





Plant essential oils against bacteria isolated from fish: an *in vitro* screening and *in vivo* efficacy of *Lippia organoides*

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ABSTRACT: *The use of natural products, such as essential oils (EOs), is a potential novel approach to treat fish bacterial infections with a lower risk of developing resistance. There has been a number of studies reporting the activity of EOs as those obtained from the species Achyrocline satureioides, Aniba parviflora, Aniba rosaeodora, Anthemis nobilis, Conobea scoparioides, Cupressus sempervirens, Illicium verum, Lippia organoides, and Melaleuca alternifolia against bacteria. However, there are few studies investigating the effect of these EOs against fish bacteria. Therefore, the aim of this study was to evaluate the in vitro antibacterial activity of EOs against the following fish bacteria, Aeromonas hydrophila, Citrobacter freundii, and Raoultella ornithinolytica. Additionally, the in vivo antibacterial activity of the EO L. organoides was evaluated against experimentally induced A. hydrophila infection of silver catfish (Rhamdia quelen). The EO of L. organoides was chosen as it showed the highest in vitro antibacterial activity, with minimum inhibitory concentrations ranging from 0.2 to 0.8 mg mL⁻¹. This EO also presented a therapeutic success of 58.33%, on a 30 day A. hydrophila infection. Therefore, we suggested that the EO of L. organoides may be a viable alternative as a treatment for A. hydrophila infection.*

Key words: *Aeromonas hydrophila, antibacterial activity, silver catfish, longevity, therapeutic success.*

Óleos essenciais de plantas contra bactérias isoladas de peixes: uma triagem *in vitro* e eficácia *in vivo* de *Lippia organoides*

RESUMO: *O uso de produtos naturais, como óleos essenciais (OEs), é uma nova abordagem potencial para o tratamento de infecções bacterianas em peixes com um baixo risco de desenvolvimento de resistência. Existem estudos reportando a atividade de OEs como aqueles obtidos das espécies Achyrocline satureioides, Aniba parviflora, Aniba rosaeodora, Anthemis nobilis, Conobea scoparioides, Cupressus sempervirens, Illicium verum, Lippia organoides e Melaleuca alternifolia contra bactérias. Porém, existem poucos estudos investigando o efeito desses OEs supracitados contra bactérias isoladas de peixes. Portanto, o objetivo desse estudo foi avaliar a atividade antibacteriana in vitro desses OEs contra bactérias isoladas de peixes (Aeromonas hydrophila, Citrobacter freundii e Raoultella ornithinolytica). Assim como avaliar a atividade antibacteriana in vivo do OE de L. organoides contra uma infecção por A. hydrophila experimentalmente induzida em jundiá (Rhamdia quelen). O OE de L. organoides foi escolhido porque apresentou a melhor atividade antibacteriana in vitro, com concentrações inibitórias mínimas variando de 0,2 a 0,8 mg mL⁻¹. Esse OE apresentou sucesso terapêutico de 58,33% no dia 30 pós-infecção. Portanto, sugerimos que o OE de L. organoides possa ser uma alternativa viável no tratamento da infecção por A. hydrophila em peixes.*

Palavras-chave: *Aeromonas hydrophila, atividade antibacteriana, jundiá, longevidade, sucesso terapêutico.*

INTRODUCTION

With the expansion of aquaculture production in recent years, fish have become exposed to adverse conditions, generating stress and consequent immunosuppression in the population. With this, the number of infections caused by opportunistic bacteria has increased, causing growth impairment, mortality,

and serious economic losses for fish producers (BAKER et al., 2013). Therefore, antibiotics are commonly used to reduce and prevent the negative impact of infections caused by opportunistic bacteria. However, their incorrect and indiscriminate utilization has led to the development of multidrug resistance, environmental contamination, and fish toxicity (EL-NAHHAL et al., 2016; GRENNI et al., 2018). The silver catfish (*Rhamdia quelen*) is the

most produced native fish species in south Brazil, being distributed widely, from the southeast of Mexico to the central region of Argentina. It shows good adaptability to different environments and artificial diets, as well as being resistant to handling required for commercial purposes (BALDISSEROTTO & RADÜNZ NETO, 2004). The majority of the diseases reported in silver catfish farms are related to bacterial pathogens, and more specifically to the genus *Aeromonas* (BALDISSEROTTO & RADÜNZ NETO, 2004; BARCELLOS et al., 2008). Furthermore, it has been shown that *Aeromonas hydrophila* and *Citrobacter freundii*, were the cause of fish injuries and mortality of naturally infected silver catfish, and *Raoultella ornithinolytica* was the cause of human intoxication by histamine due to the consumption of contaminated fish (KANKI et al., 2002; BANDEIRA JUNIOR et al., 2017).

Essential oils (EOs) isolated from plants are known for their antimicrobial properties. Specifically, their use against bacteria known to infect fish has been reported (BANDEIRA JUNIOR et al., 2017). The antimicrobial properties of a range of EOs have been studied against a range of microorganisms. These EOs included those obtained from the species *Achyrocline satureioides* (popularly known as “macela”), *Aniba parviflora* (“macacaporanga”), *Aniba roseodora* (“pau-rosa”), *Anthemis nobilis* (“Roman chamomile”), *Conoclea scoparioides* (“pataqueira”), *Cupressus sempervirens* (“Italian cypress”), *Illicium verum* (“star anise”), *Lippia origanoides* (“alecrim-d’Angola”), and *Melaleuca alternifolia* (“tea tree”) (WESELER et al., 2005; GONZÁLEZ & MARIOLI, 2010; ROSATO et al., 2010; MONCAYO, 2012; AL-SNAFI, 2016; SILVA et al., 2016; IBRAHIM et al., 2017; NIKOLIĆ et al., 2017; PERERA et al., 2017). One study reported the *in vivo* antibacterial activity of *L. origanoides* EO against an experimentally induced *A. hydrophila* infection in tambaqui (*Colossoma macropomum*) (OLIVEIRA et al., 2018). Despite this, there are few studies investigating the effect of these EOs against bacteria isolated from fish. Therefore, the aim of this study was to evaluate the *in vitro* antibacterial activity of EOs against *A. hydrophila*, *C. freundii*, and *R. ornithinolytica* isolated from fish, and to further evaluate the *in vivo* antibacterial activity of the EO *L. origanoides*, against experimentally induced *A. hydrophila* infection in silver catfish.

MATERIALS AND METHODS

Clinical isolates

The strains *A. hydrophila* MF 372510, *C. freundii* MF 565839, and *R. ornithinolytica* MF

372511 have been previously isolated from naturally infected juvenile silver catfish obtained from local fish farm (BANDEIRA JUNIOR et al., 2017). The bacteria were isolated from kidneys and skin lesions of naturally diseased fish and plated on blood and MacConkey agar. After 48 h (37 °C) of incubation bacterial growth was observed and the colonies were submitted to gram staining and biochemical tests for presumptive identification (QUINN, 1994). The DNA from single colonies was extracted by boiling method. Molecular identification was obtained by analyzing the partial sequence (~1400 base pairs) of the 16S rRNA gene amplified with universal primers (FREDRICKS & RELMAN, 1998). The PCR product were sequenced by ACT Gene Molecular Analysis (Centro de Biotecnologia, UFRGS, Brazil) using the automatic sequencer ABI-PRISM 3100 genetic analyzer (Applied Biosystems). Sequences were analyzed and their consensus sequences were assembled using 4 s Staden package programs (STADEN et al., 2000). In order to compare the consensus sequence (~1400 bp) with other sequences available on GenBank database, BLASTN tool was performed. The closest relative 16S rRNA sequences were used for the bacterial identification.

Essential oils

The EOs were obtained as follows: *A. nobilis* EO was purchased from Ferquima™ (São Paulo, Brazil), *C. sempervirens*, *I. verum*, and *M. alternifolia* EOs were purchased from Vimontti™ (Santa Maria, Brazil), *A. satureioides* EO was purchased from Mundo dos Óleos™ (Brasília, Brazil), and *A. parviflora*, *A. roseodora*, *C. scoparioides*, and *L. origanoides* EOs were extracted from the dried leaves of the plants (forced air circulation oven at 40 °C for 24 h) by hydro-distillation for 2 h using a Clevenger-type apparatus. The analysis of the chemical components of *L. origanoides* EO was determined by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu GC-17 AAF, V3, 230 LV apparatus, according to BALDISSERA et al. (2017). The five major constituents were carvacrol (40.73%), p-cimene (13.63%), thymol (8.19%), γ-terpinene (5.43%), and β-linalool (4.19%).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

Three clinical isolates (*A. hydrophila* MF 372510, *C. freundii* MF 565839, and *R. ornithinolytica* MF 372511) were used for the MIC and MBC tests. These tests were conducted using the microdilution method, in accordance with the guidelines of the

Clinical & Laboratory Standards Institute (CLSI, 2014). The EOs were diluted in 96% ethanol and incorporated into Mueller Hinton broth (Himedia Laboratories, Mumbai, India) in serial dilutions, from the concentration 6.4 to the concentration 0.0125 mg mL⁻¹, in triplicate. The bacterial inoculum at 1×10⁸ CFU mL⁻¹ (OD₆₀₀ 0.15) was prepared in a saline solution from cultures grown on Mueller Hinton agar (MHA) (Himedia Laboratories, Mumbai, India); for 24 h at 28 °C. Ten microliters (1×10⁵ CFU) of inoculum was added to each well containing the EOs. Microplates were then incubated at 28 °C for 24 h under aerobic conditions for MIC determinations, with the lowest concentration for each of the EOs that inhibited bacterial growth defined as the MIC. Ten microliters of the resazurin dye (0.1% solution) was added in each well as a visual indicator of the MIC. The same procedure was performed with an ethanol control. Values of MBC were obtained by re-plating 10 µL of each bacterial culture into MHA (28 °C/24 h), with the lowest concentration for each of the EOs showing no growth defined as the MBC.

Animals and water quality parameters

Apparently healthy (no external signs of lesions) juveniles silver catfish (n=120, 10±2 cm, 9±1 g) were obtained from a local fish farm and acclimatized for 1 week in 250 L tanks, for use in the *in vivo* experiment. The fish were fed twice a day with an extruded commercial feed, 1.7 mm in size (Supra™ Juvenil, 46% protein). Food and feces that had accumulated at the bottom of tanks were removed daily, and water renewed to 25% every two days. The following water quality parameters were maintained, temperature 20±1 °C, pH 7.3±0.5, dissolved oxygen 6.0±0.5 mg L⁻¹ and a total ammonia concentration of <0.5 mg L⁻¹.

In vivo experiment

Fish were assigned into ten groups, each with twelve animals (n=12). These groups were: uninfected (negative control), florfenicol (Maxflor™ 40%) uninfected, ethanol uninfected, *L. origanoides* EO 2.5 µL L⁻¹ uninfected, *L. origanoides* EO 5.0 µL L⁻¹ uninfected, infected (positive control), florfenicol infected, ethanol infected, *L. origanoides* EO 2.5 µL L⁻¹ infected, and *L. origanoides* EO 5.0 µL L⁻¹ infected. Concentrations greater than 5.0 µL L⁻¹ caused sedation to the animals, so this was the maximum *L. origanoides* EO concentration used. Additionally, the florfenicol concentration used (4.3 mg L⁻¹) was calculated to be equivalent to the highest *L. origanoides* EO concentration (5 µL L⁻¹) based on oil density (0.86 g mL⁻¹). As the EOs were

diluted in ethanol (1:10), a group containing the highest concentration of ethanol used (45 µL L⁻¹) was added.

For the inoculum, *A. hydrophila* MF 372510 was grown on MHA. The inoculum was prepared for the infection model in sterile saline (NaCl 0.9%) with the turbidity adjusted to an of OD₆₀₀ 0.8, equivalent to 6.4×10⁸ CFU mL⁻¹. The uninfected groups were inoculated intramuscularly into the right dorsal region with 100 µL of sterile saline, while the infected groups were inoculated with 100 µL of the inoculum, according to methods previously described (BANDEIRA JUNIOR et al., 2018a). The inoculated fish were distributed into 30 L tanks and submitted to daily baths of 1 h, with the different testing solutions, for 4-days, consecutively. After inoculation, the viability of each of the fish was verified daily for 30 days to observe acute and chronic infection as well as death.

Statistical analysis

Regarding the longevity calculation, the Shapiro-Wilk and Levene tests were used to verify the normality and homogeneity of the variances, and a two-way analysis of variance followed by the Tukey's test were performed to verify differences between groups, and *F* and *p* values were shown only if *p*<0.05. The statistical analysis was performed considering the tank as experimental unit, and the fish as observational unit.

RESULTS

EOs showed antibacterial activity against the fish pathogens

In this study it was reported that the MICs were quite similar to the MBCs, highlighting that most of these EOs were more bactericidal than bacteriostatic. However, there are exceptions (*Aniba parviflora* EO against *A. hydrophila* and *C. freundii*, *Illicium verum*, and *Melaleuca alternifolia* EOs against *A. hydrophila*, and the latter against *R. ornithinolytica*) (Table 1). The *L. origanoides* EO was the most effective, with antibacterial activity against the three tested strains observed, with MICs ranging from 0.2 to 0.8 mg mL⁻¹. The *C. scoparioides* EO showed antibacterial activity against *A. hydrophila* with a MIC and MBC of 0.2 mg mL⁻¹, but weak activity against *C. freundii* and *R. ornithinolytica*. The *A. parviflora* and *A. rosaeodora* EOs presented weak antibacterial activity against the three strains, with MICs ranging from 0.8 to 3.2 mg mL⁻¹. Additionally, the *A. nobilis*, *I. verum*, and *M. alternifolia* EOs presented weak antibacterial activity against *A. hydrophila* and did not show any

Table 1 - Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of EOs against bacteria isolated from fish.

	<i>Aeromonas hydrophila</i> MF 372510		<i>Citrobacter freundii</i> MF 565839		<i>Raoultella ornithinolytica</i> MF 372511	
	MIC mg mL ⁻¹	MBC mg mL ⁻¹	MIC mg mL ⁻¹	MBC mg mL ⁻¹	MIC mg mL ⁻¹	MBC mg mL ⁻¹
<i>Achyrocline satureioides</i>	> 6.4	> 6.4	> 6.4	> 6.4	> 6.4	> 6.4
<i>Aniba parviflora</i>	0.8	1.6	3.2	6.4	3.2	3.2
<i>Aniba rosaeodora</i>	3.2	3.2	3.2	3.2	3.2	3.2
<i>Anthemis nobilis</i>	6.4	6.4	> 6.4	> 6.4	> 6.4	> 6.4
<i>Conobea scoparioides</i>	0.2	0.2	3.2	3.2	3.2	3.2
<i>Cupressus sempervirens</i>	> 6.4	> 6.4	> 6.4	> 6.4	> 6.4	> 6.4
<i>Illicium verum</i>	1.6	3.2	> 6.4	> 6.4	> 6.4	> 6.4
<i>Lippia organoides</i>	0.2	0.2	0.8	0.8	0.8	0.8
<i>Melaleuca alternifolia</i>	3.2	6.4	> 6.4	> 6.4	6.4	> 6.4

activity against *C. freundii* and *R. ornithinolytica*. Furthermore, the *A. satureioides* and *C. sempervirens* EOs did not present any activity against all tested strains. Of the strains, *A. hydrophila* was found to be the most sensitive, with antibacterial activity against it by majority of the EOs tested.

In vivo testing of *L. organoides* EO antibacterial activity

Based on the screening results of the *in vitro* study of the antibacterial effect of EOs, *L. organoides* EO was selected for testing against experimentally induced *A. hydrophila* infection in silver catfish.

A significant interaction was observed between *A. hydrophila* infection and *L. organoides* treatment ($F_{1,0}=4.41$; $p=0.001$). No uninfected fish died during the experimental period; however, 100% of the infected control group with no treatment died. A significant increase to animal longevity was observed in the groups treated with florfenicol and *L. organoides* EO 5.0 $\mu\text{L L}^{-1}$, compared to the non-treated infected control group. Fish treated with *L. organoides* EO 5.0 $\mu\text{L L}^{-1}$ showed a similar longevity to the infected group treated with florfenicol (Table 2). However, the *L. organoides* EO 5.0 $\mu\text{L L}^{-1}$ treatment obtained the best results with a 58.33% survival rate, with treatment with florfenicol and *L. organoides* EO 2.5 $\mu\text{L L}^{-1}$ achieving a 25% survival rate (Table 2).

DISCUSSION

Bacterial diseases are considered as obstacles to the development of aquaculture, with a

global estimate of disease losses in the range of several billion US\$ per year. To control these diseases, large amounts of antibiotics are used, which often results in the development of resistance in aquaculture pathogens (DEFOIRDT, 2014). In this sense, the need arises for the discovery of new substances with activity against fish pathogenic bacteria. Among these substances, we highlighted the EOs.

In relation to the MIC and MBC assay, the *A. hydrophila* strain was more sensitive to *A. parviflora*, *A. nobilis*, *C. scoparioides*, *I. verum*, *L. organoides*, and *M. alternifolia* EOs when compared to *C. freundii* and *R. ornithinolytica* strains. BANDEIRA JUNIOR et al. (2018b) also demonstrated that the *A. hydrophila* strain was more sensitive to phytochemicals than *C. freundii* and *R. ornithinolytica* strains. However, the *A. hydrophila* strain was highly pathogenic in the *in vivo* test because it caused cutaneous ulcerations, hemorrhagic septicemia, and high mortality in experimentally infected silver catfish.

Previously, *A. satureioides* EO has been shown to be associated with antimicrobial activity against *Paenibacillus larvae* (GONZÁLEZ & MARIOLI, 2010). In the present study, this particular plant EO did not present activity against the three tested strains. Furthermore, the *C. sempervirens* EO has been previously shown to have activity against a number of different bacteria, specifically the human pathogens *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Shigella boydii* (IBRAHIM et al., 2017). In addition, the *C. sempervirens* EO also showed activity against several pathogenic bacteria for both animals and plants (MAZARI et al., 2010;

Table 2 - Mortality, longevity, and therapeutic success using *L. origanoides* EO to treat silver catfish experimentally infected with *A. hydrophila* MF 372510. Asterisk (*) indicates a significant difference between the infected and the uninfected fish submitted to the same treatment. Different letters (a-c) indicate significant differences between infected groups.

Groups	Mortality	Longevity (days)	Therapeutic success (%)
Uninfected (negative control)	0/12	30 ± 0.0	-
Uninfected + florfenicol	0/12	30 ± 0.0	-
Uninfected + ethanol	0/12	30 ± 0.0	-
Uninfected + <i>L. origanoides</i> EO 2.5 µL L ⁻¹	0/12	30 ± 0.0	-
Uninfected + <i>L. origanoides</i> EO 5.0 µL L ⁻¹	0/12	30 ± 0.0	-
Infected (positive control)	12/12	9.33 ± 1.63 ^{c*}	-
Infected + florfenicol	9/12	26.5 ± 0.88 ^a	25
Infected + ethanol	11/12	10.33 ± 1.55 ^{c*}	8.33
Infected + <i>L. origanoides</i> EO 2.5 µL L ⁻¹	9/12	15.92 ± 3.10 ^{bc*}	25
Infected + <i>L. origanoides</i> EO 5.0 µL L ⁻¹	5/12	19.25 ± 3.85 ^{ab*}	58.33

BADAWY & ABDELGALEIL, 2014). However, again in the present study this EO showed no antibacterial activity against fish pathogens.

A. parviflora EO has been previously shown to have activity against *E. coli* and *Bacillus cereus* strains (SILVA et al., 2016), but in the present study it was only reported to have weak activity against the three strains tested. Furthermore, an under studied EO *C. scoparioides* has previously been shown to have better activity against Gram-positive bacteria compared to Gram-negative bacteria (MONCAYO, 2012); however, in the present study *C. scoparioides* EO was reported to have antibacterial activity against *A. hydrophila*, but weak activity against *C. freundii* and *R. ornithinolytica*. Combination of *A. rosaeodora* EO with gentamicin has been established to have a synergistic effect against several strains of both Gram-positive and Gram-negative bacteria, but especially *Acinetobacter baumannii* (ROSATO et al., 2010). However, *A. rosaeodora* EO had only weak activity against the tested strains in the present study, possibly due to the strains being Gram-negative bacteria, which are known to be more resistant to EOs (CHAO et al., 2011).

The *A. nobilis* EO have been established to have activity against Gram-positive and Gram-negative bacteria (AL-SNAFI, 2016). Also, *I. verum* EO has been shown to have antibacterial activity against *Helicobacter pylori* (WESELER et al., 2005). However, this EO has been shown to not be effective against oral bacterial pathogens (TARDUGNO et al., 2018). Furthermore, the *M. alternifolia* EO has been previously established to have activity against *S. aureus* as well as a number

of *Streptococcus* spp. (NIKOLIĆ et al., 2017). In addition, this particular EO has been proven to be effective in the treatment of skin infections caused by *Propionibacterium acnes* and oral infections caused by *Streptococcus mutans* and *Lactobacillus* spp. (WULANSARI et al., 2017; TARDUGNO et al., 2018). Though in the present study, all these EOs, only presented weak antibacterial activity against *A. hydrophila* and did not demonstrate any activity against the two other tested bacteria.

In the present study, the most effective EO was reported to be *L. origanoides* EO with antibacterial activity against all three tested strains. Previously, *L. origanoides* EO has been shown to have activity against Gram-positive bacteria such as *S. aureus*, *E. faecalis*, and *Bacillus subtilis*, and Gram-negative bacteria such as *E. coli*, *Salmonella thyphimurium*, *Shigella sonnei*, and *Pseudomonas* spp. (CHATAING et al., 2012; PERERA et al., 2017). Due to the high *in vitro* antimicrobial efficacy of *L. origanoides* EO, this oil was then selected for an *in vivo* investigation, in which infection was experimentally induced with *A. hydrophila* in silver catfish. Strain *A. hydrophila* MF 372510 was selected for the *in vivo* assay because it was isolated from naturally infected silver catfish, which had cutaneous ulcers, hemorrhagic septicemia, and high mortality (BANDEIRA JUNIOR et al., 2017). It was reported that the most effective concentration of EO for the treatment of *A. hydrophila* was 5.0 µL L⁻¹. Another study reported that tambaquis experimentally infected with *A. hydrophila* had a high survival rate (79.2%) after therapeutic bath with 10 mg L⁻¹ of *L. origanoides* EO (OLIVEIRA

et al., 2018).

In our previous study, a screening of antibiotic susceptibility was performed and the multiple antibiotic resistance was calculated, where the bacteria used in this research were reported as multidrug-resistant (BANDEIRA JUNIOR et al., 2018b). Therefore, these results suggested that this EO may be a viable alternative for the treatment of infections by pathogens in fish, especially those caused by *A. hydrophila* resistant to conventional antimicrobials. It is thought that the bactericidal action is due to the bacterial membrane being disrupted by the lipophilic constituents of the EO (ALVIANO & ALVIANO, 2009). Recently it has been demonstrated that an *L. organoides* EO, with a similar composition to what was used in the present study, showed MIC and MBC values of 2.5 mg mL⁻¹ against *A. hydrophila* (MAJOLO et al., 2017). It was suggested that the mechanism may be associated to the rupture of the cell membrane and the subsequent release of liposaccharide, leading to an increase permeability to adenosine triphosphate. In the present study the main constituent of *L. organoides* EO was reported to be carvacrol, comprising 40.73 % of the composition. Previously, carvacrol has been shown to have the ability to cause the *A. hydrophila* membrane to rupture, causing bacterial death, reducing the mortality of channel catfish (*Ictalurus punctatus*) infected by this bacterium (ZHENG et al., 2009; SOUSA et al., 2015). Therefore, it is most likely carvacrol playing a key role in the antibacterial activity for *L. organoides* EO reported in the present study; however, synergistic activity with other compounds of this EO is also possible.

There is a shortage of treatments for bacterial infections in fish, with oxytetracycline, florfenicol, and sulfadimethoxine/ormetoprim being the only antimicrobials approved by the Food and Drug Administration for use in aquaculture (FDA, 2014). This makes treatment of *A. hydrophila* infection in fish a challenge, mortality remaining high as a consequence of cutaneous ulcers and hemorrhagic septicemia, even with treatment using conventional antimicrobials (BARCELLOS et al., 2008; NGUGI et al., 2015). Therefore, the *L. organoides* EO, with a therapeutic success of 58.33% in improving the lifespan of fish infected with *A. hydrophila*, is a promising alternative to be used in the treatment of this infection.

CONCLUSION

The *L. organoides* EO had the highest *in vitro* antibacterial activity, and *in vivo*, this EO showed the ability to have a therapeutic effect against

infection by *A. hydrophila* with lower mortality of the fish. Therefore, *L. organoides* EO represented a possible new alternative in the treatment of infections caused by *A. hydrophila* in the fish population.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All experimental protocols involving animals was approved by the Ethics Commission for Animal Use (CEUA) at Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil (Protocol 74/2014-CEUA) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the manuscript design and writing. All authors critically revised the manuscript and approved the final version.

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