BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e20459

Article

Anti-*Acanthamoeba castellanii* activity of alkaloid-enriched extracts and lycorine from the Amaryllidaceae species

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Free-living amoebae of the genus *Acanthamoeba* are the causative agents of granulomatous encephalitis and keratitis, severe human infections. Bioactive compounds from plants are recognized as an alternative source for the development of new drugs. The Amaryllidaceae is a botanical family able to synthesize a very specific and consistent group of biologically active isoquinoline-like alkaloids. The alkaloidal fractions from the Brazilian species *Hippeastrum canastrense, H. diniz-cruziae, H. puniceum*, and *Crinum x amabile*, along with the alkaloid lycorine, were investigated against *Acanthamoeba castellanii*. The *in vitro* assays were performed with distinct concentrations of lycorine and alkaloidal fractions, while the cell viability was evaluated by the MTT method upon MDCK cells. Chlorhexidine 0.02% was used as the positive control. The effect of alkaloid fractions was concentration dependent, and 2000 μg mL^{-1} of *H. canastrense* and *H. diniz-cruziae* provided a 100% inhibition. At concentrations of 250, 500, and 1000 μg mL^{-1}, the *H. diniz-cruziae* alkaloidal fraction showed the lowest cytotoxic effect (5%–7%) and remarkable anti-amoebic activity, demonstrating values of IC_{50} 285.61 μg mL^{-1}, low cytotoxicity (5%–7%), and selectivity index (7.0). Taken together, the results are indicative of the great potential that the alkaloids from *H. diniz-cruziae* have as new candidates for anti-amoebicidal compounds.

Keywords: *Acanthamoeba castellanii*. Cytotoxicity. MDCK cell. MTT. Amaryllidaceae alkaloids. Natural products.

INTRODUCTION

Species of the genus *Acanthamoeba* are free-living amobae (FLA), which are very common in the environment and considered opportunistic pathogens. These organisms are the causative agents of granulomatous amoebic encephalitis (GAE), an uncommon disease that affects the central nervous system, primarily in immunocompromised individuals (Marciano-Cabral, Cabral, 2003). *Acanthamoeba* is also involved in amoebic keratitis (AK), a corneal infection that may cause blindness and eye loss (Schuster, Visvesvara, 2004).

In recent decades, AK has gained relevance due to the increasing number of cases worldwide, which is associated with the expansion of the use of contact lenses (Brown *et al*., 2018). Contamination is facilitated by the using homemade solutions, such as saline or tap water, to wash contact lenses, showering while wearing lenses, and using disposable lenses for a longer period than recommended (Radford, Bacon, Dart, 1995). The treatment of AK is still a great challenge to be overcome due to the drug-resistance of cysts, high toxicity in the

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corneal tissue, and high cost of novel drug therapy (Alvarenga, Freitas, Hofling-Lima, 2000; Hammersmith, 2006). Pharmacokinetic parameters related to dosage schedules are also obstacles for adherence to drug therapy. Taken together, all of these factors contribute to the prevalence of AK and prompt the development of new drugs to improve the prognosis of AK.

The screening of medicinal plants has been highlighted as a promising method to discover new or alternative bioactive substances that can be used in the management of AK disease (Goze *et al*., 2009; Sifaoui *et al*., 2014). Amaryllidaceae plants have been used in traditional medicine for many years (Nair, Van Staden, 2014). The biological activities of the Amaryllidaceae species include antiprotozoal, antitumor, antiviral, immunostimulatory, antimalarial, and anti-cholinesterase activities (Sener, Orhan, Satayavivad, 2003; Bastida, Lavilla, Viladomat, 2006; Osorio *et al*., 2010; Pagliosa *et al*., 2010; Luo *et al*., 2012). The Amaryllidaceae compounds lycorine and candimine displayed anti-protozoal effects through a non-apoptotic and paraptotic cell death mechanism, respectively (Giordani *et al*., 2010; Giordani *et al*., 2011).

Considering the potential of Amaryllidaceae alkaloids as anti-parasitic agents, the enriched-alkaloid fraction from the Brazilian species *Hippeastrum canastrense*, *H. diniz-cruziae*, *H. puniceum*, and *Crinum x amabile* were obtained and evaluated as anti-*Acanthamoeba castellanii* agents. The alkaloid lycorine was also evaluated for the same biological target. The cytotoxicity of alkaloid fractions of these species and the alkaloid lycorine also are discussed in this manuscript.

MATERIAL AND METHODS

Acanthamoeba strain

An axenic strain of *Acanthamoeba castellanii*, named ALX, was used in this study. The strain was isolated from AK patients from the city of Vitória in the state of Espírito Santo, Brazil, and belongs to the T4 genotype. Trophozoites were cultivated in Proteose-peptone - yeast extract - glucose medium (PYG) at 28°C (Duarte *et al*., 2013). Trophozoites in exponential growth (48-72h) and presenting at least a 95% viability (evaluated by 0.5% trypan blue exclusion) were used in the experiments.

Cell culture

Madin-Darby Canine Kidney-MDCK (NBL_2) mammalian cells (ATCC® CCL34) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Vitrocell Embriolife) and 1% antibiotic and antimycotic-PSA solution (10,000 IU mL^{-1} penicillin, 10 mg streptomycin, and 25 µg mL^{-1} amphotericin B) (Sigma-Aldrich, St. Louis, MO, USA). Cultures were incubated at 37°C in a humidified 5% CO_2 incubator.

Plant material

Hippeastrum canastrense and *H. diniz-cruziae* were collected from the "Serra da Canastra" ecosystem in the state of Minas Gerais, Brazil. *H. puniceum* was collected in the city of Venda Nova do Imigrante, and *Crinum x amabile* was collected in the city of Vitória, both in the state of Espírito Santo (Brazil). The identification, registration, and deposit were performed in the UFES Herbarium. The Herbarium registry numbers are VIES-35305 (*H. canastrense*), VIES-39497 (*H. diniz-cruziae*), VIES-39569 (*H. puniceum*), and VIES-39506 (*C. x amabile*), and the SisGen registration number is A012E5E.

The phytochemical procedure of tested compound/ alkaloid-enriched extracts

The procedure to achieve the alkaloid-enriched extract was conducted separately for all species. Therefore, each bulb species was oven-dried at 40°C, pulverized, and macerated with methanol (MeOH) using 100 mL MeOH per 5 g of dry material, followed by filtration every 48 h. Three macerations were performed. The filtrate was pooled and evaporated in a rotary evaporator under reduced pressure to provide the methanolic extract, which was then acidified with sulfuric acid (H_2SO_4, 2% v/v) up to pH 2-3 and partitioned with diethyl ether (Et_2O, 3 times), followed by ethyl acetate (EtOAc, 2 times). The

resulting solution was then alkalinized up to pH 9-10 with ammonium hydroxide (NH_4OH) (25%) and five partitions were carried out with EtOAc to achieve the enriched-alkaloid extract. This fraction was desiccated with anhydrous sodium sulfate ($NaSO_4$) and dried by rotary evaporation. The MeOH, H_2SO_4, Et_2O, AcOEt, and NH_4OH solvents used for the extraction procedures were of analytical grade. An aliquot of the alkaloid fractions was solubilized in sterile distilled water and prepared at the appropriate concentrations for the biological tests. The remaining portion of the alkaloid fraction was used for capillary gas chromatography-mass spectrometry analysis (Capillary GC-MS, Section Capillary gas chromatography- mass spectrometry of *Hippeastrum* alkaloid fractions).

Lycorine purification

The alkaloid lycorine was purified in a previous study from the species *Worsleya procera* and *Hippeastrum aulicum*. The typical acid-base alkaloid extraction was applied in these species, and lycorine precipitated spontaneously from the AcOEt fraction (Bessa *et al.*, 2017). The structure of lycorine has been confirmed by using Nuclear Magnetic Resonance spectroscopy (Bastida *et al.*, 2006). Lycorine alkaloid was stored at our laboratory from previous phytochemical investigations in Amaryllidaceae plants (Bessa *et al*., 2017) and was used in the present study for anti-*Acanthamoeba* evaluation. Lycorine was dissolved in sterile distilled water and prepared at the appropriate concentrations for the tests.

Capillary gas chromatography-mass spectrometry of *Hippeastrum* alkaloid fractions

To characterize alkaloid fractions of *H. canastrense* and *H. diniz-cruziae*, aliquots of the extracts were suspended in MeOH (HPLC grade) at 1000 ppm (1 mg mL^{-1}). The samples were filtered through a 0.45-µm filter and subsequently analyzed individually by Capillary GC-MS. The spectra were obtained on a GC-17A Shimadzu GC-MS QP 5000, operating in EI mode at 70 eV, with a DB5 MS column (30 m × 0.25 mm × 0.25 µm). The following temperature program was used: 100–180°C at 15°C min^{-1}, 1 min hold at 180°C, 180–300°C at 5°C min^{-1}, and a 10 min hold at 300°C. The injector temperature was 280°C, the flow rate of the carrier gas (He) was 0.8 mL min^{-1}, and the split ratio was 1:20. The Amaryllidaceae alkaloids were identified by comparing their GC-MS spectra and Kovats retention indices (RI) with our library database. This library has been regularly updated with alkaloids isolated and unequivocally identified via physical and spectroscopic methods (Berkov *et al*., 2008; Giordani *et al*., 2010; De Andrade *et al*., 2016). Mass spectra were deconvoluted using AMDIS 2.64 software (NIST), and RIs recorded using a standard n-hydrocarbon calibration mixture (C9-C36). The proportion of individual components in the alkaloid fractions are expressed as a percentage of total alkaloid content. GC-MS peak areas are dependent on both the concentration of the injected alkaloid well as the intensity of its mass spectral fragmentation. Although the data is given in Tables I and II, they are not representative of a validated alkaloid quantification method; they can only be used for relative comparison purposes. Undefined compounds were referred to as "UK" (unknown) in Tables I and II. Tentative determination of the skeleton-type were based on the fragmentation mode of the Amaryllidaceae alkaloids under GC-MS conditions (Bastida, Lavilla, Viladomat, 2006; Berkov *et al*., 2008).

Amoebicidal assay

The *in vitro* anti-*A. castellanii* activity of plants' alkaloid fractions and isolated lycorine was evaluated against the trophozoites by 3-(4,5-dimethyl)-2,5-diphenyltetrazolium (MTT) assay (Mosmann, 1983), following a protocol described by Heredero-Bermejo *et al.* (2013) with some modifications. The tests were performed in quadruplicate on a 96-well cell culture microplate containing 100 µL per well of trophozoite suspension (1.0×10^6 trophozoites mL^{-1}). A hundred microliters of every plant alkaloid fraction solution was added to the wells at 250, 500, 1000, and 2000 µg mL^{-1}. The plates were incubated at 28ºC for 48 h. The isolated alkaloid lycorine was also tested at the concentrations

of 100, 200, and 400 μg mL^{-1} and incubation periods of 24, 48, and 72 h. Chlorhexidine (0.02%), a reference drug for AK, was used to compare the amoebicidal activity. After incubation, 20 μL of the MTT solution (5 mg mL^{-1}) (Life Technologies) was added to each well and the plate remained at 28ºC for 4 h. The well supernatants were then removed by aspiration, and 100 μL dimethylsulfoxide (DMSO) was added to solubilize formazan crystals. The optical density in each well was measured at 540 nm by using an ELISA microplate reader (i-Mark, Bio Rad Laboratories, Washington, USA) with a reference filter of 620 nm. Trophozoite viability was determined by the absorbance values concerning replicates containing only a PYG medium, whose absorbance value was considered to be 100% viable (Lukác *et al*., 2013). Percent inhibition was calculated by subtracting the viability value from 100. Additional controls included in the assay were constituted by the PYG medium, the solution of the alkaloid fraction without trophozoites, and the sterile distilled water (fraction diluent).

Cytotoxic assay

The MTT assay was also used to evaluate the *in vitro* cytotoxicity of the alkaloid fractions and isolated lycorine on MDCK mammalian cells. The experiments were performed in quadruplicate and on four distinct occasions following the protocols described by Sauter *et al*. (2011) with some modifications. Briefly, 100 μL of DMEM with 4.0×10^5 cells mL^{-1} were plated in each well of a 96-well cell culture microplate. After incubation for 24 h (37ºC, 5% CO_2), 100 μL of the alkaloid fractions (250, 500, 1000, and 2000 μg mL^{-1}) and isolated lycorine (100, 200, and 400 μg mL^{-1}) were added to each well. Chlorhexidine (0.02%) was also tested for comparison. The plates were incubated at 37ºC for 48h. After incubation, 20 μL of the MTT solution (5 mg mL^{-1}) (Life Technologies) was added to each well, and the plate remained at 37ºC for 4 h. The wells' supernatants were then removed by aspiration, and 100 μL dimethylsulfoxide (DMSO) was added to solubilize formazan crystals and to determine the optical density as described in the previous section. Replicates containing only DMEM were used to determine 100% viability. Percent inhibition was calculated by subtracting the viability value from 100. A solution of the alkaloid fraction without the cells and sterile distilled water (fraction diluent) were used as additional controls.

Statistical analysis

Data was analyzed by two-way ANOVA using the *Bonferroni* test as an *a posteriori* test in the *GraphPad Prism* 5.0 statistical program. Values of $p < 0.05$ were considered to be statistically significant. The 50% inhibition values in the amoeba population (IC_{50}) and the 50% cytotoxic values in the cell population (CC_{50}) for alkaloid-enriched fractions and lycorine were calculated by linear regression analysis. The selectivity index (SI) was obtained from the ratio between the value of the CC_{50} and the value of the IC_{50}.

RESULTS AND DISCUSSION

Amoebicidal assay

The alkaloid fractions of *H. canastrense, H. diniz-cruziae, H. puniceum*, and *Crinum x amabile* showed inhibitory activity on *A. castellanii* trophozoites (Figure 1). *H. canastrense* and *H. diniz-cruziae* at 2000 μg mL^{-1} caused 100% inhibition, which was significantly higher than inhibition induced by chlorhexidine (86%) ($p < 0.05$). The fractions of *H. canastrense* and *H. diniz-cruziae* at 1000 μg mL^{-1} caused 80% and 82% inhibition, respectively, while *C. x amabile* showed 72% inhibition at 2000 μg mL^{-1}. These effects and those from other plant fractions were not significantly different from the effects of chlorhexidine ($p > 0.05$).

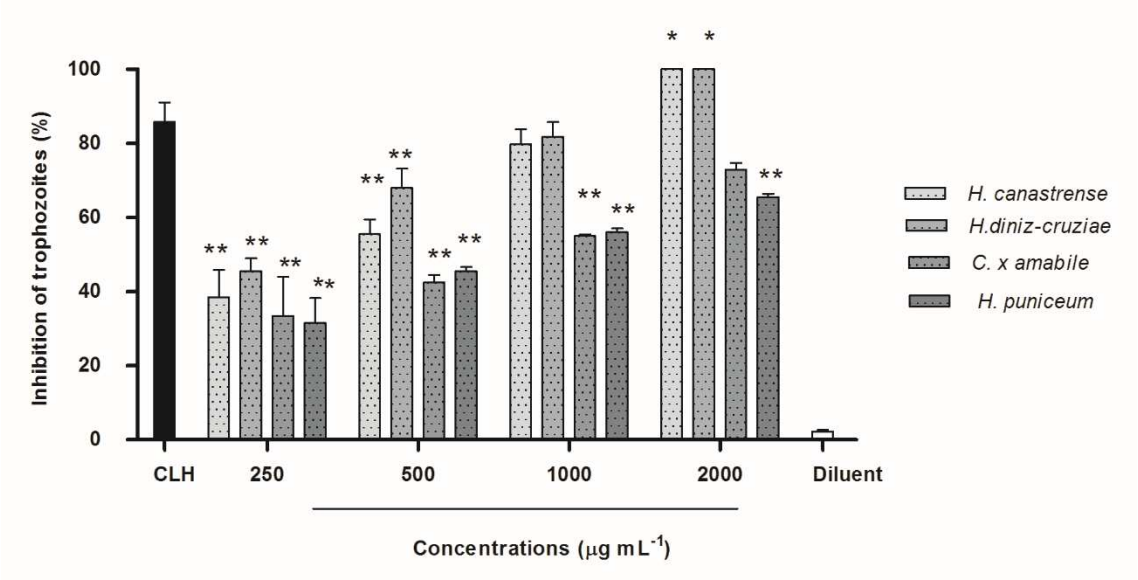

FIGURE 1 - Inhibition of *Acanthamoeba castellanii* trophozoites after exposure to various concentrations of the alkaloid fractions from the Amaryllidaceae species and chlorhexidine for 48h. Significant differences in relation to chlorhexidine effects are indicated by one ($p < 0.05$) or two ($p < 0.001$) asterisks. PYG: *Acanthamoeba* culture medium. CLH: chlorhexidine 0.02%. Diluent: Distilled water.

The alkaloid lycorine at 100 μg mL^{-1} also exhibited an inhibitory effect on the cultures of *A. castellanii* with 8%, 18%, and 20% inhibition after 24 h, 48 h, and 72 h, respectively. These percentages were significantly lower than those observed for chlorhexidine ($p< 0.001$), which caused 35%, 88%, and 93% of inhibition after 24 h, 48 h, and 72 h, respectively (Figure 2). The lycorine at 100, 200, and 400 μg mL^{-1} showed percentages of trophozoite inhibition from 18% to 44% with an exposure time of 48h. Regardless of the intensity of the amoebicidal effect displayed by the different Amaryllidaceae species, this data provides strong evidence that the isoquinolinic Amaryllidaceae alkaloids provide effective activity against *Acanthamoeba*.

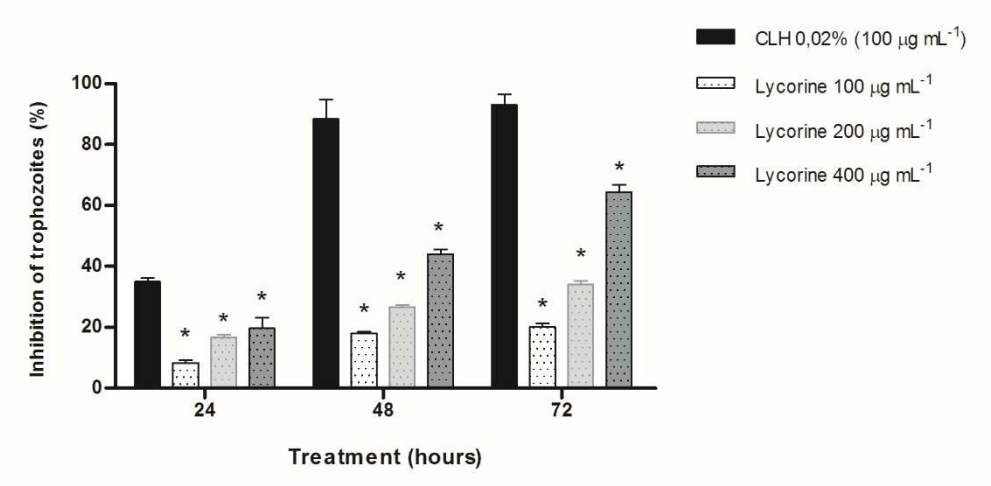

FIGURE 2 - Inhibition of *Acanthamoeba castellanii* trophozoites after exposure to the alkaloid lycorine and chlorhexidine for 24 h, 48 h, and 72 h. Significant differences ($p < 0.001$) in relation to chlorhexidine effects are indicated by one asterisk.

The investigation of anti-amoebic properties in plant compounds is an essential strategy to search for and develop potential new drugs for the treatment of *Acanthamoeba* infections. This approach has allowed for the discovery of some new bioactive compounds that exert anti-*Acanthamoeba* activity (Turner *et al*., 2000; Badria *et al*., 2014; Kuźma *et al*., 2015; Lorenzo-Morales, Khan, Walochnik, 2015). Presently, four alkaloid fractions of the Amaryllidaceae species and the alkaloid lycorine were evaluated against *A. castellanii*. Previous studies have revealed diverse biological activities of Amaryllidaceae, including antiparasitic effects on pathogenic protozoans, such as *Trypanosoma cruzi*, *T. brucei rhodesiense*, *Plasmodium falciparum*, *Leishmania infantum*, and *Trichomonas vaginalis* (Labraña *et al*., 2002; Sener, Orhan, Satayavivad, 2003; Bastida, Lavilla, Viladomat, 2006; Toriizuka *et al*., 2008; Giordani *et al*., 2010; Osorio *et al*., 2010, Kaya *et al*., 2011).

The four Amaryllidaceae species fractions and lycorine showed inhibitory effects against cultures of *A. castellanii* in distinct intensities. Previously, only one study investigated the amoebicidal activity of the Amaryllidaceae family against *Acanthamoeba* (El-Sayed *et al*., 2012). The authors found that *Pancratium maritimum* induced 75% of culture inhibition at the same concentration and exposure time used in this study to assay the alkaloid-enriched fractions from *H. canastrense* and *H. diniz-cruziae* (2000 μg mL^{-1}, 48 h). Both of these extracts and conditions showed the highest percentage of amoebic cell death (100%). Other fractions (*H. puniceum* and *C x. amabile*) caused 65% and 72% of trophozoite inhibition under similar conditions, respectively.

Cytotoxic assay

The cytotoxicity determined by *H. canastrense, H. diniz-cruziae, H. puniceum*, and *Crinum x amabile* fractions in MDCK cells is represented in Figure 3. The *H. diniz-cruziae* fraction at 250, 500, and 1000 μg mL^{-1} caused the lowest percentages of cell death (5% to 7%), which was similar to that induced by DMEM (0%) ($p > 0.05$). The other fractions (*H. canastrense, H. puniceum*, and *C. x amabile*), at all tested concentrations, presented cytotoxicity 15% to 47% higher than the control represented by samples with only DMEM ($p < 0.001$). The cytotoxicity of 0.02% chlorhexidine was significantly higher than that observed for all other fractions, inducing 97% of cell death in the cultures.

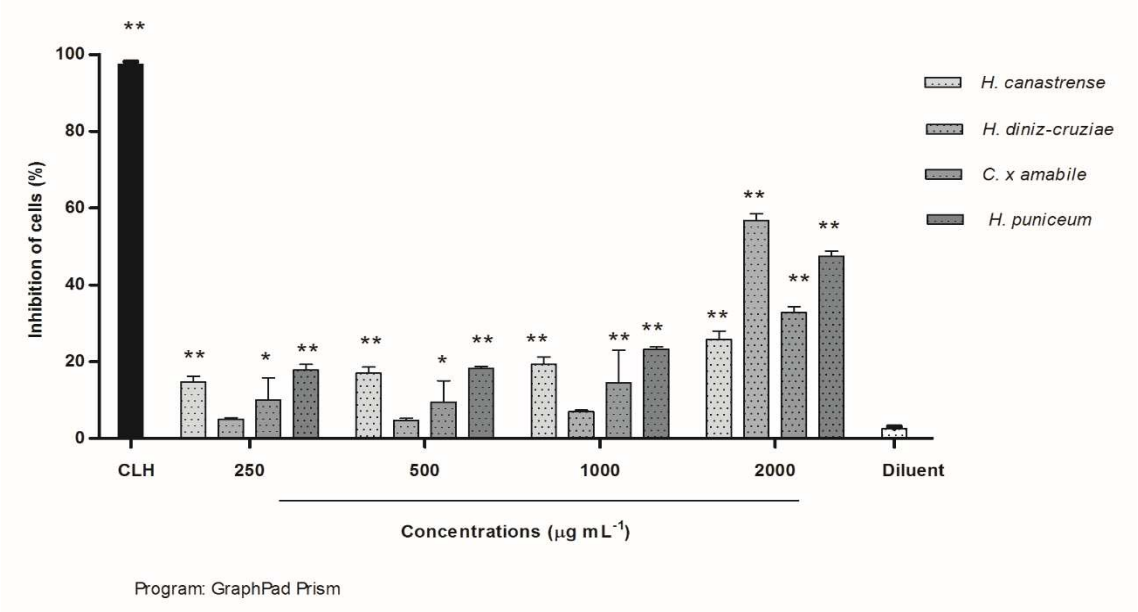

FIGURE 3 - Inhibition of MDCK cultures after 48 h exposure to the alkaloid fractions from the Amaryllidaceae species and chlorhexidine. Significant differences in relation to DMEM are indicated by one ($p < 0.05$) or two ($p < 0.001$) asterisks. DMEM: Cell culture medium. CLH: chlorhexidine 0.02%. Diluent: Distilled water.

Compared to 0.02% chlorhexidine, *H. canastrense* and *H. diniz-cruziae* showed a higher inhibitory effect, which reached 100% at 2000 μg mL^{-1}. Moreover, the prominent cytotoxicity of chlorhexidine (97%) contrasted with that exhibited by all of the alkaloid fractions (5% to 51%). Low cytotoxicity is a very relevant issue in the search for new amoebicidal targets since the current AK treatments are severely limited by aggressive effects on corneal cells. Drugs currently prescribed for treatment include biguanides (PHMB - biguanide polynexamethylene), aromatic diamines (brolene® - isethionate propamidine), and even antifungals, such as miconazole. However, even at low concentrations, these compounds may cause significant side effects and initiate keratopathy (Alvarenga, Freitas, Hofling-Lima, 2000; Lorenzo-Morales *et al*., 2013; Lorenzo-Morales, Khan, Walochnik, 2015).

Lycorine at 100, 200, and 400 μg mL^{-1} induced cytotoxic effects of 15% to 36% (Figure 4). Compared to DMEM (0%), all tested concentrations of lycorine exerted significant concentration-dependent cytotoxicity. Nevertheless, lycorine showed a significantly lower lethal effect on cells than chlorhexidine did, which caused a cytotoxicity of 97%.

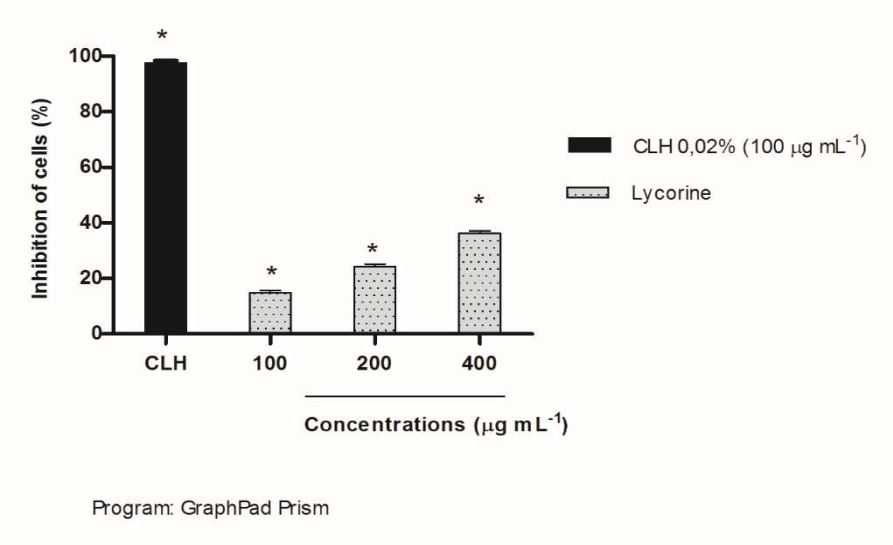

FIGURE 4 - Inhibition of MDCK cells after 48 h exposure to the alkaloid lycorine. Significant differences ($p < 0.001$) in relation to DMEM are indicated by one asterisk. DMEM: Cell culture medium. CLH: chlorhexidine 0.02%.

Chemically, lycorine is a pyrrolo[*de*] phenanthridine alkaloid (Bastida, Lavilla, Viladomat, 2006). In terms of biological activity, lycorine shows a wide range of potential as a chemotherapeutic drug due to its antiproliferative capacity against many cancer cell lines (Likhitwitayawuid *et al*., 1993; McNulty *et al*., 2009; Lamoral-Theys *et al*., 2010). Lycorine is also an antiviral, anti-inflammatory, antifungal, and anti-protozoan agent (Çitoğlu, Tanker, Gümüşel, 1998; Giordani *et al*., 2010; Reyes-Chilpa *et al*., 2011; Giordani *et al*., 2011). In this way, the great potential of lycorine as a therapeutic agent has encouraged the evaluation of its profile as an amoebicidal substance in the present work. Furthermore, the present study has sought to inquire if lycorine could be considered one of the causative agents of the activity against *A. castellani* in the enriched-alkaloid extracts. The tests indicated that lycorine was less cytotoxic than 0.02% chlorhexidine, but exerted a significantly lower lethal effect on *Acanthamoeba* trophozoites than the reference drug did. Thus, further studies with other isolated alkaloids are necessary to determine the main anti-*Acanthamoeba* compounds present in both of these Amaryllidaceae species.

Capillary gas chromatography-mass spectrometry of *Hippeastrum* alkaloid fractions

Capillary GC-MS analysis of the alkaloid fractions of *H. canastrense* and *H. diniz-cruziae* revealed the presence of alkaloid characteristics of the Amaryllidaceae family (Table I and II). Sixteen compounds were determined in *H. canastrense*, including a great number of homolycorine-type derivates. An additional four compounds were not identified in this species (Table I).

In *H. diniz-cruziae*, fourteen compounds were identified and another five were not determined (Table II). The main compound observed in *H. diniz-cruziae* was lycorine (39%). Other lycorine representatives, such as anhydrolycorine, 11, 12-dehydroanhydrolycorine, dehydrolycorine, pseudolycorine, 2-*O*-acetyllycorine, and ungiminorine were also identified in *H. diniz-cruziae* (Table II).

TABLE I – Capillary GC-MS analysis of the alkaloid fraction of *Hippeastrum canastrense*

Capillary GC-MS bulbs *H. canastrense*			
Alkaloid	**Ion Current**	**Skeleton**	**%**
Trisphaeridine	540967	Miscellaneous	0.44
Ismine	257375	Miscellaneous	0.21
Galanthamine	2553006	Galanthamine	2.08
Sanguinine	6581640	Galanthamine	5.36
Nerinine	467886	Homolycorine	0.38
Anhydrolycorine	1179138	Homolycorine	0.96
O-Methyllycorenine	1677630	Homolycorine	1.37
8-*O*-Demethylmaritidine	2237061	Haemanthamine	1.87
7-Methoxy-*O*-Methyllycorenine	23340375	Homolycorine	19.00
11,12-Anhydrodehydrolycorine	2016118	Lycorine	1.64
UK (MM 349)	1982891	Undefined	1.61
Tazettine	2847277	Tazettine	2.32
UK - Homolycorine-type compound	656310	Homolycorine	0.53
Lycorine	11282590	Lycorine	9.18
Homolycorine	3101463	Homolycorine	2.52
Albomaculine	13475858	Homolycorine	10.97
9-*O*-Demethylhomolycorine	8838237	Homolycorine	7.19
UK - Homolycorine-type compound	9510704	Homolycorine	7.74
UK - Homolycorine-type compound	5767270	Homolycorine	4.69
2-*O*-Methylcandimine	24545759	Homolycorine	19.98
Total ion current	122859555		100.00

Capillary GC-MS data for *H. canastrense*. The values are expressed as a relative percentage of total ion current (TIC).

TABLE II – Capillary GC-MS analysis of the alkaloid fractions of *Hippeastrum diniz-cruziae*

Capillary GC-MS bulbs *H. diniz-cruziae*			
Alkaloid	Ion Current	Skeleton	%
Trisphaeridine	102340	Miscellaneous	0.16
Lycoramine	64063	Galanthamine	0.10
Norlycoramine	2887235	Galanthamine	4.57
Anhydrolycorine	1523268	Lycorine	2.41
8-*O*-Demethylmaritidine	930139	Crinane	1.47
Deacetylcantabricine	1558508	Crinane	2.47
11,12-Dehydroanhydrolycorine	5572933	Lycorine	8.82
UK (MM 301)	2197548	Undefined	3.48
Lycorine	25150033	Lycorine	39.8
UK - Homolycorine-type	377994	Homolycorine	0.6
Dehydrolycorine	1729534	Lycorine	2.74
Pseudolycorine	2947111	Lycorine	4.66
2-*O*-Acetyllycorine	506424	Lycorine	0.8
UK - Possible lycorine-type compound	8035138	Lycorine	12.72
Ungiminorine	3703185	Lycorine	5.86
UK (MM 265) - Possible lycorine-type compound	2566744	Lycorine	4.06
UK (MM 299)	3051069	Undefined	4.83
2-**α**-Hydroxyhomolycorine	285748	Homolycorine	0.22
Candimine	298736	Homolycorine	0.23
Total ion current	63189014		100.00

Capillary GC-MS data for *H. diniz-cruziae*. The values are expressed as a relative percentage of total ion current (TIC).

The alkaloids are well known as active compounds, and Capillary GC-MS confirmed their presence in both *Hippeastrum* species evaluated in this study. In *H. canastrensis,* the homolycorine derivative 2-*O*-Methylcandimine is related to candimine, compounds that were purified from *H. morelianum* and showed remarkable activity against the unicellular eukaryotic protist *Trichomonas vaginalis* (Giordani *et al*., 2010). Nevertheless, another five homolycorine related compounds were also identified in *H. canastrense* (nerinine, *O*-methyllycorine, 7-methoxy-*O*-methyllycorenine, homolycorine, and albomaculine), along with three undefined compounds belonging to the homolycorine-skeleton. Unlike *H. canastrensis, H. diniz-cruziae* fractions contained more lycorine, which is biogenetically related to homolycorine-type skeletons (Bastida, Lavilla, Viladomat, 2006). *H. diniz-cruziae* also presented the trichomonicidal candimine, although at a very small relative percentage (0.23%). Collectively, the compound profile identified in Amaryllidaceae studied agrees with previous findings in the *Hippeastrum* genus, which have indicated a

consistent presence of homolycorine and lycorine representatives in their species (De Andrade *et al*., 2012; De Andrade *et al*., 2016).

The IC_{50} values were 389.31 μg mL^{-1} for *H. canastrense*, 285.61 μg mL^{-1} for *H. diniz-cruziae*, 962.75 μg mL^{-1} for *H. puniceum,* 857.61 μg mL^{-1} for *Crinum x amabile*, and 468.75 μg mL^{-1} for lycorine. The calculated CC_{50} values were 2500.02 μg mL^{-1} for *H. canastrense*, 1998.27 μg mL^{-1} for *H. diniz-cruziae*, 2297.65 μg mL^{-1} for *H. puniceum*, 2870.37 μg mL^{-1} for *C. x amabile*, and 596.47 μg mL^{-1} for lycorine. The values calculated for the SI were 6.42 for *H. canastrense,* 7.0 for *H. diniz-cruziae*, 2.38 for *H. puniceum*, 3.35 for *C. x amabile*, and 1.27 for lycorine (Table III).

TABLE III - IC_{50}, CC_{50}, and SI values of the alkaloid fractions of the Amaryllidaceae species and the alkaloid lycorine on *Acanthamoeba castellanii* trophozoites

Species of Amaryllidaceae	IC_{50}	CC_{50}	SI
Hippeastrum canastrense	389,31 μg. mL^{-1}	2.500,02 μg. mL^{-1}	6,42
Hippeastrum diniz-cruziae	285,61 μg. mL^{-1}	1.998,27 μg. mL^{-1}	7,00
Hippeastrum puniceum	962,75 μg. mL^{-1}	2.297,65 μg. mL^{-1}	2,38
Crinum x amabile	857,61 μg. mL^{-1}	2.870,37 μg. mL^{-1}	3,35
Lycorine	468,75 μg. mL^{-1}	596,47 μg. mL^{-1}	1,27
	(1633,27 μM)	(2078,29 μM)	

The SI is a parameter often used to express the safety of a compound tested and to make it possible to carry out new tests, whether *in vitro* or *in vivo* (Coen, Richman, 2007). Based on the SI values, it is possible to determine the selectivity of an extract or compound in relation to the tested cell cultures, and the higher the SI value, the more selective it will be. SI values less than 2 indicate general toxicity of the pure compound (Badisa *et al*., 2009; Koch *et al*., 2005), and values from 4 are considered positive, and the higher this value, the more promising the extract would be for further tests (Amoros *et al*., 1992). From the study of the four extracts tested, only two showed an SI value above 4, which points to two extracts, *H. canastrense* and *H. diniz-cruzie*, as being the most selective in relation to the cultures of *Acanthamoeba castellanii*. The alkaloid lycorine had an SI value of less than 2, which suggests general toxicity on cultures, requiring greater selectivity (Table III).

Lycorine is one of the main alkaloids found in Amaryllidaceae. However, the majority compound will not always be the most active in an extract since extracts are mixtures of compounds that can even act synergistically. The results found for lycorine demonstrate that this alkaloid has a role in the amebicidal activity, and this data supports future studies in the search for compounds with this anti-*Acanthamoeba* activity. However, we cannot rule out the possibility that other compounds in the extract may also have been active in the face of the reported results.

Among all of the tested fractions, *H. diniz-cruziae* combined the higher amoebicidal effect with the lowest cytotoxicity. At 1000 μg mL^{-1}, it exerted an anti-*A. castellanii* activity similar to that of chlorhexidine but with very low cytotoxicity (5%–7%). Also, the IC_{50} value of the *H. diniz-cruziae* fractions was the lowest (285.61 μg mL^{-1}), while those of the SI were the highest (7.00) among the evaluated species. Altogether, these results showed the highest selectivity for trophozoites, indicating *H. diniz-cruziae* as a very suitable candidate for the development of anti-*Acanthamoeba* drugs.

Acanthamoeba keratitis is a very serious disease due to its rapid evolution and difficulty to treat. As the disease has such clinical aggravating factors, there is great urgency in the demand for the discovery of

new effective amoebicidal therapies. Because of this, this research points to the Amaryllidaceae species as potential targets for research on therapeutic agents with anti-*Acanthamoeba* activity. The high efficacy and low cytotoxicity observed in the fractions of *H. diniz-cruziae* suggest that this species is a promising source of bioactive compounds for the treatment of acanthamoebiasis.

ACKNOWLEDGMENTS

This study was funded by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* - Brasil (CAPES) - Finance Code 001, an entity of the Brazilian Government that is focused on the formation of human resources, logged under protocol number AUX PE 1526/2011. We also wish to thank the CYTED program (416RT0511) and the Foundation of Support for Research and Innovation of Espírito Santo (FAPES Universal No. 80708382/18). J.P.A. is thankful to FAPES (n. 76192970/16) for a postdoctoral fellowship.

We send a special thanks to the VIES-UFES herbarium, under the supervision of Doctor Professor Luciana Dias Thomaz. We would also like to thank Doctor Professor Anderson Alves Araújo and Doctor Renata Souza de Oliveira. And finally, we wish to thank the Laboratory of Biological Screening of Natural Products-UFES and Doctor Professor Rodrigo Resende Kitagawa for his technical support.

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Received for publication on 16th June 2020
Accepted for publication on 03rd January 2021