ARTIGO ORIGINAL

Anesthetic-like activity of the essential oil of *Curitiba prismatica*

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ABSTRACT: This study evaluated the sedative and anesthetic potential of the essential oil (EO) of *Curitiba prismatica* (Myrtaceae) in silver catfish (*Rhamdia quelen*), and analyzed its yield and chemical composition. The EO was obtained by hydro-distillation and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The anesthetic evaluation was performed on silver catfish juveniles (7.53 ± 0.43 g; 9.6 ± 0.26 cm; n=5) by determining the time of anesthesia induction and recovery. The extractive yield obtained for the EO was 0.558 ± 0.008%, and the major identified compounds were as α-pinene (60.0%) and limonene (24.2%). At all concentrations of the EO sedative properties were observed. At 15 min an anesthetic effect was observed only at a concentration of 1000 mg/l in 40% of the animals. The use of the EO as an anesthetic is not recommended due to the occurrence of side effects. However, the sedative effect of the essential oil could be better explored without anesthetizing the fish.
Keywords: *Rhamdia quelen*, anesthesia, sedation, α -pinene, limonene

RESUMO: Atividade anestésica do óleo essencial de *Curitiba prismatica*. Este estudo avaliou o rendimento e composição química do óleo essencial (OE) de *Curitiba prismatica* (Myrtaceae), bem como seu potencial sedativo e anestésico em jundiás (*Rhamdia quelen)*. O OE foi obtido por processo de hidrodestilação em aparelho de Clevenger e analisado por cromatografia gasosa com detecção por ionização em chama e cromatografia gasosa acoplada à espectrometria de massas. Sedação e anestesia foram avaliadas em juvenis de jundiás (7,53 ± 0,43 g; 9,6 ± 0,26 cm; n=5) pela determinação do tempo de indução a esses estágios e respectiva recuperação dos mesmos. O rendimento extrativo do OE foi de 0,558 ± 0,008% e seus compostos majoritários foram α-pineno (60,0%) e limoneno (24,2%). Em todas as concentrações testadas foi possível observar a sedação induzida pelo OE. O efeito anestésico foi observado com 15 min de exposição à concentração de 1000 mg/l em 40% dos animais. O uso deste OE em concentração anestésica não é recomendado devido à ocorrência de efeitos colaterais. Entretanto, seu efeito sedativo pode ser explorado extensivamente sem a necessidade de anestesiar o animal.
Palavras-chave: *Rhamdia quelen*, anestesia, sedação, α -pineno, limoneno

INTRODUCTION

Volatile compounds are secondary metabolites produced by aromatic plants and are known to have different biological activities (Zalachoras et al., 2010). *Curitiba prismatica* (D. Legrand) Salywon & Landrum (*Myrtaceae*) was described as being the only plant species belonging to a new genera growing in the upland of the Atlantic Forest ecosystem in Southern Brazil (Salywon & Landrum, 2007; Landrum & Kawasaki, 1997). This plant species was previously misclassified as *Eugenia prismatica* and later as *Mosiera prismatica* D. Legrand (Lorenzi, 1998) but now both denominations are considered synonymies for *C. prismatica*.

The literature shows no reports about the chemical composition of the EO or about the biological activities for *C. prismatica*. However, some activities, such as anti-inflammatory, antifungal,

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antimicrobial and antioxidant, were reported for other species of *Eugenia*. (Öztürk & Özbek, 2005; Costa et al., 2000) Furthermore, it is known that clove oil, obtained from *E. aromatica* and *E. caryophyllata*, induces anesthesia in fish (Inoue et al., 2003; Simões et al., 2012).

Anesthesia is routinely used for handling and biometric procedures in aquaculture for numerous fish species (Ross & Ross, 2008). According to Zahl et al. (2011) the anesthetic process includes sedation, immobilization, unconsciousness and analgesia. Currently, the anesthetics employed most frequently in fisheries are synthetic substances, such as benzocaine, metacaine (MS-222), metomidate hydrochloride, 2-phenoxyethanol and quinaldine, which have all been associated with various systemic side effects limiting their application (Zahl et al., 2011). Some studies have been conducted with products from natural sources, which may be a safer alternative than synthetic anesthetics in aquaculture (Kristan et al., 2012). Eugenol and isoeugenol, the main components of clove oil, are considered good natural anesthetics due to their efficacy at low concentrations. However, they still produce some adverse effects and cause mortality (Zahl et al., 2011), and there is evidence of eugenol and isoeugenol having genotoxic and carcinogenic effects (National Toxicology Program, 1983; National Toxicology Program, 2010).

Silver catfish (*Rhamdia quelen)* has been used as an experimental model for the induction of anesthesia (Cunha et al., 2010; Silva et al., 2012). This species has a great potential for aquaculture due to its fast growth (Fracalossi et al. 2012). Additionally, the use of plant extracts is enabled in organic aquaculture practices (Prein et al., 2012). Therefore, the aim of this work was to assess the extractive yield and chemical composition of *C. prismatica* EO leaves, as well as to evaluate its sedative/ anesthetic potential in silver catfish.

MATERIAL AND METHODS

Plant Material and EO extraction

C. prismatica leaves were collected in Faxinal Marmeleiro de Cima (Rebouças, Paraná, Brazil) in November, 2011. A voucher specimen (SMDB 13426) was identified by Prof. Solon Jonas Longhi and deposited at the Herbarium of the Department of Biology, UFSM. The extraction of the EO of *C. prismatica* was performed in duplicate by hydrodistillation using a Clevenger type apparatus for 3 h. (European Pharmacopoeia, 2007) The essential oil was stored at -4 ºC until subjected to GC-MS analysis and the biological tests. The EO yield was calculated as w/w (%).

GC and GC-MS analysis

GC-MS TIC analysis was performed using an Agilent-6890 GC coupled with an Agilent 5973 mass selective detector, using an HP5-MS column (5% phenyl - 95% methylsiloxane, 30 m x 0.25 mm i. d. x 0.25 µm), EI-MS at 70 eV, split mode 1:100, column flow 1 ml/min, oven ramp 40-320 °C with 4 °C/min, Helium as carrier gas and a run time of 76 min. The constituents of the EO were identified by comparing the mass spectra with a mass spectral library and the Kovats retention indexes with literature data (NIST, 2005). Gas chromatography analysis were performed on an Agilent-6890 chromatograph with a flame ionization detector running with H_2 flow at 30 ml/min, synthetic air at 300 ml/min and N_2 at 25 ml/min as make up gas. The analysis was performed on a HP-5 column (5% phenyl - 95% methylsiloxane, 30 m x 0.25 mm i. d. x 0.25 µm) in split mode ratio 1:20 with the same analytical conditions as used for the GC-MS analysis.

Animals

Silver catfish were purchased from a local fish culture facility and maintained in continuously aerated 250 l tanks, under controlled water parameters. The dissolved oxygen levels (7.31 ± 0.22 mg/l) and temperature (18.53 ± 0.14 ºC) were measured with an YSI oxygen meter. The pH (6.33 ± 0.07) was determined with a DMPH-2 pH meter. Total ammonia levels (0.12 ± 0.10 mg/l) were performed using the salicylate method. (Verdouw et al., 1978) A semi-static system was used, and 50% of the water volume was changed daily. The fish were fed commercial feed containing 28% crude protein (Vicente Alimentos S.A. Presidente Prudente/SP, Brazil), once a day. The animals were fasted for a period of 24 h prior to the experiments.

Sedation and anesthesia protocol

Juveniles at 180 days of age (7.53 ± 0.43 g; 9.6 ± 0.26 cm) were transferred to aquaria containing 1 l of continuously aerated water and 87, 500 or 1000 mg/l of the EO diluted in 95% ethanol (1:10). An ethanol control was also performed using the highest concentration of ethanol (9 mL/l) used to dilute the EO. To evaluate the time required for the anesthetic induction, five animals were used for each concentration tested, and each animal was used only once. The anesthetic induction was evaluated according to the method by Gomes et al. (2011) in the following stages: sedation (stage 2), loss of equilibrium (stage 3a), total loss of equilibrium with absence of swimming ability (stage 3b) and anesthesia (stage 4). The maximum observation time for the induction of and recovery from anesthesia was 30 min. After the induction, the fish were transferred to anesthetic-free aquaria to record the recovery

time. Animals were considered to have recovered when they demonstrated normal swimming with reaction to an external stimulus. After recovery, the fish were grouped according to the anesthetic protocol and transferred to continuously aerated 40 L aquaria, where they were observed for 48 h for any signs of abnormal behavior, diseases or mortality.

Statistical analysis

Data are presented as the mean ± SEM. The relationship between the time for anesthetic induction and the concentration used was determined using the Slide Write Plus version 4.0 software. Non parametric variance analysis and Tukey tests were used to compare the time required for the induction of the stages 2, 3a with the recovery time. The analyses were performed using the SigmaPlot version 11.0 software and the minimum significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

The extractive yield obtained for the EO of *C. prismatica* (0.558 ± 0.008%) and there are not previously literature reports of this data. Nevertheless, the yield found in this work was similar to the average values found for the *Eugenia* species (Costa et al., 2000). (Souza, 2009; Zoghbi et al., 2011) The GC-MS analysis provided the identification of 91.77% of the EO composition. The major compounds were the monoterpenes α-pinene and limonene (Table 1). The high content of monoterpenes differs from the corresponding data described for most *Eugenia* species. Plants of this genus normally showed a predominance of cyclic sesquiterpenes (Stefanello et al., 2011). However, reports for the chemical composition of the EO obtained from the *Mosiera* species are highly similar to the findings of this study. A report about EO of *M. ehrenbergii* described α-pinene (33.6%), limonene (51.6%) and E-nerolidol (5.7%) as major compounds, while for the EO of *M. longipes*, α-pinene (3.8%), limonene (47.8%) and β-eudesmol (5.5%) were detected (Tucker et al., 2007).

All tested concentrations of the EO led the fish to sedation (Figure 1), and the sole application of ethanol did not produce any sedative or anesthetic effect. The effects at the concentrations of 87 mg/l and 1000 mg /l were statistically similar to each other and both induced stage 2 faster tan 500 mg/l.The similarity in the induction. times between the lowest and the highest concentrations cannot be explained from our current data. Therefore, other analysis should be performed to clarify this fact. Only 87 mg/l of the EO of *C. prismatica* may be considered adequate as a sedative for fish transport according to the criteria described in literature (Cooke et al., 2004; Ghanawi et al., 2011).

The induction time to achieve stage 3a was significantly longer at 87 mg/l of the EO of *C. prismatica* than at the highest concentrations evaluated. The concentrations of 500 and 1000 mg/ lwere similar to each other at this stage. A relationship between the concentration and effect was observed for stage 3a ($y= 666.225207 - 1.066476 x + 0.000522 x^2$; $r^2 = 1$). A similar relationship was observed for the same stage in other silver catfish studies using the EO of *Lippia alba* and *Ocimum gratissimum* (Cunha et al., 2010; Silva et al., 2012). A discontinuous loss of equilibrium was also observed in stage 3a, and additionally, the fish presented involuntary throes and jumping during this stage. A possible explanation to the occurrence of these adverse effects may be the presence of acetylcholinesterase inhibitors, such as pinene derivatives and limonene (Ghanawi et al., 2011; Miyazawa et al., 1997).

Animals subjected to 87 and 500 mg/l

TABLE 1. Chemical composition of the EO obtained from the leaves of *Curitiba prismatica.*

Peak	RT	Constituent	KI_c	KI_L	%
1	10.30	α-Pinene	931	931 [N]	60.05
2	14.13	Limonene	1027	1027 [N]	24.27
3	27.79	Longifolene	1411	1416 [N]	0.572
4	28.09	β-Caryophyllene	1421	1422 [N]	1.809
5	30.49	Bicyclogermacrene	1498	1498 [N]	0.55
6	31.28	Calamenene	1525	1529 [N]	3.03
Total identified					91.77
Monoterpene hydrocarbons					84.32
Sesquiterpene hydrocarbons					6.88

RT= retention time; KI_c= Calculated Kovats index; KI_L= Literature Kovats index, %= calculated percent using flame ionization detector, [N]= NIST (Mass spectral library) and [A]= Adams, 2001

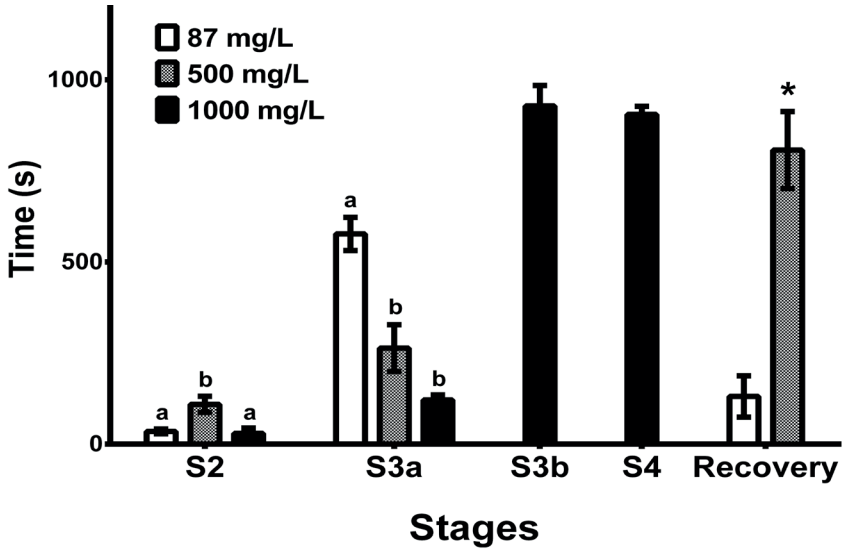

FIGURE 1. Sedative and anesthetic effects of *Curitiba prismatica* EO on silver catfish juveniles. The maximum observation time for the induction was 30 min. The time to reach each stage is given in seconds (s). The recovery of the EO 1000 mg/l group was omitted because the time was longer than 30 min. The data are presented as the mean ± SEM (N=5). Different letters indicate significant differences between the concentrations within each stage of induction and * describes a significant difference in recovery time when compared to 87 mg/l concentration ($P < 0.05$).

of the EO of *C. prismatica* did not reach stage 4. Anesthesia was achieved in only 40% of the fish exposed to 1000 mg/l in approximately 15 min. This time is higher than that considered optimal for the anesthesia of fish (lower than 3 min) (Ross & Ross, 2008). A possible explanation for this event is that the major compounds of the EO are monoterpenoids, which are known for their high volatility (Marlet & Lognay, 2010).

For silver catfish, the literature reports prompt anesthesia (approximately 4 min or less) with the EO of *Lippia alba* at 300-500 mg/l, whereas the recovery from anesthesia occurred in 4.5-7.4 min (Cunha et al., 2010). *Ocimum gratissimum* EO led to anesthesia in silver catfish at concentrations ranging from 30-300 mg/l (Silva et al., 2012). Additionally, *Aloysia triphylla* EO induces anesthesia at 100-800 mg/l, with a recovery time of 5-18 min (Parodi et al., 2014). There are also reports of anesthetic effects on this fish species using the EO of *Hesperozygis ringens* (111-554 µl/l), *Ocotea acutifolia* (300-900 µl/l) and *Hyptis mutabilis* (344 mg/l) (Silva et al., 2013; Silva et al., 2013).

Other EOs obtained from the *Myrtaceae* species showed anesthetic effects at lower concentrations when compared with the EO of *C. prismatica.* Clove oil promoted anesthesia in matrinxã (*Brycon amazonicus)* within approximately 1 min at concentrations ranging from 40 to 50 mg/l and in lambari (*Astyanax altiparanae)* in less than 1.5 min at 50-100 mg/l (Inoue et al., 2003; Pereira-da-Silva et al., 2009). Common carp (*Cyprinus carpio*) was anesthetized with 500 µl/l of tea tree oil (*Melaleuca alternifolia*) with an induction time lower than 3 min and a recovery time up to 10 min (Hajek, 2011).

It is possible that the sedative and anesthetic effects exhibited by the EO of *C. prismatica* were due to its major compound α-pinene, which presented a local anesthetic-like effect on frog nerve (Zalachoras et al., 2010). However, the action of other EO components could not be disregarded. The inhalation of 2.5% of orange EO (*Citrus aurantium* L.), which contains approximately 96% of limonene, provoked a central nervous system depressing effect in rats (Leite et al., 2008). For minor compounds, such as β-caryophyllene, anxiolytic-like effects were verified and determined as being independent of a GABAa receptor in mice (Galdino et al., 2012). Sedative-like effect of the EO could be explored owing to its large range of action without reached the anesthetic state. Therefore, this profile can be useful, since minimizes dosage mistakes.

Recovery in anesthetic-free aquaria for the concentrations of 87 and 500 mg/l occurred in approximately 2 and 14 min, respectively, which were significantly different values. At 1000 mg/l, the recovery time was longer than 30 min (Figure 1), which is outside the ideal range proposed by Ross and Ross (2008). This fact may be associated with the high lipophilicity of the EO tested. The major compounds, α-pinene and limonene, had log P

values of 4.9 and 4.2, respectively (Femenía-Font et al., 2005). These values are higher than described for MS-222 (1.8), benzocaine (1.9), metomidate (3.1) and isoeugenol (3.0) (Zahl et al., 2011). The high lipid solubility of drugs causes their accumulation in hydrophobic tissues, leading to slow elimination and, therefore, potentially longer recovery time (Zahl et al., 2011; Kiessling et al. 2009).

Additionally, the two highest concentrations of the EO of *C. prismatica* fish produced greater mucus secretion during recovery, similar to the effects observed with the anesthetic 2-phenoxyethanol (Velisek et al., 2007). There was no mortality 48 h after the test induction.

CONCLUSIONS

This study elucidated the *C. prismatica* essential oil chemical composition, unknown until nowadays. The EO showed a low effectiveness as an anesthetic for silver catfish, and its use is not recommended in this sense. However, its sedative properties can be safely explored since EO at low sedative concentration do not promote behavior side effects or mucous loss. Furthermore, other studies should be performed with this EO, aiming to evaluate other potential applications and investigate the contributions of each compound to the biological activity.

ETHICS COMMITTEE AND BIOSAFETY

The methodologies of the experiments were approved by the Ethical and Animal Welfare Committee of the Federal University of Santa Maria (Process nº 46/2010).

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