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Short communication

# Essential oil of *Piper divaricatum* induces a general anaesthesia-like state and loss of skeletal muscle tonus in juvenile tambaqui, *Colossoma macropomum*

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ABSTRACT

This study investigated the anaesthetic potential of the essential oil from the leaves of *Piper divaricatum* (EOPD) through evaluation of the behaviour and electromyographic (EMG) recordings from the fish *Colossoma macropomum* which was used as an animal model. Initially, fish (3.9 ± 0.3 g; 6.4 ± 0.49 cm, total length) were subjected to short-term anaesthetic baths in five different concentrations of the EOPD: 25, 30, 35, 40 and 45 μL.L$^{-1}$ to record the latencies for deep anaesthesia and recovery. Ten fish per concentration were used ($n$ = 10) and each animal was considered a replicate and used only once. Thereafter, for the evaluation of EMG, fish (5.6 ± 1.8 g; 8.7 ± 0.52 cm, total length) were assigned to the following groups: a) sham control (basal recordings) and b) fish exposed to the EOPD at 40 μL.L$^{-1}$ and subsequent recovery in anaesthetic-free water. Nine fish per analysis ($n$ = 9) were used. The EOPD presented a high concentration of methyleugenol (71.36%) and prompted immediate behavioural changes in fish. Initially, hyperactivity was observed, followed by loss of the righting reflex and full body immobilization. All concentrations tested were capable to promote an anaesthetic-like state in tambaqui, with 40 μL.L$^{-1}$ being the minimal concentration necessary to induce a rapid immobilization, i.e. < 3 min. The EMG showed a marked and reversible myorelaxation effect, confirming this oil as an indisputable muscle relaxant agent. The EOPD was capable of promoting a general anaesthesia-like state, with complete body immobilization of *Colossoma macropomum* at all concentrations tested. Gradual increases in frequency and amplitude of EMG tracings confirm the reversibility and uneventful resumption of normal swimming behaviour observed during recovery. Our results underscore the anaesthetic potential and myorelaxant effects of *Piper divaricatum* essential oil.

## 1. Introduction

Farmed fish are exposed to handling and confinement in several situations in which these animals, mainly those reared under intensive farming systems, are often submitted to poor management practices that could result in high levels of stress (Wendelaar Bonga, 1997). Anaesthetics have been employed to reduce the magnitude of the stress response and decrease mortality rates in aquatic organisms. They are also used in research to prevent pain and for welfare purposes (Sneddon, 2012).

In fish, general anaesthesia is achieved when there is a complete or partial loss of the body senses due to the widespread depression of the central nervous system produced by an action on nerve axons, transmitter release or membrane excitability, or a combination of these actions (Ross and Ross, 2008).

Tricaine methane-sulphonate ($C_9H_{11}O_2N + CH_3SO_3H$), also known as MS-222, is one of the most widely-applied anaesthetic agents for poikilotherm organisms worldwide Priborsky and Velisek, 2018). However, this drug can be expensive and is not available in many countries, mainly in South America. Adding to it, it has been reported that MS-222 caused aversive reaction (Readman et al., 2013) and could be considered potentially detrimental to fish physiology as it may

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contribute to hypoxemia, hypercapnia, respiratory acidosis, hyperglycaemia, increased cortisolemia, elevation in haematocrit and plasma ion loss (Sladky et al., 2001; Gressler et al., 2014).

Natural matrices, like the essential oils, which are generally formed by complex mixtures of odoriferous and liquid constituted substances, mostly represented by molecules of terpenic nature have come forward as interesting alternatives to be used as natural anaesthetics for veterinary medicine, aquaculture and animal welfare purposes (Parodi et al., 2012; Zahl et al., 2012; Sena et al., 2016; Becker et al., 2016, 2017).

The genus *Piper* is the most representative of the Piperaceae family comprising several varieties of peppers. Many species present high essential oil yield and display excellent antifungal, antioxidant and anti-inflammatory properties. Plants of this genus also produce compounds with biological activity against insects (Souto et al., 2012; da Silva et al., 2014; Erisléia-Meirele et al., 2016; de Oliveira et al., 2018).

The plant species *Piper divaricatum* has an aromatic root and a strong ginger-like flavour with a large distribution in Latin America. Its essential oil is rich in methyleugenol (ME) (da Silva et al., 2010), an important volatile compound analogous to eugenol, being commonly reported as the main essential oil phytoconstituent from a variety of plants (Lima et al., 2000; Sayyah et al., 2002; Taveira et al., 2003). Like eugenol, ME has a moderate antibacterial activity, and induces central nervous system-related effects, such as hypothermia, decreased spontaneous motor activity and loss of the righting reflex. Moreover, in higher doses, it has acted as a general anaesthetic, without compromising amine levels in the rat brain (Lima et al., 2000; Sayyah et al., 2002).

Therefore, because of the major chemical composition of ME in the essential oil of *P. divaricatum* (OEPD), the hypothesis of this study is that it will act as a natural anaesthetic, potentially capable of (i) promoting a fast and full body immobilization along with (ii) eliciting a significant and reversible loss of the skeletal muscle tonus via neuromuscular blockade.

Electromyographic recordings allow for the quantification of the muscle contraction power loss throughout anaesthesia, which, at least in part, is responsible for the complete body immobilization attained during fish anaesthesia. Such studies combining a behavioural and electrophysiological approach are scarce, with only a few reports available (Lambooij et al., 2002, 2006; Barbas et al., 2017a; Fujimoto et al., 2017; de Souza et al., 2019).

The tambaqui, *Colossoma macropomum,* is a native fish species to the Amazon basin, of great social and economic importance for the fish farming sector of northern Latin American countries (FAO, 2016). This species has been presented as a good animal model for the screening of novel anaesthetics due to its handling resistance and high sensitivity to drug testing (Barbas et al., 2016, 2017a, 2017b; Stringhetta et al., 2017; de Souza et al., 2019).

Thus, this study aimed at investigating the anaesthetic potential and myorelaxant properties of the EOPD through the assessment of the behaviour in a concentration-response trial and electromyographic (EMG) recordings from tambaqui fish, *Colossoma macropomum* used as an animal model.

## 2. Materials and methods

### 2.1. Plant material and acquisition of the essential oil

The essential oil from the fresh leaves of *P. divaricatum* was provided by the research group of "Aromatic and Oleaginous Plants of the Amazon" of the Federal University of Pará, Belém, Brazil. The EO was extracted from aerial parts of a specimen collected in Breves (Marajó Island, Pará State, Brazil), which was propagated by clones and harvested in a private area. A voucher (MG 165214) of the plant material was deposited at the herbarium of the Emílio Goeldi Museum (Belém, Pará State, Brazil) to botanical identification. The EOPD was analyzed through Gas Chromatography and Mass Spectrometry (GC-EM System; Shimadzu GCMS QP5050).

**Table 1**
Chemical composition of *Piper divaricatum* essential oil.

| Components | RI | Oil % |
|---|---|---|
| Myrcene | 1004 | 0.05 |
| β-Phellandrene | 1042 | 0.14 |
| (*Z*)-β-Ocimene | 1047 | 0.08 |
| (*E*)-β-Ocimene | 1059 | 3.01 |
| *Allo*-Ocimene | 1139 | 0.02 |
| Eugenol | **1374** | **8.82** |
| β-Elemene | 1405 | 4.45 |
| Methyleugenol | **1434** | **71.36** |
| β-copaene | 1446 | 0.08 |
| Germacrene D | 1496 | 1.38 |
| Viridiflorene | 1500 | 0.03 |
| Bicyclogermacrene | 1510 | 0.05 |
| α-Muurolene | 1512 | 0.02 |
| δ-Cadinene | 1535 | 0.04 |
| Eugenyl acetate | **1547** | **9.65** |
| Elemicin | 1569 | 0.5 |
| Total identified | | 99.68 |

Major phytoconstituents are marked in bold. RI: Retention index (DB-5 ms capillary column) based on the library of Adams (2007).

### 2.2. Oil composition analysis

The analysis of the volatile compounds was performed on a THERMO DSQ II GC–MS instrument under the experimental conditions reported by de Oliveira et al. (2016). The main chemical constituents identified in the EOPD were the phenylpropanoids methyleugenol (71.36%), eugenyl acetate (9.65%) and eugenol (8.82%) (Table 1).

### 2.3. Experimental animals

Tambaqui juveniles (1.0 ± 0.2 g and 3.2 ± 0.8 cm, initial weight and total length) were obtained from a commercial fish farm in Northern Brazil, packed in polyethylene bags (30*L*) filled with 1/4 of water and injected with 3/4 pure oxygen, and transported to laboratory facilities where they were acclimated to 250 L tanks for 30 days, in a semi-static water system maintained continuously aerated, with mechanical and biological filtration. The initial stocking density was 1 g fish.$L^{-1}$ and fish were fed twice daily with commercial feed (32% crude protein, Guabi Pirá™). Thirty minutes after feeding, the tanks were siphoned to eliminate uneaten food and faeces, and partial water changes were performed up to a maximum of 20% of the total volume daily. Photoperiod was fixed at 12 h L: 12 h D. During acclimation, the water quality parameters were monitored daily and maintained as follows: pH (7.1 ± 0.2), temperature (27.5 ± 0.7 °C) and dissolved oxygen (5.8 ± 0.2 mg $L^{-1}$), measured with a portable multi-parameter equipment (HI9828 - HANNA™). Total ammonia nitrogen – TAN ($NH_4^+$ + $NH_3$ – N mg $L^{-1}$) (0.10 ± 0.07 mg.$L^{-1}$) and total hardness (60 ± 1.08 mg $L^{-1}$ $CaCO_3$) were determined following methodology of UNESCO (1983) and through titration according to guidelines of Adad (1982), respectively. This study was approved by our Institution's Animal Experimentation Ethics Committee (CEUA/UFPA – Protocol # 7857300718/2018).

### 2.4. Biological activity

EOPD is poorly soluble in water, therefore prior to the trials, a stock solution of the oil was prepared pre-diluting it in ethanol (96%) at a ratio of 1:9, from which aliquots were taken for the anaesthesia test. The solution was stored in an amber glass bottle at 4 °C until use.

#### 2.4.1. Experiment 1: anaesthetic efficacy of EOPD

Juvenile fish (3.9 ± 0.3 g and 6.4 ± 0.49 cm, total length) were

transferred to aquaria containing 1 L of water rtand concentrations of ethanolic solution of EOPD at 25, 30, 35, 40 e 45 μL.L$^{-1}$ were tested. A sham control group was used and animals were transferred to aquaria with anaesthetic-free water and observed for 30 min. A vehicle control added with the same volume of ethanol used for the pre-dilution of the highest concentration of EOPD, i.e., 405 μL, was also evaluated. In order to record the cumulative time required to reach the different stages of induction and recovery from anaesthesia a digital stopwatch was used. Groups of 10 fish were used for each concentration and also in the control treatments ($n = 10$), being each individual used only once and considered a replicate.

Cumulative time to reach the different stages of anaesthesia and recovery were characterized according to Park et al. (2008), with modifications proposed by Barbas et al. (2016) as follows: agitation (A1), loss of equilibrium and erratic swimming (A2), and absence of or minimum opercular beating with loss of reaction to tail pinch stimulus (herein referred to as deep anaesthesia) (A3) were used as behavioural markers associated with anaesthesia induction; erratic swimming and recovery of equilibrium (R1), normal opercular beating and normal swimming (R2) were used as markers of recovery from anaesthesia.

The maximum observation time during induction was 30 min, after which fish were no longer followed for signs of anaesthesia. In the case A3 stage was achieved, fish were transferred to tanks with anaesthetic-free water, and the time elapsed for recovery was registered. After recovery, fish were observed for 48 h to check for mortalities.

#### 2.4.2. Experiment 2: evaluation of electromyography (EMG)

This experiment was conducted to evaluate electromyographic responses of fish submitted to short-term exposure to EOPD at the concentration of 40 μL.L$^{-1}$. This concentration was used because it was the minimal concentration tested capable of inducing A3 stage within 3 min, i.e., fast and reversible anaesthesia (see results in Table 2).

*2.4.2.1. Assembling of electrodes, implant and acquisition of EMG.* For the design of appropriate equipment and recording of EMG data from the dorsal muscle, the methodology of Barbas et al. (2017a) was followed with modifications to the length of the electrodes. As fish were bigger in this study, electrodes used herein were slightly longer (4.0 mm). For details on the implant procedures, acquisition and analyses of the signal, please see the aforementioned study.

*2.4.2.2. Experimental design used for the EMG monitoring.* Another group of juvenile tambaqui (5.6 ± 1.8 g, 8.7 ± 0.52 cm, total length) was assayed as follows: a) sham control (basal record), b) ethanol exposed fish (vehicle control) and c) fish exposed to EOPD and subsequent recovery in anaesthetic-free water. Nine fish per analysis were used ($n = 9$). The same group of fish was used for the evaluation of induction to and recovery from anaesthesia.

For the recording of EMG, fish were individually netted from the maintenance tanks, held in one hand out of the water and rapidly equipped with the electrodes. Animals were not anaesthetized for the attachment of the electrodes to prevent a bias from an anticipated anaesthetic effect prior to the beginning of the recordings. The time required for the handling and positioning of the equipment did not exceed 60s for each individual.

After the positioning of the electrodes, the individual was handled to the fish tank (filled with 1 L water volume from the maintenance tanks) added with EOPD at 40 μL.L$^{1}$. During induction, the field potential recordings from the dorsal muscle, including baseline profile in sham control, were performed for 5 min. After the recordings, fish were returned to their tanks of original for the observation of mortalities over a period of 48 h.

**Table 2**
Latencies to the stages of anaesthesia induction and recovery (seconds ± SD) in tambaqui, *Colossoma macropomum* exposed to five concentrations of *Piper divaricatum* essential oil.

| Concentration (μL.L$^{-1}$) | Induction stages (s) | | | Recovery stages (s) | |
|---|---|---|---|---|---|
| | A1 | A2 | A3 | R1 | R2 |
| 25 | 4 ± 1$^{b}$ | 41 ± 3$^{a}$ | 462 ± 9$^{a}$ | 25 ± 6$^{a}$ | 221 ± 3$^{a}$ |
| 30 | 3 ± 1$^{b}$ | 43 ± 11$^{a}$ | 260 ± 7$^{b}$ | 7 ± 2$^{b}$ | 149 ± 2$^{b}$ |
| 35 | 7 ± 3$^{a}$ | 42 ± 5$^{a}$ | 210 ± 1$^{b}$ | 19 ± 11$^{a}$ | 215 ± 2$^{a}$ |
| 40 | 4 ± 1$^{b}$ | 30 ± 4$^{b}$ | 156 ± 2$^{c}$ | 12 ± 4$^{b}$ | 188 ± 2$^{a}$ |
| 45 | 4 ± 1$^{b}$ | 29 ± 4$^{b}$ | 90 ± 6$^{c}$ | 17 ± 9$^{a}$ | 128 ± 1$^{b}$ |

Times to the different stages of induction or recovery are cumulative. Different superscripts in the same column denote significant differences among concentrations after ANOVA and Tukey's test ($p < .05$), $n = 10$.

### 2.5. Statistical analysis

Deviation from normal distribution and homogeneity of variances were analyzed with Kolmogorov-Smirnov's and Levene's tests, respectively. One-way ANOVA and Tukey's test were used to compare times to anaesthesia induction and recovery, and mean amplitudes in EMG throughout induction and recovery. Trends of anaesthesia (A3) and recovery (R2) stages were fitted to non-linear response models with measured time to reach the stages being the response variable and concentration of EOPD the predictor variable. Pearson correlation was performed between times to stages A3 or R2 and increasing concentrations of EOPD. The minimum significance level was set at $p < .05$ in all cases.

## 3. Results

No mortality occurred during or after exposure to EOPD. During the behaviour evaluation, no signs of anaesthesia were observed in fish exposed to anaesthetic-free water, including the vehicle control. Further, since no differences ($p > .05$) were observed in mean amplitude values of EMG between basal and ethanol controls, only mean values of the former (basal) were used for comparison purposes against the anaesthetized group.

### 3.1. Behavioural evaluation of short-term anaesthesia and recovery

Upon exposure to the EOPD, immediate behavioural changes were observed. Within the first seconds of the exposure, hyperactivity, i.e., agitated behaviour, could be noted. The mean latency to A1 was between 3 and 7 s, whereas EOPD at 35 μL.L$^{-1}$ determined the longest interval to A1 ($p < .05$) compared to the other concentrations. At this stage, fish presented faster and erratic swimming with partial loss of equilibrium, and frantic jumping towards the water surface. Time to A2 stage decreased significantly from concentration of 40 μL.L$^{-1}$ and above and all concentrations tested induced tambaqui to A3 stage within the maximum stipulated time threshold (30 min). However, EOPD was effective to promote deep anaesthesia in < 3 min (180 s) from 40 μL.L$^{-1}$ or above ($p < .05$), herein referred to as "rapid anaesthesia". Fish were considered fully recovered after resumption of the righting reflex and normal swimming. Irrespective of concentration used and some significant differences observed, all fish recovered (R2), resuming normal ventilation and swimming activity in < 5 min (300 s) (Table 2).

Non-linear patterns were observed for induction and recovery times over increasing concentrations of EOPD, which can be estimated by the equation models attained. The regression equations of the exponential type showed an inverse relationship ($p < .05$; $r^2 = 0.97$; $r = -0.98$) between increasing concentrations of EOPD and the time required to A3 stage. The same trend was observed between the time to R2 stage and the oil concentrations tested, yet, presenting a weak correlation in this case ($p < .05$; $r^2 = 0.32$; $r = -0.57$) (Fig. 1).

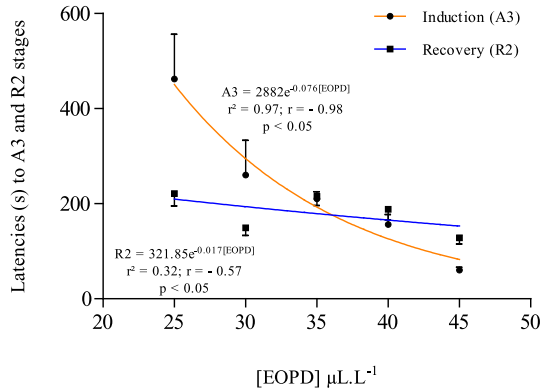


**Fig. 1.** Trends of induction (A3) and recovery (R2) times in tambaqui, *Colossoma macropomum* exposed to short-term baths with increasing concentrations of *Piper divaricatum* essential oil.

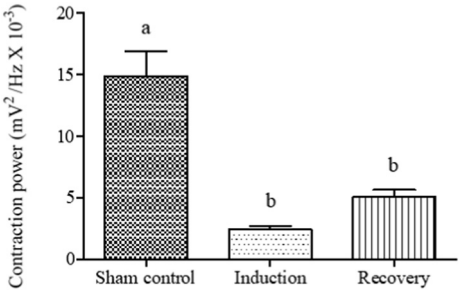


**Fig. 3.** Mean amplitude recordings (mean ± SEM) of the electromyogram (EMG) in the dorsal muscle of tambaqui, *Colossoma macropomum*. Recordings of 300 s in the basal state (Sham control), animals exposed to the essential oil of *Piper divaricatum* at 40 µL.L$^{-1}$ (Induction), and during the return (Recovery) from anaesthesia in frequencies of up to 50 Hz. Different letters in columns denote significant differences after ANOVA and Tukey's test ($p < .05$, $n = 9$).

### 3.2. Modulation of the skeletal muscle contraction power

The electromyographic data showed high amplitude of tracings in the dorsal muscle of sham control (Fig. 2A, left) and the intensity of the signal was higher in frequencies below 20 Hz (Fig. 2A, right). Upon contact with the EOPD, the tracing sharply decreased in amplitude, with transient muscle spasms between 10 and 20s, followed by less intense muscle activity with loss of contraction power (Fig. 2B, left). The spectrogram of frequency is consistent with these tracing records, whereby a more intense energy was observed at the same initial time interval (Fig. 2B, right). When the fish was allowed to recover, the

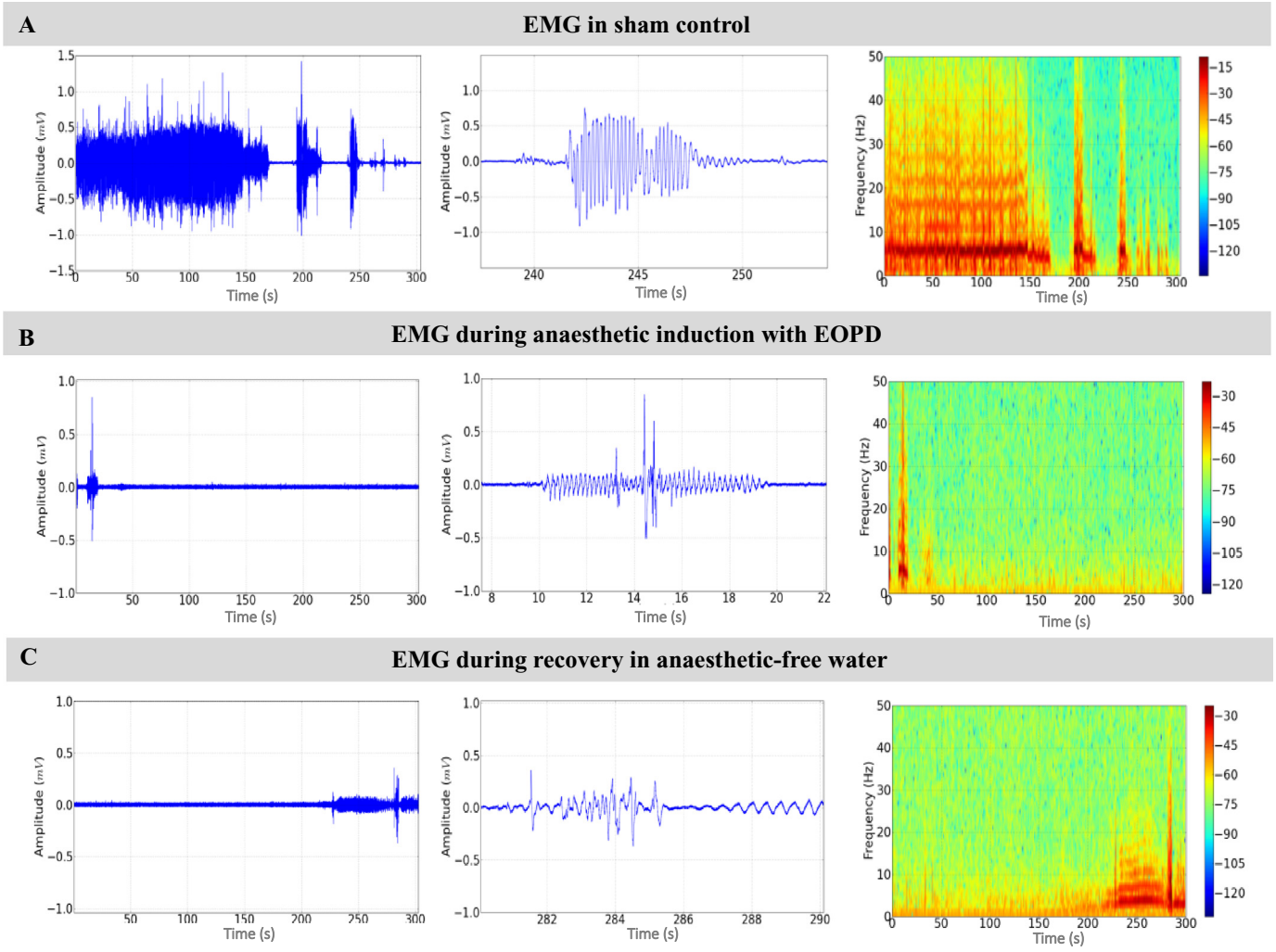


**Fig. 2.** Electromyographic tracings (EMG) of the dorsal muscle in juvenile tambaqui, *Colossoma macropomum* submitted to short-term anaesthetic bath. Recordings in sham control (A), fish exposed to the essential oil of *Piper divaricatum* (EOPD) at 40 µL.L$^{-1}$ (B), and fish in recovery after anaesthesia (C). EMG over the course of 300 s (left), amplification of fragments in EMG recordings (centre), and spectrograms of frequency (right).

record initially showed a low muscular activity which gradually increased to contraction amplitudes that were compatible with the normal swimming, indicating a reversibility of the myorelaxation effect (Fig. 2C). The mean amplitude of contractions in the control group was $14.85 \pm 5.30\,mV^2$ / Hz x $10^{-3}$ and during the exposure, the mean amplitude decreased significantly to $2.42 \pm 0.63\,mV^2$ / Hz x $10^{-3}$ with marked loss of muscle tonus. In the recovery period the animals had a mean amplitude of $5.06 \pm 1.55\,mV^2$ / Hz x $10^{-3}$, which was still lower ($p < .05$) than the control and similar ($p > .05$) to the mean amplitude of animals under anaesthesia. Despite non-significant differences observed between induction vs. recovery treatments, a gradual recovery can be anticipated, as the amplitude averages in the recovering animals were slightly above those observed in the animals during induction (Fig. 3).

## 4. Discussion

Upon contact with the anaesthetic and regardless of concentration, fish showed agitated behaviour and "coughing" with rapid opercula beating, presumably in an attempt to expel the product from the gills. Such reactions have been reported for fish during the initial stages of anaesthesia using other natural or synthetic products, and could be caused either by the irritating properties of the phytochemical constituents or by a mechanically-induced perturbation such as the coating of the gills' anatomic structures due to the oily nature of the compound (Sladky et al., 2001; Barbas et al., 2016, 2017a).

Should anaesthetics be necessary to facilitate any management procedure or handling for research activity, other than invasive interventions, which demand full body immobilization, a rapid induction is usually sought after, both for time-saving purposes and the minimization of excessive absorption through the gills that could lead to an overdose (Sladky et al., 2001; Sneddon, 2012).

Full body immobilization was attained with EOPD regardless of the concentration used. Based on the complete absence of body movements and the total loss of reaction to visual and mechanical stimuli (slight tail pinch), it could be speculated that EOPD rendered fish deeply anaesthetized as a consequence of a central nervous system (CNS) depression. In fact, depression of the CNS must be guaranteed if invasive procedures, i.e., surgeries or euthanasia are to be carried out. Apparently, EOPD exposure resulted in general anaesthesia, similarly to what has been reported for other anaesthetic-like and plant-derived agents tested in the same animal model (Barbas et al., 2016; Barbas et al., 2017a; de Souza et al., 2019).

In this sense, EOPD at concentration of $40\,\mu L.L^{-1}$ was sufficient to induce a deep and rapid anaesthesia, therefore conforming to the recommended standards of induction and recovery times for fish, which are to take place until 3 and 5 min, respectively (Bell, 1987; Ross and Ross, 2008). Similar deep and reversible anaesthesia, with rapid induction have been reported for tambaqui when other plant extracts were used, as in the case of jambu wax extract (rich in spilanthol) at $20\,mg.L^{-1}$ (Barbas et al., 2016), citronella (*Cymbopogon nardus*) essential oil (rich in citronellol and geraniol) at $600\,\mu L.L^{-1}$ (Barbas et al., 2017a), and menthol at $168\,\mu L.L^{-1}$ (Façanha and Gomes, 2005), which promoted a general anaesthesia-like effect in 173, 132 and 136 s, respectively. More recently, the essential oil of *Nepeta cataria*, rich in nepetalactone, rendered fish fully and rapidly (< 180 s) anaesthetized at $175\,\mu L\,L^{-1}$ (de Souza et al., 2019).

The trend observed for induction response over increasing concentrations of the oil followed a similar pattern as those reported by other studies with this fish species in which a strong negative correlation was attained. The higher the concentration the faster the onset of full immobilization (Barbas et al., 2016; Barbas et al., 2017a; Fujimoto et al., 2017).

Latencies to recovery from anaesthesia were all within an appropriate time interval (< 5 min), irrespective of the concentration used. However, recovery responses did not follow a linear pattern. Irregular responses during recovery are commonly reported for fish subjected to anaesthesia (Façanha and Gomes, 2005; Mylonas et al., 2005; da Cunha et al., 2010; Barbas et al., 2016), and as concentrations are increased, an oscillatory pattern is usually observed, either because of individual variations or due to an impaired clearance of the substance from the blood stream when fish are exposed to high concentrations.

Although never reported in the scientific literature, the anaesthetic effects of *P. divaricatum* essential oil, which is rich in ME, were not totally unexpected in fish, and the results attained in this study met the initially proposed hypothesis. The sedative and myorelaxant effects of ME have been reported in mice and rabbits (Sell and Carlini, 1976), in which an electroencephalographic-based and comparative study using four eugenol-derived compounds found in the volatile oil fraction of *Myristica fragans* (eugenol, ME, isoeugenol, and methyl isoeugenol) suggested such ME-related biological effects. ME was further compared with pentobarbital and propanidid, the latter being a synthetic eugenol derivative, using the intraperitoneal route in rats. It was observed that ME promoted short-term anaesthesia, which was at least comparable, and probably better, to that of pentobarbital-elicited anaesthesia. In general, ME revealed to be the most active and the less toxic compound to induce loss of the righting reflex.

Another sedative and analgesic effect was ascribed to a compound from *Piper* species. The alkamide piperovatina isolated from *Piper piscatorum* is a sialogogic and local anaesthetic compound that was evaluated for its ability to induce changes in neuronal intracellular calcium concentration. It produced marked increases in neuronal intracellular calcium, similar in duration and character to other voltage-gated sodium channel agonists (Mcferren et al., 2002).

The ME mechanism of action seems to be related to the capacity of inhibition of peripheral $Na^+$ channels. According to Wang et al. (2015) ME was able to reversibly inhibit the peak amplitude of the $Na^+$ current, specifically on the $Na^+$ channel isoform, $Na_v1.7$, preferably binding to channels in the open and/or inactivated states; displayed greater use-dependent inhibition as well as a slower rate of recovery for the inactivated channels. This implies that high concentrations of ME may effectively limit or paralyze neuronal activity to exert a local anaesthetic effect. Interestingly, in recent studies on the effect of analgesia in dental afferent neurons, the cellular mechanisms underlying the analgesic action of eugenol have also been proposed, including the inhibition of $Ca^{2+}$, $Na^+$, and $K^+$ currents and stimulation of $GABA_A$ receptors (Lee et al., 2005; Cho et al., 2008).

It is commonplace that invasive interventions, e.g. surgeries, should preferably be accompanied by effective muscle relaxation for it will facilitate handling and access to target organs. Fujimoto et al. (2017) reported a conspicuous depression of muscle contraction power in clove oil anaesthetized fish, which is in line with our findings for EOPD-exposed fish. Such findings lend more evidence-based credence to the indication of this oil as an effective skeletal muscle relaxant.

A remarkable time-dependent muscular relaxation was attained in EOPD-exposed fish, as demonstrated by the marked reduction in the frequency and amplitude of the tracings. Similarly, ME has also been studied as a muscle relaxant agent in the guinea-pig (Lima et al., 2000) where it seems to have exerted a good relaxing effect on smooth muscle tissue, however, it was not possible to state whether the mechanism of action involves a direct modulation of the muscle motor plate or if it occurs at a central level. Likewise, although our results provide indisputable evidence that EOPD blunts skeletal muscle contraction power in fish, it remains to be investigated whether it is a peripheral- or CNS-related effect.

In vitro studies have suggest that ME acts directly at the intracellular level to induce its pharmacological effects (Lima et al., 2000). Cardiovascular effects of intravenous (i.v) treatment with ME were investigated in normotensive rats. The study examined whether the autonomic nervous system was involved in the mediation of ME-induced changes in mean aortic pressure and heart rate, and whether the hypotensive effects of ME could result from its vasodilatory effects

directly upon the vascular smooth muscle. First physiological evidences attained showed that i.v. treatment with ME in either anaesthetized or conscious rats elicited hypotension; an effect that seemed to be related to an active vascular relaxation induced by the compound (Lahlou et al., 2004).

The time-frequency decomposition showing the intensity of the signal in the spectrograms of frequency coherently corroborated the amplitude oscillations observed during EMG recordings throughout induction or recovery from anaesthesia. During exposure for 5 min to EOPD, the dorsal muscle contraction power decreased by 83.7%, which points to a significant muscle relaxation effect.

These results are in line with the reduction in muscle contraction power reported by Barbas et al. (2017a) when investigating muscle relaxation in tambaqui exposed to short-term baths using essential oil of citronella at 600 $\mu L.L^{-1}$, which was capable to induce a significant depression (94%) of the dorsal muscle contraction power. Similarly, Fujimoto et al. (2017) tested different concentrations of clove oil in three different species of Amazonian fish (*Pterophyllum scalare*, *Paracheirodon axelrodi* and *Heros severus*) and concluded that this oil exerted a conspicuous depression on the muscle contraction power, of up to 91% compared to the control.

It is worth noting that although fish resumed the righting reflex after EOPD exposure, muscle contraction power was not fully re-established as per the amplitude mean values attained throughout the 5 min recordings in recovery, underscoring the need of a longer time for full recovery of the muscle contraction power. A similar outcome was reported by de Souza et al. (2019) during recovery of tambaqui after anaesthesia with essential oil of *N. cataria*.

Some natural compounds have led to undesired results, such as carvacrol and thymol which caused mortality in fish shortly after exposure (Silva et al., 2013). Under the conditions herein established, no mortality occurred in EOPD- anaesthetized fish, which makes this oil a suitable candidate for anaesthesia along with other tested agents (Soto and Burhanuddin, 1995; Roubach et al., 2005; Mylonas et al., 2005; Barbas et al., 2017a; Stringhetta et al., 2017).

Novel anaesthetic compounds should always be thoroughly tested for safety and efficacy before a broad use is recommended. There is still much to uncover about the physiological responses of fish resulting from the exposure to different anaesthetics, especially those derived from plant matrices. Further studies focusing on electroencephalographic analyses could shed light on the interplay between behaviour and the presumable depressing properties of the EOPD on the CNS.

In conclusion, the EOPD was capable of promoting a general anaesthesia-like state, with complete body immobilization of *Colossoma macropomum* at all concentrations tested. The concentration of 40 $\mu L.L^{-1}$ sufficed to induce a fast (< 3 min) and reversible (< 5 min) immobilization with loss of muscle tonus. The EOPD could be used for non-invasive procedures which require short-term immobilization and loss of muscle tonus. During recovery, there was a smooth transition to normal swimming behaviour, which indicates that this extract is capable of acting safely and reversibly as a novel skeletal muscle relaxant agent.

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## Declaration of interest

The authors declare that there is no conflict of interest.

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