Research Square

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Amaryllidaceae alkaloids with anti-*Trypanosoma cruzi* activity

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Abstract

Background: Chagas disease, caused by the protozoan Trypanosoma cruzi, is a neglected disease that affects ~7 million people worldwide. Development of new drugs to treat the infection remains a priority since those currently available have frequent side effects and limited efficacy at the chronic stage. Natural products provide a pool of diversity structures to lead the chemical synthesis of novel molecules for this purpose. Herein we analyzed the anti- T. cruzi activity of 9 alkaloids derived from plants of the Amaryllidaceae family.

Methods: the activity of each alkaloid was assessed by means of an anti- T. cruzi phenotypic assay. We further evaluated the compounds that inhibited the parasite growth on two distinct cytotoxicity assays to discard those that were toxic to host cells and assure parasite selectivity.

Results: we identified a single compound (hippeastrine 2) that was selectively active against the parasite yielding selectivity indexes of 12.7 and 35.2 against Vero and HepG2 cells, respectively. Moreover, it showed specific activity against the amastigote stage (IC 50 = 3.31 μM).

Conclusions: results reported here suggest that natural products are an interesting source of new compounds for the development of drugs against Chagas disease.

1. Background

Chagas disease (or American trypanosomiasis) is a neglected infectious disease caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*; order Kinetoplastida; family Trypanosomatidae). It is estimated that ~7 million people are affected by the disease, mainly in Latin America where *T. cruzi* infection is endemic and occur the majority of the ~10,000 deaths per year related to it (1).

The disease progresses in two phases. There is first a short acute phase that is usually asymptomatic and thus goes unnoticed. This is followed by a chronic phase characterized by absent or slow progression of clinical manifestations (2). Nonetheless, it is estimated that ~40% of those chronically infected will ultimately develop disruptive damage to the heart and/or digestive tract (esophagus and colon) tissues, which can lead to the formation of mega-syndromes and death if untreated (2,3).

Since the 1970s only two drugs are available to treat *T. cruzi* infections: benznidazole (BNZ) and nifurtimox (NFX) (1). Both have good efficacy and tolerability when administered to infected newborns (4). But their efficacy diminishes at the chronic stage, which is usually diagnosed at adulthood with serological tests that detect specific anti-*T. cruzi* type G immunoglobulins (1). Moreover, both drugs have long regimens of administration that entail the advent of frequent adverse events which often drive to treatment discontinuation (5–7). There is thus an urgent unmet need of safer and more efficacious drugs for the treatment of chronic Chagas disease, for which natural products may represent a promising approach to discover new lead compounds (8–10).

In this regards, members of the family Amaryllidaceae have attracted considerable attention in the last few years due to their unique alkaloid composition with multiple biological activities (11). Amaryllidaceae plants have been studied for their potential application as a source of anticancer, anti-inflammatory, antimicrobial, anti-parasitic and anticholinesterase activities (12). In fact they have been used for centuries as part of traditional treatments for fever, swelling, cancer or malaria (12). Remarkably, in 2001 the Food and Drug Administration (FDA) approved the use of galanthamine (trade name Razadyne), an alkaloid identified from the Amaryllidaceae plant *Galanthus woronowi*, to treat Alzheimer´s disease (13).

Alkaloid constituents found in these plants are classified in eight groups based on structure and biogenesis from the common precursor *O*-methylnorbelladine: galanthamine, lycorine, crinine, haemanthamine, homolycorine, narciclasine, tazettine and montanine (14). The unique structure of this set of alkaloids provides a viable platform for phytochemical-based drug discovery (8). With the aim to identify prospective drug development starting points that could eventually become new therapeutic solutions for Chagas disease we have adapted an in vitro anti-*T. cruzi* phenotypic assay based on the parasite Tulahuen strain engineered to express a bacterial β-galactosidase gene (15) and green monkey epithelial cells (Vero) as hosts. We evaluated the anti-*T. cruzi* activity of nine crystalized alkaloid compounds extracted from members of the Amaryllidaceae family: lycorine **1**, hippeastrine **2**, crinine **3**, haemanthamine **4**, narciclasine **5**, tazettine **6**, montanine **7**, sanguinine **8** and 1-*O*-acetylcaranine **9** (**Figure 1**) (16). In all the assays performed we always included the standard anti-parasitic drug BNZ for comparison.

In order to unveil the specific *T. cruzi* growth inhibitory capacity of those compounds that were found active in the anti-parasitic assay we further used two secondary biological assays to determine the compounds´ level of cytotoxicity. These were respectively based on the same host Vero cells and in the human hepatocellular carcinoma cell line HepG2. Finally, we determined the anti-amastigote specific activity of the only compound that was revealed to hold selective anti-parasitic activity. Results obtained were particularly promising for the compound hippeastrine **2** from *Narcissus* cv. Salome (17) and they are discussed herein.

Figure 1. Chemical structures of the alkaloids evaluated in this work.

2. Methods

- **Collection of purified alkaloid compounds from Amaryllidaceae plants.**

Lycorine **1**, hippeastrine **2**, crinine **3**, haemanthamine **4**, narciclasine **5**, tazettine **6**, montanine **7**, sanguinine **8** and 1-*O*-acetylcaranine **9** alkaloids were isolated from extracts of different *Narcissus* species (16, 18). The information of all the compounds studied can be found in the extensive chapter by Bastida et al. (16). In brief, the procedure followed to identify the alkaloids within the corresponding plant extract was as follows: plant material (60 mg) was macerated with MeOH, the mix was filtered and the solvent evaporated to dryness. After that, extracts were acidified with 500 µL of H_2SO4 (2%, *v/v*). The

neutral material was removed with Et_2O and basified with 200 µL NH_4OH (25 %, *v/v*). Then, 750 µl of Et_2O were added to separate the organic phase two times and the solvent evaporated to dryness. Finally, extracts were subjected to a combination of chromatographic techniques and alkaloids identified by GC-MS and NMR (16, 17). In order to be able to segregate the order of milligrams of product, the corresponding scale-up attending to the required proportion was made as described (19, 20).

- **Host cells cultures.**

Vero (green monkey kidney epithelial cells), LLC-MK2 (Reshus monkey kidney epithelial cells) and HepG2 (human liver epithelial cells) cultures were kept with DMEM supplemented with 1% penicillin-streptomycin (100 units/mL of penicillin and 100 µg/mL of streptomycin; P-S) and 10% heat inactivated fetal bovine serum (FBS) at 37°C, 5% CO2 and >95% humidity as described (15). HepG2 were also supplemented with 1X non-essential aminoacids (Biological Industries ref. 01-340-1B).

- **Culture of *cruzi* parasites.**

T. cruzi parasites from the Tulahuen strain (Discrete Typing Unit VI) expressing β-galactosidase were kindly provided by Dr. Fred Buckner (University of Washington, Seattle, USA) and maintained using LLC-MK2 cells as hosts in DMEM supplemented with 2% FBS and 1% P-S as described (15). Free-swimming trypomastigotes were purified by spinning the supernatants for 7 min at 2,500 rpm with a low break speed and then letting them swim out of the pellet (21). Purified trypomastigotes were used to keep the parasite cycle in LLC-MK2 cells and for the performance of the anti-parasitic assays. In the last case, an extra round of spinning was done to clean out the phenol red from the maintenance DMEM and replace it by phenol red-free assay medium, which was supplemented with 1% P-S-glutamine, 2% FBS, 1 mM sodium-pyruvate and 25 mM HEPES (21).

- **Assay to detect *cruzi* growth inhibition in 96 well plates.**

Our assay is based on Vero cells as hosts and infective trypomastigotes from Tulahuen strain that express the bacterial β-galactosidase enzyme as reporter activity (15). Firstly, alkaloids were added in the first column of a 96-well tissue culture treated plates at an initial concentration of 100 µM and diluted in assay medium into the next columns of the plate to conform dose-response plate-maps following either a 1:2 or 1:3 fold pattern. Then, Vero cells were detached from their growing flasks, counted and diluted at a concentration of $1x10^6$ cells per mL. Trypan blue staining was used to check out their viability, which had to be >95% to proceed. On the other side, purified trypomastigotes were counted as well and diluted at a concentration of $1x10^6$ cells per mL. We directly mixed Vero cells and trypomastigotes in a falcon tube in sufficient volume so as to add 100 µL of the mix per well (50,000 Vero cells and 50,000 trypomastigote

cells per well; multiplicity of infection or MOI = 1). DMSO percentage in all wells was always kept below 0.5%.

The reference drug BNZ was used as control of drug growth inhibition in each run launched, whereas each plate contained its own negative (maximum parasite growth; Vero cells plus parasites without drugs) and positive (minimum parasite growth; trypomastigote forms alone marking an enzymatic zero time or baseline galactosidase activity). Note that trypomastigotes are unable to multiply in the absence of susceptible host cells. Plates were incubated for 4 days at 37 ºC (21), and the readout was done adding 50 µL per well of a PBS solution containing 0.25% NP40 and 500 µM chlorophenol red-b-D-galactoside (CPRG) substrate as previously described (21). Upon addition of the substrate, plates were further incubated at 37 ºC for another 4 h and the absorbance read out at 590 nm using an Epoch Gene5 spectrophotometer. All experiments were performed at least in triplicate.

- **Anti-amastigote specific activity of progressed compound (hippeastrine).**

Also based on Vero cells as hosts and the recombinant *T. cruzi* strain expressing β-galactosidase, we further adapted the anti-parasitic assay described above to determine whether the anti-parasitic activity was specific against the intracellular amastigote forms. In brief, we plated 50,000 Vero cells per well in a 96-well plate and let them to attach for 1.5 hour. Then, we infected the monolayers with 50,000 purified trypomastigotes per well (MOI = 1) that were allowed 1h to adsorb and enter the cells before being PBS washed away for three times. Finally, assay medium was added and used to dilute hippeastrine and BNZ in a dose-response pattern. In each plate we included the same controls as for the previously described anti-*T. cruzi* assay (see section 2.4). Test plates were incubated for 96h and assay readout was performed as described above (see section 2.4).

- **Toxicity assays with Vero and HepG2 cells.**

For the cell toxicity assays, compounds were added to tissue culture treated 96-well plates following a dose-response dilution pattern 1:2 or 1:3 with a starting concentration of 400 or 800 µM per well in the first column of the plate. Cells viability was checked upon cell counting with Trypan blue staining and we only proceeded if it was >95%. Vero cells solution was diluted at a concentration of $5x10^5$ cells per mL before adding 100 µl per well. In the case of HepG2 cells, we used a dilution of $3.2x10^4$ cells per mL. Each test plate or run contained its own negative (untreated cells) and positive (medium alone) controls. Plates were incubated at 37º C for 4 days in the case of Vero cells and 2 days for HepG2 cells. We then added 50 µL per well of a PBS solution containing 10% Alamar Blue reagent (ThermoFischer) and incubated the plates for 6 h at 37 ºC before reading the fluorescence intensity in a Tecan Infinite M Nano$^+$ reader (excitation: 530 nm, emission: 590 nm). DMSO percentage in all wells was always kept below 0.5%. All experiments were performed at least in triplicate.

- **Data analysis.**

Absorbance and fluorescence values respectively derived from the anti-*T. cruzi* and cell toxicity assays were normalized to the controls (22). IC_{50} and TC_{50} values were determined with GraphPad Prism 7 software (version 7.00, 2016) using a non-linear regression analysis model defined by the equation:

Eq. 1: [Please see the supplementary files section to access the equation.]

These IC_{50} and TC_{50} values are the compound concentrations capable to inhibit by 50% the growth of parasites and cells, respectively. Z' values were calculated as described (23). Values provided are means ± standard deviation (SD) of at least three independent experiments.

3. Results And Discussion

- **Quality assessment of the anti- *cruzi* and cells toxicity assays.**

As part of the process of setting up the biological assays we calculated their Z' parameter to assess reproducibility and statistical robustness (23). In general, assays with a Z' between 0.5 and 1 are considered appropriate for the screening of compounds (23). Remarkably, our anti-parasitic assay had a very good performance and its Z' remained consistently >0.5 with an average value of 0.89 (CI95% = 0.73 - 1.00) (**Figure 2A**). Regarding the two cytotoxicity assays used in this work, we respectively retrieved $Z' = 0.73$ (CI95% = 0.60 - 0.93) for the assay based on Vero cells, and a $Z' = 0.71$ (CI95% = 0.65 - 0.82) for the assay based on HepG2 cells (**Figure 2B-C**).

Additionally, in every run of the *T. cruzi* growth inhibition assay and Vero cells toxicity assays performed, we included the reference drug BNZ as control, whereas the reference drug digitoxin (24) was included in all the HepG2 cells toxicity assays. Overall, averaged IC_{50} and TC_{50} values for BNZ were respectively 1.56 µM (SD ± 0.39) and 173.4 µM (SD ± 43.57) (**Figure 2A-B**), which correlate with previous reports (21, 25). Digitoxin TC_{50} mean value in the HepG2 cell assay was 0.29 µM (SD ± 0.14).

Figure 2. Quality controls of the *T. cruzi* growth inhibition assay (A-B), toxicity assay with Vero cells (C-D) and toxicity assay with HepG2 cells (E-F). Z' values for each of the rounds

launched are represented on the left (A, C, E); dashed line marks the 0.5 threshold. IC_{50} and TC_{50} values of the reference drugs BNZ and digitoxin (DTX) are represented on the right (B, D, F); straight lines indicate the average values, whereas the dashed lines indicate ± 3 SD limits.

- **Anti- *cruzi* activity of the alkaloids extracted from Amaryllidaceae.**

Same as other widely used anti-*T. cruzi* assays (21, 22, 26) we relied on the genetically robust *T. cruzi* - Tulahuen strain expressing beta-galactosidase activity as a surrogate of parasite growth (15). However, being the amastigote replicative stage of *T. cruzi* obliged intracellular, our assay relies on Vero cells as hosts. Notably, Vero cells were described to be deficient in the interferon response due to a mutation in the simian interferon beta gene (27), which makes them more susceptible to infections and thereby a good system for the discovery of active compounds against *T. cruzi*.

Results obtained from the phenotypic *T. cruzi* growth inhibition assay revealed that lycorine **1**, hippeastrine **2**, haemanthamine **4**, narciclasine **5** and montanine **7** were active, while crinine **3**, tazettine **6**, sanguinine **8** and 1-*O*-acetylcaranine **9** were inactive against the parasite (**Figure 3**). Tazettine **6**, sanguinine **8** and 1-*O*-acetylcaranine **9** have been described as poor antiprotozoal agents before (28, 29). Nonetheless, Machocho and co-workers described that compound 3-*O*-acetylsanguinine had some activity (IC_{50} = 2.3 µg/mL; i.e. 7.29 µM) against trypomastigotes from *T. cruzi* strain Tulahuen C4 (28). Although different phenotypic anti-*T. cruzi* assays were performed in each case, the results obtained by those authors could suggest that the presence of an acetyl group might increase the anti-*T. cruzi* activity of sanguinine.

On the other hand, crinine **3** and haemanthamine **4** are crinane-type alkaloids that belong to the β-crinane and α-crinane subgroups, respectively (30). Other studies evaluating the anti-parasitic potential of alkaloids have reported that the presence of a methylene-dioxi group seemed to favour a more potent anti-parasitic activity (31). This

could be the explanation for the different anti-*T. cruzi* activities observed by us between these two crinane-type alkaloids.

Figure 3. Anti-*T.cruzi* phenotypic assay dose-response curves. Graphs represent mean results and SD of at least three biological replicas.

The compounds that showed the highest anti-*T. cruzi* activities were yielded by lycorine **1** (IC_{50} = 0.70 µM; CI95% 0.65 - 0.73) and narciclassine **5** (IC_{50} = 0.495; CI95% 0.46 - 0.54), which exceeded in potency that of the reference drug BNZ (**Table 1**). Lycorine **1** was the first alkaloid described from the Amaryllidaceae plant family and one of the most commonly found amongst different genus (32). Lycorine **1** extracted from *Crinum stuhlmannii, Zhepyranthes citrina* and *Narcissus broussonetii* had been tested before against *T. cruzi* (33–35). However, in contrast to our results, it was described as inactive against the parasite; a feature that could be explained due to the use of different assays. It has been reported that major differences on compounds activity can be found depending on the host cell lines and the biological assays used (36). In this respect previous references relied on the use of L6 cells as hosts, whereas we have used Vero cells.

With respect to narciclasine **5,** which yielded the most potent activity against *T. cruzi* in this study (**Table 1**), to the best of our knowledge it has not been tested against *T. cruzi* before. Likewise, there were no previous reports on the anti-*T. cruzi* activity of montanine **7**, also shown for the first time in this study. Montanine **7** and haemanthamine **4** showed averaged IC_{50} values similar to that of BNZ, respectively 1.99 µM (CI95% 1.82 - 2.1) and 1.59 µM (CI95% 1.47 - 1.42) versus the 1.56 µM of BNZ (CI95% 0.93-2.20; see **Figure 2B**). Osorio and co-workers had previously described the high activity of haemanthamine against *T. cruzi* (IC_{50} = 1.8 µg/mL; i.e. 5.97µM) (29).

Finally, we found that hippeastrine **2** was the active alkaloid with the higher IC_{50} value (IC_{50} = 3.63 µM, CI95% 2.95 - 3.82). Its activity was poorer than that of BNZ but still fell within a range five times the IC_{50} of the standard drug (**Table 1**). Hippeastrine **2** was first isolated from the Amaryllidaceae plant *Lycoris radiata* and reported to exhibit activity

against avian influenza virus H5N1 (IC_{50} = 47.5 ± 0.37 µM) (37). Moreover, promising results against ZIKA virus infection have been recently reported with hippeastrine hydrobromide (38). This was shown to remove ZIKA virus presence from infected human neural progenitors, recover a ZIKV-induced microcephaly phenotype in human forebrain organoids and even suppress virus propagation in infected adult mice (38). Antiviral (37, 38), antibacterial and antifungal (39) activities have been reported for hippeastrine **2**, despite little information is available about its anti-parasitic activity. Cedron et al. tested twenty one hippeastrine **2** derivatives that included functional group transformations, structural simplification and dimers formation against *Plasmodium falciparum* (strain F-32 Tanzania) (40). The anti-malarial activity increased by 10-fold when dimers were evaluated compared to the single alkaloid activity, suggesting an improved binding to the related target or the hydrolysis of the dimer onto two molecules (40). To our knowledge this is the first time that anti-*T. cruzi* activity is reported for hippeastrine **2**. Results reported by Cedron and co-workers would suggest to further pursue research with hippeastrine **2** derivatives against *T. cruzi*.

- **Identification of alkaloid compounds with specific anti- *cruzi* activity.**

With the aim of further selecting those alkaloids with specific activity against the parasite and discard those that were toxic to host cells we used two secondary cell toxicity assays with monkey (Vero) and human (HepG2) cells. Since the compounds activity might vary depending on the characteristics of the cell line used, assaying cytotoxicity in two cell lines will provide a more robust readout. Moreover, HepG2 cells are a widespread cell model to anticipate potential liver toxicity of drugs metabolism (41, 42). We determined a selectivity index (SI; or TC_{50} to IC_{50} ratio) >10 to consider an alkaloid for further progression as described elsewhere (22).

Thereafter, all the alkaloids reported as active against *T. cruzi* were analysed through both cell toxicity assays. All of them were more toxic to Vero cells than to HepG2 cells (**Figure 4**). The cytotoxicity values registered against Vero and HepG2 cells indicated

that narciclasine **5** activity was not specific against *T. cruzi* (TC_{50} = 0.66 µM against Vero cells; and TC_{50} = 2.73 µM against HepG2 cells), resulting in SI < 10 in both cases (**Table 1**). In addition, montanine **7** with a SI = 2.5 in relation to Vero cells was not specific to *T. cruzi* either (**Table 1**), even though it showed low toxicity to HepG2 cells (TC_{50} = 46.1 µM; **Table 1**). Something similar occurred with lycorine **1,** which was weakly active against HepG2 cells (TC_{50} = 21.87 µM), presenting a SI versus this cell line over ten times its registered anti-*T. cruzi* activity, but turned to be toxic to Vero cells (TC_{50} = 5.21 µM) yielding a SI < 10 and thus being discarded it from further progression. We generally observed an increased sensitivity of Vero cells to the alkaloids when compared to HepG2 cells. This may have been in part due to the fact that compounds incubated for longer time (four versus two days) on Vero cells than on HepG2 cells.

Figure 4. Dose-response curves yielded by the Vero and HepG2 cell toxicity assays. Vero cells toxicity assays are represented by circles and straight lines while HepG2 cell toxicity assays are represented by triangles and dashed lines. Graphs represent mean results and SD of at least three biological replicas.

Haemanthamine **4** had been reported to present a TC_{50} = 13 µg/ml; i.e. 43.14 µM for HepG2 cells (43) which correlate with the TC_{50} value we have retrieved in our study with this cell line (TC_{50} = 42.48 µM; **Table 1**). But again, although it showed a SI > 10 with respect to HepG2 cells, when evaluated on Vero cell its TC_{50} to IC_{50} ratio was below that threshold and thus its anti-parasitic activity could not be considered specific (**Table 1**).

In contrast to all the aforementioned results, hippeastrine **2** did show low toxicities against Vero (TC_{50} = 45.99 µM) and HepG2 cells (TC_{50} = 128.1 µM), and it indeed complied with the SI window > 10 against both cell lines (**Table 1**). It was the only compound that had a SI > 10 versus Vero cells (SI = 12.7; **Table 1**). In addition, hippeastrine **2** presented the highest SI against HepG2 cells (SI = 35.3), with a TC_{50} value similar to that previously reported by Weniger et al. (TC_{50} = 40 µg/ml; i.e. 126.85 µM) (43).

We thus assessed in a subsequent anti-amastigote biological assay whether hippeastrine anti-*T. cruzi* activity was indeed specific against this replicative form of the parasite. It was found that the observed anti-amastigote activity (IC_{50}) was 3.31 µM (CI95% 2.53 - 4.28), which was again within 5x that of the reference drug BNZ in the same assay (IC_{50} = 1.2 µM, CI95% 0.69 - 1.85) (Figure 5). Moreover, there would yet be a SI > 10 for hippeastrine **2** with respect to its anti-amastigote activity (SI = 13.9 against Vero cells, and 38.7 against HepG2 cells).

Cytotoxicity results together with the in vivo assays reported by Zhou et al. (38) may predict a low toxicity in future evaluation of hippeastrine **2** anti-*T. cruzi* activity in animal models. Notwithstanding, before arriving at in vivo studies, other in vitro studies could be pursued to better qualify this alkaloid. For example, assessing its effect on *T. cruzi* CYP51 target (44) since it has been invalidated in the clinic (45, 46), and identifying whether it can kill dormant parasite forms (47). Moreover, it would be of interest to determine key in vitro pharmacokinetic (PK) parameters such as its solubility, permeability and clearance.

Figure 5. Anti-amastigote dose response curves of hippeastrine and BNZ. Graphs represent mean results and SD of at least three replicas.

Table 1. Table displaying the alkaloids´ average IC_{50}, TC_{50} and SI values for Vero and HepG2 cells.

No.	Alkaloid	IC_{50} (µM)	TC_{50}[1] (µM)	SI[1]	TC_{50}[2] (µM)	SI[2]
-	BNZ	1.56	173.4	111.15	168.76	108.18
1	Lycorine	0.70	5.21	7.5	21.87	31.2
2	Hippeastrine#	3.63	45.99	**12.7**	128.10	**35.2**
3	Crinine	57.93	-		-	
4	Haemanthamine	1.59	11.52	7.3	42.48	26.7
5	Narciclasine	0.495	0.66	1.3	2.73	5.5
6	Tazettine	83.03	-		-	
7	Montanine	1.99	5.04	2.5	46.10	23.1
8	Sanguinine	213.4	-		-	
9	1-*O*-acetylcaranine	35.49	-		-	

Superscripts [1] and [2] respectively refer to Vero and HepG2 cell toxicity assays TC_{50} and SIs.

[#]Indicate the only alkaloid evaluated in this study that showed specific anti-*T. cruzi* activity.

The standard drug BNZ is included in the first line as comparator.

4. Conclusions

Upon the evaluation of nine alkaloid compounds purified from extracts of different *Narcissus* species (family Amaryllidaceae) (16) we identified one compound that had specific anti-*T. cruzi* activity, proving that natural products are an interesting source to potentially identify new departing chemical structures for Chagas disease drug discovery. Our findings suggest that hippeastrine **2** (17) is a relevant compound to be further studied in this regards. The analysis of its capacity to kill parasite dormant forms, and identification of its main target deserve future efforts in forthcoming experiments.

5. Declarations

- **Ethics approval and consent to participate:** “Not applicable”.
- **Consent for publication:** “Not applicable”.
- **Availability of data and materials:** “Data and materials can be made available upon reasonable request to the authors”.
- **Competing interests:** “The authors declare no competing interests”.
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- **Authors' contributions:** NMP, NCS, JB and JAP conceptualized the study; NMP performed all biological assays; NCS helped with the assays; LTC and JB purified the alkaloids and provided the collection; MJP and JG provided funds; NMP and JAP wrote the article; all authors read and approved the final manuscript, and declare that there is no conflict of interests.
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Additional File Legend

Figure A. BNZ and DTX dose-response curves. Both reference drugs were included in every assay as a control of drug inhibition. Anti-*T. cruzi* assays are represented by circles while Vero and HepG2 cells

toxicity assays by squares and triangles, respectively.

Figures

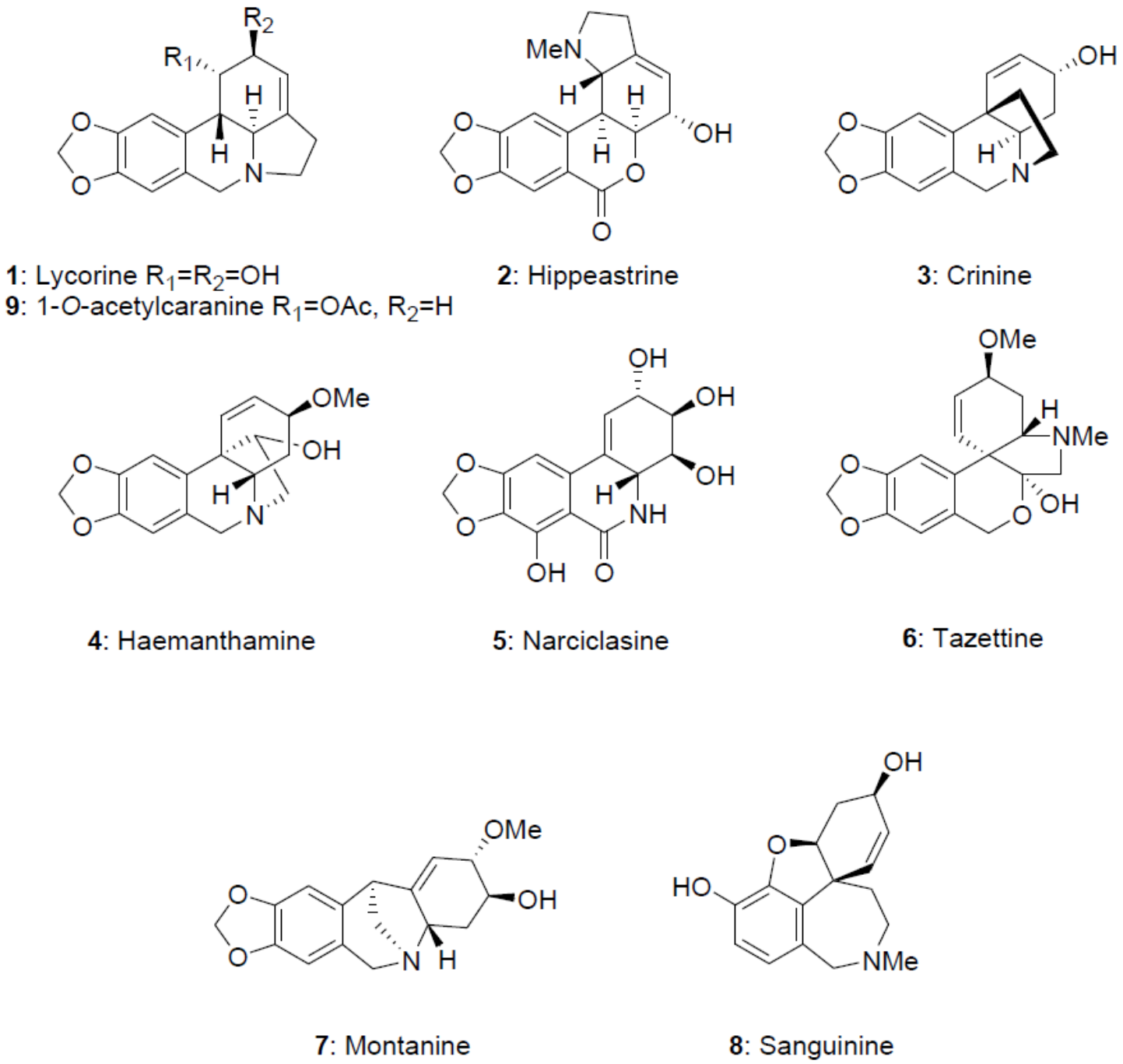

Figure 1

Chemical structures of the alkaloids evaluated in this work.

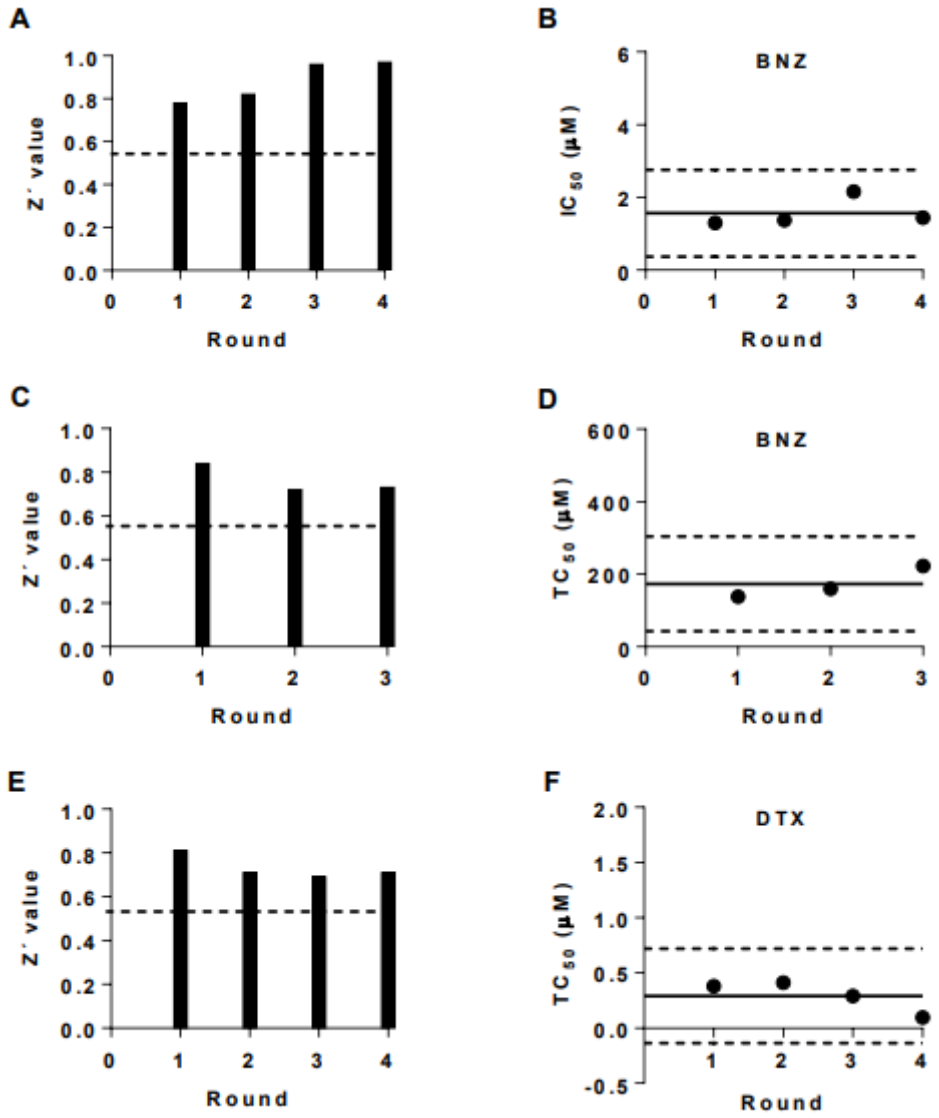

Figure 2

Quality controls of the T. cruzi growth inhibition assay (A-B), toxicity assay with Vero cells (C-D) and toxicity assay with HepG2 cells (E-F). Z´ values for each of the rounds launched are represented on the left (A, C, E); dashed line marks the 0.5 threshold. IC50 and TC50 values of the reference drugs BNZ and digitoxin (DTX) are represented on the right (B, D, F); straight lines indicate the average values, whereas the dashed lines indicate ± 3 SD limits.

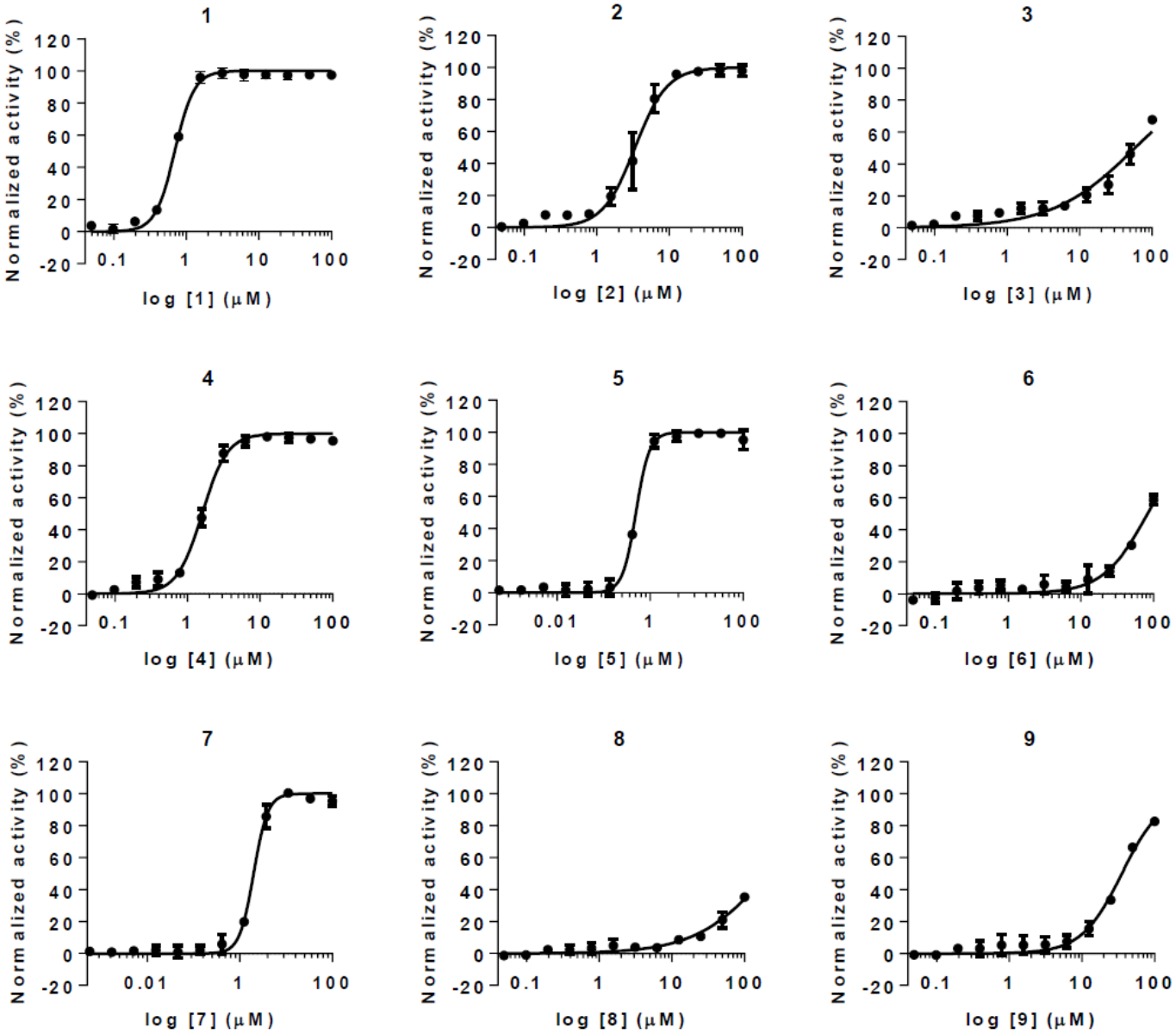

Figure 3

Anti-T.cruzi phenotypic assay dose-response curves. Graphs represent mean results and SD of at least three biological replicas.

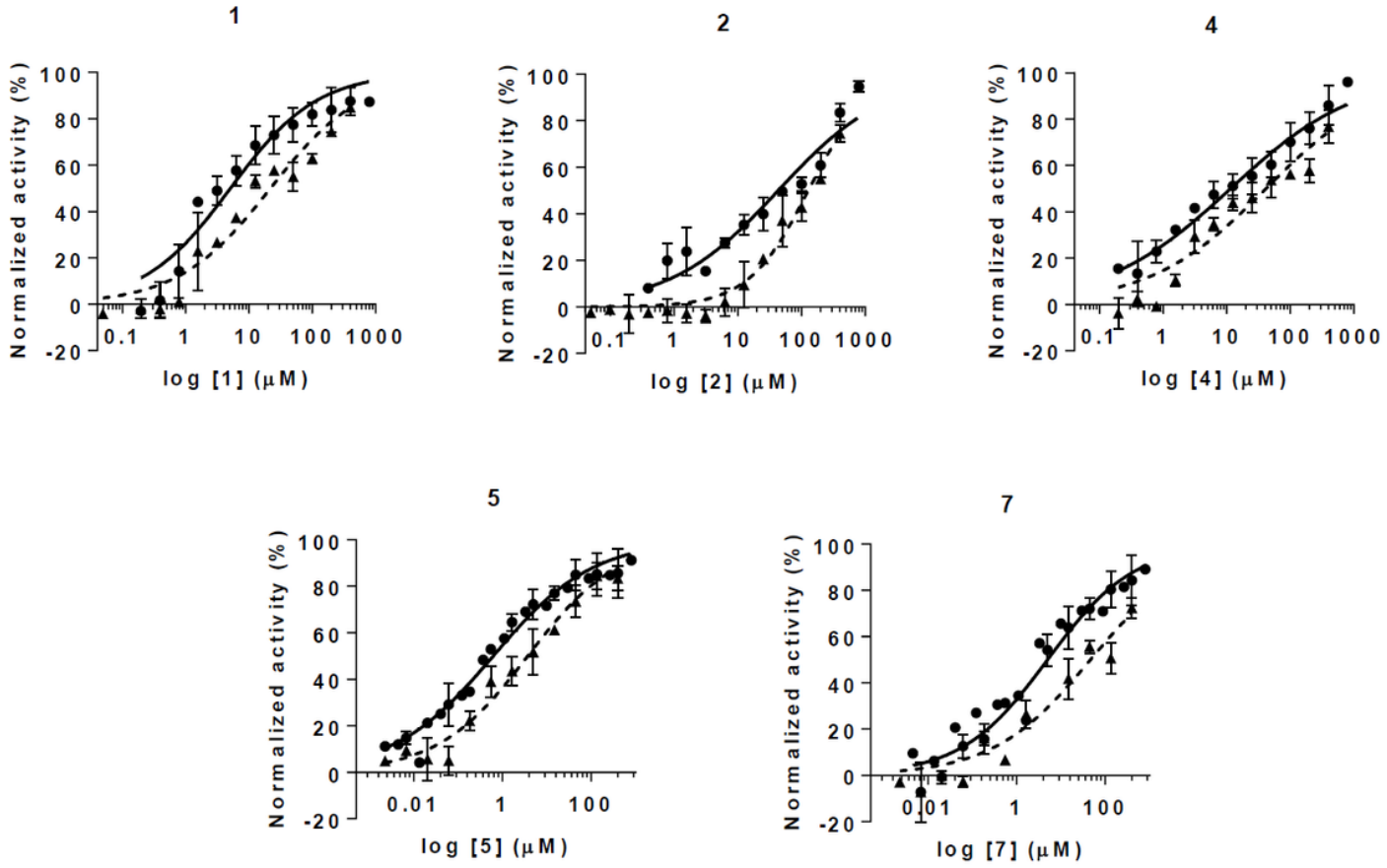

Figure 4

Dose-response curves yielded by the Vero and HepG2 cell toxicity assays. Vero cells toxicity assays are represented by circles and straight lines while HepG2 cell toxicity assays are represented by triangles and dashed lines. Graphs represent mean results and SD of at least three biological replicas.

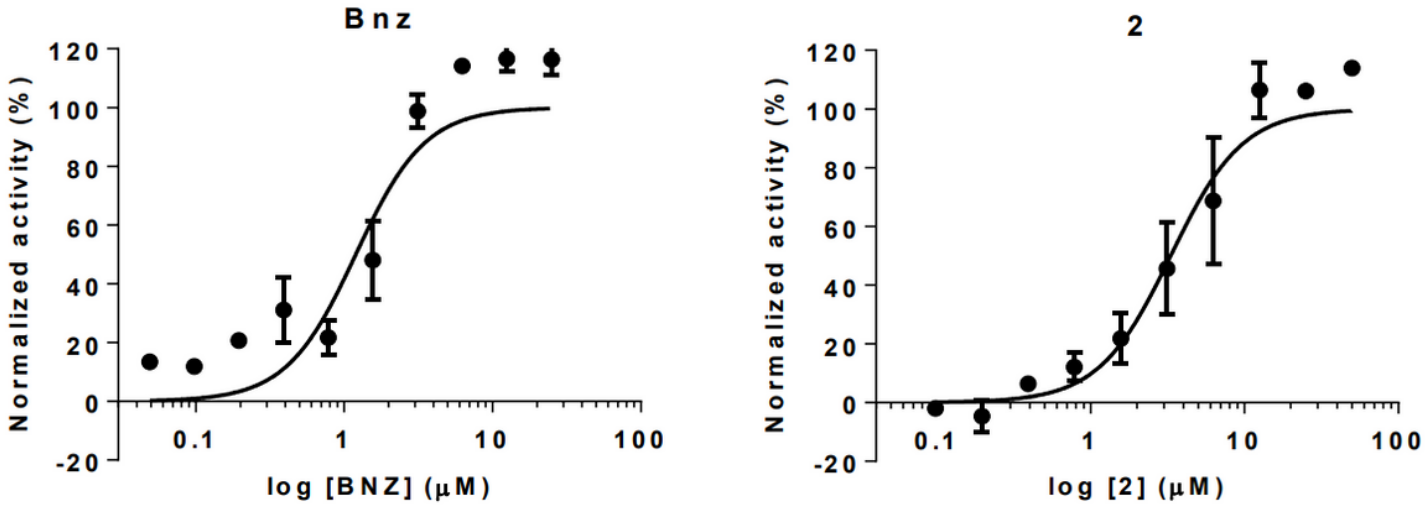

Figure 5

Anti-amastigote dose response curves of hippeastrine and BNZ. Graphs represent mean results and SD of at least three replicas.

Supplementary Files

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