz, (Ar-H)], 8.18–8.15 [d, 1H, $J = 11.76$ Hz, (Ar-H)], 8.09 (s, 1H, Ar-H), 7.85–7.83 [dd, 2H, $J = 6$ Hz, 2.08 Hz, (Pr- β -CH)]. ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 162.89, 151.28, 143.22, 140.73, 136.84, 131.09, 130.65, 129.12, 125.94, 123.99, 122.38, 118.83. MS-ESI m/z (%) - [M + Na] $^+$ 384.1 (100). Anal. Calcd for $\text{C}_{15}\text{H}_9\text{F}_6\text{N}_3\text{O}$ (361.06) C, 49.87; H, 2.51; N, 11.63; found C, 49.48; H, 2.12; N, 11.40.

(*E*)-*N'*-(2,4-dimethoxy-3-methylbenzylidene) isonicotinohydrazide (**3j**) White solid; UV (λ_{max}): 324 nm. IR (cm^{-1} ; KBr) 3207 (NH), 1654 (C=O), 1597 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 11.97 (s, 1H, -CONH), 8.78–8.76 [dd, 2H, $J = 5.88$ Hz, 2.20 Hz, (Pr- α -CH)], 8.65 (s, 1H, N=CH), 7.83–7.81 [dd, 2H, $J = 5.90$ Hz, 2.22 Hz, (Pr- β -CH)], 7.79–7.76 [d, 1H, $J = 11.64$ Hz, (Ar-H)], 6.92–6.89 [d, 1H, $J = 11.84$ Hz, (Ar-H)], 3.84 [s, 3H, (-OCH $_3$)], 3.71 [s, 3H, (-OCH $_3$)], 2.08 [s, 3H, (-CH $_3$)]. ^{13}C NMR (DMSO- d_6 , 400 MHz) δ : 162.13, 161.07, 159.28, 151.14, 145.96, 141.47, 124.98, 122.34, 120.26, 119.63, 108.29, 62.90, 56.67, 9.55. MS-ESI m/z (%) - [M + Na] $^+$ 322.1 (100). Anal. Calcd for $\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}_3$ (299.13) C, 64.20; H, 5.72; N, 14.04; found C, 64.14; H, 5.31; N, 14.13.

(*E*)-*N'*-(2-ethoxybenzylidene) isonicotinohydrazide (**3k**) White solid; UV (λ_{max}): 329 nm. IR (cm^{-1} ; KBr) 3187 (NH), 1654 (C=O), 1597 (C=N). ^1H NMR (DMSO- d_6 , 400 MHz) δ : 12.09 (s, 1H, CONH), 8.83 (s, 1H, N=CH), 8.79–8.77 [dd, 2H, $J = 5.88$ Hz, 2.20 Hz (Pr- α -CH)], 7.90–7.87 (dd, 1H, $J = 10.32$ Hz, 2.24 Hz, Ar-H), 7.85–7.83 [dd, 2H, $J = 5.86$ Hz, 2.22 Hz, (Pr- β -CH)], 7.44–7.38 [td, 1H, $J = 10.44$ Hz, 2.32 Hz, (Ar-H)], 7.10–7.08 [d, 1H, $J = 10.88$ Hz, (Ar-H)], 7.04–6.99

[t, 1H, $J = 10$ Hz, (Ar-H)], 4.15–4.08 [q, 2H, $J = 9.28$ Hz, (–CH₂)], 1.41–1.36 [t, 3H, $J = 9.28$ Hz, (–CH₃)]. ¹³C NMR (DMSO-*d*₆, 400 MHz) δ : 162.13, 158.25, 150.99, 145.77, 141.62, 132.51, 126.66, 123.54, 122.37, 121.63, 114.06, 65.02, 15.45. MS–ESI m/z (%)–[M+Na]⁺ 292.1(100). Anal. Calcd for C₁₅H₁₅N₃O₂ (269.12) C, 66.90; H, 5.61; N, 15.60; found C, 66.81; H, 5.37; N, 15.64.

(*E*)-*N'*-(2-hydroxynaphthalen-1-yl) methyleneisonicotinohydrazide (**3l**) Yellow solid; UV (λ_{\max}): 367 nm. IR (cm⁻¹; KBr) 3215 (NH), 1679 (C=O), 1626(C=N). ¹H NMR (DMSO-*d*₆, 400 MHz) δ : 12.54 (s, 1H, CONH), 12.40 (s, 1H, OH), 9.48 (s, 1H, N=CH), 8.84–8.83 [d, 2H, $J = 4.68$ Hz, (Pr- α -CH)], 8.32–8.30 [d, 1H, $J = 8.48$ Hz, (Ar-H)], 7.95–7.90 (m, 2H, Ar-H), 7.89–7.88 [d, 2H, $J = 4.6$ Hz, (Pr- β -CH)], 7.64–7.60 (t, 1H, $J = 7.64$ Hz, Ar-H), 7.43–7.39 (t, 1H, $J = 6.98$ Hz, Ar-H), 7.26–7.23 (d, 1H, $J = 8.96$ Hz, Ar-H). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ : 161.90, 159.04, 151.34, 148.45, 140.66, 134.00, 132.46, 129.85, 128.77, 128.72, 124.48, 122.30, 121.81, 119.68, 109.38. MS–ESI m/z (%)–[M+H]⁺ 292.10 (100). Anal. Calcd for C₁₇H₁₃N₃O₂ (291.10) C, 70.09; H, 4.50; N, 14.42; found C, 70.05; H, 4.17; N, 14.50.

(*E*)-*N'*-(2-fluorobenzyliden) isonicotinohydrazide (**3m**) Pale yellow solid; UV (λ_{\max}): 305 nm. IR (cm⁻¹; KBr) 3187 (NH), 1687(C=O), 1610 (C=N). ¹H NMR (DMSO-*d*₆, 400 MHz) δ : 12.19 (s, 1H, –CONH), 8.80–8.78 [d, 2H, $J = 5.94$ Hz, 2.14 Hz, (Pr- α -CH)], 8.71 (s, 1H, N=CH), 7.98–7.93 [td, 1H, $J = 10.12$ Hz, 2.24 Hz (Ar-H)], 7.84–7.82 [dd, 2H, $J = 5.90$ Hz, 2.22 Hz (Pr- β -CH)], 7.67–7.47 (m, 2H, Ar-H), 7.33–7.28 (t, 1H, $J = 11.24$ Hz, Ar-H). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ : 163.44, 160.12, 151.05, 142.93, 141.43, 133.04, 132.93, 127.49, 125.76, 122.54, 116.66. MS–ESI m/z (%)–[M+H]⁺ 244.0 (100). Anal. Calcd for C₁₃H₁₀FN₃O (243.08) C, 64.19; H, 4.14; N, 17.28; found C, 64.07; H, 3.72; N, 17.25.

(*E*)-*N'*-(2, 3, 4-trihydroxybenzylidene) isonicotinohydrazide (**3n**) Pale brown solid; UV (λ_{\max}): 331 nm. IR (cm⁻¹; KBr) 3383 (OH), 3256 (NH), 1659 (C=O), 1618 (C=N). ¹H NMR (DMSO-*d*₆, 400 MHz) δ : 12.18 (s, 1H, –CONH), 11.29 (s, 1H, –OH), 9.58 (s, 1H, –OH), 8.80–8.78 [dd, 2H, $J = 5.92$ Hz, 2.2 Hz, (Pr- α -CH)], 8.56 (s, 1H, OH), 8.49 (s, 1H, N=CH), 7.84–7.82 [dd, 2H, $J = 5.88$ Hz, 2.2 Hz, (Pr- β -CH)], 6.85–6.82 [d, 1H, $J = 11.4$ Hz (Ar-H)], 6.42–6.39 [d, 1H, $J = 11.28$ Hz, (Ar-H)]. ¹³C NMR (DMSO-*d*₆, 400 MHz) δ : 162.50, 152.36, 151.14, 149.97, 148.58, 141.05, 133.70, 122.23, 111.81, 108.76. MS–ESI m/z (%)–[M+Na]⁺ 296.10 (100). Anal. Calcd for C₁₃H₁₁N₃O₄ (273.07) C, 57.14; H, 4.06; N, 15.38; found C, 57.11; H, 4.09; N, 15.36.

(*E*)-*N'*-(2,4,6-trihydroxybenzylidene) isonicotinohydrazide (**3o**) Brown solid; UV (λ_{\max}): 338 nm. IR (cm⁻¹; KBr) 3403 (OH), 3207 (NH), 1634 (C=O), 1581 (C=N). ¹H NMR (DMSO-*d*₆, 400 MHz) δ : 12.12 (s, 1H, –CONH), 11.08 (s, 2H, –OH), 9.91 (s, 1H, OH), 8.82 (s, 1H, N=CH), 8.79–8.77 [d, 2H, $J = 7.6$ Hz, (Pr- α -CH)], 7.84–7.82 [dd, 2H, $J = 5.94$ Hz, 2.2 Hz, (Pr- β -CH)], 5.85 (s, 2H, Ar-H). ¹³C NMR (DMSO-*d*₆, 400 MHz) δ : 162.81, 161.56, 160.76, 149.21, 151.10, 141.02, 122.18, 100.06, 95.52. MS–ESI m/z (%)–[M+H]⁺ 274.10 (100). Anal. Calcd for C₁₃H₁₁N₃O₄ (273.07) C, 57.14; H, 4.06; N, 15.38; found C, 57.10; H, 4.10; N, 15.34.

Biological assays

Antimycobacterial activity

Drug solution preparation INH was obtained commercially from Sigma-Aldrich Chemical Company, United Kingdom. INH stock solution was prepared by dissolving in distilled water to obtain a concentration of 1 mg mL⁻¹. The derivatives were dissolved in DMSO to obtain a stock solution of 1 mg mL⁻¹. These stock solutions were subsequently diluted with distilled water on the day of experiment to attain the desired working concentrations (5–0.0195 μ g mL⁻¹) and then sterilised by filtration using a cellulose membrane of 0.22 μ m pore size.

Inoculum preparation *Mycobacterium tuberculosis*, strain H37Rv (ATCC 25618) and two clinical isolates *MTB* 1&2 (obtained from School of Medical Sciences, University of Science Malaysia) were used for the in vitro antimycobacterial activity. The mycobacterial inoculums were prepared from a log phase culture in Middlebrook 7H9 broth (Difco, USA) supplemented with albumin, dextrose, and catalase (ADC) (Difco, USA), and its turbidity was adjusted to McFarland standard no. 1. This bacterial suspension was then further diluted 1:25 in Middlebrook 7H9 broth with 0.2 % glycerol and OADC (oleic acid, albumin, dextrose and catalase) enrichment. (Difco, USA).

In vitro anti-tuberculosis activity screening The antimycobacterial activity was performed by a colorimetric tetrazolium microplate assay (TEMA) as described by Caviedes *et al.* (2002) with some modifications. The assay was done in 96-well sterile microplates. Each derivative was tested thrice in triplicates. First, 200 μ L of sterile distilled water was added into the outer wells to prevent dehydration, followed by the addition of 100 μ L of the Middlebrook 7H9 broth supplemented with 10 % OADC was added into wells 3, until 11 in rows B to G. An amount of 100 μ L of each derivative working solution (10 μ g mL⁻¹) was added in triplicate into wells in columns 2 and 3. The solutions

were then serially diluted with multichannel pipette from wells in columns 3 to 4 until wells 10. The last 100 μL of solutions from wells in column 10 were discarded. Finally, 100 μL of bacterial suspension was added into all the test wells. The wells in column 11 served as drug-free growth controls. Isoniazid, a standard anti-TB drug was used as a positive control. The test concentrations of drugs were ranged from 5 to 0.0195 $\mu\text{g mL}^{-1}$. The plates were sealed and incubated at 37 °C in 8 % CO_2 , for 5 days. On day 5, 50 μL of tetrazolium–tween 80 mixture [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide with a concentration of 1 mg mL^{-1} in absolute ethanol and 10 % tween 80 at 1:1] was added to well B11, and incubated for 24 h. If well B11 turned from yellow to purple, the reagent (tetrazolium–tween 80 mixtures) was added to all wells and incubated for another 24 h and the results were read visually on the following day. If the well B11 remained yellow, the plates were incubated for another 24 h, tetrazolium–tween 80 mixture was added to well C11 and incubated for additional 24 h. If the C11 remained yellow, incubation was continued, and tetrazolium–tween 80 mixture was added to wells D11, E11, F11 and G11 on day 9, 11, 13 and 15, respectively. The minimum inhibitory concentration therefore, was defined as the lowest drug concentration that prevented a colour change of tetrazolium dye from yellow to purple.

Anticancer study

Preparation of cell culture Initially, cells (HCT 116) were allowed to grow under optimal incubator conditions. Cells that have reached a confluency of 70–80 % were chosen for cell plating purposes. The old medium was aspirated out of the plate, and the cells were washed 2–3 times using sterile phosphate buffered saline (PBS) (pH 7.4). Following this, trypsin was added and distributed evenly onto cell surfaces. Cells were incubated at 37 °C in 5 % CO_2 for 1 min. Then, the flasks containing the cells were gently tapped to aid cell segregation and observed under an inverted microscope (if cells segregation is not satisfying, the cells will be incubated for another minute). Trypsin activity was inhibited by adding 5 mL of fresh complete media (10 % FBS). Cells were counted and diluted to get a final concentration of 2.5×10^5 cells mL^{-1} , and inoculated into wells (100 μL cells/well). Finally, plates containing the cells were incubated at 37 °C with an internal atmosphere of 5 % CO_2 .

MTT Assay Cancer cells (100 μL cells/well, 1.5×10^5 cells mL^{-1}) were inoculated in wells of microtitre plates. Then the plate is incubated in a CO_2 incubator for overnight in order to allow the cell for attachment. One hundred micro litre of test substance was added into each well containing the cells. Test substance was diluted with media

into the desired concentrations from the stock and the plates were incubated at 37 °C with an internal atmosphere of 5 % CO_2 for 72 h. After this treatment period, 20 μL of MTT reagent was added into each well and incubated again for 4 h, followed by the addition of 50 μL of MTT lysis solution (DMSO) into the wells. The plates were further incubated for 5 min in CO_2 incubator, and read at 570 and 620 nm wavelengths using a standard ELISA microplate reader (Ascent Multiskan). Data was recorded and analysed for the assessment of the effects of test substance on cell viability and growth inhibition. The percentage of growth inhibition was calculated from the optical density (OD) that was obtained from MTT assay, i.e. hundredth multiples of the subtracted OD value of control and survived cells over the OD of control cells.

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