



## ***Lippia alba* essential oil promotes survival of silver catfish (*Rhamdia quelen*) infected with *Aeromonas* sp.**

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### ABSTRACT

*In vitro* and *in vivo* activity of the *Lippia alba* essential oil (EO) against *Aeromonas* sp. was evaluated. In the *in vitro* assay the minimum inhibitory concentration (MIC) and a minimum bactericidal concentration (MBC) of EO for *Aeromonas* cells were determined using the microdilution method. Twenty five strains of *Aeromonas* sp. isolated from infected fish obtained from local fish farms were used. MIC and MBC values were 2862 and 5998  $\mu\text{g mL}^{-1}$  for *L. alba* EO and 0.5 and 1.2  $\mu\text{g mL}^{-1}$  for gentamicin, respectively. In the *in vivo* assay silver catfish juveniles (*Rhamdia quelen*) ( $7.50 \pm 1.85$  g and  $10.0 \pm 1.0$  cm) with typical injuries associated to *Aeromonas* infection were divided into four treatments (in triplicate  $n=10$ ): untreated fish (negative control), 10 mg  $\text{L}^{-1}$  of gentamicin, and 20 or 50  $\mu\text{L L}^{-1}$  of EO. Fish were maintained in aerated 20 L plastic boxes. After 10 days survival of silver catfish infected with *Aeromonas* sp. and treated with essential oil (50  $\mu\text{L L}^{-1}$ ) was greater than 90%.

**Key words:** antibacterial activity, essential oils, fish, gentamicin.

### INTRODUCTION

Various drugs have been used for treatment or prevention in aquaculture diseases. The abuse of these drugs is associated with several problems, such as the presence of illegal residues in meat and transmission of resistant bacteria to the environment, animals and man, a potential risk to public health (Schmidt et al. 2000). As an alternative to conventional drugs, medicinal plants have been widely used in veterinary and human medicine and nowadays also have a

significant role in aquaculture as prophylactic and therapeutic agents against fish pathogens, presenting antiviral, antibacterial, antifungal and antiparasitic potential (Citarasu 2010). Essential oils and extracts from plants could provide a viable alternative to conventional drugs minimizing the economic losses resulting from infections. These natural products contain a rich blend of highly functional molecules some of which are beneficial but others which are not, presenting toxicity. However, in the environment, this toxicity is very low compared with those of synthetic pesticides or drugs (Park et al. 2011).

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Outbreaks of bacteria of the genus *Aeromonas* in fish are associated with changes in environmental conditions and/or stress factors like parasitic infections (Barcellos et al. 2008). Usually considered a secondary pathogen, bacteria of *Aeromonas* genus can also act as a primary pathogen in some environments, causing high mortality in fish farms (Nielsen et al. 2001).

The aim of the present study was to find a natural alternative to prevent/treat infections caused by bacteria of *Aeromonas* genus in *Rhamdia quelen*, a native species suitable for fish culture in southern Brazil. *Lippia alba* was chosen because of its antimicrobial and anti-inflammatory potential (Pascual et al. 2001, Glamočlija et al. 2011).

## MATERIALS AND METHODS

### ESSENTIAL OIL

*Lippia alba* (Mill.) N. E. Brown was cultivated in São Luiz Gonzaga, Rio Grande do Sul, Brazil. The plant material was identified by botanist Dr. Gilberto Dolejal Zanetti, Departamento de Farmácia Industrial, Universidade Federal de Santa Maria (UFSM), and a voucher specimen (SMDBN<sup>o</sup>. 10050) was deposited in the UFSM herbarium. *Lippia alba* essential oil (EO) was extracted from the fresh leaves of the plant by hydrodistillation for 2 hours using a Clevenger type apparatus (European Pharmacopoeia 2007). The EO samples were stored at -4 °C in amber glass bottles and EO analysis was performed by gas chromatography-mass spectrometry (GC/MS). The major components were linalool and 1,8-cineol (Table I) (Barros et al. 2009).

### IN VITRO ASSAY

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EO for *Aeromonas* cells were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute, document M31-A3 (CLSI 2008). The EO was previously diluted in ethanol to obtain the

initial concentration of 176100 µg mL<sup>-1</sup> and two-fold dilutions were performed. Twenty five isolates of *Aeromonas* sp. from infected fish obtained from local fish farms were used. Reference samples (*Aeromonas hydrophila* ATCC 7966, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922) were used as quality control. The inoculum was prepared in saline solution from cultures grown in Mueller-Hinton agar (1x10<sup>8</sup> CFU mL<sup>-1</sup>; 0.14 OD 600 nm) and 10 µL (1x10<sup>5</sup> CFU) was added to each microplate well containing EO. The microplates were incubated at 35 °C for 24 hours under aerobic conditions. The positive control was performed with gentamicin antibiotic.

### IN VIVO ASSAY

Silver catfish juveniles (7.50 ± 1.85 g and 10.0 ± 1.0 cm) were obtained from a fish culture and transported to the laboratory where they were kept in continuously aerated 250 L tanks (23 ± 1°C, pH 6.6 - 7.0, dissolved oxygen levels 5.8 - 7.2 mg L<sup>-1</sup>). Fish arrived at the laboratory with typical injuries associated to *Aeromonas* infection. Bacterial infection of fish by *Aeromonas* was confirmed by morphological and biochemical characteristics analyzed in accordance to Quinn (1994).

To evaluate silver catfish survival, juveniles were divided into four treatments (in triplicate n=10): untreated fish (negative control); 10 mg L<sup>-1</sup> of gentamicin (Belem-Costa and Cyrino 2006), and two EO concentrations (20 and 50 µL L<sup>-1</sup>) equivalent to 16 and 40 mg L<sup>-1</sup>, respectively, because the density of this EO is approximately 0.80 g mL<sup>-1</sup>. Fish were maintained for 10 days in aerated 20 L polyethylene boxes. These concentrations were chosen because *L. alba* EO in concentrations above 100 µL L<sup>-1</sup> induce deep anesthesia in *R. quelen* (Cunha et al. 2010). Fish were fed once a day to satiety, and 30 min later feces and food remains were removed by siphoning. To avoid dilution of the EO the water volume withdrawn during each siphoning (250

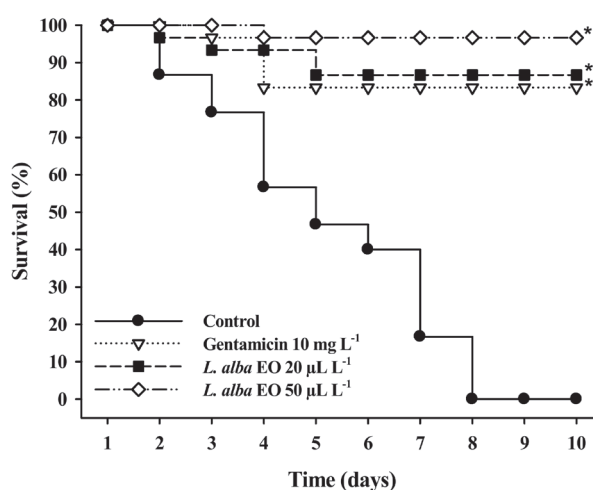
mL) was not replaced until eighth day, when all water was replaced. The methodology of this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process N. 046/2010).

Dissolved oxygen and temperature were measured with YSI oxygen meter. The water pH was verified with a DMPH-2 pH meter. Total ammonia nitrogen (TAN) levels were determined by Nessler method in accordance to Eaton et al. (2005). Un-ionized ammonia ( $\text{NH}_3$ ) levels were calculated as per Colt (2002).

Fish survival was compared by Kaplan-Meier survival analysis with Logrank Test (SPSS 18 Software). The minimum significance level was set at  $P < 0.05$ .

#### RESULTS AND DISCUSSION

In the *in vitro* test MIC and MBC values were 2862 and 5998  $\mu\text{g mL}^{-1}$  for *L. alba* EO and 0.5 and 1.2  $\mu\text{g mL}^{-1}$  for gentamicin, respectively (Table II). At the beginning of the experiment 85% of the fish from each treatment had signs of infection, including ulcerated fins and tissue loss between fin rays with haemorrhage, ulcerative dermal lesions, abnormal coloration (darkening) and lesions in the genital pore and anus. In the control group, these signs and lesions remained and gradually increased until all the fish died. Between the fourth and sixth day of treatment, 65% of fish treated with 50  $\mu\text{L L}^{-1}$  EO did not show evidence of any lesions. Fish treated with 20  $\mu\text{L L}^{-1}$  EO or gentamicin only showed the same improvement between the sixth and eighth days of the experiment. The mortality in the control group was 100% at the end of experiment. In groups treated with gentamicin (10  $\text{mg L}^{-1}$ ) and 20  $\mu\text{L L}^{-1}$  of EO survival was greater than 80%. In the group treated with 50  $\mu\text{L L}^{-1}$  of EO survival was greater than 90% (Figure 1). The survival was not significantly different between groups treated with EO and gentamicin.



**Figure 1** - Survival of juvenile silver catfish infected with *Aeromonas* sp. and treated with *L. alba* EO. (\*) indicates significant difference relative to negative control. Kaplan-Meier survival analysis with Logrank test ( $P < 0.05$ ).

The active compounds of herbs possess characteristics that could be useful in fish and shrimp culture; various herbs can stimulate growth and appetite, increase immune system responses, and have broad spectrum antimicrobial activity (Friedman et al. 2002). Different species of *Lippia* have been used against Gram-positive and Gram-negative bacteria (Pascual et al. 2001). According to Ríos and Recio (2005) only extracts or essential oils that kill or inhibit bacterial growth at concentrations below 100  $\mu\text{g mL}^{-1}$  should be considered active. Based on their statement, *L. alba* EO would not be interesting for future research because a MIC of 2862  $\mu\text{g mL}^{-1}$  was observed. However, the *in vivo* results of the test conducted with silver catfish infected with *Aeromonas* sp. and treated with this EO demonstrated higher survival compared with control group.

Plasma cortisol level is widely used as a general indicator of stressful situations in vertebrates and particularly in fish (Pickering and Pottinger 1989) and high plasma cortisol levels cause secondary immunosuppression (Wedemeyer and Mcleay 1981). The observed effect of *L. alba* EO may

be due to its effects on the fish' immune system because this EO prevented the cortisol rise in *R. quelen* submitted to acute stress (Cunha et al. 2010). Glycyrrhizin (Jang et al. 1995), aloe (Kim et al. 1999), and azadirachtin (Logambal and Michael 2001) enhance innate immunity in fishes. Oral administration of *Quillaja saponin* increased leucocyte migration in yellowtail *Seriola quinqueradiata* (Ninomiya et al. 1995). The use of diets supplemented with the medicinal plants *Cynodon dactylon*, *Aegle marmelos*, *Withania somnifera*, and *Zingiber officinale* increased non-specific immune responses and bacterial resistance against *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Vibrio campbelli*, *Vibrio vulnificus*, *Vibrio harveyi*, and *Photobacterium damsela*, and these compounds lead to lower mortality in tilapia (*Oreochromis mossambicus*) compared with control fish (Immanuel et al. 2009). The exact mechanism of action of herbal extracts on the fish immune system is still unclear (Harikrishnan et al. 2011).

The pathogenesis of *Aeromonas* genus is multifactorial and depends upon the secretion of numerous extracellular virulence factors (Yu et al. 2005). Research shows that subinhibitory concentrations of EOs from plants decrease or inhibit the production of certain virulence factors in bacteria. Essential oils of plants can inhibit biofilm formation or the production of exotoxins such as haemolysins that cause erythrocytes hemolysis thus reducing the bacteria pathogenicity (Smith-Palmer et al. 2004, Cândido et al. 2010, Millezi et al. 2013). *Thymus vulgaris* EO significantly reduced biofilm formation in *Aeromonas hydrophila*. The major compound in *T. vulgaris* EO was 1,8-cineol (Millezi et al. 2013) which is also found in *L. alba* EO. Linalool-rich EO from *L. alba* inhibited the activity of proteases and keratinases secreted from dermatophytes, which are important virulence factors in this fungus (Costa et al. 2014).

Additionally, it is desirable that antibiotic use in fish cultures be reduced and replaced by natural medicines to prevent the emergence of bacterial resistance in aquatic animals and its environment. In Brazil, antibiotics, such as oxytetracycline, are widely used in fish farms to prophylactically treat bacterial infections and as a growth promoter. Despite its widespread use, there is no regulation on this drug class in fish (Rigos and Troisi 2005).

Our results demonstrate that *L. alba* EO has weak *in vitro* activity against *Aeromonas* sp. However, the EO added to water at lower concentrations than the anesthetic concentration, promotes survival in fish infected with this bacterial genus. Further studies should be conducted to verify this activity against other pathogenic bacteria of interest in aquaculture and to confirm immune response involvement or its potential as a virulence factors inhibitor.

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#### RESUMO

Avaliou-se a atividade *in vitro* e *in vivo* do óleo essencial (OE) de *Lippia alba* contra *Aeromonas* sp. No ensaio *in vitro* foram determinadas a concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) do OE contra *Aeromonas* utilizando o método de microdiluição. Foram utilizadas 25 isolados de *Aeromonas* sp. isoladas de peixes infectados obtidos de pisciculturas locais. Os valores de CIM e CBM foram 2862 e 5998  $\mu\text{g mL}^{-1}$  para o OE de *L. alba* e

0.5 e 1.2  $\mu\text{g mL}^{-1}$  para gentamicina, respectivamente. No ensaio *in vivo* juvenis de jundiá (*Rhamdia quelen*) (7.50  $\pm$  1.85 g e 10.0  $\pm$  1.0 cm) com típicas lesões associadas à infecção por *Aeromonas* foram divididos e 4 tratamentos (em triplicata n=10): peixes não tratados (controle negativo), gentamicina 10 mg L<sup>-1</sup>, e 20 ou 50  $\mu\text{L L}^{-1}$  do OE. Os peixes foram mantidos em caixas plásticas aeradas de 20 L. Após 10 dias a sobrevivência de jundiás infectados com *Aeromonas* sp. e tratados com o OE (50  $\mu\text{L L}^{-1}$ ) foi superior a 90%.

**Palavras-chave:** atividade antibacteriana, gentamicina, óleos essenciais, peixes.

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