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Short Communication

Synthesis, characterization and biological evaluation of novel 6-ferrocenyl-4-aryl-2-substituted pyrimidine derivatives

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ABSTRACT

A new series of 6-ferrocenyl-4-aryl-2-substituted pyrimidines were synthesized and evaluated for *in vitro* antiamoebic activity against HM1:IMSS strain of *Entamoeba histolytica*. Out of 16 compounds 10 compounds have shown IC_{50} values in the range of 0.41–1.73 μM and 1.80 μM. Pyrimidine derivatives having thiomethyl group, chloro group and mono-, di-, and trimethoxy substitution, exhibited higher antiamoebic activity than the reference drug metronidazole ($IC_{50} = 1.80$ μM). The toxicological studies of these compounds on human kidney epithelial cell line showed that all compounds were non-toxic. 4-(4-Chlorophenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4f**) was found most active ($IC_{50} = 0.41$ μM) and least toxic among all the compounds.

1. Introduction

Despite continued efforts aimed at complete eradication of amoebiasis, the disease remains a major threat in many areas of the world especially tropical and subtropical countries [1]. One of the most crucial obstacles for eradicating amoebiasis is the widespread resistance of the *Entamoeba histolytica*, to almost all chemotherapeutic agents. Therefore it is very necessary to seek for new drugs attacking crucial targets in the amoebicidal pathogen in order to combat and relieve this tremendous prevalence. Pyrimidines, being an integral part of DNA and RNA in it, play an essencial role in several biological processes and have consederable chemical and pharmacological importance, particularly, the pyrimidine ring can be found in nucleoside antibiotics, antibacterial, cardiovascular as well as agrochemical and veterin products [2–10]. Pyrimidines also present an interesting group of compounds many of which possess widespread pharmacological properties such as analgesic, antiarrhythmic and anticancer activities [11–13]. Recently, some new substituted pyrimidine derivatives have been synthesized, which exhibit analgesic, anti-inflammatory, antiparkinsonian and androgenic-anabolic activities [14–19]. It is well known that incorporation of ferrocene fragment into a molecule of an organic compound often obtained unexpected biological activity, which is due to their different membrane permeation properties and anomalous metabolism. Many ferrocenyl compounds display interesting cytotoxic, anti-tumor, antimalarial, antifungal and DNA-cleaving activity [20–22]. Recently, some new ferrocenyl-substituted heterocyclic compounds have been reported as potential pharmaceuticals [23–28]. Moreover, the stability and non-toxicity of the ferrocenyl moiety is of particular interest rendering such drugs compatible with other treatment [29]. In this sense, the integration of one or more ferrocene units into a heterocyclic molecule has long been recognized as an attractive way to endow a novel molecule functionality [30]. In view of these observations and as a part of our ongoing program devoted to the synthesis of diverse heterocycles as antiamoebic agents, we had previously reported antiamoebic activity and toxicity of novel bisdioxazole derivatives [31]. In this study we report herein *in vitro* antiamoebic activity and toxicity of pyrimidine derivatives substituted with cyclic amines at the second position and ferrocene nucleus at the 4th position.

2. Chemistry

To synthesize the 2,4,6-trisubstituted pyrimidine derivatives (**3a**–**3h** and **4a**–**4h**), acetyl ferrocene was reacted with different substituted aromatic aldehydes (**a**–**h**) in KOH and ethanol to yield the corresponding ferrocenyl chalcones **2a**–**2h** [32] Scheme 1.

Abbreviations: μg, microgram; μl, microliter; μm, micromole; mL, milliliter; mg, milligram; mmol, milli mole; nm, nanometer.

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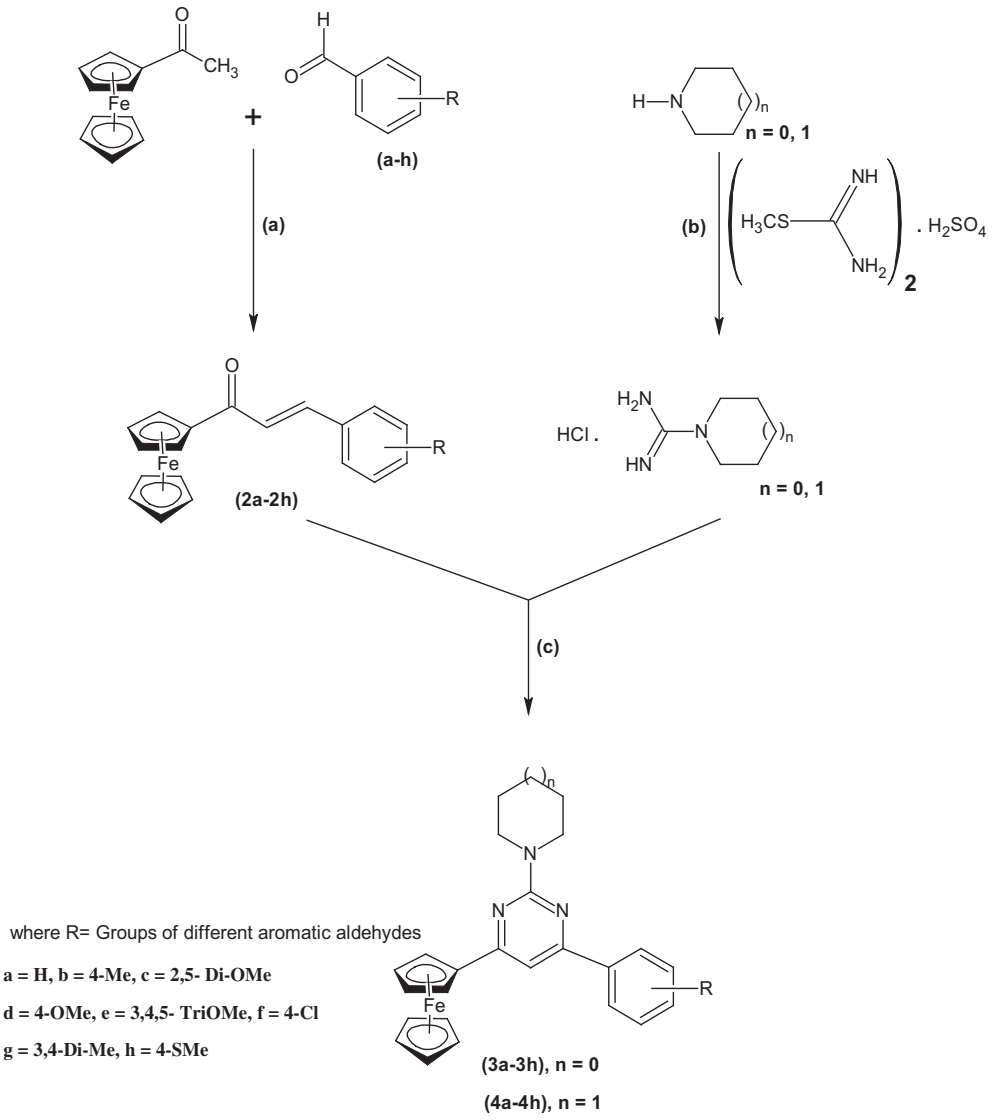


Scheme 1. General synthesis of 2,4,6-trisubstituted pyrimidines (**3a**–**3h**) and (**4a**–**4h**). Reagents and conditions: (a) different aldehydes (**a**–**h**), 10% aq. NaOH, methanol, 0 °C-rt, 30 min. (b) (i) Piperidine or pyrrolidine, *S*-methylisothiourea sulfate, water, reflux, 15 min; (ii) barium chloride, reflux, 15 min. (c) Pyrrolidine-1-carboxamidineHCl (for **3a**–**3h**) or piperidine-1-carboxamidine HCl (for **4a**–**4h**), sodiumisopropoxide, isopropanol, reflux, 8 h.

Pyrrolidine-1-carboxamidine hydrochloride and piperidine-1-carboxamidine hydrochloride were synthesized by refluxing pyrrolidine and piperidine, respectively, with *S*-methyl isothiourea sulfate in water according to reported procedure [33]. The ferrocenyl 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 pyrimidines **3a**–**3h** and **4a**–**4h** as shown in Scheme 1. All the synthesized compounds were well characterized by spectroscopic methods such as IR, NMR, Mass and elemental analysis.

3. Pharmacology

All pyrimidine derivatives (**3a**–**3h**) and (**4a**–**4h**) were screened *in vitro* against HM1:IMSS strain of *E. histolytica* by microdilution method. All the experiments were carried out in triplicate at each concentration level and repeated thrice. Toxicity of active compounds has been studied by MTT assay on human kidney epithelial cell line. The results of biological activity and toxicity are summarized in Tables 1 and 2.

4. Results and discussion

4.1. Synthesis

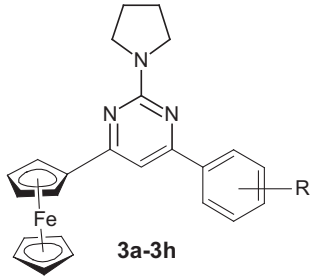
The synthesis of 2,4,6-trisubstituted pyrimidine derivatives (**3a**–**3h** and **4a**–**4h**) was performed in a manner as outlined in Scheme 1. The cyclization of corresponding ferrocenyl chalcone (**2a**–**2h**) with imidine hydrochloride in the presence of sodium isopropoxide (synthesized in situ by adding sodium metal in isopropanol) gave corresponding 2,4,6-trisubstituted pyrimidine derivatives (**3a**–**3h** and **4a**–**4h**).

The chemical structures of all the compounds **2a**–**2h**, **3a**–**3h** and **4a**–**4h** were characterized by IR, ^{1}H NMR, ^{13}C NMR and ESI-MS studies and their data are presented 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*.

Table 1
2,4,6-Trisubstituted pyrimidines (**3a**–**3h**), their antiamoebic activity against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile.



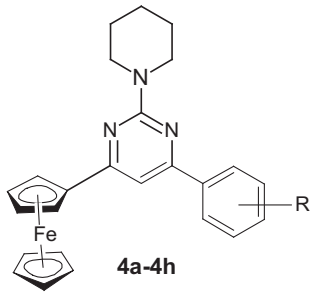
3a-3h

S.No.	R	Antiamoebic activity		Toxicity profile	
		IC_{50} (μM)	S.D.[a] (±)	IC_{50} (μM)	Safety index (SI)
3a	H	1.80	1.09	>100	>55.55
3b	4-Me	1.80	0.98	>100	>55.55
3c	2,5-DiOMe	**0.91**	0.34	>100	>109.89
3d	4-OMe	**0.62**	0.23	>100	>161.3
3e	3,4,5-TriOMe	**0.71**	0.53	>100	>140.8
3f	4-Cl	1.80	0.96	>100	>55.55
3g	3,4-DiMe	**1.73**	0.39	>100	>57.8
3h	4-SMe	**1.21**	0.65	>100	>82.6

[a] Standard deviation. The compounds with bold font IC_{50} values are more active than metronidazole.

The results are summarized in Tables 1and 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 1.80 μM in our experiments. The results showed that in pyrimidine derivatives, when the R group was a phenyl ring the pyrrolidine substituted compound (**3a**) showed IC_{50} 1.80 μM. Substitution of the phenyl ring with methyl group (**3b**) and chloro group (**3f**) also did not affect the activity. Dimethyl group (**3g**), thiomethyl group (**3h**) Mono-(**3d**), Di-(**3g**) and trisubstitution (**3e**) of the phenyl ring with methoxy group increased the activity. An almost similar trend was also observed in piperidine substituted compounds. The phenyl ring substituted compound showed IC_{50} 1.80 μM. Substitution of methyl group (**4b**) and dimethyl group (4 g) had no effect on the activity. Monomethoxy (**4d**) dimethoxy (**4c**), trimethoxy (**4e**) and thiomethyl group (**4h**) showed increased activity. On substitution of chloro group at *para* position the compound (**4f**) exhibited an exceptionally drastic increase in the activity having IC_{50} 0.41 μM. 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, 10 compounds (**3c**, **3d**, **3e**, **3g**, **3h**) and (**4c**, **4d**, **4e**, **4f**, **4h**) were found more active than the reference drug metronidazole.

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



4a-4h

S.No.	R	Antiamoebic activity		Toxicity profile	
		IC_{50} (μM) (±)	S.D.[a]	IC_{50} (μM) (SI)	Safety Index
4a	H	1.80	0.45	>100	>55.55
4b	4-Me	1.80	1.71	>100	>55.55
4c	2,5-DiOMe	**0.48**	0.28	>100	>208.3
4d	4-OMe	**0.53**	0.29	>100	>188.7
4e	3,4,5-TriOMe	**1.58**	0.68	>100	>63.3
4f	4-Cl	**0.41**	0.32	>100	>243.9
4g	3,4-DiMe	1.80	0.36	>100	>55.55
4h	4-SMe	**1.13**	0.86	>100	>88.5
Metronidazole		1.80	0.32	>100	>55.55

[a] Standard deviation. The compounds with bold font IC_{50} values are more active than metronidazole.

4.3. Toxicity profile

Compounds **3a**–**3h** and **4a**–**4h** was tested to find the toxic effects on human kidney epithelial cell line. No one inhibited cell growth at a concentration of 100 μM. 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 human (kidney epithelial) 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 and 2.

5. Conclusion

The sixteen 2,4,6-trisubstituted pyrimidines (**3a**–**3h** and **4a**–**4h**) were synthesized by the cyclization of ferrocenyl chalcones (**2a**–**2h**) with pyrrolidine-1-carboxamidine hydrochloride and piperidine-1-carboxamidine 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 10 compounds having methoxy substitution, thiomethyl group and chloro group, exhibited higher antiamoebic activity than the reference drug metronidazole ($IC_{50} = 1.80$ μM). The MTT assay revealed that all the compounds are non-toxic to human kidney epithelial (Graham) cells. 4-(4-Chlorophenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4f**) was found most active and least toxic among all the compounds. These results identified that pyrimidines are new leads in antiamoebic chemotherapy. The study suggests the beneficial potential of these leads that need to be further explored in order to discover and develop better and yet safer therapeutic agents for amoebiasis. The *in vivo* studies of these compounds are currently in progress.

6. Experimental protocol

All the chemicals were purchased from Aldrich Chemical Company (USA). Precoated aluminium sheets (silica gel 60 F_{254}, 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 ±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 spectrophotometer as KBr discs. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 400 spectrometer using $CDCl_3$ 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 ferrocenyl chalcones (**2a**-**2h**)

The acetyl ferrocene (3 mmol) and KOH (0.2 g) were dissolved in ethanol (5 mL) in a round bottomed flask and stirred at room temperature (25 °C) for 10 min. An ethanolic solution of the substituted aromatic aldehydes (3 mmol, 5 mL) was added drop wise and the mixture was stirred at room temperature. The progress of the reaction was monitored by TLC on silica gel sheets. The reaction was stopped by neutralizing the stirred solution with 2 M HCl. In most cases the product was obtained as a dark red precipitate after neutralization. It was then removed by filtration, washed with water. In the absence of a precipitate on neutralization, the solution was extracted with ethyl acetate (20 mL × 3). The organic layer was dried over anhydrous sodium sulphate and removed by evaporation under reduced pressure to give a liquid residue. The latter was passed through a column of silica gel (230–400 mesh) and eluted with THF-hexane (1:4) to yield pure compound.

6.1.1. 1-Ferrocenyl-3-phenyl-2-propen-1-one (**2a**)

Yield 70%; m.p: 122 °C; deep red solid; Anal. calc. for $C_{19}H_{16}FeO$: C 72.17, H 5.10%. Found: C 72.15; H 5.07%. IR ν_{max} (cm^{-1}): 3033 (Ar–H), 2938 (C–H), 1645 (C=O), 1448 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.89 (d, 2H, $J = 8.7$ Hz, Ar–H), 7.79 (d, 1H, $J = 16$ Hz, H_β), 7.65–7.15 (m, 4H, Ar–H, H_α), 4.92 (s, 2H, ferrocene), 4.59 (s, 2H, ferrocene), 4.22 (s, 5H, ferrocene); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.73 (C=O), 144.41 (C-β), (136.07, 134.70, 130.02, 128.51, Aromatic), 122.52 (C-α). ESI-MS *m*/*z*: [$M^+ + 1$] 316.17.

6.1.2. 1-Ferrocenyl-3-p-tolyl-2-propen-1-one (**2b**)

Yield 74%; m.p: 164 °C; deep red solid Anal. calc. for $C_{20}H_{18}FeO$: C 72.75, H 5.49%. Found: C 72.73; H 5.46%. IR ν_{max} (cm^{-1}): 3036 (Ar–H), 2931 (C–H), 1641 (C=O), 1446 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.88–7.86 (m, 2H, Ar–H), 7.79 (d, 1H, $J = 16$ Hz, H_β), 7.56–7.24 (m, 3H, Ar–H, H_α), 4.95 (s, 2H, ferrocene), 4.61 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene), 2.41 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.83 (C=O), 145.41 (C-β), (136.07, 134.70, 130.02, 128.51, Aromatic), 121.52 (C-α), 21.51 (CH_3). ESI-MS *m*/*z*: [$M^+ + 1$] 331.20.

6.1.3. 1-Ferrocenyl-3-(2,5- dimethoxyphenyl)-2-propen-1-one (**2c**)

Yield 80%; deep red liq; Anal. calc. for $C_{21}H_{20}FeO_3$: C 67.04, H 5.36. Found: C 67.05, H 5.34% IR ν max cm^{-1}: 3044 (Ar–H), 2931 (C–H), 1646 (C=O), 1453 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.81(s, 1H, Ar–H), 7.76 (d, 1H, $J = 15.6$ Hz, H_β), 7.59–7.42 (m, 2H, Ar–H), 7.36 (d, 1H, $J = 15.6$ Hz, H_α), 4.93 (s, 2H, ferrocene), 4.57 (s, 2H, ferrocene), 4.22 (s, 5H, Ferrocene), 3.91 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 190.18 (C=O), 144.65 (C-β), (135.61, 132.70, 130.02, 128.51, Aromatic), 122.23 (C-α), 55.65 (OCH_3). ESI-MS *m*/*z*: [$M^+ + 1$] 376.23.

6.1.4. 1-Ferrocenyl-3-(4-methoxyphenyl)-2-propen-1-one (**2d**)

Yield 72%; m.p: 121 °C; deep red solid; Anal. calc. for $C_{20}H_{18}FeO_2$: C 69.39, H 5.24%. Found: C 69.35, H 5.22% IR ν_{max} (cm^{-1}): 3028 (Ar–H), 2916 (C–H), 1643 (C=O), 1444 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.87–7.83 (m, 2H, Ar–H), 7.81 (d, 1H, $J = 15.6$ Hz, H_β), 7.65–7.55 (m, 2H, Ar–H,), 7.37 (d, $J = 15$ Hz, H_α) 4.92 (s, 2H, ferrocene), 4.58 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene), 3.84 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.27 (C=O), 145.44 (C-β), (139.07, 131.99, 129.92, 128.63, Aromatic), 121.93 (C-α), 56.23 (OCH_3). ESI-MS *m*/*z*: [$M^+ + 1$] 347.20.

6.1.5. 1-Ferrocenyl-3- (3,4,5-trimethoxy phenyl)-2-propen-1-one (**2e**)

Yield 78%; deep red liq; Anal. calc. for $C_{22}H_{22}FeO_4$: C 65.04, H 5.46%. Found: C, 65.06, H 5.44% IR ν_{max} (cm^{-1}): 3051 (Ar–H), 2930 (C–H), 1650 (C=O), 1455 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.71–7.68 (m, 2H, Ar–H), 7.65 (d, 1H, $J = 16$ Hz, H_β), 7.38 (d, 1H, $J = 16$ Hz, H_α), 4.96 (s, 2H, ferrocene), 4.58 (s,2H, ferrocene), 4.22 (s, 5H, ferrocene), 3.85 (s, 6H, OCH_3), 3.82 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 191.43 (C=O), 143.41 (C-β), (136.07, 134.70, 130.02, 128.51, Aromatic), 121.42 (C-α), 60.91, 56.54 (OCH_3). ESI-MS *m*/*z*: [$M^+ + 1$] 406.25.

6.1.6. 1-Ferrocenyl-3- (4-chlorophenyl)-2-propen-1-one (**2f**)

Yield 74%; m.p: 140 °C; deep red solid; Anal. calc. for $C_{19}H_{15}$ Cl Fe O: C 65.09, H 4.31%. Found: C, 65.06; H 4.28% IR ν_{max} (cm^{-1}): 3068 (Ar–H), 2932 (C–H), 1642 (C=O), 1448 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm) 7.88 (m, 2H, Ar–H), 7.62 (d, 1H, $J = 15.6$ Hz, H_β), 7.65–7.22 (m, 3H, Ar–H, H_α), 4.95 (s, 2H, ferrocene), 4.61 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.23 (C=O), 144.41 (C-β), (134.70, 130.02, 129.53,128.51, Aromatic), 122.52 (C-α). ESI-MS *m*/*z*: [$M^+ + 1$] 341.62.

6.1.7. 1-Ferrocenyl-3- (3,4-dimethyl phenyl)-2-propen-1-one (**2g**)

Yield 72%; m.p: 138 °C; deep red solid; Anal. calc. for $C_{21}H_{20}FeO$: C 73.27, H, 5.86%. Found: C 73.25, H 5.83%. IR ν_{max} (cm^{-1}): 3068 (Ar–H), 2949 (C–H), 1650 (C=O), 1447 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.63–7.61 (m, 2H, Ar–H), 7.58 (d, 1H, $J = 16$ Hz, H_β), 7.54–7.21 (m, 2H, Ar–H, H_α), 4.92 (s, 2H, ferrocene), 4.58 (s, 2H, ferrocene), 4.23 (s, 5H, ferrocene), 2.24 (s, 3H, CH_3), 2.16 (s,1H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 190.49 (C=O), 144.78 (C-β), (134.51, 132.72, 128.92, 127.63, Aromatic), 122.09 (C-α). 22.45 (methyl). ESI-MS *m*/*z*: [$M^+ + 1$] 345.23.

6.1.8. 1-Ferrocenyl-3-(4-methylthio phenyl)-2-propen-1-one (**2h**)

Yield 72%; m.p: 157 °C; deep red solid; Anal. calc. for $C_{21}H_{20}FeOS$: C 66.31, H 5.01%. Found: C 66.28, H 5.03%. IR ν_{max} (cm^{-1}): 3068 (Ar–H), 2949 (C–H), 1645 (C=O), 1446 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.65–7.62 (m, 2H, Ar–H), 7.59 (d, 1H, $J = 15.6$ Hz, H_β), 7.54–7.43 (m, 2H, Ar–H), 7.32 (d, 1H, $J = 15.6$ Hz, H_α), 4.94 (s, 2H, ferrocene), 4.59 (s, 2H, ferrocene), 4.21 (s, 5H, ferrocene), 2.42 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ (ppm): 190.89 (C=O), 143.87 (C-β), (134.51, 132.72, 128.92, 127.63, aromatic), 122.14 (C-α). 16.9 (SCH_3). ESI-MS *m*/*z*: [$M^+ + 1$] 519.46.

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 ferrocenyl 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-Phenyl-2-pyrrolidin-1-yl-6-ferrocenyl pyrimidine (**3a**)

Yield 68%; m.p: 146 °C; reddish brown solid. Anal. calc. for $C_{24}H_{23}FeN_3$:C 70.43, H 5.66, N 10.27% . Found: C 70.47, H 5.64; N

10.24%. IR ν_{max} (cm^{-1}): 3062 (Ar–H), 2913 (C–H), 1596 (C=N), 1466 (C=C), 1239 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.86 (d, 2H, $J = 8.3$ Hz, Ar–H), 7.68–7.22 (m, 3H, Ar–H), 7.38 (s, 1H, Pyrimidine), 4.96 (s, 2H, ferrocene), 4.58 (s, 2H, ferrocene), 4.22 (s, 5H, ferrocene), 2.78–2.72 (m, 4H, $-CH_2$ pyrrolidine), 1.89–1.77 (m, 4H, $-CH_2$ pyrrolidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.6 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 140.6, 138.3, 130.1, 127.7, 126.2, 123.8, 122.7, 115.5 (Ar–C), 104.9 (C–H pyrimidine), 47.1, 25.3 ($-CH_2$ pyrrolidine). ESI-MS m/z: [$M^+ + 1$] 410.30.

6.2.2. 2-Pyrrolidin-1-yl-4-ferrocenyl-6-p-tolyl-pyrimidine (**3b**)

Yield 62%; m.p: 146 °C; red solid. Anal. calc. for $C_{25}H_{25}FeN_3$:C 70.93, H 5.95, N 9.93%. Found: C 70.97, H 5.92, N 9.97%. IR ν_{max} (cm^{-1}): 3068 (Ar–H), 2933 (C–H), 1592 (C=N),1456 (C=C), 1243 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.87–7.62 (m, 3H, Ar–H), 7.46 (d, 1H, $J = 5.7$ Hz, Ar–H), 7.35 (s, 1H, Pyrimidine), 4.99 (s, 2H, ferrocene), 4.56 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene), 2.76–2.71 (m, 4H, $-CH_2$ pyrrolidine), 2.39 (s, 3H, methyl), 1.88–1.76 (m, 4H, $-CH_2$ pyrrolidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.4 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 140.2, 141.4, 130.8, 129.2, 126.9, 122.5, 117.5 (Ar–C), 104.3 (C–H pyrimidine), 47.3, 25.2 ($-CH_2$ pyrrolidine), 21.54 (-CH_3 phenyl). ESI-MS m/z: [$M^+ + 1$] 424.33.

6.2.3. 4-(2,5-Dimethoxyphenyl)-2-pyrrolidin-1-yl-6-ferrocenyl pyrimidine (**3c**)

Yield 67%; m.p: 146 °C; reddish brown solid. Anal. calc. for $C_{26}H_{27}FeN_3O_2$: C 66.53, H 5.80; N 8.95%. Found: C 66.56, H 5.77, N 8.93%. IR ν_{max} (cm^{-1}): 3065 (Ar–H), 2963 (C–H), 1598 (C=N),1468 (C=C), 1235 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.76 (s, 1H, Ar–H), 7.58–7.46 (m, 2H, Ar–H), 7.36 (s,1H, Pyrimidine), 4.91 (s, 2H, ferrocene), 4.59 (s, 2H, ferrocene), 4.24 (m, 5H, ferrocene), 3.91 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 2.78–2.72 (m, 4H, $-CH_2$ pyrolidine), 1.86–1.75 (m, 4H, $-CH_2$ pyrolidine); ^{13}C NMR (DMSO-d_6) 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), 55.7 (-OCH_3), 47.9, 25.4 ($-CH_2$ pyrrolidine). ESI-MS m/z: [$M^+ + 1$] 470.36.

6.2.4. 4-(4-Methoxyphenyl)-2-pyrrolidin-1-yl-6-ferrocenyl pyrimidine (**3d**)

Yield 68%; m.p: 146 °C; deep red solid. Anal. calc. for. $C_{25}H_{25}FeN_3O$: C 68.35, H 5.74, N 9.56% Found: C 70.56, H 5.71, N 9.52%. IR ν_{max} (cm^{-1}): 3069 (Ar–H), 2931 (C–H), 1569 (C=N), 1457 (C=C), 1293 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.88 (d, 1H, $J = 7.1$ Hz, Ar–H), 7.71–7.54 (m, 3H, Ar–H), 7.39 (s, 1H, pyrimidine), 4.98 (s, 2H, ferrocene), 4.57(s, 2H, ferrocene), 4.23 (m, 5H, ferrocene), 3.83 (s, 3H, OCH_3), 2.78–2.72 (m, 4H, $-CH_2$ pyrrolidine), 1.82–1.76 (m, 4H, $-CH_2$ pyrrolidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.5 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 140.4, 139.2, 130.6, 129.9, 126.9, 123.6, 122.7, 115.5 (Ar–C), 102.9 (C–H pyrimidine), 55.2 (-OCH_3), 47.1, 25.2 ($-CH_2$ pyrrolidine). ESI-MS m/z: [$M^+ + 1$] 440.33.

6.2.5. 2-Pyrrolidin-1-yl-4-ferrocenyl-6-(3,4,5-trimethoxyphenyl)-pyrimidine (**3e**)

Yield 69%; m.p: 146 °C; reddish brown solid. Anal. calc. for $C_{27}H_{29}FeN_3O_3$: C 64.94, H 5.85, N 8.41%. Found: C 64.96, H 5.83, N 8.42%. IR ν_{max} (cm^{-1}): 3085 (Ar–H), 2939(C–H), 1565 (C=N), 1464 (C=C), 1292 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.69–7.66 (m, 2H, Ar–H), 7.38 (s, 1H, Pyrimidine), 4.99 (s, 2H, ferrocene), 4.55 (s, 2H, ferrocene), 4.21 (s, 5H, ferrocene), 3.84 (s, 6H, OCH_3), 3.83 (s, 3H, OCH_3), 2.76–2.71 (m, 4H, $-CH_2$ pyrrolidine), 1.84–1.75 (m, 4H, $-CH_2$ pyrrolidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.0 (C=N pyrimidine), 162.3(C=C pyrimidine), 161.2 (N=C–N pyrimidine), 140.6, 130.6, 129.9, 126.9, 123.6, 122.7, 115.5 (Ar–C), 103.9 (C–H pyrimidine), 60.92 (OCH_3), 55.2 (2× OCH_3), 47.8, 25.9 ($-CH_2$ pyrrolidine). ESI-MS m/z: [$M^+ + 1$] 500.38.

6.2.6. 4-(4-Chlorophenyl)-2-pyrrolidin-1-yl-6-ferrocenyl pyrimidine (**3f**)

Yield 70%; m.p: 146 °C; reddish brown solid. Anal. calc. for. $C_{24}H_{22}ClFeN_3$: C 64.96, H 5.00, N 9.47% Found: C 64.95, H 4.98, N 9.43%. IR ν_{max} (cm^{-1}): 3088 (Ar–H), 2992 (C–H), 1557 (C=N), 1446 (C=C), 1233 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.81–7.62 (m, 3H, Ar–H), 7.49 (d, 1H, $J = 8.6$ Hz, Ar–H), 7.37 (s,1H, Pyrimidine), 4.93 (s, 2H, ferrocene), 4.62 (s,2H, ferrocene), 4.21 (s, 5H, ferrocene), 2.78–2.71 (m, 4H, $-CH_2$ pyrrolidine), 1.83–1.76 (m, 4H, $-CH_2$ pyrrolidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.3 (C=N pyrimidine), 162.9 (C=C pyrimidine), 161.2 (N=C–N pyrimidine), 140.6, 138.2, 136.8, 130.6, 129.7, 126.9, 123.8, 122.7, 115.5 (Ar–C), 104.8 (C–H pyrimidine), 47.3, 25.6 ($-CH_2$ pyrrolidine). ESI-MS m/z: [$M^+ + 1$] 443.

6.2.7. 4-(3,4-Dimethylphenyl)-2-pyrrolidin-1-yl-6-ferrocenyl pyrimidine (**3g**)

Yield 66%; m.p: 146 °C; red solid. Anal. calc. for. $C_{26}H_{27}FeN_3$:C 71.40, H 6.22, N 9.45% Found: C 71.42, H 6.23, N 9.42%. IR ν_{max} (cm^{-1}): 3078 (Ar–H), 2965 (C–H), 1592 (C=N), 1468 (C=C), 1254 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.67–7.63 (m, 2H, Ar–H), 7.53 (s, 1H, Ar–H), 7.39 (s,1H, pyrimidine), 4.94 (s, 1H, ferrocene), 4.57(s, 1H, ferrocene), 4.23 (s, 5H, ferrocene), 2.77–2.73 (m, 4H, $-CH_2$ pyrrolidine), 2.23 (s, 3H, CH_3), 1.86–1.73 (m, 4H, $-CH_2$ pyrrolidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.5 (C=N pyrimidine), 162.1 (C=C pyrimidine), 162.2 (N=C–N pyrimidine), 139.6, 138.2, 136.8, 130.6, 129.7, 126.9, 123.8, 122.7, 118.5 (Ar–C), 103.6 (C–H pyrimidine), 47.4, 25.8 ($-CH_2$ pyrrolidine). 21.54 (2× CH_3 phenyl). ESI-MS m/z: [$M^+ + 1$] 444.75.

6.2.8. 4-(4-Methylthiophenyl)-2-pyrrolidin-1-yl-6-ferrocenyl pyrimidine (**3h**)

Yield 68%; m.p: 146 °C; red solid. Anal. calc. for. $C_{25}H_{25}FeN_3S$:C 65.94, H 5.53, N 9.23, S 7.04%: Found: C 65.98, H 5.50, N 9.25%. IR ν_{max} cm^{-1}: 3088(Ar–H), 2967 (C–H), 1546 (C=N), 1486 (C=C), 1295 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.69–7.62 (m, 2H, Ar–H), 7.57- 7.53 (m, 2H, Ar–H), 7.36 (s, 1H, pyrimidine) 4.97 (s, 1H, ferrocene), 4.55 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene), 2.79–2.75 (m, 4H, $-CH_2$ pyrrolidine), 2.02 (s, 3H, CH_3), 1.86–1.72 (m, 4H, $-CH_2$ pyrrolidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 165.8 (C=N pyrimidine), 162.8 (C=C pyrimidine), 161.2 (N=C–N pyrimidine), 138.6, 138.1, 136.8, 130.6, 129.7, 126.9, 123.8, 122.7, 118.3 (Ar–C), 104.6 (C–H pyrimidine), 47.3, 25.1 ($-CH_2$ pyrolidine), 16.3 (SCH_3). ESI-MS m/z: [$M^+ + 1$] 456.40.

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 ferrocenyl 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-Phenyl-2-piperidin-1-yl-6-ferrocenyl pyrimidine (**4a**)*

Yield: 70%; m.p: 146 °C; deep red solid. Anal. calc. for $C_{25}H_{25}FeN_3$:C 70.93, H 5.95, N 9.93%. Found: C 70.97, H 5.94, N 9.90%. IR ν_{max} (cm^{-1}): 3054 (Ar–H), 2959 (C–H), 1573 (C=N), 1487 (C=C), 1243 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.89 (d, 1H, $J = 8.2$ Hz, Ar–H), 7.73–7.22 (m, 4H, Ar–H) 7.37 (s, 1H, Pyrimidine), 4.92 (s, 2H, ferrocene), 4.57 (s, 2H, ferrocene), 4.23 (s, 5H, ferrocene), 3.88–3.84 (m, 4H, piperidine), 1.66–1.54 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.7 (C=N pyrimidine), 162.5 (C=C pyrimidine), 161.9 (N=C–N pyrimidine), 141.6, 138.4, 130.6, 127.8, 126.2, 124.8, 123.7, 116.5 (Ar–C), 104.3 (C–H pyrimidine), 46.1, 27.8, 25.3 (–CH_2 piperidine). ESI-MS m/z: [$M^+ + 1$] 424.33.

*6.3.2. 4-Ferrocenyl-2-piperidin-1-yl-6-p-tolyl-pyrimidine (**4b**)*

Yield 68%; m.p: 146 °C; reddish brown solid. Anal. calc. for $C_{26}H_{27}FeN_3$:C 71.40, H 6.22, N 9.61%. Found: C 71.43, H 6.25, N 9.62%. IR ν_{max} (cm^{-1}): 3078 (Ar–H), 2953 (C–H), 1597 (C=N),1456 (C=C), 1248 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.86–7.66 (m, 3H, Ar–H), 7.49 (d, 1H, $J = 6.1$ Hz, Ar–H), 7.39 (s, 1H, pyrimidine), 4.93 (s, 2H, ferrocene), 4.58 (s, 2H, ferrocene), 4.22 (s, 5H, ferrocene), 3.87–3.83 (m, 4H, piperidine), 2.39 (s, 3H, methyl), 1.67–1.57 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.4 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 140.2, 141.4, 130.8, 129.2, 126.9, 122.5, 117.5 (Ar–C), 104.8 (C–H pyrimidine), 47.1, 27.3, 24.8 (–CH_2 piperidine), 21.54 (-CH_3 phenyl). ESI-MS m/z: [$M^+ + 1$] 438.36.

*6.3.3. 4-(2,5-Dimethoxyphenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4c**)*

Yield 72%; m.p: 146 °C; dark maroon solid. Anal. calc. for $C_{27}H_{29}FeN_3O_2$: C 67.09, H 5.05, N 8.69%. Found: C 67.11, H 5.02, N 8.70%. IR ν_{max} (cm^{-1}): 3087 (Ar–H), 2976 (C–H), 1555 (C=N), 1486 (C=C), 1254 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.78 (s, 1H, Ar–H), 7.58–7.43 (m, 2H, Ar–H), 7.37 (s, 1H, pyrimidine), 4.94 (s, 2H, ferrocene), 4.58 (s, 2H, ferrocene), 4.21 (s, 5H, Ferrocene), 3.88–3.85 (m, 4H, piperidine), 3.92 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3). 1.67–1.57 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.3 (C=N pyrimidine), 162.7 (C=C pyrimidine), 161.5 (N=C–N pyrimidine), 154.5, 139.7, 130.8, 129.7, 125.9, 123.6, 122.5, 117.8 (Ar–C), 103.6 (C–H pyrimidine), 56.7, 55.3 (-OCH_3), 47.3, 27.7, 25.2 (–CH_2 piperidine). ESI-MS m/z: [$M^+ + 1$] 484.38.

*6.3.4. 4-(4-Methoxyphenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4d**)*

Yield 66%; mp: 146 °C; reddish brown solid. Anal. calc. for. $C_{26}H_{27}FeN_3O$: C 68.88, H 6.00, N 9.27% Found: C 68.86, H 6.04, N 9.25%. IR ν_{max} (cm^{-1}): 3089 (Ar–H), 2934 (C–H), 1576 (C=N), 1455 (C=C), 1292 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.88–7.84 (m, 2H, Ar–H), 7.34 (s,1H, pyrimidine), 7.67–7.55 (m, 2H, Ar–H), 4.93 (s, 2H, ferrocene), 4.57 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene), 3.87–3.85 (m, 4 H, piperidine), 3.84 (s, 3H, OCH_3), 1.66–1.58 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.6 (C=N pyrimidine), 161.7 (C=C pyrimidine), 161.3 (N=C–N pyrimidine), 140.3, 139.2, 130.6, 129.9, 126.9, 123.6, 122.7, 115.7 (Ar–C), 102.2 (C–H pyrimidine), 56.2 (-OCH_3), 47.7, 26.7, 24.2 (–CH_2 piperidine). ESI-MS m/z: [$M^+ + 1$] 454.36.

*6.3.5. 4-Ferrocenyl-2-piperidin-1-yl-6-(3,4,5-trimethoxyphenyl)-pyrimidine (**4e**)*

Yield 69%; m.p: 146 °C; reddish brown solid. Anal. calc. for $C_{28}H_{31}FeN_3O_3$: C 65.50, H 6.09, N 8.18%. Found: C 65.47, H 6.07, N 8.21%. IR ν_{max} (cm^{-1}): 3076(Ar–H), 2945 (C–H), 1585(C=N), 1436 (C=C), 1262 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.85 (s, 2H, Ar–H), 7.37 (s, 1H, Pyrimidine), 7.57 (s, 1 H, Ar–H), 4.95 (s, 2H, ferrocene), 4.56 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene), 3.88–3.86 (m, 4 H, piperidine), 3.83 (s, 6H, OCH_3), 3.81 (s, 3H, OCH), 1.66–1.52 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.5 (C=N pyrimidine), 162.8(C=C pyrimidine), 162.6 (N=C–N pyrimidine), 141.4, 130.6, 129.9, 126.9, 123.6, 122.7, 115.5 (Ar–C), 103.6 (C–H pyrimidine), 60.9 (OCH_3), 55.8 (2× OCH_3), 47.5, 27.9, 25.8 (–CH_2 piperidine). ESI-MS m/z: [$M^+ + 1$] 514.41.

*6.3.6. 4-(4-Chlorophenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4f**)*

Yield 72%; m.p: 146 °C; reddish brown solid. Anal. calc. for. $C_{25}H_{24}ClFeN_3$: C 65.59, H 5.28, N 9.18%. Found: C 65.56, H 5.26, N 9.15%. IR ν_{max} (cm^{-1}): 3056 (Ar–H), 2976 (C–H), 1535 (C=N),1425 (C=C), 1237 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.88–7.61 (m, 3H, Ar–H), 7.53 (d, 1H, $J = 8.2$ Hz, Ar–H), 7.38 (s,1H, pyrimidine), 4.91 (s, 2H, ferrocene), 4.59 (s, 2H, ferrocene), 4.22 (s, 5H, Ferrocene), 3.89–3.82 (m, 4 H, piperidine), 2.27 (s, 3H, CH_3), 2.18 (s,1H, CH_3) 1.69–1.54 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.8 (C=N pyrimidine), 162.3(C=C pyrimidine), 162.2 (N=C–N pyrimidine), 140.7, 138.9, 136.3, 130.6, 129.7, 126.9, 123.8, 122.7, 117.5 (Ar–C), 105.4 (C–H pyrimidine), 46.4, 27.3, 25.8 (–CH_2 piperidine), ESI-MS m/z: [$M^+ + 1$] 457.78.

*6.3.7. 4-(3,4-Dimethylphenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4g**)*

Yield 72%; m.p: 146 °C; deep red solid. Anal. calc. for. $C_{27}H_{29}FeN_3$:C 71.84, H 6.48, N 9.31%. Found: C 71.86, H 6.49, N 9.35%. IR ν_{max} (cm^{-1}): 3074 (Ar–H), 2985 (C–H), 1592 (C=N), 1446 (C=C), 1259 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.68–7.64 (m, 2H, Ar–H), 7.57 (s, 1H, Ar–H), 7.36 (s, 1H, pyrimidine), 4.96 (s, 2H, ferrocene), 4.58 (s, 2H, ferrocene), 4.23 (s, 5H, ferrocene), 3.85–3.79 (m, 4H, piperidine), 2.24 (s, 3H, CH_3), 2.15 (s,1H, CH_3) 1.68–1.56 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 165.7 (C=N pyrimidine), 162.8 (C=C pyrimidine), 162.8 (N=C–N pyrimidine), 139.6, 138.2, 136.8, 130.6, 129.7, 126.9, 123.8, 122.7, 118.4 (Ar–C), 103.3 (C–H pyrimidine), 46.4, 27.3, 25.8 (–CH_2 piperidine). 21.54 (2× CH_3 phenyl). ESI-MS m/z: [$M^+ + 1$] 452.38.

*6.3.8. 4-(4-Methylthiophenyl)-6-ferrocenyl-2-piperidin-1-yl-pyrimidine (**4h**)*

Yield 66%; m.p: 146 °C; reddish brown solid. Anal. calc. for. $C_{26}H_{27}FeN_3S$:C 66.52, H 5.80, N 8.95, S 6.83%: Found: C 66.56, H 5.78, N 8.92, S 6.86%. IR ν_{max} cm^{-1}: 3076 (Ar–H), 2965 (C–H), 1587 (C=N), 1432 (C=C), 1268 (C–N); ^{1}H NMR (DMSO-d_6) δ (ppm) 7.69–7.63 (m, 2H, Ar–H), 7.57- 7.53 (m, 2H, Ar–H), 7.37 (s,1H, pyrimidine) 4.92 (s, 2H, ferrocene), 4.59 (s, 2H, ferrocene), 4.24 (s, 5H, ferrocene), 3.88–3.81 (m, 4 H, piperidine), 2.02 (s, 3H, CH_3), 1.63–1.52 (m, 6H, piperidine); ^{13}C NMR (DMSO-d_6) δ (ppm): 166.2 (C=N pyrimidine), 162.4(C=C pyrimidine), 162.5 (N=C–N pyrimidine), 137.2, 138.1, 136.8, 130.6, 129.7, 126.9, 123.8, 122.7, 119.3 (Ar–C), 102.2 (C–H pyrimidine), 46.3, 27.4, 24.8 (–CH_2 pyrolidine), 16.8 (SCH_3). ESI-MS m/z: [$M^+ + 1$] 470.42.

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 [34]. *E. histolytica* trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [35]. The test compounds (1 mg) were dissolved in DMSO (40 μl, level at which no inhibition of amoeba occurs) [36,37]. 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 μl 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 0.1 N 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 μM are reported in Tables 1 and 2.

6.5. MTT toxicity assay

For the toxicity assay, transformed human kidney epithelial (Graham) cells were continuously maintained in culture at 37 °C in 5% CO_2. The MTT (3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide, USB) cellular viability assay was used to determine the toxicity profile of the compound [38]. The trypsinized cell suspension was adjusted to 0.5 million cells/ml and plated out with the various compounds. After 44 h of incubation, 2 mM MTT was added to the plates and incubated for a further 4 h. DMSO was then added to stop the reaction and dissolved the formazan crystals. The absorbance was taken at 540 nm and references wavelength of 690 nm and the percentage cellular viability calculated with appropriated controls taken in account. The means ± S.D. value of IC_{50} values in Tables 1 and 2 are from three independent experiments.

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