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Research Article

Phytochemical and Antiplasmodial Evaluation of Five Colombian Plants with Ethnopharmacological Background of Antimalarial Use

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Abstract

Background: The study of medicinal plants has made it possible to develop products and drugs for the treatment of different diseases. Several plants in Colombia have a history of popular use for the treatment of malaria. The objective of this work was to provide information on the antiplasmodic and phytochemical activity of five neotropical native plants with a folk use for the treatment of malaria.

Methods: The ethanolic extract of each species was obtained by percolation method and characterized by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and hydrogen nuclear magnetic resonance (^{1}H-NMR). The *in vitro* antiplasmodial activity was evaluated against *Plasmodium falciparum* (strain FCR-3, chloroquine-resistant).

Results: Ethanolic extracts of *Ambelania duckey*, *Cecropia metensis*, *Cecropia membranacea*, and *Verbena littoralis* showed no activity. However, *Curarea toxicofera* extract exhibited an IC_{50} of 7.6 ± 3.9 µg/mL and was classified as moderately active. Most extracts show hemolytic concentration (CH_{50}) > 1000 µg/ mL). A preliminary phytochemical study was carried out using tube analysis, TLC, HPLC, and ^{1}H-NMR. Steroids or triterpenes, and phenolic compounds were detected by TLC in all extracts. These findings were confirmed by characteristic aliphatic and aromatic signals in ^{1}H-NMR spectra, corresponding to triterpenes and phenolics, respectively. Additionally, alkaloids were extracted from *C. toxicofera* and detected by aromatic signals in ^{1}H-NMR spectra.

Conclusion: Ethanolic extract of *C. toxicofera* showed antiplasmodial activity (IC_{50} of 7.6 ± 3.9 µg/mL); this activity may be due to the bisbenzylisoquinoline alkaloids. The ethanol extracts of *A. duckey*, *C. membranacea*, *C. metensis,* and *V. littoralis* did not present antiplasmodial activity.

Introduction

Malaria is a widely distributed infectious disease that exists in the Americas, in tropical, and subtropical regions.[1] This disease is endemic to Colombian Pacific Coast, Amazon, *Llanos Orientales* (eastern plains), and Norte de Santander regions; 3,079,472 malaria cases were registered between 1990 and 2016.[2] In week 52 of 2019, 941 cases of malaria were reported with an annual total of 78,513.[3]

Most drugs available to treat malaria have serious drawbacks in efficacy and show severe side effects that cause poor patient compliance. Resistance of infectious organisms (*Plasmodium* spp.) is also a major concern.[4,5] Hence, additional control measures, including new drugs, are urgently needed.[6-8] One important option for identifying new antimalarial compounds is medicinal plants with a history of ethnopharmacological use for the treatment of malaria.[9]

Various studies have sought to validate popular antimalarial use of different plants in Colombia.[10,11] Five plants species were selected, some with a history of traditional use for the treatment of malaria.[10] Several species of the genus *Cecropia* are reportedly used in folk medicine for the treatment fever, among other illnesses. Additionally, *C. glaziovii* and *C. hololeuca* are used to treat malaria in the Brazilian Amazon.[12,13] In Colombia, antiplasmodial activity of *C. metensis* and *C. membranacea* is reported from specimens collected in the *Llanos Orientales* (eastern plains) region.[14]

Several species of *Verbena*, including *Verbena littoralis*, are used in traditional medicine to treat fever a common symptom of malaria.[15] In Peruvian Amazon, indigenous Loreto, Yanayacu, and Chazuta use leaf decoctions, which, evaluated in *in vitro* models of chloroquine-resistant *P. falciparum*, showed promising antiplasmodial activity.[16,17]

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Plants of the Apocynaceae family produce antimalarial compounds, such as terpenes, alkaloids, and flavonoids. Some indole alkaloids show antimalarial activity against K1 strain of *P. falciparum* with a good selectivity index.[18] Plants of the genus *Ambelania* are found the Amazon region, such as *A. occidentalis,* which is used routinely in folk medicine to treat malaria.[19]

Lianas of the genus *Curarea,* are used for the treatment of parasitic diseases among the indigenous communities in Brazil, Colombia, and Peru. An ethanolic extract of *C. tecunarum* plant, used by the indigenous Loreto community in Peru, showed moderate antiplasmodial activity with an IC_{50} > 10 µg/mL. *Chondrodendron platiphyllum* is reported in the traditional Brazilian Pharmacopoeia for treatment of fever.[20-22] The indigenous Uitoto communities of the Colombian Amazon prepare a remedy from lianas of *C. toxicofera* for treatment of malaria.[23] For the screening of medicinal plants with traditional antimalarial, we use typically ethanolic extracts. Extracts were screened for antiplasmodial activity and by preliminary phytochemical analysis.

Materials and Methods

Collection and identification of plant materials

The collection of plant material was carried out in Colombia during the dry season. Leaves of *Cecropia metensis* Cuatrec (COL 581255) and *Cecropia membranacea* Trecul. (COL 581253) were collected in the province of Meta. The aerial parts of *Verbena littoralis* Kunth. (COL 581259) were collected in the province of Cundinamarca. Stems (lianas) of *Ambelania* cf *duckey* Markgr (COL 570483) and *Curarea toxicofera* (Wedd) Barneby & Krukoff (COL 591178) were collected in the province of Amazonas. Another sample of *C. toxicofera* was collected during the rainy season. Corresponding specimens were deposited in the *Herbario Nacional Colombiano* - COL (Colombian National Herbarium), where taxonomic identity was verified. Additional information on the collected plants is provided in Table S1 in Supplementary Data.

Preparation of plants extracts

All chemicals used in the present study were analytical and HPLC grade and were obtained from Merck and Sigma-Aldrich. Ethanol for extraction was pharmaceutical grade. Selected plant material was dried using a circulating air oven at temperatures between 45–50ºC. Subsequently, particle size was reduced by a semi-industrial blender. To obtain primary extracts, 96% ethanol was used to obtain a greater diversity of secondary metabolites.[24] For the ethanolic extraction, the amount of ground plant material, the solvent volume, and their ratio were provided in Table S2 in Supplementary Data.

Initially, dry material was left in solvent for 24 h to moisten the sample. Extraction then used discontinuous percolation until exhaustion, confirmed by thin-layer chromatography (TLC). Sample depletion was monitored by detecting steroid compounds for *A. duckey*, *C. membranacea*, *C. metensis,* and *V. littoralis*, using a mobile phase of ethyl acetate:hexane (2:8), the derivatization agent was vanillin in orthophosphoric acid. For *C. toxicofera*, alkaloids were detected using a mobile phase of methanol:chloroform:ammonium hydroxide (95:5:0.1), with Dragendorff's reagent as a derivatization agent. Final extracts were evaporated to dryness by a Büchi R-114 rotatory, water bath, and finally in a vacuum oven at 40°C to constant weight.

Phytochemical study of the ethanolic extracts

Preliminary phytochemical characterization used TLC and tube tests Aluminum TLC. plate, Silica gel coated with fluorescent indicator F_{254} were used as a stationary phase. Mobile phases and derivatizing agents were selected based on secondary metabolites. The preliminary phytochemical test conditions can be consulted in Table S3 in Supplementary Data.[25] Reduced or abundant quantity in precipitation tests was identified as (+), and absence of precipitate was indicated as (-). Determination of presence (+) or absence (-) on TLC plates was evidenced by the appearance of spots with colors associated with secondary metabolites. All tests were performed in parallel for each ethanolic extract. Substances isolated and purified in previous works were used as reference standards.

Phytochemical profiles of ethanolic extracts by HPLC

HPLC profiles were performed in a Shimadzu 1200 equipment with an automatic injector, binary pump, and UV-DAD detector, processing at 210 nm and 350 nm. A Lichrospher® RP-18 column (250 mm × 4 mm, 10 µm) was used with a mobile phase gradient of acetonitrile (ACN) in water from 5% to 95% ACN over 60 min, holding at 95% ACN for 10 min, and returning to initial conditions for 10 min before the next injection. The following experimental conditions were established: flow, 1 mL/min; injection volume, 10 µL, and temperature of 25°C. Assays were initially performed at a concentration of 10 mg/mL, however, HPLC profiles showed very small peaks, because of this, each sample was prepared in methanol at a concentration of 20 mg/mL for the final test.[26,27] Standards of chlorogenic acid, protocatechic acid, catechin, epicatechin, lupeol, β-sitosterol, and tetrandrine (Sigma) were used.

^{1}H-NMR spectra of ethanolic extracts

^{1}H-NMR samples were prepared by dissolving 20 mg of dried extract in 500 µL of methanol-*d*4 and transferring solutions to 5 mm NMR tubes. ^{1}H-NMR spectra were calibrated by using the 3.8 methanol-*d*4 signal. The spectra were obtained with a Bruker Avance 400 spectrometer with a resonance frequency of 400.13, a 5 mm probe, and gradient shim Z. ^{1}H-NMR spectra were used to confirm nuclei of secondary metabolite, as part of the characterization of the primary extracts. The information allows the generation of fingerprints for comparisons under different conditions.

In vitro antiplasmodial activity

In vitro antiplasmodial activity was evaluated using the *Plasmodium falciparum* FCR-3, chloroquine-resistant strain. Two independent experiments were performed for *C. metensis*, *C. membranacea*, and *V. littoralis* and one for *A. cf duckey*. Two independent experiments were performed for *C. toxicofera* with the sample collected in the drier season and another with an extract of stems collected in the rainy season.

The parasitized red blood cells (pRBC) O^+, were cultured in 25 mL flasks (Corning Costar Corporation®, USA), containing 8.1 mL of RPMI 1640 medium, 1 mL of human serum (HS), 0.4 mL of sodium bicarbonate, and 500 µL of red blood cells (RBC) (hematocrit = 5%). Parasitemia remained between 0.5 and 4% by adding healthy RBC. The parasites were incubated at 37°C in a modified atmosphere of 90% N_2, 5% CO_2, and 5% O_2. Medium was renewed every day, and a microscopic control (1000X) of parasitemia was checked by blood smear fixed with methanol and stained with Giemsa. Parasite culture was synchronized with 5% sorbitol. Extracts were dissolved in DMSO at a final plate concentration between 0.78 and 100 µg/mL (DMSO < 1%); 100 µL of pRBC culture (2.5% parasitemia) plus 100 µL of test substance were added to wells of 96-well plates and incubated for 48 h, as above. Chloroquine diphosphate (1 µg/mL) and vehicle were positive and negative controls, respectively.

Parasite density was quantified using an optimized reading method based on fluorescence of SYBR Green I. Each concentration of extracts (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL, 1.56 µg/mL and 0.78 µg/mL) was evaluated in triplicate. The results are expressed as 50% inhibitory concentration (IC_{50}) estimated by using a concentration-response logistic regression model (Microsoft Excel 365®).[28,29]

Hemolytic activity assay

An adaptation of the protocol previously described was used to assess hemolysis.[29] Samples were diluted in PBS (phosphate buffered saline, 1X, pH 7.5), further serial dilutions (from 1000 µg/mL to 0.001 µg/mL) were performed with PBS. RBCs were obtained from fresh blood from O^+ healthy voluntary donors. Blood was collected in sodium citrate buffer, washed three times with isotonic saline, and resuspended in PBS 1X, pH 7.5; 100 µL of sample and an RBC suspension with 4% hematocrit were mixed in wells of a 96-well plate. After incubation at 37°C for 1 hour, plates were centrifuged at 500 g for 10 min. Hemoglobin was quantified in an aliquot of 50 µL of supernatant by OD at 418 nm. Samples were evaluated in triplicate in three independent experiments. PBS 1X was used as a negative control and sterile 0.1% Triton X-100 as a positive control. Hemolytic concentration 50 (HC_{50}) was calculated using a regression model.

A selectivity index (SI) was calculated by the relationship between values for HC_{50} for RBC and IC_{50} for *P. falciparum*. This index reflects the ability of a sample to directly affect the parasite or indirectly affect the parasite through effects on blood cells.

Results and Discussion

Phytochemical characterization

The extraction yields obtained were between 3% and 17%, according to the plant species (Table S2 in Supplementary Data). A preliminary phytochemical evaluation of these extracts established the presence of flavonoids, tannins, terpenes, and alkaloids. Cardiotonic, anthracene, coumarin, or saponin glycosides were not detected. The presence of terpenes and steroids, phenols, tannins, and flavonoids in *A. duckey* and *C. toxicofera* is reported for the first time (Tables 1 and 2).

Table 1. Results of phytochemical characterization by color and precipitation tests for the ethanolic extracts of *A. duckey*, *C. metensis*, *C. membranacea*, *C. toxicofera,* and *V. littoralis*.

Classes of secondary metabolites	Color and precipitation tests	Plants				
		Ambelania duckey	*Cecropia metensis*	*Cecropia membranacea*	*Curarea toxicofera*	*Verbena littoralis*
Alkaloids	Dragendorff	-	-	-	+	-
	Mayer	-	-	-	+	-
	Valser	-	-	-	+	-
	Ammonium Reineckate	-	-	-	+	-
Phenolic compounds	$FeCl_3$	+	+	+	+	+
Flavonoids	Shinoda	-	+	+	-	+
	HCl	-	-	-	-	-
Cardiotonic glycosides	Bornträger-Krauss	-	-	-	-	-
Tannins	Gelatin-Salt	+	+	+	+	+
Proanthocyanidines	BuOH/HCl	-	+	+	-	-
Saponins	Foam test	-	-	-	-	-
	Hemolysis	-	-	-	-	-

Table 2. Results of the phytochemical study by TLC[a] for the ethanolic extracts of *A. cf duckey*, *C. metensis*, *C. membranacea*, *C. toxicofera,* and *V. littoralis*.

Plant extract (ethanolic extracts)	Secondary metabolite				
	Phenolic compounds (dark blue)	Flavonoids (yellow, green or orange)	Phenolic carboxylic acids (blue)	Terpenoid and steroidal compounds (Pink)	Alkaloids (orange)
Ambelania cf duckey	0.25	0.37[b]	0.30	0.25, 0.45	ND
Cecropia metensis	0.20	0.50[b], 0.60[b], 0.70[b]	0.30, 0.60	0.25, 0.45, 0.6, 0.7	ND
Cecropia membranacea	0.20	0.50[b], 0.60[b], 0.70[b]	0.30, 0.60	0.25, 0.45, 0.6, 0.7	ND
Curarea toxicofera	0.45	0.54[b]	0.7	0.25, 0.45	0.19, 0.28,0.38
Verbena littoralis	0.32	0.74[b]	0.23, 0.83	0.25, 0.45	ND

[a]Rf values obtained from the measurements of two chromatographic tests; b fluorescence 365 nm. Standards: Tannic acid: (0.30 dark blue), Rutin (0.56 orange b), Quercetin (0.35 orange b), Chlorogenic acid (0.42 blue); β-Sitosterol (0.45 pink), Quinine sulphate (0.52 orange). ND: Not detected.

Phenolic compounds

The presence of phenolic compounds was established by an intense blue color. Dark blue spots were observed on TLC plates. The presence of tannins was confirmed by gelatin-salt test. The ethanolic extracts from all species were positive in this test, indicating the presence of phenolic compounds and tannins.

Positive results with $FeCl_3$ tests, both by tube and TLC, indicate the presence of phenolic compounds, but no specific metabolite, such as, coumarins, flavonoids, proanthocyanidins, lignans, phenylpropanoids, phenolic carboxylic acids, or tannins, can be identified.[30,31] Only a small amount of precipitate and a weak blue color were observed in extracts from *C. toxicofera* and *A. duckey*, perhaps reflecting relatively low tannin content. According to the literature, *C. peltata* and *C. membranacea* contain hydrolyzable tannins and proanthocyanidines.[25] Proanthocyanidins were detected only in the ethanolic extracts of *C. metensis* and *C. membranacea*. The presence of tannins is also reported in *V. littoralis*, specifically catequic tannins.[32]

Flavonoids

Shinoda and leucoantocyanidins tube tests and TLC assay were used to confirm the presence of flavonoids. The Shinoda test with the γ-benzopyrone nucleus was positive (red coloration) only for *C. metensis*, *C. membranacea,* and *V. littoralis* extracts. The test for leucoanthocyanidins was negative for all the analyzed extracts.[25] Further, all plant extract TLC profiles showed yellow, green or orange positive fluorescent spots when observed under UV light (365 nm), after being sprayed with NP-PEG reagent.[32] For *C. metensis* and *C. membranacea,* three intense orange spots at 365 nm were observed, related to flavones, flavonols, and flavanones.[32] An intense green spot was seen for *V. littoralis* extract and for *C. toxicofera* extract, these green spots may reflect phenolic compounds. TLC chromatograms for the *A. duckey* extract showed a characteristic faint orange stain for flavonoids. The R_f for the flavonoids detected are shown in Table 2.

The obtained HPLC chromatograms of *C. metensis* and *C. membranacea* extracts (Figure 1A and 1B) exhibited similar profiles, as in TLC profiles. One major peak at a retention time of 19.2 min and UV detection at 350 nm (Figure 1A and 1B) showed the characteristic absorption pattern of flavonoids, with two maxima absorption peaks at 269 and 349 nm (Figure 1A and B).

In the region of medium polarity, a major peak was observed for the extract from *V. littoralis* with a retention time of 19.5 min and strong absorption at 202 nm and 333 nm along with a group of five peaks with retention time between 7.0 and 11.0 min. These peaks are also associated with flavonoids (Figure 1C). Additionally, the *A. duckey* chromatogram showed a main peak at a retention time of 12.4 min (Figure 1D) with two maxima at 200 nm and 333 nm. Finally, the extracts from *C. toxicofera* did not exhibit a major peak, but several small peaks with retention times between 10.0 and 36.0 min were observed. The absorption pattern of these peaks was different from a pattern associated with flavonoids (Figure 1E). The ^{1}H-NMR spectra for each extract showed signals in the low field region corresponding to aromatic protons (6.5–7.5 ppm), which are present in phenolic compounds, including flavonoids (Figure 2).[33]

The Shinoda test was positive for *C. metensis, C. membranacea*, and *V. officinalis,* establishing the presence of flavonoids with a γ-benzopyrone ring. This result matches literature reports for different species of the *Cecropia,* such as *C. peltata*, *C. obtusifolia*, *C. glaziovii,* in which isoorientin, vitexin, catechin, and epicatechin are present.[26,34,35] A previous phytochemical study using extracts of leaves of *C. metensis* and *C. membranacea*, collected in the rainy season, exhibited phenolic compounds, including flavonoids.[25] Flavonoids, littorachalcone, and verbenechalcona, are reported from *V. littoralis*.[36] The presence of flavonoids in *A. duckey* is not previously reported. However, negative results in the tube assays, the non-conclusive result in the TLC profile, and positive HPLC results suggest the presence of flavonoids in this species. The positive result in the $FeCl_3$ test for the extract from *C. toxicofera* may be associated with the presence of phenolic alkaloids, such as curine that has two phenolic groups, but not with flavonoids, since positive results were not obtained in Shinoda tests, TLC or HPLC.[37]

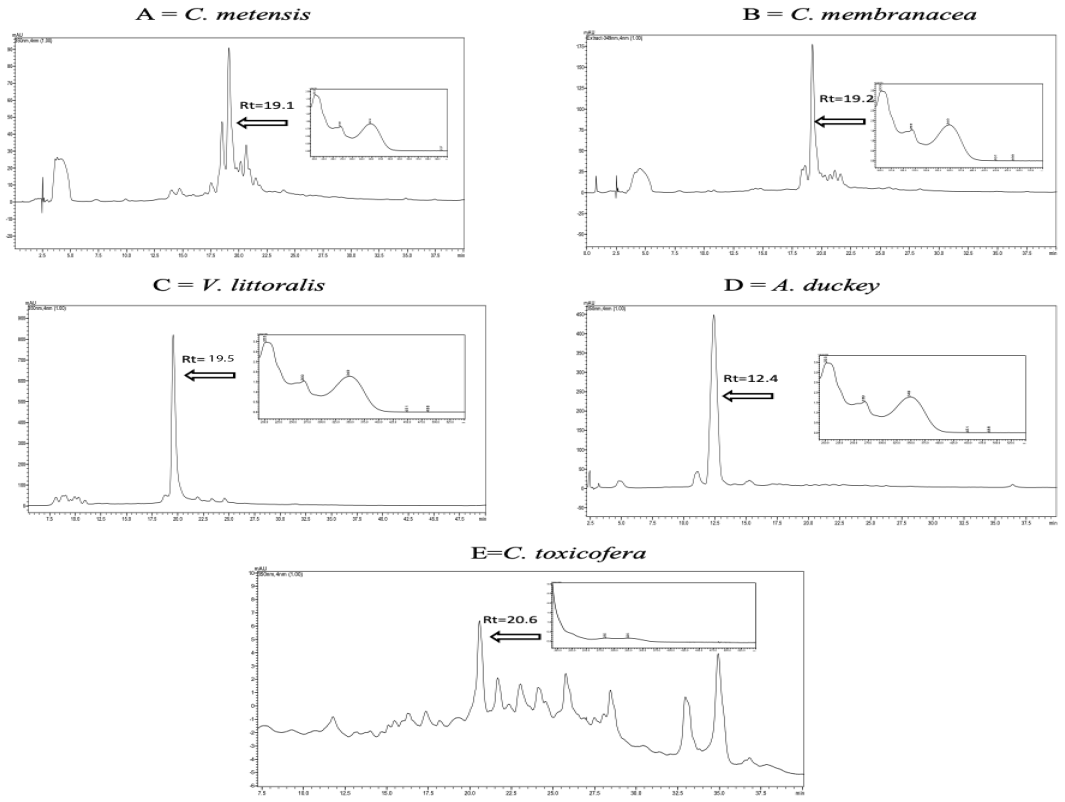

Figure 1. Chromatograms at 350 nm of the ethanolic extracts of *C. metensis*, *C. membranacea*, *V. littoralis*, *A. duckey* and *C. toxicofera*.

Phenolic carboxylic acids

The chromatograms for all the extracts in the current study showed fluorescent blue spots after sprayed with NP-PG reagent, indicating the presence of phenolic carboxylic acids. *C. metensis* and *C. membranacea* extracts exhibited two fluorescent blue spots. *V. littoralis* extract showed two intense points and *A. duckey* extract displays a single intense spot. A single low-intensity spot was observed for *C. toxicofera* extract (Table 2).

C. metensis and *C. membranacea* extracts (Figure 1A and 1B) HPLC profiles exhibited compounds with retention times between 3–5 min, corresponding to phenolic carboxylic acids. The absorption profile is characteristic for such compounds, with maximum absorption at 325 nm for chlorogenic acid.[38] The *V. littoralis* extract profile (Figure 1C) displayed a group of low absorption peaks with retention times of 7.5–10 min high polarity region, with UV maxima at 260 nm and 290 nm. Another low absorption peak with a retention time of 18.7 min showed two maxima at 200 and 350 nm. Chlorogenic acid was reported in *C. glaziovii* and *V. officinalis*.[39] The chromatogram for *A. duckey* extract (Figure 1D) displayed a group of low absorption peaks between 4 and 5 min with two maxima at 200 nm and 260 nm associated with compounds of high polarities, such as phenolic carboxylic acids. No major peak was visualized for *C. toxicofera* extracts in the polar region, but peaks at retention times between 10.0 min and 18.0 min were observed, characterized by maximum absorption at 200 nm (Figure 1E).

Extracts in which phenolic carboxylic acids were detected, showed alkene hydrogen signals (6.38–7.59 ppm) and a low field region corresponding to aromatic protons (6.96–7.09–7.17 ppm). Signals of aliphatic hydrogens corresponding to the glucosidic unit (1.9, 3.6 and 4.0) are present between others in phenolic carboxylic acids as chlorogenic acid (Figure 2).[33,40,41]

Terpenoid and steroidal compounds

Compounds with a terpene or steroidal nucleus were detected by TLC derivatized with the Libermann-Burchard reagent. Extracts of *C. membranacea* and *C. metensis* showed four small pink spots *V. littoralis* extract showed single pink spots, one with greater intensity at R_F=0.25, than the other at R_F =0.45. *A. duckey* and *C. toxicofera* extracts produced single weak pink spots (Table 2). The presence of terpene and steroidal compounds was confirmed after derivatization with vanillin / *O*-phosphoric acid by appearance of several colored spots.[42] The results of these TLC assays confirmed the presence of terpene or steroidal compounds in all five extracts.

A. duckey and *C. toxicofera* extracts exhibited no significant absorption peaks in the region of the chromatogram corresponding to lower solvent polarity (Figure 3 A-B), which might be explained by the low UV absorption of these compounds. However, the *V. littoralis* extract displayed a medium intensity peak with a retention time of 53.0 min (Figure 3C), which might corroborate with the intense spot observed by TLC. In addition, ^{1}H-NMR

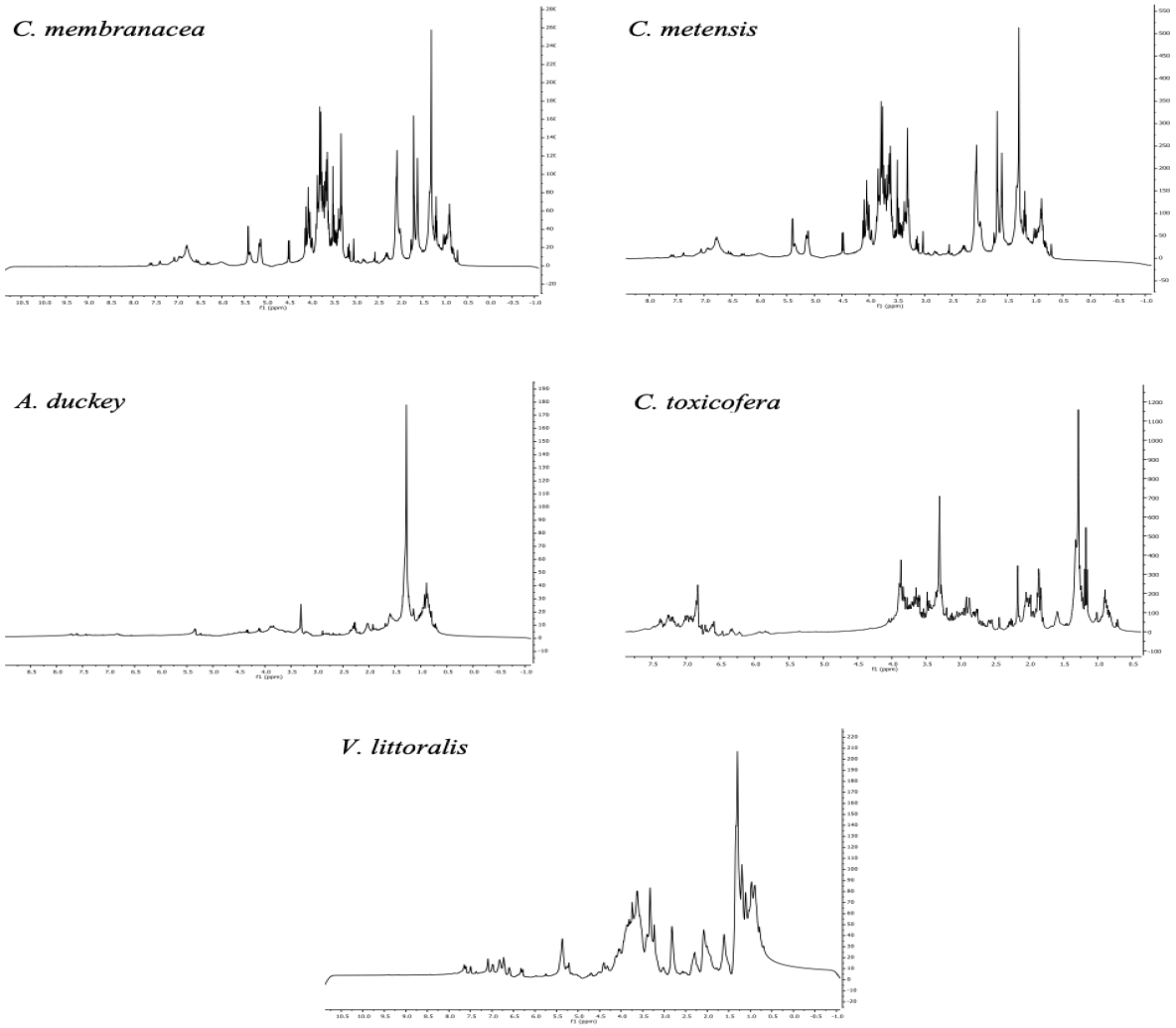

Figure 2. [1]H-NMR spectra of ethanolic plant extracts of *A. cf duckey*, *C. metensis*, *C. membranacea*, *C. toxicofera,* and *V. littoralis*.

high field signals (between 0 and 3 ppm) were observed, corresponding to aliphatic hydrogens (Figure 2).[33]

Chromatography for detection of steroids and triterpenes showed intense spots with similar R_F in extracts of *C. metensis* and *C. membranacea*, which may be associated with the presence of steroidal compounds such as β-sitosterol (R_F=0.45). Other terpenoid compounds have been reported in several species of the same genus, such as tormentic acid.[13] In a previous work, a comparable terpene profile was obtained for these same two species collected in the dry season.[13,25] β-sitosterol might be present in these plants, since a spot with the same low-intensity color and similar R_F were obtained in the extract and for this standard.

V. littoralis extract showed triterpenes/esteroids by using Liebermann-Burchard reagent, some triterpenes were reported in *V. littoralis* extract, such as the 3α, 24-dihydroxy-urs-12-en-28-oic acid.[43] The weak intensity spots observed on TLC plates derivatized with Liebermann-Burchard, indicate a low concentration such metabolites in *A. duckey* and *C. toxicofera* extracts. A more sensitive derivatization agent, vanillin / *O*-phosphoric acid showed additional spots, yet confirms the low to moderate quantity of steroidal or terpenoid compounds.

Alkaloids

Abundant precipitate formation was observed in the ethanolic extract of *C. toxicofera* after addition of reagents for confirming the presence of alkaloids (Table 1). The positive result with the Reineckate salt test can be associated with the presence of bisbenzylisoquinoline alkaloids, molecules with two quaternary ammonium groups, such as (+) - tubocurarine or chodrocurarine. These compounds were previously reported in *C. toxicofera*.[37] Other species showed no precipitation, indicating the absence of alkaloids.

This result was confirmed by TLC, using Dragendorff's reagent as a derivatizing agent. *C. toxicofera* extract showed three groups of intense orange spots. Results were negative for extracts of other species. The presence of alkaloids in *C. toxicofera* may be associated with the group of compounds with retention times between 14.0 and 20.0 min by HPLC. These peaks showed two maxima absorption at 210 and 280 nm (Figure 3B). These results are consistent with

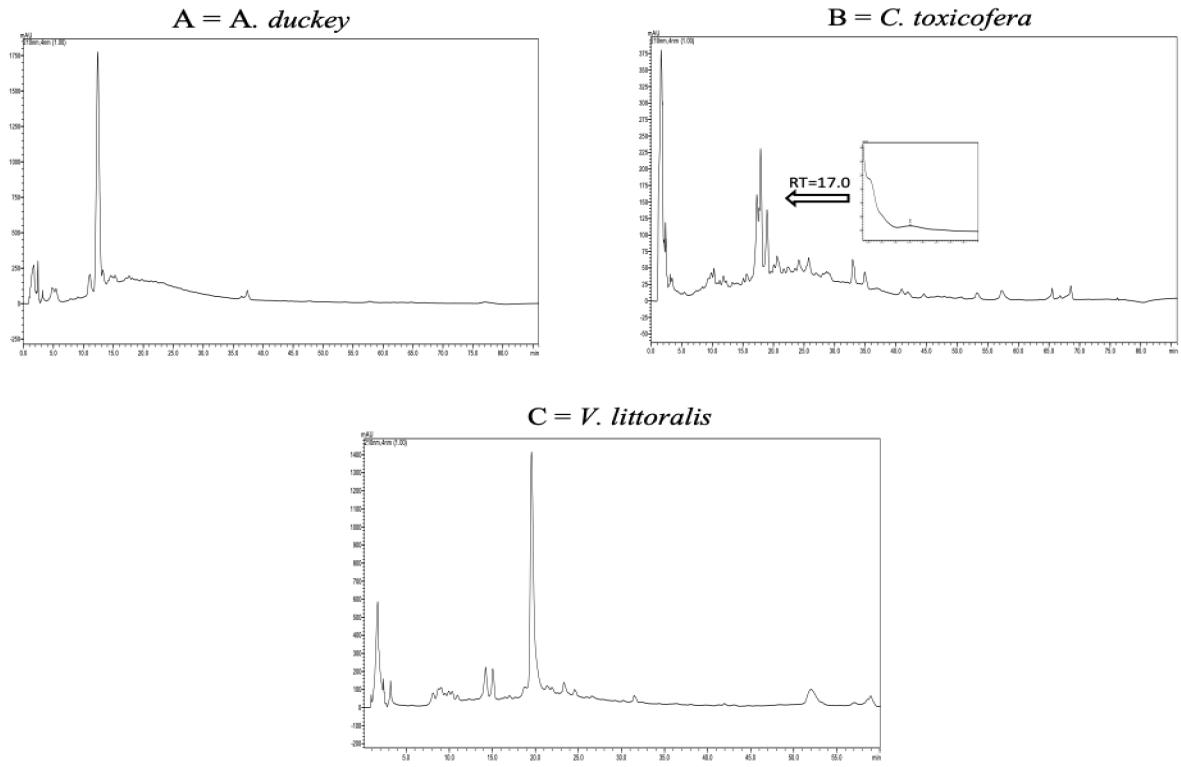

Figure 3. HPLC chromatograms at 210 nm of ethanolic extracts of *A. duckey* (A), *C. toxicofera* (B), and *V. littoralis* (C).

literature reports of the presence of bisbenzylisoquinoline alkaloid in *C. toxicofera*.[44,45]

Further, ^{1}H-NMR spectra show signals in the region corresponding to primary amine hydrogens (0.5–3.0 ppm) and signals between (6.5–7.5 ppm) corresponding to aromatic protons.[33]

The high quantity of precipitant and intensity of the TLC spots in the evaluation of alkaloids in *C. toxicofera*, suggests a high concentration of this kind of metabolites. This result is associated with the presence of bisbenzylisoquinoline alkaloids, chemotaxonomic markers for species of the Menispermaceae family of South America. For *C. toxicofera*, bisbenzylisoquinoline alkaloids such as curin, (+) - tubocurarine, isocondodendrine, and toxicoferin, are reported.[37]

For the species of the Apocynaceae family and the genus *Ambelania*, such as *A. occidentalis*, the presence of indolic alkaloids is reported.[19] However, the ethanolic extract of *A. duckey* did not display their presence. This negative result may be associated either with the season and place of collection or low concentrations of such metabolites in the extract. To our knowledge, no previous report suggests the presence or absence of alkaloids in *A. duckey*.

Antiplasmodial activity

An *in vitro* screening showed no antiplasmodial activity for extracts of *A. duckey*, *C. metensis*, *C. membranacea*, and *V. littoralis* at any tested concentration. These extracts are considered inactive against *P. falciparum* strain FCR-3. An average of IC_{50} of 7.6 ± 3.9 µg/mL was observed for *C. toxicofera* extract, suggesting moderate activity. Activity level was classified according to criteria harmonized by The Research Initiative on Traditional Antimalarial Methods (RITAM).[14,46]

The ethanolic extract of *A. duckey* and *V. littoralis* did not show antiplasmodial activity, these results do not support the traditional use of this species for the treatment of malaria, but such use might be related to other stages of the parasite life cycle, different periods of plant collection, or type of extraction. Such possibilities will require further research.[16,18]

The ethanolic extracts of *C. metensis* and *C. membranacea* do not show *in vitro* antiplasmodial activity against *P. falciparum*, strain FCR-3, at the tested conditions, up to 100 µg/mL. *C. metensis* and *C. membranacea* were collected in the dry season. In a previous work, ethanolic extracts of these two species collected in the rainy season, was tested for their antiplasmodial activity *in vitro*, with similar results. However, ethyl acetate fractions derived from inactive ethanolic extracts and containing flavonoids and terpenes, showed moderate antiplasmodial activity.[14] Other species of the genus *Cecropia* exhibited antiplasmodial activity, such as *C. pachystachya*. Authors of this latter study attribute this activity to tormentic acid and β-sitosterol.[13]

C. toxicofera extract showed antiplasmodial activity *in vitro* and was selected for further evaluation *in vivo* and for additional phytochemical characterization. The presence of alkaloids was confirmed for this species and bisbenzylisoquinoline alkaloids were previously

reported for *C. toxicofera*.[37] Such alkaloids might include (-) - curine, (+) - isochondodendrine, (-) toxicoferine or a 1:1 mixture of (-) - curine and (-) - tubocurine. Also, bisbenzylisoquinoline alkaloids such as norstephasubine, fangchinoline, hernandezine, pycnamine, berbamine, and isotetrandrine have shown antiplasmodial activity against *P. falciparum*.[47-49] Extract from stems of *Curarea tecunerum* also showed high antiplasmodial activity.[21]

C. toxicofera is used by the Uitoto community (Amazonas) to prepare an aqueous traditional remedy for malaria treatment. This remedy was previously tested to confirm antiplasmodial activity *in vitro* (IC_{50}=7.3) and antimalarial activity *in vivo* using 4-day suppression test with *P. berghei* infection (ED_{50} mg/kg/day=328.6).[23] The *in vitro* antiplasmodial activity observed for the *C. toxicofera* extract in our current research is comparable to the former one, reported by Rodriguez P, 2015.[24]

The results of the hemolytic activity indicate that most extracts show hemolytic concentration (CH_{50}) > 1000 µg/mL, ten times higher than the maximum concentration evaluated in *in vitro* antiplasmodial assays with *P. falciparum* (Table 3). Therefore, maximum concentrations used in the test allowed retention of the integrity of non-parasitized erythrocyte membranes for extracts of *C. toxicofera*, *C. membranacea*, *C. metensis,* and *V. littoralis*. The extract from *A. duckey* showed hemolysis with CH_{50} of 259 µg/mL, being the only extract with such interference.

Selectivity indices (Table 3) for *A. duckey* were larger than 3, for *C. membranacea*, *C. metensis,* and *V. littoralis* were greater than 10, and the best SI was for *C. toxicofera* (> 132). This means that the ethanolic extract of *C. toxicofera* showed antiplasmodial activity against *P. falciparum* parasites without affecting the integrity of erythrocytes.

Currently, progress is being made in the bioguided fractionation of *C. toxicofera*, with i*n vitro* and *in vivo* evaluations of the ethanolic extract and its alkaloidal fractions, in order to establish the bioactive markers for the species.

Conclusion

Accordingly, an ethanolic extract of *C. toxicofera* possesses antiplasmodial activity (IC_{50} of 7.6 ± 3.9 µg/mL) without affecting the integrity of erythrocytes; this activity may be due to the alkaloids detected through the TLC and HPLC profiles, as well as the NMR characteristic signals. The ethanolic extracts of *A. duckey*, *C. membranacea*, *C. metensis,* and *V. littoralis* do not present antiplasmodial activity.

Ethical Issues

Ethical clearance from the Ethical Committee of Faculty of Science - Universidad Nacional de Colombia, Sede Bogota, Act No. 07-2015.

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Author Contributions

JEHC was encharged of plant selection and preparation of ethanolic extracts, phytochemical screening, and data collection. MHAM and JEHC performed *in vitro* antiplasmodial activity assays and data collection. JOG provided information on the traditional treatment for malaria in the Hitoma community (Amazonas-Colombia). PELC, YAHU, GGC and, JEHC: contributed to the data analysis and interpretation, as well as the writing and revising of the article. All authors have agreed to this published version.

Table 3. Antiplasmodial screening results of ethanolic extracts against *P. falciparum* FCR-3 and *in vitro* hemolytic activity HC_{50} (µg/mL).

Ethanolic extracts	Antiplasmodial activity IC_{50} (µg/mL)± SD	RITAM classification	Hemolytic activity HC_{50} (µg/mL)	Selectivity index (HC_{50}/ IC_{50})
C. toxicofera	7.6 ± 3.9***	Moderated to Good	>1000	> 132
A. duckey	>100*	NA	259	> 3
V. littoralis	>100**	NA	>1000	> 10
C. metensis	>100**	NA	>1000	> 10
C. membranacea	>100**	NA	>1000	> 10

*Data of one independent experiment, **Data of two independent experiments, ***Data of three independent experiments, NA: Not active

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Data

Supporting information is available at https://doi.org/10.34172/PS.2022.16.

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