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**CARRERA DE MICROBIOLOGÍA**

# Activity of novel aromatic compounds against *Trypanosoma cruzi*

**Disertación previa a la obtención del título de Microbióloga**

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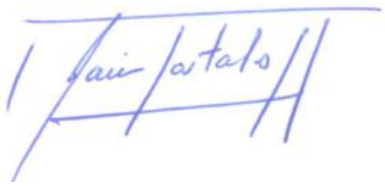
**Quito, 2023**

Yo, Dr. Jaime Costales Cordero, certifico que la disertación de Microbiología de la candidata Lesley Sharon Dávalos Ojeda ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.



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*Para mis padres Roberto y Marcia, por siempre estar.*

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**Activity of novel aromatic compounds against *Trypanosoma cruzi***

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## Abstract

**Background:** The parasitic hemoflagellate protozoan *Trypanosoma cruzi* is the causative agent of Chagas Disease (CD), a tropical infection affecting mainly low-income population segments in Latin America, where it is one of the leading parasitic infections. Nifurtimox (NFX) and benznidazole (BNZ) are the only two drugs currently approved to treat infections with *T. cruzi*; however, they frequently induce serious side effects while their efficacy is unsatisfactory. Therefore, identification of novel compounds displaying specific activity against *T. cruzi* is of high interest, as a basis for development of new drugs to treat CD.

**Methods.** Seven novel aromatic compounds were evaluated. *In vitro* activity against the recombinant Tulahuen ß-gal strain of *T. cruzi* was measured employing colorimetric assays for ß-gal activity. Cytotoxicity against cultured mammalian cells was evaluated via resazurin reduction assays. BNZ was used as a reference drug.

**Results:** All studied compounds presented activity against *T. cruzi in vitro*; however, five of them also induced significant host-cell damage, suggesting their toxicity is not specific. Compounds 3K and 3L showed no cytotoxicity measurable up to 100 µM concentrations, while displaying $IC_{50}$ values against *T. cruzi* of 1.464 and 2.717 µM, respectively. In the same assay, BNZ, displayed an $IC_{50}$ value of 1.558 µM and no cytotoxicity at concentrations up to 100 µM.

**Conclusions:** Two compounds evaluated in the study are selectively toxic against intracellular *T. cruzi* amastigotes *in vitro* and did not display cytotoxic activity against mammalian cells. Their $IC_{50}$ values are comparable to those obtained for BNZ, the reference drug. Novel compounds with specific anti-*T. cruzi* activity can be further explored in the search for options for CD treatment.


**Key words:** Chagas disease, aromatic compounds, cytotoxicity, *Trypanosoma cruzi*.

## Background

Chagas disease (CD), also known as American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi*, and mainly affects the low-income population in areas with precarious health systems [1]. This neglected tropical disease is one of the most important parasitic infections endemic to Latin America [1] and, according to the World Health Organization (WHO), approximately 6 to 7 million people are currently infected by *T. cruzi* [2,3]. In Latin America, ~30.000 new CD cases are diagnosed each year, while ~12.000 deaths occur in the same period [4].

*T. cruzi* is a flagellated parasitic protozoan belonging to the order Kinetoplastida, capable of infecting a wide range of mammals, including both domestic and wild animals, as well as humans [5]. More than 100 species of hemipteran insects belonging to the Reduviidae family, Triatominae subfamily, act as vectors, which transmit *T. cruzi* infection to more than 70 genera of mammalian hosts, including humans [6]. Vectorial transmission is stercorarian: vectors harbor the parasite in their digestive system, and when they feed on the blood of mammals, they release the parasite through their feces near the bite wound. The parasite enters the mammalian host through the bite wound, or via mucous membranes. However, transmission is also possible transplacentally, orally and, in humans, via blood transfusions [5,7]. When an uninfected triatomine feeds on the blood of an infected mammal the parasite takes up residence in the digestive tract of the bug [5].

As *T. cruzi* progresses through its life cycle, the parasite presents different developmental forms. Epimastigotes are present in the vector digestive anterior tract and midgut [8]. In the vector's hindgut, the parasite transforms into metacyclic trypomastigotes, which are infectious to mammals and are present in the vector's feces. Upon gaining access to the mammalian host's tissues, metacyclic trypomastigotes invade host cells, and transform into

amastigotes, which multiply in the host cell's cytoplasm. After several rounds of binary fission, the resulting parasites transforms back into trypomastigotes and egress the host cell [8].

Chagas disease progresses in two phases: The first two months after the initial infection are known as the acute phase, parasitemia is high and usually symptoms are barely recognizable, mild, or even absent [9]. Symptoms may include Romaña's sign (unilateral palpebral edema), fever, fatigue, fever, headache, body aches, rash [10]. Subsequently, the disease enters the chronic stage where irreversible cardiac, digestive and/or nervous damages occur in up to 30-40% of those infected [11], resulting in significant morbidity and mortality [12]. During this phase most people remain asymptomatic [10].

There are only two antiparasitic drugs currently approved to treat *T. cruzi* infection, both of which were developed almost half a century ago: NFX, which has been rejected in several countries due to its side effects, mainly linked to neurotoxicity; and BNZ, normally used during the acute phase of the disease since 76% to 100% of both children and adults have achieved seroreversion and decreased levels of parasites in blood [13]. Efficacy of BNZ during treatment of chronic CD remains uncertain. Nevertheless, some studies indicate that BNZ can modulate the phagocytic response and balance the host immune response to prevent or delay the progression of CD [14]. Studies suggest that a lower dose of BNZ during the chronic phase is just as effective, does not decrease the cure rate, and reduces side effects in patients [15].

*In vitro* axenic epimastigote cultures are many times employed for screening of trypanocidal compounds. Although microscopic parasite counting is one of the most widely used methods to select compounds with trypanocidal activity, previous research has proposed the MTT colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] that consists of the reduction of MTT in formazan to measure cell viability and metabolic activity of the parasite. Various concentrations of MTT with PMS (phenazine methosulfate)

were tested against *T. cruzi* epimastigotes of the Y-strain. This method allowed testing several compounds at the same time and detected in a simple, fast, and reliable way approximately 500.000 epimastigotes/ml, ($p < 0.01$), linear correlation absorbance/numbers ($R= 0.99$) [16].

Other studies propose the use of resazurin (RZN) to test the sensitivity of *T. cruzi* epimastigotes against certain compounds, it is a simple, fast, cost-effective, and sensitive method [17]. This quantitative colorimetric assay detects changes in cell viability through an oxidative-reductive process that measures the metabolic activity of the parasite. RZN reduction has been directly linked to the number of parasites (5 to 100 $x10^4$)/well, $R= 0.99$, $p < 0.001$) [17]. Likewise, for *T. cruzi* epimastigotes that express the *Escherichia coli LacZ* gene, it has been proposed a method that shows the activity of the enzyme ß-galactosidase on the substrate chlorophenol red ß-D-galactopyranoside (CPRG). In the assay performed, absorbance was proportional to the number of parasites ($5x10^3$ to $1.2x10^6$ parasites/ml), $R=0.98$, $p < 0.01$ [18].

However, as it was pointed out above, epimastigotes are a life cycle stage present in the vector and; therefore, they are not the best model for drugs against the life cycle stages infectious to humans. The culture of the mammalian life cycle stages of the parasite requires infection of cultured mammalian cells [19].

According to Buckner et al. [20], the drug screening technique against *T. cruzi* strains expressing *E. coli* ß-galactosidase is a method where the parasites catalyze a colorimetric chlorophenol red- ß-D-galactopyranoside as substrate. Thus, the transfected parasites can be quantified with an enzyme-linked immunosorbent assay (ELISA) reader to determine the effect of drugs on each of them. This technique is capable of determining the number of parasites in animal tissue cells, which optimizes the selection of drugs against *T. cruzi*. Likewise, Bettiol et al. [21] propose a selection technique for organic compounds with trypanocidal activity on a recombinant strain of *T. cruzi* (Tulahuen strain) that expresses ß-galactosidase.

ß-galactosidase is an enzyme that hydrolyzes D-galactosyl residues from polymers, oligosaccharides, or secondary metabolites [22]. Also, in order to know the potency of organic compounds with trypanocidal activity, the use of the Median Inhibitory Concentration ($IC_{50}$) is proposed. The $IC_{50}$ is an estimated value that indicates the necessary amount of compound to inhibit a biological process by half, that is, by 50% [23].

We aimed to identify aromatic compounds with novel structures that can be used as a basis for the development of alternative antiparasitic treatments for Chagas disease. The *in vitro* median inhibitory concentration ($IC_{50}$) against *T. cruzi* and toxicity over mammalian cells was evaluated for each of seven novel compounds, two of which presented specific anti-*T. cruzi* activity similar to that of BNZ.

# Methods

## Compounds

The seven compounds tested in this study (designated as LB01-LB05, 3K and 3L) were kindly provided by professor Eder Lenardao, from the Laboratorio de Sintese Organica Limpa, Universidade Federal de Pelotas, Brazil. Stocks (10 mM) were prepared in DMSO and maintained at -80 ºC until use. The exact identity and structure of the compounds employed in this work is withheld until a more comprehensive set of related compounds is tested and sufficient data is available for formal publication in a scientific journal.

## Mammalian cell line

LLC-MK2 monkey kidney cells from *Macaca mulatta* were cultured in Dublecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin/streptomycin (DMEM 10). Cells were maintained at 37 ºC with 5% CO2 and 98% of relative humidity in an incubator [24]. Cell passages were performed weekly.

**Parasite culture**

Trypomastigotes of Tulahuen ß-gal strain of *T. cruzi* were obtained infecting LLC-MK2 cells in 75 $cm^2$ cell culture flasks. A confluent monolayer was infected for 48 hours with $5x10^5$ trypomastigotes in 10 ml of DMEM supplemented with 2% FBS, 1% penicillin/streptomycin (DMEM 2) and incubated at 37 °C with 5% carbon dioxide and 98% relative humidity. After 48 hours, parasites were removed and the monolayer was washed with sterile PBS. Trypomastigotes were harvested from the culture medium starting at day post-infection. Parasite yield was determined using a hemocytometer [25].

**Cytotoxicity assays against mammalian cells**

A 96-well plate was seeded with $2x10^6$ LLC-MK2 cells/well and cells were allowed to attach for 24 hours. Subsequently, culture medium was removed and cells were washed with sterile phosphate-buffer saline (1X PBS). Compounds were plated in duplicate in 10 two-fold serial dilutions in DMEM without phenol-red or FBS, in concentrations ranging from 100 µM to 0.0097 µM, 20 µl of 3 mM resazurin sodium salt (RZN) in PBS were added per well and then incubated for 72 hours at 37 ºC with 5% $CO_2$ and 98% relative humidity. Cell viability was determined by RZN reduction via fluorescence (530-560 nm excitation and 590 nm emission) in a multimodal GloMax microplate reader (Promega) [20,21,25]. BNZ was used as a reference drug.

## Trypanocidal activity of compounds

A 96-well plate with $2x10^4$ LLC-MK2 cells/well was cultured for 24 hours to form cell monolayers. Thereafter, cultures were infected with five trypomastigotes per cell for 24 hours. After infection, the culture medium was removed and cells were washed 3 times with 1X PBS. Finally, 10 two-fold serial dilutions (100 µM to 0.0097 µM concentration range) of each compound in DMEM without phenol-red or FBS culture medium were applied in duplicate. The plate was incubated for 3 days at 37 ºC with 5% CO2 and 98% relative humidity [21,25].

At day 3 post-infection, 25 µl of Chlorophenol red ß-D-galactopyranoside (CPRG), containing Triton (0.5 %) were added to each well. The plate was incubated for 24 hours at 37 ºC with 5% $CO_2$ and 98% relative humidity. Absorbance was read at 490 nm in a multimodal GloMax microplate reader (Promega) [21].

## Giemsa staining of uninfected and infected mammalian cells treated with the studied compounds

Three sterile circular coverslips/well were aseptically placed in each well of six-well plates. Two ml DMEM containing $2x10^4$ LLC-MK2 cells were placed in each well; cells were cultured for 24 hours.

To obtain images of the effect of the compounds over uninfected cells, plates were washed with 1X PBS, compounds were added at its respective $IC_{50}$ concentration against mammalian cells and incubated for 72 hours at 37 ºC with 5% CO2 and 98% relative humidity. To obtain images of the effect of the compounds over intracellular parasites, cells were infected with five tissue culture derived trypomastigotes/cell in 2 ml DMEM (supplemented with 2% FBS) for 24 hours 37 ºC at 5% CO2 and 98% relative humidity. After infection, wells were

washed with PBS 1X and compounds were added at a concentration equivalent to their $IC_{50}$ against *T. cruzi.* Infection was allowed to proceed for 72 hours under the same conditions.

Finally, cells were rinsed in 1X PBS, coverslips removed, cells fixed with methanol and stained with Giemsa. Photographs were obtained under the 100X lens in a BX51 Olympus microscope equipped with a DP72 camera using Cell F imaging software.

### Statistical analysis

The half maximal inhibitory concentration ($IC_{50}$) was calculated using statistic software GraphPad prism 9.0 by log (inhibitor) vs. response dose curves also called variable slope model or four-parameter dose-response curve. This model fits the hill slope from the data rather than assuming a standard slope [26].

At least three independent replicate assays per compound were carried out to show activity against *T. cruzi.* $IC_{50}$s for each studied compound were statistically compared via Kruskall-Wallis and Dunn's *post hoc* test using GraphPad prism 9.0. A p-value of 0.05 was considered significant.

## Results

### Cytotoxicity of studied compounds against mammalian cell

Results from the cytotoxicity analysis are displayed in Table 1 and Figure 1. BNZ, which was employed as a reference drug, did not affect mammalian cells at the tested concentrations. Compounds of the LB-series were all cytotoxic, with $IC_{50}$s against mammalian cells < 9.556 µM (Table 1). These compounds caused decreased cell viability (Figure 1A) and

microscopically obvious morphological effects over the host cells (Figure 1B). Conversely, compounds 3K and 3L did not show significant cytotoxic effects at the concentrations tested (Table 1, Figure 1A and Figure 1B, panels H and I).

**Table 1.** $IC_{50}$ values of benznidazole and studied novel aromatic compounds against mammalian cells and *T. cruzi*

| **Compound** | **$IC_{50}$ against mammalian cells (± SD) 72h** | **$IC_{50}$ against *T. cruzi* (± SD) 72h** | **Selectivity index** |
|---|---|---|---|
| **Benznidazole** | >100 | 1.558 (± 0.09600) | >64.18 |
| **LB01** | 7.585 (±2.896) | 7.842 (± 2.218) | 0.97 |
| **LB02** | 9.350 (±2.588) | 1.816 (± 0.04464) | 5.15 |
| **LB03** | 9.556 (±4.669) | 4.690 (± 0.1455) | 2.04 |
| **LB04** | 6.263 (±2.305) | 5.963 (± 0.1629) | 1.05 |
| **LB05** | 3.745 (±1.450) | 0.9644 (± 0.07534) | 3.88 |
| **3K** | >100 | 1.464 (± 0.06070) | >68.31 |
| **3L** | >100 | 2.717 (± 0.1866) | >36.80 |

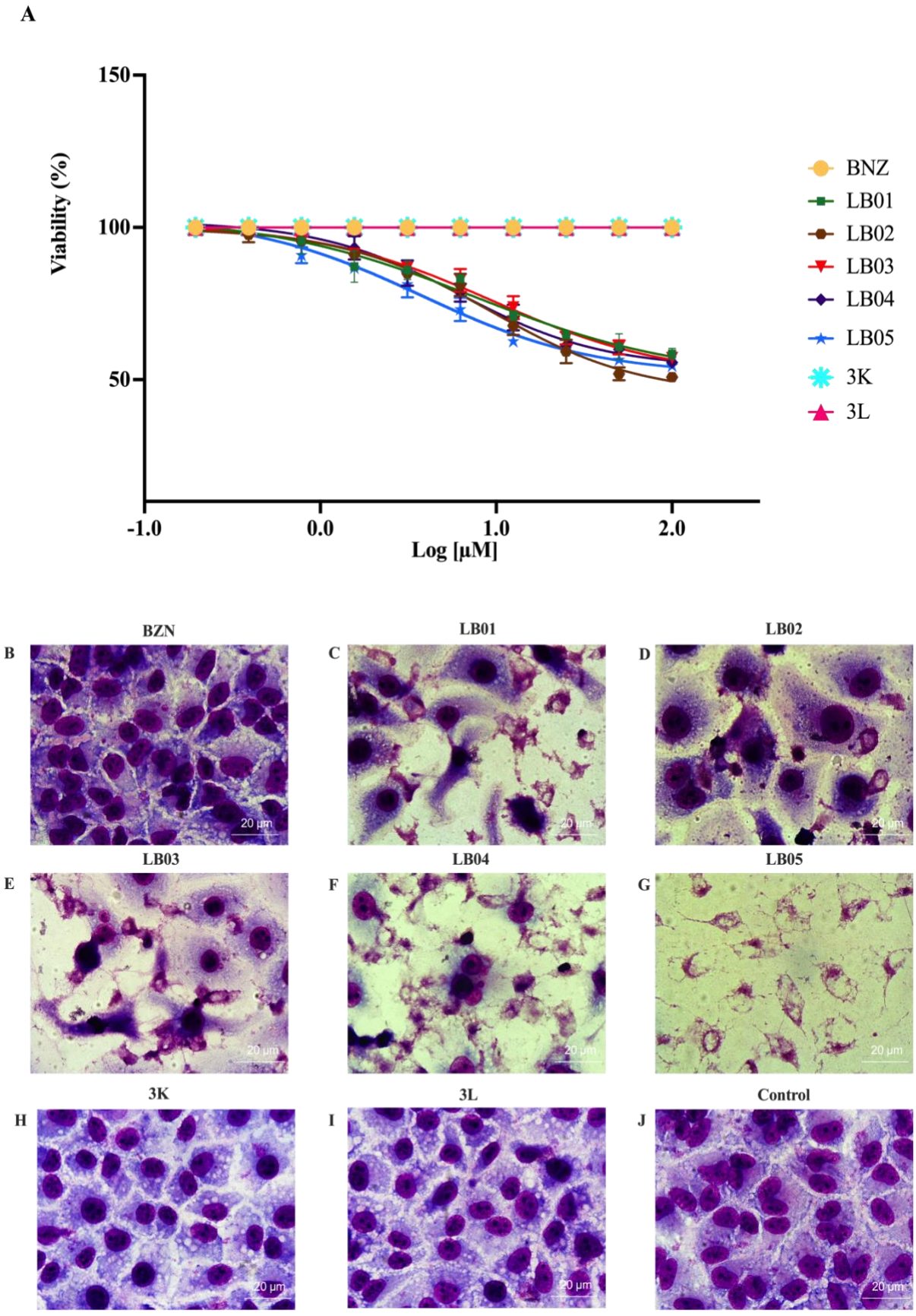
A
150
100
50
Viability (%)
-1.0
0.0
1.0
2.0
Log [µM]
BNZ
LB01
LB02
LB03
LB04
LB05
3K
3L
B
BZN
C
LB01
D
LB02
E
LB03
F
LB04
G
LB05
H
3K
I
3L
J
Control
20 µm

**Fig 1.** Cytotoxicity of studied compounds against mammalian cells. **A)** Dose- response curves for cytotoxicity of each studied compound against mammalian cells. LLC-MK2 cells were exposed to BNZ as reference drug and seven different aromatic compounds in ten two-fold serial dilutions (100-0.0097 µM concentration range) (see Methods). After 72 hours, cell viability was evaluated via RZN reduction. **B-I)** Representative images showing Giemsa-stained cells treated with BNZ (100 µM) and novel aromatic compounds (compounds were added at concentrations equivalent to their $IC_{50}$ against mammalian cells). **J)** Control: cells without aromatic compound treatment.

**Activity of studied compounds against *T. cruzi***

The data for activity of each studied compound against *T. cruzi* is shown in Table 1 and Figure 2. In our assays, BNZ presented and $IC_{50}$ of 1.558 µM. All tested compounds were found to affect the growth of intracellular amastigotes *in vitro,* with $IC_{50}$ below 5 µM, in most cases. Compounds 3K and 3L showed good anti-*T. cruzi* activity, at 1.464 and 2.717 µM, respectively. Figure 2, panels C-D show representative microscopic images the effect of compounds 3K and 3L compared to BNZ and uninfected cells. Images for compounds of the LB series are not shown, because they were previously determined to be highly cytotoxic.

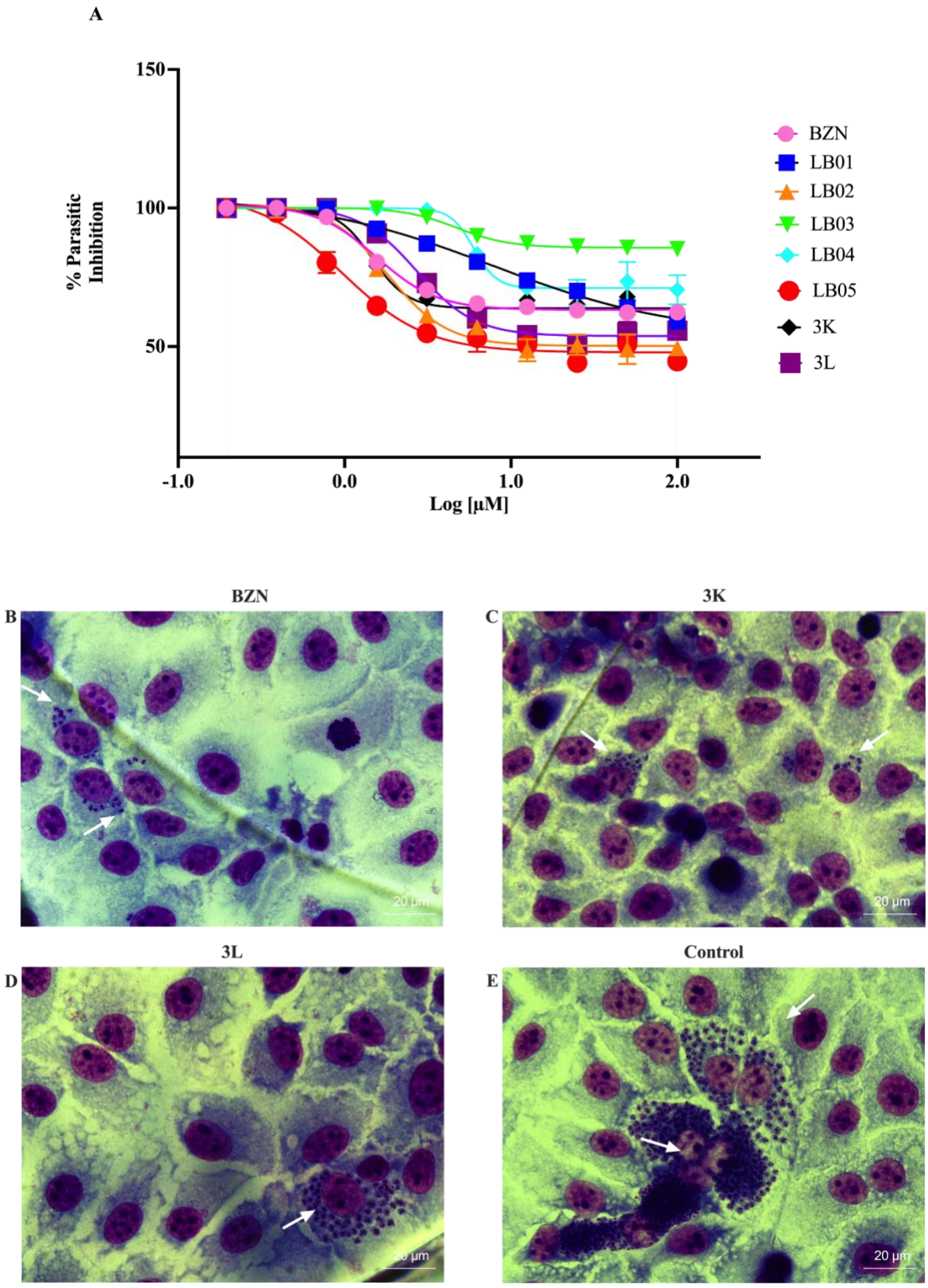
A
150
100
50
% Parasitic Inhibition
-1.0
0.0
1.0
2.0
Log [µM]
BZN
LB01
LB02
LB03
LB04
LB05
3K
3L
B
BZN
C
3K
D
3L
E
Control
20 µm

**Fig 2.** Activity of studied compounds against *T. cruzi in vitro*. **A)** Dose-response curves for each studied compound against intracellular amastigotes of the Tulahuen ß-gal strain of *T. cruzi*. LLC-MK2 cells infected for 24 hours with trypomastigotes from *T. cruzi* Tulahuen ß-gal strain (MOI 5) were exposed to ten two-fold serial dilutions (100-0.0097 µM concentration range) of BNZ (reference drug) and seven different aromatic compounds. Infected cells were in contact with the compounds for 72 hours (see Methods). The amount of intracellular parasites at 72 hours post-infection was evaluated via colorimetric measurement of ß-galactosidase activity. **B-D)** Representative images showing Giemsa-stained infected cells. **B)** BNZ (1.558 µM), reference drug. **C-D)** Non-cytotoxic compounds 3K (1.464 µM) and 3L (2.717 µM). **E)** Control: cells infected with *T. cruzi* Tulahuen ß-gal strain without compound treatment.

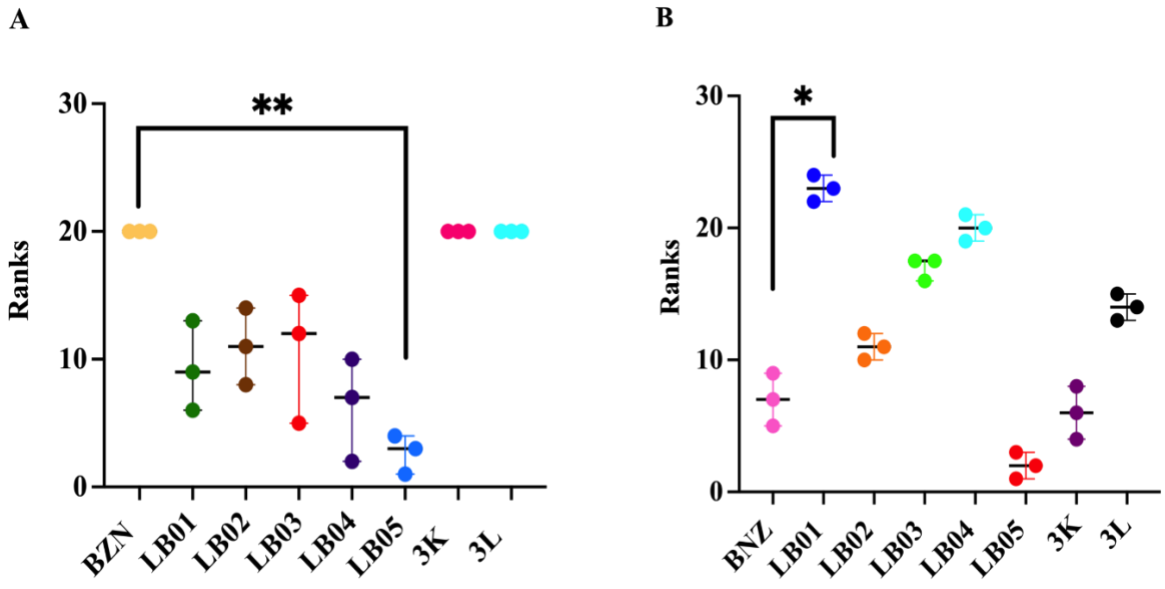


**Fig 3.** Comparison between $IC_{50}$ values for BNZ and studied compounds. **A)** Cytotoxicity against mammalian cells. Significant differences were observed between BNZ vs. LB05 (Kruskal-Wallis test, followed by Dunn's *post hoc* test. * = p<0.0143). **B)** Kruskal-Wallis test

for studied aromatic compounds against *T. cruzi.* Significant differences were observed when compared BNZ vs. LB01 ($p<0.0390$).

## Discussion

Drugs currently available for Chagas disease treatment are scarce, not effective, and toxic [25]. BNZ and NFX have poorly understood pharmacokinetic (PK) and pharmacodynamic (PD) properties and they frequently cause severe side effects, including peripheral neuropathies, severe dermatitis, nausea, vomiting, insomnia [27]. NFX has been used during acute phase of CD in children under 14 years of age, with cure rates between 88% and 100%. However, during the chronic phase and/or in adults, the cure rate is reduced to 7-8% [27]. Compared to NFX, BNZ has been more assimilable for patients and its efficacy is similar in acute infections and children under 18 years of age, as well as in congenital CD cases and women in childbearing age [27]. In recent studies, BNZ has presented rates between 6% to 50%, which do not reflect greater effectiveness, despite the fact that it is still used in the chronic phase of the disease [28].

Results from clinical trials with fexinidazole (FNZ), a very promising nitroheterocyclic drug candidate for CD treatment, have recently produced disappointing results. In 2014, in Cochabamba and Tarija, Bolivia, a double-blind, multicenter, randomized, placebo-controlled, prospective, proof-of-concept clinical trial of six FNZ regimens was conducted in adults with chronic CD [29]. The efficacy of low doses of the drug (1200 mg/day) was demonstrated with only 3 days of treatment. All patients treated with FNZ were free of parasitemia for 12 months; however, patients who received high doses for more than 14 days developed neutropenia, elevated liver enzymes, suicide due to severe depression, anxiety, headaches, and insomnia [29].

Subsequently, in 2017, in Spain, a phase II proof-of-concept study was initiated, employing reduced FNZ doses and shorter treatment courses. This clinical trial was completed at the beginning of 2022 and the results showed that, under the tested treatment regimens, the efficacy of the drug was uncertain beyond 12 months of treatment. Consequently, additional studies to determine the efficacy of FNZ in combination with other drugs or in cases of immunocompromised patients at risk of reactivation [30]. Therefore, identifying novel molecules with antiparasitic activity and less toxicity remains essential for the development of alternative treatments for Chagas disease.

Here, we have measured the *in vitro* activity of seven different novel aromatic compounds against *T. cruzi* intracellular amastigotes. Tulahuen ß-gal strain. BNZ was used as a positive control, a yielded an $IC_{50}$ value of 1.558 µM, which is comparable to values previously reported for this drug in the literature [20,21,31].

Although the five compounds in the LB series (LB01-05) were active against intracellular amastigotes of *T. cruzi*, they also presented intense cytotoxicity against mammalian cells, which indicates these compounds are broadly toxic and; therefore, they are not good candidates to be developed as antiparasitic drugs. Conversely, compounds 3K and 3L stand out due to their capacity to prevent intracellular amastigote multiplication with low $IC_{50}$ values (1.464 and 2.717 µM, respectively). However, 3K and 3L did not display significant cytotoxicity against mammalian cells, which is highly promising.

The $IC_{50}$ values we have recorded for 3K and 3L are comparable to those obtained to BNZ, tested side by side in the same assays. Additionally, they are lower than those obtained for other compounds being screened against *T. cruzi*, such as two neolignane derivates (selected form a 23-compound screen), which were tested *in vitro* against *T. cruzi* amastigotes ant trypomastigotes, using colorimetric resazurin assay and light microscopy counting [32].

Two compounds showed activity against both forms of the parasite, with $IC_{50}$ values of 64.2 ± 8.2 µM and 30.5 ± 10. 4 µM for trypomastigotes, $IC_{50}$ values for amastigotes: 8.0 ± 5.8 µM and 10.0 ± 0.8 µM; amastigotes SI of each compound: 8.1 and 7.5 [32].

Furthermore, organorutenium complexes with an $IC_{50}$ value of 8.681 ± 1.008 µM and SI of 3.01 [33], heterobimetallic nickel(II) and palladium(II) complexes with an $IC_{50}$ value of 4.9 ± 0.5 µM and SI of 40 [34], Amaryllidaceae-derived alkaloids with an $IC_{50}$ value of 3.31 µM and SI < 10 [31], and some other organic compounds have been tested as antiparasitic agents to treat Chagas disease with encouraging results. Compounds 3K and 3L tested in our assays stand out among the aforementioned compounds due to their significantly lower $IC_{50}$ values, therefore their activity against the parasite is higher without apparent cell damage.

Activity of compounds against *T. cruzi* is often tested on epimastigotes, the insect stage of the parasite life cycle, grown in axenic culture [16–18,35], since this simplifies the required culture procedures. However, our selected a screening method employs intracellular amastigotes, intracellular forms of the parasite known to multiply inside host cells and to persist in tissues in chronic infection [36]. Therefore, we tested compounds against a life cycle stage relevant for human infection and our results not only show that, besides being selectively toxic against the parasite, compounds 3K and 3L are capable of crossing the host cell's plasma membrane as well as the amastigote plasma membrane, which are highly desirable characteristics.

Future analysis of 3K and 3L compounds should include: cidality testing, given that our assays show they prevent amastigote multiplication, but do not conclusively demonstrate parasiticidal activity; testing of structurally related compounds, which might be even more selectively toxic to the parasite; testing in a set of *T. cruzi* strains representative of the broader

genetic diversity of the parasite and testing in combination with other anti-*T. cruzi* compounds, before moving to testing in experimental animal infections.

## Conclusions

We have identified two novel aromatic compounds, which are selectively toxic against intracellular *T. cruzi* amastigotes *in vitro*. They display $IC_{50}$s comparable to those of BNZ, the current standard of care drug for CD, when tested *in vitro* side by side, and lower than many other compounds reported in the literature. Additional testing and development of these compounds is warranted, including cidality testing, evaluation of structurally related compounds and over different *T. cruzi* strains.

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