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ORIGINAL PAPER

Essential oils of *Varronia curassavica* accessions have different activity against white spot disease in freshwater fish

Daniela Aparecida de Castro Nizio[1] · Rodrigo Yudi Fujimoto[2] · Alexandre Nizio Maria[2] · Paulo César Falanghe Carneiro[2] · Carina Caroline Silva França[1] · Natalino da Costa Sousa[3] · Fabiany de Andrade Brito[1] · Taís Santos Sampaio[1] · Maria de Fátima Arrigoni-Blank[1] · Arie Fitzgerald Blank[1]

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Abstract The aim of this study was to evaluate the antiprotozoal activity of essential oils from *Varronia curassavica* accessions against different stages of *Ichthyophthirius multifiliis*. Essential oils from each accession were tested in vitro at the concentrations 0, 10, 25, 50, 75, 100, and 200 mg/L. The VCUR-001, VCUR-202, VCUR-509, and VCUR-601 accessions presented the major compounds α-pinene, germacrene D-4-ol, (*E*)-caryophyllene and epiglobulol, and sabinene, respectively. These isolated compounds were tested in vitro at a concentration proportional to that found in the essential oil which caused 100% mortality of the parasite. The concentrations of 10 and 50 mg/L of the essential oil of accession VCUR-202 provided 100% mortality of trophonts and tomonts, respectively. For the accession VCUR-509, 100% mortality of trophonts and tomonts was observed at concentrations 75 and 200 mg/L of essential oil, respectively. The same mortality was observed at concentration 200 mg/L in both stages of the parasite for the other accessions. The major compounds α-pinene, sabinene, and the (*E*)-caryophyllene + epiglobulol mixture caused 100% mortality of trophonts and tomonts. The in vivo assay for white spot disease control was performed in a therapeutic bath of 1 h with the essential oil of accession VCUR-202 at concentrations of 0.5 and 2.0 mg/L. A significant reduction of about 30% of trophonts on infected fish was observed, independent of the oil concentration. The *V. curassavica* essential oil, especially the VCUR-202 accession, is a potential source of raw material for the formulation and commercialization of bioproducts to control freshwater white spot disease in fish.

Keywords Erva-baleeira · Volatile oil · Major compounds · Aquaculture · *Ichthyophthirius multifiliis*

✉ Daniela Aparecida de Castro Nizio
danielanizio@yahoo.com.br

[1] Universidade Federal de Sergipe, Av. Marechal Rondon S/N, São Cristóvão, Sergipe 49100-000, Brazil

[2] Embrapa Tabuleiros Costeiros, Av. Beira Mar 3250, Aracaju, Sergipe 49025-040, Brazil

[3] Universidade Federal do Pará, Rua Augusto Corrêa 1, Belém, Pará 6075-110, Brazil

Introduction

The medicinal species *Varronia curassavica* (*Cordia verbenacea* DC. synonymy), native from Brazil, is commonly used to treat inflammation. In the pharmaceutical industry, the essential oil of *V. curassavica* is used since 2004 in the production of a commercial phytotherapeutic medicine. In addition to its proven medicinal properties, biological activities such as antibacterial (Meccia et al. 2009; Matias et al. 2010; Pinho et al. 2012; Rodrigues et al. 2012) and antifungal (Hoyos et al. 2012; Silva et al. 2012, 2014) have been reported for this species. However, there are no studies on its antiparasitic potential.

Essential oils consist of a complex mixture of several chemicals, mostly terpenes, with wide variation in composition. In general, the most abundant compound determines the properties of essential oils, and it is likely that their mode of action involves several targets in the cell (Bakkali et al. 2008). A recent study has reported the existence of high chemical diversity of compounds in the essential oils of *V. curassavica*, depending on the plant origin. In addition, the authors verified that depending on the chemical

composition, the essential oils presented higher or lower activity against phytopathogenic fungus (Nizio et al. 2015).

For fish disease treatments, extracts of medicinal plants have been evaluated mainly to reduce or to avoid the use of chemotherapeutics which drive drug resistance in parasites and promote a negative environmental impact (Doughari et al. 2009). In this regard, several studies have been conducted, testing the use of extracts or plant compounds to control ichthyophthiriasis; these include the aqueous extract of *Capsicum frutescens* (Ling et al. 2012), methanol extracts of *Psoralea corylifolia* (Ling et al. 2013), extract and flavonoids isolated from the bark of *Morus alba* (Fu et al. 2014b; Liang et al. 2015); cynatratoside-*C* isolated from the roots of *Cynanchum atratum* (Fu et al. 2014a), and essential oils (Valladão et al. 2016a).

Ichthyophthiriasis is caused by the ciliate *Ichthyophthirius multifiliis,* popularly known as freshwater white spot disease or freshwater ich. This protozoan has caused great economic losses for the fish farming industry, in conventional and high-tech systems, or in the production of ornamental fish (Matthews 2005; Heinecke and Buchmann 2009; Martins et al. 2011). This parasite has a direct life cycle, and the water temperature dictates its life cycle which includes an infectious stage, the free-swimming theront that penetrates the skin epithelium and the gills, transforming into a trophont within a developing pustule (Shinn et al. 2012). Mature trophonts release tomonts that settle on the substrate and secrete a surrounding gelatinous cyst. The tomont multiplies by binary division producing tomites that differentiate into infectious theronts, thus repeating the cycle (Xu et al. 2012; Dickerson and Findly 2014). The objective of this study was to evaluate the antiprotozoal activity of the essential oils of different *V. curassavica* accessions against *I. multifiliis*, an important ectoparasite that causes freshwater white spot disease in fish.

Materials and methods

Fish and parasites

Tambaqui (*Colossoma macropomum*) fingerlings, naturally infected with *I. multifiliis*, were obtained from a commercial fish farm. Infected fish (4–5 g) were gently scraped to remove trophonts from their skin for in vitro and in vivo assays. The isolated trophonts were washed several times with dechlorinated tap water to remove the fish mucus. To obtain the tomonts, trophonts were incubated for 4 h at 24 °C, as described by Fu et al. (2014b).

The experimental procedures were approved by the Ethics Committee for Animals Use of Embrapa Tabuleiros Costeiros under protocol number 03.13.09.005.00.05.001.

Essential oils and major compounds

The essential oils of the following four *V. curassavica* accessions of the active germplasm bank of medicinal and aromatic plants of the Federal University of Sergipe were used: VCUR-001, VCUR-202, VCUR-509, and VCUR-601 (Table 1). Leaves were collected and dried in an oven with forced air circulation at 40 °C for 5 days (Ehlert et al. 2006). The extraction of the essential oil was carried out by hydrodistillation in a modified Clevenger apparatus, using 75 g of dry leaf samples.

Analyses of the essential oil compounds were carried out using a GC-MS/flame ionization detector (FID) (QP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler AOC-20i (Shimadzu). Separations were accomplished using an Rtx®-5MS Restek-fused silica capillary column (5% diphenyl–95% dimethyl polysiloxane) of 30 m × 0.25 mm I.D., 0.25-μm film thickness, at a constant helium (99.999%) flow rate of 1.2 mL/min. Injection volume of 0.5 μL (5 mg/mL) was employed, with a split ratio of 1:10. The oven temperature was programmed from 50 °C (isothermal for 1.5 min), with an increase of 4 °C/min, to 200 °C, then 10 °C/min to 250 °C, ending with a 5-min isothermal at 250 °C.

The MS and FID data were simultaneously acquired employing a detector splitting system; the split flow ratio was 4:1 (MS/FID). A 0.62 m × 0.15 mm i.d. restrictor tube (capillary column) was used to connect the splitter to the MS detector; a 0.74 m × 0.22 mm I.D. restrictor tube was used to connect the splitter to the FID detector. The MS data (total ion chromatogram, TIC) were acquired in the full-scan mode (m/z of 40–350) at a scan rate of 0.3 scan/s using the electron ionization (EI) with electron energy of 70 eV. The injector temperature was 250 °C, and the ion source temperature was 250 °C. The FID temperature was set to 250 °C, and the gas supplies for the FID were hydrogen, air, and helium at flow rates of 30, 300, and 30 mL/min, respectively. Quantification of each constituent was estimated by FID peak area normalization (%). Compound concentrations were calculated from the GC peak areas, and they were arranged in order of GC elution.

Table 1 Identification and origin of *Varronia curassavica* accessions of the active germplasm bank of medicinal and aromatic plants of the Federal University of Sergipe, Brazil

Accession code	Municipality/state of origin	UFS herbarium voucher no.
VCUR-001	Campinas, São Paulo	30913
VCUR-202	Tobias Barreto, Sergipe	33471
VCUR-509	Tomar do Geru, Sergipe	30916
VCUR-601	Itabi, Sergipe	30914

Retention indices were determined by the equation of Van den Dool and Kratz (1963) in relation to a homologous series of *n*-alkanes (nC9–nC18) and compared with retention indices of the literature (Adams 2007) for the identification of the compounds. Simultaneously, the three libraries WILEY8, NIST107, and NIST21 of the equipment were used, which allowed the comparison of the spectra data with the data of the libraries, using an 80% similarity index.

The major compounds of VCUR-001, VCUR-509, and VCUR-601 accessions, commercially available (Sigma-Aldrich®) and used in the tests, were (*E*)-caryophyllene, α-pinene, sabinene, and epiglobulol. The major compound of the essential oil of accession VCUR-202 (germacrene D-4-ol) was not commercially available, so it was not tested in the present study.

In vitro antiprotozoal activity

Two stages of the parasite were used, trophonts and tomonts. The experimental design was completely randomized with three replications. Essential oils were tested in vitro at the concentrations 10, 25, 50, 75, 100, and 200 mg/L, in addition to the control water + Tween 80. The essential oils and the major compounds were diluted in Tween 80 at the ratio: 2/3 essential oil/major compound: 1/3 Tween, to improve the dispersion in water. This mixture was vigorously stirred in vortex until complete homogenization. Solution containing the essential oil or the isolated compound mixture was added to 5-mL Petri dish with 15 parasites each. Incubation was carried out for 1 h.

The major compounds of the essential oil of three accessions were tested separately. Proportional concentrations were tested for each compound present in majority of each essential oil, according to the concentration of the essential oil that caused 100% mortality at each stage of the parasite.

The compounds (*E*)-caryophyllene and epiglobulol, which are the major compounds of the essential oil of the accession VCUR-509, were tested isolately and in mixture, at the concentrations 14.90 and 11.44 mg/L, respectively, for trophonts, and at the concentrations 39.74 and 30.50 mg/L for tomonts. Sabinene, the major compound of the essential oil of the accession VCUR-601, and α-pinene, the major compound of the accession VCUR-001, were tested, respectively, at the concentrations 77.34 and 84.70 mg/L for trophonts and tomonts.

Viability of parasites exposed to essential oil and to its major compounds

Viability of parasites after 1 h of incubation was measured by staining the cells with fluorescent probes SYBR-14 and propidium iodide (PI) (Molecular Probes®). Visualization was carried out in epifluorescence microscope (Nikon Eclipse 50i). This technique is an adaptation of the method described by Maria et al. (2010) for detection of sperm cell viability. Propidium iodide intercalates the DNA of the cells and can only penetrate through damaged cell membranes. SYBR-14 is a fluorescent dye which can penetrate in all cells, in intact or damaged membranes, and bind to DNA. Therefore, intact and viable parasites emit green fluorescence, while dead or damaged cells emit red fluorescence. After incubation with essential oil or isolated compounds for 1 h, the parasites were transferred with a pipette to Kline concavity slides. In each well, 2.5 μL of SYBR-14 (final concentration 250 nM) was added and incubated for 5 min. Later, 2.5 μL of iodide propidium (IP) (final concentration 30 μM) was added and incubated for an additional 5 min. The viability of the parasites was then evaluated under epifluorescence microscopy.

In vivo antiprotozoal activity

For the in vivo assay, trophonts originated from naturally infected tambaquis were collected and incubated in Petri dishes with water at 24 °C for 5 h for the formation of tomonts. In order to achieve a consistent infestation of tambaqui, tomonts were inserted in 1-L beaker at a ratio of 10 tomonts/fish. The fish ($n = 20$) were kept individually in beakers for 7 days in a semi-static system with constant temperature (24 ± 1 °C), pH (7.20 ± 0.30), and oxygenation (greater than 4 mg/L) and partial replacement of water. After this period, the fish showed high levels of parasitic infection, characterized by numerous white spots on the skin.

The experimental design was completely randomized with five treatments (oil concentrantion) and four replications (infected fish). The infected fish were submitted to a therapeutic bath for 1 h in solutions containing essential oil of the accession VCUR-202 in the concentrations 0 (control), 0.5, 1.0, 1.5, and 2.0 mg/L. The accession VCUR-202 was selected due the better results in in vitro assay.

Immediately after bath, the fish from all groups (including control) were transferred to other containers and maintained for 24 h until the trophonts were to be gently removed by scraping the skin and fins. To avoid interference of water quality in parasite survival, temperature, pH, and oxygen were monitored and maintained under the same conditions throughout the experiment. The mean intensity of infection was calculated as the number of trophonts per infected fish (Bush et al. 1997).

Previous tests showed that the tambaqui has low tolerance to the essential oil of *V. curassavica*, especially the accession VCUR-202. Therefore, only the concentrations lower than 2 mg/L were used in the therapeutic baths.

Statistical analysis

Data were analyzed with the software SPSS 17.0® and expressed as the mean ± standard deviation.

Table 2 Chemical composition of the essential oils of four *V. curassavica* accessions

Compounds	RRIo	RRIl	Content (%)			
			VCUR-001	VCUR-202	VCUR-509	VCUR-601
Tricyclene	921	921	–	–	6.17	–
α-Pinene	932	932	42.35	–	3.62	3.17
Camphene	948	946	0.26	–	4.71	0.59
Sabinene	971	969	0.43	–	0.11	38.67
β-Pinene	976	974	0.22	–	1.18	–
α-Terpinene	1017	1014	–	–	–	1.63
β-Phellandrene	1028	1025	0.53	–	–	–
Limonene	1028	1024	–	–	0.38	1.75
1,8-Cineole	1030	1026	1.08	–	0.39	0.43
γ-Terpinene	1058	1054	–	–	–	3.26
cis-Sabinene hydrato	1067	1065	–	–	–	2.57
trans-Sabinene hydrato	1100	1098	–	–	–	2.23
Terpinen-4-ol	1180	1174	–	–	–	5.21
δ-Elemene	1339	1335	4.93	0.57	0.42	0.24
α-Cubebene	1349	1345	–	–	0.41	1.39
α-Copaene	1376	1374	–	0.14	0.33	0.72
β-Elemene	1393	1389	2.45	0.87	0.44	2.25
Sesquithujene	1403	1405	1.13	–	–	–
α-Gurjunene	1412	1409	–	0.26	0.36	3.01
α-*cis*-Bergamotene	1417	1411	1.35	–	–	–
α-Santalene	1424	1416	9.17	–	–	–
E-Caryophyllene	1424	1417	9.29	4.47	19.87	7.94
Z-β-Farnesene	1444	1440	0.36	–	–	–
trans Murola-3.5-diene	1453	1451	–	–	0.54	1.93
α-Humulene	1457	1452	3.12	1.46	6.20	2.25
E-β-Farnesene	1458	1454	1.59	–	–	–
Alloaromadendrene	1468	1458	0.46	1.33	0.52	–
γ-Murolene	1482	1478	–	0.20	0.41	–
Germacrene-D	1489	1484	–	1.42	0.56	4.03
β-Selinene	1492	1489	–	–	0.67	0.49
trans Murola-4(14).5-diene	1494	1493	–	–	–	0.82
Bicyclogermacrene	1500	1500	–	4.49	4.21	3.46
β-Bisabolene	1508	1505	2.05	–	–	–
γ-Cadinene	1513	1513	–	0.48	1.57	–
Cubebol	1519	1514	–	0.40	0.95	1.41
δ-Cadinene	1528	1522	0.52	–	1.95	2.93
E-γ-Bisabolene	1533	1529	0.92	–	–	–
trans Cadina-1.4-diene	1535	1533	–	–	0.53	1.80
cis-Sesquisabinene hydrato	1550	1542	0.50	–	–	–
α-Cedrene epoxide	1567	1574	–	–	3.02	–
Germacrene D-4-ol	1584	1574	–	42.03	0.41	–
Spathulenol	1583	1577	1.47	1.25	2.02	0.84
Caryophyllene oxide	1588	1582	0.76	2.18	1.82	0.45
Epiglobulol	1596	1592	–	0.36	15.25	0.33
7-Cyclodecen-1-ona.7-methyl-3-methylene-10-(1-propyl)	1618		–	–	8.82	–
Ledol	1608	1602	–	2.48	–	0.65
Humulene epoxide II	1622	1608	0.24	–	1.12	–

Table 2 (continued)

Compounds	RRIo	RRIl	Content (%)			
			VCUR-001	VCUR-202	VCUR-509	VCUR-601
Murola-4.10(14)-dien-1-β-ol	1640	1630	2.09	–	–	–
β-Acorenol	1644	1636	0.38	–	–	–
α-Cadinol	1662	1652	0.67	4.95	0.54	–
Cubenol	1646	1645	–	–	–	0.71
Murulol epi-α	1654	1640	–	4.34	–	–
Helifolenol *B*	1682	1677	3.11	–	–	–
Bisabolone oxide-*A*-α	1692	1684	3.70	–	–	–
Shyobunol	1699	1688	–	9.81	0.78	–
Methyl farnesoate (2*E*.6*E*)	1781	1783	–	–	3.90	–
Total identified (%)			95.13	83.49	94.18	97.16

RRIo relative retention index observed, *RRIl* relative retention index literature

The data were assessed with regard to the assumptions of normality and homoscedasticity, using the Shapiro–Wilk and Levene's tests, respectively. For the data with normal distribution, analysis of variance was used, followed by the Tukey test, to make comparisons between the means. Statistical significance was set at $p < 0.05$.

Results

Table 2 shows the chemical composition of the essential oils of the four *V. curassavica* accessions. The accessions VCUR-001, VCUR-202, VCUR-509, and VCUR-601 presented the major compounds α-pinene,

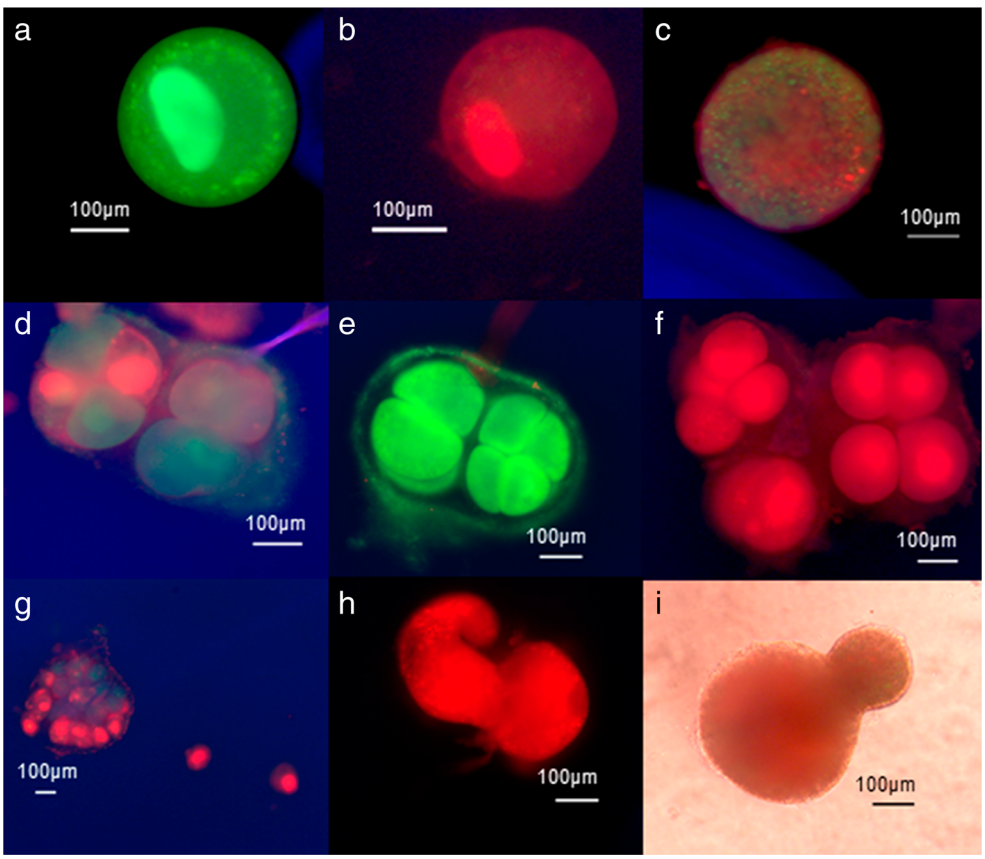

Fig. 1 *I. multifiliis* stained with fluorocromes SYBR-14 and propidium iodide (PI). Trophonts stained with SYBR-14 showing live parasite (**a**). Trophont stained with PI showing damage in cellular membrane and parasite death (**b**). Trophonts and tomonts emitting fluorescents red and green, indicating apoptosis (**c**). Tomites within tomonts (**e**, **f**). Live tomont without exposure to essential oil (**e**). Tomont exposed to 77.34 mg/L of sabinene for 1 h (**f**). Tomites released from tomont after 1 h of exposure to 75 mg/L of the essential oil of accession VCUR-509 (**g**) and extravasation of trophont cytoplasm after 1 h of exposure to 75 mg/L of the essential oil of accession VCUR-509 (**h**, **i**)

Table 3 Mortality of *I. multifiliis* at trophont and tomont stages, treated with different essential oil concentrations of four *V. curassavica* accessions, after incubation for 1 h

Concentration (mg/L)	VCUR-001	VCUR-202	VCUR-509	VCUR-601
	Trophont mortality (%)*			
0.0	3.0 ± 5.0	14.3 ± 13.7	3.0 ± 5.0	2.2 ± 3.8
10.0	12.4 ± 11.9	100.0 ± 0.0	39.9 ± 37.9	0.0 ± 0.0
25.0	18.5 ± 7.8	100.0 ± 0.0	83.2 ± 8.7	12.4 ± 13.8
50.0	57.2 ± 19.3	100.0 ± 0.0	91.5 ± 7.50	74.1 ± 6.7
75.0	72.7 ± 28.5	100.0 ± 0.0	100.0 ± 0.0	84.4 ± 9.6
100.0	77.0 ± 25.2	100.0 ± 0.0	100.0 ± 0.0	87.6 ± 7.6
200.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
	Tomont mortality (%)*			
0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10.0	52.1 ± 6.4	89.6 ± 11.2	0.0 ± 0.0	0.0 ± 0.0
25.0	48.7 ± 9.2	96.7 ± 5.77	18.5 ± 13.7	0.0 ± 0.0
50.0	90.7 ± 11.6	100.0 ± 0.0	38.3 ± 1.4	4.8 ± 8.2
75.0	97.8 ± 3.8	100.0 ± 0.0	44.5 ± 5.8	85.3 ± 5.9
100.0	95.9 ± 3.6	100.0 ± 0.0	74.6 ± 25.0	82.5 ± 11.9
200.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

*Mean ± standard deviation of three replications

germacrene D-4-ol, (*E*)-caryophyllene and epiglobulol, and sabinene, respectively.

In addition to red and green fluorescent stages (Fig. 1a, b), there were also mixed stages in which the protozoan simultaneously emitted green and red fluorescences (Fig. 1c, d). In this case, the cell membrane is damaged; therefore green–red protozoa were considered dead.

The essential oil of VCUR-202 accession exhibited more pronounced activity against the cell in the two stages, followed by VCUR-509. Concentrations of 10 and 50 mg/L of essential oil provided 100% mortality in the trophont and tomont phases, respectively. For VCUR-509 essential oil, 100% mortality was achieved for both trophonts and tomonts, respectively, at 75 and 200 mg/L of essential oil. The same mortality was observed for the other accessions at 200 mg/L for both trophonts and tomonts (Table 3). Tomites, within the tomonts, were visible and emitted green or red fluorescence (Fig. 1e, f). In all the assays, we observed cyst disruption, early release of tomites, and consequently, death (Fig. 1g).

Table 4 Mortality of *I. multifiliis* at the trophont and tomont stages, treated with the major compounds of the essential oil of *V. curassavica* accessions, after incubation for 1 h

Major compounds (accession)	Concentration (mg/L)	Mortality (%)*
		Trophont
Control	0.00	8.9 ± 3.8
α-Pinene (VCUR-001)	84.70	100.0 ± 0.0
(*E*)-Caryophyllene (VCUR-509)	14.90	0.0 ± 0.0
Epiglobulol (VCUR-509)	11.44	42.2 ± 22.5
Sabinene (VCUR-601)	77.34	100.0 ± 0.0
(*E*)-Caryophyllene + epiglobulol (VCUR-509)	26.34	100.0 ± 0.0
Major compounds (accession)	Concentration (mg/L)	Mortality (%)*
		Tomont
Control	0.00	0.0 ± 0.0
α-Pinene (VCUR-001)	84.70	100.0 ± 0.0
(*E*)-Caryophyllene (VCUR-509)	39.74	0.0 ± 0.0
Epiglobulol (VCUR-509)	30.50	30.7 ± 16.7
Sabinene (VCUR-601)	77.34	100.0 ± 0.0
(*E*)-Caryophyllene + epiglobulol (VCUR-509)	70.24	100.0 ± 0.0

*Mean ± standard deviation of three replications

It was also observed that in some trophonts incubated with the essential oil of *V. curassavica* and isolated compounds, there were disruption of the membrane and extravasation of the cellular contents (Fig. 1h, i).

The compounds α-pinene and sabinene, present in higher proportions in the VCUR-001 and VCUR-601 accessions, respectively, provided mortality similar to that shown by the essential oils at the two stages of the protozoan (Table 4). The compounds epiglobulol and (*E*)-caryophyllene, tested separately, provoked lower mortalities than those caused by the VCUR-509 essential oil, at both the trophont and tomont stages. However, when the protozoa were incubated in a solution containing both compounds simultaneously, mortality equal to that of the VCUR-509 essential oil was observed (Table 4).

A therapeutic bath with the essential oil of the VCUR-202 accession resulted in a significant reduction (about 30%) of the intensity of *I. multifiliis* on the fishes when compared to the control (Fig. 2).

Discussion

Results from in vitro and in vivo experiments show that the essential oil of *V. curassavica*, especially the accession VCUR-202, is effective against *I. multifiliis*. The in vivo test showed that therapeutic bath with essential oil reduced the *I. multifiliis* load of tambaqui significantly compared to the control, providing a decrease of about 30% of the number of trophonts per infected fish.

Tambaqui is the main cultivated Brazilian native species, corresponding to 28.1% of all fish production (IBGE 2015). Currently, there is a strong trend towards increased production of this species in intensive systems (Valladão et al. 2016b); however, the intensification of production has led to an increased incidence of parasites such as *I. multifiliis*, causing losses in fish farming. *I. multifiliis* is considered an obstacle to the productivity of tambaqui in intensive rearing, since it can present prevalence of 87 to 93% and abundance of 171,000 to 311,000 parasites per fish (Dias and Tavares-Dias 2015). In this context, the reduction in the number of trophonts promoted by the treatment with essential oil of *V. curassavica* can be considered as a relevant control option.

Although the essential oil of *V. curassavica* consists of many compounds in smaller amounts, it can be inferred that the monoterpenes α-pinene and sabinene are primarily responsible for the antiprotozoal activity presented by the essential oil of the VCUR-001 and VCUR-601 accessions, respectively. Mikus et al. (2000) also reported the antiparasitic activity of the monoterpenes α-pinene and sabinene against *Leishmania major* and *Trypanosoma brucei*. In general, the major compounds determine the biological properties of the particular essential oil and it is likely that their mode of action involves several targets in the cells (Bakkali et al. 2008).

The mortality observed in *I. multifiliis* simultaneously incubated with the compounds (*E*)-caryophyllene and epiglobulol evidences that there is a synergistic effect between the two compounds that confers antiprotozoal activity to the essential oil. These biological properties are the result of the synergy of all molecules or of those present in higher proportion (Bakkali et al. 2008). It is possible that the activity of a major compound is modulated by other compounds in minor proportions (Santana-Rios et al. 2001; Hoet et al. 2006). It should also be considered that the synergistic effect between the compounds may be linked to different modes of action of

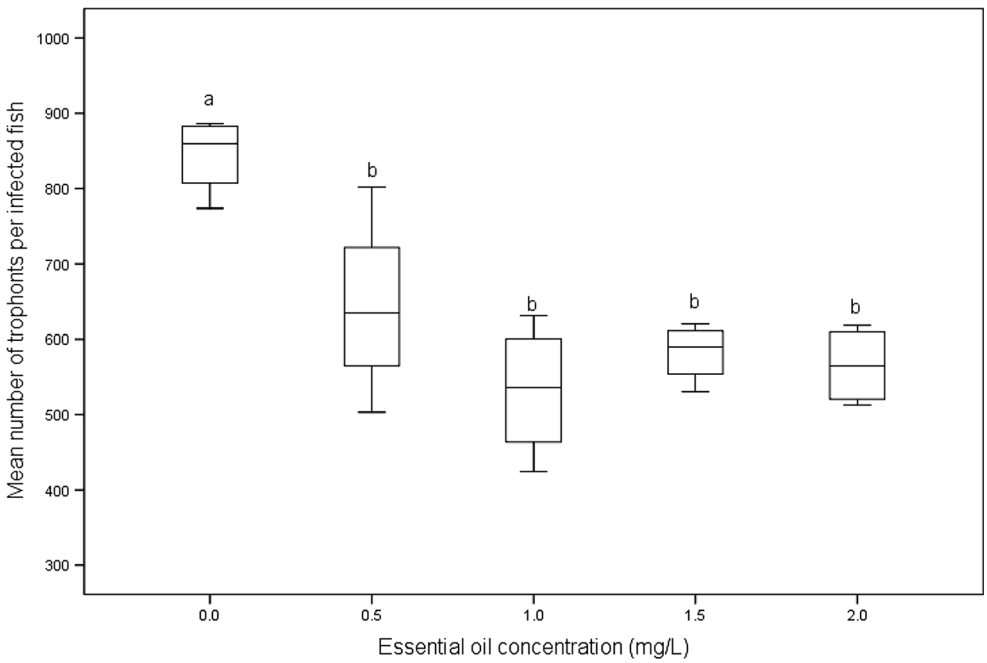

Fig. 2 Mean number of trophonts on the skin and fins of infected tambaquis after application of a therapeutic bath in different concentrations of the essential oil of *V. curassavica* accession VCUR-202 Different letters indicate differences between treatments according to the Tukey test ($p < 0.05$)

each compound. Due to the large number of components, essential oils appear to have no specific cellular targets (Carson et al. 2002).

The essential oils of *V. curassavica* and their major compounds were toxic to the two stages of *I. multifillis*. Most studies involving tests with substances and/or products with antiprotozoal activity against *I. multifiliis* use as mortality criteria the lack of motility and/or abnormal cell division (Ling et al. 2012; Fu et al. 2014a; Yao et al. 2015; Liang et al. 2015). According to De la Fuente et al. (2004), the fluorescent staining is more accurate than motility to assess protozoon viability and is recommended as a tool to quickly assess cellular damage. In the present study, we observed that damage caused to the plasma membrane of the cells of parasites has been one of the modes of action of the essential oil. At the trophont stage, disruption of the membrane and extravasation of cellular contents were visualized (Fig. 1h, i), while at the tomont stage, disruption of the cyst wall occurred, causing the release of tomites (Fig. 1g). The hydrophobicity of the essential oils allows them to pass through the cytoplasmic membrane altering its structure and permeability, leading to leakage of potassium ions and cytoplasmic content (Turina et al. 2006; Bakkali et al. 2008; Chavan and Tupe 2014).

Our study also demonstrates that the essential oils of *V. curassavica* accessions have different actions against *I. multifiliis*, all of which were directly related to the chemical composition of each essential oil. The major compounds α-pinene, sabinene, and the mixture (*E*)-caryophyllene + epiglobulol were toxic to *I. multifiliis* at the trophont and tomont stages. The major compounds tested in this study were also toxic to *I. multifiliis*.

Damage to membrane integrity at the trophont stage and cyst wall disruption at the tomont stage appear to be the main cause of death of *I. multifiliis* incubated with *V. curassavica* essential oils and isolated compounds. The confirmation of the antiprotozoal potential of the essential oils of *V. curassavica* qualifies this species as a source of promising raw material for formulation and commercialization of bioproducts to control freshwater white spot disease in fish. However, new application strategies of this essential oil should be investigated in order to improve its effectiveness.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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