PANAMJAS
Pan-American Journal of Aquatic Sciences

# Pharmacokinetic and physiological responses of *Piaractus mesopotamicus* anesthetized with the essential oil of *Lippia alba*

ARLENE SOBRINHO VENTURA[1*], TARCILA SOUSA DE CASTRO SILVA[2], RICARDO BASSO ZANON[3], LUIS ANTONIO KIOSHI AOKI INOUE[2] & CLAUDIA ANDREA LIMA CARDOSO[1]

[1] *Centro de Estudos em Recursos Naturais, Universidade Estadual de Mato Grosso do Sul, Rod. Itahum, Km 12, caixa postal 351 Jardim Aeroporto79804-970 Dourados, MS, Brasil.*
[2] *Laboratório de Aquicultura, Embrapa Agropecuária Oeste, BR 163, km 253.6, 79804-970 Dourados, MS, Brasil.*
[3] *Faculdade de Ciências Biológicas e Ambientais, Universidade Federal da Grande Dourados, Rua João Rosa Góes, Vila Progresso,1761,79825-070 Dourados, MS, Brasil.*

[*] Corresponding author: arlenesventura@gmail.com

**Abstract**. The anesthetic activity of the essential oil (EO) of *Lippia alba* and its effects on pacu (*Piaractus mesopotamicus*) metabolism were investigated. First, fish were individually (n = 54) anesthetized in different EO concentrations to evaluate the induction time to anesthesia. Second, the plasmatic responses of the fish exposed to the anesthetic were determined. Blood samples were taken 0 h and 24 h after the anesthesia procedures. Third, the presence of the major compound carvone of the EO in the fish blood was evaluated. Fish (n = 30) were exposed to the highest experimental concentration (250 mg $L^{-1}$) of the anesthetic for 10 min, and their blood was sampled 0, 0.25, 0.5, 1, 2, 4, 6, 8, 24, and 48 h after anesthesia. All fish lost equilibrium and were unable to regain the upright position irrespective of the concentration used. Plasma levels of glucose, lactate, and $K^+$ were increased in all the anesthetized fish. Plasma ammonia increased only in fish anesthetized at the concentration of 250 mg $L^{-1}$. Carvone was the main component of the EO rapidly metabolized with half-life distribution ($T_{1/2}\alpha$) of 0.06 h, and elimination half-life ($T_{1/2}\beta$) of 2.33 h. The EO of *L. alba* can hence be considered a safe anesthetic for pacu.

**Key words:** Anesthetic, induction, half-life, stress, plasma ions

**Resumo: Respostas farmacocinéticas e fisiológicas de *Piaractus mesopotamicus* anestesiados com oléo essencial de *Lippia alba*.** Foi avaliada atividade anestésica do óleo essencial de *Lippia alba* e seus efeitos no metabolismo de pacu (*Piaractus mesopotamicus*). No primeiro experimento, os peixes foram individualmente (n = 9) anestesiados em diferentes concentrações de óleo essencial para avaliação do tempo de indução anestésica. O segundo experimento determinou respostas plasmáticas de peixes expostos a anestesia com óleo essencial de *Lippia alba*. As amostras de sangue foram colhidas 0 h e 24 h após os procedimentos de anestesia experimental. A presença do composto principal carvona de EO na circulação do sangue de peixe foi avaliada no terceiro experimento. Os peixes (n = 30) foram expostos à maior concentração experimental (250 mg $L^{-1}$) de óleo essencial de *L. alba* durante 10 min, e sangue amostrado 0, 0,25, 0,5, 1, 2, 4, 6, 8, 24 e 48 horas após a anestesia. Todos os peixes perderam o equilíbrio e foram incapazes de recuperar a posição vertical, independentemente da concentração utilizada. Os níveis plasmáticos de glicose, lactato e $K^+$foram aumentados em todos os peixes anestesiados. A amônia plasmática aumentou apenas em peixes anestesiados à concentração de 250 mg $L^{-1}$. A carvona foi rapidamente metabolizado

com distribuição de meiavida (T1 / 2α) de 0,06 h, e a semi-vida de eliminação (T1 / 2β) de 2,33 h. O óleo essencial de *L. alba* pode ser considerado um anestésico seguro para pacu.

**Palavras-chave**: Anestesia, indução, meia-vida, estresse, íons plasmáticos

## Introduction

Bushy matgrass (*Lippia alba*) is an aromatic shrub, widely used in traditional medicine. Anti-inflammatory (Haldar *et al*. 2012), sedative, and antispasmodic effects (Mamun-Or-Rashid *et al*. 2013) have been reported with the use of this plant. The use of essential oil (EO) extracted from the aerial parts of the plant as fish anesthetic and as a control for fish parasites and *Aeromonas hydrophila* microbial activity have been studied (Cunha *et al*. 2010, Soares *et al*. 2016, Majolo *et al*. 2017). However, few studies describe the elimination of the EO compounds in fish (Stehly *et al*. 1998, Kiessling *et al*. 2009).

Many of the fish management practices can trigger stress as the physiological equilibrium of the fish are broken by the stimulus that is applied for biological measurements, and for procedures such as tagging and injections. Fish can be further susceptible to various diseases that result in substantial commercial losses (Moreira *et al*. 2015). The use of anesthetic agents can be useful in aquaculture to minimize physical damages and stress (Inoue *et al*. 2003). However, there is a need for new fish anesthetic substances that are effective, safe to workers and environment, and less persistent than the synthetic drugs currently available (Inoue *et al*. 2003, Guénette *et al*. 2007). The fish anesthetic, MS-222, has a 21 days withdrawal period, which is too long, especially if one or more of the previously anesthetized fish escape to nature after handling. In this regard, alternative fish anesthetics, such as *L. alba* EO, seem to be promising as natural compounds and are supposed to be less risky and rapidly eliminated (Kiessling *et al*. 2009).

Pacu *Piaractus mesopotamicus* (Holmberg, 1887, Characidae) and the hybrids with *Colossoma macropomum*, and *Piaractus brachypomus* are the most farmed tropical freshwater fish from Brazil because they show desirable aquaculture characteristics such as tolerance to low water oxygen and quick growth under artificial feeding. *L. alba* has been described as a fish anesthetic (Cunha *et al*. 2010, Toni *et al*. 2014). However, no studies have as yet reported the pharmacokinetics of the major components, which could indicate that this EO is likely to be eliminated faster than the traditional fish anesthetic. Thus, the purpose of the present work is to investigate the physiological responses, and pharmacokinetics of the EO of *L. alba* in fish, using the pacu (*Piaractus mesopotamicus*), a neotropical Characidae from the Brazilian Pantanal Basin, as the study model.

## Material and Methods

*Plant material and chemical characterization of the essential oil: Lippia alba* was cultivated in the Embrapa Western Amazon facilities, Manaus, Brazil (2°53′35.73″S; 59°58′23.36″W. A voucher specimen was deposited in the herbarium of Embrapa Eastern Amazon in Belém, Brazil, under the registration number 002\2008 (protocol 02001.002726\2013-25). The aerial parts of the plants were cut and air-dried. The crude EO was obtained by hydro-distillation in a Clevenger apparatus (European Pharmacopoeia 2007). The EO yield was 1.7% (m/v).

*Animals:* Pacu juveniles were acquired from a local commercial fish farm and transported in plastic bags to the Embrapa Western Agriculture facilities in Dourados, Brazil (22°06′49.57″S; 54°34′41.99″E). They were acclimated for seven days and stocked in tanks of 1000 L of water, fitted with a recirculating aquaculture system, and water quality parameters were kept at the species requirements. Temperature (25.6 ± 0.8 °C), oxygen dissolved (5.56 ± 0.07 mg $L^{-1}$), water conductivity (72.3 ± 4.5 µS $cm^{-1}$), and pH (7.0) were monitored daily. Fish were fed twice a day, near to satiation with commercial pellets (32% crude protein). Feeding was suspended 24 hours prior to the start of the experiment. This study was performed in accordance with the norms of the UEMS ethics committee (Protocol no. 006 /2015).

*Induction to anesthesia:* In order to evaluate the anesthetic activity of *L. alba* EO, individual juvenile pacu (n = 3; 119.3 ± 20.2 g; 18.1 ± 1.0 cm) were transferred to separate aquaria filled with eight liters of water containing the *L. alba* EO. As the EO is poorly soluble in water, it was initially dissolved in 95% ethanol at 1:10 ratio (EO:ethanol). Anesthetic solutions were prepared a few minutes before the experiment. Treatment doses were based on a preliminary experiment that was carried out in order to determine the lowest effective anesthetic concentration, based on criteria for an ideal anesthetic (Marking & Meyer 1985).

The efficacy of anesthetic agents was assessed by individual fish behavior throughout the anesthesia stages. Stage three of anesthesia in fish is observed when fish completely lose equilibrium, and is unable to regain the upright position (Woody *et al*. 2002). Induction times were recorded for 54 fish (9 fish per dose). When a fish reached the third stage of anesthesia, it was immediately netted from the anesthetic induction tank and its biometrics (119.3 ± 20.2 g; 18.1 ± 1.0 cm) was recorded. The doses employed were 100, 130, 160, 190, 220, and 250 mg $L^{-1}$. Samples of 20 mL of the water from the induction tank were collected before and after the induction process, for analysis using gas chromatography coupled with mass spectrometry (GC-MS), in order to determine the concentrations of EO present in the anesthetic induction tank. Trial was set up in a completely randomized design, with six treatments and three repetitions (n = 3). After the experiment, the animals were transferred to circular tanks containing anesthetic-free water and maintained under observation for 15 days.

*Physiological parameters:* The pacu juveniles (n = 72; 110.5 ± 25.9 g; 17.2 ± 1.5 cm) were randomly distributed into twelve 1000 L tanks (12 fish/tank) equipped with water recirculation systems. The experiment was set up in a completely randomized design, with four treatments and three repetitions (n = 3). The concentrations were chosen according to a previous experiment to evaluate the induction to anesthesia. Fish in the group that was not exposed to anesthesia were immersed in water without any EO. Fish in the groups that were exposed to anesthesia were immersed in water containing the EO at doses of 100 and 250 mg $L^{-1}$. The experimental control group of fish was exposed neither to the handling procedure nor to the EO.

After 10 min of exposure to the anesthetic agent, fish were immediately netted from the induction tank. Three fish from each repetition (i.e., nine fish per treatment) were used for blood collection. The remaining fish were returned to the initial tanks for recovery. Another three fish from each repetition were used for blood collection after 24 h. Often, due to the fact that handling procedures can involve anesthesia of a large number of fish, the exposure time of 10 min usually exceeds the induction time for individual fish. Blood was collected by puncture of the caudal vein, using syringes that were rinsed in ethylene diamine tetra acetic acid (EDTA, 10%). Blood samples were centrifuged at 75 *g* for 5 min to obtain plasma for analysis of glucose, total protein, enzymatic lactate, and chloride, using commercial kits (Labtest, Minas Gerais, Brazil). Plasma ammonia was determined according to the method described by Gentzkow & Mazen (1942). Sodium ($Na^+$) and potassium ($K^+$) ions were determined by flame photometry.

*Pharmacokinetics parameters:* Juvenile pacu individuals (n = 30) were exposed to a 250 mg $L^{-1}$ *L. alba* EO solution for 10 min. This concentration was selected based on the previous experiments. After the exposure, these individuals were transferred to anesthetic-free water. Blood samples (n = 3) were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 24, and 48 h after the exposure to the EO. Syringes rinsed in 10% EDTA were used to harvest the blood from the caudal vein. Aliquots were centrifuged at 75 *g* for 5 min, to obtain the plasma samples, which were transferred to sterile 2.0 mL vials and frozen for use in subsequent analysis by GC-MS.

*Preparation of samples for GC-MS analysis:* Samples (20 mL) of the anesthetic solutions collected before and after the inductions to anesthesia were analyzed by GC-MS. Each 10 mL of sample was extracted with 10 mL of chromatographic degree hexane employing liquid-liquid partitioning. The hexane fraction was dried and re-dissolved in 1 mL of hexane and tests were performed in triplicate. For pharmacokinetic evaluation, 500 µL of plasma were blended with 500 µL of chromatographic degree hexane. The sample was homogenized and agitated in an ultrasonic tub with a timer for one minute and the hexane fraction was then analyzed by GC-MS, in triplicate. The analytical curve was constructed using carvone at concentrations of 0.09, 0.36, 7.36, 14.73, 73.65, 147.3, 736.5, and 1473 µg $mL^{-1}$. Carvone was used because it was the main compound of the *L. alba* EO.

*GC-MS analyses:* The analyses were performed using a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) coupled with a mass spectrometer (GC MS-2010 Ultra). A DB-5 column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness) was used, with helium (99.999% purity) as the carrier gas, at a flow rate of 1.0 mL $min^{-1}$, and an injection volume of 1 µL (in split mode, 1:20). The initial oven temperature was 50 °C, with heating increased up to 280 °C at 3 °C $min^{-1}$. The injector temperature was 220 °C and the temperatures of the transfer line and the quadrupole detector were 250 °C.

The retention index was calculated using a mixture of linear alkanes ($C_8$-$C_{30}$) as an external reference. Compound identification was achieved by

comparing the mass spectra of the samples with the spectra available in the NIST21 and WILEY229 libraries, as well as with data reported in the literature (Adams 2007).

*Statistical analyses:* All data were verified for assumptions of the variance analysis (ANOVA - one-way; $\alpha = 0.05$), considering normality, variance homogeneity, and the presence of outliers. When significant differences were verified, the Tukey test was applied to compare means. The relationship between EO concentration and anesthesia induction times was calculated by linear regression analysis. Tests were performed in the SAS software. The EO concentrations in the plasma, in relation to depuration time, were analyzed with the GraphPad Prism version 6.07 (GraphPad Software Inc.; San Diego, CA, USA). The elimination speed constant (*K*), half-life ($T_{1/2}$), and total area under the curve (AUC) were determined according to Jambhekar & Breen (2009).

## Results

*Characteristics of the essential oil:* Thirty-five compounds were identified in the *L. alba* EO, corresponding to a relative proportion of 98.16% of the compounds present (Table I). The major components were carvone (52.54%), limonene (17.25%), β-copaene (4.29%), (E)-caryophyllene (3.31%), myrcene (3.02%), and β-atlantone (2.51%). The major compounds carvone, limonene, β-atlantone, and (E)-caryophyllene were detected in the anesthetic solutions before and after fish immersions in all EO tested concentrations, except in the most diluted (100 mg $L^{-1}$), where (E)-caryophyllene was not detected after the anesthetic baths.

**Table I.** Chemical composition of the essential oil of *Lippia alba*.

| Retention time (min) | IK[a] Calculated | IK literature | Components | Relative (%) |
|---|---|---|---|---|
| 7.325 | 925 | 924 | α-thujone | 0.25 |
| 8.959 | 972 | 969 | sabinene | 0.33 |
| 9.604 | 990 | 988 | myrcene | 3.02 |
| 11.174 | 1029 | 1024 | limonene | 17.25 |
| 11.241 | 1031 | 1026 | 1.8-cineole | 0.41 |
| 11.474 | 1036 | 1032 | Z-ß-ocimene | 0.27 |
| 11.903 | 1047 | 1044 | E-ß-ocimene | 1.20 |
| 12.358 | 1058 | 1054 | γ-terpinene | 0.16 |
| 14.110 | 1100 | 1095 | linalool | 1.38 |
| 17.082 | 1166 | 1165 | borneol | 0.18 |
| 18.484 | 1198 | 1200 | trans-dihydrocarvone | 0.75 |
| 19.398 | 1223 | 1215 | trans-carveol | 1.26 |
| 20.060 | 1233 | 1226 | neoiso-dihydrocarveol | 0.79 |
| 20.894 | 1252 | 1239 | carvone | 52.74 |
| 21.130 | 1257 | 1249 | piperitone | 0.45 |
| 22.773 | 1294 | 1289 | thymol | 0.11 |
| 24.874 | 1343 | 1204 | verbenone | 1.01 |
| 26.359 | 1377 | 1374 | α-copaene | 1.75 |
| 26.744 | 1386 | 1387 | ß-bourbonene | 1.01 |
| 28.220 | 1422 | 1417 | (E)-Caryophyllene | 3.31 |
| 28.598 | 1431 | 1430 | ß-copaene | 0.25 |
| 29.603 | 1455 | 1452 | α-humulene | 0.21 |

**Table I (continued).** Chemical composition of the essential oil of *Lippia alba*.

| Retention time (min) | IK[a] Calculated | IK literature | Components | Relative (%) |
|---|---|---|---|---|
| 29.695 | 1458 | 1454 | E-ß-farnesene | 0.32 |
| 29.914 | 1463 | 1458 | allo-aromadendrene | 0.48 |
| 30.566 | 1479 | 1478 | γ-muurolene | 0.49 |
| 30.765 | 1484 | 1430 | ß-Copaene | 4.29 |
| 31.514 | 1502 | 1500 | α-muurolene | 0.44 |
| 32.104 | 1517 | 1514 | cubebol | 0.39 |
| 32.433 | 1526 | 1533 | trans-Cadina-1,4-diene | 1.13 |
| 33.929 | 1564 | 1561 | E-nerolidol | 0.30 |
| 34.770 | 1586 | 1582 | caryophyllene oxide | 0.28 |
| 37.154 | 1650 | 1645 | cubenol | 0.11 |
| 37.270 | 1653 | 1653 | α-cadinol | 0.43 |
| 37.788 | 1667 | 1668 | ß-atlantona | 2.51 |
| 38.618 | 1689 | 1562 | epi-longipinanol | 0.16 |

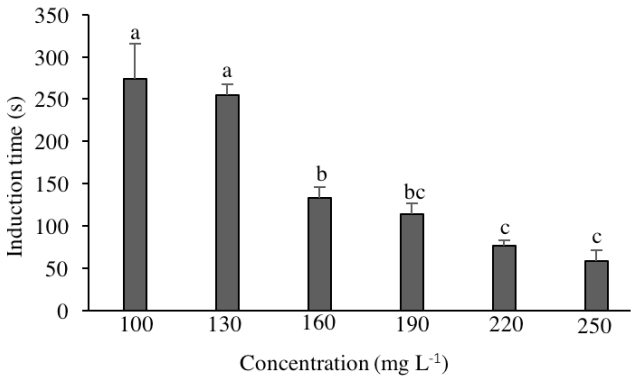


**Figure 1.** Induction time to anesthesia in *Piaractus mesopotamicus* juveniles in different essential oil concentrations of *Lippia alba*. Mean ± standard deviation (N = 9) are shown. Different letters in the induction time column indicate significant differences ($p < 0.05$).* Concentration capable of inducing anesthesia in stage three (Woody *et al*. 2002). ** Equation: Induction time = -0.585 × concentration + 263.9; $r^2 = 0.912$.

*Induction to anesthesia:* In all cases of exposure of the fish to the anesthetic, hyperactivity was observed on the first contact of fish to the solutions, which was evidenced by rapid movement in the aquarium. This decreased as the effect of the anesthetic progressed. The time for anesthesia to be induced decreased linearly with increasing concentration of the *L. alba* EO. At concentrations of 160 mg $L^{-1}$ and above fish became anesthetized in less than 3 min (Fig. 1).

*Physiological parameters:* Plasma glucose values of anesthetized fish increased significantly at time zero after the anesthetic bath. Glucose in fish exposed to 100 mg $L^{-1}$ remained high after 24 hours of recovery. There were no differences in total plasma protein concentrations between the anesthetic-free control and manipulation between treatments 100 and 250 mg $L^{-1}$ *L alba* EO at time zero. After 24 hours of the anesthetic bath with 250 mg $L^{-1}$ *L alba* EO concentration, an increase of total plasma proteins ($p < 0.05$) was found in relation to the control group. The level of enzymatic lactate in fish anesthetized with 100 and 250 mg $L^{-1}$ *L alba* EO was high when compared to the control at time zero after anesthetic bath. However, lactate returned to the initial values after the recovery period of 24 hours (Fig. 2).

The potassium ion in fish anesthetized with *L alba* EO at concentrations of 100 and 250 mg $L^{-1}$ increased when compared to the animals from the control group. A recovery period of 24 h was sufficient for the plasma potassium to return to the initial levels. The chloride and sodium ions did not differ between treatments at time zero after the anesthetic bath, and also after 24 hours of recovery. Plasma ammonia level was higher in animals anesthetized with *L. alba* EO at concentration of 250

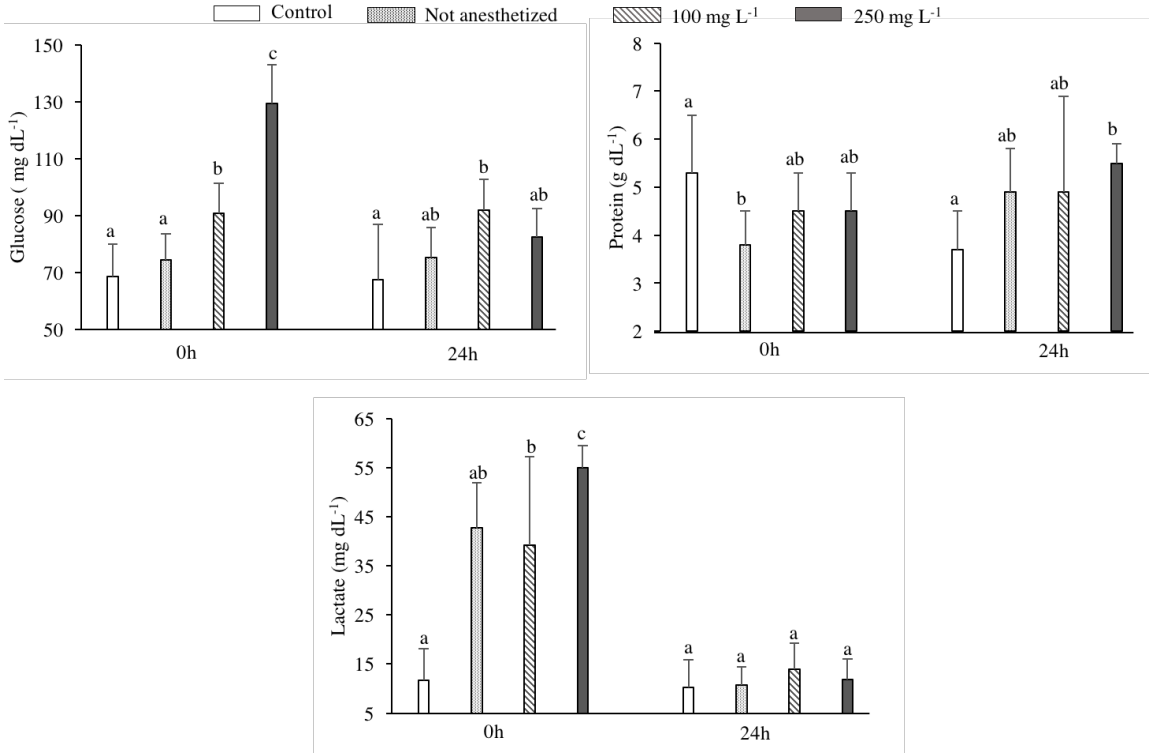


**Figure 2.** Biochemical parameters of *Piaractus mesopotamicus* subjected to anesthesia (0 h and 24 h) with essential oil of *Lippia alba*. Mean ± standard deviation (N = 9) are shown. Different lowercase letters indicate significant differences between time points within the same treatment using ANOVA one-way and Tukey test ($P < 0.05$).

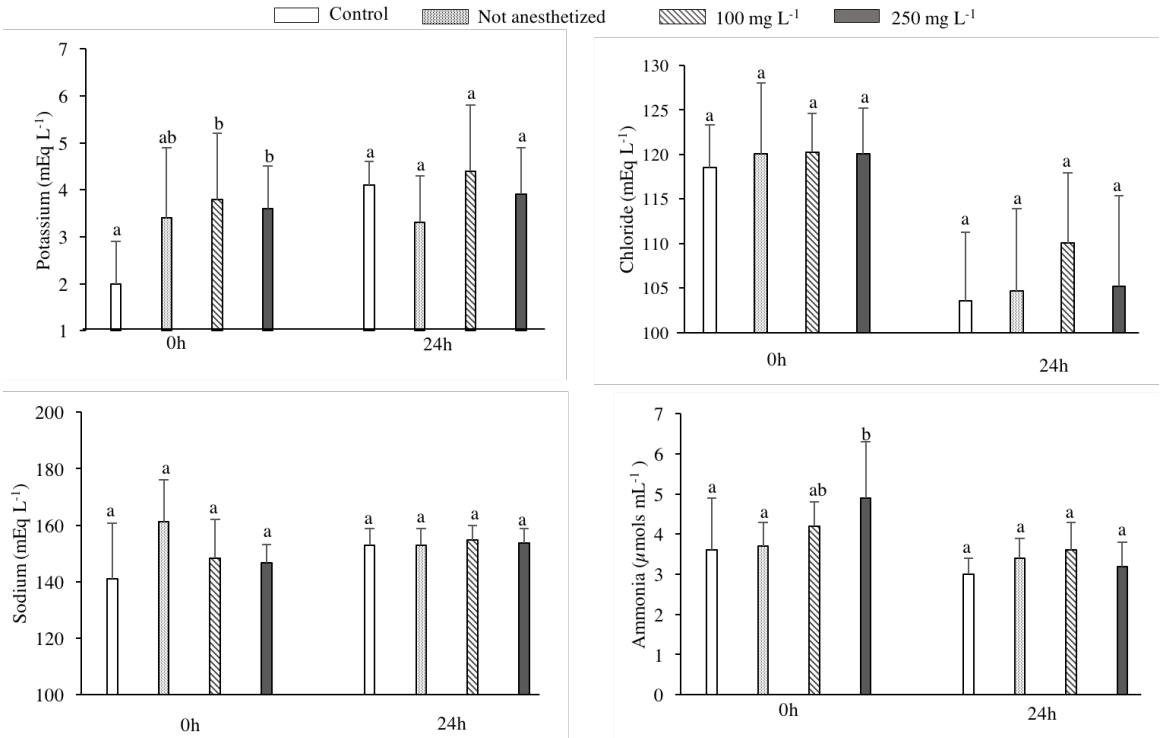


**Figure 3.** Plasma ions of *Piaractus mesopotamicus* subjected to anesthesia (0 h and 24 h) with essential oil *Lippia alba*. Mean ± standard deviation (N = 9), and different lowercase letters indicate significant differences between time points within the same treatment, using ANOVA one-way and Tukey test ($P < 0.05$).

mg $L^{-1}$ at time zero after the anesthetic bath. Plasma ammonia was similar to the initial levels after 24 hours of recovery with no significant changes observed (Fig. 3).

*Pharmacokinetic parameters:* On analysis of the *P. mesopotamicus* blood plasma, only carvone, the major component of the *L. alba* EO, was identified for all the depuration times evaluated. The concentration of carvone in the blood plasma immediately after the exposure of fish to the EO was 39.55 ± 2.22 µg $mL^{-1}$. It subsequently decreased up to six hours, after which it was relatively stable.

A biphasic elimination curve was obtained (Fig. 4), with the proportion eliminated changing over time, and an initial distribution constant (k) of 11.79 µg $h^{-1}$. As the plasma carvone concentration decreased, the relative rate of elimination also decreased. After two hours, the concentration of carvone in the blood plasma of the fish had decreased by 95.29%, to 1.79 ± 0.08 µg $mL^{-1}$, compared to the concentration at time zero.

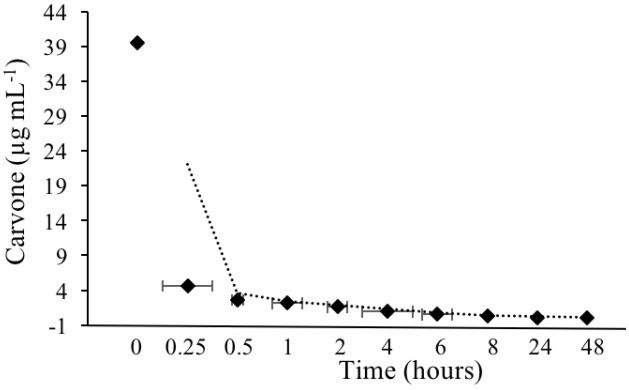


**Figure 4.** Concentration of carvone (mean ± standard deviation, µg $L^{-1}$) in *Piaractus mesopotamicus* plasma after exposure to 250 mg $L^{-1}$ of *Lippia alba* essential oil anesthetic solution, administered via a bath of 10 min duration.

## Discussion

Essential oils compositions vary depending on the origin of the plant seeds, and the environmental conditions in which they are grown (Chalchat & Özcan 2008). In this study, carvone was found to be the major constituent of the EO, accounting for 52.74%. Thus, the *L. alba* EO used in this experiment belonged to the chemotype carvone. Similar results were obtained by Soares *et al.* (2016), who also found carvone chemotype (61.7%) in *L. alba*. Souza *et al.* (2017) attributed the anesthetic activity of *L. alba* EO in fish to two different chemotypes of *L. alba* EO: citral chemotype (54.26%) and linalool (50.56%). Although these studies have been carried out with plants grown in different geographic regions, it is believed that these variations among the *L. alba* chemotypes are due to genotypic variations (Tavares *et al.* 2005).

The *L. alb*a EO carvone chemotype showed anesthetic effect for pacu. Concentrations of 160 mg $L^{-1}$ and above can be considered adequate as anesthetic for this fish species. An ideal anesthetic must induce anesthesia within 3 min, with a recovery time of up to 5 min (Marking & Meyer 1985). Fish anesthetized for a longer time reduces the safety margin of the ideal dose. However, if shorter induction times to anesthesia are necessary, our results indicated that concentration of 250 mg $L^{-1}$ is enough for rapid anesthetic effect with induction times shorter than 1 min, and without overdosing the fish. The anesthetic effects of *L. alba* EO chemotype linalool can be attributed to its action on the GABAergic system (Heldwein *et al.* 2012). However, the mechanism of action of the carvone chemotype is not well elucidated. It is believed that it could be associated with the reduction of isolated nerve excitability, possibly involving voltage-gated $Na^{+}$ channels blockade (Almeida *et al.* 2008).

Adaptive response during a stressful event possibly occurs due to catecholamine mediated glycogenolysis and cortisol-mediated gluconeogenesis, causing increase in plasma glucose levels (Pankhurst, 2011). In pacu, after the baths with the *L. alba* EO, increases in the plasma glucose levels were observed. This may be due to stress caused by anesthesia that the fish were submitted to. Both the control group and the non-anesthetized group were similar to each other, which proved that the manipulation did not cause this parameter to change. Similar results were found in Nile tilapia (*Oreochromis niloticus*) exposed to *L. alba* EO as the anesthetic (Hohlenwerger *et al.* 2016).

Mobilization of proteins is not a short-term response to stress (Mommsen *et al.* 1999). This explains why the increase in plasma protein levels occurred only 24 hours after the anesthetic bath in concentration of 250 mg $L^{-1}$. The plasma lactate increased in fish exposed to the concentration of 250 mg $L^{-1}$ soon after anesthesia with *L. alba* EO. Anaerobic metabolic reactions were evidenced in the anesthetized fish. Similar results were observed in tambaqui following exposure to eugenol in concentrations of 20 and 60 mg $L^{-1}$ (Inoue *et al.* 2011), and in silver catfish after exposure to the EO of *Hesperozygis ringens* and *L. alba* (Toni *et al.* 2014). Lactate is produced during anaerobic fermentation of glucose when the available oxygen

supply is insufficient for the metabolic requirements of the organism. Usually stress activates this response (Moreira *et al.* 2015). This increase could be attributed to the intense muscular movements of pacu under the stress, which led to the anaerobic metabolism.

The $Cl^-$ and $Na^+$ parameters were not affected by anesthesia with *L. alba* EO, which could be seen as a positive feature of the EO. However, the levels of $K^+$ at time zero after anesthetic bath increased in the fish exposed to the EO at concentrations of 100 and 250 mg $L^{-1}$. Similar results were found in the blood of silver catfish (*Rhamdia quelen*) anesthetized with two different chemotypes of *L. alba* (citral and linalool) (Souza *et al.* 2017). However, silver catfish anesthetized with 150–450 µL $L^{-1}$ of *H. ringens* and *L. alba* EO showed altered plasma $Na^+$ and $K^+$ between 30–240 min of recovery (Toni *et al.* 2014).

There was an increase in plasma ammonia levels in pacu soon after anesthesia. It indicates that anesthesia with *L. alba* EO resulted in a disturbance in nitrogen excretion. Fish in anesthetic baths have decreased opercular beats that modifies the water and blood fluxes through the gills. This results in difficulty in eliminating plasma ammonia (Inoue *et al.* 2011), as observed in the present study in fish anesthetized with *L. alba* EO in concentration of 250 mg $L^{-1}$. In the silver catfish, plasma ammonia levels were not altered when exposed to *H. ringens* and *L. alba* EO (Toni *et al.* 2014).

In the present study, the carvone concentration of 39.55 µg $mL^{-1}$ was found in the plasma of pacu just after a 10 min exposure to the *L. alba* EO at a concentration of 250 mg $L^{-1}$ at a water temperature of 24 °C. However, around 90% of the carvone disappeared from the plasma during the first hour after exposure, which was indicative of rapid elimination. The main routes of excretory metabolism in fish are via the urinary and biliary systems, although some conjugates are also excreted through the gills (Noga 2010). According to Kiessling *et al.* (2009), the gills are an important route for the elimination of anesthetics such as benzocaine, MS 222, and isoeugenol, which has been confirmed in studies of the pharmacokinetics of benzocaine in channel catfish (*Ictalurus punctatus*; Hayton *et al.* 1996) and isoeugenol in rainbow trout (*Oncorhynchus mykiss;* Meinertz *et al.* 2006).

## Conclusions

The present study demonstrated that *L. alba* EO is a safe anesthetic for tropical fish pacu. All fish lost equilibrium and were unable to regain the upright position irrespective of anesthetic concentration used. However, for fast induction purposes, i.e., < 3 min, concentrations from 160 mg $L^{-1}$ could be indicated. Most of the secondary stress parameters that were evaluated were altered during exposure to this EO. Nevertheless, the parameters recovered to control levels after 24 h. Furthermore, no fish mortality was observed during the experiments. The main component of the EO, carvone, has a short half-life metabolism in the blood plasma. Therefore, as anesthetics derived from natural products are promising sources for a sustainable aquaculture, additional studies are needed to evaluate the excretion pathway of this EO component and the possible existence of residues in the fish tissue.

## Acknowledgements

The authors are grateful to Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPQ 471655/10-4 and CNPq Proc. 310801/2015-0), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA 02.12.01.020.00.03 and 03.17.00.051.00.01.005). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT - 126/2016, SIAFEM 025886).

*Bioethics and bio-security committee approval:* This project was approved by the Ethics Committee on Animal Use under protocol no. 006/2015 Universidade Estadual de Mato Grosso do Sul (UEMS).

## References

Adams, R. P. 2007. **Identification of Essential Oil Components by Gas Chromatography/ Mass Spectroscopy**. Allured Publishing, Carol Stream, IL, 804 p.

Almeida, R. N. de, Sousa, D. P. de, Nóbrega, F. F. de F., Claudino, F. de S., Araújo, D. A. M. & Leite, J. R. 2008. Anticonvulsant effect of a natural compound α,β-epoxy-carvone and its action on the nerve excitability. **Neuroscience Letters**, 443(1):51-55.

Chalchat, J. C. & Özcan, M. M. 2008. Comparative essential oil composition of flowers, leaves and steam of basil (*Ocimum basilicum* L.) used as herb. **Food Chemistry**, 110(2): 501-503.

Cunha, M. A., Barros, F. M. C., Garcia, L. O., Veeck, A. P. L., Heinzmann, B. M., Loro, V. L. & Emanuelli, T. 2010. Essential oil of *Lippia alba*: a new anesthetic for silver catfish, *Rhamdia quelen*. **Aquaculture**, 306(1-4): 403-406.

European Pharmacopoeia. 2007. **European Pharmacopoeia** (6th edn). European Directorate for the 391 Quality of Medicines, Strasbourg, France, 3308 p.

Gentzkow, C. J. & Mazen, J. M. 1942. An accurate method for the determination of blood urea nitrogen by direct nesslerization. **The Journal of Biological Chemistry**, 143(1): 531-544.

Guénette, S. A., Uhland, F. C., Hélie, P., Beaudry, F. & Vachon, P. 2007. Pharmacokinetics of eugenol in rainbow trout (*Oncorhynchus mykiss*). **Aquaculture**, 266(1-4): 262-265.

Haldar, S., Kar, B., Dolai, N., Kumar, R. B. S., Behera, B. & Haldar, P. K. 2012. In vivo antinociceptive and anti-inflammatory activities of *Lippia alba*. **Asian Pacific Journal of Tropical Disease**, 2(2): 667-670.

Hayton, W. L., Szoke, A., Kemmenoe, B. & Vick, A. M. 1996. Disposition of benzocaine in channel catfish. **Aquatic Toxicology**, 36(1-2): 99-113.

Heldwein, C. G., Silva, L. L., Gai, E. Z., Roman, C., Parodi, T. V., Bürguer, M. E., Baldisserotto, B., Flores, E. M. M. & Heinzmann, B. M. 2014. S -(+)-Linalool from *Lippia alba*: sedative and anesthetic for silver catfish (*Rhamdia quelen*). **Veterinary Anaesthesia and Analgesia**, 41(6): 621-629.

Hohlenwerger, J. C., Copatti, C. E., Sena, A. C., Couto, R. D., Baldisserotto, B., Heinzmann, B. M., Caron, B. O. & Schmidt, D. 2016. Could the essential oil of *Lippia alba* provide a readily available and cost-effective anaesthetic for Nile tilapia (*Oreochromis niloticus*)? **Marine and Freshwater Behaviour and Physiology**, 49(2): 119-26.

Inoue, L. A. K. A., Boijink, C. L., Ribeiro, P. T., Silva, A. M. D. & Affonso, E. G. 2011. Avaliação de respostasmetabólicas do tambaquiexpostoao eugenol embanhosanestésicos. **Acta Amazonica**, 41(2): 327-332.

Inoue, L. A. K. A., Santos Neto, C. dos. & Moraes, G. 2003. Clove oil as anesthesic for juveniles of matrinxã *Brycon cephalus* (Gunther, 1969). **Ciência Rural**, 33(5): 943-947.

Jambhekar, S. & Breen, P. 2009. **Basic Pharmacokinetics**. Pharmaceutical Press, London, UK, Pp.1-75.

Kiessling, A., Johansson, D., Zahl, I. H., Samuelsen, O. B. 2009. Pharmacokinetics, plasma cortisol and effectiveness of benzocaine, MS-222 and isoeugenol measured in individual dorsal aorta-cannulated Atlantic salmon (*Salmo salar*) following bath administration. **Aquaculture**, 286(3-4): 301-308.

Majolo, C., Rocha, S. I. B. da, Chagas, E. C., Chaves, F. C. M. & Bizzo, H. R. 2017. Chemical composition of *Lippia* spp. essential oil and antimicrobial activity against *Aeromonas hydrophila*. **Aquaculture Research**, 48(5): 2380-2387.

Mamun-Or-Rashid, A. N. M., Sem, M. K., Jamal, M. A. H. M. & Nasrin, S. 2013. A comprehensive ethno-pharmacological review on *Lippia alba.* **International Journal of Biomedical Materials Research**, 1(1): 14-20.

Marking, L. L. & Meyer, F. P. 1985. Are better anesthetics needed in fisheries? **Fish**, 10: 2-5.

Meinertz, J. R., Greseth, S. L., Schreier, T. M., Bernardy, J. A. & Gingerich, W. H. 2006. Isoeugenol concentrations in rainbow trout (*Oncorhynchus mykiss*) skin-on fillet tissue after exposure to AQUI-S™ at different temperatures, durations, and concentrations. **Aquaculture**, 254(1-4): 347-354.

Mommsen, T., Vijayan, M. & Moon, T. 1999. Cortisol in teleosts: dynamics, mechanisms of actions, and metabolic regulation. **Reviews in Fish Biology and Fisheries**, 9(3): 211-268.

Moreira, A. G. L., Coelho, A. A. C., Albuquerque, L. F. G., Moreira, R. T. & Farias, W. R. L. 2015. Efeito do eugenol comoagentemitigador do estresse no transporte de juvenis de tilápia do Nilo. **Pesquisa Veterinária Brasileira**, 35(11): 893-898.

Noga, E. J. 2010. **Fish Disease: Diagnoses and Treatment**. Second Edition. Wiley-Blackwell, 536 p.

Pankhurst, N. W. 2011. The endocrinology of stress in fish: an environmental perspective. **General and Comparative Endocrinology**, 170(2): 265-275.

Soares, B. V., Neves, L. R., Oliveira, M. S. B., Chaves, F. C. M., Dias, M. K., Chagas, E. C. & Tavares-Dias, M. 2016. Antiparasitic activity of the essential oil of *Lippia alba* on ectoparasites of *Colossoma macropomum* (tambaqui) and its physiological and

histopathological effects. **Aquaculture**, 452(1): 107-114.

Souza, C. de F., Baldissera, M. D., Salbego, J., Lopes, J. M., Vaucher, R. de A., Mourão, R. H. V., Caron, B. O., Heinzmann, B. M., Silva, L. V. F. da & Baldisserotto, B. 2017. Physiological responses of *Rhamdia quelen* (Siluriformes: Heptapteridae) to anesthesia with essential oils from two different chemotypes of *Lippia alba*. **Neotropical Ichthyology**, 15(1): e160083.

Stehly, G. R., Meinertz, J. R. & Gingerich, W. H. 1998. Effect of temperature on the pharmacokinetics of benzocaine in rainbow trout (*Oncorhynchus mykiss*) after bath exposures. **Journal of Veterinary Pharmacology and Therapeutics**, 21(2): 121-127.

Tavares, E. S., Julião, L. S., Lopes, D., Bizzo, H. R., Lage, C. L. S. & Leitão, S. G. 2005. Análise do óleoessencial de folhas de trêsquimiotipos de *Lippiaalba* (Mill.) N. E. Br. (Verbenaceae) cultivadosemcondiçõessemelhantes. **Revista Brasileira de Farmacognosia**, 15(1): 1-5.

Toni, C., Becker, A. G., Simões, L. N., Pinheiro, C. G., Silva, L. L., Heinzmann, B. M., Caron, B. O. & Baldisserotto, B. 2014. Fish anesthesia: effects of the essential oils of *Hesperozygis ringens* and *Lippia alba* on the biochemistry and physiology of silver catfish (*Rhamdia quelen*). **Fish Physiology Biochemistry**, 40(3): 701-714.

Woody, C. A., Nelson, J. & Ramstad, K. 2002 Clove oil as an anaesthetic for adult sockeye salmon field trails. **Journal Fish Biology**, 60(2): 340-347.

Received: August 2018
Accepted: April 2018
Published: June 2019