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Synthesis and antiamoebic activity of 3,7-dimethyl-pyrazolo[3,4-e][1,2,4] triazin-4-yl thiosemicarbazide derivatives

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Abstract

A series of 3,7-dimethyl-pyrazolo[3,4-e][1,2,4]triazin-4-yl thiosemicarbazide derivatives **3**–**22** were prepared and evaluated in vitro against *HM1:1MSS* strain of *Entamoeba histolytica*, to identify the compounds for antiamoebic activity. They exhibited antiamoebic activity in the range (IC_{50} = 0.81–7.31 μM). The results were compared to the activity of known drug metronidazole. It is inferred from the in vitro studies that the compounds **10**, **11**, **17** and **18** were found to be significantly better inhibitors of *E. histolytica* since IC_{50} values in the μM range elicited by these compounds are much lower than metronidazole. Besides, compounds **11** and **17** have shown the most promising antiamoebic activity (IC_{50} = 0.81 μM of **11**, IC_{50} = 0.84 μM of **17** versus IC_{50} = 1.81 μM of metronidazole). The study suggests the possibility of developing triazine analogues as potential drug candidates for antiamoebic activity.

Keywords: 1,2,4-Triazine; Thiosemicarbazide; Cyclization; Antiamoebic activity

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 (Ghosh et al., 2004). *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. It can invade extra intestinal tissues such as liver and brain and result in the formation of abscesses which could be life threatening (Espinosa-Cantellano and Martinez-Palomo, 2000). 1,2,4-Triazines are a well known class of heterocyclic compounds (Neunhoffer, 1996) and posses many biological activities (Jezierska et al., 2004; Mansour et al., 2003; Haning et al., 2002; Makhlouf and Maklad, 2004; Mamolo et al., 2000) especially with condensed heterocyclic systems. Polycyclic compounds such as pyrimidine nucleic bases, antibiotics and herbicides, etc. containing triazines moiety show interesting properties (Seela et al., 2003; Yamazaki et al., 2004; Pozharskii et al., 1997; Sihver et al., 2004). Azacytidine, a synthetic triazine analogue of the natural pyrimidine nucleoside cytidine has strong antileukemic activity. When used in cancer chemotherapy, it phosphorylated in the cell, and after its incorporation into the DNA, it inhibits the methyl transferase, causing a block in the cytosine methylation in newly replicated DNA (Martin et al., 1996; Smith et al., 2000). PS-15 a prodrug of diaminotriazines maintain activity against malarial strains resistant to widely used antimalarials (Jensen et al., 2001). Triazine derivatives are target cytotoxic agents to the parasite since triazines offer excellent selectivity between host cells and parasites (Barrett and Fairlamb, 1999; De Koning and Jarvis, 1999). Triazine derivatives are also excellent drugs against trypanosomiasis (Klenke et al., 2001). In our preceding papers the in vitro antiamoebic activity of different series of compounds against *E. histolytica* were reported (Bharti et al., 2002; Shailendra et al., 2002; Singh et al., 2004; Bharti et al., 2004). The compounds found with less IC_{50} value than metronidazole, their in vivo and cytotoxicity studies are in progress. We report here the in vitro antiamoebic activity

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of newly synthesized triazine derivatives against *HM1:1MSS* strain of *E. histolytica*.

2. Experimental

Reactions were monitored by TLC using precoated aluminium Merck silica-gel 60F-254 thin layer plates. All the chemicals were purchased from Aldrich chemical company (USA). Elemental analysis (C, H, N) were performed by the Central Drug Research Institute, Lucknow, India. The results were within 0.4% of calculated values. Chlorine was estimated by standard method (Roberts et al., 1981). Melting points were recorded on a KSW melting point apparatus and are uncorrected. IR spectra were run as potassium bromide pellets on Perkin-Elmer model 1620 FT-IR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were obtained at ambient temperature using Bruker Spectrospin DPX-300 MHz spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard and chemical shifts (δ) are expressed in ppm. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

2.1. General procedure for the synthesis of triazine derivatives

2.1.1. Synthesis of 3,7-dimethylpyrazolo [4,3-e]oxadiazine (1)

The 3,7-dimethylpyrazolo[4,3-e]oxadiazine was synthesized by the method described previously (Koraiem, 1989).

2.1.1.1. Preparation of 3-methyl pyrazol-5-one. The solution of hydrazine hydrate (50 mmol) in absolute ethanol (50 ml) was added dropwise to the ethyl acetoacetate (50 mmol) with continuous stirring. The temperature of the reaction mixture was maintained below 60 °C. The reaction mixture was further stirred for about 1 h in an ice bath to complete the crystallization. The white coloured compound obtained was filtered, washed with cold ethanol and dried in vacuo. Yield: 80%, mp: 218 °C.

2.1.1.2. Preparation of 4,4-dibromo-3-methylpyrazol-5-one. To a solution of 3-methyl pyrazol-5-one (40 mmol) in acetic acid (20 ml), bromine (10 ml) in cold glacial acetic acid (25 ml) was added drop wise by keeping the reaction mixture in cold water and then refluxed at 60 °C for 4 h. It was worked up by pouring into ice-cold water. A light orange residue was separated out and it was washed with cold water and dried in vacuo. Yield: 60%; mp: 127 °C.

2.1.1.3. Preparation of 3,7-dimethylpyrazolo[4,3-e]oxadiazine (1). 4,4-Dibromo-3-methylpyrazol-5-one (25 mmol) was dissolved in acetic anhydride (25 mmol) and hydrazine hydrate (25 mmol) was added dropwise. The reaction mixture was refluxed at 80 °C for 6 h. A light yellow compound **1** was obtained. It was filtered, washed with water and dried in vacuo. Yield: 60%; mp: 150 °C, Anal. calc. For $C_6H_6N_4O$: C 48.00, H 4.00, N 37.33: found: C 47.39, H 3.77, N 37.45%; IR: ν_{max} (cm^{-1}) 1589 (C=N), 965 (N–N), 1165, 1085 (C–O–C); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.69 (3H, s, C*H*$_3$, pyrazole), 3.01 (3H, s, C*H*$_3$, oxadiazine); ^{13}C NMR ($CDCl_3$): (δ, ppm) 164.4 (C=N), 162.01 (C=N), 161.4 (C=N), 159.2 (C=N), 18.9 (CH_3), 16.2 (CH_3).

2.1.2. Synthesis of substituted thiosemicarbazide (2)

Substituted thiosemicarbazides (**2**) were prepared using a two-step synthetic route reported earlier (Sullivan et al., 1963).

2.1.2.1. Preparation of substituted thioglycolic acid. Carbon disulphide (50 mmol) was added drop wise to a solution of primary or secondary amine (50 mmol) containing potassium hydroxide (50 mmol) in water: ethanol (1:3) mixture. The temperature of the reaction was maintained below 10 °C. Sodium chloroacetate (50 mmol) was added and the reaction mixture was left overnight at room temperature. Addition of conc. hydrochloric acid (to pH ∼ 1) precipitated substituted thioglycolic acid, which was crystallized in the suitable solvent. Generally, aliphatic amines gave oil, which were extracted by one of the solvent (hexane, benzene, dichloromethane and chloroform).

2.1.2.2. Conversion of thioglycolic acid into thiosemicarbazide (2). A solution of thioglycolic acid (40 mmol) in water (15 ml) containing sodium hydroxide (40 mmol) and hydrazine hydrate (40 mmol) was refluxed for 2 h with continuous stirring. The compound separated out during the reaction or on cooling at 0 °C for 12 h. The product **2** was filtered and crystallized from suitable solvent.

2.1.3. Synthesis of triazine derivatives

The solution of 3,7-dimethylpyrazolo[4,3-e]oxadiazine **(1)** (3 mmol) in ethanol (10 ml) was mixed with the substituted thiosemicarbazide **(2)** (3 mmol) in water (10 ml) and refluxed at 60 °C for 3 h. The reaction mixture was concentrated to precipitate out the desired compound. After cooling, the solid was filtered and dried in vacuo.

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-isopropylamine thiosemicarbazide (**3**): white solid. Yield: 53%; mp: 171 °C. Anal. calc. for $C_{10}H_{15}N_7S$: C 45.27, H 5.70, N 36.95; found: C 45.34, H 5.64, N 36.87%; IR: ν_{max} (cm^{-1}) 3386 (NH), 1542 (C=N), 1301 (C–N), 1079 (C=S), 967 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.17 (6H, d, –CH(C*H*$_3$)$_2$), 3.04 (3H, s, C*H*$_3$–C=N), 3.09 (3H, s, C*H*$_3$–C=N), 4.17–4.19 (1H, m, N–C*H*), 7.84 (1H, d, –N*H*), 8.31 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 187.8 (C=S), 164.1 (C=N), 163.8 (C=N), 163.3 (C=N), 163.0 (C=N), 49.6 (CH–N), 24.3 (2 CH_3), 15.2 (CH_3), 13.2 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-diethylamine thiosemicarbazide **(4)**: white solid. Yield: 35%; mp: 204 °C. Anal. calc. for $C_{11}H_{17}N_7S$: C 47.31,

H 6.09, N 35.13; found: C 47.39, H 5.97, N 35.35%; IR: ν_{max} (cm^{-1}) 3347 (NH), 1576 (C=N), 1285 (C–N), 1038 (C=S), 942 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 1.94–1.21 (6H, m, –CH_2–CH_3), 3.04 (3H, s, CH_3–C=N), 3.08 (3H, s, CH_3–C=N), 4.17 (4H, s, –CH_2–N–CH_2), 8.94 (1H, s, –NH); ^{13}C NMR ($CDCl_3$): (δ, ppm) 189.4 (C=S), 164.7 (C=N), 163.4 (C=N), 163.1 (C=N), 162.6 (C=N), 56.5 (2 CH_2–N), 15.1 (CH_3), 13.7 (CH_3), 13.1 (2 CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-*n*-butylamine thiosemicarbazide **(5)**: white solid. Yield: 39%; mp: 145 °C. Anal. calc. for $C_{11}H_{17}N_7S$: C 47.29, H 6.13, N 35.10; found: C 47.42, H 6.28, N 35.26%; IR: ν_{max} (cm^{-1}) 3411 (NH), 1559 (C=N), 1289 (C–N), 1062 (C=S), 965 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 1.92–1.99 (3H, m, –CH_2–CH_3), 2.42–2.59 (4H, m, –CH_2), 3.06 (3H, s, CH_3–C=N), 3.11 (3H, s, CH_3–C=N), 3.90–4.01 (2H, m, –NH–CH_2), 8.21 (1H, t, –N*H*), 8.89 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.8 (C=S), 164.0 (C=N), 163.8 (C=N), 163.5 (C=N), 163.0 (C=N), 50.5 (CH_2–N), 35.3 (CH_2), 21.8 (CH_2), 14.8 (CH_3), 13.6 (CH_3), 13.4 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-isobutylamine thiosemicarbazide (**6**): white solid. Yield: 48%; mp: 182 °C. Anal. calc. for $C_{11}H_{17}N_7S$: C 47.29, H 6.13, N 35.10; found: C 47.48, H 6.33, N 35.21%; IR: ν_{max} (cm^{-1}) 3426 (NH), 1596 (C=N), 1307 (C–N), 1035 (C=S), 968 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 2.01–2.15 (6H, m, –CH–CH_3), 2.59–2.69 (1H, m, –C*H*), 3.06 (3H, s, CH_3–C=N), 3.10 (3H, s, CH_3–C=N), 4.15 (2H, d, N–CH_2), 8.35 (1H, d, –N*H*), 9.14 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 187.4 (C=S), 164.1 (C=N), 163.9 (C=N), 163.5 (C=N), 163.1 (C=N), 54.9 (CH–N), 32.6 (CH_2), 22.4 (CH_3), 14.9 (CH_3), 13.3 (CH_3), 10.2 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-*n*-methylbutyl thiosemicarbazide **(7)**: light yellow solid. Yield: 43%; mp: 162 °C. Anal. calc. for $C_{12}H_{19}N_7S$: C 49.13, H 6.53, N 33.42; found: C 49.29, H 6.24, N 33.53%; IR: ν_{max} (cm^{-1}) 3426 (NH), 1595 (C=N), 1309 (C–N), 1038 (C=S), 968 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 1.79 (3H, t, –CH_2–CH_3), 2.39–2.48 (4H, m, –CH_2), 3.04 (3H, s, CH_3–C=N), 3.10 (3H, s, CH_3–C=N), 2.65 (3H, s, N–CH_3), 3.99 (2H, t, N–CH_2), 9.23 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 187.3 (C=S), 163.8 (C=N), 163.6 (C=N), 163.4 (C=N), 163.0 (C=N), 57.6 (CH_2–N), 40.4 (CH_3–N), 32.6 (CH_2), 22.6 (CH_2), 15.6 (CH_3), 13.6 (CH_3), 13.3 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-dipropylamine thiosemicarbazide (**8**): white solid. Yield: 47%; mp: 158 °C. Anal. calc. for $C_{13}H_{21}N_7S$: C 50.79, H 6.89, N 31.89; found: C 51.05, H 6.99, N 31.63%; IR: ν_{max} (cm^{-1}) 3426 (NH), 1541 (C=N), 1303 (C–N), 1077 (C=S), 968 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 1.90–2.01 (6H, m, –CH_2–CH_3), 2.58–2.60 (4H, m, –CH_2), 3.04 (3H, s, CH_3–C=N), 3.07 (3H, s, CH_3–C=N), 4.21 (4H, s, –CH_2–N–CH_2), 9.20 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 187.1 (C=S), 164.3 (C=N), 163.9 (C=N), 163.6 (C=N), 162.9 (C=N), 57.9 (2 CH_2–N), 23.5 (2 CH_2), 14.7 (CH_3), 13.1 (CH_3), 13.0 (2 CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl) cyclopentyl thiosemicarbazide (**9**): light brown solid. Yield: 53%; mp: 132 °C. Anal. calc. for $C_{12}H_{17}N_7S$: C 49.46, H 5.88, N 33.65; found: C 49.39, H 5.94, N 33.85%; IR: ν_{max} (cm^{-1}) 3310 (NH), 1541 (C=N), 1309 (C–N), 1014 (C=S), 968 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 2.82–2.93 (8H, m, –CH_2), 3.01 (3H, s, CH_3–C=N), 3.06 (3H, s, CH_3–C=N), 4.01–4.20 (1H, m, N–C*H*), 7.86 (1H, d, –N*H*), 8.69 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 188.0 (C=S), 164.0 (C=N), 163.8 (C=N), 163.01 (C=N), 162.4 (C=N), 56.4 (CH–N), 32.6 (2 CH_2), 22.4 (2 CH_2), 15.4 (CH_3), 13.1 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl) cyclohexyl thiosemicarbazide (**10**): white solid. Yield: 51%; mp: 117 °C. Anal. calc. for $C_{13}H_{19}N_7S$: C 51.13, H 6.27, N 32.10; found: C 51.05, H 6.24, N 31.96%; IR: ν_{max} (cm^{-1}) 3221 (NH), 1526 (C=N), 1299 (C–N), 1026 (C=S), 971 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 2.29–2.50 (10H, m, –CH_2); 3.03 (3H, s, CH_3–C=N), 3.08 (3H, s, CH_3–C=N), 4.03 (1H, s, N–C*H*), 8.02 (1H, d, –N*H*), 8.91 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 187.3 (C=S), 164.0 (C=N), 163.2 (C=N), 163.0 (C=N), 162.8 (C=N), 54.2 (CH–N), 35.6 (2 CH_2), 29.5 (CH_2), 25.4 (2 CH_2), 15.3 (CH_3), 13.9 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl) cyclooctyl thiosemicarbazide (**11**): white solid. Yield: 39%; mp: 158 °C. Anal. calc. for $C_{15}H_{23}N_7S$: C 54.03, H 6.95, N 29.40; found: C 54.05, H 7.04, N 29.56%; IR: ν_{max} (cm^{-1}) 3256 (NH), 1542 (C=N), 1343 (C–N), 1058 (C=S), 978 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 2.51–2.83 (14H, m, –CH_2), 3.01 (3H, s, CH_3–C=N), 3.04 (3H, s, CH_3–C=N), 4.59–4.61 (1H, m, N–C*H*), 8.13 (1H, d, –N*H*), 9.03 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.8 (C=S), 163.9 (C=N), 163.5 (C=N), 163.2 (C=N), 162.8 (C=N), 55.8 (CH–N), 38.1 (2 CH_2), 29.5 (3 CH_2), 26.4 (2 CH_2), 14.8 (CH_3), 13.4 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl) hexamethyleneimine thiosemicarbazide (**12**): light yellow solid. Yield: 47%; mp: 135 °C. Anal. calc. for $C_{13}H_{19}N_7S$: C 51.13, H 6.27, N 32.10; found: C 51.05, H 6.24, N 32.02%; IR: ν_{max} (cm^{-1}) 3315 (NH), 1540 (C=N), 1298 (C–N), 1054 (C=S), 971 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 2.82–2.97 (8H, m, –CH_2), 3.02 (3H, s, CH_3–C=N), 3.06 (3H, s, CH_3–C=N), 4.54–4.59 (4H, m, –CH_2–N–CH_2–), 7.97 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 187.1 (C=S), 163.7 (C=N), 163.6 (C=N), 163.1 (C=N), 162.8 (C=N), 55.3 (2 CH_2–N), 30.5 (2 CH_2), 27.4 (2 CH_2), 15.2 (CH_3), 13.3 (CH_3).

3-(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-4-methylpiperidine thiosemicarbazide (**13**): light brown solid. Yield: 50%; mp: 148 °C. Anal. calc. for $C_{13}H_{19}N_7S$: C 51.13, H 6.27, N 32.10; found: C 51.15, H 6.39, N 31.97%; IR: ν_{max} (cm^{-1}) 3125 (NH), 1545 (C=N), 1297 (C–N), 1088 (C=S), 966 (N–N); ^1H NMR ($CDCl_3$): (δ, ppm) 1.99 (3H, s, –CH_3), 2.44–2.63 (4H, m, –CH(CH_2)$_2$), 3.01 (3H, s, CH_3–C=N), 3.06 (3H, s, CH_3–C=N), 3.60–3.69 (1H,

m, –C*H*), 4.71–4.75 (4H, m, –CH_2–N–CH_2), 8.94 (1H, s, –NH); ^{13}C NMR ($CDCl_3$): (δ, ppm) 187.1 (C=S), 163.9 (C=N), 163.5 (C=N), 163.3 (C=N), 162.8 (C=N), 52.8 (2 CH_2–N), 33.2 (2 CH_2), 29.4 (CH), 20.5 (CH_3), 14.6 (CH_3), 13.1 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-2-ethylpiperidine thiosemicarbazide (**14**): white solid. Yield: 45%; mp: 143 °C. Anal. calc. for $C_{14}H_{21}N_7S$: C 52.64, H 6.63, N 30.69; found: C 52.55, H 6.52, N 30.76%; IR: ν_{max} (cm^{-1}) 3310 (NH), 1537 (C=N), 1278 (C–N), 1059 (C=S), 973 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 1.73 (3H, t, –CH_2–C*H*$_3$), 2.45–2.53 (10H, m, C*H*$_2$), 3.01 (3H, s, C*H*$_3$–C=N), 3.06 (3H, s, C*H*$_3$–C=N), 4.80–4.89 (1H, m, N–C*H*), 9.21 (1H, s, –NH); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.6 (C=S), 163.8 (C=N), 163.4 (C=N), 163.3 (C=N), 162.4 (C=N), 59.2 (CH–N), 51.3 (CH_2–N), 30.8 (CH_2), 27.5 (CH_2), 26.3 (CH_2), 24.6 (CH_2), 15.1 (CH_3), 13.4 (CH_3), 10.39 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl) pyrrolidine thiosemicarbazide (**15**): light yellow solid. Yield: 41%; mp: 164 °C. Anal. calc. for $C_{11}H_{15}N_7S$: C 47.64, H 5.45, N 35.35; found: C 47.55, H 5.24, N 35.29%; IR: ν_{max} (cm^{-1}) 3426 (NH), 1543 (C=N), 1309 (C–N), 1078 (C=S), 968 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.63–2.65 (4H, m, –C*H*$_2$), 3.07 (3H, s, C*H*$_3$–C=N), 3.11 (3H, s, C*H*$_3$–C=N), 3.98–4.01 (4H, m, –C*H*$_2$–N–C*H*$_2$–), 8.94 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.6 (C=S), 163.8 (C=N), 163.7 (C=N), 163.5 (C=N), 162.9 (C=N), 53.5 (2 CH_2–N), 23.6 (2 CH_2), 14.9 (CH_3), 13.3 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-*n*-methylcyclohexyl thiosemicarbazide (**16**): Yellow solid. Yield: 65%; mp: 170 °C. Anal. calc. for $C_{14}H_{21}N_7S$: C 52.64, H 6.63, N 30.69; found: C 52.73, H 6.65, N 30.76%; IR: ν_{max} (cm^{-1}) 3176 (NH), 1537 (C=N), 1315 (C–N), 1084 (C=S), 995 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.39–2.56 (10H, m, –C*H*$_2$), 3.05 (3H, s, C*H*$_3$–C=N), 3.09 (3H, s, C*H*$_3$–C=N), 2.71 (3H, s, C*H*$_3$–N), 4.20–4.32 (1H, m, N–C*H*), 8.96 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.9 (C=S), 163.8 (C=N), 163.6 (C=N), 163.4 (C=N), 163.0 (C=N), 56.8 (CH–N), 38.6 (CH_3–N), 31.9 (2 CH_2), 29.1 (CH_2), 23.6 (2 CH_2), 14.9 (CH_3), 13.3 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-adamentamine thiosemicarbazide (**17**): white solid. Yield: 54%; mp: 192 °C. Anal. calc. for $C_{17}H_{23}N_7S$: C 57.12, H 6.49, N 27.43; found: C 57.05, H 6.24, N 27.56%; IR: ν_{max} (cm^{-1}) 3203 (NH), 1531 (C=N), 1237 (C–N), 1056 (C=S), 960 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.39–2.69 (18H, m, adamentyl ring), 3.03 (3H, s, C*H*$_3$–C=N), 3.07 (3H, s, C*H*$_3$–C=N), 4.21–4.32 (1H, m, N–C*H*), 8.83 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.8 (C=S), 163.9 (C=N), 163.6 (C=N), 163.3 (C=N), 163.4 (C=N), 57.3 (CH–N), 30.3 (4 CH_2), 29.1 (2 CH_2), 23.6 (2 CH_2), 21.9 (CH_2), 15.2 (CH_3), 13.4 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-cyclobenzylamine thiosemicarbazide (**18**): light yellow solid. Yield: 65%; mp: 125 °C. Anal. calc. for $C_{14}H_{14}N_7SCl$: C 48.34, H 4.06, N 28.19; found: C 48.05, H 4.24, N 28.39%; IR: ν_{max} (cm^{-1}) 3319 (NH), 1546 (C=N), 1298 (C–N), 1038 (C=S), 963 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 3.03 (3H, s, C*H*$_3$–C=N), 3.08 (3H, s, C*H*$_3$–C=N), 3.97 (2H, d, N–C*H*$_2$), 7.28–7.43 (4H, m, aryl), 8.46 (1H, t, –N*H*), 9.03 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.4 (C=S), 164.2 (C=N), 163.8 (C=N), 163.5 (C=N), 163.1 (C=N), 156.9, 147.8, 140.8, 135.7, 129.5, 126.8 (aryl), 58.3 (CH_2–N), 14.3 (CH_3), 13.1 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-(*n*-phenyl)piperizine thiosemicarbazide (**19**): light yellow solid. Yield: 63%; mp: 166 °C. Anal. calc. for $C_{17}H_{20}N_8S$: C 55.42, H 5.47, N 30.41; found: C 55.38, H 5.34, N 30.39%; IR: ν_{max} (cm^{-1}) 3220 (NH), 1599 (C=N), 1301 (C–N), 1021 (C=S), 932 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 3.05 (3H, s, C*H*$_3$–C=N), 3.10 (3H, s, C*H*$_3$–C=N), 2.49–2.65 (8H, m, –C*H*$_2$–N–C*H*$_2$–), 7.04–7.56 (5H, m, aryl), 9.06 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.9 (C=S), 164.0 (C=N), 163.9 (C=N), 163.6 (C=N), 163.2 (C=N), 57.5 (2 CH_2–N), 54.3 (2 CH_2–N), 148.3, 141.7, 134.9, 130.5–125.8 (aryl), 14.8 (CH_3), 13.2 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-*o*-toulidine thiosemicarbazide (**20**): light yellow solid. Yield: 62%; mp: 155 °C. Anal. calc. for $C_{14}H_{15}N_7S$: C 53.66, H 4.82, N 31.29; found: C 53.83, H 5.04, N 31.26%; IR: ν_{max} (cm^{-1}) 3330 (NH), 1526 (C=N), 1294 (C–N), 1036 (C=S), 966 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.65 (3H, s, –C*H*$_3$), 3.04 (3H, s, C*H*$_3$–C=N), 3.09 (3H, s, C*H*$_3$–C=N), 6.40–7.15 (4H, m, aryl), 8.01 (1H, s, –N*H*), 9.23 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.5 (C=S), 164.0 (C=N), 163.9 (C=N), 163.7 (C=N), 163.5 (C=N), 141.1, 135.5, 130.5, 126.8, 126.2, 125.4 (aryl), 20.6 (aryl-CH_3), 14.7 (CH_3), 13.2 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-*m*-toulidine thiosemicarbazide (**21**): white solid. Yield: 56%; mp: 144 °C. Anal. calc. for $C_{14}H_{15}N_7S$: C 53.66, H 4.82, N 31.29; found: C 53.56, H 4.73, N 31.41%; IR: ν_{max} (cm^{-1}) 3300 (NH), 1545 (C=N), 1296 (C–N), 1079 (C=S), 966 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.58 (3H, s, –C*H*$_3$), 3.03 (3H, s, C*H*$_3$–C=N), 3.08 (3H, s, C*H*$_3$–C=N), 6.42–7.14 (4H, m, aryl), 8.20 (1H, s, –N*H*), 9.27 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.5 (C=S), 164.7 (C=N), 163.8 (C=N), 163.6 (C=N), 163.4 (C=N), 140.9, 138.5, 129.5, 126.8, 126.1, 122.5 (aryl), 21.9 (aryl-CH_3), 14.3 (CH_3), 13.1 (CH_3).

3(3,7-Dimethyl-4H-pyrazolo[3,4-e][1,2,4]triazin-4-yl)-*p*-toulidine thiosemicarbazide (**22**): light brown solid. Yield: 56%; mp: 149 °C. Anal. calc. for $C_{14}H_{15}N_7S$: C 53.66, H 4.82, N 31.29; found: C 53.69, H 4.92, N 31.36%; IR: ν_{max} (cm^{-1}) 3149 (NH), 1542 (C=N), 1296 (C–N), 1079 (C=S), 966 (N–N); ^{1}H NMR ($CDCl_3$): (δ, ppm) 2.52 (3H, s, –C*H*$_3$), 3.06 (3H, s, C*H*$_3$–C=N), 3.12 (3H, s, C*H*$_3$–C=N), 6.47–7.12 (4H, m, aryl), 8.05 (1H, s, –NH), 9.31 (1H, s, –N*H*); ^{13}C NMR ($CDCl_3$): (δ, ppm) 186.5 (C=S), 164.1 (C=N), 163.9 (C=N), 163.7 (C=N), 163.3 (C=N), 138.9, 135.7, 129.5, 126.8 (aryl), 21.8 (aryl-CH_3), 14.9 (CH_3), 13.4 (CH_3).

2.2. Organism culture and in vitro testing against E. histolytica

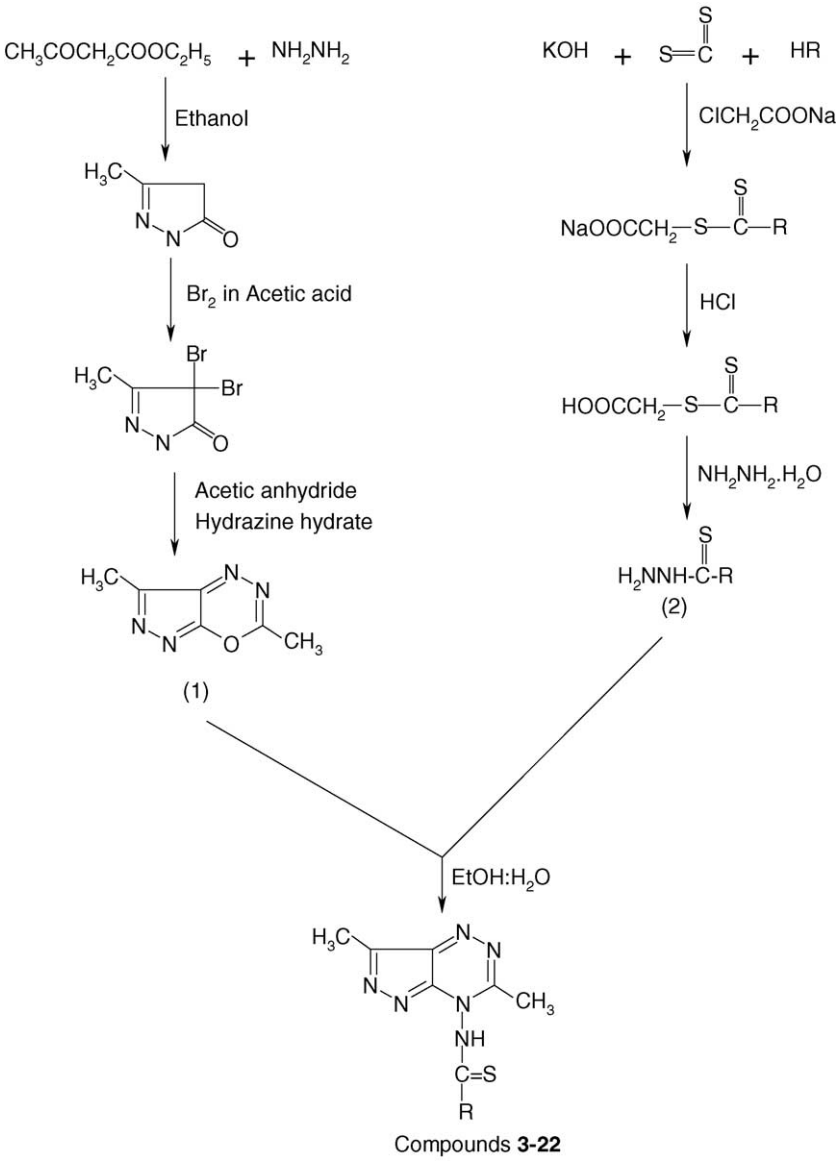
The *E. histolytica* strain HM-1:1MSS was cultured in tubes (15 cm^3) by using Diamond TYIS-33 medium (Diamond et al., 1978). Cell viability was assessed and the concentration of drug 50% inhibition (IC_{50}) was determined by the microdilution method (Wright et al., 1988). All the compounds were dissolved in DMSO (40 μl). The maximum concentration of DMSO in the test did not exceed 0.1% at which level no inhibition of amoebal growth occurred (Gillin et al., 1982; Keen et al., 1986) and is further confirmed by the experiments carried out in our laboratory, followed by adding enough culture medium to obtain concentration of 1 mg/ml. Stock solutions of the compounds were prepared freshly before use at a concentration of 0.1 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, with 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 amoebae suspension was prepared from a confluent culture by pouring off the medium at 37 °C and adding 5 ml of fresh medium, chilling the culture tube on ice to detach the organisms from the side of the flask. The number of amoeba/ml was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The suspension was diluted to 10^5 organism/ml by adding fresh medium and 170 μl of this suspension was added to the test and control wells in the plate so that the wells were completely filled (total volume, 340 μl). An inoculum of 1.7×10^4 organisms/well was chosen so that confluent, but not excessive growth, took place in control wells. Plates were sealed and gassed for 10 min with nitrogen before incubation at 37 °C for 72 h. After incubation, the growth of amoebae in the plate was checked with a low power microscope. By inverting the plate, remove the culture media with gently shaking and 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. It was allowed to dry at room temperature. After drying amoeba were fixed with chilled methanol by keeping it in ice bath for 15 min dried and stained with (0.5%) aqueous eosin for 15 min. The stained plate was washed once with tap water, then twice with distilled water and allowed to dry. A 200 μl portion of 0.1N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader. The% inhibition of amoebal growth was calculated from the optical densities of the control and test wells and plotted against the logarithm of the dose of the drug tested. Linear regression analysis was used to determine the best fitting straight line from which the IC_{50} value was found.

3. Results and discussion

3.1. Chemistry

Triazine derivatives were prepared using a multi-step synthetic route. 3,7-Dimethylpyrazolo[4,3-e]oxadiazine (**1**) the key intermediate in the synthesis of triazine was obtained in satisfactory yield. Its IR spectrum revealed two bands at 1589 and 965 cm^{-1} due to (C=N) and (N–N) groups, respectively. Bands at 1165 and 1085 cm^{-1} were assigned to the (C–O–C) group of the compound **1**. The ^1H NMR showed two singlets at 2.69 and 3.01 ppm due to the methyl protons. Substituted thiosemicarbazides (**2**) were prepared using a two-step synthetic route and obtained in 60–80% yields. Triazine derivatives were obtained by the condensation of 3,7-dimethylpyrazolo[4,3-e] oxadiazine (**1**) with the substituted thiosemicarbazide (**2**) by refluxing at 60 °C for 3 h (Scheme 1).

These compounds were characterized by elemental analysis, IR, ^1H and ^{13}C NMR. All the compounds were highly soluble in DMF, DMSO and chloroform and sparingly soluble in acetone, methanol, ethanol and insoluble in water. Analytical and spectral data were in good agreement with the composition of triazine derivatives. The IR bands of triazine derivatives appear in the region 4000–400 cm^{-1}. The IR spectra of the compounds **3**–**22** are quite similar despite the presence of different substituent, which shows that the charge delocalization was not influenced by substituting different amines. All the triazine derivatives showed a broad band in the region 3203–3426 cm^{-1} due to –NH group whereas in compounds **13**, **16** and **22** this band appears at 3125–3176 cm^{-1}, the inductive effect of the methyl group lead to lowering of frequency of the latter. The sharp band at 1526–1599 cm^{-1} can be attributed to the stretching vibrations of the C=N group. Band due to the bonding between carbon and amine present in the region 1237–1309 cm^{-1} as a sharp peak for the C–N where as the stretching vibration due to thiocarbonyl (C=S) appear in the range 1014–1088 cm^{-1}. The ^1H NMR spectrum showed a singlet at ∼7.97–9.31 ppm for the –NH proton. ^1H NMR showed that in all the compounds, both the methyl groups attached to the triazine ring resonate at 3.01–3.12 ppm while in ^{13}C NMR, these methyl carbons resonate at 12.6–15.6 ppm. The resonance of the methylene groups adjacent to the amine was found in three distinct regions around 57, 55 and 49 ppm in ^{13}C NMR. Methylene groups separated from nitrogen by at least one carbon on each side appears around 30–35 ppm. The protonation of amines causes a shielding of the carbon atoms in the vicinity of nitrogen resulting an upfield shift. Methylene groups positioned α to the amine deshielded more and therefore have larger downfield ^{13}C shift than those positioned farther away (Kemp, 1975). Methylene groups α to the secondary amine have larger downfield chemical shift than those α to primary amine. Presence of Cl atom in compound **18** attached to aromatic ring causes the down field shift of aromatic protons. In compounds **20**–**22** methyl group present on aromatic ring res-



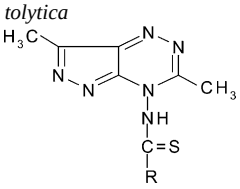
Scheme 1.

onate nearly at the same position. In ethyl piperidine derivative **8**, methylene carbons attached to nitrogen resonate exceptionally high at 59.2 ppm due to asymmetry while other carbons resonate at 51.3 ppm. All the aromatic carbons resonate in the range of 122.5–156.9 ppm.

3.2. Biological activity

The in vitro antiamoebic activities of 3,7-dimethylpyrazolo[3,4-e][1,2,4] triazin-4-yl thiosemicarbazide derivatives **3**–**22** were evaluated against *HM1:1MSS* strain of *E. histolytica* and their IC_{50} values are reported in Table 1. The metronidazole was used as reference compound with IC_{50} = 1.81 μM. The results were estimated as the percentage of 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. Compounds (**3**–**8**) showed IC_{50} values in the range 4.84–7.31 μM substituted at N^4 position with aliphatic amines. Compounds (**9**–**17**) showed IC_{50} values in the range 0.81–4.19 μM substituted at N^4 position with cyclic amines. Compounds (**18**–**22**) showed IC_{50} values in the range 1.41–4.18 μM substituted at N^4 position with aromatic amines. The biological data suggest that the compounds with substituted aliphatic amines do not show any activity. While good activities were observed for the triazine derivatives substituted with cyclic and aromatic amines. Compared to the activity of the commonly used drug metronidazole, the activities of the compounds **10**, **11**, **17** and **18** is noteworthy since they showed less IC_{50} values than Metronidazole. Compound 21 also showed IC_{50} value close to the reference drug. The compounds **11** and **17** (IC_{50} = 0.81 μM of

Table 1
In vitro antiamoebic activity of 3,7-dimethyl-pyrazolo[3,4-e][1,2.4]triazin 4-yl thiosemicarbazide derivatives against (*HM-1:1MSS*) strain of *E. histolytica*



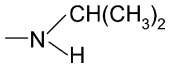
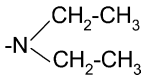
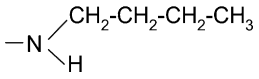
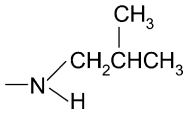
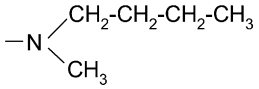
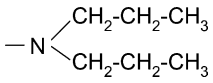
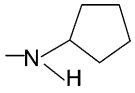
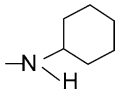
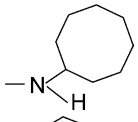
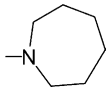
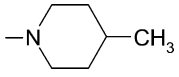
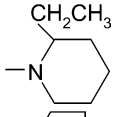
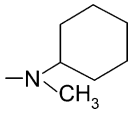
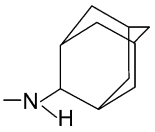
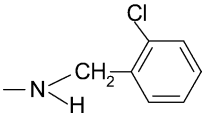
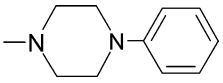
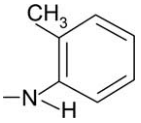
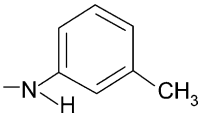
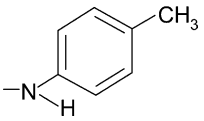
Compound	R	IC_{50} (μM)	S.D.[a]
3		5.62	0.71
4		7.31	1.02
5		6.20	0.48
6		4.84	0.44
7		5.02	0.74
8		5.44	0.64
9		1.96	0.27
10		1.28	1.77
11		0.81	0.15
12		2.09	0.16
13		2.36	0.42
14		2.04	0.26
15		4.19	0.74
16		3.92	0.58

Table 1 (*Continued*)

Compound	R	IC_{50} (μM)	S.D.[a]
17		0.84	0.11
18		1.41	0.18
19		4.18	0.65
20		2.27	0.30
21		1.85	0.26
22		2.04	0.41
Metronidazole		1.81	0.34

[a] Standard deviation.

11, IC_{50} = 0.84 μM of **17** versus IC_{50} = 1.81 μM of metronidazole) are most active of the tested compounds of the triazine series. The results were statistically evaluated by analysis of variance. The null hypothesis was tested using *t*-test. The significativity of the difference between the IC_{50} values of metronidazole and the compounds **10**, **11**, **17** and **18** 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. Studies of the toxicity towards mammalian cells as well as in vivo of these compounds (**10**, **11**, **17**, **18** and **21**) are currently underway.

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