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ORIGINAL ARTICLE

In vitro and *in vivo* antimalarial activity of the volatile oil of *Cyperus articulatus* (Cyperaceae)

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ABSTRACT

Malaria is a disease of global tropical distribution, being endemic in more than 90 countries and responsible for about 212 million cases worldwide in 2016. To date, the strategies used to eradicate this disease have been ineffective, without specific preventive measures such as vaccines. Currently, the existing therapeutic arsenal is limited and has become ineffective against the expansion of artemisinin-resistant *Plasmodium*, demonstrating the need for studies that would allow the development of new compounds against this disease. In this context, we studied the volatile oil obtained from rhizomes of *Cyperus articulatus* (VOCA), a plant species commonly found in the Amazon region and popularly used as a therapeutic alternative for the treatment of malaria, in order to confirm its potential as an antimalarial agent by *in vitro* and *in vivo* assays. We cultured *Plasmodium falciparum* W2 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) strains in erythrocytes and exposed them to VOCA at different concentrations in 96-well microplates. *In vivo* antimalarial activity was tested in BALB/c mice inoculated with approximately 10^6 erythrocytes infected with *Plasmodium berghei*. VOCA showed a high antimalarial potential against the two *P. falciparum* strains, with IC_{50} = 1.21 μg mL^{-1} for W2 and 2.30 μg mL^{-1} for 3D7. VOCA also significantly reduced the parasitemia and anemia induced by *P. berghei* in mice. Our results confirmed the antimalarial potential of the volatile oil of *Cyperus articulatus*.

KEYWORDS: antiplasmodial, artemisinin resistance, chloroquine resistance, malaria, *Plasmodium falciparum*, *Plasmodium berghei*

Atividade antimalárica *in vitro* e *in vivo* do óleo essencial de *Cyperus articulatus* (Cyperaceae)

RESUMO

A malária é uma doença de distribuição tropical, sendo endêmica em mais de 90 países, responsável por cerca de 212 milhões de casos reportados ao redor do mundo em 2016. As estratégias de erradicação dessa doença são ineficazes até o presente, sem medidas de prevenção específica, como vacinas. Atualmente, o arsenal terapêutico existente é limitado e vem se tornando ineficaz frente à expansão de plasmódios resistentes a artemisinina, evidenciando a necessidade de estudos que viabilizem o desenvolvimento de novos compostos contra a doença. Nesse contexto, estudamos o óleo essencial obtido de rizomas de *Cyperus articulatus* (VOCA), uma espécie vegetal comumente encontrada na região amazônica, utilizada popularmente como alternativa terapêutica para o tratamento de malária. Visamos confirmar o potencial antimalárico da planta através de testes *in vitro* e *in vivo*. Utilizamos cepas de *Plasmodium falciparum* W2 (cloroquina-resistente) e 3D7 (cloroquina-sensível) cultivadas em hemácias e expostas ao VOCA em microplacas de 96 poços. A atividade antimalárica *in vivo* foi testada em camundongos da linhagem BALB/c infectados com aproximadamente 10^6 eritrócitos parasitados por *Plasmodium berghei*. O VOCA apresentou alto potencial antimalárico (IC_{50} < 10 μg ml^{-1}) frente às duas cepas de *P. falciparum* testadas (IC_{50}=1,21 μg ml^{-1} para W2 e 2,3 μg ml^{-1} para 3D7). Além disso, houve redução significativa da parasitemia induzida por *P. Berghei* em camundongos tratados com EOAC, e também observamos diminuição da anemia, uma sintomatologia provocada pela infecção. Nossos resultados confirmam o potencial antimalárico do óleo essencial de *Cyperus articulatus*.

PALAVRAS-CHAVES: antiplasmódico, malária, *Plasmodium falciparum*, *Plasmodium berghei*, resistência à artemisinina, resistência à cloroquina

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INTRODUCTION

Malaria is an infectious and parasitic disease of global distribution related to prevailing seasonal factors in tropical and subtropical regions of sub-Saharan Africa, Southeast Asia and the Amazon Region (Reiners *et al.* 2010; Meneguetti *et al.* 2014). It remains a global health issue, endemic in more than 90 countries, with 212 million estimated cases worldwide in 2016 (WHO 2016).

According to the Panamerican Health Organization (PAHO 2018), after a decrease in the number of malaria cases from 2005 to 2014 in the region of the tropical Americas, an increase was observed between 2015 and 2017. In 2016, nine countries in the region (Colombia, Ecuador, El Salvador, Guyana, Haiti, Honduras, Nicaragua, Panama, and the Bolivarian Republic of Venezuela) reported an increase in malaria cases. In 2017, five countries reported an increase in cases: Brazil, Ecuador, Mexico, Nicaragua and Venezuela. Brazil reported 174,522 cases of malaria in 2017, while in 2016 the number of cases was 117,832. The states that contributed with most cases were Amazonas, Pará and Acre (North region).

Malaria is caused by a protozoan of the genus *Plasmodium* Marchiafava & Celli, 1885, which has approximately 150 species currently described, with only five species having been reported to be responsible for infecting humans: *P. falciparum* Welch, 1897*; P. vivax* Grassi & Feletti, 1890*; P. ovale* Stephens, 1922; *P. malariae* Grassi & Feletti, 1889 (França *et al.* 2008) and *P. knowlesi* Sinton and Mulligan 1933 (Rossati *et al*. 2016, Shearer *et al*. 2016). The transmission to humans occurs through the female bite of *Anopheles* Meigen 1818 mosquitoes infected with the protozoan. Most cases of severe malaria and death are caused by *P. falciparum* and its infection is responsible for the development of severe disease, affecting the central nervous system, and causing severe anemia, renal failure, pulmonary dysfunction, shock, disseminated intravascular coagulation, hypoglycemia, metabolic acidosis, and hepatic dysfunction (França *et al.* 2008; Gomes *et al.* 2011).

In the absence of an effective vaccine against malaria parasites (Tuju *et al*. 2017), appropriate treatment continues to be the basis of disease control (Landier *et al*. 2016). The therapeutic arsenal against malaria includes quinolines such as 4-aminoquinolines, 8-aminoquinolines and quinolinic alcohols including chloroquine, primaquine, quinine, mefloquine and lumefantrine (Brasil 2010), as well the endoperoxide sesquiterpene lactone artemisinin, the latter currently being the main antimalarial agent (WHO 2001). After the worldwide emergence of resistance of malaria parasites to chloroquine, the World Health Organization (WHO) recommended the use of artemisinin-based combination therapy (ACT) as the first-choice treatment for malaria (WHO 2001).

Although artemisinin remains effective in Brazil (Pinto *et al*. 2019), in the last decade artemisinin resistance has emerged and spread in Southeast Asia, impairing the therapeutic arsenal against malaria (Woodrow and White 2017). In this current scenario, the need for the identification of novel chemotherapeutic agents against resistant *Plasmodium* parasites is urgent.

Historically, natural products are the main sources for the development and production of antimalarial drugs, such as quinine, found in the bark of *Cinchona* spp. (Rubiaceae) and artemisinin isolated from *Artemisia annua* L. (Asteraceae), which represent the main existing antimalarial substances (Pohlit *et al.* 2013).

In the Amazon region, *Cyperus articulatus* L. (Cyperaceae), an aromatic plant popularly known as *priprioca,* stands out among the species used for therapeutic purposes. This native species occurs naturally in the North, Northeast and Southeast of Brazil (Flora do Brasil 2019). Cyperaceae contains small herbaceous plants (Goetghebeur 1998). The stalks of *C. articulatus* produce small tubers that, when cut, exude a fresh, woody and spicy scent, traditionally used in scented baths and in the manufacture of artisanal colonies in Northern Brazil, specially in Pará state (Nicoli *et al*. 2006). The species produces an intense, yellow volatile oil, which has economic value for perfumes and fragrance production in the cosmetic industry (Zoghbi *et al.* 2008).

Cyperaceae were cited by Bussmann and Glenn (2010) in their study on traditional treatments for malaria and fever in Northern Peru. Rhizomes (Rukunga *et al*. 2008) and leaves (Akendengue 1992) of *C. articulatus* are traditionally used to treat malaria. The congeneric *C. longus* L. has been identified as the plant named *soád* in traditional Iranian medicine books from the 11th to 18th centuries and indicated to treat malaria-like fever (Ghafari *et al.* 2013). Many other pharmacological properties have been described for *C. articulatus* such as anticonvulsant (Bum *et al*. 2001; Bum *et al*. 2003), sedative (Rakotonirina *et al.* 2001), antifungal (Duarte *et al*. 2005), antibacterial (Mongelli *et al*. 1994; Oladosu *et al*.2011; Azzaz *et al*.2014), and antioxidant (Azzaz *et al*. 2014) actions. The *in vitro* antiplasmodial potential of a methanol extract and chloroform fraction of *C. articulatus* has also been observed (Rukunga *et al.* 2008; Rukunga *et al.* 2009), yet there are no studies to date evaluating the antimalarial potential of the volatile oil of *C. articulatus*.

In this context, the objective of the present study was to analyze the chemical composition of the volatile oil of *C. articulatus* and to assess its antimalarial activity in *in vitro* and *in vivo* experimental models.

MATERIAL AND METHODS

Plant material

Cyperus articulatus was cultivated on a small scale basis in Tabocal (54°43'00.10"W; 02°37'41.10"S), km 23 of the BR-163 highway, in the municipality of Santarém, Pará, Brazil, in order to obtain raw material for our work. The rhizomes (5 kg) were collected in August 2014. The botanical material was identified by Dr. Antônio Elielson Sousa da Rocha, and a voucher specimen was deposited under registration MG-

207174 in the herbarium of Museu Paraense Emilio Goeldi (MPEG) (Belém, Pará). After collection, the rhizomes were washed in running water, dried in an oven at 40ºC for a period of three consecutive days and milled.

Extraction of the volatile oil

The milled plant material was extracted by steam distillation in a 150 L tank for 4 h in order to obtain the volatile oil of *C. articulatus* (VOCA). The VOCA yield was measured in g g^{-1} fresh weight.

Chromatographic analysis of the VOCA

The chemical composition of the volatile oil was analyzed with a model HP-6890 Agilent gas chromatograph equipped with a mass selective Agilent HP-5975 detector using an HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm) under the following conditions: injector temperature = 220 °C, column = 60 °C, heating rate = 3 °C min^{-1} up to 240 °C, and detector = 250 °C. Helium was used as a carrier gas at a flow rate of 1 ml min^{-1} with a selective mass detector operating at 70 eV, *m/z* = 30 to 500 amu. The volatile oil was solubilized in ethyl acetate at a concentration of 20 mg ml^{-1}. Quantitative data (%) were obtained by peak areas according to the software of the chromatograph. Major extract compounds were identified by comparison with the electronic library of the equipment (NIST-11) and the literature (Adams 2007).

In vitro assays

Culture of intraerythrocytic stages of P. falciparum – The W2 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) parasite strains (Laboratório da Malária da Fiocruz – Minas Gerais) were cultured in human erythrocytes *in vitro* under conditions previously established by Trager and Jensen (1976), with small modifications (Carvalho *et al.* 1991; Andrade-Neto *et al.* 2004). The parasites were cultured in triplicate in Petri dishes with 5% hematocrit using complete RPMI 1640 culture medium supplemented with 25 mM Hepes, 21 mM sodium bicarbonate, 300 µM hypoxanthine, 11 mM glucose, 40 µg ml^{-1} gentamicin and 10% inactivated human plasma. All reagents were purchased from Sigma-Aldrich (São Paulo, Brazil). The dishes were maintained at 37 ºC in desiccators in which the adequate concentration of O_2 was obtained by the combustion of a candle, and the medium was exchanged daily. Parasitemia was monitored daily in Giemsa-stained smears under an optical microscope (1000 x).

Synchronization of P. falciparum culture – The parasite cultures were synchronized by the sorbitol method (Lambros and Vanderberg 1979) and the culture with a predominance of young forms (rings), obtained soon after the synchronization, was used in the antimalarial assays.

Schizonticidal tests against P. falciparum using the traditional microtest – *Plasmodium falciparum* (W2 and 3D7) cultures synchronized with 2% parasitemia in the ring-shaped stage and 2% hematocrit were distributed in 96-well microplates. After approximately 1 h of incubation, 25 µl of RPMI medium were added to each well containing different concentrations (1.56, 3.12, 6.25, 12.5, 25 and 50 µg ml^{-1}) of the VOCA; chloroquine was used as positive control. After 24 and 48 h incubation at 37 °C in a desiccator, the culture medium was changed in each well. A blood smear of all Giemsa-stained samples was prepared after 72 h and analyzed by light microscopy to determine parasitemia. Results were expressed as IC_{50} (Carvalho *et al.* 1991). All experiments were performed in triplicate with three plates.

Human lung fibroblast culture – The WI-26 VA4 cell line (human lung fibroblast, ATCC#: CCL-75) was kindly supplied by Dr. Luciana Maria Silva (Fundação Ezequiel Dias, Belo Horizonte-MG, Brazil). Cells were thawed at 37 °C and their contents transferred to a 50-ml tube with 20 ml of incomplete RPMI medium. The cells were then centrifuged at 320 x *g* for 5 min, the supernatant was discarded and the pellet resuspended in complete RPMI medium supplemented with 5% fetal bovine serum and 40 mg l^{-1} gentamicin. Cells were then transferred to three 200-ml culture bottles and placed in a CO_2 incubator at 37 °C. The medium was changed every 2 days. After 80% confluence, the culture was used in the cytotoxicity assays.

Cytotoxicity assays – WI 26VA-4 cells in culture were trypsinized with 1 ml of trypsin, incubated at 37 °C for 5 min, resuspended in 10 ml of complete RPMI medium and centrifuged at 320 g for 5 min. The supernatant was discarded and the pellet resuspended in complete medium. Cells were distributed into 96-well microplates ($4x10^5$cells/100 µl per well) and placed in a CO_2 incubator at 37 °C for 24 h for cell adhesion to the plate. In the next step, 100 µl of complete medium containing different concentrations (0.1 to 100 µg ml^{-1}) of the test samples were added. After 24 h incubation, 20 µl of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol (MTT) bromide (5 mg ml^{-1} in phenol-free RPMI) was added to each well. After 3 h incubation, the supernatant was removed and the formed dye diluted with 50 µl DMSO. All experiments were performed in triplicate with three plates. The microplates were read in a spectrophotometer at 570 nm and the results were expressed as IC_{50} (Moreira *et al.* 2015). The selectivity index (SI) was obtained by the ratio between the IC_{50} value of WI 26VA-4 and the IC_{50} value of *P. falciparum.*

In vivo assays

Animals – The experiment was performed with healthy Balb/c mice aged 30 to 60 days, from the Animal Facility of Universidade Federal do Oeste do Pará (Campus Oriximiná). The animals were kept at a controlled temperature of 22 ± 2 ºC, on a dark-light cycle of 12 h with balanced feed (Labina®) and water *ad libitum*. The management and care of the animals followed the ethical principles of animal experimentation, according to the criteria established by the Animal Use Committee of Universidade Federal do Oeste do Pará (UFOPA), under approved protocol # 02001/2015.

Evaluation of acute toxicity – Acute oral toxicity was determined according to the OECD-423/2001 guidelines "Acute Toxic Class Method" (OECD 2001), which determine fixed doses of 5, 50, 300 and 2000 mg kg^{-1}, and the number of three animals per group. The initial dose was 5 mg kg^{-1} and the other doses were tested depending on the mortality observed during the first 24 h of exposure. Each dose was tested twice and the toxicological category was estimated according to the especifications. VOCA was solubilized in 4% Tween 80; the animals in the control group received only saline solution with 4% Tween 80. Doses were administered orally. The animals were assessed for general activity, vocal fremitus, irritability, reflex, contortion, ataxia, tremors, convulsions, hypnosis, anesthesia, lacrimation, piloerection, hypothermia, respiration, cyanosis, hyperemia, and death 30 min and 1, 2, 3 and 4 h after administration of VOCA, and thereafter, daily up to the 14th day. All animals were weighed before and on the 7th and 14th day after VOCA administration.

Induction of the experimental model in vivo – The assay was performed by the adapted test described by Peters (1985), in which the malaria model was induced by intraperitoneal inoculation of approximately 10^6 erythrocytes infected with the *Plasmodium berghei* (PbA) ANKA strain in female Balb/c mice.

Determination of parasite density – Parasitemia was determined on the 4th, 7th and 11th day after inoculation using a blood smear stained with Giemsa and reading of 1,000 erythrocytes under an optical microscope (1000 x). The percentages of parasitemia and suppression were determined using the modified model proposed by Girma *et al.* (2015) according to the equations below:

$$\% \text{ parasitemia} = \frac{\text{number of infected erythrocytes}}{\text{total number of erythrocytes}} \times 100$$

$$\% \text{ suppression} = \frac{\text{parasitemia of untreated animals} - \text{parasitemia of treated animals}}{\text{parasitemia in untreated animals}} \times 100$$

Experimental design – The animals (n= 36; six animals per group) were randomly divided into six groups: Group 1 = vehicle (PBS) control (uninfected animals); Group 2 = negative control (*P. berghei*-infected and untreated animals); Group 3 = positive control (*P. berghei*-infected animals orally treated with a daily dose of 100 mg kg^{-1} artemisinin); Groups 4, 5 and 6 = *P. berghei*-infected animals orally treated with VOCA at daily doses of 200, 100 and 10 mg kg^{-1}, respectively, diluted in 4% Tween 80 using PBS as vehicle. The selected doses corresponded to 1/200, 1/20 and 1/10 of the maximum dose tested in the acute toxicity test. The treatments were started on the 4th day after inoculation of *P. berghei* and were continued daily until the 11th day, when the animals were sacrificed for the collection of biological material by cardiac puncture.

Hematological parameters – At the end of treatment, a complete blood cell count was performed, *i.e.*, total erythrocyte count, hemoglobin quantification and hematocrit determination. The hemogram was performed on a Mindray Hematology Analyzer, BC2800 veterinary model.

Statistical analyses

IC_{50} values from *in vitro* tests were calculated using Origin Lab Corporation software (Northampton, MA, USA). Hematological parameters were compared among the *in vivo* test groups by ANOVA followed by the Tukey test using Graph Pad Software Prism 7.0® (San Diego, CA, USA), with the level of significance set at $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

RESULTS

Chromatographic analysis of the VOCA

The VOCA yield was 0.6% (g g^{-1}). Chemical characterization by GC-MS led to the identification of 37 compounds (Table 1), 9.96% of which were non-oxygenated monoterpenes, 5.03% oxygenated monoterpenes, 23.3% non-oxygenated sesquiterpenes, and 41.5% oxygenated sesquiterpenes. Major compounds were mustakone (9.9%), cyclocolorenone (7.4%), α-copaene (4.4), α-selinene (4.4) and *cis*-thujopsenal (4.0%), while 20.6% of the compounds could not be identified by molecular weigth comparison.

In vitro assays

VOCA was non-cytotoxic for human lung fibroblasts by cell viability assay with MTT (IC_{50} > 100 μg ml^{-1}). The IC_{50} values of *P. falciparum* W2 and 3D7 strains treated with VOCA were 1.21 μg ml^{-1} and 2.30 μg ml^{-1}, respectively. Chloroquine showed IC_{50} = 0.46 μg ml^{-1} and 0.21 μg ml^{-1} for W2 and 3D7 strains, respectively. The VOCA SI was greater than 80 for the W2 strain and greater than 40 for the 3D7 strain (Table 2).

In vivo assays

In the acute toxicity test, after the first dose (5.0 mg kg^{-1}), no mortality nor protocolar affection was observed for the three exposed animals in the group in the first 4 h, nor over the next 14 days. The test was repeated with the same number of animals and VOCA concentration, confirming the previous result. Thus, the concentrations of 50, 300 and 2000 mg kg^{-1} were administered following the same experimental procedures and VOCA was found to satisfy all the parameters prescribed by the OECD 423 guideline (OECD 2001) for a safe compound. The acute toxic dose of VOCA is therefore greater than 2,000 mg kg^{-1} in Balb/c mice, thus being classified as a category 5 compound in the Globally Harmonized Classification System (GHS). No clinical change or mortality was observed at any dose. No significant differences were observed in body mass (Table 3). No morbidity or any visible signs of intoxication were recorded, and all the animals were active and seemed healthy at the end of the observation period.

Treatment with VOCA at all three concentrations tested significantly reduced the parasitemia induced by *P. berghei* (Figure 1). On the 11th day after inoculation, the parasitemia suppression values were 75.2%, 74.8% and 48.9% for groups 4, 5 and 6, respectively. Treatment with 100 mg kg^{-1} artemisinin (Group 3) promoted 100% suppression of the parasites.

There was a significant increase of 144.9% and 120.1% in the number of erythrocytes, 94.2% and 90.6% in the amount of hemoglobin, and 105.5% and 82.1% in the percentage of hematocrit for Groups 4 and 5, respectively, in relation to Group 2. Results for Group 6 were not statistically relevant. Treatment with 100 mg kg^{-1} artemisinin (Group 3) promoted a significant increase of 205.5%, 121.0% and 129.7% in the number of erythrocytes, hemoglobin quantity and hematocrit percentage, respectively (Table 4).

Table 1. Chemical composition of the volatile oil of *Cyperus articulatus* from Santarém (Pará State, Brazil) by GC-MS. KI = Kovatz index [values are from Adams (2007) and were used as supporting reference for the elution order]. % rel = relative percentage.

t_R (min)	KI	Compound	% rel
5.77	933	α-pinene	5.37
6.29	953	thuja-2,4(10)-diene	0.42
6.93	977	β-pinene	3.07
8.39	1024	*p*-cymene	0.50
8.53	1028	limonene	0.60
12.13	1126	α-campholenal	0.31
12.68	1140	*trans*-pinocarveol	1.44
13.60	1162	pinocarvone	0.52
13.83	1168	*p*-mentha-1,5-dien-8-ol	0.60
15.01	1197	myrtenal	2.16
22.54	1376	α-copaene	4.42
23.48	1398	cyperene	1.70
23.74	1405	*cis*-thujupsadiene	1.18
25.08	1438	α-guaiene	0.72
25.89	1458	rotundene	0.88
26.45	1472	β-selinene	0.31
26.52	1474	4,5,9,10-dehydro-isolongifolene	0.54
26.63	1477	α-amorphene	1.04
27.04	1487	α-selinene	4.38
27.37	1495	eudesma-4(14),7(11)-diene	0.96
27.81	1506	α-bulnesene	2.16
29.26	1543	α-calacorene	1.16
30.50	1575	cedrene epoxide	2.53
30.73	1581	calamenene	1.32
30.82	1584	caryophylene oxide	3.73
31.00	1588	spathulenol	2.82
31.19	1593	β-copaen-4-α-ol	1.37
31.79	1609	humulene epoxide	1.27
32.84	1638	eudesma-3,11-dien-5-ol	1.56
33.51	1656	pogostol	2.54
34.44	1681	mustakone	9.91
34.56	1684	cyperol	2.55
34.96	1695	cyperotundone	2.79
35.51	1711	*cis*-thujopsenal	4.05
35.81	1719	14-hidroxi-α-humulene	0.55
36.98	1753	cyclocolorenone	7.42
37.63	1771	α-cyperone	0.59
Total identified (%)			79.44

Table 2. *In vitro* antiplasmodial activity, cytotoxicity (human lung fibroblast, Wi 26VA-4) and selectivity index (SI) of the volatile oil of *Cyperus articulatus* rhizomes (VOCA) against *Plasmodium falciparum* strains W2 and 3D7, and using chloroquine as control. Values are the mean ± SE.

Treatments	IC_{50} (μg ml^{-1})			SI	
	W2	3D7	Wi 26VA-4	$IC_{50Wi\ 26VA-4}$ / IC_{50W2}	$IC_{50\ Wi\ 26VA-4}$ / $IC_{50\ 3D7}$
VOCA	1.21 ± 0.05	2.30 ± 0.09	>100	>80	>40
Chloroquine	0.46 ± 0.08	0.21 ± 0.01	>100	>100	>200

Table 3. Body weight (g) of Balb/c mice (n= 6 per group) treated orally with increasing doses of volatile oil of *Cyperus articulatus* rhizomes (VOCA) (5, 50, 300 and 2,000 mg kg^{-1}) during 14 days for acute toxicity evaluation. Values are the mean ± SEM.

	Control	VOCA concentration			
		5	50	300	2,000
Day 0	24.02 ± 1.28	24.08 ± 2.56	24.18 ± 3.77	25.03 ± 0.67	24.85 ± 0.57
Day 7	25.28 ± 2.51	26.02 ± 0.59	26.08 ± 1.27	27.20 ± 1.64	26.89 ± 1.03
Day 14	25.99 ± 2.96	26.36 ± 1.75	28.19 ± 0.89	28.71 ± 1.60	29.40 ± 1.31

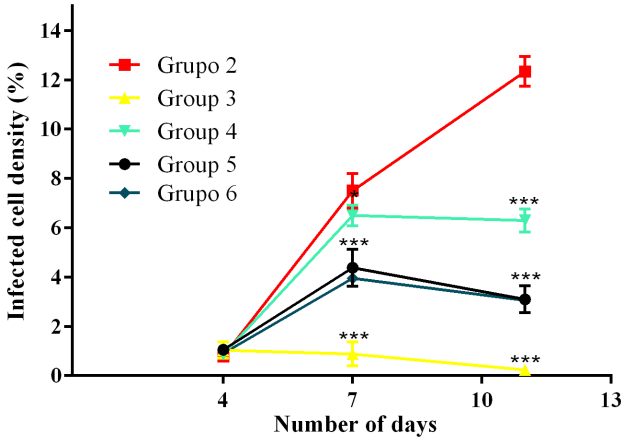

Figure 1. Parasitemia evolution in *Plasmodium berghei*-infected Balb/c mice through the percentage of infected cells. Each point represents the average ± standard error of mean (SEM) (n= 6 per group). Group 2 (untreated malaria); Group 3 (artemisinin 100 mg kg^{-1} day); Groups 4, 5 and 6 (VOCA 200, 100 and 10 mg kg^{-1} day). Asterisks indicate significant differences relative to Group 2. * $p < 0.05$; *** $p < 0.001$. VOCA = volatile oil obtained from rhizomes of *Cyperus articulatus*. This figure is in color in the electronic version.

Table 4. Hematological analysis (mean ± SEM) of *P. berghei*-infected Balb/c mice (n= 6 per group) after 11 days of treatment with volatile oil of *Cyperus articulatus* (VOCA) and artemisinin.

Treatment	Erythrocytes (10^6 µl^{-1})	Hemoglobin (g dl^{-1})	Hematocrit (%)
Group 1	9.48 ± 0.49	14.46 ± 1.21	49.50 ± 1.16
Group 2	3.28 ± 0.61***	6.16 ± 0.99***	20.92 ± 3.53***
Group 3	10.04 ± 2.03***	13.62 ± 2.50***	48.06 ± 8.3***
Group 4	8.04 ± 3.41**	14.77 ± 0.49**	43.03 ± 13.5**
Group 5	7.23 ± 2.60**	11.74 ± 2.59**	38.10 ± 11.44*
Group 6	3.60 ± 0.35	6.94 ± 0.85	25.34 ± 3.07

Group1: control; Group 2: untreated malaria; Group 3: artemisinin 100 mg kg^{-1}; Groups 4, 5 and 6: VOCA 200, 100 and 10 mg kg^{-1}. Asterisks for Group 2 indicate significant differences in relation to Group 1. Asterisks for Groups 3, 4 and 5 indicate significant differences relative to Group 2. *p < 0.05; **p < 0.01; ***p < 0.001.

DISCUSSION

Couchman *et al*. (1964) identified myrtenal, myrtenol and articulone in the volatile oil of *C. articulatus* from Nigeria. Nyasse *et al.* (1988) reported the main chemical compounds found in the hexane extract of the *C. articulatus* rhizomes from Cameroon, including mandassidione, mustakone and isopatchoul-4(5)en-3-one, all sesquiterpene diketones. These same compounds were also detected by Zoghbi *et al.* (2006) in rhizome volatile oil from Pará state (Brazil), with a 14.5% content of mustakone, as well as caryophyllene oxide (10.1%) and α-pinene (6.5%). Zoghbi *et al.*(2008) compared the chemical composition of the volatile oil of the rhizomes of *C. articulatus* var. *nodosus* collected in different localities in Pará. The main compounds were α-pinene (3.6–25.3%), β-pinene (2.2–12.5%), *trans*-pinocarolol (2.1–5.4%), myrtenal + myrtenol (2.2–5.5%), α-copaene (1.4–2.7%), cyperene (0.6–1.5%), β-selinene (0.9–2.5%), lithol (0.8–5.0%), caryophyllene oxide (3.0–8.2%), mustakone (3.3–9.8%), cyperotundone (2.5–4.0%) and α-cyperone (3.3–8.9%).

Mustakone was the major compound (9.91%) in our sample of *C. articulatus* rhizomes from Santarém, a locality that was not sampled by Zoghbi *et al*. (2008). We report other substances for the first time for VOCA (cyclocolorenone and *cis*-thujopsenal). Quali-quantitative differences in the composition of volatile oils may be due to the influence of several factors such as temperature, luminosity, seasonality, developmental stage, harvesting time, water availability, UV radiation, altitude, air pollution, nutrients, pathogens etc. (Adams 2007; Olawore *et al*. 2006; Hassanein *et al*. 2014).

Our results for the cytotoxicity tests and the acute lethal toxicity of VOCA were similar to those reported by Metuge *et al.* (2014) for *C. articulatus* from Cameroon, who found an IC_{50} = 93.7 µg ml^{-1} using monkey kidney cells to assess cytotoxicity and also determined the toxic dose in mice to be greater than 2,000 mg kg^{-1} under acute exposure conditions. Rukunga *et al.* (2008) described the antiplasmodial activity of two sesquiterpenes isolated from the chloroform extract of the rhizomes of *C. articulatus* from Kenya: corymbolone (IC_{50} = 1.07 and 1.92 µg ml^{-1}) and mustakone (IC_{50} = 0.14 and 0.25 µg ml^{-1}) against NF 54 and ENT 36 cell strains, respectively. Mustakone was about 10 times more efficient than corymbolone.

Sesquiterpenes present in tubers of *C. rotundus* from Thailand, among them patchoulenone, α-caryophyllene oxide, 10,12-peroxycalamenene and 4,7-dimethyl-1-tetralone, showed antimalarial activity (Thebtaranonth *et al.* 1995). The ethyl acetate extract of tubers of *C. rotundus* from eastern India showed antimalarial activity with an IC_{50} up to 10 µg ml^{-1}, being effective against the 3D7 and INDO (choloroquine-resistant) strains of *P. falciparum* (Kaushik *et al.* 2013).

In the development of new antimalarial drugs, substances with a high selectivity index (SI) are sought, indicating their specificity for infected erythrocytes (Basore *et al.* 2015). A drug with antimalarial potential should have a SI greater than 10 (Katsuno *et al*. 2015). According to this parameter, VOCA exhibited an excellent SI in our tests, with a value greater than 80 for the *P. falciparum* W2 strain and 40 for the *P. falciparum* 3D7 strain.

Interestingly, we observed that some of the major compounds of VOCA (mustakone and cyclocolorenone), as well some minor compounds (cyperotundone and α-cyperone), contained an α,β-unsaturated carbonyl moiety. The antimalarial potential of these compounds may be related to the tendency of the nucleic acids of *Plasmodium* to react with the α,β-unsaturated carbonyl moiety (Weenen *et al*. 1990).

In addition to the *in vitro* efficacy, our *in vivo* results also showed VOCA to be active in reducing malarial parasitemia levels, as 48.9% suppression at 10 mg kg^{-1} and >74% at 100 and 200 mg kg^{-1} are above the threshold of 40% parasite suppression *in vivo* proposed for a compound to be considered active (Coutinho *et al.* 2013; Rezende *et al.* 2013). Samy and Kadarkari (2011) evaluated the antimalarial potential of extracts from *C. rotundus* leaves from western India, alone or in combination with chloroquine, against *P. berghei* tolerant to chloroquine (NK65 strain). Mice treated with a dose of 500 mg kg^{-1} of crude extract (CE), methanolic extract (ME) and the cyclohexane (CH) and methylene chloride (MC) fractions showed 84, 87, 85 and 80% parasitemia inhibition, respectively. The survival rate was 58, 55, 59 and 50% for the animals treated with CH, MC, CE and ME, respectively. When the fractions were used in combination with chloroquine, the percentages of parasitemia inhibition were 87, 83, 87 and 90%, and the survival rate was 60, 58, 61 and 63%, respectively. Our results thus reinforce the body of evidence for the antimalarial potential of *Cyperus* species.

Anemia represents the severe phase of malaria and can be lethal (Asangha *et al.* 2017). The significantly higher values of hematological parameters (erythrocytes, hematocrit and hemoglobin) in the groups tested with VOCA relative to the negative control in our study may be strongly associated

with a decrease in hemolysis, since parasitemia was reduced, reversing the frequent anemia occurring in malaria. Our results corroborate those obtained by Ballal *et al.* (2011) for gum arabic. The reduction of parasitemia in more than 74% in mice treated with both higher VOCA concentrations also points to the inhibition of the natural progression of the infection through contention of excessive hemolysis and parasite release.

Artemisinin is one of the standard drugs used as positive control in experimental models using Balb/c mice (Agarwal *et al.* 2015; Sousa *et al.* 2017; Recuenco *et al.* 2017). The significant decrease in parasitemia and increase in the number of erythrocytes, hematocrit and hemoglobin levels in Group 3 relative to the negative control is evidence for the efficacy of the experimental model designed for our study, and showed that higher concentrations of VOCA can have a similar effect on hematological parameters as artemisinin. It is important to take into account that VOCA is a complex mixture of several volatile compounds that can act synergistically, while artemisinin is a pure isolated substance.

CONCLUSIONS

The volatile oil obtained from the rhizomes of *Cyperus articulatus* (VOCA) cultivated in Santarém (Pará state, Brazil) contained mustakone as major identified compound, and exhibited a low IC_{50} against two strains of *Plasmodium falciparum* (W2 and 3D7). We also demonstrated a significant effect of the volatile oil on the suppression of parasitemia in *P. berghei*-infected Balb/c mice, as well as significantly lower anemia in animals treated with 100 and 200 mg kg^{-1} VOCA relative to untreated infected animals, disrupting erythrocytic schizogony. VOCA also had low cytotoxicity against the WI-26 VA4 human strain and had acute toxicity *in vivo* at doses of more than 2,000 mg kg^{-1}. In comparison with the positive control artemisinin, VOCA exhibited promising results. Our results show the therapeutic potential of Amazonian *C. articulatus* for the treatment of malaria, an endemic disease in the northern region of Brazil.

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