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PARTICIPAÇÃO DO SISTEMA GABAÉRGICO NO MECANISMO DE AÇÃO ANESTÉSICA DOS ÓLEOS ESSENCIAIS DE *Aloysia triphylla E Cymbopogon flexuosus* EM JUNDIÁS (*Rhamdia quelen*)

Santa Maria, RS
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Dissertação apresentada ao Curso de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestre em Farmacologia**.

Orientador: Prof. Dr. Mauro Alves da Cunha

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RESUMO

PARTICIPAÇÃO DO SISTEMA GABAérgico NO MECANISMO DE AÇÃO ANESTÉSICA DOS ÓLEOS ESSENCIAIS DE *Aloysia triphylla E Cymbopogon flexuosus* EM JUNDIÁS (*Rhamdia quelen*)

AUTOR: Alessandro Casale dos Santos
ORIENTADOR: Mauro Alves da Cunha

Este estudo demonstrou as atividades sedativa e anestésica do óleo essencial (OE) de *Cymbopogon flexuosus* em jundiás (*Rhamdia quelen*). Os tempos de indução e recuperação relatados para esse OE foram comparados com o OE de *Aloysia triphylla* devido à semelhança entre os seus principais componentes: α-citral (geranial) e β-citral (neral). Ambos os OEs induziram anestesia nas concentrações de 150 a 300 µL L^{-1} e sedação a partir de 25 µL L^{-1}. O OE de *C. flexuosus* induziu mais rapidamente fases iniciais da anestesia, mas não houve diferença significativa para alcançar o estágio de anestesia profunda, além de induzir um tempo de recuperação significativamente mais longo. As relações cooperativas e a modulação do site benzodiazepínico (BDZ) do receptor GABAa como mecanismo de ação de ambos OEs foram testados a partir da adição de diazepam e flumazenil aos experimentos (agonista e antagonista do site BDZ do GABAa, respectivamente). A adição de diazepam (150 µM) potencializou o efeito de ambos os OEs nas concentrações de 25, 150 e 300 µL L^{-1}, sem induzir alteração significativa nos tempos de recuperação anestésica. O flumazenil (10 µM) reverteu a anestesia induzida pelo diazepam, mas não a anestesia induzida pelos OEs nas concentrações de 150 e 300 µL L^{-1}, portanto o OE de *C. flexuosus* induziu sedação e anestesia efetivas sem mortalidade a curto prazo e a modulação do site BDZ do GABAa como mecanismo de ação anestésica de ambos OEs não ficou demonstrada neste estudo.

Palavras-chave: Diazepam. Flumazenil. GABA. Peixe. Piscicultura.

ABSTRACT

PARTICIPATION OF THE GABAergic SYSTEM IN THE ANESTHETIC ACTION MECHANISM OF *Aloysia triphylla* AND *Cymbopogon flexuosus* ESSENTIAL OILS IN SILVER CATFISH (*Rhamdia quelen*)

AUTHOR: Alessandro Casale dos Santos
ADVISER: Mauro Alves da Cunha

This study demonstrated the sedative and anesthetic activity of the essential oil (EO) of *Cymbopogon flexuosus* in the silver catfish (*Rhamdia quelen*). The time for induction and recovery of this EO was compared with the EO of *Aloysia triphylla*, due to the similarity between their major components: α-citral (geranial) and β-citral (neral). Both EOs induced anesthesia at concentrations from 150 to 300 µL L^{-1} and sedation at 25 µL L^{-1}. The EO of *C. flexuosus* was faster in inducing the initial stages of anesthesia, but there was no significant difference to reach deep anesthesia, and there was a significantly longer recovery time. Cooperative relations and the modulation of the benzodiazepine (BDZ) site of GABAa as a mechanism of action of both EOs was verified from the addition of diazepam and flumazenil to experiments (BDZ site of GABAa agonist and antagonist, respectively). The addition of diazepam (150 µM) induced potentiation in concentrations of 25, 150 and 300 µL L^{-1} both EOs without significant change in anesthesia recovery time. Flumazenil (10 µM) reversed the diazepam-induced anesthesia, but not the anesthesia induced by EOs at concentrations of 150 and 300 µL L^{-1}, thus the EO of *C. flexuosus* induced effective sedation and anesthesia without short-term mortality and the modulation of the BDZ site of the GABAa in the anesthetic action mechanism of both EOs in this study was not demonstrated.

Keywords: Diazepan. Flumazenil. Fish. GABA. Pisciculture.

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1 INTRODUÇÃO

Práticas realizadas em aquicultura como biometria, coletas de sangue e outros materiais para análises, implantes hormonais e transporte, entre outros, frequentemente expõe os peixes e outros animais aquáticos a uma variedade de fatores estressantes que podem afetar o seu desempenho, bem como causar desequilíbrios que podem resultar em doenças e mortalidade (BARTON, 2000).

Tradicionalmente, fármacos sintéticos, como tricaína metanosulfonato (MS-222) e quinaldina vem sendo usados para induzir sedação e anestesia em peixes (SCHOETTGER; JULIN, 1967, 1969). Mais atualmente, etomidato, metomidato, benzocaína, barbitúricos e propofol, entre outros fármacos sintéticos, tem sido usados em diferentes espécies aquáticas (AMEND et al., 1982; GOMES et al., 2001; GRESSLER et al., 2012; MATTSON; RIPPLE, 1989; ROSS; ROSS, 2008).

Buscando alternativas a essas substâncias sintéticas, estudos tem comprovado os efeitos sedativos e anestésicos dos óleos essenciais (OEs) ou substâncias isoladas de OEs de plantas, afirmando que possuem atividades semelhantes, em relação às substâncias mais tradicionais, são os casos do eugenol e do OE de *Aloysia triphylla* (SMALL, 2003; GRESSLER et al., 2014).

Diversos OEs ou substâncias isoladas desses OEs foram relatados por apresentarem atividade depressora do sistema nervoso central (SNC) em peixes, mentol, OE de *Lippia alba* , eugenol, OE de *Ocimum gratissimum*, óleo de cravo, OE de *Hyptis mutabilis*, 1-terpinen-4-ol, (-)globulol, OE de *A. triphylla*, e *S*-(+)-linalool são exemplos disso (CUNHA et al., 2010a, b; GONÇALVES et al., 2008; HELDWEIN et al., 2014; JAVAHERY et al., 2012; PARODI et al., 2014; SILVA et al., 2012, 2013).

Recentemente foi sugerido em jundiás a modulação do sistema GABAérgico como mecanismo de ação anestésica dos OEs de *L. alba* e *O. gratissimum* (HELDWEIN et al., 2012; SILVA et al, 2012). Expressões desse sistema foram identificados em diversas espécies de animais, de peixes a mamíferos, sendo descritas evidências funcionais (Imunocitoquímicas) GABAérgicas em cérebros de zebrafish (*Danio rerio*) (DELGADO; SCHMACHTENBERG, 2008; KIM et al., 2004).

O ácido gama-aminobutírico (GABA) atua como principal neurotransmissor inibitório no SNC dos mamíferos, que apresentam altas concentrações desses aminoácidos ligando-se a receptores pós-sinápticos, que regulam uma série diversificada de processos do comportamento. As interações entre os canais iônicos, os receptores que regulam esses canais

e os neurotransmissores de aminoácidos no SNC constituem a base molecular desses processos. Existem dois tipos de receptores de GABA, os GABA ionotrópicos (GABAa e GABAc), que consistem de proteínas de membrana de múltiplas subunidades que se ligam ao GABA e que abrem um canal iônico de cloreto intrínseco e os receptores de GABA metabotrópicos (GABAb), que são receptores heterodiméricos acoplados à proteína G e que afetam as correntes iônicas neuronais através de segundos mensageiros. Os benzodiazepínicos são moduladores positivos dos receptores GABAa (site BDZ) que atuam em sítios de ligação alostéricos aumentando a neurotransmissão GABAérgica, o que a eles confere efeitos sedativos, hipnóticos, miorrelaxantes, anestésicos e ansiolíticos. Em altas doses podem causar hipnose e estupor, entretanto, quando administrados como única medicação, esses fármacos raramente provocam depressão fatal do SNC (FOSTER; KEMP, 2006).

O flumazenil (RO 15-1788) é um imidazo-benzodiazepínico dose-dependente, que antagoniza por ação competitiva, os efeitos centrais de substâncias como os benzodiazepínicos, que modulam o site benzodiazepínico (BDZ) da subunidade GABAa do complexo GABA, sem interferir no metabolismo da droga (DARRAGH et al., 1982).

O jundiá (*Rhamdia quelen*), é um peixe teleósteo noturno, de hábitos onívoros, que habita águas calmas do fundo de rios arenosos ou barrentos, podendo chegar a 50 cm de comprimento e 3 Kg de peso na natureza, amplamente distribuído da Argentina até o sul do México (GOMES et al., 2000). Por se adaptar bem as condições de cativeiro, o jundiá tem sido usado como unidade experimental para diversos estudos científicos sobre mecanismo de ação anestésica de OEs ou substâncias isoladas de OEs, como por exemplo OE de *L. alba*, OE de *O. gratissimum*, (-)globulol e *S*-(+)-linalool (HELDWEIN et al., 2012, 2014; SILVA et al., 2012, 2013).

A *A. triphylla* é um arbusto aromático perene, pertencente à família *Verbenaceae* que pode chegar até 7 metros de altura e cresce espontaneamente na América do Sul, sendo cultivada no norte da África e sul da Europa. As suas partes aéreas são relatadas por possuírem atividades digestiva, antiespasmódica, antipirética e sedativa (NEWAL et al., 1996). O seu OE, entre diversos componentes, dependendo de seu quimiotipo, pode ser constituído principalmente por citral e são relatadas atividades anestésicas em *Litopenaeus vannamei* (PARODI et al., 2012) e jundiás (GRESSLER et al., 2014; PARODI et al., 2014). O uso do OE de *A. triphylla* como anestésico de peixes foi patenteado pelo grupo de pesquisa do Laboratório de Fisiologia dos Peixes da Universidade Federal de Santa Maria (LAFIPE/UFSM) (PI 016090005905).

A espécie *Cymbopogon flexuosus*, é uma gramínea aromática pertencente à família *Poaceae* cresce naturalmente na Índia e frequentemente, assim como a *A. triphylla*, apresenta o citral como componente predominante de seu OE (TASKINEN et al., 1983). Algumas substâncias derivadas de plantas desse gênero são relatadas por apresentarem atividades contra bactérias, fungos, inflamações e neoplasias (GANJEWALA, 2009). A atividade anestésica do OE foi verificada em jundiás a partir de projeto piloto realizado no LAFIPE, embora até o momento nada ainda tivesse sido publicado.

Esse estudo objetivou documentar os tempos de indução e recuperação anestésicas induzidos a partir de diferentes concentrações do OE de *C. flexuosus* e estabelecer uma comparação com os tempos do OE de *A. triphylla* em concentrações semelhantes, bem como relatar as relações cooperativas entre ambos os OEs e o diazepam, além de investigar se os mecanismos de ação de ambos OEs poderiam envolver o site BDZ do GABAa em jundiás, através da adição de diazepam (agonista do site BDZ do GABAa) e flumazenil (antagonista do site BDZ do GABAa) aos experimentos.

2 DESENVOLVIMENTO

Neste item será apresentado um manuscrito resultante desta tese, submetido e em fase de editoração, ao periódico **Veterinary Anaesthesia and Analgesia**:

Anesthesia and anesthetic action mechanism of essential oils of *Aloysia triphylla* and *Cymbopogon flexuosus* in *Rhamdia quelen.*

Anesthesia and anesthetic action mechanism of essential oils of *Aloysia triphylla* and *Cymbopogon flexuosus* in *Rhamdia quelen*

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(XX) = Material removed by editor for blinding

Abstract

Objectives To document the time for anesthesia induction and recovery to different concentrations of essential oil (EO) of *Cymbopogon flexuosus* and *Aloysia triphylla* in silver catfish (*Rhamdia quelen*). To determine whether the mechanism of action of either EO involves the benzodiazepine (BDZ) site of the GABAa.

Study design Experimental study.

Animals Silver catfish, 144 fish, 7.5 ± 1.1 cm, 3.95 ± 0.85 g.

Methods The concentrations of EOs were 25, 150 and 300 μL L^{-1} were evaluated and a group with ethanol only (7 groups, n = 6 per group). Assessments were induction of sedation or anesthesia, and recovery. In a further 6 groups (n = 6 per group), fish were exposed to both EOs (25, 150 or 300 μL L^{-1}) with diazepam 150 μM, and a control group of diazepam (150 μM) alone was included. Flumazenil (5 or 10 μM) was added to the recovery water with fish exposed to diazepam (150 μM) or both EOs (150 and 300 μL L^{-1}) (total 10 groups, 60 fish).

Results

Both EOs induced anesthesia at concentrations from 150 to 300 µL L^{-1} and sedation at 25 µL L^{-1}. The EO of *C. flexuosus* was faster in inducing the initial stages of anesthesia, but there was no significant difference to reach deep anesthesia, and there was a significantly longer recovery time. The addition of diazepam (150 µM) induced faster anesthesia with the EOs, with no significant change in recovery times. Flumazenil (10 µM) reversed the diazepam-induced anesthesia, but not the anesthesia induced by EOs.

Conclusions and clinical relevance The EO of *C. flexuosus* induced effective sedation (25 µL L^{-1}) and anesthesia (150 and 300 µL L^{-1}) without short-term mortality and the modulation of the BDZ site of the GABAa in the anesthetic action mechanism of both EOs was not demonstrated.

Keywords diazepam, fish, flumazenil, GABA, sedation.

Introduction

Aquaculture practices such as biometry, the collection of blood and other materials for analysis, hormonal implants and transportation, often stress fish affecting subsequent performance and resulting in disorders contributing to disease and mortality (Barton 2000). Synthetic drugs, such as tricaine methanesulfonate (MS-222), quinaldine, etomidate, metomidate, benzocaine, barbiturates and propofol, have been used in various aquatic species to minimize the stress induced by these procedures (Amend et al. 1982; Mattson & Ripple 1989; Gomes et al. 2001; Ross & Ross 2008; Gressler et al. 2012).

Studies investigating alternatives to these synthetic substances have identified sedative and anesthetics effects of plant essential oils (EOs) or compounds isolated from EOs, often with similar activity (Palic et al. 2006; Gonçalves et al. 2008; Gressler et al. 2014).

The plant *Aloysia triphylla* (Verbenaceae) is an aromatic shrub with an EO that is primarily a mixture of α-citral (neral) and β-citral (geranial). Anesthetic activities of this EO were reported in white shrimp (*Litopenaeus vannamei*) (Parodi et al. 2012) and silver catfish (*Rhamdia quelen*) (Gressler et al. 2012; Parodi et al. 2014). The plant *Cymbopogon flexuosus* (Poaceae) is an aromatic grass, and also has an EO with citral as the predominant component (Taskinen et al. 1983).

Gamma-amino butyric acid (GABA) is an inhibitory neurotransmitter in the central nervous system. Modulation of the GABAergic system through benzodiazepine (BDZ) site of the GABAa as an anesthetic mechanism of action of EOs of *Lippia alba* and *Ocimum gratissimum* has recently been suggested in silver catfish (Heldwein et al. 2012; Silva et al. 2012). Expressions of this system have been identified in several species of vertebrates, from fish to mammals, with GABAergic functional evidence (immunocytochemistry) being described in the brain of zebrafish, *Danio rerio* (Kim et al. 2004; Delgado & Schmachtenberg 2008).

Benzodiazepines are positive modulators of the BDZ site of the GABAa receptors, resulting in sedative, hypnotic, muscle relaxant and anxiolytic effects in mammals (Foster & Kemp 2006). Flumazenil (RO 15-1788) is an imidazo-benzodiazepine that antagonizes the central effects of benzodiazepines by competition for the BDZ site (Darragh et al. 1982).

As both EOs have the same main compound (citral), it is possible that the EO *C. flexuosus* induces anesthesia and that the mechanism of action of both EOs may be related to the BDZ site of the GABAa. This study aimed to evaluate the anesthetic efficacy of the EO *C. flexuosus* in silver catfish and if this EO induces anesthesia, to compare the time for anesthesia induction and recovery to different concentrations with the EO of *A. triphylla*. The study also was to evaluate any potentiation offered by inclusion of diazepam and to determine involvement of the EOs in the BDZ site of the GABAa by observing responses to treatment with flumazenil. The hypotheses of this study were that the EO of *C. flexuosus* will induce anesthesia and that flumazenil would confirm that the action of both EOs is through the BDZ site of the GABAa in silver catfish.

Material and methods

Plant material

The *A. triphylla* was cultivated and identified according to Parodi et al. (2012). The *C. flexuosus* was cultivated in (XX). The species was identified by (XX) and a voucher specimen (no. 6748) was deposited in (XX).

Drugs, extraction and analysis of EOs

Flumazenil (injectable solution 0.1 mg mL^{-1}, XX) and diazepam (XX, injectable solution 5 mg mL^{-1}; XX) were obtained from local trade sources (L D T Produtos

Farmacêuticos e Hospitalares Ltda - Santa Maria, RS, Brazil). The EO of *A. triphylla* was obtained from fresh leaves and the extraction and chromatographic analysis of the constituents of EOs were performed as described by Parodi et al. (2012**).** The EO of *C. flexuosus* was obtained from fresh leaves by hydrodistillation for 2 hours, according to the European Pharmacopoeia (2007), and stored in amber bottles at -4°C. The gas chromatography coupled with mass spectrometry (GC-MS) analysis was performed on an Agilent 7890A gas chromatograph coupled to a 5975C mass spectrometer with a non-polar HP5-MS fused silica capillary column (5% phenyl - 95% methylsiloxane, 30 mm x 0.25 mm i.d. x 0.25 mm film thickness) and EI-MS of 70 eV. The operating conditions were as follows: carrier gas: He, at a flow rate of 1 mL minute^{-1}; split inlet: 1:100; injector and detector temperatures: 250ºC; Temperature program: 40ºC for 4 minutes and 40ºC - 320ºC at 4 minutes. The constituents of the EO were identified by comparison of the mass spectra and Kovats retention index with the literature and a mass spectral data bank (Adams 2001; NIST 2002). The quantification of the components was performed using Agilent 7890A with a flame ionization detector for HP5 column (30 mm x 0.25 mm i.d. x 0.25 mm film thickness) in accordance with the parameters mentioned above (carrier gas: He, at a flow rate of 1 mL minute^{-1}; the split less mode; injector and detector temperature: 300 °C; temperature program: 40ºC for 4 minutes and 40ºC - 320ºC at 4 minutes). Sample of EO was injected in triplicate. EO compounds relative percent was estimated by under peak area integration obtained from FID chromatograms.

Animals and culture conditions

Juveniles of silver catfish, both sex (144 fish, 7.5 ± 1.1 cm; 3.95 ± 0.85 g) were acquired from a local producer (Bela Vista Fish Culture, Santa Maria, RS, Brazil). They were kept for 10 days in continuously aerated recirculation system with 30 L aquaria, stocking

density of 4.94 ± 1.2 g L^{-1}. The temperature was 23.0 ± 1°C, dissolved oxygen 8.87 ± 0.51 mg L^{-1}, pH 7.40 ± 0.11 and total ammonia was 0.99 ± 0.22 mg L^{-1}. Fish were fed daily with commercial feed 28.0% crude protein and 3500 kcal kg^{-1}. The aquaria were siphoned 30 minutes after feeding to remove food debris and other impurities. Measurements of dissolved oxygen and temperature, pH and total ammonia were performed daily with the aid of an oxygen meter YSI (Model Y5512; YSI Inc. XX), DMPH-2 pH meter (Digimed, XX), and according to Eaton et al. (2005), respectively. Fish without visual alterations in the mucosa, skin, body volume and/or feeding habits and behavior were used in the study.

The methodology used in this study was approved by the Institutional Animal Care and Use of (XX) (no. 5935290715). The number of animals used in each experiment was the lowest possible in order to meet the policy of reduction, refinement and substitution of experimental animals of the institution.

Anesthesia induction and recovery

Anesthesia was evaluated according to Small (2003), which provides 4 stages: stage I - sedation and decreased responsiveness to external stimuli, stage II - partial loss of balance and erratic swimming, stage III - no movement and no response to stimuli and stage IV – respiratory failure and death. Juveniles were fasted for 24 hours before the experiments and then placed in continuously aerated 1 L aquaria. The time for anesthesia induction and recovery of the fish after exposure to EOs of *A. triphylla* and *C. flexuosus* at concentrations of 25, 150 or 300 µL L^{-1} were measured and compared (Table 1). The fish were chosen randomly and used once. Each juvenile was assessed individually and it was considered anesthetized when reached stage III. The maximum time of observation was 30 minutes. After this, juveniles were removed from the anesthetic bath and placed in an aquarium with water only to assess the anesthetic recovery, monitored until complete recovery. Juveniles were

considered recovered when normal swimming and reaction to external stimuli was observed. The EOs were previously diluted 1:10 in 95% ethanol, therefore, an ethanol group with ethanol at the highest concentration used in this study was included (2700 µL L^{-1} ethanol).

Effects of diazepam or flumazenil

To determine whether the effects of EOs were potentiated by diazepam, fish were exposed to the EOs of *A. triphylla* or *C. flexuosus* (25, 150 or 300 µL L^{-1}) with diazepam 150 µM (Table 1). Anesthetic induction and recovery time were compared between the groups with and without diazepam. The effect of diazepam (150 µM) alone, and of administration of flumazenil (5 or 10 µM) in recovery was also studied.

The impact of flumazenil (5 or 10 µM) on the recovery time from EOs anesthesia (150 or 300 µL L^{-1}) was studied in an additional 48 fish (Table 1). The results were compared with the results from fish without flumazenil administration.

Statistical analysis

All data were submitted to Levene test to check the homogeneity of variances. As data were homoscedastic, one-way ANOVA followed by Tukey test was performed. The minimum significance was 95% and the data referred to mean ± standard error (Version 9; STATISTICA; StatSoft Inc., TX, USA).

Results

EO composition

192 The major components of the EO of *A. triphylla* were α-citral (29.41%), β-citral
193 (20.78%) and limonene (11.90%). The major components of the EO of *C. flexuosus* were α-
194 citral (48.90%), β-citral (37.47%) and heptene - one <6-methyl-5> (2.67%).
195
196 EO anesthesia
197 The EOs of *A. triphylla* and *C. flexuosus* induced stage III anesthesia at 150 and 300
198 μL L^{-1} and at 25 μL L^{-1} induced up to stage II within 30 minutes. Time to anesthesia induction
199 followed a negative concentration-dependent pattern, but the recovery time demonstrated a
200 positive concentration-response relationship. As 25 μL L^{-1} of both EOs did not induce stage
201 III, recovery times were not registered.
202 The EO of *C. flexuosus* induced significantly faster stages I and II of anesthesia at the
203 three concentrations tested. The recovery times of *C. flexuosus* EO were significantly longer
204 than those of the EO of *A. triphylla*, ($p < 0.05$) (Fig. 1). Ethanol alone failed to induce
205 sedation or anesthesia.
206
207 Anesthetic effects of EOs with diazepam
208 The addition of diazepam with the EOs induced significantly faster stage I of
209 anesthesia. The association of diazepam with 25 μL L^{-1} EO of *A. triphylla* significantly
210 decreased the time to reach stage II. Diazepam also made possible the induction of stage III
211 with 25 μL L^{-1} of both EOs, but there was no significant difference in the anesthetic recovery
212 times (Fig. 2.1, 2.2) ($p < 0.05$).
213
214 Effect of flumazenil

215 Flumazenil (10 µM) reversed the diazepam-induced anesthesia, but not the anesthesia
216 induced by EOs at concentrations of 150 and 300 µL L^{-1}. No anesthetic procedure in the
217 tested groups was reversed by 5µM of flumazenil (Fig. 3) ($p < 0.05$).
218 There was no short-term mortality in all experimental period.
219

220 **Discussion**

221 Both EOs induced sedation (25 µL L^{-1}) and anesthesia (150 and 300 µL L^{-1}) at
222 different concentrations. Short-term mortality during induction or recovery was not observed,
223 similar to that reported by Cunha et al. (2010) for EO of *L. alba*, Silva et al. (2012) for EO of
224 *O. gratissimum* and Parodi et al. (2013) and Gressler et al. (2014) for EO of *A. triphylla*, in
225 concern to mortality.
226 Several authors referred to 3 and 5-10 minutes as the maximum periods for anesthesia
227 induction and recovery, respectively (Gilderhus & Marking 1987; Small 2003; Ross & Ross
228 2008). The concentration of 300 µL L^{-1} of both studied EOs was within these parameters.
229 Both EOs showed a negative concentration-response relationship for anesthesia
230 induction, but recovery times were directly proportional to concentration. Similar patterns for
231 the time of induction and recovery of anesthesia were described for the 100 to 800 µL L^{-1}
232 concentration range of EO of *A. triphylla* (Parodi et al. 2014). Cunha et al. (2010) also
233 described a negative relationship for the time of anesthesia induction from 100 to 500 µL L^{-1}
234 EO of *L. alba*, but recovery times did not follow any relationship.
235 Additional studies are needed to established the sedative and anesthetic concentrations
236 of both EOs investigated for different fish species and strains, once significant different
237 concentrations ($p < 0,05$) EO of *A. triphylla* are needed to reach sedation and anesthesia, as
238 well as anesthetic recovery in silver catfish grey or albino (Parodi et al. 2014).

The mechanisms of action of several psychoactive herbs remain undefined even after their therapeutic effects are well established. The faster anesthesia induction provoked by the EO of *C. flexuosus* compared to the EO of *A. triphylla* is probably due to the higher content of α- and β-citral in the EO of *C. flexuosus* because citral inhibited neural excitability in rats and seems to be anesthetic (Sousa et al. 2015) and induce sedation and muscle relaxation in mice (Vale et al. 2012). Additional studies are needed to understand the role of each substance isolated by chromatography and their roles in the induction of the observed effect.

Although targeted studies are needed to understand the nature of collaborative interaction between diazepam and the EOs of *A. triphylla* and *C. flexuosus*, it is noted that these associations induced significant reduction in anesthesia induction time. Diazepam also accelerated anesthesia induction time with the EOs of *O. gratissimum* (Silva et al. 2012) and *L. alba* (Heldwein et al. 2012). Furthermore, in the present study, as described by Silva et al. (2012) and Heldwein et al. (2012), the association of diazepam with concentrations of EOs that alone did not induce a certain anesthetic stage, led to a deeper anesthesia stage.

Flumazenil reversed the anesthetic effects caused by diazepam, but did not change the anesthetic recovery times from EOs of *A. triphylla* and *C. flexuosus*. Therefore, the BDZ site of the GABAa is not involved in the EOs mechanism of action, unlike that which was verified for the EOs of *L. alba* (Heldwein et al. 2012) and *O. gratissimum* (Silva et al. 2012). Some anesthetic compounds isolated from EOs as linalool (*L. alba*), and (-) globulol (*H. mutabilis*) also did not have their anesthetic effects reversed by flumazenil (Heldwein et al. 2014; Silva et al. 2013).

These results confirm the hypotheses that EO of *C. flexuosus* induces anesthesia, but does not confirm the hypotheses that the mechanism of action of both EOs involves the BDZ site of the GABAa in silver catfish.

Conclusion

The EO of *C. flexuosus* induced effective sedation (25 μL L^{-1}) and anesthesia (150 and 300 μL L^{-1}) without short-term mortality in silver catfish. In comparison with the EO of *A. triphylla*, the EO of *C. flexuosus* induced the initial stages of anesthesia (I and II) more rapidly, as well as significantly longer anesthetic recovery times. The association of diazepam with both EOs significantly decreased the time required to reach anesthesia, without changing recovery periods. Flumazenil did not reduce EO recovery times, so modulation of the BDZ site of the GABAa, was not demonstrated in the anesthetic action mechanism of EOs of *A. triphylla* and *C. flexuosus* in silver catfish.

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390 **Figure legends**

391

392 **Figure 1.** Time required for induction and recovery from anesthesia by EO of *A. triphylla*
393 (At) and EO of *C. flexuosus* (Cf). Different letters in the columns indicate a significant
394 difference between the concentrations of the same EO. * Significant difference between the
395 different EOs in similar concentrations (one-way ANOVA and Tukey, $p < 0.05$).

396

397 **Figure 2.** Time required for induction and recovery from anesthesia by (2.1) EO of *A.*
398 *triphylla* (At) and (2.2) EO of *C. flexuosus* (Cf) and the combinations EO of *A. triphylla* +
399 diazepam (At+D) (2.1) and EO of *C. flexuosus* + diazepam (Cf+D) (2.2), respectively. (D at
400 150 µM). * Significantly different from the EO of *A. triphylla* + diazepam from EO of *A.*
401 *triphylla* (2.1) and *C. flexuosus* + Diazepam from *C. flexuosus* (2.2) at same concentration
402 (one-way ANOVA and Tukey, $p < 0.05$).

403

404 **Figure 3.** Time required for recovery from anesthesia by control diazepam (D) at 150 µM,
405 and EO of *A. triphylla* (At) at 150 or 300 µL L^{-1} and EO of *C. flexuosus* (Cf) at 150 or 300 µL
406 L^{-1}. Recovery in water and flumazenil solutions at 5 or 10 µM. *Significantly different from
407 recovery in water (one-way ANOVA and Tukey, $p < 0.05$).

408 Figure 1.

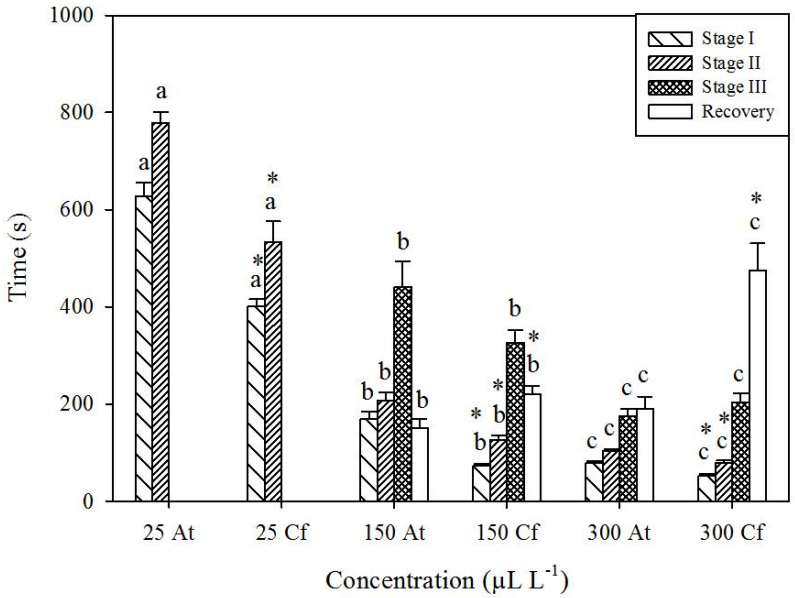

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Figure 2.1

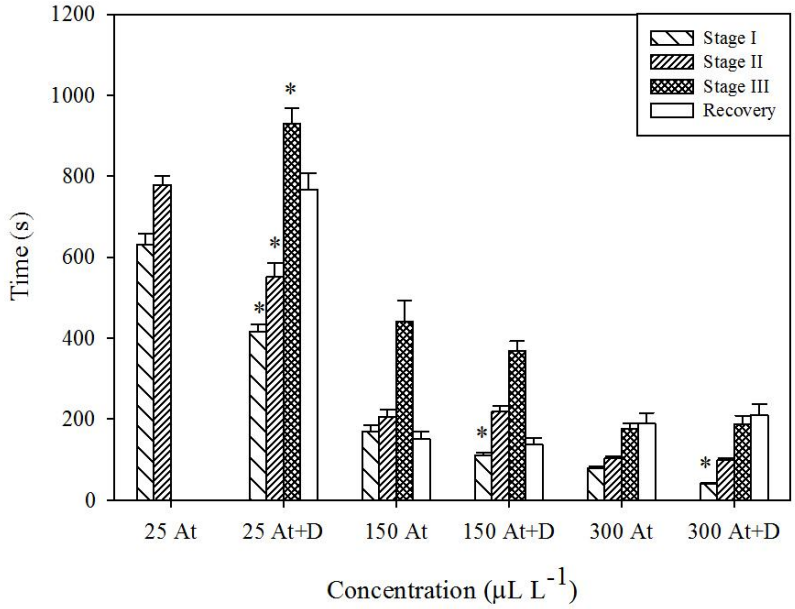

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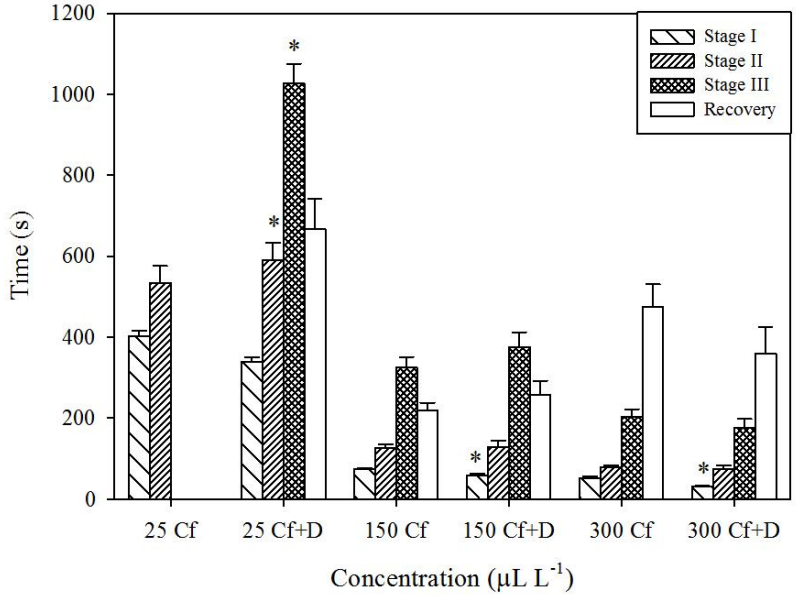
Figure 2.2


1200
1000
800
600
400
200
0
Time (s)
Stage I
Stage II
Stage III
Recovery
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25 Cf+D
150 Cf
150 Cf+D
300 Cf
300 Cf+D
Concentration (µL L-1)

Figure 3

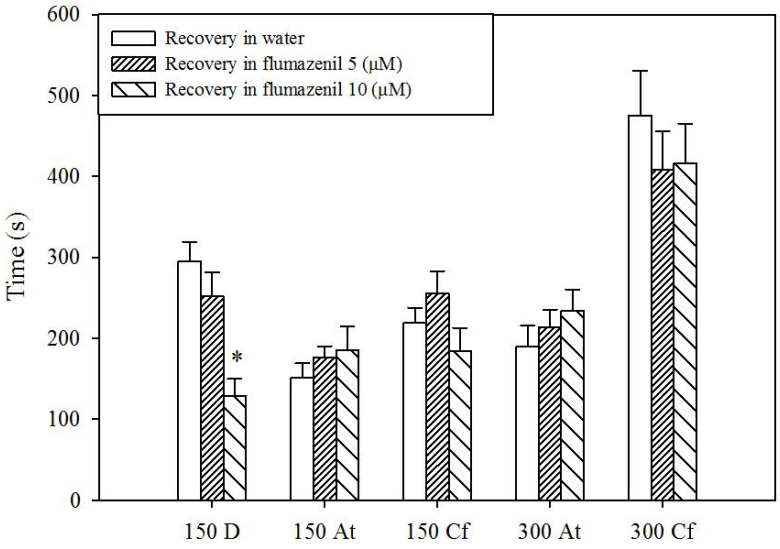

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Table 1. Sequence of experimental groups for 144 silver catfish (6 fish per group). The maximum observation period was 30 minutes after exposure to essential oil (EO), ethanol or diazepam (D), where indicated. Fish were recovered in water, with flumazenil (F) if indicated, and observed until fish were fully recovered.

Experimental sequence	Groups		
	EO of *A. triphylla*	EO of *C. flexuosus*	Controls
I. Effects of EOs			
	25 µL L^{-1}	25 µL L^{-1}	Ethanol
	150 µL L^{-1}	150 µL L^{-1}	
	300 µL L^{-1}	300 µL L^{-1}	
II. Effects of EOs and diazepam			
	25 µL L^{-1} + D 150 µM	25 µL L^{-1} + D 150 µM	D 150 µM
	150 µL L^{-1} + D 150 µM	150 µL L^{-1} + D 150 µM	D 150 µM + F 5 µM
	300 µL L^{-1} + D 150 µM	300 µL L^{-1} + D 150 µM	D 150 µM + F 10 µM
III. Effects of flumazenil on recovery from EO			
	150 µL L^{-1} + F 5 µM	150 µL L^{-1} + F 5 µM	
	150 µL L^{-1} + F 10 µM	150 µL L^{-1} + F 10 µM	
	300 µL L^{-1} + F 5 µM	300 µL L^{-1} + F 5 µM	
	300 µL L^{-1} + F 10 µM	300 µL L^{-1} + F 10 µM	

3 DISCUSSÃO GERAL

Verificou-se uma relação positiva entre as concentrações utilizadas e as intensidades das respostas fisiológicas obtidas para os dois OEs na indução de sedação e anestesia, assim como para a recuperação da anestesia ocorreu o inverso, que foi o mesmo verificado por Parodi et al. (2014) que revelou um padrão semelhante nas concentrações de 100 a 800 µL L^{-1} também para o OE de *A. triphylla* e Cunha et al. (2010a) a partir do OE de *L. alba*, em concentrações variando de 100 a 400 µL L^{-1}. O mesmo ocorreu em relação ao relato de Gressler et al. (2014) no concernente aos tempos de indução de anestesia, a partir das concentrações de 135 e 180 µL L^{-1} de *A. triphylla*, embora os tempos de recuperação verificados para a concentração de 135 µL L^{-1} tenham sido maiores.

Tanto o OE de *A. triphylla* como o OE de *C. flexuosus* induziram sedação e anestesia a partir de diferentes concentrações, sem ocorrência de efeitos colaterais indesejáveis, semelhante ao relatado por Cunha et al. (2010a), Heldwein et al. (2012, 2014) para o OE de *L. alba*, Silva et al. (2012) para o OE de *O. gratissimum* e Gressler et al. (2014) e Parodi et al. (2013) também utilizando o OE de *A. triphylla*, contrastando com o diazepam que presentemente provocou grandes quantidades de espasmos musculares durante o período de indução anestésica e com o verificado por Silva et al. (2013) que relatou hiperatividade, espasmos musculares e nado em círculos intercalados com momentos de inatividade, durante os longos períodos de recuperação induzidos pelo OE de *H. mutabilis*.

Em seus relatos publicados nos anos 60, Schoettger e Julin (1967, 1969) descrevem a indução de anestesia rápida em peixes, a partir do uso de MS-222 e benzocaína, em torno de 3 e 2 minutos, respectivamente. Gilderhus e Marking (1987), Ross e Ross (2008) e Small (2003) posteriormente referem em torno de 3 minutos como período ideal para indução rápida de anestesia. Gilderhus e Marking (1987) sugerem tempos abaixo de 10 minutos como ideais para a recuperação, já Ross e Ross (2008) abaixo de 5 minutos. No presente relato verificou-se que as médias dos tempos de indução anestésica, a partir das concentrações de 300 µL L^{-1} de ambos os OE foi possível atingir as metas preconizadas por Gilderhus e Marking (1987), Ross e Ross (2008) e Small (2003). Nenhuma indução se completou em menos de 2 minutos. Os tempos de recuperação anestésica foram compatíveis ao indicado por Ross e Ross (2008) para as concentrações de 150 e 300 µL L^{-1} do OE de *A. triphylla* e para a concentração de 150 µL L^{-1} de *C. flexuosus*, já a concentração de 300 µL L^{-1} de *C. flexuosus* induziu um tempo de recuperação maior que 5, mas menor que 10 minutos (GILDERHUS; MARKING, 1987).

Como as concentrações de 25 μL L^{-1} não alcançaram o estágio III, seus tempos de recuperação não foram computados.

Os mecanismos de ação de diversas ervas medicinais psicoativas permanecem indefinidos mesmo depois de bem estabelecidos seus efeitos terapêuticos. A complexidade das interações entre seus diversos componentes pode exibir relações cooperativas que podem explicar os diferentes níveis de resposta observados a partir de extratos de plantas (SPINELLA, 2002), o que pode explicar as diferenças verificadas em relação aos tempos de indução iniciais mais rápidos obtidos a partir do OE de *C. flexuosus*, em relação aos tempos do OE de *A. triphylla*, bem como os seus maiores tempos de recuperação. Dois tipos relações cooperativas, baseados na natureza de suas atuações podem ocorrer, o de natureza farmacodinâmica que é quando diferentes substâncias estimulam um mesmo receptor ou sistema fisiológico, ou o de natureza farmacocinética que é relativo a questões como a absorção, distribuição, biotransformação ou eliminação de um fármaco (SPINELLA, 2002). A possível interação entre os diversos componentes dos OE em estudo também foi mencionada por Silva et al. (2012) para explicar a maior atividade anestésica demonstrada pelo OE de *O. gratissimum* em relação ao eugenol. O mesmo foi mencionado por Heldwein et al. (2014) quando verificou que o OE de *L. alba* apresentou maior atividade anestésica em relação ao *S*-(+)-linalool e por Silva et al. (2013), que como os anteriores, verificou que o OE de *H. mutabilis* induziu anestesia mais rapidamente que a associação de 1-terpinen-4-ol e (-)globulol.

Benzodiazepínicos são drogas depressoras do SNC, considerados ansiolíticos e hipnóticos, estão envolvidos em overdoses acidentais e apesar de dificilmente causarem coma profundo e morte quando utilizados isoladamente, a associação a outras substâncias depressoras pode potencializar os seus efeitos (KNUDSEN et al., 1988). No presente trabalho todos os animais submetidos ao diazepam (150 μM) alcançaram o estágio III de anestesia, divergindo do registrado por Heldwein et al. (2012, 2014), Silva et al. (2012, 2013), onde não ficou evidenciada, mesmo com doses semelhantes e no mesmo modelo experimental, a indução ao estágio mais aprofundado de anestesia. As discrepâncias observadas podem estar relacionadas às diferentes formas farmacêuticas utilizadas, já que no presente estudo optou-se por utilizar o diazepam em ampolas, e não em forma de pó, diluído em Tween 80 a 0,033%. Adicionado a isso o fato da temperatura média utilizada ser maior no presente relato, que os quatro trabalhos supracitados e temperaturas mais elevadas poderem abreviar os tempos de indução e recuperação, devido ao aumento das as taxas metabólicas dos animais (MYLONAS et al., 2005).

Mortalidade ocorreu em 8,33% dos peixes levados até o estágio III de anestesia pelos 150 µM de diazepam, que ao serem retirados da solução anestésica foram demonstrando uma constante desaceleração das batidas operculares, até a cessação completa. O mesmo ocorreu a 3,22% dos animais submetidos à associação de diazepam ao OE de *C. flexuosus*, demonstrando que a associação não induziu aumento na mortalidade. Todos os animais submetidos aos OEs apenas, já se alimentavam normalmente no dia seguinte à exposição, já os animais expostos ao diazepam, ou à associação de cada OE com o diazepam, só voltaram só voltaram a se alimentar normalmente 48 horas após, assim como Silva et al. (2012) que relata que embora não tenham ocorrido óbitos durante seus experimentos, aqueles animais tratados com diazepam apenas, ou esse associado ao OE de *O. gratissimum* levaram 6 dias para voltarem a se alimentar normalmente.

Experimentos sobre sinergismo podem ser úteis na elucidação dos mecanismos de ação de fármacos (TALLARIDA, 2001). Embora estudos direcionados sejam necessários para se entender a natureza das relações cooperativas verificadas, notou-se que ambos os OEs tiveram redução significativa em seus tempos de indução anestésica quando associados ao diazepam para alcançar o estágio I, sendo que por ocasião dos estágios seguintes, II e III, os tempos verificados não tiveram diferença estatística, assemelhando-se ao relatado por Silva et al. (2012), onde também a associação acelerou os estágios iniciais em todas as diferentes concentrações testadas, chegando sem diferença significativa de tempo ao estágio de anestesia, utilizando o OE *O. gratissimum* isoladamente ou em combinação com o diazepam. Heldwein et al. (2012) relatou uma cooperação mais abrangente entre o diazepam e o OE de *L. alba*, onde todas as concentrações de OE testadas tiveram redução nos seus tempos de indução anestésica, em estágios iniciais ou mais avançados de anestesia. Além disso, no presente relato, bem como para Silva et al. (2012) e Heldwein et al. (2012) a adição do benzodiazepínico também possibilitou a indução de planos anestésicos mais aprofundados a partir das mesmas concentrações dos OEs, em nosso caso a adição de diazepam a ambos os OEs na concentração de 25 µL L^{-1} elevou a resposta anestésica de estágio I e II para III.

Resultados semelhantes aos de Heldwein et al. (2012), foram registrados aqui, relativamente a recuperação anestésica dos OE de *L. alba* (50, 100 e 300 µL L^{-1}), *A. triphylla* e *C. flexuosus* (25, 150 e 300 µL L^{-1}), onde a adição de diazepam às diferentes concentrações dos OE, não produziram alterações significativas nos tempos de recuperação. Sentido diverso foi verificado por Silva et al. (2012), onde o diazepam adicionado as concentrações de 10, 20 e 40 µL L^{-1} de OE de *O. gratissimum* induziu tempos de recuperação significantemente mais longos.

A modulação do site BDZ do GABAa a ser verificada através da reversão dos efeitos anestésicos dos OEs de *A. triphylla* e *C. flexuosus* pela adição de flumazenil à agua dos aquários de recuperação não ocorreu nesse relato, ao contrário do verificado por Heldwein et al, (2012) para o OE de *L. alba* e Silva et al. (2012), para o OE de *O. gratissimum*. Algumas substâncias anestésicas isoladas de OEs de plantas, como o S-(+)-linalool (*L. alba*), e o Globulol (*H. mutabilis*), também não tiveram seus efeitos anestésicos revertidos pelo flumazenil (HELDWEIN et al., 2014; SILVA et al., 2013).

Mortalidade não foi verificada a partir das diferentes concentrações utilizadas para induzir sedação e anestesia pelos OEs de *A. triphylla* e *C. flexuosus* isoladamente. Embora nenhum dado ainda tenha sido publicado nesse sentido para o OE de *C. flexuosus*, no concernente à *A. triphylla*, Parodi et al., (2013) submeteu jundiás a concentrações 4 vezes maiores (800 µL L^{-1}), àquela concentração indicada como a melhor dose-resposta (200 µL L^{-1}) em relação ao tempo de indução anestésica, também sem registro de morte, já o eugenol, tradicionalmente usado em aquicultura a duas décadas (GUÉNETTE et al., 2007), segundo Cunha et al., (2010) induz anestesia em Jundiás, rapidamente e sem mortalidade, a partir da concentração de 50 µL L^{-1}, embora nas concentrações de 60 e 70 µL L^{-1} a mortalidade tenha ocorrido (20 e 65%, respectivamente).

4 CONCLUSÃO

Ficou demonstrado que o OE de *C. flexuosus*, induziu sedação (25 µL L^{-1}) e anestesia eficaz e sem mortalidade a curto prazo (150 e 300 µL L^{-1}), assim como o OE de *A. triphylla* em jundiás. Em se comparando os tempos de indução e recuperação de ambos os OEs, o OE de *C. flexuosus* induziu tempos significantemente menores para obtenção dos estágios I e II (sedação), embora a indução de estágio III (anestesia) tenha ocorrido em tempos semelhantes. Os tempos de recuperação induzidos pelo OE de *A. triphylla* foram significantemente menores. A associação do diazepam a ambos os OEs potencializou os efeitos anestésicos diminuindo o tempo para alcançar o estágio I em todas as concentrações testadas, além de possibilitar o alcance de estágios mais aprofundados que não foram possíveis a partir do uso dos OEs isoladamente, sem afetar os tempos de recuperação. O Flumazenil não abreviou os tempos de recuperação de anestesia dos OE, portanto não ficou demonstrada a modulação do site BDZ do GABAa no mecanismo de ação anestésica de *A. triphylla* e *C. flexuosus* em jundiás.

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APÊNDICES

APÊNDICE A – COMPOSIÇÃO QUÍMICA DOS CONSTITUINTES DO ÓLEO ESSENCIAL DE *A. triphylla*

Constituintes	%	IKr	IKc
β-pineno	1.07	964	969
3-octanol	0.29	971	971
Limoneno	11.90	1017	1010
Cis-ocimeno	0.83	1027	1030
α-óxido de pineno	0.10	1090	1081
Linalol	0.69	1090	1084
Myrtanal	0.30	1126	1123
Citronelal	0.76	1146	1132
Isopulegol	0.19	1146	1140
2-pinen-4-ol	1.46	1155	1154
Pulegona (p-Menth-4(8)-en-3-ona)	0.44	1168	1164
α-terpineol (p-menth-1-en-8-ol)	2.24	1179	1173
Cis geraniol	0.51	1217	1220
Citronelol ou linalol acetate	1.34	1228	1225
β-citral	20.78	1240	1240
Trans- geraniol	0.55	1259	1249
α-citral	29.41	1271	1274
δ-elemeno	0.13	1331	1333
Acetato de nerilo	0.35	1364	1363
α-cubebeno	0.16	1367	1377
Acetato de geranil	2.98	1384	1385
Cariofileno	5.64	1418	1422

α-cariofileno	0.29	1459	1456
Aromadendreno	0.17	1463	1463
Acoradieno	0.18	1471	1478
Propionato de geranil	1.13	1475	1485
D Germacreno	1.55	1481	1487
Biciclogermacreno	1.16	1495	1502
β-bisaboleno	0.57	1507	1517
cis-α-bisaboleno	0.24	1509	1519
τ-cadineno	0.15	1521	1522
δ- cadineno	0.11	1527	1529
Nerolidol	0.78	1564	1575
Espatulenol	0.58	1586	1589
Óxido de cariofileno	2.33	1594	1593
Cubenol	3.29	1604	1598
Cedrol	0.16	1613	1604
Humulano-1,6-dien-3-ol	0.28	1619	1624
τ-cadinol	1.72	1648	1658
α-Bisabolol	0.38	1682	1672
Cedr-8-en-13 ol	0.23	1688	1689
Constituintes identificados	97.42		

IKr = índice retenção referencial; IKc = índice de retenção calculado; % = percentual relativo

APÊNDICE B – COMPOSIÇÃO QUÍMICA DOS CONSTITUINTES DO ÓLEO ESSENCIAL DE *C. flexuosus*

Pico	TR	Constituinte	IKr	IKc	%
1	12.62	hepten--ona<6-metil-5>	991	985[a]	2.67
2	15.95	NI	1075		0.94
3	16.95	β-linalol	1101	1101[b]	0.79
4	18.79	*E*-α-necrodol	1151	1148[a]	0.34
5	18.98	Citronelal	1156	1153[a]	0.36
6	19.39	Z-isocitral	1167	1164[a]	1.39
7	19.78	Epóxido de rosefurano	1177	1177[a]	0.29
8	20.05	*E*-isocitral	1184	1180[a]	2.35
9	20.30	NI	1191		0.25
10	20.88	NI	1207		0.31
11	21.69	NI	1231		0.24
12	22.10	β-citral	1242	1238[a]	37.47
13	22.60	Geraniol	1257	1252[a]	0.47
14	23.14	α-citral	1272	1270[b]	48.90
15	23.65	Acetato de bornila	1287	1285[a]	0.13
16	26.01	eugenol	1358	1359[b]	0.36
17	26.64	α-copaeno	1378	1376[b]	0.22
18	26.91	Acetato de geranila	1386	1381[a]	0.21
19	28.02	Cariofileno	1421	1420[b]	1.53
20	32.96	Óxido de cariofileno	1585	1583[b]	0.81
Constituintes identificados					98.26

IKr = índice retenção referencial; IKc = índice de retenção calculado; % = percentual relativo

ANEXOS

ANEXO A – ESTÁGIOS DE ANESTESIA EM PEIXES

Estagio	Caracteristicas
1	Sedação: Perda parcial da reação aos estímulos externos.
2	Perda parcial do equilíbrio, natação errática.
3	Perda total do equilíbrio e cessação da locomoção
4	Colapso medular (morte)

Fonte: Small, (2003)

ANEXO B – INSTRUÇÃO AOS AUTORES

AUTHOR GUIDELINES

Veterinary Anaesthesia and Analgesia (*VAA*) publishes original, peer-reviewed articles covering all branches of anaesthesia and the relief of pain in animals. Articles concerned with the following subjects related to anaesthesia and analgesia are also welcome:

• the basic sciences,

• pathophysiology of disease as it relates to anaesthetic management,

• equipment,

• intensive care,

• chemical restraint of animals including wildlife and exotic animals,

• welfare issues associated with pain and distress,

• education in veterinary anaesthesia and analgesia.

VAA is making an effort to avoid publication bias and will publish negative studies that have been well-designed and conducted. *VAA* may use plagiarism-detection software.

Submissions

- **Prior to submission, please read the author guidelines carefully and review the authors' checklist.**
- It is highly recommended that novice writers consult the booklet Writing for Publication in Veterinary Medicine, which is available free online.
- Manuscripts should be submitted online at http://mc.manuscriptcentral.com/vaa.
- Full instructions and support are available on the site and a user ID and password can be obtained on the first visit. Support can be contacted by clicking the Get Help Now link which appears at the top right of every ScholarOne Manuscripts page (formerly known as Manuscript Central). • If you cannot submit online, please contact the Editorial Assistant by email (VAAedoffice@wiley.com) or by phone (+44 (0)1865 476 327).
- Authors are personally responsible for obtaining permission to reproduce copyright material from other source

I. General Editorial and Ethical Issues

A. Authorship

- VAA requires a statement of the authors' individual contributions.
- VAA refers to The International Committee of Medical Journal Editors (ICMJE) for the definition of authorship.
- ICMJE defines authors as those who:
  1. Made substantial contributions to the conception and design of, or acquisition of data or analysis and interpretation of data;
  2. Drafted the article or revised it critically for important intellectual content;
  3. Approved the final version to be published.
- Authors should meet conditions 1, 2 and 3. Otherwise they should be mentioned in acknowledgements.
- Participation in the acquisition of funding alone, translation and/or editing of the manuscript alone or data collection alone does not merit authorship.
- Except in the case of complex large-scale or multi-centre research, the number of authors should normally not exceed six.
- Please provide a statement in the cover letter defining the role of each author. For example:

  M.D.: Data interpretation, statistical analysis and preparation of manuscript. R.G.: Design, data management, and preparation of manuscript.

B. Criteria for manuscript consideration

A manuscript will be ***considered*** for publication only if the work detailed therein:

1. Is written in acceptable English;
2. Adheres to the Consensus Author Guidelines on Animal Ethics and Welfare for Veterinary Journals developed by the International Association of Veterinary Editors;
3. Follows international guidelines for humane animal treatment as outlined in the Guide for the Care and Use of Laboratory Animals (NRC 2011) and/or the Euroguide;
4. Has been approved by the ethics review committee at the institution or practice at which the studies were conducted where such a committee exists;
5. When such a committee does not exist, then approval should be obtained by an independent ethical review committee (please contact the editor for more information);

6. For prospective studies using client-owned animals (public or private), demonstrates a high standard (best practice) of veterinary care and involves **informed client consent** (see editorial in VAA 2012, 39, 321-323);
7. Describes clearly the primary and any secondary objectives of the study;
8. Has made efforts in the study design to minimize the effects of subjective bias including blinding and randomization in the study design and data analysis;
9. Explains, in the manuscript, how the number of animals was arrived at;
10. Reports the number of animals used in each group for data analysis and the details of any animals or data that were not included in the analysis;
11. Reports important adverse events in each group and any modifications to the experimental protocol made to reduce these events;
12. A manuscript will be considered for publication only if the work uses the ARRIVE guidelinesas a **basis** for the design and reporting of experimental studies, the CONSORT checklist for randomized clinical trials, the STROBE statement for observational studies and PRISMA for systematic reviews and meta-analyses.

Adherence to recommended reporting guidelines and the *VAA* authors' checklist will facilitate the review of your manuscript, increase the probability of its successful publication and improve the usability of research findings from your study in further research and clinical practice. Please consult the EQUATOR network (www.equator-network.org) for up-to-date reporting guidelines and other resources for manuscript preparation.

Prior to acceptance of a manuscript, to verify compliance with the above policies, the authors must:

1. Attest that the legal and ethical requirements have been met with regards to the humane treatment of animals described in the study;
2. Specify in Materials and methods the ethical review committee approval process and the international, national, and/or institutional guidelines followed;
3. If requested, provide evidence of ethical review, such as a signed animal-use form or protocol number at the institution or practice and/or copies of the signed informed client-consent form;
4. Provide evidence in Materials and methods that the principles of reduction, refinement, and replacement have been met.

C. Criteria for immediate manuscript rejection:

1. Manuscripts and authors failing to meet the aforementioned requirements;
2. Studies that involve unnecessary death, pain, distress, suffering, or lasting harm to animals;
3. Manuscripts that do not fit the scope of VAA;
4. The Editors retain the right to reject manuscripts on the basis of the criteria stated above and the Editors' decision is final.

D. Peer Review

All submissions, once deemed appropriate for review by the Editor, will be peer reviewed by at least two independent referees and a statistician, if appropriate. We aim to give authors a decision (rejection, rejection with encouragement to re-work and re-submit, or acceptance subject to revision/copy editing) within three months of typescript submission.

II. Types of Articles

A. Original Studies. These articles usually should aim to be approximately 3500 words with a maximum word count (after review) of 5000 words. Normally there should not be more than 30-40 references and 4-6 tables and/or figures. These articles include original experimental or clinical research and meta-analyses. They require a structured abstract with a maximum of 300 words containing the following headings: Objective, Study design, Animals or Animal population, Methods, Results, Conclusions and clinical relevance.

B. Review articles. Review articles are papers which clarify, summarize and critically evaluate the current literature and should usually have ≤5000 words. These will normally be invited by the Editors or a member of the Editorial Board, although unsolicited, acceptable material will be considered for publication. Databases and literature search strategy used should be defined in the Material and methods. The abstract should contain no more than 300 words and be structured with the following headings: Objective, Databases used, Conclusions.

C. Short Reviews--"What is the Evidence?" These are short review articles designed as a platform for discussion and debate of a specific topic or question. They should be from 1500 - 3500 words with approximately 20 references and up to four tables and/or figures (if needed). The abstract should contain no more than 300 words and be structured with the following headings: Objective, Databases used, Conclusions.

D. Short communications. Short communications describe small or preliminary experiments and their results. They should have a maximum of 2000 words; have ten or fewer references and no more than one figure or table. They require a structured abstract with a maximum of 300 words containing the following headings: Objective, Study design, Animals or Animal population, Methods, Results, Conclusions and clinical relevance.

E. Case reports (case-based studies; either single or multiple animals). In general, *VAA* is no longer publishing case reports. In exceptional circumstances, they may be considered. Please contact the Editors prior to submission.

F. Letters. Letters should not exceed 800 words or 5 references. These may be descriptions of new equipment, clinical observations, short case reports or comments that the correspondent believes to be of general interest to the readership. VAA does not routinely accept letters for publication criticizing existing publications. Where a reader feels such criticism is justified, they should write (by e-mail) directly to the Editors and they should aim to make their point in an objective, positive and constructive manner. The Editors will decide if or what action is required. The Editors' decision is final.

G. Other types. Historical notes, editorials and book reviews are also published. These are generally invited by the Editors. Editorials usually should contain no more than 2500 words, 25 references and one table and/or figure. Please contact the Editors for more information.

III. Manuscript Preparation

A. Style and General Arrangement

- Manuscripts must be written in English and must conform to the guidelines on the ScholarOne Manuscripts site or they will be returned immediately to the author(s) for correction.
- The typescript should be Times New Roman 12pt.
- The manuscript should be double-spaced with a 1" or 30 mm margin on each side.
- The lines should be numbered continuously.
- Units (with some examples):
  - Blood pressure: mmHg
  - Airway pressure: cm H2O
  - Otherwise SI units, except for blood gas and vapour pressure values where both mmHg and kPA should be provided.
  - Drug dosages: mg kg^{-1}, mg kg^{-1} hr^{-1}
  - Concentration: µg mL^{-1}, L kg^{-1}
  - Flow: L $minute^{-1}$
  - Abbreviations should be defined first in the abstract and then again in the manuscript:
  *Intravenous (IV), Intramuscular (IM)
  *Respiratory frequency (f_R)
  *Tidal Volume ($\dot{V}_T$)
  *Minute Ventilation ($\dot{V}_E$)
  *Cardiac Output ($\dot{Q}t$) or (CO)
  *Arterial partial pressure of carbon dioxide ($PaCO_2$)
- Numbering:
  - Use numerals for numbers greater than 10 and words for numbers less than 10.
  - Exceptions:
  * Use numerals for things that are measured (5 weeks, 5 minutes)
  * Use words for things that are not measured (five cats, five cells)
  * Try to avoid numerals at the beginning of the sentence
  * Always write ordinal numbers in full (fourth not 4th year)
  * Use % (50% of cats not 50 percent)
- *VAA* conducts anonymous peer-review. When uploading your manuscript you will need to upload a manuscript file with no identifying author information (designate as Main Document) and a separate title page (designate as Title Page) with author details and Acknowledgements.
- Papers should be submitted in the following order:
  - Cover Letter with Authors' contributions and Funding;
  - Separate Title Page with Acknowledgements;
  - Abstract with Keywords;
  - Introduction, Materials and methods, Results, Discussion, References, Figure legends, Tables, Figures.
- Wiley now offers a service which will ensure your paper is clearly written in standard, scientific English language. Visit this site to learn about the options. Please note that using the Wiley English Language Editing Service does not guarantee that your paper will be accepted by this journal.

B. Cover Letter

The corresponding author should provide a brief cover letter. In this letter the details of the authors' contributions (see Section A - Authorship) and funding should be included.

C. Title Page

The title page should include:

1. a descriptive title for the article;
2. the names [first name (if normally used in publication), initial(s) of middle name(s), surnames] and affiliations of all authors;
3. the full postal address and e-mail of the author to whom correspondence should be addressed;
4. a telephone number and fax number where the corresponding author can be reached;
5. a suggested running title of not more than 40 characters including spaces.

This is an example of how the names and affiliations should be formatted:

Andrew Argue*, Brittany Banter* Charlotte Chatter† & Don Dolittle‡

*Department of Surgery, School of Veterinary Medicine, University of Everywhere, Everywhere, Nation

†Companion Animal Clinic, School of Veterinary Medicine, University of Anywhere, Anywhere, Nation

‡Clinic of Orderly Conduct, School of Veterinary Medicine, University of Somewhere, Somewhere, Country

Correspondence: Andrew Argue, Department of Surgery, School of Veterinary Medicine, University of Everywhere, Everywhere, State, Nation, Postal code

E-mail: arguea@ue.com

Tel: 99 999 9999

Fax: 99 999 9998

Note that the affiliation address should be the one where the work was done.

D. Abstract and Keywords

- The abstract should be on a separate page and should not exceed 300 words.
- For original research articles and short communications a structured abstract should be used with the following titles: Objective, Study design, Animals or Animal population, Methods, Results, Conclusions and clinical relevance.
- For review articles and "What is the Evidence?" articles the abstract should be structured and usually should have the following headings: Objective, Databases used and Conclusions.
- Up to five keywords or phrases should be listed after the abstract. Ideally they should beMeSH headings. Note: these keywords are not necessarily the same that you will assign to your article during the submission process on ScholarOne.
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E. Main Text

- This should begin on a separate page. Sections within the main text should be appropriately sub-headed: Introduction, Materials and methods, Results, and Discussion.
- The manufacturers of drugs and equipment used in the research that are important to the methods should be stated in parentheses immediately after the first use of that item in the text. This should include the specific identification of the equipment or the trade name for a drug followed by the name of the company, state (if USA) and the country from where it is sourced (e.g. Datex CD 200-02; Datex, UK). Abbreviations and footnotes should be avoided.

F. Statistical guidelines

The following guidelines apply to some basic approaches to statistical analysis with respect to anaesthesia and analgesia research but do not cover all eventualities. The advice and involvement of a competent statistician during the design of the research is highly recommended. The following sequence is suggested for statistical procedures:

1. Start with a testable hypothesis using comprehensive information about the biological system under test.
2. Use the best possible design for an experiment to test that hypothesis. This should include careful calculations of the number of subjects or patients needed to demonstrate clinically relevant differences (see editorial in VAA 2003, 30, 59-61).
3. Set up and conduct the experiment to conform to the specific design.
4. Analyze and interpret the data according to the specific design used and determine whether the results support the hypothesis. Do not allow the results to determine the analysis. Reasoning after the fact is only valid as a basis for future work.

5. Present the hypothesis, design, analysis, and interpretation in a clear and concise manner so that the reader can follow what was done.
6. Report the values to the same level of accuracy at which they were measured. For example blood pressure is usually measured in whole integers so it should be reported as e.g. 100 ± 10 mm Hg. *An exception to this is when whole integer scoring scales are used, where reporting to one decimal place will allow the reader to see the differences between groups (e.g. an ordinal scale of 1,2,3,4 where scores are reported as 3.3 and 3.7).*
7. Use mean and standard deviation (SD) rather than standard error (SEM) unless reporting large sets of population data. In general the 95% confidence intervals are preferred over the SEM. Many biological variables are not normally distributed so it may be more appropriate to report the median and range or interquartile range of the data (if the SD is >half the mean then the data are not likely to be normally distributed). *When using ordinal scales (e.g. pain scales) these should be reported as median and range or interquartile range.*
8. When analyzing some data it may be appropriate to use transformation techniques that "normalize" them such as logarithms, square roots or exponentials. The data should be reported as the original values even when the analysis is done in this way. Non-parametric statistical tests may need to be used on some data that fall into this category. This would be the case for most situations where ordinal scoring scales are used.
9. Report the actual P values calculated for the data e.g. p =0.034 not p <0.05 unless the statistical calculation only reports it this way.
10. When discussing the results it is important to point out the values that are statistically significant and those that are clinically significant. It is common in anaesthesia papers to measure pH and one may get statistical differences between values such as 7.425 and 7.409 but it is unlikely that this would be clinically significant.
11. Report the statistical software used to analyse the data, including the manufacturer, state (if USA), and country.

G. Acknowledgements and Conflicts of Interest

- Acknowledgements should be brief and must include reference to sources of material and logistical support.
- Author(s) should clear the copyright of material they wish to reproduce from other sources and this should be acknowledged.
- Acknowledgements should only appear on the title page and not on the main document to ensure anonymity through the peer-review process.
- Any other potential conflicts of interest should be stated.

H. References

- Abstracts that are more than two years old should not be used as references.
- Proceedings should not be used as references.
- References cited within the text that are 'unpublished observations' or 'personal communications' should not be included in the reference list. Authors are responsible for verifying that the information provided under these headings is accurate and approved by the persons concerned. Information from manuscripts that have been submitted but not accepted should be cited as unpublished observations.
- A modified Harvard style should be used in the reference list.
- Cite the author names followed by year of publication:
  - (Jones 1997; Gregory 1999).
- Where there are two authors they should both be included with an ampersand:
  - (Pascoe & Bennett 1999)
- Where there are three or more authors, the first author's name followed by et al. should be used:
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-Larenza MP, Ringer SK, Kutter AP et al. (2009a) Evaluation of anesthesia recovery quality after low-dose racemic or S-ketamine infusions during anesthesia with isoflurane in horses. Am J Vet Res 70, 710–718.

-Larenza MP, Peterbauer C, Landoni MF et al. (2009b) Stereoselective pharmacokinetics of ketamine and norketamine after constant rate infusion of a subanesthetic dose of racemic ketamine or S-ketamine in Shetland ponies. Am J Vet Res 70, 831–839.

-Hall LW, Taylor PM (1994) Anaesthesia of the Cat (1st edn), Balliere Tindall, London, UK, pp. 189-193.

-Pascoe PJ, Bennett RC (1999) Thoracic Surgery. In: Manual of Small Animal Anaesthesia and Analgesia (1st edn). Seymour C, Gleed R (eds). BSAVA, UK. pp. 183-196.

-Conde Ruiz C, Del Carro A, Rosset E et al. (2015) Alfaxalone for total intravenous anaesthesia in bitches undergoing elective caesarean section and its effects on puppies: a randomized clinical trial. Vet Anaesth Analg. doi: 10.1111/vaa.12298 [Epub ahead of print]•

-Moher D, Liberati A, Tetzlaff J et al. (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med 6, e1000097.

-[No authors listed] (2013) Notice of formal retraction of articles by Dr. Y. Fujii. Br J Anaesth 110, 669.

-Portela D, Campoy L, Otero P et al. (2015) Ultrasound-guided thoracic paravertebral injection in dog cadavers. Vet Anaesth Analg 42, A55 (abstract).

-Seeler DC, Turnwald GH, Bull KS (1999) From teaching to learning:Part III. Lectures and approaches to active learning. J Vet Med Educ 21 http://scholar.lib.vt.edu/ejournals/JVME/V21-1/Seeler1.html

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