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In Vitro and *in Vivo* Antimalarial Activity, Cytotoxicity and Phytochemical HRMS^2 Profile of Plants from the Western Pará State, Brazilian Amazon

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Ethnopharmacology and botanical taxonomy are valid criteria used to selecting plants for antimalarial bioprospection purposes. Based on these two criteria, ethanol extracts 11 plants from Santarém City vicinities, Western Pará State, Brazilian Amazonia, had their *in vitro* antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* (W2 clone) assessed by the *Pf*LDH method, whereas their cytotoxicity to HepG2-A16 cells was assessed through MTT assay. *Acmella oleracea*, *Siparuna krukovii* and *Trema micrantha* extracts disclosed the highest rate of parasite growth inhibition (90%) in screening tests. *In vivo* antimalarial assays were conducted with these species extracts against *Plasmodium berghei* (NK 65 strain) infected mice. Inhibition rate of parasite multiplication ranged from 41.4% to 60.9% at the lowest extract dose (25 mg/kg). HPLC-ESI-HRMS^2 analyses allowed the putative identification of alkylamides, fatty acids, flavonoid glycosides and alkaloids in ethanol extracts deriving from these three plant species. Results pointed towards *A. oleracea* flowers ethanol extract as the most promising potential candidate to preclinical studies aiming the development of antimalarial phytomedicine.

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Introduction

Amazonian biodiversity is internationally regarded as bioprospection hotspot that could afford therapeutic agents to treat any disease type, as well as food to fight famine besides being of fundamental importance to questions such climate change and biodiversity conservation.[1] Ethnopharmacological data of Amazonian plants are available in the literature and support the use of such information in scientific investigations[2,3,4,5] In fact, natural products have directly or indirectly contributed to the discovery of antimalarial drugs, as chemical entities, derivatives and templates for the synthesis of structurally related drugs.[6]

Therefore, it is important reminding that human malaria is an endemic disease in 84 tropical and subtropical regions in African, South-Eastern Asian, Eastern Mediterranean and South American countries. According to WHO (World Health Organization), there was a global increase in malaria cases and associated deaths from 2015 to 2020.[7] With respect to Latin America, the estimated increase in the number of malaria cases was mainly observed in Venezuela, Brazil and Colombia, accounting for 77% of cases in this region. Malaria is also endemic in the Amazonian biome in Brazil, accounting for over 99% of malaria cases in Brazil. *P. falciparum* and *P. vivax* are the main malaria parasites, *P. vivax* is the prevalent one in Brazil (68%).[7,8]

Eleven plant species occurring in the Western Pará State, which is a malaria hotspot in the Brazilian Amazonia, had their *in vitro* antiplasmodial activity and cytotoxicity as well as *in vivo* antimalarial activity aiming to contribute to a rational research on plants traditionally used to treat malaria. Phytochemical profile of the most bioactive plants was also performed. *Acmella oleracea*,[3] *Lippia origanoides*,[9] *Senna quinquangulata*,[10] and *Trema micrantha*[11] were selected based on ethnopharmacological data. Furthermore, the herein adopted taxonomic approach encouraged investigation on plants belonging to genera traditionally used by local populations, namely: *Siparuna*

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krukovii,[12,13] *Abuta sp*,[4,14,15] *Ampelozizyphus sp*,[3,4,15] *Aniba sp*,[15,16] *Aspidosperma sp*,[3,,15] *Croton sp*,[3,4,15] and *Virola sp.*[14,17]

Results

Biological Activity

Screening ethanol extracts of plants occurring in Western Pará State, Brazilian Amazonia, for in vitro antiplasmodial and cytotoxicity effects

Primary screening of ethanol extracts from the 11 selected plants was carried out to investigate for their *in vitro* antiplasmodial activity against chloroquine-resistant *P. falciparum* (W2 strain), as well as their cytotoxicity to HepG2 cell cultures. Results are shown in Table 1. Extracts with % GI >50 in antiplasmodial assay were considered potentially active. Furthermore, most extracts did not show cytotoxicity at the concentration of 100 μg/mL. *Abuta sp* ethanol extract was the only cytotoxic one (CC_{50} 31.9 ± 2.7 μg/mL), *Aniba sp* and *Virola sp* disclosed CC_{50} 57.5 ± 1.8 μg/mL and 57.6 ± 2.5 μg/mL, respectively; they were thus considered moderately cytotoxic (Table 1). Because *Abuta sp* and *Croton sp* were not taxonomically determined, their extracts were not phytochemically featured. *A. oleracea, S. krukovii* and *T. micrantha* ethanol extracts were statically equivalents with %GI close to 90 at the concentration of 50 μg/mL. Therefore, these three plant species stood out as the most promising ones of the 11 screened by *in vitro* and *in vivo* assessment herein reported support the further studies aiming to validate their antimalarial potential[18,19,20,21]

Based on the current results, *Acmella oleracea, Siparuna krukovii* and *Trema micrantha* stood out as the most active species out of the eleven assayed ones (Table 1) – their ethanol extracts had their activity *in vitro* assessed to determining their IC_{50}. Thus, the highest antiplasmodial activity was recorded for *A. oleracea* flowers extract, followed by *T. micrantha* and, finally, by *S. krukovii* – $IC_{50} = 28.93 \pm 6.97$ μg/mL, 25.05 ± 1.71 μg/mL, and 23.08 ± 8.70 μg/mL, respectively. However, differences in IC_{50} values among these species were not statistically significant ($p = 0.05$).

In vivo antimalarial activity of Acmella oleracea, Siparuna krukovii and Trema micrantha extracts against Plasmodium berghei (NK65 strain) infected mice

The inhibition rate of parasite multiplication was determined at the 4th dpi (day post infection), after the last dose of the extracts was administered to infected mice. Parasitemia levels have significantly reduced in all treated groups, mean parasite multiplication inhibition rate ranged from 41.4% to 60.9% and no dose-effect association was observed (Table 2). The highest parasite inhibition rate (60.9%) was observed for the lowest *T. micrantha* extract dose (25 mg/kg); it was close to that observed for the CQ-treated group (66% inhibition). On the 6th dpi (exactly 48 hours after the last dose of treatment) an expressive rate decrease, was observed especially in those mice that received *A. oleracea* and *T. micrantha* extracts. The lowest extract effectiveness was observed in the 6th dpi, in animals treated with *T. micrantha* extract, for which no parasites inhibition was observed (Table 2). Mean parasitemia observed for each extract at the 8th dpi was similar in both treated and non-treated No side effects were observed in any of the herein applied doses (Table 2).

Table 1. *Plasmodium falciparum* (W2) growth inhibition rate (% GI) and 50% cytotoxicity concentration (CC_{50}) of HepG2 cells observed for extracts from plants occurring in Western Pará State, Brazilian Amazonia.

Species	Plant part	% Growth inhibition		Cytotoxicity
		[100.0 μg/mL]	[50.0 μg/mL]	CC_{50} (μg/mL)
Acmella oleracea	Flowers	94.70 ± 5.10^{A}	92.30 ± 7.60^{A}	$>100.00^{C}$
Lippia origanoides	Leaves	69.00 ± 6.20^{BC}	57.00 ± 3.70^{B}	$>100.00^{C}$
Senna quinquangulata	Leaves	66.00 ± 5.00^{BC}	47.00 ± 2.00^{C}	$>100.00^{C}$
Siparuna krukovii	Leaves	91.60 ± 8.50^{A}	73.00 ± 3.60^{AB}	$>100.00^{C}$
Trema micrantha	Leaves	95.30 ± 3.50^{A}	82.00 ± 8.80^{A}	$>100.00^{C}$
Abuta sp	Leaves	91.30 ± 7.80^{A}	89.30 ± 8.40^{A}	31.90 ± 2.70^{A}
Ampelozizyphus sp	Bark	26.00 ± 6.20^{D}	17.30 ± 5.80^{E}	$>100.00^{C}$
Aniba sp	Bark	62.70 ± 6.00^{C}	46.30 ± 1.50^{C}	57.50 ± 1.80^{B}
Aspidosperma sp	Bark	71.00 ± 2.70^{B}	56.70 ± 2.30^{B}	$>100.00^{C}$
Croton sp	Bark	88.70 ± 3.20^{A}	84.70 ± 2.50^{A}	$>100.00^{C}$
Virola sp	Bark	53.00 ± 3.60^{C}	35.70 ± 6.80^{D}	57.60 ± 2.50^{B}
*Chloroquine diphosphate	–	100.00 ± 0.00*	100.00 ± 0.00*	32.10 ± 1.70^{A}

Note: Means (± SD, n = 3) followed by different letters were significantly different within columns (Tukey test's, $p = 0.05$). Statistical analyses of parasitemia reduction at 100.0 and 50.0 μg/mL were performed separately for each concentration analyzed. *Statistical analysis of the antiplasmodial activity of the chloroquine control was performed separately for each sample and concentration, and significant to $p < 0.05$.

Table 2. *In vivo* antimalarial activity of *Acmella oleracea*, *Siparuna krukovii* and *Trema micrantha* extracts against *Plasmodium berghei* (NK65 strain) infected mice.

Ethanol extract	Dose (mg/kg)	Parasitemia on 4th dpi (Mean ± SD)	Parasitemia on 6th dpi (Mean ± SD)	Parasitemia on 8th dpi (Mean ± SD)	Inhibition of parasite multiplication (%) on 4th dpi	Inhibition of parasite multiplication (%) on 6th dpi	Inhibition of parasite multiplication (%) on 8th dpi	*n*/group on 4th dpi	*n*/group on 6th dpi	*n*/group on 8th dpi
Acmella oleracea flowers	25	0.75 ± 0.10^{AB}	5.40 ± 0.55	6.70 ± 0.29	41.41	0.74	0	5/5	5/5	5/5
	50	0.72 ± 0.21^{AB}	5.00 ± 0.50	6.20 ± 0.98	43.75	8.09	0	5/5	5/5	5/5
	100	0.66 ± 0.09^{AB}	5.14 ± 1.10	6.52 ± 0.76	48.44	5.51	0	5/5	5/5	5/5
Siparuna krukovii leaves	25	0.60 ± 0.25^{AB}	4.00 ± 0.56^{A}	7.50 ± 0.69	49.15	25.95	0	5/5	5/5	5/5
	50	0.50 ± 0.11^{AB}	3.90 ± 0.44^{A}	7.70 ± 0.70	57.63	27.77	0	5/5	5/5	5/5
	100	0.60 ± 0.35^{AB}	4.10 ± 0.46^{A}	7.78 ± 0.56	49.15	24.07	0	5/5	5/5	5/5
Trema micrantha leaves	25	0.50 ± 0.09^{AB}	5.80 ± 1.22	7.90 ± 0.64	60.90	0	0	5/5	5/5	5/5
	50	0.60 ± 0.15^{AB}	6.30 ± 0.42	7.90 ± 1.52	53.13	0	0	5/5	5/5	5/5
	100	0.75 ± 0.26^{AB}	5.45 ± 0.76	8.50 ± 0.86	41.40	0	0	5/5	5/5	5/5
CQ	10	0.48 ± 0.16^{A}	0.70 ± 0.10^{A}	0.90 ± 0.16^{A}	66 %	85 %	83 %	5/5	5/5	5/5
Vehicle	mL	1.28 ± 0.16	5.44 ± 0.36	6.00 ± 0.23	–	–	–	5/5	5/5	5/5

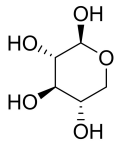
Note: CQ = Chloroquine; Vehicle = treated water; dpi = day post infection; a = p value < 0.05 (compared to the Vehicle group); b = p value > 0.05 (compared to the CQ-treated group). ANOVA + Post Tukey Test.

Phytochemical Analysis

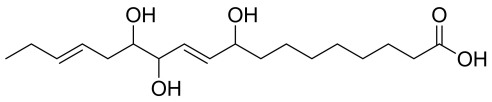
TIC's (Total Ion Chromatogram) major constituent peaks detected in HPLC-ESI-HRMS^2 acquisitions were putatively identified in ethanol extracts from *Acmella oleracea, Siparuna krukovii* and *Trema micrantha* (Table S1). LC–MS characterization is described in the literature for most putatively identified compounds.[22,23] Nine compounds belonging to different structural classes, such as alkamides, sugar, phenolic acids and fatty acids, were putatively identified in the ethanol extract from *A. oleracea* flowers, in comparison to data available in the literature (Figure 1). The alkylamide known as Spilanthol (Figure 1.8) is the major constituent of *A. oleracea* flowers. To the best of our knowledge, a new hydroxy-alkamide is herein described for the first time and its proposed fragmentations are shown in Figure 2.

Benzyltetrahydroisoquinolines, protoberberines and aporphines are often found in plants belonging to family Siparunaceae. Fragment ions detected in positive LC-HRMS^2 support the proposed structures of reticuline, coclaurine, isocorypalmine and anonaine (Figure 3) putatively identified as the major compounds in *S. krukovii* extract. This finding corroborates reports on LC–MS analyses applied to the aforementioned species,[24,25] as shown in Table S1.

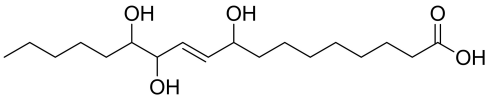
On the other hand, the supposed identification of compounds in ethanol extract from *T. micrantha* leaves was inferred based on LC-ESI-HRMS^2 data. Losses of neutral 132 Da and 150 Da fragments corresponding to ions' fragmentation at *m/z* 449.1080 $[M+H-132]^+$ and *m/z* 431.0974 $[M+H-132-18]^+$, respectively, were indicative of pentose residue in the chemical structure of this compound.[26] In addition, the association between these data and the aforementioned fragment ions, as well as lack of neutral loss of 60 Da ($[M+H-60\ Da]^+$ fragment) in the HRMS^2 spectrum, which is often generated by *C*-pentose derivatives' fragmentation, have suggested that this sugar may be an *O*-pentoside, likely one of the stereoisomers arabinoside or xyloside. Furthermore, peaks observed at *m/z* 329.0655 $[M+H-132-120]^+$, at the base peak, and the one observed at *m/z* 359.0762 $[M+H-132-90]^+$ were indicative of *C*-glycosyl residue linked to flavone genin, as well as consistent with likely regioisomers, such as orientin or isoorientin. Thus, the putative compound might be *(iso)*orientin-2″-*O*-(arabinoside or xyloside) (Figure 4.1). Based on similar analyses applied to the main peaks observed at *m/z* 433.1128 $[M+H-132]^+$ and to the base peak observed at *m/z* 313.0704 $[M+H-132-120]^+$, the second major peak detected in the extract could be attributed to *(iso)*vitexin-2″-*O*-(arabinoside or xyloside) (Figure 4.2). Negative fragment ions observed for both flavone heterosides have confirmed the structural proposals deriving from LC-HRMS^2 analyses under positive conditions (Table S1). LC–MS data about the supposed flavone heterosides were not found in the literature; thus, their likely structures were inferred based on HRMS^2 analyses, whose results have shown agreement with fragmentations observed for *(iso)*vitexin and *(iso)*orientin compounds.[27] Vitexin was isolated from ethanol extract deriving from *Trema micrantha* leaves;[28] therefore, (*iso*)vitexin-2″-O-(arabinoside or xyloside) putative identification was consistent with the reported obser-



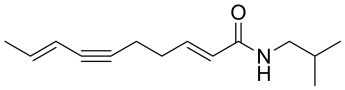
1 .1 Xylose
(*m/z* 149.0445 [M - H]$^-$)



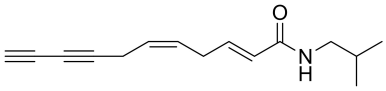
1.5 9,12,13-trihydroxyoctadeca-10,15-dienoic acid
(*m/z* 327.2177 [M - H]$^-$)



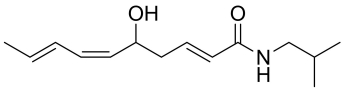
1.6 9,12,13-trihydroxyoctadeca-10-enoic acid
(*m/z* 329.2334 [M - H]$^-$)



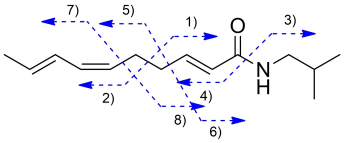
1.2 (2*E*,8*E*)-*N*-isobutyldeca-2,8-dien-6-ynamide
(*m/z* 220.1699 [M + H]$^+$)



1.7 (2*E*,5*Z*)-*N*-isobutylundeca-2,5-dien-8,10-diynamide
(*m/z* 230.1541 [M + H]$^+$)

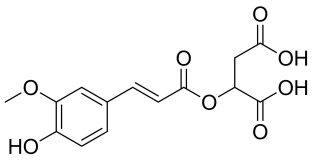


1.3 Hydroxy-spilanthol - (2*E*,6*Z*,8*E*)-5-hydroxy-*N*-isobutyldeca-2,6,8-trienamide
(*m/z* 238.1001 [M + H]$^+$)

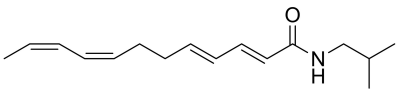


1.8 Spilanthol - (2*E*,6*Z*,8*E*)-*N*-isobutyldeca-2,6,8-trienamide (*m/z* 222.1855 [M + H]$^+$)

1) *m/z* 141.1120 [M + H]$^+$
2) Neutral loss of 81 Da
3) Neutral loss of 72 Da
4) *m/z* 149.0963 [M + H]$^+$
5) Neutral loss of 95 Da
6) *m/z* 126.0917 [M + H]$^+$
7) Neutral loss of 67 Da
8) *m/z* 154.1228 [M + H]$^+$



1.4 2-*O*-Feruloyl-malic acid
(*m/z* 309.0618 [M - H]$^-$)



1.9 (2*E*,4*E*,8*Z*,10*Z*)-*N*-isobutyldodeca-2,4,8,10-tetraenamide (*m/z* 270.1853 [M + Na]$^+$)

Figure 1. Chemical structures of compounds putatively identified by HPLC-ESI-HRMS^2 in flowers ethanol extracts from *Acmella oleracea* occurring in Pará State, Brazilian Amazonia.

vation for both vitexin and its derivatives as phytochemical markers in plant species belonging to the genus *Trema*.

Discussion

The emergence and spread of multidrug-resistant malaria parasite strains to artemisinin and ACTs[29] have raised concerns about the urgent need of finding new antimalarial drugs, as well as the rational and sustainable development of phytomedicines. The rich Amazonian biodiversity is a potential source of new drugs to be explored. Indeed, traditional and folk medicinal plants used in this biome for malaria treatment should be intensively investigated. Basic requirements that must be attended for preclinical studies for antimalarial phytomedicines activity and cytotoxicity screening, as well as active compounds' identification and quantification, standardization of extracts to be used and pre-clinical studies to develop efficient and safe phytomedicines.[30,31]

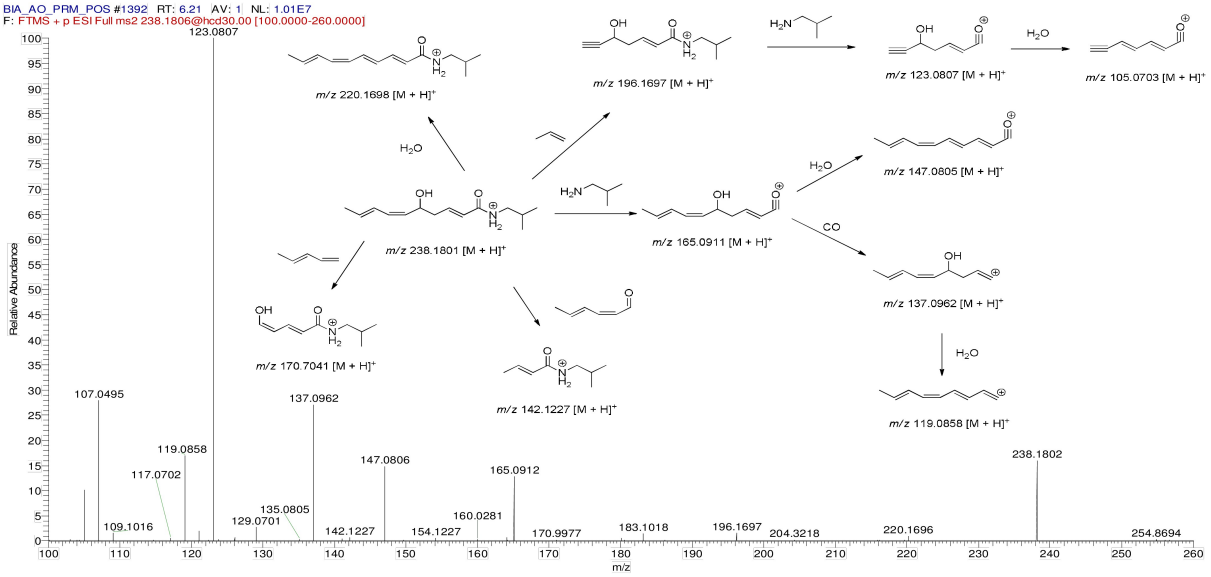
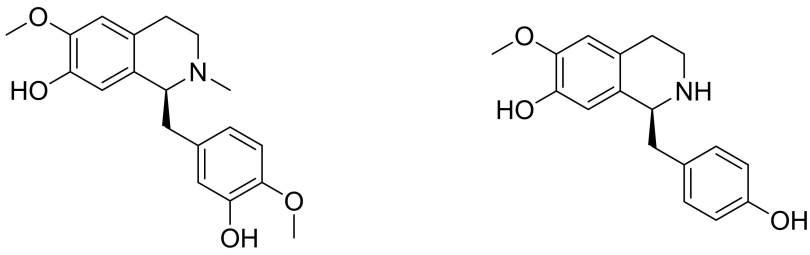
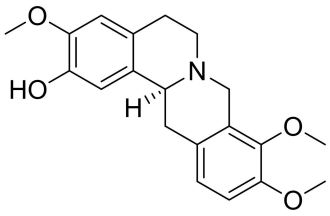


Figure 2. Proposed HRMS^2 fragmentations for hydroxy-spilanthol ((2*E*,6*Z*,8*E*)-5-hydroxy-*N*-isobutyldeca-2,6,8-trienamide), molecular ion at *m/z* 238.1801 [M + H]$^+$, a supposed new alkylamide.

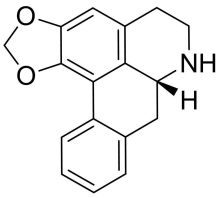


3.1 Reticuline
(*m/z* 330.1696 [M + H]$^+$)

3.3 Coclaurine
(*m/z* 286.1437 [M + H]$^+$)



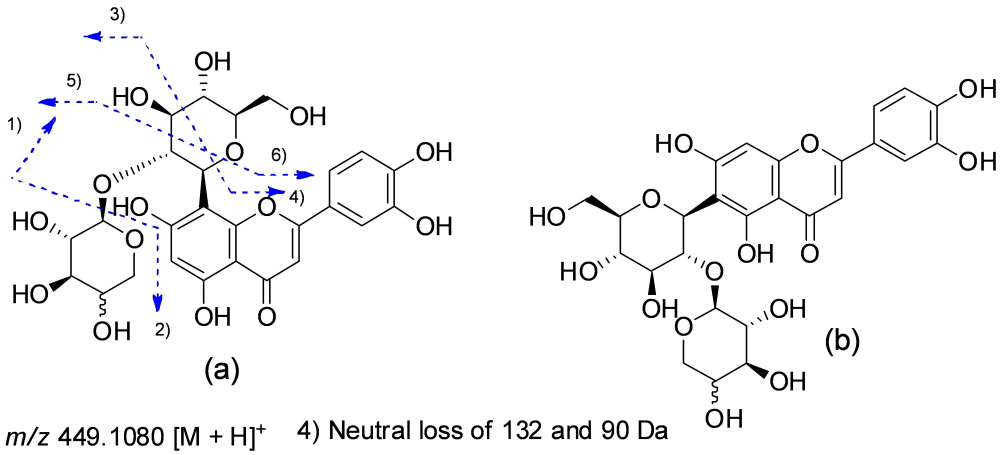
3.2 Isocorypalmine
(*m/z* 342.1702 [M + H]$^+$)



3.4 Anonaine
(*m/z* 266.1180 [M + H]$^+$)

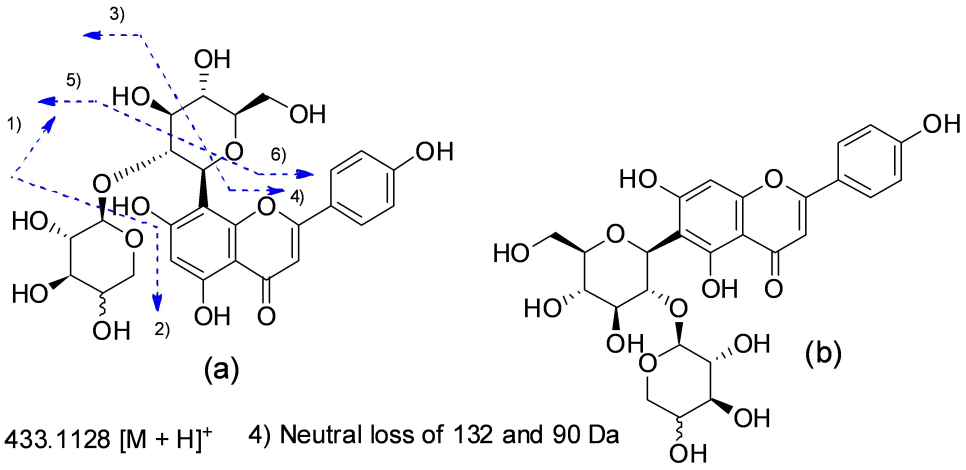
Figure 3. Chemical structures of alkaloids putatively identified through HPLC-ESI-HRMS^2 in leaves ethanol extracts from *Siparuna kurkovii* occurring in Pará State, Brazilian Amazonia.

LC–MS analysis has evidenced the simple phytochemical profile of *A. oleracea* ethanol extract, which presented spilanthol as its major metabolite; therefore, it can be considered a phytochemical marker of this species and this feature can help developing a new phytomedicine. Furthermore, the herein proposed identification of major metabolites based on HRMS^2



1) *m/z* 449.1080 $[M + H]^+$
2) Neutral loss of 132 Da
3) *m/z* 359.0762 $[M + H]^+$
4) Neutral loss of 132 and 90 Da
5) *m/z* 329.0560 $[M + H]^+$
6) Neutral loss of 132 and 120 Da

4.1 Orientin-2"-*O*-(arabinoside or xyloside) (a) or isoorientin-2"-*O*-(arabinoside or xyloside) (b) (*m/z* 581.1497 $[M + H]^+$ and *m/z* 579.1354 $[M - H]^-$)



1) *m/z* 433.1128 $[M + H]^+$
2) Neutral loss of 132 Da
3) *m/z* 343.0810 $[M + H]^+$
4) Neutral loss of 132 and 90 Da
5) *m/z* 313.0704 $[M + H]^+$
6) Neutral loss of 132 and 120 Da

4.2 Vitexin-2"-*O*-(arabinoside or xyloside) (a) or isovitexin-2"-*O*-(arabinoside or xyloside) (b) (*m/z* 565.1550 $[M + H]^+$ and *m/z* 563.1004 $[M - H]^-$)

Figure 4. Chemical structures of flavonoids putatively identified through HPLC-ESI-HRMS^2 in leaves ethanol extract of *Trema micrantha* occurring in Pará State, Brazilian Amazonia.

data can also guide phytochemical studies focused on isolating antimalarial compounds in bioactive extracts. *A. oleracea* naturally grows in tropical regions near the Equator line, mainly in Africa, Asia and South America, is called jambú in Brazil. The whole plant is used as medicinal remedy, mainly to treat toothache.[32] Pharmacological study has shown that spilanthol presented antimalarial activity against two *P. falciparum* strains (PFB strain originated from Brazil and chloroquine-resistant K1

strain originated from Thailand). Spilanthol has shown good antimalarial activity – its IC_{50} ranged from 5.8 to 41.4 μg/mL.[33] The antimalarial activity herein described for *A. oleracea* flowers may be associated with the presence of spilanthol and undeca-2*E*-ene-8,10-diynoic acid isobutylamide in its ethanol extract given their well-known antimalarial activity against *P. falciparum*.[33,34] Indeed, the current data corroborate a study conducted *in vivo*, which evidenced that water extract deriving from *S. acmella*, at the concentration of 50 mg/kg, reduced parasitemia in mice infected with *Plasmodium yoelii* by up to 53%, at the 5th dpi.[34] It is noteworthy that the *in vivo* antimalarial activity observed of *S. acmella* flowers ethanol extract described here showed, in the 4th dpi, the inhibition of parasite multiplication was of 41.4%, 43.75% and 48.44% in doses of 25, 50 and 100 mg/kg, respectively. This is a good result since, in the *in vivo* assays, a sample is considered active when it promotes a parasitemia reduction $>30\%$.[35] However, it is worth emphasizing that in the aforementioned study the extracts were administered by intraperitoneal route whereas the current study gavage (oral route) was used. However, it is worthy pinpointing that the investigated extracts did not show sterilizing-type antimalarial activity throughout the five-day treatment; besides, its activity has significantly decreased within 48 hours after the end of the treatment. This finding may be associated with the fast excretion of the most active compounds found in these extracts, although this assumption requires further investigation. On the other hand, CQ's long effect on parasite development is linked to its long half-life (20–60 days, on average) in the bloodstream.[36,37] Finally, just as few studies focused on investigating the antiplasmodial activity *in vivo* of *A. oleracea* extracts,[38] few toxicological studies conducted with *A. oleracea* extracts are available in the literature and none of them was carried out with humans. No toxic effects on animal model was observed for hydroethanol extract deriving from *A. oleracea* flowers or for its major compound, i.e., spilanthol. These findings reinforce the safety of long-term *A. oleracea* consumption.[39] Thus, the extensive use of jambú in the traditional cuisine of Northern Brazil can be also a sign of its safety for human consumption purposes.

No citation about the phytochemistry and ethnopharmacology of *Siparuna krukovii* A. C. Sm. (syn. *Siparuna langsdorfii* Tolm.), which mainly grows in the Amazonian rainforest, was found in the literature.[40] The current study is the first to report antimalarial activity by this plant species, which was selected based on taxonomic approach, since several antiplasmodial alkaloids deriving from *Siparuna* species, such as coclaurine and reticuline, were reported in the literature.[24,41,42,43] Complex LC-HRMS phytochemical profile was herein observed for *S. krukovii* extract - such a profile is capable of hindering its standardization process, which is a fundamental step in medicinal plant validation processes.

Trema micrantha (Cannabaceae) is found in the Americas, from Florida (USA) to Southern Brazil. It is the only *Trema* species growing in all Brazilian biomes[44] and its ethnopharmacological use as antimalarial drug is well known in the Brazilian Amazon.[11] To the best of our knowledge,, the current study was the first to report antiplasmodial activity of *T. micrantha* plants growing in Brazil. However, *T. micrantha* leaves have shown significant toxicity associated with both natural and experimental poisoning in goats, sheep and horses, who presented liver necrosis and encephalopathy.[45] Other *Trema* species, such as *Trema orientalis* Linn. Blume (syn. *Celtis orientalis Linn.*), are widely distributed in South Africa, Middle East, India, as well as from Southern China to Southeast Asia and Australia. This species is used in Nigeria as prophylactic agent, as well as to treat malaria, a fact that turns it into one of the most investigated *Trema* species in antimalarial assays conducted *in vitro* and/or *in vivo*.[46,47,48]

Conclusions

The present research showed that *A. oleracea* (Asteraceae), *S. krukovii* (Siparunaceae) and *T. micrantha* (Cannabaceae) were the most active out of the 11 plant extracts investigated for antimalarial activity. HPLC-ESI-HRMS^2 data of the three most bioactive extracts, showed the presence mainly of alkylamides (*A. oleracea*), alkaloids (*S. krukovii*) and flavones (*T. micrantha*). The *in vivo* assays against *P. berghei* infected mice showed that *A. oleracea* (syn. *Spilanthes acmella*) an herbal plant used in traditional medicine in Brazil and in other malarial countries, has the best profile as a potential candidate to the development of an antimalarial phytomedicine. However, the limited knowledge on *A. oleracea* preclinical studies in animals are requirements to confirming a candidate to the development of an antimalarial phytomedicine before translation to clinical trials. The results described here demonstrate that ethanol extract of *A. oleracea* flowers deserve further preclinical investigation as a potential antimalarial phytomedicine.

Experimental Section

Plant material

The 11 plant materials were collected in 2019 in the West Pará State, Brazilian Amazon (Table S2). The research with these plants was registered in Brazil, SisGEN System, under the Code A287942.

Preparation of ethanol extracts

After drying in a circulating air oven at 40 °C, the different plant parts were milled and the powders were submitted to extraction. Thus, powdered part of each plant was submitted to extraction with ethanol (96%) under heating at 50 °C for 30 min (plant:solvent ratio, 1:10 w/v). Subsequently, the extractive solutions were concentrated in a rotary evaporator affording the dry ethanol extracts (Table S2).

HPLC-ESI-HRMS^2 Acquisition

Mass detection of extract metabolites was performed in a system consisting of an Ultimate 3000 HPLC (Thermo Scientific) coupled to a QExactive high-resolution mass spectrometer (Thermo Scientific). An H-ESI (heated electrospray ionization) source in positive and negative modes was used for monitoring the compounds by HRFS

(high resolution full scan) and in the parallel reaction monitoring (PRM) experiments. Phenyl-hexyl C-18 column at 20 °C was used in liquid chromatography separation. The mobile phase consisted of water 0.1 % formic acid (solvent A) and acetonitrile 0.1 % formic acid (solvent B). The elution protocol was 0–18 min with curved gradient (5) from 30 % to 75 % B, isocratic elution with 75 % B in 18–25 min, curved gradient (5) between 25–32 min with curved gradient (5) from 75 % to 100 % B, and kept in 100 % B between 32–35 min. The flow rate was 0.3 mL min^{-1}, and the sample injection volume was 10.0 μL. The mass spectra were generated by heated electrospray ionization (HESI) that were operated in the following conditions: positive and negative ion modes; capillary temperature, 350 °C; vaporizer temperature, 250 °C; sheath gas, 30 psi; and collision energy was set at 35 eV. Analyses were run in the full scan mode (100–100 Da). The data were processed using the Xcalibur® program. The different classes of secondary metabolites were characterized based on the typical fragmentation patterns by sequential MS^2 analyses ($HRMS^2$) and comparison to the literature data.

In vitro Antiplasmodial assay

Chloroquine resistant *P. falciparum* (W2 strain) was grown and synchronized with 5.0 % sorbitol.[49] Evaluation of the *in vitro* antiplasmodial activity was performed by the quantification of the parasitic enzyme lactate dehydrogenase (*Pf*LDH).[50] Ring trophozoite stage cultures (parasitemia of 2 % and hematocrit of 1 %) and two concentrations of the extracts (50.0 and 100.0 μg/mL) were used in the screening assays. For determination of the IC_{50} (Inhibitory Concentration 50 %), 5 different concentrations (6.25, 12.5, 25.0, 50.0 and 100.0 μg/mL) of the ethanol extracts were evaluated to obtain concentration-response curves plotted with sigmoidal fit. In each experiment, wells with uninfected and infected red blood cells (RBC) without drug were included as negative and positive controls of growth, respectively. Chloroquine diphosphate salt was the standard antimalarial drug. After 48h of incubation under CO_2, plates were frozen (twice) to promote cell lysis. The lysate (15 μL) was added to Malstat reagent (100 μL) and NBT(nitroblue tetrazolium)/PES (phenazine ethosulphate) (25.0 μL), followed by incubation (1 h at 37 °C) under light and subsequent reading at 540 nm. Cell viability (%) was calculated as the ratio between non-infected (100 % viable) and infected without treatment (0 % viable) RBCs.

In vitro Cytotoxicity assay

Cell viability was determined by the MTT (3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide) method.[51] HepG2 A16 cells (human hepatocellular carcinoma) (4×10^5 cells/0.1 mL) were grown in RPMI-1640 medium, supplemented with 5 % of fetal calf serum, kept in a 5 % CO_2 atmosphere at 37 °C. The extracts were dissolved in RPMI-1640 and dimethyl sulphoxide (DMSO) (0.02 %, v/v). Chloroquine, the standard drug in the antimalarial assay was also used as reference in the cytotoxicity evaluations. After 24h, the extracts were added at the concentration of 100 μg/mL followed by 24h of further incubation. The MTT (2.0 mg/mL) was added, followed by incubation at 37 °C in an atmosphere of 5 % CO_2 for 4 h. DMSO was added to each well, and the reactions were mixed to solubilize the formazan crystals. The optical density was determined at 570 nm and 630 nm to measure the signal and background, respectively. The cell viability was expressed as a percentage of the control absorbance in the untreated cells after subtracting the appropriate background.

In vivo antimalarial assays

The *in vivo* antimalarial activity was evaluated in C57BL/6 female mice, 6–8 weeks of age, were inoculated with 10^5 *Plasmodium berghei* (NK 65 strain) infected red blood cells (iRBC) through gavage (oral route) and randomly distributed in groups of five mice each. The animals were supplied by the Biology Research Center, Federal University of Juiz de Fora after protocol approval by Ethical Committee for animal use (process # 28/2022). Afterwards the ethanol extracts of *A. oleracea flowers, S. krukovii* leaves and *T. micrantha* leaves, the three most active in the *in vitro* antiplasmodial assays (Table 1), were evaluated by the classical *in vivo* suppressive test.[52] The extracts were diluted in 3 % dimethyl sulfoxide (DMSO)/ultrapure water to the concentration of 120 mg/mL, followed by another dilution in water to obtain the treatment concentration (25, 50 or 100 mg/kg). The extracts were administrated to *P. berghei* (NK 65 strain) infected mice by gavage for five consecutive days (D0 to D4), beginning four hours after experimental infection with *P. berghei.* Two control groups were used: one receiving the standard antimalarial drug chloroquine (10 mg/kg) and the other receiving vehicle (water). Giemsa-stained blood smears were prepared from day 4 to 9 post infection and were then examined by optical microscopy for determination of parasitemia. The inhibition rate of parasite multiplication was determined by comparing treated with untreated groups by the formula $|(A-B)/A|\times100$, where A = control group parasitemia and B = test group parasitemia. The extract was considered active when promoting a parasitemia reduction major or equal to 30 %.[53]

Statistical analyses

All data were expressed as means ± standard deviations of triplicate measurements. *In vitro* antiplasmodial activity and cytotoxicity, and *in vivo* antimalarial effects of the extracts were submitted to ANOVA and the Tukey test. All statistical analyses were performed using the software SigmaPlot 12.5[54] ($p = 0.05$).

Abbreviations

ANOVA	(Analysis of Variance)
CC_{50}	(Cytotoxicity Concentration 50 %)
Da	(Dalton)
Dpi	(day post infection)
HPLC-HRMS	(High-Performance Liquid Chromatography High-Resolution Mass Spectrometry by Electron Spray Ionization)
IC_{50}	(Inhibitory Concentration 50 %)
Ip	(intraperitoneal route)
iRBC	(infected Red Blood Cells)
MTT	(3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide)
NBT	(NitroNlue Tetrazolium)
PES	(Phenazine EthoSulphate)
*Pf*LDH	(*Plasmodium falciparum* Lactate dehydrogenase)
% GI	(percentage of parasite growth inhibition)
RT	(Retention Time).

Author Contributions

MBVS was responsible for the phytochemical work. MFAN and RCP performed the *in vitro* antiplasmodial and cytotoxicity assays. JCBB and FOR performed the *in vivo* antimalarial, under the supervision of Prof Dr. KKGS. DCG and ABO performed the HPLC-ESI-MS data analyses and wrote the manuscript. KKGS, ABO, and RHVM proposed and supervised this study.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: *Acmella oleracea* · LC-HRMS^2 · malaria · *Siparuna krukovii* · *Trema micrantha*

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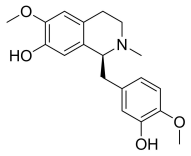
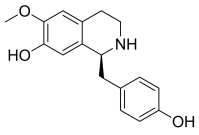
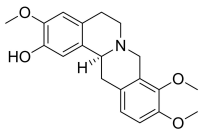


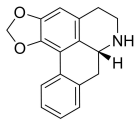
3.1 Reticuline
(*m/z* 330.1696 $[M + H]^+$)



3.3 Coclaurine
(*m/z* 286.1437 $[M + H]^+$)



3.2 Isocorypalmine
(*m/z* 342.1702 $[M + H]^+$)



3.4 Anonaine
(*m/z* 266.1180 $[M + H]^+$)

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