Original Article

Synthesis and Evaluation of Thiadiazole-based Antileishmanial Agents

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Abstract

Background and Objectives: The 1, 3, 4-thiadiazole scaffold is one of the principal structural components, in a variety of drug categories such as antimicrobial, anti-inflammatory, antineoplastic, and antileishmanial agents. Considering the reported antileishmanial effects of thiadiazole derivatives and the importance of this disease, some of the thiadiazole derivatives with modifications at sulfur atom or amine group attached to the 2-position were synthesized and evaluated for antileishmanial activity. **Materials and Methods:** Derivatives of 1,3,4-thiadiazole including 2-substituted-thio-1,3,4-thiadiazoles bearing (5-(4-nitrobenzylideneamino) or 5-amino **(II, IV, V)** and one derivative of 2-substituted-amino-1,3,4-thiadiazole bearing (5- (4-nitrophenyl) **(VII)** were synthesized and evaluated for their *in vitro* antileishmanial activity against promastigote and amastigote forms of the *Leishmania major*. **Results:** The most active compound was found to be compound **II** after 24-h incubation against promastigotes and amastigotes with the half maximal inhibitory concentration (IC_{50}) values of 44.4 µM and 64.7 µM, respectively. **Conclusion:** All of the synthesized compounds showed good antileishmanial activity against both forms of *L. major* after 48 and 72 h incubation.

Keywords: *Amastigote, antileishmanial activity, leishmania major, promastigote, thiadiazole*

Introduction

Leishmaniasis is a disease induced by the parasites of the genus *Leishmania*.[1-4] *Leishmania* parasites have two stage life cycles including: extracellular and an intracellular.[5,6] Antileishmanial effects are related with disparate heterocyclic core such as piperazine, pyrimidine, azoles (imidazole, 1, 2, 4 triazole, isoxazole, pyrazole, and thiadiazole), quinazoline, and indole[7] [Figure 1]. Thiadiazole has a constrained pharmacophore with two electron donor systems, which can act as "hydrogen binding domain."[8,9] The sulfur atom of thiadiazole increases lipophilicity resulting in good cell permeability and bioavailability.[10,11] This pharmacologically significant scaffold has shown several biological activities including anticancer, antibacterial,[9-14] anticonvulsant, antitubercular, anti-inflammatory, and antileishmanial effects.[9,13] Literature surveys have shown antileishmanial activity for 1, 3, 4-thiadiazoles; however, linkage to other heterocycles can mutate the bioactivity, contingent on the type, and the position of substituent.[1,6,15-17] One of the proposed mechanisms for antileishmanial effects of thiadiazole derivatives is affinity for attaching to the sulphydryl groups of parasit enzymes or proteins.[10,18] It has been reported that 2-mino-1, 3, 4 -thiadiazole derivatives represented antileishmanial properties through inhibition of parasitic enzymes.[10,18] Antileishmanial activity was reported for derivatives of ethyl (5-amino-1, 3, 4-thiadiazoe-2-yl) (hexahydropyrimidine or imidazolidin-2-ylidene) acetate by Ram *et al*.[7,19] The use of nitro heterocyclic structures such as 5-nitrofurans, 5-nitrothiophenes, and 5-nitroimidazoles in the expansion of antiparasitic agents has been determined.[15-17] The structure–activity relationship (SAR) study showed that the S atom joined to the 2-position of the thiadiazole ring has a high pliability for structural rectification to retain good antileishmanial activity.[16]

According to the incidence of leishmanial infection and important problems including the great cost of drugs, emergence of immunosuppressive illnesses such as AIDS and drug resistance,[1] in this research a series of thiadiazole derivatives with modifications at S-pendant or amino group joined to the 2-position were synthesized and evaluated for antileishmanial activity.

Materials and Methods

Materials

All reagents and solvents were obtained from commercial suppliers such as Merck (Germany) and Aldrich (USA). Merck silica

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gel 60 F_{254} plates (Germany) were used for analytical thin-layer chromatography (TLC) and monitoring of reactions. Proton nuclear magnetic resonance (HNMR) spectra were registered using a (Bruker 400 MHz, Germany) spectrometer. Infrared (IR) (potassium bromide discs) was recorded with a WQF-510 FT-IR spectrophotometer (China). Melting points were determined using electrothermal 9200 melting point instrument (UK) and are uncorrected. Mass spectra were registered on Agilent Technologies 5975C mass spectrometer (USA).

Chemistry

Synthesis of 2-(5-amino-1, 3, 4-thiadiazol)-1-(4-bromophenyl) ethanone (I)

To a mixture of 5-amino-1, 3, 4-thiadiazole-2-thiol (3mmole) and potassium hydroxide (3 mmol) in ethanol, 2-bromo-1 (4-bromophenyl) ethanone (3 mmol) was added. Then, the reaction mixture was allowed to stir overnight. Obtained white solid was filtered and washed with water and crystallized from ethanol to give compound **I**[16] **[**Scheme 1].

Synthesis of 2-(5-(4-nitrobenzylideneamino)-1, 3, 4-thiadiazol-2-ylthio)-1-(4-bromophenyl) ethanone (II)

Equimolar quantities of compound **I** (1.5 mmol), 4-nitrobenzaldehyde, and catalytic amount of concentrated sulfuric acid (2–5 drops) were dissolved in absolute ethanol. The reaction mixture was refluxed for 6 h until the end of the reaction that determined by TLC. Then, solvent was removed under reduced pressure, ice cold water was added, and the product was extracted using chloroform. Organic layer was evaporated to dryness. Obtained crud product was purified by preparative TLC to obtain compound **II**[20,21] [Scheme 1].

Synthesis of ethyl 2-(5-amino-1, 3, 4-thiadiazole-2-yl-thio) acetate (III)

Ethyl chloroacetate (6 mmol) was added to a stirred solution of 5-amino-1, 3, 4-thiadiazole-2-thiol (5mmol) and potassium hydroxide (5 mmol) in (20 mL) absolute ethanol. The reaction mixture was refluxed for 2 h, after completion of the reaction, solvent was evaporated in reduced pressure. Extraction was

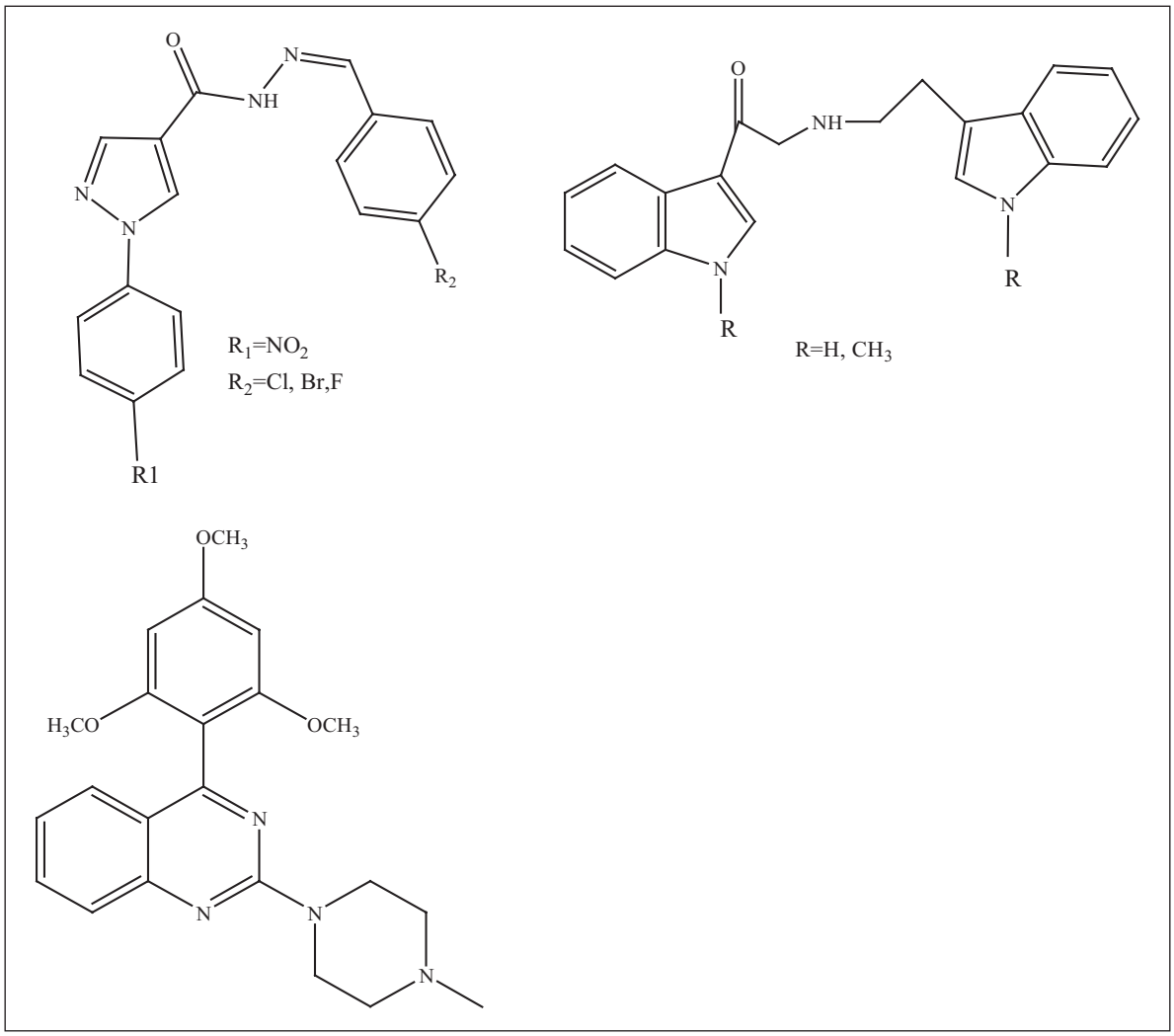


Figure 1: Different heterocyclic nucleus with antileishmanial activity

carried out with chloroform and water. Chloroform was evaporated and the remained solid was crystallized with ethanol and collected as white crystal [Scheme 1].[20,21]

Synthesis of 2-(5-amino-1, 3, 4-thiadiazole-2-ylthio) acetohydrazide (IV)

A mixture of compound **III** (3 mmol) and hydrazine hydrate (4 mmol) in ethanol (20 mL) was refluxed for 3 h. The obtained precipitate was filtered, washed with cold water, dried, and recrystallized from ethanol to give compound **IV** [Scheme 1].[14,22-24]

Synthesis of N'-(4-nitrobenzylidene)-2-(5-amino-1, 3, 4-thiadiazole-2-ylthio) acetohydrazide (V)

Compound **IV** (2 mmol) was dissolved in 20 mL of absolute methanol then, 4-nitro-benzaldehyde (2 mmol), and glacial acetic acid (3 drops) were added and refluxed for 2 h in 20°C. The reaction mixture was cooled and obtained precipitate, filtered, and crystallized from methanol [Scheme 1].[23,24]

Synthesis of 5-(4-nitrophenyl)-1, 3, 4-thiadiazole-2-amine (VI)

A mixture of 4-nitrobenzoic acid (3 mmol), thiosemicarbazide (3 mmol) and phosphorus oxychloride (20 mL) was refluxed gently for 3 h. The mixture was cooled to room temperature, then water was added (90 mL) and the suspension was refluxed for 2 h another followed by filtration. The solution was neutralized with sodium hydroxide. The new formed precipitate was filtered and crystallized from ethanol [Scheme 2].[21,25,26]

Synthesis of 2-chloro-N-(5-(4-nitrophenyl)-1, 3, 4-thiadiazole-2-yl) acetamide (VII)

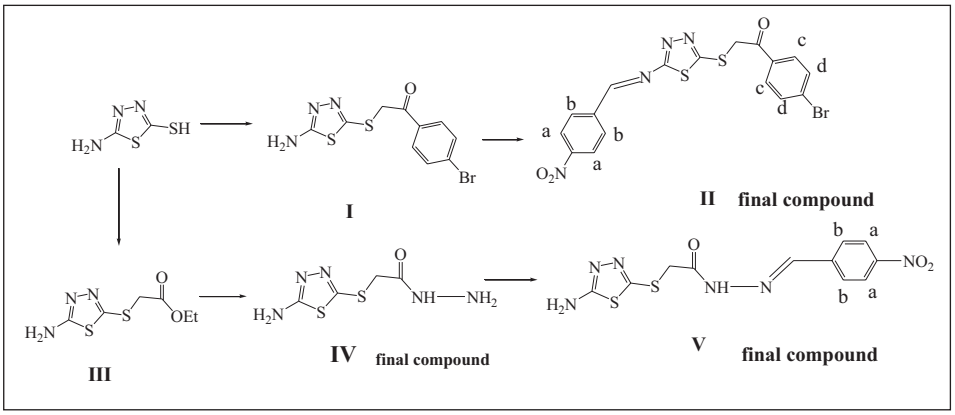
To a solution of 5-(4-nitrophenyl)-1, 3, 4-thiadiazole-2-amine **(VI)** (2 mmol) and triethylamine (2 mmol) in dichloromethane (20 mL) was added chloroacethylchloride (4 mmol). The reaction mixture was stirred at room temperature for 1 h. The resulting solid was filtered and washed with water, and then was crystallized in methanol to give compound **(VII)** [Scheme 2].[27]

Biological assays

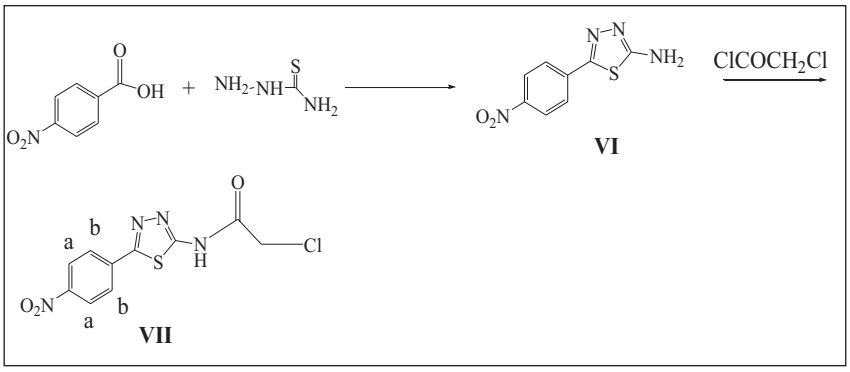
The stock solutions of compounds (1 mM and 1 mL) were prepared in dimethylsulfoxide (DMSO) and appropriately diluted with the Roswell Park Memorial Institute (RPMI) for amastigotes and phosphate-buffered saline (PBS) for promastigote to obtain 25, 50, 100, and 200 µM concentrations.

Antileishmanial activity against leishmania promastigote

The promastigote form of *L. major* (vaccine strain MRHO/IR/75/ER) obtained from the Department of Parasitological, Isfahan University of Medical Sciences, Iran was grown first



Scheme 1: Synthetic route to compounds (I–V). KOH, $BrCH_2COPhBr$, EtOH; 4-NO_2Ph-CHO, H_2SO_4, EtOH, 6h reflux; $ClCH_2COOEt$,KOH, EtOH; NH_2NH_2, EtOH, 3h reflux; 4-NO_2Ph-CHO, MeOH, Glacial CH_3COOH, 2h reflux



Scheme 2: Synthesis of the target compound VII. $POCl_3$, 3h reflux; $ClCOCH_2Cl$, DCM, Et_3N, 1 h stirred at room temperature

in Novy–MacNeal–Nicolle and then in RPMI-1640 to produce a mass of this parasite. RPMI-1640 medium was enriched with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL). The growth of promastigotes was monitored daily using microscope. Cultures were passaged during 4-day incubation at 37°C and 5% CO_2.[3,5]

100 µL of medium containing 1.125×10^6 parasites in stationary phase was poured in each well of 96-well plates. Then 100 µL of synthesized compounds at 25, 50, 100, and 200 µM concentrations were added to determine the effect on promastigote. Glucantime with the same concentrations as final compounds was used as the positive control. A well devoid of the studied compounds as a negative control was included in the study. Plates incubated at 24°C and evaluated after 24, 48, and 72 h. Afterward, neobar lamella were used for observing results and counting the number of promastigotes. Three independent tests in triplicate were done for each compound for 24, 48, and 72 h.[3,5]

Antileishmanial activity against *Leishmania* amastigotes

J774 mouse macrophage cell line was cultured in RPMI supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 37°C. Upon reaching 70% of confluence, cells were passaged.[3]

At first, 24 × 24 sterile glass cover slips were set on the bottom of each well, then 700 µL of J774 macrophage cells (5×10^5 cells/mL) were seeded in 6-well plates and the plates were incubated at 37°C and 5% CO_2 for 24–48 h to stick macrophages to the bottom of the plate and glass cover slips.[3] Then macrophages were infected with metacyclic *L. major* promastigotes at a parasite/macrophage ratio of 10:1 and incubated at 37°C in a humidified incubator with 5% CO_2 for 24 h.

After removal of extracellular parasites by washing with PBS, 1 mL of different concentrations of compounds 25, 50, 100, and 200 µM were added. Glucantime with the concentrations 25, 50, 100, and 200 µM was also added to the container as the positive control. Three sets of experiments were carried out for each compound at 24, 48, and 72 h. Afterward cover slips were fixed with methanol, stained with 10% giemsa, and the results were observed using oil-immersion light microscopy. The average number of parasites per macrophage were determined in 100 macrophages [Figure 1].[3]

Results

Chemistry

2-(5-Amino-1, 3, 4-thiadiazol)-1-(4-bromophenyl) ethanone (I)

Yield: 81%, white solid, m.p.88–89°C (lit: 88–91°C),[28] IR ν_{max}, 3330, 3122 (NH_2), 2950 (C–H), 1678 (C=O), and 1611 (C=N) cm^{-1}.

2-(5-(4-Nitrobenzylideneamino)-1, 3, 4-thiadiazol-2-ylthio)-1-(4-bromophenyl) ethanone(II)

Yield: 69.44%, yellow solid, m.p.99–101°C, IR ν_{max}, 2920 (C–H), 1711 (C=O), 1680 (C=N), 1344, 1528 (NO_2), ^{1}HNMR: (400 MHz; $CDCl_3$): δ 8.74 (1H, s, CH=N), 8.36 (2H, d, J = 8 Hz, H^a), 8.07 (2H, d, J = 8 Hz, H^b), 7.72–7.74 (2H, m, H^c), 7.54–7.57 (2H, m, H^d), 4.25 (1H, d, J = 8 Hz, CH_2), and 4.23 (1H, d, J = 8Hz, CH_2).

Ethyl 2-(5-amino-1, 3, 4-thiadiazole-2-yl-thio) acetate (III)

Yield: 57%, white solid, m.p.82–83°C (lit: 83–84°C),[8] IR ν_{max}, 3396, 3280 (NH_2), 2923 (C–H), 1735 (C = O), and 1300 (C–O) cm^{-1}.

2-(5-Amino-1, 3, 4-thiadiazole-2-ylthio) acetohydrazide (IV)

Yield: 53.78%, white solid, m.p.150–151°C, IR ν_{max}, 3300, 3274, 3029, 1555 (NH_2, NH), 1685 (C=N), 1648 (C=O) cm^{-1}, ^{1}HNMR: (400 MHz; DMSO; d_6):9.28 (1H, s, NHCO), 7.32 (2H, s, NH_2), 4.31(2H, s, CH_2), 3.71(2H, s, NH_2), MS (m/z): 205(M^+), and MW 205 g/mol.

N'-(4-Nitrobenzylidene)-2-(5-amino-1,3,4-thiadiazole-2-ylthio) acetohydrazide (V)

Yield: 49.31%, yellow solid, m.p.123–125°C, IR ν_{max}, 3441, 3189 (NH_2, NH), 1678 (C=N), 1640 (C=O), 1580, 1342 (NO_2) cm^{-1}, ^{1}HNMR: (400 MHz; DMSO; d_6): 11.94 (1H, s, CH=N), 8.28 (2H, d, J=8HZ, H^a), 8.1 (1H, s, NH), 7.93–7.99 (2H, d, J=8Hz, H^b), 7.3 (2H, s, NH_2), 4.35 (2H, s, CH_2), MS (m/z): 338 (M^+), and MW 338 g/mol.

5-(4-Nitrophenyl)-1, 3,4-thiadiazole-2-amine (VI)

Yield: 91%, yellow solid, m.p. 256–258°C (lit: 258–260 °C),[26] IR ν_{max}, 3441, 3189(NH_2), 3090(C–H), 1599, and 1336 (NO_2) cm^{-1}.

2-Chloro-N-(5-(4-nitrophenyl)-1, 3, 4-thiadiazole-2-yl) acetamide(VII)

Yield: 88%, pale white, m.p.149–150°C, IR $\nu_{max,}$ 3100(NH), 2936(C–H), 1573, 1342(NO_2) cm^{-1}, ^{1}HNMR: (400 MHz; $CDCl_3$):8.39 (2H, d, J=8Hz, H^a), 8.18(2H, d, J=8Hz, H^b),

Table 1: Half maximal inhibitory concentration (IC_{50}) values (µM) for antileishmanial activity against promastigote of the studied compounds

Compounds	24 h	48 h	72 h
II	44.47 ± 0.95	21.52 ± 0.8	6.58 ± 0.79
IV	109.9 ± 0.93	50.87 ± 0.83	3.17 ± 0.12
V	96.15 ± 0.37	52.59 ± 0.18	3.66 ± 0.11
VII	108.8 ± 0.76	50.53 ± 0.94	7.34 ± 0.73
Glucantime	119.21 ± 0.96	62.01 ± 0.81	14.87 ± 0.33

Table2: Half maximal inhibitory concentration (IC_{50}) values (µM) for antileishmanial activity against amastigote of the studied compounds

Compounds	24 h	48 h	72 h
II	64.71 ± 0.44	13.62 ± 0.26	3.38 ± 0.18
IV	106.11 ± 0.59	40.88 ± 0.14	18.56 ± 0.83
V	98.29 ± 0.61	41.27 ± 0.41	17.13 ± 0.67
VII	91.35 ± 0.29	32.85 ± 0.3	11.59 ± 0.12
Glucantime	92.27 ± 0.37	38.06 ± 0.57	17.52 ± 0.26

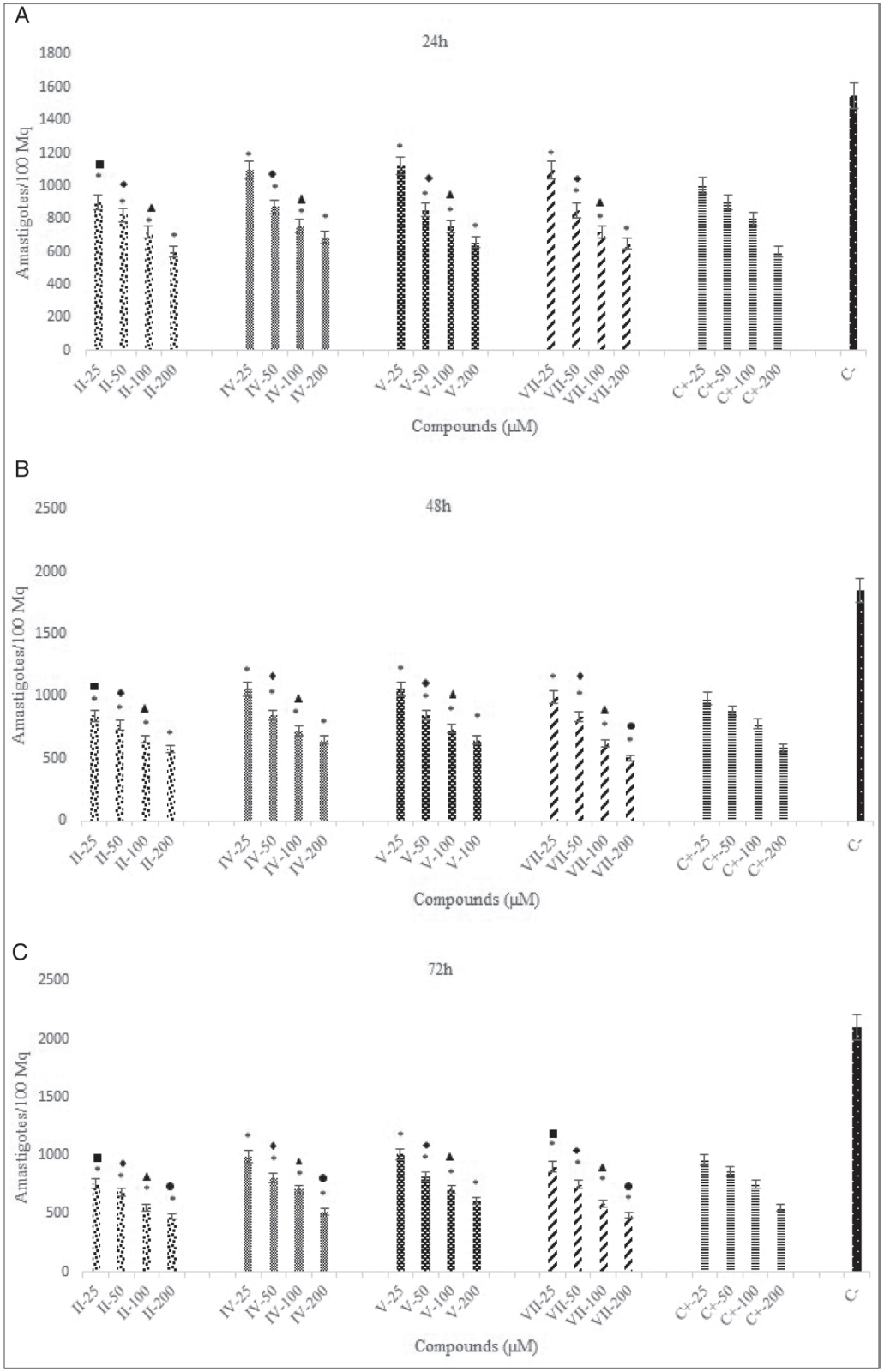


Figure 2: *In vitro* activity of target compounds against intramacrophage amastigotes of *Leishmania major*. (A) The mean number of amastigotes per 100 macrophages after treatment with target compounds for 24 h. (B) The mean number of amastigotes per 100 macrophages after treatment with target compounds for 48 h. (C) The mean number of amastigotes per 100 macrophages after treatment with the target compounds for 72 h. (*$P < 0.001$ vs. negative control; ■ $P < 0.001$ vs. Glu-25, compounds-25; ◆ $P < 0.001$ vs. Glu-50, compounds-50; ▲$P < 0.001$ vs. Glu-100, compounds-100; ●$P < 0.001$ vs. Glu-200, compounds-200

8.12(1H, s, NH), 4.42(2H, s, CH_2), MS (m/z): 298 (M^+), and MW 298.5 g/mol.

Antileishmanial activity

The half maximal inhibitory concentration (IC_{50}) values (μM) of compounds against promastigotes and amastigote after 24-, 48-, and 72-h incubation are presented in Tables 1 and 2. The lowest IC_{50} values against promastigotes belonged to the compounds **II** (in 24-, 48-, and 72-h incubation) and **IV, V** and **VII** (in 72-h incubation). The most potent compounds against amastigote were found to be **II** (in 24-, 48-, and 72-h incubation), **VII** (48 h) and **V, VI, VII** (72 h). The average number of amastigotes in 100 macrophages in three different treatments with final compounds and with glucantime and without any antileishmanial agent is shown graphically in Figure 2. Analysis of variance (ANOVA) analysis showed that the antiamastigotes effects of all compounds at all concentrations were significant in comparison with the negative control (P <0.001), as shown in Figure 2. The IC_{50} values of the test derivatives indicated that all derivatives showed better activity than glucantime as reference drug against promastigote after 72-h incubation.

Discussion

Many of 1,3,4-thiadiazoles derivatives were synthesized and evaluated in the past years in antileishmanial experiments.[29-31] In this study, a series of 2-substituted-thio-1,3,4-thiadiazoles bearing (4-nitrobenzylideneamino) or amine at the 5-position **(II, IV, V)** and one derivative of 2-substituted-amino-1,3,4-thiadiazole bearing (4-nitrophenyl) **(VII)** at the 5-position were synthesized and evaluated against promastigote and amastigote forms of *L. major*.

The resulting data revealed that all of the synthesized compounds showed good antleishmanial activity against both forms of *L. major* after 48- and 72-h incubation. IC_{50} values against promastigote forms were <60 and <10 μM after 48- and 72-h incubation, respectively. Also, these compounds showed antiamastigote activity with IC_{50} values <50 and <20 μM after 48- and 72-h incubation, respectively.

According to SAR studies, existence various nitroaryl derivatives at C-5 and large groups connected to the 2-position of thiadiazole ring were liable for the antileishmania effects.[16] Previous studies illustrated that an amine substituent at C-2 position of 5-(nitroheterocycle -2-yl)-1,3,4-thiadiazoles has profound effect in the antileishmanial activity of these compounds.[1,5]

The comparison of IC_{50} values for compounds **IV** and **V** showed nearly similar effect against both forms of the parasite. Literature surveys have shown that that the C-2 substituent in 5-(nitroheteroaryl)-1,3, 4-thiadiazoles is commutable to determine the potency and physicochemical properties of these derivatives. The substitutions of the linear in the C-2 position of 1, 3, 4-thiadiazole ring increased the *in vitro* activity against promastigotes, whereas the link of cyclic group showed lower antipromastigote activity due to a steric hindrance around the C-2 position.[4]

The comparison of IC_{50} values for compounds II and IV, V showed that the presence of bulky the substitution at 5 position could improve antileishmania activity as seen in the case of compound II. By comparing the IC_{50} values of 2-substituted-amino-1,3,4-thiadiazole **VII** with 2-substituted-thio-1,3,4-thiadiazole **II,** it was revealed that halogen substituent slightly increased the antipromastigote activity after 72-h incubation.

Conclusion

Compound **II** was the most potent compound after 24-, 48- and 72-h incubation against both forms of the parasite, whereas compounds **IV, V,** and **VII** indicated the same result after the same periods of incubation time against promastigote and amastigote forms. In addition, compound **II** was more active than the reference drug after 24-, 48-, and 72-h incubation against both forms of the parasite.

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Conflicts of interest

Nil.

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