TOXICITY OF *Lippia origanoides* ESSENTIAL OIL IN TAMBAQUI (*Colossoma macropomum*) AND ITS EFFECT AGAINST *Aeromonas hydrophila*

**ABSTRACT**

The study evaluated the toxicity of *Lippia origanoides* essential oil (EO) in tambaqui (*Colossoma macropomum*) and its in vivo activity against *Aeromonas hydrophila*. Toxicity was tested by tambaqui exposure to 0, 5, 10, 12.5, 15, 17.5 and 20 mg L\(^{-1}\) of *L. origanoides* EO for 96 h. The mean lethal concentration (LC\(_{50}\)) was 15.2 mg L\(^{-1}\). After tambaqui exposure to sublethal concentrations of *L. origanoides* EO (7.6 and 11.4 mg L\(^{-1}\)) for 96 h, only the 7.6 mg L\(^{-1}\) level caused minor hematologic and biochemical changes that include increase in mean corpuscular volume and decrease in total protein, which did not compromise fish homeostasis. Tambaqui experimentally infected with *A. hydrophila* had higher survival rate (79.2%) after therapeutic bath with 10 mg L\(^{-1}\) *L. origanoides* EO. Therefore, tambaqui exhibits moderate tolerance to *L. origanoides* EO, and this oil has potential for use in the treatment of tambaqui infected with *A. hydrophila*.

**Key words:** fish; hematology; LC\(_{50}\); pathogenic bacteria; survival.

**INTRODUCTION**

Tambaqui (*Colossoma macropomum*) is the main native fish species farmed in Brazil with a production of 137 thousand metric tons in 2016 (IBGE, 2016). In intensive production, its body weight reaches 2.62 kg, and after 10 months of growing in dugout ponds, it can yield 18,530 kg ha\(^{-1}\) (IZEL *et al*., 2013). In several regions of the country, however, the growth and intensification of fish farming systems are commonly accompanied by inadequate management practices and outbreaks of parasitic and bacterial diseases (CHAGAS *et al*., 2015; VALLADÃO *et al*., 2016; TAVARES-DIÁS and MARTINS, 2017).

In aquaculture, bacterial diseases account for economic losses worldwide (AUSTIN and AUSTIN, 2007). In Brazilian fish farms, such outbreaks are frequently recorded...
(SEBASTIÃO et al., 2015; VALLADÃO et al., 2016; TAVARES-DIAS and MARTINS, 2017) and are mainly associated with the bacteria Aeromonas hydrophila, Flavobacterium columnare and Streptococcus agalactiae, which cause hemorrhagic septicemia, columnaris and streptococcosis, respectively (FIGUEIREDO and LEAL, 2008; PILARSKI et al., 2008; SEBASTIÃO et al., 2015).

Antibiotics and other drugs are used to treat bacterial diseases in aquaculture, but their indiscriminate application has caused environmental pollution, residue accumulation in the muscle of commercialized fish and the development of drug-resistant bacterial strains (CHAKRABORTY and HANCZ, 2011). For instance, A. hydrophila isolated from pacu (Piaractus mesopotamicus) and Nile tilapia (Oreochromis niloticus) in Brazil were found to be resistant to antibiotics (BELÉM-COSTA and CYRINO, 2006). Thus, essential oils (EO) and bioactive plant extracts have been increasingly investigated as alternative therapeutic products for aquaculture (REVERTER et al., 2014; HASHIMOTO et al., 2016; SILVA et al., 2017; BRUM et al., 2017).

Studies on the control of bacterial diseases by natural products show promising results (ZHENG et al., 2009; ALSAID et al., 2010; MEEPAGALA et al., 2013). In Nile tilapia, Cratoxyllum formosum extract was found to control S. agalactiae, and therapeutic baths with Centella asiatica extract efficiently reduced mortality of fish infected with F. columnare, in a dose-dependent response (RATTANACHAIKUNSOPON and PHUMKHACHORN, 2010a,b). In addition, Lippia alba EO increased survival time of tambaqui (Rhamdia quelen) after the challenge test with A. hydrophila (SUTILI et al., 2015a).

Different Lippia species have been tested in aquaculture due to the antimicrobial potential of this genus (SUTILI et al., 2015a; MAJOLO et al., 2017; SOUZA et al., 2017a). One important species is Lippia origanoides, native of Central and South America and popularly known as salva-de-Marajó (LORENZI and MATOS, 2008). Its EO contains carvacrol, thymol and γ-terpinene, which show antimicrobial activity against Lactobacillus casei, Streptococcus mutans, Salmonella enteritidis, Escherichia coli and A. hydrophila (OLIVEIRA et al., 2007; HENAO et al., 2010; BETANCOURT et al., 2012; MAJOLO et al., 2017).

The present study evaluated the toxicity of L. origanoides EO in tambaqui and its in vivo activity against A. hydrophila.

METHODS

Plant species, extraction and chemical characterization of the essential oil

Voucher specimens of the L. origanoides (family Verbenaceae) plants were deposited in the IAN Herbarium, at Embrapa Amazônia Oriental, Belém, Pará state (PA) (No. 191734). The plants were cropped in the Medical Plants and Vegetables Division of Embrapa Amazônia Ocidental, Manaus, Amazonas state (AM). The branches were cut and the leaves separated and dried in the shadow until reaching constant weight. In the Laboratory of Medical Plants and Phytochemistry, essential oil (EO) from the dried leaves was extracted by hydro distillation for 2 h in a Clevenger-type apparatus. The EO was stored frozen in amber flasks, at -4 °C. The chemical composition of L. origanoides EO was determined by gas chromatography and mass spectrometry at Embrapa Agroindústria de Alimentos, according to POTZERNHEIM et al. (2012).

Fish acclimatization

The juvenile tambaqui were obtained from the Santo Antônio Farm (Rio Preto da Eva, AM) and transported to the experimental field at Embrapa Amazônia Ocidental (Manaus, AM), where they were held in 200 m² ponds. An YSI Pro20 dissolved oxygen meter and YSI F-1100 digital pH meter were used to measure water parameters three times a week. On average, these were kept at a temperature of 29.19 ± 0.29 °C, dissolved oxygen of 5.82 ± 0.22 mg L⁻¹ and pH of 6.32 ± 0.07. Alkalinity (17.4 ± 2.88 mg L⁻¹) and total ammonia (0.98 ± 0.10 mg L⁻¹) were measured every 15 days using EDTA and indophenol titration, respectively (APHA, 1998). In this period, fish were fed commercial food for omnivorous fish containing 32% crude protein (CP).

Acute toxicity (96-h LC50) of Lippia origanoides EO

In the assays determining mean lethal concentration (LC50) of L. origanoides EO, the tambaqui (n = 168; 139.33 ± 5.52 g and 19.94 ± 0.35 cm) were transferred to 80-L polyethylene tanks supplied with a static constant aeration system. After adjusting to the conditions for 48 h, fish were exposed to L. origanoides EO at 0, 5, 10, 12.5, 15, 17.5 and 20 mg L⁻¹ concentrations for 96 h, in triplicate.

Mortality rate and feed intake were recorded and fish behavior was observed 24, 48, 72 and 96 h after L. origanoides EO was introduced into the water, twice a day. The behaviors recorded included loss of balance, opercular movement and erratic swimming. Fish were considered dead when resting motionless on the bottom of the tank, with no operculum movement and unresponsive to mechanical stimulation.

Water quality was monitored during the tests, showing a temperature of 28.7 ± 0.5 °C, dissolved oxygen of 6.3 ± 0.3 mg L⁻¹, pH of 6.65 ± 1.41, alkalinity of 16.02 ± 0.46 mg L⁻¹ and total ammonia content of 0.41 ± 0.04 mg L⁻¹.

Sublethal effects of Lippia origanoides EO on tambaqui

After 96-h LC50 was determined, another batch of tambaqui (n = 72, 149.44 ± 7.38 g and 21.37 ± 0.33 cm) were exposed to sublethal concentrations of L. origanoides EO for the same period. The treatments, performed in triplicate, consisted of exposure to 0%, 50% and 75% of the 96-h LC50, that is, 0 (control), 7.62 and 11.43 mg L⁻¹ EO, respectively.

To evaluate hematologic and biochemical parameters, after 96 h exposure to the treatments, fish were anesthetized with 100 mg L⁻¹ of benzocaine and their blood collected by caudal vein puncture with heparinized syringes.
The hematologic parameters determined were hematocrit (HCT), hemoglobin (Hb) and red blood cells (RBC). For HCT determination, blood samples were centrifuged in heparinized capillary tubes (15,000 g for 10 min) before being read on a standardized scale. Hb was determined by the cyanmethemoglobin method, and RBC count was performed in a Neubauer chamber after blood dilution in formalin citrate fluid. These parameters were used to calculate mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) (BROW, 1988). Commercial kits (Labtest®) were used to determine plasma glucose applying the glucose oxidase method and total protein by the biuret method.

Water quality was monitored during the tests and kept at a temperature of 28.36 ± 0.84 °C, dissolved oxygen of 6.71 ± 0.60 mg L⁻¹, pH of 6.24 ± 0.80, alkalinity of 17.76 ± 0.41 mg L⁻¹ and total ammonia content of 0.50 ± 0.07 mg L⁻¹.

**Effect of Lippia origanoides EO on survival of tambaqui infected with Aeromonas hydrophila**

The juvenile tambaqui (n = 96, 90.56 ± 5.54 g and 18.01 ± 0.36 cm) were held in twelve 1000-L fiberglass tanks (12 fish per tank) supplied with water recirculation and constant aeration. Fish were allowed to adapt to tank conditions for 30 days, fed a diet containing 32% CP twice a day, to apparent satiety.

A completely randomized design was used with 4 treatments and 3 replicates. The treatments were: 1) control (fish inoculated with A. hydrophila and not treated with EO), 2) antibiotic treatment (10 mg L⁻¹ chloramphenicol), 3) EO treatment 1 (5 mg L⁻¹ L. origanoides), and 4) EO treatment 2 (10 mg L⁻¹ L. origanoides). To that end, fish were collected, anesthetized with 100 mg L⁻¹ of benzocaine and inoculated with 1.0 x 10⁸ CFU A. hydrophila. They were then transferred to 80-L tanks supplied with a static constant aeration system, and the therapeutic bath (specific for each treatment) was applied for 60-min and repeated for 5 consecutive days. Fish were then returned to 1000-L fiberglass tanks (SUTILI et al., 2015a,b). Fish mortality was assessed every 24 h for 10 days.

Water quality was monitored during the tests, showing a temperature of 29.62 ± 0.04 °C, dissolved oxygen of 6.97 ± 0.03 mg L⁻¹, pH of 7.28 ± 0.06, alkalinity of 17.22 ± 0.44 mg L⁻¹ and total ammonia content of 0.45 ± 0.04 mg L⁻¹.

**Statistical analysis**

The results obtained are expressed as mean ± standard error. LC₅₀ was calculated using the Trimmed Spearman Karber method (HAMILTON et al., 1977). The homogeneity of variances of data was tested by Levene Test and the data normality assessed by the Shapiro-Wilk test. Data on response to sublethal EO exposure and survival (data transformed) were compared by analysis of variance (one-way ANOVA), followed by a post hoc test (Tukey and Duncan, respectively) (P < 0.05).

**RESULTS**

Eighteen compounds were identified in the essential oil of L. origanoides, comprising 92.7% of the oil composition. Quantitatively, the most abundant were carvacrol (49.7%), para-cymene (13.3%) and thymol (9.9%) (Table 1).

Mortalities were not observed after 96 h exposure to the treatments with 0, 5 and 10 mg L⁻¹. On the other hand, no fish survived using a concentration of 20 mg L⁻¹ EO (Figure 1). The 96-h LC₅₀ of L. origanoides EO for tambaqui, with a 95% confidence interval, is shown in Figure 1.

### Table 1. Chemical composition of Lippia origanoides essential oil.

<table>
<thead>
<tr>
<th>Components</th>
<th>L. origanoides (%)</th>
<th>RI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-2-hexenal</td>
<td>0.5</td>
<td>853</td>
</tr>
<tr>
<td>α-thujene</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>α-pinene</td>
<td>0.5</td>
<td>936</td>
</tr>
<tr>
<td>1-octen-3-ol</td>
<td>0.6</td>
<td>977</td>
</tr>
<tr>
<td>myrcene</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>α-terpinene</td>
<td>1.1</td>
<td>1016</td>
</tr>
<tr>
<td>para-cymene</td>
<td>13.3</td>
<td>1025</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>0.9</td>
<td>1032</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>4.5</td>
<td>1059</td>
</tr>
<tr>
<td>linalool</td>
<td>2.8</td>
<td>1096</td>
</tr>
<tr>
<td>ipsdienol</td>
<td>0.4</td>
<td>1144</td>
</tr>
<tr>
<td>umbelulone</td>
<td>1.1</td>
<td>1175</td>
</tr>
<tr>
<td>thymol methyl ether</td>
<td>0.9</td>
<td>1232</td>
</tr>
<tr>
<td>thymol</td>
<td>9.9</td>
<td>1288</td>
</tr>
<tr>
<td>carvacrol</td>
<td>49.7</td>
<td>1298</td>
</tr>
<tr>
<td>carvacrol acetate</td>
<td>0.4</td>
<td>1369</td>
</tr>
<tr>
<td>(E)-β-caryophyllene</td>
<td>1.5</td>
<td>1414</td>
</tr>
<tr>
<td>carvophyllene oxide</td>
<td>1.0</td>
<td>1576</td>
</tr>
<tr>
<td>Total identified compounds</td>
<td>92.7</td>
<td></td>
</tr>
</tbody>
</table>

*Retention Index.

**Figure 1.** Mortality rate 96h after exposure of juvenile tambaqui (Colossoma macropomum) to different concentrations of Lippia origanoides essential oil.
In the acute toxicity evaluation, the EO concentrations tested (except for the control treatment) promote some behavioral alterations such as accelerated opercular movement and mucus secretion 24h after exposure. These responses were more pronounced in treatments with 17.5 and 20 mg L⁻¹ EO.

Tambaqui exposure to the two sublethal concentrations of *L. origanoides* EO (7.6 and 11.4 mg L⁻¹) for 96 h did not change HCT, Hb, RBC, MCHC or plasma glucose, but at 7.6 mg L⁻¹ it increased MCV and decreased total protein in relation to the control group (Table 2).

After the therapeutic bath assay, treatments with EO were statistically different between them, but they did not differ from the control group or those treated with antibiotics (Figure 2).

**DISCUSSION**

The major compounds of *L. origanoides* EO used in this study were carvacrol (49.7%), para-cymene (13.3%) and thymol (9.9%). These compounds were also found in the EO of *L. origanoides* collected from the Chicamocha river canyon (Santander, Colombia) by VICUNA et al. (2010), but in this study thymol (34–58%) presented a higher relative amount than that observed in the present study, suggesting that these variations can be influenced by differences in climatic conditions, geographical origin, seasonality, stage of plant development, procedures adopted for plant processing and oil extraction, and plant chemotype (SANTOS et al., 2004; MARCIAL et al., 2016).

Few studies report the toxicity of essential oils to fish (YAO et al., 2011; ABD EL-GALIL and ABOELHADID, 2012; KUMAR et al., 2012). To *L. origanoides* EO this information is scarce. To *Chenopodium abrosioides* aqueous extract the 24-h LC₅₀ for tambaqui is 2.6 ml L⁻¹ (MONTEIRO, 2012), and the 4h-LC₅₀ of Mentha piperita EO for pirarucu (*Arapaima gigas*) is 38 mg L⁻¹ (MALHEIROS et al., 2016). Compared to these studies, tambaqui was more tolerant to *L. origanoides* EO (LC₅₀ 15.24 mg L⁻¹), especially because the evaluation period was longer (96 h). The results obtained in this study with tambaqui are important given the scarcity of studies investigating the toxicity of *L. origanoides* EO or its main components, which are carvacrol, p-cymene and thymol.

In the toxicity test to tambaqui was observed an accelerated opercular movement and mucus secretion after exposure to EO *L. origanoides*, being more pronounced at higher EO concentrations (17.5 and 20 mg L⁻¹). For other *Lippia* species was observed that tambaqui treated with 30-min baths containing *L. alba* EO at higher concentrations (100 and 150 mg L⁻¹) showed an increase in opercular beat rate and mucus secretion along with severe gill lesions, such as hyperplasia and lamellar epithelial fusion, congestion, edema and necrosis, proliferation of mucous and chloride cells and lamellar hypertrophy (SOARES et al., 2016).

Tambaqui exposure at a concentration of 7.6 mg L⁻¹ of *L. origanoides* EO increased MCV and decreased total protein in relation to the control group. These results are different to...
those of SOARES et al. (2017a) who related an increased in plasma protein concentration in tambaqui exposed to 20 and 40 mg L\(^{-1}\) of \textit{L. origanoides} essential oil for 60 and 30 minutes, respectively. For other \textit{Lippia} species, increased MCV was observed in tambaqui exposed to 20 mg L\(^{-1}\) of \textit{L. sidoides} for 15 minutes because of a decrease in the number of erythrocytes, hematocrit and hemoglobin (SOARES et al., 2017b). Considering plasma glucose levels, no significant alterations were observed between treatments with \textit{L. origanoides} EO, probably due to the anaesthetic effect of this oil, as observed in others studies with tambaqui during the therapeutic baths with \textit{L. origanoides} EO (SOARES et al., 2017a). Similar pattern was observed by SENA et al. (2016) using \textit{L. alba} EO to reduce effects of stress in tambaqui (\textit{P. mesopotamicus} x \textit{C. macropomum}). Therefore, tambaqui subjected to sublethal concentrations of the \textit{L. origanoides} EO did not exhibit significant physiological responses that indicate homeostatic disturbance, as observed in others studies with others \textit{Lippia} species, since the hematologic and biochemical parameters tested in tambaqui were within the normal range for healthy fish (TAVARAS-DIAS, 2015). Thus, the minor physiological changes observed do not characterize homeostatic imbalance and do not preclude the use of \textit{L. origanoides} EO to treat bacterial and parasitic diseases.

Survival of tambaqui treated with \textit{L. origanoides} EO was above 30\% and around 80\% using 5 and 10 mg L\(^{-1}\) oil. Similar results were found in studies on silver catfish (\textit{Rhamdia quelen}) infected with \textit{A. hydrophila} and treated with different natural products in therapeutic bath protocols. Silver catfish survival was nearly 80\% in fish treated with 16 and 40 mg L\(^{-1}\) \textit{L. alba} EO (SUTILI et al., 2015a), 70 and 66\% with 20 and 40 mg L\(^{-1}\) \textit{Hesperozygis ringens} EO, respectively, 75\% with 10 mg L\(^{-1}\) \textit{Ocimum americanum} EO (SUTILI et al., 2015b), and 37 and 66\% with 5 and 10 mg L\(^{-1}\) eugenol oil, respectively (SUTILI et al., 2014).

A noteworthy point is that thymol and carvacrol are among the main components of \textit{L. origanoides} EO. These compounds are found in different \textit{Lippia} species and account for the inhibitory activity against \textit{A. hydrophila} (MAJOLO et al., 2017). This occurs because these phenolic terpenes exhibit strong antimicrobial activity, which can disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides and increasing cytoplasmic membrane permeability to AIP (HELANDER et al., 2001).

Increased survival of channel catfish (\textit{Ictalurus punctatus}) challenged with \textit{A. hydrophila} was obtained with compounds carvacrol and thymol, the main components of \textit{L. origanoides} EO, but in protocol of diet supplementation (ZHENG et al., 2009). Similar results were obtained in Nile tilapia treated with green tea (\textit{Camellia sinensis}) (ABDEL-TAWWAB et al., 2010), rainbow trout (\textit{Oncorhynchus mykiss}) treated with methanolic nettle root extracts (\textit{Urtica dioica}) (BILEN et al., 2016) and rhuo (\textit{Labeo rohita}) treated with aqueous extract of \textit{Ocimum sanctum} (DAS et al., 2015). Therefore, some EO and extract of bioactive plants are effective in promotes survival of fish in different administration protocols. However, further studies should be carried out to improve the therapeutic efficacy of \textit{L. origanoides} EO against \textit{A. hydrophila} in tambaqui as the use of nanoemulsions (THOMAS et al., 2013) and nanotechnology (SOUZA et al., 2017b).

CONCLUSIONS

Tambaqui exhibit moderate tolerance to \textit{L. origanoides} EO, showing 96-hh LC\(_{50}\) of 15.2 mg L\(^{-1}\). In addition, sublethal concentrations caused only a few hemalogic and biochemical changes that do not characterize homeostatic imbalance. \textit{L. origanoides} EO shows potential for use in the treatment of tambaqui infected with \textit{A. hydrophila}.

ACKNOWLEDGEMENTS

To Dr. Marcelo Róseo de Oliveira for helping with essential oil extraction and the laboratory assistant Iainí Morais for assisting in physiological parameter analysis.

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