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Synthesis and in vitro anti-leishmanial activity of 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-yl]- and 1-[5-(5-nitrothiophen-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines

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Abstract—The synthesis and anti-leishmanial activity of nitroheteroaryl-1,3,4-thiadiazole-based compounds including 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines and 1-[5-(5-nitrothiophen-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines were described. Most of the synthesized compounds exhibited potent anti-leishmanial activity against both promastigote and amastigote forms of *Leishmania major* at non-cytotoxic concentrations. In general, 5-nitrofuran derivatives were more active than the corresponding 5-nitrothiophene analogues.

1. Introduction

Parasitic diseases such as leishmaniasis have significant impacts on the world; especially in developing countries with infections spread over several hundred millions of people and are one of the significant causes of morbidity and mortality.[1]

The treatment options for leishmaniasis are limited and involve the administration of pentavalent antimonial agents as first line and amphotericin B and pentamidine as second line drugs.[2,3] These drugs are expensive and potentially toxic and require long-term treatment. In addition the development of drug resistance by the pathogens especially in HIV leishmania co-infected patients has aggravated public health risk.[4] The development of new effective, cheap and safe drugs for the treatment of leishmaniasis is therefore an urgent task.[5]

A great number of natural and synthetic compounds have been tested in the recent years in anti-leishmanial assays.[6–8] The use of nitroheterocycles such as 5-nitrofurans and 5-nitrothiophenes as antibacterial and antiprotozoal is well established.[9,10] On the other hand, the antiparasitc property of 1,3,4-thiadiazoles is well documented and their attachment with other heterocycles often ameliorates or diminishes the bioresponses, depending upon the type of substituent and position of attachment.[11,12] Various 2,5-disubstituted-1,3,4-thiadiazole analogues have been previously synthesized and some of them showed excellent leishmanicidal activity.[5,13,14] All of these compounds had a nitrogen heterocycle linked to the C-5 position of 1,3,4-thiadiazole ring through the heterocyclic nitrogen. Previous results demonstrated that C-5 substituent is the most adaptable site for chemical change and is an area where it determines the potency and physicochemical properties of 2-(nitroaryl)-1,3,4-thiadiazoles. Accordingly, in the preceding papers we described a number of 2-(nitroaryl)-1,3,4-thiadiazoles bearing a piperazin-1-yl substituent at the C-5 position of 1,3,4-thiadiazole nucleus. The biological activity of 1-[5-(nitroaryl)-1,3,4-thiadiazol-2-yl]piperazines indicated that the piperazine moiety of these compounds possesses enough structural flexibility

Keywords: 1,3,4-Thiadiazole; Nitrothiophene; Nitrofuran; *Leishmania major*; Anti-leishmanial activity; Promastigote; Amastigote.
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to allow product optimization.[5,14] These facts motivate our concern to N-4 substituent of 1-[5-(nitroaryl)-1,3,4-thiadiazol-2-yl]piperazines. Thus, we report here the synthesis and in vitro anti-leishmanial activity of some 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines **6**–**12a** and 1-[5-(5-nitrothiophen-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines **6**–**12b**.

2. Chemistry

The synthesis of 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines **6**–**12a** and 1-[5-(5-nitrothiophen-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines **6**–**12b** was achieved with an efficient synthetic strategy outlined in Figure 1. Intermediates 2-amino-1,3,4-thiadiazoles **3a**,**b**[15,16] were obtained from 5-nitro-2-arylidene diacetates **1**. Thus, treatment of compound **1** with thiosemicarbazide in the presence of HCl in ethanol afforded thiosemicarbazone which cyclized using $NH_4Fe(SO_4)_2$ to give 2-amino-1,3,4-thiadiazoles **3a**,**b**. Diazotization of amine **3** in hydrochloric acid in the presence of copper powder gave the 2-chloro-1,3,4-thiadiazole **4**. The reaction of compounds **4a**,**b** with piperazine in refluxing EtOH gave the corresponding *N*-piperazinyl compounds **5a**,**b**. N-aroylation of the piperazine analogues **5a**,**b** with appropriate thiophen-2-carbonyl chlorides or benzoyl chlorides afforded target compounds **6**–**12a**,**b**. The synthesis of compounds **6a** and **6b** has been reported previously.[14] Physicochemical and spectral data of compounds **7**–**12a**,**b** are shown in Tables 1 and 2, respectively.

3. Results and discussion

The leishmania life cycle consists of two developmental forms: promastigotes, flagellated extracellular parasites of the digestive tract of sand flies, and amastigotes, non-flagellated, non-motile stages that is more sensitive and live inside parasitophorous vacuoles in macrophages of mammalian hosts.[2] In the present study we describe the evaluation of anti-leishmania activity of target compounds on both Leishmania stages.

The nitrofuran and nitrothiophene analogues **6**–**12a**,**b** were tested for their in vitro activity against the promastigote form of the *Leishmania major* strain (MRHO/IR/75/ER) along with meglumine antimonate (Glucantime®), using MTT assay.[17] The IC_{50} values (in μM) against promastigotes, in comparison with Glucantime, are listed in Table 3. The most potent compounds against the promastigote form of *L. major* were found to be nitrofuran analogues **6a**, **7a**, **8a**, **11a** and **12a** with IC_{50} values between 10.73 ± 0.69 and 13.19 ± 0.96 μM. The activity profile of these compounds against promastigotes demonstrated that there is a small difference in their IC_{50} values. In particular, compound **7a** designed as a nitrofuran derivative containing 2-chlorobenzoyl on the piperazine ring, with the IC_{50} = 10.73 μM, was found to be the most active compound. Although halogen substitution (Cl or Br) on thiophen-2-carbonyl moiety seems to increase the activity in both nitrofuran and nitrothiophene analogues (compounds **10a** and **10b** when compared with **11**–**12a** and **11**–**12b**, respectively) but chloro-substitution on benzoyl containing compounds (**6a** and **6b**) cannot improve anti-leishmania activity. The effect of positional substitution was investigated by preparing all three possible chloro-substitutions on benzoylpiperazine moiety. The better results were achieved with 2-chloro- in nitrofuran series, and 4-chloro- in nitrothiophene derivatives.

Comparison between IC_{50} values of nitrofurans **6**–**12a** and nitrothiophene analogues **6**–**12b** against promastigotes revealed that nitrofurans possessed better activity with respect to corresponding nitrothiophenes, with the exception of 4-chlorobenzoyl derivatives (**9a** and **9b**), which nitrothiophene analogue **9b** showed slightly better activity than nitrofuran counterpart **9a**.

Compounds **6a, 7a, 8a, 11a** and **12a** that exhibited potent activity ($IC_{50} \leqslant 13.19$ μM) against promastigotes were also assessed for their activity against the amastigote form of *L. major* in marine peritoneal macrophages (Fig. 2).[18] All selected compounds significantly reduced intracellular amastigotes. Among them, compounds **6a** and **11a** were found to be more efficient in reducing intracellular amastigotes (Fig. 2A). Moreover, all se-

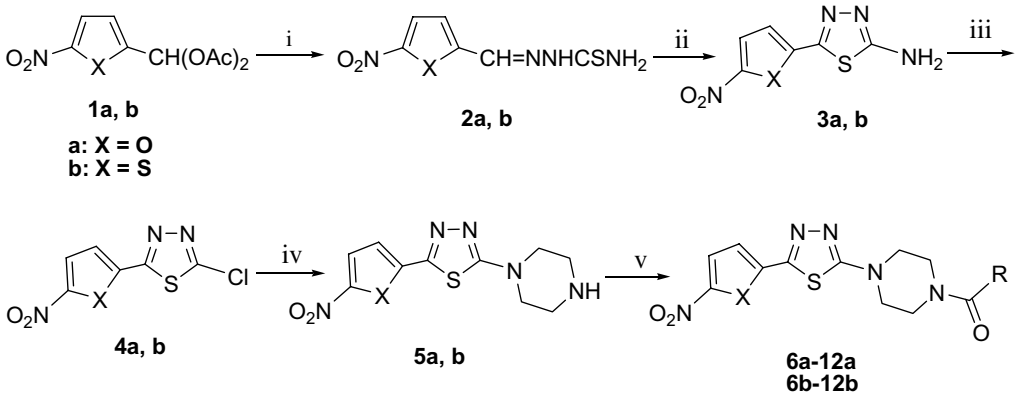
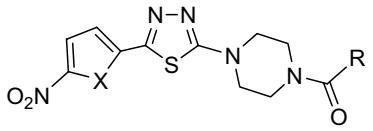
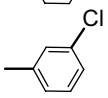
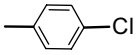
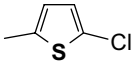
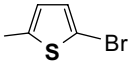
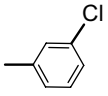
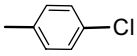
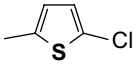
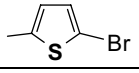


Figure 1. Synthesis of compounds **6**–**12a,b**. Reagents and conditions: (i) thiosemicarbazide, EtOH, HCl, reflux; (ii) ammonium ferric sulfate, H_2O, reflux; (iii) $NaNO_2$, HCl, Cu; (iv) piperazine, EtOH, reflux; (v) appropriate thiophen-2-carbonyl chlorides or benzoyl chlorides, benzene, pyridine, rt.

Table 1. Structures and physicochemical data of compounds **7–12a,b**



Compound	X	R	Mp (°C)	Yield[a] (%)	M_W	Formula
7a	O		225–227	78	419.84	$C_{17}H_{14}ClN_5O_4S$
8a	O		217–219	80	419.84	$C_{17}H_{14}ClN_5O_4S$
9a	O		244–246	83	419.84	$C_{17}H_{14}ClN_5O_4S$
10a	O		239–241	81	391.42	$C_{15}H_{13}N_5O_4S_2$
11a	O		223–225	73	425.87	$C_{15}H_{12}ClN_5O_4S_2$
12a	O		241–243	69	470.32	$C_{15}H_{12}BrN_5O_4S_2$
7b	S		243–245	73	435.91	$C_{17}H_{14}ClN_5O_3S_2$
8b	S		250–252	78	435.91	$C_{17}H_{14}ClN_5O_3S_2$
9b	S		260–262	81	435.91	$C_{17}H_{14}ClN_5O_3S_2$
10b	S		247–249	82	407.49	$C_{15}H_{13}N_5O_3S_3$
11b	S		250–252	73	441.94	$C_{15}H_{12}ClN_5O_3S_3$
12b	S		261–263	70	486.39	$C_{15}H_{12}BrN_5O_3S_3$

[a] Yields of the final step.

lected compounds significantly decreased the percentage of macrophage infectivity (Fig. 2B), and infectivity index (Fig. 2C).

The cytotoxicity of target compounds was also assessed using MTT colorimetric assay on macrophage cells. Macrophage cells were treated with synthesized compounds in the IC_{50} for 24 h, and side by side these were treated with the standard drug Glucantime. The results (Table 3) indicated that these compounds in the IC_{50} concentrations (against promastigote form of the *L. major*) are not remarkably toxic towards marine peritoneal macrophage cells (cell death <10%). Whereas, the reference drug Glucantime showed about 40% toxicity in host cells.

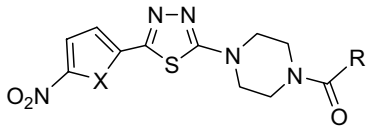
It is believed that the lipophilic character of the molecule plays an essential role in producing the biological effect. The permeability of the cell to the test compounds is one of the factors determining their activity. Drugs cross biological barriers most frequently through passive transport, which strongly depends on their lipophilicity. Therefore, hydrophobicity is one of the most important physical properties of biologically active compounds. This thermodynamic parameter describes the partitioning of a compound between an aqueous and an organic phase, and is characterized by the octanol/water partition coefficient.[19] In this context the balance of hydrophobicity would be important for such activity. Log *P*, that is, the logarithm of the partition coefficient for *n*-octanol/water, was calculated using the program HyperChem ver. 7.0.

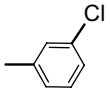
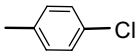
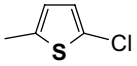
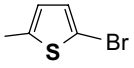
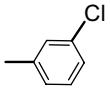
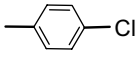
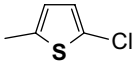
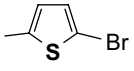
Table 2. Spectral data of compounds **7–12a,b**

Compound	IR (KBr); ν_{max}	^{1}H NMR (80 MHz, DMSO-d_6) δ (ppm)	MS (*m/e*, %)
7a	1629 (C=O), 1542 and 1342 cm^{-1} (NO_2)	7.85 (d, 1H, H_4-furan, J = 4.0 Hz), 7.35–7.55(m, 5H, H_3-furan and phenyl), 3.60–3.80 (m, 8H, piperazine).	421 ([M$^+$+2], 7), 419 (M$^+$, 18), 308 (10), 263 (28), 166 (24), 139 (100), 110 (50), 68 (40), 55 (60).
8a	1634 (C=O), 1511 and 1342 cm^{-1}(NO_2)	7.85 (d, 1H, H_4-furan, J = 4.0 Hz), 7.43–7.58 (m, 4H, phenyl), 7.4 (d, 1H, H_3-furan, J = 4.0 Hz), 3.65–3.75 (m, 8H, piperazine).	421 ([M$^+$+2], 4), 419 (M$^+$, 10), 401 (43), 376 (25), 308 (100), 196 (43), 138 (20), 90 (50), 56 (57).
9a	1634 (C=O), 1512, 1352 cm^{-1} (NO_2)	7.85 (d, 1H, H_4-furan, J = 4.0 Hz), 7.54 (s, 4H, phenyl), 7.42 (d, 1H, H_3-furan, J = 4.0 Hz), 3.69 (br s, 8H, piperazine).	421 ([M$^+$+2], 4), 419 (M$^+$, 10), 376 (25), 307 (76), 373 (25), 196 (43), 139 (91), 91 (100), 56 (73).
10a	1608 (C=O), 1539 and 1352 cm^{-1} (NO_2)	7.86 (d, 1H, H_4-furan, J = 4.0 Hz), 7.6–7.8 (m, 1H, H_5-thiophene), 7.41 (d, 1H, H_3- furan, J = 4.0 Hz), 7.45–7.55 (m, 1H, H_3-thiophene), 7.10–7.25 (m, 1H, H_4-thiophene), 3.57–4.0 (m, 8H, piperazine).	391 (M$^+$, 17), 280 (10), 166 (20), 111 (100).
11a	1602 (C=O), 1533 and 1356 cm^{-1} (NO_2)	7.85 (d, 1H, H_4-furan, J = 4.0 Hz), 7.40 (d, 2H, H_3-furan, H_3-thiophene, J = 4.0 Hz), 7.19 (d, 1H, H_4-thiophene, J = 4.0 Hz), 3.57–4.0 (m, 8H, piperazine).	426 ([M$^+$+2], 7), 424 (M$^+$, 18), 262 (34), 237 (19), 164 (24), 145 (100), 143 (95), 81 (37), 55 (40).
12a	1607 (C=O), 1540, 1361 cm^{-1} (NO_2)	7.85 (d, 1H, H_4-furan, J = 4.0 Hz), 7.40 (d, 1H, H_3-thiophene, J = 4.0 Hz), 7.34 (d, 1H, H_3-furan, J = 3.9 Hz), 7.29 (d, 1H, H_4- thiophene, J = 3.9 Hz), 3.59–3.96 (m, 8H, piperazine).	471 ([M$^+$+2], 40), 469 (M$^+$, 40), 390 (20), 281 (24), 264 (90), 239 (64), 187 (100), 167 (60), 80 (75), 54 (98).
7b	1634 (C=O), 1530, 1337 cm^{-1} (NO_2)	8.17 (d, 1H, H_4-thiophene, J = 4.0 Hz), 7.35–7.65 (m, 5H, H_3-thiophene and phenyl), 3.65–3.95 (m, 8H, piperazine).	437 ([M$^+$+2], 7), 435 (M$^+$, 18), 308 (13), 223 (18), 139 (100), 111 (56), 56 (40).
8b	1644 (C=O), 1536, 1347 cm^{-1} (NO_2)	8.18 (d, 1H, H_4-thiophene, J = 4.2 Hz), 7.40–7.60 (m, 5H, H_3-thiophene and phenyl), 3.7–4.0 (m, 8H, piperazine).	437 ([M$^+$+2], 5), 435 (M$^+$, 13), 139 (100), 111 (50), 55 (48).
9b	1634 (C=O), 1527, 1347 cm^{-1} (NO_2)	8.12 (d, 1H, H_4-thiophene, J = 4.2 Hz), 7.57 (d, 1H, H_3-thiophene, J = 4.2 Hz), 7.51 (s, 4H, phenyl), 3.66 (br s, 8H, piperazine).	437 ([M$^+$+2], 7), 435 (M$^+$, 10), 280 (18), 254 (25), 182 (25), 139 (100), 111 (50), 56 (46).
10b	1608 (C=O), 1521, 1342 cm^{-1} (NO_2)	8.13 (d, 1H, H_4-nitrothiophene, J = 4.2 Hz), 7.7–7.9 (m, 1H, H_5-thiophene, J = 4.2 Hz), 7.65 (d, 1H, H_3-nitrothiophene, J = 4.2 Hz), 7.45–7.6 (m, 1H, H_3-thiophene), 7.15–7.25 (m, 1H, H_4-thiophene), 3.52–4.0 (m, 8H, piperazine).	407 (M$^+$, 10), 279 (15), 152 (18), 111 (60), 109 (100).
11b	1605 (C=O), 1522, 1368 cm^{-1} (NO_2)	8.15 (d, 1H, H_4-nitrothiophene, J = 4.2 Hz), 7.59 (d, 1H, H_3-nitrothiophene, J = 4.2 Hz), 7.39 (d, 1H, H_3-thiophene, J = 4.0 Hz), 7.19 (d, 1H, H_4-thiophene, J = 4.0 Hz), 3.52–4.05 (m, 8H, piperazine).	443 ([M$^+$+2], 4), 441 (M$^+$, 10), 280 (15), 240 (15), 181 (22), 144 (100),69 (18), 56 (51).
12b	1609 (C=O), 1519, 1346 cm^{-1} (NO_2)	8.13 (d, 1H, H_4-nitrothiophene, J = 4.2 Hz), 7.61 (d, 1H, H_3-nitrothiophene, J = 4.2 Hz), 7.35 (d, 1H, H_3-thiophene, J = 4.0 Hz), 7.27 (d, 1H, H_4-thiophene, J = 4.0 Hz), 3.50–3.96 (m, 8H, piperazine).	488 ([M$^+$+2], 15), 486 (M$^+$, 16), 281 (37), 278 (45), 187 (100), 183 (65), 162 (24), 125 (40), 101 (76), 80 (87), 52 (98).

The results obtained are given in Table 3. The calculated values of log P for derivatives of nitrothiophene were about 0.35 higher than for the corresponding compounds with a nitrofuran moiety. It could be assumed that the increase in lipophilicity (log P) of the compounds within the series **6–12b** results in the decrease of anti-leishmanial activity. On the other hand, the observed differences in anti-leishmanial activities of nitrofurans and nitrothiophenes may be due to the reduction potential of the single-electron transfer $ArNO_2/ArNO_2^{\cdot-}$. Nitroheterocylic compounds are generally believed to exert their cytotoxic effects only after activation by single-electron reduction of their corresponding nitro anion radicals.[20–22] Under aerobic conditions, the nitro radical anion reacts with oxygen to form superoxide anion and hydroxyl radical. The resulting oxygen-derived free radicals would damage the enzyme, DNA or important structures in the surrounding cell, and result in a cytotoxic action. Under anaerobic conditions, the radical anion can be transformed into the corresponding nitroso-derivative. This

Table 3. In vitro activities of compounds **6**–**12a**,**b** against promastigote form of *L. major* and peritoneal macrophages



Compound	X	R	Log *P*[a]	Toxicity on *L. major* [IC_{50} (μM)][b]	Toxicity on macrophages [Cell death (%)][c]
6a	O		2.17	11.69 ± 1.3	7.1 ± 0.08
7a	O		1.95	10.73 ± 0.69	8.7 ± 0.06
8a	O		1.95	13.19 ± 0.96	9.7 ± 0.07
9a	O		1.95	26.22 ± 0.69	9.7 ± 0.02
10a	O		0.78	19.18 ± 1.95	2.4 ± 0.03
11a	O		1.92	11.75 ± 1.1	7.6 ± 0.09
12a	O		2.22	13.11 ± 0.86	7.7 ± 0.13
6b	S		2.52	25.00 ± 1.25	8.9 ± 0.02
7b	S		2.29	34.44 ± 1.14	6.1 ± 0.10
8b	S		2.29	>50	5.5 ± 0.12
9b	S		2.29	24.11 ± 1.14	7.2 ± 0.06
10b	S		1.13	28.26 ± 0.61	8.7 ± 0.07
11b	S		2.27	23.78 ± 0.65	9.8 ± 0.07
12b	S		2.57	24.69 ± 1.03	9.2 ± 0.02
Glucantime				30 ± 1.41[d]	40.4 ± 0.09

[a] Log *P*, that is, the logarithm of the partition coefficient for *n*-octanol/water, was calculated using the program HyperChem ver. 7.0.
[b] The values represent means ± SD.
[c] Macrophage cells were treated with compounds in the IC_{50} concentrations (observed against promastigotes) for 24 h.
[d] IC_{50} in mg/mL.

nitroso form has been put forward as an efficient scavenger of essential thiols in the cell.[23,24]

In conclusion, we have described a series of nitroheteroaryl-1,3,4-thiadiazole-based compounds including 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines and 1-[5-(5-nitrothiophen-2-yl)-1,3,4-thiadiazol-2-yl]-4-aroylpiperazines, with in vitro anti-leishmanial activity. From our biological results, it is evident that most of title compounds exhibited potent anti-leishmanial activity against both promastigote and amastigote forms of *L. major* at non-cytotoxic concentrations. However, the 5-nitrofurans were evidently more active than the corresponding 5-nitrothiophene analogues.

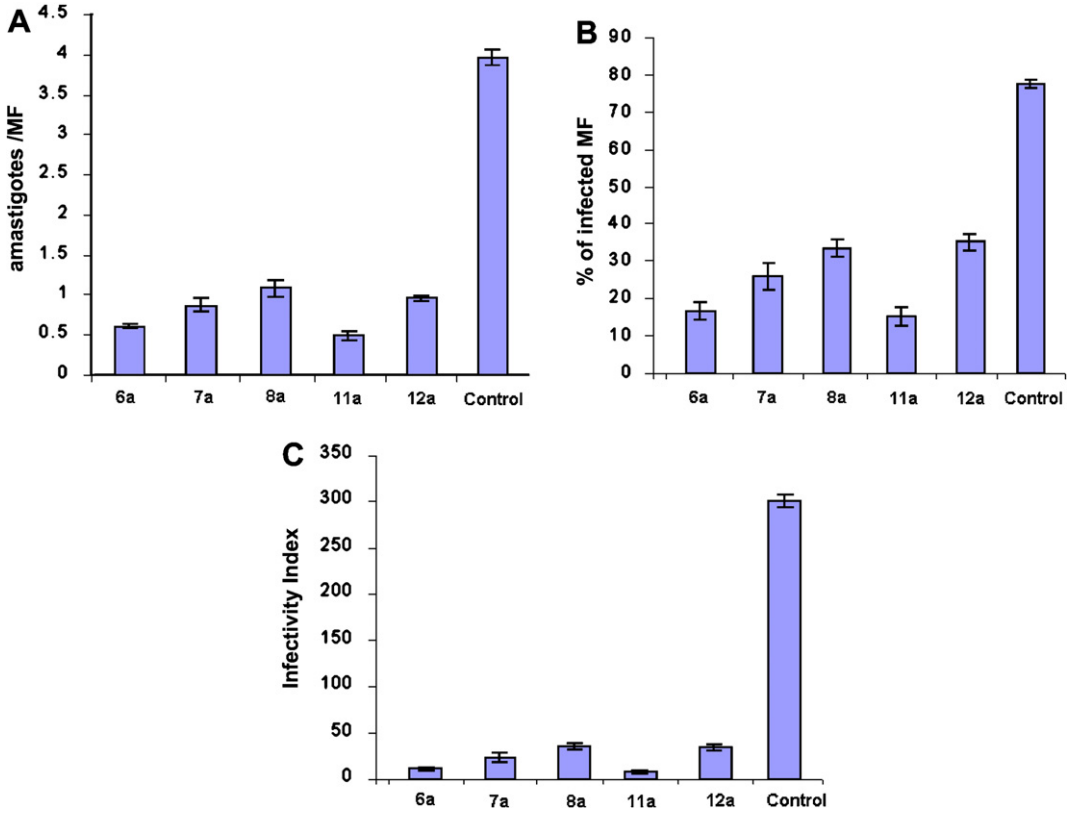


Figure 2. In vitro activity of selected 5-(5-nitrofuran-2-yl)-1,3,4-thiadiazoles against intramacrophage amastigotes of *L. major*: (A) The mean number of amastigotes per macrophage after treatment with drug for 24 h. (B) The percentage of infected macrophages after treatment. (C) Infectivity index of macrophages cultured 24 h in the presence of selected drugs.

4. Experimental

Chemicals and all solvents used in this study were purchased from Merck AG and Aldrich Chemical. Intermediates **5a**,**b** were prepared according to the literature.[14–16] Melting points were determined on a Kofler hot stage apparatus and are uncorrected. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks). ^{1}H NMR spectra were measured using a Bruker FT-80 spectrometer, and chemical shifts are expressed as δ (ppm) with tetramethylsilane as internal standard. The mass spectra were run on a Finigan TSQ-70 spectrometer (Finigan, USA) at 70 eV. Elemental analyses were carried out on a CHN–O rapid elemental analyzer (GmbH-Germany) for C, H and N, and the results are within ±0.4% of the theoretical values. Merck silica gel 60 F254 plates were used for analytical TLC. Yields are of purified product and were not optimized.

4.1. General procedure for synthesis of compounds 6–12

To a mixture of compound **5a**,**b** (2.0 mmol) in dry benzene (5 mL), pyridine (1 mL) and appropriate aroyl chloride (2.0 mmol) were added, respectively. The mixture was stirred at room temperature overnight. The solvents were removed under reduced pressure and the resulting solid was washed with water and crystallized from ethanol to give compounds **6**–**12**.

4.2. Biological activity

The strain of *L. major* used in this study was the vaccine strain (MRHO/IR/75/ER), obtained from Pasteur Institute, Tehran (Iran). The infectivity of the parasites was maintained by regular passage in susceptible BALB/C mice. The promastigote form of parasite was grown in blood agar cultures at 25 °C. The stationary parasite inoculation was 2×10^6 cells/mL. For the experiments described here, the stationary phase promastigotes were washed with phosphate buffered saline and recultured in RPMI 1640 medium (Sigma) at 2×10^6 cells/mL density, supplemented with 10% of heat-inactivated fetal bovine serum, 2 mM glutamine (Sigma), pH ~ 7.2, 100 U/mL penicillin (Sigma) and 100 μg/mL streptomycin (Sigma).

4.2.1. Anti-leishmanial activity against promastigotes form of *L. major*. The anti-leishmanial screening was performed using direct counting and MTT assay. It is noted that at the first, the growth curve of the *L. major* strain was determined daily under light microscope and counting in a Neubauer's chamber. Then, parasites (2×10^6/mL) in the logarithmic phase were incubated with a serial range of drug concentrations for 24 h at 25 °C. To determine 50% inhibitory concentrations (IC_{50}), the tetrazolium bromide salt (MTT) assay was used.[17] Briefly, promastigotes, from early log phase of growth, were seeded in 96-well plastic cell culture trays, containing serial dilution of drug and phenol red free

RPMI 1640 medium, supplemented with 10% of FBS, 2 mM glutamine, pH ~7.2 and antibiotics, in a volume of 200 μL. After 24 h of incubation at 25 °C, the media was renewed with 100 μg/well of MTT (0.5 mg/mL) and plates were further incubated for 4 h at 37 °C. The plates were centrifuged (2000 rpm × 5 min), the pellets were dissolved in 200 μL of DMSO. The samples were read using an ELISA plate reader at a wavelength of 492 nm. Two or more independent experiments in triplicate were performed for determination of sensitivity to each drug, the IC_{50} were calculated by linear regression analysis, expressed in means ± SD. Control cells were incubated with culture medium plus DMSO.

4.2.2. Anti-leishmanial activity against amastigotes form of *L. major*. Mouse peritoneal macrophages were plated in RPMI 1640, supplemented with 10% of heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin (Sigma) and 100 μg/mL streptomycin. Macrophages were placed on sterile glass cover slips in 24-well plates (1×10^6/well). After 1 h non-adherent cells were removed by washing with RPMI, the stationary phase promastigotes in RPMI were added (2×10^6 parasites/well, three parasites/macrophage) to macrophage monolayer and the plates were kept at 37 °C in a CO_2 incubator for 2 h. Extracellular parasites were removed by washing, and then new media containing IC_{50} concentration of the drug was added. Two sets of experiments were carried out for each drug at 24 h. Following these procedures, cells were fixed with methanol, stained with Giemsa stain (Sigma) and the infectivity index was determined by multiplying the percentage of macrophages that had at least one intracellular parasite by the average number of intracellular parasites per infected macrophage (60 cells were examined/ well).[18]

4.2.3. Toxicity on peritoneal macrophages. In vitro toxicity towards mouse peritoneal macrophages was assessed using MTT colorimetric assay. The macrophages were plated in 96-well plates at 2×10^5 cells/well. After cell adherence, the medium was removed and replaced by the media containing IC_{50} of each compound. The plates were incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. Control cells were incubated with culture medium plus DMSO. Cell viability was determined by MTT colorimetric assay. In vitro toxicity was calculated according to the following formula: % of cell death = (control cell viability − drug-treated cell viability)/control cell viability.

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