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S-(+)- and *R*-(-)-linalool: a comparison of the *in vitro* anti-*Aeromonas hydrophila* activity and anesthetic properties in fish

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ABSTRACT

Linalool is the main compound of many essential oils and occurs in two isomeric forms: *S*-(+)- and *R*-(-)-linalool. This study aimed to determine if linalool isomers have different antimicrobial and anesthetic properties in fish. For this purpose, these compounds were previously isolated from *Lippia alba* (Mill.) N. E. Brown and *Ocimum americanum* L. essential oils. Antimicrobial effects were evaluated through the microdilution test against *Aeromonas hydrophila*, an important fish disease etiologic agent. Induction time until sedation, anesthesia and recovery time were determined in silver catfish (*Rhamdia quelen*) through bath exposure (60, 180, 300 or 500 μL L^{-1}). The results showed different biological properties for the isomers being *S*-(+)-linalool the only active against *A. hydrophila* at 3.2 mg mL^{-1}. The sedation was induced without differences between the compounds, however *R*-(-)-linalool promoted faster anesthesia. There were no differences regarding the recovery time of the animals exposed to the linalool isomers. Although both *S*-(+)- and *R*-(-)-linalool can be used for sedative purposes, their use in *A. hydrophila* infection is inadvisable due to the high effective concentration. Considering anesthesia as the main objective, the *R*-(-)-linalool demonstrated clear advantages at lower concentration.

Key words: anesthesia, *Aeromonas hydrophila*, chirality, linalool, silver catfish.

INTRODUCTION

In 2010, the world aquaculture production amounted to 79 million tons, worth US$125 billion. It has been observed a strong and continuous growth in this industry in South America, especially in Brazil, where fish production increased 13.2% between 2010-2011 (MPA 2011, FAO 2012). It is important to observe that some key production factors, such as susceptibility to disease, growth, efficiency of food conversion, flesh quality and reproduction

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may be adversely affected by stress. Some examples of these stressors are: poor water quality, poor handling practices, inappropriate husbandry conditions and pathogens (Ellis et al. 2012, Segner et al. 2012).

Aeromonas hydrophila is an important etiologic agent being responsible for substantial economic losses in fish culture. This Gram-negative bacterium induces a hemorrhagic and ulcerative disease leading to a high mortality rate, besides it also increases the susceptibility to other diseases (Boijink and Brandão 2001, Andrade et al. 2006). Its treatment involves antimicrobial drugs such as chloramphenicol and oxytetracycline, which remain as residues in animal products and promote environmental contamination (Andrade et al. 2006). Furthermore, the use of these antibiotics has contributed to the emergence of resistant strains to available drugs (Andrade et al. 2006, Barcellos et al. 2008).

Concerning the use of anesthetic and sedative drugs, they have been used in aquaculture practices, including transport, biometry and reproduction in order to reduce fish movement promoting the welfare through stress prevention; in addition to this, and aiming to avoid synthetic drugs residual effects, the use of many natural agents, such as *Lippia alba* (Mill.)N. E. Brown, *Ocimum gratissimum* L., *Hesperozygis ringens* (Benth.) Epling and *Aloysia triphylla* (L'Herit) Britton essential oils (EO) are being suggested as effective replacements to synthetic anesthetics (Gressler et al. 2014, Silva et al. 2012a, 2013a, Toni et al. 2014). Although natural products can be a source of new drugs with less consumer health risk and fewer environmental implications, only a few studies have been conducted using isolated compounds from plants in aquatic animals (Cunha et al. 2010, Silva et al. 2013b, Heldwein et al. 2014).

The chiral compounds pharmacological assessment permits the selection of isomers with higher potentials for specific activity and fewer side effects incidences (Sousa et al. 2010). In this context, antimicrobial and anesthetic agents are classic drug examples whose chirality has greatly influenced their biological properties (see reviews of Hutt and O'Grady 1996, Mitra and Chopra 2011). However, there have been no studies involving drug chirality regarding aquaculture applications.

Linalool is a terpene alcohol, which is present in many plant species EO. It occurs naturally in two isomeric forms, also named enantiomers. The enantiomers are optically active, asymmetrical and non-overlapping isomers that are mirror images of each other. In the case of linalool, they differ according to the carbon 3 chirality; the levorotatory form, which is called 3*R*-(-)-linalool or licareol, and the dextrorotatory isomer, which is known as 3*S*-(+)-linalool or coriandrol. These enantiomers have demonstrated different industrial applications and *in vivo* biological effects (Sugawara et al. 1998, Siani et al. 2002, Höferl et al. 2006, Sousa et al. 2010). Recently, the anesthetic and sedative effects of *S*-(+)-linalool isolated from *Lippia alba* EO was described in silver catfish (*Rhamdia quelen*) (Heldwein et al. 2014). However, no mention was made about *R*-(-)-linalool in this study.

This way, this work aimed to determine if *S*-(+)- and *R*-(-)-linalool present any difference in *in vitro* antimicrobial activity against *A. hydrophila* and *in vivo* anesthetic properties in fish. For this purpose, these compounds were isolated from *Lippia alba* and *Ocimum americanum* EO, respectively, and their antimicrobial and anesthetic activities were compared.

MATERIAL AND METHODS

PLANT MATERIAL AND ESSENTIAL OIL EXTRACTION

O. americanum inflorescences were collected in December 2011 in Encantado-RS, Brazil, and leaves of *L. alba* were collected in January 2012 at the UFSM-CESNORS Campus, Frederico

Westphalen-RS, Brazil. The species were identified by Dr. Sérgio Augusto de Loreto Bordignon and Dr. Gilberto Dolejal Zanetti, respectively. Voucher specimens (nº SMDB 10050 for *L. alba*; nº SMDB 13163 for *O. americanum*) were deposited in the herbarium of the Department of Biology, at UFSM.

The EOs were obtained from the fresh plant material by hydrodistillation with a Clevenger-type apparatus for 3 hours for *O. americanum* and 2 hours for *L. alba* (European Pharmacopeia 2007). The samples were stored at -4°C until the isolation procedure.

S-(+)- AND *R*-(-)-LINALOOL ISOLATION AND ANALYSIS

S-(+)-linalool ($[\alpha]_D^{20}$ = + 1.911 (c 5.808, $CHCl_3$)) was purified from the EO *L. alba* using a chromatography column (CC) procedure based on Heldwein et al. (2014). Prior to the *O. americanum* EO fractionation process, the linalool chirality of this sample was evaluated. The analysis was carried out by chiral gas chromatography (GC) peak enrichment with a capillary column coated with heptakis-(6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin (25 m x 0.25 mm x 0.2 μm) using a Varian 3800 gas chromatograph equipped with a flame ionization detector (FID). Hydrogen was used as a carrier gas, and temperature program was chosen to be 35 °C for 20 min, 35-180 °C at 1 °C min^{-1}. Four chromatographic runs, aiming to analyze the racemic mixture (mixture of both enantiomers), the racemic mixture plus *S*-(+)-linalool, EO, and EO plus the racemic mixture were performed.

The *O. americanum* EO (3.07 g) were submitted to a CC (4 x 50 cm) on 300 g of silica-gel 60 (Merck, 70-230 mesh). The compounds were eluted with a mixture of hexane:ethyl ether (95:5 v/v) at 3 mL min^{-1}. Fractions (40 mL) were monitored by thin layer chromatography (silica gel F254, hexane:ethyl ether 95:5 v/v, detection: anisaldehyde-H_2SO_4), pooled according to their chromatographic profiles in 11 main fractions and then concentrated under reduced pressure at 40°C. Fraction 10 (1.1 g) was rechromatographed by CC (1.8 x 61 cm) using 116g of silica gel 60 impregnated with 10% silver nitrate (Williams and Mander 2001) and hexane:acetone (95:5 v/v) at 1.75 mL min^{-1}. Fractions of 40 mL were collected and pooled to produce seven main fractions (A-G); fraction F was identified as *R*-(-)-Linalool ($[\alpha]_D^{20}$ = - 15.728 (*c* 0.1208, $CHCl_3$)).

The EO chemical composition used in the isolation procedure and chromatographic fractions were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library (NIST 2005) and compared to data from literature (Adams 2001). Gas chromatograph coupled with mass spectrometer (GC-MS) was conducted using an Agilent-6890 gas chromatograph coupled to an Agilent 5973 mass selective detector according to operational conditions described by Silva et al. (2012a). Optical rotations were confirmed on a Perkin Elmer 343 polarimeter.

ANTIMICROBIAL EFFECTS

The *S*-(+)- and *R*-(-)-linalool antimicrobial activity was assayed by a broth microdilution method as established by VET01-A4 (CLSI 2013) for isolated bacteria from animals. The tested microorganisms were four strains of *Aeromonas hydrophila*: one standard (ATCC 7966) and three fish clinical isolates (SB 13/09 10, 13/09 5, 22/07) obtained by the Laboratory of Bacteriology (LABAC), Department of Veterinary Preventive Medicine, UFSM. The clinical isolates characterization was carried out by morphological and biochemical features according to Quinn et al. (1994).

Bacterial strains were grown for 24 h at 30ºC in Mueller Hinton agar (Himedia® Laboratories). The inoculum for the assays was prepared by diluting cell masses in a 0.9% NaCl solution, adjusted to McFarland scale 0.5 (1 x 10^8 CFU mL^{-1}) and confirmed by spectrophotometry at 670 nm. Cell suspensions were finally diluted in Mueller Hinton broth to 1 x 10^7 CFU mL^{-1}.

The samples were solubilized in 95% ethanol and serial dilutions were performed in culture medium to obtain concentrations of 3.2 – 0.0625 mg mL^{-1}. After the addition of 10 µL of the inoculum to the wells, the plates were incubated at 30 ºC for 24 h. All tests were performed in triplicate and included positive and negative controls (inoculum and medium). Antimicrobial activity was detected by adding 20 µL of a 1% triphenyl tetrazolium chloride aqueous solution.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the sample that prevented visible growth. The minimum bactericidal concentration (MBC) was defined as the lowest concentration that yielded negative subcultures.

ANIMALS

Juvenile silver catfish (4.64 ± 0.15 g; 7.85 ± 0.10 cm) obtained from a local fish farm were transported to the laboratory and maintained in continuously aerated 250-liter tanks under controlled water conditions for one week before the experiments. Dissolved oxygen levels (7.91 ± 0.21 mg L^{-1}) and temperature (18.48 ± 0.20 ºC) were measured with an YSI oxygen meter. The pH (7.67 ± 0.05) was determined with a pH meter (DMPH-2). Total ammonia levels (0.44 ± 0.19 mg L^{-1}) were measured by the salicylate method (Verdouw et al. 1978). A semi-static system was used in which 50% of the water volume was changed daily. The fishes were fed once a day with commercial feed (28 % crude protein). Juveniles were fasted for a period of 24 h prior experiments. All experimental protocols were approved by the Ethical and Animal Welfare Committee of the Federal University of Santa Maria (Process number 46/2010).

ANESTHESIA AND RECOVERY PROTOCOL

The *S*-(+)- and *R*-(-)-linalool sedative and anesthetic effects were evaluated on silver catfish at concentrations of 60, 180, 300 or 500 µL L^{-1}. These concentrations were chosen based on a previous study performed with the *S*-(+)-isomer (Heldwein et al. 2014). Juveniles were transferred to 1 L aquaria containing the isomer test concentration, previously diluted in 95% ethanol (1:10). Nine fish were evaluated for each concentration, and each juvenile was used only once to observe deep sedation (stage 2) or anesthesia (stage 4) (Schoettger and Julin 1967). The animals remained in the anesthetic bath until they reached stage 4 of anesthesia or for 30 min. The anesthesia level was determined by the loss of reflex activity and no reaction to strong external stimuli. After the anesthesia induction, each fish was measured, weighed and transferred to an anesthetic-free aquarium in order to recover. The fishes were considered to be recovered when their normal posture and behavior were restored. In sequence, the animals were transferred to 30-L tanks to evaluate possible side effects or mortality 24 h after exposure. The control experiments were performed using aquaria containing ethanol at the highest concentration used to dilute the samples.

STATISTICAL ANALYSIS

Data are presented as the mean ± SEM. To verify the homogeneity of variances and normality, all data were submitted to Levene and Kolmogorov-Smirnov tests, respectively. The Log transformation was performed before two-way ANOVA and Tukey test to analyze the induction time until stage 4. Scheirer-Ray-Hare extension of the Kruskal–Wallis test and Dunn test were applied to evaluate the induction time until stage 2 of anesthesia and recovery. Analyses were performed using SigmaPlot (ver. 11.0) and the minimum significance level was set at $P < 0.05$.

RESULTS

Linalool was detected in 46.6% and 46.7% in *O. americanum* and *L. alba* EO, respectively, by GC-MS analysis (Table I). The chiral GC analysis of the

racemic mixture enriched with *S*-(+)-linalool (Fig. 1a) allowed the identification of the second peak as corresponding to this isomer, because its area decreased when the racemic mixture was evaluated alone (data not shown). The *O. americanum* EO showed only one peak in this region of the chromatogram (Fig. 1c) with a similar retention time of the *R*-(-)-isomer in chiral chromatography. The presence of the *R*-(-)-isomer was confirmed by the co-injection of this sample and the racemic mixture (Fig. 1b).

Only the *S*-(+)-isomer showed *in vitro* antibacterial effects (Table II). This sample showed bactericidal activity to *A. hydrophila* ATCC 7966 and clinical isolate 22/07 at 3.2 mg mL^{-1}, but it did not act against clinical isolates 13/09 10 and 13/09

TABLE I
Chemical composition of *Lippia alba* (LA) and *Ocimum americanum* (OA) essential oils.

Constituent	LA (%)	OA (%)	RI cal	RI ref
α-pinene		0.55	931.8	933[a]
camphene		0.54	945.1	946[a]
sabinene	1.70	0.25	971.6	975[a,b]
β-pinene		0.58	973.2	976[a]
β-myrcene	0.78	0.34	991.3	992[a]
1,8-cineole	8.77	8.43	1031.4	1031[a]
E-β-ocimene	0.86	0.39	1048.5	1050[a,b]
fenchone		3.59	1085.7	1087[a,b]
β-linalool	46.69	46.61	1105.3	1101[a]
camphor	2.90	9.50	1144.8	1144[a]
α-terpineol	0.83	1.79	1190.4	1190[a]
2,6-dimethyl-3,5,7-octatrien-2-ol, *E*, *E*	1.20		1200.0	1134[b]
eugenol		3.22	1362.5	1364[a]
β-elemene	1.17	2.08	1391.8	1391[a]
β-caryophyllene	3.24	3.27	1418.9	1418[a]
γ-elemene	0.70		1430.7	1437[a]
α-guaiene		0.88	1438.3	1439[a]
α-caryophyllene	1.14	1.18	1452.9	1452[a]
germacrene D	4.66	4.76	1481.2	1480[a]
γ-amorphene	0.70		1491.5	1496[a]
τ-elemene		0.96	1495.6	1492[a]
δ-guaiene		1.72	1505.3	1505[a]
δ-amorphene	0.79		1512.0	1512[a]
τ-cadinene		1.06	1513.6	1513[a]
δ-cadinene	0.50	0.21	1523.0	1523[a,b]
germacrene B	2.57		1554.9	1561[a]
E-nerolidol	0.64	0.12	1563.7	1564[a]
germacrene-D-4-ol	1.05		1574.1	1576[a]
caryophyllene oxide	1.87	0.14	1583.8	1583[a]
1,10-di-epi-cubenol		0.47	1616.0	1619[b]
τ-cadinol		3.09	1643.1	1642[a]
Total identified (%)	86.09	99.83		

Rt: Retention time; (%): Relative percentage; RI cal: calculated retention index; RI ref: reference retention index. [a] NIST (2005); [b] Adams (2001). Compounds in amount below 0.5 % have been omitted.

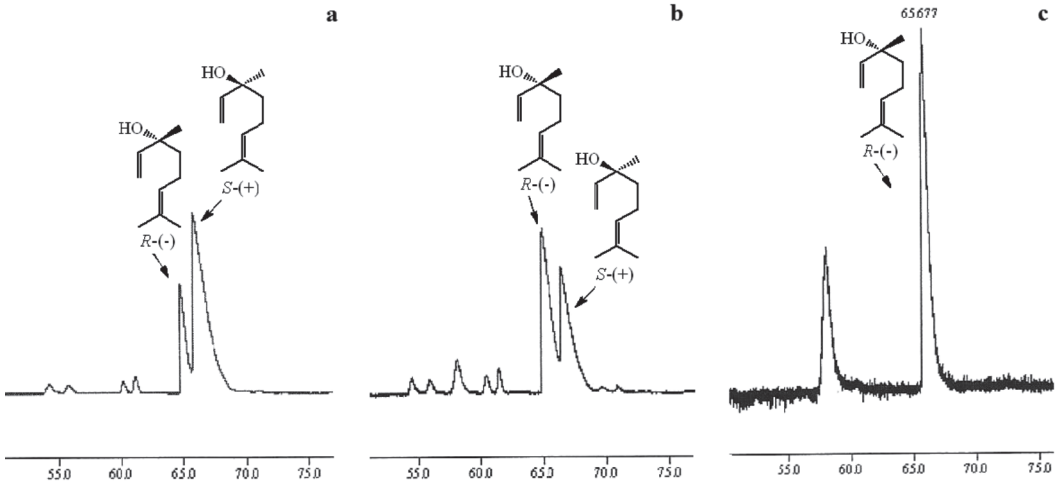

Figure 1 - Chiral chromatograms of the racemic mixture and *S*-(+)-linalool **(a)**, racemic mixture and *O. americanum* EO **(b)** and *O. americanum* EO **(c)**.

TABLE II
Anti-*Aeromonas hydrophila* activity of *S*-(+)- and *R*-(-)-linalool.

Strain	*S*-(+)-linalool		*R*-(-)-linalool	
	MIC (mg mL^{-1})	MBC (mg mL^{-1})	MIC (mg mL^{-1})	MBC(mg mL^{-1})
ATCC 7966	3.2	3.2	>3.2	>3.2
22/07	3.2	3.2	>3.2	>3.2
13/09 05	>3.2	>3.2	>3.2	>3.2
13/09 10	>3.2	>3.2	>3.2	>3.2

5. No *in vitro* activity was detected for the *R*-(-)-form.

No difference was observed in the induction time to stage 2 of anesthesia for silver catfish exposed to *S*-(+)- and *R*-(-)-linalool (Fig. 2a). Significant differences in this stage were only detected among the same sample concentrations. *S*-(+)-linalool at 60 and 180 µL L^{-1} sedated the animals in approximately 25 s while higher concentrations induced the same sedation level in less than 10 s. For *R*-(-)-isomer, an increase in concentration from 60 to 180 µL L^{-1} reduced the induction time. Fish exposed to 180 µL L^{-1} had a similar sedation time compared to those exposed at 300 and 500 µL L^{-1} of the same sample.

Within a concentration range of 180-500 µL L^{-1}, *R*-(-)-linalool generally promoted faster anesthesia compared to the *S*-(+)-isomer. The only exception to this pattern was the concentration of 300 µL L^{-1}, for which stage 4 was achieved with

TABLE III
Relationship between the time required to reach the stages of induction and recovery from anesthesia and the concentration of *R*-(-)- and *S*-(+)-linalool in silver catfish (N=9). x = concentration of essential oil (µL L^{-1}); y = time to reach the stage of induction or recovery from anesthesia (Schoettger and Julin 1967) in seconds (s).

Sample	Stage 4	Recovery
S-(+)-linalool	$1/y = 0.0091-0.1/x^{0.5}$ ($r^2 = 0.999$)	$y = 363.9-2.2x+0.02106x^2-0.000026x^3$ ($r^2 = 1$)
R-(-)-linalool	$y = 257.7+1.6x-0.00378x^2$ ($r^2 = 1$)	$1/y = 0.00381-0.000128/x^{0.5}$ ($r^2 = 0.999$)

S-(+)-isomer (5 min) in less time than the *R*-(-)-isomer (6 min) (Fig. 2b). For both samples, an increase in concentration reduced induction time until stage 4 (Table III). Ethanol alone did not produce any sedative and anesthetic effect.

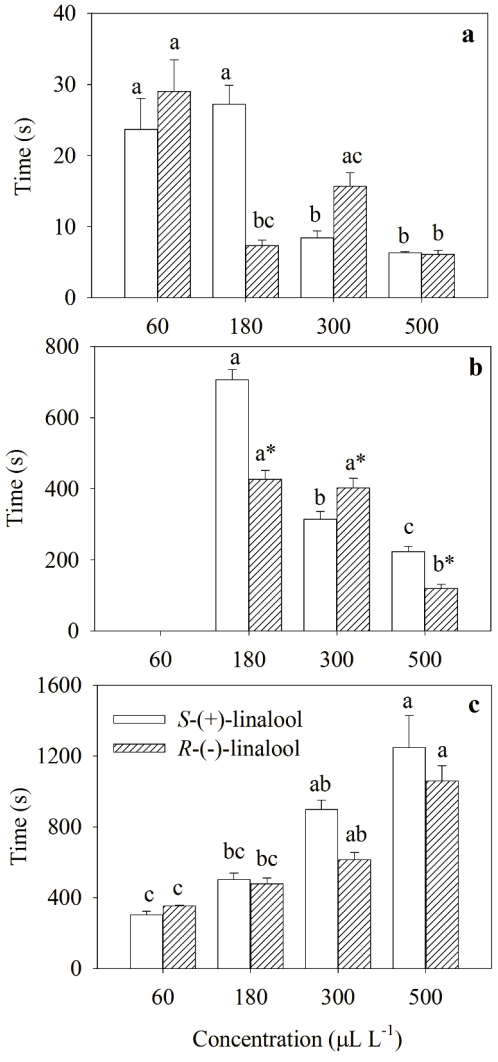

Figure 2 - Time required for anesthesia induction in silver catfish (N= 9) with *S*-(+)- and *R*-(-)-linalool: stage 2 **(a)**, stage 4 **(b)** according to Schoettger and Julin (1967), and recovery time **(c)**. Maximum observation time was 30 min. The time to reach each stage is given in seconds (s). * indicate significant differences between both samples at the same concentration; different lowercase letters correspond to significant differences between concentrations of the same sample. Two-way ANOVA and Tukey test or Scheirer-Ray-Hare extension of the Kruskal–Wallis test and Dunn test were used ($P < 0.05$).

There were no differences between the isomers regarding recovery time (Fig. 2c). However, an increase in linalool concentration was accompanied by an increase in the recovery time, which occurred independently of the evaluated sample (Table III). No side effects or mortality 24 h after exposure was observed to both compounds.

DISCUSSION

The reports about linalool antimicrobial activity are contradictory. Some authors described a wide spectrum of action for this compound (Dorman and Deans 2000, Sonboli et al. 2006) while others only detected antimicrobial activity for one strain of bacteria (Simionatto et al. 2007). These discrepancies among studies could be a consequence of the use of different techniques, growth medium, inoculum concentration or susceptibility of the tested microorganisms (Cos et al. 2006). However, as most studies did not report the chirality of the tested compound, the influence of this parameter in different reported activities may not be disregarded.

In this study, linalool chirality influenced the anti-*Aeromonas hydrophila* activity, since *S*-(+)-isomer was the only compound with antibacterial activity. Özek et al. (2010) detected similar growth inhibition for both compounds at 0.3μM against the microorganism *Botrytis cinerea,* using a microdilution broth assay. Other Gram-positive bacterial and fungal strains tested in the same study were not inhibited until 0.2 mg mL^{-1} by both enantiomers. However, studies performed with other terpenes have also indicated greater antimicrobial effect of dextrorotatory isomers. Aggarwal et al. (2002) demonstrated that (R)-(+)-limonene and (R)-(+)-carvone are the most potent isomers against bacteria and dermatophytic fungi. In other studies, only the dextrorotatory enantiomers of α- and β-pinene were active against *Candida albicans*, *Cryptococcus neoformans*, *Rhizopus oryzae* and methicillin-resistant *Staphylococcus aureus* (Silva et al. 2012b).

Regarding the anti-*Aeromonas* activity, previous reports conducted with racemic mixtures and linalool with no specified chirality verified antibacterial effects against this strain as well (Dorman and Deans 2000, Klein et al. 2013). The bactericidal property of this compound against *A. hydrophila* from dairy was observed at lower concentrations (0.72 mg mL^{-1}) (Klein et al. 2013) than those observed in this study. Similar eugenol concentrations were recently reported to have bactericidal activity against the same ATCC strain and two fish clinical isolates (Sutili et al. 2014).

The *S*-(+)-linalool sedative and anesthetic properties are similar to those described in previous reports (Heldwein et al. 2014). However, we tested other concentrations of this compound; our results allow us to propose that the concentration of 500 $\mu L\ L^{-1}$ is ideal to promote fast anesthesia (less of 4 min). The same proposition can be applied to *R*-(-)-linalool.

Distinct central nervous system effects have been described in the literature for linalool enantiomers. The sedative effects in humans and anticonvulsant activity in rodents seems to be higher for *R*-(-)-linalool than for *S*-(+)-isomer (Sugawara et al. 1998, Sousa et al. 2010). Thus, the absence of isomer differences regarding sedation and recovery time were unexpected, whereas the greater anesthetic effect of the *R*-(-)-form is in accordance to the results obtained from other species. On the other hand, Kasai et al. (2014) did not detect differences in either percentage or latency of the response in surgical anesthesia of *Danio rerio* exposed to *dl*-, *d*-, and *l*-menthol.

Biological differences between isomers have been related to differing interactions with molecular targets. Biological systems macromolecules, e.g., proteins, glycolipids and polynucleotides are formed by L-amino acids and D-carbohydrates chiral building blocks. As these molecules are involved in the pharmacokinetic and pharmacodynamic drug processing, stereoselectivity for one enantiomer can often be observed (Hutt and O'Grady 1996, Mitra and Chopra 2011).

In conclusion, *S*-(+)- and *R*-(-)-linalool showed different biological properties. The use of both isomers as antimicrobial agents against *A. hydrophila* is inadvisable due to their high effective concentration but both are appropriate for sedative purposes. When anesthesia is the main objective, *R*-(-)-linalool demonstrated clear advantages at a lower concentration. However, other studies should be conducted to evaluate if other differences are present in physiological and biochemical parameters following anesthesia or sedation.

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