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Short communication

Synthesis of new 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1, 3-thiazolino[5,4-*b*]quinoxaline derivatives and evaluation of their antiamoebic activity

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Abstract

In an effort to develop potent antiamoebic agents, we have synthesized chalcones (**1**–**8**), amino-5-substituted-(3-phenyl(2-pyrazolinyl))methane-1-thione derivatives (**1a**–**8a**) and 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-*b*]quinoxaline derivatives (**1b**–**8b**) and evaluated for their *in vitro* antiamoebic activity against HM1:IMSS strain of *E. histolytica*. All the compounds were characterized by electronic, IR, ^{1}H NMR and mass spectroscopic data. It was observed that the antiamoebic activity enhances on modifying the structure of chalcones to the pyrazolines and further to quinoxalines. The MTT assay was performed on human kidney epithelial cell line to check the cytotoxicity of the compounds and the results were compared with metronidazole. Compound **6b** showed better antiamoebic activity and less toxicity than metronidazole.

Keywords: Chalcone; Pyrazolines; Thiocarbamoyl; Quinoxaline; Antiamoebic activity

1. Introduction

Ameobiasis is the infection of the human gastrointestinal tract by *Entamoeba histolytica* [1]. *E. histolytica* causes approximately 50 million cases and approximately 100,000 deaths annually. Amoebic liver abscesses are the most frequent and severe clinical presentations of amoebiasis. Symptomatic patients, typically present abdominal pain, tenderness, diarrhea and bloody stools. The drugs used to eradicate *E. histolytica*, such as nitroimidazoles, have been shown to pose several important problems as mutagenic and toxic for the host when they are used at high doses, amoeba strains are able to develop resistance to these drugs [2]. Amoebiasis is treated with the drug metronidazole, even though significant side effects, such as neurological complication and possible selection of a resistant *E. histolytica* strain have been reported [3]. Therefore, the development of new alternative antiamoebic drugs devoid of side effects is still needed.

Among the various classes of nitrogen containing heterocyclic compounds, quinoxaline derivatives are the important components of several pharmacologically active compounds [4–9]. Although rarely described in nature, synthetic quinoxaline ring is part of number of antibiotics such as echinomycin and actinomycin, which are known to inhibit the growth of Gram-positive bacteria and are also active against various transplantable tumors [10–12]. There are many examples of biologically active quinoxalines, which showed very interesting pharmacological properties such as antibacterial, antiviral, anticancer, antifungal, anthelmintic, and insecticidal [13–18]. All literature survey reveals the pharmacological importance of quinoxalines. The new quinoxaline derivatives, **1b**–**8b**, were synthesized and screened *in vitro* for their ability to inhibit the growth of *E. histolytica*.

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2. Results and discussion

The synthetic routes of the proposed compounds **1**–**8**, **1a**–**8a** and **1b**–**8b** are outlined in Scheme 1. Claisen–Schmidt condensation between acetophenone and aromatic aldehydes in the presence of methanolic solution of sodium hydroxide resulted in the formation of chalcones (**1**–**8**) in excellent yields (49–93%). They were characterized by UV–vis, IR and ^{1}H NMR spectroscopic techniques. Cyclisation of different chalcones with thiosemicarbazide under basic condition in refluxing ethanol leads to the formation of new amino-5-substituted-(3-phenyl(2-pyrazolinyl))methane-1-thione (**1a**–**8a**) in modest yields (11–24%). They were also fully characterized using various spectroscopic techniques. The 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-*b*]quinoxaline derivatives (**1b**–**8b**) were obtained in (16–49%) yields by heating at reflux amino-5-substituted-(3-phenyl(2-pyrazolinyl))methane-1-thione (**1a**–**8a**) with 2,3-dichloroquinoxaline. The progress of each reaction was monitored by thin layer chromatography. Compounds **1b**–**8b** were characterized by IR, UV–vis, ^{1}H NMR, ^{13}C NMR and mass spectroscopy. The purity of all the compounds was checked by elemental analysis.

2.1. IR and electronic spectral studies

The positions of IR band provide significant indication for the formation of **1**–**8**, **1a**–**8a** and **1b**–**8b**. The bands due to ν(C=O) and ν(C=C) stretch at (1669–1750) cm^{-1} and (1523–1580) cm^{-1}, respectively, favors the formation of chalcone derivatives (**1**–**8**). The absence of ν(C=O) and ν(C=C) bands in the IR spectra of **1a**–**8a** obtained by cyclisation of chalcone **1**–**8** shows the formation of **1a**–**8a**. Compounds **1a**–**8a** showed intense bands in the region 1333–1370 cm^{-1} due to the ν(C=S) stretch of the thiocarbamoyl group. The IR spectra of all the compounds showed ν(C=N) stretch at 1542–1590 cm^{-1} due to the ring closure. In addition, the absorption bands at 1024–1122 cm^{-1} were attributed to the ν(C–N) stretch vibrations, which also confirm the formation of desired pyrazoline ring in all the compounds. The compounds showed sharp bands in the region 3228–3454 cm^{-1} due to the ν(NH) stretch. Compounds **1a**–**8a** were further reacted with 2,3-dichloroquinoxaline to get compounds **1b**–**8b**. The bands due to ν(NH) stretch and ν(C=S) were absent in these compounds. Selected diagnostic bands of the IR spectra of 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-*b*]quinoxaline derivatives (**1b**–**8b**) showed intense bands at 839–945 cm^{-1} due to the ν(C–S) stretch. The compounds show two strong bands at 1486–1563 and 1517–1510 cm^{-1} due to ν(C=N) stretch of azomethine nitrogen of pyrazoline ring and quinoxaline ring, respectively. In addition, the absorption band at 1078–1192 cm^{-1} attributed to the ν(C–N) stretch vibrations, which also confirm the formation of desired pyrazoline ring in all the compounds [19].

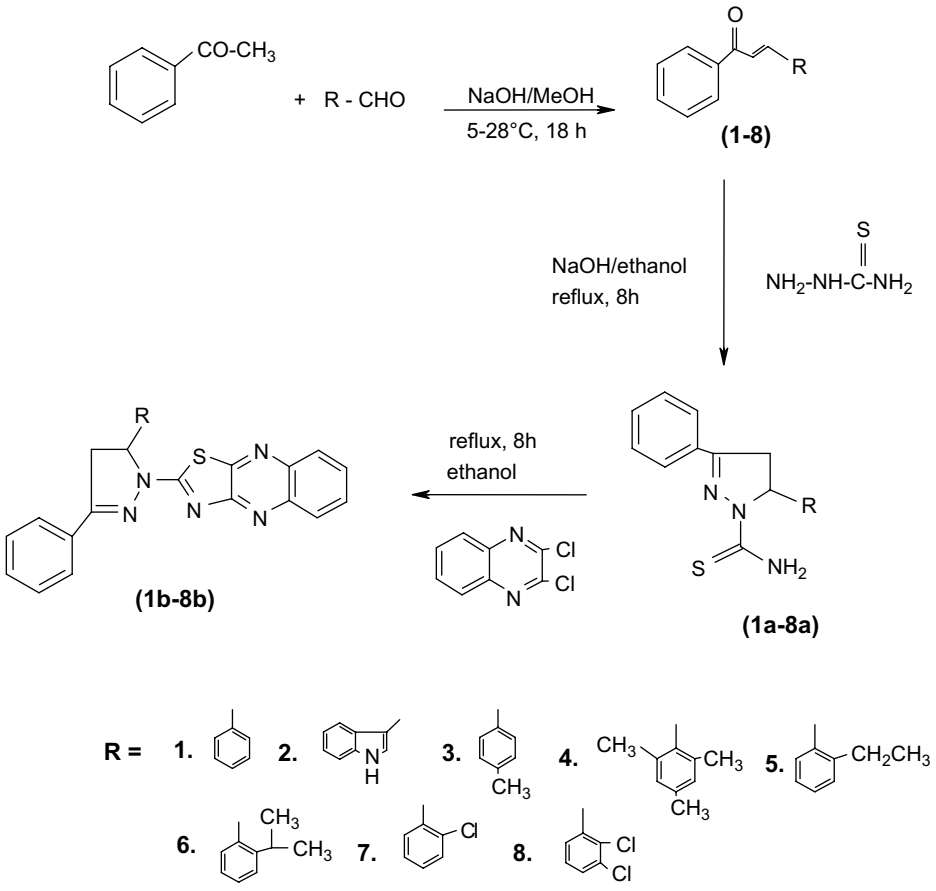
The electronic spectra of the cyclised pyrazoline analogues studied in the UV region in methanol, exhibited three absorption bands at 371–290, 270–236 and 223–205 nm assignable to n → π*, π → π* and n → σ* transitions, respectively. The band at 371–292 nm was assigned to the transition involving the thione portion (C=S) of thiocarbamoyl group. The two other absorption bands at 288–236 and 232–205 nm were due to π → π* transition of phenyl ring and n → σ* transition of azomethine nitrogen, respectively. The UV spectral data of **1b**–**8b** were also studied which showed the same type of transitions as observed in compounds **1a**–**8a**. It showed three spectral bands at 388.3–299, 287–248 and 239–204 nm assigned to n → π*, π → π* and n → σ* transitions of thiocarbamoyl group (C–S–C), aromatic ring and azomethine nitrogen, respectively.

2.2. Nuclear magnetic resonance spectral studies

Further evidence for the formation of **1**–**8**, **1a**–**8a** and **1b**–**8b** was obtained by ^{1}H NMR spectroscopy, which provide diagnostic tools for the positional elucidation of the protons. Assignment of the signals are based on the chemical shifts and intensity patterns. Two doublets in the ^{1}H NMR spectra of chalcones (**1**–**8**) in the region (6.86–7.74) and (5.82–7.66) ppm appears due to (–CO–CH=) and (=CH–Ar) protons favor their formation. In the ^{1}H NMR spectra of the cyclised product of chalcone, **1a**–**8a**, pyrazoline protons H_A and H_B are geminal protons at C_4 carbon, appears in the region 3.90–3.09 and 3.32–3.98 ppm as doublet of doublets in all compounds. The CH proton also appeared as doublet of doublets in the region of 6.32–5.40 ppm due to vicinal coupling with two non-equivalent geminal protons of C_4 carbon. These protons H_A and H_B protons at C_4 carbon were slightly shifted in case of quinoxaline compounds **1b**–**8b** and appears in the region 3.99–3.11 and 3.80–2.78 ppm as doublet of doublets in all quinoxaline compounds. The CH proton also appeared as doublet of doublets in the region of 6.58–5.11 ppm due to vicinal coupling with two non-equivalent geminal protons of C_4 carbon. The NH proton of different substituted thiocarbamoyl pyrazoline compounds showed a doublet at 9.24–7.17 ppm. Compounds **1a**–**8a** and **1b**–**8b** were additionally characterized by ^{13}C NMR spectroscopy. In the ^{13}C NMR spectra, the C_4 and C_5 carbons of the pyrazoline ring in compounds **1a**–**8a** resonate at 37.43–37.06 and 62.98–60.49 ppm, respectively. The phenyl–C resonates at 149.93–132.71 ppm. All the pyrazoline compounds showed a signal at 189.23–170.16 ppm, which was assigned to azomethine carbon of pyrazoline ring. Thiocarbamoyl carbon (C=S) displayed a signal at 169.42–204.23 ppm. The quinoxaline compounds **1b**–**8b** showed two signals at 151.66–152.36 and 140.11–143.35 ppm due to azomethine carbon of the pyrazoline ring and quinoxaline ring, respectively. Thiocarbamoyl carbon (C–S–C) displayed a signal at 143.07–139.31 ppm in all the compounds.

2.3. ESI-MS analysis

The characteristic peaks observed in the mass spectra of quinoxaline derivative (**8b**) are summarized in Scheme 2. The mass spectrum exhibits molecular ion peaks and contains fragments that confirm the quinoxaline structure in all the compounds.

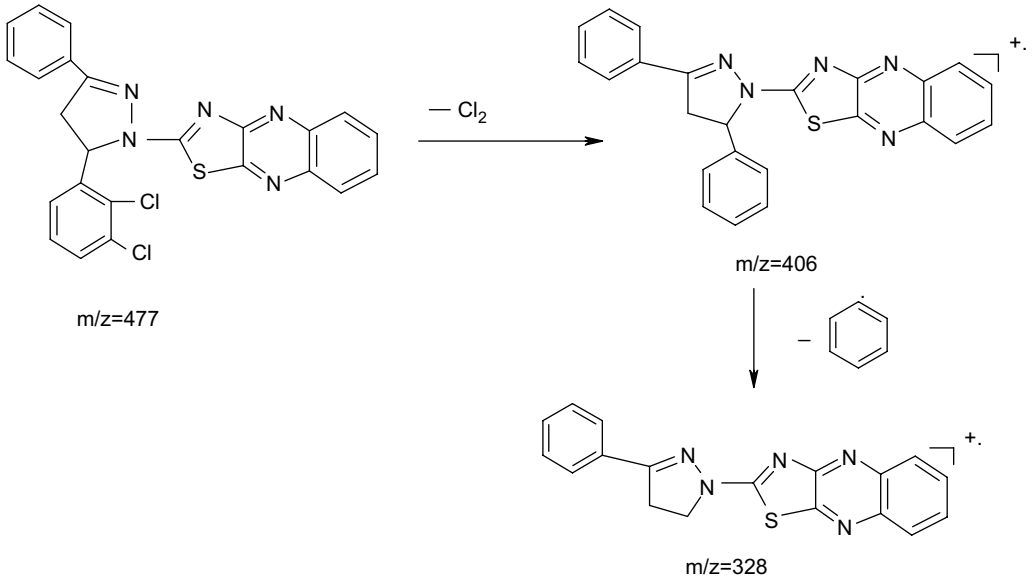


Scheme 1. Synthesis of 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-*b*]quinoxaline derivatives (**1b**–**8b**).

The fragmentation of compound **8b** showed the molecular ion peak (M + 1). The fragmentation of the compound occurs via removal of [$-Cl_2$] moiety giving desired peaks in all the compounds which correspond to the $[M - Cl_2]^+$ ion. This is followed by elimination of $-C_6H_5$ [20].

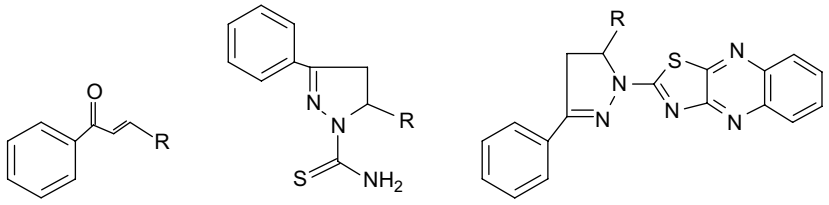
2.4. In vitro antiamoebic activity

All the compounds were evaluated for antiamoebic activity *in vitro* using HM1:IMSS strain of *E. histolytica*. The IC_{50} values in μM are shown in Table 1. The results were estimated as the

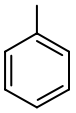
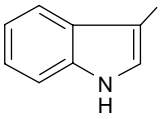
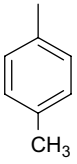
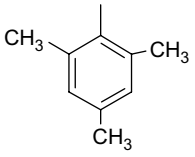
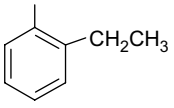
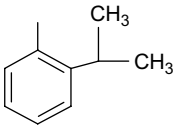
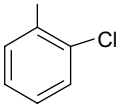
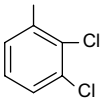


Scheme 2. Mass fragmentation pattern of compound **8b**.

Table 1
In vitro antiamoebic activity against HM1:IMSS strain of *E. histolytica* and toxicity studies



1-8 **1a-8a** **1b-8b**

Compound	R	Antiamoebic activity		Toxicity profile		
		IC_{50} (μM)	S.D.[a]	IC_{50} (μM)	Safety index	N
1		1.70	0.23	>50	>29.41	5
1a		1.61	0.34	>50	>31.05	5
1b		1.49	0.18	>50	>33.55	5
2		1.80	0.48	>44	>24.43	3
2a		1.77	0.23	>54	>30.51	5
2b		1.29	0.17	>18	>13.96	5
3		2.12	0.34	>50	>23.58	3
3a		1.58	0.18	>50	>31.68	3
3b		0.30	0.17	>14	>46.40	3
4		1.41	0.23	>50	>35.46	5
4a		4.37	0.34	>50	>11.44	5
4b		3.20	0.18	>35	>10.78	5
5		1.79	0.05	>50	>27.93	3
5a		1.61	0.04	>50	>31.05	3
5b		1.49	0.09	>50	>33.55	3
6		3.48	0.48	>46	>13.25	3
6a		0.23	0.23	>50	>217.39	3
6b		0.17	0.17	>50	>294.11	4
7		2.60	0.98	>50	>19.23	3
7a		2.23	0.02	>50	>22.42	5
7b		1.90	0.33	>50	>26.31	5
8		2.01	0.34	>50	>24.87	5
8a		1.09	0.18	>45	>41.18	5
8b		0 .89	0.17	>50	>56.17	5
	Metronidazole	1.80	0.05	>100	>55.56	5

N = number of times the experiment has been repeated.
[a] Standard deviation.

percentage of the growth inhibition compared with the untreated controls and plotted as probit values as a function of the drug concentration. The IC_{50} and 95% confidence limits were interpolated in the corresponding dose-response curve. We have synthesized chalcone derivatives (**1**–**8**) having various aromatic substituents. Chalcones have already been known for their excellent biological activity against number of diseases [21–23]. They exhibited antiamoebic activity with IC_{50} of 1.41–3.48 μM. Out of eight chalcone derivatives only two were found with more inhibition activity for *E. histolytica* than

metronidazole, the standard drug. From these chalcones we have prepared amino-5-substituted-(3-phenyl(2-pyrazolinyl))-methane-1-thione derivatives (**1a–8a**) and their activities were compared. It was observed that due to their modification as pyrazolines, their potencies were increased with IC_{50} 0.23–4.37 μM. Out of eight compounds three were found with better inhibitory tendency than metronidazole. The compound amino{5-[2-(methylethylphenyl)-3-phenyl(2-pyrazolinyl)}methane-1-thione (**6a**) having 2-methylethylphenyl substituents, $IC_{50} = 0.23$ μM was ~1.6 times more active than metronidazole ($IC_{50} = 1.8$ μM). From these very effective compounds, we have synthesized 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-*b*]quinoxaline derivatives (**1b–8b**) and their potencies were further enhanced by the introduction of quinoxaline moiety ($IC_{50} = 0.17–1.90$ μM). Out of eight compounds six had better inhibitory tendency than metronidazole as well as their parent compounds. Compound **6b** with $IC_{50} = 0.17$ μM was found to be the most active among all the compounds.

2.5. Toxicity profile

The toxic activities of compounds **1–8**, **1a–8a** and **1b–8b** were evaluated *in vitro* on human kidney epithelial cell line. The numerical results for each compound are given in Table 1. These activities were expressed as micromolar concentrations of the compounds that inhibited 50% of cell proliferation (human kidney epithelial). All the compounds inhibited cell growth at varied concentration ($IC_{50} > 14–50$ μM) while for metronidazole it is ($IC_{50} > 100$ μM). Except a few (**2b**, **3b** and **4b**) all the compounds inhibited cell growth at a concentration of >50 μM. To investigate the selectivity of the compounds, the "safety index" (SI) was calculated and defined as: 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 an estimate of which compounds might be efficacious or toxic against human cells and potentially *in vivo*. Safety index profile showed that compounds **6a**, **6b** and **8b** were less toxic than metronidazole. The results implicate that compound **6b** has less toxicity and better antiamoebic activity than metronidazole.

3. Conclusion

We report herein the synthesis and *in vitro* antiamoebic activity of chalcone (**1–8**), amino-5-substituted-(3-phenyl (2-pyrazolinyl))methane-1-thione derivatives (**1a–8a**) and 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-*b*]quinoxaline derivatives (**1b–8b**) against HM1:IMSS strain of *E. histolytica*. Modification of the compounds from chalcones, **1–8** to pyrazolines, **1a–8a** and further to quinoxalines, **1b–8b** results in an increase in antiamoebic activity. The toxic studies on human kidney epithelial cell lines showed that albeit all the compounds were not toxic but more toxic than metronidazole except **6a**, **6b** and **8b** and compound **6b** has most promising toxicity profile with potent antiamoebic activity.

4. Experimental

All the chemicals were purchased from Aldrich Chemical Company (U.S.A) and were used without further purification. The reactions were monitored by pre-coated aluminium silica gel $60F_{254}$ thin layer plates procured from Merck (Germany). The purity of compounds was confirmed by C, H and N analysis carried out at Central Drug Research Institute, Lucknow, India. Melting points were recorded on KSW melting point apparatus and are uncorrected. Electronic spectra were recorded in methanol on a Shimadzu UV-1601 PC UV–vis spectrophotometer. IR spectra on KBr disks were recorded on a Perkin Elmer model 1620 FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were obtained at ambient temperature using a BRUCKER SPECTROSCOPIN DPX-300 MHz spectrophotometer in $CDCl_3$ using tetramethylsilane as an internal standard. The ESI mass spectra of a few representative compounds were recorded on a MICRO MASS QUATTRO II triple quadrupole mass spectrometer.

4.1. Synthesis of chalcones: general procedure

A solution of acetophenones (50 mmol) and appropriate aldehyde (50 mmol) in methanolic NaOH was stirred for 18 h at 5–28 °C. Solid obtained was washed with ice-cold water and then ethanol, dried and recrystallized by the adequate solvent as indicated for each chalcone [24–26].

4.1.1. 1,3-Diphenyl prop-2-en-1-one (*1*)

Pale yellow crystals (chloroform); yield: 93%; m.p. 55 °C; Anal. calc. for $C_{15}H_{12}O$: C 86.21, H 4.86; found: C 86.50, H 4.90; UV–vis λ_{max} (nm): 371, 291, 236, 217; IR ν_{max} (cm^{-1}): 1664 (C=O), 1620 (CH=CH), 1580 (C=C); ^1H NMR ($CDCl_3$) (δ, ppm): 7.80 (m, 10H, Ar), 7.74 (d, H, $J = 15$ Hz, 1H), 7.63 (d, H, $J = 15$ Hz, 1H).

4.1.2. 3-Indol-3-yl-1-phenylprop-2-en-1-one (*2*)

Dark yellow solid (chloroform); yield: 56%; m.p. 182 °C; Anal. calc. for $C_{17}H_{12}NO$: C 77.86, H 4.58, N 5.34; found: C 77.83, H 4.52, N 5.33; IR (KBr) (cm^{-1}): 3160 (NH), 3020 (aromatic, C–H), 1710 (C=O), 1630 (–CH=CH); ^1H NMR ($CDCl_3$) (δ, ppm): 8.60 (1H, s, NH of indole), 7.11–7.89 (9H, m, Ar–H), 6.86 (1H, d, $J = 14.8$ Hz, =CH–Ar), 5.82 (1H, d, $J = 14.8$ Hz, CO–CH=).

4.1.3. 3-(4-Methylphenyl)-1-phenyl prop-2-en-1-one (*3*)

Pale yellow crystals (chloroform); yield: 54%; m.p. 175 °C; Anal. calc. for $C_{16}H_{14}O$: C 86.48, H 6.30; found: C 86.51, H 6.26; UV–vis λ_{max} (nm): 291, 236, 217; IR ν_{max} (cm^{-1}): 1710 (C=O), 1643 (CH=CH), 1550 (C=C); ^1H NMR ($CDCl_3$) (δ, ppm): 7.90 (m, 10H, Ar), 7.71 (d, H, $J = 15$ Hz, 1H), 7.66 (d, H, $J = 15$ Hz, 1H), 2.35 (s, 3H, CH_3).

*4.1.4. 1-Phenyl 3-(2,4,6-trimethylphenyl) prop-2-en-1-one (**4**)*

Pale yellow crystals (chloroform); yield: 49%; m.p. 128 °C; Anal. calc. for $C_{18}H_{18}O$: C 86.40, H 7.20; found: C 86.43, H 7.16; UV–vis λ_{max} (nm): 371, 291, 236, 217; IR ν_{max} (cm^{-1}): 1721 (C=O), 1630 (CH=CH), 1552 (C=C); ^1H NMR ($CDCl_3$) (δ, ppm): 7.81 (m, 7H, Ar), 7.70 (d, H, $J = 15$ Hz, 1H), 7.16 (d, H, $J = 15$ Hz, 1H), 2.50 (s, 9H, CH_3).

*4.1.5. 3-(2-Ethylphenyl)-1-phenylprop-2-en-1-one (**5**)*

Pale yellow crystals (chloroform); yield: 76%; m.p. 108 °C; Anal. calc. for $C_{17}H_{18}O$: C 86.44, H 6.77; found: C 86.48, H 6.80; UV–vis λ_{max} (nm): 271, 236, 217; IR ν_{max} (cm^{-1}): 1750 (C=O), 1622 (CH=CH), 1523 (C=C); ^1H NMR ($CDCl_3$) (δ, ppm): 7.71 (d, H, $J = 15$ Hz, 1H), 7.53 (d, H, $J = 15$ Hz, 1H), 7.11 (m, 10H, Ar), 1.20–2.10 (m, 2H, CH_2), 3.20 (t, 3H, CH_3).

*4.1.6. 3-(2-Methylethylphenyl)-1-phenylprop-2-en-1-one (**6**)*

Pale yellow crystals (chloroform); yield: 54%; m.p. 144 °C; Anal. calc. for $C_{18}H_{17}O$: C 86.74, H 6.82; found: C 86.70, H 6.79; UV–vis λ_{max} (nm): 291, 236, 217; IR ν_{max} (cm^{-1}): 1710 (C=O), 1613 (CH=CH), 1530 (C=C); ^1H NMR ($CDCl_3$) (δ, ppm): 7.80 (m, 9H, Ar), 7.74 (d, H, $J = 15$ Hz, 1H), 7.63 (d, H, $J = 15$ Hz, 1H), 2.10 (m, 1H, CH), 1.80 (m, 6H, CH_3).

*4.1.7. 3-(2-Chlorophenyl)-1-phenylprop-2-en-1-one (**7**)*

Pale yellow crystals (chloroform); yield: 63%; m.p. 135 °C; Anal. calc. for $C_{17}H_{13}O$: C 86.21, H 4.86; found: C 86.50, H 4.90; UV–vis λ_{max} (nm): 371, 291, 236, 217; IR ν_{max} (cm^{-1}): 1743 (C=O), 1630 (CH=CH), 1523 (C=C); ^1H NMR ($CDCl_3$) (δ, ppm): 7.78 (m, 10H, Ar), 7.67 (d, H, $J = 15$ Hz, 1H), 7.20 (d, H $J = 15$ Hz, 1H).

*4.1.8. 3-(3,4-Dichlorophenyl)-1-phenylprop-2-en-1-one (**8**)*

Pale yellow crystals (chloroform); yield: 85%; m.p. 118 °C; Anal. calc. for $C_{17}H_{13}NO_2$: C 86.21, H 4.86; found: C 86.24, H 5.16; UV–vis λ_{max} (nm): 371, 291, 236, 217; IR ν_{max} (cm^{-1}): 1710 (C=O), 1630 (CH=CH), 1550 (C=C); ^1H NMR ($CDCl_3$) (δ, ppm): 7.80 (m, 10H, Ar), 7.74 (d, 1H, $J = 15$ Hz,), 7.63 (d, 1H, $J = 15$ Hz).

*4.2. Synthesis of amino-5-substituted-(3-phenyl(2-pyrazolinyl))methane-1-thione (**1a–8a**): general procedure*

A mixture of chalcone **1–8** (10 mmol), thiosemicarbazide (10 mmol) and NaOH (25 mmol) was refluxed in ethanol (25 ml) for 8 h. The solution was poured into ice water. The precipitate was filtered and recrystallized from the suitable solvent [20].

*4.2.1. Amino[3,5-diphenyl(2-pyrazolinyl)]methane-1-thione (**1a**)*

Pale yellow powder (chloroform); yield: 24%; m.p. 175 °C; Anal. calc. for $C_{16}H_{15}N_3S$: C 68.32, H 5.33, N 14.94; found: C 68.30, H 5.31, N 14.89; UV–vis λ_{max} (nm): 371, 290, 236, 217; IR ν_{max} (cm^{-1}): 3435 (N–H), 2924 (C–H), 1558 (C=N), 1357 (C=S), 1122 (C–N); ^1H NMR ($CDCl_3$) (δ, ppm): 6.51–7.50 (m, 10H, Ar), 3.48 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 3.49 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.25), 6.32 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25), 2.13 (s, 3H, CH_3), 9.24 (d, 2H, NH_2); ^{13}C NMR ($CDCl_3$) (δ, ppm): 176.19 (C=S), 149.57 (C=N), 144.52–125.72 (Phenyl–C), 62.86 (C–H), 37.06 (CH_2).

*4.2.2. Amino(5-indol-3-yl-3-phenyl(2-pyrazolinyl))methane-1-thione (**2a**)*

Pale yellow crystal (chloroform); yield: 21%; m.p. 185 °C; Anal. calcd. for $C_{17}H_{15}N_4S$: C, 66.44, H, 4.88, N, 18.24; found: C, 66.46, H, 4.85, N, 18.39; UV–vis λ_{max} (nm): 371, 270, 236; IR: ν_{max} (cm^{-1}): 3233 (N–H), 2928 (C–H), 1590 (C=N), 1336 (C=S), 1052 (C–N); ^1H NMR ($CDCl_3$) (δ, ppm): 6.67–7.75 (m, 15H, Ar), 3.31 (dd, 1H, H_A, J_{AB}: 17.33, J_{AX}: 9.33), 3.32 (dd, 1H, H_B, J_{AB}: 16.6, J_{AX}: 9.52), 6.02 (dd, 1H, H_X, J_{AX}: 9.33, J_{BX}: 8.75), 7.89 (d, 2H, NH_2), ^{13}C NMR ($CDCl_3$) (δ, ppm): 170.16 (C=S), 149.57 (C=N), 149. 93–126.07 (Phenyl–C), 38.28 (CH), 33.86 (CH_2).

*4.2.3. Amino[5-(4-methylphenyl)-3-phenyl(2-pyrazolinyl)] methane-1-thione (**3a**)*

Pale yellow crystal (chloroform); yield: 11%; m.p. 160 °C; Anal calcd. for $C_{17}H_{17}N_3S$: C 69.15, H 5.76, N 14.23; found: C 69.21, H 5.70, N 14.19; UV–vis λ_{max} (nm): 371, 291, 245; IR ν_{max} (cm^{-1}): 3448 (N–H), 2963 (C–H), 1542 (C=N), 1364 (C=S), 1024 (C–N); ^1H NMR ($CDCl_3$) (δ, ppm): 6.70–7.60 (m, 9H, Ar), 3.80 (dd, 1H, H_A J_{AB}: 18.5, J_{AX}: 9.25 Hz), 3.60 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.5), 5.90 (dd, 1H, H_X, J_{AX}: 9.25, J_{BX}: 9.5), 2.32 (s, 3H, CH_3), 7.17 (d, 2H, NH_2).^{13}C NMR ($CDCl_3$) (δ, ppm): 172.19 (C=S), 146.57 (C=N), 144.52–125.72 (Phenyl–C), 62.86 (CH), 37.02 (CH_2), 21.09 (CH_3).

*4.2.4. Amino[3-phenyl-5-(2,4,6-methylphenyl)(2-pyrazolinyl)] methane-1-thione (**4a**)*

Pale yellow powder (chloroform); yield: 24%; m.p. 175 °C; Anal. Calc. for $C_{19}H_{21}N_3S$: C 70.58, H 6.50, N 13.00; found: C 70.54, H 6.49, N 13.03; UV–vis λ_{max} (nm): 371, 290, 236, 217; IR ν_{max} (cm^{-1}): 3435 (N–H), 2924 (C–H), 1558 (C=N), 1357 (C=S), 1122 (C–N); ^1H NMR ($CDCl_3$) (δ, ppm): 6.72–7.50 (m, 7H, Ar), 3.48 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 3.49 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.25), 6.32 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25) 2.13 (s, 3H, CH_3), 7.79 (d, 1H, NH_2); ^{13}C NMR ($CDCl_3$) (δ, ppm): 189.23 (C=S), 146.57 (C=N), 132.78–125.32 (Phenyl–C), 20.98–20.58 (CH_3), 37.43 (CH_2), 62.86 (CH).

*4.2.5. Amino[5-(2-ethylphenyl)-3-phenyl (2-pyrazolinyl)]methane-1-thione (**5a**)*

Bright orange crystal (chloroform); yield: 18%; m.p. 159 °C, Anal. calcd. for $C_{18}H_{19}$ N_3S: C 69.90, H 6.14, N 13.59; found: C 69.95, H 6.18, N 13.50; UV–vis λ_{max} (nm): 371, 291, 245, IR ν_{max} (cm^{-1}): 3228 (N–H) 2925 (C–H), 1578 (C=N), 1333 (C=S), 1066 (CN); ^1H NMR

($CDCl_3$) (δ, ppm): 6.7–7.6 (m, 9H, Ar), 3.9 (dd, 1H, H_A, J_{AB}: 18.5, J_{AX}: 9.25 Hz), 3.6 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.5), 5.4 (dd, 1H, H_X, J_{AX}: 9.25, J_{BX}: 9.5), 2.32 (s, 3H, CH_3), 7.19 (d, 2H, NH_2), ^{13}C NMR ($CDCl_3$): (δ, ppm): 172.19 (C=S), 146.50 (C=N), 144.52–125.72 (Phenyl–C), 62.86 (CH), 37.06 (CH_2), 27.90 (ethyl–CH_2), 15.70 (CH_3).

4.2.6. Amino{5-[2-(methylethylphenyl)-3-phenyl (2-pyrazolinyl)]}methane-1-thione (**6a**)

Orange crystal (chloroform); yield: 22%; m.p: 189 °C; Anal.calcd. for $C_{20}H_{23}N_3S$: C 70.80, H 6.21, N 13.04; found: C 70.83, H 6.22, N 13.10; UV–vis λ_{max} (nm): 366, 301, 223, 205, 246; IR ν_{max} (cm^{-1}): 3255 (NH) 2923 (C–H), 1545 (C=N), 1349 (C=S), 1033 (C–N); ^1H NMR (δ, ppm): 7.10–7.72 (m, 15H, Ar), 3.09 (dd, 1H, H_A), 3.5 (dd, 1H, H_B, J_{AB}: 16.6, J_{AX}: 9.52), 6.0–5.9 (dd, 1H, H_X, $J_{AX,}$ 2.58, J_{BX}: 7.75), 2.51 (s, 3H, CH_3)), 7.80 (d, 1H, NH_2); ^{13}C NMR ($CDCl_3$) (δ, ppm): 175.49 (C=S), 143.85 (C=N), 148.52–115 (Phenyl–C), 24.08 (CH_3), 62.86 (CH), 37.43 (CH_2).

4.2.7. Amino[5-(2-chlorophenyl)-3-phenyl (2-pyrazolinyl)]methane-1-thione (**7a**)

Yellow crystal (chloroform); yield: 18%; m.p. 210 °C; Anal. calc. for $C_{16}H_{14}N_3SCl$: C 60.85, H 4.43, N 13.31; found: C 60.80, H 4.40, N 13.29; UV–vis λ_{max} (nm): 371, 291, 245, 223, 211; IR ν_{max} (cm^{-1}): 3421 (NH), 2922 (C–H), 1549 (C=N), 1370 (C=S), 1076 (C–N); ^1H NMR ($CDCl_3$) (δ, ppm): 7.58–7.51 (m, 9H, Ar), 3.59 (dd, 1H, H_A, J_{AB}: 18.75 J_{AX}: 12.5), 3.98 (dd, 1H, H_B, J_{AB}: 17.5, J_{BX}: 6.77), 6.32 (dd, 1H, H_X, J_{AX}: 10.96, J_{BX}: 6.77), 7.77 (d, 2H, NH_2).^{13}C NMR ($CDCl_3$) (δ, ppm): 175.49 (C=S), 143.85 (C=N), 148.52–115 (Phenyl–C), 37.21 (CH_2), 62.98 (CH).

4.2.8. Amino[5-(2,3-dichlorophenyl)-3-phenyl (2-pyrazolinyl)]methane-1-thione (**8a**)

Yellow crystal (chloroform); yield: 13%; m.p: 190 °C; Anal. calcd. for $C_{16}H_{13}N_3SCl_2$: C 54.85, H 3.71, N 12; found: C 54.80, H 3.75, N 12.12%; UV–vis λ_{max} (nm): 371, 270, 236; IR ν_{max} (cm^{-1}): 3454 (NH), 2925 (C–H), 1585 (C=N), 1350 (C=S), 1052 (C–N); ^1H NMR ($CDCl_3$) (δ, ppm): 6.78–7.51, (m, 10H, Ar), 3.38 (dd, 1H, $H_{A,}$ J_{AB}: 17.33, J_{AX}: 9.33), 3.48 (dd, 1H, H_B, J_{AB}: 16.7, J_{BX}: 8.1), 6.21 (dd, 1H, H_X, J_{AX}: 11.6, J_{BX}: 10.2), 7.7 (d, 2H, NH_2), 1.2–2.1 (m, 10H, CH_2): ^{13}C NMR ($CDCl_3$): (δ, ppm): 177.23 (C=S), 140.66 (C=N), 136.33–125.10 (Phenyl–C), 37.43 (CH_2), 60.49 (CH).

4.3. Synthesis of 2-(5-substituted-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-b]quinoxaline derivatives (**1b–8b**): general procedure

A mixture of 1-*N*-thiocarbamoyl-3,5-diphenyl pyrazoline compounds **1a–8a** (10 mmol) and 2,3-dichloroquinoxaline (10 mmol) in absolute ethanol (15 ml) was refluxed for 8 h. The solvent was evaporated under *vacuo*. The residue was recrystallized from acetone [27].

4.3.1. 2-(3,5-Diphenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-b] quinoxaline (**1b**)

Pale yellow crystal (chloroform); yield: 43%; m.p. 181 °C; Anal. calc. for $C_{24}H_{17}N_5S$: C 70.76, H 4.18, N 17.20; found: C 70.80, H 4.16, N 17.21; UV–vis λ_{max} (nm): 371, 290, 236, 217; IR ν_{max} (cm^{-1}): 2954 (C–H), 1558 (C=N), 1542 (C=N), 1122 (C–N), 920 (C–S); ^1H NMR ($CDCl_3$) (δ, ppm): 8.72–7.50 (14H, m, Ar), 3.48 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}, 9.25), 3.80 (dd, 1H, H_B, J_{AB}: 18.5 Hz,), 6.32 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25). ^{13}C NMR ($CDCl_3$) (δ, ppm): 143.07 (C–S), 152.36 (C=N), 143.35 (C=N), 136.33–125.10 (Phenyl–C), 35.82 (CH_2), 61.60 (CH).

4.3.2. 2-(5-Indol-3-yl-3-phenyl-2-pyrazolinyl)-1, 3-thiazolino[5,4-b]quinoxaline (**2b**)

Yellow crystal (chloroform); yield: 49%; m.p. 175 °C; Anal. calc. for $C_{26}H_{17}N_6S$: C 70.11, H 3.82, N 18.87; found: C 70.12, H 3.80, N, 18.82; UV–vis λ_{max} (nm): 369, 290, 236, 215; IR ν_{max} (cm^{-1}): 2924 (C–H), 1516 (C=N), 1510 (C=N), 1099 (C–N), 862 (C–S); ^1H NMR ($CDCl_3$) (δ, ppm); 7.50–7.01 (14H, m, Ar), 3.11 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 2.80 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.25), 5.11 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25), 9.24 (d, 1H, NH), 9.24 (d, 1H, NH). ^{13}C NMR ($CDCl_3$) (δ, ppm): 140.31 (C–S), 150.23 (C=N), 140.11 (C=N), 136.33–125.10 (Phenyl–C), 35.82 (CH_2), 61.60 (CH).

4.3.3. 2-(5-(4-Methylphenyl)-3-phenyl-2-pyrazolinyl)-1,3-thiazolino[5,4-b]quinoxaline (**3b**)

Pale yellow crystal (chloroform); yield: 22%; m.p. 169 °C; Anal. calc. for $C_{25}H_{19}N_5S$: C 71.25, H 4.51, N 16.62; found: C 71.31, H 4.55, N 16.69; UV–vis λ_{max} (nm): 371, 290, 236, 217; IR ν_{max} (cm^{-1}): 2933 (C–H), 1558 (C=N), 1576 (C=N), 1122 (C–N), 915 (C–S); ^1H NMR ($CDCl_3$) (δ, ppm): 6.72–7.50 (14H, m, Ar), 3.66 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 3.49 (dd, 1H, $H_{B,}$ J_{AB}: 18.5, J_{BX}: 9.25), 6.58 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25) 2.13 (s, 3H, CH_3) ^{13}C NMR ($CDCl_3$) (δ, ppm): 140.31 (C–S), 152.01 (C=N), 141.78 (C=N), 133.33–126.10 (Phenyl–C), 33.58 (CH_2), 59.56 (CH),17.85 (CH_3).

4.3.4. 2-[3-Phenyl-5-(2,4,6-trimethylphenyl)-2-pyrazolinyl]-1,3-thiazolino[5,4-b] quinoxaline (**4b**)

Yellow crystal (chloroform); yield: 24%; m.p. 182 °C; Anal. calc. for $C_{27}H_{23}N_5S$: C 72.16, H 5.12, N 15.59; found: C 72.20, H 5.10, N 15.50; UV–vis λ_{max} (nm): 377, 289, 256, 217; IR ν_{max} (cm^{-1}): 2928 (C–H), 1509 (C=N), 1538 (C=N), 1103 (C–N), 839 (C–S); ^1H NMR ($CDCl_3$) (δ, ppm): 7.72–7.50 (14H, m, Ar), 3.48 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 3.23 (dd, 1H, $H_{B,}$ J_{AB}: 18.5, J_{BX}: 9.25), 5.69 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25). ^{13}C NMR ($CDCl_3$) (δ, ppm): 140.22 (C–S), 150.23 (C=N), 140.11 (C=N), 136.33–125.10 (Phenyl–C), 35.82 (CH_2), 61.60 (CH).

*4.3.5. 2-[5-(2-Ethylphenyl)-3-phenyl-2-pyrazolinyl]-1, 3-thiazolino[5,4-b]quinoxaline (**5b**)*

Pale yellow crystal (chloroform); yield: 18%; m.p. 187 °C; Anal. calc. for $C_{26}H_{21}N_5S$: C 71.72, H 4.83, N 16.09; found: C 71.75, H 4.77, N 16.10; UV–vis λ_{max} (nm): 373, 288, 236, 219; IR ν_{max} (cm^{-1}): 2924 (C–H),1563 (C=N), 1558 (C=N), 1192 (C–N), 945 (C–S); ^{1}H NMR ($CDCl_3$) (δ, ppm): 7.23–6.72 (14H, m, Ar), 3.48 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 3.49 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.25), 6.32 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25) 2.13 (s, 3H, CH_3), 9.24 (d, 1H, NH) ^{13}C NMR ($CDCl_3$) (δ, ppm): 140.24 (C–S), 150.11 (C=N), 141.22 (C=N), 136.33–125.10 (Phenyl–C), 35.82 (CH_2), 27.90 (CH_2) 61.60 (CH).

*4.3.6. 2-{5-[2-(Methylethyl)phenyl]-3-phenyl-2-pyrazolinyl}-1,3-thiazolino[5,4-b]quinoxaline (**6b**)*

Pale yellow crystal (chloroform); yield: 16%; m.p: 175 °C; Anal. calc. for $C_{27}H_{23}N_5S$: C 72.16, H 5.12, N 15.59; found: C 72.13, H 5.10, N 15.49; UV–vis λ_{max} (nm): 373, 288, 236, 217; IR ν_{max} cm^{-1}: 2955 (C–H), 1545 (C=N), 1516 (C=N), 1078 (C–N), 892 (C–S); ^{1}H NMR ($CDCl_3$) (δ, ppm): 6.72–7.50 (14H, m, Ar), 3.48 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 3.49 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.25), 6.32 (dd,1H, H_X, J_{AX}: 11.10, J_{Bx}: 9.25) 2.13 (s, 3H, CH_3), ^{13}C NMR ($CDCl_3$) (δ, ppm): 140.31 (C–S), 150.23 (C=N), 140.11 (C=N), 136.33–125.10 (Phenyl–C), 35.82 (CH_2), 60.56 (CH),17.85 (CH_3), 32.90 (CH).

*4.3.7. 2-[5-(2-Chlorophenyl)-3-phenyl-2-pyrazolinyl]-1, 3-thiazolino[5,4-b]quinoxaline (**7b**)*

Pale yellow crystal (chloroform); yield: 34%; m.p: 177 °C; Anal. calc. for $C_{24}H_{16}N_5SCl$: C 65.23, H 3.62, N 15.85; found: C 65.25, H 3.60; N 15.85; UV–vis λ_{max} (nm): 371, 290, 227, 216; IR ν_{max} (cm^{-1}): 2933 (C–H), 1558 (C=N), 1545 (C=N), 1162 (C–N), 920 (C–S); ^{1}H NMR ($CDCl_3$) (δ, ppm): 7.20–7.01(14H, m, Ar), 3.21 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 3.49 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.25), 5.82 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25); ^{13}C NMR ($CDCl_3$) (δ, ppm): 139.90 (C–S), 151.66 (C=N), 141.13 (C=N), 136.33–125.10 (Phenyl–C), 32.78 (CH_2), 62.33 (CH).

*4.3.8. 2-[5-(2,3-Dichlorophenyl)-3-phenyl-2-pyrazolinyl]-1,3-thiazolino[5,4-b]quinoxaline (**8b**)*

Pale yellow crystal (chloroform); yield: 22%; m.p. 170 °C; Anal. calc. for $C_{24}H_{15}N_5SCl_2$: C 60.50, H 3.15, N 14.70; found: C 60.53, H 3.15, N 14.76; UV–vis λ_{max} (nm): 371, 290, 258, 236, 217; IR ν_{max} (cm^{-1}): 2924 (C–H), 1486 (C=N), 1511 (C=N), 1122 (C–N), 945 (C–S); ^{1}H NMR ($CDCl_3$) (δ, ppm): 6.72–7.50 (14H, m, Ar), 3.99 (dd, 1H, H_A, J_{AB}: 18.5 Hz, J_{AX}: 9.25), 2.78 (dd, 1H, H_B, J_{AB}: 18.5, J_{BX}: 9.25), 6.21 (dd, 1H, H_X, J_{AX}: 11.10, J_{BX}: 9.25) 2.13 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) (δ, ppm): 139.31 (C–S), 150.23 (C=N), 140.11 (C=N), 136.33–125.10 (Phenyl–C), 35.82 (CH_2), 61.60 (CH),17.85 (CH_3), 32.90 (CH), ESI-MS: 477 (M + 1), 428, 306.

4.4. In vitro testing against E. histolytica

All the cyclised pyrazoline analogues were screened *in vitro* for antiamoebic activity against HM1:1MSS strain of *E. histolytica* by microdilution method [28]. *E. histolytica* trophozoites were cultured in TYIS-33 growth medium as described previously in wells of 96 well microtiter plate [29]. All the compounds were dissolved in DMSO (40 µl) at which level no inhibition of amoeba occurs [30–31] and 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). The number of amoeba per ml was estimated with a haemocytometer and trypan blue exclusion was used to confirm viability. The cell suspension used 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.

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.

4.5. Assessment of antiamoebic activity

After incubation, the growth of amoebae in the plate was checked with a low power microscope. Inverting the plate and shaking gently removed the culture medium. Plate was then immediately washed once in 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 dry, stained with 0.5% aqueous eosin for 15 min. Stained plate was washed once with tape water and 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 straight line from which the IC_{50} value was found. The results are reported in Table 1.

4.6. Toxicity screening

The toxic activities of compounds **1–8**, **1a–8a** and **1b–8b** were evaluated *in vitro* on human kidney epithelial cell line. For the toxicity assay, transformed human kidney epithelium (Graham) cells was continuously maintained in culture at 37 °C in 5% CO_2. The MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide, USB) cellular viability assay was used to determine the toxicity profile of these compounds [32]. The trypsinized cell suspension was adjusted to

0.5 million cells/mL and plated out with the 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 dissolve the formazan crystals. The absorbance was read at the test wavelength of 540 nm and reference wavelength of 690 nm and the percentage cellular viability calculated with appropriate controls taken into account.

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