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Short communication

Synthesis and *in vitro* evaluation of novel tetrazole embedded 1,3,5-trisubstituted pyrazoline derivatives as *Entamoeba histolytica* growth inhibitors

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ABSTRACT

A series of pyrazoline derivatives (**1a**–**15a**) was synthesized by cyclization of chalcones (**1**–**15**) with 2-[5-(4-methoxyphenyl)-1H-tetrazol-1-yl]acetohydrazide under basic conditions and were screened *in vitro*, to find out effect on the growth of HM1: IMSS strain of *Entamoeba histolytica*. The compounds **3a**, **4a**, **11a**, **13a** and **14a** showed encouraging results with IC_{50} value in the range of 0.86–1.28 μM. However compound **13a** showed most promising results with IC_{50} = 0.86 μM which is half of the metronidazole, the standard drug used for protozoal infection. Cell viability test in human hepatocellular carcinoma cell line (HepG2) revealed non-toxic nature of new synthesized compounds. Safety index calculations prevailed compound **13a** as highly antiamoebic and least cytotoxic (S.I. = >116.28), almost twice than metronidazole.

1. Introduction

Protozoan diseases stand as a major health issue in developing countries, affecting hundreds of millions of people around the world [1]. The eukaryotic origin of protozoa impedes the development of effective and selective drug, as they share many common features with their mammalian host. Amoebiasis, a protozoal disease caused by *Entamoeba histolytica* is the third prominent cause of death [2,3]. It is acquired by ingestion of *E. histolytica* cysts, which develops into motile trophozoites in ileocecal region of the intestine. The major clinical manifestations of *E. histolytica* infection are amoebic colitis and amoebic liver abscesses. Metronidazole (*2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol*), has long been used for the treatment of this disease. However reports show that metronidazole is ecotoxic [4], induces encephalopathy [5,6], neurotoxic [7], genotoxic, carcinogen [8–12] and causes spermatozoid damage [13]. Furthermore, failures in the treatment of several intestinal protozoan parasites are results of drug resistant to parasites [14–16]. Considering these facts, an unmet need to search new molecules arises, which will show better therapeutic activity and have least side effects. Serving the purpose we have synthesized a new series of pyrazoline derivatives bearing a tetrazole tail and screened the compounds for antiamoebic evaluation.

Pyrazoline skeleton has received progressive attention of many researchers because of its diverse biological properties such as antitumor, immunosuppressive, antibacterial, anti-inflammatory, anticancer, antidiabetic, and antidepressant properties [17–25]. On the other hand, tetrazole rings are shared by a number of modern drugs owing to their potential biological activities [26–33]. The recent success of pyrazoline-based antiamoebic compounds reported by our group has further highlighted the importance of these heterocycles [34].

Prompted by the above-mentioned important properties of pyrazolines and tetrazoles it was speculated that designing and synthesis of a new series of tetrazole embedded pyrazoline derivatives would be worthwhile, as an *E. histolytica* growth inhibitors (Fig. 1). Furthermore, after extensive literature search, it was observed that, till date no effort has been made to combine these vital moieties as a single molecular scaffold and to study its antiamoebic activity.

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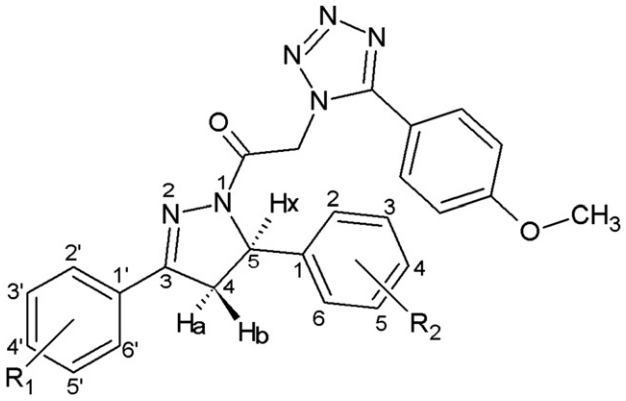
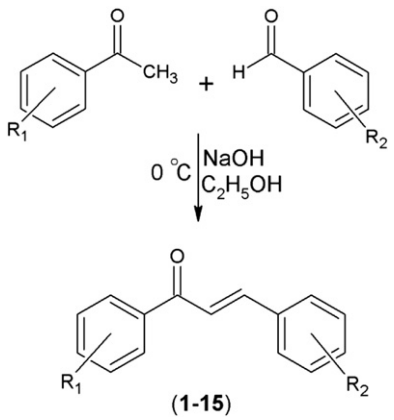


Fig. 1. General structure of 1,3,5-trisubstituted pyrazoline derivatives with a tetrazole tail.

2. Results and discussion

2.1. Chemistry

Present study was undertaken to synthesize some novel tetrazole embedded Pyrazoline derivatives and to investigate their probable antiamoebic effect. Target compounds (**1a**–**15a**) were obtained in four-step reaction process, outlined in Scheme 1–4. In the first step, synthesis of chalcones (**1**–**15**) was carried out by Claisen–Schmidt reaction and products were purified by recrystallization from methanol (70–85% yield). In the second step, 5-(4-methoxyphenyl)-1*H*-tetrazole (**2**) was synthesized starting from *p*-methoxy benzaldehyde *via* an oxime and nitrile intermediate [35,36] (Scheme 2). 2-[5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl] acetohydrazide (**3**) was synthesized from 5-(4-methoxyphenyl)-1*H*-tetrazole (**2**) following a reported reaction procedure [37] (Scheme 3). The target compounds (**1a**–**15a**) were obtained by the reaction of chalcones (**1**–**15**) with 2-[5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl]acetohydrazide (**3**) in refluxing methanol and 5% NaOH. Factors such as polarizing nature of substituents on α, β unsaturated carbon moiety profoundly influenced the rate of reaction. Halogen and nitro substituted chalcones took 10–12 h for complete conversion followed by unsubstituted (15 h), methyl substituted (16–20 h) and alkoxy substituted (24 h) groups. The detailed mechanism of the reaction involves initial formation of an aryl hydrazone with subsequent nucleophilic attack of nitrogen on the carbon–carbon double bond at β position. The rate of the reaction is determined by nucleophile attack unto β carbon and is directly proportional to the electropositive nature of β carbon. The polar nature of β carbon is controlled by inductive effect of substituted aromatic ring. The substituted halo and nitro groups in the aromatic ring increase the partial electron deficiency of β carbon, leading to quick completion of the reaction. However electron donating alkyl and alkoxy groups contributed to slow reaction.



Scheme 1. Synthesis of different substituted chalcones (**1**–**15**).

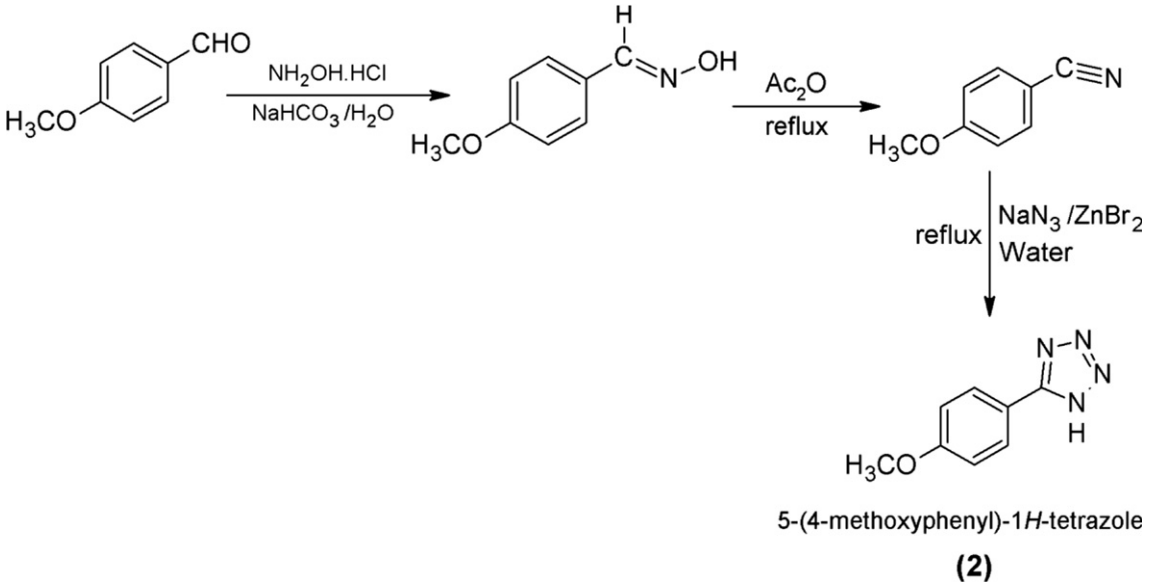
A variety of methods has been reported for the synthesis of 1,3,5-trisubstituted pyrazolines [38–42]. Among them, simplest and more efficient methods reported by E. Fischer et al. [38], involves condensation of α, β-unsaturated ketone with an acid hydrazide in glacial acetic acid (bp. 117 °C) under reflux. However, people have reported a significantly improve in the yield by using n-butanol (bp. 115 °C) as a reaction medium. In the present investigation, we found that n-butanol as a solvent not only decreased the progress of the reaction, but also froze the reaction in some cases. The reaction was refluxed in methanol and 5% NaOH, which was found to be the best solvent. Structure of compounds **1**–**15** and **1a**–**15a** were confirmed by NMR, IR and MS techniques. All of the Pyrazoline derivatives possess similar basic skeletal structure. Proton NMR signals were assigned by comparing the spectra of the products (**1a**–**15a**) with their corresponding chalcones (**1**–**15**). Signals around δ value 3.9 and 3.6 ppm recorded as doublet of doublets (dd) was assigned to **4-Ha** and **4-Hb** protons. The coupling constant "*J*" was found approximately 18 and 5 Hz for 3.9 ppm and 18 and 11 Hz for 3.6 ppm. 5-H proton (5.5 ppm) of pyrazoline ring showed a 'dd' pattern with "*J*" values 11 and 5 Hz. The coupling occurs most likely due to scalar or indirect coupling with **4-Ha** and **4-Hb** protons. This coupling strongly sports the conformational differences between Ha and Hb protons.

2.2. Pharmacology

The synthesized chalcones (**1**–**15**) and their corresponding pyrazoline derivatives (**1a**–**15a**) were screened *in vitro* against *HM1:IMSS* strain of *E. histolytica* by microdilution method [43]. All experiments were carried out in triplicate for each concentration level and repeated thrice. Cytotoxicity of all the compounds has been studied by cell viability test of human hepatocellular carcinoma cell line (HepG2). The results of antiamoebic activity and cytotoxicity are summarized in Tables 1and 2 and Fig. 2.

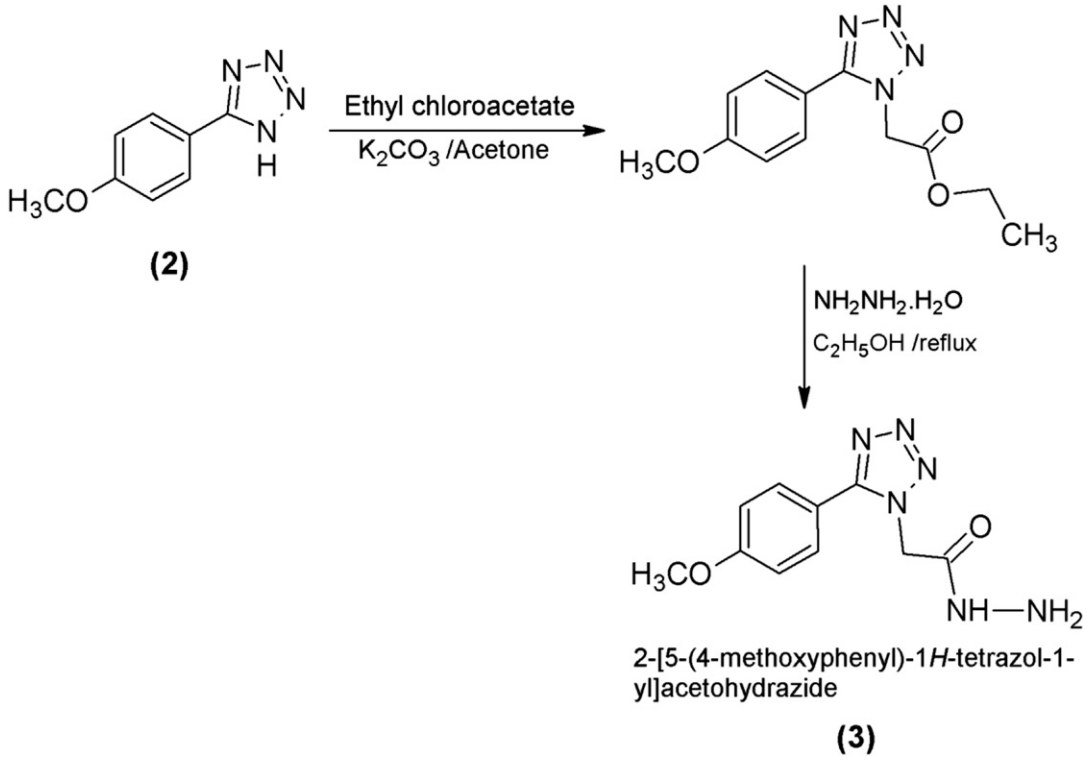
2.2.1. In vitro antiamoebic activity

Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of compounds (**1**–**15** and **1a**–**15a**) by microdilution method, using *HM1:IMSS* strain of *E. histolytica*. The results are summarized in Tables 1and 2. The data is presented in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic activity of the synthesized compounds was compared with the most widely used antiamoebic medication metronidazole (control) with 50% inhibitory concentration (IC_{50}) of 1.80 μM in our experiments. IC_{50} and 95% confidence limits were interpolated in the corresponding dose response curve. The antiamoebic activity of the test compounds was found to be substituent dependent. As depicted in Tables 1and 2 compounds exhibit an interesting inhibition pattern on *HM1:IMSS* strain of *E. histolytica*. The chalcone derivatives (**1**–**15**) showed IC_{50} value in the range of 4.19–12.86 μM, much higher than control drug. A sudden decrease in IC_{50} (0.86–5.32 μM) was observed, when chalcones (**1**–**15**) were converted into their corresponding pyrazoline derivatives (**1a**–**15a**). However the interesting inhibitory behavior of these compounds is relatively dependent on electronic nature of substituents on phenyl ring. Compound 1 bearing unsubstituted phenyl ring, showed 50% inhibition at a concentration of 12.86 μM. Presence of substituents overall increased the activity of the tested compounds.

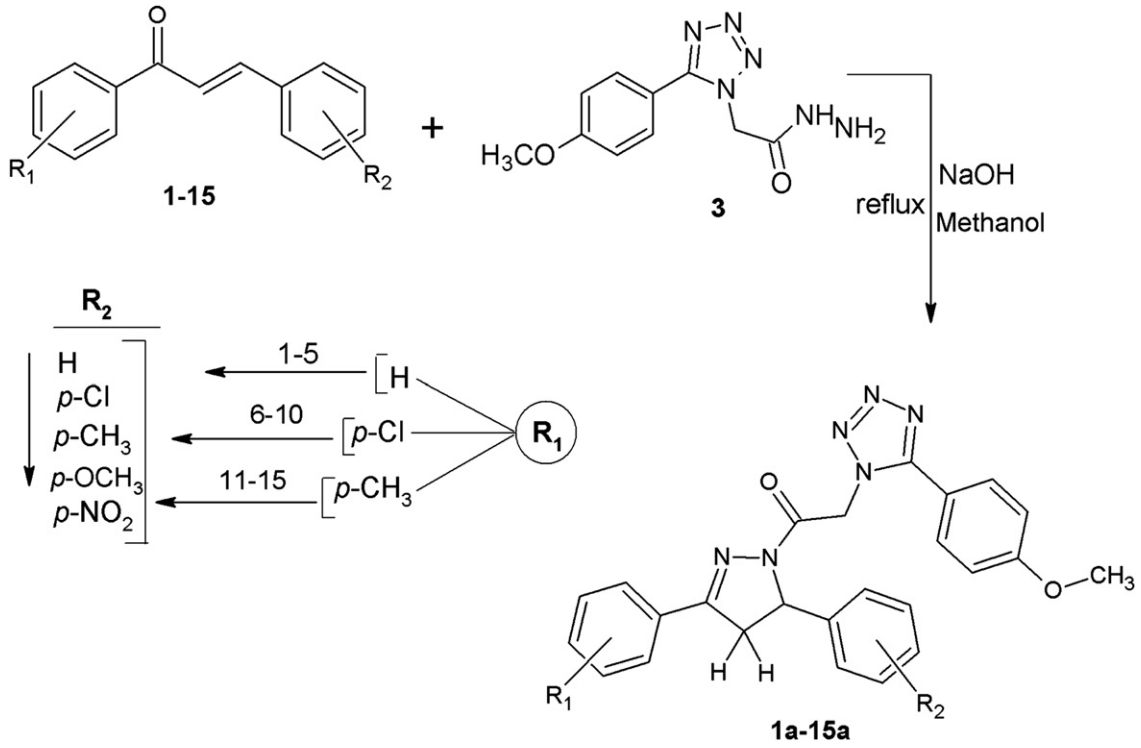


Scheme 2. Synthesis of 5-(4-methoxyphenyl)-1*H*-tetrazole (**2**).

Compounds bearing electron releasing groups, showed better activity than compounds bearing electron withdrawing groups. The pyrazoline derivatives (**1a**–**15a**) showed a similar inhibitory pattern, where activity was guided by the presence of electron releasing and electron withdrawing substituents. The results showed that compounds bearing electron withdrawing groups at para position were less active in habiting the growth of *E. histolytica* than compounds bearing electron donating/releasing groups. However, it is interested to mention that growth inhibition activity depends also on relative inductive effect of the substituents. Presence of weakly activating groups like methyl showed less activity than the methoxy group, which is strongly activating. The higher inhibition activity of methoxy group can be speculated with the effect of pi-electron cloud on lone pair of electrons than the inductive effect of the electronegative oxygen. Among all, the compounds bearing a methyl or methoxy group showed better activity than those with chloro or nitro group at para position of the phenyl rings. Presence of electron releasing and electron withdrawing group in a same compound also resulted in decreased activity. The compound **13a** bearing two methyl groups at para position (6 and 6′) of the phenyl rings showed excellent antiamoebic activity with IC_{50} value of 0.86 μM. The activity however,



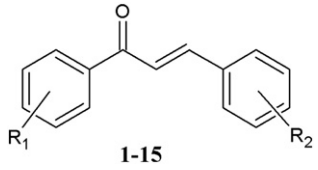
Scheme 3. Synthesis of 2-[5-(4-methoxyphenyl)-1*H*-tetrazol-1-yl]acetohydrazide (**3**).



Scheme 4. Synthesis of pyrazoline derivatives bearing a tetrazole tail (**1a**–**15a**).

was decreased when one methyl group was replaced with methoxy group in compound **14a**. The decrease in activity with electron donating substituents (OCH_3) is attributed to increased electron density, which causes more difficult diffusion through the cell membrane and substantial loss in activity [44,45]. Thus for

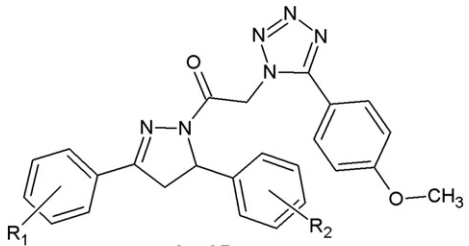
Table 1
In vitro antiamoebic activity of compounds (**1**–**15**) against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile.



Compound	R_1	R_2	Antiamoebic activity		Toxicity profile	
			IC_{50} (μM)	S.D[a]. (±)	IC_{50} (μM)	Safety index (SI)
1	H	H	12.86	0.18	>100	>7.78
2	H	Cl	7.32	0.16	97.2	13.27
3	H	CH_3	4.38	0.12	>100	>22.83
4	H	OCH_3	5.93	0.20	>100	>16.86
5	H	NO_2	6.02	0.13	64.7	10.74
6	Cl	H	6.96	0.10	94.7	13.60
7	Cl	Cl	5.86	0.16	82.8	14.13
8	Cl	CH_3	5.32	0.20	92.6	17.40
9	Cl	OCH_3	5.10	0.18	97.2	19.05
10	Cl	NO_2	6.18	0.13	40.2	6.50
11	CH_3	H	4.83	0.14	>100	>20.70
12	CH_3	Cl	5.22	0.16	74.2	14.21
13	CH_3	CH_3	4.19	0.12	>100	23.87
14	CH_3	OCH_3	4.76	0.14	>100	21.01
15	CH_3	NO_2	5.08	0.10	85.6	16.85
MNZ			1.80	0.12	>100	>55.55

[a] The value obtained in at least three separate assay done in triplicate, S.D[a]. (±) Standard deviation. MNZ stands for Metronidazole.

Table 2
In vitro antiamoebic activity of compounds (**1a**–**15a**) against HM1: IMSS strain of *Entamoeba histolytica* and toxicity profile. Compounds with bold font (IC_{50} value) are more active than metronidazole.



Compound	R_1	R_2	Antiamoebic activity		Toxicity profile	
			IC_{50} (μM)	S.D[a]. (±)	IC_{50} (μM)	Safety index (SI)
1a	H	H	5.32	0.09	85.3	16.03
2a	H	Cl	3.02	0.12	80.4	26.62
3a	H	CH_3	**1.28**	0.10	>**100**	>78.12
4a	H	OCH_3	**1.16**	0.16	>**100**	>86.20
5a	H	NO_2	3.68	0.10	79.8	21.68
6a	Cl	H	3.15	0.14	90.6	28.76
7a	Cl	Cl	4.65	0.10	80.3	17.26
8a	Cl	CH_3	2.96	0.14	92.6	31.28
9a	Cl	OCH_3	2.63	0.18	>100	>38.02
10a	Cl	NO_2	4.89	0.14	86.5	17.68
11a	CH_3	H	**1.20**	0.18	>**100**	>83.33
12a	CH_3	Cl	2.84	0.16	98.0	34.50
13a	CH_3	CH_3	**0.86**	0.16	>**100**	>**116.27**
14a	CH_3	OCH_3	**1.08**	0.10	>100	>92.59
15a	CH_3	NO_2	2.63	0.13	>100	>38.02
MNZ			1.80	0.12	>100	>55.55

[a] The value obtained in at least three separate assay done in triplicate, S.D[a]. (±) Standard deviation. MNZ stands for Metronidazole.

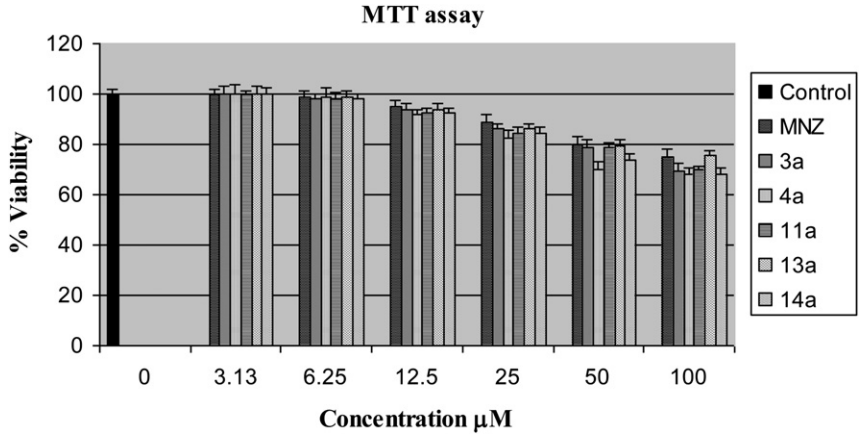


Fig. 2. Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with metronidazole, compounds **3a**, **4a**, **11a**, **13a** and **14a** as evaluated by MTT assay.

a compound an optimum electron density is inevitable so as to gain a significant activity. The compounds **2a**, **5a**, **7a**, **8a**, **9a**, **10a**, **12a** and **15a** having electron withdrawing chloro and nitro group at para position, showed a moderate activity however, it was higher than the compound **1a** containing an unsubstituted phenyl ring (IC_{50} = 5.32 μM).

As discussed above, more optimization of substitution at ortho and meta positions of the phenyl rings by electron releasing/donating or electron withdrawing groups is needed to get detailed insight in structure activity relationship. From the results it can be inferred that the compounds **3a**, **4a**, **11a**, **13a** and **14a** showed encouraging results with IC_{50} value in the range of 0.86–1.28 μM, out of which compound **13a** showed the most promising results with lower IC_{50} (0.86 μM), which is less than half of the reference drug metronidazole (IC_{50} = 1.80 μM). The results were statistically evaluated by analysis of variance. The null hypothesis was tested using *t*-test. The significance of the difference between the IC_{50} values of metronidazole and the compound **13a** was evaluated by *t*-test. The values of the calculated *T* were found higher than the Table value of *T* at 5% level, thus concluding that the character under study is said to be significantly influenced by the treatment.

2.2.2. Cell viability test

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of mitochondrial living cells to produce water insoluble purple formazan crystals [46,47], which after solubilization, can be measured spectrophotometrically. Since the amount of formazan produced is directly proportional to the number of active cells in the culture, MTT has long been used to assess the cell viability in cell proliferation and cytotoxicity tests [48–50].

A series of synthesized compounds were screened for their antiamoebic activity and then evaluated for their effect on growth of Human *hepatocellular carcinoma cell line (*HepG2) to ensure their toxic effect. Metronidazole was used as a reference drug. A subconfluent population of *HepG2* cells was treated with increasing (3.13–100 μM) concentration of test compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The viable cells (%) obtained after an exposure to the compounds for 48 h are depicted in Fig. 2. The toxicity of compounds was found to be concentration-dependent. All the compounds including the reference compound metronidazole showed viability in the range of 95–100% at the concentration of 3.13 μM, however the most active antiamoebic compounds showed a viability of 100% at this concentration as depicted in Fig. 2. On increasing the concentration, all the compounds showed a different patterns of cytotoxicity. At a concentration of 25 μM all the compounds showed viability in the range of 72–90%. On increasing the concentration range up to 50 and 100 μM the compounds showed moderate to least cytotoxicity (viability 40–75%). It is also interesting to mention that the highly antiamoebic compounds (**3a**, **4a**, **11a**, **13a** and **14a**) did not show any remarkable cytotoxicity against the *HepG2* cell line. All these compounds showed a viability of ≥68% at a concentration of 100 μM as depicted in Fig. 2. To further investigate the selectivity of the compounds, the "safety index" (SI), defined as the toxicity IC_{50}/protozoal IC_{50}, was calculated. This allows estimating the efficacy of compounds. The results are summarized in Tables 1and 2. From the results of antiamoebic activity and cytotoxicity it can be inferred that all the tested compounds **3a**, **4a**, **11a**, **13a** and **14a** are least cytotoxic and excellent *E. histolytica* growth inhibitors as compared to the reference drug metronidazole. These results also showed that the compound **13a** despite of being highly antiamoebic did not show any marked toxicity on human cell line and has safety index value of >116.28 which is better than metronidazole (>55.55).

3. Conclusion

We attempted to synthesize a novel series of pyrazoline derivatives (**1a**–**15a**) from α, β unsaturated ketones (**1**–**15**). The great potential of the new class of tetrazole embedded pyrazoline compounds as innovative antiamoebic leads (IC_{50} = 0.86–5.32 μM) as compared to their parent chalcone derivatives (IC_{50} = 4.19–12.86 μM) was studied. Through a preliminary SAR campaign, we found that presence of substituents play a dominant role on activity of the compounds, where activity seems to be guided by the electron donating and withdrawing nature of the substituents. Compound **13a** bearing methyl group at para position of the phenyl rings happened to be the most potent of all the reported compounds. The replacement of methyl group with a stronger electron releasing methoxy group resulted in decrease in activity. The results revealed that an optimum electron density is inevitable for a compound to gain a significant activity. Compounds **3a**, **4a**, **11a**, and **14a** also showed better activity (IC_{50} = 1.08–1.28 μM) than the reference compound metronidazole (IC_{50} = 1.80 μM). Cytotoxicity studies on human hepatocellular carcinoma cell line HepG2 also revealed non-toxic nature of these active compounds. The most promising results were observed for compound **13a** (1-(4,5-dihydro-3,5-dip-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone) with potent antiamoebic activity (IC_{50} = 0.86 μM), least cytotoxicity (IC_{50} = >100 μM) and safety index value of >116.28, which is better than metronidazole (>55.55).

4. Experimental protocol

4.1. Chemistry

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and the results are uncorrected. Elemental analyses were performed on HeraeusVario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ±0.4% of the theoretical values. IR spectra were recorded on Perkin–Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs/ATR mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 (300.13) MHz spectrometer using $CDCl_3$/DMSO-d_6 as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica). Visualization was achieved with UV light at 254 nm or I_2 vapor staining.

4.1.1. General method for synthesis of chalcones (**1**–**15**)

To the solution of a ketone (Acetophenone, *p*-chloro acetophenone, and *p*-methyl acetophenone 5 mmol) in 5 mL of methanol on an ice bath, freshly prepared 2 N methanolic NaOH solution (30 mL) was added and stirred for 10 min. To this 5 mmol of appropriate aldehyde was added and the reaction mixture was stirred at room temperature for 10–24 h. The reaction mixture was cooled on an ice bath and neutralized with dilute hydrochloric acid. The precipitate appeared was separated by filtration and washed three times with 50 mL distilled water to give the crude product. The product so obtained was recrystallized from methanol. The purity of the products was checked on TLC (Merck Silica gel 60 F_{254}), using mixture of hexane and ethylacetate as mobile phase (7:3 v/v).

*4.1.1.1. (2E)-1,3-diphenylprop-2-en-1-one (**1**).* Cream; Yield: 85.3% (3.20 g); mp. 48–50 °C; Anal. Calc. For $C_{15}H_{12}O$: C 86.51, H 5.81%; found: C 86.38, H 5.69%; IR ν_{max} (cm^{-1}): 3064 (Ar–H), 2939 (C–H), 1658 (C=O), 1460 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.04–7.50 (m, 10H, Ar–H), 7.45 (d, 1H, J = 12 Hz, H-β), 7.31 (d, 1H, J = 12 Hz, H-α); ^{13}C NMR($CDCl_3$) δ (ppm): 190.59 (C=O), 144.89 (C-β), (136.90, 132.84, 130.59, 128.67, 127.49), 122.09 (C-α); ESI-MS *m/z*: $[M + H]^+ = 209.09$.

*4.1.1.2. (2E)-3-(4-chlorophenyl)-1-phenylprop-2-en-1-one (**2**).* White; Yield 87.4% (3.76 g); mp. 83–85 °C; Anal. Calc. For $C_{15}H_{11}OCl$: C 74.23, H 4.57%; found: C 74.13, H 4.48%; IR ν_{max} (cm^{-1}): 3060 (Ar–H), 2931 (C–H), 1662 (C=O), 1465 (C=C), 779 (C–Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.05 (d, 2H, J = 8.4 Hz, H-2′, H-6′), 7.81 (d, 1H, J = 15.6 Hz, H-β), 7.64–7.50 (m, 7H, Ar–H), 7.43 (d, 1H, J = 15.6 Hz, H-α); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.53 (C=O), 143.47 (C-β), (136.45, 135.40, 134.28, 132.41, 130.52, 128.08), 123.54 (C-α); ESI-MS *m/z*: $[M + H]^+ = 243.05$.

*4.1.1.3. (2E)-3-(4-methylphenyl)-1-phenylprop-2-en-1-one (**3**).* White; Yield 95.4% (3.81 g); mp. 67–69 °C; Anal. Calc. For $C_{16}H_{14}O$: C 86.45, H 6.35%; found: C 86.33, H 6.24%; IR ν_{max} (cm^{-1}): 3058 (Ar–H), 2932 (C–H), 1659 (C=O), 1447 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.05 (d, 2H, J = 7.5 Hz, H-2′, H-6′), 7.85 (d, 1H, J = 15.6 Hz, H-β), 7.63–7.49 (m, 7H, Ar–H), 7.28 (d, 1H, J = 11.7 Hz, H-α), 2.42 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.98 (C=O), 144.54 (C-β), (134.34, 130.12, 129.42, 128.22, phenyl), 123.12 (C-α), 22.48 (methyl); ESI-MS *m/z*: $[M + H]^+ = 223.11$.

*4.1.1.4. (2E)-3-(4-methoxyphenyl)-1-phenylprop-2-en-1-one (**4**).* White; Yield 82.8% (3.52 g); mp. 60–63 °C; Anal. Calc. For $C_{16}H_{14}O_2$: C 80.65, H 5.92%; found: C 80.54, H 5.84%; IR ν_{max} (cm^{-1}): 3054 (Ar–H), 2938 (C–H), 1656 (C=O), 1446 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.04 (d, 2H, J = 8.1 Hz, H-2′, H-6′), 7.84 (d, 1H, J = 15.9 Hz, H-β), 7.64–7.27 (m, 7H, Ar–H), 6.97 (d, 1H, J = 15.9 Hz, H-α), 3.88 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 190.64 (C=O), 161.70 (C-β), (144.75, 138.51, 132.60, 130.27, 128.59, 127.62), 119.78 (C-α), 55.45 (OCH_3); ESI-MS *m/z*: $M + H^+ = 238.09$.

*4.1.1.5. (2E)-3-(4-nitrophenyl)-1-phenylprop-2-en-1-one (**5**).* Orange; Yield 87.7% (3.95 g); mp. 138–140 °C; Anal. Calc. For $C_{15}H_{11}NO_3$: C 71.14, H 4.38, N 5.53%; found: C 71.26, H 4.35, N 5.69%; IR ν_{max} (cm^{-1}): 3055 (Ar–H), 2923 (C–H), 1668 (C=O), 1460 (C=C), 1547, 1365 ($-NO_2$); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.31 (d, 2H, J = 8.7 Hz, H-2′, H-6′), 8.07 (d, 1H, J = 8.4 Hz, H-β), 7.86–7.62 (m, 7H, Ar–H), 7.57 (d, 1H, J = 14.7 Hz, H-α); ^{13}C NMR ($CDCl_3$) δ (ppm): 190.10 (C=O), 145.66 (C-β), (136.28, 134.25, 132.06, 128.82, 126.72), 122.08 (C-α); ESI-MS *m/z*: $[M + H]^+ = 254.08$.

*4.1.1.6. (2E)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one (**6**).* White; Yield 83% (2.80 g); mp. 81–83 °C; Anal. Calc. For $C_{15}H_{11}OCl$: C 74.23, H 4.57%; found: C 74.36, H 4.63%; IR ν_{max} (cm^{-1}): 3060 (Ar–H), 2931 (C–H), 1662 (C=O), 1465 (C=C), 793 (C–Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.98 (d, 2H, J = 8.4 Hz, H-2′, H-6′), 7.89 (d, 1H, J = 15.6 Hz, H-β), 7.66 (d, 1H, J = 15.6 Hz, H-α), 7.51–7.16 (m, 7H, Ar–H); ^{13}C NMR ($CDCl_3$) δ (ppm): 188.89 (C=O), 143.47 (C-β), (136.46, 135.03, 134.31, 130.68, 128.42), 123.18 (C-α); ESI-MS *m/z*: $[M + H]^+ = 243.05$.

*4.1.1.7. (2E)-1,3-bis(4-chlorophenyl)prop-2-en-1-one (**7**).* White; Yield 90.3% (3.45 g); mp. 96–98 °C; Anal. Calc. For $C_{15}H_{10}OCl_2$: C 65.01, H 3.64%; found: C 65.18, H 3.50%; IR ν_{max} (cm^{-1}): 3058 (Ar–H), 2930 (C–H), 1665 (C=O), 1455 (C=C), 778 (C–Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.99 (d, 2H, J = 8.4 Hz, H-2′, H-6′), 7.81 (d, 1H, J = 15.6 Hz, H-β), 7.61 (d, 1H, J = 15.6 Hz, H-α), 7.51–7.49 (m, 2H, H-3′, H-5′), 7.45–7.24 (m, 4H, H-2, H-3, H-5, H-6); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.65 (C=O), 145.47 (C-β), (140.43, 139.24, 136.46, 131.03, 129.68, 128.42), 120.18 (C-α); ESI-MS *m/z*: $[M + H]^+ = 277.01$.

*4.1.1.8. (2E)-1-(4-chlorophenyl)-3-(4-methylphenyl)prop-2-en-1-one (**8**).* White; Yield 86.5% (3.08 g); mp. 158–160 °C; Anal. Calc. For $C_{16}H_{13}OCl$: C 74.85, H 5.10%; found: C 74.79, H 5.18%; IR ν_{max} (cm^{-1}): 3028 (Ar–H), 2916 (C–H), 1657 (C=O), 1448 (C=C), 793 (C–Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.05 (d, 2H, J = 8.4 Hz, H-2′, H-6′), 7.90 (d, 1H, J = 15.6 Hz, H-β), 7.63 (d, 1H, J = 15.6 Hz, H-α), 7.56–7.49 (m, 4H, Ar–H), 7.40–7.29 (m, 2H, Ar–H) 2.47 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.31 (C=O), 145.50 (C-β), (141.39, 139.10, 136.64, 131.96, 129.78, 128.93, 128.58), 120.46 (C-α), 21.60 (methyl); ESI-MS *m/z*: $[M + H]^+ = 257.07$.

*4.1.1.9. (2E)-1-(4-chlorophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**9**).* White; Yield 98.4% (3.70 g); mp. 118–120 °C; Anal. Calc. For $C_{16}H_{13}O_2Cl$: C 70.46, H 4.80%; found: C 70.66, H 4.93%; IR ν_{max} (cm^{-1}): 3064 (Ar–H), 2934 (C–H), 1677 (C=O), 1442 (C=C), 790 (C–Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.99 (d, 2H, J = 12.9 Hz, H-2′, H-6′), 7.84 (d, 1H, J = 15.6 Hz, H-β), 7.64–7.47 (m, 4H, Ar–H), 7.41 (d, 1H, J = 15.6 Hz, H-β), 6.98 (d, 2H, J = 8.7 Hz, H-3, H-5), 3.88 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.22 (C=O), 145.25 (C-β), (138.96, 136.80, 130.37, 129.85, 128.51, 127.42), 119.13 (C-α), 55.46 (OCH_3); ESI-MS *m/z*: $[M + H]^+ = 273.06$.

*4.1.1.10. (2E)-1-(4-chlorophenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**10**).* Orange; Yield 82.5% (3.27 g); mp. 138–140 °C; Anal. Calc. For $C_{15}H_{10}NO_3Cl$: C 62.62, H 3.50, N 4.87%; found: C 62.78, H 3.43, N 4.68%; IR ν_{max} (cm^{-1}): 3064 (Ar–H), 2934 (C–H), 1677 (C=O), 1442

(C=C), 1540, 1362 (−NO_2), 789 (C−Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.32 (d, 2H, J = 12.9 Hz, H-2′, H-6′), 8.02 (d, 1H, J = 15.6 Hz, H-β), 7.82 (d, 1H, J = 15.6 Hz, H-α), 7.64–7.45 (m, 6H, Ar−H); ^{13}C NMR ($CDCl_3$) δ (ppm): 188.37 (C=O), 142.06 (C-β), (135.81, 130.02, 129.20, 129.04, 125.09), 124.29 (C-α); ESI-MS *m/z*: $[M + H]^+ = 288.04$.

*4.1.1.11. (2E)-1-(4-methylphenyl)-3-phenylprop-2-en-1-one (**11**).* White; Yield 82.2% (2.88 g); mp. 58–60 °C; Anal. Calc. For $C_{16}H_{14}O$: C 86.45, H 6.35%; found: C 86.63, H 6.48%; IR ν_{max} (cm^{-1}): 3058 (Ar−H), 2932 (C−H), 1659 (C=O), 1447 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.97 (d, 2H, J = 7.5 Hz, H-2′, H-6′), 7.85 (d, 1H, J = 15.6 Hz, H-β), 7.67–7.53 (m, 4H, Ar−H), 7.44 (d, 1H, J = 15.6 Hz, H-α), 7.34–7.27 (m, 3H, Ar−H), 2.46 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ (ppm): 189.29 (C=O), 144.47 (C-β), (134.70, 130.02, 129.53, 128.54, phenyl), 122.58 (C-α), 22.40 (CH_3); ESI-MS *m/z*: $[M + H]^+ = 223.11$.

*4.1.1.12. (2E)-3-(4-chlorophenyl)-1-(4-methylphenyl)prop-2-en-1-one (**12**).* White; Yield 88.0% (3.60 g); mp. 140–142 °C; Anal. Calc. For $C_{16}H_{13}OCl$: C 74.85, H 5.10%; found: C 74.70, H 5.18%; IR ν_{max} (cm^{-1}): 3028 (Ar−H), 2916 (C−H), 1657 (C=O), 1448 (C=C), 790 (C−Cl); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.96 (d, 2H, J = 8.1 Hz, H-2′, H-6′), 7.79 (d, 1H, J = 15.6 Hz, H-β), 7.60–7.46 (m, 4H, Ar−H), 7.42 (d, 1H, J = 15.6 Hz, H-α), 7.34–7.28 (m, 2H, Ar−H), 2.46 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 184.97 (C=O), 145.44 (C-β), (139.12, 138.15, 131.55, 130.70, 128.75, 124.82), 117.72 (C-α), 16.97 (CH_3); ESI-MS *m/z*: $[M + H]^+ = 257.07$.

*4.1.1.13. (2E)-1,3-bis(4-methylphenyl)prop-2-en-1-one (**13**).* White; Yield 87.0% (3.30 g); mp. 120–123 °C; Anal. Calc. For $C_{17}H_{16}O$: C 86.40, H 6.82%; found: C 86.32, H 6.96%; IR ν_{max} (cm^{-1}): 3032 (Ar−H), 2908 (C−H), 1650 (C=O), 1440 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.97 (d, 2H, J = 8.1 Hz, H-2′, H-6′), 7.84 (d, 1H, J = 15.6 Hz, H-β), 7.58–7.49 (m, 4H, Ar−H), 7.33 (d, 1H, J = 7.8 Hz, H-α), 7.28–7.23 (m, 2H, Ar−H), 2.45 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 190.16 (C=O), 144.53 (C-β), (143.54, 140.98, 135.15, 132.25, 129.71, 128.64, 128.47), 121.07 (C-α), 21.71 (CH_3); ESI-MS *m/z*: $[M + H]^+ = 237.12$.

*4.1.1.14. (2E)-3-(4-methoxyphenyl)-1-(4-methylphenyl)prop-2-en-1-one (**14**).* White; Yield 85.0% (3.50 g); mp. 98–100 °C; Anal. Calc. For $C_{17}H_{16}O_2$: C 80.93, H 6.39%; found: C 80.84, H 6.44%; IR ν_{max} (cm^{-1}): 3025 (Ar−H), 2920 (C−H), 1650 (C=O), 1445 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.96 (d, 2H, J = 8.1 Hz, H-2′, H-6′), 7.83 (d, 1H, J = 15.6 Hz, H-β), 7.64 (d, 2H, J = 8.7 Hz, Ar−H), 7.47 (d, 1H, J = 15.6 Hz, H-α), 7.33–7.28 (m, 2H, Ar−H), 6.97 (d, 2H, Ar−H), 3.88 (s, 3H, OCH_3), 2.45 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 188.64 (C=O), 145.04 (C-β), (138.42, 138.05, 132.65, 130.15, 128.55, 124.02), 120.82 (C-α), 54.07 (OCH_3), 21.87 (CH_3); ESI-MS *m/z*: $[M + H]^+ = 253.12$.

*4.1.1.15. (2E)-1-(4-methylphenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**15**).* Orange; Yield 85.0% (3.50 g); mp. 149–151 °C, Anal. Calc. For $C_{16}H_{13}NO_3$: C 71.90, H 4.90, N 5.24%; found: C 72.10, H 4.82, N 5.13%; IR ν_{max} (cm^{-1}): 3015 (Ar−H), 2906 (C−H), 1651 (C=O), 1445 (C=C), 1548, 1360 (−NO_2); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.30 (d, 2H, J = 8.7 Hz, H-2′, H-6′), 7.98 (d, 1H, J = 8.1 Hz, H-β), 7.85–7.78 (m, 4H, Ar−H), 7.69 (d, 1H, J = 15.6 Hz, H-α), 7.33 (d, 2H, Ar−H), 2.43 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 189.12 (C=O), 148.48 (C-β), (144.43, 141.19, 134.99, 129.55, 128.92, 125.76), 124.22 (C-α), 21.77 (CH_3); ESI-MS *m/z*: $[M + H]^+ = 268.09$.

*4.1.2. Synthesis of 5-(4-methoxyphenyl)-1H-tetrazole (**2**)*

5-(4-methoxyphenyl)-1H-tetrazole was synthesized from 4-methoxybenzonitrile by following a reported procedure [35]. 4-methoxybenzonitrile was obtained from *p*-methoxy benzal dehyde, following a standard protocol [36]. Nitrile (2.66 g, 20 mmol), sodium azide (1.43 g, 22 mmol) and zinc bromide (4.50 g, 20 mmol), were put in 60 mL of water. 5 mL of isopropanol was also added to stop the formation of clumps. The reaction mixture was refluxed for 24 h and monitored by TLC; vigorous stirring is essential. After 24 h HCl (3 N, 30 mL) and ethyl acetate (100 mL) were added, and vigorous stirring was continued until no solid was present and the aqueous layer had a pH of 1. If necessary, additional ethyl acetate was added. The organic layer was isolated and the aqueous layer extracted with 2 × 100 mL of ethyl acetate. The combined organic layers were evaporated, 200 mL of 0.25 N NaOH was added, and the mixture was stirred for 30 min, until the original precipitate was dissolved and a suspension of zinc hydroxide was formed. The suspension was filtered, and the solid washed with 20 mL of 1 N NaOH. To the filtrate was added 40 mL of 3 N HCl with vigorous stirring causing the tetrazole to precipitate. The tetrazole was filtered and washed with 2 × 20 mL of 3 N HCl and dried in a drying oven to furnish the tetrazole as a white powder.

Yield 84%; mp. 180–182 °C; Anal. Calc. For $C_8H_8N_4O$: C 54.54, H 4.58, N 31.80%; found: C 54.74, H 4.34, N 32.09%; IR ν_{max} (cm^{-1}): 3285 (NH), 3010 (C−H, Ar), 1712 (C=O), 1630 (C=N), 1584 (C=C, Ar), 1285 (C−O); ^{1}H NMR (DMSO) δ (ppm): 8.45 (1H, NH, br s), 7.54–6.84 (4H, m), 3.58 (s, 3H, OCH_3); ^{13}C NMR (DMSO) δ (ppm): 159.8 (C=N), 150.5, 129.4, 121.6, 118.5, 54.9; ESI-MS *m/z*: $[M^+ + 1] = 177.03$.

*4.1.3. Synthesis of 2-[5-(4-methoxyphenyl)-1H-tetrazol-1-yl] acetohydrazide (**3**)*

2-[5-(4-methoxyphenyl)-1H-tetrazol-1-yl]acetohydrazide was prepared by a reported method [37].

Off white; Yield 65%; mp. 193–195 °C; Anal. Calc. For $C_{10}H_{12}N_6O_2$: C 48.38, H 4.87, N 33.85%; found: C 48.22, H 4.64, N 33.68%; IR ν_{max} (cm^{-1}): 3285 (NH), 3025 (C−H, Ar), 1720 (C=O), 1630 (C=N), 1594 (C=C, Ar), 1292 (C−O); ^{1}H NMR (DMSO) δ (ppm): 8.85 (1H, NH, s), 7.24–6.72 (4H, m), 4.42 (2H, CH_2, s), 3.58 (s, 3H, OCH_3), 2.76 (2H, s, NH_2); ^{13}C NMR (DMSO) δ (ppm): 162.0 (C=N), 152.8, 128.5, 124.8, 121.6, 118.5, 68.5, 54.9, 48.2; ESI-MS *m/z*: $[M^+ + 1] = 249.10$.

*4.1.4. General method for synthesis of pyrazolines (**1a**–**15a**)*

To a solution of (5 mmol) of appropriate chalcone (**1**–**15**) in 10 mL of methanol and 5% NaOH, (5 mmol, 1.24 g) of *2-[5-(4-methoxyphenyl)-1H-tetrazol-1-yl]acetohydrazide* was added and the reaction mixture refluxed for 10–24 h. Conversion was monitored in every 60 min interval on precoated silica TLC plates (Merck, $60F_{254}$) by using mixture of Hexane and EtOAc (70:30 v/v) as mobile phase. The excess of solvent was removed under reduced pressure and the reaction mixture was cooled on an ice bath. The products precipitated out at low temperature were washed five times with 50 mL distilled water, reconstituted in minimum amount of methanol and dried under reduced pressure. In some cases the products were purified by column chromatography using hexane: ethyl acetate (70:30 v/v) as eluent.

*4.1.4.1. 1-(4,5-dihydro-3,5-diphenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**1a**).* White; Yield 45.0%; mp. 290–292 °C; Anal. Calc. For $C_{25}H_{22}N_6O_2$: C 68.48, H 5.06, N 19.17%; Found: C 68.34, H 5.14, N 19.01%; IR ν_{max} (cm^{-1}): 3035 (Ar−H), 2928 (CH_2), 1683 (C=O), 1592 (C=N), 1446, (C=C), 1238 (C−N); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.55–7.18 (m, 14H, Ar−H), 5.94 (s, 2H, CH_2), 5.58 [dd, 1H, J = 11.5, 4.8 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, J = 18.2, 5.0 Hz, Ha (pyrazoline ring)], 3.42 [dd, 1H, J = 18.0, 11.7 Hz, Hb (pyrazoline ring)], 3.20 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 154.12 (C=N), 152.85 (C-3, pyrazoline ring), (149.83, 141.35, 132.65, 128.63, 127.68, 127.76, 123.33, 120.34, 114.18, phenyl

ring), 55.45 (C-5, pyrazoline ring), 54.56 (OCH_3), 48.51 (CH_2), 42.54 (C-4, pyrazoline ring); ESI-MS *m/z*: $[M + H]^+ = 439.16$.

*4.1.4.2. 1-(5-(4-chlorophenyl)-4,5-dihydro-3-phenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**2a**).* White; Yield 45.8%; mp. 238–240 °C; Anal. Calc. For $C_{25}H_{21}ClN_6O_2$: C 63.49, H 4.48, N 17.77%; found: C 63.58, H 4.34, N 17.81%; IR ν_{max} (cm^{-1}): 3028 (Ar–H), 2930 (CH_2), 1685 (C=O), 1585 (C=N), 1440, (C=C), 1235 (C–N), 790 (C–Cl); ^1H NMR ($CDCl_3$) δ (ppm): 8.04–6.74 (m, 13H, Ar–H), 5.90 (s, 2H, CH_2), 5.67 [dd, 1H, J = 11.8, 5.8 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, J = 18.1, 5.1 Hz, Ha (pyrazoline ring)], 3.38 [dd, 1H, J = 18.0, 11.8 Hz, Hb (pyrazoline ring)], 3.25 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 168.75 (C=O), 155.58 (C=N), 152.80 (C-3, pyrazoline ring), (149.79, 142.90, 133.75, 128.45, 127.25, 127.65, 123.30, 120.46, 114.65, phenyl ring), 56.45 (C-5, pyrazoline ring), 52.10 (OCH_3), 49.55 (CH_2), 42.85 (C-4, pyrazoline ring); ESI-MS *m/z*: $[M + H]^+ = 473.14$.

*4.1.4.3. 1-(4,5-dihydro-3-phenyl-5-p-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl) ethanone (**3a**).* White; Yield 52.5%; mp. 300–302 °C; Anal. Calc. For $C_{26}H_{24}N_6O_2$: C 69.01, H 5.35, N 18.57%; found: C 69.21, H 5.44 N 18.43%; IR ν_{max} (cm^{-1}): 3035 (Ar–H), 2930 (CH_2), 1682 (C=O), 1585 (C=N), 1448, (C=C), 1235 (C–N); ^1H NMR ($CDCl_3$) δ (ppm): 7.96–6.91 (m, 13H, Ar–H), 5.89 (s, 2H, CH_2), 5.69 [dd, 1H, J = 11.0, 5.4 Hz, Hx (pyrazoline ring)], 3.94 [dd, 1H, J = 18.0, 5.1 Hz, Ha (pyrazoline ring)], 3.34 [dd, 1H, J = 11.8, 14.6 Hz, Hb (pyrazoline ring)], 3.24 (s, 3H, OCH_3), 2.82 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 154.88 (C=N), 150.44 (C-3, pyrazoline ring), (148.09, 142.34, 133.05, 128.24, 127.15, 126.45, 123.38, 120.25, 114.25, phenyl ring), 56.35 (C-5, pyrazoline ring), 54.80 (OCH_3), 48.55 (CH_2), 44.65 (C-4, pyrazoline ring), 22.85 (CH_3); ESI-MS *m/z*: $[M + H]^+ = 453.20$.

*4.1.4.4. 1-(4,5-dihydro-5-(4-methoxyphenyl)-3-phenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**4a**).* White; Yield 48.0%; mp. 198–200 °C; Anal. Calc. For $C_{26}H_{24}N_6O_3$: C 66.65, H 5.16, N 17.94%; found: C 66.53, H 5.08, N 17.83%; IR ν_{max} (cm^{-1}): 3025 (Ar–H), 2924 (CH_2), 1685 (C=O), 1580 (C=N), 1442, (C=C), 1230 (C–N); ^1H NMR ($CDCl_3$) δ (ppm): 8.27–7.15 (m, 13H, Ar–H), 5.90 (s, 2H, CH_2), 5.67 [dd, 1H, J = 11.6, 5.0 Hz, Hx (pyrazoline ring)], 3.91 [dd, 1H, J = 18.8, 5.4 Hz, Ha (pyrazoline ring)], 3.36 [dd, 1H, J = 18.0, 11.5 Hz, Hb (pyrazoline ring)], 3.23 (s, 3H, OCH_3), 3.25 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.46 (C=O), 156.22 (C=N), 151.18 (C-3, pyrazoline ring), (148.29, 142.12, 131.25, 128.77, 127.02, 126.33, 123.15, 120.15, 114.98, phenyl ring), 54.38 (C-5, pyrazoline ring), 52.16 (OCH_3), 49.50 (CH_2), 44.45 (C-4, pyrazoline ring); ESI-MS *m/z*: $[M + H]^+ = 469.19$.

*4.1.4.5. 1-(4,5-dihydro-5-(4-nitrophenyl)-3-phenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**5a**).* Yellowish; Yield 54.8%; mp. 298–300 °C; Anal. Calc. For $C_{25}H_{21}N_7O_4$: C 62.11, H 4.38, N 20.28%; found: C 62.32, H 4.26, N 20.37%; IR ν_{max} (cm^{-1}): 3038 (Ar–H), 2921 (CH_2), 1682 (C=O), 1580 (C=N), 1440, (C=C), 1230 (C–N); ^1H NMR ($CDCl_3$) δ (ppm): 8.10–6.83 (m, 13H, Ar–H), 5.95 (s, 2H, CH_2), 5.52 [dd, 1H, J = 11.4, 4.9 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, J = 18.1, 4.8 Hz, Ha (pyrazoline ring)], 3.66 [dd, 1H, J = 18.5, 11.7 Hz, Hb (pyrazoline ring)], 3.19 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.48 (C=O), 154.87 (C=N), 152.88 (C-3, pyrazoline ring), (149.10, 142.76, 133.28, 129.80, 127.42, 126.21, 123.20, 120.10, 114.35, phenyl ring), 56.45 (C-5, pyrazoline ring), 54.65 (OCH_3), 48.45 (CH_2), 43.28 (C-4, pyrazoline ring); ESI-MS *m/z*: $[M + H]^+ = 484.17$.

*4.1.4.6. 1-(3-(4-chlorophenyl)-4,5-dihydro-5-phenylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**6a**).* White; Yield 58.0%; mp. 310–312 °C; Anal. Calc. For $C_{25}H_{21}ClN_6O_2$: C 63.49, H 4.48, N 17.77%; found: C 63.35, H 4.54, N 17.83%; IR ν_{max} (cm^{-1}): 3033 (Ar–H), 2928 (CH_2), 1682 (C=O), 1585 (C=N), 1437, (C=C), 1236 (C–N), 793 (C–Cl); ^1H NMR ($CDCl_3$) δ (ppm): 8.23–7.28 (m, 13H, Ar–H), 5.90 (s, 2H, CH_2), 5.67 [dd, 1H, J = 11.4, 5.0 Hz, Hx (pyrazoline ring)], 3.85 [dd, 1H, J = 18.2, 5.1 Hz, Ha (pyrazoline ring)], 3.36 [dd, 1H, J = 11.8, 14.4 Hz, Hb (pyrazoline ring)], 3.24 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 156.22 (C=N), 151.18 (C-3, pyrazoline ring), (148.29, 142.12, 131.25, 128.77, 127.02, 126.33, 123.15, 120.15, 114.98, phenyl ring), 55.38 (C-5, pyrazoline ring), 54.10 (OCH_3), 48.50 (CH_2), 46.45 (C-4, pyrazoline ring); ESI-MS *m/z*: $[M + H]^+ = 473.14$.

*4.1.4.7. 1-(3,5-bis-(4-chlorophenyl)-4,5-dihydropyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**7a**).* Cream; Yield 61.5%; mp. 199–201 °C; Anal. Calc. For $C_{25}H_{20}Cl_2N_6O_2$: C 59.18, H 3.97, N 16.56%; found: C 59.26, H 3.87, N 16.43%; IR ν_{max} (cm^{-1}): 3025 (Ar–H), 2932 (CH_2), 1680 (C=O), 1582 (C=N), 1435, (C=C), 1230 (C–N), 790 (C–Cl); ^1H NMR ($CDCl_3$) δ (ppm): 7.83–6.85 (m, 12H, Ar–H), 5.95 (s, 2H, CH_2), 5.54 [dd, 1H, J = 11.5, 4.5 Hz, Hx (pyrazoline ring)], 3.87 [dd, 1H, J = 18.0, 4.8 Hz, Ha (pyrazoline ring)], 3.42 [dd, 1H, J = 11.4, 14.6 Hz, Hb (pyrazoline ring)], 3.28 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.35 (C=O), 154.28 (C=N), 152.10 (C-3, pyrazoline ring), (148.25, 142.05, 131.20, 128.64, 127.60, 126.11, 123.10, 120.82, 114.45, phenyl ring), 56.45 (C-5, pyrazoline ring), 54.02 (OCH_3), 49.65 (CH_2), 43.25 (C-4, pyrazoline ring); ESI-MS *m/z*: $[M + H]^+ = 507.10$.

*4.1.4.8. 1-(3-(4-chlorophenyl)-4,5-dihydro-5-p-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**8a**).* White; Yield 55.0%; mp. 208–210 °C; Anal. Calc. For $C_{26}H_{23}ClN_6O_2$: C 64.13, H 4.76, N 17.26%; found: C 64.25, H 4.65, N 17.10%; IR ν_{max} (cm^{-1}): 3032 (Ar–H), 2935 (CH_2), 1682 (C=O), 1588 (C=N), 1445, (C=C), 1230 (C–N), 779 (C–Cl); ^1H NMR ($CDCl_3$) δ (ppm): 8.20–6.91 (m, 12H, Ar–H), 5.99 (s, 2H, CH_2), 5.45 [dd, 1H, J = 11.6, 4.9 Hz, Hx (pyrazoline ring)], 3.94 [dd, 1H, J = 18.0, 5.1 Hz, Ha (pyrazoline ring)], 3.40 [dd, 1H, J = 18.0, 11.6 Hz, Hb (pyrazoline ring)], 3.25 (s, 3H, OCH_3), 2.17 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.45 (C=O), 156.22 (C=N), 151.18 (C-3, pyrazoline ring), (148.29, 142.12, 131.25, 128.77, 127.02, 126.33, 123.15, 120.15, 114.98, phenyl ring), 58.38 (C-5, pyrazoline ring), 52.10 (OCH_3), 48.50 (CH_2), 44.85 (C-4, pyrazoline ring), 22.65 (CH_3); ESI-MS *m/z*: $[M + H]^+ = 487.16$.

*4.1.4.9. 1-(3-(4-chlorophenyl)-4,5-dihydro-5-(4-methoxyphenyl)pyrazol-1-yl)-2-(5-(4-methoxy phenyl)-1H-tetrazol-1-yl)ethanone (**9a**).* Yellowish; Yield 65.0%; mp. 315–317 °C; Anal. Calc. For $C_{26}H_{23}ClN_6O_3$: C 62.09, H 4.61, N 16.71%; found: C 62.31, H 4.47, N 16.78%; IR ν_{max} (cm^{-1}): 3025 (Ar–H), 2932 (CH_2), 1680 (C=O), 1582 (C=N), 1445, (C=C), 1233 (C–N), 778 (C–Cl); ^1H NMR ($CDCl_3$) δ (ppm): 8.05–7.21 (m, 12H, Ar–H), 5.91 (s, 2H, CH_2), 5.34 [dd, 1H, J = 11.5, 4.7 Hz, Hx (pyrazoline ring)], 3.91 [dd, 1H, J = 18.4, 5.4 Hz, Ha (pyrazoline ring)], 3.60 [dd, 1H, J = 18.0, 11.7 Hz, Hb (pyrazoline ring)], 3.23 (s, 3H, OCH_3), 3.19 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 168.85 (C=O), 156.45 (C=N), 152.65 (C-3, pyrazoline ring), (148.25, 143.65, 133.20, 128.54, 127.33, 125.89, 123.45, 120.25, 114.28, phenyl ring), 56.40 (C-5, pyrazoline ring), 50.65 (OCH_3), 46.58 (CH_2), 45.35 (C-4, pyrazoline ring); ESI-MS *m/z*: $[M + H]^+ = 503.15$.

*4.1.4.10. 1-(3-(4-chlorophenyl)-4,5-dihydro-5-(4-nitrophenyl)pyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**10a**).* Orange red; Yield 52.3%; mp. 187–190 °C; Anal. Calc. For $C_{25}H_{20}ClN_7O_4$: C 57.98, H 3.89, N 18.93%; found: C 57.83, H 3.94, N 18.80%; IR ν_{max} (cm^{-1}): 3035 (Ar–H), 2934 (CH_2), 1685 (C=O), 1580 (C=N), 1445, (C=C), 1230 (C–N), 792 (C–Cl); ^1H NMR ($CDCl_3$) δ (ppm): 8.27–7.15 (m, 12H, Ar–H), 5.90 (s, 2H, CH_2), 5.51 [dd, 1H, J = 11.5, 5.0 Hz, Hx

(pyrazoline ring)], 3.86 [dd, 1H, J = 17.8, 5.0 Hz, Ha (pyrazoline ring)], 3.67 [dd, 1H, J = 17.8, 11.7 Hz, Hb (pyrazoline ring)], 3.30 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.20 (C=O), 154.98 (C=N), 151.35 (C-3, pyrazoline ring), (149.55, 144.15, 131.28, 128.34, 127.03, 125.09, 123.65, 120.18, 114.28, phenyl ring), 56.48 (C-5, pyrazoline ring), 55.06 (OCH_3), 48.24 (CH_2), 43.08 (C-4, pyrazoline ring); ESI-MS m/z: $[M + H]^+ = 518.13$.

*4.1.4.11. 1-(4,5-dihydro-5-phenyl-3-p-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl) ethanone (**11a**).* White; Yield 60.0%; mp. 302–305 °C; Anal. Calc. For $C_{26}H_{24}N_6O_2$: C 69.01, H 5.35, N 18.57%; found: C 69.13, H 5.19, N 18.48%; IR ν_{max} (cm^{-1}): 3035 (Ar–H), 2932 (CH_2), 1682 (C=O), 1580 (C=N), 1435, (C=C), 1230 (C–N); ^1H NMR ($CDCl_3$) δ (ppm): 8.04–6.90 (m, 13H, Ar–H), 5.93 (s, 2H, CH_2), 5.52 [dd, 1H, J = 11.2, 4.8 Hz, Hx (pyrazoline ring)], 3.94 [dd, 1H, J = 17.4, 4.7 Hz, Ha (pyrazoline ring)], 3.68 [dd, 1H, J = 17.8, 11.2 Hz, Hb (pyrazoline ring)], 3.25 (s, 3H, OCH_3), 2.45 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.20 (C=O), 154.98 (C=N), 151.35 (C-3, pyrazoline ring), (149.55, 144.15, 131.28, 128.34, 127.03, 125.09, 123.65, 120.18, 114.28, phenyl ring), 58.48 (C-5, pyrazoline ring), 55.45 (OCH_3), 48.24 (CH_2), 43.08 (C-4, pyrazoline ring), 20.45 (CH_3); ESI-MS m/z: $[M + H]^+ = 453.20$.

*4.1.4.12. 1-(5-(4-chlorophenyl)-4,5-dihydro-3-p-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**12a**).* Yellowish; Yield 47.0%; mp. 210–213 °C; Anal. Calc. For $C_{26}H_{23}ClN_6O_2$: C 64.13, H 4.76, N 17.26%; found: C 64.27, H 4.62, N 17.18%; IR ν_{max} (cm^{-1}): 3032 (Ar–H), 2935 (CH_2), 1680 (C=O), 1580 (C=N), 1445, (C=C), 1230 (C–N), 790 (C–Cl); ^1H NMR ($CDCl_3$) δ (ppm): 8.07–6.87 (m, 12H, Ar–H), 5.91 (s, 2H, CH_2), 5.52 [dd, 1H, J = 11.4, 5.0 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, J = 17.8, 4.8 Hz, Ha (pyrazoline ring)], 3.40 [dd, 1H, J = 18.0, 11.8 Hz, Hb (pyrazoline ring)], 3.24 (s, 3H, OCH_3), 2.21 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.28 (C=O), 154.06 (C=N), 152.30 (C-3, pyrazoline ring), (149.25, 144.29, 131.25, 128.15, 127.16, 125.04, 123.45, 120.45, 114.45, phenyl ring), 58.25 (C-5, pyrazoline ring), 55.05 (OCH_3), 48.20 (CH_2), 42.64 (C-4, pyrazoline ring) 22.60 (CH_3); ESI-MS m/z: $[M + H]^+ = 487.16$.

*4.1.4.13. 1-(4,5-dihydro-3,5-dip-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl)ethanone (**13a**).* White; Yield 56.2%; mp. 149–151 °C, Anal. Calc. For $C_{27}H_{26}N_6O_2$: C 69.51, H 5.62, N 18.01%; found: C 69.34, H 5.52, N 18.13%; IR ν_{max} (cm^{-1}): 3025 (Ar–H), 2925 (CH_2), 1682 (C=O), 1585 (C=N), 1445, (C=C), 1238 (C–N); ^1H NMR ($CDCl_3$) δ (ppm): 8.04–7.01 (m, 12H, Ar–H), 5.89 (s, 2H, CH_2), 5.52 [dd, 1H, J = 11.5, 5.0 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, J = 18.1, 5.1 Hz, Ha (pyrazoline ring)], 3.36 [dd, 1H, J = 17.4, 11.6 Hz, Hb (pyrazoline ring)], 3.25 (s, 3H, OCH_3), 2.75 (s, 3H, CH_3), 2.59 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 168.65 (C=O), 153.28 (C=N), 151.30 (C-3, pyrazoline ring), (149.25, 143.18, 131.25, 128.14, 127.63, 124.45, 123.25, 120.48, 114.20, phenyl ring), 56.40 (C-5, pyrazoline ring), 54.85 (OCH_3), 48.25 (CH_2), 42.68 (C-4, pyrazoline ring), 22.40 (CH_3); ESI-MS m/z: $[M + H]^+ = 467.21$.

*4.1.4.14. 1-(4,5-dihydro-5-(4-methoxyphenyl)-3-p-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazole-1-yl)ethanone (**14a**).* Cream; Yield 45.0%; mp. 149–151 °C, Anal. Calc. For $C_{27}H_{26}N_6O_3$: C 67.21, H 5.43, N 17.42%; found: C 67.28, H 5.40, N 17.34%; IR ν_{max} (cm^{-1}): 3032 (Ar–H), 2925 (CH_2), 1682 (C=O), 1580 (C=N), 1435, (C=C), 1232 (C–N); ^1H NMR ($CDCl_3$) δ (ppm): 7.98–6.98 (m, 12H, Ar–H), 5.81 (s, 2H, CH_2), 5.57 [dd, 1H, J = 11.6, 5.0 Hz, Hx (pyrazoline ring)], 3.86 [dd, 1H, J = 18.0, 5.1 Hz, Ha (pyrazoline ring)], 3.42 [dd, 1H, J = 18.0, 11.5 Hz, Hb (pyrazoline ring)], 3.23 (s, 3H, OCH_3), 3.19 (s, 3H, OCH_3), 2.59 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.27 (C=O), 158.48 (C=N), 152.65 (C-3, pyrazoline ring), (149.44, 142.20, 131.20, 128.11, 127.18, 125.04, 123.25, 120.15, 114.25, phenyl ring), 56.40 (C-5, pyrazoline ring), 54.16 (OCH_3), 46.25 (CH_2), 42.15 (C-4, pyrazoline ring), 21.80 (CH_3); ESI-MS m/z: $[M + H]^+ = 483.21$.

*4.1.4.15. 1-(4,5-dihydro-5-(4-nitrophenyl)-3-p-tolylpyrazol-1-yl)-2-(5-(4-methoxyphenyl)-1H-tetrazol-1-yl) ethanone (**15a**).* Orange red; Yield 46.5%; mp. 149–151 °C, Anal. Calc. For $C_{26}H_{23}N_7O_4$: C 62.77, H 4.66, N 19.71%; found: C 62.54, H 4.74, N 19.57%; IR ν_{max} (cm^{-1}): 3035 (Ar–H), 2930 (CH_2), 1680 (C=O), 1582 (C=N), 1441, (C=C), 1230 (C–N); ^1H NMR ($CDCl_3$) δ (ppm): 8.55–7.18 (m, 12H, Ar–H), 5.94 (s, 2H, CH_2), 5.57 [dd, 1H, J = 11.4, 5.0 Hz, Hx (pyrazoline ring)], 3.91 [dd, 1H, J = 17.8, 4.8 Hz, Ha (pyrazoline ring)], 3.34 [dd, 1H, J = 18.0, 11.7 Hz, Hb (pyrazoline ring)], 3.19 (s, 3H, OCH_3), 2.18 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 169.85 (C=O), 156.15 (C=N), 152.45 (C-3, pyrazoline ring), (149.28, 142.26, 131.25, 128.45, 127.25, 125.26, 123.25, 120.55, 114.20, phenyl ring), 58.28 (C-5, pyrazoline ring), 55.50 (OCH_3), 46.20 (CH_2), 42.05 (C-4, pyrazoline ring), 24.11 (CH_3); ESI-MS m/z: $[M + H]^+ = 498.18$.

4.2. In vitro antiamoebic assay

All the test compounds (**1**–**15** and **1a**–**15a**) were screened *in vitro* for antiamoebic activity against *HM1:IMSS* strain of *E. histolytica* by microdilution method [43]. *E. histolytica* trophozoites were cultured in 96-well microtiter plate by using Diamond TYIS-33 growth medium [51]. The test compounds (1 mg) were dissolved in DMSO (40 μL, level at which no inhibition of amoeba occurs) [52,53]. The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg mL^{-1}. 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 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^{-1} was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The suspension was diluted to 10^5 cells mL^{-1} 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 35.5 °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 35.5 °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 1and 2.

4.3. Cytotoxicity studies (MTT assay)

4.3.1. Cell culture

Human hepatocellular carcinoma cell line *(HepG2)* was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (heat inactivated), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B, at 37 °C in a saturated humidity atmosphere containing 95% air/5% CO_2 [54]. The cell lines were harvested when they reached 80% confluence to maintain exponential growth.

4.3.2. MTT assay

The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only [55]. For viability testing, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, M2128 from Sigma) cell proliferation assay was carried out. The cell monolayers in exponential growth were harvested using 0.25% trypsin and single-cell suspensions were obtained by repeated pipetting. Only viable cells were used in the assay. Exponentially growing cells were plated at 1.2×10^4 cells per well into 96-well plates (Costar, Corning, NY, USA) and incubated for 48 h before the addition of drugs to achieve the maximum confluency of the cells. Stock solutions were prepared by dissolving the compounds in 10% (v/v) DMSO and further diluted with fresh complete medium to achieve 1 M concentration. Cells were incubated with different concentrations of metronidazole and test compounds for 48 h at 37 °C in 5% CO_2 humidified incubator together with untreated control sample. At appropriate time points, cells were washed in PBS, treated with 50 μL MTT solution (5 mg mL^{-1}, tetrazolium salt) and incubated for 4 h at 37 °C. At the end of the incubation period, the medium was removed and pure DMSO 150 μL was added to each well. The metabolized MTT product dissolved in DMSO was quantified by measuring the absorbance at 570 nm on a Microplate reader (iMark, BIORAD, S/N 10321) with a reference wavelength of 655 nm. All assays were performed in triplicate and repeated thrice. Percent viability was defined as the relative absorbance of treated versus untreated control cells.

Conflict of interest

The authors declare no conflict of interest.

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