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Antiproliferative Effect and Ultrastructural Alterations Induced by 5-*O*-methylembelin on *Trypanosoma cruzi.*

Renata María Spina Zapata[a], Esteban Lozano[b], Patricia Andrea Barrera[a], María Belén Agüero[c,d], Alejandro Tapia[c], Gabriela Egly Feresin[c,d,1] , Miguel Ángel Sosa[a,1,*]

[a]*Facultad de Ciencias Médicas, Instituto de Histología y Embriología "Dr. Mario H. Burgos", Universidad Nacional de Cuyo-CONICET, CC 56 (5500), Mendoza, Argentina.*

[b]*Laboratorio de Inmunología y Desarrollo de Vacunas, Instituto de Medicina y Biología Experimental de Cuyo, Av. Ruiz Leal s/n Parque General San Martín, CP 5500, Mendoza, Argentina.*

[c]*Instituto de Biotecnología-Instituto de Ciencias Básicas, Universidad Nacional de San Juan, Av. Libertador General San Martín 1109 (O), CP5400, San Juan, Argentina.*

[d]*CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina*

[1]The work was co-directed by both authors

[*]*Corresponding author*

Laboratorio de Biología y Fisiología Celular, "Dr. Francisco Bertini", Instituto de Histología y Embriología, Facultad de Ciencias Médicas, Universidad de Cuyo, Mendoza, Argentina. Tel.: +54 261 4135000; fax: +54 261 4494117.
E-mail address: msosa@fcm.uncu.edu.ar (M.A. Sosa)

ABSTRACT

Background: Embelin (EMB), obtained from *Oxalis erythrorhiza* Gillies ex Hooker et Arnott (Oxalidaceae), was reported against *Trypanosoma cruzi* and *Leishmania spp.* Additionally, antiprotozoan activity against *Plasmodium falciparum* was reported for its methylated derivative (ME). *Purpose*: To evaluate the potential anti-*Trypanosoma cruzi* activity of EMB, ME and 2,5-di-*O*-methylembelin (DME) and analyze the possible mechanism of action. *Study design/Methods:* EMB was isolated by a chromatographic method from the air-dried ground whole plant. To evaluate the effects of methylation, ME and DME were synthesized and tested against *T. cruzi* epimastigotes and trypomastigotes. The most active compound ME was evaluated against amastigotes. Ultrastructural alterations, ROS generation and the effect on mitochondrial activity of ME were measured. *Results:* Compounds inhibited the proliferation of epimastigotes. ME was also active against intracellular amastigotes. Mitochondrial alterations were observed by TEM. Additionally, ME modified the mitochondrial activity, and induced an increase in ROS levels. These evidences postulate the mitochondrion as a possible target of ME. *Conclusion:* ME inhibited amastigotes proliferation, thus being a potential lead compound for the treatment of Chagas' disease.

Keywords: Embelin (EMB), 5-*O*-methylembelin (ME), 2,5-di-*O*-methylembelin (DME), *Trypanosoma cruzi*, Chagas ´disease

Abbreviations

Embelin (EMB), 5-*O*-methylembelin (ME), 2,5-di-*O*-methylembelin (DME), Benznidazole (BZN), Reactive oxygen species (ROS)

1. Introduction

Chagas' disease, which is endemic in Latin America, is a zoonotic parasitic disease caused by the protozoan parasite *Trypanosoma cruzi.* This illness affects 6 to 7 million people around the world (http://www.who.int/mediacentre/factsheets/fs340/es/). Over the last years, this disease has spread further. In fact, the number of cases increased in countries receiving immigration from endemic areas. In the United States, the CDC (Centers for Disease Control and Prevention) consider Chagas' disease as 1 of the 5 neglected parasitic infections in that country (Malik et al., 2015).

Chagas' disease comprises three phases: an acute phase, usually asymptomatic, a long silent period and finally some patients develop a chronic disease, in which the disease becomes evident with the appearance of cardiac dysfunction, and enlargement of some organs (colon and esophagus, 6-7%) (Bellini et al., 2010). Despite the intense efforts made to develop efficient antiparasitic drugs, an effective treatment for Chagas' disease is still lacking. Only nifurtimox and benznidazole are currently in use, albeit with restrictions due to the undesirable side effects and their low efficacy during the chronic phase. In addition, drug-resistant strains have been reported (Martínez et al., 2013). Consequently, new compounds against *T. cruzi* are needed. The plant kingdom is an important source of bioactive compounds, with many of them showing trypanocidal activity (Barros de Alencar et al,2017; Lozano et al., 2016). Additionally, chemical modifications of such compounds can improve their effect (Aponte et al., 2008).

Palace-Berl et al. (2013) have reported that hydrophobic compounds are highly active against *T. cruzi*. It is known that certain molecules and processes are crucial for parasite survival, thus constituting ideal targets for drug action. Parasites have different defense against free radicals compared with mammalian cells; in this way this could be an interesting goal to study (Wilkinson et al., 2003). Soeiro et al., (2011) have demonstrated

that quinones are possible antitrypanocidal candidates due to their capacity to generate reactive oxygen species. Feresin et al., (2003) have demonstrated that the benzoquinone EMB isolated from *Oxalis erythrorhiza* causes 100% lysis of *T. cruzi* when employed at 100 µg/ml. Additionally, EMB has many biological activities, such as anticancer, antiinflamatory, analgesic, antimitotic, antihelmithic, contraceptive, antispermatogenic, anticonvulsant and antidiabetic (Poojari, 2014). On the other hand, the antiprotozoal activity against *Plasmodium falciparum*, antitiviral and antitumor activities of 5-*O*-methylembelin (ME) have been reported (Hussein et al., 2000; Narayanaswamy et al., 2017; Xu et al, 2005). The aim of this work was evaluate the EMB, and its synthetic derivatives against *T. cruzi*.

2. Materials and methods

Chemicals, reagents and equipment

All reagents and solvents used were of analytical grade. Petroleum ether (PE), Chloroform (CLF), dichloromethane (DCM), ethyl acetate (EtOAc), glutaraldehyde and diazomethane (CH_2N_2) were purchased from Aldrich Chemical Co. (St Louis, MO, USA). Methanol was purchased from J. T. Baker (Phillipsburg, NJ). Sephadex LH-20 was purchased from Sigma -Aldrich Chemical Co. (St Louis, MO, USA). Thin layer chromatography (TLC) analysis was carried out on aluminum-coated silica gel (Aldrich) and cellulose F254 plates from Merck.

For the structural identification of compounds, nuclear magnetic resonance (NMR) spectra were obtained with a Bruker Avance NMR spectrometer (Bruker Elektronik GmbH, Rheinstetten, Germany), operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. $CDCl_3$ were used as solvents. Benznidazole (2-nitroimidazole), 3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and the probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich.

K_2HPO_4 (Merck- Darmstadt, Germany), tryptose (Oxoid Ltd., Hampshire, England), tryptone (ICN Biomedicals, Ohio USA), yeast extract (Merck- Darmstadt, Germany), hemin chloride (ICN Biomedicals, USA), bovine fetal serum (Internegocios, Argentina) and penicillin-streptomycin (Life technologies, USA) were used for the preparation of Diamond culture medium. The following reagents were also employed: EUKITT mounting solution (O. Kindler GmbH & Co., Freiburg, Germany), DMEM (Gibco), Giemsa stain (Merck- Darmstadt, Germany), and resins (Spurr Low Viscosity Kit, Ted Pella). A Nikon eclipse 80*i* microscope, a Multiskan FC® Microplate Photometer (Thermo Scientific), a Fluoroskan Ascent FL, (Thermo scientific) and a Zeiss EM 900 electron microscope were used.

Plant material

Oxalis erythrorhiza Gillies ex Hooker et Arnott (Oxalidaceae) whole plant specimens were collected during the blossoming period in the central Andes Region of Argentina, in December 2015. A voucher specimen was deposited at the herbarium of the Escuela de Química y Farmacia, Universidad de Chile, Santiago de Chile (no. SQF 21009).

Extracts and isolation of embelin

Extracts were obtained from the air-dried ground whole plant. A representative sample of the air-dried plant (1000 g) was successively extracted at room temperature with petroleum ether (PE), dichloromethane (DCM) and methanol (MeOH) to afford PE (30 g; 3%), DCM (40 g; 4.00%) and MeOH (100 g; .10%) extracts. EMB was isolated from DCM extract as an orange solid (5g) (Feresin et al., 2003).

Embelin derivatives

EMB (1000 mg) was treated with CH_2N_2. The reaction mixture was stirred for 30 min at 27 °C. After solvent removal under pressure, the solid was successively purified on a Sephadex LH-20 column (40 x 2.5 cm), equilibrated with PE:MeOH:Cl_2CH_2 (2:1:1) to

afford 350 mg of 5-*O*-methylembelin (2-hydroxy-5-methoxy-3-undecylcyclohexa-2,5-diene-1,4-dione, 2,35% w/w yield), and 450 mg of 2,5-di-*O*- methylembelin (3,45% w/w yield). Spectroscopic data were in agreement with those previously reported (Joshi and Kamat, 1975; Gomez et al., 1989).

Parasites and mammalian cells

T. cruzi epimastigotes (Dm28c strain) were cultured at 28 °C in Diamond medium (0.1 M NaCl, 0.05M K_2HPO_4, 0.625% tryptose, 0.625% tryptone, 0.625% yeast extract, pH 7.2), supplemented with 10% inactivated fetal bovine serum (Gibco) and 12.5 µg/ml hemin (Lozano et al., 2012). Trypomastigotes and amastigotes were maintained in Vero cells (ATCC CCL-81) with DMEM supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified incubator. Cell viability was determined by the MTT assay, according to Catunda et al.,2017.

Proliferation and viability assays

Epimastigotes (adjusted to 3×10^6 cells/ml) were incubated at 28 °C with different concentrations (3.2 - 34 µM) of EMB, 2,5-di-*O*-methylembelin or 5-*O*-methylembelin in sterile tubes, and the volume was adjusted to 1 ml with Diamond medium. Controls without compounds, with DMSO (0.2%), and BZN (19.2 µM) were used in all experiments. Aliquots were collected every 24 h, and were fixed with 2% *p*-formaldehyde in PBS. Parasites were counted in a Neubauer hemocytometer (Sülsen et al., 2010).

Other aliquots from cultures were incubated for 5 min in 2% of eosin in PBS, pH 7.2 and were analyzed under light microscopy. The percentage of dead parasites (stained cells) was determined in every treatment (Lozano et al., 2012).

To evaluate the effect of compounds on the viability of infective forms, trypomastigotes released from infected Vero cells to the culture medium were collected and incubated in

DMEM with different concentrations of ME (37 °C - 24 h). Subsequently, aliquots were stained with eosin, as previous detailed.

Ultrastructural analysis by transmission electron microscopy

The ultrastructural study was carried out according to Brengio et al. (2000). Briefly, treated or non-treated parasites were collected, centrifuged at 3000 rpm, and fixed with 2% glutaraldehyde in PBS (24 h at 4 ºC). Cells were washed twice with PBS, and then incubated overnight with 1% osmium tetroxide at 4 ºC. Parasites were washed twice and dehydrated with increasing concentrations of acetone, and pre-infiltrated in a 1:1 solution of acetone and Spurr Low Viscosity Kit, Ted Pella resin for 2 h. Cells were then centrifuged and embedded in pure Spurr Low Viscosity Kit, Ted Pella resin for 24 h at 60 ºC. Ultrathin sections were cut with a Power Tone XL ultramicrotome and contrast-stained with uranyl acetate and lead citrate. Samples were analyzed in an EM 900 Zeiss electron microscope.

Mitochondrial activity

Epimastigotes (Dm28c strain, 3×10^6/ml), were treated with 30 µM ME for 24 h. Parasites were then centrifuged at 3000 rpm for 10 min. The parasites-containing pellet was resuspended in 100 µl of Diamond medium and 10 µl MTT (5 mg/ml) with phenazine methosulfate (0.22 mg/ml) in PBS and incubated for 4 h at 28 °C. The suspension was then centrifuged, and the medium removed. The precipitate was dissolved with 100 µl DMSO and the absorbance was measured at 570 nm using a FC® Microplate Photometer, Thermo Scientific (Ormeño et al., 2016).

ROS generation

Epimastigotes (5×10^6) were incubated in Diamond medium with or without 30 µM ME for 24 and 48 h. Parasites were then incubated with 10 µM of H_2CDFA for 1 h at 28 °C in the darkness. Samples were centrifuged at 3000 rpm for 10 min, and the pellet was

suspended in PBS. The fluorescence intensity was detected in a fluorimetric microplate reader Fluoroskan Ascent FL, (Thermo scientific) with excitation and emission wavelengths of 485 and 538 nm, respectively (Barrera et al., 2013).

Compound activity against intracellular amastigotes

Vero cells were grown in T25 flasks in DMEM (supplemented with 10% FBS), and then infected with trypomastigotes at a parasite:cell ratio of 10:1 for 2 days. Subsequently, 2 x 10^4 cells were subcultured in 24 multiwell plates containing sterile coverslips. After 6 h, 19.2 µM BZN or 16 µM 5-*O*-methylembelin in DMSO were added and incubated for 24 h at 37 ºC. Controls were performed with 0.2% DMSO. After incubation, Vero cells were washed and processed for light microscopy. Three independent experiments were performed in triplicate. The coverslips containing the infected cells were washed once with PBS and cells were fixed for 1 min with pure methanol, and stained with 10% Giemsa in water for 20 min. After discarding the excess of dye, the cells were washed three times with distilled water and the coverslips were mounted on slides with EUKITT mounting solution. Cells were observed under a light microscope and the percentage of infected cells, as well as the number of parasites per cell were determined from 200 Vero cells counted per experiment (from fields taken at random). For each condition (control, BZN and ME treatment) infected cells were classified according to the number of intracellular amastigotes as: group I (1 to 10 parasites per cell), group II (11 to 20 parasites per cell) and group III (more than 20 parasites per cell). Results were expressed as the percentage of infected cells in each category. For each condition the summation of infected cells was considered 100%.

Release of trypomastigotes to the culture medium

Treated (16 µM ME) or non-treated infected Vero cells were incubated for 24 h in DMEM. Then, an aliquot of the culture medium was collected and replaced by a fresh

one. Cells were incubated for other 48 h at 37 °C. Aliquots of culture medium were collected every 24 h and counted in a Neubauer hemocytometer as described previously (Lozano et al, 2012).

Infectivity of released trypomastigotes

The trypomastigotes obtained from the culture medium of infected cells under the conditions described above were centrifuged, washed once with PBS and resuspended in fresh medium. These parasites were then added to growing Vero cells on slides at a parasite:cell ratio of 10:1. After 24 h of incubation, slides were stained with Giemsa to evaluate cell infection.

Pre-treatement of Vero cells with 5-O-methylembelin

To evaluate the sensitivity of ME pre-treated cells to infection, 2 x 10^4 cells were cultured in 24 multiwell plates containing sterile coverslips in the absence (control) or in the presence of 16 µM ME for 24 h. Vero cells were then washed with PBS, and fresh medium was added together with parasites at a parasite:cell ratio of 10:1. Cells were incubated for 24 h. Subsequently, the slides were fixed with methanol and stained with Giemsa to estimate the infection index (as percentage of infected cells).

Statistical analysis

The Student's *t* test was used to determine the statistical significance of the difference between treated and control groups. The effect of each treatment was analyzed by one-way analysis of variance (ANOVA).

3. Results

EMB, which is recommended for hepatic and heart disorders, causes lysis of *T. cruzi.* Based on this, a possible link between the traditional use and the trypanocidal effect was suggested (Feresin et al., 2003). In this work, the efficacy of embelin (EMB), and 5-*O*-methylembelin (ME) and 2,5-di-*O*-methylembelin (DME), obtained by synthesis were

tested on *T. cruzi*. The chemical structures of EMB and its derivatives are shown in **Fig. 1**.

Insert Fig. 1

As observed in the **Table 1**, EMB and its derivatives inhibit epimastigote proliferation. The inhibitory effect observed on the proliferation of *T. cruzi* epimastigotes was stronger for ME than for EMB and DME (**Table 1**).

Insert Table1

However, no significant differences were detected for neither EMB (34 µM) nor ME (16 µM), as compared to the reference drug BZN (19.2 µM) at all the timepoints tested. Moreover, the effect of ME on epimastigote proliferation was dose-dependent**.** On the first day of treatment, no significant differences were detected between the effect caused by the compounds and that of BZN. At 48 h of treatment, and for all concentrations evaluated, ME caused a significant inhibition on parasite growth, as compared to the control group. This inhibition was more marked at 72 h of incubation ($p < 0.001$). In fact, the IC_{50} values for ME and BZN were similar (21 ± 0.69 µM *vs.* 19.2 ± 1.4 µM, respectively).

As shown in the **Table 2**, both compounds (EMB and ME) exerted a cytostatic rather than a cytotoxic effect, since mortality did not exceed 2.4 ± 0.54% at 24 - 72 h of treatment at all the concentrations used. The activity of ME was also evaluated on the infective forms, showing a mild deleterious effect on trypomastigotes (12.29 ± 5.99% mortality, **Table 2**).

Insert Table 2

Additionally, the cytotoxicity of ME was assessed on Vero cells. No significant differences were observed at 24 h of treatment between 16 µM of ME and BNZ (data not shown).

Transmission electron microscopy (TEM) is an important tool to investigate the mechanism of action of trypanocidal drugs (Soeiro et al., 2011). Thus, this methodology was employed to assess the effect of ME on *T. cruzi*. Results showed that ME induces some ultrastructural alterations on epimastigotes. After 24 h of treatment with 30 µM ME, most of the parasites exhibited a cytoplasmic disorganization, mitochondrial swelling, and a kinetoplast deformity **(Fig. 2B**).

Insert Fig. 2

At 48 h of treatment, a moderate vacuolization was also observed in most of the parasites, with some of them containing electrodense material inside **(Fig. 2D**). The integrity of the plasma membrane was preserved, although some membrane blebs appeared at 48 h of treatment (**Fig. 2D**).

The fact that kinetoplastids have a single branched mitochondrion with a DNA structure named kinetoplast, which is not present in mammalian cells, makes this organelle an attractive drug target. The mitochondrial swelling and kinetoplast disruption caused by ME was also evaluated in terms of mitochondrial activity by the MTT assay. **Fig. 3** shows that after of 24 h of treatment with ME, the mitochondrial activity was reduced by 21.66 ± 3.62%, as compared to untreated parasites ($p < 0.05$). However, at 48 h of treatment, the mitochondrial activity was increased by 37.81 ± 9.94% compared to the controls. This unexpected effect could indicate the existence of a parasite population that is less sensitive to the drug, or with higher mitochondrial activity.

Insert Fig. 3

The particular defense mechanism against free radicals existing in trypanosomatids makes them particularly sensitive to oxidative stress (Jimenez et al., 2014). Thus, parasites are vulnerable to compounds with oxidative activity, such as those containing quinone groups (e.g. ME) (Soeiro et al., 2011). Considering the changes in mitochondrial activity induced by ME, the ROS production was measured using the fluorescence probe H_2CDFA. As shown in the **Fig. 4**, the ROS generation increased significantly ($p < 0.05$) after 24 h of treatment with ME. Although this trend was maintained at 48 h of treatment, no significant differences were detected at this timepoint, as compared to controls.

Insert Fig. 4

T. cruzi amastigotes (intracellular stage) are crucial for the development of Chagas' disease, and to complete its life cycle. Literature data on the activity of compounds against amastigotes are scarce (Muschietti et al, 2013). In our work, ME was found to be active against the intracellular forms of the parasite and non-toxic to the host cells. Although ME did not cause a significant change as regards the percentage of infected cells **(Fig. 5A)**, the number of intracellular parasites decreased (**Fig. 5B**).

Insert Fig. 5

Insert Fig. 6

This effect was evident as early as 24 h of incubation with ME. Thus, only 2.37 ± 0.51% of infected and treated cells contained more than 20 amastigotes, while the percentage of infected and non-treated cells containing more than 20 amastigotes was 13.87 ± 1.14%. The effect caused by ME was similar to that of BZN (**Fig. 6**).

After several cycles of cell division in the host cell cytoplasm, amastigotes transform into trypomastigotes and are released into the extracellular medium to infect other cells.

As observed in **Fig. 7**, the number of parasites released (obtained from culture supernatant) was significantly lower than that of control cultures 2 and 3 days after start of treatment with ME, ($p < 0.001$).

Insert Fig. 7

Finally, the infectivity of parasites released from ME-treated cells was evaluated. After collecting parasites from the culture medium from either control or treated cells, they were incubated with growing Vero cells for 24 h. Neither the percentage of infected cells nor the number of parasites per cell was different for parasites obtained from controls or from ME- treated cells, indicating that the compound did not alter the infectivity (data not shown).

The activity against intracellular amastigotes reflects the ability of ME to cross the plasma membrane. In this sense, Lu et al., (2013) have reported that EMB is a component of a nanomicellar carrier. In order to evaluate if the pretreatment of cells with ME affects the infection rate, cells were preincubated with this compound (16 µM) for 24 h. The medium was then removed and the culture was incubated with the parasites for 24 h in fresh medium. Results showed that ME-pretreated cells did not differ significantly from controls in neither the number of infected cells nor in the replication rate of intracellular parasites (data not shown).

4. Discussion

American trypanosomiasis (Chagas' disease) is among the most important parasitic infections belonging to the group of neglected tropical diseases. The few therapeutic alternatives available for the treatment of this disease and their side effects that these drugs cause on patients, has prompted the search for new active molecules against *T. cruzi*. In this sense, the plant kingdom offers an extensive source of compounds (Barros de Alencar et al, 2017; Gallucci et al., 2014). In addition, some molecules obtained from plant materials can be chemically modified to improve the activity (Aponte et al., 2008). Among them, several biological activities of the benzoquinone embelin (EMB) have been reported (Feresin et al., 2003; Poojari, 2014). Two methylated derivative compounds were synthesized from embelin which showed that methylation improves the bioactivity, as occurs with other molecules (Salomoa et al., 2013). It is likely that the incorporation of a methyl group conferred embelin a higher degree of lipophilicity, which favored its entrance into the parasite cell (Palace –Berl et al., 2013). However, the dimethylated derivative (DME) was less active than the monomethylated one (ME) (IC_{50} 7.07 ± 0.23 µM), which suggests that the free hydroxyl group in position 2 influences the activity of embelin derivatives. Therefore, it is likely that the active group be the carbonyl moiety present in C1. Both, EMB and ME, presented cytostatic activity on parasites at the concentrations evaluated. However ME at higher concentrations could have toxic effects as embelin (Feresin et al., 2003). The fact that ME has cytostatic rather than cytotoxic activity suggests that the drug could interfere with critical steps of the parasite's cell cycle.

ME induced ultrastructural alterations on epimastigotes, such as kinetoplast disorganization and mitochondrial swelling. Similar effects on the mitochondrion have been reported for naftoquinones (Salomao et al, 2013) and other compounds. These alterations were attributed to effects on structural components in this organelle, such as

membrane proteins or enzymes (Izumi et al., 2007). Other authors have demonstrated that quinones inhibit redox enzymes and components of the respiratory chain (Sepulveda-Boza et al., 1996). In fact, EMB is considered a weak mitochondrial uncoupler agent, producing cell death in proliferating endothelial cells (Coutelle et al. 2014). Furthermore, the mitochondrial swelling has also been attributed to alterations in the ergosterol metabolism (Kessler et al 2013).

Membrane blebs were also observed after treatment with ME. Some authors have reported these blebs as normal structures in other trypanosomatids (Diniz Atayde et al., 2015), whereas other studies suggest that such structures are produced by quinones and other compounds that affect the proliferation of amastigotes and epimastigotes (Sthal-Vieira, 2011). The ROS generation has been associated with these alterations by oxidation of lipid rafts on the plasmalemma (Oliveira dos Anjos et al. 2016).

ME also induced deformities in the kinetoplast, suggesting that the compound would exert additional effects. It is known that the kinetoplast is made of a special type of DNA (k-DNA) which is located in a specialized portion of the mitochondrion. Evidences have confirmed the presence of basic proteins (H1 histone-like) in the kinetoplast that could participate in the condensation of k-DNA in *T. cruzi* (Cavalcanti et al., 2004). ME would then interrupt such condensation.

The mitochondrial alteration was also confirmed biochemically by the MTT assay (**Fig. 3**). The reduction of MTT is mainly carried out by mitochondrial dehydrogenases, thus, the reduced levels of MTT formazan observed after 24 h of treatment with ME support the alterations observed by TEM. Conversely, the increased mitochondrial activity observed at 48 h could be explained as the development of a parasite selection process that would be less sensitive to the uncoupling effects of ME. However, it should be borne in mind that the generation of oxidative stress is one of the main mechanisms of action of

quinones, which is attributed to their structural properties (Salomao et al., 2013). This process could be initiated by the loss of one or two electrons, generating a semiquinone or a hydroquinone, respectively. These unstable molecules can produce free radicals (Soeiro et al., 2011). In this case, the population of parasites selected at 48 h could have a more efficient ROS detoxification system. On the other hand, it is important to remark that compounds like triazoles, that inhibit the sterol biosynthesis, produce accumulation of 14-α-methylsterol, which is toxic for systems like the electron transport chain. This effect could also explain the decrease in mitochondrial activity and the ROS generation (Maya et al., 2007).

In this study, we also observed that the proliferation of intracellular amastigotes was affected by ME, similarly to BZN. The latter effect constitutes a relevant finding, since currently, there are no drugs that are active on the intracellular form of *T. cruzi*. When the parasites invade cells, they are initially confined to a parasitophorous vacuole, from where they escape into the cytosol to transform into the proliferative amastigote form. After several rounds of replication in the form of amastigote, parasites differentiate into trypomastigotes, which are then released to the extracellular medium to infect new cells (Burleigh et al., 2002). Our results showed that the treatment with ME significantly reduced the number of extracellular parasites (trypomastigotes), presumably as a result of a decrease in the number of amastigotes. However, ME might also affect either the differentiation into trypomastigotes or the release of parasites from the infected cells. Interestingly, the infectivity of parasites released from ME-treated cells was preserved, since they were able to invade new cells, similarly to control non-treated parasites.

In this study, it was demonstrated that the methylation of embelin potentiates its action against *T. cruzi*, presumably by facilitating its entrance into the parasites. The drug was also found to exert its action on proliferating amastigotes. Nevertheless, effects on other

intracellular developing steps of the parasites as well as the targeting of other parasite processes cannot be ruled out.

5. Conclusions

Our work demonstrates that EMB and its derivatives are active compounds against *T. cruzi* epimastigotes. Amastigotes were also sensitive to ME. Since EMB is a potential drug against multiple diseases, further studies should be carried out in other biological models. Finally, it can be concluded that ME, the EMB methylated derivative, could be a useful hit for the development of semisynthetic drugs active against *T. cruzi*. Its capacity to reduce the amastigote load would render it a special candidate for the treatment of chronic Chagas' disease.

Conflict of interest

The authors do not have any conflict of interest.

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Table legends

Table 1. Proliferation of epimastigotes (Dm28c strain) incubated in the absence (control) or the presence of either EMB, ME, DME or BZN (positive control). Values are expressed as percentages of proliferation (means ± SEM) after 24, 48 or 72 h of incubation. (*) (**)(***) Significantly different from the control ($p < 0.05$; $p < 0.01$; $p > 0.001$, respectively).

Table 2. Cytotoxicity of compounds on epimastigotes and trypomastigotes was evaluated by the eosin exclusion method. Values are expressed as percentages of dead cells (means ± SD) after 24, 48 or 72 h of incubation. (*) Significantly different from the control, ($p < 0.05$). Results were determined in triplicate.

Figure legends

Fig. 1. Chemical structure of embelin and its derivatives

Fig. 2. Ultrastructure of the epimastigotes observed by transmission electron microscopy (TEM) after incubation for 24 or 48 h in the absence (**A** and **C**) or in the presence of ME (**B** and **D**).White asterisks: nucleus; white arrowhead: kinetoplast; white arrow: vacuole; black arrow: mitochondrion, black arrowhead: flagellum.

Fig. 3. Mitochondrial activity of epimastigotes. Parasites were treated with 30 µM ME for 24 and 48 h. Results are expressed as mean ± SEM (n = 3). (*) Significantly different from control ($p < 0.05$).

Fig. 4. ROS generation on epimastigotes after 24 and 48 h treatment with 30 µM ME. Results are expressed as mean ± SEM (n = 5). (**), significant differences, as compared to control ($p < 0.05$).

Fig. 5. Infected Vero cells treated with 16 µM ME for 24 h (**A** and **B**). After incubation, the percentage of infected Vero cells (**A**), and the number of amastigotes per cell (**B**), were estimated. (*) and (***), significantly different from the control ($p < 0.05$ and $p < 0.001$, respectively). Results are expressed as mean ± SEM (n = 3).

Fig. 6. Giemsa stained microscopic images of Vero cells. (**A**): uninfected, (**B**): infected and untreated, (**C**): infected and treated (16 µM ME for 24 h) Vero cells. Arrows indicate intracellular amastigotes.

Fig. 7. Parasites released into the culture medium at the indicated time-points after the treatment with 16 µM ME for 24 h. Results are expressed as mean ± SEM (n=3). (**), significantly different from control ($p < 0.01$).

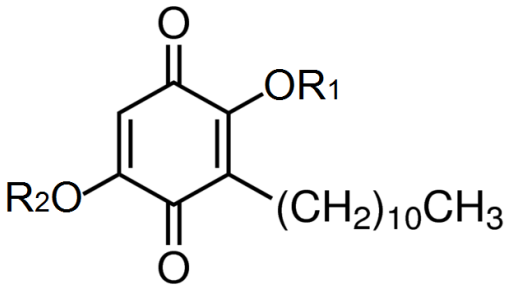
Table 1

Incubation time	Epimastigotes (1.10^6/ml)								
	Control	DMSO 0.01%	BZN 19.2 (µM)	EMB 34 (µM)	DME 31 (µM)	ME 30 (µM)	ME 16 (µM)	ME 8 (µM)	ME 3.2 (µM)
24 h	5.05 ± 0.7	3.9 ± 0.68	3.56 ± 0.52	4.08 ± 0.6	4.64 ± 0..15	2.75 ± 0.42	3.4 ± 0.53	3.5 ± 0.65	2.3 ± 0.13
48 h	10.06 ± 1.16	8.85 ± 0.61	*** 5.02 ± 0.59	7.65 ± 0.92	6.26 ± 0.13	*** 4.15 ± 0.35	*** 4.78 ± 0.44	** 5.78 ± 0.15	* 6.33 ± 0.24
72 h	14.12 ± 1.22	14.05 ± 1.46	*** 5.4 ± 0.48	12.68 ± 1.16	*** 6.64 ± 0.06	*** 4.14 ± 0.25	*** 4.43 ± 0.24	*** 7.21 ± 0.54	*** 8.43 ± 0.82

Incubation time	Epimastigotes					
	Control	ME				EMB (µM)
		30 (µM)	16 (µM)	8 (µM)	3.2 (µM)	34
24 h	1.8 ± 1.48	1.8 ± 1.09	2.4 ± 0.54	1.8 ± 1.3	1.4 ± 1.4	1.8 ± 1.09
48 h	1.4 ± 0.89	1.2 ± 0.84	1.2 ± .09	1.6 ± 1.52	1.2 ± 1.15	1.2 ± 0.84
72 h	1.4 ± 1.34	1.6 ± 1.14	1.4 ± 1.34	1.0 ± 1.41	0.8 ± 1.09	1.6 ± 1.14

	Trypomastigotes		
	Control	ME	
		240 (µM)	120 (µM)
24 h	0.66 ± 1.15	12.29 ± 5.99*	1.0 ± 1.0

Table 2



	R1	R2
Embelin	H	H
5-*O*-methylembelin	H	CH_3
2,5-di-*O*-methylembelin	CH_3	CH_3

Figure 1.

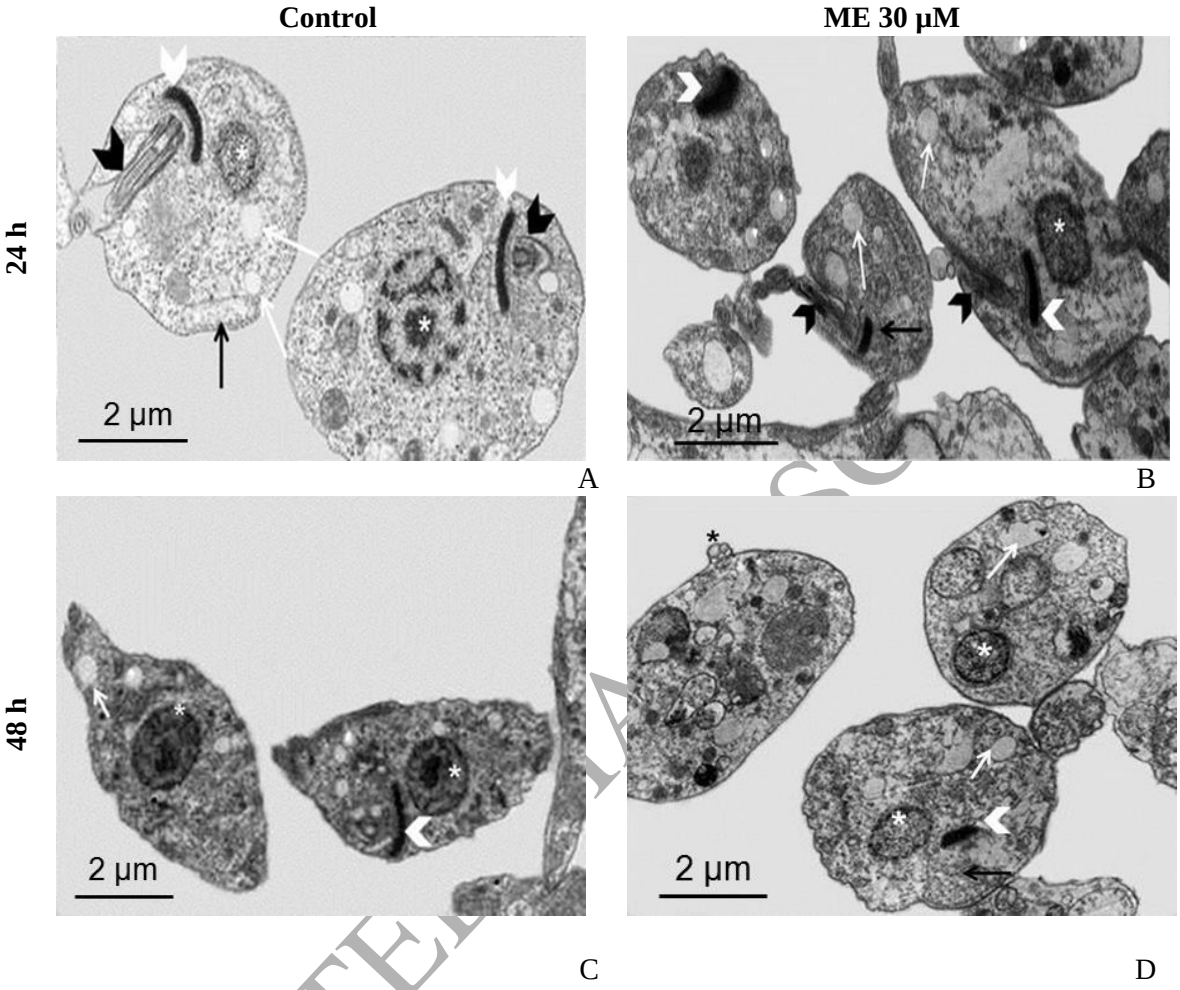
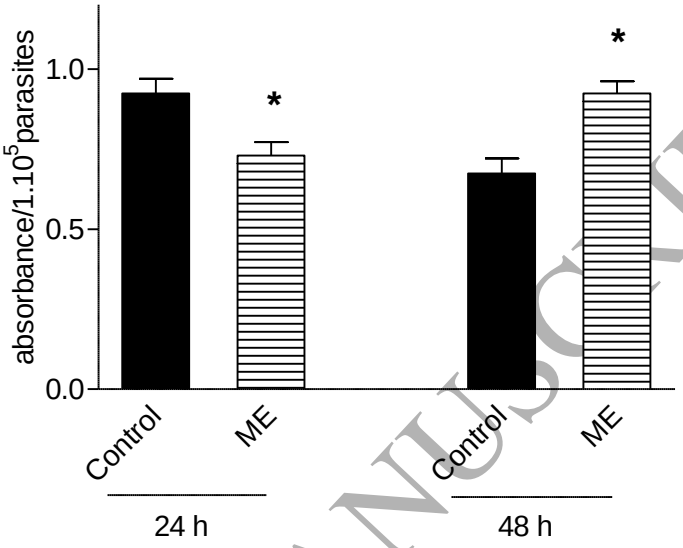


Figura 2



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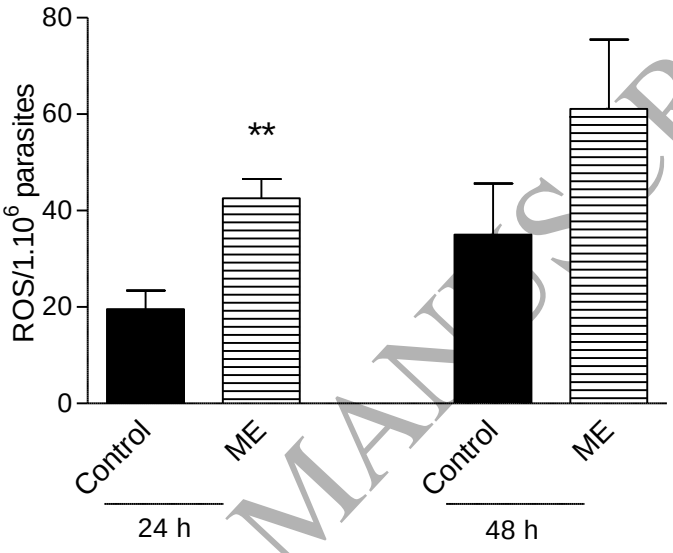

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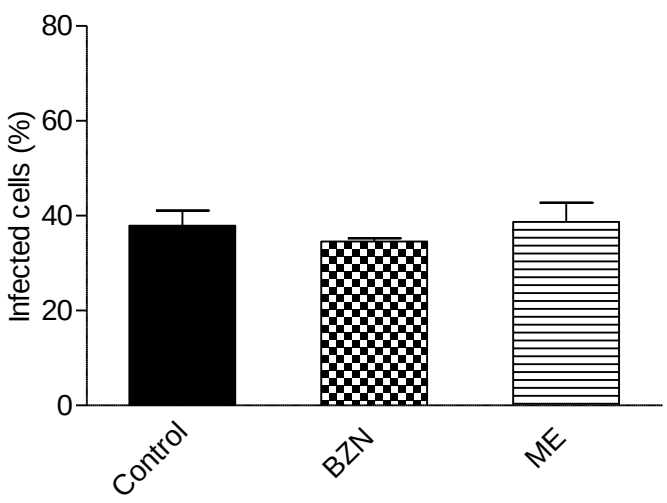
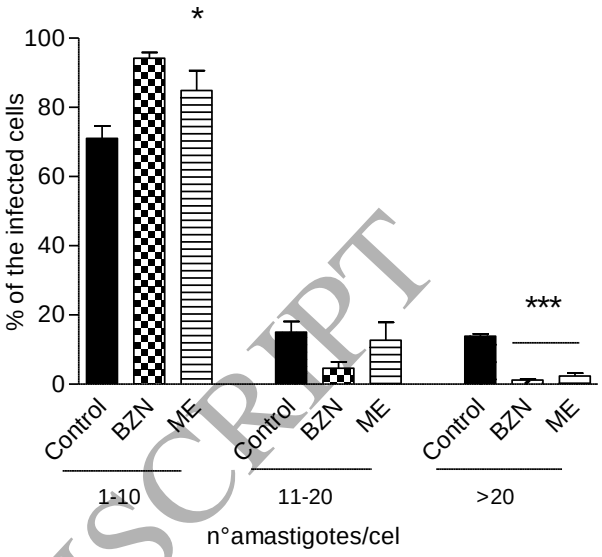




A **B**

Figura 5

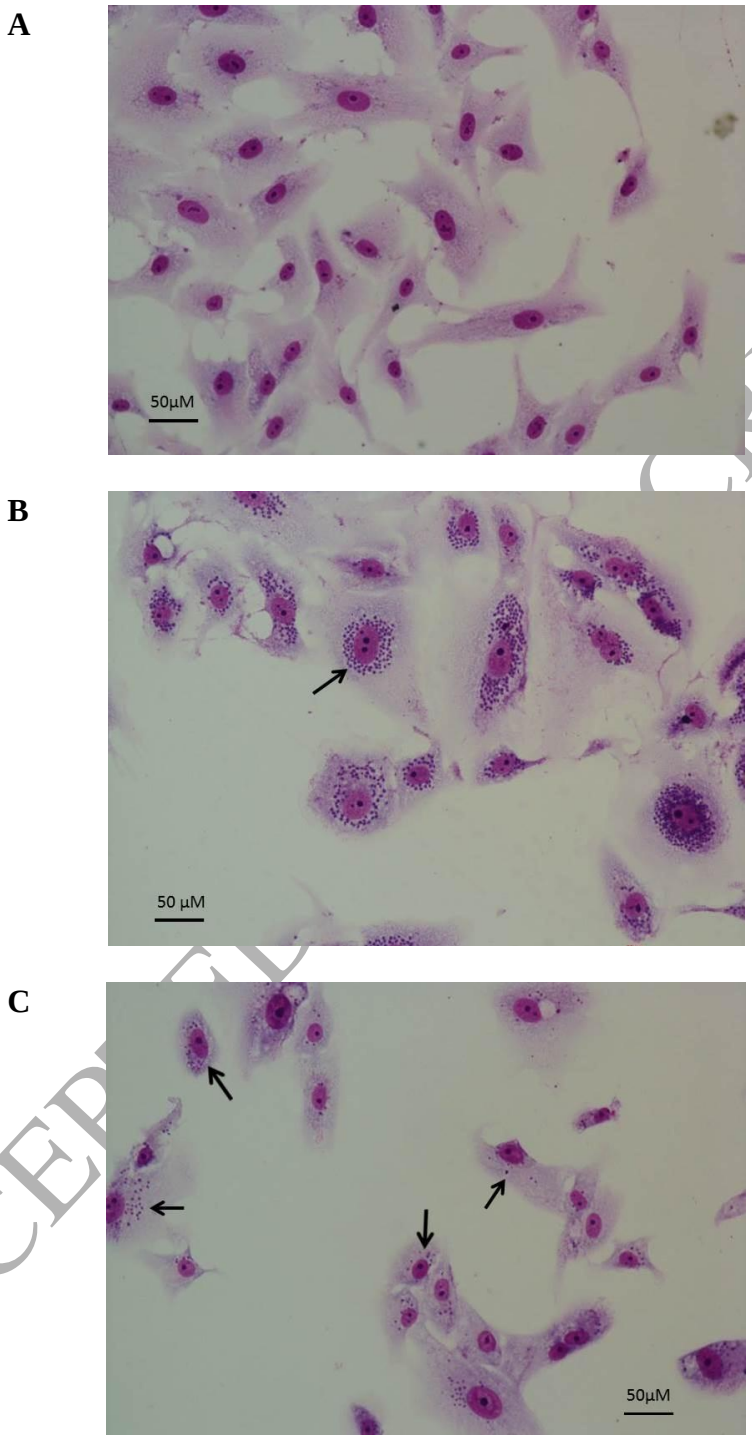
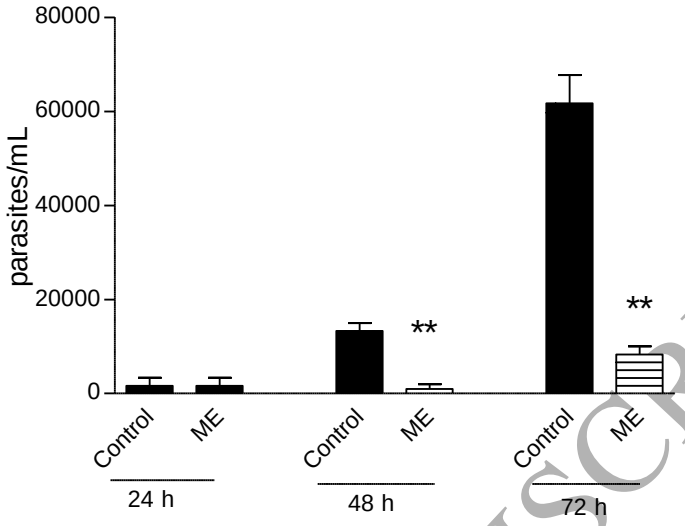


Figura 6



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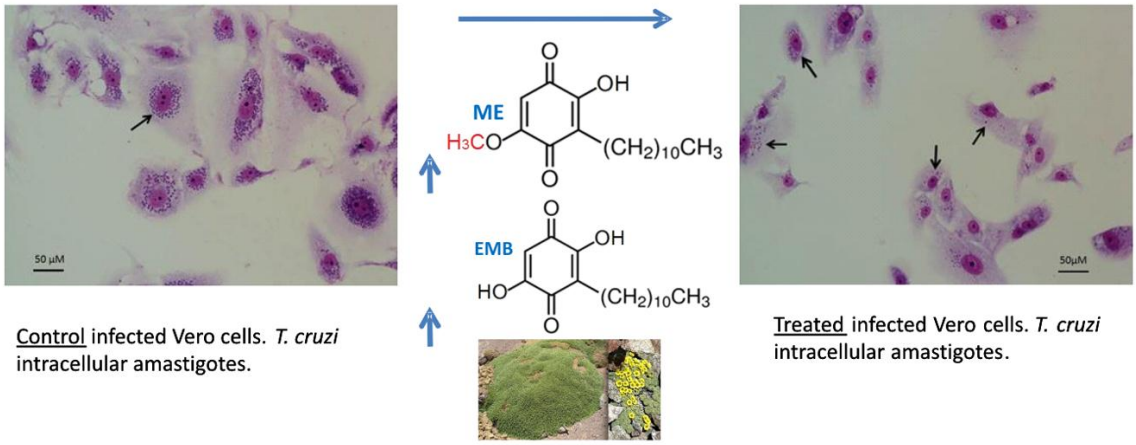

Control infected Vero cells. *T. cruzi* intracellular amastigotes.

Treated infected Vero cells. *T. cruzi* intracellular amastigotes.

Graphical Abstract