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ORIGINAL ARTICLE

Synthesis and in vitro anti-*Toxoplasma gondii* activity of a new series of aryloxyacetophenone thiosemicarbazones

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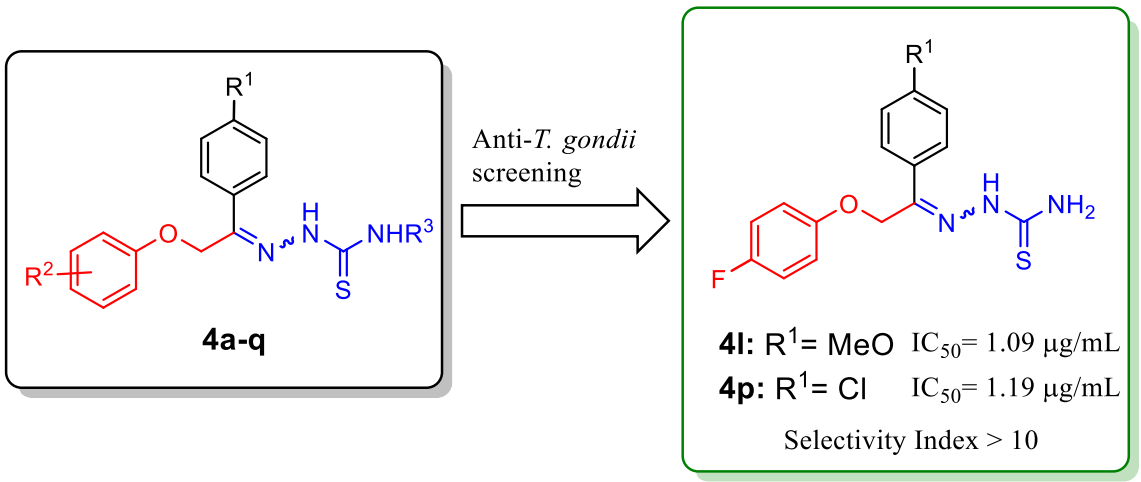
Abstract

A new series of aryloxyacetophenone thiosemicarbazones **4a–q** have been synthesized as anti-*Toxoplasma gondii* agents. All compounds showed significant inhibitory activity against *T. gondii*-infected cells (IC_{50} values 1.09–25.19 µg/mL). The 4-fluorophenoxy derivative (**4l**) was the most potent compound with the highest selectivity toward host cells (SI = 19), being better than standard drug pyrimethamine. SAR study indicated that the concurrence of proper substituents on both aryl ring of phenoxyacetophenone is important for potency and safety profile. Further in vitro experiments with the representative compounds **4l** and **4p** revealed that these compounds at the concentration of 5 µg/mL can significantly reduce the viability of *T. gondii* tachyzoites, as well as their infectivity rate and intracellular proliferation, comparable to those of pyrimethamine.

Graphic abstract



Keywords Acetophenone derivatives · Anti-apicomplexan · Thiosemicarbazones · Toxoplasmosis · *Toxoplasma gondii*

Introduction

Toxoplasma gondii (T. gondii), the ubiquitous parasitic protozoan belonging to phylum Apicomplexa, is the etiologic agent of toxoplasmosis. This successful obligate intracellular parasite can infect all warm-blooded vertebrates such as humans, livestock, birds and marine mammals [1]. It is estimated that up to one-third of the world's population is infected with *T. gondii* specifically in developing countries

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[2]. Although the infection is asymptomatic in most immunocompetent people with normal immunity, significant morbidity and mortality may especially occur in congenitally infected and immunocompromised individuals such as HIV-positive, cancer or organ transplant human patients [3].

The recommended drugs for treatment of toxoplasmosis are the combination of pyrimethamine (an inhibitor of dihydrofolate reductase) and sulfonamides such as sulfamethoxazole or sulfadiazine which inhibit dihydropteroate synthetase in the folate synthesis pathway. In addition, other drugs such as clarithromycin, azithromycin and atovaquone can be used for treatment of toxoplasmosis [4, 5]. However, current chemotherapy for toxoplasmosis is often associated with serious side effects including hypersensitivity, hematological toxicity, teratogenicity and allergic reactions. Most of chemotherapeutic agents are only effective in the acute phase of the disease and cannot eliminate intracellular parasites. Therefore, these limitations clearly indicate the need for newer, safer and more efficacious therapy for toxoplasmosis [6].

Several reports have previously described the efficacy of hydroxyurea (**A**) and thiosemicarbazones (**B**) in vitro, in eliminating the intracellular parasites of *T. gondii, Trypanosoma cruzi* and *Leishmania amazonensis* [7–9] (Fig. 1). Hydroxyurea (**A**) and thiosemicarbazones (**B**) act by arresting the cell cycle in the G1/S phase and inactivating the enzyme ribonucleotide reductase, then the DNA synthesis. However, thiosemicarbazones are known inhibitors of this enzyme, being approximately 1000 times more potent than hydroxyurea [7–10].

Thiosemicarbazones were shown to be as potential chemical leading groups for producing new therapeutic agents as antitumor, antimicrobial and anti-parasitic agents [10–13]. Biological activities of thiosemicarbazones and also avoiding of drug resistance are related to their abilities to form complex with metal cations, by bonding through the sulfur and azomethine nitrogen atoms [13, 14].

Previously, Tenório et al. have reported the synthesis and anti-*T. gondii* activity of thiosemicarbazones, substituted at arylhydrazone moiety with nitro group (structure **C**, Fig. 1). The results of their studies showed that thiosemicarbazones were effective against intracellular *T. gondii*, while the treated host cells with the thiosemicarbazones did not show any morphological alterations after 24 h of drugs treatment [11]. Inspired by these findings, our group has made efforts to develop new derivatives of thiosemicarbazone class to broaden these initial consequences with further details on anti-*T. gondii* activity to achieve more efficient drugs. In our previous study, we have synthesized a series of phenoxyacetophenones (**D**) as antimicrobial agents derived from dihydrochalcones [15]. In the current work, the thiosemicarbazone functionality was introduced in the phenoxyacetophenone framework (Fig. 1). Thus, we report here, the synthesis and anti-*T. gondii* activity of a new series of aryloxyacetophenone thiosemicarbazones.

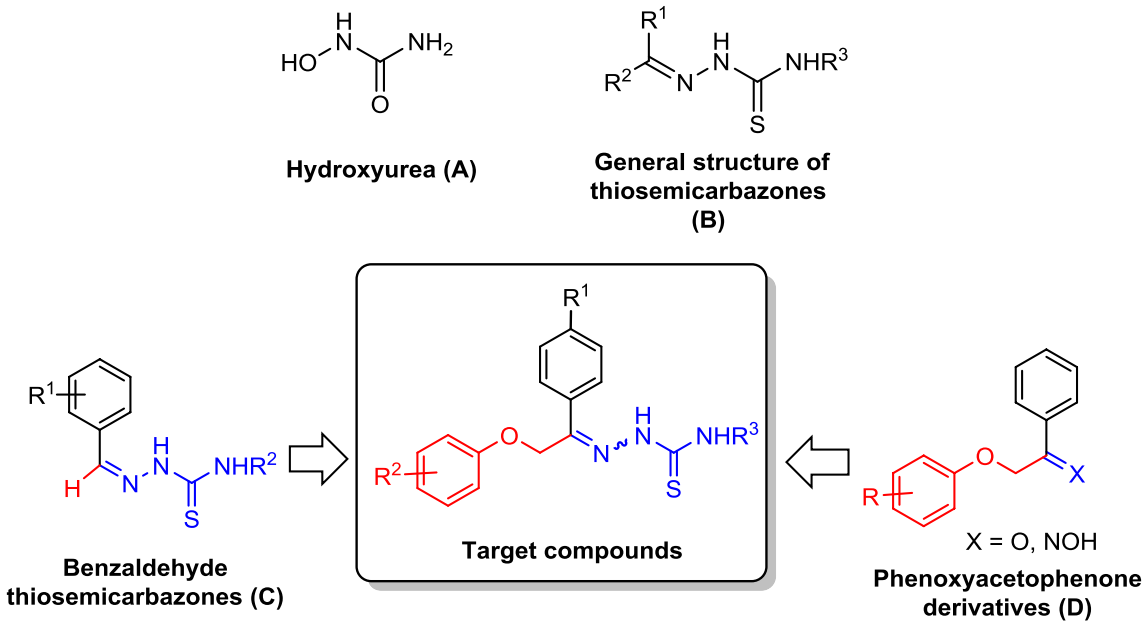


Fig. 1 Design of target compounds bearing thiosemicarbazone functionality

Results and discussion

Chemistry

The target compounds **4a–q** were synthesized through the synthetic route outlined in Scheme 1. Initially, the commercially available acetophenone derivatives **1a–c** were brominated by $CuBr_2$ in $CHCl_3$/EtOAc to give corresponding 2-bromoacetophenone derivatives **2a–c**. The intermediate compounds phenoxyacetophenones **3a–k** were obtained by treatment of **2a–c** with different phenolic derivatives in the presence of potassium carbonate in DMF at room temperature. Finally, the reaction of phenoxyacetophenones **3a–k** with thiosemicarbazide or 4-phenylthiosemicarbazide in the presence of a few drops of HCl in ethanol gave thiosemicarbazone derivatives **4a–q** in good yields. The structures of newly synthesized compounds were confirmed by spectroscopic data (IR, ^{1}H NMR and MS) and elemental analysis as interpreted in the Experimental part.

Considering the possibility for formation of *E*/*Z*-isomers in the reaction of phenoxyacetophenones **3a–k** with thiosemicarbazides, it should be noted that the exact structure of thiosemicarbazone derivatives **4a–q** is unknown. Although the formation of *E* or *Z*-isomer is controlled kinetically during the preparation of compounds, isomerization around the C=N double bond is also possible via rotation and inversion mechanisms in the absence or presence of catalysts such as acids or bases [16, 17].

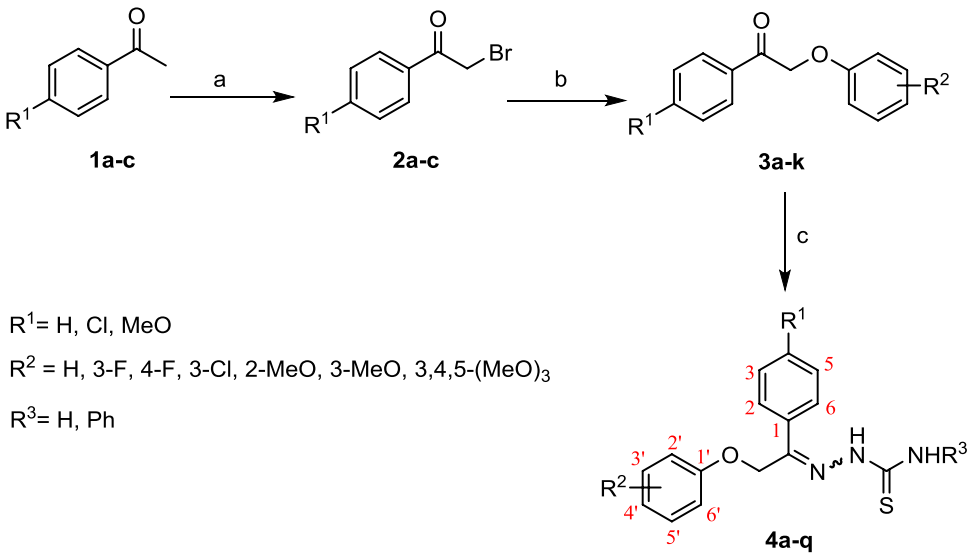
Computational studies

In the lack of spectroscopic data for distinguishing the *E*/*Z*-isomers, a computational study using density functional theory has been applied for the parent compound **4a**. Our DFT calculations with M06-D3, M06-2X, B3LYP-D3 and B97D methods demonstrated that among two possible geometric isomers, the (*Z*)-isomer is 0.4, 0.6, 0.1 and 0.7 kcal/mol, respectively, more stable than the corresponding (*E*)-isomer in term of Gibbs free energy (Fig. 2).

The optimized structure of (*Z*)-**4a** displays the intramolecular hydrogen bond between the NH and the oxygen of phenoxy with distance of 2.09 Å, which stabilizes somewhat the (*Z*)-isomer. It is clear that in all cases the nitrogen lone pair on both sides of C=S moiety can donate electrons to the π^* orbital of C=S, causing N–C bonds to get stronger, in contrast, carbon–sulfur bond of thiocarbonyl moiety tends to single bond.

The main conformers of this compound will be obtained from rotation around C–C bond (see Fig. 3). Torsion angle of O–C^C–N was chosen to explore its changing effect on the energy of the molecule and the stability of the compound in the solution phase. For this purpose, this torsion angle was scanned in the range of 360° by 5° steps. As depicted in Fig. 3, the local minimum energy points in the optimization results indicated that each of the (*Z*)- and (*E*)-isomers have two stable conformers; the (*Z*)-isomer conformers are at 56° and 301° torsion angles and the (*E*)-isomer at 125° and 235° torsion angles.

Besides configurational and conformational aspects of compounds, we explored the drug-like properties of all compounds. Molecular properties were calculated using Molinspiration web tool (www.molinspiration.com) and listed in Table S1. As seen, all compounds have acceptable properties, suggesting no problem in their bioavailability. Furthermore, the metabolism and toxicity properties of representative compounds (**4l** and **4p**) were predicted by a freely accessible web server namely pkCSM (http://structure.bioc.cam.ac.uk/pkcsm) [18]. The obtained results were presented in Table S2–S5 (Supplementary material).



Scheme 1 Synthesis of compounds **4a–q**. Reagents and conditions: a) $CuBr_2$, $CHCl_3$/EtOAc; b) appropriate phenolic derivatives, K_2CO_3, DMF, rt; c) thiosemicarbazide or phenyl thiosemicarbazide, HCl, EtOH, rt

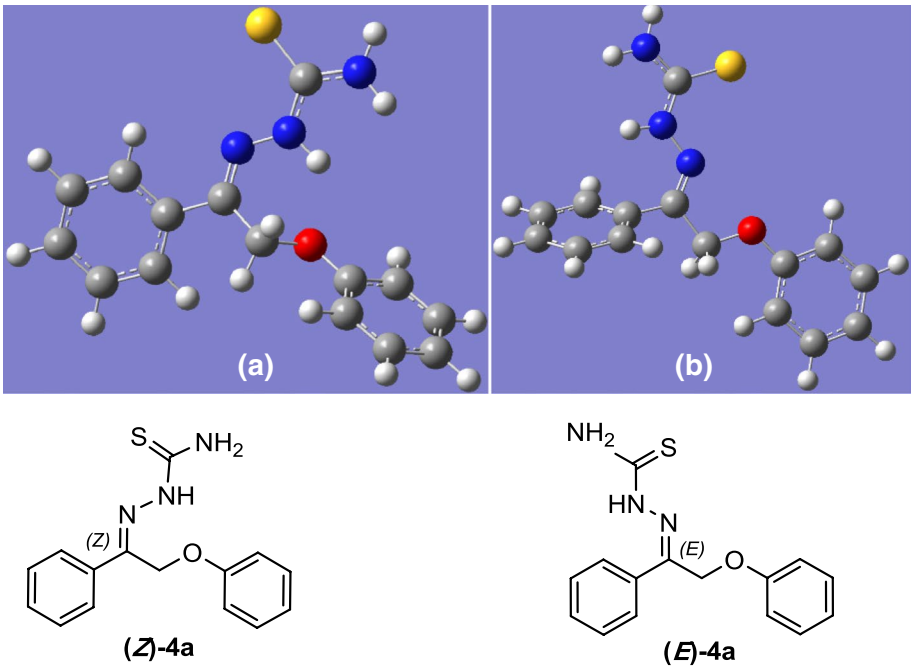


Fig. 2 Fully optimized structures of **4a**: (*Z*)-**4a** (**a**); (*E*)-**4a** (**b**)

Biological activity

In vitro anti-toxoplasma activity

The synthesized compounds **4a–q** were subjected to assess the inhibition of *T. gondii* in vitro. For this purpose, the host cells (Vero cells) were infected with intracellular parasites (tachyzoites) of RH strain, and the effect of test compounds on *T. gondii*-infected cells was evaluated. Furthermore, the cytotoxic effect of compounds was evaluated by MTT assay against uninfected Vero cells and selectivity index (SI) was determined for each compound. The obtained IC_{50} values were listed in Table 1. The IC_{50} values of compounds against *T. gondii*-infected cells were in the range of 1.09–25.19 µg/mL. The CC_{50} values of all compounds with exception of **4n** and **4o** against uninfected host cells were significantly higher than their IC_{50} values. Compounds **4c**, **4l** and **4p** with IC_{50} values of ≤ 1.38 µg/mL showed the highest inhibitory activity against infected cells, displaying also good selectivity index (SI ≥ 10). Notably, compound **4c** was 3-chlorophenoxy derivative and compounds **4l** and **4p** were derived from 4-fluorophenoxyacetophenone. Indeed, compound **4l** (R^1 = MeO, R^2 = 4-F and R^3 = H) showed the highest potency toward *T. gondii*-infected cells with more favorable safety for host cells (IC_{50} = 1.09 µg/mL, SI = 19).

Structurally, the final compounds **4a–q** were thiosemicarbazone of acetophenone, 4-methoxyacetophenone or 4-chloroacetophenone. In the acetophenone analogs **4a–i**, the introduction of 3-F or 3-Cl on the phenoxy moiety increased the activity (compare **4b** and **4c** with **4a**). In contrast, the presence of 2-MeO, 3-MeO or 4-F on the phenoxy moiety had unfavorable effect on activity; thus, compounds **4d**, **4f** and **4h** were less active than **4a**. The comparison of unsubstituted acetophenone derivative **4a** with corresponding 4-chloro analog **4n** (R^1 = Cl) revealed that the introduction of 4-chloro group diminished the activity against *T. gondii*-infected cells. Inversely, the same modification in 4-fluorophenoxyacetophenone **4h** resulted in more potent compound **4p**. Therefore, it can be concluded that the effect of substituent on the acetophenone part depends on the substitution type of phenoxy moiety.

Other modification in the target compounds was insertion of phenyl ring on the thiosemicarbazone moiety. While the *N*-phenyl-thiosemicarbazones **4g**, **4i**, **4k**, **4m** and **4q** were less active than their corresponding simple thiosemicarbazones, the *N*-phenyl analog **4o** was found to be more active than its unsubstituted thiosemicarbazone **4n**.

Effect of compounds 4l and 4p on tachyzoites

The most active compounds (**4l** and **4p**) against *T. gondii*-infected cells were selected for further evaluation against tachyzoites. Thus, *T. gondii* tachyzoites were treated with compounds **4l** and **4p** at the concentration of 5 µg/mL for 24 h. Pyrimethamine (at the concentration of 50 µg/mL) and PBS were used as positive and negative control groups. As seen in Fig. 4, while the mortality rate of tachyzoites in PBS group was 4.7%, the **4l**- and **4p**-treated tachyzoites showed 75.3 and 72.3% mortality, respectively. Therefore, the representative compounds **4l** and **4p** can significantly reduce the viability of *T. gondii* tachyzoites at the concentration of 5 µg/mL. It should be noted that the standard drug pyrimethamine could induced 80.5% mortality at the higher concentration of 50 µg/mL.

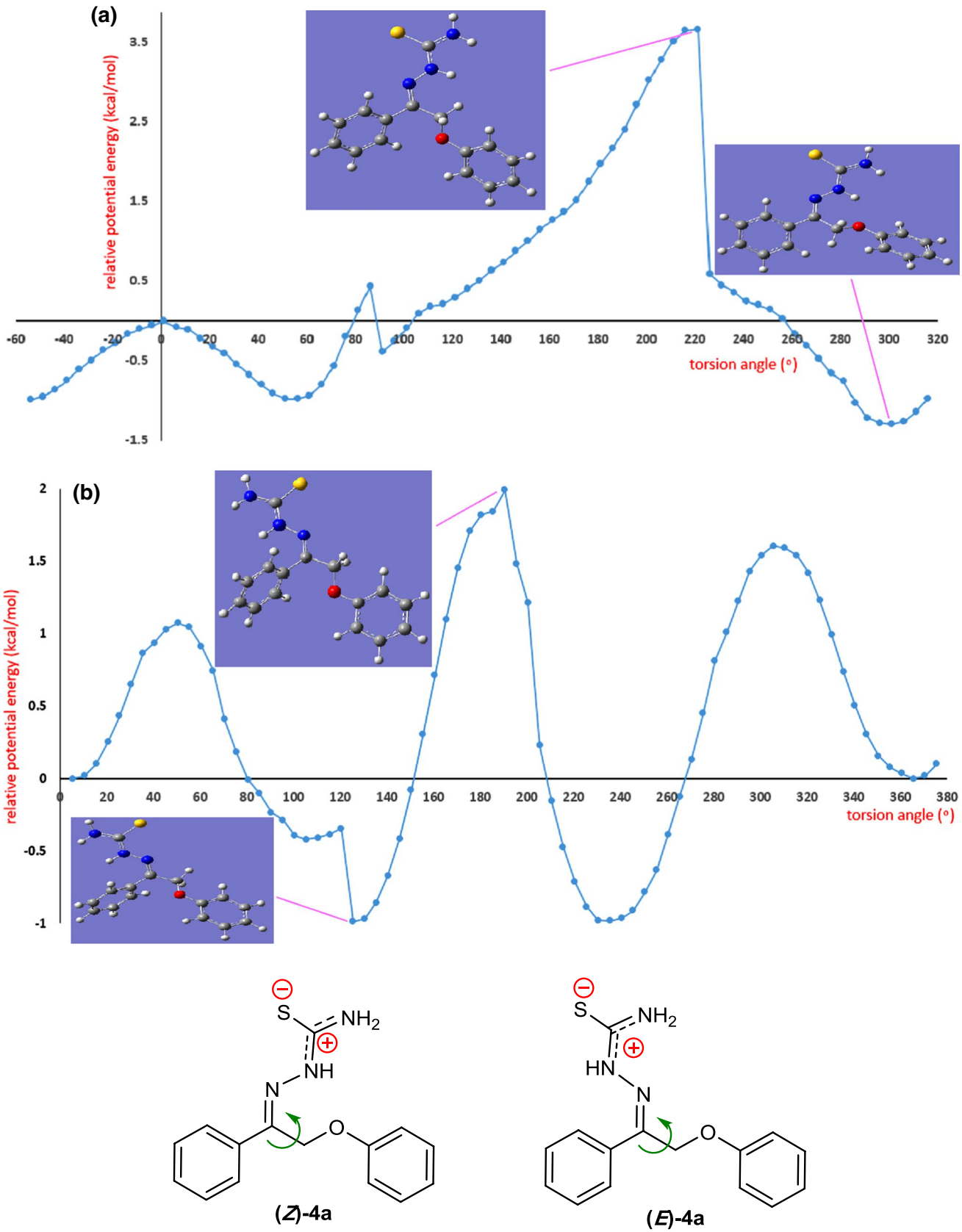
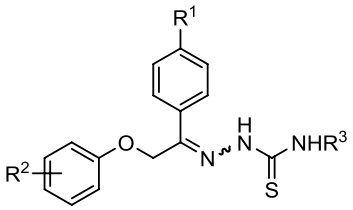


Fig. 3 Scan of potential energy surface (rotating the O–C^C–N dihedral angle in steps of 5°) at the B3LYP/6-31G(d) level **a** (*Z*)-**4a**; **b** (*E*)-**4a**

Table 1 Inhibitory activity of compounds **4a–q** against *Toxoplasma gondii*-infected Vero cells (IC_{50}s) in comparison with their cytotoxicity against Vero cells (CC_{50}s)



Compounds	R^1	R^2	R^3	IC_{50} (μg/mL)	CC_{50} (μg/mL)	SI
4a	H	H	H	4.02	5.12	1.3
4b	H	3-F	H	2.85	14.11	4.9
4c	H	3-Cl	H	1.38	13.83	10.0
4d	H	2-MeO	H	12.66	41.57	3.3
4e	H	3,4,5-$(MeO)_3$	H	15.19	36.72	2.4
4f	H	3-MeO	H	5.51	22.77	4.1
4g	H	H	Ph	5.92	7.27	1.2
4h	H	4-F	H	13.09	51.25	3.9
4i	H	4-F	Ph	25.19	34.13	1.4
4j	MeO	H	H	3.93	11.58	2.9
4k	MeO	H	Ph	7.07	15.47	2.2
4l	MeO	4-F	H	1.09	20.73	19.0
4m	MeO	4-F	Ph	4.22	13.99	3.3
4n	Cl	H	H	22.13	19.41	0.9
4o	Cl	H	Ph	7.29	6.38	0.9
4p	Cl	4-F	H	1.19	13.00	10.9
4q	Cl	4-F	Ph	8.27	35.41	4.3
Pyrimethamine				2.63	7.99	3.0

IC_{50}: half maximal inhibitory concentration, CC_{50}: half cytotoxic concentration; SI: selectivity index. $SI = CC_{50}/IC_{50}$

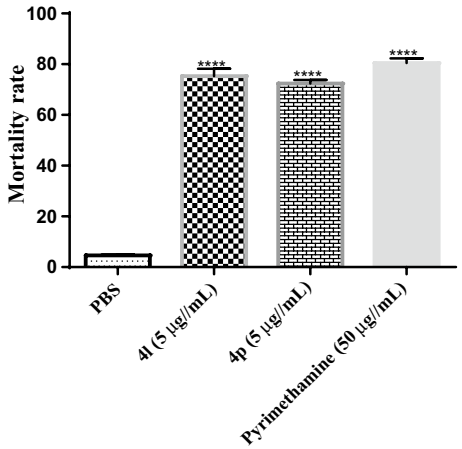


Fig. 4 The effect of compounds after 24 h on the mortality of *T. gondii* tachyzoites

Effect of compounds 4l and 4p on the invasion and intracellular proliferation of *T. gondii*

The host Vero cells were infected with tachyzoites at a ratio of 5:1 for 3 h and then treated with the compounds **4l** and **4p** or pyrimethamine (5 μg/mL). After fixing and staining, the cells were counted under a light microscope in order to determine the infection index (number of infected cell per 100 examined cells) and *T. gondii* intracellular proliferation (total number of tachyzoites per 100 examined cells). Each experiment was repeated three times; the values were determined as mean ± SEM. The obtained data were presented in Fig. 5 and compared with control group. As observed in Fig. 5, the infection index in the negative control group (the cells were infected, but not treated) was 71%. Treatment with compounds **4l** and **4p** at the concentration of 5 μg/mL resulted in the decrease in infection index to 42.3 and 47.3%, respectively. The infection index for pyrimethamine-treated cells at the same concentration was 50.7%. On the other hand, after treatment with **4l**, **4p** and pyrimethamine, the total number of tachyzoites per 100 examined cells was 352.7, 408 and 325.3, being significantly less than that of control (663.3). Based on these data, it can be concluded that compounds **4l** and **4p** can significantly reduce the infectivity rate and intracellular proliferation of *T. gondii*, comparable to those of pyrimethamine.

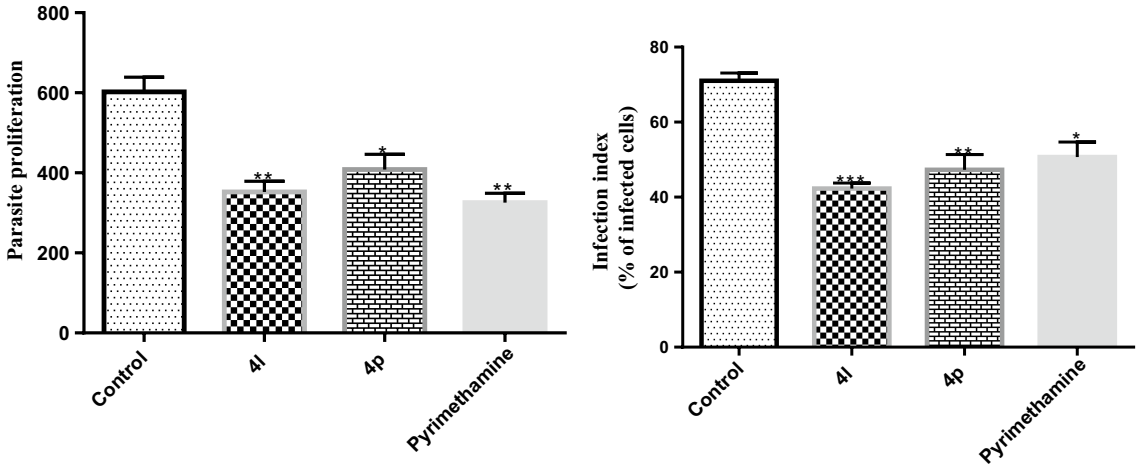


Fig. 5 Infection index (% of infected cells) and parasite proliferation (total number of tachyzoites per 100 cells) in Vero cells treated with compounds **4l**, **4p** and pyrimethamine in concentration of 5 µg/mL, staining by blue toluidine. Controls: the cells were infected, but not treated. Data were shown as mean ± SEM in triplicates. Significant difference compared to control (*$P < 0.05$)

Conclusion

We have synthesized a new series of aryloxyacetophenone thiosemicarbazones with anti-*T. gondii* activity. The in vitro assay of compounds against *T. gondii*-infected cells revealed that all thiosemicarbazones had significant inhibitory activity, displaying IC_{50} values between 1.09 and 25.19 µg/mL. The most active compound namely 2-(2-(4-fluorophenoxy)-1-(4-methoxyphenyl)ethylidene)hydrazine-1-carbothioamide (**4l**) showed also the highest selectivity (SI = 19) toward host cells (Vero cells), being better than standard drug pyrimethamine. SAR study showed that the concurrence of substituents on both aryl ring of phenoxyacetophenone is important for potency and safety profiles of promising compounds **4l** and **4p**. The representative compounds **4l** and **4p** were further tested for actions on the tachyzoite viability, invasion and intracellular proliferation. The obtained results indicated that these compounds at the concentration of 5 µg/mL can significantly reduce the viability of *T. gondii* tachyzoites, as well as their infectivity rate and intracellular proliferation, comparable to those of pyrimethamine. These findings suggest that these compounds can be considered as new leads for development of anti-*T. gondii* agents.

Experimental

Chemistry

All solvents and chemical reagents were purchased from Merck Co. and used without further purification. 2-Bromoacetophenone derivatives (**2a–c**) were prepared by using literature method [19]. The synthesis of the phenoxyacetophenones (**3a–k**) has been carried out according to the procedure previously reported by us [15]. The atom numbering of compounds **4a–q** for NMR interpretations was illustrated in Scheme 1.

General method for the preparation of final compounds 4a–q

To a solution of phenoxyacetophenone derivative **3** (1 mmol) in ethanol (4 mL), a few drops of concentrated HCl were added and stirred at room temperature. Then, thiosemicarbazide or 4-phenylthiosemicarbazide (1 mmol) was added portion-wise and stirring was continued. After consumption of starting materials (checked by TLC), the reaction mixture was left in refrigerator overnight. Subsequently, the precipitated solid was separated by filtration and washed with cool ethanol and dried to give pure compounds **4a–q**.

2-(2-Phenoxy-1-phenylethylidene)hydrazine-1-carbothioamide (4a)

White solid; yield: 88%; m.p: 121–123 °C; IR (KBr, cm^{-1}): 3412, 3248, 1599, 1472, 1290, 1212, 1119, 1075, 881, 751, 685. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.33 (s, 2H, CH_2O), 6.96–7.03 (m, 3H, H-2′, H-4′ and H-6′), 7.32 (t, 2H, $J = 7.60$ Hz, H-3′ and H-5′), 7.37–7.43 (m, 3H, H-3, H-4 and H-5), 7.89–7.96 (m, 2H, H-2 and H-6), 8.09 (br s, 1H, NH_2), 8.48 (br s, 1H, NH_2), 10.76 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 179.58, 158.05, 144.66, 136.14, 129.96, 129.76, 128.74, 127.51, 121.82, 115.28, 61.65. MS (*m/z*, %) 285 (M^+, 5), 268 (7), 195 (10), 180 (32), 178 (100), 133 (69), 105 (36), 103 (100), 94 (38), 91 (29), 77 (97), 66 (22), 60 (25). Anal. Calcd for $C_{15}H_{15}N_3OS$: C, 63.13; H, 5.30; N, 14.73. Found: C, 63.09; H, 5.39; N, 14.70.

2-(2-(3-Fluorophenoxy)-1-phenylethylidene)hydrazine-1-carbothioamide (4b)

White solid; yield: 84%; m.p: 158–160 °C; IR (KBr, cm^{-1}): 3357, 3262, 3179, 1643, 1604, 1538, 1486, 1384, 1281, 1144, 802, 553. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.33 (s, 2H, CH_2O), 6.79–6.86 (m, 2H, H-5′ and H-6′), 6.88 (dt, 1H, J = 11.2 and 2.4 Hz, H-2′), 7.31–7.37 (m, 1H, H-4′), 7.38–7.41 (m, 3H, H-3, H-4 and H-5), 7.90–7.96 (m, 2H, H-2 and H-6), 8.10 (br s, 1H, NH_2), 8.49 (br s, 1H, NH_2), 10.85 (br s, 1H, NH). MS (*m/z*, %) 303 (M^+, < 1), 133 (17), 112 (100), 103 (27), 92 (15), 83 (37), 76 (18), 73 (7), 64 (15), 51 (6). Anal. Calcd for $C_{15}H_{14}FN_3OS$: C, 59.39; H, 4.65; N, 13.85; Found: C, 59.50; H, 4.57; N, 13.66.

2-(2-(3-Chlorophenoxy)-1-phenylethylidene)hydrazine-1-carbothioamide (4c)

Yellow solid; yield: 91%; m.p: 111–113 °C; IR (KBr, cm^{-1}): 3123, 3049, 1968, 1872, 1498, 1072, 1049, 681, 572. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.33 (s, 2H, CH_2O), 6.92 (dd, 1H, J = 8.4 and 1.6 Hz, H-6′), 7.05 (dd, 1H, J = 7.6 and 1.2 Hz, H-4′), 7.10 (t, 1H, J = 2.0 Hz, H-2′), 7.33 (t, 1H, H-5′), 7.36–7.42 (m, 3H, H-3, H-4 and H-5), 7.88–7.95 (m, 2H, H-2 and H-6), 8.11 (br s, 1H, NH_2), 8.49 (br s, 1H, NH_2), 10.88 (br s, 1H, NH). MS (*m/z*, %) 319 (M^+, < 1), 256 (3), 178 (23), 160 (2), 131 (100), 128 (9), 116 (59), 89 (12), 60 (31), 57 (25). Anal. Calcd for $C_{15}H_{14}ClN_3OS$: C, 56.34; H, 4.41; N, 13.14. Found: C, 56.15; H, 4.53; N, 13.10.

2-(2-(2-Methoxyphenoxy)-1-phenylethylidene)hydrazine-1-carbothioamide (4d)

White solid; yield: 85%; m.p: 114–117 °C; IR (KBr, cm^{-1}): 3288, 2961, 1596, 1498, 1477, 1263, 1130, 996, 731. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.83 (s, 3H, OCH_3), 5.23 (s, 2H, CH_2O), 6.88–6.96 (m, 1H, H-3′), 6.98–7.06 (m, 2H, H-4′ and H-5′), 7.15 (d, 1H, J = 8.4 Hz, H-6′), 7.45–7.44 (m, 3H, H-3, H-4 and H-5), 7.95–8.01 (m, 2H, H-2 and H-6), 8.14 (br s, 1H, NH_2), 8.53 (br s, 1H, NH_2), 10.75 (br s, 1H, NH). MS (*m/z*, %) 315 (M^+, 1), 298 (7), 233 (4), 210 (11), 178 (100), 133 (30), 124 (90), 110 (54), 103 (58), 81 (27), 77 (48), 51 (14). Anal. Calcd for $C_{16}H_{17}N_3O_2S$: C, 60.93; H, 5.43; N, 13.32. Found: C, 60.89; H, 5.61; N, 13.21.

2-(1-Phenyl-2-(3,4,5-trimethoxyphenoxy)ethylidene) hydrazine-1-carbothioamide (4e)

White solid; yield: 84%; m.p: 178–180 °C; IR (KBr, cm^{-1}): 3413, 3016, 2964, 1610, 1592, 1508, 1493, 1235, 1130, 1018, 808, 762. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.57 (s, 3H, OCH_3), 3.71 (s, 6H, 2 × OCH_3), 5.32 (s, 2H, CH_2O), 6.28 (s, 2H, H-2′ and H-6′), 7.37–7.43 (m, 3H, H-3, H-4 and H-5), 7.93–7.99 (m, 2H, H-2 and H-6), 8.12 (br s, 1H, NH_2), 8.51 (br s, 1H, NH_2), 10.74 (br s, 1H, NH). MS (*m/z*, %) 375 (M^+, 2), 358 (7), 284 (6), 233 (6), 193 (9), 184 (80), 178 (100), 159 (6), 141 (41), 124 (53), 103 (83), 77 (54), 51 (16). Anal. Calcd for $C_{18}H_{21}N_3O_4S$: C, 57.58; H, 5.64; N, 11.19. Found: C, 57.44; H, 5.60; N, 11.30.

2-(2-(3-Methoxyphenoxy)-1-phenylethylidene)hydrazine-1-carbothioamide (4f)

White solid; yield: 76%; m.p: 125–127 °C; IR (KBr, cm^{-1}): 3406, 3160, 2954, 1606, 1584, 1494, 1482, 1288, 1279, 1194, 1158, 1066, 807, 758. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.71 (s, 3H, OCH_3), 5.32 (s, 2H, CH_2O), 6.50–6.58 (m, 3H, H-2′, H-4′ and H-6′), 7.19 (t, 1H, J = 8.00 Hz, H-5′), 7.36–7.42 (m, 3H, H-3, H-4 and H-5), 7.89–7.98 (m, 2H, H-2 and H-6), 8.10 (br s, 1H, NH_2), 8.49 (br s, 1H, NH_2), 10.78 (br s, 1H, NH). MS (*m/z*, %) 315 (M^+, < 1), 284 (6), 240 (8), 207 (16), 178 (100), 159 (8), 141 (17), 124 (77), 103 (95), 77 (81), 51 (29). Anal. Calcd for $C_{16}H_{17}N_3O_2S$: C, 60.93; H, 5.43; N, 13.32. Found: C, 61.09; H, 5.31; N, 13.28.

2-(2-Phenoxy-1-phenylethylidene)-*N*-phenylhydrazine-1-carbothioamide (4g)

White solid; yield: 69%; m.p: 133–135 °C; IR (KBr, cm^{-1}): 3273, 3182, 2889, 1594, 1492, 1228, 1180, 1030, 794, 686. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.40 (s, 2H, CH_2O), 6.97–7.05 (m, 3H, H-2′, H-4′ and H-6′), 7.23 (t, 1H, J = 7.2 Hz, H-4″), 7.30–7.39 (m, 4H, H-3′, H-5′, H-3″ and H-5″), 7.39–7.44 (m, 3H, H-3, H-4 and H-5), 7.56 (dd, 2H, J = 7.2 and 1.2 Hz, H-2″ and H-6″), 7.97–8.04 (m, 2H, H-2 and H-6), 10.20 (br s, 1H, NH), 10.92 (br s, 1H, NH). MS (*m/z*, %) 361 (M^+, < 1), 268 (8), 254 (7), 210 (15), 195 (15), 169 (7), 152 (7), 135 (21), 107 (13), 105 (86), 98 (22), 93 (100), 77 (81), 51 (27). Anal. Calcd for $C_{21}H_{19}N_3OS$: C, 69.78; H, 5.30; N, 11.63. Found: C, 69.55; H, 5.17; N, 11.80.

2-(2-(4-Fluorophenoxy)-1-phenylethylidene)hydrazine-1-carbothioamide (4h)

Yellow solid; yield 81%; m.p: 103–105 °C; IR (KBr, cm^{-1}): 3358, 2765, 1601, 1597, 1497, 1320, 1240, 1226, 1128, 1004, 829, 751, 554. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.58 (s, 2H, CH_2O), 7.69–7.31 (m, 4H, H-2′, H-3′, H-5′ and H-6′), 7.62–7.75 (m, 3H, H-3, H-4 and H-5), 8.31 (d, 2H, J = 7.6 Hz, H-2 and H-6), 8.59 (br s, 1H, NH), 8.83 (br s, 1H, NH). MS (*m/z*, %) 303 (M^+, < 1), 256 (4), 230 (10), 179 (10), 178 (100), 160 (4), 133 (27), 112 (24), 105 (51), 103 (52), 77 (41), 60 (19), 51 (11). Anal. Calcd for $C_{15}H_{14}FN_3OS$: C, 59.39; H, 4.65; N, 13.85. Found: C, 59.33; H, 4.39; N, 14.01.

2-(2-(4-Fluorophenoxy)-1-phenylethylidene)-*N*-phenylhydrazine-1-carbothioamide (4i)

White solid; yield 73%; m.p: 125–127 °C; IR (KBr, cm^{-1}): 3302, 3273, 2972, 1551, 1508, 1497, 1441, 1287, 1213, 1178, 897, 689, 566. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.38 (s, 2H, CH_2O), 7.04 (dd, 2H, J = 9.2 and 4.4 Hz, H-2′ and H-6′), 7.18 (t, 2H, J = 8.8 Hz, H-3′ and H-5′), 7.30 (t, 1H, J = 7.2 Hz, H-4″), 7.38 (t, 2H, J = 7.6 Hz, H-3″ and H-5″), 7.39–7.45 (m, 3H, H-3, H-4 and H-5), 7.56 (d, 2H, J = 7.2 Hz, H-2″ and H-6″), 7.96–8.03 (m, 2H, H-2 and H-6), 10.19 (br s, 1H, NH), 10.78 (br s, 1H, NH). MS (*m*/*z*, %) 379 (M^+, < 1), 286 (21), 254 (36), 178 (28), 133 (41), 112 (30), 103 (100), 94 (84), 77 (53), 51 (17). Anal. Calcd for $C_{21}H_{18}FN_3OS$: C, 66.47; H, 4.78; N, 11.07. Found: C, 66.40; H, 4.74; N, 10.89.

2-(1-(4-Methoxyphenyl)-2-phenoxyethylidene)hydrazine-1-carbothioamide (4j)

White solid; yield 74%, m.p: 156–158 °C; IR (KBr, cm^{-1}): 3343, 3433, 2807, 1598, 1587, 1502, 1481, 1296, 1257, 1234, 1171, 1114, 835, 760, 630, 555. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.81 (s, 3H, OCH_3), 5.31 (s, 2H, CH_2O), 6.94 (d, 2H, J = 8.4 Hz, H-3 and H-5), 6.97–7.06 (m, 3H, H-2′, H-4′ and H-6′), 7.33 (t, 2H, J = 8.0 Hz, H-3′ and H-5′), 7.91 (d, 2H, J = 8.8 Hz, H-2 and H-6), 8.07 (br s, 1H, NH_2), 8.43 (br s, 1H, NH_2), 10.70 (br s, 1H, NH). MS (*m*/*z*, %) 315 (M^+, < 1), 242 (12), 135 (100), 121 (7), 107 (6), 92 (7), 77 (18), 51 (3). Anal. Calcd for $C_{16}H_{17}N_3O_2S$: C, 60.93; H, 5.43; N, 13.32. Found: C, 61.20; H, 5.29; N, 13.16.

2-(1-(4-Methoxyphenyl)-2-phenoxyethylidene)-*N*-phenylhydrazine-1-carbothioamide (4k)

White solid; yield 84%; m.p: 162–164 °C; IR (KBr, cm^{-1}): 3281, 3220, 2838, 1591, 1544, 1487, 1256, 1183, 1033, 759, 518. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.82 (s, 3H, OCH_3), 5.39 (s, 2H, CH_2O), 6.97 (d, 2H, J = 8.8 Hz, H-3 and H-5), 7.00–7.08 (m, 3H, H-2′, H-4′ and H-6′), 7.24 (t, 1H, J = 7.2 Hz, H-4″), 7.34 (d, 2H, J = 7.6 Hz, H-3, and H-5), 7.40 (t, 2H, J = 8.0 Hz, H-3″ and H-5″), 7.58 (d, 2H, J = 8.0 Hz, H-2″ and H-6″), 8.00 (d, 2H, J = 8.4 Hz, H-2 and H-6), 10.17 (br s, 1H, NH), 11.04 (br s, 1H, NH). MS (*m*/*z*, %) 391 (M^+, < 1), 298 (59), 240 (27), 225 (6), 210 (8), 163 (4), 133 (100), 122 (81), 107 (28), 93 (96), 77 (72), 51 (16). Anal. Calcd for $C_{22}H_{21}N_3O_2S$: C, 67.50; H, 5.41; N, 10.73. Found: C, 67.46; H, 5.77; N, 10.79.

2-(2-(4-Fluorophenoxy)-1-(4-methoxyphenyl)ethylidene) hydrazine-1-carbothioamide (4l)

White solid; yield 75%; m.p: 140–142 °C; IR (KBr, cm^{-1}): 3420, 2998, 1605, 1589, 1466, 1257, 1212, 1014, 809, 569. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.80 (s, 3H, OCH_3), 5.29 (s, 2H, CH_2O), 6.94 (d, 2H, J = 8.8 Hz, H-3 and H-5), 7.00 (dd, 2H, J = 9.2 and 4.4 Hz, H-2′, H-6′), 7.17 (t, 2H, J = 8.8 Hz, H-3′ and H-5′), 7.90 (d, 2H, J = 8.8 Hz, H-2 and H-6), 8.07 (br s, 1H, NH_2), 8.44 (br s, 1H, NH_2), 10.70 (br s, 1H, NH). MS (*m*/*z*, %) 333 (M^+, 6), 316 (12), 259 (3), 222 (2), 208 (100), 180 (4), 163 (28), 148 (12), 133 (100), 122 (55), 112 (24), 95 (19), 77 (16), 60 (14). Anal. Calcd for $C_{16}H_{16}FN_3O_2S$: C, 57.64; H, 4.84; N, 12.60. Found: C, 57.31; H, 4.81; N, 12.73.

2-(2-(4-Fluorophenoxy)-1-(4-methoxyphenyl) ethylidene)-*N*-phenylhydrazine-1-carbothioamide (4m)

White solid; yield 91%; m.p: 156–158 °C; IR (KBr, cm^{-1}): 3210, 2935, 1610, 1596, 1542, 1445, 1470, 1253, 1180, 1016, 829, 650, 556. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.82 (s, 3H, OCH_3), 5.37 (s, 2H, CH_2O), 6.97 (d, 2H, J = 8.8 Hz, H-3, and H-5), 7.04 (dd, 2H, J = 8.8 and 4.0 Hz, H-2′ and H-6′), 7.20 (t, 2H, J = 8.8 Hz, H-3′ and H-5′), 7.24 (t, 1H, J = 7.2 Hz, H-4″), 7.40 (t, 2H, J = 7.6 Hz, H-3″and H-5″), 7.58 (d, 2H, J = 7.6 Hz, H-2″ and H-6″), 7.99 (d, 2H, J = 8.8 Hz, H-2 and H-6), 10.17 (br s, 1H, NH), 11.04 (br s, 1H, NH). MS (*m*/*z*, %) 409 (M^+ < 1), 316 (52), 284 (28), 258 (7), 205 (10), 163 (4), 147 (18), 133 (100), 122 (95), 118 (9), 93 (68), 77 (25), 51 (8). Anal. Calcd for $C_{22}H_{20}FN_3O_2S$: C, 64.53; H, 4.92; N, 10.26. Found: C, 64.50; H, 5.04; N, 10.33.

2-(1-(4-Chlorophenyl)-2-phenoxyethylidene)hydrazine-1-carbothioamide (4n)

White solid; yield 46%; m.p: 164–166 °C; IR (KBr, cm^{-1}): 3259, 3164, 2911, 1619, 1607, 1508, 1210, 1199, 1130, 831, 556. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.31 (s, 2H, CH_2O), 6.93–7.07 (m, 3H, H-2′, H-4′ and H-6′), 7.17 (t, 2H, J = 8.4 Hz, H-3′ and H-5′), 7.45 (d, 2H, J = 8.0 Hz, H-3 and H-5), 7.97 (d, 2H, J = 8.4 Hz, H-2 and H-6), 8.19 (br s, 1H, NH_2), 8.54 (br s, 1H, NH_2), 10.89 (br s, 1H, NH). MS (*m*/*z*, %) 320 (M + 1, 9), 262 (2), 247 (2), 226 (8), 214 (62), 212 (100), 184 (4), 167 (43), 137 (75), 125 (22), 102 (22), 75 (22), 60 (7). Anal. Calcd for $C_{15}H_{14}ClN_3OS$: C, 56.34; H, 4.41; N, 13.14. Found: C, 56.61; H, 4.30, N, 13.16.

2-(1-(4-Chlorophenyl)-2-phenoxyethylidene)-*N*-phenylhydrazine-1-carbothioamide (4o)

White solid; yield 63%; m.p: 154–156 °C; IR (KBr, cm^{-1}): 3181, 3058, 1614, 1597, 1549, 1490, 1232, 1178, 1023, 985, 822, 744. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.40 (s, 2H, CH_2O), 6.98–7.06 (m, 3H, H-2′, H-4′ and H-6′), 7.24 (t, 1H, J = 7.6 Hz, H-4″), 7.35 (t, 2H, J = 8.0 Hz, H-3′ and H-5′), 7.41 (t, 2H, J = 7.6 Hz, H-3″ and H-5″), 7.48 (d, 2H, J = 8.4 Hz, H-3 and H-5), 7.55 (d, 2H, J = 7.6 Hz, H-2″

and H-6″), 8.06 (d, 2H, $J = 8.8$ Hz, H-2 and H-6), 10.26 (br s, 1H, NH), 11.23 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 177.81, 158.01, 144.42, 139.49, 134.89, 134.62, 130.00, 129.65, 128.71, 128.63, 126.68, 126.16, 121.89, 115.30, 61.69. MS (*m/z*, %) 395 (M^+, < 1), 378 (3), 302 (21), 290 (36), 288 (100), 244 (26), 229 (22), 212 (21), 180 (12), 167 (19), 137 (100), 125 (20), 111 (12), 102 (29), 93 (57), 77 (73), 66 (28), 51 (20). Anal. Calcd for $C_{21}H_{18}ClN_3OS$: C, 63.71; H, 4.58; N, 10.61. Found: C, 63.65; H, 4.77; N, 10.46.

2-(1-(4-Chlorophenyl)-2-(4-fluorophenoxy)ethylidene) hydrazine-1-carbothioamide (4p)

White solid; yield 45%; m.p: 159–161 °C; IR (KBr, cm^{-1}): 3421, 3060, 1610, 1596, 1486, 1437, 1287, 1241, 1092, 823, 753, 519. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.33 (s, 2H, CH_2O), 6.90–7.08 (m, 2H, H-2′ and H-6′), 7.33 (t, 2H, $J = 8.0$ Hz, H-3′ and H-5′), 7.45 (d, 2H, $J = 8.4$ Hz, H-3 and H-5), 7.99 (d, 2H, $J = 8.8$ Hz, H-2 and H-6), 8.20 (br s, 1H, NH_2), 8.54 (br s, 1H, NH_2), 10.90 (br s, 1H, NH). MS (*m/z*, %) 337 (M^+, < 1), 302 (2), 244 (3), 212 (100), 184 (4), 167 (32), 137 (60), 125 (16), 102 (6), 77 (33), 60 (25). Anal. Calcd for $C_{15}H_{13}ClFN_3OS$: C, 53.34; H, 3.88; N, 12.44. Found: C, 53.27; H, 4.01; N, 12.40.

2-(1-(4-Chlorophenyl)-2-(4-fluorophenoxy) ethylidene)-*N*-phenylhydrazine-1-carbothioamide (4q)

White solid; yield 72%; m.p: 168–170 °C; IR (KBr, cm^{-1}): 3295, 3063, 1606, 1595, 1547, 1490, 1204, 1179, 1096, 824, 662, 535. ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.38 (s, 2H, CH_2O), 7.04 (dd, 2H, $J = 8.8$ and 4.4 Hz, H-2′ and H-6′), 7.20 (t, 2H, $J = 8.4$ Hz, H-3′ and H-5′), 7.26 (t, 1H, $J = 7.6$ Hz, H-4″), 7.41 (t, 2H, $J = 7.6$ Hz, H-3″ and H-5″), 7.48 (d, 2H, $J = 8.4$ Hz, H-3 and H-5), 7.55 (d, 2H, $J = 8.4$ Hz, H-2″ and H-6″), 8.05 (d, 2H, $J = 8.4$ Hz, H-2 and H-6), 10.26 (br s, 1H, NH), 11.23 (br s, 1H, NH). MS (*m/z*, %) 413 (M^+, < 1), 320 (29), 288 (25), 262 (8), 247 (5), 209 (11), 180 (8), 155 (5), 139 (34), 137 (100), 125 (30), 111 (16), 93 (70), 75 (27), 66 (22), 51 (8). Anal. Calcd for $C_{21}H_{17}ClFN_3OS$: C, 60.94; H, 4.14; N, 10.15. Found: C, 61.15; H, 4.10; N, 10.23.

Biological evaluations

Anti-*T. gondii* assay using MTT

Tachyzoites (RH strain of *T. gondii*) were collected from the peritoneal cavity of Balb/c mice after intraperitoneal injection with 1×10^5 of the parasite. Six-week-old inbred female Balb/c mice weighing 20–22 g were used for this experiment. All experimental animals were housed in cages under standard laboratory conditions (with an average temperature of 20 to 25 °C) and were given drinking water and a regular mouse diet. This research was performed according to institutional animal ethics guidelines of the Animal Research Center, Mazandaran University of Medical Sciences (ARC-MUMS). The experimental protocols for animal use in this study were approved by the Mazandaran University of Medical Sciences Ethics Committee (MUMSEC).

Vero cells (ATCC No. CCL-81) were cultivated in RPMI-1640 medium with the addition of 10% inactivated FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin. For cytotoxicity assay, 2×10^4 Vero cells/mL were seeded in 96-well plates. The compounds were initially dissolved in dimethyl sulfoxide (DMSO) to form a stock solution. Then, this stock solution was diluted in PBS as required [7].

After 24 h, the cells were exposed to compounds and pyrimethamine (positive control) at the final concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µg/mL. In the next day, the viabilities of the Vero cells were evaluated by using a colorimetric MTT assay [9]. For effects of the compounds on *T. gondii* infection, 2×10^4 Vero cells/mL (suspended in RPMI-1640 supplemented with 10% FBS) were seeded in 96-well plates. After 24 h of seeding, the cells were infected with RH strain of *T. gondii* (2×10^5 tachyzoites/mL) and placed in a 37 °C incubator maintained at 5% CO_2 for 24 h. After that, the cells were exposed to the compounds and pyrimethamine at final concentrations of 1.56–100 µg/mL. After 24 h, the viability of *T. gondii*-infected Vero cells was evaluated by the MTT assay [20]. The 50% inhibitory concentrations (IC_{50}s) were calculated by the GraphPad Prism 6.0.

In vitro evaluation of compounds against tachyzoites

To evaluate the effect of compounds **4l** and **4p** on tachyzoites, a concentration (5 µg/mL) of compounds were assessed for 24 h on mortality of *T. gondii* tachyzoites. Equal proportions of each compound and 2×10^5 fresh tachyzoites were placed in test tube. After slow mixing, the contents of the tubes were incubated at 37 °C for 24 h. Then, 0.5 mL of 0.5% trypan blue stain was added to the settled tachyzoites. The percentage of dead tachyzoites was determined by counting 100 tachyzoites with hemocytometer under a light microscope. PBS and pyrimethamine (50 µg/mL) were considered as negative and positive control groups, respectively. The experiments were performed in triplicate [21].

Anti-parasitic evaluation of compounds against the invasion and intracellular proliferation of *T. gondii*

Vero cells were cultured on 13-mm round glass slides in 24-well plates (1×10^4 cells/200 µL per well). After 72 h incubation in 37 °C and 5% CO_2, the cells were infected with tachyzoites at a ratio of 5:1 for 3 h incubation. Then,

the cells were washed to remove extracellular parasites, and the cells were treated with the compounds or pyrimethamine (5 µg/mL). The slides with adherent cells were washed with sterile cold PBS, fixed with buffered formalin 10% for 24 h and stained with 1% toluidine blue for 10 s [22]. After that, the cells were counted under a light microscope (Nikon, Japan) in order to determine the infection index (number of infected cell per 100 examined cells) and *T. gondii* intracellular proliferation (total number of tachyzoites per 100 examined cells).

Computational studies

Gaussian 09 was used to fully optimize the structures at the B3LYP level of theory [23]. For all the calculations, solvent effects were considered using the SMD solvation model with water as the solvent [24]. The 6-31G(d) basis set was used for all atoms [25]. Frequency calculations were carried out at the same level of theory as those for the structural optimization. To further refine the energies obtained from the B3LYP-SMD/6-31g(d) calculations and to consider dispersive interactions [26], we carried out single-point energy calculations using the M06-D3 functional method for all of the structures with a larger def2-TZVP basis set on all atoms [27].

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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