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Parasitology

Antiparasitic effect of (−)-α-bisabolol against *Trypanosoma cruzi* Y strain forms

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ABSTRACT

Chagas disease is caused by *Trypanosoma cruzi* and affects about 7 million people worldwide. Benznidazole and nifurtimox have low efficacy and high toxicity. The present study was designed to identify the trypanocidal effect of (−)-α-Bisabolol (BIS) and investigate its mechanism. Epimastigotes and trypomastigotes were cultured with BIS and the viable cells were counted. BIS antiamastigote effect was evaluated using infected LLC-MK2 cells. MTT assay was performed to evaluate BIS cytotoxicity. Growth recovery was assessed to evaluate BIS effect after short times of exposure. BIS mechanism was investigated through flow cytometry, with 7-AAD and Annexin V-PE. DCFH-DA, rhodamine 123 (Rho123) and acridine orange (AO). Finally, enzymatic and computational assays were performed to identify BIS interaction with *T. cruzi* GAPDH (*tc*GAPDH). BIS showed an inhibitory effect on epimastigotes after all tested periods, as well on trypomastigotes. It caused cytotoxicity on LLC-MK2 cells at higher concentrations, with selectivity index (SeI) = 26.5. After treatment, infected cells showed a decrease in infected cells, the number of amastigotes per infected cell and the survival index (SuI). Growth recovery demonstrated that BIS effect causes rapid death of *T. cruzi*. Flow cytometry showed that BIS biological effect is associated with apoptosis induction, increase in cytoplasmic ROS and mitochondrial transmembrane potential, while reservosome swelling was observed at a late stage. Also, BIS action mechanism may be associated to *tc*GAPDH inhibition. Altogether, the results demonstrate that BIS causes cell death in *Trypanosoma cruzi* Y strain forms, with the involvement of apoptosis and oxidative stress and enzymatic inhibition.

1. Introduction

Chagas disease (CD) is considered by the World Health Organization as one of the 13 most neglected diseases, with approximately 6 to 7 million infected people worldwide (WHO, 2015). Although CD was initially restricted to Latin America, people's migration has caused its incidence to increase in non-endemic regions, such as the USA, Canada and Europe (Afonso et al., 2012).

CD is caused by *Trypanosoma cruzi*, a hemoflagellate protozoan of the *Kinetoplastida* order (Costa et al., 2011). It has a heteroxenous life cycle, with a phase inside the invertebrate triatomine hosts and another phase in the mammalian species (Afonso et al., 2012). Humans are accidental hosts, and in CD transmission, the invertebrate hosts play the role of vectors.

CD has two distinct clinical phases, based on symptoms and complications. Acute CD is characterized by symptoms such as fever, asthenia and headache, but it can also be asymptomatic (WHO, 2015). In the acute phase, trypomastigotes can be found in peripheral blood. Untreated patients will progress to the chronic phase a few weeks after become infected, which shows no detectable parasitemia and can remain asymptomatic for several years or develop cardiac and/or digestive complications, such as myocardial hypertrophy, fibrosis, dysrhythmia and megacolon. This phase is characterized by the accumulation of intracellular amastigote forms (Costa et al., 2011).

Abbreviations: 7-AAD, 7-aminoactinomycin D; AO, acridine orange; Ax/PE, phycoerythrin-conjugated annexin V; BIS, (−)-α-bisabolol; CC_{50}, drug concentration able to inhibit the growth of 50% of LLC-MK2 cells; CD, Chagas disease; DCFH-DA, 2′,7′-Dichlorofluorescin diacetate; DMEM, Dulbecco's Modified Eagle Medium; FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; IC_{50}, drug concentration able to inhibit the growth of 50% of epimastigotes; LC_{50}, drug concentration able to kill 50% of trypomastigotes; LIT, liver infusion tryptose medium; Rho 123, rhodamine 123; SeI, selectivity index; SEM, standard error mean; SuI, survival index; $\Delta\psi m$, mitochondrial transmembrane potential; *tc*GAPDH, glyceraldehyde-3-phosphate dehydrogenase from *Trypanosoma cruzi*.

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The pharmacological treatment of CD includes two drugs, benznidazole and nifurtimox, which have been used for many years and have several limitations. The most important problems associated to these drugs are their low efficacy in chronic CD and their side effects, which include dermatitis, polyneuritis, bone marrow depression, anorexia, nausea, vomiting and lymphoma (WHO, 2015). Therefore, one of the strategies to improve CD outcomes is the research of new trypanocidal drugs with differential bioactivities.

(−)-α-Bisabolol (BIS) ($C_{15}H_{26}O$) is a monocyclic sesquiterpene alcohol found in some plants, mainly in *Matricaria chamomilla* flowers. It is the main component of chamomile essential oil and has several bioactivities, such as antimicrobial, leishmanicidal, anti-inflammatory, cytotoxic, apoptotic, gastroprotective and anti-nociceptive actions (Bonifacio et al., 2012; Leite et al., 2011; Rocha et al., 2011; Uno et al., 2016).

Recently, *T. cruzi* glyceraldehyde-3-phosphate dehydrogenase (*tc*GAPDH) has been considered a valuable target for the development of new trypanocidal drugs (Harris et al., 2014). Since this enzyme is involved in glucose degradation and ATP synthesis, its inhibition causes cell death through ROS accumulation (Maugeri et al., 2011). Thus, the aim of this study was to investigate the antiparasitic effect of (−)-α-bisabolol on *Trypanosoma cruzi* forms and evaluate its trypanocidal mechanism.

2. Materials and methods

2.1. Drugs and chemicals

(−)-α-Bisabolol (BIS) was purchased from Sigma-Aldrich (St. Louis, USA) (purity >95%). Benznidazole (BZN) (purity 99.93%) was kindly donated by LAFEPE (Laboratório Farmacêutico de Pernambuco). BIS and BZN were separately diluted in 200 mM in sterile DMSO. Serial dilutions were performed in sterile PBS to produce working solutions for both drugs. All dilutions were performed to guarantee that maximum DMSO concentration was 0.5% in experimental groups.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), annexin V-PE, 7-AAD (7-aminoactinomycin D), DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate), rhodamine 123 (Rho123) and FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) were purchased from BD Biosciences (BD Biosciences, New Jersey, USA).

2.2. Trypanocidal evaluation

2.2.1. Assessment of cytotoxic effect on epimastigote forms

T. cruzi epimastigote Y strain forms were kindly donated by the Biochemistry Laboratory from São Paulo University (USP). The parasites were cultured at 28 °C in Liver Infusion Tryptose (LIT) medium supplemented with antibiotics and 10% fetal bovine serum (FBS) until a logarithmic grow phase. Then, 10^6 parasites/mL were cultured with BIS (1000–31.25 μM) in sterile 96-well plates. This concentration range was selected based on previous experiments performed by our group (Sampaio et al., 2016), and subtoxic concentrations (125–3.9 μM)on host cells were selected for *T. cruzi* treatment. Cell density was determined by counting cells in a Neubauer chamber after 24, 48, 72 and 96 hours, as previously described (Menezes et al., 2012). Only parasites with mobility and typical format preserved were considered viable. BZN was tested in the same concentrations as reference drug. The data were analyzed by non-linear regression to determine the concentration able to inhibit parasite growth by 50% (IC_{50}).

2.2.2. Cytotoxic assessment on trypomastigote and amastigote forms

Trypomastigote forms of *T. cruzi* were obtained from the supernatant of infected LLC-MK2 cells, as described elsewhere (Lima et al., 2016). The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium), supplemented with glutamine and 10% FBS. When confluent, the cells were dislocated with trypsin/EDTA and plated on 25 cm^2 sterile flasks at 2×10^5 cells/mL. After 24 hours, the media was replaced by DMEM with 2% FBS and 10^7 trypomastigotes were added. The medium was replaced every two days, and after 5–6 days, the medium was collected and centrifuged. Trypomastigote forms were counted to infect new cells or for experimental protocols.

BIS effect on trypomastigotes was evaluated by adding the drug (125–3.9 μM) to 10^6 trypomastigotes/mL. After 24 hours, cell density was determined by counting cells. LC_{50} (concentration able to kill 50% of trypomastigotes) was estimated as explained above.

To assess BIS effect on *T. cruzi* intracellular amastigotes, 10^5 LLC-MK2 cells/mL were cultured on the surface of 1.8 cm^2 culture chamber slides (Sigma-Aldrich, St Louis, USA) and, 24 hours later, 5×10^6 trypomastigotes/mL were added. After 48 hours, the medium was removed, and the cells were treated with BIS (10, 20 and 40 μM). BIS antiamastigote effect was evaluated after 24 hours of incubation. Untreated infected cells were used as control. The slides were fixed with Bouin's solution and stained with Giemsa. The number of infected cells, the number of amastigotes per infected cell and the survival index (SuI), calculated as the number of infected cells × amastigote/infected cells (Marín et al., 2015) were evaluated in each group. For trypomastigote and amastigote experiments, BZN was used as reference drug.

2.3. Cytotoxicity assessment

BIS cytotoxic effect on host cells was investigated by MTT reduction assay in LLC-MK2 cells, cultured in 10% FBS DMEM. Briefly, 10^5 cells/mL were cultured in sterile plates overnight at 37 °C and 5% CO_2, and then incubated with BIS for 24 hours. The experimental groups (control and BIS-treated epimastigotes) were then incubated with 10 μL of MTT (2.5 mg/mL) and, after 4 hours of incubation, 90 μL of 10% SDS solution was added to solubilize the formazan salt produced. The plates were incubated for 17 hours to complete solubilization and finally the absorbance was read at 570 nm. Absorbance values were used to determine cell viability and estimate CC_{50} (concentrations of drug required to reduce cell viability by 50%). The selectivity of BIS on trypomastigotes in comparison to LLC-MK2 cells was verified by assessing the Selectivity Index (SeI), calculated as CC_{50}/LC_{50} (Kessler et al., 2013). For reference purpose, BZN was also tested.

2.4. Recovery experiments

10^6 epimastigote/mL were exposed to BIS at high concentrations (1000 and 500 μM) for short periods of time (15, 30, 60, and 120 min). Then, the cells were washed twice with PBS and cultured in fresh, drug-free LIT medium. Growth recovery was assessed daily for 4 consecutive days, by cell counting (Kessler et al., 2013).

2.5. Flow cytometry experiments

2.5.1. Annexin V-PE and 7-AAD labeling

Cell death pathway was evaluated by flow cytometry, using 7-AAD (7-Aminoactinomycin D) and Annexin V as necrosis and apoptosis markers, respectively. 7-AAD enters cells with damaged membranes and binds to DNA, while Annexin V binds to phosphatidylserine in apoptotic cells (Krysko et al., 2008).

Briefly, epimastigotes untreated and treated with BIS (285 μM, IC_{50} estimated by counting, as described on section *2.2*) for 6 and 24 hours were washed twice in binding buffer and labeled with 7-AAD and Annexin V-PE for 15 min in the dark, as recommended by the manufacturer. Finally, the cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences, New Jersey, USA) using FL_2 and FL_3 channels. The percentages of labeled and unlabeled cell populations were analyzed to determine the cell death mechanisms involved in BIS trypanocidal effect.

2.5.2. Cytoplasmic ROS measurement

The increase in cytoplasmic reactive oxygen species (ROS) induced by (−)-α-bisabolol on *T. cruzi* was determined by flow cytometry,

using the fluorescent probe DCFH-DA (2′,7′-Dichlorofluorescin diacetate). The cells were labeled with DCFH-DA (20 μM) 3 hours after addition of BIS (285 μM) and the plates were incubated, while protected from light, for 15 and 30 minutes; 1, 2, 6 and 24 hours. These time points were selected aiming to compare with the recovery experiments. FL_1 labeling level was evaluated by the fold-change (ratio of treated/untreated cells) of the geometric mean ($[(X_1).(X_2)\ldots(X_n)]^{1/n}$, where X is the fluorescence intensity of each event and *n* is the number of events) (Kessler et al., 2013). FCCP (39 μM) was used as positive control.

2.5.3. ΔΨm measurement

The effect of BIS on mitochondrial transmembrane potential was assessed in epimastigotes using rhodamine 123 (Rho 123). Briefly, epimastigotes treated with BIS (285 μM) were washed with PBS and labeled with Rho 123 (10 μg/mL) for 30 minutes in the dark. As positive control, it was used FCCP (39 μM). The cells were washed again and analyzed by flow cytometry in an FL_1 channel. The decrease in Rho 123 accumulation in treated groups was analyzed by the fold-change in fluorescence emission.

2.5.4. Acridine orange staining

To evaluate reservosome swelling in *T. cruzi*, epimastigotes were treated with BIS (285 μM) for different periods, washed twice and labeled with AO (5 μg/mL) for 30 minutes at the dark. After washing, the cells were analyzed for the measurement of fold-change in fluorescence (Atale et al., 2014).

2.6. Molecular docking

The geometric optimization of the bisabolol structure (ChemSpider ID390796) was performed using semiempirical Hamiltonian PM7 in MOPAC2012 program (http://openmopac.net). The crystallographic structure of the *tc*GAPDH-chalepin complex was obtained from the RCSB Protein Data Bank (PDB code: 1K3T) elucidated by Pavão et al. (Pavão et al., 2002). To identify potential binding sites, molecular docking was carried out using automated docking in AutoDocking Vina program (Molecular Graphics Lab) (Trott and Olson, 2010). The ligand used was the optimized bisabolol, and the target was the *tc*GAPDH enzyme after the removal of the chalepin of the *tc*GAPDH–chalepin complex. The best bisabolol pose was selected based on the best-ranked conformation of the ligand in the enzyme and used for the construction of the *tc*GAPDH-bisabolol complex. The *tc*GAPDH-chalepin complex was used to compare the position of the ligand in the binding site. Molecular graphics were performed using the UCSF Chimera 1.8 package (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco) (Pettersen et al., 2004).

2.7. Enzymatic assay

After incubation with BIS (285 μM) for different periods of time (15 min, 30 min, 1 h and 2 h), epimastigotes were lysed to obtain BIS-treated tcGAPDH. Then, the samples were analyzed using a commercial kit (Abcam®, Cambridge, UK) to quantify GAPDH activity.

2.8. Statistical analysis

All experiments were performed in triplicate ($n = 3$) in 3 independent experiments. The data are presented as mean ± SEM (standard error mean) and the comparison between groups was assessed by ANOVA with Dunnett's post-test, using $P < 0.05$ as significance criteria. All graphs and analyses were generated using Microsoft Excel 2010 and GraphPad Prism version 5.0.

3. Results

The present study was designed to study the trypanocidal effect of (−)-α-bisabolol, as well as to investigate its mechanism of action on *T. cruzi*. Epimastigotes were cultured in the presence of BIS for 24, 48, 72 and 96 hours. A time- and concentration-dependent effect was observed, as shown in Fig. 1A. After 24 hours of treatment, BIS showed

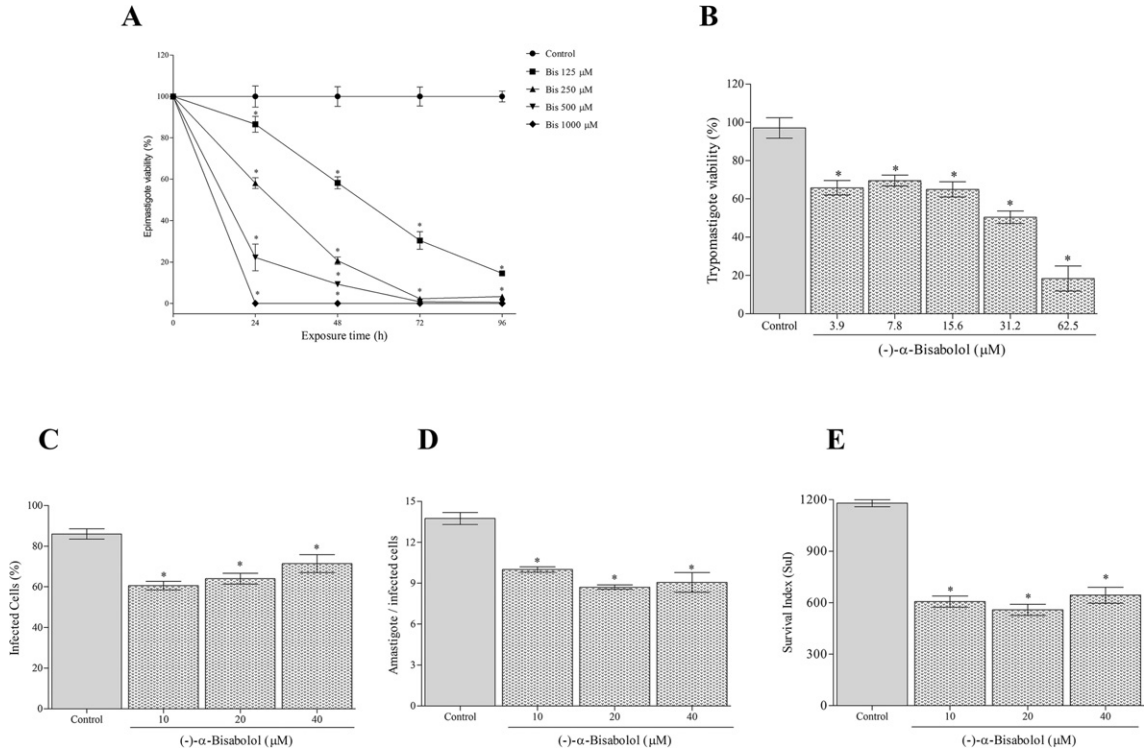

Fig. 1. Trypanocidal effect of (−)-α-bisabolol. [A] Effect of (−)-α-bisabolol (BIS) on epimastigote forms after 24, 48, 72 and 96 hours of incubation; [B] Effect of BIS on trypomastigote forms of *T. cruzi*; [C–E] Antiamastigote effect of BIS, demonstrated as [C] Percentage of infected cells, [D] number of amastigotes per infected cell and [E] Survival Index (SuI). The data are presented as mean ± SEM of three independent experiments performed in triplicate. *$P < 0.05$ vs. control group.

Table 1
IC_{50}, LC_{50}, CC_{50} and selectivity index for (−)-α-bisabolol and benznidazole.

	Exposure time			
	24 h	48 h	72 h	96 h
(−)-α-Bisabolol				
IC_{50} (Epimastigote)	285 ± 20 μM	145 ± 8.6 μM	97.3 ± 3.25 μM	59.8 ± 4.5 μM
LC_{50} (Trypomastigote)	20 ± 3.2 μM	-	-	-
CC_{50} (LLC-MK2)	528 ± 34 μM	-	-	-
SeI	26.5	-	-	-
Benzonidazol				
IC_{50} (Epimastigote)	218 ± 55 μM	61 ± 10 μM	16.5 ± 3 μM	12.7 ± 2 μM
LC_{50} (Trypomastigote)	256 ± 77 μM	-	-	-
CC_{50} (LLC-MK2)	616 ± 51 μM	-	-	-
SeI	2.4	-	-	-

an inhibitory effect at 1000, 500 and 250 μM, with IC_{50}/24 h = 285 ± 20 μM. This effect is enhanced throughout incubation time, with IC_{50}/48 h = 145 ± 8.6 μM, IC_{50}/72 h = 97.3 ± 3.25 μM and IC_{50}/96 h = 59.8 ± 4.5 μM.

BIS was also able to kill trypomastigotes, as shown in Fig. 1B. BIS reduced the number of viable trypomastigotes at all tested concentrations. For these cells, an LC_{50}/24 h = 20 ± 3.2 μM was obtained. Benznidazole also showed effect on epimastigotes and trypomastigotes, but with less potency, as demonstrated by CC_{50} and LC_{50} values (Table 1).

The antiamastigote effect was assessed with BIS at 10, 20 and 40 μM. These concentrations were selected because of their with trypanocidal activity on trypomastigotes, but no cytotoxicity on LLC-MK2 cells. As shown in Fig. 1C, D and E, BIS was able to reduce the percentage of infected cells, the number of amastigotes per infected cells and the survival index. Altogether, these results demonstrate a potential effect of BIS on the intracellular forms of *T. cruzi*. Benznidazole caused similar effect, but in much higher concentrations (Table 2).

To evaluate the selectivity of BIS on *T. cruzi* in comparison with host cells, LLC-MK2 cells were treated and the cell viability was analyzed by MTT reduction assay (Fig. 2). It was observed that BIS causes cytotoxicity only at high concentrations (1000 and 500 μM), with CC_{50}/24 h = 529 ± 34.8 μM. With this result, it was possible to calculate a SeI = 26.5. BZN was also tested, showing SeI = 2.4. This data demonstrates that, in comparison to BZN, BIS presents high selectivity to *T. cruzi*.

Growth recovery experiments were performed to verify the effect of BIS after short periods of time. Epimastigotes were incubated with high concentrations of BIS (1000 and 500 μM) for 15, 30, 60 and 120 minutes and then transferred to fresh drug-free medium. It was observed that both concentrations were able to inhibit parasite growth, even when incubated for a few minutes. The cells treated with 1000 μM showed partial inhibition after 15 minutes and 100% of cell death after 30, 60 and 120 minutes. The group treated with 500 μM showed a partial inhibitory effect at these 4 time points. These results are shown in Fig. 3.

The flow cytometry analyses were performed to investigate cell death mechanisms involved with the BIS effect, using epimastigote forms (Fig. 4). The experiments using 7-AAD and Annexin V-PE showed that epimastigotes treated with BIS (6 and 24 hours) showed an increase on Annexin labeling, as demonstrated by the presence of a cell population on down right quadrant. These findings indicate phosphatidylserine externalization, at both exposure times and suggest that BIS has an apoptotic effect on *T. cruzi* epimastigotes.

To investigate the oxidative stress induced by (−)-α-bisabolol, treated and untreated cells were submitted to DCF and rhodamine 123-labeling. DCF is a fluorescent probe able to identify the induction of cytoplasmic ROS. The results demonstrated that BIS caused enhancement in DCF fluorescence after 2, 6 and 24 hours (Fig. 5), showing cytoplasmic oxidative stress induced by bisabolol.

Rho123 analysis showed a reduction in fluorescence after 6 and 24 hours of treatment (Fig. 6). This result indicates that parasites treated with BIS have mitochondrial transmembrane potential dysfunction, which can help to explain cell death. AO was also used to investigate reservosome swelling. It was observed that BIS caused an increase in AO labeling only after 24 hours of incubation (Fig. 7).

We used molecular docking analysis to suggest a bisabolol mechanism of action on the parasite through the study of its interaction with the *tc*GAPDH enzyme. The best-ranked bisabolol pose in the docking simulation is shown in Fig. 8a, together with the chalepin pose, a known inhibitor of *tc*GAPDH (obtained from crystallographic data of *tc*GAPDH –chalepin complex) for comparison (Pavão et al., 2002). It can be observed that the docking pose of bisabolol is similar to that occupied by the chalepin molecule in the *tc*GAPDH active site. The region of nearest neighbors (< 3.0 Å) between bisabolol and the amino acid residues of the binding site is shown in Fig. 8b. In this neighborhood, the residues involved are: ILE13, SER165, CYS166, THR167, HIS194, ASN335.

Table 2
Antiamastigote effect of (−)-α-bisabolol and Benznidazole.

	Infected cells (%)	Amastigote/infected cell	Survival index (SuI)
Control	86 ± 2.52	13.7 ± 0.44	1179 ± 21
(−)-α-Bisabolol			
10 μM	61 ± 2.1*	10 ± 0.19*	606 ± 32*
20 μM	64 ± 2.7*	8.7 ± 0.16*	558 ± 33*
40 μM	71 ± 4.4*	9.1 ± 0.72*	644 ± 46*
Benznidazole			
257 μM	64.4 ± 3.6*	9.4 ± 0.39*	603 ± 67*
514 μM	48.8 ± 5.5	7.0 ± 0.8*	371 ± 27*

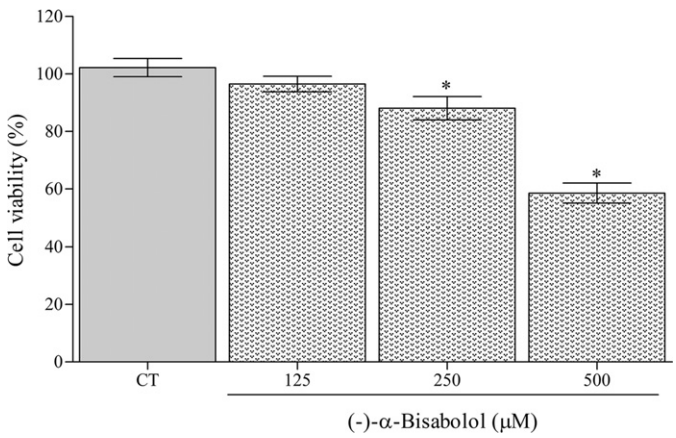

Fig. 2. Cytotoxic effect of BIS on LLC-MK2 cells. The cells were incubated with BIS for 24 hours and submitted to MTT reduction assay to verify cell viability. The data are presented as mean ± SEM of three independent experiments performed in triplicate. *$P < 0.05$ vs. control group.

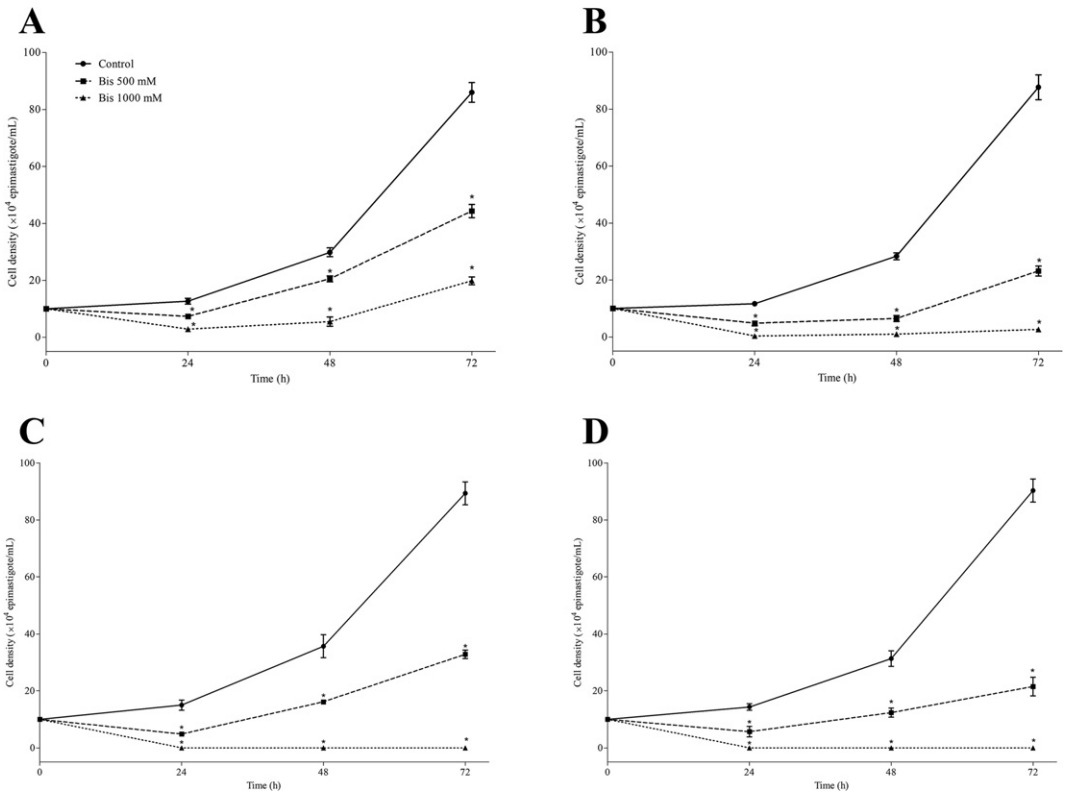

Fig. 3. Growth recovery experiments. Epimastigotes were cultured with BIS at 1000 and 500 μM for [A] 15 minutes, [B] 30 minutes, [C] 60 minutes and [D] 120 minutes and counted for 4 days. The data are presented as mean ± SEM of three independent experiments performed in triplicate. *$P < 0.05$ vs. control group.

To confirm this result, tcGAPDH was obtained from treated epimastigotes and submitted to enzymatic activity assay. As a result, BIS inhibited *tc*GAPDH activity after 1 and 2 hours of incubation (Fig. 9). This data corroborates molecular docking simulations.

4. Discussion

Recently, (−)-α-bisabolol has been explored by some researchers as a possible new leishmanicidal drug, showing good results in both in vitro and in vivo models (Corpas-López et al., 2015, 2016; Morales-Yuste et al., 2010; Rottini et al., 2015). Also, its inhibitory effect on *Trypanosoma evansi* has been demonstrated (Baldissera et al., 2016). These findings led our research group to investigate the effects of this sesquiterpene alcohol on *T. cruzi* forms, since *Trypanosoma* sp. and *Leishmania* sp. have several similar features regarding their biology, cell cycle and morphology (Wheeler et al., 2016).

In this study, we investigated the potential effect of BIS on *T. cruzi* three forms, as well the mechanisms involved in this trypanocidal effect. We performed the assays using *T. cruzi* Y strain, of which main characteristic is a partial resistance do benznidazole (Cherkesova et al., 2014). The present study was performed using in vitro methods, without the use of animals. This strategy is highly recommended due to ethical questions, which have been aiming to diminish the number of animals needed for the development of new drugs and therapeutic systems (Unciti-Broceta et al., 2015).

The initial screening of BIS trypanocidal effect was performed using epimastigote forms, which are found in insect vectors (Porto-Carreiro et al., 2000). Although it is not an infective form, it is widely used to study the trypanocidal effect of new drugs, because of its morphological and biochemical similarities to trypomastigotes (Zingales et al., 1997). Moreover, it is easily cultured, without the necessity of cell infection or coculture with other cell lineages. Additionally, this cell lineage is easily applied to the investigation of trypanocidal mechanisms of substances.

It was observed that BIS was able to inhibit parasite growth after 24, 48, 72 and 96 hours. Moreover, IC_{50} values decreased throughout the time period, which demonstrates an increasing biological effect. These results have been found for other sesquiterpenes, such as artesunate (Olivera et al., 2015), dehydroleucodine, helenalin (Jimenez et al., 2014) and others (Saúde-guimarães and Raslan, 2014).

The confirmation of BIS inhibitory effect on epimastigote forms is useful to delineate the experiments with the other *T. cruzi* forms, defining the starting points and concentrations to be tested. Thus, LLC-MK2 cells were cultured and infected to obtain the trypomastigote forms used in this study. These cells are well established and are widely used to induce trypomastigote infection (Lima et al., 2016), due to their adherence to plates and coverslips, high infection percentage and internalization rate (Costarelli et al., 2010).

Trypomastigotes were tested against (−)-α-bisabolol for 24 hours, and the trypanocidal effect was observed for all tested concentrations, up to 3.9 μM. LC_{50} value, demonstrating that BIS has a higher effect on trypomastigote than on epimastigote forms. It has been previously reported that BIS has an antiparasitic effect on promastigotes of *Leishmania infantum* (Morales-Yuste et al., 2010), *L. tropica* (Corpas-López et al., 2016), *L. amazonensis* (Rottini et al., 2015) and *L. donovani* (Corpas-López et al., 2015, 2016), with similar LC_{50} values. These results are important, since trypomastigotes are the infective forms and are mainly present in the acute phase of CD (Pays, 2016).

Aiming to identify BIS selectivity over *T. cruzi*, LLC-MK2 cells were incubated with BIS for 24 hours and submitted to MTT reduction assay. This method is very useful, as it detects the metabolic capacity of cells exposed to bioactive substances (Costa Torres et al., 2010) and it is easy to perform. It was observed that BIS caused cytotoxicity in this cell lineage only at 1000 and 500 μM concentrations.

The authors suggest that drugs with a SeI > 10 are considered appropriate for further evaluation (Kessler et al., 2013). The selectivity index (SeI) obtained here, 26.5, shows that this substance has suitable effectivity and toxicity to become a new trypanocidal drug or prototype.

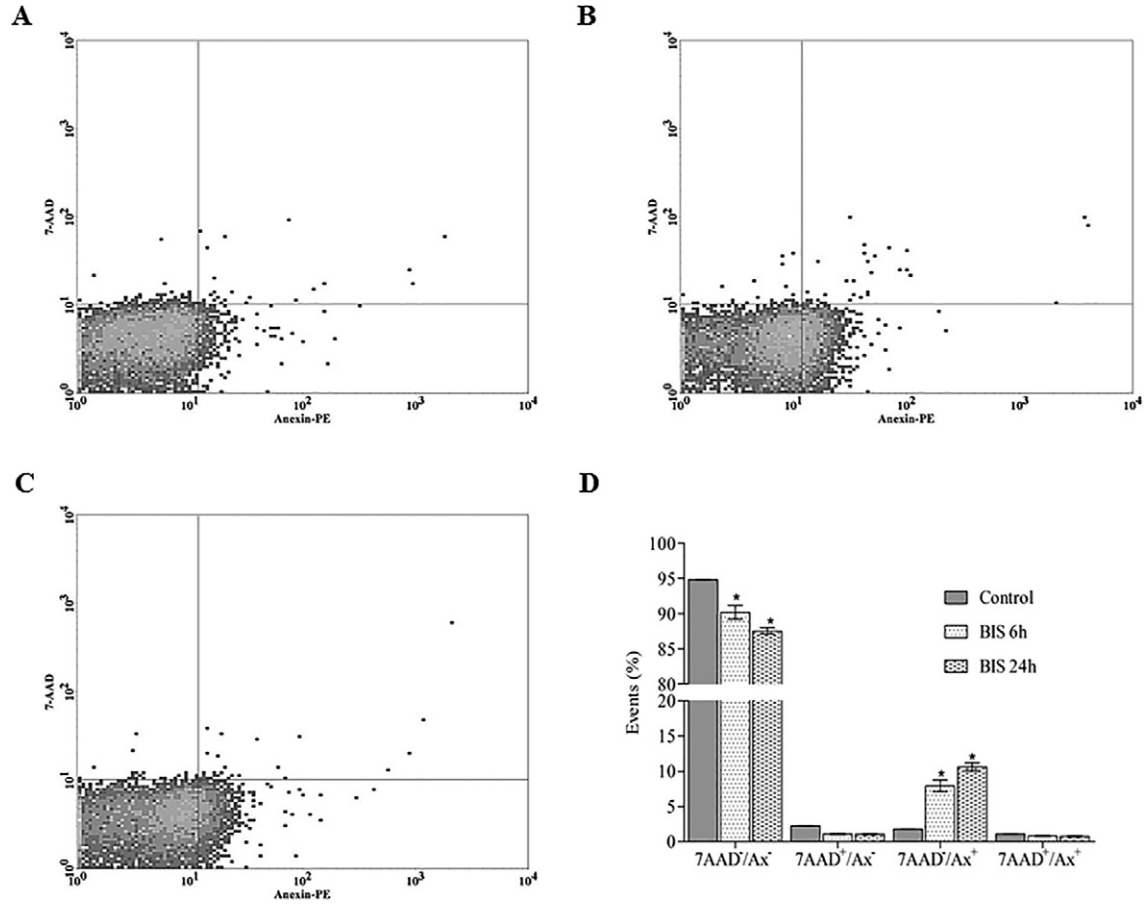

Fig. 4. 7-AAD and Annexin V-PE labeling assays. [A – C] Density plot representations of [A] Control, [B] treatment with BIS for 6 hours and [C] for 24 hours. Down left quadrant = viable cells; upper left quadrant = $7AAD^+$ population (necrosis); down right quadrant = Ax^+ population (early apoptosis); and upper right quadrant = $7AAD^+/Ax^+$ population (late apoptosis). [D] Percentage of labeled and unlabeled cells in the experiment. The data are presented as mean ± SEM of three independent experiments performed in triplicate. $^*P < 0.05$.

The antiamastigote effect was verified by a previously reported method (Massarico Serafim et al., 2014). This assay is intended to select new bioactive substances as options for the treatment of chronic CD, avoiding the excessive use of animal models. Infectivity levels can be mostly assessed by observing the decrease in the number of infected cells and the average number of amastigotes present in the infected cells.

BIS was able to reduce the number of infected cells and the number of amastigotes in cells at all tested concentrations, demonstrating that BIS was able to impair *T. cruzi* intracellular proliferation without host

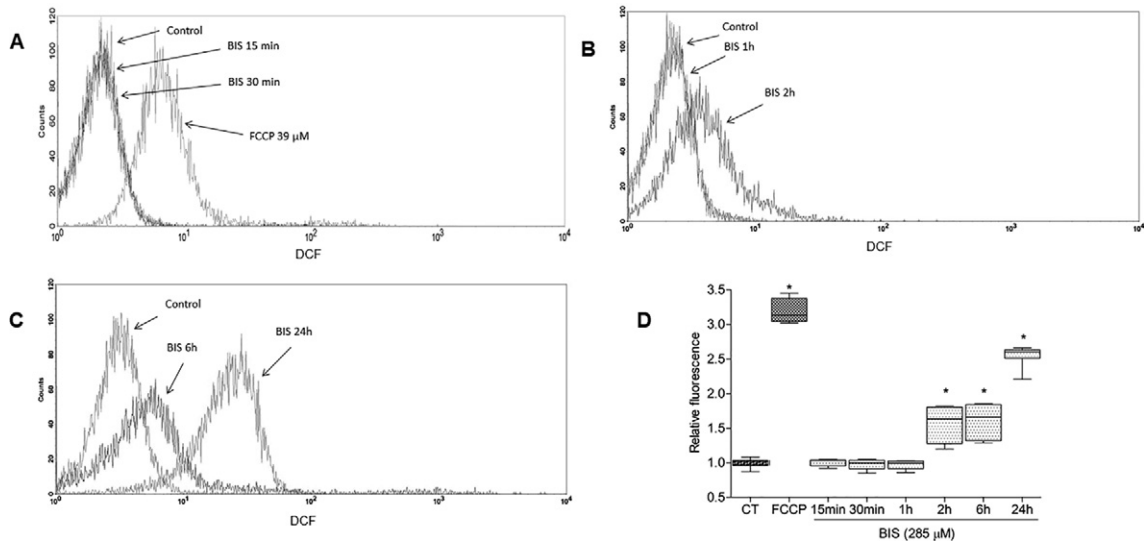

Fig. 5. Flow cytometry analyses for cytoplasmic ROS measurement. [A, B and C] Overlay flow cytometry histogram of BIS-treated cultures, after 15 and 30 minutes; 2, 6 and 24 hours of incubation, for DCF labeling; [D] Fold-change in geometric means on DCF. The data are presented as mean ± SEM of three independent experiments performed in triplicate. FCCP (39 µM) = positive control. The data are presented as mean ± SEM of three independent experiments performed in triplicate. $^*P < 0.05$.

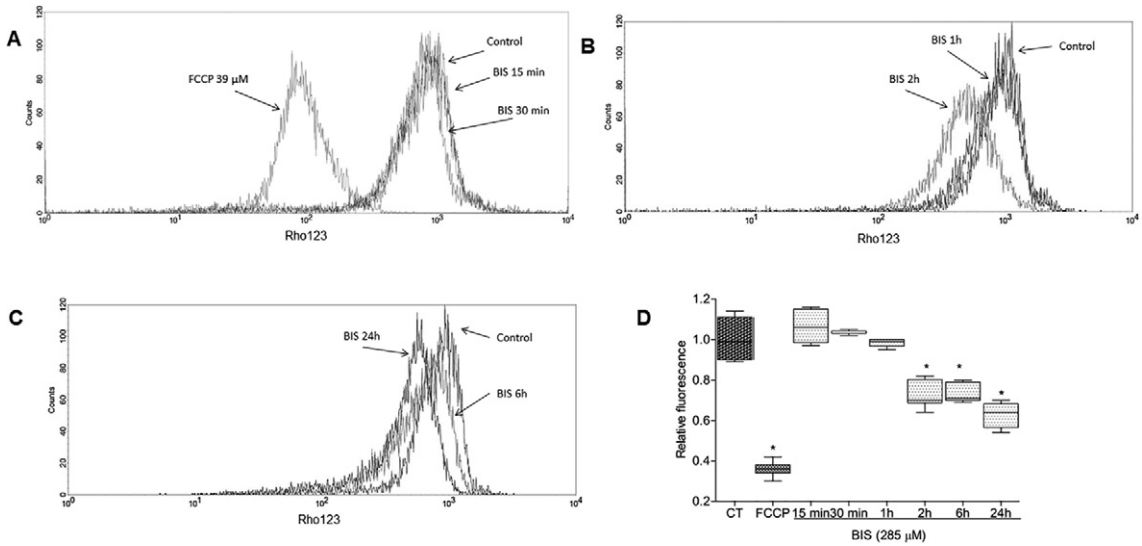

Fig. 6. Flow cytometry analyses for $\Delta\psi m$. [A, B and C] Overlay flow cytometry histogram of BIS-treated cultures, after 15 and 30 minutes; 2, 6 and 24 hours of incubation, for Rho 123 labeling; [D] Fold-change in geometric means on Rho 123. FCCP (39 μM) = positive control. The data are presented as mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.05$.

cell damage. Also, BIS antiamastigote effect was expressed by a reduction in the Survival Index (SuI), which has been used to compare trypanocidal and leishmanicidal drugs, by assessing the ability of a substance to remove a parasite from a host cell (Dutra et al., 2005).

The time of drug exposure to which biological targets are submitted is considered important to guarantee the expected biological effects. In this study, we performed a growth recovery assay, aiming to identify BIS ability to quickly induce cell death mechanisms. It was verified that, after a few minutes of incubation, BIS triggered epimastigote death, which continued even in the absence of BIS in the culture media. These results suggest that BIS is quickly internalized by epimastigotes and/or is able to initiate irreversible cell death pathways in *T. cruzi*. These data are supported by recent studies, which verified that BIS enters leukemic cells via lipid rafts and disrupts mitochondrial function (Cavalieri et al., 2011).

Necrotic and apoptotic pathways were evaluated using 7-AAD and annexin V-PE (Kumar et al., 2015). After 6 and 24 hours of incubation, BIS caused annexin V-PE labeling, which indicates phosphatidylserine (PS) externalization. This molecule is a phospholipid present in the inner cell membrane of viable cells. When apoptotic induction starts, PS is externalized to the outer cell membrane, aiming to signalize this cell for further phagocytosis (Atale et al., 2014).

In agreement with our results, we verified that BIS has been screened as an apoptotic substance in several experimental models. BIS is able to induce apoptosis in HEPG2 cells (5–10 μM) through extrinsic and intrinsic pathways (Chen et al., 2010). Primary leukemia cells were also tested against BIS (6.37 μM), showing mitochondrial damage and apoptosis (da Silva et al., 2010). BIS causes DNA ladder formation, mitochondrial disruption and poly(ADP-ribose) polymerase (PARP) cleavage in glioma cells (4.5 μM) (Cavalieri et al., 2004), also characteristic of apoptosis. In parasites, BIS apoptotic effect was observed in *Leishmania major* and *L. tropica* (with IC_{50} values of 25.2 and 33.7 μM, respectively) (Corpas-López et al., 2016), through mitochondrial impairment and oxidative stress.

Some authors have shown that oxidative stress and changes on mitochondrial structure and metabolism are commonly associated to BIS effect. Thus, flow cytometry analyses were performed to verify BIS ability to change these parameters in *T. cruzi*. DCFH-DA is an

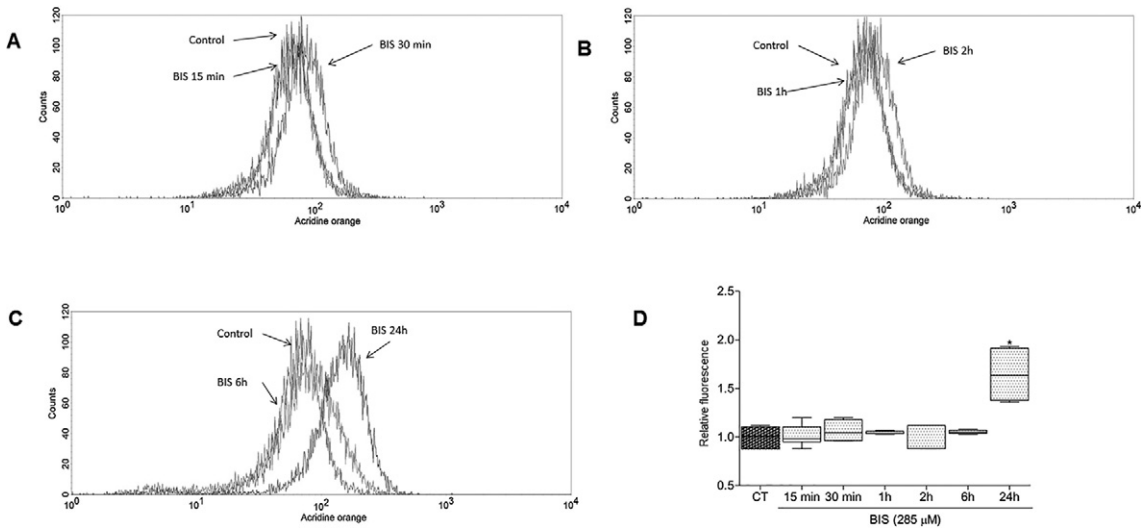

Fig. 7. Flow cytometry analyses for acridine orange staining. [A, B and C] Overlay flow cytometry histogram of BIS-treated cultures, after 15 and 30 minutes; 2, 6 and 24 hours of incubation, for AO labeling; [D] Fold-change in geometric means on AO. The data are presented as mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.05$.

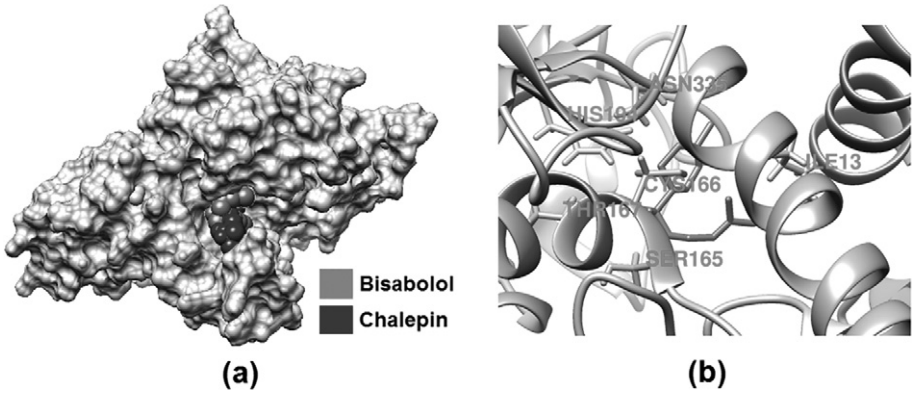

Fig. 8. Molecular docking of bisabolol in the TcGAPDH enzyme. [A] The highest score pose of the bisabolol in the TcGAPDH active site. The chalepin pose obtained from crystallographic data of TcGAPDH–chalepin complex is also shown for comparison. [B] The nearest amino acids residues (with contact distance <3.0 Å) of bisabolol with highest score in the binding site of TcGAPDH enzyme.

unspecific marker of cytoplasmic ROS, which is used in many cell-based assays.

T. cruzi has to deal with several redox environments during its life cycle, so this parasite has some defense mechanisms that are vital to its survival, including distinct enzymatic ROS detoxification pathways (Irigoín et al., 2008), mediated by trypanothione (Irigoín et al., 2008), peroxiredoxins and superoxide dismutase (Piacenza et al., 2012). In our experiments, an increase in DCF labeling was observed after 6 and 24 hours of incubation with BIS, indicative of cytoplasmic oxidative stress.

BIS caused mitochondrial depolarization after 6 and 24 hours, verified by a decrease in Rho123 accumulation. This organelle is related to apoptosis induction by intrinsic pathways, with mitochondrial outer membrane permeabilization, cytochrome c release and consequent apoptosis signaling (Yamada and Yoshida, 2019). Thus, we suggest that bisabolol induces apoptosis in *T. cruzi*. Our hypothesis is supported by other authors, which have shown that BIS causes apoptosis with mitochondrial involvement in different cell lineages (Basurco et al., 1995; Bonifacio et al., 2012; Cavalieri et al., 2011; Darra et al., 2008; Rigo and Vinante, 2016; Seki et al., 2011). For instance, BIS caused mitochondrial damage with pore formation in the outer membrane in Jurkat cells at 4.5 and 6.7 μM after 96 hours. Also, Corpas-López et al. (2016) have previously shown that BIS is able to induce mitochondrial depolarization on *Leishmania tropica* promastigotes at 25 and 100 μM.

Acridine Orange (AO) is an useful marker for reservosome labeling, since it is capable of emitting specific fluorescence within acidic compartment. In our experiments, it was observed AO labeling only after 24 h of incubation with BIS. This result suggests that, after this period, it is observed progressive damage on other cell organelles as consequence of BIS effect.

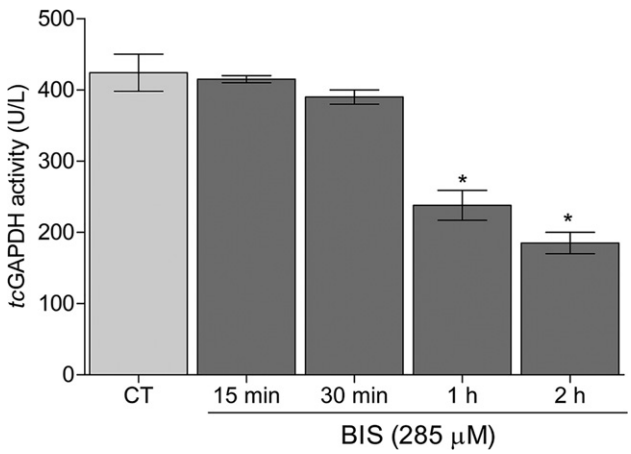

Fig. 9. Inhibitory effect of BIS on *tc*GAPDH. The data are presented as mean ± SEM of three independent experiments performed in triplicate. *$P < 0.05$ vs. control group.

In this work, it is demonstrated for the first time a possible BIS interaction with *tc*GAPDH. This enzyme is a homotetramer (approximately 150 kDa). Each subunit contain 2 folding domains, consisting of a substrate binding domain and a cofactor binding domain (Maluf et al., 2019). Although *tc*GAPDH catalytic mechanism is not completely elucidated, some authors have been investigating this enzyme as a new target for Chagas disease treatment. There is been reported for different authors that CYS166 residue is key to ensure its activity, since it is the first amino acid residue to interact with the substrate (Castilho et al., 2003; Menezes et al., 2003; Reis et al., 2013). Also, new *tc*GAPDH inhibitors have been studied, showing promising results (Maluf et al., 2019; Silva et al., 2010).

Our molecular docking simulations showed a possible interaction between BIS and tcGAPDH. SER165 and CYS166, amino acid residues interacting with BIS, which participate in the catalytic mechanism of *tc*GAPDH oxidoreductase activity (Seidler, 2013). Thus, the bisabolol pose with the highest score obtained from the molecular docking with *tc*GAPDH suggests the possibility of inhibition of this enzyme.

Since molecular docking is a computational method, it is not able to confirm a real interaction between a drug and an enzymatic target. Therefore, an enzymatic assay was performed, demonstrating that BIS can act as a partial *tc*GAPDH inhibitor, with approximately 50% of enzyme inhibition. This could be a mechanism for the biological action of the drug on the parasite.

In conclusion, the present study demonstrated, for the first time, the trypanocidal effect of (−)-α-bisabolol on the three main *Trypanosoma cruzi* forms. Also, the results suggest that bisabolol induces cell death by apoptosis, with cytoplasmic and mitochondrial oxidative stress. The data shown in this study provides useful information for further biological investigations, aiming at the development of new drugs and/or strategic therapeutic approaches.

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Conflict of interest

None to declare.

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