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ATIVIDADE ANESTÉSICA DE ÓLEOS ESSENCIAIS E CONSTITUINTES ISOLADOS DE PLANTAS MEDICINAIS BRASILEIRAS EM *Rhamdia quelen*

TESE DE DOUTORADO

Lenise de Lima Silva

Santa Maria, RS, Brasil
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Lenise de Lima Silva

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Orientadora: Prof[a]. Dr [a]. Berta Maria Heinzmann
Co-orientador: Prof. Dr. Bernardo Baldisserotto

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elaborada por
Lenise de Lima Silva

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À todos aqueles que direta ou indiretamente contribuiram
para a realização deste trabalho.

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“Nunca deixe que lhe digam que
não vale a pena acreditar nos sonhos que se tem...
quem acredita sempre alcança”
(Renato Russo).

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria

ATIVIDADE ANESTÉSICA DE ÓLEOS ESSENCIAIS E CONSTITUINTES ISOLADOS DE PLANTAS MEDICINAIS BRASILEIRAS EM *Rhamdia quelen*

AUTOR: LENISE DE LIMA SILVA
ORIENTADORA: BERTA MARIA HEINZMANN
DATA E LOCAL DA DEFESA: Santa Maria, 13 de janeiro de 2014.

Este trabalho visou obter anestésicos e sedativos para peixes a partir de plantas medicinais brasileiras, nomeadamente *Ocimum gratissimum* (OG), *Ocimum americanum* (OA) e *Hyptis mutabilis* (HM). Os óleos essenciais destas espécies foram obtidos por hidrodestilação e analisados por cromatografia gasosa acoplada à espectrometria de massas (CG-EM). A quiralidade de um dos componentes do óleo de OA também foi determinada. Os óleos de OA e HM foram submetidos ao processo de isolamento de seus constituintes, os quais foram caracterizados por métodos espectroscópicos. A determinação do tempo de indução aos estágios de anestesia e de recuperação foi realizada em jundiás juvenis (*Rhamdia quelen*) por meio de banhos contendo os óleos essenciais ou constituintes isolados. Para o óleo de OG e o (-)-globulol também foram avaliados o mecanismo de ação via sítio benzodiazepínico do receptor gabaérgico por meio de associação com diazepam (150 μM) e reversão com flumazenil (5μM). O desenvolvimento de tolerância foi avaliado para o óleo de OG através de re-exposição sucessiva após 30 dias ou com intervalos semanais. Os efeitos da exposição ao óleo de OA (300 e 500 mg L^{-1}) em parâmetros de estresse (níveis plasmáticos de cortisol, glicose e sódio) após manuseio também foram testados. Os constituintes majoritários dos óleos essenciais corresponderam ao eugenol (73,6%) em OG, 1,8-cineol (21% nas folhas) e linalol (20,2% nas folhas e 46,6% nas inflorescências) em OA, globulol (26,6% nas folhas) e germacreno D (15% nas inflorescências) em HM. Todos os óleos apresentaram atividade anestésica em jundiás juvenis em concentrações entre 30-300 mg L^{-1} de OG, 200-500 mg L^{-1} de OA e 344 mg L^{-1} de HM. O fracionamento do óleo essencial de HM forneceu os compostos (+)-1-terpinen-4-ol e (-)-globulol, os quais demonstraram ação sedativa em 10 mg L^{-1}. (-)-Globulol foi capaz de induzir anestesia entre 83-190 mg L^{-1}, contudo perda de muco e mortalidade foram observados nos peixes expostos. Ação similar aos benzodiazepínicos no receptor GABA foi detectada para o óleo de OG, inclusive no que se refere ao desenvolvimento de tolerância. Para o (-)-globulol o mesmo não pode ser observado. A avaliação da quiralidade do linalol presente no óleo de OA demonstrou a presença do isômero *R*-(-), o qual demonstrou maior efeito anestésico que o *S*-(+)-linalol isolado do óleo de *Lippia alba* em jundiás. Os isômeros também apresentaram diferenças em relação à ação bactericida frente a *Aeromonas hydrophila*, sendo apenas o *S*-(+)-linalol ativo. Em relação aos efeitos sobre parâmetros de estresse, a exposição prévia ao óleo essencial de OA preveniu o estresse induzido pelo manuseio.

Palavras-chaves: *Ocimum*, *Hyptis mutabilis*, globulol, GABA, cortisol, glicemia, jundiá.

ABSTRACT

Doctoral Thesis
Post-Graduating Program in Pharmacology
Federal University of Santa Maria

ANESTHETIC ACTIVITY OF ESSENTIAL OILS AND ISOLATED CONSTITUENTS OF BRAZILIAN MEDICINAL PLANTS IN *Rhamdia quelen*

AUTHOR: LENISE DE LIMA SILVA
ADVISOR: BERTA MARIA HEINZMANN
DATE AND PLACE: Santa Maria, 13th January 2014.

The aim of this study was to obtain new fish anesthetic and sedative drugs from Brazilian medicinal plants: *Ocimum gratissimum* (OG), *Ocimum americanum* (OA) e *Hyptis mutabilis* (HM). Essential oils from these plants were obtained by hydrodestilation and analysed by gas chromatography coupled to mass spectrometry (GC-MS). The chirality of one constituent of OA oil was also determined. Isolation process from essential oil of OA and HM was conduced and the isolated compounds were analyzed by spectroscopic methods. To determine the point at which anesthesia was induced and the length of the recovery period, juvenile silver catfish (*Rhamdia quelen*) were placed in aquaria containing essential oil or isolated compounds. The mechanism of action of the essential oil of OG and (-)-globulol was evaluated through association with diazepam (150 µM) and reversion with flumazenil (5µM). The evaluation of tolerance development was performed to OG oil using re-exposure after 30 days or at week intervals. The effects of OA oil (300 and 500 mg L^{-1}) exposure on stress parameters (plasma cortisol, glucose and sodium levels) after handling were also assayed. The major compounds of the essential oils were eugenol (73.6%) in OG, 1,8-cineol (21% in the leaf) and linalool (20.2% in the leaf; 46.6% in the inflorescence) in OA, globulol (26.6% in the leaf) and germacrene D (15% in the inflorescence) in HM. Anesthetic effects were detected to all samples in silver catfish in concentration ranges of 30-300 mg L^{-1} of OG, 200-500 mg L^{-1} of OA and 344 mg L^{-1} of HM. Fractionation of the essential oil of HM furnished (+)-1-terpinen-4-ol and (-)-globulol, which demonstrated sedative effect at 10 mg L^{-1}. Anesthesia was obtained with 83-190 mg L^{-1} of (-)-globulol, but animals showed loss of mucus during induction and mortality at these concentrations. Benzodiazepinic-like action on receptor $GABA_A$ was detected to the essential oil of OG, as well as tolerance development. The same pattern could not be observed to (-)-globulol. The *R*-(-)-linalool detected in essential oil of OA demonstrated higher anesthetic effect in silver catfish in relation to the *S*-(+)-form isolated from the essential oil of *Lippia alba*, which was the only active against *Aeromonas hydrophila in vitro*. Regarding to the stress parameters, previous exposure to the essential oil of OA was able to prevent handling-induced stress.

Key-words: *Ocimum*, *Hyptis mutabilis*, globulol, GABA, cortisol, glicemia, silver catfish.

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APRESENTAÇÃO

O presente trabalho consiste na apresentação dos resultados obtidos para fins de defesa de Tese de Doutorado. Inicialmente é apresentada uma breve introdução sobre o assunto, a fim de fornecer uma explanação sobre as espécies vegetais em estudo e descrever aspectos relevantes relacionados à atividade biológica avaliada. Logo em seguida são descritos os objetivos desta proposta.

Os resultados encontram-se organizados na forma de artigos científicos, que englobam trabalhos já publicados, e manuscritos a serem submetidos a periódicos da área. O primeiro artigo trata da atividade anestésica do óleo essencial de *O. gratissimum*, seu mecanismo de ação e o desenvolvimento de tolerância a sua utilização. Já o segundo artigo reporta a atividade anestésica e sedativa dos óleos das diferentes partes de *H. mutabilis*, o processo de isolamento de seus constituintes, a avaliação da atividade dos mesmos e a determinação do envolvimento do receptor GABA via sitio benzodiazepínico no efeito sedativo de seu constituinte majoritário.

O primeiro manuscrito descreve os efeitos depressores centrais do óleo essencial de diferentes órgãos vegetais de *O. americanum* e a avaliação de parâmetros relacionados ao estresse após manuseio de animais anestesiados com a amostra mais ativa. No segundo manuscrito é realizado um comparativo das atividades antimicrobiana e anestésica do *R*-(-)-linalol, isolado a partir do óleo de *O. americanum*, em relação a seu *S*-(+)-enantiômetro.

Subsequentemente a estes será apresentada uma discussão geral, a fim de realizar uma ampla interpretação dos resultados obtidos correlacionando-os com a literatura e entre si. A tese é, então, finalizada pelas conclusões, referências bibliográficas e anexos.

1 INTRODUÇÃO

A utilização de produtos naturais como recurso terapêutico é tão antiga quanto à civilização humana (RATES, 2001). Na medicina popular, assim como na terapêutica, plantas contendo derivados terpênicos e fenilpropanóides têm sido usadas como sedativas, tranquilizantes e anticonvulsivantes (BROWN, BRADEN, 1987; PASSOS et al., 2009). Estes dois grupos biossinteticamente distintos de constituintes fazem parte da composição dos óleos essenciais, os quais, por definição, correspondem a misturas complexas de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas. Na mistura, tais compostos apresentam-se em diferentes concentrações; normalmente, um deles é o composto majoritário, existindo outros em menores teores e alguns em baixíssimas quantidades (traços) (SIMÕES, SPITZER, 2004; BAKKALI et al., 2008).

Em relação à distribuição dos óleos essenciais, as plantas capazes de produzir seus constituintes correspondem a um número limitado de espécies, classificadas em determinadas famílias vegetais, como Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae, Piperaceae, entre outras (BRUNETON, 2001). Dentro da família Lamiaceae, a subfamília Nepetoideae, composta por aproximadamente 3400 espécies em 105 gêneros, engloba a maioria das espécies ricas em óleos essenciais. A tribo Ocimeae, pertencente a esta subfamília, corresponde a um grupo predominantemente tropical de 35 gêneros e 1060 espécies, com grande importância econômica e medicinal. Dentro desta tribo encontram-se as subtribos Ociminae e Hyptidinae, que englobam, respectivamente, plantas dos gêneros *Ocimum* e *Hyptis* (GRAYER et al., 2003; PATON et al., 2004).

O gênero *Ocimum* L. é formado por aproximadamente 30 espécies, das quais 16 são originárias da África. Sua área de distribuição engloba as regiões tropical e subtropical, contudo encontram-se no Brasil como espécies naturalizadas ou cultivadas: *Ocimum basilicum* L., *O. americanum* L., *O. gratissimum* L., *O. minimum* L. e *O. tenuiflorum* L. (ALBUQUERQUE, ANDRADE, 1998a). Em nosso país, estas espécies de modo geral são denominadas de alfavaca, o qual corresponde a um nome de origem européia difundido pelos africanos em suas migrações dentro do Brasil (ALBUQUERQUE, ANDRADE, 1998b).

Ocimum gratissimum L. (Figura 1) corresponde a uma erva de 50-250 cm, de base lenhosa e aromática (ALBUQUERQUE, ANDRADE, 1998a). Suas folhas e flores são utilizadas na medicina popular como digestivo, expectorante, calmante, carminativo, no

tratamento de flatulência, gripe, tontura, sinusite, tosse, bronquite, prurido, dor de cabeça e estômago,topicamente em micoses, bem como nos casos de estresse, fadiga e com finalidade mágico-religiosa (DI STASI et al., 2002; CHANWITHEESUK et al., 2005; ALBUQUERQUE et al., 2007a; 2007b).

Figura 1 – *Ocimum gratissimum* – aspecto geral da planta.

A maioria dos estudos com *O. gratissimum* foi realizado com o seu óleo essencial. Sua composição sugere a existência de dois grandes grupos, um rico em timol (VIEIRA et al., 2001; YAYI et al., 2004) e outro rico em eugenol (SILVA et al., 1999; VIEIRA et al., 2001; JIROVETZ et al., 2003; MADEIRA et al., 2005; FREIRE, MARQUES, COSTA, 2006). Outros quimiotipos também foram relatados como ricos em cinamato de etila (DUBEY et al., 2000) e o geraniol (VIEIRA et al., 2001). Em relação às atividades biológicas, a mais descrita na literatura é a antimicrobiana (JEDLIČKOVÁ, MOTTL, ŠERÝ, 1992; NAKAMURA et al., 1999; DUBEY et al., 2000; CIMANGA et al., 2002; ACOSTA et al., 2003; IWALOKUN et al., 2003). Contudo, efeitos hipoglicemiante (AGUIYI et al., 2000), antinociceptivo (RABELO et al., 2003), larvicida frente a *Aedes aegypti* (CAVALCANTI et al., 2004), relaxante em íleo isolado de cobaio (MADEIRA et al., 2005), anticonvulsivante (FREIRE, MARQUES, COSTA, 2006) e hipotensivo (INTERAMINENSE et al., 2007) também foram descritos para o óleo essencial desta espécie.

Outra espécie aromática deste gênero corresponde a *Ocimum americanum* L. (sinonímia: *O. canum* Sims). Esta espécie (Figura 2) é uma erva de cerca de 50 cm, anual ou perene e de base lenhosa (ALBUQUERQUE, ANDRADE, 1998a). Suas folhas são empregadas na medicina popular como analgésico, rubefasciente, carminativo,

hipocolesterolemiante, estomáquico, antigripal, no controle do diabetes, no tratamento da insônia e ansiedade (NYARKO et al., 2002; CHANWITHEESUK et al., 2005;ALBUQUERQUE et al., 2007a; HASSANE et al., 2011).

Figura 2 – *Ocimum americanum* – aspecto geral da planta.

Em relação a *O. americanum*, diversos quimiotipos foram detectados segundo a composição do seu óleo essencial. Entre eles destacam-se o eugenol, fenchona, linalol, (*E*)-α-bergamoteno, 1,8-cineol, terpinen-4-ol, cânfora, citral, timol, cinamato de metila, limoneno, anisol e metilchavicol como majoritários (LAWRENCE, 1989; SANDA et al., 1998; CIMANGA et al., 2002; MONDELLO et al., 2002; JIROVETZ et al., 2003; VASCONCELOS SILVA et al., 2003; CAVALCANTI et al., 2004; DJIBO, SAMATÉ, NACRO, 2004; MURILLO et al., 2004; NGASSOUM et al., 2004; OUSSOU et al., 2004; VIEIRA, SIMON, 2006; CAROVIC-STANKO et al., 2010; HASSANE et al., 2011; NASCIMENTO et al., 2011; SELVI, THIRUGNANASAMPANDAN, SUNDARAMMAL, 2012).

Atividade antibacteriana de largo espectro foi verificada para os óleos essenciais de *O. americanum* ricos em timol (CIMANGA et al., 2002), citral (CAROVIC-STANKO et al., 2010), 1,8-cineol (HASSANE et al., 2011) e metilchavicol (NASCIMENTO et al., 2011). Já o óleo do quimiotipo cinamato de metila demonstrou propriedades larvicidas contra *Aedes aegypti* (CL_{50} = 67 ppm) (CAVALCANTI et al., 2004). Também foi descrito para o óleo essencial das sementes e das folhas citotoxicidade frente a linhagens de células tumorais *in vitro* (MANOSROI, DHUMTANOM, MANOSROI, 2006; SELVI, THIRUGNANASAMPANDAN, SUNDARAMMAL, 2012).

O gênero *Hyptis* é o segundo maior entre as Lamiaceas e apresenta cerca de 300 espécies. A maioria das espécies ocorre no continente americano, desde o sul dos Estados Unidos até a Argentina. Algumas são encontradas espontaneamente também na África, Ásia e norte da Austrália (KISSMANN, GROTH, 1995). Dentre estas, *Hyptis mutabilis* (Rich.) Briq. (Figura 3) corresponde a uma espécie nativa do continente americano, com ampla distribuição geográfica no Brasil e de ocorrência frequente na região sul (KISSMANN, GROTH, 1995). É uma planta perene, de crescimento espontâneo da primavera ao verão (GOLENIOWSKI et al., 2006; ALBUQUERQUE et al., 2007b), que pode ser encontrada na beira de matas, em terrenos abandonados e como infestante em lavouras (KISSMANN, GROTH, 1995).

Figura 3 – *Hyptis mutabilis* – aspecto geral da planta.

No Brasil esta espécie é conhecida popularmente como acauã-tajá, alfavacão, alfavaca, sambacuité, alfavaca-de-caboclo, sambaité, bamburral e manjericão (LUZ et al., 1984; AGUIAR et al., 2003; COELHO-FERREIRA, 2009). O chá das folhas frescas de *H. mutabilis* é utilizado popularmente no tratamento de doenças da mucosa uterina cervical, gastrite, úlcera gástrica, lesões de pele infectadas e conjuntivite (BARBOSA, RAMOS, 1992), enquanto que as raízes são usadas em casos de reumatismo (COELHO-FERREIRA, 2009). Outros usos populares relatados são em casos de inflamação uterina, tosse, dor de cabeça, como expectorante, cicatrizante, sedativo e para indução do parto (ALBUQUERQUE et al., 2007b; AMORIM, 2009; SILVA, BÜNDCHEN, 2011).

O óleo essencial obtido de *H. mutabilis* tem demonstrado grandes variações no rendimento e na composição química, de acordo com o local de coleta (LUZ et al., 1984; BAILAC et al., 1999; AGUIAR et al., 2003). Constituintes como α-felandreno, *p*-cimeno

(LUZ et al., 1984), *E*-cariofileno (BARBOSA, RAMOS, 1992; GILLIJ, GLEISER, ZYGADLO, 2008; DAMBOLENA et al., 2009;), *E*-diidrocarvona (VELASCO-NEGUERUELA et al., 1995), timol, δ-3-careno e *E*-cinamato de metila (AGUIAR et al., 2003) foram descritos como majoritários. Em relação as suas propriedades biológicas, atividades anti-ulcerogênica (BARBOSA, RAMOS, 1992), antimicrobiana (DEMO et al., 2005; OLIVA et al., 2006) e repelente para *Aedes aegypti* (GILLIJ, GLEISER, ZYGADLO, 2008) encontram-se reportadas na literatura.

Considerando que muitos óleos voláteis e seus constituintes possuem uma grande variedade de atividades farmacológicas relacionadas aos seus efeitos sobre o sistema nervoso central (BROWN, BRADEN, 1987; PASSOS et al., 2009), a sua utilização na piscicultura corresponde a um campo a ser melhor explorado. Nesta área, substâncias com propriedades anestésicas e sedativas são utilizadas em animais aquáticos para prevenir injúrias físicas, reduzir o metabolismo e promover a imobilização do animal (COYLE, DURBOROW, TIDWELL, 2004). Além disso, o uso de substâncias com estas propriedades visa também reduzir ou prevenir o desencadeamento de uma resposta ao estresse no animal, decorrente das atividades de manejo e transporte (COYLE, DURBOROW, TIDWELL, 2004; VIDAL et al., 2007).

O estresse em peixes tem sido definido como um estado resultante das condições ambientais que ameaçam sua sobrevivência (ROSS, ROSS, 2008). A resposta ao estresse é descrita em três níveis: primário, secundário e terciário. A resposta primária envolve a ativação de vias neuroendócrinas como o sistema nervoso autônomo e o eixo hipotálamo-pituitária-interrenal (HPI), que ocasionam a liberação de catecolaminas e corticosteróides (cortisol) para a circulação. Estes hormônios promovem alterações em nível secundário no metabolismo, respiração, equilíbrio acido-básico, balanço hidromineral e hematócrito. Caso o estímulo estressor persista, as alterações estendem-se para o nível de organismo e populacional (resposta terciária) ocasionando efeitos como inibição de crescimento, da reprodução e da resposta imune, além da redução da capacidade de tolerância a agentes estressores adicionais (HONTELA, 1998; BARTON, 2002; OBA, MARIANO, SANTOS, 2009; WEBER, 2011).

Anestesia é um estado biológico induzido por um agente externo, que resulta em parcial ou completa perda sensorial e perda do controle motor voluntário, através da utilização de meios químicos e não químicos (BRESSLER, RON, 2004). Por razões práticas, a anestesia pode ser descrita em três fases: a indução, a manutenção e a recuperação (ROSS, ROSS, 2008). Quando a indução é lenta, uma série de estágios pode ser observada nos animais. Estes

estágios incluem a sedação, a perda parcial e total do equilíbrio, a anestesia e o colapso medular, que poderá levar a morte (SCHOETTGER, JULIN, 1967; COYLE, DURBOROW, TIDWELL, 2004; ROSS, ROSS, 2008). O estágio alcançado geralmente depende da concentração/dose e da extensão da exposição (COYLE, DURBOROW, TIDWELL, 2004). A manutenção corresponde ao prolongamento do estágio anestésico alcançado em um nível estável, sem detrimento da saúde do animal. Nesta fase, a condição dos animais deve ser visualmente monitorada de forma contínua (ROSS, ROSS, 2008). Durante a fase de recuperação, o peixe é retirado do contato com o anestésico a fim de que retorne ao seu estado normal. Para reduzir o tempo de recuperação, a indução deve ser rápida e tempo de manuseio deve ser mínimo. Dependendo da espécie animal e do anestésico administrado, o início da recuperação poderá levar de alguns segundos a vários minutos, enquanto que a completa recuperação ocorrerá de minutos a horas (COYLE, DURBOROW, TIDWELL, 2004).

A via de administração mais comum de anestésicos em animais aquáticos é a inalatória. Neste caso, o fármaco é adicionado à água onde o animal se encontra, sendo então absorvido pelas brânquias durante o processo de respiração e, em menores proporções, por via cutânea. O efeito da substância é avaliado através da determinação do tempo de indução e recuperação, por meio da observação de alterações na atividade natatória, equilíbrio, frequência respiratória e responsividade a estímulos externos (ZAHL, SAMUELSEN, KIESSLING, 2012).

Segundo Ross e Ross (2008), o processo anestésico ocorre devido a uma difusa depressão do sistema nervoso central, a qual pode ser produzida através da estabilização da propagação neuronal, bloqueio da liberação de neurotransmissores ou de seus receptores ou pela combinação destas ações. O mecanismo de ação de anestésicos em peixes não é bem conhecido e tem sido geralmente extrapolado a partir de estudos realizados em vertebrados superiores (ZAHL, SAMUELSEN, KIESSLING, 2012).

A eficácia de fármacos anestésicos em animais aquáticos pode ser afetada por muitos fatores. Dentre os fatores biológicos temos a idade, sexo, estágio de desenvolvimento, peso corporal, taxa de crescimento, composição corporal, condição fisiológica e de saúde do animal. Fatores ambientais, como temperatura da água, salinidade, pH e nível de oxigênio dissolvido, também devem ser considerados como fonte de variação (COYLE, DURBOROW, TIDWELL, 2004; ROSS, ROSS, 2008; ZAHL, SAMUELSEN, KIESSLING, 2012).

Dentre os agentes anestésicos empregados na área da piscicultura, a grande maioria dos fármacos disponíveis é de origem sintética. Os mais comuns compreendem o metanossulfonato de tricaina (MS-222), a benzocaína, o metomidato, o 2-fenoxietanol e a

quinaldina (ZAHL, SAMUELSEN, KIESSLING, 2012). Estas substâncias, apesar de serem efetivas como anestésicas, são capazes de promover efeitos colaterais nos animais, como perda de muco, irritação nas brânquias e olhos, e alguns incômodos aos trabalhadores, como a necessidade do uso de luvas (INOUE, SANTOS NETO, MORAES, 2003). Adicionalmente, seu emprego deve ser seguido por um determinado período de depuração, a fim de mininizar os níveis de resíduos presentes no filé do animal abatido (COYLE, DURBOROW, TIDWELL, 2004).

Segundo Façanha e Gomes (2005), no Brasil não existe uma legislação que especifique quais os anestésicos permitidos na área de piscicultura. Desta forma, muitos produtores seguem as recomendações de organismos de outros países, como a FDA (*Food and Drug Administration*). Este fato dificulta e encarece o processo anestésico, uma vez que o MS-222, único anestésico químico aprovado pelo FDA para uso em peixes, não é produzido em nosso país (FAÇANHA, GOMES, 2005).

Por outro lado, a instrução normativa interministerial nº 28, de 8 de junho de 2011, especifica que o transporte, o pré-abate e o abate dos organismos aquáticos, inclusive aqueles doentes ou descartados, deve atender ao princípios de respeito ao bem-estar, à redução de processos dolorosos e aos procedimentos de abate humanitário. A mesma normativa também recomenda a utilização de anestésicos na eutanásia de animais e permite o emprego de fitoterápicos e extratos vegetais na prevenção e tratamento de enfermidades de organismos aquáticos (BRASIL, 2011).

Considerando-se que o desenvolvimento de algumas patologias em peixes como, por exemplo, as infecções promovidas pela bactéria *Aeromonas hydrophila* e pelo protozoário *Ichthyophtirius multifiliis*, são favorecidas por uma situação de manejo estressante (ANDRADE et al., 2006; SEGNER et al., 2012), a utilização de anestésicos e sedativos a fim de promover o bem-estar do animal pode ser considerada uma medida preventiva para estas enfermidades. Neste sentido, alguns óleos essenciais e constituintes isolados a partir de espécies vegetais têm se mostrado efetivos em diversas espécies de peixes no que se refere ao seu efeito anestésico e sedativo (FAÇANHA, GOMES, 2005; VIDAL et al., 2007; GONÇALVES et al., 2008; ROSS, ROSS, 2008; SIMÕES, GOMES, 2009; CUNHA et al., 2010a; 2010b; 2011). Os mais amplamente estudados tem sido os óleos essenciais de cravo (constituído por 70-90% de eugenol) e de *Lippia alba*, bem como o mentol, o eugenol e seu isômero, o isoeugenol (WOOD, NELSON, RAMSTAD, 2002; INOUE, SANTOS NETO, MORAES, 2003; IVERSEN et al., 2003; PALIĆ et al., 2006; GUÉNETTE et al., 2007; VIDAL et al., 2007; FAÇANHA, GOMES, 2005; GONÇALVES et al.; 2008; ROSS, ROSS,

2008; SILVA et al., 2009; SIMÕES, GOMES, 2009; CUNHA et al., 2010a, 2010b; 2011; IMANPOOR, BAGHERI, HEDAYATI, 2010). Outros produtos naturais menos estudados, mas com bioatividade relevante neste contexto, correspondem aos óleos essenciais de *Aloysia triphylla*, *Hesperozygis ringens*, *Lippia sidoides*, *Melaleuca alternifolia*, *Nectandra megapotamica* e *Ocotea acutifolia* (HAJEK, 2011; GRESSLER et al., 2012; SILVA et al., 2013; TONDOLO et al., 2013).

2 OBJETIVOS

2.1 Objetivo Geral

Obter novos anestésicos e sedativos a partir de *Ocimum gratissimum* L., *Ocimum americanum* L. e *Hyptis mutabilis* (Rich.) Briq. para uso em piscicultura.

2.2 Objetivos específicos

- A avaliação da atividade anestésica e/ou sedativa dos óleos essenciais de *Ocimum gratissimum* L., *Ocimum americanum* L. e *Hyptis mutabilis* (Rich.) Briq. em jundiás juvenis (*Rhamdia quelen*);
- A análise da composição química dos óleos essenciais das espécies nomeadas acima;
- O isolamento de constituintes dos óleos essenciais de *H. mutabilis* e *O. americanum*;
- A avaliação da atividade anestésica e/ou sedativa dos constituintes isolados dos óleos essenciais *H. mutabilis* e *O. americanum* em jundiás juvenis;
- A avaliação do envolvimento do receptor GABAérgico no mecanismo de ação do óleo essencial de *O. gratissimum* e o constituinte majoritário do óleo essencial de *H. mutabilis* em jundiás;
- Determinação do desenvolvimento de tolerância para as amostras que atuarem via receptor GABAérgico;
- A avaliação de parâmetros de estresse em peixes anestesiados e/ou sedados, com o óleo essencial de *O. americanum*.

3 ARTIGOS PUBLICADOS

2.1 Artigo 1

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Essential oil of *Ocimum gratissimum* L.: Anesthetic effects, mechanism of action and tolerance in silver catfish, *Rhamdia quelen*

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ABSTRACT

The aim of this study was to determine the time until anesthetic induction and recovery in silver catfish (*Rhamdia quelen*) exposed to the essential oil of *Ocimum gratissimum* L. (EO), its potential mechanism of action via GABAergic transmission and the development of tolerance. The EO was obtained from the aerial parts of *O. gratissimum* L. by hydrodistillation and analyzed by gas chromatograph coupled to mass spectrometer (GC–MS). Eugenol (73.6%) and β-bisabolene (18.3%) are the major compounds of the EO. Juvenile silver catfish were placed in aquaria containing different concentrations of the EO (10, 20, 30, 40, 50, 60, 70, 150 and 300 mg L^{-1}) to determine the point at which anesthesia was induced and the length of the recovery period. In the following experiments, the anesthetic effect of the EO (10, 20 and 40 mg L^{-1}) in association with benzodiazepine (BDZ) was determined. The GABAergic-like action and development of tolerance were assessed in fish exposed to BDZ (diazepam 150 μM), EO (40 mg L^{-1}) or both EO and BDZ (EO + BDZ) at the same concentrations. After the induction of anesthesia, juveniles were transferred to an anesthetic-free aquarium containing either the classic BDZ antagonist flumazenil (5 μM) or water to assess their recovery. The development of tolerance was evaluated in two experiments. In the first, juveniles were exposed to the EO twice in a 30-day interval, and in the second five exposures, weekly intervals were performed. Fish exposed to concentrations above 30 mg L^{-1} of the EO were effectively anesthetized without experiencing side effects or mortality. Fast anesthesia (<4 min) could be obtained using 150 and 300 mg L^{-1} of the EO. Synergism was detected for EO + BDZ at the lower concentrations tested. Juveniles exposed to all tested drugs showed a faster recovery in flumazenil than those that recovered in water. The development of tolerance was observed in fish exposed to the EO or BDZ separately after the fifth exposure. Two exposures were sufficient to sensitize the fish to the action of these drugs. However, repeated exposure to EO + BDZ did not induce tolerance and/or sensitization. In conclusion, the EO of *O. gratissimum* is an effective and safe anesthetic for silver catfish, and its mechanism seems to be related to an interaction with the $GABA_A$-benzodiazepine receptor.

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1. Introduction

Anesthetics are used in aquaculture to reduce the stress response and prevent physical injury during handling procedures (Bressler and Ron, 2004; Façanha and Gomes, 2005). Drugs, such as benzocaine, tricaine methanesulfonate (MS-222), quinaldine and 2-phenoxyethanol, have been used to achieve anesthesia in fish (Bressler and Ron, 2004; Mylonas et al., 2005; Zahl et al., 2009). However, highly variable anesthetic efficiency and side effects, such as the loss of mucus, gill irritation and corneal damage, have been observed (Guénette et al., 2007; Inoue et al., 2003). These undesirable side effects have led to a search for new safer and effective anesthetic drugs. In this context, the investigation of natural sources has produced good results. Essential oils (EOs) obtained from cloves (*Eugenia caryophyllata* and *Eugenia aromatica*) and *Lippia alba* as well as isolated compounds from plants (eugenol and menthol) are examples of anesthetics obtained from natural products that were effective in different fish species (Bressler and Ron, 2004; Cunha et al., 2010a,b; Façanha and Gomes, 2005; Guénette et al., 2007; Inoue et al., 2003).

The genus *Ocimum* (Lamiaceae), collectively called basil, belongs to an economically important group of herbaceous plants that are distributed in the tropical areas of Africa, America and Asia (Orafidiya et

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al., 2004; Tchoumbougnang et al., 2006). One of the most important species is *Ocimum gratissimum* L., commonly known as alfavaca or tree basil. This species is widely used as a condiment and is also used in traditional medicine as a sedative and treatment for stress and headaches, among other ailments (Albuquerque et al., 2007; Di Stasi et al., 2002). Orafidiya et al. (2004) described a dose-dependent sedative effect of timol-rich *Ocimum* oil in rodents during an investigation of acute and subchronic toxicity. In other studies, EOs rich in eugenol were shown to be an effective antinociceptive (Rabelo et al., 2003), anticonvulsant and sedative (Freire et al., 2006) agents in mice.

The sedative and anesthetic properties of drugs have been related to their effect on receptor for the γ-aminobutyric acid (GABA), which respond to the most abundant inhibitory neurochemical in the adult vertebrate brain (Mueller et al., 2006; Reynolds et al., 2003). $GABA_A$ receptors are ligand-gated ion channels surrounded by five protein subunits; this complex mediates fast neurotransmission via activation by GABA, which makes the central pore permeable to chloride ions (Johnston et al., 2006). The binding sites for GABA and benzodiazepine (BDZ) in the fish brain are quite similar to those in the mammalian brain in terms of density and pharmacological regulation (Betti et al., 2001; Oggier et al., 2010). BDZs increase the opening frequency of the GABA-gated chloride channel. Their therapeutic properties (anxiolytic, anticonvulsant, sedative, and muscle-relaxant) and the development of tolerance may result from the action and successive activation of different $GABA_A$ receptor subtypes, respectively (Bateson, 2002; Fernandes et al., 1999; Johnston et al., 2006; van Rijnsoever et al., 2004).

Data concerning the anesthesia of silver catfish (*Rhamdia quelen*) and whether the anesthesia functions through a mechanism involving GABAergic transmission are limited (Heldwein et al., in press). This fish species is commonly found in South American rivers and has been considered to be a good alternative for fish production due to its fast growth rate in warmer months (Barcellos et al., 2001; Gomes et al., 2000). Because this fish species has been used for intensive aquaculture, the knowledge of the anesthetic effects of the EO of *O. gratissimum* and its mechanism of action might be helpful for its production.

The aim of this work was to determine the time of anesthetic induction and recovery in silver catfish that were exposed to the EO of *O. gratissimum*. Additionally, a possible mechanism of action via GABAergic transmission was assessed through treatment in combination with BDZ and recovery in the presence of flumazenil, a classic BDZ antagonist. The development of tolerance was also evaluated in fish submitted to successive anesthesia procedures using standard BDZ, EO or the combination of EO and BDZ.

2. Material and methods

2.1. Animals

Juvenile silver catfish (9.0 ± 0.2 g; 10.3 ± 0.1 cm) were purchased from a local fish hatchery and transported to the laboratory, where they were maintained in continuously aerated 250 L tanks with controlled water parameters (22.5 ± 0.2 °C, pH: 7.97 ± 0.05, dissolved oxygen levels: 7.21 ± 0.17 mg L^{-1}, total ammonia levels: 1.16 ± 0.15 mg L^{-1}). The dissolved oxygen levels and temperature were measured with an YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA). The pH was determined with a DMPH-2 pH meter (Digimed, São Paulo, SP, Brazil). Total ammonia levels were measured by nesslerization (Eaton et al., 2005). A semi-static system was used, and 50% of the water volume was changed daily. Fish were fed once a day with commercial feed (Vicente Alimentos S.A. Presidente Prudente/SP, Brazil) containing 8% lipids, 28.0% crude protein and 3500 kcal kg^{-1} digestible energy according to the manufacturer. Juveniles were fasted for a period of 24 h prior to the experiments.

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).

2.2. Plant material

O. gratissimum L. were grown in Jardinópolis, São Paulo, Brazil. The aerial parts of the plant material were collected in March 2007, dried for 3 days in a ventilated drying oven at 45 °C and stored in closed dark packages until extraction. A voucher specimen identified by Dr. Lin Chan Ming was deposited in the Biotechnology Department of the University of Ribeirão Preto (voucher no. 1329).

2.3. Essential oil extraction and analysis

The EO was obtained from the aerial parts of the plant without flowers by hydrodistillation for 3 h using a Clevenger-type apparatus (European Pharmacopoeia, 2007) and was stored for some days at −20 °C in amber glass bottles until gas chromatograph coupled to mass spectrometer (GC–MS) analysis and biological tests. The EO was extracted in triplicate, and the yield was calculated as w/w (%).

GC–MS TIC analysis was performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector using an HP5-MS column (5% phenyl, 95% methylsiloxane, 30 m × 0.25 mm i.d. × 0.25 μm) and EI-MS of 70 eV. The operating conditions were as follows: split inlet 1:100; temperature program, 40–320 °C at 4 °C min^{-1}; carrier gas He; flow rate 1 mL min^{-1}; and injector and detector temperature 250 °C. The constituents of the EO were identified by comparison of the mass spectra with a mass spectral library (NIST, 2002) and the Kovats retention index with literature data (Adams, 2001).

2.4. Anesthesia induction and recovery

Juvenile fish were transferred to aquaria containing 1 L of continuously aerated water and EO at concentrations of 10, 20, 30, 40, 50, 60, 70, 150 and 300 mg L^{-1}, first diluted 1:10 in 95% ethanol. These concentrations were corrected based on the density of the EO (1.09 g mL^{-1}). Control experiments were performed using aquaria containing ethanol alone at the same concentration used for the dilution of the highest EO concentration. To evaluate the time required for the induction of anesthesia, 10 juveniles were used for each concentration tested, and each juvenile was used only once, based on the procedure reported by Schoettger and Julin (1967). This method involves six stages, in which the following parameters were observed: light and deep sedation (stages 1 and 2, respectively), partial and total loss of equilibrium (stage 3a and b, respectively), deep anesthesia (stage 4) and medullar collapse (stage 5). The maximum observation time was 30 min. After the induction of anesthesia, juveniles were transferred to anesthetic-free aquaria to measure the recovery time. Animals were considered to have recovered when they demonstrated normal swimming and reaction to external stimuli.

2.5. Anesthetic effect of EO in association with BDZ

Different concentrations (10, 20 and 40 mg L^{-1}) of the EO (diluted 1:10 in 95% ethanol) were tested along with standard BDZ (150 μM diazepam obtained from DEG, Brazil, in Tween 80 at 0.033%) as previously described (Heldwein et al., in press), in aquaria containing 1 L of water. A control group received ethanol in the same proportions used to dilute the EO. Fish (n = 10 for EO; n = 6 for BDZ and combination) were individually tested at each concentration. The stages of the induction of anesthesia and recovery time were evaluated as described in Section 2.4. The maximum observation times were 30 min for induction and 60 min for recovery. After recovery, the fish were grouped according to the anesthetic protocol and

transferred into continuously aerated 40 L aquaria, where they were observed for 1 week for any signs of abnormal behavior, diseases or mortality.

2.6. Evaluation of the GABAergic mechanism of action

To evaluate the GABAergic action of the EO, the protocol standardized by Heldwein et al. (in press) was used. Anesthesia was induced in the fish by treatment with BDZ (150 μM of diazepam), EO (40 mg L^{-1}) or the combination (EO + BDZ, at the same concentrations). These concentrations were chosen due to their long recovery time. A longer recovery time was required to better determine the efficacy of the antagonist in the reversal of the anesthetic effects. To induce anesthesia, the same experimental conditions described in Sections 2.4 and 2.5 were used. After induction, juveniles were separated into two aquaria: one was an anesthetic-free aquarium with 1 L water and the other contained 5 μM flumazenil (Flumazil®, Cristália, Brazil) in 1 L water. Fish (n = 8 for EO and BDZ individually; n = 7 for EO + BDZ) were tested individually.

In treatments, the fish behavior was scored after 1, 5, 10, 15 and 20 min. Recovery scores were as follows: 0, total loss of equilibrium, without swimming ability; 0.5, total loss of equilibrium, without swimming ability, but response to pressure on the caudal peduncle; 1, swimming ability returns, but with partial loss of equilibrium; 1.5, without movement after erratic swimming, with partial loss of equilibrium; 2, normal swimming, but without reaction to external stimuli; 2.5, without movement and reaction to external stimuli, followed by normal swimming; and 3, normal swimming with reaction to external stimuli. At the end of 20 min, the scores of each fish were added. An additional 0.5 was added to the score when the fish appeared agitated. As juveniles tend to remain stationary during recovery, each observation included external stimuli that corresponded to three successive hits of a glass rod against the bottom of the aquarium. A higher sum of the scores corresponds to faster recovery.

2.7. Tolerance development evaluation

To determine the development of tolerance, two experiments were carried out with the following experimental groups: BDZ (diazepam 150 μM), EO (40 mg L^{-1}) or the combination (EO + BDZ, at the same concentrations). In the first experiment, juveniles (n = 6–10) were exposed to the drugs twice, with an interval of 30 days between each exposure. In the second experiment, fish (n = 6–9) were exposed to the drugs five times over 30 days at weekly intervals. The experimental conditions of the induction of anesthesia and the recovery were the same as those described in Sections 2.4 and 2.5. After recovery, juveniles were grouped according to their exposure time and the anesthetic received and placed into a continuously aerated 40 L aquarium. They were observed between exposures for any signs of abnormal behavior, diseases or mortality. Water parameters were controlled weekly for each experimental group due to the low variation observed between them.

2.8. Statistical analysis

Data are presented as the mean ± SEM or median and interquartile range (Q1–Q3). To verify the homogeneity of variances, all data were submitted to the Levene test. The results of the induction of and recovery from anesthesia using the EO, BDZ or both were analyzed by a one-way ANOVA followed by the Tukey test or the Kruskal–Wallis test followed by the Mann–Whitney test when appropriate. A two-way ANOVA and the Tukey test were used to analyze the recovery scores of the mechanism test. Data from the evaluation of tolerance were analyzed by a paired *t*-test or the Wilcoxon signed rank test. Statistical analysis was performed using the software SPSS, version 17 and SigmaPlot, version 11.0, and the minimum significance level was set at $P<0.05$.

3. Results

The extraction of the EO resulted in a 1.12 ± 0.02% yield. The major compounds of the EO were eugenol (73.6%) and β-bisabolene (18.3%) (Table 1). In regard to the anesthetic action of the EO, the fish exposed to 10 and 20 mg L^{-1} of the EO did not show evidence of deep anesthesia (stage 4) during the 30 min evaluation period. Additionally, only 60% of the fish reached stage 4 when exposed to 30 mg L^{-1} of the EO. For all juveniles tested, 40 to 300 mg L^{-1} of the EO was effective in causing a stage 4 response without side effects or mortality. In this study, ethanol did not produce an anesthetic effect when applied alone. The time of recovery was significantly longer following exposure to the highest concentrations of EO tested (150 and 300 mg L^{-1}). No significant differences in recovery time were detected between concentrations of 30–70 mg L^{-1}; all recovered within 7–14 min. There was a significant positive relationship between the EO concentration and the time required for the induction of anesthesia for all stages except stage 4. No significant relationship was found between the EO concentration and the recovery time from the anesthesia (Table 2).

The increase of EO concentration in combination with BDZ promoted a proportional decrease in the time required for the induction of anesthesia up to stages 2 (Fig. 1A) and 4 (Fig. 1D). The same relationship did not occur in the stages of induction that were characterized by a total loss of equilibrium (stage 3a and b) (Fig. 1B and C). Furthermore, the recovery time of the fish exposed to EO + BDZ was significantly longer than those found with concentrations of EO alone (Fig. 1E). However, when the recovery time after treatment with EO + BDZ was compared with the recovery time after treatment with BDZ alone, we observed that a shorter time was required at 10 and 20 mg L^{-1} EO + BDZ, while the situation was reversed at the highest concentration.

Faster induction of anesthesia was obtained with the EO + BDZ treatment compared to BDZ or EO alone except for stage 4. To reach this stage, 40 mg L^{-1} of EO did not differ statistically from EO + BDZ at the same concentration (Fig. 1D). However, a clear synergism could be observed in the EO + BDZ treated group at the other tested concentrations. The application of only 10 or 20 mg L^{-1} of EO leads to stage 2 and 3a anesthesia, respectively (Fig. 1A and B), while fish exposed to the combination with BDZ reached deep anesthesia (Fig. 1D). Mortality was not observed during the 7-day period following exposure in this experiment. However, animals exposed to BDZ and EO + BDZ returned to normal feed intake 6 days after anesthetic exposure, while those exposed to EO fed normally after their recovery from anesthesia.

To evaluate whether the EO has a GABAergic mechanism of action, scores were compared after recovery in flumazenil or water. Juveniles exposed to BDZ had a higher total score when 5 μM of flumazenil was added to the water compared to those that recovered in water alone ($P = 0.045$). This result demonstrates the pharmacological validation

Table 1
Chemical composition of *Ocimum gratissimum* L. essential oil.

Peak	Rt (min)	Constituents	(%)	RI calc	RI ref
1	26.697	Eugenol	73.6	1361	1359
2	27.247	α-Ylangene	1.2	1368	1375
3	27.570	β-Bourbonene	0.8	1386	1388
4	31.648	β-bisabolene	18.3	1510	1509
5	33.816	Spathulenol	1.4	1581	1580
6	34.0	β-Caryophyllene oxide	4.8	1587	1585
		Total	100		

%: relative percentage; Rt: retention time; RI calc: calculated Kovats retention index; RI ref: reference Kovats retention index.

Table 2
Time required for the induction of and recovery from anesthesia using the essential oil of *Ocimum gratissimum* in juvenile silver catfish. Stages are defined according to Schoettger and Julin (1967). Maximum observation time was 30 min. Time to reach each stage is given in seconds (s). n = 10 for each concentration tested.

Concentration ($mg\ L^{-1}$)	Induction				Time to recovery (s)
	Stage 2 (s)	Stage 3a (s)	Stage 3b (s)	Stage 4 (s)	
10	726 (511.8–827.5)	–	–	–	293 (234.3–313.5)[e]
20	132 (116.8–170.5)	337.5 (282.8–356.3)	–	–	434.5 (352.3–482.3)[d]
30	89.5 (58.3–91.8)	128.5 (96.3–143.8)	664 (519.8–778.8)	854 (776–950.8)[a]	581.5 (527.5–664.5)[c]
40	49 (34.3–60.5)	74 (49.3–95.5)	500.5 (478.8–660.5)	759 (680.3–896.5)[ab]	444 (332–776.5)[cd]
50	51.5 (46.3–59.3)	79.5 (68.8–101.3)	471 (409.5–538)	681.5 (562.3–839.8)[b]	551 (522.8–644.5)[c]
60	50.5 (38.5–56.3)	59.5 (47.8–68)	320 (308–344.3)	467 (397–490.5)[c]	827 (500–958.5)[bc]
70	32.5 (24–36.5)	49.5 (44.5–52)	304 (293.5–318)	376.5 (349–477.8)[c]	587 (528.5–740.3)[c]
150	19.5 (17–23.7)	28 (24.3–31.8)	88 (83.8–94)	153 (146.3–160)[d]	1031 (900.5–1155)[ab]
300	17 (10.8–18)	21.5 (18–24.8)	33.5 (30.3–44.5)	65.5 (48.3–78.3)[e]	1181 (1150–1689)[a]
Equations	$Ln(y) = 0.0595 + 14.8399/ln(x)$ $r^2 = 0.9895$	$1/y = -0.0146 + 0.0019\ ln(x^2)$ $r^2 = 0.9915$	$y = 42.95 + 1364.7072exp^{-x/41.2725}$ $r^2 = 0.9938$		

Data are expressed as the median and interquartile range (Q1–Q3). Different letters in the columns indicate significant difference between concentrations based on Kruskal–Wallis and Mann–Whitney tests ($P<0.05$).

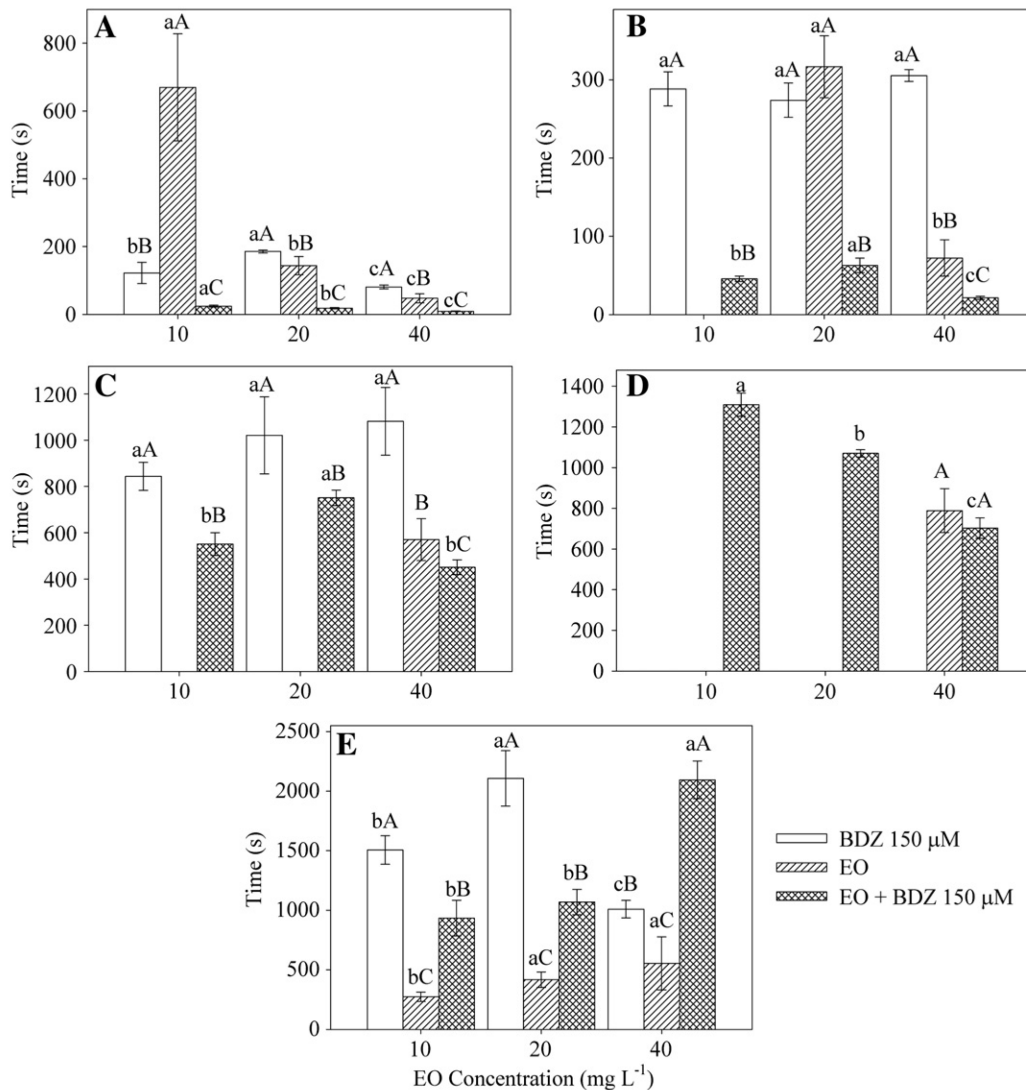

Fig. 1. Anesthetic effect of the essential oil of *Ocimum gratissimum* L. (EO) in association with benzodiazepine (BDZ) in juvenile silver catfish: (A) stage 2, (B) stage 3a, (C) stage 3b and (D) stage 4, based on the definitions of Schoettger and Julin (1967). (E) Recovery time. Maximum observation time for induction was 30 min. Time to reach each stage is given in seconds (s). n = 10 for EO and n = 6 for BDZ and the combination (EO + BDZ). The control group of vehicle treatment is omitted because it did not produce any stage of anesthesia. Data are expressed as the median and interquartile range (Q1–Q3). Capital letters indicate significant differences between groups (EO; BDZ; EO + BDZ) at the same concentration (Kruskal–Wallis and Mann–Whitney tests; $P<0.05$). Lowercase letters indicate significant differences between concentrations of the same group (Kruskal–Wallis and Mann–Whitney tests for stages of EO, stage 2 of BDZ and stage 3a of EO + BDZ; one-way ANOVA and Tukey test for stages 3a, 3b and recovery of BDZ and stages 2, 3b, 4 and recovery of EO + BDZ; $P<0.05$).

Table 3
Sum of recovery scores for silver catfish exposed to the essential oil of *Ocimum gratissimum* (EO), benzodiazepine (BDZ) and the combination (EO + BDZ).

Group	Recovery treatment	
	Water	Flumazenil
BDZ (n = 8)	0.64 ± 0.32^{bB}	2.43 ± 0.35^{aB}
EO (n = 8)	5.25 ± 0.86^{bA}	7.87 ± 0.58^{aA}
EO + BDZ (n = 7)	4.50 ± 0.65^{bA}	6.25 ± 0.44^{aA}

Data are presented as the mean ± SEM. Capital letters in the columns indicate significant differences between groups, and lowercase letters in the rows indicate significant differences between the recovery treatments. Two-way ANOVA and Tukey test were used ($P<0.05$).

of the test used in this work. A similar pattern was verified in silver catfish exposed to EO ($P = 0.002$) and EO + BDZ ($P = 0.036$). Fish exposed to BDZ presented the slowest recovery regardless of whether they recovered in water alone or with flumazenil (Table 3).

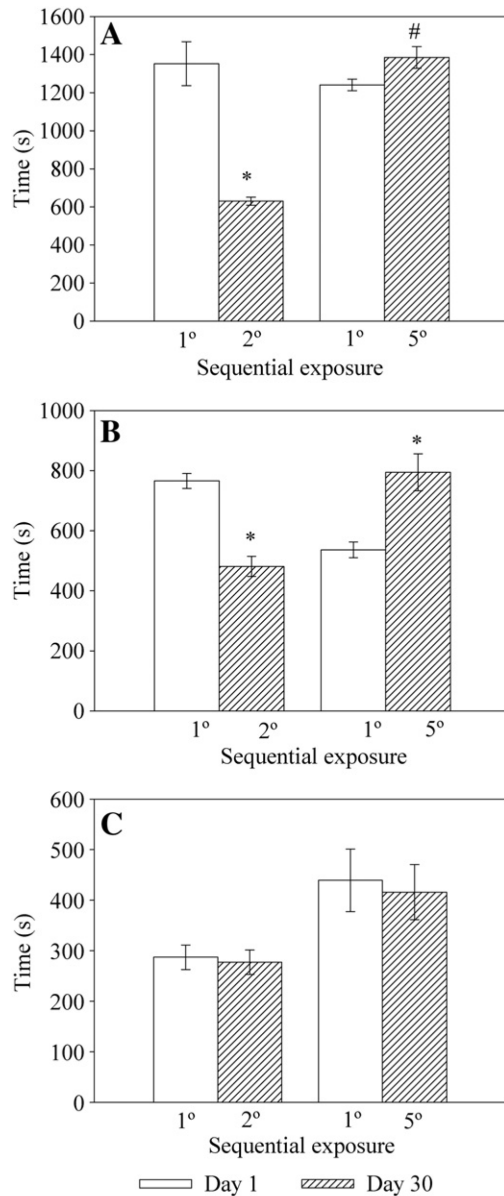

Fig. 2. Induction of tolerance of silver catfish juveniles to (A) 150 μM benzodiazepine (BDZ), (B) 40 mg L^{-1} essential oil of *Ocimum gratissimum* (EO), and (C) combination (EO + BDZ). Data are presented as the mean ± SEM (n = 5–9). * $P<0.05$ compared with first exposure (day 1) using paired *t*-test. # $P<0.05$ compared with first exposure (day 1) using Wilcoxon signed rank test.

Anesthesia at stage 3b or total loss of equilibrium was chosen to represent tolerance when performing the statistical analysis of the tolerance of juvenile silver catfish after successive exposures to anesthesia with BDZ, EO or EO + BDZ, because the standard BDZ treatment leads to this induction stage. BDZ and EO need less time to reach stage 3b in the second exposure after 30 days when compared to the first exposure. An opposite pattern was observed in fish exposed to the same drugs five times in a 30-day period (Fig. 2A and B). At the fifth exposure, exposure to BDZ and EO took longer to cause stage 3b response than in the first exposure. However, EO + BDZ did not demonstrate significant differences between successive exposures ($P > 0.05$) (Fig. 2C).

Mortality was evaluated in silver catfish exposed to BDZ (20% juveniles) and EO + BDZ (10% juveniles) one day after the anesthetic procedure during the sequential two-exposure protocol. Infectious diseases occurred in 20% of the fish in the BDZ group and 10% of the fish in the EO + BDZ group of this experiment and also in 16.7% of the juveniles of the EO + BDZ group in the sequential five-exposure protocol. These animals were excluded from the study. The water parameters during the exposure protocols did not differ statistically from the initial values (data not shown).

4. Discussion

The chemical composition and yield obtained for the EO were similar to those of the eugenol chemotype of the same plant species previously described (Freire et al., 2006; Rabelo et al., 2003; Vieira et al., 2001). However, the eugenol and β-bisabolene contents were higher in the EO than previously described (Vieira et al., 2001) and suggest a new chemical profile for this plant. These changes in chemical composition of EO can be due to geographic, physiological and genetic variations, as well as environmental conditions (Figueiredo et al., 2008).

Previously, reports on *O. gratissimum* described its sedative properties in both folk medicine treatments and animal models using rodents (Albuquerque et al., 2007; Di Stasi et al., 2002; Freire et al., 2006; Orafidiya et al., 2004). Thus, this plant was expected to have a sedative and anesthetic effect in silver catfish. The EO at 30 mg L^{-1} or higher concentrations caused deep anesthesia in silver catfish without side effects and mortality even at 300 mg L^{-1}. In the same species, eugenol, the major compound of EO, leads to stage 4 anesthesia at a concentration range of 20–50 mg L^{-1}, and higher concentrations induced mortality (Cunha et al., 2010a). The essential oil of *L. alba* at 300–500 mg L^{-1} induced rapid deep anesthesia (<4 min) in silver catfish (Cunha et al., 2010b), while EO leads quickly to the same stage at 150–300 mg L^{-1}. These results show that the EO is an effective alternative to currently used anesthesia for this species and is safer than eugenol because the effective EO concentration range that does not cause mortality is wider than that of eugenol. According to Freire et al. (2006), the sedative activity of the EO in rodents is not accompanied by effects upon the exploratory behavior or the motor system. The same authors did not find a relationship between the amount of eugenol and the sedative activity of the EO. These reports, when analyzed together with our results and a previous study of the anesthetic activity of eugenol (Cunha et al., 2010a), indicate a possible synergic effect of the EO constituents that promotes sedation and anesthesia in silver catfish.

Experiments using synergism may be a basis for investigating the mechanism of action (Tallarida, 2001). In the present study, clear synergic behavior was verified through treatment with the combination of BDZ and EO at the lower concentrations tested, given that both drugs alone did not induce deep anesthesia in silver catfish. Although studies attempting to determine the type of synergic interaction were not performed here, an additive effect between the compounds could have occurred. Additivity means that the addition of a more potent drug results in a treatment that acts like a more concentrated form

of the less potent drug (Tallarida, 2001). An example of this behavior may be observed in the time required to reach stage 4, where the EO and the combination were not significantly different at 40 mg L^{-1}.

Due to its relatively short half-life, flumazenil, a benzodiazepine antagonist, is therapeutically used to speed recovery from the effect of longer half-life benzodiazepines, such as diazepam (Bateson, 2002; Betti et al., 2001; Johnston et al., 2006). In this study, the possible involvement of $GABA_A$ receptors in the EO mechanism of action was investigated through recovery in a bath containing flumazenil. Similar to what was observed in fish treated with BDZ alone, fish exposed to the EO or the combination of EO + BDZ recovered from anesthesia faster in the presence of flumazenil. These results suggest a benzodiazepine-like action of the EO.

In regard to the central depressant effects of BDZ, a longer induction time until stage 3a and higher depression level (fish reached stage 3b) was verified in this study when compared to the previous effects described in silver catfish (Heldwein et al., in press). One possible reason for this discrepancy could be the higher water temperature used in the present study. Water temperature changes the eugenol induction time in silver catfish, and this effect is concentration dependent (Gomes et al., 2011). In addition, a temperature increase decreased the induction and recovery time in other fish species exposed to different anesthetic agents, such as clove oil, 2-phenoxyethanol, benzocaine, MS-222 and metomidate (Imanpoor et al., 2010; Mylonas et al., 2005; Zahl et al., 2009). This phenomenon is probably related to the acceleration of ventilation rates and gill blood flow due to the increased basal metabolism of fish maintained at higher temperatures (Mylonas et al., 2005; Zahl et al., 2009).

Tolerance is defined as a decrease in the ability of a drug to produce the same degree of pharmacological effect over time (Bateson, 2002). The evaluation of tolerance to the EO and BDZ was determined after a seven-day interval between exposures because silver catfish exposed to BDZ or the combination (EO + BDZ) only returned to normal feeding behavior 6 days after exposure. In the present study, tolerance was observed in fish exposed successively to the EO and BDZ for 30 days at weekly intervals. Two exposures, however, were enough to sensitize the fish to the action of these drugs. In this context, the combination of EO + BDZ has a better pharmacological profile because repeated exposures did not induce tolerance and/or enhance sensitivity. To our knowledge, this is the first time that tolerance to an essential oil and BDZ has been verified in fish after long administration intervals for both central depressor agents. The development of a slight tolerance was described previously for European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) anesthetized with clove oil after three exposures repeated daily (Mylonas et al., 2005).

According to Fernandes et al. (1999), in some animal tests, continuous receptor occupancy in minimal amounts favors the development of tolerance. Kildea et al. (2004) in their studies of the accumulation and clearance of anesthetics from the edible tissue of silver perch (*Bidyanus bidyanus*) concluded that repeated exposure to clove oil may lead to a decreased capacity to completely clear tissues of the residues of this anesthetic in fish. The long half-life of eugenol (12.14 h) in rainbow trout (*Oncorhynchus mykiss*) also suggests that accumulation is a possible consequence of repeated administration (Guénette et al., 2007). Additionally, long recovery times, as observed for all drugs tested, are indicative of slow clearance and elimination (Zahl et al., 2009). Therefore, the observed tolerance to the EO and BDZ could be the result of their pharmacokinetic characteristics. However, more studies must be performed to confirm this hypothesis. Our current knowledge does not allow us to explain why the combination did not cause development of tolerance.

Tolerance, in terms of the sedative effects of classical benzodiazepines, has been widely reported in animals (Bateson, 2002). However, the mechanism of tolerance development is not completely understood. Down-regulation of the number of $GABA_A$ receptors as a consequence of prolonged exposure to benzodiazepine positive modulators is an obvious potential mechanism (Bateson, 2002; van Rijnsoever et al., 2004). There is increasing experimental evidence for a number of molecular processes that include the uncoupling of the allosteric linkage between GABA and benzodiazepine sites as well as changes in receptor subunit turnover and in receptor gene expression (Bateson, 2002; Oggier et al., 2010).

The infections and inhibition of appetite observed in the fish could be the result of concomitant stress from handling or confinement in addition to anesthesia. Fish react to challenges with a series of neuroendocrine adjustments, known as the stress response. This involves the release of hormones through the activation of the hypothalamic–pituitary–interrenal axis, which results in changes in secondary physiological responses, including immunodepression and the inhibition of feed intake (Bressler and Ron, 2004; Hontela, 1998; Kulczykowska and Vázquez, 2010; Schreck, 2010).

Barcellos et al. (2001) described an increase in cortisol and glucose levels for silver catfish after net capture and tank transference. Zahl et al. (2010) observed stress in response to anesthesia with benzocaine, MS-222, metomidate and isoeugenol in Atlantic salmon (*Salmo salar*), Atlantic halibut (*Hippoglossus hippoglossus*), and Atlantic cod (*Gadus morhua*). However, in the present study, only the groups anesthetized with BDZ or the combination (EO + BDZ) showed signs of infection or a reduced feed intake. As all animals were submitted to capture and tank transference, the EO seems to play a role in preventing the development of stress in silver catfish when applied alone. This hypothesis is partially supported by the study carried out with eugenol, the major constituent of EO, which prevented an increase of cortisol in silver catfish due to handling and air exposure (Cunha et al., 2010a). However, further studies are being performed to confirm this property of the EO.

The inhibition of feed intake after exposure to BDZ and EO + BDZ may also be related to the action of diazepam on melatonin secretion. According to Meissl and Yáñez (1996), the addition of diazepam increased melatonin production in the mesopic and partly in the photopic range of illumination of cultured trout pineal organs. In their review on the melatonin system in teleost fish, Falcón et al. (2010) described that pineal (melatonin) control plays a role in the regulation of physiological processes as growth, feed intake and digestion.

5. Conclusion

In conclusion, the EO of *O. gratissimum* is an effective and safe anesthetic for silver catfish. Its mechanism of action seems to be related to the $GABA_A$–benzodiazepine receptor. Similar to BDZ, it is able to establish tolerance after successive exposures even after a long period of time. However, further studies are required to determine whether the pharmacokinetic characteristics of the EO will influence its acceptability to consumers of fish subjected to anesthesia using this essential oil. Regarding human safety concerns, this plant having been widely used as a food flavor and in traditional medicine (Di Stasi et al., 2002) may facilitate its approval as an anesthetic in fish destined for human consumption.

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2.2 Artigo 2

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Sedative and anesthetic activities of the essential oils of *Hyptis mutabilis* (Rich.) Briq. and their isolated components in silver catfish (*Rhamdia quelen*)

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Abstract

This study evaluated the sedative and anesthetic effects of the essential oils (EO) of *Hyptis mutabilis* (Rich.) Briq. and their isolated components on silver catfish (*Rhamdia quelen*). Quantitative chemical differences between the EOs obtained from leaves and inflorescences were verified, and a new chemotype rich in globulol was described. Although there were no significant differences in the time of induction for sedation and anesthesia between the EOs, only the leaf EO at 344 mg/L anesthetized all fish without side effects. Fractionation of the leaf EO was carried out by column chromatography. The isolated compounds [(+)-1-terpinen-4-ol and (-)-globulol] showed different activity from that detected for the leaf EO in proportional concentrations and similar sedation to a eugenol control at 10 mg/L. However, fish exposed to 1-terpinen-4-ol (3 and 10 mg/L) did not remain sedated for 30 min. Anesthesia was obtained with 83-190 mg/L globulol, but animals showed loss of mucus during induction and mortality at these concentrations. Synergism of the depressor effects was detected with the association of globulol and benzodiazepine (BDZ), compared with either drug alone. Fish exposed to BDZ or globulol+BDZ association showed faster recovery from anesthesia in water containing flumazenil, but the same did not occur with globulol. In conclusion, the use of globulol in aquaculture procedures should be considered only at sedative concentrations of 10 and 20 mg/L, and its mechanism of action seems not to involve the $GABA_A$-BDZ system.

Key words: Silver catfish; (+)-1-Terpinen-4-ol; (-)-Globulol; Sedation; GABA

Introduction

Anesthetics are used in aquaculture procedures to immobilize the animals and to prevent stress and pain (1,2). However, some commonly used synthetic compounds induce significant side effects in fish, such as depression of cardiovascular and respiratory function and immunosuppressive effects (3). In this context, essential oils (EOs) and their constituents offer an alternative as a sedative and anesthetic for aquatic animals, and their use has been spreading in recent years (4-8).

The most frequent representative of the genus *Hyptis* (Lamiaceae) in Southern Brazil is *Hyptis mutabilis* (Rich.) Briq., known as alfavacão and basil (9,10). In folk medicine, it is used for the treatment of gastritis, headache, and as a healing product, expectorant, and sedative (11,12). Until now, only the antiulcerogenic (13) and antimicrobial (14) activities have been reported for its EO. Distinct major chemical constituents that have been described for the EO of this plant include α-phellandrene, *p*-cymene, *E*-caryophyllene, *E*-dihydrocarvone, thymol, δ-3-carene, and *E*-methyl cinnamate (9,13,15,16).

In the present study, we evaluated the sedative and anesthetic properties of *H. mutabilis* EOs and their isolated compounds in silver catfish (*Rhamdia quelen*) in order to identify alternative products for use in aquaculture. In addition, involvement of the GABAergic system in depressor activities of the major isolated compound was evaluated.

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Material and Methods

Plant material

Aerial parts of *H. mutabilis* were collected in March 2010, January 2011, and March 2012 in Santa Maria (RS, Brazil). A voucher specimen (No. SMDB 13076) identified by Dr. Solon Jonas Longhi was deposited in the Herbarium of the Departamento de Biologia, Universidade Federal de Santa Maria.

Animals

Silver catfish (*R. quelen*) juveniles were purchased from a local fish farm and transported to the laboratory, where they were maintained in continuously aerated 250-L tanks with controlled water parameters. The dissolved oxygen levels (experiment 1: 8.65 ± 0.07 mg/L; experiments 2 and 3: 7.18 ± 0.12 mg/L; experiments 4 and 5: 7.10 ± 0.17 mg/L) and temperature (experiment 1: 15.90 ± 0.14°C; experiments 2 and 3: 19.91 ± 0.13°C; experiments 4 and 5: 21.02 ± 0.11°C) were measured with a YSI-dissolved oxygen meter (YSI Inc., USA). The pH (experiment 1: 7.37 ± 0.11; experiments 2 and 3: 6.70 ± 0.12; experiments 4 and 5: 7.11 ± 0.06) was measured with a DMPH-2 pH meter (Digimed, Brazil). Total ammonia levels (experiment 1: 2.43 ± 0.81 mg/L; experiments 2 and 3: 0.51 ± 0.17 mg/L; experiments 4 and 5: 1.08 ± 0.12 mg/L) were measured by the salicylate method (17). A semi-static system was used, where 50% of the water volume was changed daily. Fish were fed once a day with commercial feed (28.0% crude protein). Juveniles were fasted for a period of 24 h prior to the experiments. The methodologies were approved by the Ethics and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process No. 46/2010). The number of fish used in each experiment was the lowest possible in order to satisfy the policy of reduction of experimental animals of the institution.

Phytochemical analysis

Essential oil extraction and analysis. Inflorescence and leaves were submitted separately to a hydrodistillation procedure for 3 h in a Clevenger-type apparatus (18) and percentage extractive yield was determined (w/w). Essential oils were stored at -4°C in amber glass bottles until gas chromatography and mass spectrometry (GC-MS) analysis, fractionation, and biological testing.

GC-MS was performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector using an HP5-MS column (5% phenyl, 95% methylsiloxane, $30\ m \times 0.25\ mm\ ID \times 0.25\ \mu m$) as described by Silva et al. (8). The EO constituents were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library (19), and published data (20).

Isolation and identification. Leaf essential oil obtained in January 2011 was fractionated by column chromatography (CC) in three repetitions. Each repetition was performed with 2 g EO added to a column (3×27 cm) containing 100 g silica gel 60 (70-230 mesh, Macherey-Nagel, Germany) and eluted with 98:2 (v/v) hexane-acetone at 2 mL/min. The 20-mL fractions obtained were collected in 15 main fractions (fractions A-O) based on thin-layer chromatography (TLC) profile (silica gel F254; detection: anisaldehyde-H_2SO_4) and concentrated under reduced pressure at 40°C. The compound (+)-1-terpinen-4-ol [32.9 mg, yellow oil, EI-MS m/z (%): 154 (M^+); $(\alpha)_D^{20} = +8.7°$ (*c* 0.023, $CHCl_3$)] was obtained from fraction G of the first repetition.

Fraction M (405 mg) was applied to a 2×48 cm column containing 46 g silica gel 60 impregnated with 10% silver nitrate (21) and eluted with 95:5 (v/v) hexane-acetone at 1 mL/min. This CC gives 12 fractions of 20 mL (M1-12), where M4 corresponded to (-)-globulol [252 mg; white crystalline solid; m.p. 86.9-87°C; EI-MS m/z (%): 222 (M^+); $(\alpha)_D^{20} = -41.8°$ (*c* 0.11, $CHCl_3$)]. A larger quantity of this compound (634.8 mg) was purified by CC (1.9×61.5 cm) under the same conditions described above using the remaining part of fractions M (755.9 mg), M3, and M5 (67.3 mg).

Identification of the isolated compounds was confirmed by GC-MS, ^1H and ^{13}C nuclear magnetic resonance (NMR). NMR spectra were recorded in $CDCl_3$ on a Bruker HPX 400 FT-NMR at 400 MHz for ^1H and 100 MHz for ^{13}C with tetramethylsilane (TMS) as an internal standard.

Evaluation of the depressor properties

Two experimental sets were performed to evaluate the sedative and anesthetic potential of the EOs of *H. mutabilis* and their isolated compounds in juvenile silver catfish. In experiment 1, the anesthetic activity of the leaf and inflorescence EOs extracted in March 2010 were evaluated. Six juveniles (4.96 ± 0.56 g; 7.95 ± 0.25 cm) were used for each concentration tested (172 and 344 mg/L).

The second experiment measured the activity of the isolated compounds in concentrations proportional to those detected in 344 mg/L leaf EO obtained in March 2012 (3 mg/L 1-terpinen-4-ol and 83 mg/L globulol) in comparison to the same EO. Next, the same substances were evaluated at other concentrations (10 mg/L 1-terpinen-4-ol; 10, 20, 50, and 190 mg/L globulol) to determine the effective concentrations as a sedative and an anesthetic. These concentrations were chosen to allow comparison of the results with other, previously tested, fish anesthetics (5,6). Each concentration was tested in 7 fish (8.58 ± 0.21 g; 10.20 ± 0.13 cm). The EO concentrations were corrected from the density of inflorescence and leaf EOs (March 2010), 0.86 g/mL, and leaf EO (March 2012), 0.90 g/mL, before adding to the aquarium water.

Fish were transferred to aquaria containing 1 L water and the sample to be tested, previously diluted in 95%

ethanol (1:10 for EO and 1-terpinen-4-ol, 1:5 for globulol), to evaluate the time required to induce anesthesia (22). Each juvenile was used only once for observation of deep sedation (S2), partial and total loss of equilibrium (S3a and S3b), anesthesia (S4), and/or medullary collapse (S5) (22). The animals remained in the anesthetic bath for 30 min or until reaching S4. Anesthesia was determined by loss of reflex activity and lack of reaction to strong external stimuli. After induction of anesthesia, each fish was measured, weighed, and transferred to an anesthetic-free aquarium to recover. The fish were considered to have recovered if their normal posture and behavior were restored by 30 min. Subsequently, the animals were transferred to 30-L tanks to evaluate possible side effects or mortality until 48 h after exposure. Control experiments were performed using aquaria containing ethanol at the higher concentration used to dilute the EO and eugenol (Fluka, Switzerland) at 10 and 50 mg/L. Eugenol was used in this study as positive control because it corresponds to the only compound isolated of natural source with anesthetic activity in silver catfish at low concentrations (5).

Involvement of the GABAergic system

Association with benzodiazepine (BDZ). Sedative concentrations (10 and 20 mg/L) of globulol (diluted in 1 mL 95% ethanol) were tested along with BDZ (150 μM diazepam obtained from DEG, in Tween 80 at 0.033%) in aquaria containing 1 L water in this fourth experiment. Controls of BDZ and globulol at the same concentrations were also performed. Eight fish (32.93 ± 0.95 g; 15.23 ± 0.21 cm) were tested individually at each concentration. The stages of the induction of anesthesia and recovery time were evaluated as described for the evaluation of the depressor properties. The maximum observation times were 30 min for induction and 60 min for recovery.

Reversion of the depressor effects. Fish (28.34 ± 1.19 g; 14.60 ± 0.21 cm) were placed in water containing BDZ (150 μM diazepam), 20 mg/L globulol, or globulol plus BDZ, at the same concentration as described in Evaluation of the depressor properties and Association with benzodiazepine sections. After induction, animals were transferred to an anesthetic-free aquarium containing either the classic BDZ antagonist flumazenil (5 μM, Flumazil®, Cristália, Brazil) or water. Fish behavior was scored 1, 5, 10, 15, and 20 min after the transfer according to the protocol described by Heldwein et al. (23). After 20 min, scores for each fish (n = 5 for each recovery treatment) were summed.

Statistical analysis

Data are reported as means ± SE. The homogeneity of variances and normality of data were verified with Levene and Kolmogorov-Smirnov tests, respectively. Log transformations were performed before statistical analysis when appropriate. Comparisons of activity of the leaf and inflorescence EOs at the same concentration, between concentrations of the isolated compound and between recovery treatments in the reversion experiment were performed with the *t*-test or the Mann-Whitney test. Anesthetic effects of EO or eugenol compared with the isolated compounds were analyzed by one-way ANOVA and Tukey or Kruskall-Wallis and Dunn tests. Two-way ANOVA and the Tukey test or Scheirer-Ray-Hare extension of the Kruskal-Wallis test and the Dunn test were used to evaluate the association in comparison to globulol and BDZ alone. Analysis was performed using the SigmaPlot version 11.0 software, and the minimum significance level was set at $P<0.05$.

Results

Phytochemical analysis

In the extractive yield of *H. mutabilis*, the EO was higher from inflorescences (0.28%) than from leaves (0.25%). Quantitative differences in composition were detected between the EOs of the different parts of the plant. Globulol (26.61%) was the major compound of leaf EO in the two collection periods evaluated, while inflorescence EO was rich in germacrene D (14.97%; Table 1). The fractionation of leaf EO resulted in isolation of two compounds that were identified as 1-terpinen-4-ol and globulol according to the literature (19,20,24-26).

Evaluation of depressor properties

Silver catfish exposed to leaf and inflorescence EOs did not show significant differences in the induction time for deep sedation and anesthesia (Figure 1). However, only 33% of fish were anesthetized with 344 mg/L inflorescence EO, whereas all animals reached this stage with leaf EO. At 172 mg/L, leaf EO promoted S3b anesthesia in all animals exposed, but only 1 fish (17%) reached this stage with the inflorescence EO (1578 s). Ethanol alone did not produce any anesthetic or sedative effect.

Total recovery was observed in all animals within 2 h. During the 30 min of observation, about half the fish exposed to leaf EO and two animals (33%) exposed to 344 mg/L inflorescence EO returned to normal behavior. Side effects were observed in 30% of the fish exposed to inflorescence EO during recovery time, and they were independent of the concentration tested. There were hyperactivity episodes, where spasms and/or ‘‘corkscrew-like’’ circling swimming behavior could be observed, followed by an immobile period and a return to swimming, with partial loss of equilibrium. Mortality or other side effects were not observed in either sample 48 h after exposure.

The induction time to stages of anesthesia was shorter for fish exposed to 344 mg/L EO than to the isolated compounds in proportional concentrations. The only

Table 1. Chemical composition of essential oils (EO) of *Hyptis mutabilis*.

Components	Relative percentage			RI cal	RI ref
	IEO	LEO 1	LEO 2		
α-thujene	4.258	1.649	6.001	925	925[a]
α-pinene	7.27	1.096	3.713	930	933[a]
4(10)-thujene			2.027	970	968[a]
β-pinene	7.906	2.897	1.915	972	970[a,b]
1-octen-3-ol	0.774	1.233	1.27	980	979[a,b]
Limonene	1.34	1.731	1.636	1025	1029[a]
τ-terpinene	0.385	0.199	0.715	1056	1056[a]
(+)-1-terpinen-4-ol	**0.712**	**0.395**	**0.886**	**1175**	**1174[a]**
α-copaene	2.364	2.647	1.694	1377	1377[a,b]
β-bourbonene	0.787	1.492	1.532	1386	1384[a]
β-cubebene	0.875	1.172	0.693	1391	1389[a]
(-)-β-elemene	0.789	0.552	0.554	1393	1393[a]
E-caryophyllene	12.424	10.839	13.948	1423	1419[a,b]
α-caryophyllene	2.434	3.95	2.54	1455	1455[a,b]
Germacrene D	14.968	6.936	8.697	1484	1485[a]
Bicyclogermacrene	10.895	7.845	9.048	1499	1500[b]
Germacrene A	0.906	0.874	0.591	1506	1509[b]
γ-cadinene			0.994	1513	1514[a]
Cubebol	1.085	2.06		1517	1515[b]
(+)-δ-cadinene	2.166	1.966	1.188	1525	1523[a]
Spathulenol			1.401	1577	1578[a,b]
Germacrene D-4-ol	0.875			1580	1576[a,b]
(-)-globulol	**11.604**	**26.61**	**24.232**	**1587**	**1585**
				1593	**1592[a,b]**
Viridiflorol	0.664	1.14	0.835	1592	1593
				1597	1595[a,b]
Humulene epoxide II	0.426	1.336		1613	1608[b]
1-epi-cubenol	0.419	0.837	0.388	1632	1629[b]
Caryophylla-4(14),8(15)-dien-5α-ol		0.797	0.37	1636	164[b]
Caryophylla-4(14),8(15)-dien-5β-ol		1.127	0.811	1640	1641[b]
τ-muurolol	1.109	1.434	0.597	1645	1645[a]
(-)-δ-cadinol	0.384	0.589	0.396	1649	1648[a]
α-cadinol	1.359	1.759	0.82	1658	1654[a]
Total identified (%)	95.679	95.882	91.126		

LEO: leaf EO collected in March 2010 (1) and March 2012 (2); IEO: inflorescence EO; RI cal: calculated Kovats retention index; RI ref: reference Kovats retention index. [a]NIST (19); [b]Adams (20). Bold type indicates isolated constituents. Components in amount below 0.5% have been omitted.

exception to this pattern occurred with globulol in stage 3a (Table 2). The exposure to 1-terpinen-4-ol (3 and 10 mg/L) was able to promote only deep sedation in the juveniles, with significant differences between concentrations (Table 2). However, the fish showed signs of recovery by 30 min of exposure to both concentrations of 1-terpinen-4-ol. Higher concentrations of 1-terpinen-4-ol were not evaluated in this study due to the small quantity obtained from the isolation process and its high volatility from the anesthetic bath.

Concentrations of 10 and 20 mg/L globulol induced to stages 2 and 3a, but 190 mg/L anesthetized (stage 4) all exposed fish in about 13 min. Intermediate concentrations (50 and 83 mg/L) promoted stages 3b and 4 in 14-71% of the fish tested. A positive relationship between an increase of drug concentration and a decrease in the time required for anesthesia induction was observed for all stages (Table 2).

Only fish exposed to 10 mg/L globulol recovered during the observation period (195.4 ± 25.6 s). For the other concentrations, the time of recovery exceeded 30 min (data not shown). ''C-shaped'' contractions and/or circular swimming followed by a motionless period were observed in the recovery period after exposure to 20 and

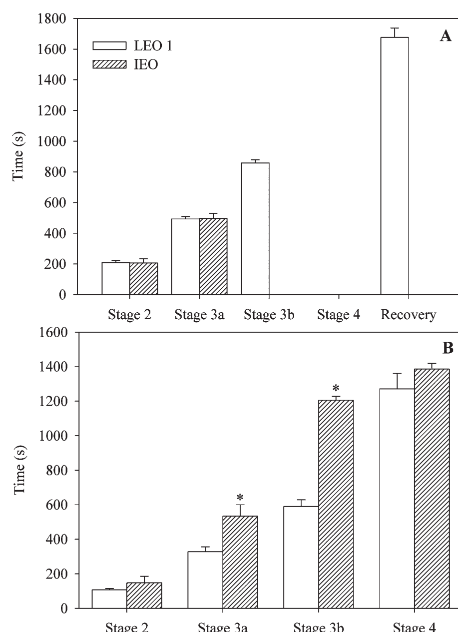

Figure 1. Anesthetic activity of essential oils (EO) of *Hyptis mutabilis* obtained from leaf (LEO 1) and inflorescence (IEO) at concentrations of 172 mg/L (*A*) and 344 mg/L (*B*) in silver catfish juveniles. Stages of anesthesia: deep sedation (stage 2), partial loss of equilibrium (stage 3a), total loss of equilibrium (stage 3b), and anesthesia (stage 4) (22). Maximum observation time was 30 min. Time to reach each stage is given in seconds (s). Data are reported as means ± SE (n = 6). *P<0.05, compared to LEO 1 (*t*-test or Mann-Whitney U-test).

50 mg/L globulol. Mortality within 48 h after testing occurred in animals exposed to 83 and 190 mg/L globulol (29 and 57%, respectively), as well as loss of mucus during induction.

Statistically significant differences between 1-terpinen-4-ol and eugenol controls in induction time were not detected until stage 2 at 10 mg/L. Fish exposed to globulol (10 and 50 mg/L) needed more time to reach this stage than those exposed to eugenol or 1-terpinen-4-ol at equal concentrations (Table 2). Recovery occurred faster with juveniles exposed to 10 mg/L globulol than those sedated with eugenol at the same concentration (411.4 ± 73.0 s). At 50 mg/L, only animals exposed to eugenol recovered during the observation time (508.4 ± 11.4 s).

Involvement of the GABAergic system

A reduction in the time required to reach most of the stages of anesthesia and a higher depression level were observed with the association of BDZ and globulol (Figure 2). Among the fish exposed to 150 μM BDZ and to 10 mg/L globulol, 75 and 25%, respectively, reached stage 3a during the observation time. However, some fish exposed to the association reached stage 3b (37.5% with 10 mg/L globulol + BDZ, and 75% with 20 mg/L globulol + BDZ) and stage 4 (50% with 20 mg/L globulol + BDZ, in 1617.5 ± 94.0 s). An irregular recovery pattern was also observed in this experiment. Only fish exposed to BDZ presented 100% recovery within 60 min, whereas return to normal behavior occurred in 43.7-75% of the animals exposed to globulol or its association with BDZ.

During the reversal of depressive effect, a higher total score corresponds to faster recovery. Fish exposed to BDZ had a higher total score when 5 μM flumazenil was added to the water compared with those fish that recovered in water alone (Figure 3). The same pattern occurred when the two agents were combined, but not with globulol alone.

Discussion

Phytochemical analysis

The EO content extracted from both parts of the plant was within the range described for EOs obtained from aerial parts of *H. mutabilis* collected in different localities (0.1-2.1%) (9). The same pattern was not verified in relation to the chemical composition of the EOs from the leaves and inflorescences (9,13-16,27). Globulol was one of the major compounds in leaf and inflorescence EOs. It was also detected in high amounts (11.9%) in the EO obtained from aerial parts of this species collected in Retiro das Pedras (State of Amapá, North of Brazil). However, those samples contained δ-3-carene (25.5%) and terpinolene (24.7%) as additional major components (9), which characterize them as a chemotype distinct from those detected in this study.

Evaluation of the depressor properties

The anesthetic activity detected for the EO was not surprising, since *H. mutabilis* is used as a sedative in folk medicine (12). Silver catfish reached the anesthesia stage around 21-23 min with 344 mg/L EO obtained from leaves and inflorescences. Other fish anesthetics in similar concentrations are able to induce anesthesia in less time. For example, the EO of *Lippia alba* (300-500 mg/L) anesthetized silver catfish in 1-4 min (4).

There are only a few reports on the anesthetic and sedative properties of isolated plant compounds in aquatic animals, and those only evaluated the effects of eugenol and menthol (5,6,28,29). In this study, the depressor effects of 1-terpinen-4-ol and globulol obtained from EO of *H. mutabilis* were characterized in silver catfish. These effects did not correspond to the leaf EOs from which they were isolated, which suggests a synergic action between the EO constituents.

The sedative effect of 1-terpinen-4-ol is reported to be higher or similar to that of eugenol at the same concentrations in silver catfish (28). However, the use of 1-terpinen-4-ol in place of eugenol is not advisable, because it was not able to maintain this stage for

Table 2. Time required for induction of anesthesia in silver catfish juveniles with globulol and 1-terpinen-4-ol, in comparison with EO of *Hyptis mutabilis* (LEO 2) and eugenol (positive control).

Compound (mg/L)	Induction time (s)			
	Stage 2	Stage 3a	Stage 3b	Stage 4
Globulol				
10	1331.4 ± 124.8*			
20	325.3 ± 28.0	717.9 ± 70.6		
50	107.3 ± 1.6*	139.3 ± 9.1*	1041.5 ± 25.5*	#
83	44.7 ± 5.3^{B}	116.6 ± 19.0^{A}	1056.2 ± 63.7^{A}	1247.0 ± 95.7^{Aa}
190	14.1 ± 2.5	148.0 ± 10.6	502.0 ± 7.9	779.1 ± 55.0^{b}
Equation	$y = 115.27 + 6732.71 \exp^{-0.17x} - 0.56x$ ($r^2 = 0.999$)	$y = 89.68 + 4297.40 \exp^{-0.0966x} + 0.31x$ ($r^2 = 1.0$)	$y = 852.49 + 5.79x - 0.04x^2$ ($r^2 = 1.0$)	
1-terpinen-4-ol				
3	946.8 ± 65.0^{Aa}			
10	300.9 ± 42.8^{b}			
LEO 2				
344	25.3 ± 3.7^{C}	93.0 ± 16.6^{A}	566.4 ± 66.6^{B}	796.3 ± 68.2^{B}
Eugenol				
10	165.7 ± 19.7	226.0 ± 19.1		
50	35.7 ± 6.0	60.6 ± 2.9	363.7 ± 18.6	549.7 ± 26.5

Stages are according to Schoettger and Julin (22). Maximum observation time was 30 min. Data are reported as means ± SE (n = 7). #Only 1 fish reached this stage of induction (1767s) until 30 min. Different lowercase letters in the columns indicate significant difference among concentrations of the same compound using the *t*-test ($P < 0.05$). Different uppercase letters in the columns indicate significant differences between LEO 2 and isolated compound at proportional concentrations (83 mg/L of globulol and 3 mg/L 1-terpinen-4-ol) using one-way ANOVA and the Tukey test ($P < 0.05$). * Statistically different from eugenol at the same concentration by Kruskall-Wallis and Dunn tests ($P < 0.05$). In equations, x: concentration of compound (mg/L); y: time to reach the stage of induction or recovery from anesthesia in seconds (s).

30 min. In this context, globulol at 10 mg/L seems to have a better pharmacological profile. Despite its induction time being about 10-fold higher than eugenol, its low concentration effectiveness and fast recovery after exposure are positive points for its use as a sedative. Low concentrations of anesthetics are used for long exposures during fish transport, aiming to reduce stress and injury (28,30).

Anesthetic activity of globulol was observed at concentrations from 83 to 190 mg/L with an induction time of 13-21 min. A similar concentration range of menthol (100-200 mg/L) induces anesthesia within 10 min in tambaqui (*Colossoma macropomum*) and pacu

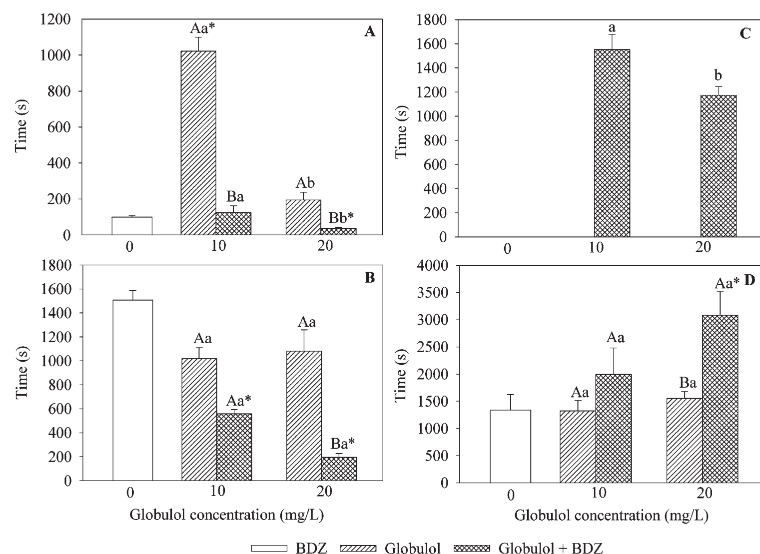

Figure 2. Time required for induction of anesthesia in silver catfish with globulol in association with benzodiazepine (BDZ): stage 2 (*A*), stage 3a (*B*) and stage 3b (*C*) according to Schoettger and Julin (22), and recovery time (*D*). Maximum observation time was 30 min to induction and 60 min to recovery. Time to reach each stage is given in seconds (s). Data are reported as means ± SE (n = 8). *Significantly different compared to BDZ; different uppercase letters indicate significant differences between globulol and association (globulol + BDZ) at the same concentration; different lowercase letters indicate significant differences between concentrations of the same group. Two-way ANOVA and the Tukey test or Scheirer-Ray-Hare extension of the Kruskal-Wallis test and the Dunn test were used ($P < 0.05$). The control group of vehicle treatment is omitted because it did not produce any stage of anesthesia.

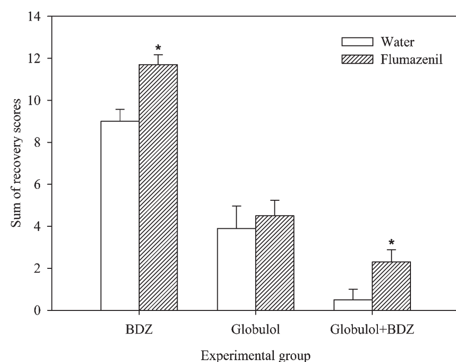

Figure 3. Sum of recovery scores for silver catfish exposed to 20 mg/L globulol, 150 μM benzodiazepine (BDZ), and the association (globulol + BDZ) at the same concentrations. Data are reported as means ± SE (n = 5). *$P < 0.05$, compared to treatment with water (*t*-test).

(*Piaractus mesopotamicus*) (6,29). Eugenol requires a lower concentration and less time to induce this depression level in silver catfish (20-50 mg/L; 1.6-15 min) and pacu (25-100 mg/L; 0.8-3.5 min) (5,6).

Loss of mucus during induction and mortality after exposure were observed at the highest globulol concentrations, but these effects are also commonly observed with some synthetic anesthetics used in aquaculture, such as benzocaine (1,31-33).

The irregular pattern of anesthetic induction observed with some anesthetic concentrations or samples can result from individual differences in fish responsiveness. The presence of high and low responders within the same fish family was previously described in relation to cortisol release (34). However, other studies should be performed to confirm this hypothesis.

The long recovery time detected for all the drugs tested could be due to their hydrophobic characteristics. Kiessling et al. (2) confirmed that isoeugenol, a lipophilic compound, exhibits slower clearance than the hydrophilic drug MS-222 in Atlantic salmon (*Salmo salar*). Slow clearance may be associated with drug accumulation in adipose tissue, which in turn would increase the recovery time after long exposure (2,3).

Involvement of the GABAergic system

The evaluation of a drug's mechanism of action may include experiments using synergism and/or reversal of effect by a pharmacological antagonist (35,36). In this study, these two strategies were used to assess the involvement of the GABAergic system in globulol's mechanism. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the vertebrate brain, and its ionotropic receptors are widely recognized as targets of action of sedative and anesthetic compounds. Benzodiazepine drugs, such as diazepam, are positive allosteric modulators of the $GABA_A$ receptor, and they increase the opening frequency of the GABA-gated chloride channel (37).

Clear synergic behavior was detected when globulol and diazepam were associated at sedative concentrations. Similar results were observed for sedation induced by the EOs of *L. alba* and *O. gratissimum* using the same protocol (8,23). However, as opposed to those results, globulol alone seems to act independently of flumazenil-sensitive benzodiazepine sites on the GABA receptor. Thus, the sedative properties of globulol could be the result of action at another site on the GABA receptor or at a different receptor.

On the other hand, the abnormal behavior observed in fish exposed to globulol at 20-50 mg/L may be indicative of anti-glutamatergic activity. Riehl et al. (38) reported circular swimming behavior without spiraling rotations in zebrafish (*Danio rerio*) exposed to sub-anesthetic doses of ketamine, and related these effects to antagonist action on N-methyl-D-aspartate glutamate receptors. As globulol behavior seems to be distinct from that found for inflorescence EO, additional studies should be performed to explain these effects and to study globulol action on glutamate receptors.

In conclusion, sedative and anesthetic properties of EOs of *H. mutabilis* and their isolated compounds (1-terpinen-4-ol and globulol) were verified in silver catfish. The biological action and chemical composition of the EOs were influenced by the part of the plant from which they were obtained. Of the samples tested, globulol was the only one with potential application as a sedative for aquatic animals. Its effect seemed not to involve the $GABA_A$-benzodiazepine receptor.

Acknowledgments

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4 MANUSCRITOS

3.1 Manuscrito 1

SILVA, L. L.; GARLET, Q. I.; KOAKOSKI, G.; ABREU, M. S.; MALLMANN, C. A.; BALDISSEROTTO, B.; BARCELLOS, L. J. G.; HEINZMANN, B. M. Anesthetic activity of essential oil of *Ocimum americanum* in silver catfish (*Rhamdia quelen*) and its effects on stress parameters. A ser submetido ao periódico “Aquaculture”.

Anesthetic activity of essential oil of *Ocimum americanum* in silver catfish (*Rhamdia quelen*) and its effects on stress parameters

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Abstract

The aim of this study was to evaluate the anesthetic activity of the essential oil (EO) of *Ocimum americanum* L. in silver catfish (*Rhamdia quelen*). In the first experiment, the depressor effects and chemical composition of the leaf EO (LEO) and inflorescence EO (IEO) were compared. The EO were obtained separately from the distinct vegetal organs by hydrodistillation and analyzed by gas chromatograph coupled to mass spectrometer (GC-MS). Juveniles (n = 10 per concentration) were placed in aquaria containing different concentrations of the EO (25, 50, 100, 200, 300 and 500 mg L^{-1}) to determine the point at which anesthesia was induced and the length of the recovery period. In the following experiment, the effects of 300 and 500 mg L^{-1} LEO exposure on stress parameters (plasma cortisol, glucose and sodium levels) after air exposure for 1 min were assayed. Fish (n = 10 per sampling time) were sampled immediately or transferred to anesthetic-free aquaria until sampling (15, 30, 60 or 240 min). LEO was composed mainly by β-linalool and 1,8-cineole in similar proportions, whereas IEO showed β-linalool as major compound. Anesthesia was obtained in silver catfish with 200-500 mg L^{-1} between 4-8 min for LEO and 6-16 min for IEO. Lower EO concentrations did not reach anesthetic stage up to 30 min. The LEO use as anesthetic prevented the cortisol increase and sodium loss induced by aerial exposure. Glucose levels were raised in catfish exposed to LEO compared to basal group (not air exposed) in almost all observation times. EO of *O. americanum* obtained from leaves was considered suitable to anesthetic procedures due to its fast induction and handling-induced stress prevention.

Keywords: anesthesia, cortisol, glucose, sodium, basil.

1. Introduction

Anesthetic and sedative drugs are used in aquaculture to improve fish welfare, minimize movement, handling trauma and pain, and to attenuate the physiological response to stress (Neiffer and Stamper, 2009; Zahl et al., 2012). Some essential oils (EO) and their constituents have shown promising results in this context due their effectiveness, low incidence of side effects and ability to mitigate stress effects (Benovit et al., 2012; Cunha et al., 2010a, b; Gressler et al., 2012a; Heldwein et al., in press; Silva et al., 2012, 2013a; Wagner et al., 2003). Stress response in fish develops initially from activation of neuroendocrine pathways, which promote the release of catecholamines and corticosteroids (cortisol). These hormones lead to physiological and behavioral changes that allow response/adaptation to the stressor, but leads to adverse whole-animal effects if extreme or sustained (Ellis et al., 2012; Zahl et al., 2012).

The plants of the *Ocimum* genus (Lamiaceae), collectively called basil, are considered good sources of essential oils used to flavor foods, in oral products, fragrances and traditional medicines (Vieira and Simon, 2006). Recently, the anesthetic and sedative effects of the EO of *Ocimum gratissimum* L. in fish and its potential application in aquaculture have been reported (Benovit et al., 2012; Silva et al., 2012). One important species of this genus due to its high EO content is *Ocimum americanum* L. (synonymy *O. canum* Sims), known as hairy basil (Chanwitheesuk et al., 2005; Vasconcelos Silva et al., 2003). Preparations obtained from its aerial parts are widely used in folk medicine in the treatment of insomnia and anxiety (Hassane et al., 2011). However, until this moment, only the antimicrobial and antioxidant activities were confirmed *in vitro* for its EO (Hassane et al., 2011; Nascimento et al., 2011; Selvi et al., 2012).

Silver catfish (*Rhamdia quelen*) is a nocturnal bagrid of the Heptapteridae family commonly found in South American rivers. It has been considered a good alternative for fish production due to its fast growth rate in warmer months (Barcellos et al., 2001, 2012; Gomes et al., 2000). However, this species is susceptible to stress resulting from confinement and handling procedures, such as capture, tank transference, and air exposure (Barcellos et al. 2001, 2006; Cunha et al. 2010a, b), which makes the use of anesthetics and sedatives with attenuation of endocrine secretion advantageous for its production.

Thus, the aim of this study was to evaluate the anesthetic activity of the EO of *Ocimum americanum* L. in silver catfish. In the first moment, the depressor effects and

Abbreviations: EO: essential oil; LEO: leaf EO; IEO: inflorescence EO

35 chemical composition of the leaf EO (LEO) and inflorescence EO (IEO) were
36 compared. Following, the effects of LEO exposure on stress parameters after handling
37 was assayed.
38
39 **2. Material and Methods**
40 *2.1. Plant material*
41 Aerial parts of *O. americanum* were collected in December 2011 in Encantado
42 (RS, Brazil). Voucher specimen (no. SMDB 13163) identified by Dr. Sérgio Augusto de
43 Loreto Bordignon was deposited in the Herbarium of the Department of Biology,
44 UFSM.
45
46 2.2. *Essential oil extraction and analysis*
47 Leaves and inflorescences were separately hydrodistillated for 3 h in a
48 Clevenger-type apparatus (European Pharmacopoeia, 2007). Extractive yield was
49 determined as % (w/w). The EOs were stored at -4ºC in amber glass bottles until
50 analysis by gas chromatograph coupled to mass spectrometer (GC-MS) and biological
51 tests.
52 GC-MS TIC analysis was performed using an Agilent-6890 gas chromatograph
53 coupled with an Agilent 5973 mass selective detector using an HP5-MS column (5%
54 phenyl, 95% methylsiloxane, 30 m x 0.25 mm i. d. x 0.25 μm) and EI-MS of 70 eV
55 according to operational conditions described by Silva et al. (2012). The constituents
56 were identified by comparison of the Kovats retention index and mass spectra with a
57 mass spectral library (NIST, 2005) and with literature data (Adams, 2001).
58
59 *2.3. Animals*
60 Four month-old juvenile silver catfish were maintained in continuously aerated
61 250 L (experiment 1) and 95 L (experiment 2) tanks with controlled water parameters at
62 loading density of 3.5 g L^{-1} and 4.5 g L^{-1}, respectively. The dissolved oxygen levels and
63 temperature were measured with an YSI oxygen meter. The pH was determined with pH
64 meters DMPH-2 and Solar SL110. Total ammonia levels were measured by the
65 salicylate method (Verdouw et al., 1978). A semi-static system was used with
66 independent tanks, where 50% of the water volume was changed daily. Fish were fed
67 once a day with commercial feed (28 % crude protein). Juveniles were fasted for a

68 period of 24 h prior to the experiments. The methodologies of the experiments were
69 approved by the Ethical and Animal Welfare Committee of the Federal University of
70 Santa Maria (Process no. 46/2010).
71
72 *2.4. Experiment 1: Anesthesia induction and recovery*
73 Sedative and anesthetic effects of LEO and IEO were evaluated on silver catfish
74 at concentrations of 25, 50, 100, 200, 300 and 500 mg L^{-1}. These concentrations were
75 corrected by the density of the EO (LEO: 0.9273 g mL^{-1}; IEO: 0.8884 g mL^{-1}) and
76 diluted in 95% ethanol (1:10) before bath administration. Control experiments were
77 performed using aquaria containing ethanol at the highest concentration used to dilute
78 the EO.
79 Juveniles of silver catfish (8.09 ± 0.22 g; 9.89 ± 0.11 cm) were transferred to 1 L
80 aquaria containing the EO concentration to be tested. Ten fish were evaluated in each
81 concentration, and each juvenile was used only once to observation of deep sedation
82 (S2), partial and total loss of equilibrium (S3a and S3b, respectively) and/or anesthesia
83 (S4) (adapted from Schoettger and Julin, 1967). The animals remain in anesthetic bath
84 until reaching S4 or for 30 min. The anesthesia was determined by loss of reflex activity
85 and no reaction to strong external stimuli. After the induction of anesthesia, each fish
86 was measured, weighed and transferred to an anesthetic-free aquarium to recover. The
87 fish were considered to have recovered if their normal posture and behavior were
88 restored. Following, the animals were transferred to 30 L tanks to evaluate possible side
89 effects or mortality after 24 h of exposure.
90
91 *2.5. Experiment 2: Evaluation of stress parameters*
92 Fish (21.80 ± 1.00 g; 13.18 ± 0.19 cm) were subjected to one of the following
93 treatments: water control, 300 or 500 mg L^{-1} of LEO. Ten fish were used by treatment
94 in each collection time (0, 15, 30, 60 or 240 min), and each animal was used only once.
95 To prevent cumulative stress of the repeated captures, the fish used in each collection
96 time were maintained in tanks of 96 L for one week before experiment without
97 additional handling and simultaneously captured.
98 Experimental protocol consisted in fish capture with a hand net and their
99 transference to continuously aerated aquaria containing only 1 L of water (water
100 control) or the samples to be tested. The time between capture and release did not

101 exceed 30 seconds. Juveniles stayed in the aquarium until reached stage 4 of anesthesia
102 induction (Schoettger and Julin, 1967) with LEO, or for 8 minutes in case of the water
103 control. This time was chosen for the controls because it corresponds to the highest
104 induction time observed for the LEO concentrations in the experiment described in
105 section 2.4.

106 After these procedures, the fish were exposed to air for 1 minute. Aerial
107 exposure is a protocol previously described as able to induce stress in silver catfish
108 (Barcellos et al. 2006; Cunha et al. 2010a, b). Basal group was carried out with
109 unmanipulated fish. Animals were sampled immediately or transferred to anesthetic-free
110 aquaria containing 50 L of water until sampling. Blood was collected (0.1-0.3 mL)
111 using heparinized capillaries from caudal peduncles. Afterwards, the fish were
112 euthanized by severing the spinal cord.

113 Capillaries were centrifuged (3000-g, 15 minutes) in a microhematocrit
114 centrifuge, and plasma was transferred to 1.5 mL eppendorf tubes and stored at –25°C
115 until analysis. Cortisol was measured in unextracted plasma samples, using
116 commercially available EIA kits (EIAgenTM Cortisol, Adaltis Italy S.p.A), previously
117 validated for the species (Barcellos et al., 2006). Glucose was analyzed by a
118 colorimetric test based on the oxidase/peroxidase reaction (Glicose – PP, Gold Analisa
119 Diagnostica Ltda, Brasil). Plasma Na^+ concentrations were measured in appropriate
120 diluted samples against four standard solutions of NaCl using flame photometry
121 (Micronal B262). All measures were performed in duplicate.

122

123 *2.6. Statistical analysis*

124 Data are presented as median and interquartile range (Q1–Q3) or mean ± SEM.
125 To verify the homogeneity of variances and normality, all data were submitted to
126 Levene and Kolmogorov-Smirnov tests, respectively. Extractive yields and water
127 parameters between experiments were compared using Student’s t-test. The results of
128 time of induction and recovery and stress parameters were analyzed by Scheirer-Ray-
129 Hare extension of the Kruskal–Wallis followed by the Dunn test or two-way ANOVA
130 and Tukey test. Minimum significance level was set at $P < 0.05$.

131

132 **3. Results**

133

134 *3.1. Essential oil analysis*

135 Extractive yields of LEO (0.67 ± 0.02 %) and IEO (0.62 ± 0.02 %) did not differ
136 statistically. Qualitative differences in the chemical composition of the EOs obtained
137 from different parts of the plant were detected to some minor compounds (0.02-0.51%).
138 In relation to the major compounds, lower content of β-linalool was observed in LEO
139 (20.18%) when compared to IEO (46.61%). LEO contained 1,8-cineole, eugenol and
140 camphor in higher proportions than the IEO (Table 1).

141

142 *3.2. Water parameters*

143 Experiments 1 and 2 were performed at similar conditions of dissolved oxygen
144 (7.31 ± 0.22 mg L^{-1}) and total ammonia levels (0.26 ± 0.16 mg L^{-1}). Water temperature
145 (experiment 1: 18.53 ± 0.14 ºC; experiment 2: 24.57 ± 0.41 ºC) and pH (experiment 1:
146 6.33 ± 0.07; experiment 2: 7.00 ± 0.09) were statistically different between experiments.

147

148 *3.3. Experiment 1: Anesthesia induction and recovery*

149 Sedative and anesthetic effects were verified in fish exposed to both EOs of *O.*
150 *americanum*. Concentrations of 25-100 mg L^{-1} were not able to induce anesthesia
151 during 30 min of exposure, whereas both EOs at 200-500 mg L^{-1} led to this depression
152 level in all animals exposed (Table 2). A positive relationship was detected to stages 3b
153 and 4 in both samples, where an increment of EO concentration caused a reduction in
154 the time required for anesthesia induction. LEO (4-8 min) anesthetized (stage 4) silver
155 catfish in significantly less time than IEO (6-16 min) at the same concentration. Ethanol
156 alone did not produce any sedative and anesthetic effects.

157 The EO samples tested did not show differences in the recovery time. Fish
158 exposed to 25 - 200 mg L^{-1} of both EOs recovered quickly (within 1-6 min) without
159 significant differences between concentrations. Larger times of recovery (within 11-14
160 min) were observed to the highest concentrations tested (300 and 500 mg L^{-1}). No
161 significant relationship was found between EO concentration and recovery time from
162 anesthesia.

163

164 *3.4. Experiment 2: Evaluation of stress parameters*

165 In this experiment, anesthesia was reached with 300 and 500 mg L^{-1} of LEO in
166 274.84 ± 5.79 s (4.5 min) and 197.26 ± 9.63 s (3 min), respectively. Lower cortisol

167 levels were verified in fish exposed to 300 and 500 mg L^{-1} of LEO in comparison to
168 control group up to 15 min after handling. Animals anesthetized previously with LEO
169 did not change their cortisol concentrations during the evaluation time, and were able to
170 maintain similar values to those of basal group. The same did not occur with fish of the
171 control group, which showed a rise of cortisol in 15 min followed by a decrease to
172 levels below the basal group at 240 min (Fig. 1A).

173 Blood glucose levels increased in all experimental groups in relation to basal
174 group for almost all observation times. Glycemia only returned to baseline levels at 240
175 min for the control group and 300 mg L^{-1} of LEO. There were no differences in the
176 blood glucose contents between LEO and control groups, as well as during the
177 evaluation time to animals of the control group or exposed to 500 mg L^{-1} of LEO (Fig.
178 1B).

179 Significant decrease in plasma Na^+ levels occurred in the control group 30 min
180 after handling compared to basal group and previous observation times. At this time,
181 fish anesthetized with LEO showed similar plasma Na^+ levels to basal group.
182 Differences in the ionic concentration were only verified between LEO concentrations
183 immediately after handling (data statistic not shown) and during the observation time
184 for fish exposed to 300 mg L^{-1} LEO (Fig. 1C).

185

186 **4. Discussion**

187 Intermediary extractive yield was detected to EOs of *O. americanum* in
188 comparison to previous reports (Hassane et al., 2011; Lawrence, 1989; Nascimento et
189 al., 2011; Ngassoum et al., 2004; Vasconcelos Silva et al., 2003; Vieira and Simon,
190 2006). The similar oil content between parts of the plant found in the present study
191 corresponded to different profile from that described by Vasconcelos Silva et al. (2003),
192 where leaves and inflorescences supplied, respectively, 2.1% and 0.3% of EO.

193 The chemical composition of the EOs of *O. americanum* were distinct from
194 those reported for the same species growing in Brazil, where only *E*-methyl cinnamate
195 and methylchavicol chemotypes were detected (Nascimento et al., 2011; Vasconcelos
196 Silva et al., 2003; Vieira and Simon, 2006). Furthermore, the presence of similar
197 contents of 1,8-cineole and β-linalool in the LEO composition also did not permit its
198 classification in any other chemotype reported to this plant. Lawrence (1989) described
199 that the β-linalool-chemotype of *O. canum* contained 43.9-69.3% of this compound,

200 0.1-11.4% of eugenol, 0.2-7.1% of 1,8-cineole and trace-0.5% of camphor. On the other
201 hand, oils rich in 1,8-cineole (18-34%) showed as common pattern low amounts of β-
202 linalool (0.4-0.9%) and absence of eugenol in their chemical profiles (Hassane et al.,
203 2011; Ngassoum et al., 2004). Thus, the present study detected a distinct chemical
204 profile for the EOs of *O. americanum*.

205 The differences in the anesthetic effect of the EOs of *O. americanum* can be
206 related to their distinct chemical compositions. In this context, the high contents of β-
207 linalool and eugenol in the LEO, the most active sample, must be highlighted. The
208 results suggest that both compounds, which had their anesthetic effect in the same fish
209 species recently reported (Cunha et al., 2010a; Heldwein et al., in press), may act in
210 synergistic form. Regarding to the other constituents, only 1,8-cineole had its anesthetic
211 activity evaluated, and did not show any depressor action up to 17 mg L^{-1} for the same
212 fish species (Heldwein, 2011).

213 In relation to induction time, other eugenol or β-linalool-rich oils promoted fast
214 anesthesia (about 4 min) at lower concentrations than LEO. Concentrations range of
215 100-150 mg L^{-1} of EO of *O. gratissimum*, an eugenol-rich sample, are required to
216 anesthetize silver catfish and Brazilian flounder (*Paralichthys orbignyanus*) (Benovit et
217 al., 2012; Silva et al., 2012). For EO of *Lippia alba* with high β-linalool content,
218 anesthesia was obtained in silver catfish at 300 mg L^{-1} in comparable period (Cunha et
219 al., 2010b).

220 Synthetic compounds, such as MS-222 and propofol, and also the natural
221 substance eugenol are able to induce quicker anesthesia at lower concentrations than the
222 EOs of *O. americanum* for same fish species (Cunha et al., 2010a; Gressler et al.,
223 2012b). On the other hand, β-linalool and EOs obtained from *Ocotea acutifolia*, *Lippia*
224 *sidoides*, *Aloysia triphylla* or *Hyptis mutabilis* required higher induction times and/or
225 concentrations to promote fast anesthesia (Heldwein et al., in press; Gressler et al.,
226 2012a; Silva et al., 2013a, b). Similar concentration and anesthetic induction time from
227 LEO was only described to EO of *Hesperozygis ringens* in silver catfish (Silva et al.,
228 2013a).

229 A clear relationship between concentration of EOs of *O. americanum* and
230 recovery time was not observed in silver catfish, which seems to be similar to that
231 described to other EOs (Cunha et al., 2010b; Silva et al., 2012; 2013a). However, fish
232 exposed to 300 mg L^{-1} of EOs of *O. americanum* had an intermediary recovery time

233 (about 12 min) when compared to those anesthetized at the same concentration of EO of
234 *L. alba* (about 6 min) and EO of *O. gratissimum* (about 20 min) (Cunha et al., 2010b;
235 Silva et al., 2012). These differences between samples may be resultant of distinct
236 accumulation levels in the fish tissues, and other studies should be performed to
237 evaluate this question.

238 The cortisol level has been considered the main indicator of the hormonal
239 response in face to stressors (Ellis et al., 2012). In this context, the experimental
240 protocol used was considered adequate to induce a stress event. The cortisol peak in
241 control group occurred at the same time course and with similar magnitude to that
242 described to juveniles of silver catfish after persecution with a pen net for 1 min
243 (Barcellos et al., 2012). The previous exposure to LEO was able to prevent the cortisol
244 rise due to handling. Preventive effect was also verified in silver catfish anesthetized
245 with EO of *L. alba* and eugenol after 1 and 4 h of the aerial exposure for 1 min (Cunha
246 et al., 2010 a,b).

247 Gressler et al. (2012a) detected increases in the cortisol levels of silver catfish
248 immediately after anesthesia with 150 mg L^{-1} of MS-222 using a protocol without
249 application of additional stressor. Similar pattern was not verified to both concentrations
250 of LEO after stress event, which can be considered a positive point to its use.

251 Secondary effects of stress can be evaluated from changes in glycemic and ionic
252 levels (Ellis et al., 2012). Silver catfish exposed to LEO showed hyperglycemia after
253 handling, which was not accompanied by ionoregulatory changes. The maintenance of
254 the plasma Na^+ levels in LEO groups corroborates with its effect on cortisol levels. This
255 hormone acts increasing Na^+/K^+-ATPase density in gill membranes, which favors the
256 ion loss (Dang et al., 2000).

257 Effect on plasma glucose levels, but not in cortisol contents, was also detected in
258 anesthesia of rainbow trout (*Oncorhynchus mykiss*) with clove oil, an eugenol rich
259 sample (Wagner et al., 2003). According to the authors, this phenomenon occurs due to
260 fish perception to anesthetic presence. It promotes cathecolamine release, which induces
261 liver glycogenolysis increasing, thus, the plasma glucose levels.

262 The hyperglycemic effect detected to LEO seems to be transient mainly at the
263 lowest concentration tested. Toni et al. (2013) also verified glucose increase and
264 baseline levels return only with the lowest concentration of EO of *H. ringens* tested
265 (150 μL L^{-1}) under the same experimental protocol. Opposite pattern was reported to

266 linalool-rich EO of *L. alba* in the same study, since it did not induce glycemic changes
267 after exposure (Toni et al., 2013).
268
269 **5. Conclusion**
270 The EOs of *O. americanum* demonstrated sedative and anesthetic effects in
271 silver catfish between 25-50 mg L^{-1} and 200-500 mg L^{-1}, respectively. The compounds
272 β-linalool and 1,8-cineole were detected in similar proportions in the LEO, together
273 with great amounts of eugenol and camphor. LEO corresponded to the most active
274 sample, which was considered suitable to the anesthetic procedures due to its fast
275 induction and handling-induced stress prevention. However, other studies should be
276 performed to evaluate its bioaccumulation in fish tissues aiming its approval as
277 anesthetic prior to slaughter.
278
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288
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401 **Table 1**

402 Chemical composition of the essential oils of *Ocimum americanum* obtained from

403 leaves (LEO) and inflorescences (IEO).

Rt (min)	**Constituent**	**LEO (%)**	**IEO (%)**	**RI cal**	**RI ref**
9.23	tricyclene	0.02	0.02	919.8	920[1]
9.51	α-thujene	0.04	0.02	926.7	925[1]
9.72	α-pinene	1.03	0.55	931.8	933[1]
10.28	camphene	0.76	0.54	945.1	946[1]
11.37	sabinene	0.88	0.25	971.6	975[1,2]
11.44	β-pinene	1.53	0.58	973.2	976[1]
11.78	1-octen-3-ol	0.06	0.03	981.5	980[1]
12.19	β-myrcene	1.29	0.34	991.3	992[1]
12.61	α-phellandrene	0.08	0.07	1001.5	1003[1,2]
13.13	α-terpinene	0.12	0.08	1014.3	1017[1,2]
13.46	*o*-cymene		0.02	1022.5	1022[1]
13.83	**1,8-cineole**	**21.00**	**8.43**	**1031.4**	**1031[1]**
14.13	*Z*-β-ocimene	0.03	0.03	1038.8	1038[2]
14.31	benzene acetaldehyde	0.02	0.02	1043.1	1045[1]
14.53	*E*-β-ocimene	0.29	0.39	1048.5	1050[1,2]
14.88	τ-terpinene	0.26	0.16	1057.2	1056[1]
15.22	bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1α,2α,5α)-	0.34	0.12	1065.5	1065[1]

15.53	1-octanol	0.17		1073.1	1070[1]
16.04	fenchone	1.41	3.59	1085.7	1087[1,2]
16.84	**β-linalool**	**20.18**	**46.61**	**1105.3**	**1101[1]**
17.22	exo-fenchol		0.11	1115.2	1115[1]
17.46	bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1α,2β,5α)-	0.05	0.08	1121.2	1121[1]
17.66	α-campholenal		0.05	1126.3	1127[1]
18.38	**camphor**	**11.96**	**9.50**	**1144.8**	**1144[1]**
18.45	*Z*-β-terpineol	0.09	0.08	1146.5	1144[1]
19.13	isoborneol	0.08	0.11	1164.0	1162[1,2]
19.21	δ-terpineol	0.49	0.20	1165.9	1166[2]
19.59	1-terpinen-4-ol	0.74	0.41	1175.7	1177[1]
20.16	α-terpineol	3.12	1.79	1190.4	1190[1]
20.35	myrtenol	0.06		1195.2	1196[1]
21.00	acetic acid, octyl ester	0.17		1212.6	1210[1]
21.24	fenchyl acetate		0.07	1219.0	1223[1]
22.57	chavicol	0.09		1255.2	1250[2]
23.67	bornyl acetate	0.21	0.28	1285.2	1284[1]
25.11	myrtenyl acetate	0.10	0.03	1325.7	1327[1,2]
25.49	δ-elemene		0.04	1336.8	1338[2]
25.91	α-cubebene		0.05	1349.1	1351[2]

26.38	**eugenol**	**17.17**	**3.22**	**1362.5**	**1364[1]**
26.84	α-copaene	0.24	0.21	1375.8	1376[1,2]
27.14	β-bourbonene	0.28	0.45	1384.6	1384[1]
27.40	β-elemene	0.60	2.08	1391.8	1391[1]
28.30	β-caryophyllene	1.46	3.27	1418.9	1418[1]
28.62	β-copaene	0.08	0.10	1428.5	1432[2]
28.85	α-bergamotene	2.06		1435.7	1436[1]
28.94	α-guaiene	0.11	0.88	1438.3	1439[1]
29.19	*Z*-muurola-3,5-diene	0.14	0.19	1445.9	1450[2]
29.42	α-caryophyllene	0.46	1.18	1452.9	1452[1]
29.54	β-farnesene	0.08		1456.3	1456[1]
29.74	*Z*-muurola-4(14), 5-diene		0.35	1462.6	1467[2]
30.35	germacrene D	3.49	4.76	1481.2	1480[1]
30.51	valencene		0.18	1485.8	1485[1]
30.83	τ-elemene	0.53	0.96	1495.6	1492[1]
31.10	germacrene A	0.51		1504.2	1509[2]
31.14	δ-guaiene		1.72	1505.3	1505[1]
31.39	τ-cadinene	0.97	1.06	1513.6	1513[1]
31.68	δ-cadinene		0.21	1523.0	1523[1,2]
32.92	*E*-nerolidol		0.12	1563.7	1564[1]
33.53	caryophyllene oxide		0.14	1583.8	1583[1]

34.50	1,10-di-epi-cubenol	0.49	0.47	1616.0	1619[2]
35.29	τ-cadinol	3.36	3.09	1643.1	1642[1]
35.55	β-eudesmol	0.12	0.26	1652.0	1651[1]
35.67	α-cadinol	0.22	0.28	1656.0	1657[1]
Total identified (%)		**99.16**		**99.83**	

Rt: Retention time; (%): Relative percentage; RI cal: calculated retention index; RI ref: reference retention index. [1] NIST (2005); [2] Adams (2001). Bold type indicates major components.

407 **Table 2**

408 Time required for induction and recovery from anesthesia using the EO of *Ocimum americanum* obtained from leaves (LEO) and inflorescences
409 (IEO) in juvenile silver catfish. Stages are defined according to Schoettger and Julin (1967). Maximum observation time was 30 min. Time to
410 reach each stage is given in seconds (s). n=10 for each concentration tested.

Sample	Concentration (mg L^{-1})	Induction time (s)				Time to recovery (s)
		Stage 2	Stage 3a	Stage 3b	Stage 4	
LEO	25	392 (367-430)[a]				100 (43-110)[b]
	50	146 (120-157)[ab]	229 (195-266)[a]			290 (230-344)[bc]
	100	66 (56-82)[abc]	88 (77-96)[ab]	1120 (824.7-1529.2)		290 (265-332)[bc]
	200	15 (9-19)[bcd]	63 (46-68)[bc]	349.5 (323-394)	501.5 (472-584)*	401 (322-448)[ac]
	300	7 (6-11)[cd]	22.5 (17-26)[cd]	160.5 (116-194)	362 (316-431)*	730 (600-900)[a]
	500	5 (4-5)[d]	10 (9-17)[d]	68 (63-90)	240 (179-308)*	813 (622-952)[a]
	Equation	-	-	$y = 45.061+7992.389 \exp(-0.5(\ln(x/9.089)/1.209)^2)$	$\ln y = 1.311+26.188/\ln(x)$ ($r^2 = 0.999$)	-

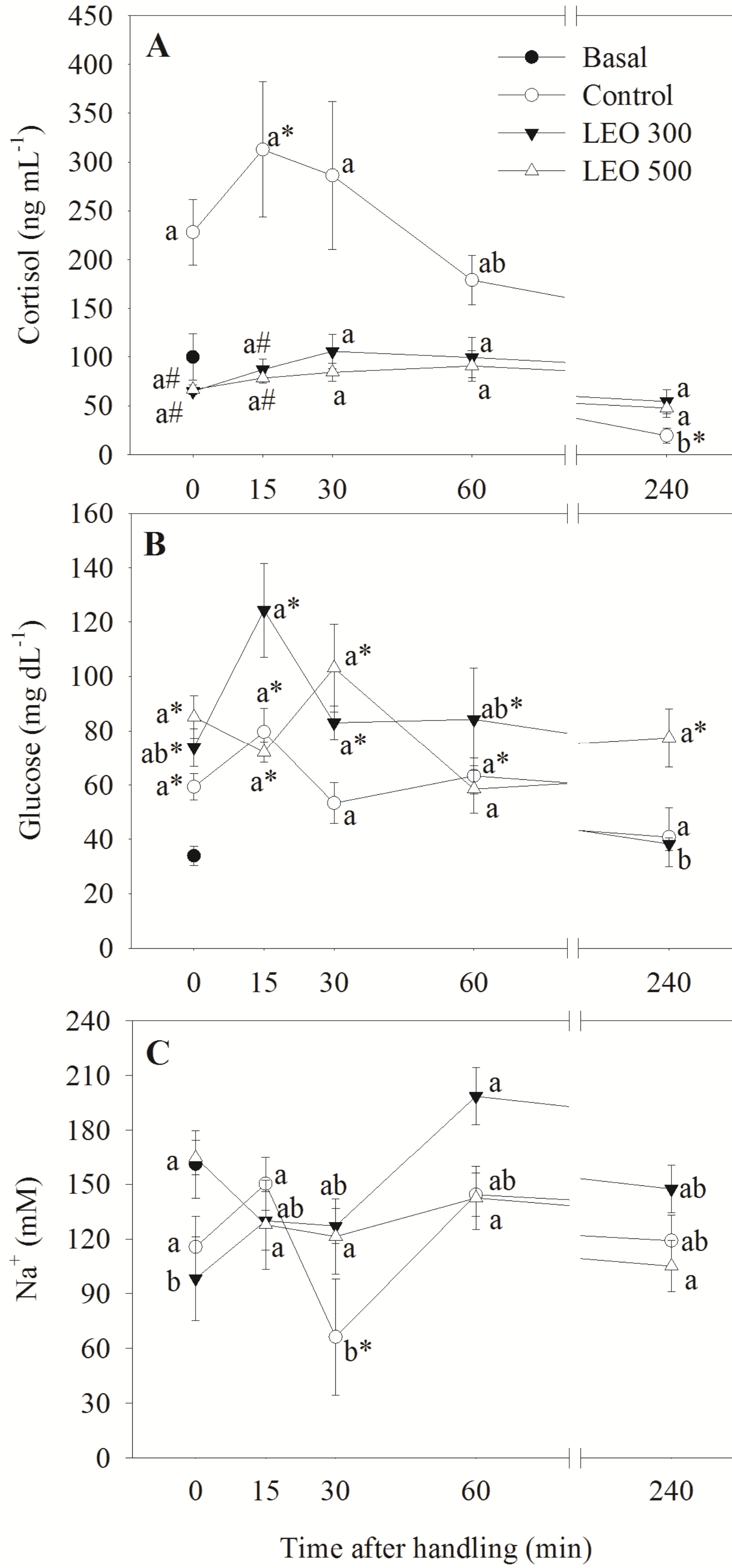
				(r^2 = 0.999)		
IEO	25	157 (123-186)[a]				100 (58-116)[b]
	50	117 (100-132)[ab]	246 (194-282)[a]			81 (61-123)[b]
	100	77 (25-128)[abc]	127 (91-142)[ab]	773 (710-810)		392 (324-440)[ab]
	200	49 (19-56)[bcd]	70 (65-79)[b]	448 (410-515)	970 (696-1050)	395 (325-502)[ab]
	300	10.5 (8-19)[cd]	41 (37-54)[bc]	249 (230-270)	487 (468-492)	705 (619-1294)[a]
	500	5 (4-10)[d]	25 (24-30)[c]	142 (110-160)	413 (325-420)	891 (679-989)[a]
	Equation	-	-	$y = 82.436 + 697.608 \exp(-0.5(\ln(x/84.545)/0.776)^2)$ (r^2 = 0.999)	$y = 2462.8 - 10.281x + 0.012x^2$ (r^2 = 1)	-

Data are presented as the median and interquartile range (Q1–Q3). Different letters in the columns indicate significant difference between concentrations of the same sample, while * represents significant difference between samples at the same concentration based on Scheirer-Ray-Hare extension of the Kruskal–Wallis followed by the Dunn test ($P < 0.05$). In the equations, x= concentration of essential oil (mg L^{-1}); y = time to reach the stage of induction or recovery from anesthesia in seconds (s).

Figure captions

Fig. 1. Effects of the essential oil of *Ocimum americanum* (LEO) on cortisol (A), glucose (B) and Na^+ (C) levels of silver catfish after handling.

Data are presented as the mean ± SEM. Lowercase letters indicate significant differences between times after handling within same experimental group, # represents statistical differences in comparison to water control at the same time after handling, and * corresponds to differences in relation to basal level. Scheirer-Ray-Hare extension of the Kruskal–Wallis test followed by the Dunn test or two-way ANOVA and Tukey test were used ($P < 0.05$).


A
B
C
Basal
Control
LEO 300
LEO 500
Cortisol (ng mL-1)
Glucose (mg dL-1)
Na+ (mM)
Time after handling (min)

Highlights

- Anesthetic effect of the essential oil of *O. americanum* was verified in *R. quelen*.
- Deep anesthesia was obtained at concentrations of 200-500 mg L^{-1}.
- Different induction time until anesthesia was verified between plant organs.
- The most effective anesthetic was leaf EO composed by β-linalool and 1,8-cineole.
- LEO prevented the cortisol increase and sodium loss induced by handling.

3.2 Manuscrito 2

SILVA, L. L.; BALCONI, L. S.; GRESSLER, L. T.; GARLET, Q. I.; SUTILI, F. J.; MALLMANN, C. A.; VARGAS, A. P. C.; BALDISSEROTTO, B.; MOREL, A. F.; HEINZMANN, B. M. *S*-(+)- and *R*-(-)-linalool: a comparison of the *in vitro* anti-*Aeromonas hydrophila* activity and anesthetic properties in fish. A ser submetido ao periódico "Aquaculture Research".

S-(+)- and *R*-(-)-linalool: a comparison of the *in vitro* anti-*Aeromonas hydrophila* activity and anesthetic properties in fish

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Running title: *S*-(+)- and *R*-(-)-linalool: aquaculture application

Keywords: chirality, linalool, *Aeromonas hydrophila*, anesthesia, silver catfish

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30 **Abstract**

31

32 Linalool is the main compound of many essential oils. It occurs in two isomeric forms: the *S*-
33 (+)- and *R*-(-)-linalool. The aim of this study was to determine if linalool isomers can show
34 differences on antimicrobial and anesthetic properties in fish. For this purpose, these
35 compounds were previously isolated from the essential oils of *Lippia alba* and *Ocimum*
36 *americanum*. Antimicrobial effects were evaluated through the microdilution test against
37 *Aeromonas hydrophila*, an important etiologic agent of fish disease. Induction time until
38 sedation and anesthesia and recovery time were determined in silver catfish (*Rhamdia quelen*)
39 through bath exposure (60, 180, 300 or 500 μL L^{-1}). The results showed different biological
40 properties for the isomers. Only *S*-(+)-linalool was active against *A. hydrophila* at 3.2 mg mL^{-}
41 ^{1}. Sedation was induced without differences between the compounds, whereas *R*-(-)-linalool
42 promoted anesthesia in less time. There are no differences in recovery time of animals
43 exposed to linalool isomers. Thus, the use of the *S*-(+)- and *R*-(-)-linalool in *A. hydrophila*
44 infection is inadvisable due their high effective concentration, while for sedative purposes any
45 one can be used. When anesthesia is the main objective, the *R*-(-)-linalool demonstrated clear
46 advantages at lower concentration.

47

48 # Introduction

49

50 World aquaculture production in 2010 obtained 79 million tons, worth US$125 billion.
51 In this context, a strong and continuous growth has been observed in South America,
52 particularly in Brazil where the fish production increased 13.2% between 2010-2011 (MPA
53 2011; FAO 2012). However, it is worth to note that some key production factors such as
54 disease susceptibility, growth, food conversion efficiency, flesh quality and reproduction can
55 be affected negatively by stressful agents. Examples of stressor factors in this sense are the
56 poor water quality, handling practices, inappropriate husbandry conditions and pathogen
57 occurrences (Ellis, Yildiz, López-Olmeda, Spedicato, Tort, Overli & Martins 2012; Segner,
58 Sundh, Buchmann, Douxfils, Sundell, Mathieu, Ruane, Jutfelt, Toften & Vaughan 2012).
59 *Aeromonas hydrophila* is an important etiologic agent responsible for substantial
60 economic losses in fish culture. This Gram-negative bacteria induces a hemorrhagic and
61 ulcerative disease, which causes high mortality rate and also increase susceptibility to other
62 diseases (Boijink & Brandão, 2001; Andrade, Andrade, Becker & Baldisserotto 2006). The
63 treatment involves antimicrobial drugs such as chloramphenicol and oxytetracycline, which
64 are able to remain as residues in animal products and to promote environmental contamination
65 (Andrade *et al.* 2006). Furthermore, the antibiotic use has conducted to emergence of
66 resistance strains to available drugs (Andrade *et al.* 2006; Barcellos, Kreutz, Rodrigues,
67 Santos, Motta, Ritter, Bedin & Silva 2008).
68 In a similar way, anesthetic and sedative drugs have been used during aquaculture
69 practices, such as transport, biometry and reproduction, to reduce fish movement and to
70 promote welfare through stress prevention. In order to avoid residual effects of synthetic
71 drugs, many natural agents, such as essential oils (EOs) of *Lippia alba*, *Ocimum gratissimum*,
72 *Hesperozygis ringens* and *Aloysia triphylla* were proposed in the last years as effective
73 anesthetics in replacement to synthetic compounds (Gressler, Riffel, Parodi, Saccol,
74 Koakoski, Costa, Pavanato, Heinzmann, Caron, Schmidt, Llesuy, Barcellos & Baldisserotto
75 2012; Silva, Silva, Garlet, Cunha, Mallmann, Baldisserotto, Longhi, Pereira & Heinzmann
76 2013a; Silva, Parodi, Reckziegel, Garcia, Bürger, Baldisserotto, Malmann, Pereira &
77 Heinzmann 2012a; Toni, Becker, Simões, Pinheiro, Silva, Heinzmann & Baldisserotto 2013).
78 Although natural products can be a source of new drugs with less risk to consumers' health
79 and environmental implications, few studies have been conducted until this moment with
80 isolated compounds from plants in aquatic animals (Cunha, Zeppenfeld, Garcia, Loro,

81 Fonseca, Emanuelli, Veeck, Copatti & Baldisserotto 2010; Silva, Garlet, Benovit, Dolci,
82 Mallmann, Bürger, Baldisserotto, Longhi & Heinzmann 2013b; Heldwein, Silva, Gai, Roman,
83 Parodi, Bürger, Baldisserotto, Flores, & Heinzmann in press).

84 Pharmacological assessment of chiral compounds permits to select the isomer with
85 higher potential for specific activity and fewer side effects incidence (Sousa, Nóbrega, Santos
86 & Almeida 2010). In this sense, antimicrobial and anesthetic agents are classical examples of
87 drugs whose chirality has demonstrated great influence in their biological properties (see
88 reviews of Hutt & O'Grady 1996; Mitra & Chopra 2011). However, until this moment, there
89 are no studies involving chirality of drugs in relation to aquaculture applications.

90 Linalool is a terpene alcohol present in the essential oil of many plant species. It
91 occurs naturally in two isomeric forms, also called enantiomers. Enantiomers are optical
92 active asymmetrical non-overlapping isomers, which are the mirror image of each other. In
93 the linalool case, they differ according to the chirality of carbon 3: the levorotatory form
94 called 3*R*-(-)-linalool or licareol, and the dextrorotatory isomer known as 3*S*-(+)-linalool or
95 coriandrol. These enantiomers have demonstrated different industrial applications and
96 biological effects *in vivo* (Sugawara, Hara, Tamura, Fujii, Nakamura, Masujima & Aoki 1998;
97 Siani, Tappin, Ramos, Mazzei, Ramos, Aquino Neto & Frighetto 2002; Höferl, Krist &
98 Buchbauer 2006; Sousa *et al.* 2010). Recently, the anesthetic and sedative effects of *S*-(+)-
99 linalool isolated from essential oil of *Lippia alba* was described in silver catfish (*Rhamdia*
100 *quelen*) (Heldwein *et al.* in press). However, no mention was made to *R*-(-)-linalool in this
101 study.

102 Thus, the aim of this work was to determine if *S*-(+)- and *R*-(-)-linalool can show
103 differences in antimicrobial activity *in vitro* against *A. hydrophila* and fish anesthetic
104 properties. For this purpose, these compounds were isolated from essential oils of *Lippia alba*
105 and *Ocimum americanum*, respectively, and had their antimicrobial and anesthetic activities
106 compared.

107

108 Material and Methods

109

110 Obtention and analysis of *S*-(+)- and *R*-(-)-linalool

111

112 *Plant material and essential oil extraction*

113 Inflorescences of the *O. americanum* were collected in December 2011 in Encantado-
114 RS, Brazil, whereas leaves of *L. alba* were collected in January 2012 in UFSM-CESNORS
115 Campus, Frederico Westphalen-RS, Brazil. The species were identified by Dr. Sérgio
116 Augusto de Loreto Bordignon and Dr. Gilberto Dolejal Zanetti, respectively. Voucher
117 specimens (nº SMDB 10050 for *L. alba*; nº SMDB 13163 for *O. americanum*) were deposited
118 in the herbarium of the Department of Biology, UFSM.
119 The EOs of *O. americanum* and *L. alba* were obtained from the fresh plant material by
120 hydrodistillation process with a Clevenger type apparatus for 3 and 2 hours, respectively
121 (European Pharmacopeia 2007). The samples were stored at -4°C until the isolation
122 procedure.
123

124 *Isolation and analysis*

125 *S*-(+)-linalool ($[\alpha]_D^{20}$ = + 1.911 (c 5.808, $CHCl_3$)) was obtained from chromatography
126 column (CC) procedure of the EO of *L. alba* according to Heldwein *et al.* (in press).
127 Previously to isolation process from the EO of *O. americanum*, the linalool chirality of this
128 sample was evaluated. The analysis was carried out by peak enrichment in gas
129 chromatography (GC) with capillary column coated with heptakis-(6-O-methyl-2,3-di-O-
130 pentyl)-β-cyclodextrin (25 m x 0.25 mm x 0.2 µm) using a Varian 3800 gas chromatography
131 equipped with a flame ionization detector (FID). Hydrogen was used as carrier gas, and 35 °C
132 for 20 min, 35-180 °C at 1 °C min^{-1} was chosen as temperature program. Four
133 chromatographic runs, aiming to analyze the racemic mixture (mixture of both enantiomers),
134 racemic mixture plus *S*-(+)-linalool, EO, EO plus racemic mixture were performed.
135 The EO of *O. americanum* (3.07 g) was submitted to a CC (4 x 50 cm) on 300 g of
136 silica-gel 60 (Merck, 70-230 mesh). The compounds were eluted with a mixture of hexane:
137 ethyl ether (95:5 v/v), at 3 mL min^{-1}. Fractions (40 mL) were monitored by TLC (silica gel
138 F254, hexane:ethyl ether 95:5 v/v, detection: anisaldehyde-H_2SO_4), pooled according to their
139 chromatographic profiles in 11 main fractions and then concentrated under reduced pressure,
140 at 40°C. Fraction 10 (1.1 g) was rechromatographed by CC (1.8 x 61 cm) using 116 g of silica
141 gel 60 impregnated with 10% silver nitrate (Williams & Mander 2001) and hexane:acetone
142 (95:5 v/v) at 1.75 mL min^{-1}. Fractions of 40 mL were collected and pooled together to give
143 seven main fractions (A-G), where fraction F was identified as *R*-(-)-Linalool ($[\alpha]_D^{20}$ = -
144 15.728 (*c* 0.1208, $CHCl_3$)).
145 The samples were identified by comparison of the Kovats retention index and mass
146 spectra with a mass spectral library (NIST, 2005) and with literature data (Adams 2001). GC-

147 MS was performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973
148 mass selective detector according to operational conditions described by Silva *et al.* (2012a).
149 Optical rotations were confirmed on a Perkin Elmer 343 polarimeter.
150
151 **Antimicrobial effects**
152
153 Antimicrobial activity of *S*-(+)- and *R*-(-)-linalool was assayed by broth microdilution
154 method as established by VET01-A4 (CLSI, 2013) for bacteria isolated from animals. The
155 microorganisms tested included four strains of *Aeromonas hydrophila*: one standard (ATCC
156 7966) and three fish clinical isolates (SB 13/09 10, 13/09 5, 22/07) obtained by the
157 Laboratory of Bacteriology (LABAC), Department of Veterinary Preventive Medicine,
158 UFSM. The characterization of the clinical isolates was carried out by morphological and
159 biochemical features, according to Quinn, Carter, Markey & Carter (1994).
160 Bacterial strains were grown 24 h at 30ºC in Mueller Hinton agar (Himedia®
161 Laboratories). The inoculum for the assays was prepared by diluting cell mass in 0.9% NaCl
162 solution, adjusted to McFarland scale 0.5 (1 x 10^8 CFU mL^{-1}) and confirmed by
163 spectrophotometrical reading at 670 nm. Cell suspensions were finally diluted in Mueller
164 Hinton broth to 1 x 10^7 CFU mL^{-1}.
165 Samples were solubilized in ethanol 95% and serial dilutions were performed in
166 culture medium to obtain concentrations of 3.2 – 0.0625 mg mL^{-1}. After inoculum (10 µL)
167 addition to the wells, the plates were incubated at 30 ºC for 24h. All tests were performed in
168 triplicate, including positives and negatives controls (inoculum and medium). Antimicrobial
169 activity was detected by adding 20 µL of 1% triphenyl tetrazolium chloride aqueous solution.
170 The minimum inhibitory concentration (MIC) was defined as the lowest concentration
171 of the sample that prevented visible growth. Minimum bactericidal concentration (MBC) was
172 defined as the lowest concentration yielding negative subcultures.
173
174 **Anesthesia and recovery**
175
176 *Animals*
177 Juvenile silver catfish (4.64 ± 0.15 g; 7.85 ± 0.10 cm) obtained from a local fish farm
178 were transported to the laboratory, where they were maintained in continuously aerated 250-
179 liter tanks with controlled water parameters for one week before the experiments. Dissolved
180 oxygen levels (7.91 ± 0.21 mg L^{-1}) and temperature (18.48 ± 0.20 ºC) were measured with an

181 YSI oxygen meter. The pH (7.67 ± 0.05) was determined with pH meter DMPH-2. Total
182 ammonia levels (0.44 ± 0.19 mg L^{-1}) were measured by the salicylate method (Verdouw, Van
183 Echteld & Dekkers 1978). A semi-static system was used, where 50% of the water volume
184 was changed daily. Fish were fed once a day with commercial feed (28 % crude protein).
185 Juveniles were fasted for a period of 24 h prior to the experiments. The methodologies of the
186 experiments were approved by the Ethical and Animal Welfare Committee of the Federal
187 University of Santa Maria (Process no. 46/2010).
188
189 *Experimental protocol*
190 Sedative and anesthetic effects of *S*-(+)- and *R*-(-)-linalool were evaluated on silver
191 catfish at concentrations of 60, 180, 300 or 500 µL L^{-1}. These concentrations were chosen
192 from previous study performed with *S*-(+)-isomer (Heldwein *et al.* in press). Juveniles were
193 transferred to 1L aquaria containing the concentration to be tested, previously diluted in
194 ethanol 95% (1:10). Nine fish were evaluated in each concentration, and each juvenile was
195 used only once to observe deep sedation (stage 2) or anesthesia (stage 4) (Schoettger & Julin
196 1967). The animals remained in anesthetic bath until to reach stage 4 or for 30 min. The
197 anesthesia was determined by loss of reflex activity and no reaction to strong external stimuli.
198 After the induction of anesthesia, each fish was measured, weighed and transferred to an
199 anesthetic-free aquarium to recover. The fish were considered to have recovered if their
200 normal posture and behavior were restored. Following, the animals were transferred to 30 L
201 tanks to evaluate possible side effects or mortality after 24 h of exposure.
202
203 **Statistical analysis**
204
205 Data are presented as mean ± SEM. To verify the homogeneity of variances and
206 normality, all data were submitted to Levene and Kolmogorov-Smirnov tests, respectively.
207 Log transformation was performed before two-way ANOVA and Tukey test to analyze the
208 time of induction until stage 4. Scheirer-Ray-Hare extension of the Kruskal–Wallis test and
209 Dunn test were used to evaluate the induction time until stage 2 and recovery. Analyses were
210 performed using the software SigmaPlot ver. 11.0, and the minimum significance level was
211 set at $P < 0.05$.
212
213 **Results**

214

215 Chiral GC analysis of the racemic mixture enriched with *S*-(+)-linalool (Fig. 1A)
216 allowed the identification of the second peak as corresponding to this isomer, since its area
217 decreased when racemic mixture alone was evaluated (data not show). EO of *O. americanum*
218 showed only one peak in this region of the chromatogram (Fig. 1C) with similar retention
219 time of the *R*-(-)-isomer in chiral chromatography. The *R*-(-)-isomer presence was confirmed
220 by the co-injection of this sample and racemic mixture (Fig. 1B).

221 Antibacterial effects *in vitro* were only verified to the *S*-(+)-isomer. This sample
222 showed bactericidal activity to *A. hydrophila* ATCC 7966 and clinical isolate 22/07 at 3.2 mg
223 mL^{-1}, but it did not act against clinical isolates 13/09 10 and 13/09 5. No activity was detected
224 to the *R*-(-)-form *in vitro*.

225 Similar induction time until stage 2 was verified in silver catfish exposed to *S*-(+)- and
226 *R*-(-)-linalool (Fig. 2A). Statistical differences in this stage were only detected among
227 concentrations of the same sample. *S*-(+)-linalool at 60 and 180 µL L^{-1} sedated the animals in
228 about 25 s, whereas higher concentrations induced the same depressor level in less of 10 s.
229 For *R*-(-)-isomer, a concentration increase from 60 to 180 µL L^{-1} promoted a reduction in the
230 induction time. Fish exposed to 180 µL L^{-1} sedated in similar time than those exposed at 300
231 and 500 µL L^{-1} of the same sample.

232 At concentration range of 180-500 µL L^{-1}, in general, *R*-(-)-linalool promoted faster
233 anesthesia in comparison to *S*-(+)-isomer. The only exception to this pattern was
234 concentration of 300 µL L^{-1}, where stage 4 was achieved with *S*-(+)-isomer (5 min) in less
235 time than *R*-(-)-isomer (6 min) (Fig. 2B). For both samples, an increase of the concentration
236 promoted a reduction in the induction time until stage 4 (Table 1).

237 In relation to recovery time, there were no differences between the isomers (Fig. 2C).
238 However, an increase in linalool concentration was accompanied by additional rise in
239 recovery time, which occurred independent of the sample evaluated (Table 1).

240

241 **Discussion**

242

243 Antimicrobial activity reported to linalool is contradictory. Some authors described
244 great spectrum of action for this compound (Dorman & Deans 2000; Sonboli, Babakhani &
245 Mehrabian 2006), while others detected antimicrobial activity for only one strain (Simionatto,
246 Porto, Stüker, Dalcol & Silva 2007; Özek, Tabanca, Demirci, Wedge & Baser 2010). These

247 discrepancies between studies could be a consequence of the use of different techniques,
248 growth medium, inoculum concentration and susceptibility of the microorganisms tested
249 (Cos, Vlietinck, Berghe & Maes 2006). However, as most works did not report the chirality of
250 the compound used, the influence of this parameter in the different reported activities cannot
251 be discarded.

252 In this study, the linalool chirality was able to influence the anti-*Aeromonas*
253 *hydrophila* activity, being *S*-(+)-isomer the only active compound. Özek *et al.* (2010) detected
254 similar growth inhibition for both compounds at 0.3µM against a distinct microorganism,
255 named *Botrytis cinerea,* using the microdilution broth assay. Other Gram-positive bacterial
256 and fungal strains tested in the same work were not inhibited until 0.2 mg mL^{-1} by both
257 enantiomers. However, studies performed with other terpenes also indicated the higher
258 antimicrobial effect of dextrorotatory isomers. Aggarwal, Khanuja, Ahmad, Kumar, Gupta &
259 Kuma (2002) demonstrated that (R)-(+)-limonene and (R)-(+)-carvone are the most potent
260 isomers against bacteria and dermatophytic fungi. In other work, only dextrorotatory
261 enantiomers of α- and β-pinene were active against *Candida albicans*, *Cryptococcus*
262 *neoformans*, *Rhizopus oryzae* and methicillin-resistant *Staphylococcus aureus* (Silva, Lopes,
263 Azevedo, Costa, Alviano & Alviano 2012b).

264 Regarding to anti-*Aeromonas* activity, previous reports conduced with racemic
265 mixture and linalool of no specified chirality also verified antibacterial effects against this
266 strain (Dorman & Deans 2000; Klein, Ruben & Upmann 2013). Bactericidal property of this
267 compound against *A. hydrophila* of dairy origin was verified at lower concentrations (0.72 mg
268 mL^{-1}) (Klein *et al.* 2013) than detected in this study. Similar bactericidal concentrations were
269 reported recently to eugenol against the same ATCC strain and two fish clinical isolates
270 (Sutili, Kreutz, Noro, Gressler, Heinzmann, Vargas & Baldisserotto in press).

271 The sedative and anesthetic properties of the *S*-(+)-linalool were similar to described
272 in previous report (Heldwein *et al.* in press). However, as other concentrations were tested for
273 this compound, our results permit to propose the concentration of 500 µL L^{-1} as the ideal to
274 promote fast anesthesia (less of 4 min). The same proposition can also be stated regarding *R*-
275 (-)-linalool.

276 Distinct central nervous system effects were described to linalool enantiomers in the
277 literature. The sedative effect in humans and anticonvulsant activity in rodents seems to be
278 higher in *R*-(-)-linalool than *S*-(+)-isomer (Sugawara *et al.* 1998; Sousa *et al.* 2010). Thus, the
279 absence of isomers differences in relation to sedation and recovery time were unexpected

280 results, while the higher anesthetic effect of *R*-(-)-form is in accordance with the results
281 detected in other species.
282 The biological differences between isomers have been related to distinct interactions
283 with molecular targets. Macromolecules of biological systems, e.g. proteins, glycolipids and
284 polynucleotides, are formed by chiral building blocks of L-amino acids and D-carbohydrates.
285 As these molecules are involved in the pharmacokinetic and pharmacodynamic processes of
286 the drugs, the stereoselectivity for one enantiomer can often be observed (Hutt & O'Grady
287 1996; Mitra & Chopra 2011).
288 In conclusion, *S*-(+)- and *R*-(-)-linalool showed different biological properties. The use
289 of both isomers as antimicrobial against *A. hydrophila* is inadvisable due their high effective
290 concentration, but for sedative purposes any one can be used. When anesthesia is the main
291 objective, the *R*-(-)-linalool demonstrated clear advantages at lower concentration. However,
292 other studies should be conducted to evaluate if these differences also occur in physiological
293 and biochemical parameters after anesthesia or sedation.
294

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296

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424 ## Figure legends

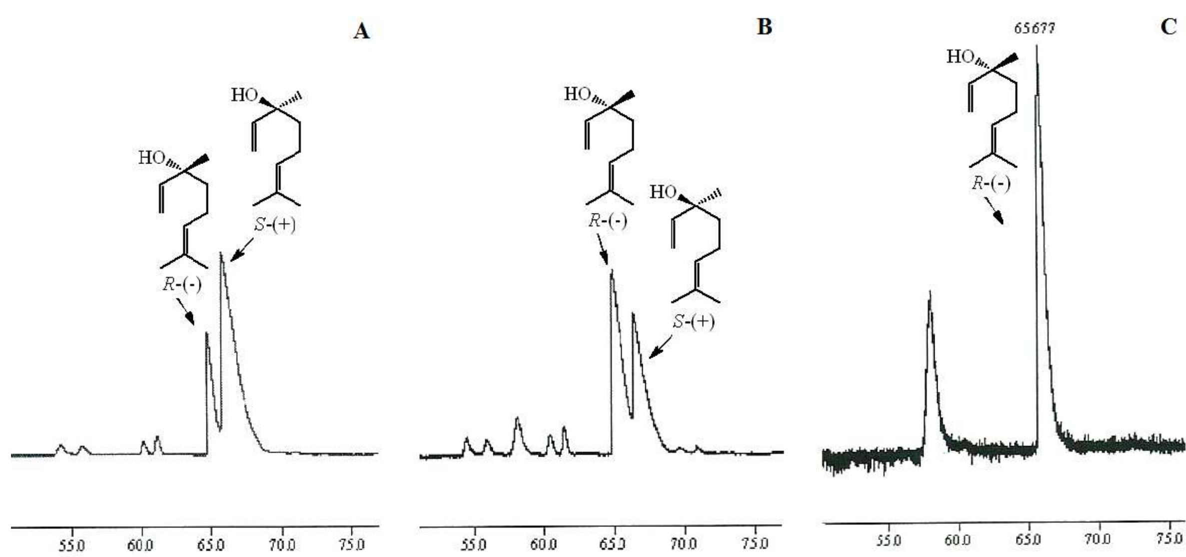
425

426 **Figure 1** Chiral chromatograms of the racemic mixture and *S*-(+)-linalool (A), racemic
427 mixture and EO of *O. americanum* (B) and EO of *O. americanum* (C).

428

429 **Figure 2** Time required for induction to anesthesia in silver catfish with *S*-(+)- and *R*-(-)-
430 linalool: stage 2 (A), stage 4 (B) according to Schoettger & Julin (1967), and recovery time
431 (C). Maximum observation time was 30 min. Time to reach each stage is given in seconds (s).
432 * indicate significant differences between both samples at the same concentration; different
433 lowercase letters correspond to significant differences between concentrations of the same
434 sample. Two-way ANOVA and Tukey test or Scheirer-Ray-Hare extension of the Kruskal–
435 Wallis test and Dunn test were used ($P < 0.05$).

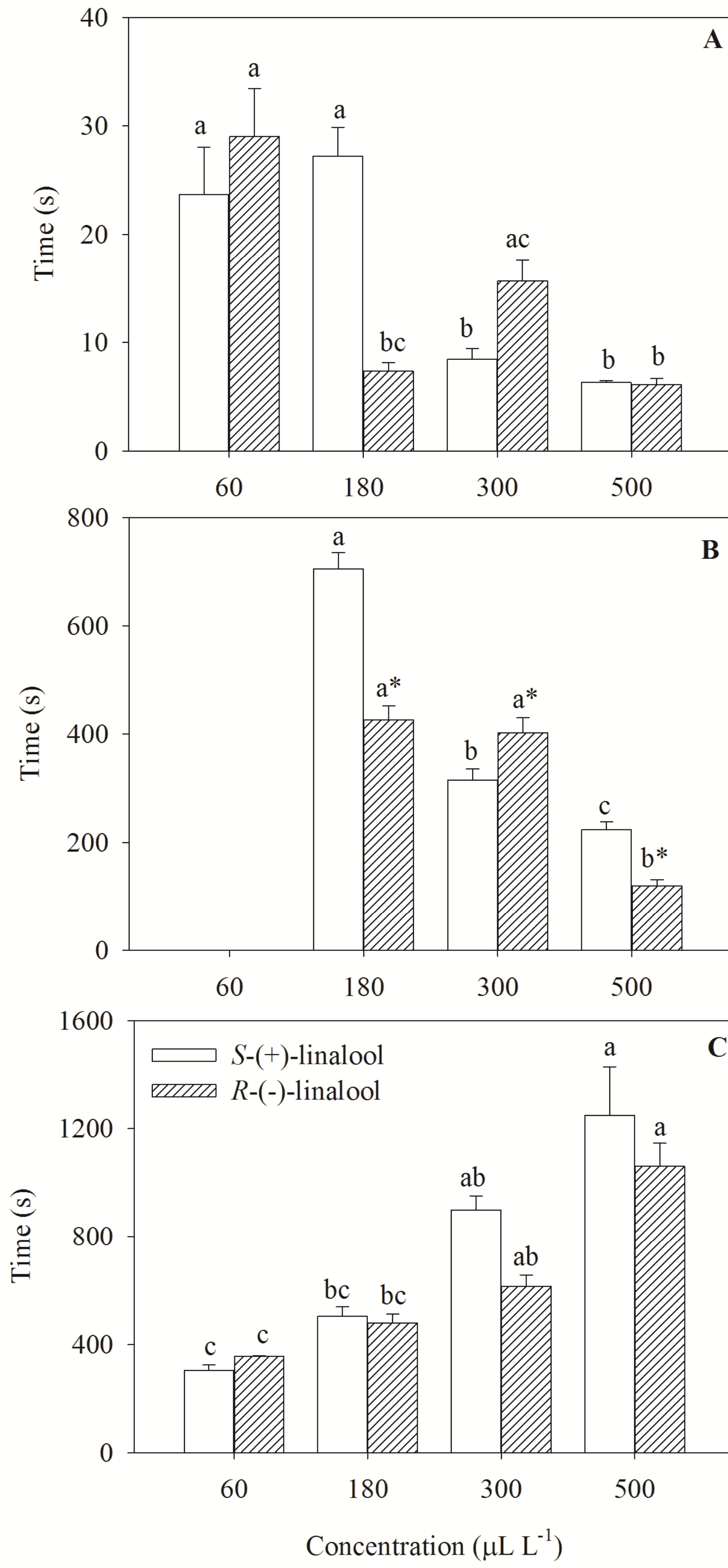
436



437

438 Figure 1

439



440

441 Figure 2

442

443 **Table 1** Relationship between the time required to reach the stages of induction and recovery
444 from anesthesia and the concentration of the *R*-(-)- and *S*-(+)-linalool in silver catfish. Where
445 x=concentration of essential oil (µL L^{-1}); y=time to reach the stage of induction or recovery
446 from anesthesia (Schoettger & Julin 1967) in seconds (s).

Sample	Stage 4	Recovery
S-(+)-linalool	$1/y=0.0091-0.1/x^{0.5}$ ($r^2=0.999$)	$y=363.9-2.2x+0.02106x^2-0.000026x^3$ ($r^2=1$)
R-(-)-linalool	$y=257.7+1.6x-0.00378x^2$ ($r^2=1$)	$1/y=0.00381-0.000128/x^{0.5}$ ($r^2=0.999$)

5 DISCUSSÃO GERAL

A presença, rendimento e composição química de óleos essenciais podem ser influenciados por fatores genéticos, fisiológicos e ambientais. Dentre os fatores fisiológicos temos o estágio de desenvolvimento, idade e órgão vegetal, tipo de estrutura secretora, reação à ocorrência de injúrias mecânicas ou químicas e sazonalidade. Em relação às condições ambientais devem ser consideradas: temperatura, disponibilidade hídrica, nutrientes, poluição, estação do ano, intensidade de radiação solar e altitude como influenciadoras do metabolismo secundário em espécies vegetais (GOBBO-NETO, LOPES, 2007; FIGUEIREDO et al., 2008).

Possíveis reflexos da influência destes fatores foram observados neste trabalho. Composições químicas distintas daquelas reportadas na literatura foram verificadas para os óleos essenciais de *O. americanum* e *H. mutabilis* (LUZ et al., 1984; LAWRENCE, 1989; BARBOSA, RAMOS, 1992; VELASCO-NEGUERUELA et al., 1995; SANDA et al., 1998; BAILAC et al., 1999; AGUIAR et al., 2003; VASCONCELOS SILVA et al., 2003; NGASSOUM et al., 2004; OLIVA et al., 2006; DAMBOLENA et al., 2009; HASSANE et al., 2011). Além disso, variações quantitativas na composição dos óleos destas espécies também ocorreram entre os diferentes órgãos vegetais e estas diferem daquelas descritas previamente (VASCONCELOS SILVA et al., 2003; NGASSOUM et al., 2004).

Todos os óleos essenciais avaliados apresentaram atividade anestésica e sedativa em juvenis de jundiá. Variações nestes efeitos e na presença ou não de reações adversas foram observadas com a modificação do órgão vegetal utilizado para obtenção do óleo essencial de *O. americanum* e *H. mutabilis*. Alteração da atividade biológica (antioxidante e antimicrobiana) de acordo com a parte do vegetal utilizada foi verificada previamente para os óleos essenciais de *Eucalyptus oleosa*, *Juniperus phoenicea* e *Myrtus communis* var. *italica* (ENNAJAR et al., 2010; WANNES et al., 2010; MARZOUG et al., 2011). Estes resultados demonstram que a determinação da composição química concomitantemente à atividade de um extrato vegetal é um fator extremamente relevante, tanto na caracterização de seus efeitos quanto para fins de controle de qualidade do fitomedicamento eventualmente gerado a partir do material vegetal em estudo.

Dentre as amostras de óleos essenciais testadas, aquelas obtidas a partir de *O. gratissimum* e *O. americanum* foram as que se mostraram mais efetivas e seguras como

anestésicos em jundiás. Suas atividades possivelmente sejam resultantes de seus constituintes majoritários, linalol e eugenol, que correspondem a substâncias de reconhecidas atividades depressoras centrais em animais aquáticos (CUNHA et al., 2010a; HELDWEIN et al., no prelo). Desta forma, estes óleos não foram inicialmente submetidos ao processo de isolamento de seus constituintes, mas sim avaliados quanto a outros parâmetros, como mecanismo de ação, no caso do óleo de *O. gratissimum*, e efeitos fisiológicos quando aplicados em situações estressantes para o animal, no caso de óleo de *O. americanum.*

Já para o óleo essencial de *H. mutabilis* foi detectada situação distinta tanto no que se refere à efetividade e incidência de efeitos adversos, quanto à ausência de prévia descrição de atividade central de seus constituintes. Em virtude disso, o mesmo foi submetido ao processo de fracionamento para obtenção de compostos isolados, que neste caso corresponderam ao (-)-globulol e (+)-1-terpinen-4-ol.

O sesquiterpenóide globulol, independentemente de sua atividade óptica, pode ser obtido também por isolamento a partir das espécies *Angelica sylvestris*, *Eucalyptus globulus* e *Hyptidendron canum* (sinonímia: *Hyptis cana*) (VINOKUROVA et al., 1999; TAN et al., 2008; LEMES, FERRI, LOPES, 2011), bem como por síntese química e microbiológica (MARSHALL, RUTH, 1974; GIJSEN et al., 1992; STODULKOVÁ et al., 2008). Para esta substância, Tan e colaboradores (2008) verificaram atividade antimicrobiana frente à fitopatógenos com valores de IC_{50} entre 21,4 - 53,4 µg/mL. Além disso, patentes englobando a sua utilização como aditivo alimentar em ração animal para alivio, cura ou prevenção de doenças causadas por *Clostridium* sp. e como agente capaz de regular o sistema nervoso autônomo promovendo, assim, ações sedativas e anti-estresse também foram registradas (EP 1 170 005 B1, 2009; US 0173986 A1, 2010).

Para o 1-terpinen-4-ol, constituinte minoritário do óleo essencial de *H. mutabilis*, inúmeras atividades biológicas foram comprovadas para seus isômeros ópticos e/ou mistura racêmica. Dentre elas merecem destaque a supressão da produção de mediadores inflamatórios por monócitos, a inibição do crescimento de células de melanoma humano (sensíveis e resistentes a adriamicina), bem como as ações antimicrobiana, hipotensora e anticonvulsivante. Este fitoconstituinte pode ser obtido em maiores proporções a partir dos óleos essenciais de *Alpinia zerumbet* e *Melaleuca alternifolia* (HART et al., 2000; LAHLOU et al., 2002; CALCABRINI et al., 2004; LOUGHLIN et al., 2008; SOUSA et al., 2009). A atividade anestésica do óleo de *M. alternifolia* foi detectada em carpa comum (HAJEK, 2011). Contudo, esta não foi relacionada à ação sedativa de seu composto majoritário 1-

terpinen-4-ol em animais aquáticos, o que confirma o carater inédito dos dados apresentados neste estudo.

A literatura relata uma distinta atividade depressora central para os diferentes enantiômeros do linalol tanto em seres humanos quanto em roedores (SUGAWARA et al., 1998; SOUSA et al., 2010). Adicionalmente a este fato, apenas o *S*-(+)-linalol havia sido avaliado em jundiás quanto a seu caráter anestésico e sedativo (HELDWEIN et al., no prelo). Estas considerações tornaram, por conseguinte, a determinação da quiralidade do linalol presente no óleo de *O. americanum* um parâmetro relevante a ser avaliado dentro do contexto deste trabalho.

Segundo Ravid e colaboradores (1997), algumas espécies de *Ocimum* podem ser diferenciadas pela presença de determinado isômero do linalol em seu óleo essencial. Estes autores verificaram que no óleo de *O. canum* proveniente da Tailândia com 0,6% de linalol, a forma predominante é o *S*-(+)-isômero (RAVID et al., 1997). Estes resultados contrastam com o verificado neste estudo onde foi detectada apenas a presença do *R*-(-)-linalol. Estas discrepâncias entre trabalhos podem ser resultantes da expressão de diferentes genes em consequência de distintos fatores fisiológicos e ambientais ao qual a espécie foi submetida (GOBBO-NETO, LOPES, 2007; FIGUEIREDO et al., 2008). Contudo, maiores estudos devem ser conduzidos para confirmar esta hipótese.

Em relação aos óleos essenciais propostos como anestésicos neste trabalho, significativas diferenças nas atividades farmacológicas das amostras foram verificadas. Em concentrações entre 300-500 mg L^{-1}, o óleo essencial de *O. gratissimum* promoveu anestesia em até 1 min, enquanto que os óleos de *O. americanum* e *H. mutabilis* necessitaram de um período de exposição entre 4-8 min e 21-23 min, respectivamente, para induzir o mesmo efeito depressor. Desta forma, o óleo de *O. gratissimum* pode ser considerado o mais potente em função do tempo de indução à anestesia em jundiás dentre os óleos em estudo, bem como em comparação ao óleo essencial de *L. alba* (CUNHA et al., 2010b).

A maior atividade anestésica e ausência de efeitos adversos (perda de muco) do óleo essencial das folhas de *H. mutabilis* em comparação ao (-)-globulol, assim como as amplas margens terapêuticas verificadas para os óleos essenciais de *O. gratissimum* e *O. americanum* em relação ao eugenol (CUNHA et al., 2010a), permite-nos considerar que a utilização desses óleos essenciais como anestésicos para peixes apresenta vantagens em relação ao uso dos constituintes isolados até agora avaliados.

Uma das vantagens mais importantes abordadas na literatura para os fitoterápicos é exatamente o maior índice terapêutico destas preparações, em comparação com as substâncias

isoladas. Um aspecto adicional a ser considerado é o fato de que alguns constituintes do fitocomplexo poderem influenciar positivamente a atividade do mesmo, através da melhora na sua estabilidade ou da solubilidade (HÄNSEL, STICHER, 2007). Por este motivo, a ANVISA, agência governamental responsável pela liberação dos medicamentos no país, tem exigências menos severas para o registro de fitoterápicos, do que no caso de substâncias isoladas de plantas (fitofármacos), que recebem o mesmo tratamento dos fármacos de origem sintética (BRASIL, 2004). Adicionalmente, o Ministério da Agricultura e Pecuária e o Ministério da Pesca permitem o uso de compostos naturais não tóxicos em sistemas de aquicultura orgânica sem necessidade de estudos de degradabilidade no ambiente (BRASIL, 2011), o que facilita de forma considerável a aprovação de novos produtos nesta área.

Além disso, os efeitos anestésicos e sedativos dos constituintes individuais descritos na literatura (CUNHA et al., 2010a; HELDWEIN et al., no prelo) ou avaliados neste estudo não explicam completamente a ação dos óleos essenciais testados. Por exemplo, a concentração considerada como ideal pra anestesia com óleo das folhas de *O. americanum* (500 mg L^{-1}) apresenta em torno de 100 mg L^{-1} de *R*-(-)-linalol, o que corresponde à concentração inferior àquela determinada experimentalmente como capaz de induzir efeito anestésico em jundiás (180-500 μL L^{-1} ou 162-450 mg L^{-1}). Em virtude de todos estes aspectos, os resultados obtidos neste trabalho sugerem que a interação entre diferentes fitoconstituintes possivelmente influenciou a atividade final dos óleos essenciais, bem como a sua segurança farmacológica.

Isto vem de encontro ao descrito por Galindo, Pultrini e Costa (2010) em relação aos constituintes majoritários do óleo essencial de *O. gratissimum* (eugenol, 1,8-cineol e *E*-cariofileno). Estes autores verificaram que estes constituintes isoladamente ou em associação, não alteraram os episódios convulsivos em modelos experimentais com roedores quando avaliados em concentrações proporcionais às existentes no óleo. Além disso, somente a associação foi capaz de aumentar o tempo de duração do sono, o que demonstra que o efeito desta espécie vegetal deve-se a interação sinérgica entre fitocompostos (GALINDO, PULTRINI, COSTA, 2010).

Harris (2002), em sua revisão sobre sinergismo de óleos essenciais, descreveu inúmeros exemplos onde pode ser observado o mesmo tipo de comportamento. Segundo este autor, a interação pode ser resultante tanto de compostos majoritários e minoritários, quanto entre os majoritários (HARRIS, 2002). Os efeitos sinérgicos ocorrem devido ação dos fitocomponentes em diferentes sítios ou receptores, bem como pela melhor solubilidade, absorção e biodisponibilidade apresentada por estes na mistura. Além disso, a interação

sinérgica poderia também explicar a eliminação ou neutralização de efeitos adversos de constituintes isolados (WAGNER, ULRICH-MERZENIC, 2009) quando esses fazem parte de um fitocomplexo, o que mais uma vez está de acordo com o verificado neste estudo.

Em relação ao tempo de recuperação, os jundiás submetidos à anestesia com os óleos essenciais de *O. americanum* recuperam-se mais rapidamente do que aqueles expostos às outras amostras de óleos testadas em concentrações similares. Adicionalmente, os óleos desta espécie vegetal promovem um menor tempo de recuperação que o observado em jundiás expostos ao *R*-(-)-linalol em similares concentrações, o que demonstra mais uma vez o efeito da interação dos constituintes presentes na amostra no efeito biológico observado.

Por outro lado, para os óleos essenciais de *H. mutabilis* e seu constituinte isolado, (-)-globulol, os tempos de recuperação em alguns casos foram superiores a 30 min. Essas diferenças observadas entre as amostras testadas podem ser decorrentes das suas diferentes lipossolubilidades e comportamentos farmacocinéticos. Alguns estudos têm demonstrado que anestésicos mais lipofílicos, como eugenol e isoeugenol, apresentam um prolongado tempo de meia-vida plasmática e uma lenta depuração em peixes, em virtude da sua bioacumulação nos tecidos (GUÉNETTE et al., 2007; KIESSLING et al., 2009; MEINERTZ, SCHREIER, 2009; ZAHL, SAMUELSEN, KIESSLING, 2012). Desta forma, estudos adicionais devem ser conduzidos para avaliar a possivel acumulação tecidual destes agentes anestésicos, a fim de determinar um período de depuração prévio ao abate de animais expostos aos mesmos.

A avaliação do mecanismo de ação de anestésicos e sedativos em animais aquáticos tem sido conduzida em poucos estudos, sendo que modelos comportamentais com esta finalidade são bem escassos (POWER, FUENTES, HARRISON, 2010; HELDWEIN et al., 2012). Power, Fuentes e Harrison (2010) descreveram a utilização de rutênio vermelho para avaliação do envolvimento do receptor vanilóide 1 (TRPV1) na ação do óleo de cravo em *Xiphosphorus maculatus*. Posteriomente, Heldwein e colaboradores (2012) padronizaram um modelo experimental para avaliação da participação do sítio benzodiazepínico do complexo receptor GABAérgico no mecanismo de ação de *L. alba*. Este protocolo experimental foi o mesmo utilizado para o óleo essencial de *O. gratissimum* e o globulol neste trabalho. O mesmo permitiu a verificação de um mecanismo similar ao de benzodiazepínicos para o óleo de *O. gratissimum*, inclusive no que se refere ao desenvolvimento de tolerância farmacológica.

Para o eugenol, constituinte majoritário do óleo essencial de *O. gratissimum*, inúmeros estudos de mecanismo de ação já foram realizados tentando comprovar suas propriedades anestésicas e analgésicas. No entanto, os relatos da literatura sobre a ação do eugenol como

anestésico local são controversos. Ghelardini, Galeotti e Mazzanti (2001) não verificaram efeitos *in vivo* no teste do reflexo conjuntival de coelho e *in vitro* na redução das contrações da preparação nervo frênico-diafragma de rato, os quais justificassem a ação anestésica local deste composto. Estudos posteriores, entretanto, demonstraram que o eugenol é capaz de promover a inibição da corrente de canais de Na^+ voltagem dependentes de uma forma concentração-dependente em neurônios ganglionares da raiz dorsal de ratos. Este composto não alterou a voltagem de ativação do canal, porém promoveu o deslocamento da curva de inativação do estado de equilíbrio para a direção hiperpolarizada e reduziu a corrente de Na^+ máxima do canal. O efeito inibitório sobre a corrente de Na^+ ocorreu através da interação do composto com os canais inativados e em repouso, sendo que o mesmo tornou mais lenta à recuperação da inativação (CHO et al., 2008). Adicionalmente, o eugenol demonstrou inibir os receptores NMDA e potencializar os receptores $GABA_A$ (CHO et al., 2008), o que viria a explicar sua ação depressora central.

Segundo Cho et al. (2008), o mecanismo da analgesia promovida pelo eugenol vai desde uma ação antiinflamatória, por inibição da cicloxigenase e lipoxigenase, até a interação com neurotransmissores envolvidos na sensação de dor. Experimentos *in vivo* têm demonstrado que eugenol apresenta propriedades antinociceptivas comparáveis à da capsaicina e que seu efeito é revertido pelo pré-tratamento com capsazepina, um antagonista competitivo do receptor TRPV1. A dessensibilização destes receptores, gerada pela interação com a capsaicina, tem sido descrita como o mecanismo da analgesia produzida por esta substância (CALIXTO et al., 2005; CHO et al., 2008). Os efeitos analgésicos do eugenol também têm sido relacionados à inibição de canais de cálcio ativados por alta voltagem, em neurônios aferentes dentais sensíveis e insensíveis à capsaicina (LEE et al., 2005).

Em relação ao composto 1-terpinen-4-ol, isolado neste trabalho a partir do óleo de *H. mutabilis*, a literatura relata a potencialização da ação do GABA em seus receptores (AOSHIMA et al., 2001) e a atividade inibitória sobre a butirilcolinesterase *in vitro* (BONESI et al., 2010) como possiveis mecanismos de ação sobre o sistema nervoso central. Já para o (-)-globulol, nenhum estudo sobre a avaliação de seu possível mecanismo de ação tinha sido reportado até o momento. (+)-Viridiflorol, um isômero deste composto, foi descrito como fraco agonista do sítio benzodiazepínico do receptor GABAérgico (JÄGER et al., 2007), o que nos levou a supor uma possível interação do (-)-globulol com este receptor. Contudo, os resultados encontrados não confirmaram esta hipótese e outros sítios deste receptor ou outros receptores ainda podem ser investigados.

A determinação do envolvimento do sistema gabaérgico no mecanismo de ação do óleo essencial de *O. americanum* não foi realizada em virtude do mecanismo de ação de seus constituintes majoritários (eugenol e linalol) já ter sido explorado em outros trabalhos (CHO et al., 2008; HELDWEIN et al., no prelo). Adicionalmente, sua composição química é intermediária àquela descrita para os óleos de *L. alba* (rico em linalol) e *O. gratissimum* (rico em eugenol), para os quais já tinha sido comprovado o envolvimento GABAérgico em seu mecanismo de ação (HELDWEIN et al., 2012).

Em relação à avaliação do estresse, resultados contraditórios foram reportados na literatura no que se refere ao caráter estressor dos anestésicos e suas influências sobre o manuseio de peixes, uma vez que diferentes espécies de animais podem apresentam distintas respostas frente a um mesmo agente (DERIGGI et al., 2006; ROSS, ROSS, 2008; CUNHA et al., 2010a,b; ZAHL et al., 2010). Um aumento da glicemia foi observado na anestesia com óleo de cravo em *Sparus aurata* (BRESSLER, RON, 2004) e com mentol em *Oreochromis niloticus* (SIMÕES, GOMES, 2009). No que se refere aos níveis sanguíneos de cortisol, aumentos foram descritos mesmo com a utilização de óleo de cravo em *Sparus aurata* como anestésico (BRESSLER, RON, 2004) e de eugenol em *Pimephales promelas* como sedativo (PALIĆ et al., 2006). Já estudo realizado por Deriggi e colaboradores (2006) verificou que a anestesia com eugenol não reduziu ou promoveu algum efeito adicional nos níveis de cortisol e íons plasmáticos em *Oreochromis niloticus* após o manuseio.

Efeito preventivo no aumento dos níveis plasmáticos de cortisol foi observado em *Salmo salar* com concentrações iguais ou superiores a 20 mg L^{-1} de óleo de cravo e isoeugenol. Por outro lado, estes anestésicos demonstraram, também nesta espécie, aumentos significativos na concentração plasmática de lactato e não promoveram alterações detectáveis na glicemia dos animais (IVERSEN et al., 2003). Em *Ictalurus punctatus* anestesiado com óleo de cravo (100 mg L^{-1}) a concentração de cortisol permaneceu dentro dos níveis basais durante os 30 minutos de exposição ao anestésico (SMALL, 2003). Por outro lado, Park e colaboradores (2009) verificaram que os níveis plasmáticos de cortisol e glicose de *Oplegnathus fasciatus* anestesiados com 100 mg L^{-1} de óleo de cravo somente retornaram aos níveis pré-exposição anestésica após dois dias.

Estes estudos reforçam o discutido com relação ao óleo essencial de *O. americanum* no que se refere a ser vantajosa sua aplicação como anestésico previamente ao manuseio de jundiás. Neste contexto, ainda devem ser realizados outros estudos com esta mesma amostra no que se refere a sua exposição durante procedimentos com longo tempo de exposição, como o transporte. O mesmo pode ser dito com relação ao óleo essencial de *O. gratissimum*, ao

(-)-globulol e aos enantiômeros de linalol.

6 CONCLUSÕES

- O óleo essencial de *O. gratissimum* L. rico em eugenol pode ser empregado como sedativo (10-20 mg L^{-1}) e anestésico (30-300 mg L^{-1}) para jundiás. Seu mecanismo de ação anestésico envolve a interação com o sítio benzodiazepínico do complexo receptor GABAérgico e seu uso é capaz de induzir tolerância de forma similar ao diazepam.
- A utilização do óleo essencial das folhas de *H. mutabilis* (Rich.) Briq. com alto conteúdo de globulol apresenta vantagens em comparação ao óleo das inflorescências da mesma espécie vegetal, rico em germacreno D, em virtude do seu efeito anestésico superior e ausência de efeitos adversos.
- Os compostos (-)-globulol e (+)-1-terpinen-4-ol, isolados a partir do óleo essencial das folhas de *H. mutabilis*, apresentam efeitos depressores inferiores ao óleo quando testados em concentrações equivalentes àquelas encontradas no mesmo.
- O (-)-globulol deve ser empregado apenas como sedativo em jundiás (10-20 mg L^{-1}), uma vez que concentrações anestésicas (83-190 mg L^{-1}) induzem perda de muco e mortalidade nos animais expostos. Adicionalmente, a sedação mediada por este composto não envolve a interação com o sítio benzodiazepínico do complexo receptor GABAérgico.
- O (+)-1-terpinen-4-ol promove apenas sedação em jundiás entre 3-10 mg L^{-1}. Contudo, o seu emprego com este propósito não é recomendado devido a esta substância não ser capaz de manter seu efeito durante o período de exposição.
- Os óleos essenciais obtidos a partir de folhas e inflorescências de *O. americanum* L. apresentam distintos constituintes majoritários (1,8-cineol, linalol e eugenol nas folhas; linalol nas inflorescências) e tempos de indução a anestesia em jundiás entre 200-500 mg L^{-1}. Neste sentido, as folhas correspondem ao órgão vegetal de escolha para obtenção de óleo essencial para utilização como anestésico.
- O uso do óleo essencial das folhas de *O. americanum* como anestésico previamente ao manuseio é capaz de prevenir a liberação de cortisol e seus efeitos fisiológicos em jundiás.
- O óleo essencial de *O. americanum* apresenta *R*-(-)-linalol em sua composição. Esta substância demonstra um similar efeito sedativo (60 µL L^{-1}) e superior propriedade anestésica (180-500 µL L^{-1}) em jundiás em comparação ao seu isômero dextrorotatório, obtido partir do óleo de *Lippia alba*.

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ANEXOS

Anexo A - ESPECTROS DE RMN DO (+)-1-TERPINEN-4-OL

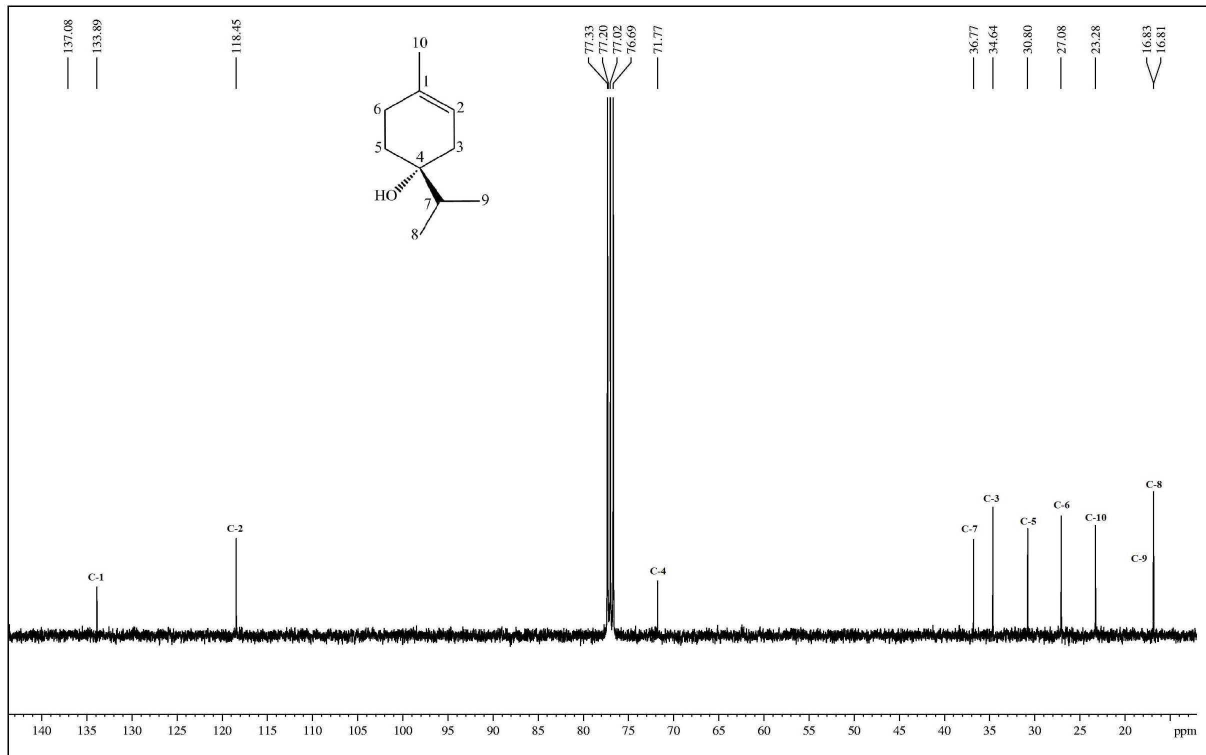

Figura 4 - Espectro de RMN ^{13}C do (+)-1-terpinen-4-ol (100 MHz, $CDCl_3$).

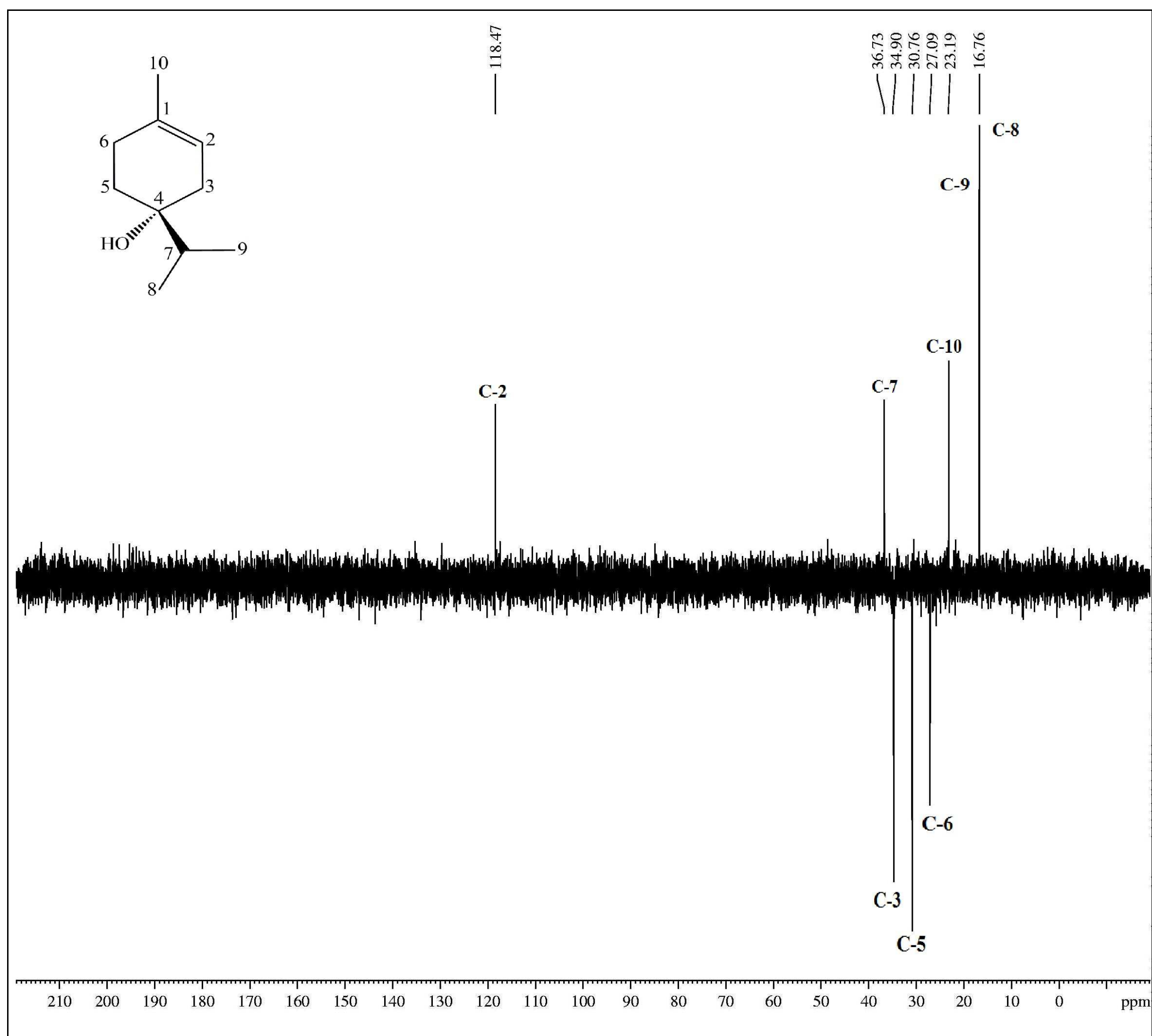

Figura 5 - Espectro de RMN ^{13}C-DEPT do (+)-1-terpinen-4-ol (100 MHz, $CDCl_3$).

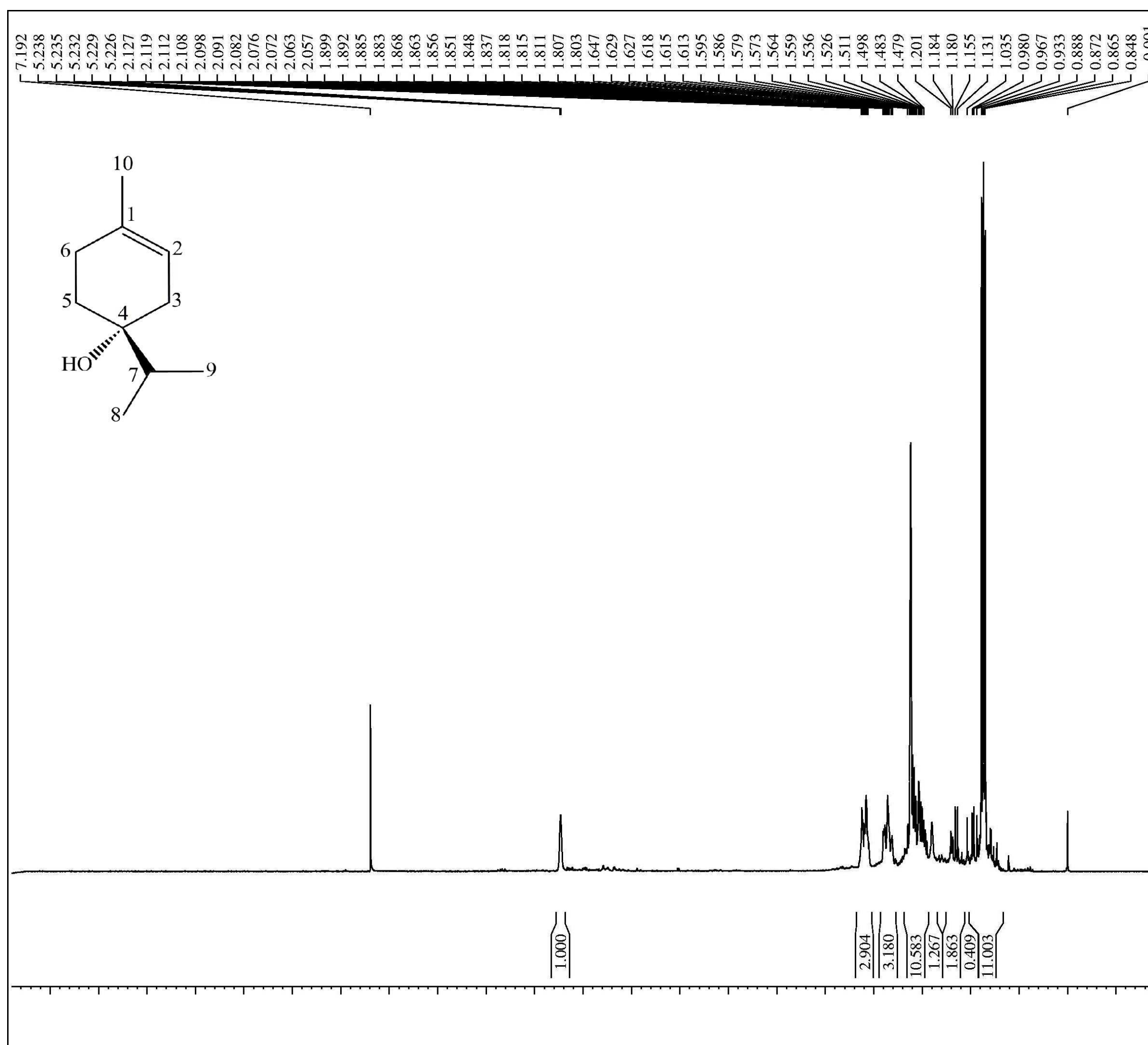

Figura 6- Espectro de RMN ^{1}H do (+)-1-terpinen-4-ol (400 MHz, $CDCl_3$).

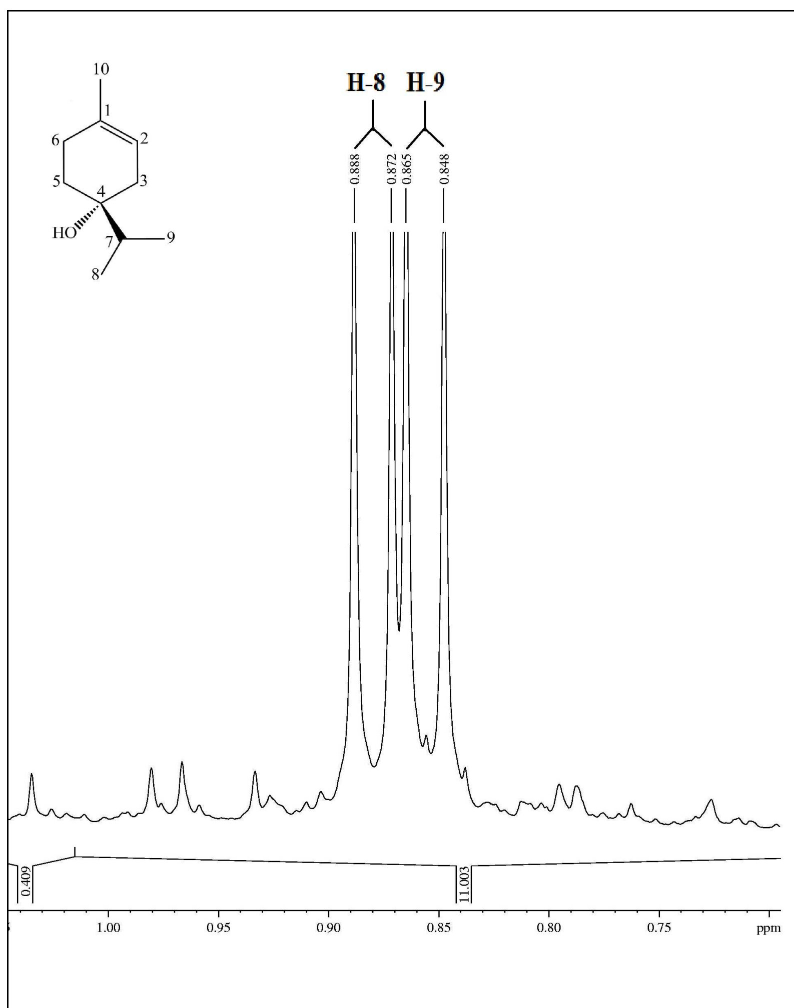

Figura 7- Ampliação do espectro de RMN ^{1}H do (+)-1-terpinen-4-ol na região de δ 0,7 a 1,04 ppm (400 MHz, $CDCl_3$).

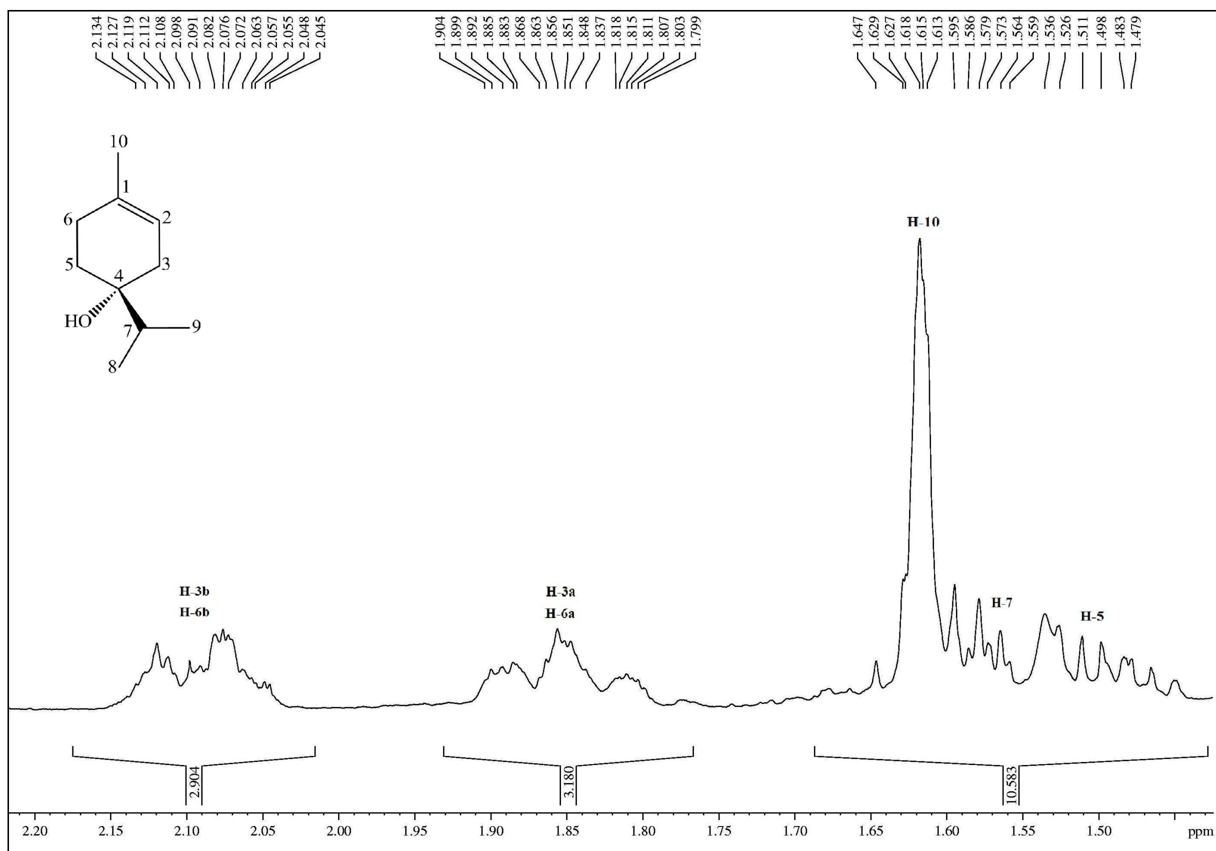

Figura 8 - Ampliação do espectro de RMN ^{1}H do (+)-1-terpinen-4-ol na região de δ 1,38 a 2,21 ppm (400 MHz, $CDCl_3$).

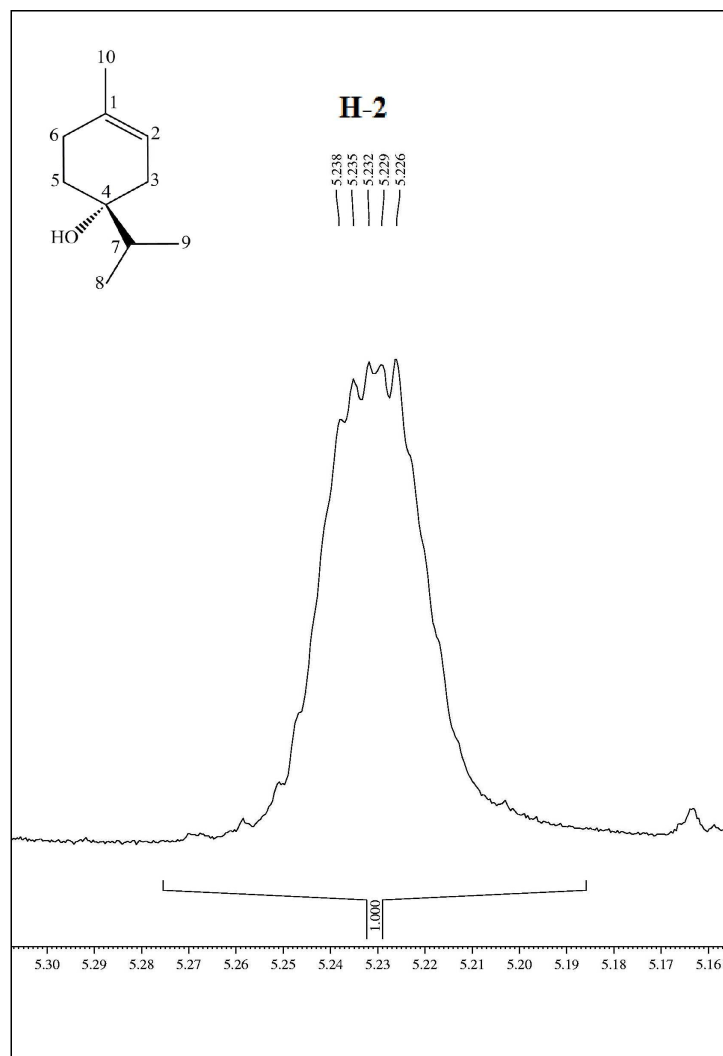

Figura 9 - Ampliação do espectro de RMN ^{1}H do (+)-1-terpinen-4-ol na região de δ 5,16 a 5,30 ppm (400 MHz, $CDCl_3$).

Anexo B - ESPECTROS DE RMN DO (-)-GLOBULOL

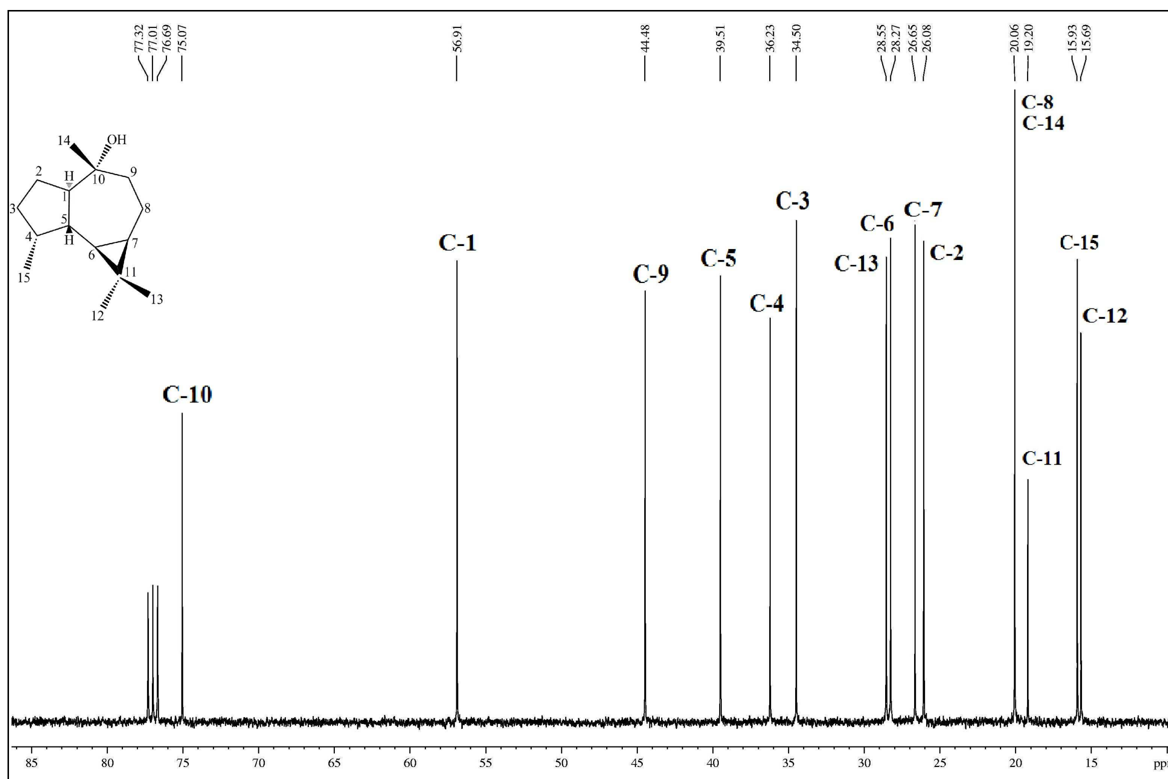

Figura 10 - Espectro de RMN ^{13}C do (-)-globulol (100 MHz, $CDCl_3$).

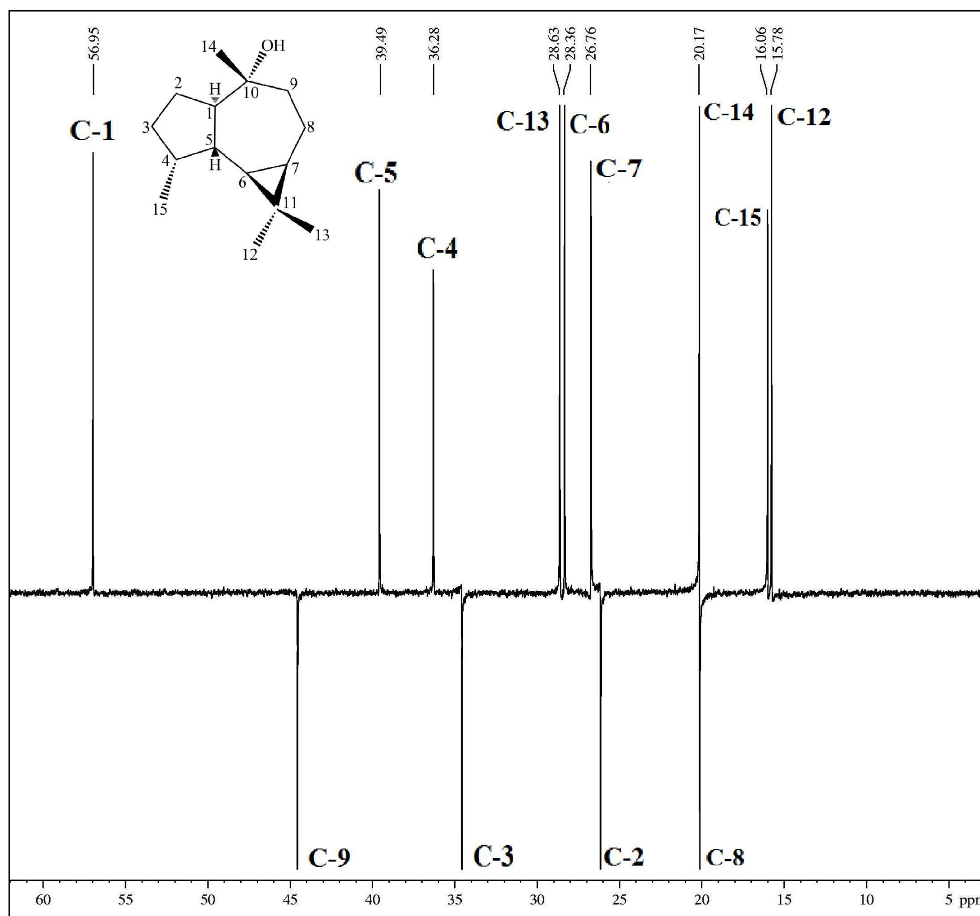

Figura 11 - Espectro de RMN ^{13}C-DEPT do (-)-globulol (100 MHz, $CDCl_3$).

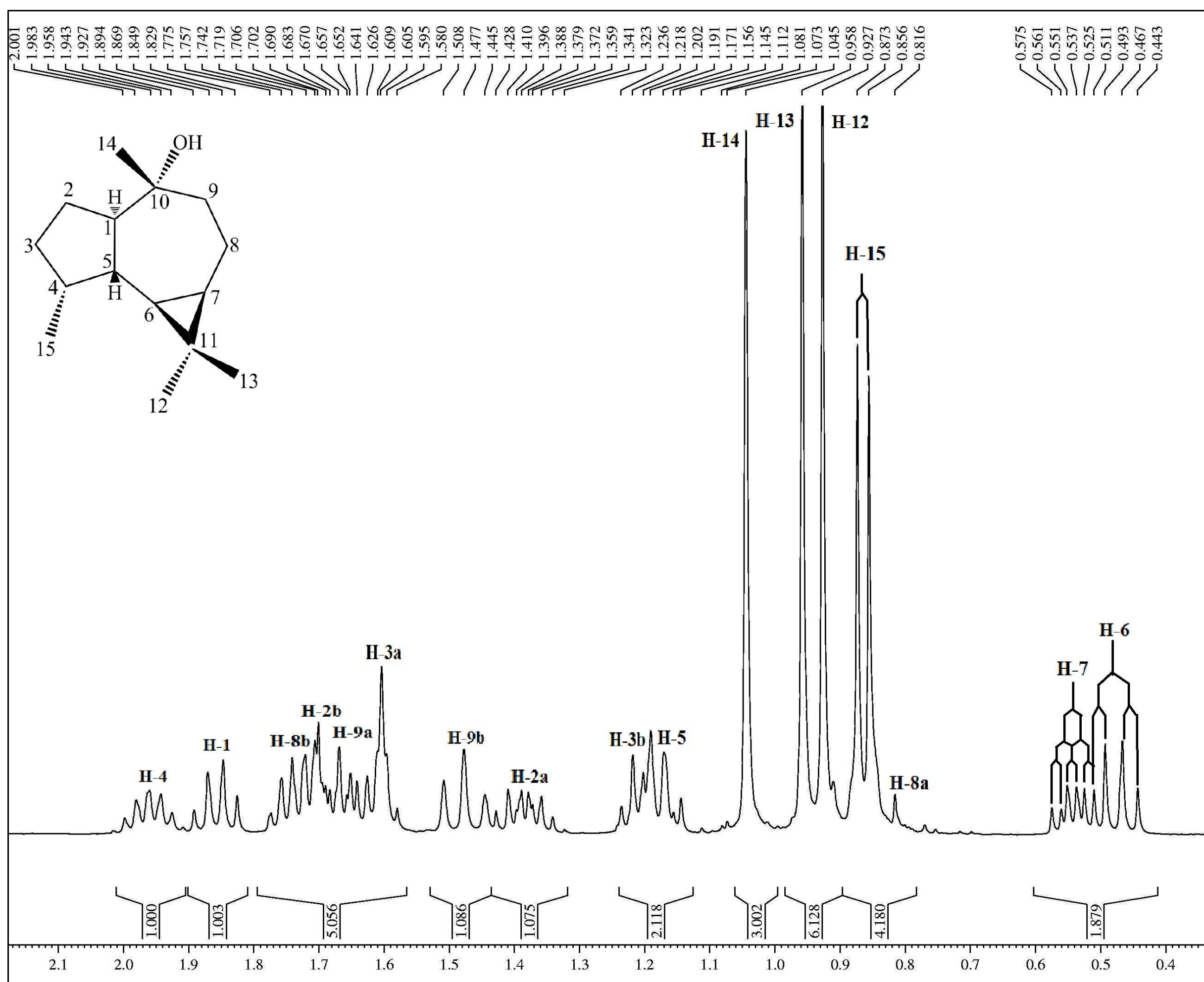

Figura 12 - Espectro de RMN ^{1}H do (-)-globulol (400 MHz, $CDCl_3$).