



Anesthetic induction of juveniles of *Rhamdia quelen* and *Ctenopharyngodon idella* with *Ocimum micranthum* essential oil

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ABSTRACT: Anesthetics are effective to reduce or minimize stress in handling operations related to fish cultures. Aiming to avoid residual effects of synthetic drugs, several natural agents, such as essential oils (EOs), have been tested. The aim of this study was to determine the optimal concentration of the EO of *Ocimum micranthum* for induction of anesthesia in juveniles of silver catfish (*Rhamdia quelen*) and grass carp (*Ctenopharyngodon idella*). Results demonstrated that the most suitable concentration to sedate silver catfish and carp is 25 µL/L, while to anesthetize 200 µL/L is more suitable for silver catfish and 100 µL/L for grass carp, because these concentrations induce deep anesthesia in less than three min and recovery in less than five min. Analysis of the EO constituents showed methyl chavicol, a compound with carcinogenic potential, as the major component (58.2%). Therefore, the EO of *O. micranthum* is not recommended for fish anesthesia if the fish are to be used as food.

Key words: anesthesia, silver catfish, grass carp, sedation.

Indução anestésica de *Rhamdia quelen* e *Ctenopharyngodon idella* com o óleo essencial de *Ocimum micranthum*

RESUMO: Os anestésicos são eficazes para reduzir ou minimizar o estresse nas operações de manuseio relacionadas à piscicultura. Com o objetivo de evitar efeitos residuais de fármacos sintéticos, vários agentes naturais, como óleos essenciais (OEs), têm sido testados. O objetivo deste estudo foi determinar a concentração ótima do OE de *Ocimum micranthum* para a indução de anestesia em juvenis de jundiá (*Rhamdia quelen*) e carpa capim (*Ctenopharyngodon idella*). Os resultados demonstram que a concentração mais adequada para sedar jundiás e carpas capim é 25 µL/L. Enquanto que para anestésiar 200 µL/L é mais adequado para jundiás e 100 µL/L para carpas capim, pois essas concentrações induzem anestesia profunda em menos de três minutos e recuperação em menos de cinco minutos. A análise dos constituintes do OE identificou o metil chavicol, um composto com potencial carcinogênico, como componente majoritário (58,2%). Portanto, o OE de *O. micranthum* não é recomendado para anestesia de peixes usado como alimento.

Palavras-chave: anestesia, jundiá, carpa-capim, sedação.

INTRODUCTION

Anesthetics have been used in aquaculture to minimize the stress that occurs in fish management operations, but some may cause adverse effects on fish, manipulators or in the environment. The choice of an anesthetic also depends on availability, cost, ease use and physical state (CHO & HEATH, 2000). Several substances and combinations of substances, such

as alcohol, ether, barbiturates, quinaldine, tricaine methanesulfonate (MS 222), chlorbutanol and benzocaine have been used to induce anesthesia in fish. However, each of these agents has been associated with undesirable systemic side effects and limited safety margins to the extent that their use has either been limited or rejected altogether (GILDERHUS & MARKING, 1987; PALIĆ et al., 2006). Consequently, there is a search for natural anesthetics with little or no toxicity and low cost.

Some essential oils (EOs), such as those from *Lippia alba* (chemotype linalool) and *Aloysia triphylla*, are efficacious to anesthetize fish, not aversive and have almost no side effects (BANDEIRA JUNIOR et al., 2018; PARODI et al., 2014; SOUZA et al., 2017; ZEPPENFELD et al., 2014). However, in zebrafish, *Danio rerio*, the EO of *A. triphylla* was stressful and did not attenuate cortisol increase after stress (BANDEIRA JUNIOR et al., 2018). The EO of plants from the genus *Ocimum* also have presented anesthetic activity (BENOVIT et al., 2012).

Ocimum micranthum (Lamiaceae), an herb native of Central Lowlands, South America and West Indies, is used locally to flavor beverages and soups, and for the treatment of fever, stomach disturbances and dysentery. A decoction of the plant is also used for nervous disorders, earaches, colic and convulsions in children, as well as for painful menstruation (LINO et al., 2005). Therefore, the aim of this study was to determine the anesthetic effect of another species of this genus, *Ocimum micranthum*, in two fish species: *Rhamdia quelen* (silver catfish) and *Ctenopharyngodon idella* (grass carp).

MATERIALS AND METHODS

Fish and culture conditions

Experiments were conducted in the Fish Physiology Laboratory at the Universidade Federal de Santa Maria (UFSM), Rio Grande do Sul state (RS), Brazil. Juvenile silver catfish (*Rhamdia quelen*) and grass carp (*Ctenopharyngodon idella*) with average weights of 10.14 ± 0.70 g and 7.20 ± 0.33 g, respectively, were obtained from a local supplier and kept for one week for acclimation in 250L tanks. The fish were given a diet of commercial feed with 28.0% crude protein ZEPPENFELD et al. (2014). Juveniles were fed once a day, at 8:00 a.m. at a ratio of 5.0% body mass. Juveniles were fasted for a period of 24h prior to experiments. Water parameters were checked daily (temperature, pH and dissolved oxygen) or weekly (alkalinity, hardness, total ammonia and nitrite) as described by ZEPPENFELD et al., (2014). Water parameters remained stable and in the desired range throughout the experimental period. Temperature was maintained at 23.20 ± 0.20 °C, pH at 7.00 ± 0.01 , dissolved oxygen at 6.50 ± 0.40 mg/L, hardness at 23.0 ± 1.7 mg CaCO₃/L, alkalinity at 41.0 ± 0.7 mg CaCO₃/L, nitrite at 0.05 ± 0.01 mg/L, total ammonia at 0.81 ± 0.60 mg/L and non-ionized ammonia at 0.0053 ± 0.0500 mg/L.

Plant material and essential oil extraction

EO analysis was performed on a GCMS-QP2010 Ultra system (Shimadzu Corporation,

Tokyo, Japan) equipped with an AOC-20i auto-injector and the GCMS-Solution software containing the Adams, NIST11 and FFNSC2 libraries. Rxi-5ms (30m x 0.25mm; 0.25µm film thickness) silica capillary column (Restek Corporation, Bellefonte, PA, USA) was used. Conditions of analysis were: injector temperature of 250°C; oven temperature programming of 60-240°C (3°C/min); helium as carrier gas, adjusted at a linear velocity of 36.5cm/s (rate of 1.0mL/min); injection in the split mode of 1µl of the sample (2µL of the essential oil in 500µL of hexane); split ratio 1:20; ionization by electronic impact at 70 eV; ionization source and transfer line temperatures of 200°C and 250°C, respectively. Mass spectra were obtained by automatic scanning every 0.3s, with mass fragments in the range of 35-400m/z. Retention index was calculated for all volatile components using a homologous series of C8-C20 n-alkanes (Sigma-Aldrich), according to the linear equation of VAN DEN DOOL and KRATZ (1963). Quantitative data regarding the volatile constituents were obtained by peak-area normalization using a Shimadzu GC 2010 Ultra system, coupled to FID Detector, operated under similar conditions as the GC-MS system except nitrogen was used as carrier gas. Constituents were identified by comparing their retention indices and mass spectra (molecular mass and fragmentation pattern) with those existing in the GCMS-Solution system libraries, and also with spectra from literature (ADAMS, 2007).

Anesthesia induction and recovery

After the acclimation period, fish were transferred to tanks containing 1L of water and EO of *O. micranthum* at concentrations of 25, 50, 100, 200 and 300µL/L, previously dissolved in ethanol [1:10]. Grass carp were also exposed to the highest concentration of ethanol used to dilute the EO. This ethanol concentration did not have any sedative or anesthetic effect in silver catfish (SOUZA et al., 2017). Ten fish were used to evaluate anesthetic induction times in each concentration, each fish being used only once. Induction of anesthesia was evaluated according to an adaptation of the stages described in SMALL (2003): Stage 1 – sedation (decreased response to external stimuli), Stage 2 – slight anesthesia (partial loss of equilibrium; erratic swimming) and Stage 3 – deep anesthesia (total loss of equilibrium and no reaction to caudal peduncle pressure). Maximum observation time was 30min. After the induction tests, fish were transferred to anesthetic free aquaria for checking recovery time. Fish were considered recovered when they presented

normal swimming behavior and were responsiveness to external stimuli.

Statistical analyses

To verify the homogeneity of variances, all data were submitted to Levene's test. Evaluation of anesthetic activity was performed by regression analysis (concentration of the EO x time to induce sedation, slight and deep anesthesia, or recovery) using the Sigma Plot 11.0 software. When no significant relationship was reported, concentrations were compared by one-way ANOVA followed by Tukey's post hoc test using the Statistica software 7.0. Differences were considered significant at $P < 0.05$. Data are presented as the mean \pm SEM.

RESULTS

The major components of EO *Ocimum micranthum* were determined to be methyl chavicol (58.2 %) and linalool (29.8%).

The increased EO concentrations of *O. micranthum* proportionally decreased the time required for light and deep anesthesia in silverfish and carp. Time to induce sedation also decreased at higher concentrations, but the relationship was significant only for grass carp. No significant relationship was observed between concentration and time required for recovery in both species. Fish of both species submitted to 25 μ L/L did not reach the slight or deep anesthesia stages during the 30min. observation. Silver catfish exposed 50 μ L/L EO *O. micranthum* showed slower recovery than those exposed to 100 and 200 μ L/L (Table 1). However, grass carp exposed

to 50 μ L/L presented faster recovery than those exposed to 100 and 200 μ L/L (Table 2). Ethanol did not provoke any sedative or anesthetic effect in grass carps. There was no mortality during the trial period.

DISCUSSION

Methylchavicol and linalool are the main compounds of the EO of *O. micranthum*. Similar results were reported by OLIVEIRA et al. (2013). Methylchavicol, or estragole, is a natural constituent of a number of plants, including tarragon (*Artemisia dracunculus*), sweet basil (*Ocimum canum*) and sweet fennel (*Pimpinella anisum*), and their EOs have been widely used in foodstuffs as flavoring agents (VINCENZI et al., 2000).

The efficacy of a fish anesthetic depends on short latency time (approximately three min.) and rapid recovery of the fish (approximately five min.) (MARKING & MEYER, 1985). The recommended concentrations for deep anesthesia are 200 μ L/L for silver catfish and 100 μ L/L for grass carp, because they induce deep anesthesia in less than three min and recovery in less than five min. However, to maintain the fish only sedated (Stage 1) for a long period, as in fish transport for some hours, the concentration indicated is 25 μ L/L for both species.

Similar results for anesthetic induction of silver catfish were found using other EOs, such as of *Lippia alba*, chemotype linalool (300mg/L) (CUNHA et al., 2010). Linalool, one of the main compounds of the EO of *O. micranthum*, was used in *Cyprinus carpio* (753 μ LL⁻¹ L) to induce deep anesthesia within three min (MIRGHAED et al., 2016).

Table 1 - Relationship between the essential oil concentration of *Ocimum micranthum* and the time required to reach each stage of anesthetic induction and recovery in *Rhamdia quelen*. x=concentration of essential oil (μ L/L); y=time required for the stages of anesthetic induction in seconds. Different letters in the column "Recovery" indicate significant difference between concentrations. Evaluation of anesthetic activity was performed by regression analysis (concentration of the EO x time to induce sedation, slight and deep anesthesia, or recovery). When no significant relationship was reported, concentrations were compared by one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $P < 0.05$. Data are presented as the mean \pm SEM.

	Sedation	Slightanesthesia	Deepanesthesia	Recovery
25 μ L/L	163.30 \pm 13.79 ^a	-	-	-
50 μ L/L	121.60 \pm 11.39 ^a	483.33 \pm 8.49	1361.20 \pm 97.36	227.50 \pm 7.20 ^a
100 μ L/L	50.90 \pm 4.84 ^b	182.80 \pm 12.82	686.80 \pm 92.51	82.89 \pm 19.49 ^b
200 μ L/L	42.45 \pm 1.63 ^b	60.00 \pm 2.53	89.73 \pm 4.03	92.09 \pm 10.01 ^b
300 μ L/L	42.89 \pm 1.16 ^b	68.70 \pm 4.27	119.20 \pm 9.64	200.70 \pm 22.47 ^{ab}
	-----y = -52.0 + (26077.48/x)-----		-----y = 211.023 + (78964.55/x)-----	
	-----r ² = 0.9583-----		-----r ² = 0.8751-----	

Table 2 – Relationship between the essential oil concentration of *Ocimum micranthum* and the time required to reach each stage of anesthetic induction and recovery in *Ctenopharyngodon idella*. x =concentration of essential oil ($\mu\text{L/L}$); y =time required for the stages of anesthetic induction in seconds. Different letters in the column “Recovery” indicated significant difference between concentrations. Evaluation of anesthetic activity was performed by regression analysis (concentration of the EO x time to induce sedation, slight and deep anesthesia, or recovery). When no significant relationship was reported, concentrations were compared by one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $P<0.05$. Data are presented as the mean \pm SEM.

	Sedation	Slightanesthesia	Deepanesthesia	Recovery
25 $\mu\text{L/L}$	903.60 \pm 76.98	-	-	-
50 $\mu\text{L/L}$	147.44 \pm 11.61	324.10 \pm 21.09	704.80 \pm 58.2	40.56 \pm 4.96 ^a
100 $\mu\text{L/L}$	60.70 \pm 2.43	121.40 \pm 3.95	108.44 \pm 8.82	67.80 \pm 4.99 ^{ab}
200 $\mu\text{L/L}$	51.90 \pm 2.46	97.70 \pm 3.55	114.06 \pm 4.11	142.50 \pm 10.03 ^b
300 $\mu\text{L/L}$	41.40 \pm 1.55	68.44 \pm 1.89	103.40 \pm 3.38	162.90 \pm 14.84 ^b
	----- $y=-124.5405+(23352.7995/x)$ -----	---- $y=15.7675+(14353.8272/x)$ ----	----- $y=-387.3652+(96399.7606/x)$ -----	
	----- $r^2=0.8083$ -----	----- $r^2=0.7757$ -----	----- $r^2=0.8386$ -----	

The EO of *O. micranthum* has demonstrated anti-nociceptive effects in mice, and pretreatment with naloxone did not reverse the anti-nociception, indicating that the opioid system is not involved (LINO et al., 2005). Estragole (methylchavicol) presents some controversial results regarding its mutagenicity and carcinogenic effect. However, the formation of hepatic DNA adducts *in vivo* and *in vitro* by metabolites of estragole has been demonstrated (VINCENZI et al., 2000). The authors reported that in order to better assess the risk associated with exposure to estragole long-term carcinogenicity studies and a wide range of dose levels are needed.

CONCLUSION

The EO of *O. micranthum* leaves could be considered an effective anesthetic for silver catfish and grass carp, since induction and recovery occur rapidly and safely without causing mortality to fish. However, analysis of the EO constituents showed the presence of estragole (methylchavicol) as one of the major components and this compound has carcinogenic potential. Therefore, the EO of *O. micranthum* is not recommended for fish anesthesia.

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COMMITTEE ON ETHICS AND BIOSAFETY

The experimental protocol was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (UFSM) under registration n 74/2014.

DECLARATION OF CONFLICTING 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

Carla C. Zeppenfeld, Mauro A. Cunha, and Bernardo Baldisserotto designed the experiments. Carla C. Zeppenfeld and Gisela Cavalcante carried out the experiments. Carla C. Zeppenfeld and Mauro A. Cunha performed the statistical analysis of experimental data. Lenise V.F. Silva and Rosa H. Mourão provided the essential oil its chromatography analysis. Carla C. Zeppenfeld, Mauro A. Cunha and Bernardo Baldisserotto wrote the manuscript. All authors critically reviewed the manuscript and approved the final version.

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