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Short communication

Synthesis, characterization and structure optimization of a series of thiazolidinone derivatives as *Entamoeba histolytica* inhibitors

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ABSTRACT

A series of thiazolidinone derivatives were synthesized by sodium acetate assisted cyclization of 1-isobutyl-3-phenylthiourea with chloroacetic acid followed by the piperidine facilitated substitution of the resulting thiazolidinone with different substituted aldehydes. The ethene and imine double bonds adopt (*Z,Z*) configuration as indicated by ^{1}H–^{1}H COSY and 2D-NOESY ^{1}H NMR and further confirmed by the crystal structure studies. The *in vitro* antiamoebic activity of these compounds was evaluated against HM1:IMSS strain of *Entamoeba histolytica*. Eight compounds exhibited promising activity with IC_{50} values (0.11–0.172 μM) lower than the standard drug metronidazole (IC_{50} 1.64 μM). *In vitro* cytotoxicity results revealed low cytotoxic up to the concentration of 25 μM.

1. Introduction

Amoebiasis, caused by the enteric dwelling protozoan parasite *Entamoeba histolytica* is one of the major health problems in developing countries, surpassed only by malaria and schistosomiasis [1–3]. Colon is the primary location where *E. histolytica* is found as inactive, but after a certain period of time it becomes dangerous and fatal to the human being by causing extreme conditions like dysentery, colitis and liver abscess [4–6]. The antiamoebic drug, 5-nitroimidazole or metronidazole, (*2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol*) has long been used for the treatment of this disease. But some studies have reported that this drug is ecotoxic [7], induces encephalopathy [8,9], showed neurologic toxicity [10], genotoxicity, carcinogenicity [11–13], spermatozoid damage [14] and lack of selectivity [15,16]. Although metronidazole and its derivatives are currently employed in therapy, the paucity of effective drugs and potential clinical resistance necessitate the development of novel drug with a good therapeutic activity and without these side effects. Five-membered heterocyclic compounds natural as well as synthetic are important for their biological activities. Previously, we reported the synthesis and evaluation of series of azole derivatives that have exhibited potent *in vitro* antiamoebic activity comparable or superior to metronidazole, the reference drug (Fig. 1) [17–22]. Compounds with thiazolidinone ring are of interest due to their broad spectrum of biological activities such as bactericidal [23] fungicidal [24,25] antimicrobial [26–29] antiproliferative [30,31], antiviral [32], anticonvulsant [33,34], anticancer [35–39] and anti-inflammatory [40–42] activities. It was reported that 5-arylidene unsubstituted nitrogen of 2-phenylimino thiazolidin-4-one derivatives (**2a**) (Fig. 2) showed promising anticancer activity but when nitrogen of 5-arylidene 2-phenylimino thiazolidin-4-one derivatives was substituted by alkyl group showed less activity [39]. It was shown that by substituting propyl group at nitrogen of 5-arylidene 2-phenylimino thiazolidin-4-one (**2b**) (Fig. 2) has potent anti-inflammatory activity [42]. It has been reported that N-atom of thiazolidinone ring was substituted with several groups including isobutyl (**2c**, **2d**) (Fig. 2) were found active against N-type calcium channel blocker [43].

In view of the versatile pharmacological significances of phenyliminothiazolidinone derivatives, we herein report the synthesis, characterization, antiamoebic activity and cytotoxicity of thiazolidinone derivatives. To the best of our knowledge, this is the first report of thiazolidinone derivatives showing promising *in vitro* activity against *E. histolytica*.

2. Results and discussion

2.1. Synthesis

Present study was undertaken to synthesize some thiazolidinone derivatives by reported methods [44] to investigate their

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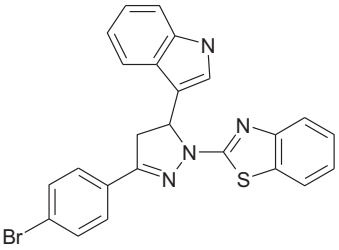
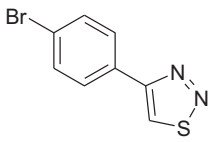
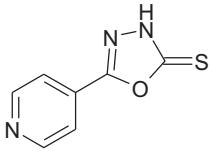


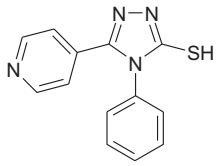
(1a) IC_{50} = 1.05 μM



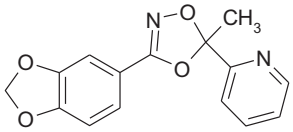
(1b) IC_{50} = 0.24 μM



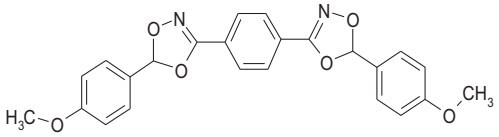
(1c) IC_{50} =0.558 μM



(1d) IC_{50} =0.160 μM



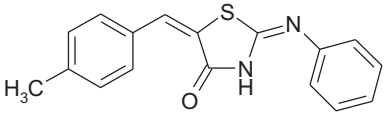
(1e) IC_{50} = 1 μM



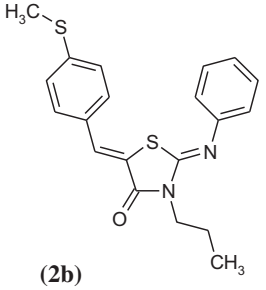
(1f) IC_{50} = 1 μM

Fig. 1. Azole based antiamoebic compounds.

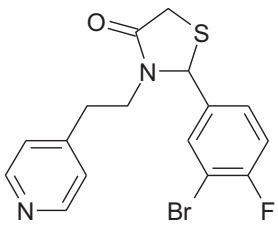
probable antiamoebic effect. Target compounds (**3**–**18**) were obtained in a three step reaction procedure as outlined in Scheme 1. In the first step, synthesis of 1-(2-methylpropyl)-3-phenylthiourea (**1**) was carried out by the reaction of phenylisothiocyanate and 2-methylpropan-1-amine in toluene. In the second step, 3-(2-methylpropyl)-2-(phenylimino)-1,3-thiazolidin-4-one (**2**) was synthesized by sodium acetate assisted cyclization of **1** with chloroacetic acid. The target compounds (**3**–**18**) were obtained in the third step by Knoevenagel condensation of 3-(2-methylpropyl)-2-(phenylimino)-1,3-thiazolidin-4-one (**2**) with different



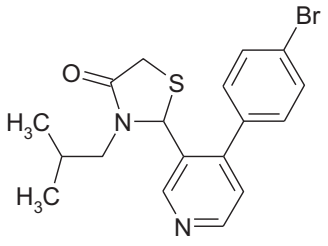
(2a)



(2b)

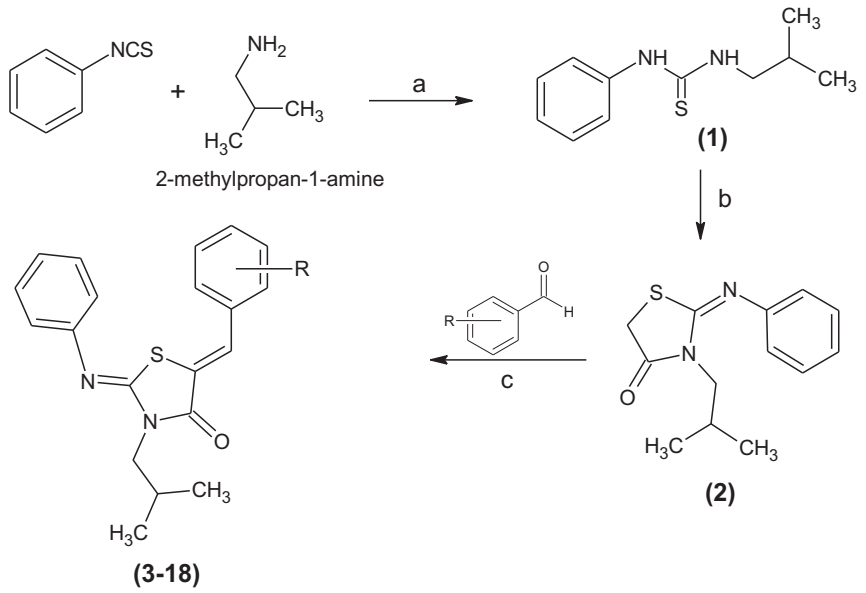


(2c)



(2d)

Fig. 2. Thiazolidinone derivative.



Scheme 1. Synthesis of thiazolidinone derivatives. Reagents and conditions: (a) Toluene, RT, 1 h. (b) Anhydrous Sod. acetate, chloroacetic acid, EtOH, reflux. (c) Piperidine, EtOH, reflux; where R represents different substituted aldehydes.

substituted aldehydes in ethanol. The purity of the compounds was confirmed by CHNS analysis. All the compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR and Mass spectral studies. The stereochemistry (*E* or *Z* form) of the target compounds was confirmed by ^{1}H–^{1}H COSY, 2D-NOESY ^{1}H NMR spectroscopy and single crystal structure.

Characteristic IR spectra showed significant bands for the formation of 1-isobutyl-3-phenylthiourea (**1**) where the appearance of two characteristic bands at 3244 cm^{-1} and 1225.68 cm^{-1} was assigned to N–H and C=S bond respectively. The structure of (**1**) was confirmed by ^{1}H NMR and ^{13}C NMR. The appearance of (–N–H) and (S=C–NH) protons at 8.52 ppm and 6.14 ppm as broad singlet and the signal for C=S appeared at 180.37 ppm in ^{13}C NMR indicated the formation of the compound **1**. For the compound **2**, the characteristic bands appeared at 1650–1720 (O=C–N–), 1627.89 (C=N), 1350–1400 (tert. N) and 750–800 (C–S–C) cm^{-1}. In ^{1}H NMR spectra (S–CH$_2$–) protons appeared as singlet at 3.80 ppm with disappearance of NH signal indicates the formation of the compound **2**. The ^{13}C NMR spectra strongly supported the structure of **2** as the signals appeared at 166.88 (O=C–N–), 150.20 (–C=N) and 132.28 (S–C=C). For the series of thiazolidinone derivatives (**3**–**18**), the appearance of one characteristic band at 1550–1600 cm^{-1} was assigned to the C=C which suggested the condensation of different aromatic aldehydes with **2**. In all the compounds (**3**–**18**), alkenic (H–C=C) protons appeared as singlet at 7.65–8.35 ppm. In ^{13}C NMR, the alkenic carbon (C=C) signal appeared at 148 ppm confirmed the formation of thiazolidinone derivatives. The values are given in the experimental section.

2.2. Stereochemistry and conformational properties

To confirm the stereochemistry (*E* or *Z* isomer), due to the presence of exocyclic C=N and C=C bonds, ^{1}H–^{1}H COSY and ^{1}H-NOESY NMR spectroscopy were carried out. The ^{1}H–^{1}H COSY NMR showed the presence of strong coupling of off-diagonal peaks. The doublet of C(9,11) at δ 1.00 (ppm) couples with multiplet of C(10) at δ 2.39–2.31 (ppm) and the multiplet of C(10) at δ 2.39–2.31 (ppm) couples with doublet of C(8) at δ 3.83 (ppm) (see Supporting information) [for the position of the atom see crystal structure]. There is no off-diagonal peak present in the region of the aliphatic and exocyclic N-substituted aromatic protons, which could show the correlation among them, indicating that the position of aromatic moiety as *Z*-configuration with respect to exocyclic nitrogen. Furthermore, there is strong interaction between substituted aromatic protons at N(1) and substituted aromatic protons C(14) at δ 6.91–7.36 and 7.21–7.40 (ppm) indicating the *Z* configuration of exocyclic 5-arylidene. *Z* configuration of the exocyclic C=C bond in the thiazolidinone derivatives was further confirmed by the signal of a methine proton, which resonated at higher chemical shift at δ 7.69 (ppm) as a singlet in the ^{1}H NMR spectrum. The downfield chemical shift of the methine proton was due to deshielding effect of carbonyl group. If it were in *E* configuration, it would resonate upfield at less than δ 6.64 (ppm) [42,45]. In 2D NOESY ^{1}H NMR, the C=N imino exocyclic double bond showed strong NOE signals at δ 6.91–7.41 (ppm) due to the interaction of the protons of two aromatic rings attached to N(1) and C(14) positions indicating the *Z* configuration while there is no NOE signal observed for N(1) substituted aromatic proton and aliphatic protons C(8 or 9 or 10 or 11) to show *E* configuration. Above discussed result was further, supported by the X-ray crystal structure.

2.2.1. Discussion of crystal structure

Fig. 3 shows a perspective view of the crystal structure of the (2*Z*,5*Z*) 2-(4-methoxybenzylidene)-3-isobutyl-2-(phenylimino) thiazolidin-4-one (**9**). The C(13)–C(14) and N(1)–C(7) bonds show a typical double-bond character corresponding to ethene and imine bonds, with bond lengths 1.334(5) and 1.262(4) Å, respectively. C(14)–C(15) bond shows a partial double bond character, with bond lengths 1.448(5) Å, that seems to indicate a certain electronic delocalization between C(13)–C(14) and C(14)–C(15) bonds. The double bonds C(13)–C(14) and N(1)–C(7) adopt a *Z*,*Z* configuration. The central thiazolidinone ring, S(1), C(7), N(2), C(12) and C(13) is planar [mean deviation from planarity 0.0183(17) Å] and keeps the planarity across of the ethene bond with the methoxybenzylidene group [mean deviation from planarity S(1), C(7), N(2), C(12), C(13), C(14), C(15), C(16), C(17), C(20) and C(21) except C(18), which is disordered 0.0480(31) Å]. The other phenyl ring is not planar with respect to the rest of the molecule. This ring C(1), C(2), C(3), C(4), C(5), C(6) forms an angle of 58.34(11)° with respect to the thiazolidinone ring. The crystal packing is controlled by van der Waals

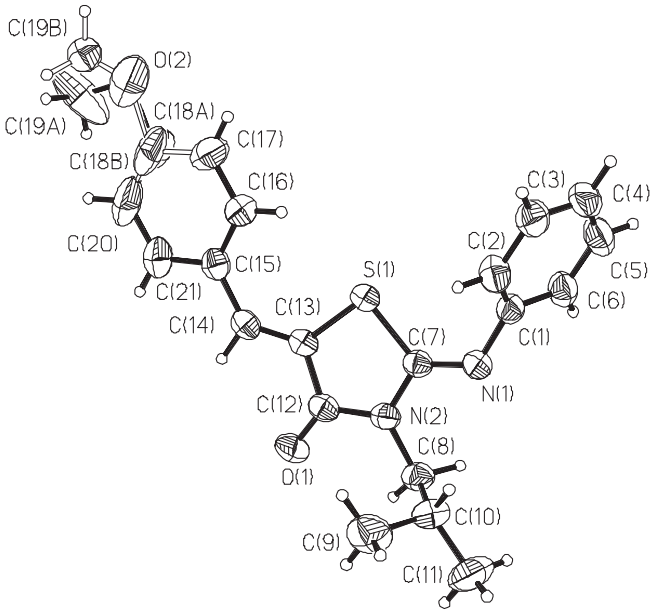


Fig. 3. Crystal structure of compound **9**.

forces while π–π stacking interactions are absent in the structure. The crystal data and structure refinement for compound **9** are given in Table 1.

Table 1
Crystal data and structure refinement for compound **9**.

Formula	$C_{21}H_{22}N_2O_2S$
Formula weight	366.47
Temperature, K	298(2)
Wavelength, Å	0.71073
Crystal system	Monoclinic
Space group	$P2_1/n$
a/Å	6.3200(2)
b/Å	18.4428(6)
c/Å	16.8208(5)
β/°	90.705(2)
V/Å^3	1960.46(11)
Z	4
D_{calcd}/g cm^{-3}	1.242
Absorption coefficient/mm^{-1}	0.182
F_{000}	776
Crystal size/mm^3	0.34 × 0.26 × 0.22
θ range for data collection ($\theta_{Min}/\theta_{Max}$)/(°)	1.64–25.11
Index ranges	$-6 \le h \le 7$ $-21 \le k \le 21$ $-20 \le l \le 20$
Reflections collected	19,416
Independent reflections	3493
R_{int}	0.0384
Completeness %/(θ)	99.6/(25.11°)
Max./min. transmission	0.9619 and 0.9416
Refinement method	Full-matrix least-squares on F^2
Restraints/parameters	3493/66/275
Goodness-of-fit on F^2	1.019
R_1[a]	0.0635
wR_2 (all data)[b]	0.2121
Extinction coefficient	0.035(4)
Largest differences peak and hole (eÅ^{-3})	0.645 and −0.322

[a] $R_1 = \sum||F_0| - |F_c||/\sum|F_0|$.
[b] $wR_2 = \{\sum[w(|F_0|^2 - |F_c|^2|)^2]/\sum[w(F_0^4)]\}^{1/2}$.

2.3. Pharmacology

All the synthesized compounds (**3**–**18**) were screened *in vitro* against HM1:IMSS strain of *E. histolytica* by microdilution method [46]. All the experiments were carried out in triplicate at each concentration level and repeated thrice. Cytotoxicity of all the compounds has been studied by MTT assay on human hepatocellular carcinoma cell line (HepG2). The results of antiamoebic activity and cytotoxicity are summarized in Table 2.

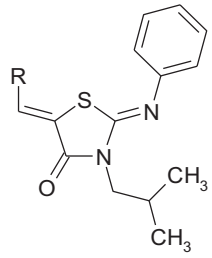
2.3.1. Antiamoebic activity

Preliminary experiments were carried out to determine *in vitro* antiamoebic activity of all the synthesized compounds (**3**–**18**) by microdilution method using HM1:IMSS strain of *E. histolytica* and their IC_{50} values are reported in Table 2. Metronidazole was used as reference drug having $IC_{50} = 1.64$ μM in our experiment. 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. IC_{50} and 95% confidence limits were interpolated in the corresponding dose response curve. The antiamoebic activity of the test compounds seems to depend upon by the presence and position of the electron donating and withdrawing substituents. Furthermore, the ring activating and deactivating nature of the substituents also play a dominant role on the activity of the synthesized compounds. The thiazolidinone derivatives (**3**–**18**) showed IC_{50} values in the range (0.11–16.8 μM). The presence of strong deactivating groups on the phenyl ring showed an interesting pattern of activity. Presence of trifluoromethyl group (strong deactivating group) at ortho position of the phenyl ring in compound **4**, showed less activity ($IC_{50} = 3.71$ μM) as compared to nitro group at para position of the phenyl ring in compound **6** ($IC_{50} = 0.138$ μM). Presence of weak deactivating (chloro group) at meta position in compound **16** showed better activity ($IC_{50} = 0.66$ μM) than compound **3** having chloro group at para position of the phenyl ring ($IC_{50} = 1.92$ μM). Methyl group, a weak activating group, increases the electron density through hyperconjugation to the aromatic ring. The methyl group present at para position of the phenyl ring (compound **10**) provides less activity ($IC_{50} = 3.21$ μM) than the compound **18** ($IC_{50} = 0.96$ μM), which has ethyl group at para position. The presence of methyl group at ortho position of thiophene (compound **14**) showed better activity ($IC_{50} = 0.11$ μM) than compounds **10** and **18**. When a strong activating group is present at ortho position (compound **11**) it shows moderate activity ($IC_{50} = 2.04$ μM). It is also observed that when it is present at para position in compounds **7** & **9**, it does not affect the activity ($IC_{50} = 2.45$ & 2.72 μM) of compound too much. But it has also been observed that, when N,N-dimethyl group (compound 5) is present at same position, it enhances the activity ($IC_{50} = 0.172$ μM). Insertion of two methoxy groups at positions 3 & 4 of the phenyl ring in compound **8** increase the activity ($IC_{50} = 0.44$ μM). However, the activity decreased in compound **17**, by inserting two methoxy groups at positions 2 & 5 of the phenyl ring ($IC_{50} = 1.95$ μM). The presence of methoxy and hydroxyl group together in compound **15** also showed better activity ($IC_{50} = 0.64$ μM). It is worth to mention that the replacement of the substituted phenyl ring with heteroaromatic rings and more bulky piperonal ring resulted in a dramatic change in the activity of the compounds. Presence of a piperonal ring in compound **12**, showed a precipitous decrease in activity. This may be attributed to the significantly reduced aromaticity of the group or its bulky nature might be imposing steric hindrance thereby, drastically reducing its efficacy. From the above discussion, it can be concluded that compound **14** seems to be the most potent of all the compounds screened. Also, an optimum electron density may be helpful for a compound to gain better activity.

2.3.2. Relation of pharmacological activity with hydrophobicity index (log P) & HBA/HBD

Octanol–water partition coefficient, log *P*, tells about the compound's ability to dissolve into hydrophobic (non-aqueous)

Table 2
In vitro antiamoebic activity of thiazolidinone derivatives (**3**–**18**) against HM1:IMSS strain of *E. histolytica* and cytotoxicity profile.



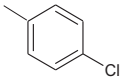
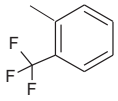
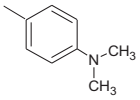
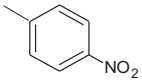
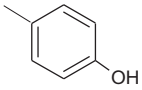
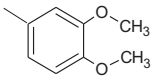
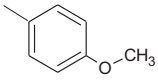
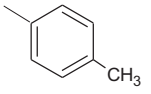
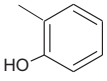
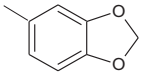
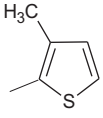
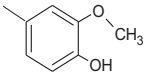
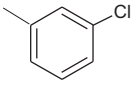
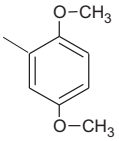
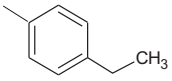
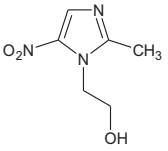
Compound	R	Antiamoebic activity		Cytotoxicity profile	
		IC_{50} (μM) ± SD		IC_{50} (μM) ± SD	
3		1.92	0.006	N.D	N.D
4		3.71	0.008	N.D	N.D
5		0.172	0.007	>100	0.12
6		0.138	0.007	>100	0.07
7		2.45	0.0045	N.D	N.D
8		0.44	0.008	>100	0.12
9		2.72	0.009	N.D	N.D
10		3.21	0.007	N.D	N.D
11		2.04	0.003	N.D	N.D
12		16.68	0.01	N.D	N.D
13		0.60	0.01	>100	0.17
14		0.11	0.01	89.2	0.17

Table 2 (*continued*)

Compound	R	Antiamoebic activity		Cytotoxicity profile	
		IC_{50} (μM) ± SD		IC_{50} (μM) ± SD	
15		0.64	0.03	89.5	0.06
16		0.66	0.03	88.1	0.09
17		1.95	0.009	N.D	N.D
18		0.96	0.001	84.7	N.D
MNZ		1.64	0.008	>100	0.07

SD = Standard deviation, ND = Not done.

medium and is used in rational drug design. Hydrophobicity is needed for compounds to permeate through various biological membranes. Hydrophobicity affects drug absorption, bioavailability, hydrophobic drug–receptor interactions, metabolism of molecules, as well as their toxicity. It is expressed as a 10-base logarithm of the concentration ratios between the two phases. In this paper, we have discussed 2 important parameters, log *P* and HBA (Hydrogen Bond Acceptor)/HBD (Hydrogen Bond Donor), of the synthesized compounds to check their drug likeness properties. To evaluate drug-likeness better, several modifications have been made to the Lipinski rule. Ghose et al. [47] set the range −0.4 to 5.6 for log *P* for a compound behaving as drug.

The log *P* and HBA/HBD value of the synthesized compounds has been determined using Marwin sketch [48] and is given in Table 3. From this table one can see that compounds **13** and **15** have log $P < 5$, with moderate activity ($IC_{50} = 0.60$ & 0.64 μM respectively), with high toxicity. Surprisingly, despite having log $P = 5$, compound **12** has exhibited lowest activity among the series of the compounds and hence was not selected for cytotoxic study. Out of the two compounds **14** and **16**, former has lower log *P* than latter and have better antiamoebic activity with low cytotoxicity. As per the rule, molecules having HBD greater than 5 and HBA greater than 10 are more hydrophilic, i.e. poor permeation through the membrane. There is no HBD in compound **14**, while compound **15** has one; indicating compound **14** is following the rule better than compound **15**. In terms of HBA, compound **14** has 3 acceptors while compound **15** has 5 acceptors which again indicate that compound **14** has greater chance of permeation through the biological membranes rather than **15**, which has more HBA and HBD. Thus it can be concluded that compound **14** is the best throughout the series.

2.3.3. Cytotoxicity studies

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

Table 3
Values of hydrophobicity index (log *P*) and HBA/HBD.

S. No	log *P*	No. of	
		HBD	HBA
3	5.98	0	3
4	6.26	0	3
5	5.49	0	4
6	5.32	0	7
7	5.08	1	4
8	5.06	0	5
9	5.22	0	4
10	5.89	0	3
11	5.08	1	4
12	5.00	0	5
13	4.40	0	4
14	5.81	0	3
15	4.92	1	5
16	5.98	0	3
17	5.06	0	5
18	6.34	0	3
MNZ	−0.46	1	6

crystals [49,50] 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 [51–53].

In the present study, only those compounds were chosen for their cytotoxicity against human hepatocellular carcinoma cell line (HepG2) which has good activity against *E. histolytica* to ensure their toxic effect. Metronidazole was used as a positive control. A sub-confluent population of HepG2 cells was treated with increasing concentration of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.13–100 μM. The cell viability (%) obtained with continuous exposure for 48 h is depicted in Fig. 4. The cytotoxicity of all the compounds was found to be concentration-dependent. Fig. 4 depicts that all the compounds including the reference compound metronidazole showed a viability of 100% at the concentration range of 3.13 μM and up to a concentration of 25 μM all the compounds showed a viability of ≥75%. On increasing the concentration range up to 50 μM the compounds showed moderate to high cytotoxicity against HepG2 cell line. Compounds **5**, **6**, **8** and **14** (viability 70–80%) showed least cytotoxicity among all the compounds screened. At 100 μM only two compounds, **6** and **14** showed maximum viability. Out of two, compound **14** displayed better antiamoebic activity and hence chosen as best. The cytotoxicity (IC_{50}) of the compounds screened including the reference drug metronidazole is given in Table 2.

3. Conclusion

In summary, thiazolidinone derivatives (**3**–**18**) were synthesized, characterized and screened *in vitro* against HM1:IMSS strain of *E. histolytica*. The preliminary results showed that out of sixteen compounds, eight compounds **5**, **6**, **8**, **13**–**16** and **18** exhibited better antiamoebic activity than the reference drug metronidazole. The MTT assay revealed that compounds **5**, **6**, **8** and **13** showed low cytotoxicity.

4. Experimental protocol

All the required chemicals were purchased from Merck and Aldrich Chemical Company (USA). Precoated aluminium sheets (Silica gel 60 F254, Merck Germany) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. Elemental analysis was carried out on CHNS Elementar analyzer (Vario EL-III) and the results were within ±0.3% of the theoretical values. 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 Spectrospin DPX 300 MHz and Bruker Spectrospin DPX 75 MHz spectrometer respectively using $CDCl_3$ as a solvent and trimethylsilane (TMS) as an internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; m, multiplet. Chemical shift values are given in ppm. The TOF-MS-ES$^+$ spectra of the compounds were recorded on Waters micromass-LCT Mass spectrometer. X-ray data were collected on Bruker SMART Apex CCD diffractometer (SAI, Universidade da Coruña).

*4.1. General procedure for the synthesis of 1-isobutyl phenylthiourea (**1**)*

Phenylisothiocyanate (15 g, 111.11 mmol) was dissolved in toluene, and then isobutyl amine (8.11 g, 111.11 mmol) was added slowly at room temperature, and after a while plenty of white precipitation appeared. Stirring was continued for 1 h. The precipitate was collected by filtration, washed with toluene, and dried to afford product (**1**) as white powder.

*4.1.1. 1-Isobutyl-3-phenylthiourea (**1**)*

Yield 95%. M.p: 112 °C; Anal. calc. $C_{11}H_{16}N_2S$: C 63.42, H 7.74, N 13.45, S 15.39%. Found: C 63.39, H 7.26, N 12.99, S 15.30%. IR ν_{max} (cm^{-1}): 3244.71 (NH), 3059.93 (C–H, Ar–H), 1225.68 (C=S); ^{1}H

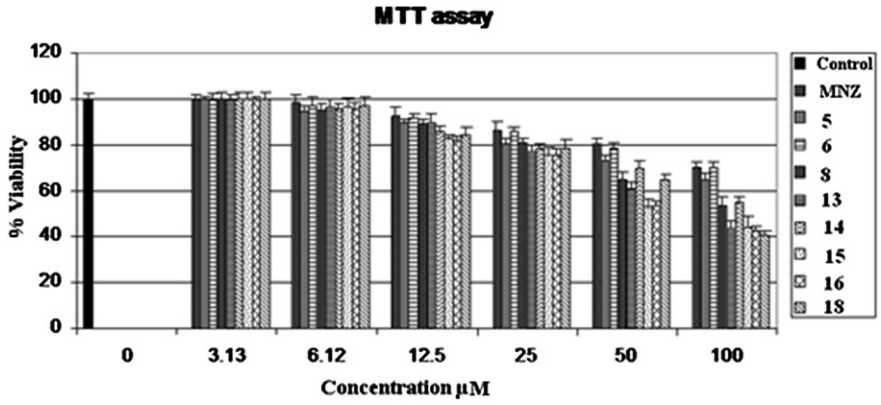


Fig. 4. Percentage of viable cells after 48 h pre-treatment of human hepatocellular carcinoma cell line (HepG2) with compounds **5**, **6**, **8**, **13**, **14**, **15**, **16**, **18** and metronidazole evaluated by the MTT assay.

NMR ($CDCl_3$) δ (ppm): 7.46 (t, 2H, J = 7.5 Hz, Ar–H), 7.32–7.22 (m, 3H, Ar–H), 8.52 (s, 1H, NH), 6.14 (s, 1H, S=C–NH), 3.47 (t, 2H, J = 6.0 Hz, CH_2), 1.95–1.86 (m, 1H, CH), 0.91 (d, 6H, J = 6.6 Hz, $(CH_3)_2CH$); ^{13}C NMR ($CDCl_3$) δ (ppm): 180.37 (C=S), 136.15, 130.02, 127.03, 125.07 (aromatic), 52.58 (C–N), 27.88 (CH), 20.03 (CH_3).

4.2. General procedure of synthesis of 3-isobutyl-2-(phenylimino) thiazolidin-4-one

1-Isobutyl-3-phenylthiourea (12 g, 48.07 mmol) was dissolved in ethanol, and then anhydrous sodium acetate (7.88 g, 96.14 mmol) and chloroacetic acid (5.65 g, 60.08 mmol) were added in sequence. The suspension was refluxed for 12 h, and then the solvent was evaporated. Water was added and the aqueous layer was back-extracted with ethyl acetate. The organic layer was washed with saturated sodium bicarbonate and brine. The combined organic extracts were dried over Na_2SO_4 filtered through cotton, and concentrated *in vacuo*, after the concentration the yellowish-white solid product was obtained.

4.2.1. 3-Isobutyl-2-(phenylimino)thiazolidin-4-one (**2**)

Yield: 90%; m.p: 130 °C; Anal. calc. $C_{11}H_{16}N_2S$: C 62.87, H 6.49, N 11.28, S 12.91%. Found: C 62.85, H 6.44, N 11.25, S 12.90%. IR ν_{max}(cm^{-1}): 1715.37 (–N–C=O), 1627.89 (C=N), 766.64 (C–S–C), 1370.67 (tert. N); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.36–7.23 (m, 2H, Ar–H), 7.16–7.10 (m, 1H, Ar–H), 6.95–6.92 (m, 2H, Ar–H), 3.81 (s, 2H, CH_2), 3.80 (t, 2H, J = 7.5 Hz, CH_2), 2.32–2.23 (m, 1H, CH), 0.97 (d, 6H, J = 5.1 Hz, $(CH_3)_2CH$).

4.3. General procedure of synthesis of thiazolidinone derivatives

A mixture of 3-isobutyl-2-(phenylimino)thiazolidin-4-one (1 mmol), different aldehydes (1 mmol), hexahydropyridine (1.15 mmol), and 35 mL of ethanol were refluxed for 11–12 h. The reaction mixture was cooled to room temperature and the precipitated solid was collected by filtration, washed with ethanol.

4.3.1. (2Z,5Z)-5-(4-Chlorobenzylidene)-3-isobutyl-2-(phenylimino) thiazolidin-4-one (**3**)

Yield: 85%; m.p: 162 °C; Anal. calc. $C_{20}H_{19}ClN_2OS$: C 64.77, H 5.16, N 7.55, S 8.64%. Found: C 64.70, H 5.11, N 7.49, S 8.60%; IR ν_{max} (cm^{-1}): 1706 (–N–C=O), 1625 (C=N), 758 (C–S–C), 1370 (tert. N), 1585 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.00 (t, 2H, J = 7.2 Hz, Ar–H), 7.23 (t, 1H, J = 7.2 Hz, Ar–H), 7.42 (t, 6H, J = 8.4 Hz, Ar–H), 7.68 (s, 1H, CH=C), 3.85 (d, 2H, J = 7.5 Hz, CH_2), 2.39–2.30 (m, 1H, CH), 1.02 (d, 6H, J = 6.6 Hz, $CH(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 166.88 (C=O), 150.20 (C=N), 132.28 (S–C=C), 148.20 (C=C), 135.60, 130.99, 129.37, 129.24, 129.15, 124.87, 122.29,121.03 (aromatic), 50.29 (C–N), 26.95 (CH), 20.09 (CH_3); TOF MS ES$^+$ m/z: [M^+] 370 (100%).

4.3.2. (2Z,5Z)-5-(2-(Trifluoromethyl)benzylidene)-3-isobutyl-2(phenylimino)thiazolidin-4-one (**4**)

Yield: 85%; m.p: 165 °C; Anal. calc. $C_{21}H_{19}F_3N_2OS$: C 62.36, H 4.74, N 6.93, S 7.93%. Found: C 62.31, H 4.71, N 6.90, S 7.90%. IR ν_{max} (cm^{-1}): 1703 (–N–C=O), 1635 (C=N), 766 (C–S–C), 1377 (tert. N), 1586 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.73 (d, 1H, J = 7.8 Hz, Ar–H), 7.55 (t, 2H, J = 3 Hz, Ar–H), 7.45–7.33 (m, 3H, Ar–H), 7.20 (t, 1H, J = 7.5 Hz, Ar–H), 6.96 (d, 2H, J = 7.2 Hz, Ar–H), 8.00 (s, 1H, CH=C), 3.86 (d, 2H, J = 7.5 Hz, CH_2), 2.40–2.31 (m, 1H, CH), 1.03 (d, 6H, J = 6.6 Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 166.14 (C=O), 150.26 (C=N), 132.12 (S–C=C), 148 (C=C), 129.58, 129.35, 129.17, 129.06, 128.89, 26.56, 126.49, 126.38, 125.53 (aromatic), 121.00 (CF_3), 50.37 (C–N), 27.02 (CH), 20.18 (CH_3); TOF MS ES$^+$ m/z: [M^+] 404 (100%).

4.3.3. (2Z,5Z)-5-(4-(Dimethylamino)benzylidene)-3-isobutyl-2-(phenylimino)thiazolidin-4-one (**5**)

Yield: 85%; m.p: 163 °C; Anal. calc. $C_{22}H_{25}N_3OS$: C 69.6, H 6.62, N 11.07, S 8.45%. Found: C 69.98, H 6.53, N 11.23, S 8.46%. IR ν_{max} (cm^{-1}): 1687 (–N–C=O), 1620 (C=N), 749 (C–S–C), 1332 (tert. N), 1573 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.41–7.33 (m, 4H, Ar–H), 7.19–7.15 (t, 1H, J = 7.2 Hz, Ar–H), 7.02 (t, 2 H, J = 7.2 Hz, Ar–H), 6.68 (d, 2H, J = 8.71 Hz, Ar–H), 7.67 (s, 1H, CH=C), 3.01 (s, 6H, $N(CH_3)_2$), 3.83 (d, 2H, J = 7.5 Hz, CH_2), 2.41–2.32 (m, 1H, CH), 1.01 (d, 6H, J = 6.6 Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 166.44 (C=O), 151.26 (C=N), 156.35 (SC=C), 148.31 (C=C), 131.59, 129.38, 128.60, 126.97, 124.82, 121.38, 121.12, 120.35, 120.22 (aromatic), 116.26 (H_3C–N), 50.26 (C–N), 27.06 (CH), 20.20 (CH_3); TOF MS ES$^+$ m/z: [M^+] 379 (100%).

4.3.4. (2Z,5Z)-3-(2-Methylpropyl)-5-(5-nitrobenzylidene)-2-(phenylimino)-1,3-thiazolidin-4-one (**6**)

Yield: 85%; m.p: 185 °C; Anal. calc. $C_{20}H_{19}N_3O_3S$: C 62.98, H 5.05, N 11.02, S 8.41%. Found: C 63.37, H 4.96, N 11.13, S 8.31%. IR ν_{max} (cm^{-1}): 1701 (–N–C=O), 1633 (C=N), 752 (C–S–C), 1376 (tert. N), 1581 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 8.26 (d, 2H, J = 8.7 Hz, Ar–H), 7.60 (d, 2H, J = 8.7 Hz, Ar–H), 7.44 (t, 2H, J = 7.8 Hz, Ar–H), 7.26 (t, 1H, J = 7.2 Hz, Ar–H), 7.00 (d, 2H, J = 7.5 Hz, Ar–H), 7.75 (s, 1H, CH=C), 3.88 (d, 2H, J = 7.2 Hz, CH_2), 2.40–2.31 (m, 1H, CH), 1.03 (d, 2H, J = 6.6 Hz, $CH(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 166.42 (C=O), 149.33 (C=N), 124.17 (S–C=C), 148.93 (C=C), 140.03, 130.28, 129.47, 127.41, 126.5, 125.20, 120.96 (aromatic), 50.58 (C–N), 27.01 (CH), 20.10 (CH_3); TOF MS ES$^+$ m/z: [M^+] 381 (100%).

4.3.5. (2Z,5Z)-5-(4-Hydroxybenzylidene)-3-isobutyl-2-(phenylimino) thiazolidin-4-one (**7**)

Yield: 85%; m.p: 190 °C; Anal. calc. $C_{20}H_{20}N_2O_2S$: C 68.16, H 5.72, N 7.95, S 9.10%. Found: C 68.12, H 5.68, N 7.92, S 9.07%. IR ν_{max} (cm^{-1}): 3495 (OH), 1702 (–N–C=O), 1630 (C=N), 751 (C–S–C), 1370 (tert. N), 1581 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.38–7.34 (m, 3H, Ar–H), 7.25–7.16 (m, 2H, Ar–H), 7.00 (d, 2H, J = 8.1 Hz, Ar–H), 6.93 (t, 2H, J = 6.9 Hz, Ar–H), 8.35 (s, 1H, OH), 7.41 (s, 1H, CH=C), 3.89 (d, 2H, J = 7.5 Hz, CH_2), 2.41–2.32 (m, 1H, CH), 1.03 (d, 6H, J = 6.6 Hz, $CH(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.61 (C=O), 151.26 (C=N), 124.63 (S–C=C), 148.53 (C=C), 159.35, 154.35, 132.20, 131.15, 129.32, 129.24, 125.20, 124.57, 121.18, 117.59, 116.54 (aromatic), 50.14 (C–N), 26.95 (CH), 20.05 (CH_3); TOF MS ES$^+$ m/z: [M^+] 381 (100%).

4.3.6. (2Z,5Z)-5-(3,4-Dimethoxybenzylidene)-3-isobutyl-2-(phenylimino)thiazolidin-4-one (**8**)

Yield: 85%; m.p: 115 °C; Anal. calc. $C_{22}H_{24}N_2O_3S$: C 66.64, H 6.10, N 6.07, S 8.09%. Found: C 66.69, H 5.96, N 6.04, S 8.06%; IR ν_{max} (cm^{-1}); 1709 (–N–C=O), 1633 (C=N), 768 (C–S–C), 1379 (tert. N), 1593 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.40 (t, 2H J = 7.5 Hz, Ar–H), 7.20 (t, 1H, J = 7.5 Hz, Ar–H), 7.07–7.04 (m, 1H, Ar–H), 7.01–6.99 (m, 2H, Ar–H), 6.94 (d, 1H, J = 1.8 Hz, Ar–H), 6.89 (d, 1H, J = 8.4 Hz, Ar–H), 7.67 (s, 1H, CH=C), 3.85 (s, 6H, $(OCH_3)_2$), 3.85 (d, 2H, J = 8.4 Hz, CH_2), 2.40–2.31 (m, 1H, CH), 1.01 (d, 6H, J = 6.6 Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.16 (C=O), 150.75 (C=N), 124.6 (S–C=C), 148.36 (C=C), 149.09, 30.66, 129.63, 129.25, 128.88, 127.1, 126.80, 123.28, 121.10, 119.30 (aromatic), 55.92 (OCH_3), 50.10 (C–N), 27.14 (CH), 20.08 (CH_3); TOF MS ES$^+$ m/z: [M^+] 396 (100%).

4.3.7. (2Z,5Z)-2-(4-Methoxybenzylidene)-3-isobutyl-2-(phenylimino) thiazolidin-4-one (**9**)

Yield: 85%; m.p: 149 °C; Anal. calc. $C_{21}H_{22}N_2O_2S$: C 68.83, H 6.05, N 7.64, S 8.75%. Found: C 68.79, H 6.02, N 7.61, S 8.70%. IR ν_{max} (cm^{-1}): 1707 (–N–C=O), 1625 (C=N), 762 (C–S–C), 1372 (tert. N), 1556 (C=C); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.41 (t, 4H, J = 8.4 Hz, Ar–H), 7.21 (t, 1H, J = 7.2 Hz, Ar–H), 7.01 (t, 2H, J = 7.2 Hz, Ar–H), 6.93 (d, 2H, J = 8.7 Hz, Ar–H), 7.67 (s, 1H, CH=C), 3.84 (s, 3H, CH_3), 3.80 (d,

2H, $J = 7.2$ Hz, CH_2), 2.40–2.17 (m, 1H, CH), 1.01 (d, 6H, $J = 6.6$ Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.35 (C=O), 150.99 (C=N), 150.99 (S–C=C), 148.49 (C=C), 131.78, 130.53, 129.32, 126.48, 124.67, 121.14, 118.79, 114.49 (aromatic), 55.37 (OCH_3), 50.11 (C–N), 26.94 (CH), 20.11 (CH_3); TOF MS ES$^+$ *m/z*: [M$^+$] 366 (100%).

*4.3.8. (2Z,5Z)-3-(5-Methylbenzylidene)-3-(2-methylpropyl)-2-(phenylimino)-1,3-thiazolidin-4-one (**10**)*

Yield: 8%; m.p: 104 °C; Anal. calc. $C_{21}H_{22}N_2O_2S$: C 68.38, H 6.05, N 6.05, S 8.75%. Found: C 68.79, H 6.02, N 7.61, S 8.75%. IR ν_{max} (cm^{-1}): 1690 (–N–C=O), 1620 (C=N), 760 (C–S–C), 1371 (tert. N), 1559 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.41–7.32 (m, 6H, Ar–H), 7.00–6.92 (m, 3H, Ar–H), 7.71 (s, 1H, CH=C), 3.84 (d, 2H, $J = 7.5$ Hz, CH_2), 2.43–2.30 (m, 1H, CH), 1.01 (d, 6H, $J = 6.6$ Hz, $CH(CH_3)_2$), 2.28 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.20 (C=O), 150.04 (C=N), 124.23 (S–C=C), 148.40 (C=C), 151.65, 133.73, 131.53, 130.32, 129.95, 129.48, 127.14, 126.45 (aromatic), 50.05 (C–N), 26.94 (CH), 23.54 (CH_3), 20.01 $(CH_3)_2$CH; TOF MS ES$^+$ *m/z*: [M$^+$] 350 (100%).

*4.3.9. (2Z,5Z)-5-(2-Hydroxybenzylidene)-3-isobutyl-2-(phenylimino) thiazolidin-4-one (**11**)*

Yield: 85%; m.p: 213 °C; Anal. calc. $C_{20}H_{20}N_2O_2S$: C 68.16, H 5.72, N 7.95, S 9.10%. Found: C 68.13, H 5.72, N7.91, S 9.05%. IR ν_{max} (cm^{-1}): 3510 (OH), 1675 (–N–C=O), 1623 (C=N), 745 (C–S–C), 1339 (tert. N), 1582 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.38–7.33 (t, 3H, $J = 7.2$ Hz, Ar–H), 7.22–7.15 (m, 2H, Ar–H), 7.00 (d, 2H, $J = 8.1$ Hz, Ar–H), 6.91–6.87 (m, 2H, Ar–H), 8.30 (s, 1H, OH), 7.40 (s, 1H, CH=C), 3.87 (d, 2H, $J = 7.5$ Hz, CH_2), 2.40–2.31 (m, 1H, CH), 1.02 (d, 6H, $J = 6.6$ Hz, $CH(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.18 (C=O), 150.09 (C=N), 124.20 (S–C=C), 148.42 (C=C), 155.34, 151.12, 133.73, 131.53, 130.32, 129.95, 129.48, 127.14, 126.45, 122.34 (aromatic); TOF MS ES$^+$ *m/z*: [M$^+$] 352 (100%).

*4.3.10. (2Z,5Z)-5-(Benzo[d][1,3]dioxol-4-ylmethylene)-3-isobutyl-2(phenylimino)thiazolidin-4-one (**12**)*

Yield: 85%; m.p:146 °C; Anal. calc. $C_{21}H_{20}N_2O_3S$: C 66.30, H 5.30, N 7.36, S 8.43%. Found: C 66.25, H 5.27, N 7.33, S 8.40%. IR ν_{max} (cm^{-1}): 1694 (–N–C=O), 1632 (C=N), 756 (C–S–C), 1373 (tert. N), 1589 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.41 (t, 2H, $J = 7.8$ Hz, Ar–H), 7.21 (t, 1H, $J = 7.2$ Hz, Ar–H), 7.00 (t, 4H, $J = 10.2$ Hz, Ar–H), 6.84 (d, 1H, $J = 8.1$ Hz, Ar–H), 7.65 (s, 1H, CH=C), 6.00 (s, 2H, CHO_2), 2.17 (d, 2H, $J = 6.9$ Hz, CH_2), 2.39–2.30 (m, 1H, CH), 1.01 (d, 6H, $J = 6.6$ Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ(ppm): 167.22 (C=O), 150.70 (C=N), 124.75 (S–C=C), 148.37 (C=C), 148.94, 148.26, 130.57, 129.36, 128.10, 125.86, 121.08, 119.45 (aromatic), 109.05 (CHO_2), 50.16 (C–N), 26.94 (CH), 20.11 (CH_3); TOF MS ES$^+$ *m/z*: [M$^+$] 380 (100%).

*4.3.11. (2Z,5Z)-3-Isobutyl-2-(phenylimino)-5-(pyridine-2-ylmethylne) thiazolidin-4-one (**13**)*

Yield: 85%; m.p: 196 °C: Anal. calc. $C_{19}H_{19}N_3OS$: C 67.63, H 5.68, N 12.45, S 9.50%. Found: C 67.97, H 5.65, N 12.57, S 9.27%. IR ν_{max} (cm^{-1}): 1700 (–N–C=O), 1634 (C=N), 763 (C–S–C), 1342 (tert. N), 1577 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 8.65 (d, 1H, $J = 4.5$ Hz Ar–H), 7.72–7.70 (m, 1H, Ar–H), 7.45–7.38 (m, 3H, Ar–H), 7.20–7.15 (m, 2H, Ar–H), 7.03 (t, 2H, $J = 8.7$ Hz, Ar–H), 7.73 (s, 1H, CH=C), 3.85 (d, 2H, $J = 7.5$ Hz, CH_2), 2.40–2.31 (m, 1H, CH), 1.01 (d, 6H, $J = 6.9$ Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 1679.22 (C=O), 149.28 (C=N), 124.47 (S–C=C), 148.26 (C=C), 154.18, 152.49, 136.65, 129.28, 127.31, 126.51, 126.27, 122.69, 121.22 (aromatic), 9.58 (C–N), 27.02 (CH), 20.13 (CH_3); TOF MS ES$^+$ *m/z*: [M$^+$] 337 (100%).

*4.3.12. (2Z,5Z)-3-isobutyl-5-((3-Methylthiophene-2-yl)methylene-2-(phenylimino)thiazolidin-4-one (**14**)*

Yield: 85%; m.p: 172 °C; Anal. calc. $C_{19}H_{20}N_2OS_2$: C 64.01, H 5.65, N 7.86, S 17.99%. Found: C 64.00, H 5.63, N 7.84, S 17.95%. IR ν_{max} (cm^{-1}): 1694 (–N–C=O), 1629 (C=N), 763 (C–S–C), 1370 (tert. N), 1582 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.44–7.36 (m, 3H, Ar–H), 7.20 (t, 1H, $J = 7.5$ Hz, Ar–H), 7.01 (t, 2H, $J = 7.5$ Hz, Ar–H), 6.96 (d, 1H, $J = 5.1$ Hz, Ar–H), 7.97 (s, 1H, CH=C), 2.40 (s, 3H, CH_3), 3.84 (d, 2H, $J = 7.5$ Hz, CH_2), 2.38–2.26 (m, 1H, CH), 1.01 (d, 6H, $J = 6.9$ Hz, $CH(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.15 (C=O), 150.44 (C=N), 124.76 (S–C=C), 148.32 (C=C), 142.19, 132.57, 130.97, 129.35, 129.16, 123.50, 122.08, 121.15, 118.85 (aromatic), 50.3 (C–N), 26.99 (CH), 20.1 (CH_3), 14.42 (CH_3); TOF MS ES$^+$ *m/z*: [M$^+$] 356 (100%).

*4.3.13. (2Z,5Z)-5-(4-Hydroxy-3-methoxybenzylidene)-3-isobutyl-2 (phenylimino)thiazolidin-4-one (**15**)*

Yield: 85%; m.p: 163 °C; Anal. calc. $C_{21}H_{22}N_2O_3S$: C 65.95, H 5.80, N 7.32, S 8.38%. Found: C 66.30, H 6.09, N 7.7, S 7.89%. IR ν_{max} (cm^{-1}): 3513.49 (OH), 1690 (–N–C=O), 1624 (C=N), 756.45 (C–S–C), 1363.13 (tert. N), 1579.24 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.40 (t, 2H, $J = 7.5$ Hz, Ar–H), 7.20–7.15 (m, 1H, Ar–H), 7.03 (t, 3H, $J = 7.5$ Hz, Ar–H), 6.98 (d, 2H, $J = 4.5$ Hz, Ar–H), 7.67 (s, 1H, CH=C), 3.88 (s, 3H, OCH_3), 3.84 (d, 2H, $J = 7.5$ Hz, CH2), 2.39–2.30 (m, 1H, CH), 1.01 (d, 6H, $J = 6.9$ Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.10 (C=O), 150.82 (C=N), 124.71 (S–C=C), 148.40 (C=C), 147.4, 146.74, 130.90,129.31, 126.38, 123.89, 121.14, 118.92, 115.06, 112.64 (aromatic), 5 6.02 (OCH_3), 50.3 (C–N), 26.96 (CH), 20.12 (CH), 15.12 (CH_3); TOF MS ES$^+$ *m/z*: [M$^+$] 382 (100%).

*4.3.14. (2Z,5Z)-5-(3-Chlorobenzylidene)-3-isobutyl-2-(phenylimino) thiazolidin-4-one (**16**)*

Yield: 85%; m.p: 145 °C; Anal calc. $C_{20}H_{19}ClN_2OS$: C 64.77, H 5.16, N 7.55, S 8.64%. Found: C 64.53, H 5.58, N7.93, S 8.60%. IR ν_{max} (cm^{-1}): 1701 (–N–C=O), 1612 (C=N), 768 (C–S–C), 1374 (tert. N), 1581 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.41 (t, 4H, $J = 7.5$ Hz, Ar–H), 7.20 (t, 1H, $J = 7.2$ Hz, Ar–H), 7.00 (d, 2H, $J = 7.2$ Hz, Ar–H), 6.92 (d, 2H, $J = 8.7$ Hz, Ar–H), 7.69 (s, 1H, CH=C), 3.84 (d, 2H, $J = 7.8$ Hz, CH_2), 2.37–2.33 (m,1H, CH), 1.01 (d, 6H, $J = 6.6$ Hz, CH $(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.36 (C=O), 151.00 (C=N), 124.69 (S–C=C), 148.53 (C=C), 133.32, 131.80, 130.55, 129.35, 127.85, 126.53, 122.67, 121.17, 118.85, 114.53 (aromatic), 50.14 (C–N), 26.97 (CH), 20.14 (CH_3); TOF MS ES$^+$ *m/z*: [M$^+$] 370 (100%).

*4.3.15. (2Z,5Z)-5-(2,5-Dimethoxybenzylidene)-3-isobutyl-2-(phenylimino)thiazolidin-4-one (**17**)*

Yield: 85%; m.p: 135 °C; Anal. calc. $C_{22}H_{24}N_2O_3S$: C 66.64, H 6.10, N 7.06, S 8.09%. Found: C 67.09, H 6.01, N 7.10, S 7.95%. IR ν_{max} (cm^{-1}): 1695 (–N–C=O), 1615 (C=N), 771 (C–S–C), 1372 (tert. N), 1581 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.38–7.33 (m, 2H, Ar–H), 7.18 (t, 1H, $J = 7.2$ Hz, Ar–H), 6.98 (t, 2H, $J = 7.2$ Hz, Ar–H), 6.92 (d, 1H, $J = 8.4$ Hz, Ar–H), 6.85–6.80 (m, 2H, Ar–H), 8.08 (s, 1H, CH=C), 3.84 (s, 6H, $(OCH_3)_2$), 3.81 (d, 2H, $J = 7.2$ Hz, CH_2), 2.37–2.32 (m, 1H, CH), 1.01 (d, 6H, $J = 6.9$ Hz, $CH(CH_3)_2$); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.06 (C=O), 150.88 (C=N), 124.70 (S–C=C), 148.30 (C=C), 153.37, 152.27, 129.30, 125.90, 123.79, 122.18, 121.11, 115.84, 114.60, 111.96 (aromatic), 56.07 (OCH_3), 55.80 (OCH_3), 50.14 (C–N), 26.98 (CH_2), 20.16 (CH_3); TOF MS ES$^+$ *m/z*: [M$^+$] 396 (100%).

*4.3.16. (2Z,5Z)-5-(4-Ethylbenzylidene)-3-isobutyl-2-(phenylimino) thiazolidi-4-one (**18**)*

Yield: 85%; m.p: 125 °C; Anal. calc. $C_{22}H_{24}N_2OS$: C 72.49, H 6.64, N 7.69, S 8.80%. Found: C 72.73, H 6.562, N 7.74, S 8.566%. IR ν_{max} (cm^{-1}): 1706 (–N–C=O), 1625 (C=N), 759 (C–S–C), 1372 (tert. N), 1586 (C=C); ^1H NMR ($CDCl_3$) δ (ppm): 7.41 (t, 4H, $J = 7.8$ Hz, Ar–H), 7.25–7.15 (m, 3H, Ar–H), 7.00 (t, 2H, $J = 7.2$ Hz, Ar–H), 7.72 (s, 1H, CH=C), 3.84 (d, 2H, $J = 7.5$ Hz, CH_2), 2.40–2.31 (m, 1H, CH), 1.01 (d, 6H, $J = 6.9$ Hz, $CH(CH_3)_2$), 2.69 (q, 2H, $J = 7.5$ Hz, CH_2), 1.24 (t, 3H, $J = 7.5$ Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 167.27 (C=O), 150.94 (C=N), 124.74 (S–C=C), 148.44 (C=C), 146.49, 131.30, 130.84,

129.36, 128.56, 121.56, 120.57 (aromatic), 50.18 (C–N), 26.99 (CH), 20.15 (CH_3), 28.82 (CH_2), 15.24 (CH_3); TOF MS ES$^+$ m/z: [M$^+$] 364 (100%).

4.4. X-ray crystal structure determination

Three-dimensional X-ray data for compound **9** were collected on a Bruker SMART Apex CCD diffractometer at 298(2) K, using a graphite monochromator and Mo-K_α radiation ($\lambda = 0.71073$ Å) by the φ–ω scan method. The measurement was not done at low temperature because the crystals disintegrated. Reflections were measured from a hemisphere of data collected of frames each covering 0.3° in ω. Of the 19,416 reflections measured, all of which were corrected for Lorentz and polarization effects, and for absorption by semi-empirical methods based on symmetry-equivalent and repeated reflections, 2323 independent reflections exceeded the significance level $|F|/\sigma(|F|) > 4.0$. Complex scattering factors were taken from the program package SHELXL [54]. The structures were solved by direct methods and refined by full-matrix least-squares methods on F^2. The non-hydrogen atoms were refined with anisotropic thermal parameters in all cases. All hydrogen atoms were refined to carbon atoms, which were placed in idealized positions and refined by using a riding mode, except for C(8), C(10) and C(14) which were left to refine freely. The crystal presents a slight disorder on methoxybenzylidene group. This disorder has been observed and refined with anisotropic atomic displacement parameters. The site occupancy factor was 0.67324 for C(18A) and C(19A). A final difference Fourier map showed no residual density outside: 0.645 and −0.322 e Å^{-3}. CCDC No. 872074 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk.

4.5. In vitro antiamoebic assay

All the compounds (**3**–**18**) were screened *in vitro* for anti-amoebic activity against HM1:IMSS strain of *E. histolytica* by microdilution method. *E. histolytica* trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [55]. The test compounds (1 mg) were dissolved in DMSO (40 μL, level at which no inhibition of amoeba occurs) [56,57]. The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg/mL. Two-fold serial dilutions were made in the wells of 96-well microtiter plate (costar). Each test includes metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoeba) 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 amoeba 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 2.

4.6. Cytotoxicity studies (MTT assay)

4.6.1. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle's medium with 10% foetal bovine serum (heat inactivated), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B, at 37 °C in a saturated humidity atmosphere containing 95% air/5% CO_2 [58]. The cell lines were harvested when they reached 80% confluence to maintain exponential growth.

4.6.2. 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 [59]. For viability testing, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, M2128 Sigma–Aldrich) 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) 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) and incubated for further 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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in online version at http://dx.doi.org/10.1016/j.ejmech.2012.06.052.

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