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Short communication

Synthesis, characterization and biological evaluation of novel 2,4,6-trisubstituted bis-pyrimidine derivatives

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1. Introduction

Amoebiasis is the most aggressive protozoal disease and considered to be the second or third leading cause of death amongst the parasitic diseases [1]. Entamoeba histolytica, a protozoan parasite, is the causative agent of amoebiasis and amoebic dysentery. Though ubiguitous in distribution, this parasite is more prevalent in tropical and subtropical regions [2]. Metronidazole is known to be highly effective amoebicide and is considered to be the drug of choice for the treatment of amoebiasis, but this drug has been shown to be mutagenic in a microbiological system and carcinogenic to rodents [3-5]. Repeated treatment of E. histolytica infection with commonly used antiamoebic drugs results in not only increasing the toxicity potential but also leads to the development of clinical resistance. Therefore, new effective agents with less toxicity against amoebiasis are urgently required. Five membered heterocyclic compounds natural as well as synthetic are important for their biological activities. Compounds with pyrimidine ring are of interest due to their broad

ABSTRACT

A new series of 2,4,6-trisubstituted bis-pyrimidines were synthesized and evaluated for *in vitro* antiamoebic activity against HM1:IMSS strain of *Entamoeba histolytica*. Out of 16 compounds 8 compounds have shown IC_{50} values in the range of 0.10–1.86 μ M. Bis-pyrimidine having methyl-, methoxy-, thiomethyl- and dimehyl-phenyl substituents, exhibited higher antiamoebic activity than the reference drug metronidazole ($IC_{50} = 1.9 \ \mu$ M). The toxicological studies of active compounds on PC12-rat pheochoromocytoma cell line showed that all compounds were non-toxic at a concentration of 100 μ M. 4-4'-Benzene-1,3-diylbis[6-(4-methylphenyl-2-(piperidin-1-yl)pyrimidine] (4c) was found most active ($IC_{50} = 0.10 \ \mu$ M) and least toxic among all the compounds.

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spectrum of biological activities [6–25]. In view of these observations and as a part of our ongoing program devoted to the synthesis of diverse heterocycles as antiamoebic agents [26,27], we have synthesized a new series of organic molecules containing two pyrimidine rings and have evaluated the same against HM1:IMSS strain of *E. histolytica*, a protozoan responsible for amoebiasis. On the basis of their activity and favourable therapeutic indexes, these compounds were identified as viable leads for further studies.

2. Chemistry

To synthesize the 2,4,6-trisubstituted bis-pyrimidine derivatives (3a-3h and 4a-4h), teraphthaldicarboxaldehyde was reacted with different substituted aromatic acetophenone (a-h) in NaOH and methanol to yield the corresponding bis-chalcones 2a-2h [28] Scheme 1. Pyrrolidine-1-carboxamidine hydrochloride and piperidine-1-carboxamidine hydrochloride and piperidine-1-carboxamidine hydrochloride by refluxing pyrrolidine and piperidine, respectively, with S-methyl isothiourea sulphate in water according to reported procedure [29]. The bis-chalcones 2a-2h were further cyclized with imidine hydrochlorides in the presence of sodium isopropoxide (synthesized in situ by adding sodium metal in isopropanol) to afford 2,4,6-trisubstituted bis-pyrimidines 3a-3h and 4a-4h as shown in Scheme 1. All the synthesized compounds were well characterized

Abbreviation: µM, Micromole.

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Scheme 1. General synthesis of 2,4,6-trisubstituted bis-pyrimidines (**3a-3h**) and (**4a-4h**). Reagents and conditions : (a) Different acetophenones (**a-h**), 10% aq NaOH, methanol, 0 °Crt, 30 min. (b) (i) Piperidine or pyrrolidine, *S*- methylisothiourea sulphate, water, reflux, 15 min (ii) Barium chloride, reflux, 15 min. (c) Pyrrolidine-1-carboxamidine hydrochloride (for **3a-3h**) or piperidine-1-carboxamidine hydrochloride (for **4a-4h**), sodium isopropoxide, isopropanol, reflux, **8h**.

by spectroscopic methods such as IR, NMR, Mass and elemental analysis.

3. Pharmacology

All newly synthesized bis-pyrimidine derivatives (**3a–3h**) and (**4a–4h**) were screened *in vitro* against HM1:IMSS strain of *E. histolytica* by microdilution method [30]. All the experiments were carried out in triplicate at each concentration level and repeated thrice. Cytotoxicity of active compounds has been studied by MTT assay on PC12-rat pheochoromocytoma cell line [34]. The results of biological activity are summarized in Tables 1 and 2.

4. Results and discussion

4.1. Synthesis

The synthesis of 2,4,6-trisubstituted bis-pyrimidine derivatives (**3a–3h** and **4a–4h**) was performed in a manner as outlined in Scheme 1. In the IR spectra of bis-chalcones (**2a–2h**) the appearance of characteristic bands at 1650–1661 cm⁻¹ and 1572–1589 cm⁻¹ due to α , β unsaturated carbonyl group and C=C, respectively,

suggested the condensation of substituted acetophenones with terephthaldicarboxaldehyde. In ¹H NMR spectra, the proton of *α*, *β* unsaturated carbonyl compounds showed two doublets in the range of δ 7.51–7.69 ppm for H-*α* and δ 7.77–7.88 ppm for H-*β* with coupling constant in the range of 15.6–16.2 Hz, it is inferred that they are trans isomers. The rest of signals for aromatic protons have appeared in the expected regions. The structures of all these compounds (**2a**–**2h**) were further confirmed by ¹³C NMR spectra. A characteristic signal for the chalcone (C=O) appeared in the range of δ 189.85–190.83 ppm. The signals at δ 121.56–122.87 ppm and δ 143.41–145.66 ppm revealed the presence of *α*, *β*-unsaturated keto function in bis-chalcones (**2a**–**2h**).

Assignment of selected characteristic IR bands provides significant indications for the formation of the bis-pyrimidine (**3a–3h** and **4a–4h**). All the compounds showed sharp bands in the region 1533–1598 cm⁻¹ due to C=N strech which confirmed the formation ring closure. In addition, the absorption bands at 1233–1295 cm⁻¹ were attributed to C–N stretch vibrations, which also confirm the formation of desired pyrimidine ring in all the compounds **3a–3h** and **4a–4h**. The structure of the compounds was further confirmed by ¹H NMR and ¹³C NMR. The appearance of singlet at δ 7.35–7.41 ppm showed the C–H proton of desire pyrimidine ring in

Table 1

2,4,6-Trisubstituted Bis-Pyrimidines (**3a**–**3h**), their antiamoebic activity against HM1:IMSS strain of *Entamoeba histolytica* and toxicity profile.



S. No.	R	Antiamoebic activity		Toxicity Profile	
		<i>IC</i> ₅₀ (μM)	S.D. ^a (±)	<i>IC</i> ₅₀ (μM)	Safety Index
3a	Н	4.76	0.012	N.D. ^b	N.D.
3b	4-Cl	9.57	0.028	N.D.	N.D.
3c	4-Me	0.16	0.013	>100	>625
3d	3,4-DiMe	0.89	0.012	>100	>112.36
3e	4-SMe	1.54	0.009	>100	>64.94
3f	4-OMe	0.88	0.011	>100	>113.64
3g	2,5-DiOMe	5.81	0.031	N.D.	N.D.
3h	3,4,5-TriOMe	2.12	0.006	N.D.	N.D.

^a Standard Deviation. The compounds with bold font IC_{50} values are more active than metronidazole.

^b Not detected.

all the compounds **3a**–**3h** and **4a**–**4h**. Pyrrolidine and piperidine functionality appeared in the ¹H NMR spectra as multiplets at δ 1.52–3.86 ppm for the compounds **3a**–**3h** and **4a**–**4h**. Further evidence for the formation of pyrimidines analogues was obtained from ¹³C NMR spectra. A signal at δ 164.4–166.5 and δ 161.8–162.8 ppm was attributed to C=N and C=C respectively in all the compounds **3a**–**3h** and **4a**–**4h**. The characteristic signal for C–H of pyrimidine ring found in the range of δ 102.1–105.7 ppm clearly favoured the formation of pyrimidine nucleus in all the

Table 2

2,4,6-Trisubstituted Bis-pyrimidines (**4a**–**4h**), their antiamoebic activity against HM1:IMSS strain of *Entamoeba histolytica* and toxicity profile.



S. No.	R	Antiamoebic activity		Toxicity Profile	
		<i>IC</i> ₅₀ (μM)	S.D. ^a (±)	<i>IC</i> ₅₀ (μM)	Safety Index (SI)
4a	Н	2.86	0.009	N.D. ^b	N.D.
4b	4-Cl	5.33	0.004	N.D.	N.D.
4c	4-Me	0.10	0.014	>100	>1000
4d	3,4-DiMe	0.53	0.005	>100	>188.68
4e	4-SMe	1.86	0.006	>100	>53.76
4f	4-OMe	0.52	0.010	>100	>192.31
4g	2,5-DiOMe	7.39	0.008	N.D.	N.D.
4h	3,4,5-TriOMe	2.41	0.005	N.D.	N.D.
	Metronidazole	9	0.020	>100	>52.63

^a Standard Deviation. The compounds with bold font IC_{50} values are more active than metronidazole.

^b Not detected.

compounds. The signals due to the phenyl ring, pyrimidine and piperidine ring resonate at their usual position, and the values are given in the experimental section.

4.2. Antiamoebic activities

Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of all the compounds **3a–3h** and **4a–4h** by microdilution method using HM1:IMSS strain of E. histolytica. The results are summarized in Tables 1 and 2. The data are present in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic effect was compared with the most widely used antiamoebic medication metronidazole which had a 50% inhibitory concentration (IC_{50}) of 1.9 μ M in our experiments. The results showed that in bis-pyrimidine derivatives, when the R group was a phenyl ring the pyrrolidine substituted compound (**3a**) showed IC_{50} 4.76 μ M. Substitution of the phenyl ring with chloro group (3b) dimethoxy group (**3g**) and trimethoxy group (**3h**) also did not affect the activity. Substitution with thiomethyl group (3e), monomethoxy group (3f), mono-(3c) and dimethyl group (3d), increased the activity. An almost similar trend was also observed in piperidine substituted compounds. The phenyl ring substituted compound showed IC50 2.86 μ M. Substitution with chloro group (4b), dimethoxy group (4g) and trimethoxy group (4h) had no effect on the activity. Monomethyl (4c) dimethyl (4d), thiomethyl group (4e) and monomethoxy (4f) showed increased activity. In general the activity profiles in both the pyrrolidine and piperidine substituted compounds are almost similar. Therefore out of sixteen compounds screened in vitro for antiamoebic activity, 8 compounds (3c-3f) and (4c-4f) were found more active than the reference drug metronidazole.

4.3. Toxicity profile

Compounds **3c**–**3f** and **4c**–**4f** was tested to find the toxic effects on PC12-rat pheochoromocytoma cell line. No one inhibited cell growth at a concentration of 100 μ M. To investigate the selectivity of the compounds, the "safety index" (SI) was calculated and defined as the toxicity *IC*₅₀/protozoal *IC*₅₀, where toxicity *IC*₅₀ is defined as the concentration of compound that kills 50% of the PC12-rat pheochoromocytoma cell line and protozoal *IC*₅₀ is the concentration that kills 50% of amoeba protozoa. This allows to estimate which compound might be efficacious or toxic against human cells and potentially in vivo. The numerical results for each compound are given in Tables 1–2.

5. Conclusion

The sixteen 2.4.6-trisubstituted bis-pyrimidines (**3a–3h** and 4a-4h) were synthesized by the cyclization of bis-chalcones (2a-2h) with pyrrolidine-1-carboxamidine hydrochloride and piperidine-1carboxamidine hydrochloride respectively. The in vitro antiamoebic activity was examined using HM1:IMSS strain of E. histolytica and results showed that out of the 16 compounds 8 compounds having monomethoxy substitution, thiomethyl group and mono and dimethyl group, exhibited higher antiamoebic activity than the reference drug metronidazole ($IC_{50} = 1.9 \mu$ M). The MTT assay revealed that all the compounds are non-toxic to PC12-rat pheochoromocytoma cells. 4-4'-Benzene-1,3-diylbis[6-(4-methylphenyl-2-(piperidin-1-yl)pyrimidine] (4c) was found most active and least toxic among all the compounds. The present study suggested that the newly synthesized 2,4,6-trisubstituted bis-pyrimidines are new leads in antiamoebic chemotherapy. These molecules can be very useful for further optimization work in amoebicidal chemotherapy, in order to discover and develop better and yet safer therapeutic agents for amoebiasis.

6. Experimental protocol

All the chemicals were purchased from Aldrich Chemical Company (USA). Precoated aluminium sheets (silica gel 60 F₂₅₄, Merck Germany) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. Elemental analyses were performed on Heraeus Vario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within $\pm 0.3\%$ of the theoretical values. Melting points were determined on MEL-TEMP capillary melting point apparatus and are uncorrected. IR spectra were recorded on Perkin–Elmer model 1600 FT-IR RX1 spectro-photometer as KBr discs. ¹H NMR and ¹³C NMR spectra were recorded on Bruker AVANCE 400 spectrometer using CDCl₃ or DMSO-*d*₆ as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer.

6.1. General procedure for the preparation of bis-chalcones (2a-2h)

An aqueous solution of sodium hydroxide (60%, 150 mL) was added slowly to a methanolic solution (200 mL) of appropriate acetophenone (33 mmol). After cooling the solution to room temperature, a methanolic suspension (50 mL) of terephthaldicarboxaaldehyde (2.2 g, 16.4 mmol) was added. The reaction mixture was stirred at room temperature for 20 h, and then it was poured into a mixture of ice and hydrochloric acid (pH was adjust to about 2). The resulting solid was filtered, dissolved in dichloromethane (150 mL), and washed with a saturated solution of sodium hydrogen carbonate (2 \times 100 mL). The solvent was evaporated to dryness and the residue was purified by column chromatography using dichloromethane as eluent.

6.1.1. (2E,2'E)-3,3'-benzene-1,4-diylbis(1-phenylprop-2-en-1-one) (**2a**)

Yield 88%; solid (Ethanol); mp: 100–110 °C; Anal. calc. for C₂₄H₁₈O₂ : C 85.18, H 5.36%; found: C 85.09, H 5.29%; IR v_{max} (cm⁻¹): 3064 (Ar–H), 2921 (C–H), 1650 (C=O), 1589 (C=C); ¹H NMR (CDCl₃) δ (ppm): 8.00–7.93 (m, 4H, Ar–H), 7.88 (d, 2H, *J* = 15 Hz, H_β), 7.76–7.72 (m, 4H, Ar–H), 7.69 (d, 2H, *J* = 15 Hz, H_α), 7.57–7.35 (m, 6H, Ar–H); ¹³C NMR (CDCl₃) δ (ppm): 190.83 (C=O), 143.41 (C-β), (136.07, 134.70, 130.02, 128.51, Aromatic), 122.52 (C-α). ESI-MS m/z: [M⁺1] 339.1.

6.1.2. (2E,2'E)-3,3'-benzene-1,4-diylbis[1-(4-chlorophenyl)prop-2en-1-one] (**2b**)

Yield 76%; solid (Ethanol); mp: 220 °C; Anal. calc. for C₂₄H₁₆O₂Cl₂ : C 70.77, H 3.96%; found: C 70.88, H 3.86%; IR ν_{max} (cm⁻¹): 3068 (Ar–H), 2929 (C–H), 1655 (C=O), 1575 (C=C); ¹H NMR (CDCl₃) δ (ppm): 8.16–8.09 (m, 4H, Ar–H), 7.94–7.89 (m, 3H, Ar–H), 7.83 (d, 2H, J = 15.6 Hz, H_β), 7.77 (d, 2H, J = 7.6 Hz, Ar–H),7.71 (d, 2H, J = 15.6 Hz, H_α), 7.53–7.46 (m, 4H, Ar–H); ¹³C NMR (CDCl₃) δ (ppm): 190.65 (C=O), 144.56 (C-β), (138.17, 136.50, 134.12, 127.51, Aromatic), 122.66 (C-α). ESI-MS m/z: [M⁺1] 408.0.

6.1.3. (2E,2'E)-3,3'-benzene-1,4-diylbis[1-(4-methylphenyl)prop-2en-1-one] (**2c**)

Yield 76%; solid (Ethanol); mp: 165–170 °C; Anal. calc. for $C_{26}H_{22}O_2$: C 85.22, H 6.05%; found: C 85.30, H 6.00%; IR v_{max} (cm⁻¹): 3066 (Ar–H), 2922 (C–H), 1661 (C=O), 1583(C=C); ¹H NMR (CDCl₃) δ (ppm): 7.89–7.80 (m, 4H, Ar–H), 7.77 (d, 2H, *J* = 16.2 Hz, H_β), 7.72–7.71(m, 4H, Ar–H), 7.69 (d, 2H, *J* = 16.2 Hz, H_α), 7.57–7.18 (m,

4H, Ar–H), 2.47 (s, 3H, CH₃), 2.34 (s, 3H, CH₃); ¹³C NMR (CDCl₃) δ (ppm): 189.66 (C=O), 144.76 (C- β), (138.77, 136.67, 133.42, 127.76, Aromatic), 121.67 (C- α), 21.50 (CH₃); ESI-MS m/z: [M⁺1] 367.1.

6.1.4. (2E,2'E)-3,3'-benzene-1,4-diylbis[1-(3,4-dimethylphenyl)prop-2-en-1-one] (**2d**)

Yield 82%; solid (Ethanol); mp: 200 °C; Anal. calc. for $C_{28}H_{26}O_2$: C 85.25, H 6.64%; found: C 85.28, H 6.63%; IR v_{max} (cm⁻¹): 3061 (A–H), 2931 (C–H), 1656 (C=O), 1579 (C=C); ¹H NMR (CDCl₃) δ (ppm): 8.01–7.91 (m, 4H, Ar–H), 7.86–7.81 (2H, m, Ar–H), 7.79 (d, 2H, *J* = 15.6 Hz, H_β), 7.77–7.71 (m, 2H, Ar–H),7.68 (d, 2H, *J* = 15.6 Hz, H_α), 7.62–7.55 (m, 4H, Ar–H), 2.47 (s, 6H, CH₃), 2.28 (s, 6H, CH₃); ¹³C NMR (CDCl₃) δ (ppm): 190.75 (C=O), 145.66 (C-β), (138.22, 136.76, 134.11, 128.88, Aromatic), 122.87 (C-α); ESI-MS m/z: [M⁺1] 395.2.

6.1.5. (2E,2'E)-3,3'-benzene-1,4-diylbis[(1-(4-methylthio)phenyl) prop-2-en-1-one] (**2e**)

Yield 74%; solid (Ethanol); mp: 185–190 °C; Anal. calc. for $C_{26}H_{22}O_{2}S_{2}$: C 72.52, H 5.15%; found: C 72.54, H 5.18%; IR ν_{max} (cm⁻¹): 3068 (Ar–H), 2917 (C-H), 1653 (C=O), 1589 (C=C); ¹H NMR (CDCl₃) δ (ppm): 8.12–7.94 (m, 4H, Ar–H), 7.84 (d, 2H, J = 16.2 Hz, H_β), 7.79–7.71 (m, 4H, Ar–H),7.68 (d, 2H, J = 16.2 Hz, H_α), 7.31–7.23 (m, 4H, Ar–H), 2.42 (s, 3H, –SCH₃); ¹³C NMR (CDCl₃) δ (ppm): 189.85 (C=O), 144.84 (C-β), (138.22, 136.78, 134.22, 127.52, Aromatic), 121.64 (C-α), 14.98 (SCH₃), ESI-M m/z: [M⁺1] 431.1.

6.1.6. (2E,2'E)-3,3'-benzene-1,4-diylbis[1-(4-methoxyphenyl)prop-2en-1-one] (**2f**)

Yield 78%; solid (Ethanol); mp: 220 °C; Anal. calc. for $C_{26}H_{22}O_4$: C 78.37, H 5.57%; found: C 78.31, H 5.52%; IR ν_{max} (cm⁻¹): 3069 (Ar–H), 2914 (C–H), 1657 (C=O), 1583 (C=C); ¹H NMR (CDCl₃) δ (ppm) 8.20–7.92 (m, 4H, Ar–H), 7.86 (d, 2H, J = 16 Hz, H_β), 7.76–7.73 (m, 4H, Ar–H), 7.71 (d, 2H, J = 16 Hz, H_α), 7.45 (d, 1H, J = 6Hz, Ar–H), 7.03–6.95 (m, 3H, Ar–H), 3.84 (s, 3H, –OCH₃), 3.80 (s, 3H, –OCH₃); ¹³C NMR (CDCl₃) δ (ppm): 190.87 (C=O), 143.76 (C- β), (137.87, 136.80, 134.92, 127.23, Aromatic), 122.45 (C-α), 55.19 (OCH₃), ESI-MS m/z: [M⁺1] 399.1.

6.1.7. (2E,2'E)-3,3'-benzene-1,4-diylbis[1-(3,4-dimethoxyphenyl) prop-2-en-1-one] (**2g**)

Yield 78%; solid (Ethanol); mp: 189 °C; Anal. calc. for $C_{28}H_{26}O_6$: C 73.35, H 5.72%; found: C 37.31, H 5.78%; IR v_{max} (cm⁻¹): 3068 (Ar–H), 2927(C–H), 1654 (C=O), 1572 (C=C); ¹H NMR (CDCl₃) δ (ppm) 8.18–7.96 (m, 4H, Ar–H), 7.84 (d, 2H, *J* = 15.6 Hz, H_β), 7.68–7.53 (m, 3H, Ar–H), 7.51 (d, 2H, *J* = 15.6 Hz, H_α), 7.27–7.18 (m, 3H, Ar–H), 3.83 (s, 6H, –OCH₃), 3.80 (s, 6H, –OCH₃); ¹³C NMR (CDCl₃) δ (ppm): 189.76 (C=O), 143.87 (C-β), (138.44, 136.60, 134.82, 127.56, Aromatic), 122.09 (C-α), 56.87 (OCH₃), ESI-MS m/z: [M⁺1] 459.1.

6.1.8. (2E,2'E)-3,3'-benzene-1,4-diylbis[1-(3,4,5-trimethoxyphenyl) prop-2-en-1-one] (**2h**)

Yield 78%; solid (Ethanol); mp: 180 °C; Anal. calc. for $C_{30}H_{30}O_8$: C 69.49, H 5.83%; found: C 69.42, H 5.88%; IR ν_{max} (cm⁻¹): 3067(Ar–H), 2925 (C–H), 1658 (C=O), 1576 (C=C); ¹H NMR (CDCl₃) δ (ppm) 8.12–7.91 (m, 4H, Ar–H), 7.86 (d, 2H, *J* = 15.6 Hz, H_β), 7.66–7.55 (m, 2H, Ar–H), 7.52 (d, 2H, *J* = 15.6 Hz, H_α), 7.25–7.22 (m, 2H, Ar–H), 3.92 (s, 6H, OCH₃), 3.88 (s, 6H, OCH₃); ¹³C NMR (CDCl₃) δ (ppm): 190.46 (C=O), 144.67 (C-β), (138.87, 136.44 134.82, 127.57, Aromatic), 121.56 (C-α), 61.84 (OCH₃), 55.67 (OCH₃). ESI-MS m/z: [M⁺1] 519.2.

6.2. General procedure for the synthesis of compounds (**3a**–**3h**)

To a solution of 1.0 equiv. of pyrrolidine-1-carboxamidine hydrochloride in 50 mL of isopropanol, 1.1 equiv. of sodium metal was added. The reaction mixture was refluxed for 2 h and then

different bis-chalcones **(2a–2h**, 1.0 equiv.) were added to it and refluxed for 8 h. The solvent was removed from the reaction mixture under reduced pressure. Water was added and the aqueous phase was extracted with chloroform and washed with brine solution. The organic phase was dried over anhydrous sodium sulphate, filtered and concentrated. The crude product was purified by crystallization from methanol or ethanol or sometimes by column chromatography on silica gel (2% methanol in chloroform to afford the pure compounds.

6.2.1. 4-4'-Benzene-1,3-diylbis[6-phenyl-2-(pyrrolidin-1-yl) pyrimidine] (**3a**)

Yield 71%; solid (Ethanol); mp: 123 °C; Anal. calc. for $C_{34}H_{32}N_6$: C 77.83, H 6.15, N 16.02%; found: C 77.89, H 6.13, N 16.05%; IR v_{max} (cm⁻¹): 3056 (Ar–H), 2973 (C-H), 1576 (C=N),1436, (C=C), 1286 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.12–7.76 (m, 4H, Ar–H), 7.68–7.61 (m, 4H, Ar–H), 7.54–7.33 (m, 6H, Ar–H), 7.37 (s, 2H, pyrimidine), 3.81–3.72 (m, 8H, pyrrolidine), 2.02–1.78 (m, 8H, –CH₂ pyrrolidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.7 (C=N pyrimidine), 162.3 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 140.1, 138.7, 130.5, 127.8, 126.9, 123.7, 122.5, 115.6 (Ar–C), 104.7 (C–H pyrimidine), 47.2, 25.3 (CH₂ pyrrolidine). ESI-MS m/z: [M⁺1] 525.2.

6.2.2. 4-4'-Benzene-1,3-diylbis[6-(4-chlorophenyl-2-(pyrrolidin-1-yl) pyrimidine] (**3b**)

Yield 73%; solid (Ethanol); mp: 180 °C; Anal. calc. for $C_{34}H_{30}N_6Cl_2$: C 68.80, H 5.09, N 14.16%; found: C 68.85, H 5.12, N 14.11%; IR ν_{max} (cm⁻¹): 3067 (Ar–H), 2973 (C–H), 1596 (C=N), 1476, (C=C), 1279 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.14–7.99 (m, 4H, Ar–H), 7.97–7.83 (m, 4H, Ar–H), 7.49–7.43 (m, 4H, Ar–H), 7.35 (s, 2H, pyrimidine), 3.83–3.74 (m, 8H, pyrrolidine), 2.05–1.76 (m, 8H, –CH₂ pyrrolidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.6 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.2 (N=C–N pyrimidine), 140.7, 138.7, 136.1, 130.6, 129.6, 126.2, 123.6, 122.6, 115.1 (Ar–C), 104.5 (C–H pyrimidine), 47.4, 25.7 (–CH₂ pyrrolidine). ESI-MS m/z: [M⁺1] 593.1.

6.2.3. 4-4'-Benzene-1,3-diylbis[6-(4-methylphenyl-2-(pyrrolidin-1-yl) pyrimidine] (**3c**)

Yield 69%; solid (Ethanol); mp: 150 °C; Anal. calc. for $C_{36}H_{36}N_6$: C 78.23, H 6.57, N 15.21%; found: C 78.25, H 6.59, N 15.26%; IR v_{max} (cm⁻¹): 3087 (Ar–H), 2997(C-H), 1543 (C=N), 1413, (C=C), 1278 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.09–7.96 (m, 4H, Ar–H), 7.72–7.68 (m, 4H, Ar–H), 7.37 (s, 2H, pyrimidine), 7.22–7.11 (m, 4H, Ar–H), 3.82–3.76 (m, 8H, pyrrolidine), 2.03–1.77 (m, 8H, CH₂ pyrrolidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.5 (C=N pyrimidine), 162.8 (C=C pyrimidine), 161.3 (N=C–N pyrimidine), 140, 141.4, 130.6, 129.3, 126.9, 122.4, 117.7 (Ar–C), 104.8 (C–H pyrimidine), 47.3, 25.6 (CH₂ pyrrolidine), 21.3 (CH₃ phenyl). ESI-MS m/z: [M⁺1] 553.3.

6.2.4. 4-4'-Benzene-1,3-diylbis[6-(3,4-dimethylphenyl-2-(pyrrolidin-1-yl)pyrimidine] (**3d**)

Yield 71%; solid (Ethanol); mp: 180 °C; Anal. calc. for $C_{38}H_{40}N_6$: C 78.59, H 6.94, N 14.47%; found: C 78.55, H 6.91, N 14.49%; IR v_{max} (cm⁻¹): 3056 (Ar–H), 2986 (C-H), 1597 (C=N), 1432 (C=C), 1235 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.15–7.96 (m, 4H, Ar–H), 7.75–7.73 (m, 4H, Ar–H), 7.38 (s, 2H, pyrimidine), 7.23–7.14 (m, 4H, Ar–H), 3.83–3.78 (m, 8H, pyrrolidine), 2.35 (s, 6H, CH₃) 2.01–1.76 (m, 8H, CH₂ pyrrolidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.4 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 140.3, 141.6, 130.5, 129.4, 126.7, 122.8, 117.4 (Ar–C), 104.6 (C–H pyrimidine), 47.4, 25.7 (–CH₂ pyrrolidine), 21.7 (CH₃ phenyl). ESI-MS m/z: [M⁺1] 581.3.

6.2.5. 4-4'-Benzene-1,3-diylbis[6-(4-methylthiophenyl-2-(pyrrolidin-1-yl)pyrimidine] (**3e**)

Yield 72%; solid (Ethanol); mp: 105–110 °C; Anal. calc. for $C_{36}H_{36}N_6S_2$: C 70.10, H 5.88, N 13.62%; found: C 70.15, H 5.91, N 13.66%; IR v_{max} (cm⁻¹): 3066 (Ar–H), 2954(C–H), 1575 (C=N),1435, (C=C), 1285 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.13–7.96 (m, 4H, Ar–H), 7.78–7.69 (m, 4H, Ar–H), 7.37 (s, 2H, pyrimidine) 7.33–7.23 (m, 4H, Ar–H), 3.83–3.71 (m, 8H, pyrrolidine), 2.41 (m, 6H, SMe), 2.04–1.74 (m, 8H, pyrrolidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.5 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.4 (N=C–N pyrimidine), 140.1, 141.5, 130.7129.3, 126.6, 122.7, 117.6 (Ar–C), 104.4 (C–H pyrimidine), 47.6, 25.7 (CH₂ pyrrolidine), 16.7 (SCH3 phenyl). ESI-MS m/z: [M⁺1] 617.8.

6.2.6. 4-4'-Benzene-1,3-diylbis[6-(4-methoxyphenyl-2-(pyrrolidin-1-yl)pyrimidine] (**3f**)

Yield 66%; solid (Ethanol); mp: 131 °C; Anal. calc. for $C_{36}H_{36}N_6O_4$: C 73.95, H 6.21, N 14.37%; found: C 73.91, H 6.29, N 14.39%; IR v_{max} (cm⁻¹): 3075 (Ar–H), 2945 (C–H), 1567 (C=N),1436, (C=C), 1286 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.19–7.97 (m, 4H, Ar–H), 7.94–7.83 (m, 4H, Ar–H), 7.35 (s, 2H, pyrimidine) 7.18–7.02 (m, 4H, Ar–H), 3.89–3.83 (m, 8H, pyrrolidine), 3.84 (s, 3H, OCH₃), 2.06–1.72 (m, 8H, pyrrolidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.5 (C=N pyrimidine), 162.4 (C=C pyrimidine), 161.6 (N=C–N pyrimidine), 140.7, 139.2, 130.7, 129.2, 126.1, 123.4, 122.6, 115.3 (Ar–C), 102.2 (C–H pyrimidine), 55.5 (–OCH₃), 47.5, 25.6 (–CH₂ pyrrolidine). ESI-MS m/ z: [M⁺1] 585.2.

6.2.7. 4-4'-Benzene-1,3-diylbis[6-(2,5-dimethoxyphenyl-2-(pyrrolidin-1-yl)pyrimidine] (**3g**)

Yield 70%; solid (Ethanol); mp: 148 °C; Anal. calc. for C₃₈H₄₀N₆O₄: C 70.79, H 6.25, N 13.03%; found: C 70.81, H 6.29, N 13.09%; IR v_{max} (cm⁻¹): 3077 (Ar–H), 2976 (C–H), 1535 (C=N),1465, (C=C), 1244 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.17–7.99 (m, 4H, Ar–H), 7.92–7.81 (m, 4H, Ar–H), 7.41(s, 2H, pyrimidine) 7.25–7.08 (m, 4H, Ar–H), 3.84 (s, 6H, 2XOCH₃), 3.86 (s, 6H, 2XOCH₃), 3.81–3.72 (m, 8H, pyrrolidine); 2.05–1.70 (m, 8H, pyrrolidine) ¹³C NMR (DMSO-d₆) δ (ppm): 164.8 (C=N pyrimidine), 162.1 (C=C pyrimidine), 160.5 (N=C–N pyrimidine), 154.0, 139.2, 130.8, 129.7, 125.9, 123.6, 122.5, 117.5 (Ar–C), 103.2 (C–H pyrimidine), 55.4 (–OCH3), 47.9, 25.3 (–CH₂ pyrrolidine). ESI-MS m/z: [M⁺1] 645.3.

6.2.8. 4-4'-Benzene-1,3-diylbis[6-(3,4,5-trimethoxyphenyl-2-(pyrrolidin-1-yl) pyrimidine] (**3h**)

Yield 71%; solid (Ethanol); mp: 114 °C; Anal. calc. for C₄₀H₄₄N₆O₄: C 68.16, H 6.29, N 11.92%; found: C 68.19, H 6.25, N 11.98%; IR v_{max} (cm⁻¹): 3055 (Ar–H), 2917 (C–H), 1587 (C=N),1464, (C=C), 1243 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.11–7.94 (m, 4H, Ar–H), 7.66 (s, 4H, Ar–H), 7.38 (s, 2H, pyrimidine) 3.88–3.82 (m, 8H, pyrrolidine), 3.84 (s, 12H, OCH3), 3.82 (s, 6H, OCH₃), 2.03–1.74 (m, 8H, pyrrolidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.5 (C=N pyrimidine), 162.7(C=C pyrimidine), 161.4 (N=C–N pyrimidine), 140.3, 130.6, 129.9, 126.9, 123.6, 122.7, 115.5 (Ar–C), 103.9 (C–H pyrimidine), 60.9 (OCH₃), 55.5 (2XOCH₃), 47.5, 25.3 (–CH₂ pyrrolidine). ESI-MS m/z: [M⁺1] 705.3.

6.3. General procedure for the synthesis of compounds (4a–4h)

To a solution of 1.0 equiv. of piperidine-1-carboxamidine hydrochloride in 50 mL of isopropanol, 1.1 equiv. of sodium metal was added. The reaction mixture was refluxed for 2 h and then different bis-chalcones (**2a**–**2h**, 1.0 equiv.) were added to it and refluxed for 8 h. The solvent was removed from the reaction mixture under reduced pressure. Water was added and the aqueous phase was extracted with chloroform and washed with brine solution. The organic phase was dried over anhydrous sodium sulphate, filtered and concentrated. The crude product was purified by crystallization from methanol or ethanol or sometimes by column chromatography on silica gel (2% methanol in chloroform to afford the pure compounds.

6.3.1. 4-4'-Benzene-1,3-diylbis[6-phenyl-2-(piperidin-1-yl) pyrimidine](**4a**)

Yield 68%; solid (Ethanol); mp: 133 °C; Anal. calc. for $C_{36}H_{36}N_6$: C 78.23, H 6.57, N 15.21%; found: C 78.20, H 6.53, N 15.28%; IR v_{max} (cm⁻¹): 3077 (Ar–H), 2953 (C–H), 1576 (C=N), 1486, (C=C), 1234 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.14–7.74 (m, 4H, Ar–H), 7.67–7.63 (m, 4H, Ar–H), 7.57–7.36 (m, 6H, Ar–H), 7.35 (s, 2H, pyrimidine), 3.88–3.82 (m, 8H, piperidine), 1.71–1.56 (m, 12H, piperidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.5 (C=N pyrimidine), 162.2 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 141.4, 138.5, 130.6, 127.1, 126.7, 124.3, 123.5, 116.3 (Ar–C), 104.6 (C–H pyrimidine), 46.4, 27.6, 25.4 (–CH₂ piperidine). ESI-MS m/z: [M⁺1] 553.

6.3.2. 4-4'-Benzene-1,3-diylbis[6-(4-chlorophenyl-2-(piperidin-1-yl) pyrimidine](**4b**)

Yield 72%; solid (Ethanol); mp: 133 °C; Anal. calc. for $C_{36}H_{34}N_6Cl_2$: C 69.56, H 5.51, N 13.52%; found: C 69.51, H 5.55, N 13.58%; IR v_{max} (cm-¹): 3065 (Ar–H), 2923 (C–H), 1545 (C=N),1465, (C=C), 1254 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.10–8.04 (m, 4H, Ar–H), 7.95–7.84 (m, 4H, Ar–H), 7.46–7.41 (m, 4H, Ar–H), 7.38 (s, 2H, pyrimidine), 3.87–3.83 (m, 8H, piperidine), 1.69–1.53 (m, 12H, piperidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.3 (C=N pyrimidine), 162.5 (C=C pyrimidine), 162.1 (N=C–N pyrimidine), 140.6, 138.3, 136.5, 130.5, 129.7, 126.8, 123.9, 122.4, 117.4 (Ar–C), 105.7 (C–H pyrimidine), 46.4, 27.5, 25.7 (–CH₂ piperidine). ESI-MS m/z: [M⁺1] 622.2.

6.3.3. 4-4'-Benzene-1,3-diylbis[6-(4-methylphenyl-2-(piperidin-1-yl) pyrimidine] (4c)

Yield 70%; solid (Ethanol); mp: 140 °C; Anal. calc. for $C_{38}H_{40}N_6$: C 78.59, H 6.94, N 14.47%; found: C 78.56, H 6.93, N 14.48%; IR v_{max} (cm⁻¹): 3068 (Ar–H), 2943 (C–H), 1576 (C=N), 1456, (C=C), 1244 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.12–7.99 (m, 4H, Ar–H), 7.74–7.71 (m, 4H, Ar–H), 7.36 (s, 2H, pyrimidine), 7.24–7.16 (m, 4H, Ar–H), 3.87–3.84 (m, 8H, piperidine), 2.36 (s, 6H, CH₃) 1.68–1.56 (m, 12H, piperidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.7 (C=N pyrimidine), 162.6 (C=C pyrimidine), 161.4 (N=C–N pyrimidine), 140.1, 139.4, 130.4, 1296, 126.2, 122.5, 117.5 (Ar–C), 104.6 (C–H pyrimidine), 47.2, 27.6, 24.7 (–CH2 piperidine), 21.5 (–CH3 phenyl). ESI-MS m/z: [M⁺1] 581.3.

6.3.4. 4-4'-Benzene-1,3-diylbis[6-(3,4-dimethylphenyl-2-(piperidin-1-yl)pyrimidine] (**4d**)

Yield 22%; solid (Ethanol); mp: 146 °C; Anal. calc. for C₄₀H₄₄N₆: C 78.91, H 7.28, N 13.80%; found: C 78.95, H 7.23, N 13.85%; IR v_{max} (cm⁻¹): 3066 (Ar–H), 2913 (C–H), 1566 (C=N), 1426, (C=C), 1239 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.12–7.99 (m, 4H, Ar–H), 7.74–7.71 (m, 4H, Ar–H), 7.37(s, 2H, pyrimidine), 7.24–7.16 (m, 4H, Ar–H), 3.85–3.83 (m, 8H, piperidine), 2.36 (s, 6H, CH₃) 1.70–1.58 (m, 12H, piperidine); ¹³C NMR (DMSO-d₆) δ (ppm): 165.7 (C=N pyrimidine), 162.4 (C=C pyrimidine), 162.6 (N=C–N pyrimidine), 139.7, 138.7, 136.9, 130.4, 129.5, 126.6, 123.2, 122.6, 118.7 (Ar–C), 103.6 (C–H pyrimidine), 46.2, 27.1, 25.5 (–CH₂ piperidine), 21.4 (2 × CH₃ phenyl). ESI-MS m/z: [M⁺1] 609.3.

6.3.5. 4-4'-Benzene-1,3-diylbis[6-(4-methylthiophenyl-2-(piperidin-1-yl)pyrimidine] (**4e**)

Yield 68%; solid (Ethanol); mp: 147 °C; Anal. calc. for $C_{38}H_{40}N_6S_2$: C 70.77, H 6.25, N 13.03%; found: C 70.75, H 6.23, N 13.05%; IR v_{max} (cm⁻¹): 3056 (Ar–H), 2978 (C–H), 1598 (C=N),1446, (C=C), 1233 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.13–7.96 (m, 4H, Ar–H), 7.78–7.69 (m, 4H, Ar–H), 7.37 (s, 2H, pyrimidine) 7.33–7.23 (m, 4H, Ar–H), 3.89–3.84 (m, 8H, piperidine), 2.41 (m, 6H, -SMe), 1.69–1.53 (m, 12H, piperidine); ¹³C NMR (DMSO-d₆) δ (ppm): 166.5 (C=N pyrimidine), 162.7 (C=C pyrimidine), 162.2 (N=C–N pyrimidine), 137.7, 138.4, 136.8, 130.9, 129.5, 126.8, 123.3, 122.5, 119.4 (Ar–C), 102.1 (C–H pyrimidine), 46.5, 27.6, 24.7 (–CH₂ piperidine)), 16.9 (SCH₃). ESI-MS m/z: [M⁺1] 645.2.

6.3.6. 4-4'-Benzene-1,3-diylbis[6-(4-methoxyphenyl-2-(piperidin-1-yl)pyrimidine] (4f)

Yield 61%; solid (Ethanol); mp: 155 °C; Anal. calc. for $C_{38}H_{40}N_6O_2$: C 74.48, H 6. 58, N 13.71%; found: C 74.45, H 6.53, N 13.75%; IR v_{max} (cm⁻¹): 3022(Ar–H), 2965 (C–H), 1567 (C=N),1489, (C=C), 1234 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.19–7.97 (m, 4H, Ar–H), 7.94–7.83 (m, 4H, Ar–H), 7.39 (s, 2H, pyrimidine) 7.18–7.02 (m, 4H, Ar–H), 3.89–3.83 (m, 8H, piperidine), 3.81 (s, 3H, OCH₃), 1.66–1.52 (m, 12H, piperidine ¹³C NMR (DMSO-d₆) δ (ppm): 164.5 (C=N pyrimidine), 161.8 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 140.7, 139.5, 130.7, 129.4, 126.8, 123.3, 122.9, 115.2 (Ar–C), 102.4 (C–H pyrimidine), 56.5 (–OCH₃), 47.8, 26.6, 24.9 (–CH₂ piperidine). ESI-MS m/z: [M⁺1] 613.3.

6.3.7. 4-4'-Benzene-1,3-diylbis[6-(2,5-dimethoxyphenyl-2-(piperidin-1-yl)pyrimidine] (**4g**)

Yield 68%; solid (Ethanol); mp: 141 °C; Anal. calc. for C₄₀H₄₄N₆O₄: C 71.41, H 6. 59, N 12.49%; found: C 71.45, H 6.53, N 12.50%; IR v_{max} (cm⁻¹): 3055 (Ar–H), 2965 (C–H), 1533 (C=N),1485, (C=C), 1236 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.17–7.99 (m, 4H, Ar–H), 7.92–7.81 (m, 4H, Ar–H), 7.41(s, 2H, pyrimidine) 7.25–7.08 (m, 4H, Ar–H), 3.87–3.86 (m, 8H, piperidine), 3.84 (s, 6H, 2XOCH₃), 3.83 (s, 6H, 2XOCH₃), 1.68–1.55 (m, 12H, piperidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.6 (C=N pyrimidine), 162.5 (C=C pyrimidine), 161.7 (N=C–N pyrimidine), 154.3, 139.7, 130.8, 129.5, 125.3 1237, 122.5, 117.1 (Ar–C), 103.3 (C–H pyrimidine), 56.6, 55.7 (–OCH₃), 47.7, 27.5, 25.6 (–CH₂ piperidine), ESI-MS m/z: [M⁺1] 673.3.

6.3.8. 4-4'-Benzene-1,3-diylbis[6-(3,4,5-trimethoxyphenyl-2-(piperidin-1-yl) pyrimidine] (**4h**)

Yield 77%; solid (Ethanol); mp: 142 °C; Anal. calc. for $C_{42}H_{48}N_6O_6$: C 68.83, H 6.60, N 11.47%; found: C 68.85, H 6.63, N 11.45%; IR v_{max} (cm⁻¹): 3044 (Ar–H), 2963 (C–H), 1534 (C=N),1487, (C=C), 1241 (C–N); ¹H NMR (DMSO-d₆) δ (ppm) 8.11–7.94 (m, 4H, Ar–H), 7.66 (s, 4H, Ar–H), 7.34 (s, 2H, pyrimidine) 3.88–3.82 (m, 8H, piperidine), 3.83 (s, 12H, OCH₃), 3.81 (s, 6H, OCH₃), 1.67–1.55 (m, 12H, piperidine); ¹³C NMR (DMSO-d₆) δ (ppm): 164.4 (C=N pyrimidine), 162.7 (C=C pyrimidine), 162.7 (N=C–N pyrimidine), 141.5, 130.8, 129.3, 126.6, 123.7, 122.1, 115.3 (Ar–C), 103.5 (C–H pyrimidine), 60.3 (OCH₃), 55.7 (2 × OCH₃), 47.4, 27.7, 25.7 (–CH₂ piperidine), ESI-MS m/z: [M⁺1] 733.3.

6.4. In vitro antiamoebic assay

All the compounds **3a**–**3h** and **4a**–**4h** were screened *in vitro* for antiamoebic activity against HM1:IMSS strain of *E. histolytica* by microdilution method [30]. *E. histolytica* trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [31]. The test compounds (1 mg) were dissolved in DMSO (40 μ L, level at which no inhibition of amoeba occurs) [32,33]. The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg/mL. Two-fold serial dilutions were made in the wells of 96-well microtiter plate (costar). Each test includes metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoebae) and a blank (culture medium only). All the experiments were carried out in triplicate at each concentration level and repeated thrice. The amoeba suspension was prepared from a confluent culture by pouring off the medium at 37 °C and adding 5 mL of fresh medium, chilling the culture tube on ice to detach the organisms from the side of the flask. The number of amoeba/mL was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The suspension was diluted to 10^5 organism/ml by adding fresh medium and 170 uL of this suspension was added to the test and control wells in the plate so that the wells were completely filled (total volume, 340 µL). An inoculum of 1.7×10^4 organisms/well was chosen so that confluent, but not excessive growth, took place in control wells. Plates were sealed and gassed for 10 min with nitrogen before incubation at 37 °C for 72 h. After incubation, the growth of amoeba in the plate was checked with a low power microscope. The culture medium was removed by inverting the plate and shaking gently. Plate was then immediately washed with sodium chloride solution (0.9%) at 37 °C. This procedure was completed quickly and the plate was not allowed to cool in order to prevent the detachment of amoebae. The plate was allowed to dry at room temperature and the amoebae were fixed with methanol and when dried, stained with (0.5%) aqueous eosin for 15 min. The stained plate was washed once with tap water, then twice with distilled water and allowed to dry. A 200 µL portion of 0.1N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader. The % inhibition of amoebal growth was calculated from the optical densities of the control and test wells and plotted against the logarithm of the dose of the drug tested. Linear regression analysis was used to determine the best fitting line from which the IC_{50} value was found. The IC_{50} values in μ M are reported in Tables 1 and 2.

6.5. MTT toxicity assay

PC12-rat pheochoromocytoma cell line, used in the experiment was originally procured from Indian Institute for Toxicological Research (IITR), Lucknow as a gift from Dr. A. B. Pant and since then it has been maintained in Neurotoxicology laboratory, Department of Toxicology, Jamia Hamdard. The cells were suspended which was made adherent on poly-L-lysine coated plates. Cultured cells were maintained as monolayer in F-12 Hams (Gibco) supplemented with 2.5% foetal bovine serum (Gibco), 15% horse serum (Gibco), 0.2% sodium bicarbonate and 1.5% (100 \times solution) of antibiotics and antimycotic (Gibco). All cells were cultured in T-75 corning culture flasks at 37 °C in the 100% humidity atmosphere and 5% of CO₂. In brief, 1.0×10^4 cells were plated per well into 96-well plates and were left for 48 h to achieve the maximum confluency of the cells. The compounds were dissolved in 20% DMSO (v/v) and were further diluted with fresh medium to make stock solution of 100 µM. The effect of these compounds on cell viability was measured by MTT assay. Tetrazolium (10 µL/well; 5 mg/mL of stock in PBS) salt was added 4 h prior to completion of incubation periods. Thereafter, the reaction mixture was carefully taken out and 200 µL of DMSO was added to each well by pipetting up and down several times unless the content gets homogenized. After 10 min, the colour was read at 550 nm on an ELISA plate reader (Labsystems Multiskan RC, Helsinki, Finland) with a reference wavelength of 655 nm. Percentage cellular viability calculated with appropriated controls taken in account. All assays were performed in triplicate and repeated thrice.

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