The *in vivo* trypanocidal effect of the diterpene 5-epi-icetexone obtained from *Salvia gilliesii*

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The *in vivo* trypanocidal effect of the diterpene 5-epi-icetexone obtained from *Salvia gilliesii*.

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Abstract

The search for new compounds with trypanocidal activity is crucial for the treatment of Chagas' disease. Previous *in vitro* studies have shown that the diterpene 5-epi-icetexone (ICTX) is active against *T. cruzi*. The aim of this work was to evaluate the effect of ICTX on the parasites in infected mice, in an experimental model that mimics the acute phase of the disease. Swiss Albino mice were infected with *T. cruzi* and treated daily with 10 mg/kg/day ICTX (i.p.). Infected mice and injected with either saline or the vehicle DMSO were used as controls. Animal´s survival and parasitemia were monitored once a week and histological studies were made at necropsy by the 5th week after infection. It was observed that the administration of ICTX increased survival of mice infected, and induced a significant decrease in the parasitemia, as compared to controls. A similar protective effect was observed when animals were treated orally with benznidazole (BZN, used as a control of antiparasitic effect). By the 5th week post-infection, the presence of amastigotes nests was observed within the fibers of the cardiac and skeletal muscle in controls, but not in animals treated with either ICTX or BZN. In addition, inflammatory infiltrates were observed in the tissues of controls, but not in animals treated with the drugs. We conclude that ICTX has an antiparasitic effect against *T. cruzi*, thus constituting an interesting option for the treatment of Chagas' disease, alone or combined with other drugs.

Keywords. Chagas´ disease, *Trypanosoma cruzi*, natural compounds, diterpenes.

The monoflagellate parasite *Trypanosoma cruzi* is the agent causative of Chagas' disease, which is widespread in Latin America. The disease has a short acute phase spanning one or two months, generally asymptomatic, followed by a long silent period [1], and finally the patients enter the chronic phase in which around two-thirds of them remain in an asymptomatic indeterminate stage, and one-third become symptomatic, with the appearance of severe cardiovascular disorders, and/or gastrointestinal dysfunctions (with visceromegaly) [1-3]. Despite the fact that intense efforts are being made toward the development of novel drugs for the treatment of Chagas' disease, only the nitro derivatives nifurtimox and benznidazole (BZN) are currently in use, albeit with restrictions due to the undesirable side effects on patients and the uncertain efficacy during the chronic phase of the disease [4-8]. In addition, the search for drugs for the chronic phase has been hindered by the lack of animal models reproducing the human disease. However, some experimental models employing animal cell lines have been successful [9,10] as well as models employing mice maintained under controlled experimental conditions [9,11-14], which are being used to study the mechanism of infection, the survival of intracellular parasites and the evolution of the disease. For decades, numerous natural or synthetic compounds have proved to be active against *T. cruzi*, albeit a successful therapy for Chagas' disease is still lacking, since important side effects have been observed in patients treated with these compounds [4, 15].

Terpenoids from the aerial parts of the large genus *Salvia* (Labiatae) are currently considered to be used against parasitic diseases, due to their multiple biological activities and their abundance in the plant kingdom [16]. In fact, the diterpene 5-epi-icetexone (ICTX) obtained from *Salvia gilliesii* Benth has proved to be effective against *T. cruzi*

epimastigotes [17] and the infective forms of the parasite (trypomastigotes and intracellular amastigotes), and with low cytotoxicity on the host's cells [10]. It is possible that the deleterious effect on parasites was due to an oxidative action of the compound through the quinone groups [17]. Since ICTX appears to be an attractive candidate for treating Chagas disease, this justifies extending the studies to animal models.

In this study we observed that ICTX is also effective against *T. cruzi* in an experimental model that mimics the acute phase of Chagas` disease. Swiss albino mice were infected with *T. cruzi* (Tulahuen strain, Tc VI) under controlled conditions [18, 19]. All the experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (revised 2011) and had been approved by the Institutional Committee for the Care and Use of Laboratory Animals from the School of Medicine, National University of Córdoba, Argentina [18,19]. Starting on day 1 post-infection, when parasites can be found in the bloodstream [18,19], ICTX (99.8 % purity) was administered daily (i.p.) at 10 mg/kg/day, a dose that has proved to be effective against *T. cruzi in vitro* and without exerting toxic effects on mammalian cells [10].

It was observed that the survival of mice after infection increased from ~50% in controls (treated with either saline or 0.1% DMSO) to ~75% when treated with ICTX (Figure 1A). This effect was not observed when treated with a dose of 1 mg/kg/day of ICTX (data not shown). Moreover, when benznidazole (BZN, L AFEPE, Brazil) was administered orally at a dose of 0.1 g/kg/day, the survival of mice also increased significantly (~80 %) (Figure 1A). However, care must be taken in the comparison of the results obtained with ICTX and BZN, since they had been administered through different routes.

During the acute phase of the disease, parasites are usually released to the bloodstream, and the mortality of mice is, in part, due to the circulating parasites. Based on the increased survival of mice achieved by the treatment with ICTX, we also monitored parasitemia weekly, and observed a significant decrease of bloodstream parasites after treatment with either ICTX or BZN (Figure 1B). In all cases, the maximum parasitemia was observed two weeks after infection, to decrease to undetectable levels by the 5th week (Figure 1B). At this time point, the invasion of tissues by trypomastigotes occurs, leading to the formation of intracellular nests of proliferating amastigotes. These nests are typically formed within the cardiac fibers or in other organs, causing the symptoms mentioned above.

Employing the hematoxilin-eosin stain, tissues were also evaluated on the 5th week after infection. A typical inflammatory response in the cardiac and skeletal muscles with nests of amastigotes within muscle fibers (Figures 2 and 3) was observed. These nests were found in these tissues of all controls that had survived by the 5th week. However, treatment with ICTX prevented nest formation and the tissue architecture of the cardiac and skeletal muscles tissues was similar to that observed in non-infected mice, thus indicating that the compound may prevent tissue invasion by the parasites. A similar effect was observed in the group of animals treated with BZN (Figures 2 and 3). At this time point, we did not observe invasion of other organs, such as kidney or spleen (data not shown). It is worth mentioning that DMSO, the solvent for ICTX, did not have any toxic effects in the animals, at least during the experimental period.

Taking into account these observations, it can be hypothesized that ICTX exerts its effect while the parasite is in the bloodstream. These results are in line with those reported by Lozano et al. [10] who have found trypomastigotes to be highly sensitive to ICTX.

However, effects of this terpenoid on the other phases of the infection, that is, tissue invasion and multiplication, cannot be ruled out, since ICTX has also been found to be active against the intracellular forms of the parasite [10]. Although the mechanism of action of ICTX also remains to be determined, the generation of an oxidative stress status has been proposed [20]. Probably, the ICTX activity lies in the quinone functional group that could be chemically modified to change the compound solubility, and thus be administered by another route.

In conclusion, in this study we have confirmed that ICTX is also active against *T. cruzi* in an animal model, without apparent side effects. This molecule and/or derivatives could be an important drug in the treatment of Chagas' disease.

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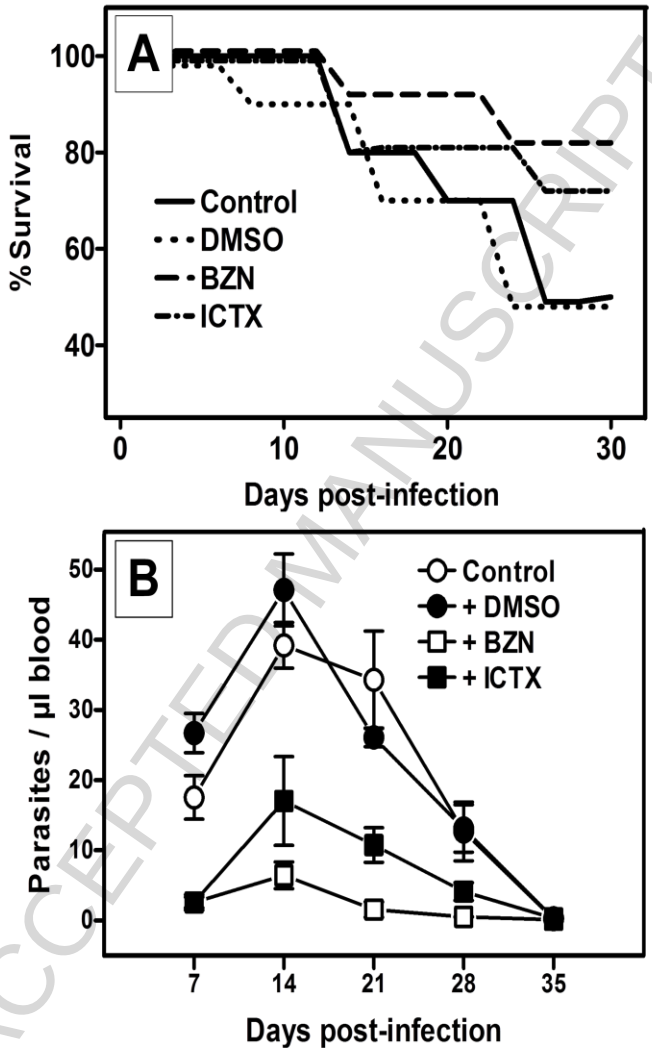
Legends to figures

Figure 1. (A) Survival of mice infected with *T. cruzi* and treated with either BZN or ICTX. (B) Parasitemia in mice inoculated with *T. cruz*i and treated with the compounds. Untreated mice or treated with DMSO were used as controls at the indicated time points. a, b and c: significantly different from control ($p < 0.001$; $p < 0.01$ and $p < 0.05$ respectively, ANOVA followed by Dunnett's multiple comparisons test) at each time point (n = 5-15 animals/time point).

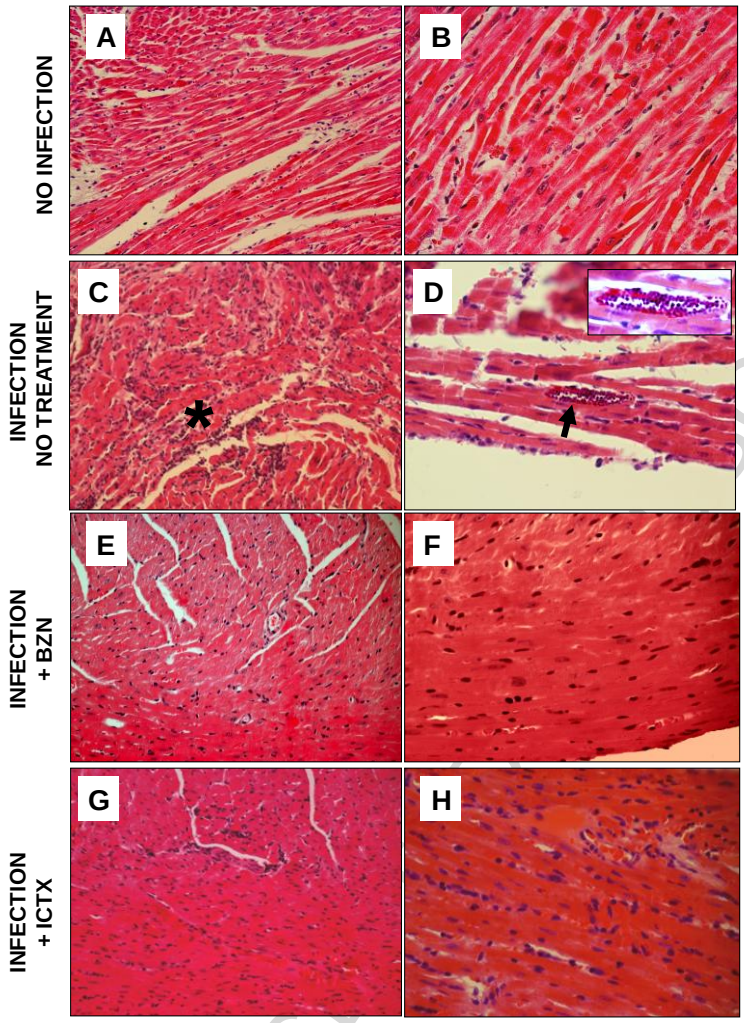
Figure 2. Histological analysis of cardiac muscle in mice inoculated with *T. cruzi* and treated or not with either BZN or ICTX on day 35 after infection. Typical inflammatory infiltrates (C, asterisk) and amastigote nests (D, arrow and inset) can be observed in

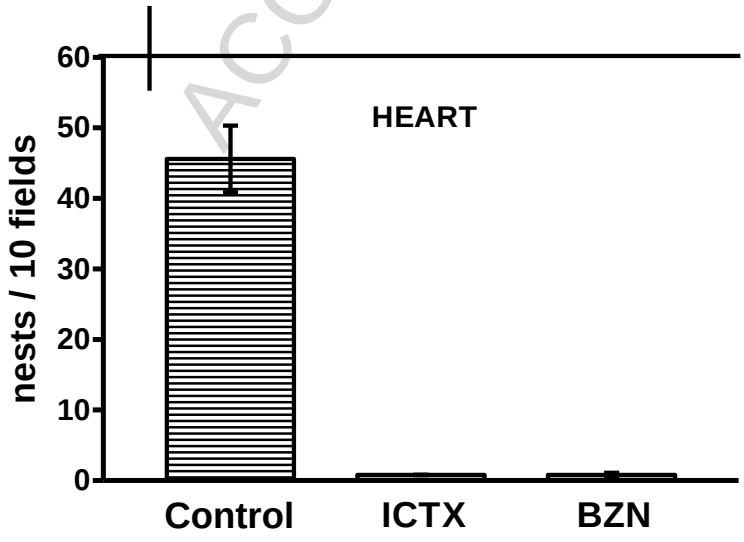
controls. Magnification: 200 x (A, C, E, and G), or 400 x (B, D, F and H). Values in the graphic represent the means ± SD from 5 animals under each condition).

Figure 3. Histological analysis of skeletal muscle of the hind leg from mice inoculated with *T. cruzi* and treated either with BZN or ICTX on day 35 after infection. Typical inflammatory infiltrates (C, asterisk) and nest of amastigotes (C and D, arrows) can be observed. Magnification: 200 x (A, C, E, and G), 400 x (B, D, F, and H). Values in the graphic represent the means ± SD from 5 animals under each condition)



Lozano et al., Figure 1





Lozano et al., Figure 2

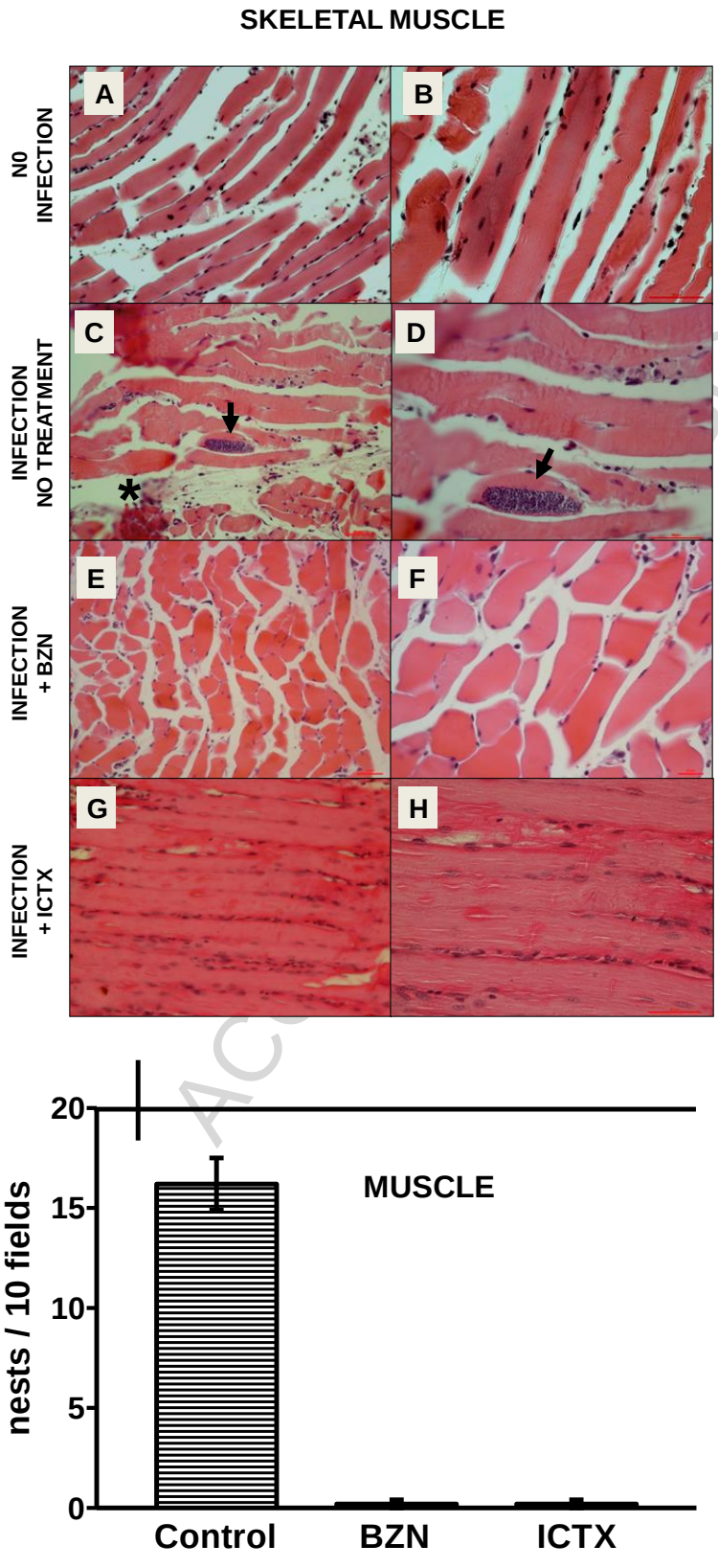
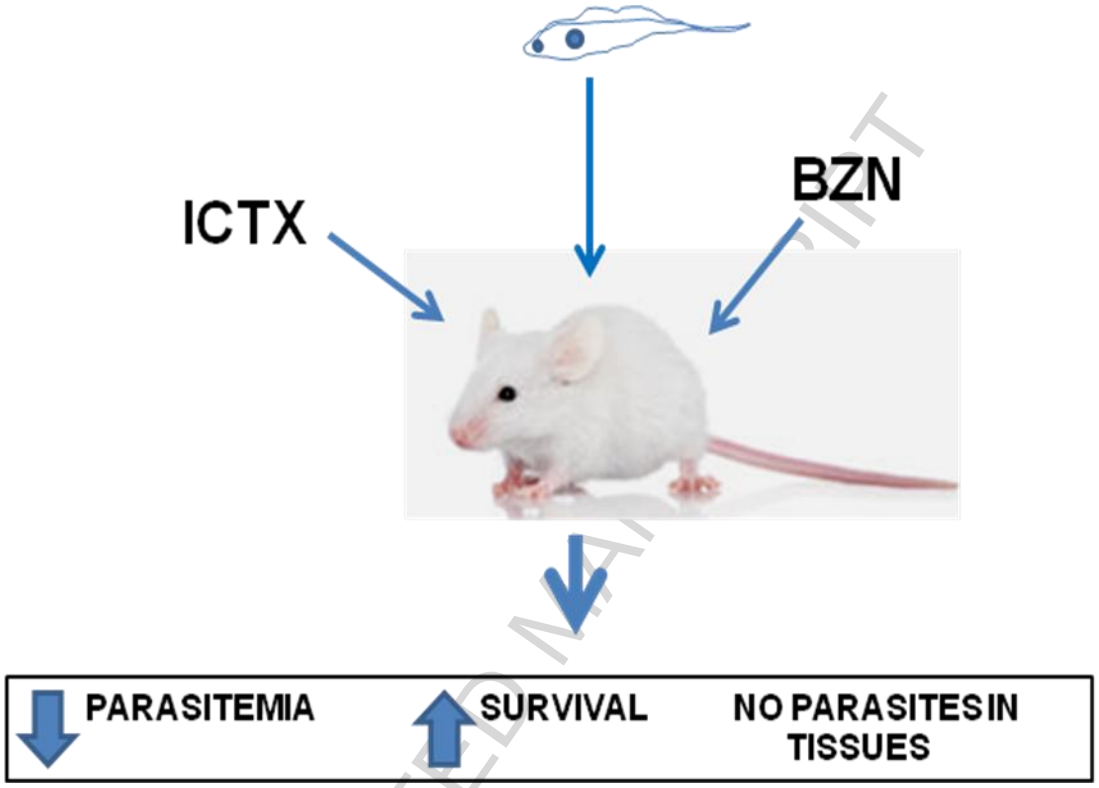


Figure 3



LOZANO ET AL., GRAPHICAL ABSTRACT