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Anti-trypanosomatid and Antiplasmodial Activities of Alkaloids from *Hippeastrum* Species

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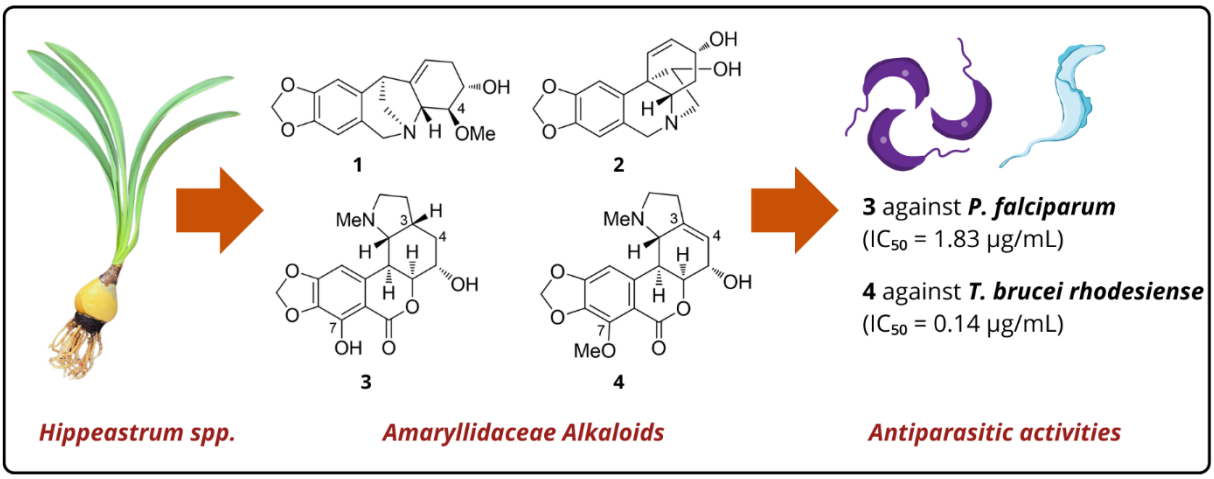
§ Dedicated to the memory of Prof. Jaume Bastida (April, 1958–November 2024), whose invaluable contributions were essential in the development of this work.

Abstract: Diseases caused by trypanosomatid parasites like human African trypanosomiasis (HAT), Chagas disease (CD), leishmaniasis, and malaria, are persistent health problems in developing countries that still demand for new drug development. The species of the Amaryllidoideae subfamily (Amaryllidaceae) represent a vast source of alkaloids with a wide range of bioactive properties, including antiparasitic effects. The aim of this study was to evaluate the antiparasitic activity of the alkaloids hamayne, 7-hydroxyclivonine, 4-*O*-methylnangustine, and candimine against *Trypanosoma brucei rhodesiense, T. cruzi*, *Leishmania donovani*, and *Plasmodium falciparum* parasites. The alkaloids were isolated from the leaves of *Hippeastrum argentinum* and *H. escoipense* using several chromatographic techniques then identified by GC-MS, UPLC-MS/MS, and NMR data. The compounds were assessed against different life cycle stages of these four parasites. Furthermore, the cytotoxic activity of the alkaloids against L6 rat skeletal myoblast cells, was tested. *P. falciparum* was very sensible to 7-hydroxyclivonine. Candimine showed significant antiparasitic activity against all the evaluated parasites, especially *T. brucei rhodesiense*. Candimine merits deeper research regarding its effect against trypanosomatid parasites as a lead compound for the development of alternative treatments for HAT, CD, and malaria.

Keywords: *Trypanosoma*; Trypanosomiasis; Malaria; Candimine; Amaryllidaceae

Graphical abstract



1. Introduction

Trypanosomiasis and leishmaniasis are classified among neglected tropical diseases and represent a serious health problem that causes significant mortality and morbidity, particularly in low- and middle-income countries, affecting some of the poorest countries in the world [1,2]. The etiological agents responsible for these illnesses are trypanosomatids of the genera *Trypanosoma* and *Leishmania* (Trypanosomatidae family), which are obligatorily dixenous, have zoonotic or anthroponotic life cycles and are transmitted by hematophagous insects. The genus *Trypanosoma* comprises approximately 20 species distributed throughout the world, and includes *T. cruzi* and *T. brucei*, the protozoan parasites that cause Chagas disease (CD) (also known as American trypanosomiasis) and human African trypanosomiasis (HAT), respectively [3,4]. It is estimated that between 6 and 7 million people worldwide, mainly in Latin America, are infected with *T. cruzi*. Regarding HAT, it is endemic in sub-Saharan Africa and, without treatment, is usually fatal. Depending on the subspecies of the infecting parasite, HAT takes two forms: *T. brucei gambiense* and *T. brucei rhodesiense*, 92% and 8% of reported cases, respectively. *T. brucei rhodesiense* is found in 13 countries in eastern and southern Africa and causes a rapid-onset acute form of the disease [1,5]. Leishmaniasis is an endemic disease in African and American countries such as Algeria, Somalia, Yemen, and Brazil, which is caused by about 20 species of parasites corresponding to the genus *Leshmania* [3]. Malaria, on the other hand, is an acute febrile disease caused by parasites of *Plasmodium* genus (Plasmodiidae family), mainly *P. falciparum* and *P. vivax*. Currently, malaria remains one of the major global health problems affecting millions of people every year, especially in non-Mediterranean Africa, South Asia, and countries of Latin America such as Brazil. According to WHO, approximately 263 million malaria cases have been reported in 2023, of which 597,000 died, and 76 % of these deaths are represented by children under five years of age [1,6].

New treatments have been developed to combat HAT, however, yet there is still a critical need for new drugs to address CD, as well as both, cutaneous, mucocutaneous, and Post kala-azar dermal leishmaniasis.

The persistence, resistance, and tissue distribution of parasites represent additional challenges when it comes to finding new drugs to successfully treat these parasitic diseases, making it an even more critical health issue that requires urgent attention [2,7].

Amaryllidaceae alkaloids possess a wide variety of biological properties, including antiparasitic activities [8]. The montanine-type alkaloid pancracine showed weak activity against *T. cruzi*, *T. brucei*, and *P. falciparum* [9]. Likewise, the alkaloid extract from *Habranthus brachyandrus* and ismine showed trypanocidal activity against *T. cruzi* [10]. Montanine alkaloid, was reported to have a high antiproliferative anti-*T. cruzi* activity against amastigotes and epimastigotes while hippeastrine showed significant selectivity against *T. cruzi* activity [10,11]. The alkaloidic extract of *H. escoipense* and the isolated alkaloid candimine showed high and specific anti-*T. cruzi* activity against epimastigotes, trypomastigotes, and amastigotes forms with active-concentration values similar to benznidazole, displaying ultrastructural changes in epimastigotes such as vacuolization, membrane vesicles, and increased mitochondrial activity [12].

In our continuous search for novel natural antiparasitic compounds, four Amaryllidaceae alkaloids were isolated from the leaves of two species of the *Hippeastrum* genus, these compounds were then tested for their antiparasitic activities against *T. brucei rhodesiense*, *T. cruzi*, *L. donovani,* and *P. falciparum*.

2. Results and discussion

2.1. Alkaloids Identification

The compounds **1-4** were identified as 4-*O*-methylnangustine, hamayne, 7-hydroxyclivonine, and candimine, respectively. The chemical structures of the isolated alkaloids are shown in Fig. 1. The compounds were identified by GC-MS analysis (Fig. 2), which has proven to be a useful tool in the identification of new, known, or unusual structures from alkaloid-rich extracts by comparing their mass fragmentation patterns and retention indexes with standard reference spectra in homemade databases and by direct comparison of their NMR (1D and 2D) spectroscopic properties with those of authentic samples obtained from the same and other plant sources.

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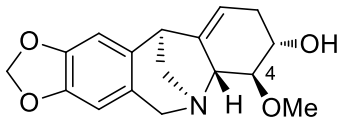
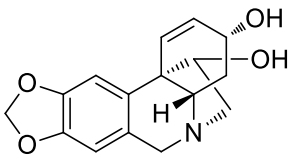
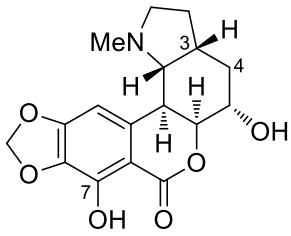


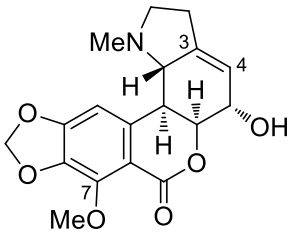
4-*O*-Methylnangustine (**1**)



Hamayne (**2**)



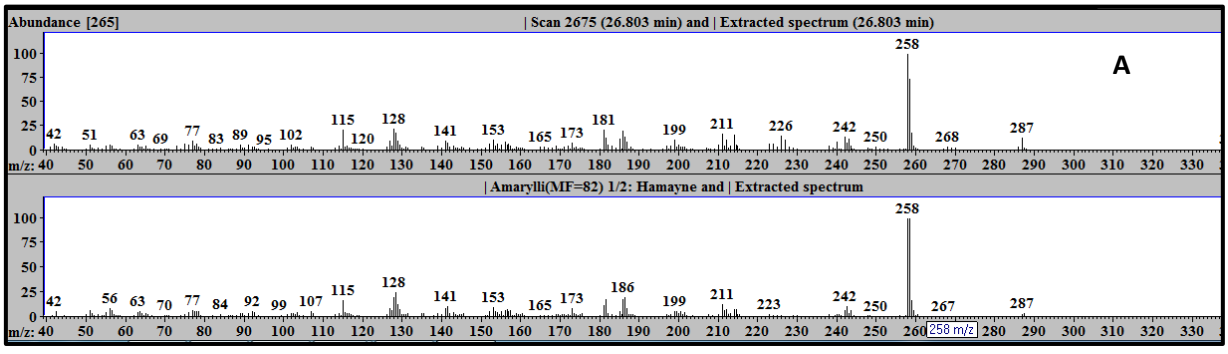
7-Hydroxyclivonine (**3**)

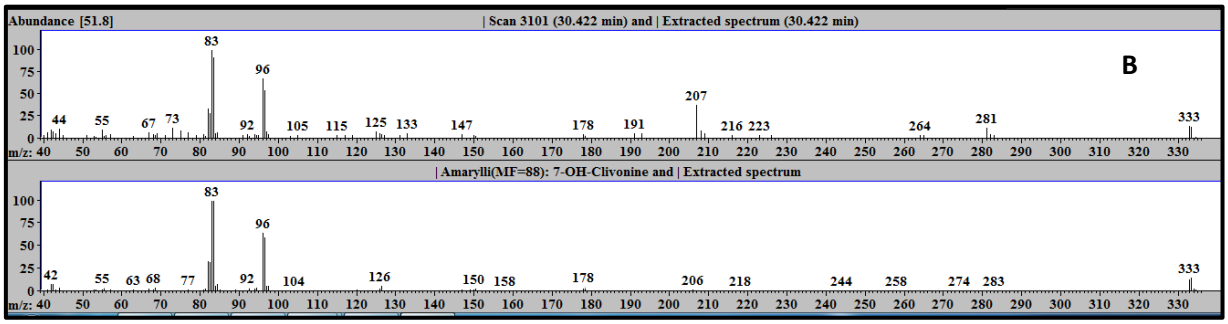


Candimine (**4**)

Fig. 1. Isolated alkaloids from *H. argentinum* and *H. escoipense.*

Likewise, UPLC-ESI-MS/MS analysis showed a different fragmentation patterns of the analyzed alkaloids compared to those from GC-MS, providing additional information for chemical characterization. Finally, the NMR spectroscopic results were in close agreement with previously reported data (Supporting information: S1-8) [8, 12, 13, 14].





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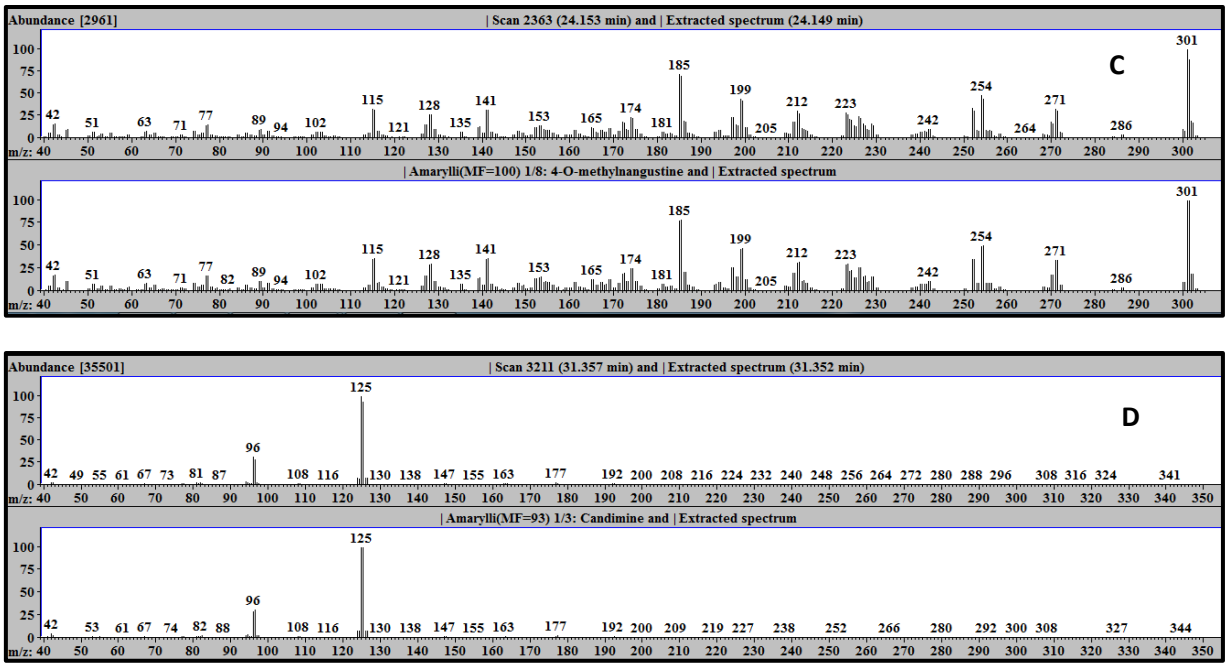

Fig. 2. GC-MS spectra of isolated alkaloids compared against Amaryllidaceae alkaloids library. A) hamayne, B) 7-hydroxyclivonine, C) 4-O-methylnangustine, and D) candimine

2.2. Antiparasitic activities

Antiparasitic results of the isolated alkaloids (**1-4**) are summarized in Table 1. The IC_{50} values >100 µg/mL are considered inactive for antiparasitic activity, and non-toxic for cytotoxicity. Regarding *T. b. rhodesiense* trypomastigotes assay, compound **4** showed strong activity with a IC_{50} value = 0.14 µg/mL, while **2** recorded moderate activity (IC_{50} = 5.28 µg/mL), followed by **3** and **1** with low activity (IC_{50} = 10.13 and 37.45 µg/mL, respectively). For *T. cruzi* amastigotes antiparasitic assess, **4** was again the most active alkaloid with a IC_{50} value of 1.55 µg/mL while the rest of the alkaloids showed low activity. Among the evaluated alkaloids against amastigotes forms of *L. donovani*, the alkaloid **4** exhibited anti-trypanosomatid activity with an IC_{50} value of 7.27 µg/mL, while **1**, **2** and **3** alkaloids were inactive (IC_{50} >100). In regard to *P. falciparum* antiparasitic evaluation, all alkaloids assayed were active to different extents being **4** strongly active (0,85 µg/mL), **3** (1,83 µg/mL) and **2** (2,91 µg/mL) moderately active, and **1** (9,17 µg/mL) slightly active.

On the other hand, to test the specific activity of alkaloids against parasites, cytotoxicity was evaluated in rat skeletal myoblasts (L6 cells). The compounds **1** and **3** were non-cytotoxic, and their TC_{50} values were 79.05 and 90.9 µg/mL, respectively. However, alkaloid **3** showed only a good selectivity index (SI) for *P.*

falciparum (49.67). Finally, **4** was the alkaloid with the lower TC_{50} against L6 cells. Although, for *T. cruzi* amastigotes showed better SI than benznidazole.

Table 1. *In vitro* antiparasitic assays for the isolated alkaloids

Parasite **Stage** **Strain**	***T. b. rhodesiense*** **trypomastigotes** **STIB 900**		***T. cruzi*** **amastigotes** **Tulahuen C4**		***L. donovani*** **amastigotes** **MHOM-ET-67/L82**		***P. falciparum*** **trophozoites** **NF54**		**Cytotoxicity** **L-6 cells**
Standard/compound	IC_{50} [1]	SI [3]	IC_{50} [1]	SI [3]	IC_{50} [1]	SI [3]	IC_{50} [1]	SI [3]	TC_{50} [1]
4-*O*-methylnangustine (**1**)	37.45±10.3	2.11	56.15±2.47	1.4	>100	-	9.17±1.88	8.62	79.05±5.40
Hamayne (**2**)	5.28 ± 1.71	2.55	17.35±1.70	0.77	>100	-	2.91±0.17	4.63	13.5±3.67
7-hydroxyclivonine (**3**)	10.13±2.70	8.07	42.45±0.21	1.92	>100	-	1.83±0.19	49.67	90.9±3.25
Candimine (**4**)	**0.14±0.07**	13.28	**1.55**±0.12	1.2	**7.27**±0.28	0.25	**0.85**±0.09	2.18	**1.86**±1.02
Melarsoprol[2]	0.0035±0.002	2.29							
Benznidazole[2]			0.66±0.045	68.94					
Miltefosine[2]					0.085±0.031	3.18			
Chloroquine[2]							0.002±0.0005	124	
Podophyllotoxin[2]									0.005±0.0007

[1] IC_{50}/TC_{50} values are expressed as µg/mL [2] Reference compound. [3] Selectivity Index (TC_{50} cytotoxicity/IC_{50} antiparasitic activity)

In general, candimine (**4**) showed to be the alkaloid with the lowest IC_{50} values in all antiparasitic tests. Previously, reported data demonstrated the antiparasitic properties of **4** against *Trichomonas vaginalis*, the parasite causing trichomoniasis, through a mechanism that exhibits features similar to those of paraptosis [15, 16]. Among the observed results presented in Table 1, stands out the inhibitory activity of **4** over *T. b. rhodesiense*, displaying a strong antiparasitic activity (IC_{50} equal to 0.14 µg/mL). A recent trypanosomatid research that evaluated the anti-*T. cruzi* activity of the **4** against epimastigotes, amastigotes, and trypomastigotes forms indicated IC_{50} values similar to benznidazole [12]. Regarding the activity over L6 cells, **4** showed the highest cytotoxicity (IC_{50} of 1.865 µg/mL) among the evaluated alkaloids. However, its selectivity index (SI) towards *T. b. rhodesiense* trypomastigotes (13.28), is in agreement with the minimum hit selection criterion for promising HAT compounds, which proposes at least a SI ≥ 10 [17-19]. Regarding *L. donovani* trypanosomatid inhibition assay, it is remarkable that compound **4** showed selectivity against the amastigote form highlighting this alkaloid as a promising candidate for further leishmaniasis research. To the best of our knowledge, this is the first report of a homolycorine-type alkaloid with antiparasitic activity against *L. donovani*.

The difference in the antiparasitic activities between the alkaloid candimine (**4**) and 7-hydroxyclivonine (**3**) against *Tb rhodesiense* is remarkable, being approximately 72 times. Regarding *T. cruzi* antiparasitic activity, was 27 times more potent than **3**. Although they are structurally similar alkaloids (homolycorin-type), these results could be related to the only two structural differences between them, a double bond between C3 and C4 and the substituent in C7 (methoxyl or hydroxyl). Similarly, this would also explain the absence of antiparasitic activity of **3** against *L. donovani* [13, 20]. Similar situation occurs with the structurally related montanine-type alkaloids nangustine previously reported as active against T. *b. rhodesiense* (IC50 = 9.6 μg/mL) [9], and *4-O*-methylnangustin (**1**) here reported as inactive. The structural difference of these alkaloids is localized in the C-4 position (methoxyl or hydroxyl group), suggesting this position as relevant in the activity towards *T. b. rhodesiense* [13]. These arguments could be considered to develop semi-synthetic derivatives using these alkaloids as a starting point to improve their antiparasitic activities.

Regarding *P. falciparum* the findings are quite interesting; all the alkaloids demonstrated low IC_{50} values, suggesting this parasite has a high sensitivity pattern against these compounds with the sequence being **4**>**3**>**2**>**1**. Concurrently, the general cytotoxicity in L6-cells was low for alkaloids **3**, **2**, and **1**, whereas alkaloid **4** was the most cytotoxic. *P. falciparum* exhibited high sensitivity to compounds **3** (1.83 μg/mL) and **4** (0.85 μg/mL); notably, compound **3** demonstrated the lowest cytotoxicity while maintaining high antiplasmodial efficacy and an excellent SI value (49.67). These attributes establish the alkaloid **3** as a promising candidate for further malaria research.

In order to highlight the SI values significance of the screened compounds, it is important to remind that the success on the continuous screening for new leading antiparasitic nitroheterocycles including Amaryllidaceae alkaloids, not only depends on the toxicity but the selectivity index, since fexinidazole (a drug recently approved for HAT treatment) displayed an IC_{50} value against different strains of *T. brucei* close to 1 μg/mL, and yet meeting all the conditions to be incorporated into the market as an effective drug against HAT [21, 22]. In this regard, lycorine, another well know alkaloid belonging to this plant family, has been reported to show high activity against *P. falciparum*, however, it has shown high cytotoxicity against normal human cells with a low SI value [10, 23]. Thus, **3** and **4** are ranked among the

Amaryllidaceae alkaloids with the highest anti-*P. falciparum* activity, standing out **3** for its low cytotoxicity.

3. Materials and Methods

3.1. General experimental procedures

NMR spectra were performed on Mercury 400 MHz (Palo Alto, CA, USA) and a Varian 500 MHz (Palo Alto, CA, USA) instrument using $CDCl_3$ and CD_3OD as solvents. All 2D NMR experiments were performed using standard pulse sequences. GC-MS analysis of alkaloid extracts and pure compounds were performed on an Agilent 6890N GC 5975 instrument (Agilent Technologies, Santa Clara, CA, USA) operating in EI mode at 70 eV. A DB-5 MS column (30 m x 0.25 mm x 0.25 μm) was used. The temperature program was: 100-180 °C at 15 °C min^{-1}, 1 min hold at 180 °C, 180-300 °C at 5 °C min^{-1}, and 1 min hold at 300 °C. The injector temperature was 280 °C. The flow rate of carrier gas (He) was 0.8 ml min^{-1}. The split ratio was 1:20. The results obtained were analyzed using AMDIS 2.64 software. Compounds were identified through comparing their mass spectral patterns and retention index (RI) with those of more than 300 previously isolated alkaloids spectra in homemade databases (a private Amaryllidaceae alkaloid library) [8]. RIs were calculated through calibration with a standard *n*-hydrocarbon mixture (C9−C36). The LC-MS analysis was performed in an ACQUITY H–Class UPLC instrument equipped with a XEVO TQ-S micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) with electrospray ionization (ESI). An UPLC ACQUITY BEH C18 (1.7 μm, 2.1 mm × 100 mm) column was used for separation at 35 °C. The mobile phase consisted of A (0.1% formic acid), B (acetonitrile, 0.1% formic acid), and C (methanol) with a flow rate of 0.2 mL/min. The gradient conditions were as follows: initially, 95%A - 5%B and hold for 2min; 5 min, 85%A - 15%B; 10 min, 80%A - 10%B – 10%C and hold for 7min; 18 min, 95%A - 5%B and hold for 2 min; completing 20 min. The *H. argentinum* and *H. escoipense* alkaloid extracts were prepared at 100 ppm. The samples were dissolved in a mixture of methanol:water (50:50) and filtered through a membrane filter (0.22 μm). The injection volume was 10 μL. The capillary, cone, and collision energies were 2 kV, 43 V, and 30 eV, respectively. The data were acquired in ESI positive mode, MS2 scan function (50-1000Da), and processed using MassLynx Software V4.2 (Waters, Milford, MA, USA).

3.2. Plant material

Whole plants of *Hippeastrum argentinum* (Pax) Hunz. and *Hippeastrum escoipense* Slanis & Huaylla were collected in the Provinces of Tucumán and Salta, Argentine, in February 2021 during the flowering period. The plant species were taxonomically classified by botanists Alberto C. Slanis and Hibert Huaylla and then cultivated in greenhouse conditions for research purposes. In this regard, the leaves of these plants were harvested in late summer period and dried for further research studies. A voucher of each specimen was deposited at the Instituto de Biotecnología, Universidad Nacional de San Juan, with the code IBT-UNSJ-Arg.15 (*H. argentinum*) and at the Herbario Miguel Lillo (*H. escoipense*) (holotipo, LIL-617,830 [004,008], isotipos LIL- 617,830 [004, 009, 004, 010, 004, 011, 004, 012]) according to [24].

3.3. Extraction and Isolation

Dried plant material was extracted three times with MeOH under reflux for 1 h each. The solvent was evaporated under reduced pressure to obtain the crude MeOH extract. The extract was dissolved in 2% H_2SO_4 (v/v), and neutral material was removed three times with Et_2O. Then, the aqueous solution was basified with 25% NaOH to pH 10-11 and extracted three times with EtOAc to give the alkaloid extracts. Compounds **1-4** were isolated following the main chromatographic steps described by Ortiz *et al.* [12,13].

3.3.1. *H. argentinum*

The dried leaves (112,5 g) yielded 250 mg of alkaloid extract. It was roughly separated by SiO_2 flash CC using an *n*-hexane/EtOAc/CH_2Cl_2/MeOH gradient to give six fractions (A–F): fraction A (*n*-hexane/EtOAc, 1:4), B (EtOAc), C (EtOAc/CH_2Cl_2, 1:1), D (EtOAc/MeOH, 9.5:0.5), E (EtOAc/MeOH, 9:1), and F (MeOH). Column chromatography on Sephadex LH–20 of fraction A in MeOH gave three subfractions. Fraction A1 was consecutively chromatographed on a silica gel column (CC) using gradient elution from *n*-hexane/EtOAc (2:8–0:10) to EtOAc/MeOH (9:1) to give six subfractions (A1a–A1f). Column fractions were monitored by TLC, and similar ones were combined and evaporated to dryness. Crystallization of fraction A1c afforded 7-hydroxyclivonine (**3**) (12,4 mg). Column chromatography on Sephadex LH-20 of fraction B in MeOH gave three subfractions. Subfraction B1 was subjected to prep. TLC (silica gel with *n*-hexane/EtOAc/$CHCl_3$/MeOH, 3:4:2:1, in NH_3 atmosphere) to give 4-*O*-

methylnangustine (**1**) (15,2 mg). Subfraction B2 was subjected to CC on silica gel eluted with $CHCl_3$/MeOH (95:5), to afford hamayne (**2**) (9 mg).

3.3.2. *H. escoipense*

The dried leaves (80 g) yielded 110 mg of alkaloid extract. Column chromatography on Sephadex LH–20 of the alkaloid extract in MeOH gave three fractions (A-C). Fraction B was filtered (0.22 μm) and 13,5 mg of candimine (**4**) was obtained by direct crystallization.

3.4. *In Vitro* Assays

3.4.1 *Trypanosoma brucei rhodesiense.*

Serial drug dilutions in Minimum Essential Medium (MEM) supplemented according to Baltz *et al.* [25] were added to microplates. Trypomastigotes of *T. brucei rhodesiense* (STIB 900 strain) were added to each well and the plates incubated for 72 h. Viability was assessed by Alamar Blue leading to a colour reaction which was read in a fluorescence scanner (Millipore Cytofluor 2300) [26]. Fluorescence development was expressed as percentage of the control, and IC_{50} values determined.

3.4.2 *Trypanosoma cruzi.*

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 μL in RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h 5000 trypomastigotes of *T. cruzi* (Tulahuen C2C4 strain, containing the β-galactosidase (Lac Z) gene) were added in 100 μL per well with 2x of a serial drug dilution. The plates were incubated at 37 °C in 5% CO_2 for 4 days. For measurement of the IC_{50} the substrate CPRG/Nonidet was added to the wells. The colour reaction which developed during the following 2– 4 h was read photometrically at 540 nm. From the sigmoidal inhibition curve IC_{50} values were calculated. Considering robustness, reproducibility, and selectivity criteria, this assay was repeated for candimine under laboratory conditions similar to those previously reported by our group [12]. Thus, similar results are presented again in this article just for candimine against *T. cruzi*.

3.4.3 *Leishmania donovani.*

Mouse peritoneal macrophages were seeded in RPMI 1640 medium with 10% heat inactivated FBS into Lab-tek 16-chamber slides. After 24 h, *L. donovani* amastigotes (MHOM-ET-67/L82 strain) were added at a ratio of 3:1 (amastigotes to macrophages). The medium containing free amastigotes was replaced by fresh medium 4 h later. Next day the medium was replaced by fresh medium containing different drug concentrations. The slides were incubated at 37 ºC under a 5% CO_2 atmosphere for 96 h. Then the medium was removed, the slides fixed with methanol and stained with Giemsa. The ratio of infected to non-infected macrophages was determined microscopically, expressed as percentage of the control and the IC_{50} value calculated by linear regression.

3.4.4 *Plasmodium falciparum.*

Antiplasmodial activity was determined using trophozoites of *P. falciparum* (NF54 strain). A modification of the [3H]-hypoxanthine incorporation assay was used [27]. Briefly, infected human red blood cells were exposed to serial drug dilutions in microtiter plates for 48 h. Viability was assessed by measuring the incorporation of [3H]-hypoxanthine by liquid scintillation counting. From the sigmoidal inhibition curves, IC_{50} values were calculated.

3.4.5 Cytotoxic activity for L-6 cells.

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 µL in RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h 100 µL of drug dilutions were added per well. Viability was assessed by Alamar Blue leading to a colour reaction which was read in a fluorescence scanner (Millipore Cytofluor 2300) [26]. Fluorescence development was expressed as percentage of the control, and IC_{50} values determined. All the experiments were performed at least in triplicate. Melarsoprol, benznidazole, miltefosine, chloroquine, and podophyllotoxin were used as reference drugs for *T. b. rhodesiense*, *T. cruzi*, *L. donovani*, and *P. falciparum*, respectively.

4. Conclusions

The two *Hippeastrum* species of the Amaryllidoideae subfamily (Amaryllidaceae) species consolidate as sources of bioactive alkaloids for a variety of parasites including different trypanosomatids in the screening for new drugs candidates. Concerning this, homolycorine-type alkaloids, emerge as promising

scaffolds for antiparasitic drug development, among which, candimine highlights as a new lead compound against trypanosomatids and other parasites related to neglected tropical diseases, deserving further study as a scaffold in order to apply different strategies to obtain similar compounds with similar or more activity but especially less toxicity. Finally, given the selectivity properties of 7-hydroxyclivonine against *P. falciparum*, additional assays and comprehensive studies involving medicinal chemistry and organic semi-synthesis, among other fields, are necessary to enhance its bioactivity.

Supplementary Material

Supporting information for this article is available

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Author Contributions

Conceptualization, J.E.O., J.B., and G.E.F.; methodology, J.E.O., M.P., M.K., and P.M.; formal analysis, J.E.O., M.P., J.B., and G.E.F.; investigation, J.E.O., M.P., and G-E.F.; writing-original draft preparation, J.E.O, and G.E.F.; writing—review and editing, G.E.F. and J.B.; supervision, M.K., P.M.; funding acquisition, J.B. and G.E.F. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials.

Data will be made available on request.

Declarations

Competing interests

The authors declare no competing interests.

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