The antiparasitic effect of the norditerpenoid yaretol on *Trypanosoma cruzi*

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Abstract:

Chagas disease is caused by the intracellular parasite *Trypanosoma cruzi.* Approximately seven million people are infected worldwide, and more of 10,000 deaths occur annually. Due to the increase in migration from Latin America to the rest of the world, mainly the United States and Europe, Chagas disease has emerged in countries where it was unknown. The effect on the proliferation and viability of *T. cruzi* epimastigotes of the eight terpenes, AZ1: azorellolide, AZ2: mulinol, AZ3: stachytriol, AZ4:1α,10β,4β,5α-diepoxy-7β-germacran-6β-ol, AZ5:1β,10α,4β,5α-diepoxy-7β-germacran-6β- ol, AZ6: 1,2,3,3α,4,5,6,7,8,8α-decahydro-7-(1-hydroxy-1-methylethyl)-1,4- dimethylazulene-3α,8α-diol, AZ7: madreporanone, and AZ8: yaretol, previously isolated of the aerial parts from *Azorella cryptantha* were tested. The norditerpenoid yaretol inhibited epimastigotes proliferation, the IC_{50} value was 6.38 ± 0.47 µM, more effective than benznidazole (9.6 µM) and less cytotoxic in Vero cell line (8.87 µM) at 48 hs incubation. Studies to evaluate the effect of yaretol on other forms of the parasite are currently being performed.

Keywords: *anti-proliferative activity, epimastigotes, Trypanosoma cruzi, yaretol Azorella cryptantha*

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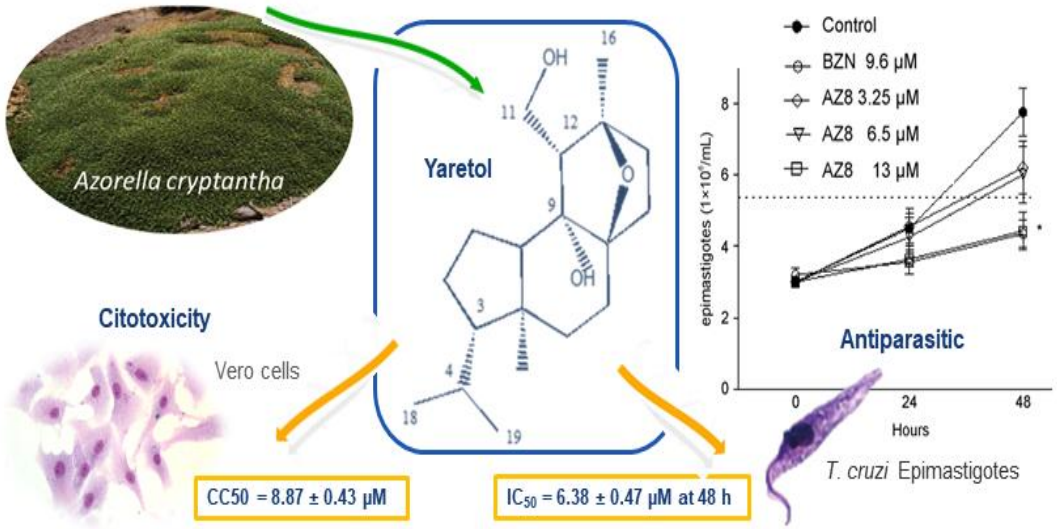
Introduction

Chagas disease is caused by the intracellular parasite *Trypanosoma cruzi*. Approximately seven million people are infected worldwide, and more of 10,000 deaths occur annually. Due to the increase in migration from Latin America to the rest of the world, mainly the United States and Europe, Chagas disease has emerged in countries where it was unknown (WHO 2022). It courses initially in an acute phase, usually asymptomatic (Perez-Molina & Molina 2018). Around 30% of chronically infected people develop Chagas disease, with severe clinical manifestations (Fonseca-Berzal et al. 2018). Currently, only two drugs are available for the treatment of Chagas disease, benznidazole (BZN) and nifurtimox, which have significant side effects and low effectiveness, mainly during the chronic phase (WHO 2022; Castro et al. 2006; Bermudez et al. 2016; Sueth-Santiago et al. 2017). Therefore, there is an urgent need for new alternatives and effective treatments to combat the causal agent of Chagas disease, *T. cruzi*, from different approaches. Natural products are gaining ground (Manikandan et al. 2022), around 30 species belonging to the *Azorella* genus grow in the Andean Mountains range, out of which, 15 grow in Argentina (Martínez 1989). Biological activities have been reported for azorellane and mulinane diterpenoids such as antiplasmodial, trichomonacidal, antituberculosis, antiprotozoal and antibacterial activity (Araya et al. 2003; Dzul-Beh et al. 2020). The main goal of this work was to evaluate the effect of eight terpenoids previously isolated from *A. cryptantha* against *T. cruzi* epimastigotes.

A graphical abstract is presented self-explanatory that shown the scientific data and the main research findings of the yaretol (**AZ8**) on proliferative effect against *T. cruzi* parasites.

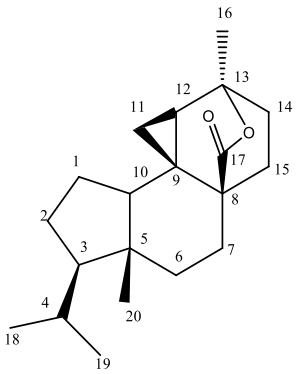


Graphical Abstract

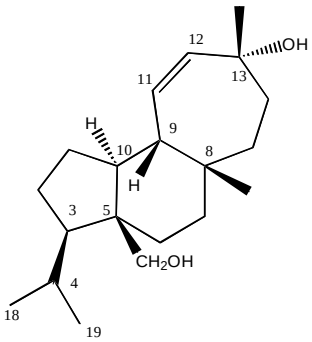
Materials and Methods

Compounds

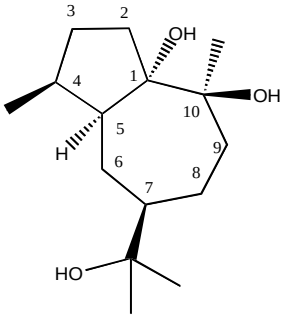
The compound assayed were azorellolide (**AZ1**), mulinol (**AZ2**), stachytriol (**AZ3**), 1α,10β,4β,5α-diepoxy-7β-germacran-6β-ol (**AZ4**), 1β,10α,4β,5α-diepoxy-7β-germacran-6β-ol (**AZ5**), 1,2,3,3α,4,5,6,7,8,8α-decahydro-7-(1-hydroxy-1-methylethyl)-1,4-dimethylazulene-3α,8α-diol (**AZ6**), madreporanone (**AZ7**), and yaretol (**AZ8**) (**Fig. 1**). The eight compounds were previously identified and isolated from *A. cryptantha* aerial parts collected in Andean mountains by Lima et al. (2015).



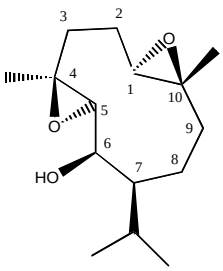
AZ1: azorellolide



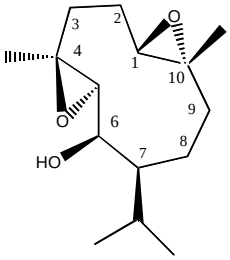
AZ2: mulinol



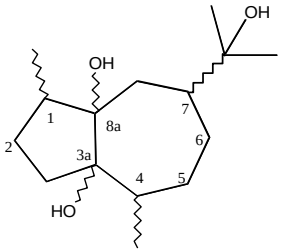
AZ3: stachytriol



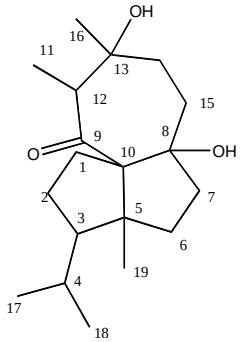
AZ4: 1α, 10β, 4β, 5α-diepoxy-7β-germacran-6β-ol



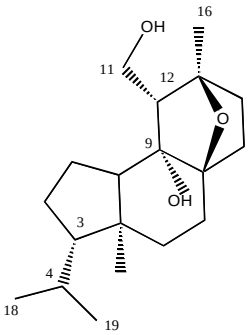
AZ5: 1β, 10α, 4β, 5α-diepoxy-7β-germacran-6β-ol



AZ6: 1,2,3,3a,4,5,6,7,8,8a-decahidro-7-(1-hydroxy-1-methylethyl)-1,4-dimetilazulene-3a,8a-diol



AZ7: madreporanone



AZ8: yaretol

Figure 1. Compounds **AZ1-AZ8**

Parasites and mammalian cells

T. cruzi epimastigotes (Dm28c strain, DTU: TcI) were cultured at 28 °C in Diamond medium, supplemented with 10% inactivated fetal bovine serum (Gibco) and 12.5 µg/mL hemin, according to Spina et al. (2018). Vero cell line (ATCC CCL-81) was maintained in DMEM supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified incubator.

Proliferation and viability assays

Epimastigotes (3×10^6 cells) were incubated at 28 °C in sterile tubes with 10 µg/mL of each terpenoids (**AZ1**-**AZ8**) and adjusted to 1 mL with Diamond medium. Aliquots were collected every 24 h, and fixed with 2% paraformaldehyde in PBS. Parasites were counted in a Neubauer haemocytometer (Sülsen et al. 2010). The most active compound (**AZ8**) was then assayed at concentrations of 3.25, 6.5 and 13 µM. Control cultures either without any compound were used in all experiments. BZN was used at 9.6 µM as a reference drug. To determine the viability of epimastigotes, aliquots of the treated culture were taken and placed on slides for 3 minutes with 2% eosin in PBS. The MTT assay (Abdallah et al. 2020) was employed to determine Vero cell line viability (Catunda et al. 2017).

Ultrastructural analysis by transmission electron microscopy

The ultrastructural study was carried out according to Spina et al. (2018). Ultrathin sections with interference grey colour were cut with an ultramicrotome Ultracut R Leica (Wien, Austria) and counterstained with uranyl acetate and lead citrate. Samples were analysed in EM 900 Zeiss (Jena, Germany) electron microscope.

Statistical analysis

The Student's t-test was used to determine the statistical. The effect of each treatment was analysed by one-way ANOVA. The level of significance was set at $p<0.05$. All statistical analyses were performed using the software Statistica 5.1 (StatSoft, Inc.). Results were expressed as the mean ± S.D.

Results

Activity of terpenoids on proliferation and viability of T. cruzi epimastigotes

The *in vitro* inhibitory effect on *T. cruzi* epimastigotes of terpenoids **AZ1**–**AZ8** (10 µg/mL) was measured. At 48 h of incubation, compounds **AZ1-3, 5** and **6** caused a slight inhibition in the proliferation of the parasites (no-significant differences were observed). In contrast, the terpenoids **AZ4** and **AZ7** stand out with moderate activity, while **AZ8** showed powerful antiproliferative activity on *T. cruzi* (Fig. 2A). Parasites treated with the most active terpenoids (**AZ7** and **AZ8**) were further incubated for 72 h (Fig. 2B). Both compounds, **AZ7** and **AZ8**, exerted a significant inhibitory activity on the proliferation of epimastigotes.

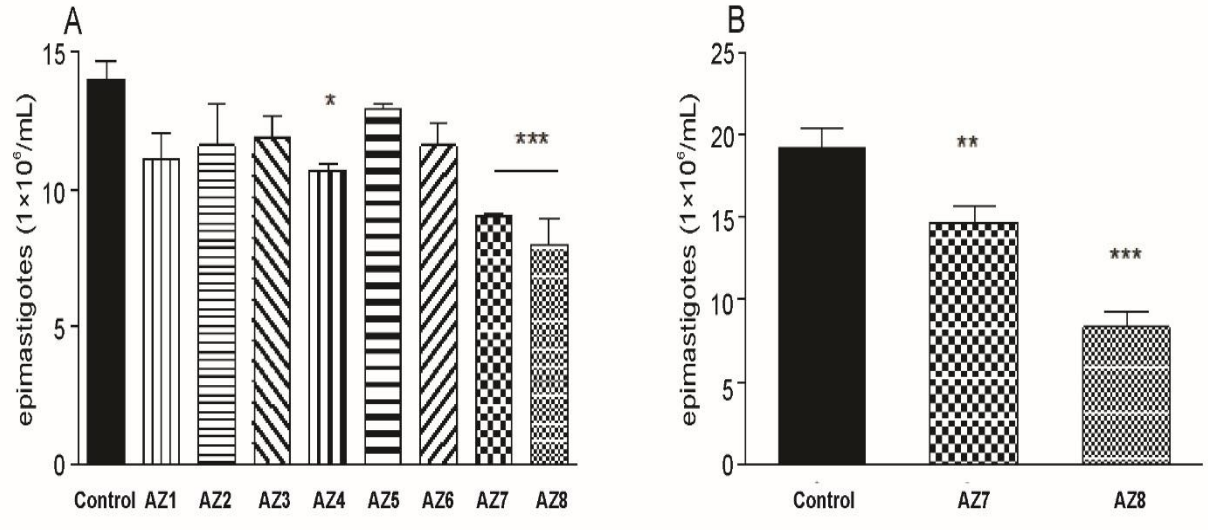


Figure 2. A. Epimastigotes treated with **AZ1-AZ8** (10 µg/mL, 48 h). **B.** Epimastigotes treated with **AZ7** and **AZ8** (10 µg/mL, 72 h.) Values are expressed as number of epimastigotes (means ± SD) from three independent experiments. Significant differences as compared to control cultures (*p < 0.05, **p < 0.01, and ***p < 0.001)

Since **AZ8** showed higher activity than the rest of the terpenoids, lower concentrations (3.25, 6.5 and 13 µM) were evaluated, and the IC_{50} was calculated, obtaining a value of 6.38 ± 0.47 µM at 48 h (Fig. 3).

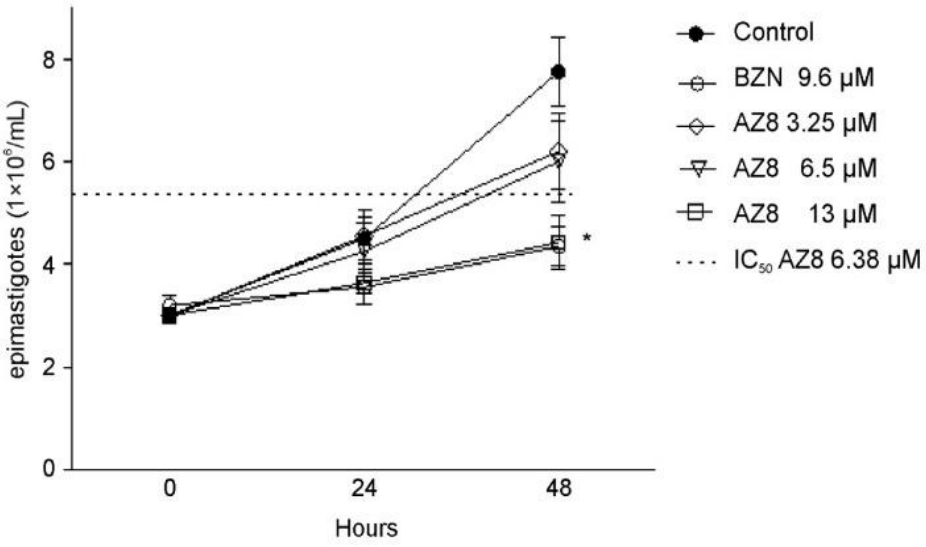


Figure 3. Proliferation of epimastigotes with different concentrations of **AZ8**. Values are expressed as number of epimastigotes (means ± SD) from three independent experiments. Significant difference from control cultures (*p < 0.05).

The cytotoxic activity of **AZ8** on epimastigotes was evaluated by employing the eosin exclusion method (Table 1). **AZ8** did not affect parasite viability. The mammalian cell viability was measured on Vero cell line by the MTT method, obtaining a CC_{50} value for **AZ8** of 8.87 ± 0.43 µM.

Table 1. Effect of **AZ8** on viability of the epimastigotes and Vero cells, using the eosin exclusion method and MTT assay, respectively.

AZ8		Viability (%)	
µM	**(µg/mL)**	**Epimastigotes**	**Vero cell line**
3.25	2.5	98 ± 0.58	63.85 ± 6.62
6.5	5.0	98 ± 0.80	57.12 ± 2.46
13	10.0	99.27 ± 0.58	42.69 ± 0.62

Values are expressed as percentages of viable cells (means ± SD) after 48 h of incubation.

Ultrastructural analysis in epimastigotes of T. cruzi treated with Yaretol (AZ8)

Transmission electron microscopy (TEM) is used as an approach to determining the cellular targets of bioactive compounds (Menna–Barreto et al. 2010). Figure 4, shows that the treatment of epimastigotes for 48 h with 13 µM of AZ8 did not induce major ultrastructural changes on parasites.

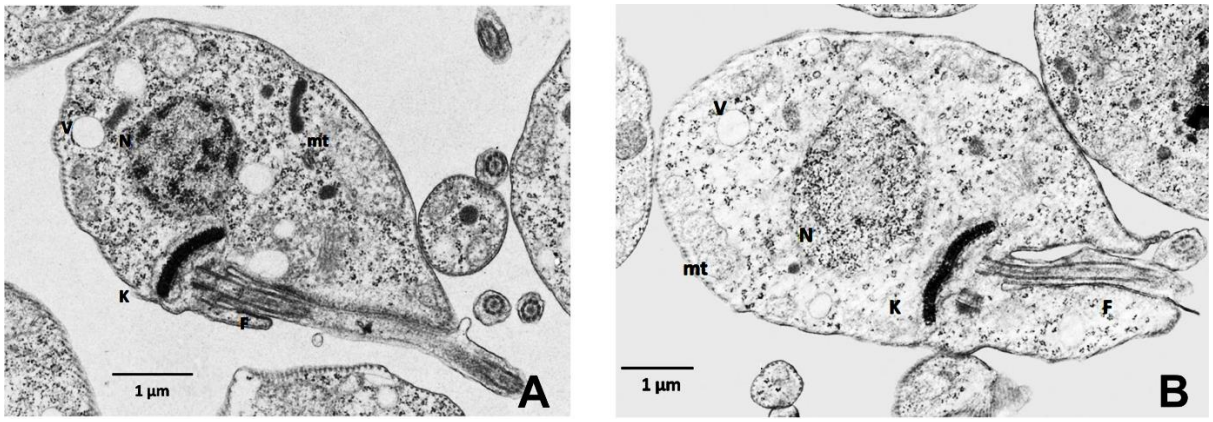


Figure 4. Transmission electron microscopy of *T. cruzi* epimastigotes untreated (A) and treated (B) for 48 h with 13 µM of **AZ8**. V: vacuoles; N: nucleus; K: kinetoplast; mt: mitochondrion; F: flagellum.

Discussion

The few currently available therapeutic alternatives for Chagas disease, together with their side effects, have motivated the search for new active compounds against *T. cruzi*. Dzul-Beh et al. (2020) reported the pharmacology of remarkably interesting diterpenoids isolated form *Azorella* genera. Herein, eight terpenoids, previously isolated from *A. cryptantha* (Lima et al. 2015) were assayed *in vitro* against *T. cruzi* epimastigotes. The compounds **AZ7** and **AZ8** affecting parasites proliferation but not viability. Similarly, the cytostatic rather than cytotoxic effect of **AZ8** against epimastigotes form has also been reported for other compounds (Spina et al. 2018). Likewise, compounds with low cytotoxicity but high inhibition of epimastigotes proliferation have reported strong activity against trypomastigotes and amastigotes (Lozano et al. 2012). Even the IC_{50} value of yaretol against epimastigotes was considerably more effective than that reported for BZN in the same strain of *T. cruzi* (Dm28c) (6.38 and 19.2 µM, respectively). On the other hand, **AZ8** was not able to induce any change on the ultrastructure of parasite, similarly to other compounds, including BZN (Lozano et al. 2012; 2015; Frank et al. 2013; Palace-Berl et al. 2013).

On the other hand, the parasite *T. cruzi* is a very heterogeneous species presenting a high genetic variability. There is into six discrete typing units (DTUs) for *T cruzi*, TcI-TcVI and one more Seventh TcBat, basically found in bats (Zingales and Bartholomeu 2021). Among the DTUs, TcI is the lineage that shows the highest genetic heterogeneity. TcI is ubiquitous in the sylvatic cycle in which about 50 mammalian genera are naturally infected. In regions like South America where this lineage is prevalent, parasites isolated from patients linked to acute and fatal cases in oral transmission were identified as TcI (Grisard et al. 2014; Dario et al. 2016; Faria et al. 2018). De Souza and Barrias (2021) proposed a new scheme of the life cycle of *T. cruzi*, where epimastigotes-type forms with infective and proliferative capacity are described, highlight the importance of taking measures on these stages of the parasite because they are a new target for treat during the course of Chagas disease. In this new scenario, the greater anti-epimastigote activity of yaretol (**AZ8**) over BZN and low concentrations of cytotoxicity in Vero cell line, make **AZ8** a promising candidate for the treatment of Chagas disease or for the development of semi-synthetic drugs active against *T. cruzi*. As a result, further research is necessary to explore potential of **AZ8**.

Conclusion

The norditerpenoid yaretol (AZ8) isolated from *A. cryptantha*, presented interesting anti-proliferative activity against *T. cruzi* epimastigotes-type forms. Studies aimed at evaluating the effects of this compound on the infective forms of the parasite are in progress, in order to support the usefulness of yaretol as a potential lead compound for the treatment of Chagas disease.

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Author contributions

RMS-Z, P.B., M.P. and P.B. carried out experiments; A.T., M.A.S., P.B. and G.E.F. performed the design of the study. RMS-Z, M.P and G.E.F. wrote the manuscript.

RMS-Z, M.P. M.A.S. and G E.F. analysed experimental results and revised the whole manuscript.

Funding details

The authors are grateful to Consejo Nacional de Investigaciones Científicas y Técnicas, CICITCA-UNSJ Argentina., and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT). SECYTP UNCuyo. PICT 2020-SERIEA-I-03883. Red 223RT0140 (RENATEC) CYTED.

Arabian Journal of Medicinal and Aromatic Plants,

ISSN 2458-5920, www.ajmap.info