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Original Article

The efficacy of new 2,5-dihydroxybenzyl derivatives against *Trypanosoma cruzi, Leishmania infantum and Leishmania braziliensis*

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Abstract

Introduction: Chagas disease and Leishmaniasis are among the most important parasitic diseases. They are considered to be within the most relevant group of neglected tropical diseases and have been included as priorities for searching new drugs due to their several treatment limitations. These parasitic diseases caused by flagellated protozoans affect more than 20 million people predominantly in developing countries.
Methodology: In this study, we prepared a series of 2-substituted 1,4-benzenediols by an efficient, green, and lithium salt-free synthesis in water/ethanol as solvent to test their anti-parasitic activity. All 36 phenolic derivatives were evaluated *in vitro* for their activity against *T. cruzi* epimastigotes, *L. infantum*, and *L. braziliensis* promastigotes, as well as their cytotoxicity on macrophage and fibroblast cell lines.
Results: Based on the results obtained, the compounds that presented a methyl, trifluoromethyl or bromo group at the para-position of the second benzene ring were found the most active analogs, with higher selective index values on the three parasites assayed.
Conclusion: This evidence suggests that the anti-parasitic activity observed in these analogs is affected by the size of the group at the 4-position of the second ring, but not related with electronic factors.This study identified hit compounds with the potential to target several kinetoplastid parasites.

Key words: Phenolic derivatives; *Trypanosoma cruzi*; *Leishmania infantum*; *Leishmania braziliensis*;antiprotozoal activity.

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Introduction

The effort to find more effective and affordable forms of treatment for Chagas disease and Leishmaniasis is framed in the priority list of the World Health Organization (WHO) [1]. Currently, the range of drugs used to treat these diseases is severely limited, for several reasons: they are expensive, their efficacy is questionable, they require prolonged treatment and regular medical supervision, and they have significant side effects [2-6]. Chagas disease, also known as American trypanosomiasis, caused by the kinetoplastid parasite *Trypanosoma cruzi* [7]. It represents a major public health problem in South America, affecting at least 8-9 million people, whilemore than 25 million are at risk of infection [8]. The incidence has been calculated to be about 300,000 new cases per year in the absence of control interventions; about 14,000 deaths occur every year [9,10]. Many infected individuals have moved from the rural zones to cities and to other countries; a few hundred thousand chagasic individuals are estimated to live today in the U.S.A., Europe, and Asia [11]. Under natural conditions, the flagellate *T. cruzi* infects over 100 mammalian species from different orders. It is transmitted by hemiptera ('true bugs') from the family *Reduviidae*, such as *Triatoma*, *Rhodnius* and *Panstrongylus*, in which the parasites are carried in the hind gut of the insect. During blood sucking, the insect defecates on the skin, and the protozoa in the feces, in the form of metacyclic trypomastigotes, enter the mammalian hosts when they scratch their skin or rub their eyes. Once inside the mammalian host, the trypomastigotes penetrate cells and transform into amastigotes that multiply intracellularly. Amastigotes transform into blood trypanomastigotes that are released together with some amastigotes during cell rupture. The released amastigotes infect new cells and the trypomastigotes re-enter the blood stream. *Reduviidae* bugs are infected by ingesting trypomastigotes during feeding on infected blood. In the insect's stomach, trypomastigotes change into two replicative forms, epimastigotes and spheromastigotes. These proliferate by cell division

and, on reaching the rectum, differentiate into the infective metacyclic trypomastigote form, thus completing the life cycle [12]. Although spread primarily by blood-sucking triatomine bugs, other modes of transmission have been described: blood transfusion, organ transplantation, contaminated food or drink, and there are rare cases of sexual transmission. Congenital transmission from mother to child is gaining in importance, especially in non-endemic regions [13,14]. The disease has two clinically defined phases: the acute phase, which is often asymptomatic and unrecognized, lasting around two months, during which *T. cruzi* can easily be detected by blood examination, and the chronic phase, during which *T. cruzi* is very hard to detect [15]. Whilst current treatment options targeting the parasite are limited to two rather old and suboptimal nitro-heterocyclic drugs, the newer options benznidazole (Abarax/ELEA and Rochagan/LAFEPE) and nifurtimox (Lampit/Bayer) have been shown to be effective in the acute phase of the disease and evidence is mounting for their efficacy in the chronic phase. Especially in children, their use is limited by poor access and substantial side effects, as well as the lack of a test of cure in chronically infected adults [16-18]. This highlights a clear need for new, better, and safer drugs for Chagas disease.

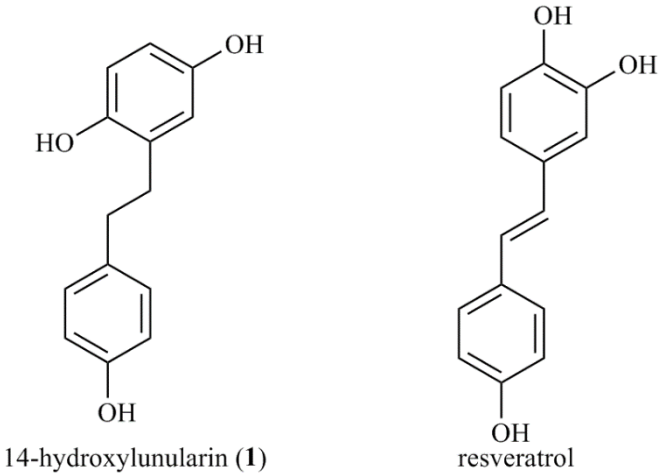
Leishmaniasis is a vector-borne disease caused by a parasite protozoon from the genus *Leishmania*, that has infected more than 12 million people worldwide; 350 million are at risk of acquiring it [19]. Leishmaniasis is transmitted by the bite of an infected female phlebotomine (sandfly). Currently there are more than 90 known species of sandflies in the world that are capable of transmitting the parasite [20]. *Leishmania* has a digenetic life cycle, involving both invertebrate (phlebotominae sandflies) and vertebrate (mammals, including humans) hosts. It presents in two very distinct stages: promastigotes (extracellular and flagellated forms found in the insect gut) and amastigotes (intracellular and round forms that multiply within phagocytic immune cells). Mammals are infected by the bite of female sandflies that regurgitate infective promastigotes during a blood meal. Upon host infection, promastigotes are phagocytosed mainly by macrophages, where they differentiate into amastigotes inside phagolysosomal compartments. After successive multiplication, amastigotes are released from macrophages and re-infect new cells, such as macrophages, dendritic cells and fibroblasts. Occasionally, sandflies become infected by ingesting infected cells during the next blood meal [21]. The three major manifestations of the disease are: visceral (VL), cutaneous (CL) and mucocutaneous (MCL) Leishmaniasis. Patients illustrate clinical symptoms ranging from simple cutaneous lesions to mucocutaneous ulcers or systemic disease affecting vital body organs such as the liver and spleen. They may show a confounded severe clinical manifestation complicated by co-infections such as HIV [22]. Visceral Leishmaniasis (VL) is the most severe form of *Leishmania* infection. Its annual incidence is estimated at 500,000 new cases, with 60,000 deaths occurring each year [23,24]. The fatality rate of VL can be as high as 100% within two years if left untreated. The disease is characterized by fever, weight loss, anemia and swelling of the spleen and liver [25]. Although the fatality rates of CL and MCL are lower compared to VL, the skin and mucous membrane lesions are debilitating, and can lead to serious disability and stigma, leaving patients permanently scarred, unable to be employed, and sometimes cast out from society [25,26]. The therapeutic arsenal to treat leishmaniasis is far from ideal. Pentavalent antimonials including sodium stibogluconate (SSG) and N-methylglucamineantimoniate remain the first line treatment for VL [27]. As resistance to antimonials is becoming more common, limiting their use, emerging second-line treatments involve pentamidine and Amphotericin B (AmB). AmB administered parenterally is highly effective in VL and is available as an affordable micellar formulation (Fungizone), which is however highly nephrotoxic, which limits the dose that can be administered [28]. A liposomal formulation Ambisome, the first line treatment in the developed world. Also available are lipidic complexes (Amphocil and Abelcet) whose use is limited further by their high cost compared to Ambisome. Cost of therapy with AmB is also further increased by the need for cold transport and refrigeration. Pentamidine, another second-line drug, has been shown to cause insulin-dependent diabetes which is irreversible and potentially fatal [29].

The parasites *T. cruzi* and *Leishmania sp* are the focus of worldwide research efforts, with the aim to find effective and less toxic drugs than those few currently available, and to control the spread of the two diseases. For these reasons, the primary objective of this project was to establish a sequential and rational screening of new compounds by using *in vitro* assays on the parasites *Trypanosoma cruzi*, *Leishmania infantum* and *Leishmania braziliensis*, to decrease the time needed to develop new trypanocidal and leishmanicidal drugs.

We have studied phenols and their analogs, which are widely distributed in nature and display a

remarkable range of potent biological, biochemical and pharmacological activities [30-33] in the *in vitro* assays. In particular, hydroxybibenzyl and hydroxystilbene derivatives are endowed with antifungal, antimicrobial, and cytotoxic activities, among others (Figure 1) [34-38]. The potent *in vitro* and *in vivo* pharmacological activity shown for 14-hydroxylunularin (2,5,4´-trihydroxybibenzyl) (**1**) [39], a natural phenol, against *Leishmania sp.*, combined with its low cytotoxicity on mammalian cells, motivated us to find more active analogs of this compound. According to our previous work [39], the presence of two hydroxyl groups in positions 2,5 of the bibenzyl moiety is indispensable for the biological activity.

Figure1. Phenolic compounds with antileishmanial activity.



14-hydroxylunularin (**1**) resveratrol

Methodology

Chemistry

All commercially available reagents, K_2CO_3, 1,4-cyclohexanedione and the corresponding aldehyde, were purchased and used without further purification (Sigma-Aldrich, St.Louis, USA). All solvents, EtOH, AcOEt, and n-hexanes were purified by distillations (Sigma-Aldrich, St.Louis, USA). All reactions were monitored by thin layer chromatography (TLC) performed on 0.25 mm silica gel (60 F_{254}, polyester sheet, Macherey-Nagel, Düren, Germany) using UV light, iodine and Brady solution as visualizing agents. Flash column chromatography was carried out with silica gel (spherical, neutral, 63-210 μm grade, Macherey-Nagel, Düren, Germany). Yields refer to chromatographically and spectroscopically homogeneous materials. Melting points were measured on a melting point apparatus (Gallenkamp, London, England) and were uncorrected. Mass spectra (EI MS) were obtained on a Shimadzu (Kyoto, Japan) GC-EI MS QP 1100 EX spectrometer. ^{1}H-NMR spectra (400 MHz) and ^{13}C-NMR (100 MHz) were recorded in the indicated solvent on a Bruker Advance DPX 400 MHz spectrometer (Rheinstetten, Germany) Chemical shifts (δ) are reported in delta (δ) units, parts per million (ppm). Chemical shifts for ^{1}H-NMR spectra are given relative to signals for internal tetramethylsilane (0 ppm) or residual nondeuterated solvents, i.e., methanol (3.30 ppm). Chemical shifts for ^{13}C-NMR spectra are given relative to the signal for chloroform-d (77.0 ppm) or dimethyl sulphoxide-6d (39.5 ppm). Multiplicities are reported using the following abbreviations: s (singlet), d (doublet), t (triplet), p (pentet), m (multiplet), dd (double doublet), dt (double triplet), br-s (broad singlet). Coupling constants (*J*) are represented in hertz (Hz). Compounds **8-18**, **20** and **22-38** were previously synthesized and characterized according to Peixoto *et al.* [40] (Figure 2). Compounds **3** and **5** were synthesized under our general methods and previously reported by Ozaki *et al.* [41,42]. They were described in details in the supporting information.

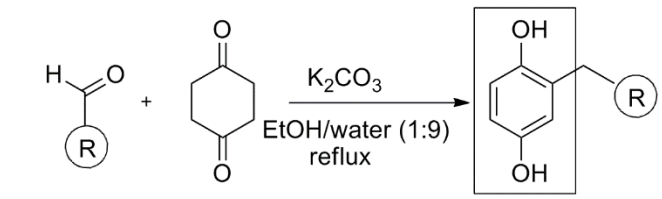
General procedure for the synthesis of 2-alkylated 1,4-benzenediol derivatives

A solution of K_2CO_3 (1 mmol) in a 20 mL water-ethanol mixture (9:1 by volume) is heated with stirring. Once the solution is under reflux, 1,4-cyclohexanedione (1 mmol) is added slowly. Afterwards, the aldehyde (2 mmol) is added slowly in portions. When the reaction is complete (approx. 4h), a 10% HCl solution is added dropwise while still hot, up to a neutral pH (especially with amphoteric compounds) or slightly acidic pH. The aqueous solution is extracted with ethyl acetate, and the organic extract is washed with brine, dried and evaporated *in vacuo*. Purification is performed by column chromatography.

Structural characterization of new products

2-(4-quinolinylmethyl)-1,4-benzenediol (6): ^{1}H-RMN (400MHz, $(CD_3)_2SO$) δ (ppm):4,28 (s, 2H) 6,30 (d, *J*=2,9 Hz, 1H) 6,45 (dd, J_1=2,9 Hz J_2=8,6 Hz, 1H) 6,69 (d, *J*=8,6 Hz, 1H) 7,25 (d, *J*=4,4 Hz, 1H) 7,59 (ddd,

Figure 2. General synthesis of 2,5-dihydroxybenzyl derivatives under green chemistry conditions.



R= Aryl and alkyl derivatives Pharmacophore

J_1=1,3 Hz J_2=6,8 Hz J_3=8,3 Hz, 1H) 7,73 (ddd,J_1=1,3 Hz J_2=6,8 Hz J_3=8,3 Hz, 1H) 8,02 (dd, *J1*=0,8 Hz *J2*=8,4 Hz, 1H) 8,16 (dd, *J1*=0,8 Hz *J2*=8,4 Hz, 1H) 8,56 (s, 1H) 8,79 (d, *J*=4,4 Hz, 1H) 8,94 (s, 1H); ^{13}C-NMR (100 MHz, $(CD_3)_2SO$)δ (ppm): 31.3, 114.0, 115.8, 116.6, 121.7, 124.4, 125.7, 126.4, 127.3, 129.1, 129.6, 147.0, 147.2, 147.8, 149.8, 150.3; EI MS *m/z* : 251 (M^+, 100); 250 (29); 234 (27); 130 (32); MP=229-232°C; Yield: 57%

2-(5-isoquinolidinylmethyl)-1,4-benzenediol (7): ^{1}H-RMN (400MHz, $(CD_3)_2SO$) δ (ppm): 4,24 (s, 2H) 6,21 (d, *J*=2,9 Hz, 1H) 6,42 (dd, J_1=2,9 Hz J_2=8,6 Hz, 1H) 6,68 (d, *J*=8,6 Hz, 1H) 7,61 (m, 2H) 7,90 (d, *J*=5,9 Hz, 1H) 7,99 (d, *J*=7,8 Hz, 1H) 8,49 (m, 2H) 8,93 (s, 1H) 9,30 (s, 1H);^{13}C-NMR (100 MHz, $(CD_3)_2SO$)δ (ppm): 31.2, 113.6, 115.7, 116.4, 117.2, 126.1, 126.8, 127.2, 128.6, 130.9, 134.2, 136.7, 142.9, 147.1, 149.8, 152.9; EI MS *m/z*: 251 (M^+, 80); 130 (100); MP=222-225°C; Yield: 81%

2-(4-(1H-imidazol-1-yl)bencyl)-1,4-benzenediol (19): ^{1}H-NMR (400 MHz, $(CD_2)_2SO$) (ppm): 3,82 (s, 1H) 6,42 (dd, J_1=8,3 Hz, J_2=2,9 Hz, 1H) 6,45 (d, *J*=2,9 Hz, 1H) 6,61 (d, *J*=8,3 Hz, 1H) 7,08 (s, 1H) 7,32 (d, *J*=8,5 Hz, 2H) 7,52 (d, *J*=8,5 Hz, 2H) 7,67 (t, *J*=1,3 Hz, 1H) 8,18 (s, 1H) 8,58 (s, 1H) 8,72 (s, 1H); ^{13}C-NMR (100 MHz, $(CD_3)_2SO$) (ppm): 34.7, 113.5, 115.6, 116.8, 118.1, 120.4, 127.7, 129.7, 129.9, 134.8, 135.5, 140.2, 147.3, 149.8; EI MS *m/z* : 60 (43); 69 (43); 71 (36); 73 (49); 83 (32); 145 (56); 266 (M^+, 100). MP=191-193°C; Yield: 73%

2-(3-(3-trifluorometil)-fenoxibencil)-1,4-bencenodiol (21): ^{1}H-RMN (400MHz, $(CD_3)_2SO$) δ (ppm): 3,79 (s, 2H) 6,41 (dd, J_1=8,3 Hz J_2=2,8 Hz, 1H) 6,43 (d, *J*=2,8 Hz, 1H) 6,59 (d, *J*=8,3 Hz, 1H) 6,86 (dd, J_1=8,1 Hz J_2=2,4 Hz, 1H) 6,95 (s, 1H) 7,06 (d, *J*=8,1 Hz, 1H) 7,24-7,27 (m, 2H) 7,32 (t, *J*=7,9 Hz, 1H) 7,47 (d, *J*=8,8 Hz, 1H) 7,60 (t, *J*=8,1 Hz, 1H) 8,58 (s, 1H) 8,70 (s, 1H); ^{13}C-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm): 35.2, 113.6, 114.5 (d, *J*=4.0 Hz), 115.7, 116.5, 116.9, 119.6 (d, *J*=4.0 Hz), 119.7,121.9, 123.8 (q, *J*=272.5 Hz), 124.9, 127.5, 130.0, 130.8 (d, *J*=32.1 Hz), 131.4, 144.2, 147.3, 149.8, 155.4, 157.5; IR (NaCl): ν (cm^{-1}): 3375, 1490, 1325, 1250, 1150, 800, 700; EI MS *m/z* : 57 (M^+, 50); 149 (36); 125 (31); 111 (54); 97 (87); 96 (43); 95 (39); 85 (49); 84 (34); 83 (84); 82 (43); 81 (47); 71 (75); 70 (40); 69 (100); 68 (32); MP=100-102ºC; Yield: 81%.

Drugs and reagents

Resazurin Sodium Salt was obtained from Sigma-Aldrich (St. Louis, MO), stored at 4ºC protected from light. The solution of resazurin was prepared in phosphate buffered saline solution (PBS), pH 7.4, and filtered through a 0.22 µm filter before use. Chlorophenol red-β-D-galactopyranoside (CPRG; Roche, Indianapolis, Ind.) was dissolved in 0.9% Triton X-100 (pH 7.4). Reference drugs Miltefosine and Benznidazole were purchased from Sigma-Aldrich. The stock solution was prepared in sterile distilled water.

Parasites, cells and culture procedure

Leishmania sp. promastigotes.Twog species of *Leishmania* were used: *L. infantum* (MCAN/ES/92/BCN83) and *L. braziliensis* (MHOM/CO/88/UA301). Promastigotes were cultured at 26 °C in Schneider's Insect Medium (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 100 U/mL of penicillin plus 100 µg/mL of streptomycin (Sigma-Aldrich) in 25 mL culture flasks.

T. cruzi epimastigotes.For *in vitro T. cruzi* studies, the clone CL-B5 was used. The parasites, stably transfected with the *Escherichia coli*β-galactosidase gene (lacZ), were kindly provided by Dr. F. Buckner through the Universidad Complutense de Madrid (Spain). The epimastigotes were grown at 28 ºC in liver infusion tryptose broth (LIT) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin and harvested during the exponential growth phase.

Cell culture. The cell lines used were NCTC clone 929 and murine J774 macrophages. NCTC clone 929 cells were grown in Minimal Essential Medium (Sigma), and J774 macrophages were grown in RPMI 1640 medium (Sigma). Both media were supplemented with 10% heat-inactivated FBS (30 minutes at 56ºC), penicillin G (100 U/ml) and streptomycin (100 µg/ml). For the experiments, cells in the pre-confluence phase were harvested with trypsin. Cell cultures were maintained in a humidified incubator at 37ºC in a 5% CO_2 atmosphere.

Leishmania sp. promastigote susceptibility assay

The screening assay was performed on both *L. infantum* and *L. braziliensis* promastigotes in 96-well microplates cultured in Schneider medium that had not reached the stationary phase, as described [43]. Promastigotes were seeded at 2×10^6 and 2.5×10^6 parasites per milliliter for *L. infantum* and *L. braziliensis*, respectively, in a 200 µL volume in the presence of the compounds in different dilutions at 26º C for 72 hours. Then, 20 µL of 1mM resazurin solution

was added and returned to the incubator for other 4 h. Growth controls were also included. Miltefosine was used as reference drug. The oxidation-reduction was quantified at 570 and 595 nm. Each concentration was assayed three times.

The efficacy of each compound was estimated by calculating the IC_{50}. These values were calculated by the sigmoidal dose-response curve adjustment, using the statistical software program Graph-Pad Prism 3.0.

T. cruzi epimastigote susceptibility assay

The screening assay was performed in 96-well microplates using cell cultures that had not reached the stationary phase, as described [44]. Briefly, epimastigotes were seeded at 1 x 10^5 parasites per milliliter in 200 µL of Schneider medium. The plates were then incubated with the compounds at 28 ºC for 72 h, followed by adding of 50 µL of the substrate solution, CPRG to give a final concentration of 200 µM. The plates were incubated at 37 ºC for an additional 6 hours and were then read at 595 nm. Benznidazole was used as reference drug. Each concentration was tested in triplicate. Each experiment was performed twice separately. The efficacy of each compound was estimated by calculating the IC_{50}.

Cytotoxicity assay

Macrophages. J774 macrophages were seeded (5 × 10^4 cells/well) in 96-well flat-bottom microplates with 100 µL of RPMI 1640 medium. The cells were allowed to attach for 24 hours at 37ºC, 5% CO_2 and the medium was replaced with different concentrations of the compounds in 200 µL of medium, and exposed for other 24 h. Growth controls were also included. Afterwards, 20 µL of 1mM resazurin solution was added, and plates were returned to the incubator for 3 h. to evaluate cell viability. The reduction of resazurin was determined by dual wavelength absorbance measurement at 490 nm and 595 nm. The background was subtracted. Each concentration was assayed three times. Medium and drug controls were used in each test as blanks [45].

Fibroblasts. NCTC clone 929 cells were plated in 96-microtitre plates at 3 × 10^4 cells per well in 100 µL growth medium. The cells were grown overnight at 37ºC, 5% CO_2. After that, the medium was removed, and the compounds were added in 200 µL medium for 24 hours. After incubation, 20 µL 2mM resazurin solution was added to each well. The plates were incubated for 3 hours to allow optimal oxidation-reduction. The plates were read at 570 and 595 nm on a microplate reader. Each concentration was assayed three times. Medium and drug controls were used in each test as blanks [45].

Results Analysis

Statistical analysis:All *in vitro* assays were carried out in triplicate for each experimental condition. The mean percentage of parasites obtained in three independent experiments was used to estimate the fifty percent inhibitory concentration (IC_{50}). These values were calculated by the sigmoidal dose-response curve adjustment using the statistical software program Graph-Pad Prism 3.0.

Selectivity index (SI): It was expressed as the ratio (IC_{50} cell cultures / IC_{50} *T. cruzi* or *Leishmania sp*. parasites). The selection criteria for hit compounds used is based on the DnDi guidelines for Human African Trypanosomiasis and Visceral Leishmaniasis [46-50]:

If the IC50 is ≤ 10 µM and SI ≥ 10, the compound is classified as active;

If the IC50 is >10 µM and SI ≥ 10, the compound is classified as moderately active;

If the IC50 is > 10 µM and SI < 10, the compound is classified as inactive.

Results

Based on the above discussion, we synthesized a library of 2,5-dihydroxybenzyl derivatives to assay their biological activity against *Leishmania sp*. and *T. cruzi* to complement structure-activity relationship studies. In the course of our study, we synthesized these phenolic derivatives following green chemistry principles [40] using water/ethanol as solvent and potassium carbonate as base. This strategy involves a tandem aldolic condensation/isomerization/aromatization between 1,4-cyclohexanedione (**2**) and the appropriate aldehyde (Figure 2). We used several alkyl, heteroaryl and aryl aldehydes with a wide variety of substituents, including electron-donor and electron-withdrawing groups.

This method provides a rapid and efficient route to prepare 2,5-dihydroxybenzyl derivatives with moderate to excellent yields. A total of thirty-six phenolic derivatives were tested *in vitro* against *T. cruzi* epimastigotes, and *L. infantum* and *L. braziliensis* promastigotes. In addition, the cytotoxicity of the compounds on J774 macrophage and NCTC 929 fibroblast cell lines was determined (Supplementary Tables 1 to 5).

The compounds were grouped into five tables according to their structural features attached to the pharmacophore (PHA). The results are presented in selectivity indexes (SI) and IC_{50}values (µM) (see

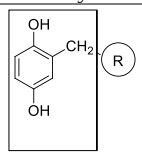
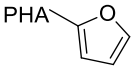
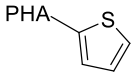
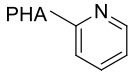
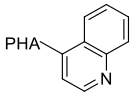
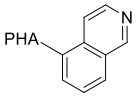
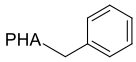
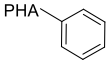
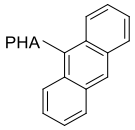
Supplementary Tables 1 to 5) on both species of parasites (SI = CC_{50} of fibroblasts NCTC 929 / IC_{50} *T. cruzi* and CC_{50} ofmacrophages J774 or fibroblasts NCTC 929* / IC_{50} *Leishmania sp*. parasites).

The first set of compounds (Table 1) incorporates a second aromatic heterocyclic ring in the molecule **3**-**7**. Especially compound **4** exhibited good activity against all of the assayed species. Coincidentally, in both species the SI of **4** increased with a sulfur atom in the second ring, while replacing it with oxygen or nitrogen (**3**,**5**), resulted in a decline of activity. However, compound **6**, a quinoline derivative, exhibited good activity against *T. cruzi* with an SI of 24. It was also determined that an unsubstituted aromatic hydrocarbon derivative (anthracene) showed activity **10**. This same compound, unlike others in this group, presented an interesting SI against *L. infantum* and *T. cruzi* and significant loss of activity against *L. braziliensis*. The increasing of spatial volume occupied by the second ring, seems to improve the activity (compound **10** compared with **8)**. However, compound **9** with a methylene group as a bridge presented the best SI against *T. cruzi* and *L. infantum*.

The data described in Table 2 showed the selectivity index of substituted benzene ring with electron donating group (EDG) derivatives. Compound **17**,with a methyl group in *para* position, resulted in an outstanding selectivity index against all tested species. Surprisingly, compound **26** (Table 3) with an trifluoromethyl (EWG) in *para* position also presented very good activity. This result made us suspect that the biological activity of these derivatives appeared relatively independent of electronic factors in the molecules. The related *para*-methyl derivative **17 was** evenmore active than **26** against *L. infantum*, with SI at 60 and 36, respectively.

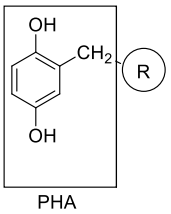
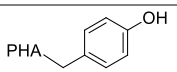
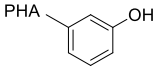
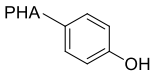
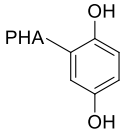
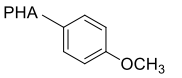
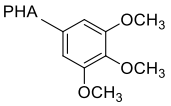
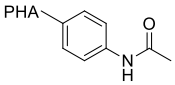
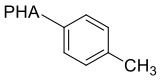
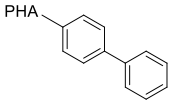
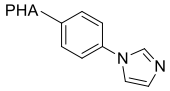
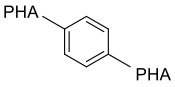
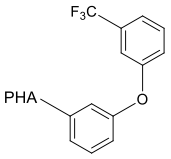
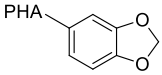
In Table 4, it can be seen that the compounds containing one halogen atom at *para* position increased the selectivity index from fluorine to bromine **30**-**32**, accompanying the increase in atomic size. It was also determined that the *ortho*-bromo-substituted analog **29** increased its anti-parasitic activity but also its cytotoxicity. These results showed that compound **32** with bromine at *para* position exhibited moderated selective anti-parasitic activity against the three assayed microorganisms.

Table 1. Selectivity index of pharmacophore and aromatic heterocycle and unsubstituted benzene ring structure derivatives.

Cs	 PHA	Selectivity index		
		***T. cruzi* epimastigotes**	***L. infantum* promastigotes**	***L. braziliensis* promastigotes**
3		13	1	2
4		23	25	36*
5		5	2	1
6		24	10*	7*
7		10	10*	7*
8		10	1	N/D
9		34	46*	N/D
10		23	21*	5*

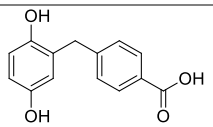
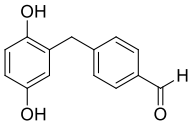
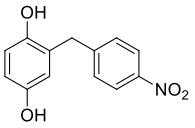
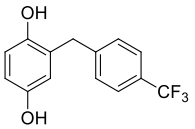
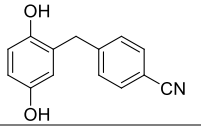
* SI = (CC_{50} NCTC 929 / IC_{50}*Leishmania sp.*), N/D = not determined.

Table 2. Selectivity index of pharmacophore and substituted benzene ring with electron donating group (EDG) derivatives.

Cs.		Selectivity index		
		T. cruzi **epimastigotes**	***L. infantum*** **promastigotes**	***L.braziliensis*** **promastigotes**
1		16	5	5
11		7	3*	1*
12		3	2	2
13		2	1	1
14		4	3	6
15		8	1*	2*
16		22	9	10
17		86	60	80
18		4	5*	8*
19		11	3*	2*
20		11	1	1
21		9	7	9
22		5	2	4

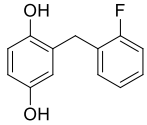
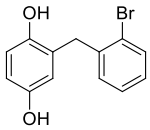
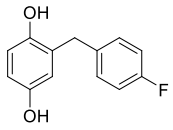
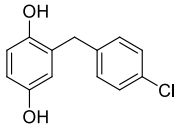
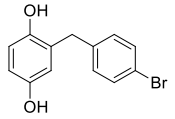
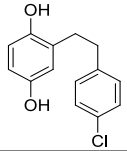
* SI = (CC_{50} NCTC 929 / IC_{50}*Leishmania sp.*).

Table 3. Selectivity index of pharmacophore and substituted benzene ring with electron withdrawing group (EWG) derivatives.

Cs.		Selectivity index		
		T. cruzi **epimastigotes**	***L. infantum*** **promastigotes**	***L braziliensis*-promastigotes**
23		8	3*	2*
24		13	7*	12*
25		11	9*	14*
26		71	36*	66*
27		15	13*	8*

* SI = (CC_{50} NCTC 929 / IC_{50}*Leishmania sp.*).

Table 4. Selectivity index of pharmacophore and mono-substituted halo aromatic derivatives.

Cs		Selectivity index		
		T. cruzi **epimastigotes**	***L. infantum*** **promastigotes**	***L. braziliensis*** **promastigotes**
28		9	3	6
29		5	4	8
30		5	3	6
31		7	2	5
32		29	28	40*
33		43	16	14

* SI = (CC_{50} NCTC 929 / IC_{50}*Leishmania sp.*).

These results confirmed that the presence of substituents at the "*para*" position of the second aromatic ring significantly increase the selectivity index. An excellent result was obtained for compound **33** against *T. cruzi* with an SI of 43, but, unfortunately we have not detected activity on *L.infantum* or *L. braziliensis.*

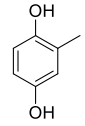
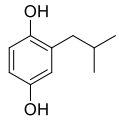
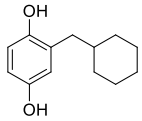
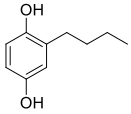
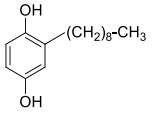
In Table 5, we show the anti-parasitic activity of the PHA bonded to five different linear or cyclic hydrocarbons. These compounds exhibited good IC_{50} but a dramatic decrease in SI. However, compound **37** showed good activity against *T. cruzi* and *L. infantum* with an SI higher than 25and 23, respectively; but there was decreased selectivity for *L. braziliensis*.

Discussion

The first stage in the discovery or repurposing of antimicrobial agents is target identification. It usually involves the screening of collections of compounds against a molecular target, typically an enzyme (target-based screening), or against whole organisms (cell-based or phenotypic screening) [51]. All candidates must then be refined through a cyclical process of structure modifications, until they achieve significant activity, typically in an animal model of infection. Subsequently, the biological activity, pharmacokinetics, and safety profile of the series are optimized by a process that leads to the selection of candidate drugs [51]. Selected drugs are then submitted to a process of regulatory toxicology and scale-up that enables their evaluation in human studies [52]. Unfortunately, the probability of a drug entering the clinical testing phase and its eventual approval is only about 12%, with an estimated out-of-pocket cost per approved new compound of US $ 1,395 million [53]. Because of the cost of development of new drugs, the majority of pharmaceutical companies have shown little interest in the development of new drugs for the treatment of parasitic diseases [54]. In the absence of adequate funding it is vital to design research projects that take advantage of available biological, bioinformatics, structural, and chemical data that is being incorporated in large publicly available databases. With the intention of identifying new candidate drugs for Chagas Disease and Leishmaniasis, we present in this work the process of green chemical synthesis of analogs of phenolic compounds and their subsequent primary screening in the replicative parasitic forms in order to of determining the parasitic activity and the possible structure-activity relationship of the compounds.

We have extended the green method for the synthesis of 2-alkyl-1,4-benzenediols using 1,4-cyclohexanedione for a variety of alkyl and aryl aldehyde in hydroalcoholic medium and K_2CO_3 as a base under reflux. The reaction times were short

Table 5. Selectivity index of pharmacophore and alkyl derivatives.

Cs.		Selectivity index		
		T. cruzi **epimastigotes**	***L. infantum*** **promastigotes**	***L. braziliensis*** **promastigotes**
34		2	1	2
35		4	2	4
36		5	1	5
37		> 25	> 23	> 3
38		1	1	1
39	Miltefosine	-	110	43
40	Benznidazole	14	-	-

(approximately 4 hours) in the majority of cases, except 3,4-dihydroxybenzaldehyde which needed 12 hours to reach completion. We assayed several alkyl and arylaldehydes with a wide variety of substituents, including electron-donor and electron-acceptor groups. Moderate to excellent yields were obtained with a broad substrate scope. The reaction only failed with nitro substrates.

The biological activity of these compounds was then evaluated in search of new antiprotozoal agents. The efficacy of new 2,5-dihydroxybenzyl derivatives were tested *in vitro* against *T. cruzi*, *L. infantum*, and *L. braziliensis* parasites. According to our current knowledge, very few compounds show good trypanosomaticidal activity against *Leishmania sp.* and *T. cruzi* simultaneously. A few compounds have already shown a demonstrated activity against these organisms. We report herein three possible candidates with biological activity against these three trypanosomatids that deserve further investigation.

A concise structure-activity relationship (SAR) suggests that sterically similar but electronically very different groups like *p*-methyl and *p*-trifluoromethyl substituents are essential to increase the biological activity against the assayed parasites. We also observed a good selectivity index of the *para*-bromobenzene derivative. It is important to note that natural 14-hydroxylunularin (**1**), which gave origin to derivatives evaluated in this study, has yielded different results of activity than those previously reported. Such variations are possible because the batches of parasite species tested in this study are not the same as those evaluated in the previous study.

Conclusion

In summary, we have described a library of phenolic derivatives, which was tested *in vitro* for activity against *T. cruzi, L. infantum* and *L. braziliensis* parasites, as well as for cytotoxicity on macrophage and fibroblast cell lines. Interestingly, compounds **17** and **26,** having methyl and, trifluoromethyl group at the "*para*" position of the second benzene ring, presented high antiprotozoal activity against *T. cruzi, L. infantum* and *L. braziliensis* with an SI $\geq$ 36. We also observed a high activity of the *para*-bromobenzene derivative compound **32** on all parasites tested, with IS $\geq$ 28. This fact suggests that anti-parasitic activity in these analogs depends upon the size of the group at the "*para*" position of the second ring, but not on electronic factors. On the other hand, it is important to mention that the highly anti-parasitic activity of compounds **17, 26** and **32** were simultaneously observed on both *Leishmania* speciesand *T. cruzi*. We report herein three dihydroxybenzyl derivatives compounds with potential anti-parasitic drugs against three kinetoplastid parasites. With these results in hand, we continue our studies to understand the mechanism of action of these compounds.

Acknowledgements

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Characterization data

2-(2-furanylmethyl)-1,4-benzenediol (3): ^{1}H-RMN (400MHz, $(CD_3)_2SO$) δ (ppm): 3,79 (s, 2H) 6,04 (d, *J*=3,1 Hz, 1H) 6,35 (dd, J_1=3,0 Hz J_2=1,9 Hz, 1H) 6,40 (d, *J*=2,8 Hz, 1H) 6,44 (dd, J_1=8,4 Hz J_2=2,9 Hz, 1H) 6,60 (d, *J*=8,4 Hz, 1H) 7,51 (d, *J*=1,8 Hz, 1H) 8,59 (s, 1H) 8,71 (s, 1H); ^{13}C-NMR (100 MHz, $(CD_3)_2SO$) δ(ppm): 28.1, 106.5, 110.8, 114.1, 116.0, 116.8, 125.4, 141.8, 147.7, 150.0, 154.5; Oil; Yield: 80% [41].**2-(2-thiophenylmethyl)-1,4-benzenediol (4)**: ^{1}H-RMN (400MHz, $(CD_3)_2SO$) δ (ppm): 3,95 (s, 2H) 6,43 (dd, J_1=2,9 Hz J_2=8,5 Hz, 1H) 6,47 (d, *J*=2,9 Hz, 1H) 6,60 (d, *J*=8,5 Hz, 1H) 6,84 (dd, J_1=1,2 Hz J_2=3,4 Hz, 1H) 6,91 (dd, J_1=3,4 Hz J_2=5,1 Hz, 1H) 7,27 (dd, J_1=1,2 Hz J_2=5,1 Hz, 1H) 8,61 (s, 1H) 8,77 (s, 1H);^{13}C-NMR (100 MHz, $(CD_3)_2SO$) δ(ppm): 29.5, 113.8, 115.7, 116.5, 123.9, 125.1, 126.7,127.5, 143.9, 147.2, 149.8; EI MS *m/z*: 206 (M^+, 73); 122 (100); 94 (36); Mp=82-84°C; Yield: 79%.% [41].

2-(2-pyridinylmethyl)-1,4-benzenediol (5): ^{1}H-RMN (400MHz, $CDCl_3$) δ (ppm):4,02 (s, 2H) 6,57 (dd, J_1=8,5 Hz J_2=3,0 Hz, 1H) 6,61 (d, *J*=2,9 Hz, 1H) 6,73 (d, *J*=8,5 Hz, 1H) 7,19 (ddd, J_1=7,5 Hz J_2=5,0 Hz J_3=1,1 Hz, 1H) 7,29 (d, *J*=7,8, 1H) 7,67 (td, J_1=7,7 Hz J_2=1,8 Hz, 1H) 8,40 (ddd, J_1=5,0 Hz J_2=1,7 Hz J_3=0,8 Hz, 1H); Mp=249-251°C; Yield: 66% [41].

2-(4-quinolinylmethyl)-1,4-benzenediol (6):^{1}H-RMN (400MHz, $(CD_3)_2SO$) δ (ppm):4,28 (s, 2H) 6,30 (d, *J*=2,9 Hz, 1H) 6,45 (dd, J_1=2,9 Hz J_2=8,6 Hz, 1H) 6,69 (d, *J*=8,6 Hz, 1H) 7,25 (d, *J*=4,4 Hz, 1H) 7,59 (ddd, J_1=1,3 Hz J_2=6,8 Hz J_3=8,3 Hz, 1H) 7,73 (ddd,J_1=1,3 Hz J_2=6,8 Hz J_3=8,3 Hz, 1H) 8,02 (dd, *J1*=0,8 Hz *J2*=8,4 Hz, 1H) 8,16 (dd, *J1*=0,8 Hz *J2*=8,4 Hz, 1H) 8,56 (s, 1H) 8,79 (d, *J*=4,4 Hz, 1H) 8,94 (s, 1H); ^{13}C-NMR (100 MHz, $(CD_3)_2SO$)δ (ppm): 31.3, 114.0, 115.8, 116.6, 121.7, 124.4, 125.7, 126.4, 127.3, 129.1, 129.6, 147.0, 147.2, 147.8, 149.8, 150.3; EI MS *m/z* : 251 (M^+, 100); 250 (29); 234 (27); 130 (32); Mp=229-232°C; Yield: 57%

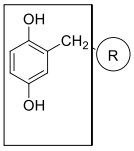
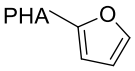
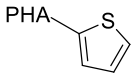
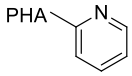
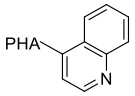
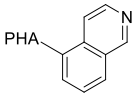
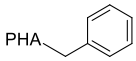
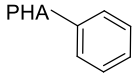
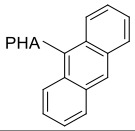
2-(5-isoquinolidinylmethyl)-1,4-benzenediol (7): ^{1}H-RMN (400MHz, $(CD_3)_2SO$) δ (ppm): 4,24 (s, 2H) 6,21 (d, *J*=2,9 Hz, 1H) 6,42 (dd, J_1=2,9 Hz J_2=8,6 Hz, 1H) 6,68 (d, *J*=8,6 Hz, 1H) 7,61 (m, 2H) 7,90 (d, *J*=5,9 Hz, 1H) 7,99 (d, *J*=7,8 Hz, 1H) 8,49 (m, 2H) 8,93 (s, 1H) 9,30 (s, 1H);^{13}C-NMR (100 MHz, $(CD_3)_2SO$)δ (ppm): 31.2, 113.6, 115.7, 116.4, 117.2, 126.1, 126.8, 127.2, 128.6, 130.9, 134.2, 136.7, 142.9, 147.1, 149.8, 152.9; EI MS *m/z*: 251 (M^+, 80); 130 (100); Mp=222-225°C; Yield: 81%.

2-(4-(1H-imidazol-1-yl)bencyl)-1,4-benzenediol (19):^{1}H-NMR (400 MHz, $(CD_2)_2SO$) (ppm): 3,82 (s, 1H) 6,42 (dd, J_1=8,3 Hz, J_2=2,9 Hz, 1H) 6,45 (d, *J*=2,9 Hz, 1H) 6,61 (d, *J*=8,3 Hz, 1H) 7,08 (s, 1H) 7,32 (d, *J*=8,5 Hz, 2H) 7,52 (d, *J*=8,5 Hz, 2H) 7,67 (t, *J*=1,3 Hz, 1H) 8,18 (s, 1H) 8,58 (s, 1H) 8,72 (s, 1H); ^{13}C-NMR (100 MHz, $(CD_3)_2SO$) (ppm): 34.7, 113.5, 115.6, 116.8, 118.1, 120.4, 127.7, 129.7, 129.9, 134.8, 135.5, 140.2, 147.3, 149.8; EI MS *m/z* : 60 (43); 69 (43); 71 (36); 73 (49); 83 (32); 145 (56); 266 (M^+, 100). MP=191-193°C; Yield: 73%.

2-(3-(3-trifluorometil)-fenoxibencil)-1,4-bencenodiol(21):^{1}H-RMN(400MHz, $(CD_3)_2SO$) δ (ppm): 3,79 (s, 2H) 6,41 (dd, J_1=8,3 Hz J_2=2,8 Hz, 1H) 6,43 (d, *J*=2,8 Hz, 1H) 6,59 (d, *J*=8,3 Hz, 1H) 6,86 (dd, J_1=8,1 Hz J_2=2,4 Hz, 1H) 6,95 (s, 1H) 7,06 (d, *J*=8,1 Hz, 1H) 7,24-7,27 (m, 2H) 7,32 (t, *J*=7,9 Hz, 1H) 7,47 (d, *J*=8,8 Hz, 1H) 7,60 (t, *J*=8,1 Hz, 1H) 8,58 (s, 1H) 8,70 (s, 1H); ^{13}C-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm): 35.2, 113.6, 114.5 (d, *J*=4.0 Hz), 115.7, 116.5, 116.9, 119.6 (d, *J*=4.0 Hz), 119.7,121.9, 123.8 (q, *J*=272.5 Hz), 124.9, 127.5, 130.0, 130.8 (d, *J*=32.1 Hz), 131.4, 144.2, 147.3, 149.8, 155.4, 157.5; IR (NaCl): ν (cm^{-1}): 3375, 1490, 1325, 1250, 1150, 800, 700; EI MS *m/z* : 57 (M^+, 50); 149 (36); 125 (31); 111 (54); 97 (87); 96 (43); 95 (39); 85 (49); 84 (34); 83 (84); 82 (43); 81 (47); 71 (75); 70 (40); 69 (100); 68 (32); MP: 100-102ºC; Yield: 81%.

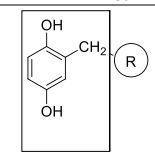
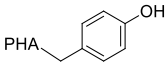
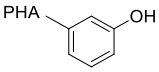
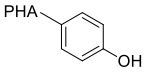
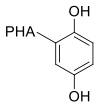
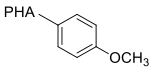
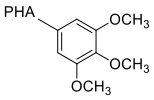
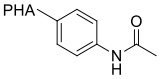
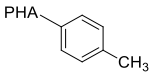
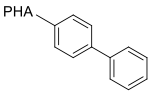
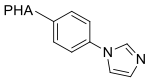
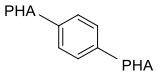
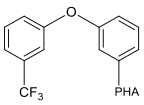
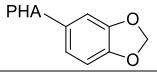
3. Tables of antiparasitic and cytotoxicity activity

Supplementary Table 1. IC_{50} and CC_{50} data of pharmacophore and aromatic heterocycle and unsubstituted benzene ring structure derivatives.

Cs		**IC_{50} (µM)**	**CC_{50} (µM)**	**IC_{50} (µM)**		**CC_{50} (µM)**
		Epimastigotes *T.cruzi*	**Fibroblasts NCTC 929**	**Promastigotes *L. infantum***	**Promastigotes *L. braziliensis***	**Macrophages J774**
3		8	98	40	17	30
4		11	2.5	10	7	N/D
5		35	188	47	71	80
6		10	245	33	24	N/D
7		9	91	13	9	N/D
8		6.5	64	8	N/D	11.2
9		0.7	23.7	1.4	N/D	>64
10		3	72	15	4	N/D

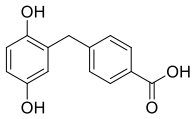
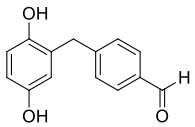
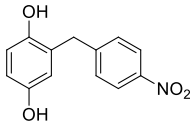
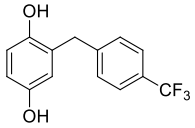
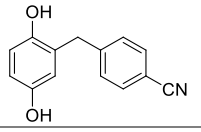
N/D = not determined.

Supplementary Table 2. IC_{50} and CC_{50} data of pharmacophore and substituted benzene ring with electron donating group (EDG) derivatives.

Cs.		IC_{50} (µM)	CC_{50} (µM)	IC_{50} (µM)		CC_{50} (µM)
		Epimastigotes *T.cruzi*	**Fibroblasts NCTC 929**	**Promastigotes *L. infantum***	**Promastigotes *L.braziliensis***	**Macrophages J774**
1		10	163	32	34	279
11		14	94	69	37	N/D
12		38	103	63	65	105
13		61	129	169	172	135
14		32	131	25	14	87
15		13	105	46	76	N/D
16		18	392	42	41	N/D
17		6	482	8	6	N/D
18		4	50	6	11	N/D
19		11	116	47	34	N/D
20		12	135	59	92	1
21		12	101	6.5	5	44
22		41	214	67	40	154

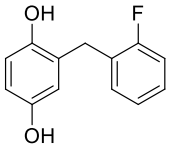
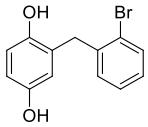
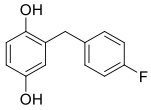
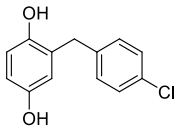
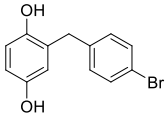
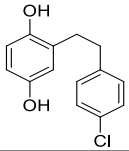
N/D = not determined.

Supplementary Table3. IC_{50} and CC_{50} data of pharmacophore and substituted benzene ring with electron withdrawing group (EWG) derivatives.

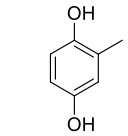
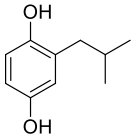
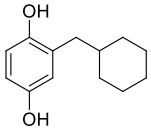
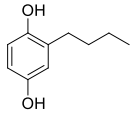
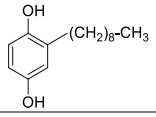
Cs		**IC_{50} (µM)**	**CC_{50} (µM)**	**IC_{50} (µM)**		**CC_{50} (µM)**
		Epimastigotes *T.cruzi*	Fibroblasts NCTC 929	Promastigotes *infantum*	Promastigotes *L. braziliensis*	Macrophages J774
23		39	295	91	140	N/D
24		13	173	25	14	N/D
25		12	128	14	9	N/D
26		6	393	11	6	N/D
27		6	89	11	7	N/D

N/D = not determined.

Supplementary Table 4. IC_{50} and CC_{50} data of pharmacophore and mono-substituted halo aromatic derivatives.

Cs		**IC_{50} (µM)**	**CC_{50} (µM)**	**IC_{50} (µM)**		**CC_{50} (µM)**
		Epimastigotes *T.cruzi*	**Fibroblasts NCTC 929**	**Promastigotes *L. infantum***	**Promastigotes *L. braziliensis***	**Macrophages J774**
28		38	351	29	15	84
29		41	194	26	11	91
30		36	182	28	16	97
31		3	20	10	5	23
2		12	359	13	9	N/D
33		0.9	37	0.2	0.8	11

Supplementary Table 5. IC_{50} and CC_{50} data of pharmacophore and alkyl derivatives.

Cs.		IC_{50} (µM)	CC_{50} (µM)	IC_{50} (µM)		CC_{50} (µM)
		Epimastigotes *T.cruzi*	**Fibroblasts NCTC 929**	**Promastigotes *L.infantum***	**Promastigotes *L. braziliensis***	**Macrophages J774**
34		37	60	43	23	39
35		38	159	48	21	93
36		39	190	89	17	96
37		4	> 64	3	20	> 64
38		50	30	8	9	9