New phenoxyacetohydrazones against *Trypanosoma cruzi*

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Abstract

Herein, we reported the design, synthesis, antitrypanosomal and cytotoxic evaluation of a new phenoxyacetohydrazones series. All derivatives were assayed against bloodstream trypomastigote forms of *T. cruzi* (Y strain) and intracellular amastigotes using the model of L-929 cells infected with trypomastigotes of the *Tulahuen* strain. Compound (*E*)-*N*-(3,4-dihydroxybenzylidene)-2-phenoxyacetohydrazide (**11**) showed an activity against trypomastigotes ($IC_{50}/24\text{ h} = 10.3\text{ }\mu\text{M}$) equivalent to that of benznidazole and with selectivity index (SI) = 46. Against infected cultures, (*E*)-*N*-((5-nitrofuran-2-yl) methylene)-2-phenoxyacetohydrazide (**19**) was active at the nanomolar range ($IC_{50}/96\text{ h} = 100\text{ nM}$), being about 15-fold more active than the standard drug and with SI equal to 1000. Thus, derivatives **11** and **19** could be considered good prototype for the development of new candidates for Chagas disease therapy.

Introduction

Chagas disease is a parasitic illness resulting from infection by the hemoflagellate parasite *Trypanosoma cruzi*. It is endemic in 21 countries in Latin America and affects 6 to 7 million people worldwide, causing 10,000 deaths per year [1, 2]. It is estimated that 752,000 working days per year are lost due to premature deaths and U\$ 1.2 billion in lost productivity in seven southernmost American countries. Brazilian absenteeism of workers affected by Chagas disease represented an estimated minimum loss of \$5.6 million per year. Due to the migration of *T. cruzi*-infected people to other continents, Chagas disease is also reported in non-endemic regions, such as the European continent [3–5].

The treatment of Chagas disease is restricted to the nitro derivatives Nifurtimox and Benznidazole and both are effective in reducing the duration and clinical severity of the disease, being the second most used in the Brazilian context. The available chemotherapy is unsatisfactory as a result of severe side effects and restricted efficacy in the chronic phase of the disease [6–8].

The *N*-acylhydrazone (NAH) framework has a special advantage in medicinal chemistry, the presence of an imine vicinal group to the amide is unique characteristic to be explored by the insertion of groups linked to its surroundings, allowing the modulation of multiple targets described in bioactive compounds such as cysteinyl protease inhibitors [9–12]. Compound **1** showed excellent cruzain inhibitory activity ($IC_{50} = 200\text{ nM}$) and a considerable *in vitro* potency against *T. cruzi* ($IC_{50} = 16.2\text{ }\mu\text{M}$) [13]. Our group reported compound **2** containing a hydrazone-cathecol subunit which demonstrated a pharmacophoric character over *T. cruzi*, possibly interfering in the oxidative metabolism [14].

In our effort to develop new potent trypanocidal compounds, we constructed a new class of phenoxyacetohydrazones designed by molecular hybridization between a potent cruzain inhibitor represented by the 2-phenoxyacetamide derivative (**1**) ($IC_{50} = 200\text{ nM}$) [13] and the 1,3,4-thiadiazole-2-arylhydrazone derivative (**2**) ($IC_{50} = 5.3\text{ }\mu\text{M}$) [14]. The design concept of this compounds generates a NAH subunit (A, Fig. 1) with the aim to potentialize the cruzain inhibitor framework of (**1**) (B, Fig. 1) and

explore the introduction of a catechol moiety (C, Fig. 1) a potential radical scavenger group [15]. The planned compounds were synthesized with substituents with different stereoelectronic properties attached to the phenyl subunit D (**3–16**) (Fig. 1) and heterocycles derivatives (**17–19**). Derivative (**10**) was planned due the ability of the *o*-hydroxybenzylidene *N*-acylhydrazone framework to form an electrophilic quinone methide intermediate that could interact with nucleophilic sites in target enzymes of *T. cruzi*, e.g. cysteine proteases [10]. We have elected two types of *in vitro* assays, the direct effect on bloodstream trypomastigote form after 24 h of treatment and the effect on the intracellular proliferation of amastigotes after 96 h. Benznidazole was used as a positive control against *T. cruzi* and the cytotoxicity was determined in mouse peritoneal macrophages (24 h) and mouse fibroblast L929 cells (96 h).

Results And Discussion

Chemistry

The synthetic route planned to achieve the NAH targets compounds (**3–19**) consists in three steps. To a suspension of phenol (**20**) and potassium carbonate in acetonitrile, was added methyl bromoacetate (**21**) and the reaction was warmed till reflux to furnish methyl 2-phenoxyacetate (**22**) in 96% yield [21]. The hydrazinolysis of the ester furnished the key *N*-acylhydrazide intermediate (**23**) in 95% yield [22]. Finally, the target compounds (**3–19**), were obtained by addition to carbonyl followed by the elimination of water between the *N*-acylhydrazide (**23**) and the corresponding aromatic aldehydes (Het/ArCHO) in water, using hydrochloric acid as catalyst described in Scheme 1 [17,23].

The HPLC and the ¹H NMR analysis was consistent with only one geometric isomer at the imine bond level, which X-ray diffraction data indicated as having the relative configuration (*E*) (Fig. 2), according to previous results [17, 24]. Phenoxyacetohydrazones NAH exists in equilibrium between the two stable conformers synperiplanar (*sp*) and antiperiplanar (*ap*) in DMSO solution. It is described that the signal from the imine hydrogen being more down shield matches the *ap* conformer [25].

Crystallography

The compound **3** was recrystallized from ethanol solution by slow evaporation at room temperature. Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre, deposit number 2050640. The angle between the two phenyl rings is 69.33(8)°. The geometry of the exo-C=N bond is (*E*), while the H and O1 atoms in the H1-N1-C8=O1 unit have a cis arrangement, which allows the formation of centrosymmetric dimers, formed from pairs of classical N1-H1—O1 hydrogen bonds. Additional contacts between molecules are generated from C-H—O hydrogen bonds and $\pi(\text{C}=\text{N})-\pi(\text{Ph})$ stacking interactions.

Antitrypanosomal activity

We started our analysis by evaluating the effect of the compounds against bloodstream trypomastigotes of *T. cruzi* (Y strain), a representative of Discrete Typing Unit (DTU) II. The *in vitro* screening using trypomastigote revealed **11**, with an 3,4-dihydroxyphenyl group attached to the imine unit, as the most active compound with an $IC_{50}/24\text{ h} = 10.3\text{ }\mu\text{M}$, equivalent to benznidazole, the reference drug. In order to evaluate the contribution of its hydroxyl groups at C-3 and at C-4, we compared its activity with different analogues [4-OH (**9**); 3,4-OCH₃ (**12**); 3-OCH₃, 4-OH (**13**); 3-OH, 4-OCH₃ (**14**) and -O-CH₂-O- (**16**)]. We observed an important decrease in the activity, indicating that the two hydroxyl groups in the catechol subunit present a pharmacophoric characteristic against *T. cruzi*.

Among the phenyl substituted derivatives, the most active four derivatives presented a hydroxyl group (**9**, **10**, **11** and **13**), three of them in the *para* position. An attractive bioprofile was also identified for 5-nitrofurane derivative (**19**), which showed an $IC_{50}/24\text{ h} = 19.11\text{ }\mu\text{M}$. The *para* substituted derivatives (**4** - **9**) presenting different electronic and lipophilic properties and the trisubstituted derivative (**15**) showed no trypanocidal activity, except for the 4-OH derivative (**9**) with an $IC_{50}/24\text{ h} = 53.3\text{ }\mu\text{M}$ (Table 1).

Additionally, to expand this study, another strain of *T. cruzi* was employed, *Tulahuen* (DTU VI), considered susceptible to nitro derivatives. All phenoxyacetohydrazones were also evaluated on intracellular amastigote forms for *Tulahuen* strain (Table 1). The most active phenoxyacetohydrazone was 5-nitrofurane derivative (**19**), which showed an $IC_{50}/96\text{ h} = 100\text{ nM}$, fifteen times more potent than Benznidazole. The *para* substituted compounds **4-9** and **17** and the disubstituted ones, **12-14** and **16**, presented only moderated activity. The trisubstituted derivative (**15**) was inactive and *o*-OH derivative (**10**), planned to increase the interaction with nucleophilic sites in the cruzain, presented moderate activity ($IC_{50}/96\text{ h} = 22.7\text{ }\mu\text{M}$) (Table 1). Although the intracellular location of the amastigote form difficulties the access of a given drug, in this study we observed that the best results were observed in amastigotes forms. The distinct behavior of the *Tulahuen* and Y parasites could be also due to intrinsic differences between both strains and to the time of treatment of the standardized protocols [19], requiring the assay with the intracellular form 96 h of contact with the drug.

The cytotoxic results of the most active compounds **11** for bloodstream trypomastigotes and **19** for intracellular amastigotes were determined and the selectivity indexes (SI) (LC_{50} for cytotoxicity divided by IC_{50} for antitrypanosomal activity) were 46 and 1000 respectively. In this context, the two compounds with $SI \geq 50$ were considered as good candidates for subsequent studies on antitrypanosomal activity in a murine model [19,26].

Conclusions

We described here in the synthesis, antitrypanosomal and cytotoxic evaluation of new phenoxyacetohydrazones against both infective forms of *T. cruzi*, bloodstream trypomastigotes and intracellular amastigotes. The synthetic methodology practiced was reproducible, with global yields ranging from 43–74%. The assay against trypomastigote form of *T. cruzi* reported compound **11** ($IC_{50}/24$

h = 10.3 μ M) equivalent to benznidazole with the selectivity index (SI) = 46. The assay against *T. cruzi*-infected cultures presented compound **19** (IC₅₀/96 h = 100 nM) with the selectivity index (SI) = 1000. Then phenoxyacetohydrazones derivatives **11** and **19** furnishes supporting data for forward *in vivo* studies of these compounds in pertinent animal models for Chagas disease.

Materials And Methods

General experimental details

Melting points were determined on a Buchi (B-545) and are uncorrected. The gas chromatography coupled to the mass detector (GC-MS) was performed using the Agilent chromatograph model 6890 with Agilent masses model 5973 at 70 eV. The fragmentation and molecular ion values were expressed in terms of mass/load (m/z). The Agilent column was used DB-5MS (5% diphenyl: 95% dimethylpolysiloxane). The chromatographic analyzes were performed by split injection method by using a ratio 10:1 and temperature ramp of 50°C to 325°C in the rate 10°C/min for 35 min with an initial flow rate of 0.5 ml/min. The spectra in the infrared (IV) region were obtained in a spectrophotometer from the Thermo Scientific brand, model Nicolet 6700 FTIR by ATR (Attenuated Total Reflectance). The absorption values were reported in wave number (ν) whose unit is cm⁻¹. ¹H NMR and ¹³C NMR spectra were recorded at room temperature on a Bruker Avance 500 and BrukerAvance 400 spectrometers operating at 500 and 400MHz (¹H)/125 and 100MHz (¹³C), respectively. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) used as an internal standard and coupling constant (J) values are given in Hertz (Hz). The chromatographic purity of the final products was determined by a Shimadzu (VP) apparatus with model LC-20ADXR pumps, DGU-20A5R degasser, CBM20A controller and SPD-M20A model photodiode array detector (DAD). Data acquisition and control were performed using Shimadzu Labsolutions. Chromatographic analyzes were monitored by scanning from 225 nm to 489 nm. In the analyzes, the mobile phase used as eluent (A) water, pH 5.8 adjusted with 0.025 Mol/L ammonium acetate, and eluent (B) methanol, isocratic elution for 70 minutes at 30 °C; the flow of the mobile phase was 1 mL/min and the volume injected was 1000 μ L. The separation was obtained on a Supelcosil LC 18-3 reverse phase column, 200 x 4.6 mm, with a particle diameter of 5 μ m. The progress of all reactions was monitored by TLC (Thin Layer Chromatography). which was performed on 2.0-6.0 cm aluminum sheets precoated with silica gel 60 (HF254. Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light (254 and 265 nm).

General procedures for the preparation of methyl phenoxyacetate (22)

To a suspension of **20** (0.07 mol; 1.0 eq.) and potassium carbonate (1.2 eq.) in 100 mL of acetonitrile was added slowly **21** (1.0 eq.). The reaction mixture was stirred under reflux for 4 h and after complete reaction was cooled and added distilled water until solubilized the mixture. The solution was extracted with ethyl acetate (3 x 50 mL) and the organic phase was dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo providing the ester used in the next step.

Methyl 2-phenoxyacetate (22). Light brown oil (AcOEt); MW: 166.17; 90% yield; IR (ATR. cm^{-1}): ν_{max} 1758 and 1738 ($\text{C}=\text{O}_{\text{ester}}$); 1599 ($\text{C}=\text{C}_{\text{Ar}}$); 1494 ($\text{C}-\text{H}_{\text{sp}^2_{\text{Ar}}}$); 1200 and 1173 ($\text{C}-\text{O}_{\text{ether}}$); ^1H NMR (400 MHz. $\text{DMSO}-d_6$): δ 3.70 (3H; s; $\text{O}-\text{CH}_3$); 4.79 (2H; s; H2); 6.90 – 6.98 (3H; m; H6; H7; H8); 7.27 – 7.32 (2H; m; H5; H9); ^{13}C NMR (100 MHz. $\text{DMSO}-d_6$): δ 51.7 ($\text{O}-\text{CH}_3$); 64.3 (C2); 114.3 (C5; C9); 121.1 (C7); 129.4 (C6; C8); 157.4 (C4); 169.2 (C1).

General procedure for the preparation of 2-phenoxyacetohydrazide (23)

To an ethanolic solution of **22** (0.06 mol; 1.0 eq.) was added slowly hydrazine hydrate 80% (aq.) (5.0 eq.) and the reaction mixture was stirred for 3 h under reflux. The mixture was cooled, the precipitate was collected by vacuum filtration, washed with cold ethanol and dried under vacuum.

2-phenoxyacetohydrazide (23). White crystal (EtOH); MW: 166.18; 95% yield; m.p. 113.5 – 114.2 °C; IR (ATR. cm^{-1}): ν_{max} 3400 ($\text{N}-\text{H}_2$); 1598 ($\text{C}=\text{O}_{\text{amide}}$); 1496 ($\text{C}=\text{C}_{\text{Ar}}$); 1241 and 1069 ($\text{C}-\text{O}_{\text{ether}}$); ^1H NMR (400 MHz. $\text{DMSO}-d_6$): δ 4.33 ($\text{N}-\text{H}_2$); 4.47 (2H; s; H2); 6.94 – 6.97 (3H; m; H6; H7; H8); 7.27 – 7.31 (2H; m; H5; H9); 9.33 (1H; s; $\text{N}-\text{H}$); ^{13}C NMR (100 MHz. $\text{DMSO}-d_6$): δ 66.0 (C2); 114.5 (C6; C8); 121.0 (C7); 129.3 (C5; C9); 157.7 (C4); 166.5 (C1).

General procedure for the preparation of NAH (3-19)

To a solution of **23** (2.41 mmol; 1.0 eq.) in 30 mL of water, were added the corresponding aromatic aldehydes (2.43 mmol; 1.01 eq.) and hydrochloric acid in catalytic quantity. After the complete reaction, the mixture was cooled and the precipitate was collected by vacuum filtration and recrystallized in distilled water. The products were obtained in yields that varied 50-86%.

(E)-N-Benzylidene-2-phenoxyacetohydrazide (3). White solid (H_2O); MW: 254.28; yield 80%; m.p. 156.2 – 157.0 °C; IR (ATR. cm^{-1}): ν_{max} 1678 ($\text{C}=\text{O}_{\text{amide}}$); 1403 ($\text{C}=\text{C}_{\text{Ar}}$); 1259 ($\text{N}=\text{C}$); 1219 and 1086 ($\text{C}-\text{O}_{\text{ether}}$); ^1H NMR (400 MHz. $\text{DMSO}-d_6$): δ 4.66 and 5.14 (2H; s; H2); 6.92 – 7.01 (3H; m; H6; H7; H8); 7.27 – 7.35 (2H; m; H5; H9); 7.42 – 7.47 (3H; m; H13; H14; H15); 7.69 – 7.72 (2H; m; H12; H16); 8.02 and 8.34 (1H; s; H10); 11.57 and 11.60 (1H; s; $\text{N}-\text{H}$); ^{13}C NMR (100 MHz. $\text{DMSO}-d_6$): δ 64.4 and 66.3 (C2); 114.3 and 114.5 (C6; C8); 120.6 and 121.2 (C7); 126.8 and 127.0 (C12; C16); 128.7 (C13; C15); 129.3 and 129.4 (C5; C9); 129.8 and 130.1 (C14); 133.8 and 134.0 (C11); 143.7 and 147.8 (C10); 157.6 and 158.1 (C4); 164.2 and 168.9 (C1); HPLC-UV nm (%): 254 (100). ESI-MS: m/z 277.0947 [$\text{M}+\text{Na}$] $^+$, calcd. for $\text{C}_{15}\text{H}_{14}\text{N}_2\text{NaO}_2$: 277.0946.

(E)-N-(4-Chlorobenzylidene)-2-phenoxyacetohydrazide (4). White solid (H_2O); MW: 288.73; yield 50%; m.p. 175.7 – 177.8 °C; IR (ATR. cm^{-1}): ν_{max} 1677 ($\text{C}=\text{O}_{\text{amide}}$); 1401 ($\text{C}=\text{C}_{\text{Ar}}$); 1259 ($\text{N}=\text{C}$); 1218 and 1088 ($\text{C}-\text{O}_{\text{ether}}$); ^1H NMR (400 MHz. $\text{DMSO}-d_6$): δ 4.68 and 5.14 (2H; s; H2); 6.92 – 7.01 (3H; m; H6; H7; H8); 7.27 – 7.34 (2H; m; H5; H9); 7.49 – 7.52 (2H; m; H13; H15); 7.71 – 7.75 (2H; m; H12; H16); 8.02 and 8.35 (1H; s; H10); 11.73 (1H; s; $\text{N}-\text{H}$); ^{13}C NMR (100 MHz. $\text{DMSO}-d_6$): δ 64.5 and 66.3 (C2); 114.4 and 114.5 (C6; C8); 120.6 and 121.1 (C7); 128.5 and 128.6 (C13; C15); 128.8 and 128.8 (C12; C16); 129.3 and 129.4 (C5; C9);

132.8 and 133.0 (C11); 134.2 and 134.5 (C14); 142.4 and 146.4 (C10); 157.6 and 158.0 (C4); 164.3 and 169.0 (C1); HPLC-UV nm (%): 254 (100); ESI-MS: m/z 311.0543 $[M+Na]^+$, calcd. for $C_{15}H_{13}ClN_2NaO_2$: 311.0557.

(*E*)-*N*-(4-Bromobenzylidene)-2-phenoxyacetohydrazide (**5**). White solid (H_2O); MW: 333.18; yield 50%; m.p. 173.7 – 174.8 °C; IR (ATR. cm^{-1}): ν_{max} 1677 ($C=O_{amide}$); 1398 ($C=C_{Ar}$); 1260 ($N=C$); 1219 and 1068 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.67 and 5.13 (2H; s; H2); 6.92 – 7.00 (3H; m; H6; H7; H8); 7.27 – 7.34 (2H; m; H5; H9); 7.62 – 7.68 (4H; m; H12; H13; H15; H16); 7.99 and 8.32 (1H; s; H10); 11.68 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.5 and 66.3 (C2); 114.4 and 114.5 (C6; C8); 120.6 and 121.1 (C7); 123.0 and 123.3 (C14); 128.7 and 128.9 (C13; C15); 129.3 and 129.4 (C5; C9); 131.7 and 131.7 (C12; C16); 133.2 and 133.3 (C11); 142.5 and 146.5 (C10); 157.6 and 158.0 (C4); 164.3 and 169.0 (C1); HPLC-UV nm (%): 254 (100); ESI-MS: m/z 355.0022 $[M+Na]^+$, calcd. for $C_{15}H_{13}BrN_2NaO_2$: 355.0052.

(*E*)-*N*-(4-Fluorobenzylidene)-2-phenoxyacetohydrazide (**6**). White solid (H_2O); MW: 272.27; yield 50%; m.p. 155.7 – 159.3 °C; IR (ATR. cm^{-1}): ν_{max} 1674 ($C=O_{amide}$); 1495 ($C=C_{Ar}$); 1220 ($N=C$); 1210 and 1086 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.66 and 5.13 (2H; s; H2); 6.92 – 7.01 (3H; m; H6; H7; H8); 7.26 – 7.34 (4H; m; H5; H9; H13; H15); 7.74 – 7.80 (2H; m; H12; H16); 8.02 and 8.35 (1H; s; H10); 11.62 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$. ppm): δ 64.5 and 66.3 (C2); 114.4 and 114.5 (C6; C8); 115.7 and 115.9 (C13; C15; d; J_{C-F} = 6.1 Hz); 120.6 and 121.1 (C7); 129.0 and 129.2 (C12; C16; d; J_{C-F} = 8.4 Hz); 129.3 and 129.4 (C5; C9); 130.5 and 130.6 (C11; d; J_{C-F} = 2.9 Hz); 142.5 and 146.7 (C10); 157.6 and 158.1 (C4); 161.7 and 164.2 (C14; d; J_{C-F} = 14.0 Hz); 164.2 and 169.0 (C1); HPLC-UV nm (%): 254 (100); ESI-MS: m/z 295.0830 $[M+Na]^+$, calcd. for $C_{15}H_{13}FN_2NaO_2$: 295.0853.

(*E*)-*N*-(4-Methoxybenzylidene)-2-phenoxyacetohydrazide (**7**). White solid (H_2O); MW: 284.31; yield 80%; m.p. 136.1 – 138.3 °C; IR (ATR. cm^{-1}): ν_{max} 1671 ($C=O_{amide}$); 1487 ($C=C_{Ar}$); 1245 ($N=C$); 1204 and 1031 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 3.79 and 3.80 (1H; s; H17); 4.64 and 5.11 (2H; s; H2); 6.92 – 6.96 (2H; m; H13; H15); 6.99 – 7.02 (3H; m; H6; H7; H8); 7.27 – 7.34 (2H; m; H5; H9); 7.63 – 7.66 (2H; m; H12; H16); 7.96 and 8.28 (1H; s; H10); 11.44 and 11.47 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 55.2 (C17); 64.5 and 66.3 (C2); 114.2 (C6; C8); 114.3 and 114.6 (C13; C15); 120.6 and 121.1 (C7); 126.5 and 126.5 (C11); 128.4 and 128.7 (C12; C16); 129.3 and 129.4 (C5; C9); 143.6 and 147.7 (C10); 157.7 and 158.1 (C4); 160.6 and 160.8 (C14); 163.9 and 168.7 (C1); HPLC-UV nm (%): 254 (100); 274 (100); ESI-MS: m/z 307.1038 $[M+Na]^+$, calcd. for $C_{16}H_{16}N_2NaO_3$: 307.1053.

(*E*)-*N*-(4-Nitrobenzylidene)-2-phenoxyacetohydrazide (**8**). White solid (H_2O); MW: 299.28; yield 83%; m.p. 186.5 – 188.3 °C; IR (ATR. cm^{-1}): ν_{max} 1676 ($C=O_{amide}$); 1587 and 1339 ($Aryl-NO_2$); 1517 ($C=C_{Ar}$); 1256 ($N=C$); 1089 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.71 and 5.19 (2H; s; H2); 6.94 – 7.02 (3H; m; H6; H7; H8); 7.28 – 7.35 (2H; m; H5; H9); 7.95 – 8.00 (2H; m; H12; H16); 8.12 and 8.45 (1H; s; H10); 8.26 – 8.31 (2H; m; H13; H15); 11.90 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.5 and 66.3 (C2); 114.4 and

114.6 (C6; C8); 120.7 and 121.2 (C7); 123.9 and 124.0 (C13; C15); 127.8 and 128.0 (C12; C16); 129.3 and 129.5 (C5; C9); 140.2 and 140.3 (C11); 141.3 and 145.3 (C10); 147.7 and 147.8 (C14); 157.6 and 158.0 (C4); 164.7 and 169.4 (C1); ESI-MS: m/z 322.0810 $[M+Na]^+$, calcd. for $C_{15}H_{13}N_3NaO_4$: 322.0798.

(*E*)-*N*-(4-Hydroxybenzylidene)-2-phenoxyacetohydrazide (**9**). White solid (H_2O); MW: 270.28; yield 50% (H_2O); m.p. 215.9 – 216.7 °C; IR (ATR. cm^{-1}): ν_{max} 3316 (O-H); 2988 (CH_{2sp3}); 1672 ($C=O_{amide}$); 1604 ($C=C_{Ar}$); 1256 ($N=C_{imine}$); 1245 and 1220 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.62 and 5.09 (2H; s; H2); 6.82 (2H; dd; J = 8.6 Hz; H13; H15); 6.91 – 7.00 (3H; m; H6; H7; H8); 7.27 – 7.34 (2H; m; H5; H9); 7.53 (2H; dd; J = 8.6 Hz; H12; H16); 7.91 and 8.22 (1H; s; H10); 9.91 (1H; s; O-H); 11.36 and 11.39 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.4 and 66.3 (C2); 114.3 and 114.5 (C6; C8); 115.5 and 115.6 (C13; C15); 120.6 and 121.1 (C7); 124.9 and 124.9 (C11); 128.5 and 128.8 (C12; C16); 129.3 and 129.4 (C5; C9); 144.0 and 148.1 (C10); 157.7 and 158.1 (C4); 159.2 and 159.4 (C14); 163.7 and 168.5 (C1); HPLC-UV nm (%): 254 (96.6); 274 (99.8); 292 (99.8); ESI-MS: m/z 293.0899 $[M+Na]^+$, calcd. for $C_{15}H_{14}N_2NaO_3$: 293.0896.

(*E*)-*N*-(2-Hydroxybenzylidene)-2-phenoxyacetohydrazide (**10**). White solid (H_2O); MW: 270.28; yield 77%; m.p. 172.6 – 174.9 °C; IR (ATR. cm^{-1}): ν_{max} 1673 ($C=O_{amide}$); 1413 ($C=C_{Ar}$); 1219 ($N=C$); 1086 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.69 and 5.10 (2H; s; H2); 6.85 – 6.91 (3H; m; H6; H7; H8); 6.93 – 7.02 (2H; m; H13; H15); 7.22 – 7.35 (4H; m; H5; H9; H14; H16); 7.52 (1H; d; J = 6.88 Hz; O-H); 7.71 (1H; d; J = 6.44 Hz; O-H); 8.32 and 8.57 (1H; s; H10); 11.49 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.5 and 66.2 (C2); 114.3 and 114.6 (C6; C8); 116.0 and 116.3 (C13); 118.5 and 119.2 (C15); 119.9 and 126.3 (C16); 120.6 and 121.2 (C7); 129.1 (C14); 129.3 and 129.4 (C5; C9); 131.1 and 131.4 (C11); 141.3 and 148.1 (C10); 156.4 and 157.3 (C12); 157.6 and 158.1 (C4); 164.1 and 168.6 (C1); HPLC-UV nm (%): 254 (99.5); ESI-MS: m/z 271.1075 $[M+H]^+$, calcd. for $C_{15}H_{15}N_2O_3$: 271.1077; m/z 293.089428 $[M+Na]^+$, calcd. for $C_{15}H_{14}N_2NaO_3$: 293.0896.

(*E*)-*N*-(3,4-Dihydroxybenzylidene)-2-phenoxyacetohydrazide (**11**). White solid (H_2O); MW: 286.28; yield 50%; m.p. 129.9 – 133.2 °C; IR (ATR. cm^{-1}): ν_{max} 3521 and 3257 (O-H); 1653 ($C=O_{amide}$); 1590 ($C=C_{Ar}$); 1299 ($N=C$); 1257 and 1062 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.62 and 5.08 (2H; s; H2); 6.77 (1H; d; J = 8.1 Hz; H15); 6.89 – 7.00 (4H; m; H6; H7; H8; H16); 7.15 and 7.19 (1H; d; J = 2.0 Hz; H12); 7.27 – 7.34 (2H; m; H5; H9); 7.83 and 8.13 (1H; s; H10); 9.19 and 9.27 (1H; s; O-H); 9.40 (1H; s; O-H); 11.32 and 11.35 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.4 and 66.3 (C2); 112.6 and 112.6 (C15); 114.3 and 114.5 (C6; C8); 115.4 and 115.5 (C12); 120.0 and 121.1 (C7); 120.5 and 120.6 (C16); 125.3 and 125.4 (C11); 129.3 and 129.4 (C5; C9); 144.3 and 148.3 (C10); 145.5 and 145.6 (C13); 147.7 and 147.9 (C14); 157.7 and 158.1 (C4); 163.7 and 168.4 (C1); HPLC-UV nm (%): 254 (100); 274 (100); ESI-MS: m/z 309.0837 $[M+Na]^+$, calcd. for $C_{15}H_{14}N_2NaO_4$: 309.0845.

(*E*)-*N*-(3,4-Dimethoxybenzylidene)-2-phenoxyacetohydrazide (**12**). White solid (H_2O); MW: 314.34; yield 53%; m.p. 153.6 – 155.2 °C; IR (ATR. cm^{-1}): ν_{max} 1675 ($C=O_{amide}$); 1498 ($C=C_{Ar}$); 1406 ($C-H_{sp^2_{Ar}}$); 1262

(N=C); 1162 (C-O_{ether}); ¹H NMR (400 MHz. DMSO-*d*₆): δ 3.79 – 3.80 (6H; m; H17; H18); 4.65 and 5.14 (2H; s; H2); 6.91 – 7.03 (4H; m; H6; H7; H8; H15); 7.17 – 7.21 (1H; m; H16); 7.27 – 7.34 (3H; m; H5; H9; H12); 7.93 and 8.26 (1H; s; H10); 11.44 and 11.50 (1H; s; N-H); ¹³C NMR (100 MHz. DMSO-*d*₆): δ 55.3 and 55.4 (C17; C18); 64.5 and 66.3 (C2); 108.1 and 108.5 (C15); 111.3 and 111.4 (C12); 114.3 and 114.6 (C6; C8); 120.6 and 121.8 (C7); 121.1 and 121.1 (C16); 126.6 and 126.7 (C11); 129.3 and 129.4 (C5; C9); 143.7 and 148.0 (C10); 148.9 and 148.9 (C13); 150.5 and 150.7 (C14); 157.7 and 158.1 (C4); 163.9 and 168.8 (C1); HPLC-UV nm (%): 254 (100); 274 (100); ESI-MS: *m/z* 337.1185 [M+Na]⁺, calcd. for C₁₇H₁₈N₂NaO₄ 337.1158.

(*E*)-*N*-(4-Hydroxy-3-methoxybenzylidene)-2-phenoxyacetohydrazide (**13**). White solid (H₂O); MW: 300.31; yield 65%; m.p. 156.3 – 158.7 °C; IR (ATR. cm⁻¹): ν_{max} 3200 (O-H); 1670 (C=O_{amide}); 1586 (C=C_{Ar}); 1511 (C-Hsp²_{Ar}); 1295 (N=C); 1240 and 1211 (C-O_{ether}); ¹H NMR (400 MHz. DMSO-*d*₆): δ 3.80 and 3.81 (3H; s; H17); 4.63 and 5.12 (2H; s; H2); 6.81 – 6.84 (1H; m; H15); 6.90 – 7.00 (3H; m; H6; H7; H8); 7.05 – 7.10 (1H; m; H12); 7.27 – 7.34 (3H; m; H5; H9; H16); 7.90 and 8.21 (1H; s; H10); 9.51 and 9.56 (1H; s; O-H); 11.38 and 11.43 (1H; s; N-H); ¹³C NMR (100 MHz. DMSO-*d*₆): δ 55.4 and 55.4 (C17); 64.5 and 66.3 (C2); 115.3 and 115.4 (C15); 114.3 and 114.6 (C6; C8); 108.9 and 109.4 (C12); 120.5 and 122.1 (C7); 121.1 and 121.2 (C16); 125.3 and 125.3 (C11); 129.3 and 129.4 (C5; C9); 144.1 and 148.3 (C10); 147.8 and 147.9 (C13); 148.7 and 149.0 (C14); 157.7 and 158.1 (C4); 163.7 and 168.6 (C1); HPLC-UV nm (%): 254 (98.2); 274 (98.6); ESI-MS: *m/z* 323.0996 [M+Na]⁺, calcd. for C₁₆H₁₆N₂NaO₄: 323.1002.

(*E*)-*N*-(3-Hydroxy-4-methoxybenzylidene)-2-phenoxyacetohydrazide (**14**). White solid (H₂O); MW: 300.31; yield 60%; m.p. 143.9 – 145.9 °C; IR (ATR. cm⁻¹): ν_{max} 1678 (C=O_{amide}); 1496 (C=C_{Ar}); 1269 (N=C); 1254 and 1216 (C-O_{ether}); 792 and 754 (Ar_{mono}); ¹H NMR (400 MHz. DMSO-*d*₆): δ 3.80 (3H; s; H17); 4.63 and 5.09 (2H; s; H2); 6.91 – 7.04 (4H; m; H6; H7; H8; H15); 7.20 – 7.23 (1H; m; H12); 7.27 – 7.34 (3H; m; H5; H9; H16); 7.87 and 8.18 (1H; s; H10); 9.23 and 9.30 (1H; s; O-H); 11.38 and 11.42 (1H; s; N-H); ¹³C NMR (100 MHz. DMSO-*d*₆): δ 55.5 (C17); 64.4 and 66.3 (C2); 111.7 (C15); 112.1 and 112.2 (C12); 114.3 and 114.6 (C6; C8); 119.9 and 120.3 (C16); 120.6 and 121.1 (C7); 126.7 and 126.8 (C11); 129.3 and 129.4 (C5; C9); 144.0 and 147.9 (C10); 146.7 and 146.7 (C13); 149.5 and 149.8 (C14); 157.7 and 158.1 (C4); 163.8 and 168.6 (C1); HPLC-UV nm (%): 254 (100); 274 (100); ESI-MS: *m/z* 323.1022 [M+Na]⁺, calcd. for C₁₆H₁₆N₂NaO₄: 323.1002.

(*E*)-2-phenoxy-*N*-(3,4,5-trimethoxybenzylidene) acetohydrazide (**15**). White solid (H₂O); MW: 344.36; yield 86%; m.p. 162.2 – 163.1 °C; IR (ATR. cm⁻¹): ν_{max} 1679 (C=O_{amide}); 1411 (C=C_{Ar}); 1232 (N=C); 1127 and 1003 (C-O_{ether}); ¹H NMR (400 MHz. DMSO-*d*₆): δ 3.69 and 3.70 (3H; s; H18); 3.81 and 3.82 (6H; s; H17; H19); 4.66 and 5.15 (2H; s; H2); 6.91 – 7.02 (5H; m; H6; H7; H8; H12; H16); 7.27 – 7.35 (2H; m; H5; H9); 7.93 and 8.26 (1H; s; H10); 11.55 and 11.63 (1H; s; N-H); ¹³C NMR (100 MHz. DMSO-*d*₆): δ 55.8 (C17; C19); 60.0 (C18); 64.6 and 66.3 (C2); 104.1 and 104.2 (C12 and C16); 114.3 and 114.6 (C6; C8); 120.6 and 121.2 (C7); 129.3 and 129.4 (C5; C9); 129.4 and 129.5 (C11); 138.9 and 139.2 (C14); 143.5 and 147.8

(C10); 153.0 and 153.1 (C13; C15); 157.6 and 158.1 (C4); 164.1 and 169.0 (C1); HPLC-UV nm (%): 254 (98.5); 274 (99.5); ESI-MS: m/z 367.1266 $[M+Na]^+$, calcd. for $C_{18}H_{20}N_2NaO_5$: 367.1264.

(*E*)-*N'*-(Benzo[d][1,3]dioxol-5-ylmethylene)-2-phenoxyacetohydrazide (**16**). White solid (H_2O); MW: 298.29; yield 84%; m.p. 198.9 – 200.8 °C; IR (ATR. cm^{-1}): ν_{max} 1670 ($C=O_{amide}$); 1447 ($C=C_{Ar}$); 1250 ($N=C$); 1204 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.64 and 5.11 (2H; s; H2); 6.08 and 6.09 (2H; s; H17); 6.92 – 7.00 (4H; m; H6; H7; H8; H13); 7.12 – 7.16 (1H; m; H16); 7.25 – 7.35 (3H; m; H5; H9; H12); 7.92 and 8.24 (1H; s; H10); 11.47 and 11.48 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.5 and 66.3 (C2); 101.4 and 101.5 (C17); 105.0 and 105.1 (C15); 108.2 and 108.4 (C12); 114.4 and 114.5 (C6; C8); 120.6 and 121.1 (C7); 123.1 and 123.3 (C16); 128.3 and 128.4 (C11); 129.3 and 129.4 (C5; C9); 143.4 and 147.9 (C10); 147.6 (C13); 148.8 and 149.1 (C14); 157.6 and 158.1 (C4); 164.0 and 168.8 (C1); HPLC-UV nm (%): 254 (100); 274 (99.6); ESI-MS: m/z 321.0834 $[M+Na]^+$, calcd. for $C_{16}H_{14}N_2NaO_4$: 321.0845.

(*E*)-2-Phenoxy-*N*-(pyridin-4-ylmethylene) acetohydrazide (**17**). White solid (H_2O); MW: 255.27; yield 50%; m.p. 141.3 – 143.4 °C; IR (ATR. cm^{-1}): ν_{max} 1676 ($C=O_{amide}$); 1598 ($C=C_{Ar}$); 1225 ($N=C$); 1089 ($C-O_{ether}$); 816 (pyridin 4-subs); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.71 and 5.18 (2H; s; H2); 6.94 – 7.01 (3H; m; H6; H7; H8); 7.28 – 7.33 (2H; m; H5; H9); 7.63 – 7.68 (2H; d; $j=5.8$ Hz; H12; H15); 8.00 and 8.34 (1H; s; H10); 8.64 (2H; d; $j=5.1$ Hz; H13; H14); 11.86 and 11.88 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.5 and 66.3 (C2); 114.4 and 114.6 (C6; C8); 120.7 and 121.2 (C7); 120.8 and 120.9 (C11); 129.3 and 129.5 (C5; C9); 141.0 and 145.4 (C10); 141.2 and 141.2 (C12; C15); 150.1 and 150.2 (C13; C14); 157.6 and 158.0 (C4); 164.7 and 169.4 (C1); HPLC-UV nm (%): 254 (100); 274 (100); ESI-MS: m/z 256.1100 $[M+H]^+$, calcd. for $C_{14}H_{14}N_3O_2$: 256.1080; m/z 278.0920 $[M+Na]^+$, calcd. for $C_{14}H_{13}N_3NaO_2$: 278.0899.

(*E*)-*N*-((5-Bromothiophen-2-yl) methylene)-2-phenoxyacetohydrazide (**18**). White solid (H_2O); MW: 339.21; yield 81%; m.p. 151.8 – 154.1 °C; IR (ATR. cm^{-1}): ν_{max} 1672 ($C=O_{amide}$); 1425 ($C=C_{Ar}$); 1221 ($N=C$); 1085 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.64 and 5.02 (2H; s; H2); 6.89 – 7.00 (3H; m; H6; H7; H8); 7.25 – 7.34 (4H; m; H5; H9; H12; H13); 8.10 and 8.48 (1H; s; H10); 11.61 and 11.65 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.2 and 66.3 (C2); 114.2 and 114.8 (C14); 114.3 and 114.6 (C6; C8); 120.7 and 121.2 (C7); 129.3 and 129.4 (C5; C9); 130.9 and 131.6 (C10 ou C13); 131.0 and 131.6 (C10 ou C13); 137.9 (C12); 140.5 and 140.6 (C11); 131.2 and 142.2 (C10); 157.6 and 157.9 (C4); 164.2 and 168.6 (C1); HPLC-UV nm (%): 254 (100); 274 (100); ESI-MS: m/z 360.9623 $[M+Na]^+$, calcd. for $C_{13}H_{11}BrN_2NaO_2S$ 360.9616.

(*E*)-*N*-((5-Nitrofuran-2-yl) methylene)-2-phenoxyacetohydrazide (**19**). White solid (H_2O); MW: 289.24; yield 60%; m.p. 169.9 – 172.7 °C; IR (ATR. cm^{-1}): ν_{max} 1695 ($C=O_{amide}$); 1587 ($C=C_{Ar}$); 1479 ($C-H_{sp^2_{Ar}}$); 1350 ($Aryl-NO_2$); 1239 ($N=C$); 1116 ($C-O_{ether}$); 1H NMR (400 MHz. $DMSO-d_6$): δ 4.72 and 5.12 (2H; s; H2); 6.91 – 7.01 (3H; m; H6; H7; H8); 7.25 – 7.35 (3H; m; H5; H9; H13); 7.78 – 7.81 (1H; m; H12); 7.97 and 8.32 (1H; s; H10); 11.99 (1H; s; N-H); ^{13}C NMR (100 MHz. $DMSO-d_6$): δ 64.3 and 66.3 (C2); 114.4 and 114.6 (C6; C8); 114.5 and 114.8 (C13); 115.5 (C12); 120.8 and 121.3 (C7); 129.3 and 129.5 (C5; C9); 131.9 and 135.7

(C10); 151.3 and 151.4 (C11); 151.7 and 151.9 (C14); 157.5 and 157.9 (C4); 164.9 and 169.3 (C1); HPLC-UV nm (%): 254 (98.1); 274 (98.3); ESI-MS: m/z 312.0577 $[M+Na]^+$, calcd. for $C_{13}H_{11}N_3NaO_5$: 312.0590.

Experimental procedure for biological activity

The use of animals in our trial was performed in accordance with the Brazilian Law 11.794/2008 and regulations of the National Council of Animal Experimentation Control under the license L038/2018 from the Ethics Committee for Animal Use of the Oswaldo Cruz Institute (CEUA/IOC). The assays were carried out using the bloodstream trypomastigotes of *Y* strain obtained from infected mice at peak parasitemia [16]. The stock solutions of the phenoxyacetohydrazones and Benznidazole were prepared in dimethyl sulfoxide, with the final concentration of the solvent never exceeding 1%, which has no deleterious effect on the parasite. The compounds were diluted, in series 1:2, in decreasing concentrations, with an initial concentration of 1 mM. The assays were performed with the parasites (5×10^6 cells/ml) incubated for 24 h at 37°C and 5% CO₂ atmosphere [17,18]. Untreated and Benznidazole-treated parasites were used as controls. The parasites were quantified in Neubauer's chamber. The results were analyzed by plotting % lysis of *T. cruzi* against the concentration of the test compound and activity of compounds was expressed as the IC₅₀/24 h corresponded to the concentration that led to 50% lysis of the parasite and are summarized in Table 1. Next, to evaluate the effects of the compounds on intracellular parasites, L929 fibroblasts (ATCC® CCL-1™) were infected with trypomastigotes of *Tulahuen* strain, expressing *Escherichia coli* β -galactosidase as reporter gene according to the method described previously [19]. Briefly, for the bioassay, 4,000 L929 cells were added to each well of a 96-well microtiter plate. After an overnight incubation, 40,000 trypomastigotes were added to the cells and incubated for 2 h. The culture was maintained for 48 h to establish the infection and then it was treated with the compounds at serial decreasing dilutions for further 96 h at 37°C. After this period the chlorophenol red- β -D-galactopyranoside (CPRG) reagent (Roche) was added in Nonidet P40 solution (Sigma-Aldrich) and the plate was incubated for another 18 h and the absorbance was measured at 570 nm. Controls with uninfected cells, untreated infected cells, and infected cells treated with benznidazole at 3.8 μ M (positive control) or DMSO 1% were used. The results were expressed as the percentage of *T. cruzi* growth inhibition in compound-tested cells as compared to the infected cells and untreated cells. The IC₅₀ values were calculated by linear interpolation. Quadruplicates were run in the same plate, and the experiments were repeated at least once. The active compounds were tested *in vitro* for the determination of cellular toxicity against uninfected L-929 cells and primary cultures of peritoneal macrophages obtained from Albino Swiss mice, using the AlamarBlue® dye [19]. The cells were exposed to compounds at increasing concentrations starting at IC₅₀ value for *T. cruzi*. After 24 or 96 h of incubation, the AlamarBlue® was added and the absorbance at 570 and 600 nm was measured after 4-6 h for L-929 cells and after 2 h for peritoneal macrophages. The cell viability was expressed as the percentage of difference in the reduction between treated and untreated cells being the LC₅₀ value, corresponding to the concentration that leads to lysis of 50% of the mammalian cells [20]. The selectivity index (SI) was determined based on the ratio of the LC₅₀ value in the host cell and the IC₅₀ value of the parasite. Quadruplicates were run in the same plate, and the experiments were repeated at least twice.

Declarations

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary data

Supplementary data to this article can be found online at...

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Table

Due to technical limitations, the table is only available as a download in the supplementary files section.

Figures

Figure 1

Design of phenoxyacetohydrazone derivatives 3 - 19.

Figure 2

(A) ORTEP representation of compound 3 in the solid state: probability ellipsoids are drawn at the 50% level. (B) View of the conformation of compound 3 looking across the phenyl ring containing C13.

Supplementary Files

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