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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, antiprotozoal activity and cytotoxicity in U-937 macrophages of triclosan–hydrazone hybrids

Sebastian Vergara[1] · Miguel Carda[2] · Raül Agut[2] · Lina M. Yepes[3] · Iván D. Vélez[3] · Sara M. Robledo[3] · Wilson Cardona Galeano[1]

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Abstract The synthesis and biological activities (cytotoxicity, leishmanicidal, and trypanocidal) of 11 triclosan–hydrazone hybrids are described herein. The structure of the products was elucidated by spectral data (NMR, IR) and mass spectrometric analyses. The synthesized compounds were evaluated against amastigotes forms of *L. (V) panamensis*, which is the most prevalent *Leishmania* species in Colombia, and against *Trypanosoma cruzi*, which is the major pathogenic species to Chagas disease in humans. In addition, the cytotoxic activity of the synthesized compounds was evaluated against human U-937 macrophages. Hydrazone hybrids were obtained as *E*-synperiplanar and *E*-antiperiplanar conformers. Nine of them were active against *L. (V) panamensis* (**5a–5d**, **5f–5j**) and eight of them against *T. cruzi* (**5a**, **5c**, **5d**, **5f–5j**), with EC_{50} values lower than 40 µM. The compounds **5c**, **5e**, and **5h** exhibit the best selectivity index against both *L. (V) panamensis* and *T. cruzi*, with values ranging from 5.90 to 16.55, thus showing potential as starting compounds for the eventual development of drugs against these parasites. The presence of hydroxy or methoxy groups in positions 2 and 4 of the aromatic ring of the benzylidene moiety increases both activity and cytotoxicity. There is no clear relationship between the antiprotozoal activity and the methylation pattern of the hydroxy groups, since in some cases methylation decreases the activity (**5d** vs. **5g**) while in other cases the activity is increased (**5c** vs. **5f** and **5i** vs. **5j**).

Keywords Leishmaniasis · Chagas disease · *Trypanosoma cruzi* · Antiprotozoal activity · Cytotoxicity · Triclosan-Hydrazone Hybrids

✉ Sara M. Robledo
sara.robledo@udea.edu.co

✉ Wilson Cardona Galeano
wilson.cardona1@udea.edu.co

[1] Chemistry of Colombian Plants, Institute of Chemistry, Exact and Natural Sciences School, University of Antioquia-UdeA, Calle 70 No. 52-21, Medellín AA 1226, Colombia

[2] Department of Inorganic and Organic Chemistry, Jaume I University, Castellón E-12071, Spain

[3] PECET-Medical Research Institute, School of Medicine, University of Antioquia-UdeA, Calle 70 No. 52-21, Medellín AA 1226, Colombia

Introduction

Protozoal diseases are a cause of mortality in various developing countries of tropical and subtropical regions. For leishmania and Chagas-endemic countries these diseases cause significant health problems affecting more than one billion people worldwide (WHO 2002, 2013; Alvar et al. 2012; Nouvellet et al. 2015). This situation is aggravated by increasing treatment failures with available drugs (Bhutta et al. 2014). Chagas disease (American trypanosomiasis) and leishmaniasis are parasitic diseases caused by the parasitic protozoan *Trypanosoma cruzi* (*T. cruzi*) and *Leishmania* species, respectively.

The Leishmaniasis involves a wide spectrum of clinical manifestations in which *L. (V) panamensis* is one of the most prevalent *Leishmania* species involved in human cases of cutaneous leishmaniasis in Colombia (Alvar et al. 2012). Chagas disease (also named American trypanosomiasis) is produced by the protozoan parasite *T. cruzi* that is transmitted to the mammalian host through the bite of triatomine

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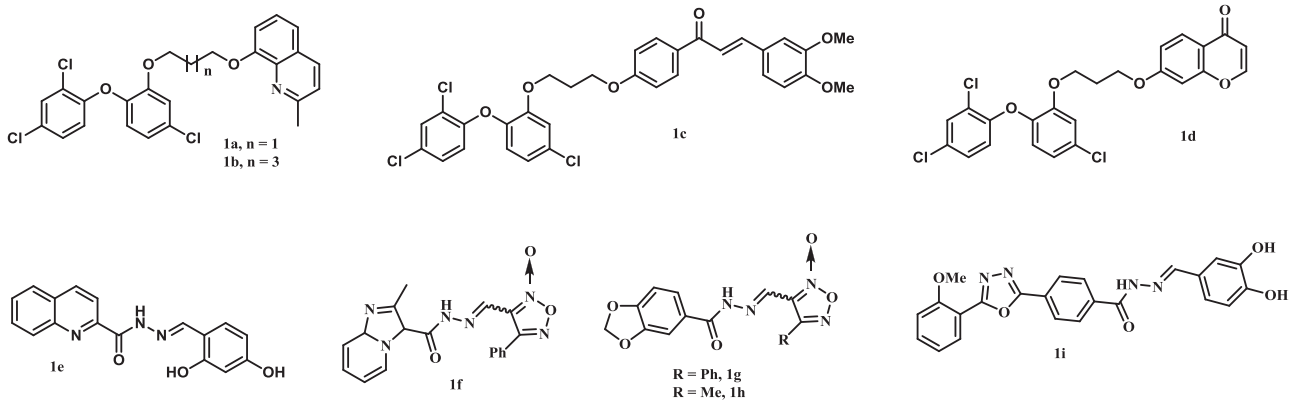


Fig. 1 Compounds with antiprotozoal activity

bugs belonging to *Triatoma*, *Rhodnius*, and *Panstrongylus* genus (Nouvellet et al. 2015). Current treatments for cutaneous leishmaniasis are based on pentavalent antimonials (meglumine antimoniate and sodium stibogluconate). For the treatment of Chagas disease nitroaromatic compounds (benznidazole and nifurtimox) are usually employed. However, these treatments are not without significant side effects, particularly in patients undergoing high-dose and long-term treatments. In addition, the development of drug resistance has significantly increased the health problems associated with these diseases (Chatelain and Ioset 2011; Den Boer et al. 2011; Keenan and Chaplin 2015).

Triclosan is an uncompetitive inhibitor of purified enoyl-acyl carrier protein reductase (ENR), which has demonstrated in vitro inhibitory activity against *Plasmodium falciparum* (Kapoor et al. 2004; McLeod et al. 2001; Surolia and Surolia 2001; Perozzo et al. 2002). A previous study showed that triclosan has in vitro anti-leishmanial activity against axenic amastigotes of *L. panamensis* with an effective concentration (EC_{50}) of 39 µM.

Further, hydrazones constitute an important type of biologically active compounds (Rollas and Küçükgüzel 2007; Singh and Raghav 2011; Verma et al. 2014) with high ability to elicit anti-leishmanicidal (Bernardino et al. 2006; Rando et al. 2008; Taha et al. 2014) and trypanocidal activity (Carvalho et al. 2012; Porcal et al. 2008; Jorge et al. 2013; Massarico Serafim et al. 2014).

In recent years a promising strategy has emerged based on hybrid molecules, which bear in their structures two distinct pharmacophores having, for example, anti-protozoal, anti-inflammatory, anti-fungal, or anti-cancer activity, thus showing a dual mode of action (Keith et al. 2005; Meunier 2008). These hybrid molecules may display dual activity, but do not necessarily act on the same biological target (Opsenica et al. 2008; Roth et al. 2004; Walsh et al. 2007).

Triclosan–quinoline hybrids with shorter methylene units spacers (**1a** and **1b**) have in vitro activity against intracellular amastigotes of *L. panamensis* with effective concentrations (EC_{50}) of 13.1 and 4.7 µM, respectively (Arango et al. 2012). Triclosan–chalcone (**1c**) and triclosan–chromone (**1d**) hybrids showed no cytotoxicity against U-937 cells but were active against *L. panamensis* amastigotes ($LC_{50} = > 326.7$ µM, $EC_{50} = 15.4$ and 5.5 µM, respectively) (Otero et al. 2014) (see Fig. 1). Quinoline–hydrazone hybrid **1e** showed activity against *L. panamensis* and against *T. cruzi* with EC_{50} of 2.6 and 4.6 µM, respectively (Coa et al. 2015). Some furoxanyl *N*-acylhydrazone derivatives were evaluated in vitro against amastigote form of *L. panamensis* and against *T. cruzi*. The compound **1f** exhibited excellent profile as anti-*T. cruzi* with an $IC_{50} = 0.91$ µM. On the other hand, compounds **1g** and **1h** showed very good anti-Leishmania activity with $IC_{50} = 1.3$ and 1.7 µM, respectively. In addition, compounds **1g** and **1h** displayed higher selectivity than the reference drug Amph (Hernández et al. 2013). Finally, antileishmanial activity of several phenyl-linked oxadiazole–phenylhydrazone hybrids was evaluated, compound **1i** being the most potent antileishmanial agent among this type of hybrids, displaying an IC_{50} of 0.95 ± 0.01 µM (Taha et al. 2017) (Fig. 1).

In the search for new therapeutic alternatives to treat cutaneous leishmaniasis and Chagas disease a number of triclosan–hydrazone hybrids have been designed and synthesized. Their leishmanicidal and trypanocidal activities, as well as their cytotoxicity in U-937 macrophages, have been evaluated in vitro (Fig. 2).

Materials and methods

Chemical synthesis

General remarks

Microwave reactions were carried out in a CEM Discover microwave reactor in sealed vessels (monowave, maximum power 300 W, temperature control by IR sensor, fixed

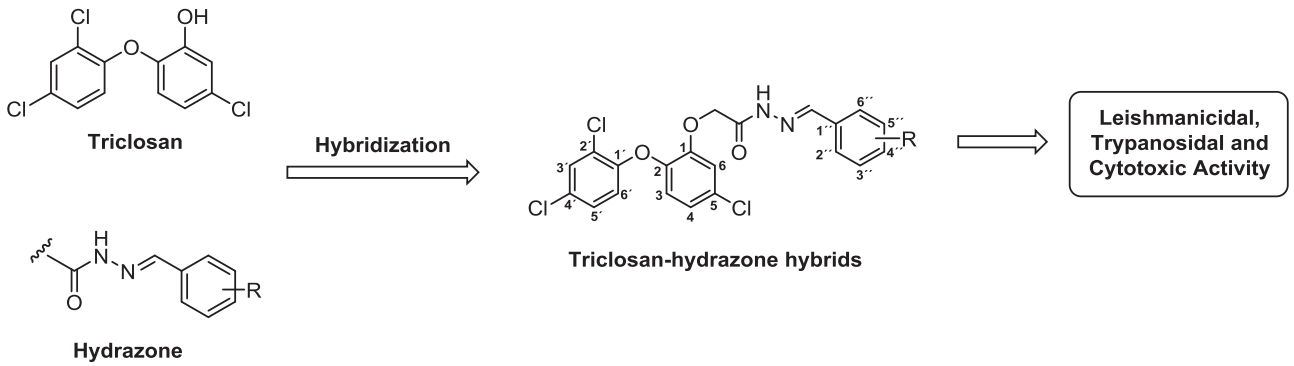


Fig. 2 Design of triclosan–hydrazone hybrids as antiprotozoal agents

temperature). ^{1}H and ^{13}C NMR spectra were recorded on a Varian instrument operating at 500 and 125 MHz, respectively. The signals of the deuterated solvent ($CDCl_3$) were used as reference (the singlet at $\delta = 7.27$ ppm for ^{1}H NMR and the triplet centered at $\delta = 77.00$ ppm for ^{13}C NMR). Carbon atom types (C, CH, CH_2, CH_3) were determined by using the DEPT or APT pulse sequence. Signals were assigned using two-dimensional heteronuclear correlations (COSY and HSQC). High-resolution mass spectra were recorded using electrospray ionization-mass spectrometry (ESI-MS). A QTOF Premier instrument with an orthogonal Z-spray–electrospray interface (Waters, Manchester, UK) was used for operating in the W-mode. The drying and cone gas was nitrogen-set to flow rates of 300 and 30 L/h, respectively. Methanol sample solutions (ca. 1×10^{-5} M) were directly introduced into the ESI spectrometer at a flow rate of 10 μL/min. A capillary voltage of 3.5 kV was used in the positive scan mode, and the cone voltage was set to $U_c = 10$ V. For accurate mass measurements, a 2 mg/L standard solution of leucine enkephalin was introduced via the lock spray needle at a cone voltage set to 85 V and a flow rate of 30 μL/min. IR spectra were recorded on a Spectrum RX I FT-IR system (Perkin-Elmer, Waltham, MA, USA) in KBr disks. Silica gel 60 (0.063–0.200 mesh, Merck, Whitehouse Station, NJ, USA) was used for column chromatography, and precoated silica gel plates (Merck 60 F254 0.2 mm) were used for thin layer chromatography (TLC).

Synthetic procedure for ethyl 2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)acetate Triclosan **1** (6.0 g, 0.021 mol), potassium hydroxide (1.7 g, 0.03 mol), and acetonitrile (20 mL) were placed in a 50 mL flat-bottomed flask equipped with a magnetic stirring bar. The mixture was stirred and heated under microwave irradiation to reflux for a period of 5 min. Then, ethyl bromoacetate (2.4 mL, 3.67 g, 0.022 mol) was added to the reaction mixture, which was then refluxed for 30 min (200 W). The crude reaction mixture was concentrated on a rotatory evaporator, and the residue was purified by column chromatography over silica gel eluting with hexane–ethyl acetate (9:1 ratio) to obtain the ester **2** with 75% yield (0.016 mol, 5.9 g). Monitoring of the reaction progress and product purification were carried out by TLC. White solid, m.p. 58–60 °C; IR (cm^{-1}): ν_{max} max 2985 (C–H), 1745 (C=O), 1492 (C=C_{Ar}), 1215 ((C=O)–O), 829 (C–H_{Ar}), 723 (C–Cl). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.28 (CH_3, t, $J = 7.1$ Hz), 4.23 (–OCH_2–, q, $J = 7.1$ Hz), 4.66 (–OCH_2C=O–, s), 6.79 (H_3, d, $J = 8.8$ Hz), 6.91 (H_6, d, $J = 8.8$ Hz), 6.94–7.00 (H_4, H_6, m), 7.14 ($H_{5'}$, dd, $J = 8.8$, 2.5 Hz), 7.44 ($H_{3'}$, d, $J = 2.5$ Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 14.12 (CH_3), 61.49 (–OCH_2–), 66.29 (–OCH_2–), 115.91 (C_6), 118.77 (C_3), 121.81 ($C_{6'}$), 122.54 (C_4), 124.76 ($C_{4'}$), 127.82 ($C_{2'}$), 128.31 ($C_{5'}$), 130.19 (C_5), 130.22 ($C_{3'}$), 143.61 (C_2), 149.78 (C_1), 151.90 ($C_{1'}$), 168.00 (C=O).

Synthetic procedure for 2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)acetohydrazide Hydrazine monohydrate (3 mL of a 80% solution) was added to a solution of **2** (5 g, 0.013 mol) in ethanol (15 mL). The reaction mixture was submitted to microwave irradiation and maintained under reflux for 30 min. Then, the reaction mixture was poured on ice and the resulting precipitate was filtered out, affording the title compound **3** in 83% yield (3.90 g, 0.011 mmol). White solid, m.p. 148–150 °C; IR (cm^{-1}): ν_{max} max 3298 (N–H), 1656 (C=O), 1473 (C=C_{Ar}), 1232 ((C=O)–N), 975 (C–H_{Ar}), 796 (C–Cl). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 4.55 (–OCH_2C=O–, s), 6.86 (H_3, d, $J = 8.7$ Hz), 6.99–7.08 (H_4, $H_{6'}$, m), 7.20 (H_6, d, $J = 2.0$ Hz), 7.33 ($H_{5'}$, dd, $J = 8.7$, 2.3 Hz), 7.70 ($H_{3'}$, d, $J = 2.3$ Hz), 8.57 (NH), 9.10 (NH_2); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 55.25 (–OCH_2–), 115.75 (C_6), 119.50 (C_3), 122.24 ($C_{6'}$), 122.38 (C_4), 123.98 ($C_{4'}$), 127.71 ($C_{2'}$), 128.94 ($C_{5'}$), 129.75 (C_5), 130.24 ($C_{3'}$), 143.07 (C_1), 150.50 (C_2), 152.01 ($C_{1'}$), 166.62 (C=O).

Synthetic procedure for hydrazones

A triclosan–carbohydrazide **3** (0.5 g, 1.38 mmol) solution in methanol (2 mL) was sonicated for 2 min and then

benzaldehyde **4** (1.38 mmol) and acetic acid (0.1 mL) were added dropwise to the reaction mixture. Upon completion of the reaction (5–10 min), the product was filtered, sequentially washed with water (20 mL) and ethyl ether (5 mL), dried in vacuo and recrystallized from ethanol, affording the corresponding hydrazones in yields ranging from 42 to 93%.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N′*-(2-hydroxybenzylidene)acetohydrazide (**5a**) Yield 91% (1.256 mmol, 585 mg); white solid, m.p. 178–180 °C; IR (cm^{-1}): ν_{max} max 3450 (Ar–OH), 3100 (N–H), 1680 (C=O), 1494 (C=N), 1409 (C=C_{Ar}), 1259 ((C=O)–N), 829 (C–H_{Ar}), 754 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 4.78, 5.23 (~1:1, –OCH_2, s), 6.82–6.94 (H_3, $H_{3''}$, $H_{5''}$, $H_{6'}$, m), 7.01–7.10 (H_4, $H_{4''}$, m), 7.19–7.31 (H_6, $H_{6'}$, m), 7.34 ($H_{5'}$, dd, $J = 8.9$, 2.0 Hz), 7.18–7.38 ($H_{3'}$, m), 8.28, 8.43 (~1:1, N=C–H, s), 9.98, 10.92 (OH), 11.50, 11.71 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.23, 4.79 (~2:1 –OCH_2, s), 8.28, 8.42 (~2:1, N=C–H, s), 11.53, 11.75 (~2:1, NH, s). ^{13}C-NMR (DMSO-d_6): δ 66.10, 67.34 (–OCH_2), 115.58, 116.07 (C_6), 116.54, 116.80, ($C_{3''}$), 119.08, 119.38 ($C_{5''}$), 121.68, 122.38 (C_3), 122.62, 122.80 ($C_{6'}$), 123.68, 123.94 (C_4), 127.06, 127.26 ($C_{2'}$), 127.28, 127.61 ($C_{5'}$), 128.74, 128.90 (C_5), 129.55, 129.80 ($C_{4'}$), 129.93, 130.09 ($C_{6''}$), 130.11, 130.22 ($C_{3'}$), 131.65, 131.99 ($C_{4''}$), 142.07, 142.66 (C_2), 148.08, 148.25 (C_1), 150.63, 151.10 (N=CH), 152.13, 152.35 ($C_{1'}$), 156.83, 157.73 ($C_{2''}$), 163.83, 168.42 (C=O). EIMS: *m/z* 465.0176 $[M + H]^+$, calcd for $C_{21}H_{15}Cl_3N_2O_4$: 465.0172.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N′*-(2,3-dihydroxybenzylidene)acetohydrazide (**5b**) Yield 52% (0.718 mmol, 345 mg); yellow solid, m.p. 182–186 °C; IR (cm^{-1}): ν_{max} max 3468 (Ar-OH), 3290 (N–H), 1680 (C=O), 1494 (C=N), 1475 (C=C_{Ar}), 1271 ((C=O)–N), 810 (C–H_{Ar}), 732 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 4.79, 5.22 (~1:1, –OCH_2, s), 6.67, 6.72 ($H_{5''}$, tapp, $J = 7.8$ Hz), 6.82, 6.85 (H_3, d, $J = 7.8$ Hz), 6.88, 6.90 ($H_{4''}$, d, $J = 8.8$ Hz), 6.96, 7.12 ($H_{6'}$, d, $J = 7.8$ Hz), 7.03 ($H_{6''}$, dd, $J = 8.6$, 2.0), 7.05–7.09 (H_4, m), 7.23, 7.30 (H_6, d, $J = 2.0$ Hz), 7.28, 7.34 ($H_{5'}$, dd, $J = 8.9$, 2.3 Hz), 7.68, 7.70 ($H_{3'}$, d, $J =$ 2.3 Hz), 8.28, 8.39 (~1:1, N=C–H, s), 9.22, 10.66 (OH), 11.49, 11.71 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.30, 4.86 (~2:1 –OCH_2, s), 8.27, 8.35 (~2:1, N=C–H, s), 11.51, 11.39 (~2:1, NH, s). ^{13}C-NMR (DMSO-d_6): δ 65.39, 66.39 (–OCH_2), 114.93, 115.42 (C_6), 116.44, 116.83, ($C_{4''}$), 116.83, 117.24 ($C_{1''}$), 118.46, 118.51 (C_3), 118.76, 118.95 ($C_{5''}$), 119.49, 120.23 ($C_{6'}$), 121.02, 121.73 ($C_{6''}$), 121.92, 122.12 (C_4), 123.03, 123.31 ($C_{2'}$), 126.63, 126.97 ($C_{5'}$), 128.09, 128.25 ($C_{4'}$), 129.13, 129.26 ($C_{3'}$), 129.46, 129.58 (C_5), 142.12, 142.48 (C_2), 145.01 ($C_{3''}$), 145.37, 145.77 (N=CH), 148.37 ($C_{2''}$), 149.95, 150.44 (C_1), 152.13, 152.35 ($C_{1'}$), 151.45, 151.69 ($C_{2'}$), 163.14, 167.68 (C=O). EIMS: *m/z* 481.0125 $[M + H]^+$, calcd for $C_{21}H_{15}Cl_3N_2O_5$: 481.0115.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N′*-(3,4-dihydroxybenzylidene)acetohydrazide (**5c**) Yield 60% (0.828 mmol, 398 mg); brown solid, m.p. 216–218 °C; IR (cm^{-1}): ν_{max} max 3433 (Ar–OH), 3300 (N–H), 1658 (C=O), 1598 (C=N), 1475 (C=C_{Ar}), 1269 ((C=O)–N), 802 (C–H_{Ar}), 730 (C–Cl). ^{1}H-NMR (DMSO-d_6)**:** δ 4.72, 5.20 (~1:1, –OCH_2, s), 6.76, 6.77 ($H_{5''}$, d, $J = 8.8$ Hz), 6.85–6.94 (H_3, $H_{6'}$, m), 7.00–7.10 (H_4, $H_{6''}$ m), 7.13, 7.19 (H_6, d, $J = 2.0$ Hz), 7.26–7.35 ($H_{5'}$, $H_{2''}$, m), 7.68, 7.70 ($H_{3'}$, d, $J = 2.0$ Hz), 7.81, 7.98 (~1:1, N=C–H, s), 9.27 (OH), 11.20, 11.32 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.28, 4.77 (~3:1 –OCH_2, s), 7.92, 7.99 (~3:1, N=C–H, s), 10.31, 9.94 (~3:1, NH, s). ^{13}C-NMR (DMSO-d_6): δ 65.93, 67.32 (–OCH_2), 113.14, 113.38, ($C_{2''}$), 115.12, 115.44 (C_6), 115.82, 115.98 ($C_{5''}$), 119.09, 119.26 (C_3), 120.59, 121.19 ($C_{6''}$), 121.66, 122.25 ($C_{6'}$), 122.75, 121.85 (C_4), 123.66, 123.82 ($C_{2'}$), 125.80 ($C_{5'}$), 127.29, 127.53 ($C_{4'}$), 128.80, 128.92 ($C_{1''}$), 129.89, 129.96 (C_5), 130.15, 130.24 ($C_{3'}$), 142.60, 142.90 (C_2), 145.11, 145.53 ($C_{3''}$), 146.06, 146.16 ($C_{4''}$), 148.29, 148.58 (C_1), 150.73, 151.15 (N=CH), 152.18, 152.37 ($C_{1'}$), 163.46, 168.30 (C=O). EIMS: *m/z* 481.0125 $[M + H]^+$, calcd for $C_{21}H_{15}Cl_3N_2O_5$: 481.0126.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N′*-(2,4-dihydroxybenzylidene)acetohydrazide (**5d**) Yield 64% (0.883 mmol, 425 mg); light yellow solid, m.p. 222–226 °C; IR (cm^{-1}): ν_{max} max 3448 (Ar–OH), 3344 (N–H), 1705 (C=O), 1496 (C=N), 1471 (C=C_{Ar}), 1251 ((C=O)–N), 866 (C–H_{Ar}), 770 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 4.75, 5.18 (~1:1, –OCH_2, s), 6.28–6.32 ($H_{5''}$, m), 6.35, 6.33 ($H_{3''}$, d, $J = 2.2$ Hz), 6.87, 6.89 (H_3, d, $J = 8.9$ Hz), 7.01–7.10 (H_4, $H_{6'}$ m), 7.20 (H_6, d, $J = 2.0$ Hz), 7.27, 7.35 ($H_{5'}$, $H_{6''}$, m), 7.67, 7.70 ($H_{3'}$, d, $J = 2.4$ Hz), 8.15, 8.28 (~1:1, N=C–H, s), 11.07, 9.94 (OH), 11.31, 11.51 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.23, 5.31 (~1:2 –OCH_2, s), 8.16, 8.25 (~1:2, N=C–H, s), 11.39, 10.38 (~1:2, NH, s). ^{13}C-NMR (DMSO-d_6): δ 66.89, 67.77 (–OCH_2), 102.28, 102.52 ($C_{3''}$), 110.30, 111.41 ($C_{5''}$), 115.07, 115.56 ($C_{1''}$), 118.62, 118.89 (C_6), 121.16, 121.87 (C_3), 122.12, 122.30 ($C_{6'}$), 123.19, 123.45 (C_4), 126.79, 127.13 ($C_{2'}$), 128.26, 128.41 ($C_{5'}$), 129.31, 129.43 ($C_{4'}$), 129.59, 129.62 (C_5), 129.73, 129.76 ($C_{3'}$), 130.98, 131.00 ($C_{6''}$), 142.17, 142.62 (C_2), 148.78 (C_1), 150.16, 150.64 (N=CH), 152.64, 151.87 ($C_{1'}$), 158.01, 159.26 ($C_{2''}$), 160.45, 160.82 ($C_{4''}$), 162.87, 167.45 (C=O). EIMS: *m/z* 481.0125 $[M + H]^+$, calcd for $C_{21}H_{15}Cl_3N_2O_5$: 481.0130.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N′*-(2,5-dihydroxybenzylidene)acetohydrazide (**5e**) Yield 90% (1.242 mmol, 597 mg); yellow solid, m.p. 220–224 °C; IR

(cm^{-1}): ν_{max} max 3325 (Ar–OH), 3167 (N–H), 1676 (C=O), 1492 (C=N), 1471 (C=C_{Ar}), 1255 ((C=O)–N), 910 (C–H_{Ar}), 785 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 4.77, 5.22 (~1:1, –OCH_2, s), 6.67–6.74 (H_3, $H_{4''}$, m), 6.89 ($H_{3''}$, d, $J = 8.8$ Hz), 6.94–7.11 (H_4, $H_{6''}$, $H_{6'}$, m), 7.21, 7.29 (H_6, d, $J = 2.0$ Hz), 7.30, 7.34 ($H_{5'}$, dd, $J = 8.9$, 2.2 Hz), 7.68, 7.69 ($H_{3'}$, d, $J = 2.2$ Hz), 8.22, 8.35 (~1:1, N=C–H, s), 8.86, 8.94, 9.29, 10.07, (OH), 11.46, 11.59 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.28, 4.84 (~1:2 –OCH_2, s), 8.21, 8.28 (~1:2, N=C–H, s), 11.54, 10.62 (~1:2, NH, s). ^{13}C-NMR (DMSO-d_6): δ 65.50, 66.86 (–OCH_2), 111.39, 113.35 ($C_{6''}$), 115.00, 115.54 (C_6), 116.91, 117.00 ($C_{4''}$), 118.64, 118.77 ($C_{3''}$), 118.88, 119.09 (C_3), 120.12 ($C_{1''}$), 121.19, 121.85 ($C_{6'}$), 122.12, 122.28 (C_4), 123.21, 123.44 ($C_{2'}$), 126.81, 127.12 ($C_{5'}$), 128.26, 128.40 ($C_{4'}$), 129.32, 129.44 (C_5), 129.61, 129.72 ($C_{3'}$), 142.51, 142.60 (C_2), 146.79, 147.10 (N=CH), 149.36, 149.84 ($C_{5''}$), 149.87, 150.04 (C_1), 150.15, 150.61 ($C_{1'}$), 151.64, 151.86 ($C_{2''}$), 163.23, 167.83 (C=O). EIMS: *m/z* 481.0125 $[M + H]^+$, calcd for for $C_{21}H_{15}Cl_3N_2O_5$: 481.0119.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N*′-(4-hydroxy-3-methoxybenzylidene) acetohydrazide (**5f**) Yield 63% (0.870 mmol, 430 mg); white solid, m.p. 148–150 °C; IR (cm^{-1}): ν_{max} max 3431 (Ar–OH), 3084 (N–H), 1681 (C=O), 1496 (C=N), 1473 (C=C_{Ar}), 1251 ((C=O)–N), 806 (C–H_{Ar}), 780 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 3.30, 3.80 (–OCH_3, s), 4.73, 5.24 (~1:1, –OCH_2, s), 6.81, 6.83 (H_3, d, $J = 8.5$ Hz), 6.88 ($H_{6'}$, d, $J = 8.7$ Hz), 7.03 ($H_{6''}$, dd, 8.5, 2.0 Hz), 7.05–7.10 (H_4, $H_{5''}$ m), 7.19 (H_6, d, $J = 2.0$ Hz), 7.27 ($H_{2''}$, $s_{apparent}$), 7.29, 7.34 ($H_{5'}$, dd, $J =$ 8.7, 2.3 Hz), 7.68, 7.70 ($H_{3'}$, d, $J = 2.3$ Hz), 7.87, 8.05 (~1:1, N=C–H, s), 9.46, 9.52 (OH), 11.26, 11.41 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.30, 4.78 (~2:1 –OCH_2, s), 7.97, 8.12 (~2:1, N=C–H, s), 10.37, 10.10 (~2:1, NH, s). ^{13}C-NMR (DMSO-d_6): δ 55.99 (–OCH_3, s), 66.09, 67.25 (–OCH_2), 119.46, 119.95 ($C_{2''}$), 115.44, 115.86 (C_6), 119.05, 119.21 ($C_{5''}$), 121.61, 121.93 (C_3), 122.24, 122.69 ($C_{6''}$), 121.76, 122.85 ($C_{6'}$), 123.65, 123.78 (C_4), 125.74, 125.83 ($C_{2'}$), 127.28, 127.50 ($C_{4'}$, $C_{5'}$), 128.79, 128.91 ($C_{1''}$), 129.94, 130.15 ($C_{3'}$), 130.23, 131.07 (C_5), 142.56, 142.86 (C_2), 143.71, 144.78 ($C_{3''}$), 148.37, 148.46 (N=CH), 149.26, 149.57 (C_1), 150.74, 151.16 ($C_{4''}$), 152.20, 152.36 ($C_{1'}$), 163.52, 168.50 (C=O). EIMS: *m/z* 495.0281 $[M + H]^+$, calcd for $C_{22}H_{17}Cl_3N_2O_5$: 495.0290.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N*′-(2-hydroxy-4-methoxybenzylidene) acetohydrazide (**5g**) Yield 80% (1.104 mmol, 547 mg); light yellow solid, m.p. 148–150 °C; IR (cm^{-1}): ν_{max} max 3400 (Ar–OH), 3250 (N–H), 1666 (C=O), 1496 (C=N), 1475 (C=C_{Ar}), 1274 ((C=O)–N), 806 (C–H_{Ar}), 712 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 3.74, 3.76 (–OCH_3, s), 4.76, 5.20 (~1:1, –OCH_2, s), 6.42–6.53 ($H_{3''}$, $H_{5''}$, m), 6.88, 6.89 (H_3, d, $J = 8.9$ Hz), 6.98–7.11 (H_4, $H_{6'}$ m), 7.21, 7.28 (H_6, d, $J = 2.0$ Hz),7.30, 7.34 ($H_{5'}$, dd, $J = 8.8$, 2.4 Hz), 7.42, 7.57 ($H_{6''}$, d, $J = 8.6$ Hz), 7.68, 7.70 ($H_{3'}$, d, $J = 2.4$ Hz), 8.19, 8.33 (~1:1, N=C–H, s),10.15 (OH), 11.25, 11.41 (~1:1, NH). ^{1}H (Acetone-d_6): δ 4.82, 5.25 (~3:1 –OCH_2, s), 8.30, 8.20 (~3:1, N=C–H, s), 10.58, 10.64 (~3:1, NH, s). ^{13}C-NMR (DMSO-d_6): δ 55.37, 55.62 (–OCH_3, s), 66.00, 67.70 (–OCH_2), 101.30, 101.55 ($C_{3''}$), 106.80, 106.99 ($C_{5''}$), 112.07, 113.36 ($C_{1''}$), 114.46, 115.95 (C_6), 119.04, 119.32 (C_3), 121.66, 122.34 ($C_{6'}$), 122.68, 122.87 (C_4), 123.64, 123.89 ($C_{4'}$), 127.26, 127.59 ($C_{2'}$), 128.59, 128.79 ($C_{5'}$), 128.95, 129.22 (C_5), 129.84, 129.96 ($C_{3'}$), 130.13, 130.25 ($C_{6''}$), 142.56, 143.02 (N=CH), 148.78, 148.86 (C_1), 150.67, 151.14 (C_2), 152.17, 152.37 ($C_{1'}$), 158.41, 159.69 ($C_{2''}$), 162.36, 162.66 ($C_{4''}$), 163.56, 168.14 (C=O). EIMS: *m/z* 495.0281 $[M + H]^+$, calcd for $C_{22}H_{17}Cl_3N_2O_5$: 495.0278.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N*′-(2,3,4-trihydroxybenzylidene)acetohydrazide (**5h**) Yield 82% (1.132 mmol, 562 mg); yellow solid, m.p. 184–186 °C; IR (cm^{-1}): ν_{max} max 3462 (Ar–OH), 3310 (N–H), 1689 (C=O), 1494 (C=N), 1475 (C=C_{Ar}), 1265 ((C=O)–N), 804 (C–H_{Ar}), 715 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 4.76, 5.19 (~1:1, –OCH_2, s), 6.36, 6.38 ($H_{5''}$, d, $J = 8.6$ Hz), 6.78, 6.87 (H_3, d, $J = 8.4$ Hz), 6.89, 6.94 ($H_{6''}$, d, $J = 8.6$ Hz), 6.99–7.10 (H_4, $H_{6'}$, m), 7.18–7.37 ($H_{5'}$, H_6, m), 7.67, 7.70 ($H_{3'}$, d, $J = 2.4$ Hz), 8.13, 8.25 (~1:1, N=C–H, s), 8.45 9.32, 9.47, 11.06 (OH), 11.36, 11.56 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.26, 4.82 (~1:3 –OCH_2, s), 8.23, 8.15 (~1:3, N=C–H, s), 11.36, 10.36 (~1:3, NH, s). ^{13}C-NMR (DMSO-d_6): δ 65.90, 67.28 (–OCH_2), 107.62, 108.15 ($C_{5''}$), 111.11, 112.51 ($C_{1''}$), 115.51, 115.98 (C_6), 119.09, 119.38 (C_3), 121.46, 121.66 ($C_{6'}$), 122.36, 122.50 ($C_{6''}$), 122.63, 122.83 (C_4), 123.65, 123.93 ($C_{2'}$), 127.27, 127.62 ($C_{5'}$), 128.78, 128.94 ($C_{4'}$), 129.82, 129.97 (C_5), 130.13, 130.26 ($C_{3'}$), 133.12, 133.16 ($C_{3''}$), 142.57, 143.06 (C_2), 144.43, 147.05 (N=CH), 147.85, 148.79 (C_1), 149.34, 150.35 ($C_{2''}$), 150.65, 151.13 ($C_{4''}$), 152.12, 152.36 ($C_{1'}$), 163.43, 167.87 (C=O). EIMS: *m/z* 497.0074 $[M + H]^+$, calcd for $C_{21}H_{15}Cl_3N_2O_6$: 497.0075.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N*′-(3,4-dihydroxy-5-methoxybenzylidene) acetohydrazide (**5i**) Yield 78% (1.076 mmol, 550 mg); yellow solid, m.p. 106–110 °C; IR (cm^{-1}): ν_{max} max 3466 (Ar–OH), 3280 (N–H), 1659 (C=O), 1495 (C=N), 1475 (C=C_{Ar}), 1269 ((C=O)–N), 806 (C–H_{Ar}), 716 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 3.31–3.79 (–OCH_3, s), 4.73, 5.22 (~1:1, –OCH_2, s), 6.76–6.82 (H_3, m), 6.89 ($H_{6'}$, d, $J = 8.8$ Hz), 7.02, 7.04 ($H_{2''}$, $H_{6''}$, d, $J = 2$ Hz), 7.07 (H_4, $d_{apparent}$, $J = 9.5$ Hz), 7.19, 7.27 (H_6, d, $J = 2.0$ Hz), 7.29, 7.34 ($H_{5'}$, dd, $J = 8.9$, 2.5 Hz),

7.69, 7.71 ($H_{3'}$, d, $J = 2.5$ Hz), 7.85, 8.03 (~1:1, N=C–H, s), 8.58, 8.87, 8.93, 9.08 (OH), 11.36, 11.50 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.29, 4.77 (~3:1 –OCH_2, s), 7.91, 7.97 (~3:1, N=C–H, s), 10.37, 10.01 (~3:1, NH, s). ^{13}C-NMR (DMSO-d_6): δ 56.31, 56.41 (–OCH_3), 65.96, 67.43 (–OCH_2), 102.99, 103.05 ($C_{5''}$), 108.52, 108.90 ($C_{1''}$), 115.41, 115.80 (C_6), 119.08, 119.23 (C_3), 121.62, 122.24 ($C_{6'}$), 122.80, 122.85 ($C_{6''}$), 123.66, 123.79 (C_4), 124.62, 124.68 ($C_{2'}$), 127.29, 127.51 ($C_{4'}$), 128.79, 128.91 ($C_{5'}$), 129.90, 129.94 (C_5), 130.16, 130.23 ($C_{3'}$), 137.01, 137.28 ($C_{4''}$), 142.57, 142.87 (C_2) 145.14, 145.39 ($C_{3''}$), 146.28, 146.40 (N=CH), 148.89, 148.91 ($C_{5''}$), 150.73, 151.15 (C_1), 152.19, 152.36 ($C_{1'}$), 163.39, 168.41 (C=O). EIMS: *m/z* 511.0230 $[M + H]^+$, calcd for $C_{22}H_{17}Cl_3N_2O_6$: 511.0152.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N*′-(4-hydroxy-3,5-dimethoxybenzylidene) acetohydrazide (**5j**) Yield 93% (1.283 mmol, 674 mg); white solid, m.p. 166–170 °C; IR (cm^{-1}): ν_{max} max 3383 (Ar–OH), 3224 (N–H), 1662 (C=O), 1494 (C=N), 1480 (C=C_{Ar}), 1269 ((C=O)–N), 803 (C–H_{Ar}), 712 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 3.79, 3.80 (–OCH_3, s), 4.75, 5.26 (~1:1, –OCH_2, s), 6.86, 6.88 (H_3, d, $J = 8.9$ Hz), 6.96 ($H_{6'}$, d, J=8.6 Hz), 7.01, 7.04 ($H_{2''}$, $H_{6''}$, d, $J = 2.0$ Hz), 7.07 (H_4, d, $J = 8.9$ Hz), 7.19, 7.27 (H_6, d, $J = 8.9$, 2.5 Hz), 7.29, 7.34 ($H_{5'}$, dd, J = 8.9, 2.5 Hz), 7.69, 7.71 ($H_{3'}$, d, $J = 2.5$ Hz), 7.85, 8.03 (~1:1, N=C–H, s), 8.87, 8.43 (OH), 11.36, 11.50 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.30, 4.78 (~3:1 –OCH_2, s), 7.96, 8.03 (~3:1, N=C–H, s), 10.39, 9.98 (~3:1, NH, s). ^{13}C-NMR (DMSO-d_6): δ 56.45, 66.07 (–OCH_2), 105.00, 105.13 ($C_{2''}$, $C_{6''}$), 115.00, 115.43 (C_6), 116.53, 116.59 (C_3), 119.05, 119.18 ($C_{6'}$), 120.98, 121.60 (C_4), 122.74, 122.85 ($C_{4'}$), 123.66, 123.78 ($C_{2'}$), 124.58, 124.67 ($C_{1''}$), 127.29, 127.49 ($C_{5'}$), 128.79, 128.91 (C_5), 129.93, 130.14 ($C_{3'}$), 137.39, 138.20 ($C_{4''}$), 138.50, 139.15 (C_2), 142.55, 142.84 (C_1), 144.84, 145.02 ($C_{3''}$, $C_{5''}$), 148.52, 148.74 (N=CH), 151.15, 152.36 ($C_{1'}$), 161.11, 162.88 (C=O). EIMS: *m/z* 525.0387 $[M + H]^+$, calcd for $C_{23}H_{19}Cl_3N_2O_6$: 525.0376.

(*E*)-2-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)-*N*′-(2,4,6-trihydroxybenzylidene)acetohydrazide (**5k**) Yield 42% (0.580 mmol, 288 mg); light yellow solid, m.p. 210–214 °C; IR (cm^{-1}): ν_{max} max 3425 (Ar–OH), 3310 (N–H), 1687 (C=O), 1495 (C=N), 1473 (C=C_{Ar}), 1259 ((C=O)–N), 827 (C–H_{Ar}), 702 (C–Cl). ^{1}H-NMR (DMSO-d_6): δ 4.74, 5.14 (~1:1, –OCH_2, s), 5.83 ($H_{3''}$, $H_{5''}$, m), 6.87 (H_3, $H_{6'}$, d, $J = 8.9$ Hz), 6.99–7.09 (H_4, m), 7.25–7.30 (H_6, m), 7.34 ($H_{5'}$, dd, $J = 8.9$, 2.5 Hz), 7.69, 7.68 ($H_{3'}$, d, J = 2.5 Hz), 8.35, 8.55 (~1:1, N=C–H, s), 10.92, 10.25, 9.85 (OH), 11.38, 11.58 (~1:1, NH). ^{1}H (Acetone-d_6): δ 5.22, 4.80 (~1:4 –OCH_2, s), 8.54, 8.63 (~1:4, N=C–H, s), 10.24, 10.37 (~1:4, NH, s). ^{13}C-NMR (DMSO-d_6): δ 56.48, 67.24 (–OCH_2), 94.80 (3″,5″), 99.14, 99.25 (1″), 116.01, 116.08 (C_6), 119.05, 119.35 (C_3), 121.69, 122.35 ($C_{6'}$), 122.63, 122.79 (C_4), 123.91, 123.64 ($C_{2'}$), 127.26, 127.61 ($C_{5'}$), 128.77, 128.94 ($C_{4'}$), 129.82, 129.97 (C_5), 130.11, 130.26 ($C_{3'}$), 142.52, 143.05 (N=CH), 144.59, 147.26 (C_2), 150.07, 151.03 (C_1), 152.13, 152.35 ($C_{1'}$), 159.67, 160.13 ($C_{2''}$, $C_{6''}$), 162.21, 163.09 ($C_{4''}$), 163.53, 167.12 (C=O). EIMS: *m/z* 497.0074 $[M + H]^+$, calcd for $C_{21}H_{15}Cl_3N_2O_6$: 497.0075.

Biological activity assays

The compounds were subjected to in vitro evaluation as regards their cytotoxicity, leishmanicidal, and trypanocidal activity against U-937 human cells and intracellular amastigotes of *L. (V) panamensis* and *T. cruzi*, respectively.

In vitro cytotoxicity

The cytotoxic activity of the compounds was assessed based on the viability of the human promonocytic cell line U-937 (ATCC CRL-1593.2^{TM}) evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following the methodology described elsewhere (Pulido et al. 2012). Briefly, cells grown in tissue flasks were harvested and washed with phosphate buffered saline (PBS) by centrifuging. Cells were counted and adjusted at 1×10^6 cells/mL of RPMI-1640 supplemented with complete 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). One hundred µL was dispensed into each well of a 96-well cell-culture plate and then 100 mL of RPMI-1640 and the corresponding concentrations of the compounds were added, starting at 200 µg/mL in duplicate. Plates were incubated at 37 °C, 5% CO_2 for 72 h in the presence of compounds. The effect of compounds was determined by measuring the activity of the mitochondrial dehydrogenase by adding 10 µL/well of MTT solution (0.5 mg/mL) and incubated at 37 °C for 3 h. The reaction was stopped by adding 100 µL/well of 50% isopropanol solution with 10% sodium dodecyl sulfate and 30 min incubation. The cell viability was determined based on the quantity of formazan produced according to the intensity of color (absorbance) registered as optical densities (O.D.) obtained at 570 nm in a spectrophotometer (Varioskan™ Flash Multimode Reader—Thermo Scientific, USA). Cells cultured in the absence of compounds were used as control of viability (100% viability), while amphotericin B (AmB) was used as non-cytotoxic and cytotoxic drug control, respectively. Assays were conducted in two independent runs with three replicates per each concentration tested.

In vitro anti-leishmanial activity

The activity of the compounds was evaluated on intracellular amastigotes of *L. (V) panamensis* transfected with the

green fluorescent protein gene (MHOM/CO/87/UA140-EGFP) (Taylor et al. 2011). The effect of each compound was determined according to the inhibition of the infection evidenced by the decrease of the infected cells and parasite inside the cells. Briefly, U-937 human cells at a concentration of 3×10^5 cells/mL in RPMI 1640 and 0.1 µg/mL of phorbol-12-myristate-13-acetate (PMA) were dispensed into each well of a 24-well cell culture plate and then infected with 5 days old promastigotes in a 15:1 parasites per cell ratio. Plates were incubated at 34 °C, 5% CO_2 for 3 h and cells were washed two times with PBS to eliminate internalized parasites. One mL of fresh RPMI 1640 supplemented with 10% FBS and 1% antibiotics was added into each well, cells were incubated again to guarantee multiplication of intracellular parasites. After 24 h of infection, culture medium was replaced by fresh culture medium containing each compound at four-fold dilutions (100–25–6.25 and 1.56 µg/mL) and plates were incubated at 37 °C, 5% CO_2. After 72 h, inhibition of the infection was determined. Shortly, cells were removed from the bottom plate with a trypsin/EDTA (250 mg) solution, recovered cells were centrifuged at 1100 rpm for 10 min at 4 °C, the supernatant was discarded and cells were washed with 1 mL of cold PBS by centrifuging at 1100 rpm for 10 min at 4 °C. The supernatant was discarded and cells were suspended in 500 µL of PBS and analyzed by flow cytometry (FC 500MPL, Cytomics, Brea, CA, USA). All determinations for each compounds and standard drugs were carried out in triplicate, in two independent experiments (Pulido et al. 2012). Activity of the tested compounds was carried out in parallel with infection progress in culture medium alone and in culture medium with AmB as antileishmanial drugs.

In vitro anti-trypanosomal activity

Compounds were tested on intracellular amastigotes of *T. cruzi*, Tulahuen strain transfected with β-galactosidase gene (donated by Dr. F. S. Buckner, University of Washington) (Buckner et al. 1996). The activity was determined according to the ability of the compounds to reduce the infection of U-937 cells by *T. cruzi*. In this case, 100 µL of U-937 human cells at a concentration of 2.5×10^5 cells/mL in RPMI-1640, 10% SFB, and 0.1 µg/mL of PMA were placed in each well of 96-well plates and then infected with phase growth epimastigotes in 5:1 (parasites per cell) ratio and incubated at 34 °C, 5% CO_2. After 24 h of incubation four-fold dilutions of each compound (100–25–6.25 and 1.56 mg/mL) were added to infected cells. After 72 h of incubation, the effect of all compounds on viability of intracellular amastigotes was determined by measuring the β-galactosidase activity by spectrophotometry adding 100 µM CPRG and 0.1% nonidet P-40 to each well. After 3 h of incubation, plates were read at 570 nm in a spectrophotometer (Varioskan™ Flash Multimode Reader —Thermo Scientific, USA) and intensity of color (absorbance) was registered as O.D. Infected cells exposed to benznidazol (BNZ) were used as control for anti-trypanosomal activity while infected cells incubated in culture medium alone were used as control for infection. Non-specific absorbance was corrected by subtracting the O.D. from the blank. Determinations were done by triplicate in at least two independent experiments (Buckner et al. 1996; Insuasty et al. 2015).

Statistical analysis

Cytotoxicity was determined according to viability and mortality percentages obtained for each isolated experiment (compounds, amphotericin B, Benznidazole, and culture medium alone). The results were expressed as 50 lethal concentrations (LC_{50}) that corresponds to the concentration necessary to eliminate 50% of cells and calculated by Probit analysis (Finney 1978). Percentage of viability was calculated by Eq. 1, where the O.D. of control corresponds to 100% of viability. In turn, mortality percentage corresponds to 100%–%viability:

$$\%\,\text{Viability} = (\text{O.D Exposed cells}) / (\text{O.D unexposed cells}) \times 100 \quad (1)$$

The degree of toxicity was graded according to the LC_{50} value using the following scale: high cytotoxicity: $LC_{50} <$ 200 µM; moderate cytotoxicity: $LC_{50} > 200$–<400 µM and potential non-cytotoxicity: $LC_{50} > 400$ µM.

Anti-leishmanial activity was determined according to the percentage of infection (amount of parasites in infected cells) obtained for each experimental condition by flow cytometry. The percentage of infected cells was determined as the number of positive events by double fluorescence (green for parasites and red for cells) using dotplot analysis. On the other hand, the amount of parasites in the infected cells was determined by analysis of mean fluorescence intensity (MFI) in fluorescent parasites (Pulido et al. 2012). The parasite inhibition was calculated by Eq. 2, where the MFI of control corresponds to 100% of parasites. In turn, inhibition percentage corresponds to 100%–%Parasites. Results of anti-leishmanial activity were expressed as EC_{50} determined by the Probit method (Finney 1978):

$$\%\,\text{Parasite} = (\text{MFI Exposed parasites}) / (\text{MFI unexposed parasites}) \times 100 \quad (2)$$

Similarly, anti-trypanosomal activity was determined according to the amount of parasites in infected cells obtained for each experimental condition by colorimetry.

The parasite inhibition was calculated by Eq. 3, where the O.D. of unexposed parasites corresponds to 100% of parasites. In turn, percentage of inhibition corresponds to 100%−% Parasites. Results of anti-trypanosomal activity were also expressed as EC_{50} determined by the Probit method (Finney 1978):

$$\%\,\text{Parasite} = (\text{O.D Exposed parasites}) / (\text{O.Dunexposed parasites}) \times 100 \quad (3)$$

The anti-leishmanial and anti-trypanosomal activities were graded according to the EC_{50} value using the following scale: high activity: $EC_{50} < 40\,\mu M$, moderate activity: $EC_{50} > 40$–$<80\,\mu M$; and potential non-activity: $EC_{50} > 80\,\mu M$.

The selectivity index (SI), was calculated by dividing the cytotoxic activity and the anti-leishmanial or anti-trypanosomal activity using the following formula: $SI = LC_{50}/EC_{50}$.

Results and discussion

Chemistry

Microwave-assisted Williamson etherification of triclosan **1** with ethyl bromoacetate gave rise to ester **2** in 75% yield (Otero et al. 2014). Nucleophilic reaction of hydrazine hydrate on compound **2** gave rise to acylhydrazide **3** in 83% yield. Coupling compound **3** with a number of aldehydes in alcoholic medium provided hydrazones **5a–6h** in 42–93% yields (Coa et al. 2015). This synthetic strategy involves ultrasound-assisted or microwave-assisted reactions which allows to achieve the compounds with shorter reaction times than the conventional heating methods. In addition, the products were obtained in very good to excellent yields and without appreciable by-product formation (Scheme 1).

The structures and stereochemistry of all hydrazones have been established by a combined study of IR, ESI-MS, ^{1}H-NMR, ^{13}C-NMR, COSY, and NOESY spectra. IR spectra exhibited characteristic absorption peaks corresponding to Ar–OH (3325–3468 cm^{-1}), N–H (3100–3290 cm^{-1}), C=O (1658–1705 cm^{-1}), C=N (1492–1598 cm^{-1}), $C{=}C_{Ar}$ (1409–1480 cm^{-1}), (C=O)–N (1251–1274 cm^{-1}), $C{-}H_{Ar}$ (802–910 cm^{-1}), and C–Cl (702–780 cm^{-1}). ESI-MS spectra showed characteristic $[M+1]^+$ peaks corresponding to their molecular weights. The assignments of all the signals to individual H-atoms or C-atoms have been performed on the basis of typical δ-values and J-constants. The ^{1}H-NMR and ^{13}C-NMR spectra of these compounds dissolved in DMSO-d_6 showed double signals. For example, for compounds **5a** we have the following ^{1}H-NMR signals: C(=O)–NH– (11.50, 11.71 ppm), –N=CH (8.28, 8.43 ppm), and Ar–O–CH_2– (4.78, 5.23 ppm). ^{13}C-NMR spectra showed signals at 150.63 and 151.10 ppm due to the presence of CH=N, signals at 163.83 and 168.42 ppm corresponding to C=O group and signals to 66.10 and 67.34 due to Ar–O–CH_2−, which indicated that these molecules are present in two stereoisomeric forms (e.g., geometric or conformational isomers) (see Fig. 3).

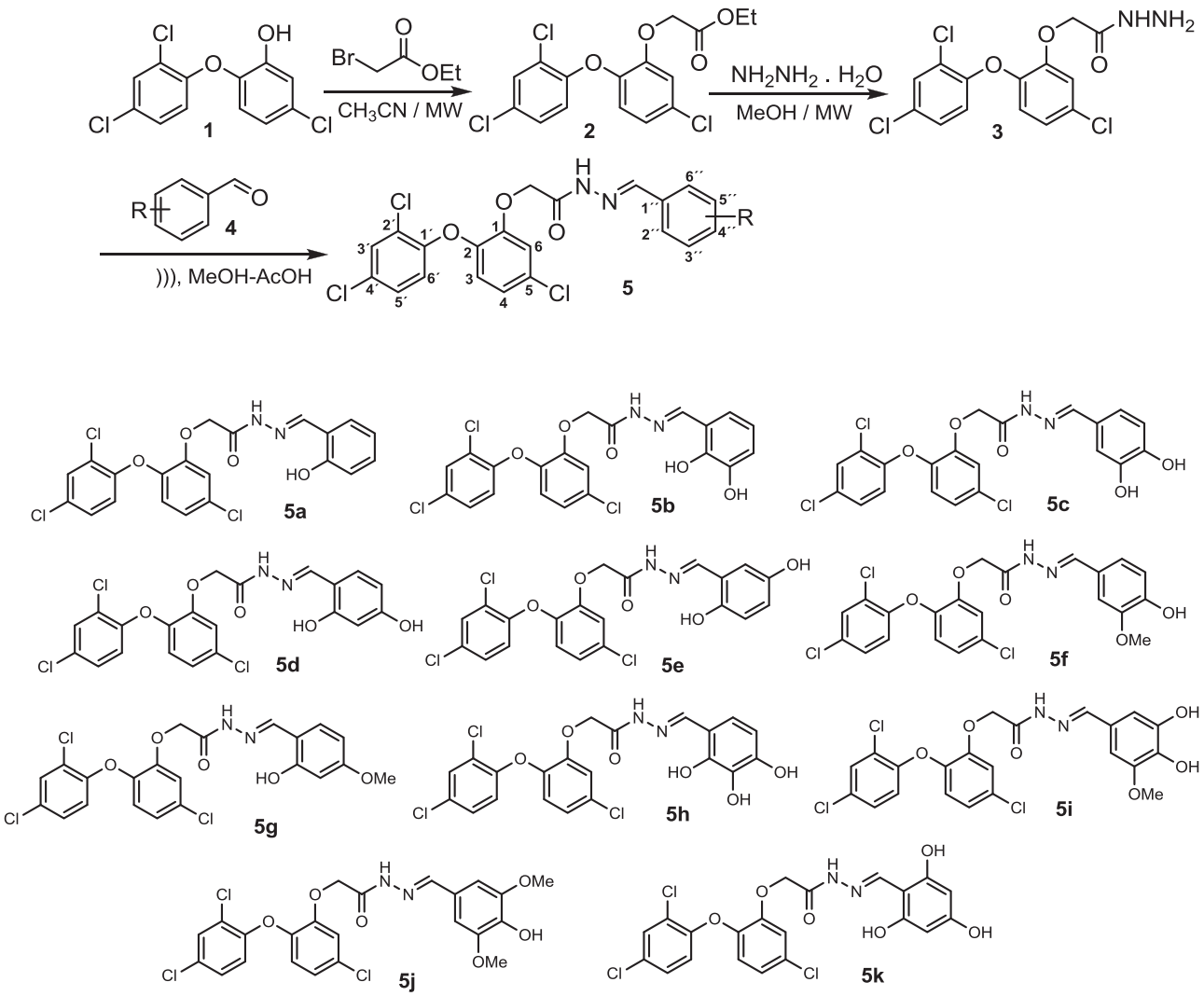
Integration values of these signals showed a ~1:1 ratio between the two stereoisomers when compounds were dissolved in DMSO-d_6. However, a ~2:1 ratio was obtained when ^{1}H-NMR spectra of hydrazones were taken in acetone-d_6. This result shows the presence of an equilibrium system that is dependent on the polarity of the solvent (Rahman et al. 2005). NOESY experiment showed coupling between C(=O)–NH– and –N=CH, and between –N=CH and Ar–O–CH_2− hydrogens. These couplings can be explained for the presence of *E*-antiperiplanar isomer, which is depicted in Fig. 3 (Basilio et al. 2013). Therefore, the other constituent of the isomeric mixture must be the *E*-synperiplanar isomer (see Fig. 3).

Biological activities

The effect of hydrazones on cell growth and viability was assessed in human macrophages (U-937 cells) (Pulido et al. 2012), which are the host cells for *L. (V) panamensis* and *T. cruzi* parasites. On the other hand, the antiparasite activity of these compounds was tested on intracellular amastigotes of *L. (V.) panamensis* (Taylor et al. 2011) and *T. cruzi* (Buckner et al. 1996; Insuasty et al. 2015) according to the ability of these compounds to reduce the amount of parasite inside infected macrophages. Results are summarized in Table 1.

All synthetic hydrazones, with the exception of **5c**, **5e**, and **5h**, were highly cytotoxic to U-937 cells showing $LC_{50} < 200.0\,\mu M$ (Table 1). Compounds **5c**, **5e**, and **5h** showed no cytotoxicity ($LC_{50} > 400\,\mu M$). In turn, amphotericin B and benznidazole showed high and moderate and no cytotoxicity, respectively.

The anti-leishmanial and anti-trypanosomal activities were measured by determining the effective concentration 50 (EC_{50}) that corresponds to the concentration of drug that gives the half-maximal reduction of the amount of intracellular parasites (Table 1). Dose–response relationship showed that compounds **5a–5d**, **5f–5j**, and triclosan were active against intracellular amastigotes of *L. (V) panamensis* with $EC_{50} < 40\,\mu M$. The most active hybrid compounds were **5a**, **5d**, **5g**, **5f**, **5j**, and **5i** with an EC_{50} of >1.29, 1.64, 2.36, 6.88, 9.30, and 11.92 μM, respectively, followed by **5b**, **5c**, triclosan and **5h** with EC_{50} of 24.70, 25.08, 38.61, and 39.24 μM, respectively. Compounds **5k** and **5e** showed moderate activity with a $EC_{50} > 40\,\mu M$. As expected, the anti-leishmanial drug amphotericin B showed activity with low EC_{50} values.



Scheme 1 Synthetic pathway to triclosan–hydrazone hybrids

On the other hand, compounds **5d**, **5a**, **5f**, **5g**, and **5i** were highly active against intracellular amastigotes of *T. cruzi* with EC_{50} of 2.28, 2.36, >5.04, 2.95, and 11.31 μM, respectively, followed by compounds **5j**, **5c**, **5h**, and triclosan with an EC_{50} of 25.11, 31.31, 37.11, and 48.97 μM, respectively. In this case, benznidazole showed activity with an EC_{50} of 40.3 μM.

In general, the anti-leishmanial and anti-trypanosomal activity of the hydrazones were higher than their cytotoxicity. Thus, the SI values calculated for these compounds were >1. Compounds **5c**, **5e**, and **5h** showed the best SI with values from 5.90 to 16.55 (Table 1). Amphotericin B has very high SI values. Benznidazole exhibited a SI of 17.0. Although several hybrid compounds showed better activity than benznidazole, the SI of these compounds is affected by their cytotoxicity. These results suggest that biological activity of the triclosan–hydrazone hybrids is selective, being more active against *T. cruzi* parasites than U-937 cells.

On a structure–activity relationship basis, it is worth noting the synergistic effect of the parent subunits in the hybrids in comparison with the unlinked cases. For example, triclosan is by itself less potent than their hybrids **5a**, **5d**, **5f**, **5g**, **5i**, and **5j**. The presence of hydroxy or methoxy groups in positions 2 and 4 on the benzylidene moiety increases both the activity and cytotoxicity (**5d** and **5g** vs. **5b**, **5c**, and **5e**), which could be explained by a better molecular recognition ability towards target bioreceptors. There is not a clear relationship between the antiprotozoal activity and the methylation of the hydroxy groups, since in some cases methylation decreases the activity (**5d** vs. **5g**) while in other cases an increase in activity is measured (**5c** vs. **5f** and **5i** vs. **5j**). Furthermore, increasing the number of hydroxy groups decreases the activity (**5a** vs. **5b** and **5h**; **5a** vs. **5d** and **5k**). The biological activity of these compounds could be explained by their action as iron chelators (Walcourt et al. 2004; Coa et al. 2015) or/and as alkylating

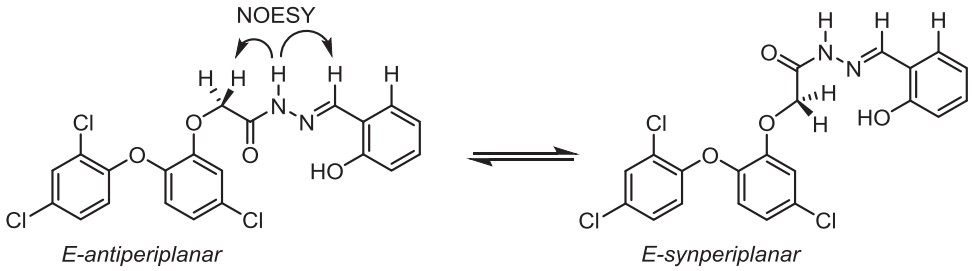


Fig. 3 Triclosan–hydrazone hybrids stereoisomers

Table 1 In vitro cytotoxicity and antiprotozoal activity of triclosan–hydrazone hybrids

Compound	Cytotoxicity	Anti-leishmanial activity		Anti-trypanosomal activity	
	LC_{50} (Mean ± SEM) [μM]	EC_{50} (Mean ± SEM) [μM]	SI	EC_{50} (Mean ± SEM) [μM]	SI
5a	2.79 ± 0.65	>1.29	<2	2.36 ± 0.13	1.18
5b	91.96 ± 9.38	24.70 ± 1.54	3.72	47.81 ± 4.24	1.92
5c	>415.19	25.08 ± 0.44	>16.55	31.31 ± 1.27	>13.26
5d	2.08 ± 0.13	1.64 ± 0.27	1.31	2.28 ± 0.21	0.95
5e	>415.19	70.33 ± 12.48	>5.90	58.48 ± 6.85	>7.10
5f	10.89 ± 0.81	6.88 ± 0.36	1.58	>5.04	<2.16
5g	5.45 ± 0.20	2.36 ± 0.12	2.28	2.95 ± 0.49	1.83
5h	>401.84	39.24 ± 2.18	>10.24	37.11 ± 6.43	>10.83
5i	36.15 ± 5.10	11.92 ± 0.82	3.03	11.31 ± 0.27	3.20
5j	23.78 ± 5.15	9.30 ± 0.52	2.55	25.11 ± 4.31	0.95
5k	62.08 ± 1.61	45.61 ± 6.19	1.36	42.39 ± 6.73	1.46
Triclosan	193.41 ± 32.81	38.61 ± 2.38	5.01	48.97 ± 4.21	3.95
Amphotericin B	45.60 ± 2.16	0.054 ± 0.011	842	NA	NA
Benznidazole	687.8 ± 16.14	NA	NA	40.3 ± 6.92	17.0

Data represent mean value ± standard deviation

LC_{50} lethal concentration 50 in μM, *EC_{50}* effective concentration 50 in μM, *SI* selectivity index = LC_{50}/EC_{50}, *NA* not applicable

agents (Michael acceptor) (Cardona et al. 2014). Studies in vitro have shown that chelating agents are able to inhibit parasite growth and proliferation by deprivation of iron, which is an essential nutrient for cell growth and division (Richardson et al. 1995). An electrophilic-conjugated system could be generated from *o*-hydroxybenzylidene-*N*-acylhydrazone framework due to the ability of this system to be converted into an electrophilic quinone methide intermediate through a pericyclic rearrangement (Ifa et al. 2000). The generation of such a system would allow conjugate addition of nucleophilic amino acid residues such as those found in *Leishmania* cysteine proteases (Mottram et al. 2004).

Conclusions

The synthesis, cytotoxicity, and activity against *L. (V) panamensis* and *T. cruzi* amastigotes of 11 triclosan–hydrazone hybrids are reported. Hydrazones were obtained as two *E*-synperiplanar and *E*-antiperiplanar conformers. Nine of them were active against *L. (V) panamensis* (**5a–5d**, **5f–5j**) and eight of them were active against *T. cruzi* (**5a**, **5c**, **5d**, **5f–5j**), with EC_{50} values lower than 40 μM. Compounds **5c**, **5e**, and **5h** showed the best SI against both *L. panamensis and T. cruzi*, with values between 5.90 and 16.55. These results suggest that these compounds have potential as templates for drug development against protozoal diseases. The presence of hydroxy or methoxy groups in positions 2 and 4 on the benzylidene moiety increases both activity and cytotoxicity. There is no clear relationship between the antiprotozoal activity and the methylation pattern of the hydroxy groups, since in some cases methylation decreases the activity (**5d** vs. **5g**), while in other cases the activity is increased (**5c** vs. **5f** and **5i** vs. **5j**). The mechanism of action of these compounds needs to be adressed and will be the objective of further studies.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

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