



Anesthetic activity and bio-guided fractionation of the essential oil of *Aloysia gratissima* (Gillies & Hook.) Tronc. in silver catfish *Rhamdia quelen*

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ABSTRACT

This work aimed to determine the efficacy of the essential oil of *A. gratissima* as anesthetic for silver catfish, and to perform the bio-guided fractionation of essential oil aiming to isolate compounds responsible for the noted effects. Fish were submitted to anesthesia bath with essential oil, its fractions and isolated compounds to determine time of anesthetic induction and recovery. Eugenol (50 mg L⁻¹) was used as positive control. Essential oil of *A. gratissima* was effective as an anesthetic at concentrations of 300 to 900 mg L⁻¹. Fish presented involuntary muscle contractions during induction and recovery. The bio-guided fractionation of essential oil furnished *E*-(-)-pinocamphone, (-)-caryophyllene oxide, (-)-guaïol and (+)-spathulenol. *E*-(-)-pinocamphone caused the same side effects observed for essential oil. (-)-Caryophyllene oxide, (-)-guaïol and (+)-spathulenol showed only sedative effects at proportional concentrations to those of the constituents in essential oil. (+)-Spathulenol (51.2 mg L⁻¹) promoted deep anesthesia without side effects. A higher concentration of (+)-spathulenol, and lower or absent amounts of *E*-(-)-pinocamphone could contribute to increase the activity and safety of the essential oil of *A. gratissima*. (+)-Spathulenol showed potent sedative and anesthetic activities in silver catfish, and could be considered as a viable compound for the development of a new anesthetic.

Key words: anesthesia, (-)-caryophyllene oxide, *E*-(-)-pinocamphone, (-)-guaïol, silver catfish, (+)-spathulenol.

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INTRODUCTION

There are different handling practices in fish farming, such as biometrics and transport. These practices can cause stress, which can be detrimental to animal performance. Anesthetics are used in this context to maintain fish welfare, minimizing stressful situations and facilitating handling (Coyle et al. 2004, Zahl et al. 2012). Natural products have been evaluated as an alternative to synthetic anesthetics, such as 2-phenoxyethanol, quinaldine sulfate and benzocaine, which may cause some side effects in fish and handlers (Inoue et al. 2003, Velisek et al. 2007). Some compounds isolated from plants, such as eugenol (Cunha et al. 2010a, Gomes et al. 2011, Becker et al. 2012) and menthol (Simões and Gomes 2009), and also some essential oils, such as the ones obtained from *Eugenia caryophyllata* (Inoue et al. 2003), *Lippia alba* (Cunha et al. 2010b, 2011), *Ocimum gratissimum* (Benović et al. 2012, Silva et al. 2012) and *Melaleuca alternifolia* (Hajek 2011) have shown anesthetic activity on different fish species.

The genus *Aloysia* (Verbenaceae) is originally from South America, with 34 species cataloged, among which 12 can be found in Brazil (Cordo and Deloach 1995). Certain species of *Aloysia* are known to exhibit central activity. *A. gratissima* (Gillies & Hook) Tronc. is known in Brazil as *erva-santa* (Souza and Wiest 20007), and has been used in folk medicine due to its sedative properties (Goleniowski et al. 2006). The essential oil of *A. triphylla* showed potent anesthetic activity in white shrimp (*Litopenaeus vannamei*) (Parodi et al. 2012) and silver catfish (*Rhamdia quelen*) (Gressler et al. 2014). Benović et al. (2012) verified anesthetic activity of essential oil of *A. gratissima* in Brazilian flounder (*Paralichthys orbignyanus*). However, there are no studies correlating its depressant effect with some chemical constituent.

This work aimed to determine the efficacy of the essential oil obtained from leaves of *A. gratissima* (EOA) as anesthetic for silver catfish

(*Rhamdia quelen*), and to perform the bio-guided fractionation of EOA aiming to isolate the active compounds responsible for the noted effects. Acetylcholinesterase (AChE) activity under the influence of EOA was also investigated.

MATERIALS AND METHODS

PLANT MATERIAL

Aerial parts of *A. gratissima* were collected in April 2010 in Santa Maria, Rio Grande do Sul, Brazil. A voucher specimen (SMDB 13.077) was kept in the Biology Department of the Universidade Federal de Santa Maria and identified by Professor Marcos Sobral, UFSJ, and Professor Solon Jonas Longhi, UFSM.

ESSENTIAL OIL EXTRACTION

The essential oil was obtained from fresh leaves of *A. gratissima* by hydrodistillation with a Clevenger type apparatus for 3h (British Pharmacopoeia 2007). Samples of the essential oil were stored at -4°C in amber glass bottles prior to the tests or isolation.

GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS) AND NUCLEAR MAGNETIC RESONANCE (NMR) ANALYSIS

GC-MS analysis of EOA and fractions were performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector, with an HP5-MS column (5% phenyl - 95% methylsiloxane, 30 m x 0.25 mm i.d. x 0.25 µm) and EI-MS of 70 eV. The isolated compounds were analyzed by a Varian CP-3800 gas chromatograph coupled to a Saturno mass selective detector, using VF-5 MS column (Varian, 30 m x 0.25 mm x 0.25 µm). Conditions of analysis: injector 1177 (MS), 250°C, split 1:20; injector 1093 (FID), 250°C. The operating conditions were: split inlet 1:100; temperature program, 40-320°C at 4°C min⁻¹; carrier gas He; flow rate 1.3 mL min⁻¹; injector and detector temperature 250°C. The constituents were identified by comparing their Kovats retention

index and mass spectra with a mass spectral library (NIST 2005) and literature data (Adams 2001).

Optical rotations were obtained on a Perkin Elmer 343 polarimeter. NMR spectra were recorded in CDCl_3 on Bruker HPX 300 FT-NMR at 400 MHz for ^1H and 100 MHz for ^{13}C with TMS as internal standard.

FRACTIONATION AND ISOLATION OF ESSENTIAL OIL (EOA)

EOA (6.03 g) was first submitted to fractionation by column chromatography (CC, 3.5 x 52 cm) with 300 g silica gel 60 (Macherey-Nagel, 70-230 mesh) as stationary phase and hexane:acetone (99:1 v/v) as mobile phase. The fractions obtained (100 mL each) were gathered in 16 main fractions (Fractions A-P) based on TLC profile (silica gel F254; detection: anisaldehyde H_2SO_4). The fractions G and J were subsequently fractionated.

Fraction G (910.4 mg) was submitted to CC (1.9 x 61 cm) with 89 g of silica gel 60 and eluted with hexane:ethyl ether (99:1 v/v) to obtain fractions of 20 mL, resulting in 23 main fractions (G_{1-23}). The fraction G_9 (167.7 mg) was rechromatographed by CC (1 x 31.5 cm) using 18 g of silica gel 60 impregnated with 10% silver nitrate (Williams and Mander 2001) and hexane:acetone (99:1 v/v). Fractions of 10 mL were collected and pooled together to render 10 main fractions (G_{9A-J}). Purification of the main constituent of G_{9B} (99.0 mg) was performed with CC (1 x 28.5 cm) using 15 g silica gel 60 and eluted with chloroform:hexane:ethyl acetate (78:20:2 v/v/v), to obtain *E*-(-)-pinocamphone [76.1 mg; EI-MS m/z (%): 152 (M^+); $[\alpha]_{\text{D}}^{20} = -24.54^\circ$ (c 0.22, CHCl_3)] Fractions $\text{G}_{17} - \text{G}_{20}$ were gathered (112.7 mg) and chromatographed by CC (1 x 30 cm) using 16.31 g of silica gel 60 impregnated with 10% silver nitrate and hexane:ethyl acetate (97:3 v/v) to provide (-)-caryophyllene oxide [91.1 mg; EI-MS m/z (%): 220 (M^+); $[\alpha]_{\text{D}}^{20} = -62.5^\circ$ (c 0.24, CHCl_3)]. Fraction J (493.3 mg) was submitted to CC (1.6 x 47.5 cm) with 48 g of silica gel 60 impregnated

with 10% silver nitrate, using hexane:ethyl ether (92:8 v/v) as eluent, and furnished (-)-guaiaol [107.0 mg; EI-MS m/z (%): 222 (M^+); $[\alpha]_{\text{D}}^{20} = -28.125^\circ$ (c 0.16, CHCl_3)] and (+)-spathulenol [91.3 mg; EI-MS m/z (%): 220 (M^+); $[\alpha]_{\text{D}}^{20} = +8.66^\circ$ (c 0.15, CHCl_3)].

E-(-)-pinocamphone: ^1H NMR, δ (ppm): 0.84 (s, 3H, H9); 1.05 (d, 3H, H10); 1.12 (d, 1H, H7a); 1.29 (s, 3H, H8); 1.88 (dt, 1H, H1); 2.06 (m, 1H, H5); 2.38* (m, 1H, H4a); 2.41* (m, 1H, H7b); 2.57 (m, 1H, H2); 2.58 (m, 1H, H4b) (* These values can be exchanged). ^{13}C NMR (ppm), δ : 14.96 (C10); 19.70 (C9); 26.28 (C8); 29.04 (C7); 38.24 (C5); 39.36 (C6); 44.29 (C4); 44.39 (C1); 46.41 (C2); 215.34 (C3).

(-)-Caryophyllene oxide: ^1H NMR, δ (ppm): 0.9 (m, 1H, H); 0.91 (s, 3H, H13); 0.93 (s, 3H, H12); 1.13 (s, 3H, H14); 1.25 (m, 1H, H6a); 1.35 (m, 1H, H2a); 1.58 (m, 3H, H2b, H10a, H10b); 1.7 (m, 1H, H1); 2.02 (m, 1H, H3b); 2.03 (m, 1H, H7b); 2.17 (m, 1H, H6b); 2.27 (m, 1H, H7a); 2.54 (q, 1H, H9); 2.80 (dd, 1H, H5); 4.89 (s, 1H, H15a); 4.97 (s, 1H, H15b). ^{13}C NMR (ppm), δ : 16.94 (C14); 21.59 (C13); 27.18 (C2); 29.78 (C7); 29.84 (C12); 30.14 (C6); 33.96 (C11); 39.12 (C3); 39.72 (C10); 48.68 (C9); 50.74 (C1); 59.74 (C4); 63.68 (C5); 112.69 (C15); 151.76 (C8).

(-)-Guaiaol: ^1H NMR, δ (ppm): 0.94 (d, 3H, H14); 0.98 (d, 3H, H15); 1.14 (s, 3H, H12); 1.17 (s, 3H, H13); 1.27, 1.95 (both m, 1H each, H3); 1.52 (m, 1H, H7); 1.70, 1.55 (both m, 1H each, H9); 1.80, 1.44 (both m, 1H each, H8); 2.13, 1.87 (dd, m respectively, 1H each, H6); 2.28 (m, 1H, H10); 2.41, 2.05 (both m, 1H each, H2); 2.52 (dd, 1H, H4). ^{13}C NMR, δ (ppm): 19.69 (C15); 19.88 (C14); 25.93 (C12); 27.26 (C8); 27.31 (C13); 27.80 (C6); 30.91 (C3); 33.64 (C10); 33.73 (C9); 35.34 (C2); 46.27 (C4); 49.59 (C7); 73.48 (C11); 138.69 (C5); 140.07 (C1).

(+)-Spathulenol: ^1H NMR, δ (ppm): 0.39 (dd, 1H, H6); 0.64 (m, 1H, H7); 0.92 (m, 1H, H8b); 0.97 (s, 3H, H13); 0.98 (s, 3H, H12); 1.21 (s, 3H, H15); 1.25 (dd, 1H, H5); 1.48 (m, 1H, H3b); 1.55 (m, 1H,

H2b); 1.70 (m, 1H, H3a); 1.83 (m, 1H, H2a); 1.91 (m, 1H, H8a); 1.98 (m, 1H, H9a); 2.14 (m, 1H, H1); 2.35 (dd, 1H, H9b); 4.60 (d, 2H, H14a/H14b). ^{13}C NMR, δ (ppm): 16.29 (C13); 20.21 (C11); 24.74 (C8); 26.03 (C15); 26.68 (C2); 27.46 (C7); 28.62 (C12); 29.90 (C6); 38.83 (C9); 41.71 (C3); 53.37 (C1); 54.27 (C5); 80.90 (C4); 106.22 (C14); 153.38 (C10).

ANIMALS

Silver catfish were purchased from local producers. Fish were kept in continuously aerated 250 L tanks for seven days for acclimatization under the same conditions of each experiment performed in sequence. A semi-static system was used, where 50% of the water volume was exchanged once a day. In experiment 1, the water parameters were maintained at the following values: temperature, $15.90 \pm 0.14^\circ\text{C}$; pH, 7.37 ± 0.11 ; dissolved oxygen, $8.65 \pm 0.07 \text{ mg L}^{-1}$; total ammonia, $2.43 \pm 0.81 \text{ mg L}^{-1}$ and unionized ammonia, 0.016 mg L^{-1} . In the other experiments, the values were $19.33 \pm 0.69^\circ\text{C}$; pH, 7.19 ± 0.56 ; dissolved oxygen, 6.61 ± 0.95 ; total ammonia, $1.06 \pm 0.39 \text{ mg L}^{-1}$ and unionized ammonia, 0.006 mg L^{-1} . Dissolved oxygen and temperature were measured with a YSI oxygen meter (model Y5512). The pH was verified with a DMPH-2 pH meter. Total ammonia levels were determined by the salicylate method (Verdouw et al. 1978). Fish were fed once a day until satiation with commercial feed for juveniles containing 28.0% of crude protein. The juveniles were fasted for 24h prior to the experiments. The experimental methodologies were approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (process No. 46/2010).

EXPERIMENTAL PROCEDURE

Three groups of experiments were performed to evaluate the sedative and anesthetic potential, as well as the recovery of silver catfish exposed to EOA, fractions and isolated compounds. Animals were transferred to aquaria containing 1 L of

water and the samples to be tested, these samples were previously diluted in ethanol 95% (1:10), to evaluate the time required to reach stages of anesthesia (Gomes et al. 2011). In this method, each juvenile was used only once in order to observe deep sedation (S2), partial (S3a) and total loss (S3b) of equilibrium, anesthesia (S4) and/or medullar collapse (S5). Anesthesia was determined by loss of reflex activity and no reaction to strong external stimuli (pressure on caudal peduncle). The animals remained in the anesthetic bath until they reached S4 or for 30 min. After anesthesia induction, each fish was measured, weighed and transferred to an anesthetic-free aquarium to recover. The fish were considered to have recovered if their normal posture and behavior were restored, and the maximum time observed for this to occur was 30 min. After that, the animals were transferred to 30 L aquariums, where possible side effects (spasms, contortions, secretion of mucus, seizures) or mortality was examined after 24h of exposition. Control experiments were performed using aquarium containing ethanol at the highest concentration used to dilute the EOA.

In experiment 1, the anesthetic activity of EOA was evaluated. Ten silver catfish juveniles ($6.82 \pm 2.04 \text{ g}$; $9.10 \pm 0.91 \text{ cm}$) were used for each concentration of EOA (90, 300, 450, or 900 mg L^{-1}). Experiment 2 was performed to compare the activity among 450 mg L^{-1} EOA and the fractions with higher chromatographic yield obtained by column chromatography (B, D, F, G and J). Six juveniles ($8.66 \pm 2.32 \text{ g}$; $9.52 \pm 0.86 \text{ cm}$) were used for testing each fraction, at proportional concentrations to those of the major constituents in the EOA. In experiment 3, the sedative and anesthetic activities of the isolated compounds were evaluated. Six juveniles ($6.99 \pm 2.24 \text{ g}$; $9.44 \pm 1.03 \text{ cm}$) were used for each concentration. The isolated compounds were evaluated at proportional concentrations to those of the constituents in the EOA at concentrations of 450, 675 and 900 mg L^{-1} , which corresponded to: 19.06, 28.60 and 38.14 mg

L⁻¹ of *E*-(-)-pinocamphone; 10.00, 15.00, 20.00 mg L⁻¹ of (-)-caryophyllene oxide; 32.65, 48.96 and 65.30 mg L⁻¹ of (-)-guaiol; and 12.80, 19.20 and 25.60 mg L⁻¹ of (+)-spathulenol. Additional concentrations of (-)-caryophyllene oxide (40 mg L⁻¹) and (+)-spathulenol (51.20 mg L⁻¹) were tested. (-)-Guaiol and (+)-spathulenol were also evaluated together (32.65 and 12.80 mg L⁻¹, respectively). Eugenol at concentration of 50 mg L⁻¹ was used as positive control (Cunha et al. 2010a).

LONG EXPOSURE TIME

This experiment evaluated the effects of long exposure time (24h) of silver catfish to EOA and (+)-spathulenol at sedative concentrations. Considering the sedative concentrations determined in the experiments described in the previous sections, four experimental groups were evaluated: EOA at 90 mg L⁻¹, (+)-spathulenol at 2.6 mg L⁻¹ (proportional concentration of the constituent in the EOA at 90 mg L⁻¹), ethanol (at the concentration used to dilute the EOA) and water.

Silver catfish (6.72 ± 2.01 g; 8.75 ± 1.02 cm) were transferred to aquaria containing 1 L of water and the sample to be tested. Six animals were used for each group to determine the time it would take to reach stage S3a. Initially, animal condition was evaluated in the first 30 min of exposure to confirm the occurrence of sedation and then after 24h, aiming to evaluate the toxicity of the substances during this time. After exposure, fish were transferred to anesthetic-free 7 L aquariums, where possible signs of intoxication or mortality were examined for an additional 24h.

ACETYLCHOLINESTERASE ASSAY

Six silver catfish juveniles (20.49 ± 6.71 g; 16.50 ± 1.33 cm) were exposed to each concentration of EOA (90, 300 or 450 mg L⁻¹), firstly diluted in ethanol 95% (1:10). Control experiments were performed using water alone or ethanol at the highest concentration used to dilute the EOA.

Fish were euthanized by severing the spinal cord after they reached S4 or after 30 min of exposure (control group and concentrations that did not promote anesthesia). Whole brain and muscle samples were removed, packed in eppendorf tubes and kept at -20°C for AChE analysis. AChE assay was performed in homogenates of brain and muscle according to the method described by Ellman et al. (1961) and modified by Villescas et al. (1981). Homogenates (50-100 µL) were incubated with 0.8 mM of acetylthiocholine and 1.0 mM of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) at 25°C for 2 min. Protein content was determined according to Bradford (1976). Enzyme activity was expressed as micromoles of acetylthiocholine hydrolyzed per minute per milligram of protein.

STATISTICAL ANALYSIS

Values are presented as mean $\pm$ SEM (standard error of the mean). A Levene test was used with all data to verify the homogeneity of variances. The results were analyzed by one-way ANOVA and Tukey test or Kruskal-Wallis and Dunn test, when appropriate. Comparison between spathulenol and eugenol was performed with t-test. The minimum significance level was set at $P < 0.05$. Regression analyses were performed to EOA and isolated compounds.

RESULTS

The yield of EOA was $1.94 \pm 0.03\%$ (w/w). EOA showed 73 constituents, and 91.86% of the chemical composition was identified. The major compounds of EOA were 1,8-cineole (18.54%), sabinene (9.5%), guaicol (6.79%) and bicyclogermacrene (5.12%) (Table I).

Silver catfish reached anesthesia with EOA at concentrations ranging from 300 (approximately 18 min) to 900 mg L⁻¹ (approximately 12 min). Fish exposed to 90 mg L⁻¹ reached stage 3a after 8 min and they remained in this stage for 30 min of observation. There were no significant differences in the induction times to anesthesia

TABLE I
Chemical composition of essential oil of *Aloysia*
***gratissima* and fractions of higher yield.**

RT (min)	Constituents	Essential oil (%)	Fractions (%)					RI calc	RI ref
			B	D	F	G	J		
9.756	α -pinene	2.74	7.91					931.0	932
11.459	sabinene	9.50	44.26					973.0	972
11.518	β -pinene	3.69	15.76					974.1	973
12.205	β -myrcene	1.27						990.9	990
13.679	limonene	2.34	9.90					1027.1	1029
13.820	1,8-cineole	18.54			31.19	9.51		1030.5	1031
15.232	<i>Z</i> -sabinene hydrate	1.13						1065.2	1070
16.477	<i>E</i> -sabinene hydrate	0.60						1095.8	1098
18.056	<i>E</i> -pinocarveol	0.89						1136.1	1139
18.345	<i>E</i> -verbenol	0.50						1143.5	1145
18.951	<i>E</i> -pinocamphone	3.99			14.40	30.21		1159.0	1163
19.455	<i>Z</i> -pinocamphone	1.33				8.96		1171.9	1175
20.136	α -terpineol	0.63						1189.3	1189
20.328	myrtenol	0.63						1194.2	1196
24.215	<i>E</i> -pinocavyl acetate	2.84			34.37	12.05		1299.6	1298
25.520	δ -elemene	0.85						1337.2	1338
27.409	β -elemene	0.63						1391.8	1391
28.323	β -caryophyllene	2.82	9.30					1419.2	1419
28.806	γ -elemene	0.60						1433.8	1437
29.440	α -caryophyllene	0.87		5.82				1453.1	1455
30.372	germacrene D	3.91		7.16				1481.4	1482
30.883	bicyclogermacrene	5.12		49.38				1496.9	1497
31.456	cubebol	0.68						1515.4	1515
32.529	elemol	0.73						1550.6	1550
32.772	germacrene B	4.15		21.14				1558.6	1560
33.426	spathulenol	2.71					30.53	1580.0	1582
33.586	caryophyllene oxide	2.16				18.96		1585.3	1585
34.063	guaial	6.79					30.87	1601.0	1601
36.095	bulnesol	2.38					9.12	1670.4	1672
	Total identified	91.86							

*(%): Relative percentage; Rt: Retention time; RI cal: calculated Kovats retention index; RI ref: reference Kovats retention index. Components in amounts below 0.5% in the essential oil have been omitted. Only major constituents of the fractions of higher yield obtained from EOA were shown.

between the concentrations of 450 and 900 mg L⁻¹, but these induction times (approximately 12 min) were significantly faster than those of 300 mg L⁻¹. A significant relationship between the EOA concentration and the time required for anesthesia induction was noted for all stages (Table II), except for recovery time. Time of recovery was

significantly faster at 900 mg L⁻¹ (approximately 12 min) when compared to 300 and 450 mg L⁻¹. During this experiment, and 24h after it, no mortality occurred. However, involuntary muscle contractions were observed in the fish submitted to EOA during induction and recovery. The group exposed to ethanol showed light sedation (S2 in

TABLE II
Time (s) required for anesthesia induction and recovery using different concentrations of EOA in *Rhamdia quelen*.

Concentration (mg L ⁻¹)	Time of induction to anesthesia (s)				Time of recovery (s)
	Stage 2	Stage 3a	Stage 3b	Stage 4	
90	89.17 ± 25.92 ^a	519.20 ± 30.91 ^a	-	-	956.60 ± 78.46 ^{ab}
300	39.90 ± 7.12 ^a	163.10 ± 12.35 ^b	875.10 ± 29.95 ^a	1085.70 ± 45.54 ^a	1083.14 ± 91.35 ^a
450	16.60 ± 1.18 ^b	74.40 ± 4.32 ^c	669.10 ± 38.58 ^b	776.10 ± 33.68 ^b	1188.12 ± 57.19 ^a
900	15.70 ± 1.86 ^b	36.30 ± 1.94 ^d	593.00 ± 54.50 ^b	720.90 ± 36.11 ^b	746.60 ± 54.81 ^b
Equations	$y = 12.56 + 128.64(-x/175.71)$ $r^2 = 0.984$	$\ln y = 8.37 - 0.104 \ln(x)^2$ $r^2 = 0.988$	$y = 0.002x^2 - 2.88x + 1558.05$ $r^2 = 1$	$y = 0.003x^2 - 4.49x + 2141.7$ $r^2 = 1$	-

*Values are means ± SEM. Different letters in the columns indicate significant differences among concentrations by one-way ANOVA and Tukey tests (S3b, S4 and recovery) or by Kruskal-Wallis and Dunn tests (S2 and S3a) ($P < 0.05$). (–) indicates that the stage was not reached within 30 min or that there was no significant relationship.

approximately 7.9 min), but it was significantly different from all EOA-treated groups.

AChE activity did not show any significant difference among treatments (Table III).

The fractions B, D, F, G and J showed higher yield from the EOA chromatography process. Similar chemical composition was verified for fractions F and G, while the others showed different major constituents (Table I). No sedative effect was observed for fraction D in silver catfish within 30 min of exposure. Fish exposed to fractions B and F reached only S3a (Table IV). Both fractions G and J resulted in anesthesia (S4) in 50% of fish with no significant differences between them. However,

fraction G caused involuntary muscle contractions similar to those verified with the EOA. Fraction J caused no side effects, but it resulted in increased recovery time in relation to the other fractions. Since fractions G and J showed anesthetic or side effects, we chose to isolate their constituents in order to identify the possible active compounds. *E*-(-)-pinocamphone and (-)-caryophyllene oxide were obtained from fraction G, while purification of fraction J resulted in the isolation of (-)-guaicol and (+)-spathulenol. The isolated constituents were identified by comparing the MS and NMR data with literature reports (Coxon et al. 1984, Raharivelomana et al. 1995, Chaves and Santos 2002, Höld et al. 2002, Bolzan 2007).

TABLE III
Brain and muscle specific AChE activity ($\mu\text{mol min}^{-1}$ mg protein⁻¹) of *Rhamdia quelen* exposed to EOA.

Tissue	Treatments				
	Water control	Ethanol control	EOA (mg L ⁻¹)		
			90	300	450
Brain	0.693 ± 0.098 ^a	0.702 ± 0.082 ^a	0.672 ± 0.093 ^a	0.612 ± 0.064 ^a	0.645 ± 0.028 ^a
Muscle	0.853 ± 0.130 ^a	0.723 ± 0.047 ^a	0.743 ± 0.064 ^a	0.943 ± 0.128 ^a	1.080 ± 0.151 ^a

*Values are means ± SEM. Data was submitted to one-way ANOVA ($P < 0.05$).

Juveniles submitted to *E*-(-)-pinocamphone showed different behavior of sedated or anesthetized fish. In the first minutes of exposure, a period of

hyperactivity could be noted. Animals tended to present faster and erratic swimming with partial loss of equilibrium, and sudden jumping towards the

TABLE IV
Time (s) required for induction and recovery from anesthesia using EOA (at concentration of 450 mg L⁻¹) and their fractions of higher yield (at proportional concentrations to those of the major constituents in the EOA) in *Rhamdia quelen*.

Fraction	Time of induction to anesthesia (s)				Time of recovery (s)
	Stage 2	Stage 3a	Stage 3b	Stage 4	
EOA	6.67±0.99 ^b	37.33±2.86 ^b	930.60±290.64 ^a	1035.33±226.44 ^a	894.00±67.79 ^{ab}
B	83.33±19.11 ^a	762.50±52.50 ^a	-	-	213.00±16.18 ^c
D	-	-	-	-	-
F	99.33±32.87 ^a	608.00±91.38 ^a	-	-	272.50±15.73 ^c
G	23.83±2.09 ^{ab}	67.33±10.60 ^b	279.00±96.77 ^a	894.67±150.36 ^a	587.50±95.97 ^b
J	37.00±15.01 ^{ab}	92.67±32.46 ^b	1138.33±210.90 ^a	1457.00±94.12 ^a	1119.00±220.40 ^a

*Values are means ± SEM. Different letters in the columns indicate significant difference among treatments by one-way ANOVA and Tukey tests (stages 3a, 3b, 4 and recovery) or Kruskal-Wallis and Dunn tests (stage 2) ($P < 0.05$). (–) indicates that the stage was not reached within 30 min.

water surface. Afterwards, there was a period of total loss of equilibrium, animals tumbled on the bottom of the aquarium and remained immobile even after pressure on caudal peduncle, but they responded to external stimuli made with a glass rod in the bottom of the aquarium. Then, fish went back to swimming erratically, and, at that moment, involuntary muscle contractions were noted. Fish writhed to the right and left, or remained contracted in “C or S-shape”. There was a significant relationship between the concentration of *E*-(-)-pinocamphone and the time required for hyperactivity stage ($y = 283.198 - 15.111x + 0.228x^2$; $r^2 = 1$) and tipping stage ($y = -13.117 + 18.650x - 0.406x^2$; $r^2 = 1$). Three juveniles were found dead 24h after the experiment.

The other isolated compounds reached stages S2 and S3a at proportional concentrations to EOA. Sedation (S2) obtained with (-)-caryophyllene oxide was not significantly different from EOA, except at the lowest concentration tested, or (+)-spathulenol (Fig. 1a). EOA was the most effective for loss of equilibrium (S3a), except at concentration of 675 mg L⁻¹, where it did not differ from (+)-spathulenol. (-)-Caryophyllene oxide and (-)-guaïol did not present statistical differences to reach stage S3a, except at the highest concentration, in which (-)-caryophyllene oxide was more

effective (Fig. 1b). In addition, both compounds showed different induction times from EOA.

(-)-Caryophyllene oxide ($\ln y = 0.769 + 35.066/x$; $r^2 = 0.995$; $N = 6$), (-)-guaïol ($\ln y = 1.18 + 0.039x$; $r^2 = 0.999$; $N = 6$), and (+)-spathulenol ($y = 28.28 + 6.333x - 0.377x^2 + 0.005x^3$; $r^2 = 1$; $N = 6$) showed a significant relationship between the concentrations and the time required to reach stage S2. The regression equations to reach stage S3a were $y = -3873.656 + 874.635x - 48.433x^2 + 0.725x^3$; $r^2 = 1$; $N = 6$ to (-)-caryophyllene oxide, $\ln y = 3.42 + 17.2/x^{0.5}$; $r^2 = 0.999$; $N = 6$ to (-)-guaïol and $y = 453.198 - 11.955x - 0.285x^2 + 0.007x^3$; $r^2 = 1$; $N = 6$ to (+)-spathulenol. (-)-Caryophyllene oxide ($y = 2901.2 - 495.21x + 27.753x^2 - 0.411x^3$; $r^2 = 1$; $N = 6$) and (-)-guaïol ($y = 1676.76 - 59.58x + 0.68x^2$; $r^2 = 1$; $N = 6$) also showed significant relationships between the concentrations and recovery times. There was no significant difference in the recovery time of juveniles submitted to concentrations of 12.8 to 25.6 mg L⁻¹ of (+)-spathulenol (data not shown). (-)-Caryophyllene oxide, (-)-guaïol and (+)-spathulenol did not promote mortality during induction and recovery and also 24h after exposition, while four fish exposed to EOA were found dead 24h after the experiment. Fish submitted

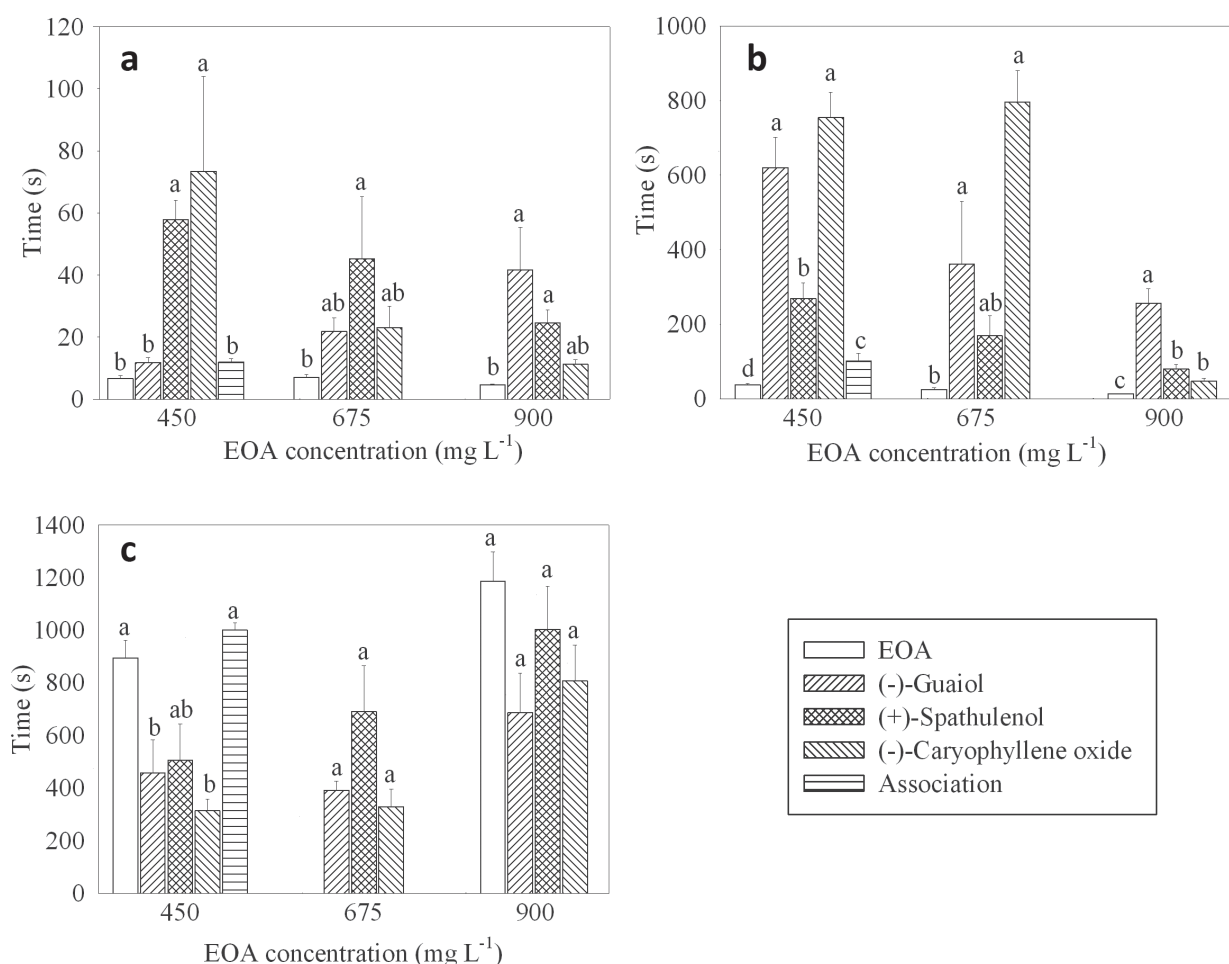


Figure 1 - Time required for the induction and recovery from sedation in silver catfish submitted to EOA and the isolated compounds at proportional concentrations to those of the constituents in EOA: (a): Stage 2; (b): Stage 3a; (c): Recovery. For 450 mg L⁻¹ EOA: (-)-guaiol (32.65 mg L⁻¹), (+)-spathulenol (12.8 mg L⁻¹), (-)-caryophyllene oxide (10 mg L⁻¹) and association of (-)-guaiol and (+)-spathulenol (32.65 and 12.8 mg L⁻¹); For 675 mg L⁻¹ EOA: (-)-guaiol (48.96 mg L⁻¹), (+)-spathulenol (19.2 mg L⁻¹) and (-)-caryophyllene oxide (15 mg L⁻¹); For 900 mg L⁻¹ EOA, (-)-guaiol (65.3 mg L⁻¹), (+)-spathulenol (25.6 mg L⁻¹) and (-)-caryophyllene oxide (20 mg L⁻¹). Fish submitted to 675 mg L⁻¹ EOA did not recover within 30 min, so it was not shown in this figure. Stages are according to Gomes et al. (2011). Maximum observation time was 30 min. N = 6 for each concentration tested. Data are expressed as mean ± SEM. Different letters indicate a significant difference between the different products at the same stage. Stage S3a and recovery time of 675 mg L⁻¹ EOA and S2 of 900 mg L⁻¹ EOA were submitted to Kruskal-Wallis and Dunn tests (P<0.05). The other stages were submitted to ANOVA and Tukey tests (P<0.05).

to (-)-caryophyllene oxide (40 mg L⁻¹) and (-)-guaiol (48.96 and 65.30 mg L⁻¹) showed secretion of mucus during exposition, whereas (-)-caryophyllene oxide (15 - 40 mg L⁻¹) caused light spasms in the animals.

(-)-Caryophyllene oxide did not promoted anesthesia even at the highest concentration tested (40 mg L⁻¹). Stage S2 and S3a were reached at 5.50±0.22 s and 25.50±4.29 s, respectively, with 40

mg L⁻¹ of this compound. Moreover, fish recovered after 1168.00±126.08 s. Fish exposed to 51.2 mg L⁻¹ of (+)-spathulenol reached S2 and S3a faster than with the positive control eugenol (Fig. 2). (+)-Spathulenol promoted deep anesthesia in approximately 22 min, which was a significantly different time, than that obtained with eugenol, which was of approximately 13 min, to reach the same stage. Recovery after

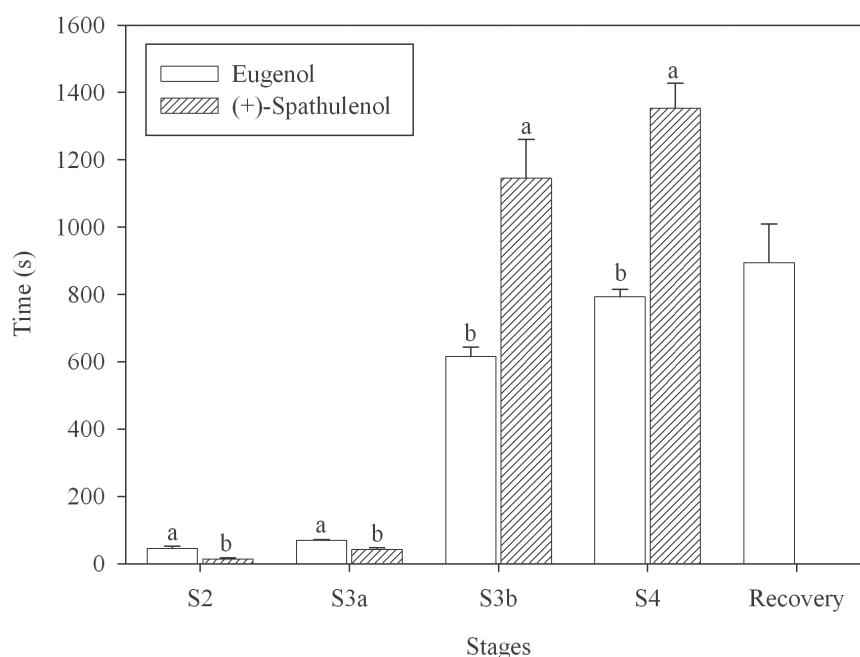


Figure 2 - Time required for the induction of sedation and anesthesia in silver catfish submitted to (+)-spathulenol (51.20 mg L^{-1}) in comparison with eugenol (50 mg L^{-1}). Stages are according to Gomes et al. (2011). Maximum observation time was 30 min. $N=6$ for each concentration tested. Data are expressed as mean $\pm$ SEM. Different letters indicate a significant difference between the different products at the same stage using t-test ($P<0.05$).

eugenol exposure occurred in approximately 15 min, while fish exposed to (+)-spathulenol did not recover within 30 min.

The association of (-)-guaïol and (+)-spathulenol resulted in decrease of induction time until stage S3a, when compared to the isolated compounds (Fig. 1b). However, animals submitted to this association showed mucus secretion and greater recovery time than with the isolated compounds (Fig. 1c). When compared with EOA, the recovery time of the association did not differ statistically. The three isolated compounds did not show statistical differences in the recovery time at all concentrations. Moreover, only one fish submitted to EOA recovered within 30 min at the concentration of 675 mg L^{-1} .

Regarding the long exposure time (24h), fish reached S3a at $241.50 \pm 20.28 \text{ s}$ with EOA and $1752.50 \pm 70.94 \text{ s}$ with (+)-spathulenol. After 24h of exposure, no mortality was observed, and the

animals submitted to EOA and (+)-spathulenol were not sedated. Fish submitted to (+)-spathulenol did not present signs of intoxication or mortality after 24h in anesthetic-free aquaria. However, 33.33% of mortality was observed with EOA.

DISCUSSION

Previous studies showed quantitative differences in the chemical composition of the EO obtained from leaves of *A. gratissima*. Damolena et al. (2010) identified 1,8-cineole (45.5%) and sabinene (8.3%) as major constituents. However, the proportion of these compounds found by these authors was different from the corresponding data found in the present study. Similar proportion of guaïol (6.27%) was found by Franco et al. (2007). Varied amounts of bicyclogermacrene (0 - 12.8%) were described by Ricciardi et al. (2006), and this variation depended on sites and period of collection.

Anesthesia (S4) is achieved when fish lose reflex activity and do not show a reaction to strong external stimuli (Gomes et al. 2011). Silver catfish exposed to concentrations of 300-900 mg L⁻¹ EOA reached this stage within 12-18 min. The concentration of 900 mg L⁻¹ was the most effective, because it showed lower anesthetic induction and recovery time. Similar EOA concentrations (270-900 mg L⁻¹) also provoked deep anesthesia in Brazilian flounder (Benovit et al. 2012). To reach the same level of anesthesia in a similar period of time (approximately 12 min) in silver catfish, 30 mg L⁻¹ of eugenol and 100 mg L⁻¹ of EO of *Lippia alba* are made necessary (Cunha et al. 2010a, b). Moreover, 200 µL L⁻¹ of EO of *Melaleuca alternifolia* and 250 mg L⁻¹ of menthol lead to S4 in common carp, *Cyprinus carpio*, and in Nile tilapia, *Oreochromis niloticus*, respectively (Simões and Gomes 2009, Hajek 2011).

The fractionation process showed that compounds present in fraction D did not contribute to the depressor effects of EOA, while constituents of the fractions B and F could be involved in its sedative effect. Previous reports described central activity for some compounds present in fractions B and F, such as limonene, 1,8-cineol, α - and β -pinene (Kasanen et al. 1998, Santos and Rao 2000, Vale et al. 2002), which need to be confirmed in aquatic animals. On the other hand, fractions G and J promoted anesthesia in 50 % of fish, which indicates that the compounds present in these fractions contribute to the anesthetic effect of EOA.

The involuntary muscle contractions and mortality observed in fish exposed to EOA and/or fraction G may be explained by the presence of *E*-(-)-pinocamphone in these samples. Additionally, with this isolated compound the fish showed two distinct stages, hyperactivity and tipping. Similar effects were characterized as seizure in zebrafish, *Danio rerio*, exposed to caffeine, pentylenetetrazole (PTZ) and picrotoxin (Wong et al. 2010). *E*- and *Z*-pinocamphone are known antagonists of gamma-

aminobutyric acid type A (GABA_A) receptor (Höld et al. 2002). These compounds are able to promote convulsions in mouse that can be alleviated by diazepam, and their signs of intoxication are similar to picrotoxin (Höld et al. 2002). As fish submitted to *E*-(-)-pinocamphone showed more intense side effects than EOA, the compounds with sedative/anesthetic activity present in EOA may have reduced their effects.

The results obtained for *E*-(-)-pinocamphone are not inconsistent with the results of the AChE assay of EOA. Action in AChE was researched because the muscle inhibition of this enzyme leads to increased acetylcholine levels, resulting in excessive muscular stimulation (Kirby et al. 2000). However, AChE activity was not affected by EOA at the tested concentrations and, therefore, the contractures observed on the fish were not due to alterations in the activity of this enzyme. On the other hand, this response could be attributed, as reported earlier, to the GABAergic system, and future studies should be conducted to clarify this issue.

(-)-Caryophyllene oxide and (-)-guaiaol promoted sedation at all tested concentrations. Higher concentrations of these compounds were not used because they caused mucus secretion on the animals. Additionally, (-)-caryophyllene oxide showed light spasm in fish. Secretion of mucus has been observed with some synthetic anesthetics for fish, such as 2-phenoxyethanol, quinaldine sulfate and benzocaine (Inoue et al. 2003, Velisek et al. 2007); as well as 2,4-D [(2,4-dichlorophenoxy) acetic acid], a herbicide (Sarıkaya and Yilmaz 2003). Caryophyllene oxide, isolated from the *Psidium guajava*, showed depressant effects on the central nervous system of mice, through potencialization of the pentobarbital induced-sleeping time (Meckes and Calzada 1996). However, studies on the central activity of (-)-guaiaol were not found.

Sedation induced by (+)-spathulenol is in accordance with those described for the EO obtained from *Baccharis uncinella*, which showed sedative

effects in mice and contains spathulenol as one of the major constituents (Ascari et al. 2012). There were no other studies related to the central activity of (+)-spathulenol. Since this compound did not cause any side effects at sedative concentrations, one higher concentration was tested, which resulted in anesthesia. In this concentration, fish submitted to (+)-spathulenol showed faster sedation, slower anesthesia and recovery, than eugenol. Eugenol is largely used as anesthetic for fish and its activity has been proven in different species (Vidal et al. 2007, Honczaryk and Inoue 2009, Cunha et al. 2010a, Gomes et al. 2011, Becker et al. 2012). On the other hand, (+)-spathulenol promoted anesthesia in lower concentrations (51.2 mg L^{-1}) than *S*-(+)-linalool (153 mg L^{-1}) isolated from the EO of *L. alba* in silver catfish (Heinzmann et al. 2011). Additionally, with *S*-(+)-linalool, fish did not recover within 30 min, similar to (+)-spathulenol in the present study.

(+)-Spathulenol at 2.6 mg L^{-1} was shown to be a safe compound to sedate silver catfish for 24h. An opposite pattern occurred with EOA, because mortality was noted after exposure for the same period of time. Spathulenol at 8 mg L^{-1} for 24h was classified by Ito et al. (2004) as ichthyotoxic in medaka, *Oryzias latipes*. It is probable that this concentration of spathulenol led to S5 of anesthesia induction (medullar collapse) and thus, caused mortality to this fish. An anesthetic may induce different results due to variations between fish species (Zahl et al. 2012).

A relationship between concentration and recovery time was noted in fish exposed to (-)-caryophyllene oxide and (-)-guaiol, while the other tested compounds did not show the same relation. Studies with eugenol and EO of *L. alba* in silver catfish also showed that the concentration of these substances did not influence in the recovery time (Cunha et al. 2010a, b).

The fractionation of EOA resulted in a decrease of the anesthetic potential. The isolated compounds did not promote anesthesia

at proportional concentrations to those of the constituents in the EOA. Moreover, the association of (-)-guaiol and (+)-spathulenol caused a decrease in the time required to reach stage S3a. These evidences indicate that the anesthetic and sedative effects are due to the synergism of the constituents present in EOA. Similar results were observed regarding the anticonvulsive activity of the EO of *Ocimum gratissimum* in mice. Moreover, the EO of this plant was more effective, than their major constituents tested alone or in association, eugenol, 1,8-cineole and *E*-caryophyllene, which were evaluated in the same proportion found for the EO (Galindo et al. 2010).

Conversely, the hypothesis that the minor constituents are responsible for the anesthetic activity of EOA in silver catfish cannot be ruled out, since the fractions of lower yield were not tested. Essential oils contain several substances, which can show different mechanisms of action. These different mechanisms can act in synergy resulting in improved activity (Harris 2002), but antagonism can also occur (Savelev et al. 2003). Pharmacokinetic synergism can also be observed, where absorption, distribution, biotransformation or elimination of a compound can be influenced by other substance, obtaining positive results (Spinella 2002).

EOA showed anesthetic properties, however, it should be used carefully in silver catfish due to the observed side effects. Additionally, EOA had a higher induction time than ideal, of 3-5 min (Cunha et al. 2010b). The presence of *E*-(-)-pinocamphone and (-)-caryophyllene oxide contributed to the side effects of EOA, whereas anesthetic activity was partially related to (+)-spathulenol. Then, higher concentration of (+)-spathulenol, and lower or absent amounts of *E*-(-)-pinocamphone, could contribute to improve the anesthetic activity and safety of the EOA. (+)-Spathulenol was considered a promising anesthetic and sedative for aquatic animals.

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RESUMO

Este trabalho tem como objetivo determinar a eficácia do óleo essencial de *A. gratissima* como anestésico para jundiás, bem como realizar o fracionamento bio-guiado do óleo essencial a fim de isolar os compostos responsáveis pelos efeitos observados. Os peixes foram submetidos ao banho-anestésico com óleo essencial, suas frações e compostos isolados para determinar o tempo de indução e recuperação da anestesia. O eugenol (50 mg L⁻¹) foi utilizado como controle positivo. O óleo essencial de *A. gratissima* promoveu anestesia em concentrações de 300 a 900 mg L⁻¹. Os peixes apresentaram contrações musculares involuntárias durante indução e recuperação. Com o fracionamento bio-guiado do óleo essencial foram obtidos *E*-(-)-pinocanfona, (-)-óxido de cariofileno, (-)-guaíol e (+)-espatulenol. A *E*-(-)-pinocanfona promoveu os mesmos efeitos adversos observados com o óleo essencial. (-)-Óxido de cariofileno, (-)-guaíol e (+)-espatulenol demonstraram apenas efeitos sedativos nas concentrações proporcionais ao óleo essencial. O (+)-espatulenol (51.2 mg L⁻¹) promoveu anestesia profunda sem efeitos adversos. Uma maior concentração de (+)-espatulenol e menores quantidades ou ausência de *E*-(-)-pinocanfona poderiam contribuir para melhorar a atividade anestésica e segurança do óleo

essencial de *A. gratissima*. O (+)-espatulenol apresentou potente atividade sedativa e anestésica em jundiás, e poderia ser considerado um composto viável para o desenvolvimento de um novo anestésico.

Palavras-chave: anestesia, (-)-óxido de cariofileno, *E*-(-)-pinocanfona, (-)-guaíol, jundiá, (+)-espatulenol.

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