

# Extraction and Fractionation Effects on Antiplasmodial Activity and Phytochemical Composition of *Palicourea hoffmannseggiana*



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## Key words

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## ABSTRACT

The present study on *Palicourea hoffmannseggiana*, which was collected at Marapanim, state of Pará, Brazil, comprises the preparation of different stem and leaf extracts and fractions. Ethanol, hydroethanol, and water extracts were prepared by several methods and evaluated for *in vitro* activity against resistant *Plasmodium falciparum* (W2 strain), disclosing a low parasite growth inhibition effect (< 50%). Dereplication by UPLC-DAD-ESI<sup>-</sup>MS of the leaf ethanol extract showed the presence of two known alkaloids, lyalosidic and strictosidinic acids, along with a sinapoyl ester of lyalosidic acid, with *m/z* 719.33 [M + H]<sup>+</sup>, which is possibly a new monoterpene indole alkaloid representative. Sequential liquid-liquid acid-base alkaloid separations from the leaf ethanol extract as well as directly from leaf powder afforded fractions of increased parasite growth inhibition, reaching up to 92.5 ± 0.7%. The most bioactive fractions were shown to contain the  $\beta$ -carboline alkaloids harmine and 4-methyl- $\beta$ -carboline, along with *N*-methyl-tryptamine and *N*-acetyl-tryptamine, while monoterpene indole alkaloids were detected in inactive fractions of these processes. The present results demonstrate that these preliminary fractionation methods can lead to significantly active fractions supporting an adequate scale-up to carrying out the isolation of anti-plasmodial compounds.

## Introduction

*Palicourea hoffmannseggiana* (Schult.) Borhidi (Rubiaceae) is a recently accepted name of this specimen for which *Psychotria hoffmannseggiana* (Willd. ex Schult.) Müll. Arg. is a synonym according to Flora do Brasil 2020 [1]. On the other hand, different taxonomic data can be found in other open access databases such as The Plant List and TROPICOS. In the The Plant List database, *P. hoffmannseggiana* is pointed out as a synonym of *P. hoffmannseggiana* (Willd. Ex Schult.) Müll. Arg., which is the accepted name along with 20 more *Psychotria* names [2]. In TROPICOS, the accepted name is *P. hoffmannseggiana* (Roem. & Schult.) Müll. Arg. along with *P. hoffmannseggiana* (Roem. & Schult.) Borhidi [3]. This complex taxonomic situation is a consequence of the huge number of taxa in the Rubiaceae family that are distributed worldwide (13 100), particularly in the Neotropics (5000) where they are present in most biomes. This family has the highest number of species in the order Gentianales, with about 66% of the species (about 115 genera and 1600 species) occurring in Brazil. However, considering that many species have not yet been collected in the country due to its vast unknown areas, especially in the Amazon Region and the Atlantic Forest, the number of species of this family might be up to 2000 [4].

*Palicourea* and *Psychotria* species are both known for the presence of alkaloids of high structural diversity, including those biosynthetically derived from tryptophan, such as monoterpene indole alkaloids (MIAs),  $\beta$ -carboline alkaloids, quinoline and isoquinoline alkaloids, and iridoids as well as other terpenoids, flavonoids, coumarins, and cyclic peptides (cyclotides). Some of these are responsible for a wide range of biological activities (cytotoxicity, analgesics, antivirals, antifungals, and modulators of the activity of the central nervous system) [5–7]. However, the number of MIAs and  $\beta$ -carboline alkaloids of known antimalarial/anti-plasmodial activity is relatively low. This fact motivated a systematic investigation of *Palicourea* and *Psychotria* taxa aiming to find potential antimalarial natural products [8]. In a recent review of the taxonomy, phytochemistry, biological activities, and antimalarial activity of *P. hoffmannseggiana* (Schult.) Borhidi, only psychorubrine, a quinone, from *P. hoffmannseggiana* Roem. & Schult, and *Psychotria barbiflora* DC., as well as harmine from *Psychotria rubra* (not determined author) were recognized as showing moderate anti-plasmodial activity out of a total of 20 described natural products [8].

The impact of natural products in malaria chemotherapy is no doubt a valuable weapon to fight malaria. Malaria is still a global health problem and, according to the WHO's estimation, the number of cases worldwide were 251, 231, and 228 million in 2010, 2017 and 2018, respectively [9]. Overall, these data represent a significant decrease in the number of cases. However, in the Region of the Americas, an increase in cases, attributed to higher malaria transmission in Brazil, Nicaragua, and Venezuela, was recorded [9].

The development of new antimalarials is of great relevance due to the resistance of the malaria parasites to nearly all available antimalarial agents [10]. Therefore, the discovery of new antimalarial hits by investigation of plants of traditional use and those taxonomically related is still a valid strategy, particularly in Brazil, because of its rich biodiversity. Diverse strategies are available for the development of novel antimalarial drugs. The cellular screening for evaluation of antimalarial efficacy of pure compounds and extracts was generally carried out by the traditional radioisotopic assay

using the tritiated hypoxanthine method ( $^3\text{H}$ -hypoxanthine) to determine the level of *Plasmodium falciparum* growth inhibition (GI) [11]. Presently, the protocol most frequently used is a colorimetric enzymatic assay determined by an ELISA method based on the determination of the parasite lactate dehydrogenase (*Pf*LDH) for testing antimalarial drug susceptibility. It is cheap and requires minimum instrumentation. This method is also presently used on a large scale: in robotic equipment, high-throughput screening (HTS), in big pharma, and for screening millions of chemicals against multidrug-resistant *P. falciparum* [12, 13].

HTS, associated with artificial intelligence, is revolutionizing diverse areas of RD&I, but not without the usual problems related to the acquisition and analyses of required data [14]. However, usually, academic researchers do not have access to these sophisticated HTS equipment. The usual bioguided fractionation of an active extract can be exploited with different strains of *P. falciparum*. When combined with powerful analytical methodologies, a rapid online identification of several known compounds is achieved. The profiling methods, employing spectroscopic methods in conjunction with chromatography, i. e., HPLC to DAD and MS (HPLC-DAD-ESI-MS<sup>n</sup>), are increasingly being used.

Against this backdrop, a bioguided screening of *P. hoffmannseggiana* (Schult.) Borhidi (Rubiaceae), a species with a complex taxonomic history, was extensively investigated with the aim of discovering antimalarial metabolites of minor content. Usual extracts, prepared by extractions with ethanol, ethanol/water, and hot water (tea), and preliminary fractionations by liquid-liquid partitions and alkaloid chemical separations were evaluated *in vitro* against the resistant *P. falciparum* W2 strain by the *Pf*LDH method. In addition, intense dereplication, based on tandem mass spectrometry (UPLC-DAD-ESI-MS<sup>n</sup>) of different extracts and fractions, was also carried out. Further investigations on a preparative scale are expected to afford promising compounds.

## Results and Discussion

The results of the *in vitro* antimalarial activity determination of different extracts and fractions of *P. hoffmannseggiana* leaves and stems are shown in ► **Table 1** and are displayed in groups according to extraction procedures. Extracts of Group 1 included the use of different extractive liquids and procedures: 1) maceration of stems and leaves, separately, with ethanol; 2) sonication of stems and leaves with ethanol-water (7/3 and 1/1) at room temperature; 3) decoction of stems and leaves with water. All these fractions showed a low percentage of parasite inhibition (GI < 50%) and, therefore, were considered inactive. Indeed, no statistical difference was observed among the Group 1 extracts regardless of the plant part used, leaves or stems. In addition, a significant similarity was observed for the UPLC-DAD chromatogram profiles of these extracts, although greater intensity of some peaks was observed in the leaf extracts. Practically, no effect was noted in the antimalarial activity, as corroborated by the statistical data (**Fig. 1S**, Supplementary Material). These results are surprising since it is widely known that solvents and techniques used in extraction processes have been reported to influence the nature and quantity of secondary metabolites extracted [15]. Taken together, the selection of the best extraction method is of great relevance in chemical and biological in-

**► Table 1** Percentage of *P. falciparum* (W2) (PfLDH) growth inhibition of extracts and acid-base fractions of *P. hoffmannseggiana*.

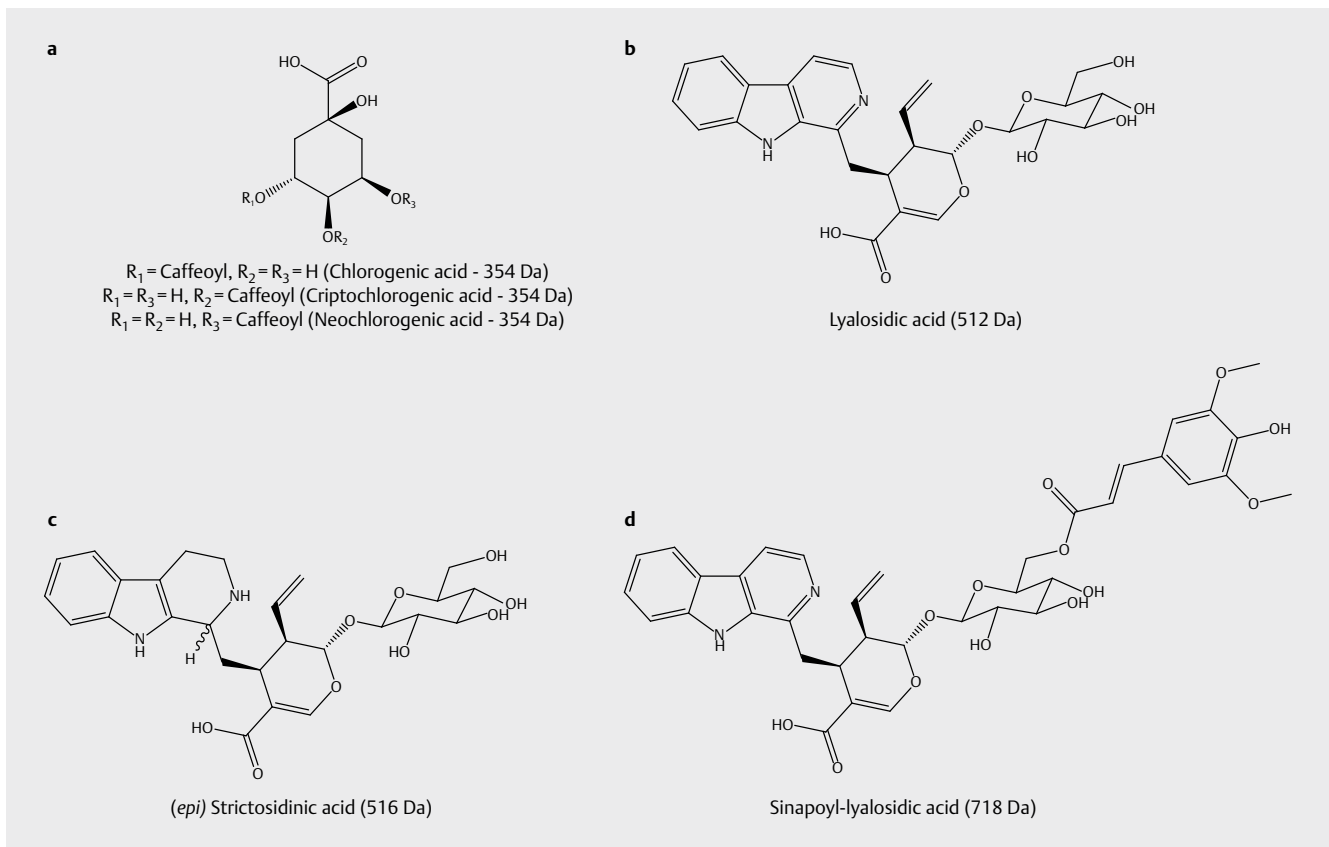
Samples	% Growth inhibition	
	[100.0 µg/mL]	[50.0 µg/mL]
<sup>1</sup> Group 1		
<sup>1.1</sup> Stems EtOH Ext.	24.0 ± 9.9 <sup>A</sup>	24.0 ± 9.9 <sup>A</sup>
<sup>1.2</sup> Stems EtOH/H <sub>2</sub> O 7/3 Ext.	33.0 ± 7.0 <sup>A</sup>	30.0 ± 4.2 <sup>A</sup>
<sup>1.2</sup> Stems EtOH/H <sub>2</sub> O 1/1 Ext.	37.0 ± 0.0 <sup>A</sup>	28.0 ± 2.8 <sup>A</sup>
<sup>1.3</sup> Stems H <sub>2</sub> O Ext.	39.5 ± 0.7 <sup>A</sup>	25.5 ± 12.0 <sup>A</sup>
<sup>1.1</sup> Leaves EtOH Ext.	26.0 ± 4.2 <sup>A</sup>	25.0 ± 5.6 <sup>A</sup>
<sup>1.2</sup> Leaves EtOH/H <sub>2</sub> O 7/3 Ext.	31.0 ± 1.4 <sup>A</sup>	27.5 ± 3.5 <sup>A</sup>
<sup>1.2</sup> Leaves EtOH/H <sub>2</sub> O 1/1 Ext.	37.0 ± 7.0 <sup>A</sup>	35.5 ± 6.3 <sup>A</sup>
<sup>1.3</sup> Leaves H <sub>2</sub> O Ext.	33.0 ± 1.4 <sup>A</sup>	25.5 ± 2.1 <sup>A</sup>
<sup>2</sup> Group 2		
Leaves EtOH Ext.	26.0 ± 4.2 <sup>D</sup>	25.0 ± 5.6 <sup>C</sup>
Leaves Acid CHCl <sub>3</sub> Fr.	80.0 ± 8.4 <sup>B</sup>	73.5 ± 6.3 <sup>A</sup>
Leaves Neutral CHCl <sub>3</sub> Fr.	78.5 ± 2.1 <sup>B</sup>	76.5 ± 0.7 <sup>A</sup>
Leaves Basic CHCl <sub>3</sub> Fr.	92.5 ± 0.7 <sup>A*</sup>	67.0 ± 1.4 <sup>A, B</sup>
Leaves Basic EtOAc Fr.	65.0 ± 7.0 <sup>C</sup>	24.5 ± 7.7 <sup>C</sup>
Leaves <i>n</i> -BuOH Fr.	23.0 ± 2.8 <sup>D</sup>	16.5 ± 0.7 <sup>C</sup>
<sup>3</sup> Group 3		
Stems EtOH Ext.	24.0 ± 9.9 <sup>C</sup>	24.0 ± 9.9 <sup>B, C</sup>
Stems Basic DCM Fr.	65.5 ± 10.6 <sup>A</sup>	60.5 ± 3.5 <sup>A</sup>
Stems <i>n</i> -BuOH Fr.	52.5 ± 2.1 <sup>B</sup>	37.5 ± 2.1 <sup>B</sup>
Leaves EtOH Ext.	26.0 ± 4.2 <sup>C</sup>	25.0 ± 5.6 <sup>C</sup>
Leaves Basic DCM Fr.	64.0 ± 1.4 <sup>A</sup>	59.5 ± 4.9 <sup>A</sup>
Leaves <i>n</i> -BuOH Fr.	27.5 ± 3.5 <sup>C</sup>	25.0 ± 1.4 <sup>C</sup>
Positive Control		
* Chloroquine diphosphate salt	100.0 ± 0.0 <sup>*</sup>	100.0 ± 0.0 <sup>*</sup>
Note: Means (±SD, n=3) followed by different letters were significantly different within columns (Tukey test, α=0.05). Statistical analyses of parasitemia reduction at 100.0 and 50.0 µg/mL were performed separately for each concentration as well as for each group analyzed. * Statistical analysis of the anti-plasmodial activity of the chloroquine control was performed separately with each sample and concentration, and significant to p < 0.05. Ext.: Extract; Fr.: fraction; EtOH: ethanol; H <sub>2</sub> O: water; CHCl <sub>3</sub> : chloroform; EtOAc: ethyl acetate; <i>n</i> -BuOH: butanol; DCM: dichloromethane. <sup>1.1</sup> Extraction by maceration; <sup>1.2</sup> extraction by sonication; <sup>1.3</sup> extraction by decoction; <sup>2</sup> fractionation by acid base (acetic acid, Method A); <sup>3</sup> acid-base extraction (chloridric acid, Method B). Conditions of extraction and fractionation: see Experimental Section. A, B, and C represent statistical differences between analysed results, with no corresponding indicators.		

vestigations [16]. Given the numerous taxonomic depictions for *P. hoffmannseggiana* and the various metabolites described, evaluation of the effects of different extractive methods on antimalarial activity is important, making viable use of more appropriate parts of the plants, solvents, isolation, and purification processes.

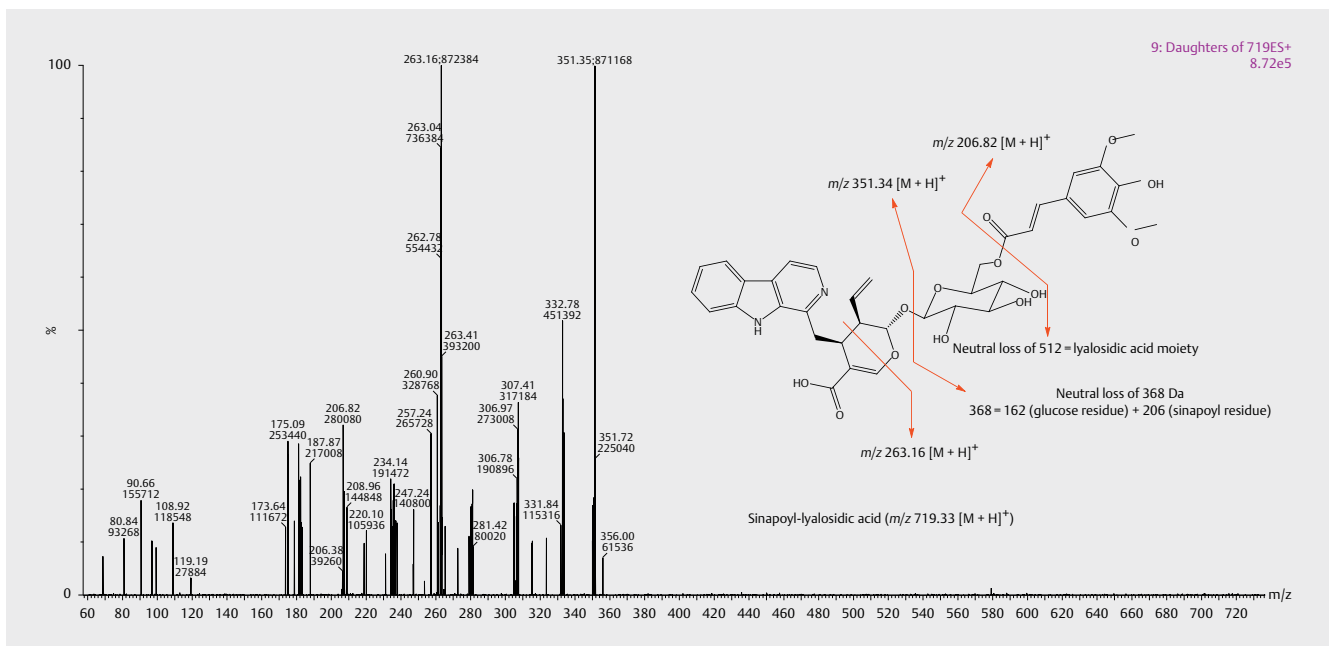
Taking into account that the various extracts showed phytochemical similarity, the characterization of the leaf ethanol extract by UPLC-ESI-MS<sup>2</sup>, in the positive and negative ionization modes, presented here is closely related to the other extracts (**Table 1S, Figs. 2S and 3S**, Supplementary Material). Dereplication of extracts and fractions of *P. hoffmannseggiana* leaves and stems was carried out on the basis of UV absorption and typical fragmentation patterns

by sequential MS analyses (UPLC-DAD-ESI-MS<sup>2</sup>) in comparison to literature data. Considerations on the main fragmentations registered by tandem mass spectrometry for the compounds detected in the *P. hoffmannseggiana* extracts are discussed here.

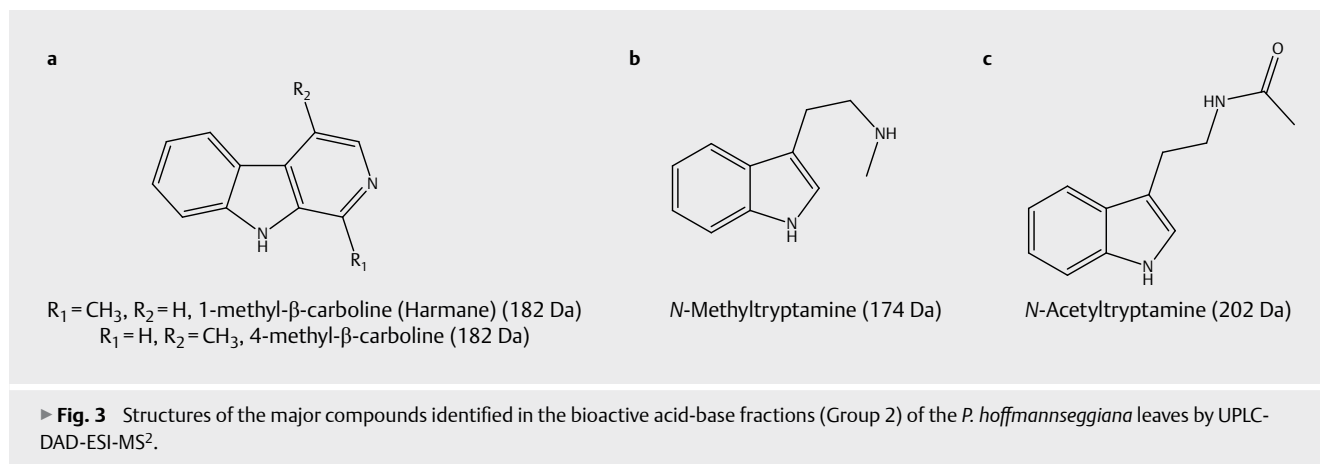
For three intense peaks in the total ion chromatogram in the negative mode (TIC ESI<sup>-</sup>) of *P. hoffmannseggiana* leaves ethanol extract (**Fig. 3S**, Supplementary Material), corresponding in the UPLC-DAD chromatogram to peaks retention times (RT) 1.46, 1.82, and 1.95 min, the same UV and MS data were observed: λ<sub>max</sub> 217, 280sh, 325 nm; *m/z* 353.09 [M – H]<sup>-</sup> (**Fig. 3S**, Supplementary Material). These data led to the identification of chlorogenic, neochlorogenic, and cryptochlorogenic acids, all of them showing fragmentation ions *m/z* 191.40 [M – H]<sup>-</sup> referring to quinic acid while *m/z* 161.26 [M – H]<sup>-</sup> is related to the caffeoyl residue (**► Fig. 1a**). Correlation of these isomeric chlorogenic acids with the registered observed RTs cannot be done due to their structural similarity. Three other intense peaks are shown in the UPLC-DAD chromatogram at RTs 2.67, 2.73, and 3.09 min. The first two were putatively identified as lyalosidic and strictosidinic acids, two known MIAs [17–19]. The first one, at RT 2.67 min, was identified as lyalosidic acid on the basis of the following UV and MS data (ESI<sup>+</sup> and ESI<sup>-</sup>): λ<sub>max</sub> 217, 303, 350sh nm; *m/z* 513.28 [M + H]<sup>+</sup> and *m/z* 511.38 [M – H]<sup>-</sup>, respectively (**Table 1S, Figs. 2S and 3S**, Supplementary Material). Indeed, the MS<sup>2</sup> data for the compound with an RT of 2.67 min is consistent with lyalosidic acid, with fragments corresponding to the loss of a glucose residue *m/z* 351.38 [M + H – 162]<sup>+</sup> followed by the loss of CO<sub>2</sub> *m/z* 307.19 [M + H – 162 – 44]<sup>+</sup>, and *m/z* 182.06 [M + H – 331]<sup>+</sup>, which can be attributed to the indole unity after the loss of the secologanin-glucose residue (**► Fig. 1b**). The second MIA, at RT 2.73 min, was attributed to the stereoisomer of strictosidinic acid: λ<sub>max</sub> 221, 300sh, 350sh nm; *m/z* 517.33 [M + H]<sup>+</sup> and *m/z* 515.37 [M – H]<sup>-</sup> (**Table 1S, Figs. 2S and 3S**, Supplementary Material). The similar fragmentations to the compound described above led to the loss of glucose *m/z* 355.26 [M + H – 162]<sup>+</sup>. A retro-Diels-Alder fragmentation of the secologanin moiety gave *m/z* 284.94 [M + H – 232]<sup>+</sup>. The *m/z* 187.20 [M + H – 330]<sup>+</sup> must correspond to the indole unity after the loss of the secologanin-glucose moiety, besides *m/z* 144.24 corresponding to C<sub>10</sub>H<sub>10</sub>N<sup>+</sup> [M + H – 373]<sup>+</sup>, which is probably derived by a heterocyclic ring fission (HRF) of the indole nucleus (**► Fig. 1c**). The presence of lyalosidic and strictosidinic acids or any isomer of those known as an MIA in different extracts of *P. hoffmannseggiana* leaves and stems is in accordance with literature data for several *Palicourea* species [4, 17, 18]. By contrast, the metabolite detected at RT 3.09 min is proposed to be a sinapoyl ester of lyalosidic acid, as inferred from the UV spectrum (λ<sub>max</sub> 221, 307, 345sh nm), the ESI<sup>+</sup> and ESI<sup>-</sup> MS data *m/z* 719.33 [M + H]<sup>+</sup> and *m/z* 717.44 [M – H]<sup>-</sup>, as well as MS fragmentations pointing to the loss of a sinapoyl moiety to give *m/z* 513.25 [M + H – 206]<sup>+</sup> along with the loss of a glucosylsinapoyl unity at *m/z* 351.22 [M + H – 368]<sup>+</sup> for which 368 = 162 (glucose residue) + 206 (sinapoyl residue), in addition to a diagnostic fragment for the sinapoyl acilium ion at *m/z* 206.92 [M + H – 512]<sup>+</sup> derived by the loss of the lyalosidic acid residue itself (**► Figs. 1d and ► 2**). Furthermore, the MS<sup>3</sup> for the *m/z* 206.92 [M + H]<sup>+</sup> characteristic ions were observed for the sinapoyl residue at *m/z* 191.67 [M + H – 15]<sup>+</sup>, *m/z* 175.84 [M + H – 31]<sup>+</sup>, and *m/z* 145.48 [M + H – 31–31]<sup>+</sup>, related to the losses of a methyl and methoxy radicals.



► **Fig. 1** Chemical structures of the major compounds identified in the ethanol extract (Group 1) of *P. hoffmannseggiana* leaves by UPLC-DAD-ESI-MS<sup>2</sup>.



► **Fig. 2** MS/MS spectrum and chemical structure (main fragmentations) of the sinapoyl-lyalosidic acid identified in the ethanol extract of *P. hoffmannseggiana* leaves by UPLC-DAD-ESI-MS<sup>2</sup>.



No reference was found for this putative alkaloid of MW 718 Da. Thus, this is possibly a new MIA, a sinapoyl-lyalosidic acid. Isolation and structural elucidation by NMR of this new MIA derivative to confirm the stereochemistry and localization of the sinapoyl moiety is ongoing. Similar MIA cinnamoyl-lyalosides (methyl esters of lyalosidic acid), such as (*E*)-*O*-6'-feruloyl-lyaloside and (*E*)-*O*-6'-sinapoyl-lyaloside, have already been detected in *Palicourea* and *Psychotria* species, while (*E*)-*O*-6'-sinapoyl-lyaloside was isolated from *Palicourea adusta* [17–20].

Because of the promising biological applications of alkaloids, there is continuous research for the development of new extraction and purification techniques of alkaloids [15]. Thus, a significant increase in the antimalarial activity was observed for Group 2 fractions (► **Table 1**) that were obtained after the fractionation of the leaf ethanol extract by acid-base separation of the alkaloids (Method A). In this procedure, only the Leaves *n*-BuOH Fr. (Fraction) was inactive (*GI* < 50%) and all other fractions showed moderate to high activity with a parasite *GI* in the range of 65 to 92%. Interestingly, differences in the UPLC-DAD and the full scan TIC profiles were observed for these fractions (**Figs. 4S–6S**, Supplementary Material). However, from a bioactive point of view, the antimalarial activities of the Group 2 fractions are very similar, except for the Leaves *n*-BuOH Fr. whose phytochemical profile and bioactivity are similar to the ethanol extract (► **Table 1**).

The UPLC-DAD chromatograms of the five Group 2 bioactive acid-base fractions from *P. hoffmannseggiana* leaves are shown in **Fig. 4S**, Supplementary Material, and the RTs for the more prominent peaks along with their UV and MS data in positive and negative modes are discussed here (**Table 1S**, **Figs. 5S** and **6S**, Supplementary Material). Attention is called to the UV spectra, which show characteristics of an indole nucleus, such as in tetrahydro- $\beta$ -carbolines and tryptamines ( $\lambda_{\text{max}}$  220, 279 nm), as well as  $\beta$ -carboline alkaloids ( $\lambda_{\text{max}}$  246, 299, 365 nm) [21, 22]. Identification of some constituents of this group is discussed in the following topics.

Leaves Neutral  $\text{CHCl}_3$  Fr. (**Table 1S**, **Figs. 4SC**, **5SB**, and **6SB**, Supplementary Material): RT 2.40 min;  $\lambda_{\text{max}}$  246, 299, 365 nm;  $m/z$  183.10  $[\text{M} + \text{H}]^+$  and  $m/z$  180.88  $[\text{M} - \text{H}]^-$ .

The UV spectrum for this metabolite is indicative of a  $\beta$ -carboline. A methyl- $\beta$ -carboline compound was inferred from the protonated molecular ion  $m/z$  183.10  $[\text{M} - \text{H}]^-$ , and by the following fragment

ions at  $m/z$  115.05  $[\text{M} + \text{H} - 68]^+$  derived from the  $\beta$ -carboline nucleus by an HRF radical loss of  $\text{C}_3\text{H}_4\text{N}_2 \cdot$ . However, the broad peak at RT 2.40 might be related to the coelution of isomeric compounds with  $m/z$  183.10  $[\text{M} + \text{H}]^+$  (**Fig. 4S**, Supplementary Material). Indeed, three natural methyl- $\beta$ -carbolines supporting a methyl group in the 1, 2, or 4 position are known (► **Fig. 3a**) [23] and the present data do not allow to make a distinction for two out of the three possible constitutional isomers. The presence of the deprotonated molecular ion  $m/z$  180.88  $[\text{M} - \text{H}]^-$  supports the exclusion of 2-methyl- $\beta$ -carboline as one of the three possible isomers, since this isomer would not show significant negative ionization because of the NH bond absence.

Leaves Basic  $\text{CHCl}_3$  Fr. (**Table 1S**, **Figs. 4SD**, **5SC**, and **6SC**, Supplementary Material): RT 1.83 min;  $\lambda_{\text{max}}$  220, 279 nm;  $m/z$  174.61  $[\text{M} + \text{H}]^+$ .

This peak was already attributed here to *N*-methyl-tryptamine ( $m/z$  174.61  $[\text{M} + \text{H}]^+$ ) and fragment ions of  $m/z$  115.09  $[\text{M} + \text{H} - 60]^+$  for the loss of  $\text{C}_3\text{H}_{10}\text{N} \cdot$ ,  $m/z$  143.05  $[\text{M} + \text{H} - 32]^+$  due to the loss of  $\text{CH}_6\text{N} \cdot$ ,  $m/z$  91.17 corresponding to  $[\text{M} + \text{H} - 84]^+$  derived from the loss of  $\text{C}_5\text{H}_{10}\text{N} \cdot$ , and  $m/z$  77.25  $[\text{M} + \text{H} - 98]^+$  related to different HRF fragmentations of the indole nucleus (► **Fig. 3b**) [24, 25].

Leaves Basic  $\text{CHCl}_3$  Fr. (**Table 1S**, **Figs. 4SC**, **5SC**, and **6SC**, Supplementary Material): RT 2.64 min;  $\lambda_{\text{max}}$  246, 299, 365 nm;  $m/z$  183.10  $[\text{M} + \text{H}]^+$  and  $m/z$  180.88  $[\text{M} - \text{H}]^-$ .

Identification of the metabolite at RT 2.64 min ( $m/z$  183.10  $[\text{M} + \text{H}]^+$ ) was discussed above in the analysis of the Leaves Neutral  $\text{CHCl}_3$  Fr. (item a) on the basis of the fragment ion at  $m/z$  141.24  $[\text{M} + \text{H} - 42]^+$  resulting from the loss of  $\text{C}_2\text{H}_4\text{N} \cdot$  that was taken as an indication of the localization of the methyl group at C-1, corresponding, therefore, to the known harmine (► **Fig. 3a**). Other fragments such as  $m/z$  167.80  $[\text{M} + \text{H} - 15]^+$ ,  $m/z$  115.05  $[\text{M} + \text{H} - 68]^+$ ,  $m/z$  88.99  $[\text{M} + \text{H} - 94]^+$ , and  $m/z$  65.07  $[\text{M} + \text{H} - 118]^+$  are also in accordance for typical  $\beta$ -carbolines [26].

Leaves Basic  $\text{CHCl}_3$  Fr. (**Table 1S**, **Figs. 4SC**, **5SC**, and **6SC**, Supplementary Material): RT 3.85 min;  $\lambda_{\text{max}}$  220, 279 nm;  $m/z$  203.23  $[\text{M} + \text{H}]^+$  and  $m/z$  201.33  $[\text{M} - \text{H}]^-$ .

The RT at 3.85 min was attributed to *N*-acetyl-tryptamine ( $m/z$  203.23  $[\text{M} + \text{H}]^+$ ), characterized by the fragment ions at  $m/z$  159.84

$[M + H - 43]^+$  for the loss of an acetyl moiety and  $m/z$  143.97  $[M + H - 59]^+$  due to the loss of the radical  $\text{CH}_3\text{COHN} \cdot$  (► **Fig. 3c**).

Peaks of lower intensity in the UPLC-DAD chromatograms of the Group 2 fractions of *P. hoffmannseggiana* leaves extract (**Fig. 4S**, Supplementary Material), disclosing similar UV spectra, were observed in other bioactive fractions, and some of them clearly overlap to major peaks identified in the leaf ethanol extract as discussed in the last part. Furthermore, some  $\beta$ -carboline alkaloids such as harmine and harmaline showed moderate *in vitro* anti-plasmodial activity ( $\text{IC}_{50}$  8.0 and 25.1  $\mu\text{g}/\text{mL}$ , respectively) against *P. falciparum* [27]. Harmane and related analogues were also isolated from *Guiera senegalensis* (Combretaceae), a plant from Mali, with traditional use for malaria treatment that afforded harmine and tetrahydroharmine with significant antimalarial activity ( $\text{IC}_{50} < 4 \mu\text{g}/\text{mL}$ ) [28, 29]. These simple  $\beta$ -carboline alkaloids were also found in South American plants such as *Banisteriopsis caapi* (Malpighiaceae) and *Psychotria viridis* (Rubiaceae), which are key ingredients in the hallucinogenic beverage Ayahuasca, used by Amazonian indigenous people and found in other *Psychotria* and *Palicourea* species (Rubiaceae) [22]. In addition, a wide review on antimalarial activity of natural  $\beta$ -carboline alkaloids against different *P. falciparum* strains, including simple diversifications of the basic tricyclic structure, initially reported on their potent cytotoxicity and potential antimalarial activity [30]. However, as far as we are concerned, there is no report on the antimalarial/antimalarial activity of simple  $\beta$ -carbolines and tryptamines from *P. hoffmannseggiana* (Schult.) Borhidi and its synonyms [7, 8]. The described results stimulated to carry on the classical acid-base separation procedure of alkaloids, starting by the extraction of *P. hoffmannseggiana* leaf and stem powders with 1N HCl (Method B) as shown in Group 3 (► **Table 1**). Stems and Leaves Basic DCM Frs. were moderately active ( $\text{GI} \sim 64\%$ ) and disclosed statistical similarity data in both assayed concentrations (Group 3) (► **Table 1**). The Stems *n*-BuOH Fr. showed low parasite GI ( $52.5 \pm 2.1 \mu\text{g}/\text{mL}$ , at  $100.0 \mu\text{g}/\text{mL}$ ) and the Leaves *n*-BuOH Fr. was inactive ( $\text{GI} < 50\%$ ) in both concentrations. Therefore, the Leaves and Stems Basic DCM Frs. of this process were about three times more active in relation to the respective ethanol extracts. However, Group 3 fractions disclosed significantly lower activity than Group 2 (► **Table 1**). Marked distinctions in the UPLC-DAD and TIC profiles of Group 2 and Group 3 fractions might therefore be related to different active compounds in these fractions that disclosed different ranges of the parasite GI (**Figs. 7S–10S**, Supplementary Material).

Phytochemically, the Leaves and Stems DCM Frs. showed weak peaks in the UPLC-DAD chromatograms that were not identified (**Figs. 7S and 8S**, Supplementary Material). On the other hand, the positive TIC of the Leaves and Stems Basic DCM for the major constituents disclosed pairs of peaks differing by 44 Da (**Table 1S and Fig. 9S**, Supplementary Material), similar to the observed profiles of the ethanol extracts of *Palicourea sessilis* leaves and stems, in which hydroxycinnamic acid amides derived from *p*-coumaric, caffeic, and/or ferulic acids with amines such as putrescine, agmatine, and/or spermine were identified for the first time as major components of a *Palicourea* species (**Figs. 9S and 10S**, Supplementary Material). However, they are of greater molecular masses than the ones described for *P. sessilis* [31] and were not yet identified. In addition, despite TIC chromatograms profiles of the Group 3 fractions

being significantly different from Group 2, the presence of a peak at  $m/z$  183.10  $[M + H]^+$  relative to methyl- $\beta$ -carbolines (► **Fig. 3a**) was also observed in the leaves and stems of Group 3 basic DCM fractions. Thus, here these compounds could also be associated with the moderate antiplasmodial activity of these fractions.

Taken together, these results show that the increase in the polarity of the extractive solvents ( $\text{EtOH} \rightarrow \text{EtOH}/\text{H}_2\text{O} 7/3 \rightarrow \text{EtOH}/\text{H}_2\text{O} 1/1 \rightarrow \text{H}_2\text{O}$ ) as well as the different extractive methods (maceration, sonication, and decoction) did not have a significant change on the antiplasmodial activity of Group 1 extracts (► **Table 1**). However, a significant increase in the antiplasmodial activity of fractions obtained from the leaf ethanol extract fractionation of *P. hoffmannseggiana* was observed, as can be seen by comparing the results for Groups 1 and 2 (► **Table 1**). Furthermore, the chlorogenic acid isomers as well as the MIA presently identified in the leaf ethanol extract were not detected in the bioactive fractions of *P. hoffmannseggiana* leaves and stems. These compounds are responsible for the major peaks in the *n*-BuOH fractions (Groups 2 and 3), which did not show antiplasmodial activity (parasite  $\text{GI} < 50\%$ ) (► **Table 1 and Figs. 4S and 7S**, Supplementary Material). Thus, one might infer that lyalosidic acid and its sinapoyl derivative as well as strictosidinic acid and the chlorogenic acid isomers might not possess relevant antiplasmodial action.

Traditionally, alkaloid extraction is usually performed by acid-base liquid-liquid procedures starting with methanol or ethanol extracts, or even directly from the powdered plant material, followed by alkalization and extraction with solvents of increasing polarity. Sequential variation of both the strength of the base as well as the polarity of the extractive solvents can lead to special alkaloid separations [16]. Variations in this process are necessary given the diversity of alkaloids and the complexity of samples, especially for the discovery of minor and structurally similar compounds [32]. Indeed, the fractionation aiming at obtaining alkaloid-rich fractions allowed the detection of  $\beta$ -carboline alkaloids that were not detected in the different crude extracts containing MIAs as major components. Besides, significant variations were observed in the phytochemical composition between fractions originating from different fractionation procedures.

In conclusion, the LC-MS analyses of extracts and fractions obtained from *P. hoffmannseggiana* by acid-base extractions and liquid-liquid partitions bioguided by *in vitro* antiplasmodial activity allowed for the identification of major compounds occurring in this species as well as to infer those that may be potentially related to the observed bioactivity. In this sense, the extracts and fractions containing mostly MIAs disclosed low antiplasmodial activity. On the other hand, fractions containing mostly  $\beta$ -carboline alkaloids and tryptamine derivatives disclosed significant activity that was previously demonstrated for harmine, presently detected in this *Palicourea* species. In addition, phytochemical reports on several plants of synonyms attributed to this accepted name supports our assumption that several metabolites described for these plants might be found in a further analysis of the complex chromatographic profile of this species. Furthermore, the present screening, which was guided by *in vitro* antiplasmodial bioassays and LC-MS analyses, has demonstrated that preliminary fractionations of inactive crude extracts are recommended in order not to neglect the presence of minor active compounds. Scale-up of the processes

here described with *P. hoffmannseggiana* will certainly afford promising active fractions of interest for phytomedicine development and/or isolation of new antimalarial hits.

## Materials and Methods

### Plant material

Aerial parts of *P. hoffmannseggiana* were collected at Crispin beach in the municipality of Marapanin, state of Pará (S 0° 35' 35.23" W 47° 39' 28.92"), an area of a *restinga* ecosystem (sandbank vegetation). The plant was taxonomically identified by Dr. L. F. Margalho, Instituto Federal do Pará, Belém, Brazil. A voucher specimen was deposited at the Herbarium of Museu Paraense Emílio Goeldi under the code MG 226057.

### Preparation of ethanol, hydroethanol, and aqueous extracts of *Palicourea hoffmannseggiana* stems and leaves

After drying in a circulating air oven at 37 °C, the dried leaves and stems were milled, and the powder was kept in dark flasks. The different extracts were prepared as follows (Fig. 11S, Supplementary Material): 1) Preparation of ethanol extracts: powdered leaves (200 g) and stems (500 g) were submitted to maceration with ethanol (96 GL) for 6 days (plant:solvent ratio, 1:20 w/v). Subsequently, the extractive solutions were concentrated in a rotary evaporator affording the dry ethanol extract of the leaves (Leaves EtOH Ext., 16 g) and ethanol extract of the stems (Stems EtOH Ext., 13 g), which were kept in a desiccator under vacuum. 2) Preparation of hydroethanol extracts: powdered leaves (10 g) and stems (10 g) in two proportions (7/3 and 1/1, plant:solvent ratio, 1:20 w/v) were obtained by sonication for 5 min at room temperature. The extractive solutions were concentrated in a rotary evaporator and subsequently in a freeze dryer affording the dry hydroethanol extracts of the leaves (Leaves EtOH/H<sub>2</sub>O 7/3 Ext. 0.5 g; Leaves EtOH/H<sub>2</sub>O 1/1 Ext. 0.8 g) and hydroethanol extracts of the stems (Stems EtOH/H<sub>2</sub>O 7/3 Ext. 0.4 g; Stems EtOH/H<sub>2</sub>O 1/1 Ext. 0.5 g), which were kept in a desiccator under vacuum. 3) Preparation of aqueous extracts: powdered leaves (10 g) and stems (10 g) were prepared by decoction with distilled water at 90 °C (plant:solvent ratio, 1:20 w/v) for 10 min. The extractive solutions were concentrated in a freeze dryer affording the dry aqueous extract of the leaves (Leaves H<sub>2</sub>O Ext. 0.8 g), and aqueous extract of the stems (Stems H<sub>2</sub>O Ext. 0.45 g), which were kept in a desiccator under vacuum.

### Fractionation of *Palicourea hoffmannseggiana* ethanol leaves extract by acid-base procedure (Method A)

To an aliquot of Leaves EtOH Ext. (10 g), an aqueous solution of 10 % acetic acid (v/v) pH 1–2 (100 mL) was added, and the mixture was kept under sonication for 40 min. Subsequently, the acidic aqueous layer was separated by filtration following extraction with chloroform (4 × 50 mL). The combined chloroform phases were dried (anhydrous Na<sub>2</sub>SO<sub>4</sub>) and after concentration, gave the leaves acid chloroform fraction (Leaves Acid CHCl<sub>3</sub> Fr., 200 mg). Afterwards, the acidic aqueous layer was neutralized with conc. NH<sub>4</sub>OH to pH 7 and was then extracted with chloroform (4 × 50 mL), gi-

ving the leaves a neutral chloroform fraction (Leaves Neutral CHCl<sub>3</sub> Fr., 31 mg). Finally, conc. NH<sub>4</sub>OH was added to the neutral phase to pH 9–10 and was then sequentially extracted with chloroform, ethyl acetate, and *n*-butanol (4 × 50 mL each solvent), giving the following fractions, leaves basic chloroform fraction (Leaves Basic CHCl<sub>3</sub> Fr., 16 mg), leaves basic ethyl acetate fraction (Leaves Basic EtOAc Fr., 76 mg), and leaves *n*-butanol fraction (Leaves *n*-BuOH Fr., 1.3 g), respectively (Fig. 12S, Supplementary Material).

### Acid-base extraction of *Palicourea hoffmannseggiana* stems and leaves (Method B)

Aliquots of stem and leaf powders (10 g each) were separately submitted to maceration with 50 mL of aqueous solution of 1N HCl (3 × 50 mL), and the acidic aqueous layers were extracted with *n*-hexane (2 × 100 mL), giving the respective *n*-hexane fractions that were not assayed due to their low quantities (< 2 mg). Subsequently, the acidic aqueous layers were alkalized with conc. NH<sub>4</sub>OH to pH 9–10, and then the basic aqueous phases were extracted with dichloromethane (2 × 100 mL), giving the stems dichloromethane fraction (Stems DCM Fr., 85 mg) and the leaves dichloromethane fraction of (Leaves DCM Fr., 90 mg). Finally, the basic aqueous phases (pH 9–10) were extracted with *n*-butanol (2 × 100 mL), giving the stems *n*-butanol fraction (Stems *n*-BuOH Fr., 11 mg) and the leaves *n*-butanol fraction (Leaves, *n*-BuOH Fr., 30 mg) (Fig. 13S, Supplementary Material).

### Chemical analysis

#### UPLC-DAD

Analyses were performed using an ACQUITY UPLC H-Class Core System (Waters) with an ACQUITY UPLC PDA Detector, according to Gontijo et al. [33], in the Faculdade de Farmácia, UFMG, equipped with a reversed-phase column ACQUITY UPLC BEH (1.7 μm, 50 × 2 mm i.d.; Waters). The mobile phase consisted of water 0.1 % formic acid (solvent A) and acetonitrile 0.1 % formic acid (solvent B). The elution protocol was 0–13 min, linear gradient from 5 to 95 % B. The flow rate was 0.3 mL min<sup>-1</sup>, and the sample injection volume was 4.0 μL. The UV spectra were registered from 200 to 400 nm.

#### Mass spectrometry analysis

Mass spectrometry analysis by electron spray ionization (ESI-MS) was performed in an UPLC Acquity (Waters) equipped with a tandem triple quadrupole detector (TQqQD). The ESI source was operated in the following conditions: positive and negative ion modes; ion spray voltage -4 kV; orifice voltage -60 V, with helium as the collision gas; collision energy was set at 30 eV; capillary voltage 3500 V; capillary temperature 320 °C; source voltage 5 kV; vaporizer temperature 320 °C; corona needle current 5 mA; and sheath gas, nitrogen 27 psi. Analyses were run in the full scan mode (100–2000 Da). Data acquisition was achieved with MassLynx V4.1 software (Waters).

#### Antiplasmodial assay

Evaluation of the *in vitro* anti-plasmodial activity against chloroquine-resistant *P. falciparum* (W2 strain) was carried out for two concentrations (100 and 50 μg/mL) of the different extracts and fractions by the quantification of the pFLDH according to methodology routinely performed in our laboratory at the Faculdade de Farmá-

cia, UFMG [17, 34]. Chloroquine diphosphate salt ( $\geq 98.5\%$  purity; Sigma-Aldrich) was the standard antimalarial drug.

## Statistical analysis

All data are expressed as means  $\pm$  standard deviations of triplicate measurements. Antiplasmodial activity was determined for two concentrations (100 and 50  $\mu\text{g/mL}$ ) of the different extracts and fractions and the results were submitted to ANOVA and Tukey tests. All statistical analyses were performed using the software Sigma-Plot 12.5 with  $\alpha = 0.05$  [35].

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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