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Short communication

Synthesis, characterization, antiamoebic activity and toxicity of novel bisdioxazole derivatives

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ABSTRACT

Cyclization of benzene-1,4-dicarbaldehyde dioxime **1** with different aromatic aldehydes in inert atmosphere yielded the corresponding new bisdioxazoles **2**–**11**. The structure of **2**–**11** was elucidated by spectral data. *In vitro* antiamoebic activity was performed against HM1:IMSS strain of *Entamoeba histolytica*. The results showed that the compounds **3** ($IC_{50} = 1.22$ μM), **4** ($IC_{50} = 1.41$ μM), **7** ($IC_{50} = 1.05$ μM) and **10** ($IC_{50} = 1.01$ μM) exhibited better antiamoebic activity than the standard drug metronidazole ($IC_{50} = 1.80$ μM). The compounds **3**, **4**, **7** and **10** were tested for toxicity by MTT assay on H9c2 cardiac myoblasts and the results showed that the compounds **3**, **4**, **7** and **10** offered remarkable viability of 96.2%, 83.5%, 82% and 89%, respectively at a concentration of 12.5 μg/ml.

1. Introduction

Amoebiasis is the most aggressive protozoal disease and considered to be the second or third leading cause of death amongst the parasitic diseases [1]. *Entamoeba histolytica*, a protozoan parasite, is the causative agent of amoebiasis and amoebic dysentery. Though ubiquitous in distribution, this parasite is more prevalent in tropical and subtropical regions [2]. It can invade extra intestinal tissues such as liver and brain and result in the formation of abscesses which could be life threatening [3]. Metronidazole is known to be highly effective amoebicide and is considered to be the drug of choice for the treatment of amoebiasis, but this drug has been shown to be mutagenic in a microbiological system and carcinogenic to rodents [4–6]. In addition, this drug has several adverse effects for which the most common are gastrointestinal disturbances, especially nausea, vomiting and diarrhoea or constipation may also occur [7]. Due to its adverse effects and the emergence of drug resistance [8,9], it is desirable to search for discovering and developing newer antiamoebic agents so that the limitation as presented by available therapeutic agents can be minimized and more safer, effective antiamoebic drugs or vaccines can come into existence.

Dioxazoles belong to the azole family which has long been targets of synthetic investigation because of their known biological properties like cognitive-enhancing and anxiolytic-like activity [10–13]. The antifungal agents of azoles are useful drugs and are widely used for the treatment of topic or inner mycoses and AIDS-related mycotic pathologies [14–16]. Imidazoles representing one class of antifungal azole derivatives have shown a broad spectrum of antifungal activities both *in vitro* and *in vivo* [17,18]. Oxazole an important member of the azole family also contains a number of biologically active molecules, which play an important role in the drug chemistry. A number of compounds were screened for antituberculosis agents including dihydrophenazines [19], indoles and ureas [20]. Oxybenzylglycine, possessing an oxazoline group, is currently in clinical development for the treatment of type II diabetes and dyslipidemia [21]. Recently a number of dioxazole derivatives were synthesized and their potential antiamoebic activity has been studied in our laboratory [22].

Considering the facts that nearly all the classes of the azole family are biologically active and as a part of our continuous efforts towards the development of more potent amoebicidal agents, we herein report the synthesis, characterization, antiamoebic activity and toxicity of a new series of bisdioxazoles **2**–**11** to find an efficacy better than metronidazole, a member of azole family and the commercially available drug for amoebiasis.

2. Chemistry

The synthesis of the bisdioxazole derivatives (**2**–**11**) was performed in a manner as outlined in Scheme 1 [22]. The reaction of

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benzene-1,4-dicarbaldehyde dioxime in the presence of sodium hypochlorite and triethylamine in ethyl acetate gave the bisdioxazole derivatives. The solid compounds showed sharp melting points and the elemental analysis was found in accordance with ±0.3%. The compounds were stable in solid state and were soluble in DMSO, methanol and ethanol.

3. Pharmacology

Benzene-1,4-dicarbaldehyde dioxime (**1**) and dioxazole derivatives (**2**–**11**) were screened *in vitro* against HM1:IMSS strain of *E. histolytica* by microdilution method. All the experiments were carried out in triplicate at each concentration level and repeated thrice. Toxicity of active compounds has been studied by MTT assay on H9c2 cardiac myoblasts. The results of biological activity and toxicity are summarized in Table 1 and Fig. 1.

4. Results and discussion

4.1. Synthesis

The bisdioxazoles **2**–**11** were prepared by treating benzene-1,4-dicarbaldehyde dioxime **1** with different aldehydes as shown in Scheme 1. The bisdioxazole derivatives **2**–**11** were recrystallized from appropriate solvents and the yield was in the range of 23–60%. All the compounds were highly soluble in methanol and DMSO. The bisdioxazole derivatives were characterized by electronic, IR, ^{1}H and ^{13}C NMR spectra. The purity of the compounds was confirmed by elemental analysis and data were found in accordance with ±0.3%.

Characteristic IR bands provide significant indications for the formation of the dioxime **1** and bisdioxazoles **2**–**11**. The absence of a band at/or around 2670 cm^{-1} due to aldehydic proton and the appearance of characteristic bands at 3250–3267 cm^{-1}, 1623–1634 cm^{-1} due to ν(NO–H) and ν(C=N) respectively, confirmed the formation of dioxime **1**. The absence of a band at 3250–3267 cm^{-1} in all the bisdioxazoles **2**–**11** confirmed the conversion of dioxime into bisdioxazole. The ν(C=N) band in bisdioxazoles was in the range of 1630–1664 cm^{-1} and a new band in the range of 1125–1193 cm^{-1} arised due to (C–O–C) group, which was splitted in some cases. In addition to ν(C=C) of aromatic region, the respective bands of substituted benzene were also present.

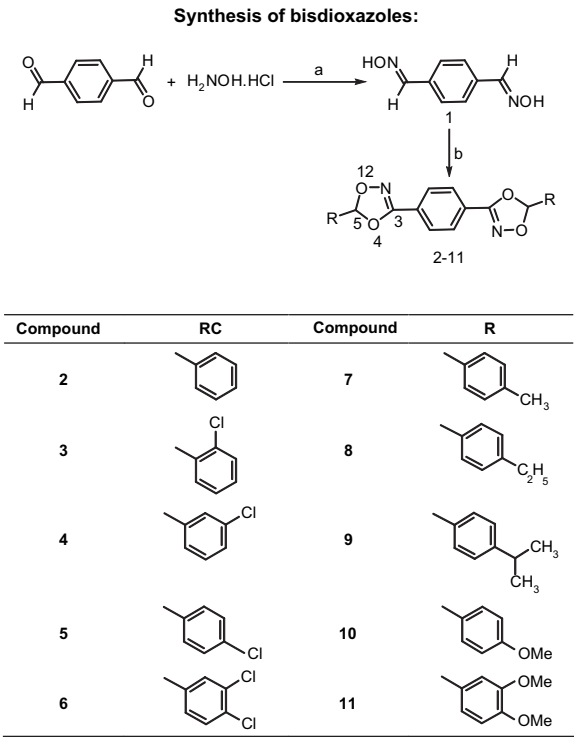
The electronic spectral data of the compounds **2**–**11** studied in the UV region, exhibited three absorption bands at 317–356 nm, 299–318 nm and 250–290 nm assigned to $n \rightarrow \pi^*$, $\pi \rightarrow \pi^*$ and $n \rightarrow \sigma^*$ transitions respectively. The band at 317–356 nm was assigned to the transition involving the azomethine group (C=N). The two other absorption bands at 299–318 nm and at 250–290 nm were due to $\pi \rightarrow \pi^*$ transition of dioxazole ring and $n \rightarrow \sigma^*$ transition of azomethine nitrogen respectively.

The structure of the dioxime **1** was further confirmed by ^{1}H NMR spectra. A singlet at 8.13 ppm due to (CH=N) proton showed the occurrence of condensation between the (CH=O) and (H_2N–OH) groups. The signal at 11.13 ppm due to (N–OH) proton further confirmed the formation of the dioxime. The structure of dioxime **1** was further supported by ^{13}C NMR spectra. The absence of aldehydic carbon signal at 191 ppm and the presence of a signal at 147.58 ppm due to (C=N) confirmed the formation of dioxime. The formation of bisdioxazoles was also supported by the absence of a signal at

Table 1
Dioxime (**1**) and bisdioxazoles (**2**–**11**), their *in vitro* antiamoebic activity against HM1:IMSS strain of *E. histolytica* and toxicity profile of compounds **3**, **4**, **7**, **10** and metronidazole.

Compound	Antiamoebic activity		Toxicity profile		Safety Index
	IC_{50} (μM)	S.D	IC_{50} (μM)	S.D	
1	5.73	0.62	N.D	N.D	N.C
2	3.96	0.13	N.D	N.D	N.C
3	**1.22**	**0.16**	**34**	**0.19**	**27.86**
4	**1.41**	**0.27**	**8**	**0.21**	**5.67**
5	4.21	0.29	N.D	N.D	N.C
6	6.10	0.16	N.D	N.D	N.C
7	**1.05**	**0.18**	**49**	**0.11**	**46.66**
8	2.78	0.25	N.D	N.D	N.C
9	4.65	0.28	N.D	N.D	N.C
10	**1.01**	**0.14**	**>17**	**0.13**	**>16.83**
11	4.38	0.13	N.D	N.D	N.C
Metronidazole	**1.80**	**0.33**	**>116**	**0.33**	**>64.44**

S.D. = Standard deviation, N.D. = Not done, N. C. = Not calculated.



Scheme 1. Synthesis of bisdioxazoles: a = Pyridine, C_2H_5OH, reflux 24 h, b = aq. NaOCl, Et_3N, EtOAc, different aldehydes. Where R = aryl group of different aldehydes.

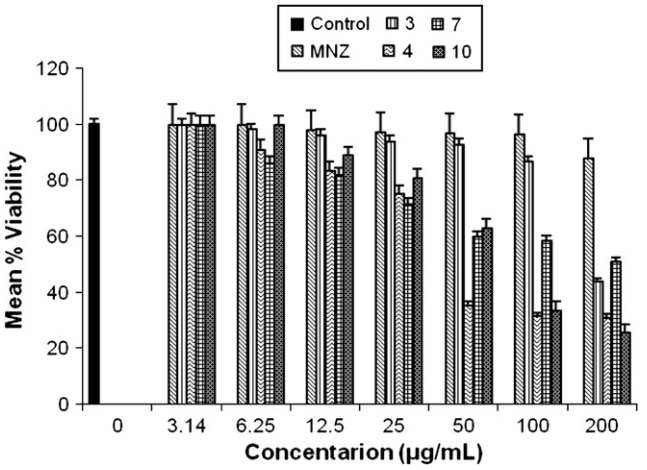


Fig. 1. Percentage of viable cells after 48 h pre-treatment of H9c2 myoblasts with metronidazole (MNZ), compounds **3**, **4**, **7** and **10**, evaluated by MTT assay.

11.13 ppm and 8.13 ppm due to (N–OH) and (N=CH) respectively in all the compounds **2**–**11**. The singlet at 5.96–6.24 ppm, which arised due to (CH) group present at C-5 of the dioxazole ring, confirmed the condensation of dioxime with different aromatic aldehydes. For methyl group, a singlet at 2.21–2.43 ppm appeared in compound **7** while for ethyl group, a quartet at 1.87–2.66 ppm for (CH_2) protons and a triplet at 1.11–1.19 ppm for (CH_3) protons appeared for compound **8**. For methine proton, a multiplet at 3.01–3.11 ppm appeared for compound **9**. In addition, the signals for aromatic region appeared in the range of 6.67–7.99 ppm for all the compounds. The structures of the compounds **2**–**11** were further supported by ^{13}C NMR spectra. The (C=N) signal in the range 158.1–164.6 ppm and the characteristic signal for (–OCO–) found in the range of 88.4–98.7 ppm clearly favored the formation of dioxazole rings. The signals due to the aromatic and aliphatic carbons resonate at their usual positions and the values are given in Experimental section.

4.2. Antiamoebic activities

Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of all the compounds **1**–**11** by microdilution method using HM1:IMSS strain of *E. histolytica*. The results are summarized in Table 1. The data are present in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic effect was compared with the most widely used antiamoebic medication metronidazole which had a 50% inhibitory concentration (IC_{50}) of 1.80 μM in our experiments. The results showed that the compounds **3** ($IC_{50} = 1.22$ μM) and **4** ($IC_{50} = 1.41$ μM) having chloro group at *ortho* and *meta* positions respectively, compound **7** ($IC_{50} = 1.05$ μM) having methyl group at *para* position and compound **10** ($IC_{50} = 1.01$ μM) having methoxy group at *para* position exhibited better antiamoebic activity than the standard drug metronidazole. Therefore out of eleven compounds screened *in vitro* for antiamoebic activity, four compounds **3**, **4**, **7** and **10** were more active than the standard drug metronidazole.

4.3. Toxicity profile

To ensure the toxicity of the compounds **3**, **4**, **7** and **10** with better IC_{50} values than metronidazole, they were tested against H9c2 cardiac myoblasts. A subconfluent population of H9c2 cells was treated with increasing concentrations of compounds **3**, **4**, **7** and **10** and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of compounds **3**, **4**, **7** and **10** was 3.125–200 μg/ml. Fig. 1 depicted that metronidazole, compounds **3**, **4**, **7** and **10** exhibited >80% viability at the concentration of 12.5 μg/ml. The toxicity IC_{50} values along with the safety index values of compounds **3**, **4**, **7** and **10** are given in Table 1.

5. Conclusion

This research involves the synthesis of benzene-1,4-dicarbaldehyde dioxime, which was cyclized to give novel bisdioxazoles **2**–**11**. The antiamoebic activity was examined using HM1:IMSS strain of *E. histolytica* and results showed that the compounds **3** and **4** having chloro group at *ortho* and *meta* positions respectively, compound **7** having methyl group at *para* position and compound **10** having methoxy group at *para* position exhibited better antiamoebic activity than the standard drug metronidazole. The MTT assay results showed that the compounds **3**, **4**, **7** and **10** offered remarkable viability (>80% at 12.5 μg/ml).

6. Experimental protocol

All the chemicals were purchased from Aldrich Chemical Company (USA). Precoated aluminium sheets (silica gel 60 F_{254}, Merck Germany) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. Elemental analyses were performed on Heraeus Vario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ±0.3% of the theoretical values. Electronic spectra were recorded on a Shimadzu UV 1601 PC UV–vis spectrophotometer. IR spectra were recorded on Perkin–Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 400 spectrometer using DMSO-d_6 as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer.

6.1. Preparation of benzene-1,4-dicarbaldehyde dioxime (*1*)

A solution of terephthalaldehyde (0.12 mol) and hydroxylamine hydrochloride (0.26 mol) in a solution of ethanol and pyridine (2:1) was refluxed with stirring for 24 h. After cooling, the mixture was concentrated and then poured on 600 ml of ice cold water. The solid mass was collected, washed with water, crystallized from methanol and gave the corresponding dioxime.

Yield 86%; cream coloured crystals; m.p.: 190–192 °C; Anal. calc. For $C_8H_8N_2O_2$: C 58.53, H 4.87, N 17.07%; found: C 58.51, H 4.89, N 17.06%; IR ν_{max} (cm^{-1}): 3263 (NO–H), 3033 (Ar–H), 2864 (C–H), 1624 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.13 (s, 1H, N–OH), 8.31 (s, 1H, CH=N), 7.40–7.98 (m, 4H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 147.58 (C=N), 133.65 (2 × Ar–C), 126.48 (4 × Ar–C); ESI-MS *m/z*: [$M^+ + 1$] 165.

6.2. General procedure for the synthesis of bisdioxazoles (*2*–*11*)

A 13% aqueous solution of NaOCl (3.2 equiv) was added to a solution of aldehydes (2 equiv) and triethylamine (0.2 equiv) in ethyl acetate under argon atmosphere. The dioxime (1 equiv) in ethyl acetate was added dropwise (over a period of 1 h) at 0 °C to the above solution and stirred at room temperature for 24 h and refluxed for additional 24 h, water was added to the reaction mixture and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with water, brine, dried over $MgSO_4$, filtered and concentrated under *vacuo*. The compounds were crystallized using ethyl acetate–hexane (85:15) solution [22].

6.2.1. 5-Phenyl-3-(4-(5-phenyl-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (*2*)

Yield 60%; Oil; Anal. calc. For $C_{22}H_{16}N_2O_4$: C 71.73, H 4.34, N 6.52%; found: C 71.70, H 4.33, N 6.54%; UV/vis λ_{max} (nm): 319, 299, 261; IR ν_{max} (cm^{-1}): 3030 (Ar–H), 2840 (C–H), 1632 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.86 (s, 4H, Ar–H), 7.13–7.19 (m, 10H, Ar–H), 6.12 (s, 2H, C–H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ (ppm): 162.4 (C=N), 142.2, 136.2, 132.3, 131.1, 129.3, 128.1 (Ar–C), 94.7(O–C–O); ESI-MS *m/z*: [$M^+ + 1$] 369.

6.2.2. 5-(2-Chlorophenyl)-3-(4-(5-(2-chlorophenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (*3*)

Yield 30%; Oil; Anal. calc. For $C_{22}H_{14}N_2O_4Cl_2$: C 60.41, H 3.20, N 6.40%; found: C 60.39, H 3.17, N 6.43%; UV/vis λ_{max} (nm): 333, 308, 251; IR ν_{max} (cm^{-1}): 3041 (Ar–H), 2853 (C–H), 1624 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.85 (s, 4H, Ar–H), 7.01–7.23 (m, 8H, Ar–H), 5.89 (s, 2H, C–H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ (ppm): 158.9

(C=N), 142.6, 135.4, 134.9, 132.2, 131.9, 130.8, 129.2, 127.6 (Ar–C), 96.3 (O–C–O); ESI-MS *m/z*: [$M^+ + 1$] 438.

*6.2.3. 5-(3-Chlorophenyl)-3-(4-(5-(3-chlorophenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (**4**)*

Yield 27%; Oil; Anal. calc. for $C_{22}H_{14}N_2O_4Cl_2$: C 60.41, H 3.20, N 6.40%; found: C 60.42, H 3.23, N 6.39%; UV/vis λ_{max} (nm): 340, 301, 255; IR ν_{max} (cm^{-1}): 3054 (Ar–H), 2880 (C–H), 1660 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.89 (s, 4H, Ar–H), 7.03–7.34 (m, 8H, Ar–H), 5.99 (s, 2H, C–H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ (ppm): 162.4 (C=N), 138.3, 136.2, 132.3, 129.5, 128.2, 127.2 (Ar–C), 96.3 (O–C–O); ESI-MS *m/z*: [$M^+ + 1$] 438.

*6.2.4. 5-(4-Chlorophenyl)-3-(4-(5-(4-chlorophenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (**5**)*

Yield 42%; m.p.: 150–152 °C; Anal. calc. for $C_{22}H_{14}N_2O_4Cl_2$: C 60.41, H 3.20, N 6.40%; found: C 60.42, H 3.19, N 6.43%; UV/vis λ_{max} (nm): 347, 305, 265; IR ν_{max} (cm^{-1}): 3026 (Ar–H), 2867 (C–H), 1664 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.97 (s, 4H, Ar–H), 7.19 (d, 4H, $J = 7.6$ Hz, Ar–H), 7.14 (d, 4H, $J = 7.6$ Hz, Ar–H), 6.24 (s, 2H, C–H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.8 (C=N), 139.1, 137.5, 134.7, 132.9, 130.7, 128.2 (Ar–C), 96.4 (O–C–O); ESI-MS *m/z*: [$M^+ + 1$] 438.

*6.2.5. 5-(3,4-Dichlorophenyl)-3-(4-(5-(3,4-dichlorophenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (**6**)*

Yield 39%; m.p.: 131–133 °C; Anal. calc. for $C_{22}H_{12}N_2O_4Cl_4$: C 52.17, H 2.37, N 5.53%; found: C 52.19, H 2.35, N 5.50%; UV/vis λ_{max} (nm): 330, 317, 291; IR ν_{max} (cm^{-1}): 3032 (Ar–H), 2873 (C–H), 1666 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.88 (s, 4H, Ar–H), 7.30 (d, 2H, $J = 7.3$ Hz, Ar–H), 7.16 (d, 2H, $J = 7.3$ Hz, Ar–H), 7.13 (s, 2H, Ar–H), 6.17 (s, 2H, C–H (dioxazole ring)); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.95 (C=N), 140.7, 136.9, 135.9, 133.5, 132.1, 131.1, 130.6, 128.4 (Ar–C), 98.2 (O–C–O); ESI-MS *m/z*: [$M^+ + 1$] 507.

*6.2.6. 5-p-Tolyl-3-(4-(5-p-tolyl-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (**7**)*

Yield 35%; m.p.: 115–117 °C; Anal. calc. for $C_{24}H_{20}N_2O_4$: C 70.00, H 5.00, N 7.00%; found: C 70.03, H 5.01, N 7.03%; UV/vis λ_{max} (nm): 329, 309, 254; IR ν_{max} (cm^{-1}): 3018 (Ar–H), 2930 (CH_3), 2858 (C–H), 1648 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.94 (s, 4H, Ar–H), 7.10 (d, 4H, $J = 7.4$ Hz, Ar–H), 6.99 (d, 4H, $J = 7.4$ Hz, Ar–H), 6.19 (s, 2H, C–H (dioxazole ring)), 2.33 (s, 6H, $2 \times CH_3$); ^{13}C NMR (DMSO-d_6) δ (ppm): 159.4 (C=N), 143.2, 142.9, 134.8, 131.4, 129.3, 128.1 (Ar–C), 96.7 (O–C–O), 24.9 (CH_3); ESI-MS *m/z*: [$M^+ + 1$] 401.

*6.2.7. 5-(4-Ethylphenyl)-3-(4-(5-(4-ethylphenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (**8**)*

Yield 35%; Oil; Anal. calc. for $C_{26}H_{24}N_2O_4$: C 72.28, H 5.60, N 6.54%; found: C 72.30, H 5.58, N 6.55%; UV/vis λ_{max} (nm): 347, 312, 250; IR ν_{max} (cm^{-1}): 3040 (Ar–H), 2913 (CH_3), 2893 (CH_2), 2860 (C–H), 1655 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.92 (s, 4H, Ar–H), 7.76 (d, 4H, $J = 7.6$ Hz, Ar–H), 7.34 (d, 4H, $J = 7.6$ Hz, Ar–H), 6.12 (s, 2H, C–H (dioxazole ring)), 2.60 (q, 4H, $2 \times CH_2$), 1.17 (t, 6H, $2 \times CH_3$); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.5 (C=N), 148.6, 142.3, 134.2, 131.6, 129.2, 128.4 (Ar–C), 94.3 (O–C–O), 28.13 (CH_2), 14.48 (CH_3); ESI-MS *m/z*: [$M^+ + 1$] 429.

*6.2.8. 5-(4-Isopropylphenyl)-3-(4-(5-(4-isopropylphenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (**9**)*

Yield 23%; Oil; Anal. calc. for $C_{28}H_{28}N_2O_4$: C 73.66, H 6.18, N 6.14%; found: C 73.31, H 5.90, N 6.36%; UV/vis λ_{max} (nm): 337, 308, 269; IR ν_{max} (cm^{-1}): 3046 (Ar–H), 2917 (CH_3), 2881 (C–H), 1661 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.94 (s, 4H, Ar–H), 7.63 (d, 4H, $J = 6.2$ Hz, Ar–H), 7.12 (d, 4H, $J = 6.2$ Hz, Ar–H), 5.96 (s, 2H, C–H (dioxazole ring)), 3.09 (m, 2H, $2 \times$ C–H), 1.02 (d, 12H, $4 \times CH_3$); ^{13}C NMR (DMSO-d_6) δ (ppm): 163.8 (C=N), 148.9, 138.4, 134.6, 132.3, 129.9, 126.7 (Ar–C), 92.6 (O–C–O), 34.4 (C–H), 21.7 (CH_3); ESI-MS *m/z*: [$M^+ + 1$] 457.

*6.2.9. 5-(4-Methoxyphenyl)-3-(4-(5-(4-methoxyphenyl)-1,4,2-dioxazol-3-yl)phenyl)-1,4,2-dioxazole (**10**)*

Yield 33%; Oil; Anal. calc. for $C_{24}H_{20}N_2O_6$: C 66.66, H 4.62, N 6.48%; found: C 66.65, H 4.64, N 6.50%; UV/vis λ_{max} (nm): 340, 302, 277; IR ν_{max} (cm^{-1}): 3036 (Ar–H), 2929 (CH_3), 2880 (C–H), 1639 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.97 (s, 4H, Ar–H), 7.64 (d, 4H, $J = 7.1$ Hz, Ar–H), 6.88 (d, 4H, $J = 7.1$ Hz, Ar–H), 5.98 (s, 2H, C–H (dioxazole ring)), 3.79 (s, 6H, $2 \times OCH_3$); ^{13}C NMR (DMSO-d_6) δ (ppm): 162.8 (C=N), 158.01, 133.6, 132.1, 131.4, 129.1, 128.2 (Ar–C), 95.8 (O–C–O), 55.25 (OCH_3); ESI-MS *m/z*: [$M^+ + 1$] 433.

*6.2.10. 5-(3,4-Dimethoxyphenyl)-3-(4-(5-(3,4-dimethoxyphenyl)-1,4,2-dioxazol-3-yl) phenyl)-1,4,2-dioxazole (**11**)*

Yield 26%; Oil; Anal. calc. for $C_{26}H_{24}N_2O_8$: C 63.41, H 4.91, N 5.69%; found: C 63.43, H 4.85, N 5.70%; UV/vis λ_{max} (nm): 322, 306, 287; IR ν_{max} (cm^{-1}): 3049 (Ar–H), 2960 (CH_3), 2857 (C–H), 1663 (C=N); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.95 (s, 4H, Ar–H), 7.47 (d, 2H, $J = 6.4$ Hz, Ar–H), 7.25 (d, 2H, $J = 6.4$ Hz, Ar–H), 6.88 (s, 2H, Ar–H), 6.23 (s, 2H, C–H (dioxazole ring)), 3.75–3.76 (s, 12H, $4 \times OCH_3$); ^{13}C NMR (DMSO-d_6) δ (ppm): 158.8 (C=N), 154.6, 149.6, 134.8, 133.0, 129.4, 112.3, 111.5, 109.4 (Ar–C) 95.9 (O–C–O), 55.83 (OCH_3), 55.50 (OCH_3); ESI-MS *m/z*: [$M^+ + 1$] 493.

6.3. In vitro antiamoebic assay

All the compounds **1**–**11** were screened *in vitro* for antiamoebic activity against HM1:IMSS strain of *E. histolytica* by microdilution method [23]. *E. histolytica* trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [24]. The test compounds (1 mg) were dissolved in DMSO (40 μl), level at which no inhibition of amoeba occurs [25,26]. The maximum percentage of DMSO used in serial dilution of compounds is 0.016%. The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg/ml. Twofold serial dilutions were made in the wells of 96-well microtiter plate (costar). Each test includes metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoebae) and a blank (culture medium only). All the experiments were carried out in triplicate at each concentration level and repeated thrice. The amoeba suspension was prepared from a confluent culture by pouring off the medium at 37 °C and adding 5 ml of fresh medium, chilling the culture tube on ice to detach the organisms from the side of the flask. The number of amoeba/ml was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The suspension was diluted to 10^5 organisms/ml by adding fresh medium and 170 μl of this suspension was added to the test and control wells in the plate so that the wells were completely filled (total volume, 340 μl). An inoculum of 1.7×10^4 organisms/well was chosen so that confluent, but not excessive growth, took place in control wells. Plates were sealed and gassed for 10 min with nitrogen before incubation at 37 °C for 72 h. After incubation, the growth of amoeba in the plate was checked with a low power microscope. The culture medium was removed by inverting the plate and shaking gently. Plate was then immediately washed with sodium chloride solution (0.9%) at 37 °C. This procedure was completed quickly and the plate was not allowed to cool in order to prevent the detachment of amoebae. The plate was allowed to dry at room temperature and the

amoebae were fixed with methanol and when dried, stained with (0.5%) aqueous eosin for 15 min. The stained plate was washed once with tap water, then twice with distilled water and allowed to dry. A 200 μl portion of 0.1 N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader. The % inhibition of amoebal growth was calculated from the optical densities of the control and test wells and plotted against the logarithm of the dose of the drug tested. Linear regression analysis was used to determine the best fitting line from which the IC_{50} value was found. The IC_{50} values in μM are reported in Table 1.

6.4. MTT assay

H9c2 rat cardiac myoblasts were cultured and maintained as monolayer in Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with 10% fetal bovine serum (heat inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, at 37 °C in humidified incubator with 5% CO_2 [27].

Cells were incubated with different concentrations of metronidazole, compounds **3**, **4**, **7** and **10** 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 600 μl MTT solution (5 mg/ml, tetrazolium salt) and incubated for 45 min at 37 °C. After 45 min of incubation at 37 °C, the cell supernatants were discarded, MTT crystals were dissolved with acid isopropanol and the absorbance measured at 570 nm. All assays were performed in triplicate. Percent viability was defined as the relative absorbance of treated versus untreated control cells. Plates were analyzed in an ELISA plate reader (Labsystems Multiskan RC, Helsinki, Finland) at 570 nm with a reference wavelength of 655 nm.

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