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Bioprospection for new larvicides against *Aedes aegypti* based on ethnoknowledge from the Amazonian São Sebastião de Marinaú riverside community

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ABSTRACT

Ethnopharmacological relevance: Vector-borne diseases represent a huge global burden impacting health systems. *Aedes aegypti* is the main vector of arboviral diseases including dengue, Zika, chikungunya and urban yellow fever in both tropical and subtropical areas. Ethnopharmacological investigations provide potential avenues for developing new vector control strategies.

Aim of the study: The objective of this study is to document the São Sebastião de Marinaú riverside community's ethnoknowledge of local plants used to control mosquitoes and perform bioguided fractionation to isolate the compounds active against the arboviral disease vector *Ae. aegypti*.

Materials and methods: Semi-structured interviews were conducted with residents of the Marinaú community located in the Caxiuanã National Forest, in the Amazon biome, Pará, Brazil. The plants used to control mosquitoes were subjected to phytochemical studies guided by *Ae. aegypti* assays. Extracts were obtained from seven species using distinct organic solvents. Active extracts and fractions were separated by chromatographic techniques. Isolated compounds were characterized by NMR, LC/MS and GC/MS. Sample activity against *Ae. aegypti* larvae and pupae was evaluated after 24, 48 and 72 h exposure. The extracts were also investigated against adult female mosquitoes. The LC_{50} values were determined by diluting each sample to obtain different concentrations in the respective activity range.

Results: The Marinaú community uses more than ten plants as a repellent, most of which are trees native to the region. The primary applications of these plants to protect against insect bites were: burning plants (fumigation), application of body oils and bathing in macerated plants. *Carapa guianensis* is the predominant species used as a repellent. Extracts from *Diospyros guianensis* fruits, *Carapa guianensis* seed shells and *Aspidosperma nitidum* wood demonstrated *Ae. aegypti* larvicidal activity. The *C. guianensis* seed shell extract demonstrated a residual larvicidal effect. Plumbagin, stigmasterol, β-sitosterol, betulinic, ursolic and oleanolic acids, and betulin were identified in the *D. guianensis* extract. The plumbagin, ursolic and oleanolic acids displayed larvicidal activity. Oleanolic, ursolic and betulinic acids, and betulin were considered pupicidal. Aricine, the major alkaloid isolated from *A. nitidum* wood, also presented larvicidal activity.

Conclusions: Ten plant species traditionally used by the Marinaú community to afford protection against mosquitoes were reported. *C. guianensis, D. guianensis* and *A. nitidum* extracts were considered larvicidal against *Ae. aegypti*. Four triterpenes stood out as very active compounds against pupae. Aricine, an indole alkaloid, displayed larvicidal activity. Therefore, traditional knowledge of Amazonian plants combined with bioguided fractionation constitutes a strategy for the development of eco-friendly insecticides to control *Ae. aegypti*, an arbovirus vector.

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1. Introduction

Abbreviations

GC/MS	gas chromatography mass spectrometry
HPLC	high-performance liquid chromatography
LC_{50}	50% lethal concentration
LC_{90}	90% lethal concentration
LC/MS	liquid chromatography-mass spectrometry
NMR	nuclear magnetic resonance
TLC	thin-layer chromatography
UPLC-PDA-MS/MS	ultra-performance liquid chromatography coupled with high resolution mass spectrometry with electrospray ionization and quadrupole time-of-flight

The mosquito *Aedes aegypti* L. (Diptera: Culididae) constitutes a main public health concern as the major transmitter of arboviral diseases such as dengue, Zika, chikungunya and urban yellow fever. The Health Information Platform for the Americas (PLISA) confirmed 2737 cases of Zika and 1,007,900 cases of dengue in 2021 (PLISA, 2021). Furthermore, 2.9 million suspected cases of chikungunya were documented, with 296 confirmed deaths (Higuera and Ramírez, 2019). In 2019, Brazil confirmed 1376 human yellow fever cases, including 483 deaths (PAHO/WHO, 2019). A number of these patients reported health complications including congenital and adverse pregnancy outcomes (Torres et al., 2016), chronic neurological diseases (Alvis-Kakzuk et al., 2018; Levob et al., 2018) and the emergence of co-infections (Mercado-Reyes et al., 2019), including SARS-CoV-2/DENV (Schulte et al., 2021).

The capacity of *Ae. aegypti* to develop resistance to commercially available insecticides led us to research alternatives based on traditional knowledge. Ethnopharmacology constitutes an economical and viable means for the selection of natural products (Gou et al., 2020). Most importantly, not only do these ethnoknowledge-based studies support autochthonous development, they can also yield products which benefit the entire population. Reported examples of plants selected using ethnopharmacological concepts for *Ae. aegypti* control include: Orozco et al., 2005 (Peru); Dos Santos et al. (2012) (Brazil); Cantrell et al. (2016) (Africa); Banumathi et al. (2017) and Anoopkumar et al. (2017) (India); Gou et al. (2020) (China), and Falkowski et al., (2020) (French Guiana).

The Brazilian Amazon region spans nine Brazilian states (Legal Amazon), corresponding to approximately 60% of the national territory (IBGE, 2019). Conservation Units were established in the Legal Amazon to safeguard socio-biodiversity, contain deforestation and maintain climate balance. In addition, these Units are of strategic importance in terms of developing research in partnership with local populations for bioprospecting (ICMBio, 2019).

The Caxiuanã National Forest, officially decreed a conservation unit in 1961, is considered one of the richest and densest of the Eastern Amazon plain. This important Conservation Unit is reported as being home to numerous species: 2400 plants, 134 lichens, 233 fungi, 1803 animals and 55 culicids, including arboviral disease vectors (Confalonieri and Neto, 2012; Lisboa et al., 2013; Fernandes et al., 2020). The riverside populations that have inhabited this forest for generations possess invaluable know-how to protect themselves against insect bites, thus enabling a narrowed search for eco-friendly insecticides. Therefore, this study (i) documents the Marinaú community's knowledge of local plants to protect against mosquitoes, and (ii) utilizes this insight to direct the isolation of compounds with the potential to control *Ae. aegypti*.

2. Materials and methods

2.1. Study area characteristics

Geographic information about the Caxiuanã National Forest is presented in Fig. 1. Located in northern Brazil, in the State of Pará, it is located 328 km from the capital Belém and has mainly fluvial access. More specifically, Caxiuanã is in the northeast of Pará, covering 317,946.37 ha, next to the Xingu and Amazon rivers, and two municipalities known as Melgaço (01°48'21.44"S, 50°43'0"W) and Portel (01°56'9"S, 50°49'15"W) (Fig. 1). In addition, the forest borders the Anapu river, the mouth of which is 11 km wide (ICMBio, 2012) opening to form the Caxiuanã Bay. The regional topography is flat, slightly undulating, with land elevations ranging from 19 to 47 m above sea level. The soil is a deep yellow latossoil of tertiary origin, an oligotrophic acidic sandy clay (Ferreira et al., 2013). According to the Köppen classification, the Caxiuanã climate is an "Am" tropical zone: a monsoon, with annual rainfall ranging from 2500 to 3000 mm, an average temperature of 25.7 ± 0.8 °C, relative humidity near 80% and 62 m above sea level (Alvares et al., 2014; Oliveira et al., 2008). The vegetation is predominantly composed of dense ombrophilous lowland forests or upland forests covering 85% of the area, with alluvial dense ombrophilous lowland (*igapó*) forests comprising the remaining area. There are also enclaves of grassland, scrub vegetation, and patches of secondary vegetation resulting from anthropogenic activity. The area boasts plant species of Fabaceae, Sapotaceae, Chrysobalanaceae, Burseraceae and Lecythidaceae (Fernandes et al., 2020).

The data documents 1600 families living in the Caxiuanã region which are distributed among 14 communities (Lisboa, 2011). In the present study, factors including resident availability, accessibility, and time-frame activities led to the selection of the São Sebastião de Marinaú community (Fig. 1), adjacent to the designated conservation area (10 min by wooden riverboat). This community consists of close to 30 families who have lived in wooden houses located along the Marinaú riverside (Fig. 2) for generations, either in unflooded forest or floodplain areas, in an agricultural economic self-sufficient society. In addition, the community develops extractivist activities. There is no electricity, basic sanitation or assistance from health posts.

2.2. Field study conditions and species collection

Four field surveys were conducted between June 2018 and May 2019 after meetings with the São Sebastião de Marinaú community administrators informing them of the purpose of our work. The surveys documented local information regarding the traditional use of plants. Ethnoknowledge regarding the use of plants was gathered by documenting interviewee responses to a list of standard questions including: Do insects transmit diseases? If so, which ones? Who uses/knows the plants used as insect repellents? What parts of the plant are used? What are the methods of preparation? What insects are the plants used against? Each interviewee gave his/her written consent to cooperate with the present study, which was approved by the Research Ethics Committee (CEP) of the Universidade Federal do Pará (UFPA/ICS n. 3.965.175) and registered with the National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen n. AE259B4). The participants selected consisted of at least one representative aged ≥18 years, from each of the 30 households in the study area, male/female and head of the family. The information documented included: sociocultural profile, mosquito control strategies, repellent plant species, plant parts used, together with mode of preparation.

The vascular plants indicated were collected with the study participants. The identification of all specimens was confirmed by the parataxonomists: Luis C. B. Lobato and Carlos A. S. da Silva. Voucher specimens were deposited in the Herbarium and Ethnobotanical Collection of the Museu Paraense Emílio Goeldi (Herbário MG). Species were classified according to the Angiosperm Phylogeny Group - APG IV

2016 with scientific names/geographical origin checked using http://www.tropicos.org and the List of Brazilian flora species databases (http://www.floradobrasil.jbrj.gov.br/reflora). All species names were cross-checked using The Plant List (www.theplantlist.org).

2.3. Plant material and extracts

Plant parts of: *Rolandra fruticosa* (L.) Kuntze; *Homalolepis cedron* (Planch.) Devecchi and Piran; *Aspidosperma nitidum* Benth. ex Müll.Arg.; *Geissospermum argenteum* Woodson; *Carapa guianensis* Aubl.; *Diospyros guianensis* L. (Aubl.) Gürke and *Annona exsucca* DC (Fig. 3). were extracted using different polarity solvents (Table 1). The resulting crude extracts were assayed against *Aedes aegypti* larvae and pupae (section 2.4).

For dried plant materials of >500 g, 20 g portions were extracted using hexane, ethyl acetate or 70% hydroethanolic solvent. For dried plant materials of less than 500 g, the entire quantity was extracted with hexane:ethyl acetate:dichloromethane (45:45:10). A total of 29 extracts were prepared by 3 successive 60-min solvent exposures in an ultrasonic bath, with the solvent renewed for each extraction. Extractive solutions were concentrated using a rotary evaporator (Buchi, Flawil, Switzerland) and each respective yield determined (Table 1).

The species *Hymenaea courbaril, Montrichardia linifera* and *Piper klotzschianum* were also collected in the São Sebastião de Marinaú region, however, the small quantities available were only sufficient for voucher specimen deposits in the Herbarium.

2.4. Biological assays

The *Aedes aegypti* colony (Rockefeller strain) was maintained at 28 ± 2 °C, 70 ± 10% relative humidity and a 12-h light/dark cycle in the Laboratório de Farmacognosia Insectarium at the Universidade de Brasília without exposure to any insecticide. Adult insects were fed with 10% sugar solution-soaked filter paper (Whatman, Canterbury, UK), changed twice a week. An equine blood meal (Hospital Veterinário of the Universidade de Brasília) was given 3 times a week by an artificial feeder to allow egg production. The blood was added on the surface of an undulated metal plate covered with parafilm in the upper part of the cages and heated to body temperature. Egg hatching occurred in shallow tap water and fish food was added. *Aedes aegypti* third-instar larvae (L3)

Fig. 2. A typical residence situated along the São Sebastião de Marinaú riverside, Amazonia, viewed from a wooden riverboat.

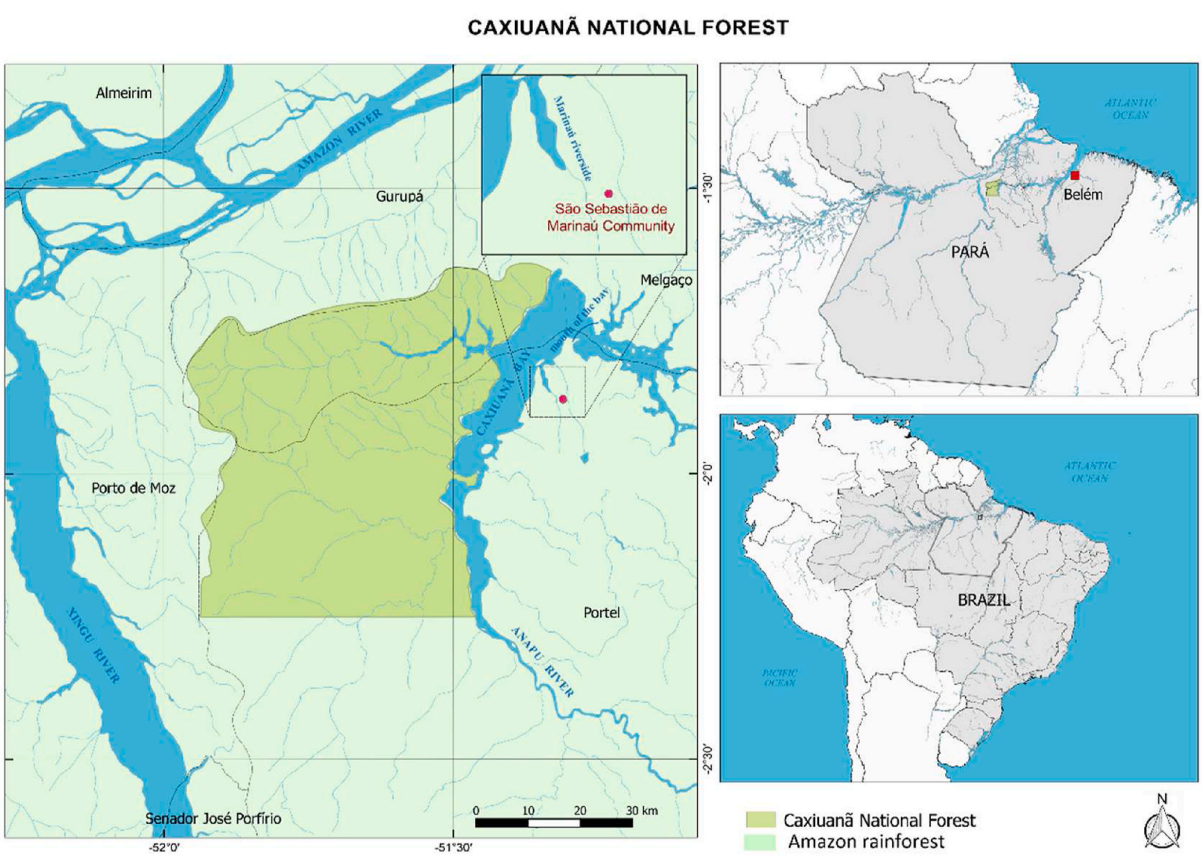

Fig. 1. Geographic localization of the São Sebastião de Marinaú Community in relation to the Caxiuanã National Forest (01°42′30″S and 51°31′45″W), State of Pará, northern Brazil where the ethnopharmacological knowledge and associated samples were collected.

Fig. 3. Plant parts collected with the São Sebastião de Marinaú study participants from the Marinaú Forest, Amazonia: a, b, c) *Annona exsucca* stem bark; d, e, f) *Aspidosperma nitidum* stem wood, stem bark, leaves and rachis; g, h, i) *Carapa guianensis* stem wood and seed shells (discarded during *andiroba* oil extraction); j, l, m) *Diospyros guianensis* fruits.

Table 1
List of extracts obtained from plants used to protect the São Sebastião de Marinaú community against mosquitoes.

Plant	Coordinates	Plant part	Solvent	Yield (%)
Rolandra fruticosa	1°52′7″S	aerial parts	hed	2.19
	51°18′33″W	root	hed	1.82
Homalolepis cedron	1°52′10″S	stem	hex	0.08
	51°18′37′W	stem	ea	0.14
		stem	he	2.44
		stem bark	hex	0.56
		stem bark	ea	0.67
		stem bark	he	1.52
		leaves	hed	2.68
Aspidosperma nitidum	1°52′9″S	stem bark	hex	0.06
	51°18′34″W	stem bark	ea	0.15
		stem bark	he	0.15
		rachis	hed	1.22
		leaves	hed	6.95
		wood	hed	0.42
Geissospermum argenteum	1°52′4″S	stem bark	hex	0.20
	51°18′31″W	stem bark	ea	0.42
		stem bark	he	26.7
		wood	hex	0.19
		wood	ea	0.89
		wood	he	0.82
		leaves	hed	0.18
		rachis	hed	3.30
Carapa guianensis	1°52′8″S	stem wood	hed	0.30
	51°18′35″W	seed shells	hed	13.0
Diospyros guianensis	1°52′7″S	Fruits	hed	3.10
	51°18′33″W			
Annona exsucca	1°52′12″S	wood	hex	0.13
	51°18′28″W	wood	ea	0.47
		wood	he	0.32

hed - hexane:ethyl acetate:dichloromethane (45:45:10); hex - hexane; ea - ethyl acetate; he - 70% hydroethanolic solvent.

aged 72–96 h and pupae older than 168 h were collected from the mosquito colony, without any insecticide exposure.

Extracts, fractions and compounds that caused ≥80% of larval and/or pupal mortality in 250, 100 and 25 μg/mL, respectively, were considered active. Plant extracts with moderate activity, 40–79% mortality over 72 h (larvae) and 48 h (pupae) (Silva et al., 2020; Melo et al., 2021), were also evaluated because of their traditional use by the local population. The samples were dissolved in dimethylsulfoxide (DMSO). For each bioassay, performed in quadruplicate, 10 larvae or 10 pupae were transferred to 12-well plates containing a final volume of 3 mL tap water and the sample at the test concentration. Mortality percentages were recorded after 24, 48 and 72 h exposure. LC_{50} values determined for samples which caused ≥80% mortality. Temephos (0.045 μg/mL) and DMSO in tap water (1.66%, in 12-well plate) were used as positive (larvae only) and negative controls, respectively. Acetone was used in place of DMSO for insoluble samples, with acetone in tap water (1.66%) as the negative control. The LC_{50} values were determined by diluting each sample to obtain different concentrations in the respective activity range.

LC_{50} values from the *Carapa guianensis* hexane:ethyl acetate:dichloromethane seed shell extract were also determined utilizing 200 mL plastic cups of water (approximately 5 cm height) containing 25 L3 larvae per cup (WHO, 2005). This sample was dissolved in DMSO (0.83%, in cup) and the final concentration range was 250, 200, 150, 100, 75, 50 and 25 μg/mL. The final volume of each cup was 120 mL. Each sample concentration and negative control was performed in quadruplicate, using a total of 700 larvae. Temephos (0.045 μg/mL) was used as the positive control.

As the *C. guianensis* is widely available and its extract was the lowest concentration (150 μg/mL) to cause 100% mortality after 72 h of exposure, it was selected for residual activity investigation. The cups containing a final volume of 120 mL tap water received an additional 25 larvae and the mortality was monitored for a further 72 h. This procedure was repeated until the mortality effect was <20% (WHO, 2005).

The adulticidal tests were conducted with extracts only and performed using the bottle assay protocol (Centers for Disease Control and Prevention, 2019). The assays were realized with a total of 80 mosquitoes per sample (10 mosquitoes/250 mL bottle) (Wheaton Science, Millville, NJ, USA). The extracts were solubilized in acetone and tested at 500 μg/bottle. Mosquito knockdown was observed at time 0 and at 15-min intervals up to 120 min. The negative control bottles used were internally coated with 1 mL of acetone. All bottles were dried overnight, partially-capped to allow acetone evaporation, at room temperature in a horizontal position. The assays were carried out with the bottles in a vertical position. Malathion (50 μg/bottle) was used as the positive control.

The average larvae mortality data were analyzed by nonlinear regression with four parameters in the GraphPad Prism 8 software to calculate the LC_{50}, LC_{90} and 95% fiducial limits of lower and upper confidence.

2.5. Instruments and materials

Initial analyses were performed using thin-layer chromatography (TLC, Merck 60 GF_{254}, 0.25 mm) visualized at UV 254/366 nm and with phosphomolybdic acid. Glass chromatography columns with silica gel 60 (0.062–0.200 mm, Merck) were utilized for separation procedures. Thermally stable samples were analyzed by a gas chromatography mass spectrometer (Shimadzu GCMS-QP2010 Plus). Chemical structures and elucidation analyses were performed by: (i) nuclear magnetic resonance operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C (NMR Avance III HD 600, Bruker, Rheinstetten, Germany); and (ii) ultra-performance liquid chromatography coupled with high resolution mass spectrometry with electrospray ionization and quadrupole time-of-flight (UPLC-PDA-MS/MS, Bruker Daltonics, Bremen, Germany). For the NMR experiments the samples were dissolved in chloroform-*d* or methanol-*d4* according to the polarity of each purified compound. Tetramethylsilane (TMS) was used as the internal reference standard. The samples identified as mixtures of (i) betulinic acid/betulin and (ii) ursolic/oleanolic acids were acquired as highly pure (>90%) commercial standards (Sigma Aldrich, Buchs, Switzerland).

2.6. Compound separation

The *C. guianensis* hexane:ethyl acetate:dichloromethane seed shell extract (40 g) (Fig. 3i - discarded during *andiroba* oil extraction) was submitted to glass column chromatography (85/6 cm, 380 g silica gel) using hexane-ethyl acetate (9:1) as the mobile phase, with increasing polarity, finalized by 100% methanol. A total of 88 fractions (500 mL each) were analyzed by TLC. Fractions were combined and dried at room temperature: (i) fractions 24–40 (25 g), a white solid mixture of **1**, **2** and **3** and (ii) fractions 63–82 (1.8 g), further separated chromatographically in a similar manner resulting in: 25 mg crystalized compound (**4**) and a white solid (**5**).

The *Diospyros guianensis* hexane:ethyl acetate:dichloromethane fruits extract (2.5 g) (Fig. 3m) was also fractionated by glass column chromatography (68/3.5 cm, 65 g silica gel) with a linear gradient mobile phase composed of hexane-ethyl acetate (9.8:0.2), followed by ethyl acetate and ethyl acetate-methanol (1:1). A total of 90 fractions (250 mL each) were analyzed by TLC. Fractions were grouped as follows: 7–8 (16 mg, **DG1b**), 13–24 (22 mg, **DG1d**) and 61–64 (40 mg, **DG1s**). Fraction **DG1b** was purified by high-performance liquid chromatography (HPLC, Luna Phenomenex C_{18}(2) column - 150 × 21.2 mm, 5 μm particle size), mobile phase containing 0.5% formic acid in both channels: A (water) and B (methanol), with a linear gradient starting at 90% water over 29 min. The injection volume was 100 μL and the flow rate was 1.5 mL/min. After HPLC separation, a 3.5 mg yellow solid (**6**) was obtained. Fraction **DG1d** was submitted to solid-phase extraction (SPE 2.7 g silica gel cartridge), eluted sequentially with 50 mL hexane and 50 mL hexane-ethyl acetate (8.5:1.5), yielding 5 mg (a mixture of **7** and **8**). Fraction **DG1s** was rechromatographed resulting in 10 mg (a mixture of **9** and **10**) and 12 mg (a mixture of **11** and **12**) samples.

The *Aspidosperma nitidum* hexane:ethyl acetate:dichloromethane wood extract (2.7 g) (Fig. 3e) was dissolved in 250 mL 90% hydromethanol and submitted to sequential liquid-liquid partitioning with 250 mL (x 3): hexane, dichloromethane and ethyl acetate, yielding 80 mg, 1300 mg and 13 mg, respectively, together with 58 mg in the hydromethanol fraction. The dichloromethane fraction was chromatographed in a similar manner as described previously for the *Diospyros guianensis* extract, resulting in the isolation of a 5 mg compound (**13**).

3. Results and discussion

3.1. Field data collection

During the field studies, 30 São Sebastião de Marinaú community residents were interviewed, 60% of which were women aged between 21 and 68 years. Half of the participants had attended the local elementary school. The participants reported that the community was established in 1979 and the main economic resources are cassava and flour production. For routine subsistence, the men practice extractivist activities including fishing, hunting and plant material extraction. The women also fish and are occupied with the housework.

Around 30% of the interviewees were knowledgeable about leishmaniasis, malaria and dengue, and 40% associated mosquitoes with diseases. The community recognized that some insect bites cause dengue, malaria or leishmaniasis. The local concepts are that: leishmaniasis is caused by the bite of a red mosquito (*pium*) or another mosquito locally named *maruim*; malaria and yellow fever by the bite of a small mosquito (*black carapanã*) that appears especially during intense deforestation, while dengue is associated with the bite of another common mosquito (*carapanã*) that lives in stagnant water or drinking water contaminated with these insects. All of the interviewees referred to these insects using the local terminology only. Furthermore, there was no expressive knowledge about habits or morphological characteristics that clearly distinguished these vectors from other mosquitoes. The aforementioned local insect names have an indigenous origin. In the Tupi language, *Carapanã* means blood-sucking mosquitoes, *Pium* refers to mosquitoes imperceptible to the naked eye with irritable bites, while *Marium* are named mangrove or biting powder mosquitoes that measure 1–4 mm.

The Amazonian people also use the expression *carapanã infected*. Studies in the Brazilian, Colombian and Peruvian Amazon regions showed that the local inhabitants possess different knowledge of vector-borne disease morphology than the WHO (Pineda and Agudelo, 2005; Odonne et al., 2011; Veiga, 2011), possibly due to the high diversity of these insects in forest areas. In addition, these relatively isolated populations living far from urban centers or in vulnerable areas frequently receive limited information regarding the health care provided by government programs. This lack of awareness about the prevention, diagnosis and primary care of diseases contributes to the persistent association of poverty with poor health. Therefore, it is necessary to improve health sector activities, together with education, housing, water and basic sanitation (Chan et al., 2020).

3.2. Plants indicated as mosquito repellents

The ethnoknowledge gathered from the São Sebastião de Marinaú community indicated that ten plant species, belonging to ten genera and nine families, are employed as mosquito repellents, five of which are endemic to the Amazon biome (Table 2), comprising 60% trees and 40% shrubs. In general, these trees are large which accounts for greater consumption of stem bark (50%), among the most accessible organs, while the remaining 50% consisted of leaves, seed/seed oil, fruits, resin and roots. Organoleptic and morphological characteristics explain the organ selection of these species (Medeiros et al., 2015).

The principal modes of use to afford protection against insect bites, suggested by 70% of the interviewees, were: burning plants, spreading oils and maceration baths. These insect repellent practices are common among Amazon populations (Kidane et al., 2013; Ribeiro et al., 2017). The Caxiuanã forest area has mosquitoes year-round, with elevated numbers during periods of increased precipitation. The community burn the listed plants (Table 2) mixed with other flammable firewood in a packaged format tied with vines, which are positioned in rooms or around the house to provide protection. Another instance cited was topical use of these listed species as repellents when venturing into the forest in search of resources.

More specifically, regarding the repellent effects of local species, 15

Table 2
Plants used as insect repellents and/or to treat malaria and fever indicated by the São Sebastião de Marinaú community, Caxiuanã Forest, Amazonia.

Family	Plant Species	Voucher Number[a]	Vernacular Name	Habit	Part Used	Mode of Use	Indication ([c])
Annonaceae	*Annona exsucca* DC.	MG239777	Envira-preta	tree	stem bark	maceration bath	repellent (4) malaria (5)
Apocynaceae	*Geissospermum argenteum* Woodson	MG239444	Taquarirana	tree[b]	stem bark	maceration bath	repellent (7) malaria (14) fever (15)
	Aspidosperma nitidum Benth. ex Müll.Arg.	MG239156	Carapanã	tree[b]	stem bark	maceration bath	repellent (5)
Araceae	*Montrichardia linifera* (Arruda) Schott	MG215476	Aninga	shrub	leaves	fumigation/smoke	repellent (3)
Asteraceae	*Rolandra fruticosa* (L.) Kuntze	MG239800	Carica-á	shrub	roots	tea (decoction) bath (infusion)	repellent (3) malaria (5)
Ebenaceae	*Diospyros guianensis* L. (Aubl.) Gürke	MG239801	Maria preta	shrub[b]	fruits	topical: minced fruits applied as poultice	repel insects from fungal wounds (4)
Fabaceae	*Hymenaea courbaril* L.	MG239333	Jatobá or Breu jutaicíca	tree	stem bark or resin	maceration bath fumigation/smoke	repellent (3)
Meliaceae	*Carapa guianensis* Aubl.	MG239791	Andiroba	tree[b]	seed, seed oil	topical: oil application fumigation/ smoke: burning seed shells	repellent (15)
Piperaceae	*Piper klotzschianum* (Kunth) C. DC.	MG239121	Jambú do mato	shrub	leaves	topical: crushed and applied	repellent (3)
Simaroubaceae	*Homalolepis cedron* (Planch.) Devecchi and Piran	MG777791	Pau-para- tudo	tree	stem bark	maceration bath	repellent (5)

[a] Recorded at the Museu Paraense Emílio Goeldi Herbarium.
[b] endemic species to Amazonia.
[c] number of interviewee citations.

interviewees stated *Carapa guianensis*, 7 referred to *Geissospermum argenteum,* while 5 participants specified *Homalolepis cedron* and *Aspidosperma nitidum*. The other 6 species listed in Table 2 received at least 3 recommendations. The community reported that *Geissospermum argenteum* and *Aspidosperma nitidum* stems have invaginations containing ant houses. For them, this plant-insect interaction gives the tree bark a bitter taste, and when consumed confers a bitter taste to the blood, thus avoiding mosquito bites. Repellency was also attributed to the smell of *Hymenaea courbaril* and *Annona exsucca* resins. The burning of *Montrichardia linifera* leaves, an abundant riverbank species, releases a potent mosquito repellent. The residents also believe that drinking tea or bathing in water prepared using the bitter roots of *Rolandra fruticosa* confers protection against insect bites. A similar bitter-tasting insect repellent effect was cited for bathing in water prepared using *Homalolepis cedron* stem bark. The *Diospyros guianensis* fruit resembles a furuncle, so it is used topically by the community to help heal skin wounds and prevent mosquitoes from contacting the affected area. The study participants reported that rubbing *Piper klotzschianum* on the legs results in numb and fresh sensations, and consequently ticks and insects do not adhere to or pierce the skin. Half of the interviewees specified the use of *Carapa guianensis* (known as *andiroba*) oil, seeds and seed shell residues/oil extraction waste (fumigation) as repellents. Furthermore, andiroba oil is routinely used on a broader scale by Amazonian residents. Indeed, specialists have been recommending this oil as an ingredient in mosquito control formulations (Frausin et al., 2015; Maia and Moore, 2011).

Our findings about the use of these wild plant resources by the São Sebastião de Marinaú community as repellents differ from other field-based ethnopharmacological studies involving exotic and herbaceous species (Gou et al., 2020). The diversity and abundance of these wild species, together with the difficulty in maintaining vegetable gardens in backyards prone to flooding, explains the local practices and forest management expertise. There are numerous reports in the literature of Amazonian inhabitants, especially indigenous and riverside populations, employing endemic tree species either as repellents or for the treatment of malaria and leishmaniasis (Rocha et al., 2013; Frausin et al., 2015; Kffuri et al., 2016). Our study adds to the literature by registering the traditional use of *G. argenteum*, *H. cedron*, *A. nitidum*, *A. exsucca* and *D. guianensis* as mosquito repellent strategies.

3.3. Extract bioassays

A total of 29 extracts obtained from 7 plants traditionally used as insect repellents were investigated for their activity against *Ae. aegypti.* These extracts, from *R. fruticosa, H. cedron, A. nitidum, G. argenteum, C. guianensis, D. guianensis and A. exsucca* (see Table 1), were assayed for larvicidal/pupicidal activity. Extracts (250 μg/mL) were considered highly active with ≥80% mortality or moderately active with 40–79% mortality over 72 h (larvae)/48 h (pupae). The plant extracts with moderate activity were also investigated because of their traditional use by the local population. None of the extracts tested showed activity against the adult mosquito.

LC_{50} values were determined for 4 extracts, prepared from *H. cedron* and *A. exsucca*, which presented a moderate (≥40–79%) level of mortality in the initial screening. The corresponding high LC_{50} values indicated no activity. Three extracts caused ≥80% larvae mortality after 72 h exposure, and the LC_{50} values were determined: (i) *A. nitidum* wood (LC_{50} 137 μg/mL); (ii) *C. guianensis* seed shells (LC_{50} 70 μg/mL), and (iii) *D. guianensis* fruits (LC_{50} 62 μg/mL), all extracted using hexane:ethyl acetate:dichloromethane (Table 3).

Regarding the percentage of mortality determined against pupae, 4 extracts demonstrated moderate activity (40–79% mortality, as specified by the Laboratório de Farmacognosia at the Universidade de Brasília): hexane:ethyl acetate:dichloromethane extracts of aerial parts and roots of *R. fruticosa* and *A. nitidum* rachis and a hydroethanolic extract of *A. exsucca* wood, all with 60% mortality.

Table 3
Aedes aegypti larval/pupal mortality (%, at 250 μg/mL) and lethal concentrations (LC_{50} μg/mL) of extracts from plant species employed by the São Sebastião de Marinaú community to protect themselves against mosquitoes.

Plant species	Plant part (solvent)[a]	Larval mortality 24/48/72 h	LC_{50} larvae 24/48/72 h	Pupal mortality 24/48 h
Rolandra fruticosa	aerial parts (hed)	5/10/10	–	47/60
	root (hed)	5/8/10	–	47/60
Homalolepis cedron	stem (hex)	8/15/15	–	[b]
	stem (ea)	0/5/10	–	[b]
	stem bark (hex)	[b]	–	[b]
	stem bark (ea)	0/0/15	–	[b]
	stem bark (he)	0/8/13	–	[b]
	leaves (hed)	13/70/73	–	[b]
	wood (hed)	45/48/60	–	[b]
Aspidosperma nitidum	stem bark (hex)	[b]	–	[b]
	stem bark (ea)	25/25/25	–	[b]
	stem bark (he)	[b]	–	[b]
	rachis (hed)	8/10/12	–	47/60
	leaves (hed)	5/5/10	–	[b]
	wood (hed)	25/90/100	-/136.5/137	[b]
Geissospermum argenteum	stem bark (hex)	30/30/30	–	[b]
	stem bark (ea)	[b]	–	[b]
	stem bark (he)	0/10/10	–	[b]
	wood (hex)	0/8/8	–	[b]
	wood (ea)	[b]	–	[b]
	wood (he)	0/10/10	–	[b]
	leaves (hed)	0/8/10	–	[b]
	rachis (hed)	0/10/10	–	[b]
Carapa guianensis	stem wood (hed)	10/10/10	–	[b]
	seed shells (hed)	100	135/96/70	[b]
Diospyros guianensis	fruits (hed)	100	141/130/62	[b]
Annona exsucca	wood (hex)	40/40/40	–	[b]
	wood (ea)	15/40/63	–	15/20
	wood (he)	13/13/13	–	30/60

[a] hed - hexane:ethyl acetate:dichloromethane (45:45:10); hex - hexane; ea - ethyl acetate; he - 70% hydroethanolic solvent.
[b] Extract was assayed but was inactive. – LC_{50} value only reported for extracts with mortality ≥80% (according to adopted parameter). DMSO (negative control): larvae mortality <20%. According to the WHO guideline, tests with control mortality >20% were discarded. Temephos: positive control.

The larval toxicity LC_{50} values determined for *A. nitidum, C. guianensis* and *D. guianensis* extracts were consistent with those of other traditionally used plants (Luna et al., 2005; Orozco et al., 2005; Dos Santos et al., 2012; Anoopkumar et al., 2017). The extracts obtained from the aforementioned plants could be promising for the development of formulations to control *Ae. aegypti*, particularly *C. guianensis* (*andiroba*) given its wide availability, widespread application, including ingestion in the Brazilian Amazon region. Furthermore, a study in female Swiss mice supports non-toxic properties of *andiroba* oil as no clinical or behavioral alterations were reported (Milhomem-Paixão et al., 2016). It is probable that the larvae filter-feed during the development stages, so they may retain compounds, possibly increasing their susceptibility (Da Silva Costa et al., 2018). Pupae do not feed and are therefore less susceptible to harmful agents. This characteristic, together with the relatively short pupae stage duration, may help explain the relatively low number of commercially available pupicidal products in comparison to larvicidal formulations (Silverio et al., 2020). It is probable that the *R. fruticosa*, *A. nitidum* and *A. exsucca* extracts partially permeated the pupae chitin exoskeleton resulting in the moderate pupicidal activity.

3.4. Active sample investigation

The *Carapa guianensis* seeds, *Diospyros guianensis* fruits and *Aspidosperma nitidum* wood hexane:ethyl acetate:dichloromethane extracts presented LC_{50} < 250 μg/mL after 72 h exposure. These 3 extracts with activity against *Ae. aegypti* larvae were submitted to bioguided fractionation.

The LC_{50} of the *C. guianensis* hexane:ethyl acetate:dichloromethane seed shell extract were 135 μg/mL (24 h), 96 μg/mL (48 h) and 70 μg/mL (72 h) (Table 3), values similar to those previously obtained for *C. guianensis* oil (LC_{50} 140 μg/mL) also tested against *Ae. aegypti* Rockefeller larvae (Prophiro et al., 2012a). NMR analyses of the initial grouped fractions (24–40) revealed signals compatible with fatty acid derivatives and was therefore submitted to derivatization reactions (Milhomem-Paixão et al., 2016). Subsequent GC/MS analysis allowed the identification of a conventional mixture of 46% palmitic (**1**), 42% oleic (**2**) and 10% stearic (**3**) acids (Fig. S1). Another fraction grouping (63–82) resulted in the isolation of the limonoids 6α-acetoxygedunin (**4**) and 7-deacetoxy-7-oxogedunin (**5**), elucidated by NMR, confirmed by high resolution mass spectrometry and reference to the literature (Silva et al., 2012) (Figs. S2–S9). These isolated compounds were inactive against *Ae. aegypti* larvae and pupae.

The cytotoxic action of *C. guianensis* oil against pathogenic fungi was attributed to a mixture of fatty acids and limonoids (Nascimento et al., 2019). In the present study, as the initial *C. guianensis* seed shell extract caused 100% larval mortality (Table 3), the fatty acid mixture was pooled with the limonoids **4** and **5** (1:1:1, 65 μg/mL) and submitted to the same assays, resulting in: 0% (24 h), 48% (48 h) and 83% (72 h) larval mortality. The mixture was inactive against pupae. The aforementioned larvicidal activity could be due to a combined, if not synergistic, effect of the different constituents. Large quantities of *C. guianensis* seed shell residues are underused/discarded following the artesanal or industrial extraction of *andiroba* oil, an important traditional and commercial product in Amazonia (Mendonça and Ferraz, 2007; Nardi et al., 2016). *Andiroba* seeds therefore constitute a promising alternative large scale eco-friendly larvicide.

The residual activity of the initial *C. guianensis* seed shell extract was investigated against *Ae. aegypti* Rockefeller larvae (Fig. 4), achieving 100% mortality by Day 6 (250 μg/mL), which decreased to 62% on Day 9 and was inactive on Day 10. Interestingly, Prophiro et al. (2012b) reported that the residual effect of a higher concentration of andiroba oil persisted until Day 12 (500 μg/mL) against wild *Ae. aegypti* larvae. The difference between the residual activity reported in these studies could be attributed to the higher concentration employed and/or the different mosquito strains tested.

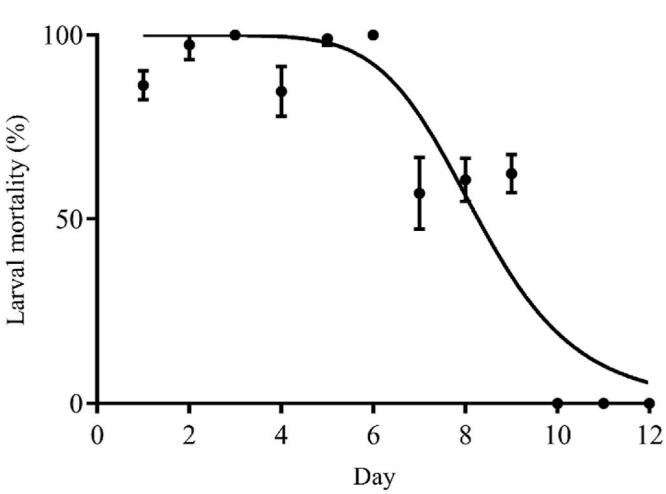

Fig. 4. Residual effect of the *Carapa guianensis* (*andiroba*) hexane:ethyl acetate: dichloromethane seeds extract (250 μg/mL) against *Ae. aegypti* larvae.

The larvicidal LC_{50} values of the *D. guianensis* hexane:ethyl acetate: dichloromethane fruits extract were 141 μg/mL (24 h), 130 μg/mL (48 h) and 62 μg/mL (72 h). This extract was inactive against pupae. Subsequent fractionation resulted in the isolation of plumbagin (**6**), stigmasterol (**7**) and *β*-sitosterol (**8**) (1:1), ursolic (**9**) and oleanolic (**10**) acids (8:2) and betulinic acid (**11**) and betulin (**12**) (9:1), all of which were characterized by NMR, LC/MS or GC/MS and compared to the literature (Sanhueza et al., 2019; Baek et al., 2010; Mahato and Kundu, 1994; Prakash and Prakash, 2012) (Figs. S10–S20, Table S1). The larval LC_{50} values (24 h) were 12.5 μg/mL for plumbagin (**6**) and 49.8 μg/mL for the ursolic (**9**)/oleanolic (**10**) acid mixture, while the other mixtures were inactive. Regarding pupicidal activity, the LC_{50} values (24 h) were 9.2 μg/mL for **9**/**10** mixture and 16.8 μg/mL for the betulinic acid (**11**)/betulin (**12**) mixture, while **6** and the stigmasterol (**7**)/*β*-sitosterol (**8**) mixture were inactive.

D. guianensis fruits are used by the São Sebastião de Marinaú community as a repellent, antifungal, anti-inflammatory and antibacterial agents, while the bark and leaves are employed to treat fever and dermatoses (Tropical Plants Database, 2021). The *Diospyros* genus were reported concerning naphthoquinone that relieves certain skin lesions, together with the terpenes lupeol, betulinol and betulinic acid for larvicidal activity (Rauf et al., 2017).

The aforementioned triterpenes isolated in this mixture (betulin, betulinic acid, ursolic acid and oleanolic acid) from *D. guianensis* fruits were commercially acquired and assayed individually against *Ae. aegypti* larvae and pupae (Table 4). The ursolic and oleanolic acids were individually active against larvae at 24 h, with LC_{50} > 200 μg/mL and LC_{50} 27.7 μg/mL, respectively. Interestingly, ursolic acid was 4 times less active than the ursolic:oleanolic acid mixture (8:2) after 24 h (LC_{50} 49.8 μg/mL), while ursolic acid proved twice as active (LC_{50} 24.6 μg/mL) after 72 h. Therefore, oleanolic acid was considerably more larvicidal and, more significantly, presented the highest pupicidal activity after 24 h (LC_{50} 0.37 μg/mL), followed by ursolic acid (LC_{50} 2.47 μg/mL), betulinic acid (LC_{50} 5.93 μg/mL) and betulin (LC_{50} 25.59 μg/mL) (Table 4). Betulinic acid was almost 3 times more active than the betulinic acid:betulin mixture (9:1, LC_{50} 16.8 μg/mL). It is probable that the presence of stigmasterol and *β*-sitosterol (1:1), could interfere with the activity of the *D. guianensis* fruits extract (LC_{50} 141 μg/mL, 24 h).

There is a lack of commercially available pupicidal agents to control the mosquito vector. Our findings support a number of triterpenes, most notably oleanolic acid, as candidates to address this gap.

The *A. nitidum* hexane:ethyl acetate:dichloromethane wood extract exhibited larvicidal activity (LC_{50} 137 μg/mL, 72 h). After separation, only the dichloromethane fraction was active, with 100% larval mortality when using 100 μg/mL (48 h). Subsequent fractionation resulted in the isolation of a major compound – aricine (**13**) – a known indole alkaloid, elucidated by NMR, LC/MS and reference to the literature (Verpoorte et al., 1983; Flores-Sanchez et al., 2016) (Figure S21- S25). Aricine (Fig. 5) was active against *Ae. aegypti* larvae with: LC_{50} values of 77 (24 h), 45 (48 h) and 17 μg/mL (72 h); and LC_{90} of 87 (24 h), 77 (48 h) and 72 μg/mL (72 h). A literature search did not find any reports of aricine larvicidal activity.

4. Conclusion

We documented ten plant species traditionally used as insect repellents by the São Sebastião de Marinaú riverside community in Brazilian Amazonia, together with their respective modes of preparation and application. Ethnoknowledge was gathered from 30 interviewees representing all the family units in the community, each possessing expertise in local practices and forest management. Our targeted study investigated the activity of plant parts extracted using different solvent polarities against larvae and pupae of the arbovirus vector *Ae. aegypti.*

Of the 10 plants indicated by the Marinaú community, our results add to the literature by registering the traditional use of *G. argenteum*, *H. cedron*, *A. nitidum*, *A. exsucca* and *D. guianensis* to protect against

Table 4
Larvicidal and pupicidal activity of triterpenes identified in the *Diospyros guianensis* hexane:ethyl acetate:dichloromethane fruits extract.

Assay	Time (h)	n[a]	LC_{50} [μg/mL] (95% CI)[b]	LC_{90} [μg/mL] (95% CI)	Slope (SE)[c]	R^2
Larvicidal						
ursolic acid	24	720	>200.0	>200.0	–	–
	48	720	>200.0	>200.0	–	–
	72	720	24.61 (23–27)	71.58 (61–85)	2.05 (0.14)	0.93
oleanolic acid	24	600	27.71 (25–30)	92.54 (51–98)	2.01 (0.15)	0.90
	48	600	20.44 (18–23)	82.31 (61–105)	2.54 (0.13)	0.95
	72	600	20.40 (18–23)	82.30 (61–105)	2.00 (0.14)	0.93
Temephos	24	240	0.023 (0.02–0.03)	0.033 (0.031–0.040)	3.58 (0.47)	0.96
	48	240	0.017 (0.016–0.018)	0.039 (0.032–0.052)	4.84 (0.36)	0.98
	72	240	0.0052 (0.0049–0.006)	0.0094 (0.0082–0.012)	3.24 (0.21)	0.95
Pupicidal						
ursolic acid	24	720	2.47 (2–3)	7.13 (6–9)	2.07 (0.15)	0.93
oleanolic acid	24	1200	0.37 (0.34–0.39)	0.99 (0.85–1.2)	2.24 (0.15)	0.92
betulinic acid	24	960	5.93 (5–8)	60.08 (29–128)	0.94 (0.10)	0.74
betulin	24	600	25.59 (23–29)	63.48 (48–85)	2.41 (0.28)	0.87

[a] Number of larvae/pupae tested.
[b] CI: Confidence interval.
[c] SE standard error. Temephos: positive control. DMSO negative control: larval mortality <20%.

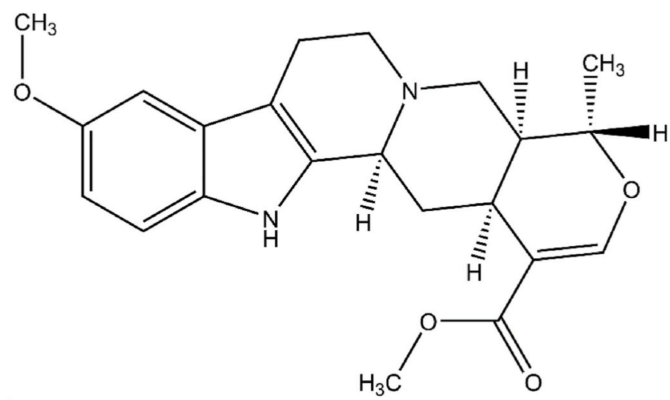

Fig. 5. Aricine (**13**), an *Ae. aegypti* larvicide isolated from the *Aspidosperma nitidum* hexane:ethyl acetate:dichloromethane wood extract.

mosquitoes. Extracts of *C. guianensis*, *D. guianensis* and *A. nitidum* were larvicidal against *Ae. aegypti* after 72 h exposure, while extracts of *R. fruticosa, A. nitidum* and *A. exsucca* presented moderate pupicidal activity. The *C. guianensis* (*andiroba*) hexane:ethyl acetate:dichloromethane seed shell extract is of particular interest due to the significant residual larvicidal effect observed - 100% by Day 6 at 250 μg/mL. Fractionation of this extract led to the identification of a mixture of: palmitic (**1**), oleic (**2**) and stearic (**3**) acids, together with the isolation of the limonoids 6α-acetoxygedunin (**4**) and 7-deacetoxy-7-oxogedunin (**5**), none of which demonstrated toxicity individually. Large-scale upcycling of the aforementioned underused/discarded *andiroba* seed shell residues therefore constitutes a feasible sustainable alternative insecticide. Plumbagin (**6**); a stigmasterol (**7**)/*β*-sitosterol (**8**) mixture; and the triterpenes: (i) an ursolic (9)/oleanolic (**10**) acid mixture, together with (ii) a betulinic acid (**11**)/betulin (**12**) mixture were isolated from the *D. guianensis* fruits extract. Plumbagin was active against larvae (LC_{50} 12.5 μg/mL), the betulin/betulinic acid mixture was active against pupae (LC_{50} 16.8 μg/mL), while the ursolic/oleanolic acid mixture was active against both *Ae. aegypti* forms (LC_{50} 49.8 μg/mL, larvae; LC_{50} 9.2 μg/mL, pupae). Individual assays of these 4 triterpenes (commercially acquired) showed that oleanolic acid was the most potent pupicide (LC_{50} 0.37 μg/mL), followed by ursolic acid (LC_{50} 2.47 μg/mL). These compounds constitute candidates to develop formulations to address the shortage of commercial pupicides. Aricine (**13**), an indole alkaloid, isolated as a major compound from the *A. nitidum* wood extract, demonstrated previously unreported larvicidal activity (LC_{50} 17 μg/mL, 72 h).

Our ethno-directed bioprospecting approach sourced natural products to control *Aedes aegypti.* This combined knowledge partnership between the scientific community and local Amazonian inhabitants presents an eco-friendly opportunity to address significant public health concerns. At the same time, this strategy protects the rainforest and rights of currently threatened traditional communities, in addition to promoting local economic opportunities and social inclusion.

Declaration of competing interest

The authors declare no competing interest associated with the study.

CRediT authorship contribution statement

Paula Maria Correa de Oliveira Melo: Conceptualization, conducted ethnopharmacological field survey and collected plant materials, depositing samples in the herbarium, Methodology, Investigation, Formal analysis, data acquisition and interpretation, drafted manuscript. **João Paulo Barreto Sousa:** Conceptualization, Methodology, Investigation, Formal analysis, data acquisition and interpretation, Supervision, drafted manuscript. **Lorena C. Albernaz:** Methodology, Investigation, Formal analysis, data interpretation. **Márlia Coelho-Ferreira:** Conceptualization, and formulation of the ethnopharmacological field survey, depositing samples in the herbarium, Supervision, Investigation, Formal analysis, data interpretation, drafted manuscript. **Laila Salmen Espindola:** Formal analysis, data interpretation, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115284.

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