

## Dereplication of Aporphine Alkaloids by UHPLC-HR-ESI-MS/MS and NMR from *Duguetia lanceolata* St.-Hil (Annonaceae) and Antiparasitic Activity Evaluation

Erick P. Dantas,<sup>a</sup> Jackson Monteiro,<sup>a</sup> Lívia S. de Medeiros,<sup>a</sup> Maiara M. Romanelli,<sup>b</sup> Maiara Amaral,<sup>b</sup> Andre G. Tempone,<sup>b</sup> João Henrique G. Lago,<sup>b,c</sup> Marisi G. Soares<sup>d</sup> and Patricia Sartorelli<sup>b,\*a</sup>

<sup>a</sup>Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, 09972-270 Diadema-SP, Brazil

<sup>b</sup>Centro de Parasitologia e Micologia, Instituto Adolfo Lutz, 01246-000 São Paulo-SP, Brazil

<sup>c</sup>Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, 09210-180 Santo André-SP, Brazil

<sup>d</sup>Instituto de Química, Universidade Federal de Alfenas, 37130-000 Alfenas-MG, Brazil

Although the genus *Duguetia* is well known for producing alkaloids as chemical constituents, there are no reports of alkaloids identified in the species *D. lanceolata*. Thus, aiming to identify the chemical composition of this species, the dereplication of alkaloidic phase was performed by use of ultra-high performance liquid chromatography high resolution electrospray ionization tandem mass spectrometry (UHPLC-HR-ESI-MS/MS) and <sup>1</sup>H nuclear magnetic resonance (NMR). The chromatographic fractionation of the alkaloid extract from *Duguetia lanceolata* (Annonaceae) leaves afforded four fractions (I-IV) that were shown to be composed of aporphine alkaloids. <sup>1</sup>H NMR analysis and UHPLC-HR-ESI-MS/MS based dereplication allowed the identification of eight alkaloids: glaucine (**1**), norglaucine (**2**), isocorydine (**3**), N-methylaurotetanine (**4**), oxoglucine (**5**), liriodenine (**6**), lanuginosine (**7**), dehydroglaucine (**8**). Compounds **2**, **3**, **4**, **6** and **7** were described for the first time in this species, while alkaloids **1**, **5** and **8** are newly discovered in the genus *Duguetia*. Additionally, the antiparasitic activity of the four fractions was evaluated *in vitro* against *Leishmania infantum* and *Trypanosoma cruzi*. Fraction I, composed exclusively by **1**, displayed activity against *Leishmania infantum* and *Trypanosoma cruzi* intracellular amastigotes, with half maximal inhibitory concentration (IC<sub>50</sub>) values of 7.5 and 28.6 µg mL<sup>-1</sup>, respectively. Fraction IV (constituted by **2**, **3** and **4**) showed activity against promastigotes of *Leishmania infantum* with IC<sub>50</sub> value of 50.0 µg mL<sup>-1</sup>, while fraction II (constituted by **5** and **6**) showed activity against trypomastigotes of *Trypanosoma cruzi*, with IC<sub>50</sub> values of 83.0 µg mL<sup>-1</sup>. This work showed that fragmentation in UHPLC-HR-ESI-MS/MS combined with <sup>1</sup>H NMR analysis of fractions is useful for identifying alkaloids in mixtures. Additionally, it was also demonstrated the potential of aporphine alkaloids from *Duguetia lanceolata* St.-Hil (Annonaceae) in the search for new drug candidates for neglected diseases.

**Keywords:** *Duguetia lanceolata*, Annonaceae, aporphine alkaloids, dereplication, UHPLC-HR-ESI-MS/MS, antiparasitic activity

### Introduction

Annonaceae is composed of 135 genera and 2500 species.<sup>1</sup> Among these genera, 34 can be found in South America and 29 occur in Brazil, including *Duguetia*.<sup>2</sup> The main chemical compounds isolated from Annonaceae are alkaloids, diterpenes, flavonoids, steroids and acetogenins.<sup>3-6</sup>

*Duguetia lanceolata* St.-Hil is popularly known as “pindaiva” or “ateira-da-mata”.<sup>7</sup> This species occurs in Brazil in the states of São Paulo, Minas Gerais, Mato Grosso do Sul, Paraná, Rio Grande do Sul and Santa Catarina.<sup>8</sup> Until now, there are few chemical reports about *D. lanceolata* in literature, and some studies describe identification of *trans*-asarone, 2,4,5-trimethoxystyrene, stigmasterol, sitosterol and campesterol from its leaves.<sup>9,10</sup> Furthermore, the chemical composition of its essential

\*e-mail: psartorelli@unifesp.br

oil from bark and its biological activities has also been reported.<sup>11,12</sup>

Ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) has been widely used as an important tool for dereplication of natural product extracts, including alkaloidal fractions.<sup>13-16</sup> In addition, fragmentations observed by UHPLC high resolution electrospray ionization tandem mass spectrometry (UHPLC-HR-ESI-MS/MS) may be different from those observed with other mass spectrometric methods and it is important to understand these fragmentation mechanisms for the identification of such compounds.<sup>17,18</sup> Besides that, nuclear magnetic resonance (NMR) spectroscopy can be used to directly analyze compounds in mixtures.<sup>19</sup>

The aim of the present work was the characterization of alkaloids obtained from the leaves of *D. lanceolata* through dereplication by UHPLC-HR-ESI-MS/MS and <sup>1</sup>H NMR. Additionally, the antiparasitic activity of fractions was evaluated *in vitro* against *Leishmania infantum* and *Trypanosoma cruzi*.

## Experimental

### Plant material

Leaves of *Duguetia lanceolata* St.-Hil were collected at the Atlantic Forest region in Alfenas, Minas Gerais, Brazil (21°22'53.8"S, 45°55'46.4"W). A voucher specimen has been identified and deposited at the herbarium of the Instituto de Biociências of the University of Sao Paulo (No. J.P.C.03.2017). The research project was registered in the Sistema de Patrimônio Genético e Conhecimento Tradicional Associado (SisGen) platform, with registration No. A90708B.

### General experimental procedures

All solvents were analytical reagents (AR) grade. Silica gel 60 F254 plates (Merck, USA) were used for comparative thin layer chromatography (TLC). TLC spots were visualized under UV light (254 nm) or using Dragendorff reagent for alkaloid detection. NMR spectra were recorded on a spectrometer Ultrashield 300 Bruker Avance III (USA, 300 MHz).

### General HPLC parameters

HPLC analyses were performed in Dionex (Ultimate 3000 model, USA) equipped with a Kinetex column (EVO, 5 μm, C18 of 250 mm long × 4.60 mm internal diameter

for analytical system), a spectrophotometric detector with a diode array (model UVD-170U-DAD3000) and quaternary pump on analytical scale under the following conditions: gradient elution system, 15% MeOH (B) and 85% H<sub>2</sub>O acidified 0.1% formic acid (A) at 0.0 min; 15% B and 85% A at 2.0 min; 90% B and 10% A at 25.0 min; and 100% B and 0% A at 30 min. Flow was 1.0 mL min<sup>-1</sup>, sample concentration 2.0 mg mL<sup>-1</sup>, injection volume 20.0 μL and total run time 28.0 min.

### UHPLC-HR-ESI-MS/MS experiments

UHPLC-HR-ESI-MS/MS data were acquired on a Shimadzu Nexera X2 system (Shimadzu, Japan) equipped with a SPD-M20A Proeminence diode array detector and coupled to a quadrupole time-of-flight mass spectrometer (MicroTOF-QII; Bruker Daltonics, USA) using an ESI ion source operating in positive ion mode. The applied nebulizer gas was 58 psi, flow and temperature of the drying gas (N<sub>2</sub>) were 9 mL min<sup>-1</sup> and 220 °C, respectively. Capillary voltage was set to 4500 V and end plate offset of 500 V. The mass analyzer was operated in SCAN mode in the range *m/z* 50-1200 and AutoMS mode with maximum of 5 precursors *per* cycle and collision energies varying from 15 to 70 eV. Accurate mass calibration was obtained using a solution of sodium formate (10 mM) as internal standard. Separation was performed applying a Kinetex column (2.1 × 100.0 mm × 2.6 mm, Phenomenex) at 50 °C using linear gradient of solvents starting from H<sub>2</sub>O:CH<sub>3</sub>CN (8.5:1.5) to H<sub>2</sub>O:CH<sub>3</sub>CN (1:9) in 12 min at a flow rate of 0.4 mL min<sup>-1</sup>. Both eluents were buffered with 20 mM of formic acid.

### Extraction and semi-purification procedures

Dried leaves of *D. lanceolata* (150 g) were powdered and extracted with EtOH (5 × 500 mL at room temperature) affording (10 g) a crude extract after evaporation of the solvent under reduced pressure. Part of the EtOH extract (5 g) was dissolved in 3% HCl in water and then extracted with *n*-hexane. The aqueous phase (pH 1) was alkalized until it reached pH 10 with NH<sub>4</sub>OH and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The CH<sub>2</sub>Cl<sub>2</sub> phase was concentrated under reduced pressure to afford 1 g. The alkaloid phase (1 g) was chromatographed over silica gel eluted with CH<sub>2</sub>Cl<sub>2</sub> containing increasing amounts of MeOH (up to 100%) to afford seven fractions (I-VII). Fraction I (4.0 mg), II (11.9 mg), III (10.8 mg) and IV (9.7 mg) showed positive results for Dragendorff reagent and were analyzed by HPLC. These fractions were subsequently analyzed by <sup>1</sup>H NMR and UHPLC-HR-ESI-MS/MS allowing the identification of the alkaloids glaucine

(**1**,  $t_r = 15.1$  min) from fraction I (Figure S2, Supplementary Information (SI) section); liriodenine (**6**,  $t_r = 18.2$  min) and oxoglaucone (**5**,  $t_r = 19.9$  min) from fraction II (Figure S6, SI section); oxoglaucone (**5**,  $t_r = 19.9$  min), lanuginosine (**7**,  $t_r = 22.4$  min) and dehydroglaucon (**8**,  $t_r = 25.4$  min) from fraction III (Figure S12, SI section) and norglaurine (**2**,  $t_r = 19.8$  min), isocorydine (**3**,  $t_r = 9.6$  min) and N-methylaurotitanine (**4**,  $t_r = 18.1$  min) from fraction IV (Figure S18, SI section).

#### Parasite maintenance

Promastigotes of *Leishmania (L.) infantum* (MHOM/BR/1972/LD) were maintained in M-199 medium supplemented with 10% calf serum and 0.25% hemin at 24 °C. Trypomastigotes of *T. cruzi* (Y strain) were maintained in LLC-MK2 (ATCC CCL 7) cells using Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2% calf serum at 37 °C in a 5% CO<sub>2</sub> incubator.<sup>20</sup>

#### Antileishmanial activity

Promastigotes of *Leishmania (L.) infantum* were seeded at  $1 \times 10^6$  *per well* in 96 well microplates and incubated with different fractions for 48 h at 24 °C. Then, the viability of promastigotes was measured by the mitochondrial activity using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.<sup>21</sup>

#### Antitrypanosomal activity

Trypomastigotes of *T. cruzi* obtained from LLC-MK2 cells were seeded at  $1 \times 10^6$  *per well* in 96-well microplates and incubated with the fractions for 48 h. The viability of trypomastigotes was determined using the colorimetric resazurin assay at 570 nm.<sup>21</sup> Benznidazole was used as the standard drug.

#### Intracellular amastigotes of *T. cruzi*

Macrophages were collected from the peritoneal cavity of BALB/c mice by washing with RPMI-1640 medium supplemented with 10% FBS and maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub> in a humidified incubator. Macrophages ( $1 \times 10^5$  cells *per well*) were dispensed in 16-well chamber slides (NUNC, Thermo, USA) and maintained for 24 h in the same medium at 37 °C in a 5% CO<sub>2</sub> humidified incubator for attachment. Non-adherent cells were removed by two-step washings with medium. After 24 h, these cells were infected with  $1 \times 10^6$  culture

trypomastigotes for 4 h (parasite-to-macrophage ratio 10:1). Subsequently, infected cells were incubated with fraction I at 100 to 0.78 µg mL<sup>-1</sup> and with fractions II-IV at 200 to 0.39 µg mL<sup>-1</sup> for 48 h. Finally, the slides were fixed with methanol, stained with Giemsa, and observed using light microscopy. The parasite load was defined by counting 200 macrophages *per well* using the formula: number of infected macrophages × number of intracellular amastigotes *per total number of macrophages*.<sup>22</sup>

This study was approved by the Research Ethics Committees on Animals of Instituto Adolfo Lutz under No. CEU-IAL 05/2018.

#### Intracellular amastigotes of *L. (L.) infantum*

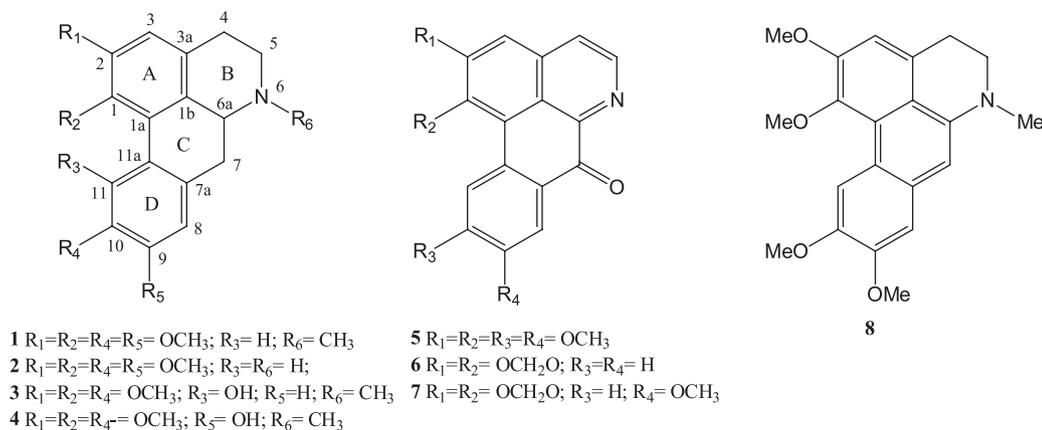
Murine peritoneal macrophages were collected as described above and were seeded at  $1 \times 10^5$  *per well* and incubated for 24 h in a 16-well slide chamber (NUNC, Thermo, USA). Amastigotes were obtained from the spleens of previously infected hamsters by differential centrifugation and seeded at a 1:10 ratio (macrophage: amastigotes) and kept at 37 °C in a 5% CO<sub>2</sub>-humidified incubator for 24 h. Fraction I (at 100 to 0.78 µg mL<sup>-1</sup>) and fractions II-IV (at 200 to 0.39 µg mL<sup>-1</sup>) were incubated for 96 h with infected macrophages. Subsequently, the cells were fixed with MeOH, stained with Giemsa (Merck KGaA, Darmstadt, Germany), and observed using a light microscope. The parasite load was defined by counting 200 macrophages *per well* using the formula: number of infected macrophages × number of intracellular amastigotes *per total number of macrophages*.<sup>23</sup>

## Results and Discussion

The EtOH extract from leaves of *D. lanceolata* was submitted to an acid-base extraction, according to literature, in order to afford alkaloidic phase.<sup>24</sup> After column chromatography separation procedure, the Dragendorff positive fractions (I-IV) were analyzed by <sup>1</sup>H NMR and UHPLC-HRMS/MS and allowed the identification of eight aporphinoid alkaloids, including four aporphine *sensu stricto*: glaucine (**1**), norglaurine (**2**), isocorydine (**3**) and N-methylaurotitanine (**4**), three oxoaporphines: oxoglaucone (**5**), liriodenine (**6**) and lanuginosine (**7**), and one aporphine derivative dehydroglaucone (**8**) (Figure 1).

#### Preliminary NMR analyses in mixture of alkaloids

<sup>1</sup>H NMR spectrum of fraction I showed four singlets of methoxyl protons at  $\delta_H$  3.63, 3.85, 3.86 and 3.88 (Figure S1, SI section). Multiplets between  $\delta_H$  2.55-3.04



**Figure 1.** Structures of the aporphine alkaloids identified in *Duguetia lanceolata*.

with integration for six hydrogens (H-4 and H-5, H-7) can be assigned to hydrogens of rings B and C of aporphine alkaloids and a singlet at  $\delta_{\text{H}}$  2.55, with integration for three hydrogens was assigned to hydrogens of the methyl group linked to nitrogen atom. Furthermore, three singlets at 6.71, 6.91 and 7.99 can be attributed to aromatic hydrogens H-3, H-8 and H-11, respectively, (Figure S1, SI section). This data made the identification of glaucine (**1**) ( $\text{C}_{21}\text{H}_{25}\text{NO}_4$ )  $[\text{M} + \text{H}]^+$  356.1861 (0.0 ppm) possible after comparison of  $^1\text{H}$  NMR data with those reported in literature.<sup>25</sup>

Fraction II was composed of two substances as indicated by HPLC retention times of 18.1 and 19.9 min (Figure S6, SI section). The  $^1\text{H}$  NMR spectrum of this mixture showed duplicated characteristic signals of oxoaporphine alkaloids, including two singlets at  $\delta_{\text{H}}$  7.39/7.51, attributed to H-3, besides aromatic hydrogens with two doublets at  $\delta_{\text{H}}$  7.74/7.79 and two at  $\delta_{\text{H}}$  8.87/8.92 that could be attributed to H-4 and H-5, respectively, of two different alkaloids (Figure S5, SI section). Also, two singlets at 8.03 and 8.75 were observed that can be attributed to H-8 and H-11 for one of the alkaloids.<sup>26</sup> In addition, the  $^1\text{H}$  NMR spectrum showed four aromatic protons at  $\delta_{\text{H}}$  8.47 (1H, m), 7.63 (1H, m), 7.82 (1H, m) and 8.56 (1H, m) which were attributed to the protons of the unsubstituted D ring of the aporphine nucleus like liriodenine.<sup>27,28</sup> There was also one singlet at  $\delta_{\text{H}}$  6.46 corresponding to the presence of one methylenedioxy group,  $\text{OCH}_2\text{O}$  and four methoxy signals with integration for three hydrogens each at  $\delta_{\text{H}}$  4.03, 4.08, 4.09 and 4.11 (Figure S5, SI section). Comparing with literature these signals may indicate the presence of oxoaporphine alkaloids **5** and **6**.<sup>26,29-31</sup>

Fraction III showed to be composed of three substances as indicated by HPLC retention times of 19.9, 22.4 and 25.4 min (Figure S12, SI section). In its  $^1\text{H}$  NMR spectrum, duplicated signals were also observed, suggesting the presence of two alkaloids of analogous

structures, being a set of signals similar to that observed in fraction II (assigned to compound **5**). In this way signals at  $\delta_{\text{H}}$  7.15/7.21 (H-3), and doublets at  $\delta_{\text{H}}$  7.80/7.99 (H-4) and 8.92/9.04 (H-5) were observed, with integration for one hydrogen each and can be attributed to the hydrogens  $\alpha$  and  $\beta$  of the pyridine ring (Figure S11, SI section), characteristic of alkaloids with an aromatic ring B.<sup>30,31</sup> Additionally, multiplets were also observed at  $\delta_{\text{H}}$  3.10 and 3.20 which may refer to signals H-4 and H5 of non-aromatic ring B from another alkaloid. These signals combined with others observed in  $\delta_{\text{H}}$  2.75 (N- $\text{CH}_3$ ), and  $\delta_{\text{H}}$  7.04 (H-7) suggest the presence of a dehydroaporphine alkaloid.<sup>32</sup> Other signals were observed in the region of aromatic hydrogens such as doublets at  $\delta_{\text{H}}$  7.33 (H-10); 8.56 (H-11) and a singlet at  $\delta_{\text{H}}$  8.04 (H-8) as well as at  $\delta_{\text{H}}$  6.42 ( $-\text{OCH}_2\text{O}$ ) and comparative analyzes with data from literature suggest an aporphine alkaloid trisubstituted in ring D.<sup>30</sup> The others signals were at  $\delta_{\text{H}}$  8.02/8.33 (H-8) and 7.52/8.75 (H-11) of singlets with integration for one hydrogen each referring to the two tetrasubstituted alkaloids in ring D. Additionally, the signals at  $\delta_{\text{H}}$  4.09 (OMe-C9) and 4.14 (OMe-C10), with integration for three hydrogens each, confirm the existence of the tetrasubstituted benzene ring (ring D) for two of the alkaloids (Figure S11, SI section). Comparison of NMR data with literature suggests the presence of oxoaporphine alkaloids **5** and **7** and one dehydroaporphine alkaloid **8** as constituents of fraction III.<sup>30-33</sup>

The  $^1\text{H}$  NMR spectra of fraction IV (Figure S17, SI section), whose HPLC chromatogram analysis suggested the presence of three compounds Figure S18, SI section, was very similar to the spectrum of fraction I, composed exclusively of glaucine, suggesting presence of analogous alkaloids. Singlets were observed at the range of  $\delta_{\text{H}}$  6.64-6.97 (H-3) and multiplets between  $\delta_{\text{H}}$  2.88-3.00 (H-4 and H-5, H-7), besides signals at  $\delta_{\text{H}}$  4.05 (H-6a) that show the existence of non-aromatic rings B and C. The

several methoxyl groups at  $\delta_{\text{H}}$  3.66-3.91, corroborate the existence of a benzene ring tetrasubstituted (ring D) for the three compounds but with different substitution pattern for one of them. Singlets at  $\delta_{\text{H}}$  6.76/6.80 (H-11) and 8.07/8.10 (H-8) evidenced the D ring substitution pattern for two of them,<sup>34,35</sup> whereas the presence of two doublets in 6.82 and 6.87 revealed that one of the alkaloids has a substitution pattern that locates the remaining hydrogens in the *ortho* position as in the case of isocorydine.<sup>36</sup> Additionally, the presence of two singlets at  $\delta_{\text{H}}$  2.64 (NMe) proves the existence of a methyl group linked to nitrogen for two of them and suggests that in one of the alkaloids there is no N substitution (Figure S17, SI section), in the case of norglaucine.<sup>34</sup> Comparison with literature data suggested the presence of alkaloids **2**, **3** and **4**.<sup>34-36</sup>

#### UHPLC-HR-ESI-MS/MS analysis

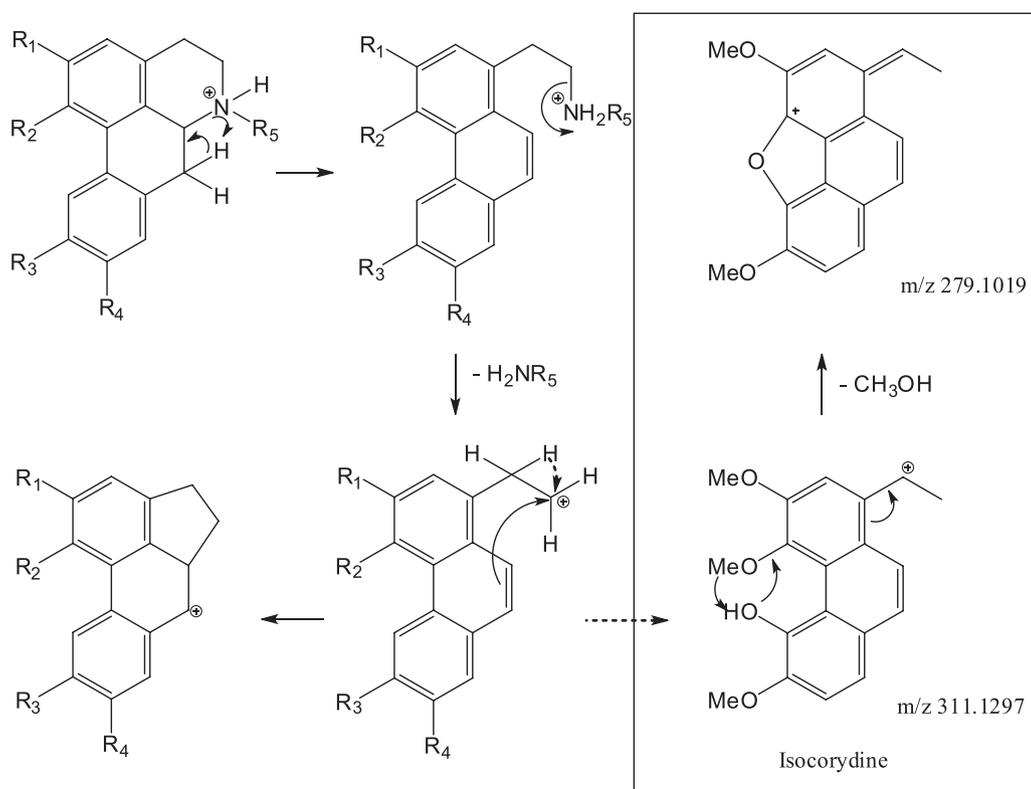
All UHPLC-HR-ESI-MS in positive mode spectra showed  $[\text{M} + \text{H}]^+$  ions as the main peak. The most diagnostic fragment ions,  $[\text{M} + \text{H} - 17]^+$  or  $[\text{M} + \text{H} - 31]^+$  are commonly observed for aporphine alkaloids depending on the nature of substituent of the amino group ( $-\text{NH}_2$  or  $-\text{N}-\text{CH}_3$ ) (Figure 2). These losses come from key fragments of aporphine alkaloids.<sup>37</sup> In order to characterize aporphine alkaloids in the *D. lanceolata* extract, MS/MS experiment was performed to obtain more chemical structural information about the alkaloids produced by the species, allowing to distinguish the different types of aporphine alkaloids present in the plant's leaves. Thus, depending on substitution, pattern losses of  $m/z$  15 (Me); 31 (OMe), 28 (CO) or 32 (MeOH) can be observed.<sup>13</sup>

For glaucine (**1**), present in fraction I, in addition to the loss of  $\text{NH}_2\text{CH}_3$  ( $m/z$  31.0419), the fragment at  $m/z$  294.1237 is relative to the loss of the  $\text{OCH}_3$  radical ( $m/z$  31.0183) and the fragment at  $m/z$  310.1186 corresponds to a loss of  $\text{CH}_3$  radical from fragment  $m/z$  325.1433 (Table 1). The loss of radical methyl or methoxy groups seem to be favored by the presence of an aromatic ring with one or more non-*vicinal* methoxy groups to an OH or one which does not have any OH.<sup>14</sup> This is also observed for norglaucine (**2**) whose MS/MS spectra showed a peak at  $m/z$  325.1427 corresponding to  $[\text{M} + \text{H} - \text{NH}_3]^+$ , confirming loss of  $\text{NH}_3$  ( $m/z$  17.0265). Isocorydine (**3**), in turn, contains the hydroxy and a methoxy group in the *vicinal* positions, so this alkaloid has characteristic losses of  $\text{CH}_3\text{OH}$  followed by CO.<sup>14</sup> In UHPLC-HR-ESI-MS, fragments at  $m/z$  296.1031  $[\text{M} + \text{H} - \text{CH}_3]^+$ ; 279.1019  $[\text{M} + \text{H} - \text{MeOH}]^+$  were observed. The proposed fragmentation for alkaloids **1-4** is shown in Figure 2. Additional fragmentations of **3** are in the box of Figure 2.

Although **2**, **3** and **4** are isomers (molecular formula  $\text{C}_{20}\text{H}_{24}\text{NO}_4$ ) with  $[\text{M} + \text{H}]^+$  at  $m/z$  342.1687, the fragmentation pattern is different, since norglaucine (**2**) fragmentation leads to loss of  $\text{NH}_3$  ( $m/z$  17.0265) generating the product ion  $m/z$  325.1427, observed in the MS/MS spectrum, while isocorydine (**3**) and N-methylauroretanine (**4**) fragmentation afforded  $\text{CH}_3\text{NH}_2$  neutral loss corresponding to  $m/z$  311.1267. Additionally, loss of  $\text{CH}_3$  radical gives rise to  $m/z$  296.1060 for **3** and **4** (Figure 2). Distinction between **3** and **4** can be verified by the fragment obtained from loss of methoxyl group at  $m/z$  280.1093 from fragment  $m/z$  311.1267 (Table 1) to **4**,<sup>17</sup> while for **3** there is the loss of a molecule of methanol that is only possible by the position of the hydroxyl group (Figure 2).

In fraction II it was possible to identify two oxoaporphine alkaloids: oxoglaucine ( $[\text{M} + \text{H}]^+$  at  $m/z$  352.1180) and liriodenine ( $[\text{M} + \text{H}]^+$  at  $m/z$  276.0654). These compounds, being oxoaporphines, do not allow the loss of the amino group, since the nitrogen atom is part of an aromatic nucleus.<sup>16</sup> Thus, the fragmentations observed in the MS/MS spectra for these alkaloids correspond to the loss of methyl substituents of the methoxyl groups or loss of CO from a methylenedioxy group. Liriodenine (**6**) ( $\text{C}_{17}\text{H}_{19}\text{NO}_3$ ) is an alkaloid containing one methylenedioxy group and the fragmentation of this alkaloid showed a fragment at  $m/z$  248.0705 for the  $[\text{M} + \text{H} - \text{CO}]^+$  ion,<sup>16</sup> while oxoglaucine (**5**) showed in its MS/MS spectrum the fragment  $[\text{M} + \text{H} - \text{CH}_3]^+$  at  $m/z$  337.0943 and at  $m/z$  306.0762 corresponding to loss of an additional methoxyl radical.<sup>38</sup> Another oxoaporphine alkaloid, identified in fraction IV, lanuginosine ( $\text{C}_{18}\text{H}_{12}\text{NO}_4$ ) showed a  $[\text{M} + \text{H}]^+$  at  $m/z$  306.0769 (calcd. 306.0766) containing, besides a methylenedioxy and a methoxy group, a peak at  $m/z$  291.0522 in the MS/MS spectrum, corresponding to loss of a  $\text{CH}_3$  radical and from this another peak at  $m/z$  263.0576 corresponding to loss of CO of one methylenedioxy group (Table 1). Dehydroglaucine (**8**) (present in fraction III) like oxoaporphine alkaloids does not allow the loss of amino group, due to the lack of one of the hydrogens in position 7. Thus, for **8**, only loss of methyl radicals was observed in MS/MS spectrum including peak at  $m/z$  339.1472 and 324.1236, corresponding to multiple loss of methyl radicals.

The literature reports chemical analysis of approximately 16 *Duguetia* species, resulting in the characterization of more than 100 different alkaloids.<sup>4</sup> Despite of this information, in the present work eight related aporphine alkaloids were identified, with **1**, **5** and **8** being described for the first time in *Duguetia* genus while the occurrence of alkaloids **2**, **3**, **4**, **6** and **7** have been described for the first time in *D. lanceolata*.



**Figure 2.** Proposed fragmentation mechanism for aporphine alkaloids **1-4**. In box additional fragmentation of isocorydine (**3**).

**Table 1.** Elemental composition of aporphine alkaloids (**1-8**) observed in UPLC-ESI-HRMS ( $[M + H]^+$ ) and MS/MS spectra from *D. lanceolata* fractions; calculated exact mass (calcd.) and measured accurate mass (acc.) for each indicated precursor and product ion

Alkaloid	MF	$[M + H]^+$ calcd. ( $m/z$ )	Error values / ppm	$[M + H]^+$ acc. ( $m/z$ )	MS/MS acc. ( $m/z$ )
Glaucine ( <b>1</b> )	$C_{21}H_{25}NO_4$	356.1861	0.0	356.1861	325.1433
					310.1186
					294.1237
Norglaucine ( <b>2</b> )	$C_{20}H_{23}NO_4$	342.1705	5.2	342.1687	325.1427
					310.1183
					294.1237
Isocorydine ( <b>3</b> )	$C_{20}H_{23}NO_4$	342.1705	6.4	342.1683	311.1297
					296.1031
					279.1019
N-methylaurotetanine ( <b>4</b> )	$C_{20}H_{23}NO_4$	342.1705	4.7	342.1689	311.1267
					296.1060
					280.1093
Oxoglaucine ( <b>5</b> )	$C_{21}H_{25}NO_4$	352.1184	1.1	352.1180	337.0943
Liriodenine ( <b>6</b> )	$C_{17}H_{19}NO_3$	276.0660	1.1	276.0654	248.0705
Lanuginosine ( <b>7</b> )	$C_{18}H_{11}NO_4$	306.0766	0.9	306.0769	291.0522
Dehydroglaucine ( <b>8</b> )	$C_{21}H_{23}NO_4$	354.1705	1.7	354.1699	339.1472
					324.1236

MF: molecular formula.

#### Antiparasitic activity

Fractions containing alkaloids were incubated with *T. cruzi* trypomastigotes and promastigotes of

*Leishmania (L.) infantum* and the viability detected by colorimetric assays. The most promising activity was observed for fraction I, constituted by glaucine. Additionally, this fraction demonstrated activity against

**Table 2.** Alkaloidal fractions I-IV from *D. lanceolata* were assayed against *L. infantum* (promastigotes and amastigotes) and against *T. cruzi* (trypomastigotes and amastigotes) and expressed as effective concentration 50% (IC<sub>50</sub>) for parasites

Fraction (alkaloids)	IC <sub>50</sub> trypomastigote <i>T. cruzi</i> (± SD) / (µg mL <sup>-1</sup> )	IC <sub>50</sub> amastigote <i>T. cruzi</i> / (µg mL <sup>-1</sup> )	IC <sub>50</sub> promastigote <i>L. infantum</i> (± SD) / (µg mL <sup>-1</sup> )	IC <sub>50</sub> amastigote <i>L. infantum</i> / (µg mL <sup>-1</sup> )
Fraction I (1)	46.0 (± 11.0)	28.6	> 100.0	7.5
Fraction II (5 + 6)	83.0 (± 39.0)	n/a	> 100.0	n/a
Fraction III (5 + 7 + 8)	> 100.0	n/a	> 100.0	n/a
Fraction IV (2 + 3 + 4)	> 100.0	n/a	50.0 (± 1.8)	n/a
Benznidazole	4.6 ± 0.5	1.3 ± 0.4	–	–
Miltefosine	–	–	2.1 ± 0.2	2.5 ± 1.2

n/a: not active; SD: standard deviation; Parasites without treatment were used as negative controls. Benznidazole was used as a positive control for *T. cruzi* and miltefosine for *Leishmania infantum*.

the clinically relevant forms of *L. infantum* intracellular amastigotes, with a half maximal inhibitory concentration (IC<sub>50</sub>) value of 7.5 µg mL<sup>-1</sup> (Table 2). Furthermore, glaucine also showed anti trypanosomal activity against intracellular amastigotes of *T. cruzi* and resulted in an IC<sub>50</sub> value of 28.6 µg mL<sup>-1</sup>. No mammalian cytotoxicity was observed up to 70.0 µg mL<sup>-1</sup>, confirming the potential of glaucine to be used as a future scaffold for the design of new antiprotozoan derivatives. Fraction IV, constituted by norglaurine (2), isocorydine (3) and N-methylaurotetanine (4), showed an IC<sub>50</sub> value of 50.0 µg mL<sup>-1</sup> against promastigotes of *L. infantum*, but no activity was detected against *T. cruzi* trypomastigotes (Table 2). Fraction II, constituted by oxoglaurine (5) and liriodenine (6), showed an IC<sub>50</sub> value of 83.0 µg mL<sup>-1</sup> against *T. cruzi* trypomastigotes (Table 2). Glaucine has been also described showing activity against epimastigote forms of *Trypanosoma cruzi* with an IC<sub>50</sub> value of 90 µM.<sup>39</sup> Previous studies reported the antileishmanial and antitrypanosomal activity of crude extracts from isoquinoline alkaloid-producing families, including *D. lanceolata*,<sup>40</sup> but no information about the activity of the identified compounds have been described.

Chagas disease and leishmaniasis affect more than 20 million people in developing countries.<sup>41,42</sup> Considering the limited efficacy of the current therapies and highly toxic drugs, new safe and affordable treatments are needed. Our work investigated for the first time in literature the *in vitro* antitrypanosomal and antileishmanial efficacy of the aporphine alkaloid glaucine and other fractions against clinically relevant intracellular forms of the parasites and also demonstrate the potential of *D. lanceolata* in the search for bioactive compounds against neglected diseases.

## Conclusions

The approach used for the dereplication of the alkaloid fraction extracted from the leaves of

*D. lanceolata* assisted in the identification of four aporphine alkaloids, one derivative as well as three oxoaporphine alkaloids. Additionally, glaucine showed activity against the clinical forms of the protozoan intracellular amastigotes of parasites *Leishmania infantum* and *Trypanosoma cruzi* and no mammalian cytotoxicity and fraction IV constituted by isocorydine (3), norglaurine (2) and N-methylaurotetanine (4) showed activity against promastigotes of *Leishmania infantum*. This data demonstrates the potential of aporphine alkaloids from *D. lanceolata* St.-Hil (Annonaceae) in the search for new drug candidates for neglected diseases.

## Supplementary Information

Supplementary data are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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## Author Contributions

EPD was responsible for experimental work, data collection and evaluation, literature search and manuscript preparation, investigation, writing original draft; JM for experimental work, data collection and evaluation, literature search and manuscript preparation, investigation, writing original draft; MMR for experimental work, data collection and evaluation; MA for experimental work, data collection and evaluation; PS for experimental work, data collection and evaluation, conceptualization, literature search and

manuscript preparation, data curation, funding acquisition, project administration, spectral analysis and interpretations, writing original draft, writing review and editing; MGS for data curation, writing review and editing; AGT for funding acquisition, writing review and editing; LSM for spectral analysis and interpretations, writing review and editing; JHGL for spectral analysis and interpretations, writing review and editing.

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