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Sodium diethyldithiocarbamate antiparasitic activity against different *Trypanosoma cruzi* strains: Insights of its biological activity.

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Abstract: Chagas disease is caused by *Trypanosoma cruzi* that affects thousands of people. The drugs currently used in therapy are toxic and have therapeutic limitations. In addition, the genetic diversity of *T. cruzi* represents an important variable and challenge in treatment. Sodium diethyldithiocarbamate (DETC) is a compound with pharmacological versatility acting as metal chelators and ROS generation. Thus, the objective is to characterize the antiparasitic action of DETC against different strains and forms of *T. cruzi* and their mechanism. The different strains of *T. cruzi* were grown in LIT medium. To evaluate the antiparasitic activity of DETC, epimastigote and trypomastigote forms of *T. cruzi* were used by resazurin reduction methods and by counting. Different response patterns were obtained between the strains and an IC₅₀ of DETC ranging from 9.44 ± 3.181 µM to 60.49 ± 7.62 µM. Cell cytotoxicity against 3T3 and RAW cell lines and evaluated by MTT, demonstrated that DETC in high concentration (2,222.00 µM) present low toxicity. DETC yet cause mitochondrial damage in *T. cruzi*, as well as disruption in parasite membrane. DETC has antiparasitic activity against different genotypes and forms of *T. cruzi*, therefore, representing a promising molecule as a drug for the treatment of Chagas disease.

Keywords: Chagas disease, *Trypanosoma cruzi*, Sodium diethyldithiocarbamate, Antiparasitic activity, DTU's, Neglected tropical disease, drug discovery.

1. Introduction

Chagas Disease (CD) is a Neglected Tropical Disease (NTD) caused by flagellated protozoan *Trypanosoma cruzi*. This disease affects more than 8 million people worldwide, causing more than 10,000 deaths per year and has more than 80 million people living in risk zone [1]. Chagas disease has an acute asymptomatic phase or nonspecific clinical signs,

and in the chronic phase, individuals may be asymptomatic or progress to cardiac and/ or digestive complications [2]. This disease can be transmitted in different ways and the two main transmission mechanisms are vector transmission, during the blood meal of triatomines, and ingestion of food contaminated with the faeces of these vectors [3]. However, other transmission mechanisms also contribute to Chagas disease, such as blood transfusion, organ transplants, and vertical congenital transmission responsible for the dissemination of disease in other countries through the emigration for non-endemic countries [4].

T. cruzi is a flagellated protozoan that belongs to the trypanosomatid family. This parasite has a complex heteroxenic life cycle that maintains its biological cycle between the invertebrate host of the *Triatominae* family and the mammalian host (humans, dogs, and wild animals) passing by metacyclogenesis during this cycle suffering alteration in different forms [5]. Although *T. cruzi* is represented by a single species, the diversity and genetic variability of this parasite is made up of different strains, grouped based on their genetic characteristics, named Discrete Typing Units (DTUs) [6]. Thus, *T. cruzi* can be classified into seven different genetic variants or genotypes, in which several strains were included, called from TcI to TcVI, and, more recently, the variant called TcBat [6,7]. These genetic variants of *T. cruzi* are important from an epidemiological point of view, as they are involved in different aspects of Chagas disease, mainly in the context of the transmission cycle, clinical manifestations, pathogenesis, reservoir and geographical distribution of these different strains of *T. cruzi* [8].

In the context of pharmacological therapy for Chagas disease, the drugs currently used are benznidazole and nifurtimox [9]. These two nitroaromatic-compounds act on the parasite by producing reactive oxygen species (ROS), thus presenting antiparasitic activity. However, from a clinical point of view, these drugs have limitations regarding their use, since they are responsible for several adverse reactions like liver damage, pruritus, spots on the skin, and itchy in eyes [10]. Additionally, these drugs have low pharmacological efficacy in the chronic phase of infection with *T. cruzi* [11]. This fact has led to the search for new compounds that may be more effective in the treatment of Chagas disease.

Diethyldithiocarbamate (DETC) is a compound that belongs to the class of dithiocarbamates, formed by two ethyl substituents linked to an amine group, which in turn is linked to carbon disulfide. The chemical structure of DETC has two distinct portions; the first, an amine portion formed by nitrogen and the two ethyl groups that stimulate the production of reactive oxygen species. A second portion, a carbon disulfide end that can chelate on metals, thus attributing an essential biological activity of this compound against metalloproteases, enzymes necessary for parasitic biology [12–14]. Due to the structural characteristics of DETC, several pharmacological applications were observed in this compound [15–21]. In addition, we recently published a review that addresses the different justifications for enhancing DETC as a potential antiparasitic drug [15].

However, little is known about the antiparasitic activity of DETC against *T. cruzi*. Thus, the aim of this study is to evaluate the antiparasitic activity of DETC *in vitro* against the different strains (different DTUs) and forms of *T. cruzi*. Thus, in this work we report, for the first time, the antiparasitic activity of DETC against different strains of *T. cruzi*, as well as insights into its mechanisms of action as an antiparasitic drug.

2. Results

2.1. DETC *in vitro* antiparasitic activity towards *T. cruzi*

The parasite's ability to reduce resazurin after exposure to different conditions is shown in Figure 1. In general, this parasite's ability was affected depending on the time (it was most affected after 48 h) and the concentration of DETC. In addition, the effect of DETC also varied according to each strain on different forms; epimastigotes forms were more affected than trypomastigotes forms.

The *T. cruzi* Y strain was the most sensitive to DETC exposure, suffering a significant decrease in the resazurin reduction capacity. QMM9 strain of *T. cruzi*, in both forms, epimastigote and trypomastigote of all strains, was less affected with the ability to reduce resazurin, after 24 hours of exposure at DETC. Among all experimental conditions evaluated with the DM28c strain of *T. cruzi*, the greatest resistance of the parasites was observed when the epimastigote forms of DM28c were exposed to DETC during 24 h of treatment. Interestingly, after 48 hours of exposure to DETC, all strains of *T. cruzi* tested, in both evolutionary forms, showed the ability to reduce resazurin practically to zero.

To confirm the antiparasitic activity of DETC against *T. cruzi*, the viability of parasites treated with DETC was also determined by counting in a light chamber, being considered viable parasites only those in movement, due to the presence of the flagellum. These results are shown in Figure 1S. When comparing the results presented in Figure 1 with the data in Figure 1S, the same profile of DETC antiparasitic activity against *T. cruzi* is observed in both assays.

Figure 1: Evaluation of resazurin reduction inhibition by different strains and forms of *Trypanosoma cruzi* treated with DETC. Parasitic inhibition of resazurin reduction (%) of DETC against different forms (epimastigotes and trypomastigotes) and strains (Y, Dm28c, CL Brener, and QMM9) of *T. cruzi*, after 24 and 48 hours of culture and analysed by resazurin assay. A: Epimastigotes for 24 hours of treatment; B: Trypomastigotes for 24 hours of treatment; C: Epimastigotes for 48 hours of treatment; D: Trypomastigotes for 48 hours of treatment. Results presented as mean $\pm$ standard deviation of the percentage of parasitic inhibition in a triplicate system and for the statistical analysis of the Anova Test, together with the Tukey Post-test ($P < 0.01$ (**); $P < 0.001$ (***), $P < 0.0001$ (****)). In order to verify differences, the profile of each strain was compared against others treated with the same concentration of DETC.

The IC_{50} of DETC was determined using the data from the resazurin assay and the values are shown in Table 1. The IC_{50} of DETC towards each strain of *T. cruzi* was different in the conditions tested, and the trypomastigote profile presented a higher IC_{50} , when compared with epimastigote, except for QMM9. When compared with IC_{50} of benznidazole, tested in the same conditions of DETC, the IC_{50} of benznidazole was always higher for all strains in both forms.

The strain QMM9 was less affected, by exposure to DETC, in the capacity of reducing resazurin since it showed the highest IC_{50} among the tested strains. Meanwhile, with the smallest IC_{50} , the strain Y was the most affected by the exposure to DETC. The strains Dm28c and CL Brener have similar profiles in epimastigotes, and different responses in trypomastigotes. Furthermore, the strains Dm28c, Y and CL Brener have a higher IC_{50} in trypomastigote form when compared with the result in epimastigote form. At the same time, QMM9 had a reduced in IC_{50} of epimastigote form compared with the trypomastigote form.

In addition, the correlation between each IC_{50} result of DETC and benznidazole against all strains in different forms was also determined. Based on the results obtained, an R square > 0.88 to all strains tested was observed through the Pearson Correlation, representing a strong correlation between results of DETC and benznidazole. The effectiveness of the anti-parasitic activity of DETC was also compared with the effectiveness of benznidazole for each strain and evolutionary form evaluated in our study.

Table 1: DETC antiparasitic activity, expressed in IC_{50} values $\pm$ standard deviation, against the different strains and forms of *Trypanosoma cruzi* during 24 hours of cultivation.

<i>Trypanosoma cruzi</i> strains (DTUs)	IC ₅₀ epimastigote		IC ₅₀ trypomastigote	
	DETC (μM)	BZN (μM)	DETC (μM)	BZN (μM)
Strain Dm28c (TcI)	15.94±7.43	69.32±8.42	25.00±5.34	89.95±4.87
Strain Y (TcII)	9.44±3.181	92.66±7.78	23.35±5.41	98.74±3.98
Strain QMM9 (TcIII)	60.49±7.62	99.38±9.41	53.49±8.47	108.71±6.74
Strain CL Brener (TcVI)	15.18±3.64	62.86±8.47	43.18±7.61	82.43±5.83
BZN - benznidazole				

9

0

1 In addition, in order to validate the effectiveness of the DETC antiparasitic activity against *T. cruzi* strains, the para-
2 sites treated with DETC and with 100% resazurin reduction capacity were re-cultivated in a new culture medium, and
3 after 7 days of culture these parasites had no cell viability.

4

5 2.2. Visualization of DETC damage towards *T. cruzi*

6 In order to observe if the exposure to DETC by different strains of *T. cruzi* causes morphological changes in the para-
7 site membrane structure after treatment, the parasites had their membrane structure analysed by scanning electron
8 microscopy (SEM); results are shown in Figure 2. The Figure 2 shows four columns, the first column shows the images
9 1, 5, 9, and 13 corresponding the control of strain Y, Dm28c, QMM9 and CL Brener, respectively. These images show
0 the normal morphology of parasite without treatment with DETC. The others images present in second, third and
1 fourth columns show the parasites treated with DETC. Three images of each strain treated with DETC were used to
2 visualize the damage suffered by *T. cruzi* treated with DETC. Based on the images it was possible to observe differ-
3 ent alterations suffered by the parasite. In the strain Y of *T. cruzi* (images 2, 3, and 4) we observed pores in the mem-
4 brane in large scale. In the strain of *T. cruzi* Dm28c (images 6, 7, and 8) we observed a large damage in the parasite
5 membrane and the presence of pores, but they are fewer than in the strain Y of *T. cruzi*. In the strain QMM9 of *T. cruzi*
6 (images 10, 11, and 12) we observed alterations in the membrane morphology, lacks in the membrane, disruptions and
7 small pores. Lastly, in the strain CL Brener of *T. cruzi* (images 14, 15, and 16) we observed big pores and alterations in
8 the parasite membrane morphology.

9 EDS analysis was used as a quality control for the structures shown in the SEM images (Figure 2S). The carbon struc-
0 ture of the parasites inserted in the correct place on the silicon plate and low salt concentration were observed in the
1 samples used in the SEM analysis. For the images used in the SEM analysis, an EDS analysis was performed to verify
2 and confirm the results obtained.

Figure 2: Morphological changes of *Trypanosoma cruzi* epimastigotes treated with diethyldithiocarbamate and analysed by scanning electron microscopy. Images 1-4 corresponding to the strain Y; images 5-8 corresponding to the strain Dm28c; images 9-12 corresponding to the strain QMM9; and images 13-16 corresponding to the strain CL Brenner of *T. cruzi*. Images 1, 5, 9, and 13 corresponding the controls used in MEV and the others images present the treatment using DETC show the alterations in membrane of parasite. The yellow arrows in the figure indicate the presence of pore in parasite structure.

2.3. DETC cytotoxicity *in vitro* cell culture

The next step was to assess the DETC effect on the ability of mammalian cells to reduce MTT. Therefore, RAW and 3T3 cells were submitted to DETC (from 4.44 to 2222.0 μM) for 24 hours, as described in methods section. As shown in Figure 3, DETC affected the cells' ability to reduce MTT. Low concentrations of DETC affected RAW more than 3T3 cell lines. From the concentration of 11.1 μM to 111.0 μM , the RAW cells was more sensitive, statically observed from concentration 222.0 μM , RAW and 3T3 cell lines had similar profiles of response against DETC. In this case, the ability of both cells to reduce MTT decreased to around 50%, and did not decrease, even with an increase in the concentration (from 1111.0 to 2222.00 μM) of DETC. The IC_{50} of each cell line was determinated based on the decrease of reduce MTT when exposed to DETC. Then, based on the MTT metabolism curve, present in Figure 3, for each cell line, the IC_{50} values were calculated, 859.90 μM and 698.80 μM for cellular lineages, 3T3 and RAW, respectively.

Figure 3: Evaluation of the MTT reduction in 3T3 and RAW cell lines exposed to diethyldithiocarbamate. Viability curves of the 3T3 and RAW cell lines obtained through the MTT reduction assay when treated with different concentrations of DETC for 24 hours. 100 % of MTT reduction represents cells untreated. The curves present in this image were constructed based on mean $\pm$ standard deviation of the percentage of MTT reduction for each cell line (RAW and 3T3). To compare the MTT metabolization of each cell lines against same concentration of DETC treatment, a statistical analysis was performed using T de student Test. The statistical analysis showed difference in metabolization on range concentrations from 11.1 μ M to 44.4 μ M $P < 0.001$ (***)

The selectivity index (SI) was determined based on the data from the resazurin and the MTT assays, as described in the Methods section. Table 2 shows the different SI of DETC obtained with different strains in trypomastigote form of *T. cruzi*. In addition, the different strains associated with each cell line presented different SI. The selectivity of DETC for the strain QMM9 towards the cell lines 3T3 and RAW, presented the smaller values of SI for both forms and cellular lineages. Meanwhile, selectivity for the strain Y towards cell lines presented higher value of SI and cellular lineages. In addition, the SI of DETC for the strains CL Brener and QMM9 presented a similar profile.

5 **Table 2:** Selectivity index (SI) of diethyldithiocarbamate in different strains in trypomastigote form of *Trypanosoma*
6 *cruzi* after 24 hours of cultivation. **Note:** Different forms of *T. cruzi* epimastigote (Epi) and trypomastigotes (Trypo) and cell
7 lines (3T3 and RAW cells).

<i>Trypanosoma cruzi</i> strains (DTUs)	3T3: Trypo	RAW: Trypo
Strain Dm28c (TcI)	34.396	27.952
Strain Y (TcII)	36.827	29.927
Strain Qmm9 (TcIII)	16.076	13.064
Strain CL Brener (TcVI)	19.914	16.183

8

9 **2.4. Mechanisms of cellular death in *T. cruzi* after treatment with DETC**

0 Annexin/PI assay, markers of cellular death, was analyzed towards different forms and strains of *T. cruzi* when treat-
1 ed with DETC. The profile of trypomastigote forms treated with DETC is shown in Figure 4 and the epimastigote
2 form in Figure 3S. Based on these results it is possible to observe the absence of death cellular markers Annexin/PI in
3 all strains of *T. cruzi*, when exposed to different concentrations of DETC (4.44 μ M, 44.40 μ M, 444.00 μ M). These con-
4 centrations were used based on results of resazurin and counting of parasites, seeking to identify the mechanism of
5 death related to the stain Annexin/PI by flow cytometry.

6 Data for strains Y and Dm28c of *T. cruzi* when treated with DETC was similar to those observed with untreated par-
7 asites, that is, more 97% of parasites were considered viable, even when DETC was used at higher concentration
8 (444.00 μ M). However, a low fluorescence response of death cellular markers Annexin/PI was verified to strains CL
9 Brener and QMM9 of *T. cruzi*, when exposed to highest concentration of DETC. 71 % and 80% of viability was identi-
0 fied for both strains after exposure, respectively.

Figure 4: Analysis of the cell death mechanism of trypomastigotes from different *Trypanosoma cruzi* strains treated with DETC. The parasites death cell mechanism was evaluated by flow cytometry as to the capacity for Annexin/PI stain after 24 hours of treatment with different DETC concentrations; 4.44 μ M; 44.44 μ M; and 444.00 μ M.

2.5. Mitochondrial damage in *T. cruzi* after treatment with DETC

A second possible death cell mechanism was evaluated by the mitochondrial activity assay using rhodamine-123. In this case, the parasites were treated with different concentrations of DETC (44.4 μ M; 111.0 μ M; and 222.0 μ M) and subsequently, the fluorescence rhodamine-123 stain was detected by flow cytometry. These concentrations were used to visualize the effect of DETC towards the parasite exposed for 24 hours. The concentration of 444.0 μ M DETC was not used to prevent the death of 100% of the parasites and thus making the rhodamine-123 assay unfeasible.

The fluorescent profile of rhodamine-123 in each strain of *T. cruzi* when exposed to DETC is shown in Figure 5.A. The results shown in figure 5.A were quantified and used to produce Figure 5.B. All *T. cruzi* strains treated with DETC, when compared to the control, show a reduction in the fluorescence profile for rhodamine-123. The increase in DETC concentration affects the fluorescence intensity of the parasite for rhodamine-123 by flow cytometry. The Y and QMM9 strains of *T. cruzi* were the least susceptible to DETC, with a similar profile of reduction in fluorescence emission, and the Dm28c strain was the most susceptible to DETC, resulting in smaller fluorescence when compared with control.

Figure 5: Evaluation of the mechanism of mitochondrial damage caused by DETC in *Trypanosoma cruzi*. A: Fluorescence intensity (rhodamine-123) in different strains in trypomastigote form of *T. cruzi* untreated (control), represents 100% of fluorescence, and treated with different concentrations of DETC (44.4 μ M, 111.0 μ M, 222.0 μ M) for 24 hours. (1) Y strain; (2) Dm28c strain; (3) CL Brener strain; (4) Qmm9 strain. 10,000 events were used for each analysis to obtain the data, which was carried out in triplicate. B: Fluorescence intensity (%) of rhodamine-123 inhibition of different strains of *T. cruzi* treated with different concentrations of DETC. The results were presented based on mean $\pm$ standard deviation of the percentage of parasitic inhibition. The statistical analyses were performed using the Anova Test with the Tukey Post-test ($P < 0.01$ (**); $P < 0.001$ (***)). The statistical analyses were used to compare the profile of each strain treated with the same DETC concentration.

3. Discussion

Our work evaluated the antiparasitic activity of DETC against four different strains of *T. cruzi*, the strain Dm28c (TcI), strain (TcII), strain QMM9 (TcIII) and strain CL Brener (TcVI). When observed the results referred the antiparasitic activity of DETC towards *T. cruzi* showed in Figure 1, Figure 1S and Table 1, we notice the strains present a different profile of response when exposed to DETC. DETC is a dithiocarbamate that present an elevate profile of antiparasitic activity, these results was expected, once, in literature dithiocarbamate was classified as a group compounds with high activity against *T. cruzi*, a study demonstrated the activity of different dithiocarbamate compounds toward *T. cruzi* and explained the mechanism of the group to inhibiting of SOD and others molecules in oxidative pathway of parasite in *in vitro* system [22,23].

Furthermore, the different inhibitory resazurin profile and counting for different strains after treatment with DETC is in agreement with others authors, they notice that different patterns of response should be associated with phylogenetic and evolutionary process that act making the strains resistant or susceptible at compound [24–26].

In addition, was showed different profile of inhibition when occurs the alteration in evolutionary form of *T. cruzi*, in this case epimastigote and trypomastigote forms. The different profile in evolutionary forms towards treatment with DETC are associated with metacyclogenesis process, in this process occurs the alteration in protein expression, RNA and changes in parasite structure, that acting directly or indirectly in strain resistance at compound [27–29].

Worth mentioning that strain QMM9 showed the more resistant profile in DETC treatment and the same was observed when exposed the conventional drug used in Chagas disease treatment benznidazole [30]. However, this strain was recently isolated and characterized and due that don't have studies with mechanisms or causes for this high resistance.

Our results show that DETC has a better antiparasitic activity against *T. cruzi* than benznidazole, when compared *in vitro* and in the experimental conditions established in this study. In other study, it was observed that benznidazole presents an $IC_{50} < 10\mu M$ against Y strain of *T. cruzi* [31]. However, here we used a higher concentration of parasites for the tests of antiparasitic assay (107 parasites/mL), while in the other study the antiparasitic activity is determined in a lower concentration of parasites (106 parasites/mL). In addition, we analysed the antiparasitic activity of DETC and benznidazole in shorter exposure times (24 and 48 hours), while many other authors use benznidazole treatment in 72 hours [31,32].

To evaluate the damage cause by DETC in parasite structure, was utilized the SEM analysis after treatment with DETC for 24 hours. Was observed that DETC cause severe damage in parasite structure and acting in pore formation (Figure 2). In literature, DETC was characterized as inhibitor of antioxidative enzymes, occasioning the accumulation of reactive species inside cell or parasite. The excessive presence of reactive species causes a lot of damage and leading a pore formation. In others works that using compound able to trigger the same increase of reactive species, were observed the pore formation in SEM analysis and damage in membrane [33,34].

With regard to the DETC cytotoxic profile, in this study we observed the viability of the 3T3 and RAW cell lines treated with DETC for 24 hours (Figure 3). Only in high concentrations (1111.0 μM and 2222.0 μM), DETC was able to significantly reduce the viability of the 3T3 and RAW cell lines, but not less than 40% of cell viability. Even at high concentrations, DETC was not toxic enough to cause 100% mortality of cell lines. However, even in low concentrations, DETC has 100% antiparasitic activity. With these data it was also possible to determine the selectivity index (SI) of DETC of different strains and forms of *T. cruzi* (Table 2). Based on the SI, DETC showed less toxicity for fibroblast cell line when compared to benznidazole and derivatives [35]. In addition, DETC has less toxicity than benznidazole when evaluated in RAW cell line [36]. These results corroborated the evaluation of *in vitro* efficacy of DETC compared with conventional drug used in treatment of Chagas disease.

In This work, we making an approach showing DETC as potent inhibitor of *T. cruzi* activity. In others jobs utilizing DETC against parasites of Trypanosomatidae family [20,21], DETC showed an important Fe-SOD inhibitor resulting in accumulation of O₂ and cause the death of parasite. *In vitro* studies showed either that DETC can inhibit Fe-SOD of *T. cruzi* in *in vitro* essay [22,23]. Due that, when analyzed the stain of Annexin/PI in figure 4 and figure 3S, was observed the absence or low staining for both evolutionary form and for different strains of parasite. This result was expected, because was noticed that SOD inhibitors can blocked apoptosis pathway and denied the cascade signalization to expose phosphatidylserine in membrane, due that the stain was not detected [37,38]. Furthermore, studies with dithiocarbamates showed the capacity of compound in inhibition of apoptosis pathway and induce non-specific necrosis [39].

The structure of DETC may act producing reactive species and causing inhibition of antioxidative enzymes. Inside cells and parasites, the local more common associated with reactive species production and neutralization is the mitochondria. Because of this, in our work we evaluate if DETC could attack and damaged the mitochondria. When observe the results present in Figure 1 and Figure 5, both resazurin and rhodamine-123 are metabolized inside mitochondria. It is known that the metabolism of these two compounds by the parasite is directly related to the functioning of complexes I, II and III of the inner membrane of the mitochondria [40]. The reduction phenomenon of the resazurin and rhodamine-123 compounds may be related to the damage of the mitochondrial inner membrane of *T. cruzi* due to the action of DETC. Consequently, causing a malfunction in the regulation of the protonation gradient carried out by the mitochondrial complexes I, II and III, and impairing ATP synthesis, and consequently causing the death of the parasites [40–43]. Interestingly, it can be seen in Figure 5 that the highest concentration of DETC (222.0 μM) is able to reduce the metabolism of rhodamine-123 in all strains of *T. cruzi* analysed in this study, probably associated with the reduction of the membrane potential mitochondrial, potentiating the mitochondria as a possible target for the anti-*T. cruzi* activity presented by DETC.

T. cruzi treated with DETC shows damage and pore formation in the cytoplasmic membrane. This phenomenon can also be a consequence of mitochondrial damage induced by DETC. Some studies report the capacity of mitochondrial

damage caused by the production of reactive species, a possible explanation for the antiparasitic mechanism presented by the DETC [44–46].

In conclusion, in this work we demonstrated the antiparasitic activity of DETC against different forms (epimastigotes and trypomastigotes) and four different strains of *T. cruzi*, the etiological agent of Chagas disease. When we compared the antiparasitic activity of DETC with benznidazole, the first-line drug for the treatment of Chagas disease, DETC showed better antiparasitic activity *in vitro*. Finally, new assays and validations are necessary in order to enhance the use of DETC as a promising candidate in the treatment of Chagas disease.

4. Methods

4.1. Axenic cultivation of *Trypanosoma cruzi*

Different strains of *T. cruzi* TcI (strain Dm28c) [47]; TcII (strain Y) [48]; TcIII (QMM9 strain) [30] and TcVI (CL Blener strain) [49] were grown in LIT (Liver Infusion Tryptose) medium supplemented with 10% inactivated fetal bovine serum (FBS) (v/v) and 5% of antibiotic streptomycin/penicillin (100 UI/mL). All strains were donated by *Laboratório de Parasitologia* (FCFAr), Araraquara, Brazil. The epimastigote forms of *T. cruzi* were kept at 27 °C in a BOD oven (incubator chamber, ASP, SP-500). The trypomastigote forms of *T. cruzi* were obtained through the nutritional stress method, from epimastigote forms of the parasite for a period of 25 days, according to the methodology developed by Camargo et al., 1964 [50]. Briefly, 20 mL of cell culture were kept in 50 mL flasks. The flask was kept closed and the parasites were subsequently analysed for cell viability and differentiation morphological changes. The morphological changes of the parasites were analysed by optical microscopy and a parasitic concentration greater than 75% of the trypomastigote forms, was considered.

4.2. Cells

RAW 264.7 macrophages (ATCC number TIB-71) and 3T3 fibroblast (ATCC CRL-1658) cell lines were cultured in DMEM (Cultilab, Campinas, SP, Brazil) supplemented with 10% (v/v) fetal serum bovine (FBS) (Cultilab, Campinas, SP, Brazil) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). The cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. For maintenance of the cells, the culture medium was changed every three days, and the cells were further subcultured at 80% confluence using a cell scraper (RAW cells) or trypsin/EDTA (3T3 cells).

4.3. Determination of DETC antiparasitic activity against different strains and forms of *Trypanosoma cruzi*

The different strains and forms of *T. cruzi*, mentioned above, were used in this study. The parasites were diluted in a concentration of 1.0×10^7 parasites/mL and then 200 µL kept in 96-well plates together with different concentrations of sodium diethyldithiocarbamate (DETC) solved in LIT medium, ranged from 4.44 to 444.00 µM. The antiparasitic activity of DETC was determined through the viability of the parasites after a treatment period of 24 and 48 hours. Benznidazole was used as a positive control of antiparasitic activity against *T. cruzi*, in different concentrations that ranged from 3.84 to 500.00 µM. This range was selected due to an existing variability response against the different strains of *T. cruzi* and to determinate the IC₅₀ of benznidazole to compare with DETC.

Parasite viability was determined by the resazurin (Sigma Aldrich, Laramie, WY, USA) reduction assay at a concentration of 1 mM and subsequently measured by spectrophotometer (Epoch, BioTek Instruments, Winooski, VT, USA), at 570 nm and 600 nm wavelengths [51]. The antiparasitic activity of DETC was expressed as % of resazurin reduction $= 100 - [(A_{test570} - (A_{test600} \times Ro)) / (A_{control570} - (A_{control600} \times Ro))] \times 100$, in which A_{test} corresponds to the absorbance of the experimental group, and A_{control} corresponds to the absorbance of the negative control, 570 and 600 are the wavelengths that correspond to 570 nm and 600 nm, Ro represents the index of correction between the medium and resazurin, that allows analyse if DETC interfere in the resazurin activity used during the test. It was observed that DETC does not interfere in the resazurin activity. Based on the percentage of reduction in resazurin, the IC₅₀ of the compounds were calculated, representing the concentration of the drug needed to reduce the viability of a parasitic population by 50%.

The viability of the parasites was also determined by microscopy in a clear camera through direct counting in a Neubauer camera. In this assay, parasites with some movement were considered viable. Cell viability results were compared between the two methodologies. In some conditions, when the DETC concentration produced 100% of par-

asite mortality, they were removed from the culture medium and later centrifuged (2,000 rpm, 10 min, 4 ° C) to remove the drug. Afterwards, the parasites received a new-LIT medium, supplemented with 10% (v/v) FBS and 5% of antibiotic streptomycin/penicillin (100 UI/mL). After 7 days, the culture was analysed by conventional optical microscopy. The absence of live parasites confirms 100% mortality.

4.4. Evaluation of the membrane structure alteration in *Trypanosoma cruzi* submitted to DETC by scanning electron microscopy

To evaluate possible morphological changes in the membrane of different strains of *T. cruzi* caused by DETC, the parasite membrane structure was analysed by scanning electron microscopy after treatment with DETC, according to a protocol established by Amorim-Carmo et al., 2019 [52]. Briefly, the epimastigote forms of the different strains of *T. cruzi* (1.0×10^7 parasites/mL), in the same cultivation conditions described previously in topic 2.2, were treated with DETC (44.40 μ M and 222.00 μ M), also in identical conditions described previously. After 24 hours, the parasites were centrifuged for 10 min at 2000 rpm (4 °C) and washed twice in saline solution (0.9% NaCl) pH 7.4. The parasites were then fixed in solution of 2.5% glutaraldehyde in PBS (v/v) for 4 hours. After this step, the parasites were dehydrated being exposed to different ethanol concentrations. First, to dehydrated the parasite, ethanol at 25% was added and left to stand for 10 minutes, followed by washes in saline solution (0.9% NaCl) pH 7.4 for 10 minutes at 1500 rpm room temperature. The same operation was repeated for 50%, 80%, and 100% ethanol concentrations to dehydrate the parasite for next steps. Then, the parasites were resuspended in absolute ethanol, discarded on silicon plates and placed to dry at room temperature. Finally, after dried, the parasites were placed on stubs and subjected to metallization with gold using the sputtering (Bal-Tec SCD-005 Sputter Coater, Schalksmühle, NWR, GER) in an argon atmosphere for 30 seconds with a current of 30 mA, and then analysed by a scanning electron microscopy under a FEG microscope (Model augira, Brand Carl Zeiss, Oberkochen, WB, GER). In addition, in order to validate the results in the SEM, an X-ray spectroscopy for energy dispersion (SED) was performed. This technique allows the visualisation of the elements present in the samples, the structure and components of parasites, and allows identifying the presence or absence of residues and impurities.

4.5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) reduction assay on cell viability submitted to DETC

The ability of RAW 264.7 macrophages and fibroblast 3T3 to reduce MTT was evaluated according to the previously described method of Mosmann [53]. Initially, the cells were seeded in 96-well plates at a density of 5×10^3 /well and kept at culture condition for 12 hours. Then, the culture medium was replaced by another medium containing DETC in the different concentrations (from 4.444 to 2.222 μ M). After 24 h, the culture medium was replaced with 100 μ L of MTT (1 mg/mL dissolved in DMEM). Then, the cells were incubated for 4 h at 37 °C. Subsequently, the culture supernatant was discarded, and the crystals of formazan were solubilized in ethanol, 100 μ L/well. Absorbance was measured with an Epoch microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA) at 570 nm. Cell viability was calculated in relation to the negative control using the formula: % viability = (A_{test}/A_{control}) $\times$ 100, in which A_{test} corresponds to the absorbance of the experimental group, and A_{control} corresponds to the absorbance of the negative control. Based on % MTT reduction, IC₅₀, that represents the drug concentration necessary to reduce by fifty per cent the cell population, was calculate. In order to analyse a possible chemical incompatibility between DETC and MTT, wells were analysed containing only the DMEM, DETC and MTT culture medium. It was observed that DETC does not interact chemically with MTT.

4.6. Determination of Selective Index of DETC

To assess the predilection of DETC between the parasite and cell, the Selective Index (SI) was used. This Index represents the preference of drug calculated by: $SI = IC_{50\text{parasite}}/IC_{50\text{cell}}$; in which $IC_{50\text{parasite}}$ represents the drug concentration to reduce by fifty per cent the parasite population in *vitro* and $IC_{50\text{cell}}$ represents the drug concentration to reduce by fifty per cent the *in vitro* cell population.

4.7. Evaluation of death mechanism in *Trypanosoma cruzi* caused by DETC

Annexin V/propidium iodide (PI) was used to evaluate the DETC death mechanism at *T. cruzi*. Briefly, the epimastigotes and trypomastigotes forms of the different strains of *T. cruzi*, in the same culture conditions described previously in topic 2.2, were exposed to DETC (4.44 μ M, 44.40 μ M or 444.00 μ M) for 24 hours. Then, the parasites were

centrifuged (2000 rpm for 5 minutes, 4 °C), rinsed with PBS 1x pH 7.4 (Phosphate-Saline Buffer), and suspended in 300 µL bond buffer 1x. Then, 5 µL of Annexin V-FITC (fluorescein isothiocyanate) was added to the system, let reacting for 10 minutes. Then, the parasites were centrifuged (2000 rpm for 5 minutes, 4 °C) and resuspended again in 200 µL Bind Buffer 1x and 10 µL of PI. All these steps are described in the protocol proposed by the manufacturer (Annexin V FITC Apoptosis Detection Kit, Invitrogen, Carlsbad, CA, USA). After 10 minutes, the fluorescence intensity was determined using a flow cytometer (FACSCanto II, BD Biosciences, Eugene, OR, USA) with FACSDiva software, version 6.1.2 (Becton Dickson, Franklin Lakes, NJ, USA). For the analysis, we counted 10,000 events for each strain and evolutionary form of *T. cruzi*. A solution of 100% DMSO was used as positive control using the same protocol of Annexin/PI stain of DETC. The solution was added in the wells and the parasite was exposed for 24 hours. Conditions were the same used in topic 2.2.

4.8. Evaluation of mitochondrial damage in *Trypanosoma cruzi* by DETC

In order to analyse the mitochondrial damage induced in different strains and forms of *T. cruzi* by DETC, the mitochondrial potential marker, rhodamine-123 (Invitrogen, Carlsbad, CA, USA) was used, following the manufacturer's recommendations. Briefly, the different strains and forms of *T. cruzi* were treated with different concentrations of DETC (44.40 µM; 111.00 µM; or 222.00 µM) for 24 hours in same conditions described previously. After treatment, the parasites were centrifuged at 2000 rpm for 6 min at 4 °C and rinsed with PBS pH 7.4. Then, the parasites were suspended and 200 µL of PBS and 0.5 µL of rhodamine-123 (5 mg/mL) were added, as described by Sulsen et al., 2016 [54]. After 30 minutes, the parasites were rinsed twice in PBS and analysed by flow cytometry at wavelengths of 488 and 633 nm. Finally, the fluorescence intensity was determined by flow cytometer (FACSCanto II, BD Biosciences, Eugene, OR, USA) with FACSDiva software, version 6.1.2 (Becton Dickson, Franklin Lakes, NJ, USA). The parasites mitochondrial activity was calculated according to the formula: % of reduction rhodamine-123 stain = (fluorescence intensity of the parasites treated with DETC)/(fluorescence intensity of negative control) * 100. A total of 10,000 events were analysed for each strain and form of *T. cruzi*.

4.9. Statistical analysis of the data

All experiments were carried out in triplicate and independently. The results are presented in the form of arithmetic mean ± standard deviation. Data were submitted to the Shapiro-Wilk normality test. Parametric data were analysed using the ANOVA test associated with the Tukey-T post-test for more of 2 groups and t of student for 2 groups and Pearson correlation. Tests were performed using the software GraphPad Prism v. 7.0 (2016) and P.A.S.T v. 2.17 (2012).

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Supplementary information:

Figure 1S – Inhibition in different strains of *T. cruzi* after treatment with DETC with range concentration 4.44 μM until 444.0 μM analysed by count. A) Epimastigote 24 hours, B) Trypomastigote 24 hours, C) Epimastigote 48 hours and D) Trypomastigote 48 hours. Results presented as mean $\pm$ standard deviation of the percentage of parasitic inhibition in a triplicate system and for the statistical analysis the Anova Test together with the Tukey Post-test ($P < 0.01$ (**); $P < 0.001$ (***); $P < 0.0001$ (****)). In statistical analysis was compared the response in different strains submitted to same concentration of DETC.

Figure 2S: Spectroscopy of energy dispersion (SED) of different MEV images after treatment with DETC to prove the mains chemical elements that compose the image. A) Image of parasite strain Y after treatment with DETC corresponding the images 3 in figure 2, respectively ; B) and C) images to refer strain Dm28c after treatment with DETC corresponding the images 7 and 8 in figure 2, respectively; D) and E) images to refer strain QMM9 after treatment with DETC corresponding the images 10 and 11 in figure 2, respectively; F) and G) images to refer strain CL Brener after treatment with DETC corresponding the images 16 and 15 in figure 2, respectively.

Figure 3S: Analysis of the cell death mechanism of epimastigote from different *Trypanosoma cruzi* strains treated with DETC. The parasites death cell mechanism was evaluated by flow cytometer as to the capacity for Annexin/Pi stain after 24 hours of treatment with different DETC concentrations; 4.44 μ M; 44.44 μ M; and 444.00 μ M.

Figure Legends

Figure 1: Evaluation of resazurin reduction inhibition by different strains and forms of *Trypanosoma cruzi* treated with DETC. Parasitic inhibition of resazurin reduction (%) of DETC against different forms (epimastigotes and trypomastigotes) and strains (Y, Dm28c, CL Brener, and QMM9) of *T. cruzi*, after 24 and 48 hours of culture and analysed by resazurin assay. A: Epimastigotes for 24 hours of treatment; B: Trypomastigotes for 24 hours of treatment; C: Epimastigotes for 48 hours of treatment; D: Trypomastigotes for 48 hours of treatment. Results presented as mean $\pm$ standard deviation of the percentage of parasitic inhibition in a triplicate system and for the statistical analysis of the Anova Test, together with the Tukey Post-test ($P < 0.01$ (**); $P < 0.001$ (***), $P < 0.0001$ (****)). In order to verify differences, the profile of each strain was compared against others treated with the same concentration of DETC.

Figure 2: Morphological changes of *Trypanosoma cruzi* epimastigotes treated with diethyldithiocarbamate and analysed by scanning electron microscopy. Images 1-4 corresponding to the strain Y; images 5-8 corresponding to the strain Dm28c; images 9-12 corresponding to the strain QMM9; and images 13-16 corresponding to the strain CL Brenner of *T. cruzi*. Images 1, 5, 9, and 13 corresponding the controls used in MEV and the others images present the treatment using DETC show the alterations in membrane of parasite. The yellow arrows in the figure indicate the presence of pore in parasite structure.

Figure 3: Evaluation of the MTT reduction in 3T3 and RAW cell lines exposed to diethyldithiocarbamate. Viability curves of the 3T3 and RAW cell lines obtained through the MTT reduction assay when treated with different concentrations of DETC for 24 hours. 100 % of MTT reduction represents cells untreated. The curves present in this image were constructed based on mean $\pm$ standard deviation of the percentage of MTT reduction for each cell line (RAW and 3T3). To compare the MTT metabolization of each cell lines against same concentration of DETC treatment, a statisti-

cal analysis was performed using T de student Test. The statistical analysis showed difference in metabolization on range concentrations from 11.1 μ M to 44.4 μ M P <0.001 (**).

Figure 4: Analysis of the cell death mechanism of trypomastigotes from different *Trypanosoma cruzi* strains treated with DETC. The parasites death cell mechanism was evaluated by flow cytometry as to the capacity for Annexin/PI stain after 24 hours of treatment with different DETC concentrations; 4.44 μ M; 44.44 μ M; and 444.00 μ M.

Figure 5: Evaluation of the mechanism of mitochondrial damage caused by DETC in *Trypanosoma cruzi*. A: Fluorescence intensity (rhodamine-123) in different strains in trypomastigote form of *T. cruzi* untreated (control), represents 100% of fluorescence, and treated with different concentrations of DETC (44.4 μ M, 111.0 μ M, 222.0 μ M) for 24 hours. (1) Y strain; (2) Dm28c strain; (3) CL Brener strain; (4) Qmm9 strain. 10,000 events were used for each analysis to obtain the data, which was carried out in triplicate. B: Fluorescence intensity (%) of rhodamine-123 inhibition of different strains of *T. cruzi* treated with different concentrations of DETC. The results were presented based on mean $\pm$ standard deviation of the percentage of parasitic inhibition. The statistical analyses were performed using the Anova Test with the Tukey Post-test (P <0.01 (**); P <0.001 (***)). The statistical analyses were used to compare the profile of each strain treated with the same DETC concentration.

Tables

Table 1: DETC antiparasitic activity, expressed in IC₅₀ values $\pm$ standard deviation, against the different strains and forms of *Trypanosoma cruzi* during 24 hours of cultivation.

<i>Trypanosoma cruzi</i> strains (DTUs)	IC ₅₀ epimastigote		IC ₅₀ trypomastigote	
	DETC (μ M)	BZN (μ M)	DETC (μ M)	BZN (μ M)
Strain Dm28c (TcI)	15.94 $\pm$ 7.43	69.32 $\pm$ 8.42	25.00 $\pm$ 5.34	89.95 $\pm$ 4.87
Strain Y (TcII)	9.44 $\pm$ 3.181	92.66 $\pm$ 7.78	23.35 $\pm$ 5.41	98.74 $\pm$ 3.98
Strain QMM9 (TcIII)	60.49 $\pm$ 7.62	99.38 $\pm$ 9.41	53.49 $\pm$ 8.47	108.71 $\pm$ 6.74
Strain CL Brener (TcVI)	15.18 $\pm$ 3.64	62.86 $\pm$ 8.47	43.18 $\pm$ 7.61	82.43 $\pm$ 5.83

BZN - benznidazole

Table 2: Selectivity index (SI) of diethyldithiocarbamate in different strains in trypomastigote form of *Trypanosoma cruzi* after 24 hours of cultivation**Note:** Different forms of *T. cruzi* epimastigote (Epi) and trypomastigotes (Trypo) and cell lines (3T3 and RAW cells).

<i>Trypanosoma cruzi</i> strains (DTUs)	3T3: Trypo	RAW: Trypo
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Strain Dm28c (TcI)	34.396	27.952
Strain Y (TcII)	36.827	29.927
Strain Qmm9 (TcIII)	16.076	13.064
Strain CL Brener (TcVI)	19.914	16.183

Figures

Figure 1

Evaluation of resazurin reduction inhibition by different strains and forms of *Trypanosoma cruzi* treated with DETC. Parasitic inhibition of resazurin reduction (%) of DETC against different forms (epimastigotes and try-pomastigotes) and strains (Y, Dm28c, CL Brener, and QMM9) of *T. cruzi*, after 24 and 48 hours of culture and analysed by resazurin assay. A: Epimastigotes for 24 hours of treatment; B: Trypomastigotes for 24 hours of treatment; C: Epimastigotes for 48 hours of treatment; D: Trypomastigotes for 48 hours of treatment. Results presented as mean $\pm$ standard deviation of the percentage of parasitic inhibition in a triplicate system and for the statistical analysis of the Anova Test, together with the Tukey Post-test ($P < 0.01$ (**); $P < 0.001$ (***), $P < 0.0001$ (****)). In order to verify differences, the profile of each strain was compared against others treated with the same concentration of DETC.

Figure 2

Morphological changes of *Trypanosoma cruzi* epimastigotes treated with diethyldithiocarbamate and analysed by scanning electron microscopy. Images 1-4 corresponding to the strain Y; images 5-8 corresponding to the strain Dm28c; images 9-12 corresponding to the strain QMM9; and images 13-16 corresponding to the strain CL Brener of *T. cruzi*. Images 1, 5, 9, and 13 corresponding the controls used in MEV and the others images present the treatment using DETC show the alterations in membrane of parasite. The yellow arrows in the figure indicate the presence of pore in parasite structure.

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