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Jambu, *Spilanthes acmella* as a novel anaesthetic for juvenile tambaqui, *Colossoma macropomum*: Secondary stress responses during recovery

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ABSTRACT

The aim of this study was to evaluate the efficacy of the waxy extract of jambu flowers, *Spilanthes acmella* as an anaesthetic for fish, using juvenile tambaqui *Colossoma macropomum* as a model. The times to induction and recovery after short-term anaesthesia were evaluated by concentration-response trials and secondary stress responses. Juveniles were placed in aquaria containing five different concentrations of jambu extract (5, 10, 15, 20, 25 mg L^{-1}) and the times of anaesthetic induction and recovery were determined. Sham control fish and fish exposed to ethanol-added water were used as controls. The secondary stress responses of fish following anaesthesia with jambu extract (20 mg L^{-1}) were investigated through an assessment of whole blood variables: glucose, ions (Na^+, K^+, and Ca^{++}), osmolality, haematocrit (Htc), haemoglobin (Hb), partial pressures of CO_2 (pCO_2) and O_2 (pO_2), bicarbonate concentration (HCO_3^-), and pH. Deep anaesthesia was observed at all concentrations tested in this study. The use of 20 mg L^{-1} of this extract is recommended for rapid induction (<3 min) and uneventful recovery (<5 min) from deep anaesthesia; while the concentration of 2 mg L^{-1} is sufficient to promote sedation. Only transient changes in secondary stress responses were observed in tambaqui during recovery, with most parameters returning to initial values within 48 h post-anaesthesia. Therefore, the extract of jambu flowers may be considered an efficient anaesthetic for tambaqui and other fish species.
Statement of relevance: This is the first study using extract of Jambu, *Spilanthes acmella* as an anaesthetic for aquatic organisms.

1. Introduction

Anaesthetics are important in aquaculture to reduce handling stress and mortality and have also been used for research and veterinary medicine purposes (Sneddon, 2012). In addition to the advantages of using anaesthetics to mitigate stress and decrease mortality rates, recent studies have raised awareness about the importance of using anaesthetics for aquatic organisms from an ethical perspective. Since there is reasonable evidence that fish are capable of nociception or pain perception (Ashley et al., 2007; Roques et al., 2010), welfare and pain are important aspects to be addressed.

Tambaqui *C. macropomum* is the most widely farmed native fish species in Brazil and in several other countries in South and Central America (FAO, 2014). Therefore, this species has the potential to be selected as a model to establish studies with anaesthetics for tropical species.

Alternative anaesthetics such as menthol and eugenol have been previously tested on juvenile tambaqui (Façanha and Gomes, 2005; Roubach et al., 2005), the latter being largely used for other tropical teleost species (Vidal et al., 2007, 2008; Inoue et al., 2011). Essential oils of *Lippia alba*, *Ocimum gratissimum* and *Aloysia triphylla* have been recently investigated for their anaesthetic properties and were presented as new natural compounds for anaesthesia of aquatic organisms (Cunha et al., 2010; Azambuja et al., 2011; Benovit et al., 2012; Parodi et al., 2012; Silva et al., 2012).

The genus *Spilanthes* is comprised of approximately 60 species widely distributed over tropical and subtropical regions of the world, namely in Africa, America and Asia (Chung et al., 2008; Tiwari et al., 2011). *Spilanthes acmella* var. *oleracea* is commonly known as jambu, toothache plant and Brazil cress. This species has long been used in traditional cuisines and medicines of different civilizations (Hind and Biggs, 2003).

Previous studies have assessed *S. acmella* extract bioactivity as an analgesic and local anaesthetic in rats and mice (Ansari et al., 1988; Chakraborty et al., 2002; Nomura et al., 2013). Ethanolic extract of the leaves was investigated and significant peripheral analgesic activity was observed in experimental animal models (Barman et al., 2009).

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Other studies report the extract also proved to possess anti-nociceptive activity against continuous inflammatory pain and anti-hyperalgesic activity, possibly by inhibiting prostaglandin synthesis (Ratnasooriya and Pieris, 2005). The presence of N-alkylamides in jambu, namely "spilanthol" (N-isobutyl-2E,6Z,8E-decatrienamide) suggests that the therapeutic effect observed is a result of its marked anaesthetic activity (Nomura et al., 2013).

Frequently, the use of anaesthetics to reduce handling stress in fish is advantageous based on comparisons of plasma cortisol levels, glycemia, plasma lactate concentration, haematology, and osmolality between anaesthetized and non-anaesthetized animals (Bressler and Ron, 2004; Small, 2005; Crosby et al., 2006). Contradictorily, anaesthetics themselves can also induce stress responses by potentializing catecholamine release into the blood stream (Bressler and Ron, 2004; Zahl et al., 2010; Weber, 2011). It has been reported that MS-222 caused a 'detrimental physiological impact' in silver catfish, *Rhamdia quelen* (Gressler et al., 2014).

To date, there are no reports about sedative and/or anaesthetic efficacy of jambu, *S. acmella* on fish. The aim of this study was to evaluate the anaesthetic activity of jambu on juvenile tambaqui, through determination of time to induction and recovery by concentration-response trials, and secondary stress responses after short-term anaesthesia.

2. Materials and methods

2.1. Animals

Tambaqui juveniles were purchased from a commercial fish farm in Brazilian Amazon and transported to the laboratory where they were maintained in continuously aerated 250 L tanks, with controlled water parameters. Fish were acclimated for 15 days in two recirculation systems prior to the beginning of the experiments. Photoperiod was fixed at 12 L/12 D. Fish were fed twice a day at 2% of biomass with commercial feed (28% crude protein). Juveniles were fasted for a period of 24 h prior to the experiments.

The experiments were approved by the Ethical and Animal Welfare Committee of the Federal University of Rio Grande — FURG.

2.2. Water quality

The parameters (mean ± S.D) were maintained as follows: dissolved oxygen (DO) (experiment 1: 6.45 ± 0.39 mg L^{-1}; experiment 2: 6.82 ± 0.09 mg L^{-1}) and temperature (experiment 1: 25.6 ± 0.7 °C; experiment 2: 25.7 ± 0.1 °C) were measured using an oxygen metre (Yellow Springs Instruments, Yellow Springs, OH, USA); pH (experiment 1: 7.1 ± 0.2; experiment 2: 7.05 ± 0.1) was determined with a Five Easy FE20, Switzerland. Total ammonia nitrogen (TAN) (experiment 1: 0.80 ± 0.08 mg L^{-1} $NH_4^+ + NH_3^- - N$; experiment 2: 0.90 ± 0.05 mg L^{-1} $NH_4^+ + NH_3^- - N$) was quantified according to Unesco (1983), and nitrite was determined according to Bendschneider and Robinson (1952) (experiment 1: 0.03 ± 0.01 mg L^{-1}; experiment 2: 0.02 ± 0.01 mg L^{-1}). Total alkalinity was evaluated by titration in accordance with Eaton et al. (2005) guidelines (experiment 1: 41.7 ± 0.09; experiment 2: 52.6 ± 0.05 mg $CaCO_3$ L^{-1}, respectively).

2.3. Plant materials

Extract of the flowers of *Spilanthes acmella* Var. *oleracea* (L.) was obtained by means of fractionated supercritical fluid extraction methodology with the use of CO_2 [SFE(CO_2)] to remove less polar compounds from the flowers of the plant. For details on the methodology of extraction, global and spilanthol yields of the extract see Dias et al. (2012).

Vegetal extracts, in the form of a wax, such as those obtained from jambu flowers through [SFE(CO_2)] extraction methodology are poorly diluted in water and therefore it is necessary to pre-dilute them in ethanol before using in anaesthetic baths for fish. The stock solution of *S. acmella* extract was prepared by weighing and diluting the jambu flowers extract in commercial alcohol (96%) yielding a 2.24 g L^{-1} solution which was stored in an amber glass bottle at 4 °C until its use.

2.4. Biological activity

2.4.1. Experiment 1: anaesthetic efficacy of extract of jambu, S. acmella

Juvenile fish (46.6 ± 6.2 g; 14.6 ± 0.8 cm, total length) were transferred to aquaria containing 30 L of continuously aerated water. Concentrations of the extract at 5, 10, 15, 20 and 25 mg L^{-1} were used in this experiment. A sham control group was used and animals (n = 5) were transferred to aquaria with anaesthetic-free water and observed for 30 min. A vehicle control added with the same volume of ethanol to reach the concentration of 25 mg L^{-1} of jambu extract in the water was also evaluated. In order to evaluate the cumulative time required to reach the different stages of induction and recovery from anaesthesia a digital stopwatch was used. Groups of 10 juveniles were used for each concentration tested and each juvenile was used only once, observed individually and considered a replicate. All animals were starved for 24 h prior to the tests.

Cumulative time to reach the different stages of anaesthesia and recovery were characterized according to Park et al. (2008) with modifications 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 (A3) were used as behavioural indicators 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 was 30 min. After induction, juveniles were transferred to tanks with anaesthetic-free water, and the time elapsed for recovery was registered. Animals were considered to have recovered when they showed normal swimming behaviour. After recovery, fish were grouped according to the anaesthetic concentration and transferred to continuously aerated 250 L tanks, where they were observed for two weeks to check for mortalities.

In order to investigate the effectiveness of a sedation state (slight anaesthesia), a group of fish (n = 10) was exposed for 10 min to 2 mg L^{-1} of jambu extract which corresponded to 10% of the anaesthetic concentration (20 mg L^{-1}) considered adequate to induce deep anaesthesia of juvenile tambaqui (see results).

2.4.2. Experiment 2: evaluation of secondary stress responses

This experiment was conducted to verify stress response of fish in recovery after short-term exposure (3 min) to *S. acmella* extract at the concentration of 20 mg L^{-1}. This concentration was used because it was the minimal concentration tested capable of inducing stage A3 within 3 min (see results in Table 1). Tambaqui juveniles (50.5 ± 3.9 g; 15.0 ± 0.4 cm, total length) were assayed in five groups (n = 10 per sampling time post-anaesthesia). Instead of using the same specimens for blood collection over time, groups of 10 animals were used for each sampling time in order to avoid cumulative stress due to handling. A sham control group (CT) and an ethanol control (EC) were used (n = 10 in each control group). In the EC fish were exposed to water added with ethanol at the same volume used to provide the concentration of 20 mg L^{-1} of jambu extract.

Fish were captured with a dip net and individually transferred to continuously aerated 30 L aquaria previously added with jambu extract at 20 mg L^{-1} where they were exposed to anaesthetic baths of 3 min. Immediately following anaesthesia, all fish were handled for biometric measurements and transferred to their respective anaesthetic-free 100 L recovery tanks. Similarly, CT and EC groups were transferred to identical aquaria and were also maintained for 3 min in their respective anaesthetic-free water tanks, simulating the same handling procedures of anaesthetized fish.