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Original Article

Anti-*Trypanosoma cruzi* activity of alkaloids isolated from *Habranthus brachyandrus* (Amaryllidaceae) from Argentina

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ABSTRACT

Background: Chagas disease, caused by the parasite *Trypanosoma cruzi*, affects over six million people worldwide, mainly in Latin American countries. Currently available drugs have variable efficacy in the chronic phase and significant side effects, so there is an urgent need for safer chemotherapeutic treatments. Natural products provide privileged structures that could serve as templates for the synthesis of new drugs. Among them, Amaryllidaceae plants have proved to be a potential natural source of therapeutical agents due to their rich diversity in alkaloids.

Purpose: To identify alkaloids with anti-*T. cruzi* activity from *Habranthus brachyandrus* (Baker) Sealy (Amaryllidaceae, subfamily Amaryllidoideae) collected in Argentina.

Methods: An *H. brachyandrus* alkaloid extract was tested against *T. cruzi*, and its cytotoxicity profile was evaluated against two mammalian cell lines to ascertain its selectivity against the parasite and potential liver toxicity. It was also assessed by a stage-specific anti-amastigote assay and analysed by GC/MS to determine its alkaloid profile. The isolated alkaloids were also tested using the aforementioned assays.

Results: The extract showed high and specific activity against *T. cruzi*. The alkaloids lycoramine, galanthindole, 8-*O*-demethylmaritidine, 8-*O*-demethylhomolycorine, nerinine, trisphaeridine, deoxytazettine, and tazettamide were identified by means of GC-MS. In addition, hippeastidine (also named aulicine), tazzetine, ismine, and 3-epimacronine were isolated. The alkaloid ismine was specifically active against the parasite and had low toxicity against HepG2 cells, but did not show anti-amastigote activity.

Conclusion: The extract had specific anti-*T. cruzi* activity and the isolated alkaloid ismine was partially responsible of it. These results encourage further exploration of *H. brachyandrus* alkaloids in search of novel starting points for Chagas disease drug development.

Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is an anthropozoonosis affecting over six million people worldwide. It is endemic in Latin America, where it is the parasitic disease with the greatest socioeconomic impact, causing over 800,000 disability-adjusted life years and ~7500 deaths *per* year (Kratz, 2019; WHO, 2021). In the last decades, the disease has spread beyond its natural geographical boundaries due to migratory movements and is now a global health issue (WHO, 2021).

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The clinical course of Chagas disease usually comprises two phases. The initial acute phase is generally asymptomatic and thus frequently goes undiagnosed and untreated. This is followed by a chronic phase that can be clinically silent for life. Nevertheless, around 30–40% of chronically infected individuals will develop digestive and/or cardiac symptomatology, cardiac tissue alterations being the most frequent and leading cause of morbidity and mortality.

There is no vaccine available for Chagas disease and current specific chemotherapy for *T. cruzi* infections relies only on benznidazole (BNZ) and nifurtimox (NFX). Both drugs, in use since the early 1970s, have several drawbacks, including variable efficacy in the chronic phase and frequent toxic side-effects that often lead to treatment interruption (Kratz, 2019). Therefore, new drugs with improved efficacy and safety profiles are urgently needed.

In this regard, natural products constitute a source of highly underexplored chemical entities with privileged structures and bioactivity, which could serve as templates for the synthesis of new drugs. In fact, 61% of new chemical entities approved as anti-infective drugs over the period 1981–2019 were of natural origin (Newman and Cragg, 2020).

Almost 400 species belonging to more than 100 plant families have been studied for a possible Chagas disease treatment (Santos et al., 2020; Varela et al., 2018). Several compounds with anti-trypanosome activity have been extracted from plants, including alkaloids, terpenoids, flavonoids, and quinonoids (Salem and Werbovetz, 2006). Alkaloids are of particular interest in biomedicine and drug discovery research due to their structural diversity and specific biological potential (Daley and Cordell, 2021). The Amaryllidaceae is a plant family that contains an exclusive, large and still expanding alkaloids group, which is characterized by unique skeleton arrangements and a broad spectrum of biological activities (Berkov et al., 2020).

Overall, Amaryllidaceae alkaloids are represented by four major types (lycorine, crinine, haemanthamine and galanthamine (GAL)), three minor types (tazettine, homolycorine and montanine), as well as less-conspicuous congeners such as cherylline, trisphaeridine and ismine (Berkov et al., 2020).

Amaryllidaceae are distributed in tropical and subtropical regions, coinciding with areas conventionally associated with a rich culture in folk medicine. For instance, they have been used for their therapeutic activity in cancer, fever, swelling, and some parasitic diseases (Berkov et al., 2020). Indeed, around 15 species of Amaryllidaceae have been indicated for their use against parasitic infections (Nair and van Staden, 2019). Among them, extracts or alkaloids isolated from the genera *Crinum, Hippeastrum, Rhodophiala,* and *Zephyranthes* have been previously reported for their anti-parasitic activity (Martinez-Peinado et al., 2021a, 2020b; Nair and van Staden, 2019). In Argentina, the Amaryllidaceae family includes around 61 species belonging to ten genera (*Chlidanthus, Crinum, Habranthus, Haylockia, Hieronymiella, Hippeastrum, Phycella, Rhodophiala, Stenomesson* and *Zephyranthes*).

Habranthus brachyandrus (Baker) Sealy (synonym *Hippeastrum brachyandrum* Baker & Beknopte, *Zephyranthes brachyandra* (Baker) Backer, *Zephyranthes porphyrospila* E. Holmb.) (WFO, 2021), an endemic species from South America (Arroyo-Leuenberger, 1996) has been previously studied for its chemical composition. Among the reported alkaloids there were lycorine, lycorenine, habranthine haemanthamine, haemanthidine, (+)-bulbispermine, galanthine, 10-*O*-demethylgalanthine and pancratistatine (Boit and Döpke, 1959; Jitsuno, 2009; Wildman and Brown, 1968). In this work, the alkaloid extract obtained from the bulbs of *H. brachyandrus* and four isolated alkaloids were evaluated for their activity against *T. cruzi*.

Material and methods

Plant material

H. brachyandrus was cultivated under greenhouse conditions, kindly provided for research in May 2017 and authenticated by German Roitman, MSc, (Facultad de Turismo y Urbanismo, Universidad Nacional de San Luis, Av. del Libertador San Martín 721 (D5881DFN) Villa de Merlo, San Luis, Argentina). A sample was deposited in the herbarium of Universidad de Buenos Aires under the code: IBT-UNSJ-Arg.19.

Alkaloid extraction and isolation

Dry powdered bulb material (200 g) was macerated in H_2SO_4 2% for 4 h in an ultrasonic bath (3 × 1000 ml). Subsequently, samples were centrifuged at 5000 x g (10 min), and the supernatant was transferred to another flask where it was defatted with di-ethyl-ether (3 × 500 ml). The aqueous solution was led to pH 11–12 with 10% NaOH and the alkaloids were extracted with dichloromethane (3 × 500 ml). The organic phase was dried with anhydrous sodium sulfate and then evaporated to obtain the basic alkaloid extract, named AE-Hbr (194 mg).

AE-Hbr (120 mg) was roughly separated by SiO_2 (100 g) flash column chromatography (CC) using an *n*-hexane/EtOAc/MeOH gradient to give three fractions (A − C): 24 mg A (*n*-hexane), 58.5 mg B (EtOAc), 32.25 mg C (MeOH). CC on Sephadex LH-20 (30 cm length, 2.5 cm i.d.) of fraction A gave 3 subfractions (6.75 mg A1, 11.55 mg A2, and 2.25 mg A3). Subfraction A2 was then subjected to preparative TLC using *n*-hexane/EtOAc/MeOH 4.5:5:0.5 as the mobile phase in NH_3 atmosphere to give 3.9 mg of ismine (**1**). Likewise, fraction B was permeated through a Sephadex LH-20 column using MeOH as the eluent to give four subfractions (9.6 mg B1, 18.1 mg B2, 23.25 mg B3, and 6.52 mg B4). Crystallization of subfraction B3 afforded tazettine (**9**) (14.2 mg). Subfraction B2 was subjected to preparative TLC using EtOAc/MeOH 9.5:0.5 as the mobile phase in NH3 atmosphere to give 10.3 mg of hippeastidine (**8**). Finally, a silica gel CC using gradient elution from EtOAc/MeOH (8:2 − 1:9) was applied to fraction C to give four subfractions (2.5 mg C1, 3.1 mg C2, 20.4 mg C3, and 3.6 mg C4). Subfraction C3 was subjected to preparative TLC using EtOAc/MeOH 9:1 as the mobile phase in NH_3 atmosphere to give 2.8 mg of 3-epimacronine (**11**). To monitor the isolation process, column fractions were subjected to analytical TLC. Then column fractions were analyzed, applying UV light, iodine vapors resublimed and/or Dragendorff's reagent, and similar ones were combined. Their structures were confirmed by NMR spectroscopy, the results being in agreement with literature data (Bastida et al., 2006). Based on the ^{1}H and ^{13}C NMR data, the purities of the isolated alkaloids ismine (**1**), tazettine (**9**), hippeastidine (**8**), and 3-epimacronine (**11**) were 96%, 94%, 96%, and 95%, respectively (Figs. S3–6).

GC-MS analysis

The alkaloids were identified by comparing their GC-MS spectra and Kovats retention index (RI) values against authentic Amaryllidaceae alkaloids previously isolated and identified. Spectral data were processed with AMDIS 2.64 software. Alkaloids were identified by comparing their fragmentation pattern and RI with those of the Amaryllidaceae alkaloids of our laboratory library, in which the isolated compounds were identified by NMR and other spectroscopic techniques (UV, CD, MS), as well as the NIST database and literature data. RI values were calibrated with an *n*-hydrocarbon calibration mixture (C9-C36), and compounds were semi-quantified according to Torras-Claveria et al. (2014) and expressed as μg GAL/mg AE-Hbr as well as μg GAL/100 mg of bulb dry weight.

Host cells and T. cruzi parasite cultures

Vero (green monkey kidney epithelial cells), LLC-MK2 (Rhesus monkey kidney epithelial cells) and HepG2 (human liver epithelial cells) were cultivated as previously described (Martinez-Peinado et al., 2020b). *T. cruzi* Tulahuen-β-galactosidase parasites (Discrete Typing Unit VI) were kept in culture by infection of LLC-MK2 cells as described (Martinez-Peinado et al., 2020b).

T. cruzi growth inhibition assay

The anti-*T. cruzi* assay was performed as previously described (Martinez-Peinado et al., 2020b). The assay test plates were prepared with starting concentrations of 754 μg/ml of extract and 500 μM of alkaloid, which were diluted following a dose-response pattern. The percentage of DMSO was kept below 0.5% in all the wells. Vero cells and purified trypomastigotes were diluted at a concentration of 1×10^6 *per* ml and mixed at a volume/volume (1:1). Then, 100 μl of the solution was added *per* well (50,000 Vero cells and trypomastigotes), the multiplicity of infection being 1 (MOI) (Martinez-Peinado et al., 2020b). Each run contained the reference drug BNZ as well as positive and negative controls, as described (Martinez-Peinado et al., 2020b). Plates were incubated for 4 days at 37 °C. Assay read out was performed by adding 50 μl *per* well of a PBS solution containing 0.25% NP40 and 500 μM chlorophenol red-β-D-galactoside (CPRG) substrate, incubating for 4 h at 37 °C and recording the absorbance at 590 nm, as described (Buckner et al., 1996). All experiments were performed at least in triplicate.

Anti-amastigote specific activity

An anti-amastigote assay was performed as previously reported (Martinez-Peinado et al., 2021b). Briefly, 5×10^6 Vero cells were seeded in a T-175 flask and cultured for 24 h. Then, cells were washed and infected with 1×10^7 trypomastigotes (MOI ~ 1). After 18 h, infected cell monolayers were washed with PBS and detached from the flask. Cells were counted and diluted to a concentration of 5×10^5 cells *per* ml, before adding 100 μl *per* well to test plates already containing the extract or alkaloids as previously described. We included BNZ, positive and negative controls, as described (Martinez-Peinado et al., 2021b).

Cell toxicity assays

Vero and HepG2 cell toxicity assays were performed as described (Martinez-Peinado et al., 2020b). Briefly, Vero and HepG2 cells were respectively diluted at a concentration of 5×10^5 and 3.2×10^5 cells *per* ml, before adding 100 μl *per* well. Each run contained its own negative and positive controls as described (Martinez-Peinado et al., 2020b). Plates were incubated at 37 °C for 4 days in the case of Vero cells, and 2 days for HepG2 cells. Then, 50 μl of a PBS solution containing 10% AlamarBlue was added to each well and the plates were incubated for another 6 h at 37 °C before reading the fluorescence intensity (excitation: 530 nm, emission: 590 nm) as described (Martinez-Peinado et al., 2020b). All experiments were performed at least in triplicate.

Statistical analysis

The absorbance and fluorescence values were normalized to the controls as described (Martinez-Peinado et al., 2020b). IC_{50} and TC_{50} values were determined with GraphPad Prism 7 software (version 7.00, 2016) using a non-linear regression analysis model ([Inhibitor] versus normalized response – Variable slope) (Martinez-Peinado et al., 2020b). Values provided are means and standard deviation (mean ± SD) of at least three independent experiments.

Results

Alkaloid profile analysis

To elucidate the alkaloid profile, AE-Hbr was analyzed by GC/MS, comparing the fragmentation patterns of the compounds in the extract against those of the alkaloids belonging to the GC-MS library (Fig. S1). Twelve alkaloids were identified from AE-Hbr by GC-MS analysis, involving nine Amaryllidaceae skeleton types (Berkov et al., 2020). The GC-MS results are presented in Table 1, which shows the alkaloid composition of the extract, as well as the retention index and semi-quantitation expressed as μg GAL/mg AE-Hbr and μg GAL/100 mg of bulb dry weight for each alkaloid.

Table 1
GC-MS data of the alkaloid extract from *H. brachyandrus* (AE-Hbr). Values are expressed as μg GAL/mg AE-Hbr and μg GAL/100 mg of dry weight (DW).

Alkaloid	RI[1]	μg GAL/mg AE-Hbr	μg GAL/100 mg DW
Crinine-type			
Hippeastidine (**8**)	2650.9	54.77	18.13
Galanthamine-type			
Lycoramine (**3**)	2457.4	0.91	0.30
Galanthindole-type			
Galanthindole (**5**)	2535.2	2.68	0.89
Haemanthamine-type			
8-*O*-demethylmaritidine (**6**)	2540.6	1.15	0.38
Homolycorine-type			
8-*O*-Demethylhomolycorine (**10**)	2781.3	1.01	0.33
Nerinine (**4**)	2491.1	1.04	0.35
Ismine-type			
Ismine (**1**)	2306.0	3.99	1.32
Narciclasine-type			
Trisphaeridine (**2**)	2328.8	1.91	0.63
Pretazettine-type			
Deoxytazettine (**7**)	2570.9	0.98	0.33
Tazettine (**9**)	2689.2	42.77	14.16
3-epimacronine (**11**)	2840.8	3.49	1.16
Miscellaneous-type			
Tazettamide (**12**)	2961.2	0.91	0.30

[1] RI: Kovats retention index.

Based on the semi-quantitation analysis, the alkaloids found in highest abundance were hippeastidine (also named aulicine), tazettine and, to a lesser extent, ismine and 3-epimacronine (see μg GAL/mg AE-Hbr in Table 1). Alkaloid structures are shown in Fig. 1 (MS data of the alkaloids are provided in Table S1).

Anti-T cruzi activity of AE-Hbr

AE-Hbr was tested against *T. cruzi* Tulahuen-β-galactosidase parasites, using Vero cells as hosts (Martinez-Peinado et al., 2020b). AE-Hbr showed an IC_{50} value of 0.56 ± 0.03 μg/ml, which is similar to that of BNZ (IC_{50} = 0.4 ± 0.01 μg/ml) (Table 2, Fig. 2A).

AE-Hbr was then evaluated in a Vero cell toxicity assay to discern whether the activity observed was specific against the parasite. Compared to BNZ, the extract displayed higher toxicity against Vero cells, showing a TC_{50} value of 14.24 ± 4.53 μg/ml (Table 2, Fig. 2B). As previously described, a selectivity index (SI, or TC_{50} to IC_{50} ratio) > 10 was considered for progression of the extract (Martinez-Peinado et al., 2020b). AE-Hbr committed to this threshold with a SI value of 25.42, so it was tested in a HepG2 cell toxicity assay, a model used to predict acute liver toxicity. Digitoxin was included in every round and an average TC_{50} value of 0.19 ± 0.06 μM [i.e., 0.15 ± 0.04 μg/ml] was obtained. As in the Vero cell toxicity assays, AE-Hbr was more toxic than BNZ against HepG2 cells, with a TC_{50} value of 16.20 ± 3.56 μg/ml (Table 2, Fig. 2C).

Amastigotes, the intracellular replicative forms infecting mammals in the parasite life cycle, are likely the main target for any prospective drug to treat chronic *T. cruzi* infections. Thus, AE-Hbr was evaluated by means of a biological assay specifically targeting amastigotes and found to have a potent IC_{50} value of 0.66 ± 0.02 μg/ml and a SI window > 10 (SI = 21.57), indicating specific anti-amastigote activity (Table 2, Fig. 2D).

AE-Hbr exhibited highly specific anti-parasitic activity and low toxicity in both mammalian cell lines. In addition, we assessed the anti-parasitic effects of the alkaloids isolated from AE-Hbr: hippeastidine (**8**), tazettine (**9**), ismine (**1**) and 3-epimacronine (**11**).

Ismine exhibited an IC_{50} value of 31.13 ± 1.10 μM (Table 2), which was less potent than that of BNZ (Fig. 3A). After progressive testing in the Vero and HepG2 cell toxicity assays, it showed TC_{50} values ≥ 300

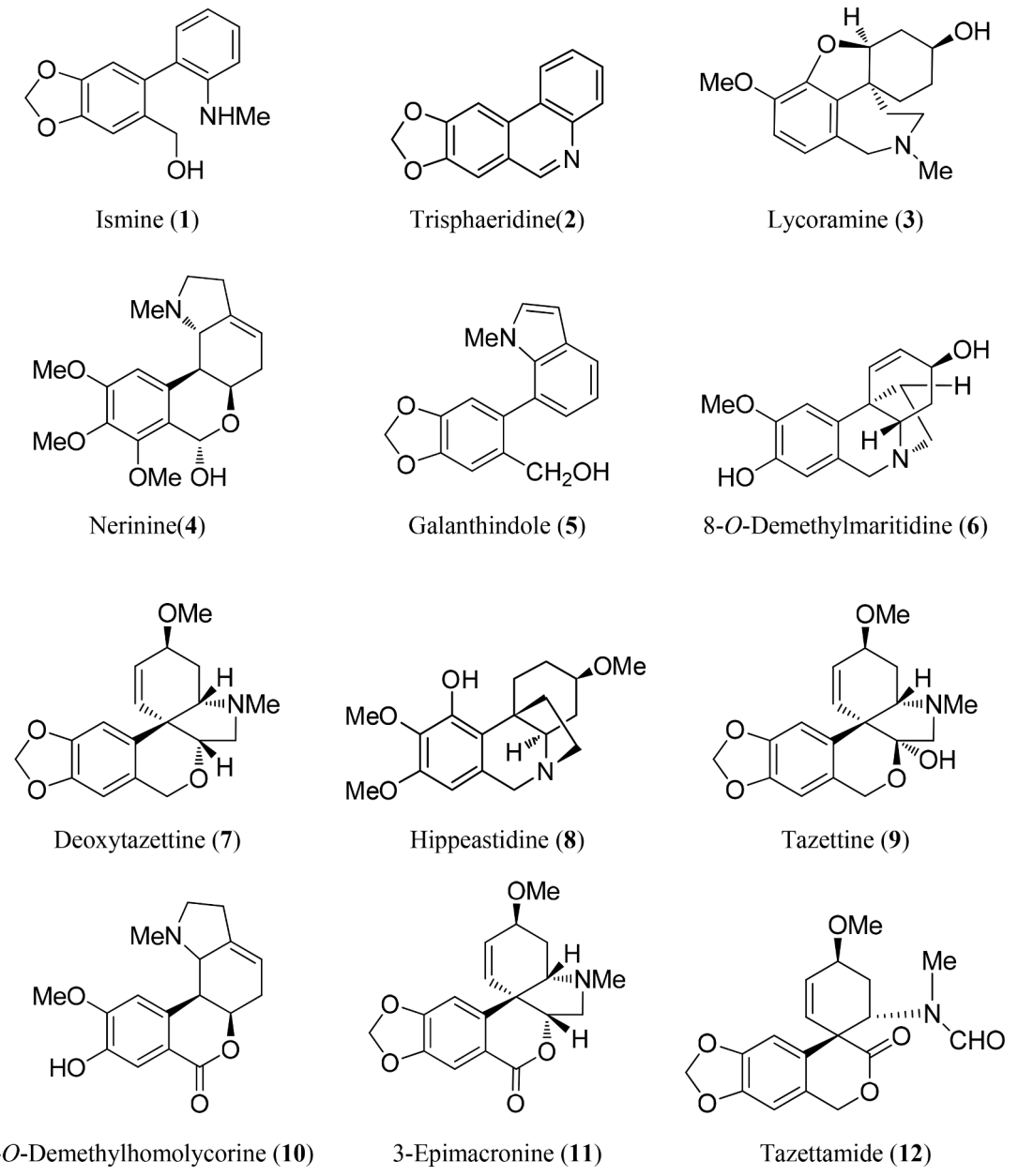

Fig. 1. Isolated alkaloids: Ismine (**1**), Hippeastidine (**8**), Tazettine (**9**), and 3-Epimacronine (**11**). Alkaloids identified by means of GC-MS: Trisphaeridine (**2**), Lycoramine (**3**), Nerinine (**4**), Galanthindole (**5**), 8-*O*-demethylmaritidine (**6**), Deoxytazettine (**7**), 8-*O*-demethylhomolycorine (**10**).

Table 2
Antiparasitic and cytotoxic activities of the extract from *H. brachyandrus* (AE-HBr) and isolated alkaloids.

Extract / Compounds	Assays					
	Anti-*T. cruzi*	Toxicity Vero Cells		HepG2	Anti-amastigote	
	IC_{50} [a]	TC_{50} [b]	SI [c]	TC_{50} [b]	IC_{50} [a]	SI [c]
HBr-AE	**0.56 ± 0.03** µg/ml	**14.24 ± 4.53** µg/ml	**25.42**	**16.20 ± 3.56** µg/ml	**0.66 ± 0.02** µg/ml	**21.57**
Ismine (**1**)	31.13 ± 1.10 µM	> 300 µM	> 10	> 300 µM	191.7 ± 3.75 µM	1.56
Hippeastidine (**8**)	> 150 µM	–	–	–	–	–
3-Epimacronine (**11**)	> 400 µM	–	–	–	–	–
BNZ[d]	1.55 ± 0.05 µM	269.4 ± 14.39 µM	174.05	193.8 ± 28.92 µM	2.02 ± 0.09 µM	131.36

[a] Half-maximal inhibitory concentration,.
[b] half-maximal toxic concentration,.
[c] selectivity index,.
[d] positive control. (-) Not assayed.

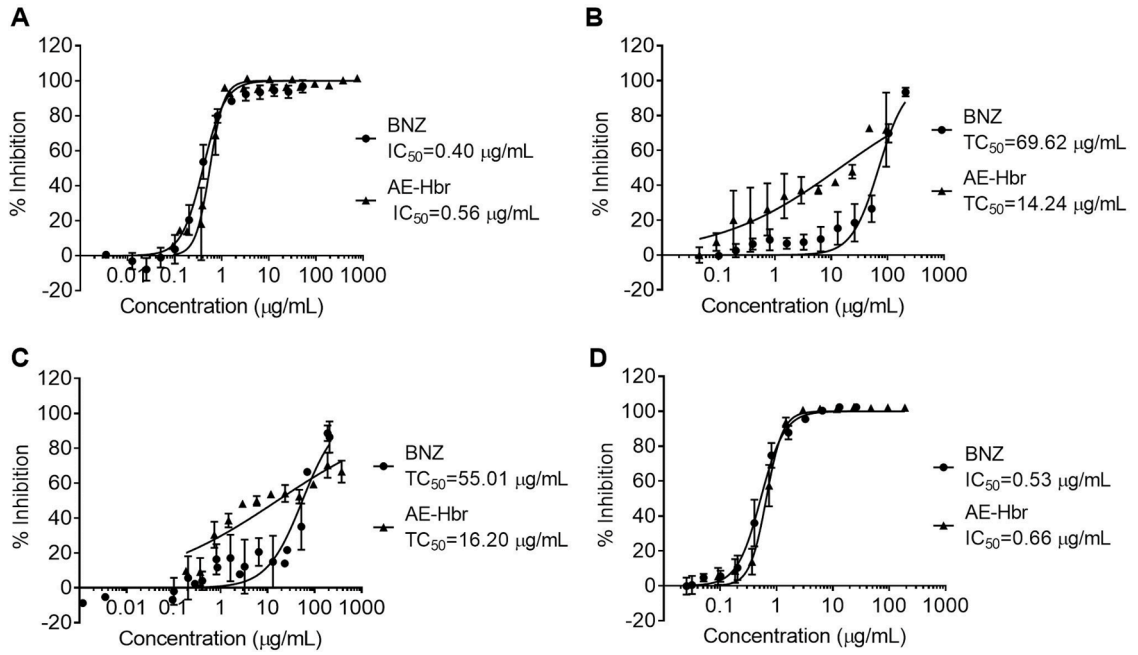

Fig. 2. Dose-response curves of AE-Hbr (triangles) and BNZ (circles). (A) Anti-*T. cruzi* assay. (B) Vero cells toxicity assay. (C) HepG2 cells toxicity assay. (D) Anti-amastigote assay.

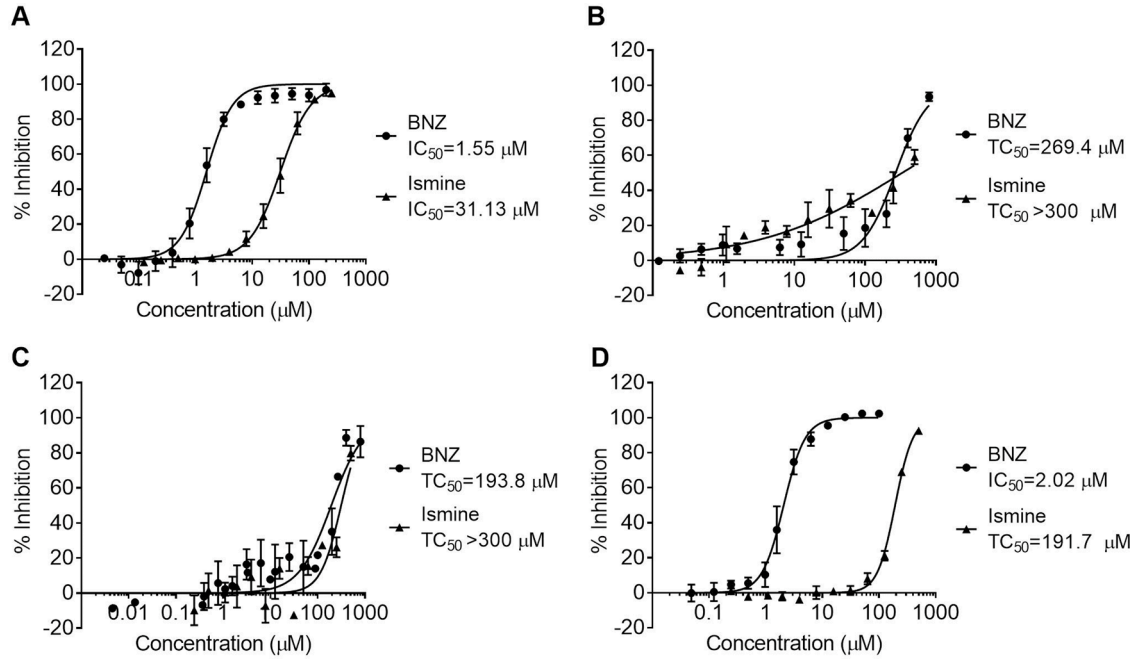

Fig. 3. Dose-response curves of ismine (triangles) and BNZ (circles). (A) Anti-*T. cruzi* assay. (B) Vero cells toxicity assay. (C) HepG2 cells toxicity assay. (D) Anti-amastigote assay.

μM, with low toxicity against both cell lines and specificity against the parasite. In addition, ismine was less toxic than BNZ against both cell lines (Table 2, Fig. 3B and C). However, it was found to be inactive against the amastigotes (Table 2, Fig. 3D).

Hippeastidine and 3-epimacronine, tested for the first time against *T. cruzi* in this study, were both found to be inactive (IC_{50} values > 150 μM) (Table 2, Fig. S2). Tazettine, which has been evaluated previously, was also inactive against *T. cruzi* (Martinez-Peinado et al., 2020b).

Discussion

The chemical profile of AE-Hbr showed the presence of twelve alkaloids, four of which, ismine (**1**), hippeastidine (**8**), tazettine (**9**) and 3-epimacronine (**11**), were isolated and characterized by means of NMR data. In other phytochemical studies of *H. brachyandrus*, using samples obtained in the Netherlands, Boit and Döpke (1959) and Wildman and Brown (1968) found a different alkaloid profile. Likewise, Jitsuno et al. (2009) reported a different alkaloidal composition of a *H. brachyandrus* bulb alkaloidal extract obtained from a sample purchased from a garden

center in Heiwaen, Japan. Causes of alkaloid diversity can be genetic or environmental, or an interaction between both factors, as well as the geographical origin of the species (Berkov et al., 2011). Finally, a mistake in taxonomic characterization is possible.

In this study, AE-Hbr was found to specifically inhibit the parasite growth. It also showed low toxicity against HepG2 cells and Vero cells, and was active against amastigote forms, with an IC_{50} value of 0.66 ± 0.02 μg/ml and an SI value of 21.57 (Table 2). The low toxicity observed against both mammalian cell lines is of interest and could be related to the absence of lycorine- and crinine-type alkaloids, which are reported to have cytotoxic properties (Nair et al., 2012; Nair and van Staden, 2014).

To determine their individual contributions to the biological activity of the extract, the alkaloids hippeastidine (**8**), ismine (**1**), and 3-epimacronine (**11**) were isolated and tested against the parasite. Ismine had specific activity against the *T. cruzi* mammalian forms with IC_{50} values equal to 31.13 ± 1.10 μM. Notably, it showed very low toxicity against Vero and HepG2 cells, which contributed to a good selectivity index.

Gasca et al. (2020) conducted *in silico* studies with Amaryllidaceae alkaloids, showing that ismine is highly hydrophobic, and therefore able to penetrate biological membranes such as the human intestinal and blood-brain barrier, and a substrate of the human P-glycoprotein. These pharmacokinetic properties could allow effective distribution of the compound if administrated orally, which is an important requirement for new antichagasic drugs.

Information on the mechanisms of action involved in the anti-protozoal effects of Amaryllidaceae alkaloids is scant in the literature. Amaryllidaceae alkaloids have shown potent antioxidant activities due to the presence of enol or phenol groups able to stabilize reactive oxygen species and reactive nitrogen species (Cortes et al., 2018). Since the 1980s, it has been known that several synthetic antioxidants inhibit the respiration and growth of *T. cruzi* in culture (Aldunate et al., 1986). Some aphorphine alkaloids with antioxidant properties have also shown anti-*T. cruzi* activity (Barbosa et al., 2021; Morello et al., 1994). For instance, Morello et al. (1994) found that boldine and other aporphine alkaloids were able to inhibit *T. cruzi* epimastigote growth. The most active compounds also inhibited cell respiration, suggesting that these alkaloids may act by blocking mitochondrial electron transport. Moreover, their anti-*T. cruzi* activity appears to be correlated with their antioxidative properties (Morello et al., 1994). Recently, another aporphine alkaloid, dicentrine-β-N-oxide, was described to have specific activity against *T. cruzi* trypomastigotes (IC_{50} = 18.2 μM, SI = 11) and the mechanism of parasite death was mitochondrial depolarization (Barbosa et al., 2021). Aporphine and Amaryllidaceae alkaloids, which belong to the isoquinoline class, are characterized by a benzene ring fused to a pyridine ring, and share antioxidant properties. Taking these previous works into consideration, it could be hypothesized that ismine is acting through a similar mechanism of action. In addition, genes encoding enzymes involved in mitochondrial oxidative phosphorylation are upregulated in amastigotes compared to trypomastigotes, suggesting that the respiratory capacity differs between these two parasite life stages (Li et al., 2016). Thus, this mechanism of action would also account for the lack of activity of ismine against amastigote forms. However, other mechanisms of action reported for other Amaryllidaceae alkaloids in cancer cell lines, such as apoptosis induction and/or interference with DNA, RNA, or protein synthesis, should not be discarded (Nair and van Staden, 2019). The identification of the molecular mode of action or potential targets of ismine is a major challenge that might be explored with *in silico* or chemical genomics approaches.

In any case, ismine was non-active when tested specifically against *T. cruzi* amastigotes and thus it would only be partially responsible for the anti-parasitic effects observed in AE-Hbr. Its lack of activity against amastigotes suggests that the anti-parasitic properties of the extract are caused by other less abundant alkaloid/s, or by a synergic action among them. Hippeastidine and 3-epimacronine were found to be inactive. Regarding the alkaloids with a lower content (< 1 μg GAL/mg AE), only 8-*O*-demethylmaritidine and 8-*O*-demethylhomolycorine have been tested against *T. cruzi* and both proved inactive (de Andrade et al., 2012; Kaya et al., 2011). Thus, the composition of AE-Hbr deserves further study, focusing on the anti-*T. cruzi* activity of the remaining alkaloids. Lycoramine is a GAL-type alkaloid that structurally differs from GAL in the absence of a double bond between C4 and C4a. GAL has been found to be inactive against *T. cruzi* (Osorio et al., 2010). Thus, hypothesizing that this small structural difference would not have a modifying effect, lycoramine is unlikely to contribute to the AE-Hbr anti-*T. cruzi* activity. Similarly, we would expect nerinine to be a poor antiprotozoal agent, based on previous work by de Andrade et al. (2012), in which three homolycorine-type alkaloids (homolycorine, 8-*O*-demethylhomolycorine and 6-*O*-methyllycorenine) were inactive against the *T. cruzi* strain Tulahuen C2C4. Deoxytazettine would also be expected to lack anti-*T. cruzi* activity, as tazettine and 3-epimacronine are reported to be inactive (Martinez-Peinado et al., 2020a). Therefore, the anti-*T. cruzi* effects of AE-Hbr may be due to the presence of trisphaeridine, galanthindole and/or tazettamide. Structure–antiprotozoal activity relationships have been poorly studied in Amaryllidaceae alkaloids. However, some results suggest that the presence of a methylendioxy group, a feature common to the three aforementioned alkaloids, and a tertiary non-methylated nitrogen, found in trisphaeridine, induces a higher anti-parasitic activity (Osorio et al., 2008). In any case, the alkaloids found in AE-Hbr in low amounts (<1 μg GAL/mg AE) warrant testing against *T. cruzi* to elucidate their potential contribution to the extract activity. To the best of our knowledge, this is the first time AE-Hbr and its isolated alkaloids have been evaluated for their anti-*T. cruzi* properties.

Conclusions

H. brachyandrus specifically inhibited *T. cruzi* growth, showed low toxicity to HepG2 cells and was active against amastigote forms. The chemical profile of AE-Hbr showed the presence of twelve alkaloids and four of them were isolated. Among them, ismine was shown to be partially responsible for the anti-parasitic effects of AE-Hbr. The results obtained in the present work encourage us to continue exploring the alkaloids that are also present in AE-Hbr in different amounts, including those that are present in minor proportions.

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Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

CRediT authorship contribution statement

Nieves Martinez-Peinado: Conceptualization, Writing – original draft. **Javier E. Ortiz:** Conceptualization, Writing – original draft. **Nuria Cortes-Serra:** . **Maria Jesus Pinazo:** Funding acquisition. **Joaquim Gascon:** Funding acquisition. **Alejandro Tapia:** . **German Roitman:** . **Jaume Bastida:** Conceptualization, Writing – original draft. **Gabriela E. Feresin:** Conceptualization, Writing – original draft. **Julio Alonso-Padilla:** Conceptualization, Writing – original draft.

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Supplementary materials

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References

Aldunate, J., Ferreira, J., Letelier, M.E., Repetto, Y., Morello, A., 1986. t-Butyl-4-hydroxyanisole, a novel respiratory chain inhibitor. Effects on *Trypanosoma cruzi* epimastigotes. FEBS Lett. 195, 295–297. https://doi.org/10.1016/0014-5793(86)80180-6.

Arroyo-Leuenberger, S.C., Zuloaga, F.O., Morrone, O., 1996. Amaryllidaceae. Catálogo de las plantas vasculares de la República Argentina I. Missouri Botanical Garden Press, St Louis, MO, USA, pp. 90–100.

Barbosa, H., Costa-Silva, T.A., Alves Conserva, G.A., Araujo, A.J., Lordello, A., Antar, G. M., Amaral, M., Soares, M.G., Tempone, A.G., Lago, J., 2021. Aporphine alkaloids from *Ocotea puberula* with anti-*Trypanosoma cruzi* potential - activity of dicentrine-β-N-oxide in the plasma membrane electric potentials. Chem. Biodivers. 18, e2001022 https://doi.org/10.1002/cbdv.202001022.

Bastida, J., Lavilla, R., Viladomat, F., Cordell, G.A., 2006. Chemical and biological aspects of *Narcissus* alkaloids. In: The Alkaloids, 63. Elsevier Inc. Press, Amsterdam, Netherlands, pp. 87–179. https://doi.org/10.1016/S1099-4831(06)63003-4.

Berkov, S., Bastida, J., Sidjimova, B., Viladomat, F., Codina, C., 2011. Alkaloid diversity in *Galanthus elwesii* and *Galanthus nivalis*. Chem. Biodivers. 1, 115–130. https://doi.org/10.1002/cbdv.200900380.

Berkov, S., Osorio, E., Viladomat, F., Bastida, J., Knölker, H.J., 2020. Chemodiversity, chemotaxonomy and chemoecology of Amaryllidaceae alkaloids. In: The Alkaloids, 83. Elsevier Inc. Press, Amsterdam, Netherlands, pp. 113–185. https://doi.org/10.1016/bs.alkal.2019.10.002.

Boit, H.G., Döpke, W., 1959. Alkaloide aus *Hippeastrum brachyandrum* und *Hippeastrum rutihm*. Chem. Ber. 92, 2582–2584.

Buckner, F.S., Verlinde, C.L., La Flamme, A.C., Van Voorhis, W.C., 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. Antimicrob. Agents Chemother. 40, 2592–2597. https://doi.org/10.1128/AAC.40.11.2592.

Cortes, N., Castañeda, C., Osorio, E.H., Cardona-Gomez, G.P., Osorio, E., 2018. Amaryllidaceae alkaloids as agents with protective effects against oxidative neural cell injury. Life Sci. 203, 54–65. https://doi.org/10.1016/j.lfs.2018.04.026.

Daley, S., Cordell, G.A., 2021. Alkaloids in contemporary drug discovery to meet global disease needs. Molecules 26, 3800. https://doi.org/10.3390/molecules26133800.

de Andrade, J.P., Pigni, N.B., Torras-Claveria, L., Berkov, S., Codina, C., Viladomat, F., Bastida, J., 2012. Bioactive alkaloid extracts from *Narcissus broussonetii*: mass spectral studies. J. Pharm. Biomed. Anal. 70, 13–25. https://doi.org/10.1016/J.JPBA.2012.05.009.

Gasca, C.A., Moreira, N.C.S., de Almeida, F.C., Dutra Gomes, J.V., Castillo, W.O., Fagg, C. W., Magalhães, P.O., Fonseca-Bazzo, Y.M., Sakamoto-Hojo, E., de Medeiros, Y.K., de Souza Borges, W., Silveira, D., 2020. Acetylcholinesterase inhibitory activity, anti-inflammatory, and neuroprotective potential of *Hippeastrum psittacinum* (Ker Gawl.) herb (Amaryllidaceae). Food Chem. Toxicol. 145, 111703 https://doi.org/10.1016/j.fct.2020.111703.

Jitsuno, M., Yokosuka, A., Sakagami, H., Mimaki, Y., 2009. Chemical constituents of the bulbs of *Habranthus brachyandrus* and their cytotoxic activities. Chem. Pharm. Bull. 57 (10), 1153–1157. https://doi.org/10.1248/cpb.57.1153.

Kaya, G.I., Sarikaya, B., Onur, M.A., Somer, N.U., Viladomat, F., Codina, C., Bastida, J., Lauinger, I.L., Kaiser, M., Tasdemir, D., 2011. Antiprotozoal alkaloids from *Galanthus trojanus*. Phytochem. Lett. 4, 301–305. https://doi.org/10.1016/j.phytol.2011.05.008.

Kratz, J.M., 2019. Drug discovery for Chagas disease: a viewpoint. Acta Trop. 198, 105107 https://doi.org/10.1016/j.actatropica.2019.105107.

Li, Y., Shah-Simpson, S., Okrah, K., Belew, A.T., Choi, J., Caradonna, K.L., Padmanabhan, P., Ndegwa, D.M., Temanni, M.R., Corrada Bravo, H., El-Sayed, N.M., Burleigh, B.A., 2016. Transcriptome remodeling in *Trypanosoma cruzi* and human cells during intracellular infection. PLoS Pathog. 12, e1005511 https://doi.org/10.1371/journal.ppat.1005511.

Martinez-Peinado, N., Cortes-Serra, N., Losada-Galvan, I., Alonso-Vega, C., Urbina, A.J., Rodriguez, A., VandeBerg, J.L., Pinazo, M.J., Gascon, J., Alonso-Padilla, J., 2020a. Emerging agents for the treatment of Chagas disease: what is in the preclinical and clinical development pipeline? Expert Opin. Investig. Drugs 29, 947–959. https://doi.org/10.1080/13543784.2020.1793955.

Martinez-Peinado, N., Cortes-Serra, N., Tallini, L.R., Pinazo, M.J., Gascon, J., Bastida, J., Alonso-Padilla, J., 2021a. Amaryllidaceae plants: a potential natural resource for the treatment of Chagas disease. Parasites Vectors 14, 337. https://doi.org/10.1186/s13071-021-04837-9.

Martinez-Peinado, N., Cortes-Serra, N., Torras-Claveria, L., Pinazo, M.J., Gascon, J., Bastida, J., Alonso-Padilla, J., 2020b. Amaryllidaceae alkaloids with anti-*Trypanosoma cruzi* activity. Parasites Vectors 13, 299. https://doi.org/10.1186/s13071-020-04171-6.

Martinez-Peinado, N., Martori, C., Cortes-Serra, N., Sherman, J., Rodriguez, A., Gascon, J., Alberola, J., Pinazo, M., Rodriguez-Cortes, A., Alonso-Padilla, J., 2021b. Anti- *Trypanosoma cruzi* activity of metabolism modifier compounds. Int. J. Mol. Sci. 22, 688. https://doi.org/10.3390/ijms22020688.

Morello, A., Lipchenca, I., Cassels, B.K., Speisky, H., Aldunate, J., Repetto, Y., 1994. Trypanocidal effect of boldine and related alkaloids upon several strains of *Trypanosoma cruzi*. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 107, 367–371. https://doi.org/10.1016/1367-8280(94)90063-9.

Nair, J.J., Bastida, J., Viladomat, F., van Staden, J., 2012. Cytotoxic agents of the crinane series of Amaryllidaceae alkaloids. Nat. Prod. Commun. 7, 1677–1688.

Nair, J.J., van Staden, J., 2014. Cytotoxicity studies of lycorine alkaloids of the Amaryllidaceae. Nat. Prod. Commun. 9, 1193–1210.

Nair, J.J., van Staden, J., 2019. Antiprotozoal alkaloid principles of the plant family Amaryllidaceae. Bioorg. Med. Chem. Lett. 29, 126642 https://doi.org/10.1016/j.bmcl.2019.126642.

Newman, D.J., Cragg, G.M., 2020. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. J. Nat. Prod. 83, 770–803. https://doi.org/10.1021/ACS.JNATPROD.9B01285.

Osorio, E.D., Berkov, S., Brun, R., Codina, C., Viladomat, F., Cabezas, F., Bastida, J., 2010. *In vitro* antiprotozoal activity of alkaloids from *Phaedranassa dubia* (Amaryllidaceae). Phytochem. Lett. 3, 161–163. https://doi.org/10.1016/j.phytol.2010.06.004.

Osorio, E.D., Robledo, S.M., Bastida, J., Cordell, G.A., 2008. Alkaloids with antiprotozoal activity. In: The Alkaloids, 66. Elsevier Inc. Press, Amsterdam, Netherlands, pp. 113–190. https://doi.org/10.1016/S1099-4831(08)00202-2.

Salem, M., Werbovetz, K., 2006. Natural products from plants as drug candidates and lead compounds against leishmaniasis and trypanosomiasis. Curr. Med. Chem. 13, 2571–2598. https://doi.org/10.2174/092986706778201611.

Santos, S.S., de Araújo, R.V., Giarolla, J., El Seoud, O., Ferreira, E.I., 2020. Searching for drugs for Chagas disease, leishmaniasis and schistosomiasis: a review. Int. J. Antimicrob. Agents 55, 105906. https://doi.org/10.1016/j.ijantimicag.2020.105906.

Torras-Claveria, L., Berkov, S., Codina, C., Viladomat, F., Bastida, J., 2014. Metabolomic analysis of bioactive Amaryllidaceae alkaloids of ornamental varieties of Narcissus by GC-MS combined with k-means cluster analysis. Ind. Crop. Prod. 56, 211–222. https://doi.org/10.1016/j.indcrop.2014.03.008.

Varela, J., Cerecetto, H., González, M., Nissapatorn, V, Oz, H.S., 2018. Slowed development of natural products for Chagas disease, how to move forward? Chapter 10. Chagas Disease: Basic Investigations and Challenges. Intech Open Limited Press., London, UK https://doi.org/10.5772/intechopen.69020.

WHO Chagas disease (American trypanosomiasis). URL: https://www.who.int/en/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis) (accessed on 28th September 2021).

WFO (The world flora online) plant list (2021), Published on the Internet; https://wfoplantlist.org/plant-list/taxon/wfo-0000645851-2021-12 (accessed on 15th February 2022).

Wildman, W.C., Brown, C.L., 1968. The structure of habranthine. Tetrahedron Lett. 43, 4573–4576.