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Antiplasmodial activity and phenolic composition of Brazilian *Salix humboldtiana* Willd. extract and fractions

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***Salix humboldtiana* Willd. (*Salicaceae*) colonizes Brazilian riverine forests and, besides other congeneric species, is traditionally used to treat fever. In folk medicine, it is even used as a Quinine substitute to treat malaria fever. Although its occurrence worldwide was largely reduced between 2000-2015, in Brazil, the case numbers induced by *Plasmodium falciparum* have increased. Thus, new tools and strategies are necessary against malaria according to the World-Malaria-Report. In the present study the characterization of flavonoids, phenolic acids, phenolic glycosides, and salicylates detected in the ethanol extract of willow leaves, and the antiplasmodial activity of extract of *S. humboldtiana* and its fractions are firstly reported. Chromatography, UV-Visible spectroscopy, and mass spectrometry were used to characterize substances, and the antiplasmodial activity was accessed by cellular DNA fluorescence measurement with ethidium bromide on a flow cytometer. Kaempferol, apigenin, salicylic acid, and salicylates were found in the extract among other phenolics. The bioassays revealed that lyophilized extract of *S. humboldtiana* Willd showed antiplasmodial activity. Hexane, AcOEt, and Methanol fractions were active against *P. falciparum* at IC_{50} of 0.655, 0.929 and 0.688 µg/mL, respectively. Quinine, used as a reference, showed an IC_{50} of 0.147 µg/mL. The polyphenols - salicylates and flavonoids, especially kaempferol and catechin, detected in LESh, exhibit biochemical interference on the plasmodial metabolism, as already reported, and they could also promote the antiplasmodial activity detected. This alleged medicinal use has been already reported for some *Salix* species. Moreover, this work presents scientific evidence for the antiplasmodial activity of *S. humboldtiana*, which can be helpful in the development of antiplasmodial phytomedicine.**

Key words: *Salix humboldtiana* Willd, extracts, phenolic compounds, antiplasmodial activity.

INTRODUCTION

The genus *Salix* is a species-rich taxon comprising five subgenres, and includes about 400 species of deciduous trees and shrubs (Freischmidt et al., 2012). *Salix humboldtiana* Willd. is the single willow species native to South America, belonging to the subgenus *Protitea* according to molecular data (Argus, 2011) and settles in riparian floodplains as a pioneer tree species in a wide distribution area, ranging from southern North America to Patagonia, in southern South America. The willow species is adapted to tropical and subtropical climates (Hernandez-Leal et al., 2019).

In Europe, “the success of the willow bark in the cure of the ague” was already highlighted by Stone (1763) and he provided the first convincing demonstration of a potent antipyretic effect of the willow containing salicylates. Moreover, in Europe at that time, the most problematic ‘plague’ (intermittent fever) to be effectively cured, with white willow (*Salix alba* L.) bark, was probably, caused by malaria (Wood, 2015). In folk medicine, leaves and bark of some *Salix* species are used to treat fever, including malaria (Frei et al., 2009; Deharo et al., 2001), rheumatism (Leporatti and Ivancheva, 2003), and constipation (Scarpa, 2004). In South American folk medicine, Salix is even used as a Quinine substitute to treat fever associated with malaria indicating that *S. humboldtiana* could be included in the research for new natural active pharmaceutical ingredients to treat malaria as reported by Milliken (1997) and Felix-Cuenca et al., (2022).

According to the recent World-Malaria-Report (WHO, 2019) new malaria-facing agents and strategies will be critical to accelerate the pace of malaria-eliminating progress. This is necessary since, despite the malaria burden reduction in the period of 2000-2015, the rate of malaria eliminating progress has slowed in recent years and, e.g., in Brazil the casuistics of *Plasmodium falciparum* malaria even increased. Willows are known for their medicinal properties for millennia, and from them, in 1828, salicylic acid was isolated, becoming the precursor of the anti-thermic and anti-inflammatory drug, aspirin (FAO, 2014).

Generally, secondary metabolites, like phenolics, are biosynthesized, aiming mainly to defend plants against infections and predation. Thus, they can be employed as anti-infectious agents and insecticides (Zaynab et al., 2018). In this way, phenolics of willow seem to exert a protective function, since high concentrations of them, in the leaves, present a negative effect on mammalian herbivory (Stolter et al., 2013). Particularly, phenolic glycosides can be very abundant in willows compared to other secondary metabolites. Concentrations of up to 30% of plant dry weight have been reported (Donaldson et al., 2006) and extracts containing phenolic glycosides can be analyzed by GC, TLC, and HPLC, which is the most frequently reported analytical method using gradient elution and UV detection (Boeckler et al., 2011).

The sixth edition of the Brazilian Pharmacopoeia (2019) presents a monograph of *S. alba* and the second edition of the Brazilian Pharmacopoeia Formulary of Phytomedicines (2021) describes formulations obtained from *S. alba* and other *Salix* species. Also, the World Health Organization (WHO, 2004) and European Medicines Agency (EMA, 2020) report monographs on *Salix* species and preparations, respectively.

Considering the information found in the relevant literature and presented in this paper, the central aim of this work is to detect and characterize the main phenolic substances found in *S. humboldtiana* by chromatographic and spectrometric techniques, and secondarily to assess the antiplasmodial activity of its ethanolic extract.

MATERIALS AND METHODS

Plant material

The plant material was collected from willow formations on the sandy levees of the Aquidauana River, Anastácio, Mato Grosso do Sul, Brazil, at the site 20º28’42.55” S 55º48’9.46”0 with original coordinates Lat: -20.478486 Long: -55.812778 WGS84 Alt: 146 m on August 23rd, 2015. Ten of these willow samples were identified as *S. humboldtiana* Willd. by both botanists Geraldo Damasceno Junior and Heike Markus-Michalczyk. A voucher specimen is deposited in herbarium at the Federal University of Mato Grosso do Sul under number GGMS 52608. Willow branches with leaves were collected from these ten specimens.

The plant material was stored in plastic boxes to keep them fresh until further analyses. This material was cleaned, dried, and ground to produce the herbal derivative as a semi-coarse powder.

Analytical methods

All the reported phytochemical analyses of the present study were performed in the Laboratory of Chromatography and Mass Spectrometry (LCMS) of the Pharmacy Faculty at the Para Federal University on a freeze-dried extract of dried leaves of *S. humboldtiana* Willd. (LESh). For detecting different classes of secondary metabolites, common chemical reactions were performed, in triplicate, on the herbal extract. Among these classes of secondary metabolites are saponins, organic acids, reducing sugars, polysaccharides, proteins and amino acids, phenols and tannins, flavonoids, cardiac glycosides, catechins, sesquiterpenes and other lactones, alkaloids, purines, steroids, and triterpenoids,

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azulenes, depsides and dapsones, coumarin derivatives and anthraquinones, according to Barbosa et al. (2020).

Extraction and fractionation

The extract of *S. humboldtiana* Willd. was prepared by macerating about 200 g of ground leaves in 1000 mL 96ºGL Ethanol (RDD 1:5). After 7 days, maceration was filtered and concentrated on a rotary evaporator under reduced pressure until the complete removal of ethanol. The aqueous residue was then frozen and lyophilized, furnishing a lyophilized extract of *S. humboldtiana* Willd. (LESh). The dry weight of the extract was 23.6 g (yield 11.8%).

An aliquot of 5 g LESh was then treated with solvents of increasing polarity: Hexane, Dichloromethane, Ethyl Acetate, and Methanol. After evaporation of the solvents, the following fractions were obtained: HFSh, from Hexane; DFSh, from Dichloromethane; AFSh, from Ethyl Acetate; MFSh, from Methanol.

Thin layer chromatography (TLC)

The qualitative analysis of the extract (10 mg/mL), polar fractions (10 mg/mL), and standard catechin (1 mg/mL), were performed by TLC using silica gel chromatoplates of 0.20 mm thickness. The qualitative analysis of the extract (10 mg/mL), polar fractions (10 mg/mL), and standard catechin (1 mg/mL), were also performed as the mobile phase ethyl acetate, toluene, and formic acid (85:10:5) with observation under ultraviolet radiation (254 nm and 365 nm) and after sprayed with vanillin sulfuric (ethanolic vanillin 1% followed by ethanolic sulfuric acid 10% with heating at 110ºC for 5 min) (Wagner and Blat, 2001) and pulverization with a methanolic solution of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) 0.05% (Wang et al., 2012).

Thin layer chromatography chromatoplates were sized 20 cm x 20 cm, covered with a 0.2 mm thick 60G - F254 silica gel layer, containing fluorescence indicator (MERCK®). Catechin, Rutin, Chlorogenic acid, and DPPH solution, and all other chemicals, including solvents HPLC grade, used in this work were purchased from Sigma-Aldrich (Darmstadt, Germany).

High-pressure liquid chromatography (HPLC)

Preparation of samples: Standard substances, extract, and fractions

High-pressure liquid chromatography was conducted as LC-DAD analysis and as LC-MS analysis. These are analytical techniques for the separation of phenolic compounds in high-performance reverse-phase liquid chromatography, with diode array detection (DAD) and mass spectrometry (MS) detection, respectively. This is a combination of techniques commonly used for separation, identification, and quantification of phenolic compounds (Kähkönen et al., 2001).

Aliquots of LESh were dissolved in MeOH-HPLC at 20 mg/mL and 1 mg/mL for LC-DAD and LC-MS analyses, respectively. All samples were centrifuged for 10 min at 14,000 rpm (Centrifuge 5418, Eppendorf) before each injection. The substances used as standard in the LC-DAD analyses were dissolved at 1 mg/mL (m:v), also in MeOH-HPLC.

LC-DAD analyses

LC–DAD analyses were performed using an Agilent 1260 model G1361A Infinity series instrument coupled to a Diode Array Detector G1315C, managed by Open Lab ChemStation software version A.01.05 (Agilent Technologies).

The sample injection volume was 10 µL and separation occurred on a C18 column (Zorbax Eclipse XDB-C18) with particle size 5 µm, 4.n6 x 150 mm maintained at 35ºC. Two solvents form the mobile phase in a gradient elution system using H_2O at pH 3.2 by formic acid (0.03% v/v) (A) and acetonitrile (B) at a flow of 0.8 mL/min. The gradient proportions were: 0 min: 90% A; 10 min: 75% A; 20 min: 10% A; 30 min: 0% A, 32 min: 90% A. The UV–vis spectra were recorded between 200 and 600 nm and the chromatograms were registered at 280 and 330 nm, respectively.

LC-MS analyses

The LC-MS system used was an Agilent 1260 model 6460 Triple Quad LC/MS managed by Agilent MassHunter Qualitative Analysis Software Version B.07.00. The same conditions as sample injection volume, column, analysis time, and flow rate were used for the UPLC–MS analysis. For the ESI (negative mode) source, the following inlet conditions were employed: scan spectra from m/z 100 to 1500, the gas temperature used was 350ºC; the Nitrogen flow rate, 6 L/min; nebulizer pressure, 20 psi and capillary voltage, 3500 V. The applied fragmentation energy values were: 60, 80, 120, and 150 V, where the 120 V generated the best profiles.

The sample injection volume was 4 µL and the separation occurred on a C8 column (Zorbax Eclipse XDB-C8) with particle size 5 µm, 4.6 x 150 mm maintained at 27ºC. Two solvents form the mobile phase in a gradient elution system using H_2O at pH 3.2 by Formic Acid (0.03% v/v) (A) and Acetonitrile (B) at a flow of 0.4 mL/min. The gradient proportions were: 4 min: 80% A; 6 min: 60% A; 9-20 min: 5% A. The UV–vis spectra were recorded between 200 and 600 nm, respectively and the chromatograms were registered at 254, 280, 315, 330, and 350 nm, respectively.

The relevant peaks of the samples were identified by comparison of retention time of authentic reference substance (catechin and chlorogenic acid) and UV-Vis spectrum with the same experimental data. Some peaks of the sample were also identified based on of data reported in the literature, including the results obtained by mass spectrometry.

Evaluation of antiplasmodial activity

The FRC3 *P. falciparum* strains were provided by Leônidas and Maria Deane Institute – FIOCRUZ-Amazônia. The parasites were cultivated according to Trager and Jensen (2005) and the tests carried out based on Rabelo et al. (2014) and Bolson et al. (2019). The samples were dissolved in Dimethylsulfoxide (DMSO), in a serial dilution (1:2), providing concentrations from 0.39 to 50 µg/mL, and added to the RPMI 1640 culture medium (Gibco, Waltham, USA). Correspondingly, the final solvent concentration in the wells was ≤ 0.5%, a concentration that did not interfere with the parasite's viability.

Blood samples containing *P. falciparum* at 1% parasitemia and 2% hematocrit were equally distributed in the test wells, which were completed with culture medium up to 100 µL / well. The plates were incubated for 72 h at 37°C in a low concentration of oxygen and carbon dioxide, following the traditional microaerophilic technique of candle burning in a desiccator. After this time, the plates were centrifuged at 800 rpm for 5 min. Subsequently, each well was treated with 50 µL of an ethidium bromide solution (1:50) for 30 min. Thereafter, the plates were washed twice with 200 µL of PBS buffer 1X. After that, the samples were resuspended in 200 µL of PBS buffer. Finally, the samples were analyzed on a BD FACSCanto II

flow cytometer (BD Biosciences, San Jose, USA), on channel FL-1 using software Getting Started with BD FACSDiva™ and FlowJo™ version 10. The parasitic growth in 0.5% DMSO was used as a positive control and non-parasitized erythrocytes as a negative control. Quinine was used as a reference substance, in the same concentrations as phytochemical samples (Dose/Concentration).

The percentage of parasite growth inhibition was determined using the formula by Lopes et al. (2009):

$$\%\ \text{Inhibition} = 100 - \frac{(\%\ \text{of Fluorescence of the Sample} - \%\ \text{of Fluorescence of Healthy Erythrocytes})}{\%\ \text{of Control Fluorescence} - \%\ \text{of Fluorescence of Healthy Erythrocytes})} \times 100$$

The concentration responsible for 50% inhibition of total parasitemia (IC_{50}) was calculated using the GraphPad Prism 8 software based on a logarithmic graph of dose versus inhibition (expressed as a percentage in relation to the control) by non-linear regression analysis.

RESULTS AND DISCUSSION

Previous phytochemical tests on the lyophilized extract of *S. humboldtiana* - LESh – implemented to characterize the presence of secondary metabolites, following an analytic protocol described by Barbosa et al. (2020) showed the presence of phenols and tannins, catechins, steroids, triterpenoids, and coumarin derivatives. These metabolic classes were described by Evans et al. (1995) and Jeon et al. (2008), besides flavonoids, which have been connected to different biological activities, including antiplasmodial (Miliken, 1997; Felix-Cuenca et al., 2022).

Thin layer chromatography (TLC)

The presence of a phenolic substance, like catechin, in LESh was confirmed by TLC, since both shows remarkably close Rf values and the same red color. When the chromatogram was treated with vanillin, according to Wagner and Blat (2001), and Stahl and Schild (1981), flavonoids were also detected. Moreover, these spots reacted with DPPH reagent, indicating their antioxidant capacity. The same reagent indicated the presence of salicylates by discoloration (Wagner and Bladt, 2001).

The results agree with the study on phenolics and flavonoids in extracts of *S. aegyptiaca* of Enayat and Banerjee (2009), which were described as significant amounts of catechin and rutin in the aqueous extract of the leaves and in the ethanolic extract of bark, respectively.

High-pressure liquid chromatography (HPLC-DAD)

The results of the conducted HPLC-DAD on LESh are shown in Figures 1 and 2. Peak 1, shown in Figure 2, at 7.22 min, corresponds to a UV-Visible spectrum of catechin showing λ_{max} = 280 nm. By overlying the chromatograms registered under the same conditions, both for the catechin standard and the sample, the retention times are remarkably close. In addition, the absorption maxima at 280 nm observed in both UV–Visible spectra are superimposed, depicting the structural similarity (Figure 2A). "In addition, the LC-MS analyses show a signal at m/z 288.9 that corroborate with data reported by Pohjamo at al (2003)."

Peaks 2, 3, 4 shown in Figure 2 at 18.30 min; 19.40 min; 25.38 min, respectively, correspond to UV-Vis spectra, and present band 1 at 272 nm, and band 2 at 220 nm, Figure B; band 1 at 272 nm, and band 2 at 213 nm, Figure C; and band 1 at 272 nm, and band 2 at 220 nm, Figure D (Figure 2B, C, D). These spectra are similar to those described by Abreu et al. (2011) for salicylates, as phenolic glycoside type, present spectra with band 1, between 215 nm and 220 nm and a weaker band 2 around 270 nm.

Figure 3 shows a chromatogram of LESh registered at 330 nm by HPLC-DAD. Peak 1 shown in Figure 3 at 5.24 min, corresponding to UV-Vis spectrum presents absorption maxima at 217, 245, and 322 nm, respectively.

This is similar to that obtained from chlorogenic acid, a standard substance (Figure 3-A), a derivative of hydroxycinnamic acid, and an isomer of caffeoylquinic acid. These data combined with that of the LC-MS analyses (Figure 3), a peak in m/z = 352.9 $[M-H]^-$ (Table 1), allow inferring that chlorogenic acid is present in this sample, although slight differences in retention times and in the resolution of the UV-Vis spectrum, at low wavelength, may be due to the different purity grade of the acid as isolated standard and in the mixture of a complex matrix.

Peak 2 shown in Figure 3 at 13.02 min corresponds to a UV-Vis spectrum (Figure 3-B) with the band I at 350 nm and band II around 250 nm, characteristic of flavonoids. As described by Mabry et al. (1970) and Merker and Beecher (2000), flavone and flavanols spectra exhibit two absorption maxima, band I (300-380 nm) and band II (240-280 nm).

Investigation on the polyphenol content of six different *Salix* species pointed out that luteolin and apigenin, along with their derivatives, are the main flavones present in their leaves, while the main flavonols are myricetin and quercetin, and their derivatives, along with with isorhamnetin-3-glucoside (Nyman and Julkunen-Tiitto, 2005).

Furthermore, according to El-Wakil et al. (2015), the methanolic extract of *Salix tetrasperma* and its fractions show antioxidant activity and high content of phenolic compounds, especially in the ethyl acetate fraction.

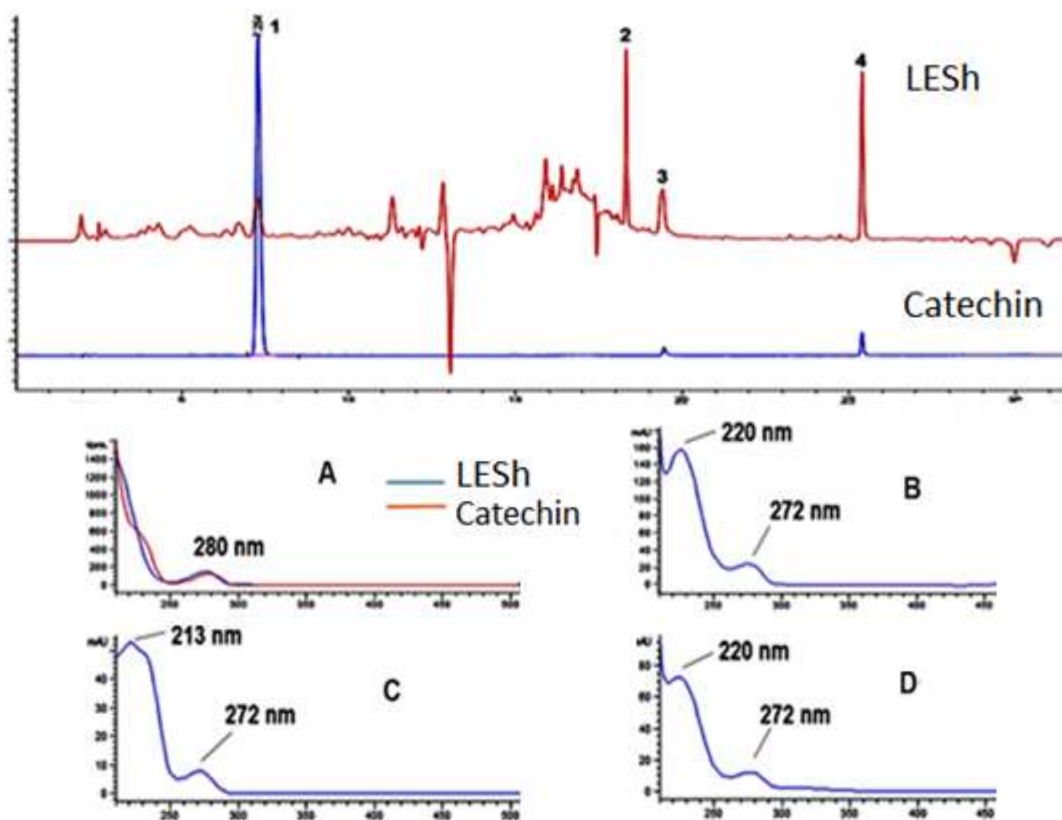

Figure 1. The overlay of the LESh chromatograms (red) and the reference substance Catechin (blue), recorded by HPLC-DAD at 280 nm, is shown at the top of the figure. Below, the UV-Vis spectra of peak 1 (blue) and catechin standard (red), (A), and peaks 2, (B), 3, (C), and 4, (D), are shown, respectively.

Liquid chromatography electrospray ionization mass spectroscopy (LC-ESI-MS)

Results of the analyses on LESh using Electrospray Ionization (ESI) Mass Spectroscopy (ESI-MS) are shown in Figures 3 and 4.

Figure 3 shows the Total Ion Chromatogram - TIC -, using ESI-MS, with peaks of phenolic compounds, like phenolic acids, phenolic glycosides, and polyphenols. The electrospray source (ESI) in negative mode $[M-H]^-$ generated ions that produced peaks 1, 2, 5, 8, 11, 12 and 13 corresponding to the following ions m/z: 178.9; 330.9; 352.9; 288.9; 468.9; 285.9; 572.9, respectively.

Caffeic acid (peak 1) is a constituent of the extract, derived from caffeoylquinic acid, identified by ESI-MS in the crude extract based on m/z= 170.9 $[M-H]^-$. This acid and its derivatives have been widely reported in the leaf and bark of *Salix* species (Enayat and Banerjee, 2009; Poblocka-Olech et al., 2010; El-Wakil et al., 2015).

In this research, catechin was characterized by CCD and by HPLC-DAD in LESh. This substance (peak 5) is suggested as a constituent of the extract, identified by ESI-MS based on m/z= 288.9 $[M-H]^-$. Catechin is a polyphenol, precisely a flavan-3-ol, and is known for its antioxidant (Karakaya et al. 2001; Kim et al., 2003), and antiplasmodial activities (Tasdemir et al., 2006). Catechin was identified in bark extracts of other *Salix* species (Tawfeek et al., 2021). Other flavonoids identified by ESI-MS in negative mode are kaempferol, peak 12, (Plazonic et al., 2009; Abu-Reidah et al., 2015), also an antiplasmodial substance (Tasdemir et al., 2006), and an apigenin derivative, peak 6, (Plazonic et al., 2009).

Figure 2 shows the chromatogram of LESh (A) and chlorogenic acid (B) obtained by LC-DAD-EM registeredat 315 nm. Peak 4 (Rt= 9.46 min) was recorded at 315 nm. In comparison with the retention times of this peak and the chlorogenic acid pattern obtained under the same conditions, it is inferred that both show high equivalence. In addition, the corresponding mass spectrum, with a base peak if m/z= 352.9 [M-H]- characteristic of chlorogenic acid, confirms the identity of the substance. This acid was also identified by El-Wakil et al. (2015) in the leaves of *S. tetrasperma*.

Other salicylates were identified by ESI-MS in LESh

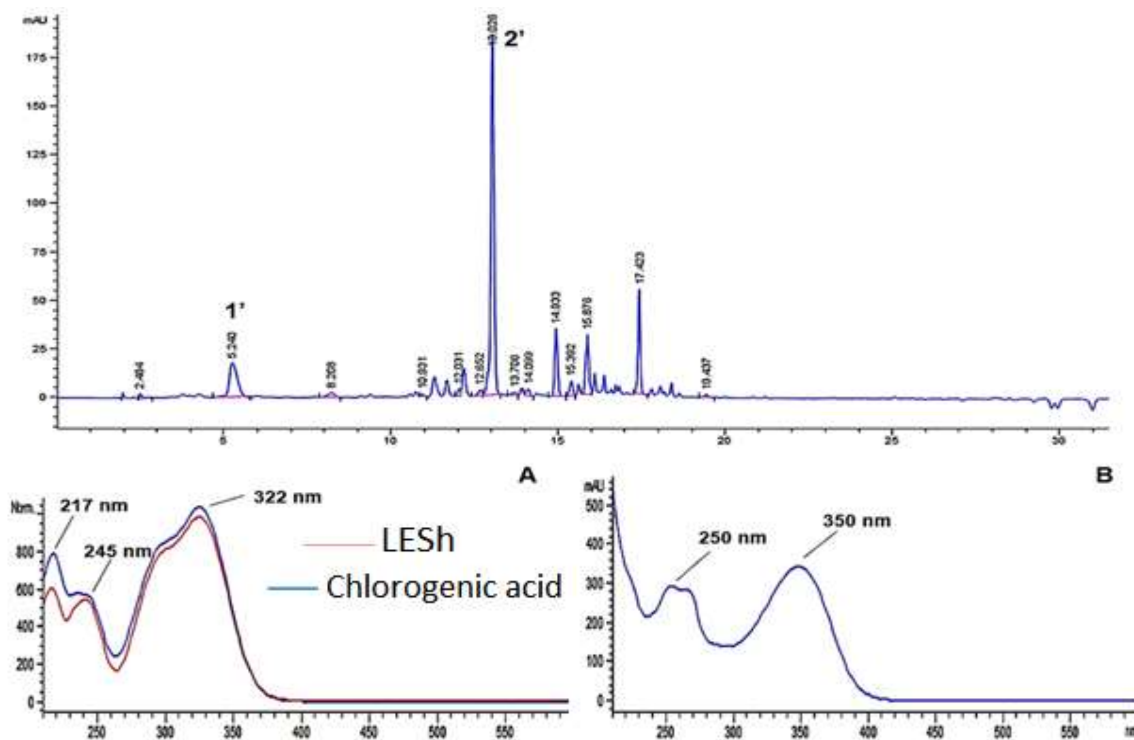

Figure 2. Chromatogram of LESh obtained by HPLC-DAD, registered at 330nm, highlighting peak 1' and Peak 2' and their respective spectra, in A and B.

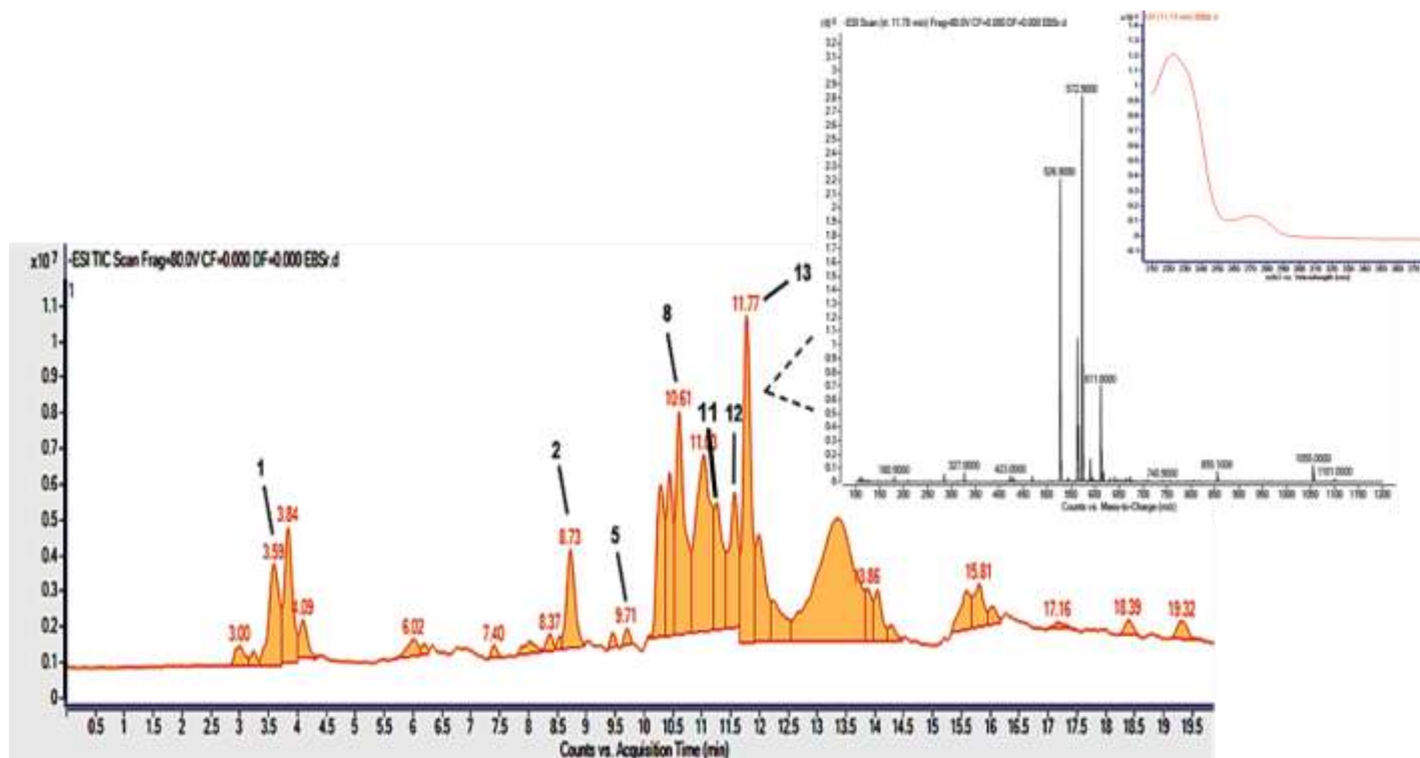

Figure 3. Total ion chromatogram by LC-ESI-MS from LESh, featuring peaks 1 (Rt 3.58 min), 2 (Rt 8.73 min), 5 (Rt 9.71 min), 8 (Rt 10.61 min), 11 (Rt 11.31 min), 12 (Rt 11.56 min) and 13 (Rt 11.73 min). mass and UV-vis spectra of Peak 13 are highlighted.

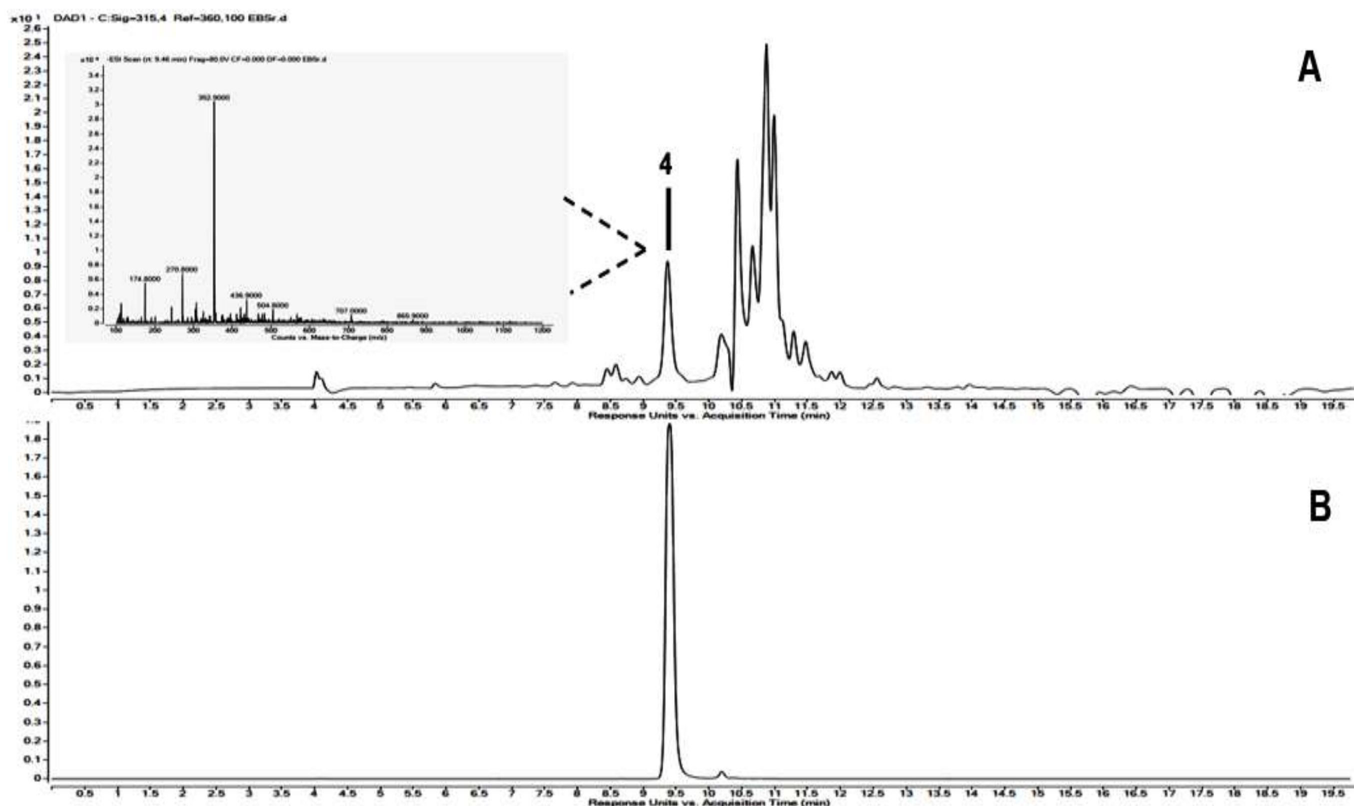

Figure 4. Chromatograms of LESh (A) and Chlorogenic acid (B) obtained by LC-DAD-EM registered at 315 nm highlighting Peak 4 and, in detail, its mass spectrum.

Table 1. Inhibitory concentrations (IC_{50}) of the extract - LESh, and its fractions -HFSh, DFSh, AFSh, MFSh against *Plasmodium falciparum* (FRC3).

Fractions	IC_{50} (μg/mL)
LESh	58.790
HFSh	0.655
DFSh	2.278
AFSh	0.929
MFSh	0.688
Quinine-Std	0.147

(Figure 3). These are: salicin 2 (m/z 330.9 [M-H + HCCOH]-), Salicortin 8 (m/z 468.9 [M -H + HCCOH]-), Tremuloidine 11 (m/z 434.9 [M-H + HCCOH]-) and Tremulacin 13 (m/z 572.9 [M-H + HCCOH]$^-$). Finally, salicylic acid 14 and hydroxybenzoic acid were identified by their mass in the subfraction AFSh6 (Figure 4).

Salicin, the main phenolic glycoside present in bioactive extracts of *Salix* species, is considered the pharmacologically active substance due to its aspirin-like structure. In animal models, extracts of leaves of *S. aegyptiaca* and male flowers showed anti-inflammatory effects in Carrageenan-induced paw edema and hot plaque tests (Karawya et al., 2010; Rabbani et al., 2010).

Antiplasmodial activity

The bioassay model used in this work revealed that fractions of a lyophilized extract of *S. humboldtiana* leave - LESh - treated with solvents of increasing polarity

(Hexane - HFSh; Dichloromethane - DFSh; Ethyl acetate. - AFSh; Methanol - MFSh) showed antiplasmodial activity after evaporating the solvents. HFSh presented the best result regarding the antiplasmodial activity against *P falciparum* showing an IC_{50} of 0.655 μg/mL, better than MFSh and AFSh, which showed IC_{50} of 0.688 μg/mL and IC_{50} of 0.929 μg/mL, respectively (Table 1).

The antiplasmodial activity of HFSh and MFSh, fractions of LESh, against *P. falciparum* is encouraging even when compared to the standard substance used (quinine), considering that the samples are complex matrices containing many substances, among which are flavonoids and phenolics. Ogunlanaa et al. (2015) assayed the antiplasmodial activity of an ethanolic extract and of an Ethyl Acetate derivative and found IC_{50} values higher than those here reported: (>92.0±0.04) and (16.0±0.01), respectively. These samples contain flavonoids derivatives, which were isolated and tested. The phytochemical investigation of AFSh (ethyl acetate fraction), which is here reported in detail, provides a remarkably interesting phenolic profile, which results from the detection and characterization of salicylates and flavonoids, like apigenin, catechin, and kaempferol. Soré et al. (2018) reported the antiplasmodial activity of such substances derivatives. Additionally, five phenolic glycosides detected in a *Salix* species present antiplasmodial activity as described by Kim et al. (2014), and their IC_{50} ranged from 6.6 to 20.5 μM.

Conclusion

The hydroethanolic extract of *S. humboldtiana* leaves presents antiplasmodial activity, which according to published data can be connected to the flavonoids kaempferol, and catechin, whose presence in the analyzed samples was evidenced by TLC and LCMS. This attribute justifies upcoming efforts aiming at innovative *Salix* formulations to treat malaria, as urged by WHO, and to accelerate the pace of malaria-fighting. Also, six salicylates were characterized in the samples, which can be considered quimiomarker of the genus *Salix*, useful to standardize extracts and control the quality of herbal ingredients. Further investigations aiming to identify the antiplasmodial activity detected for HFSh and MFSh and the substances of this species that allow the standardization of the active pharmaceutical substance are recommended. This may support the development of a phytomedicine based on *S. humboldtiana* Willd., and the elucidation of the action mechanism of the active pharmaceutical substance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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