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Adriane Erbice Bianchini

MECANISMO DE AÇÃO DE MONOTERPENOIDES COM ATIVIDADE SEDATIVA E ANESTÉSICA EM JUNDIÁS (*Rhamdia quelen*)

Santa Maria, RS
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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Farmacologia**

Orientador: Bernardo Baldisserotto
Co-orientadora: Berta Maria Heinzmann

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Santa Maria, RS
2017

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“Conheça todas as teorias, domine todas as técnicas,
mas ao tocar uma alma humana, seja apenas outra alma humana.”
(Carl Jung)

RESUMO

MECANISMO DE AÇÃO DE MONOTERPENOIDES COM ATIVIDADE SEDATIVA E ANESTÉSICA EM JUNDIÁS (*Rhamdia quelen*)

AUTOR: ADRIANE ERBICE BIANCHINI
ORIENTADOR: BERNARDO BALDISSEROTTO

Os monoterpenoides estão presentes em grande proporção em óleos essenciais de plantas aromáticas e são fontes importantes de moléculas bioativas. Timol, carvacrol e linalol são exemplos destes compostos e dentre suas propriedades biológicas destacam-se a atividade depressora no sistema nervoso central. Neste contexto, o objetivo deste estudo foi avaliar o potencial anestésico do timol e do carvacrol e a influência na atividade da acetilcolinesterase (AChE) no músculo e no cérebro de jundiás (*Rhamdia quelen*). A atividade da AChE em jundiás expostos ao S-(+)-linalol também foi avaliada. Posteriormente, avaliamos a ação do timol e do S-(+)-linalol no sistema GABAérgico. Os peixes foram expostos a timol e carvacrol (25, 50, 75 e 100 mg/L) para avaliar o tempo de anestesia e recuperação. Ambos os compostos induziram sedação com 25 mg/L e anestesia com 50-100 mg/L. No entanto, os peixes expostos ao carvacrol apresentaram fortes contrações musculares e mortalidade. A atividade da AChE aumentou cerca de duas vezes no cérebro de peixes expostos a 50 mg/L de carvacrol e cerca de três vezes com 100 mg/L de timol, contudo a atividade da AChE diminuiu (cerca de cinco vezes) no músculo de jundiás expostos à 100 mg/L de carvacrol. O S-(+)-linalol não alterou a atividade da AChE. A anestesia com timol foi revertida por exposição à picrotoxina (antagonista de $GABA_A$), semelhante ao controle positivo propofol, mas não foi revertida pelo flumazenil (antagonista do sítio de ligação benzodiazepínico), como observado para o diazepam, controle positivo. A picrotoxina não reverteu o efeito de S-(+)-linalol. Timol 50 mg/L é mais adequado que carvacrol para anestesia em jundiás, uma vez que observamos um bom desempenho anestésico e nenhuma interferência com a atividade da AChE. O efeito anestésico do timol parece envolver os receptores $GABA_A$, mas não está relacionado com o sítio de ligação benzodiazepínico. Contudo, o efeito anestésico de S-(+)-linalol em jundiás não parece envolver os receptores $GABA_A$.

Palavras-chave: Benzodiazepínico. Carvacrol. $GABA_A$. S-(+)-linalol. Timol.

ABSTRACT

MECHANISM OF ACTION OF MONOTERPENOIDS WITH SEDATIVE AND ANESTHETIC ACTIVITY IN SILVER CATFISH (*Rhamdia quelen*)

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Monoterpenoids are present in a large proportion in essential oils of aromatic plants and are important sources of bioactive molecules. Thymol, carvacrol and linalool are examples of these compounds and among their biological properties the depressant activity in the central nervous system stands out. In this context, the objective of this study was to evaluate the anesthetic potential of thymol and carvacrol, and their influence on acetylcholinesterase (AChE) activity in the muscle and brain of silver catfish (*Rhamdia quelen*). The AChE activity of S-(+)-linalool was also evaluated. We subsequently assessed the effects of thymol and S-(+)-linalool on the GABAergic system. Fish were exposed to thymol and carvacrol (25, 50, 75 and 100 mg L^{-1}) to evaluate time for anesthesia and recovery. Both compounds induced sedation at 25 mg L^{-1} and anesthesia with 50–100 mg L^{-1}. However, fish exposed to carvacrol presented strong muscle contractions and mortality. AChE activity increased about two-fold in the brain of fish exposed to 50 mg L^{-1} carvacrol and about three-fold at 100 mg L^{-1} thymol, however AChE activity decreased (about five-fold) in the muscle of silver catfish exposed to 100 mg L^{-1} carvacrol. S-(+)-linalool did not alter AChE activity. Anesthesia with thymol was reversed by exposure to picrotoxin ($GABA_A$ antagonist), similar to the positive control propofol, but was not reversed by flumazenil (antagonist of benzodiazepine binding site), as observed for the positive control diazepam. Picrotoxin did not reverse the effect of S-(+)-linalool. Thymol 50 mg L^{-1} is more suitable than carvacrol for anesthesia in silver catfish, as we observed good anesthetic performance and no interference with AChE activity. Its anesthetic effect appeared to involve the $GABA_A$ receptors, but was not related to the benzodiazepine site. Therefore, the anesthetic effect of S-(+)-linalool in silver catfish does not appear to involve the $GABA_A$ receptors.

Key-words: Benzodiazepine. Carvacrol. $GABA_A$. S-(+)-linalool. Thymol.

LISTA DE FIGURAS

INTRODUÇÃO

MANUSCRITO

LISTA DE TABELAS

INTRODUÇÃO

MANUSCRITO

SUMÁRIO

1 INTRODUÇÃO

1.1 ESPÉCIE EM ESTUDO

O jundiá (*Rhamdia quelen*) (Fig. 1) é um teleósteo de água doce pertencente à ordem Siluriformes e família Heptapteridae. Sua distribuição é neotropical e se estende do sudeste do México ao norte e do centro da Argentina ao sul, sendo encontrado em todo o Brasil. Outros nomes populares descritos para a espécie aqui no Brasil são jundiá-tinga, jandiá, jandiá-tinga, mandi e sapipoca (GOMES et al., 2000). Atualmente, o jundiá é a terceira espécie com maior produção no Rio Grande do Sul, perdendo apenas para carpa e tilápia, contudo é a de maior produção entre as espécies nativas (EMATER/ASCAR, 2015). Essa espécie tem sido constantemente utilizada por nosso grupo de pesquisa como modelo experimental em trabalhos de melhoramento de dietas (SOUZA et al., 2015; ZEPPENFELD et al., 2016), tratamento de infecções (SOUZA et al., 2016; SUTILI et al., 2015; 2016), estudos de toxicologia (GOLOMBIESKI et al., 2016), descoberta de compostos com propriedades sedativas e anestésicas (BENOVIT et al., 2015; SANTOS et al., 2016; SILVA et al., 2015), transporte (SALBEGO et al., 2015; ZEPPENFELD et al., 2014), além de servir como modelo animal para estudos em farmacologia (GARLET et al., 2016).

Figura 1 – Exemplar de *Rhamdia quelen*

Fonte: Autor

1.2 ANESTESIA NA AQUICULTURA

Na aquicultura o uso de anestésicos visa atenuar o estresse gerado durante procedimentos de manejo e transporte, além de possibilitar aqueles mais invasivos como

cirurgias (ROSS; ROSS, 2008). Apesar das afirmações que peixes são incapazes de sentir a experiência da dor (ROSE et al., 2014), sabe-se que teleósteos possuem nociceptores capazes de detectar estímulos nocivos (ASHLEY et al., 2007). Por conseguinte, a percepção destes estímulos poderá desencadear respostas comportamentais e fisiológicas que afetarão o bem estar animal. Neste contexto, o uso de anestésicos em procedimentos estressantes na aquicultura e também na experimentação torna-se indispensável (SNEDDON, 2012).

A via de administração mais comum para anestesia em peixes é por inalação. Neste caso o anestésico de escolha é disperso na água e então absorvido pelas brânquias. Tratando-se de compostos mais lipossolúveis como óleos essenciais, muitas vezes é necessário a pré-diluição com um solvente orgânico como etanol (ZAHL; SAMUELSEN; KIESSLING, 2012). A profundidade da anestesia (sedação ou anestesia profunda, bem como colapso medular) dependerá da concentração do anestésico e do tempo de exposição. Em peixes, um método fácil de controlar a profundidade da indução anestésica é de acordo com as mudanças na atividade de natação, equilíbrio e reações a estímulos externos (Tab. 1) (GOMES et al., 2011). A frequência respiratória também pode ser avaliada (ZAHL; SAMUELSEN; KIESSLING, 2012). Para procedimentos rápidos e não invasivos a indução de sedação apenas pode ser suficiente. Contudo, para procedimentos invasivos e mais prolongados a anestesia profunda (cirúrgica) é aconselhada. Nestes casos também pode ser necessária a ventilação artificial das brânquias (SNEDDON, 2012).

Tabela 1 – Estágios de indução anestésica em peixes.

Estágios	Descrição	Resposta comportamental
1	Sedação leve	Perda parcial da reação a estímulos externos
2	Sedação profunda	Perda parcial de equilíbrio e ausência de reação a estímulos externos
3a	Perda total de equilíbrio	O peixe costuma virar, mas mantém a natação
3b	Perda total de equilíbrio	O peixe perde a capacidade de nadar, mas responde à pressão sobre o pedúnculo caudal
4	Anestesia	Perda de atividade reflexa e ausência de reação a estímulos externos fortes
5	Colapso medular	O movimento respiratório cessa (morte)

Fonte: GOMES et al. (2011).

Dentre os fatores que podem interferir na anestesia de peixes estão os fatores biológicos e ambientais. Os biológicos são aqueles relacionados ao animal, como peso corporal, estado nutricional, patológico ou condição de estresse, sexo e maturidade sexual, além da variabilidade inter- e intra-espécies. Já os fatores ambientais incluem aqueles referentes às características físico-químicas da água como temperatura, pH e salinidade (ROSS; ROSS, 2008). Estudos com anestésicos em diferentes espécies mostram que na maioria dos casos em temperaturas mais elevadas ocorre aumento da velocidade de absorção, distribuição e a depuração do anestésico, uma vez que o aumento da temperatura da água aumenta a taxa metabólica e consequentemente aumenta a perfusão sanguínea em órgãos como brânquias, fígado e rins (GOMES et al., 2011; STEHLY; MEINERTZ; GINGERICH, 1998; ULKA et al., 2015; ZAHL et al., 2011).

Os anestésicos utilizados usualmente na aquicultura são o metanossulfonato de trícaina (MS-222), benzocaína, metomidato, 2-fenoxietanol a quinaldina e isoeugenol. Segundo Ross e Ross (2008) os anestésicos gerais promovem depressão generalizada do sistema nervoso central por mecanismos que envolvem a ação sobre os axônios nervosos, a liberação de neurotransmissores, a alteração na excitabilidade celular ou uma combinação dessas ações. Contudo, estes conceitos são extrapolados a partir de informações que se tem em mamíferos e pouco se sabe sobre o modo de ação preciso em invertebrados e peixes (ROSS; ROSS, 2008). O metomidato parece atuar em receptores $GABA_A$, enquanto o MS-222 e a benzocaína são bloqueadores de canais de Na^+, e por isso os dois últimos são considerados anestésicos locais. Porém em peixes estes anestésicos são aplicados por banhos de imersão e os mecanismos responsáveis pelo efeito anestésico sistêmico permanecem desconhecidos (SNEDDON, 2012; ZAHL; SAMUELSEN; KIESSLING, 2012).

Uma problemática atribuída ao uso destes anestésicos em peixes são os seus efeitos colaterais. O MS-222, por exemplo, é o único aprovado pelo FDA (*Food and Drug Administration*), mas a anestesia profunda causa o aumento dos níveis plasmáticos de glicose, cortisol e lactato. Além disso, peixes expostos a este anestésico necessitam de um período de 21 dias de carência antes do abate. Este período deve ser respeitado para permitir que todos os resíduos do MS-222 sejam eliminados pelo peixe (PALIC et al., 2006; ROSS; ROSS, 2008; SMALL, 2003). Outros produtos como a quinaldina e o 2-fenoxietanol possuem algumas limitações devido ao poder irritante nos olhos e pele do manipulador (ROSS; ROSS, 2008). Neste contexto, estão sendo feitos estudos que visam à pesquisa de compostos com menos efeitos colaterais para uso na aquicultura e o uso de produtos de origem natural surgem como alternativa.

1.3 RECEPTORES $GABA_A$ NA ANESTESIA GERAL

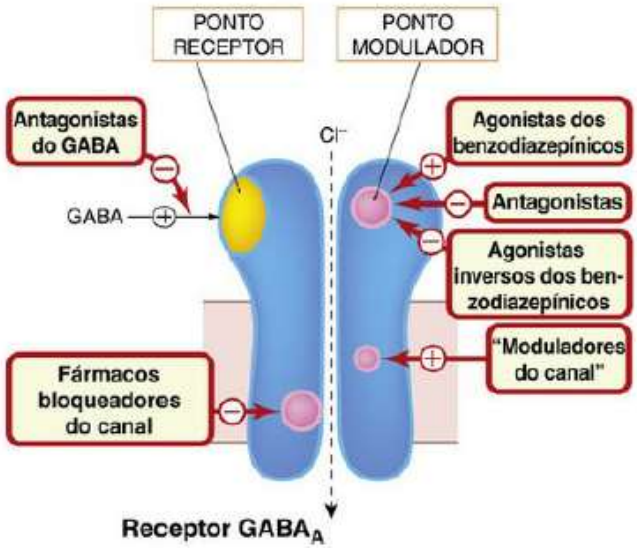
O ácido γ-aminobutírico (GABA) é o principal neurotransmissor inibitório e seus receptores estão expressos na maioria dos neurônios do sistema nervoso central (SNC) de vertebrados. Este neurotransmissor produz inibição neuronal através de sua ligação com receptores do tipo $GABA_A$ e $GABA_C$ ligados à membrana celular, os quais são canais de cloreto regulados por ligantes, chamados também de receptores ionotrópicos, ou através de receptores acoplados à proteína G, conhecidos como receptores $GABA_B$ ou metabotrópicos (BRUNTON, 2012). Contudo, alguns autores consideram os receptores $GABA_C$ um subtipo de $GABA_A$ (RANG et al., 2016). Atualmente o baclofeno, um análogo estrutural de GABA utilizado como relaxante muscular, é o único fármaco de uso clínico cuja ação é exercida sobre os receptores do tipo $GABA_B$, enquanto para $GABA_C$ ainda não existe nenhum composto utilizado clinicamente. Consequentemente os receptores $GABA_A$ são o principal alvo de fármacos que atuam na modulação GABAérgica (GOLAN et al., 2009).

Fármacos com afinidade por $GABA_A$ ligam-se a ele através de diferentes locais, incluindo o mesmo local de ligação de GABA ou sítios de modulação alostérica. Também há aqueles que atuam no canal iônico (Fig. 2). Contudo, o principal modo de ação de fármacos terapeuticamente importantes que atuam sobre os receptores $GABA_A$ é através da modulação alostérica. Estes fármacos ao se ligarem a sítios distintos dos de ligação de GABA potencializam as correntes de cloreto evocadas por GABA, causando hiperpolarização e consequentemente diminuindo a excitabilidade celular. A maioria dos fármacos usados como anestésicos gerais intravenosos atua dessa forma sobre os receptores $GABA_A$, mas ao contrário dos benzodiazepínicos, o sítio de ligação específico desses fármacos ainda permanece desconhecido. Tais anestésicos incluem barbitúricos como tiopental e pentobarbital, o etomidato e o propofol (JOHNSTON, 2005; RANG et al., 2016).

Há ainda muitas dúvidas sobre o real mecanismo de ação dos anestésicos gerais, pois diferente de outras classes de fármacos, a maioria não apresenta relação estrutural. Além disso, acredita-se que os anestésicos gerais atuam através de inúmeros mecanismos de ação que diferem entre um anestésico e outro (RANG et al., 2016). No caso do propofol, apesar de grande parte da literatura relatar que ele atua como um modulador alostérico positivo de receptores $GABA_A$, um estudo *in vitro* descreve sua capacidade de ativar correntes de cloreto mesmo na ausência de GABA (MOHAMMADI et al., 2001). Além disso, propofol *in vitro* interage com constituintes lipídicos de membranas biológicas, sugerindo que a sua atividade anestésica poderia ser o resultado combinado da sua interação com proteínas receptoras

específicas, neste caso os receptores $GABA_A$ e com as moléculas lipídicas que modulam a organização molecular do ambiente do receptor (REINER; LABUCKAS; GARCÍA, 2013; TSUCHIYA; MIZOGAMI, 2014). Estes estudos corroboram a teoria lipídica dos anestésicos, que foi uma das primeiras a surgir, uma vez que a única característica comum destes compostos é a lipossolubilidade. Contudo, hoje se sabe que este fator não é o único responsável pela ação dos anestésicos gerais (RANG et al., 2016). Adicionalmente, a descoberta de novos subtipos de $GABA_A$ e as subunidades constituintes têm fornecido informações importantes para um melhor entendimento sobre os mecanismos dos anestésicos gerais (OLSEN; LI, 2011).

Figura 2 – Principais locais propostos para ligação de GABA e substâncias que se ligam ao sítio benzodiazepínico nos receptores $GABA_A$.



Fonte: RANG et al. (2016)

1.4 COMPOSTOS NATURAIS COMO ANESTÉSICOS EM PEIXES

Estudos com objetivo de descobrir novos anestésicos para aquicultura cresceram muito nos últimos anos e estão direcionados a produtos de origem natural como óleos essenciais (OEs) e/ou seus compostos isolados. Frente ao jundiá os OEs de *Lippia alba, Ocimum gratissimum, Ocimum americanum, Ocotea acutifólia, Aloysia triphylla, Hesperozygis ringens* e *Cymbopogon flexuosus* obtiveram resultados satisfatórios como anestésicos (GRESSLER et al., 2014; HELDWEIN et al., 2012; SANTOS et al., 2016; SILVA et al., 2012; 2013; 2015; TONI et al., 2014). Contudo, para aprofundar os conhecimentos sobre os OEs é necessária a identificação e o

isolamento do(s) constituinte(s) responsável(s) pela(s) atividades(s) biológica(s). Uma vez que OEs são misturas complexas de constituintes em diferentes proporções (BAKKALI et al., 2008), o uso de substâncias isoladas promove atividades biológicas mais específicas, além de permitir estudos mais avançados, como os de mecanismo de ação. O eugenol e mentol são os compostos isolados já conhecidos por suas atividades anestésicas em espécies aquáticas (FAÇANHA GOMES, 2005; GONÇALVES et al., 2008; GUÉNETTE et al., 2012; PARODI et al., 2012; ROTILI et al., 2012) incluindo o jundiá (BECKER et al., 2011; CUNHA et al., 2010a). Outros trabalhos mais recentes também descrevem a atividade anestésica de (+)-espatulenol, (+)-dehidrofuquinona, R-(-)-linalol e S-(+)-linalol em jundiás (BENOVIT et al., 2015; GARLET et al., 2016; HELDWEIN et al., 2014; SILVA, 2014). Esses compostos são terpenoides, principal grupo de metabólitos secundários encontrado nos OEs, dos quais a maioria possui ações sobre o SNC (MANAYI et al., 2016; PASSOS et al., 2009).

1.5 MONOTERPENOIDES EM ESTUDO

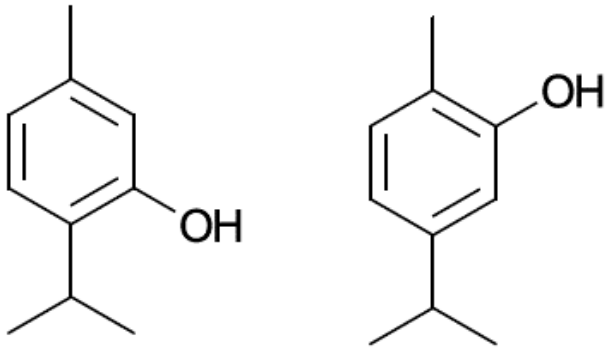
Terpenoides são moléculas oxigenadas cuja origem biossintética deriva da condensação de um número variável de unidades isoprênicas (C5). De acordo com o número de unidades de isopreno condensadas irão se originar monoterpenoides (C_{10}), sesquiterpenoides (C_{15}), diterpenoides (C_{20}) e triterpenoides (C_{30}). Contudo, são os monoterpenoides as moléculas mais representativas e constituem 90% dos óleos essenciais (BAKKALI et al., 2008). Dentre muitos dos monoterpenoides disponíveis na natureza com propriedades farmacológicas estão o timol, o carvacrol e o linalol, cujas características serão discutidas a seguir.

1.5.1 Timol e carvacrol: aspectos gerais

Timol (5-metil-2-(1-metiletil)-fenol) e carvacrol (2-metil-5-(1-metiletil)-fenol) (Fig. 3) são monoterpenoides biosintetizados a partir dos precursores ɣ-terpineno e *p*-cimeno. Estes dois compostos possuem a mesma fórmula química elementar, diferindo entre si pela posição do grupo hidroxila no anel fenólico, sendo por isso considerados isômeros de posição (LIMA et al., 2013). Eles estão presentes como metabólitos secundários de plantas aromáticas do gênero *Thymus* (TOHIDI; RAHIMMALEK; ARZANI, 2017) e em espécies como *Lippia sidoides* (CAVALCANTI et al., 2010; GUIMARÃES et al., 2014) e *Origanum vulgares*

(LICATA et al., 2015), entre outras. Ambos podem estar presentes de forma concomitante em um mesmo exemplar da espécie (TOHIDI; RAHIMMALEK; ARZANI, 2017) ou a espécie pode apresentar um quimiotipo definido (SILVA et al., 2013). Contudo, fatores como idade da planta, sazonalidade, índice pluviométrico e temperatura da região de cultivo podem influenciar a o conteúdo dos metabólitos secundários da planta (GOBBO-NETO; LOPES, 2007).

Figura 3 – Estrutura química do timol (esquerda) e do carvacrol (direita).



Fonte: Autor

1.5.2 Timol e carvacrol: atividades biológicas

Em vários estudos o timol e o carvacrol demostraram atuação sobre componentes do SNC. Em receptores $GABA_A$ de rato expressos em células HEK 293 o timol atua como agonista, induzindo correntes internas de cloreto sem a presença do agonista natural (GABA). Tal efeito foi atribuído ao grupo hidroxila ligado ao anel benzeno e grupo isopropila, substituinte alifático em posição *orto* presentes na estrutura química do timol. Estas mesmas características estruturais estão presentes na molécula de propofol, que foi utilizado como controle positivo no estudo (MOHAMMADI et al., 2001). O timol também atua como modulador positivo de receptores $GABA_A$ *in vitro* (GARCÍA et al., 2006; 2008), porém seu local de ação no complexo $GABA_A$ ainda não é bem conhecido, embora um estudo *in vitro* descarte o local de ação de benzodiazepínicos e esteroides (PRIESTLEY et al., 2003). Estudos mais recentes também descrevem a atividade do carvacrol sobre receptores $GABA_A$ *in vitro* (TONG; COATS, 2010; TRAILOVIĆ et al., 2015).

O fato de timol e carvacrol apresentarem afinidade pelos receptores $GABA_A$ contribui para muitas aplicações farmacológicas, algumas delas evidenciadas em modelos *in vivo*. Em

roedores, por exemplo, timol (50 e 100 mg/kg, i.p.) e carvacrol (200 mg/kg, i.p) apresentaram atividade anticonvulsivante em modelos de epilepsias induzidas por pentilenotetrazol e eletrochoques (QUINTANS-JÚNIOR et al., 2010; SANCHETI et al., 2014) e corroborando o estudo de Priestley et al. (2003), a ação anticonvulsivante do carvacrol não foi antagonizada pelo flumazenil, antagonista do sítio de ligação de benzodiazepínicos no receptor $GABA_A$ (QUINTANS-JÚNIOR et al., 2010). Contudo, resultados contrastantes foram descritos por Melo et al. (2010), os quais relataram que o efeito ansiolítico do carvacrol (12.5, 25 e 50 mg/kg, v.o.) em camundongos foi antagonizado na presença de flumazenil.

Timol e carvacrol também parecem atuar no sistema nervoso periférico, pois a literatura contém informações sobre a atuação de ambos sobre os canais de Na^+ operados por voltagem. Estes canais iônicos são importantes no controle da excitabilidade celular e são o principal alvo de agentes utilizados como anestésicos locais (RANG et al., 2016). Em neurônios do gânglio da raiz dorsal isolados de ratos o carvacrol inibe a geração do potencial de ação devido o bloqueio dos canais de Na^+ voltagem-dependentes (JOCA et al., 2012). Da mesma forma timol e carvacrol inibiram os potenciais de ação compostos (CAPs) em nervo ciático de rãs (KAWASAKI et al., 2013). Devido a essas atividades ambos foram sugeridos para aplicação como anestésicos locais pelos autores

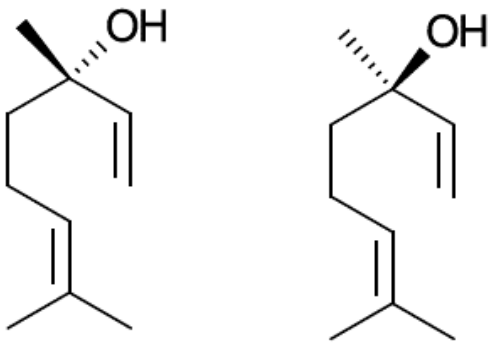
Outra atividade farmacológica já descrita para timol e carvacrol é a inibição da atividade da enzima acetilcolinesterase (AChE). Essa enzima é responsável por hidrolisar a acetilcolina (ACh) nas sinapses colinérgicas. Segundo Jukic et al. (2007), o carvacrol é 10 vezes mais potente que o timol em estudo de inibição *in vitro* e sugerem que a posição do grupo hidroxila na sua estrutura molecular desempenha papel crucial para o efeito inibitório da AChE. Além disso, substâncias com atividade anticolinesterásica têm potencial para o tratamento de doenças neurológicas como a doença de Alzheimer (RANG et al., 2016). Contudo, em peixes a exposição a compostos que inibem a atividade da AChE pode causar contrações musculares e nado errático, além de prejudicar a alimentação, fuga e comportamento reprodutivo (DUTTA; ARENDS, 2003). A AChE está presente em grande quantidade no tecido muscular e cerebral de peixes e consequentemente, funções exercidas por esses tecidos serão prejudicadas em caso de desregulação da função normal desta enzima (BRETAUD; TOUTANT; SAGLIO, 2000). Devido a esses fatores, além do fato de vários terpenoides apresentarem ação sobre a atividade da AChE (LóPEZ et al., 2015), torna-se importante a investigação da atividade enzimática da AChE em peixes expostos a estes compostos.

Na aquicultura, até o momento timol e carvacrol foram empregados apenas como aditivos na ração de peixes. A adição de timol e carvacrol na ração (6 e 12 g/kg de ração, respectivamente) de truta arco-íris (*Oncorhynchus mykiss*) exerceu efeito benéfico na conversão alimentar, na microbiota intestinal e em parâmetros antioxidante (GIANNENAS et al., 2012). Parâmetros de crescimento e imunidade também melhoraram em esturjão-branco (*Huso huso*) alimentado com dieta suplementada com esses compostos (AHMADIFAR et al., 2014).

1.5.3 Linalol: aspectos gerais

O linalol é um álcool monoterpênico presente no OE de diversas espécies de plantas aromáticas na forma de enantiômeros: S-(+)-linalol e R-(-)-linalol (Fig. 4). O enantiômero R-(-)-linalol puro pode ser encontrado em diferentes proporções no OE de algumas plantas do gênero *Origanum* (*O. basilicum, O. floribundum, O. majorana* e *O. onites*) e *Thymus* (*T. migricus, T. revolutus, T. sibthorpii* e *T. zygioides*) e também em outras espécies como *Achillea grandifolia* e *Ocimum basilicum.* O S-(+)-linalol está presente no OE de plantas do gênero *Nepeta* (*N. cadmea, N. cataria, N. conferta, N. flavida* e *N. italica*) (ÖZEK et al., 2010), além de ser constituinte majoritário do OE de partes aéreas de *Lippia alba* cultivada no sul do Brasil e na Índia (CHANOTIYA; YADAV, 2009; HELDWEIN, et al., 2012). No entanto, em algumas plantas a presença dos dois enantiômeros ocorre simultaneamente. *Aniba rosaeodora*, por exemplo, apresenta as duas formas quirais do linalol, porém a porcentagem de cada um difere entre as partes da planta, sendo que a casca, o caule e raiz apresentam somente R-(-)-linalol (CHANTRAINE; DHÉNIN; MORETTI, 2009).

Figura 4 – Estrutura química do S-(+)-linalol (esquerda) e R-(-)-linalol (direita).



Fonte: Autor

1.5.4 Linalol: atividades biológicas

Estudos farmacológicos com linalol descrevem uma variedade de atividades farmacológicas, principalmente sobre o SNC (APROTOSOAIE et al., 2014). Contudo, grande parte destes estudos é referentes à mistura racêmica de linalol (*R*-*S*-(±)-linalol) ou ao isômero R-(-)-linalol. Atividade sedativa em humanos e roedores de *R*-*S*-(±)-linalol e R-(-)-linalol foi evidenciado em vários estudos. Contudo, em humanos o enantiômero S-(+)-linalol causou efeito contrário (LINCK et al, 2009; SUGAWARA at al., 1998; KURODA et al., 2005). Estes autores concluíram que a atividade sedativa do linalol em humanos é influenciada pela sua quiralidade, mas não descrevem possíveis mecanismos de ação envolvidos.

O linalol em concentrações baixas também promove sedação em peixes, enquanto concentrações mais elevadas provocam indução anestésica. Em carpa comum (*Cyprinus carpio*) a natureza enantiomérica do linalol e o(s) mecanismo(s) de ação envolvido(s) não foram descritos (MIRGHAED; GHELICHPOUR; HOSEINI, et al., 2016). Em jundiás o S-(+)-linalol promove sedação e anestesia por mecanismos que não envolvem a afinidade pelo sítio benzodiazepínico do complexo $GABA_A$ (HELDWEIN et al., 2014), contudo a interação com receptores $GABA_A$ por outros locais de ligação ainda não foi investigado. Adicionalmente, o R-(-)-linalol também promoveu sedação e anestesia nesta espécie, mas seu mecanismo de ação não é conhecido (SILVA, 2014).

A atividade anestésica local de linalol foi relatada primeiramente por Ghelardini et al. (1999). Logo após, foi descoberto que o linalol inibe a liberação de acetilcolina e altera a cinética de abertura do canal na junção neuromuscular de ratos, mecanismo responsável em parte pela atividade anestésica local (RE et al., 2000). Linalol também bloqueou potenciais de ação em estudo *ex vivo* (ZALACHORAS et al., 2010), que segundo Leal-Cardoso et al. (2010), ocorre devido ao bloqueio dos canais de sódio voltagem-dependentes. Porém, nenhum destes estudos especifica a natureza enantiomérica do linalol. Mais recentemente, Venâncio et al. (2016) descreveram o potencial do R-(-)-linalol em inibir a excitabilidade de nervo ciático de ratos, o que condiz com outros resultados já descritos na literatura. Contudo, pouco se sabe sobre a atividade anestésica local do S-(+)-linalol.

Estudos com linalol indicam seu potencial como ansiolítico. Em camundongos a inalação de linalol a 3% (racemato ou enantiômero não identificado) foi responsável pela atividade ansiolítica no teste de claro/escuro, além de diminuir o comportamento agressivo dos animais, efeito semelhante ao diazepam (LINCK et al., 2010). Cheng et al. (2015) também enfatizam o uso do linalol no tratamento de transtornos de ansiedade. Nesse estudo

foi constatado que ambos enantiômeros, R-(-)-linalol e S-(+)-linalol, após administração oral de 500 mg/kg em camundongos por 14 dias consecutivos produziram potente atividade ansiolítica sem alteração das atividades locomotoras nos modelos de campo aberto, claro/escuro e labirinto em cruz elevado. Além disso, os autores relacionaram a atividade ansiolítica dos isômeros de linalol à diminuição nos níveis de monoaminas (serotonina, dopamina e norepinefrina) em regiões do cérebro dos camundongos tratados com R-(-)-linalol e S-(+)-linalol.

Atividade anticonvulsivante do linalol é atribuída principalmente à ação sobre a transmissão glutamatérgica. Em estudo *in vitro* o R-S-(±)-linalol inibiu por antagonismo competitivo a ligação de [^{3}H] glutamato e [^{3}H] MK801 às membranas do córtex cerebral de ratos, assim como *in vivo* retardou convulsões induzidas por *N*-metil-*D*-aspartato (NMDA) e bloqueou aquelas causadas por ácido quinolínico em camundongos (ELISABETSKY; SILVA-BRUM; SOUZA, 1999; SILVA-BRUM; ELISABETSKY; SOUZA, 2001). A atividade anticonvulsivante dos enantiômeros R-(-)-linalol e S-(+)-linalol também foi evidenciado em modelo de epilepsia induzida por picrotoxina, porém o R-S-(±)-linalol foi mais potente, o pode ser resultado de uma sinergia (efeito aditivo ou potenciação) entre as duas formas quirais do linalol (SOUSA et al., 2010).

A ação antinociceptiva e anti-hiperalgésica do R-(-)-linalol, investigada através de diferentes modelos de dor, foi atribuída a mecanismos que envolvem a ativação de receptores opioides, muscarínicos M2, dopaminérgicos D2, canais de potássio sensíveis ao ATP e receptores A1 e A2 de adenosina (PEANA et al., 2003; 2004; 2006a). Outros mecanismos prováveis são a inibição da formação de óxido nítrico (PEANA et al., 2006b) e inibição de receptores ionotrópicos de glutamato (α-amino-3-hidroxi-metil-5-4-isoxazolpropionato (AMPA), NMDA e kainato) (BATISTA et al., 2008). Sabe-se também que o R-(-)-linalol foi eficaz em inibir o comportamento nociceptivo de camundongos após a administração intratecal de mediadores inflamatórios (IL-1β e TNF-a) (BATISTA et al., 2010). A mistura racêmica de linalol também foi eficaz em reduzir nocicepção, hiperalgesia e alodinia. Estas atividades foram antagonizadas pelo metionato de naloxona, um antagonista μ-opioide periférico (KATSUYAMA et al., 2012; 2015; SAKURADA et al., 2011). Outro mecanismo descrito para a atenuação da alodinia por R-S-(±)-linalol é via inibição da fosforilação da proteína quinase (ERK) (KUWAHATA et al., 2013). Por fim, não foram encontrados relatos sobre atividade antinociceptiva do enantiômero S-(+)-linalol isolado.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial de diferentes monoterpenoides como sedativos e/ou anestésicos e a influência sobre na atividade da acetilcolinesterase (AChE) e no sistema GABAérgico de jundiás (*Rhamdia quelen*).

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o potencial do timol e carvacrol como sedativos e anestésicos em jundiás;

- Determinar a atividade da enzima acetilcolinesterase (AChE) no músculo e cérebro de jundiás expostos ao timol, carvacrol e S-(+)-linalol;

- Investigar o possível envolvimento de monoterpenoides que apresentarem atividade anestésica frente à espécie estudada com receptores $GABA_A$ e com o sítio benzodiazepínico do complexo $GABA_A$.

3 MANUSCRITO

O manuscrito “Monoterpenoids (thymol, carvacrol and S-(+)-linalool) with anesthetic activity in silver catfish (*Rhamdia quelen*): evaluation of AChE and GABAergic activity” foi submetido ao periódico **Brazilian Journal of Medical and Biological Research.**

Monoterpenoids (thymol, carvacrol and S-(+)-linalool) with anesthetic activity in silver catfish (*Rhamdia quelen*): evaluation of acetylcholinesterase and GABAergic activity

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Abstract

The objective of this study was to evaluate the anesthetic potential of thymol and carvacrol, and their influence on acetylcholinesterase (AChE) activity in the muscle and brain of silver catfish (*Rhamdia quelen*). The AChE activity of S-(+)-linalool was also evaluated. We subsequently assessed the effects of thymol and S-(+)-linalool on the GABAergic system. Fish were exposed to thymol and carvacrol (25, 50, 75 and 100 mg/L) to evaluate time for anesthesia and recovery. Both compounds induced sedation at 25 mg/L and anesthesia with 50–100 mg L^{-1}. However, fish exposed to carvacrol presented strong muscle contractions and mortality. AChE activity was increased about two-fold in the brain of fish exposed to 50 mg/L carvacrol and about three-fold at 100 mg/L thymol, however AChE activity decreased (about five-fold) in the muscle of silver catfish exposed to 100 mg/L carvacrol. S-(+)-linalool did not alter AChE activity. Anesthesia with thymol was reversed by exposure to picrotoxin ($GABA_A$ antagonist), similar to the positive control propofol, but was not reversed by flumazenil (antagonist of benzodiazepine binding site), as observed for the positive control diazepam. Picrotoxin did not reverse the effect of S-(+)-linalool. Thymol 50 mg/L is more suitable than carvacrol for anesthesia in silver catfish, as we observed good anesthetic performance and no interference with AChE activity. Its anesthetic effect appeared to involve the $GABA_A$ receptors, but was not related to the benzodiazepine site. Therefore, the anesthetic effect of S-(+)-linalool in silver catfish does not appear to involve the $GABA_A$ receptors.

Key words: AChE; Anesthesia; Benzodiazepine; $GABA_A$; terpenoids.

Running title: Interaction of monoterpenoids with the $GABA_A$ receptor.

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Introduction

Anesthetics have many applications in aquaculture, used to improve animal welfare during and after management practices, during transport, and also for surgery. The most commonly used anesthetics for fish are tricaine methanesulfonate (MS-222), benzocaine, metomidate, 2-phenoxyethanol and quinaldine. However, undesirable effects due to the use of these anesthetics have been reported (1). Studies have investigated the use of essential oils (EOs) from plants and their isolated compounds, with the objective of finding new safe and effective anesthetics with fewer side effects (2,3).

One important characteristic of EOs related to their effectivity as anesthetics is their lipophilic properties, as high lipid solubility contributes to rapid diffusion through biological membranes (1). Furthermore, EOs are primarily made up of terpenoids, substances whose pharmacological activities on the central nervous system (CNS) are frequently described (4). Such activities are connected with the ability of terpenes to cross the blood-brain barrier, where they modulate brain function (5).

Monoterpenoids (C_{10}) are the predominant constituents found in EOs (6). Among these, thymol and carvacrol isomers (Figure 1) are found in the EOs of plants including *Origanum vulgare* (7) and *Lippia sidoides* (8). Both isomeric forms have demonstrated anticonvulsant activity in mice, mediated by GABA (ɣ-aminobutyric acid) receptor activity, the main inhibitory neurotransmitter in the brain (9). These data suggest that there may be a possible sedative and/or anesthetic activity of thymol and carvacrol in fish via a mechanism similar to that reported in rodents, as modulation of $GABA_A$ is frequently involved in the mechanism of action for anesthetics. For example, the anesthetic propofol is a widely used $GABA_A$ agonist for inducing anesthesia in human and veterinary medicine, which also has proven effects in fish (10). Furthermore, EOs of *L. sidoides* containing the thymol and carvacrol chemotypes have been reported to act as anesthetics in silver catfish (*Rhamdia quelen*), which further strengthens this hypothesis (8).

Another monoterpenoids that deserves attention is linalool, as recent studies have suggested a potential anesthetic activity in fish (3,11). Linalool is present in EOs from aromatic plants in both enantiomeric forms, such as EOs derived from *Ocimum basilicum* L.

(69.54% R-(-)-linalool) (12) and *Lippia alba* (59.66% S-(+)-linalool, Figure 1) (13). However, the biological activities and mechanism of action of S-(+)-linalool are poorly understood. S-(+)-linalool acts as a sedative and anesthetic in silver catfish, but these activity are not associated with the benzodiazepine site of $GABA_A$ receptors (11). No studies have investigated whether S-(+)-linalool interacts with other $GABA_A$ sites, which has encouraged further investigation.

Some monoterpenoids have the ability to positively or negatively modulate the enzymatic activity of acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine (ACh) (14). In fish, inhibition of AChE results in the accumulation of ACh in nervous terminations, and consequently, hyperstimulation of muscarinic and nicotinic receptors, which can cause muscle contractions and erratic swimming, in addition to impaired feeding and reproduction (15). Therefore, studies evaluating AChE activity in fish exposed to these compounds are useful for identifying any potential negative effects associated with the modulation of AChE activity.

The objectives of this study were to evaluate the anesthetic potential of thymol and carvacrol, their action on $GABA_A$ receptors, and their influence on the activity of AChE in silver catfish. We also investigated the mechanism of action for the anesthetic effect of S-(+)-linalool on $GABA_A$ receptors and the influence on AChE activity, as this monoterpenoid has been proposed as a promising anesthetic for use in this species (11).

Materials and methods

Animals

Juvenile silver catfish were obtained from a fish farm in Cruz Alta (southern Brazil) and transported to the Fish Physiology Laboratory at the Federal University of Santa Maria (UFSM). Fish were allowed to acclimatize for 1 week in continuously aerated 250 L tanks prior to the experiments. Dissolved oxygen and temperature (oximeter 550A; YSI, Yellow Springs, OH, USA), pH (microprocessor pH meter, AT-315; Alfakit, Florianópolis, Brazil) and total ammonia (16) were monitored daily, and maintained within the recommended values for this species (temperature 19.76 ± 0.11°C, pH 7.15 ± 0.12, dissolved oxygen 6.86 ± 0.33 mg/L and total ammonia 0.12 ± 0.02 mg/L). Animals were fed to satiation with commercial feed (42% crude protein, Supra®) once per day, and fasted for 24 h before the experiments. Each animal was used only once. The protocol was approved by the Ethics and Animal Welfare Committee of UFSM (process no. 074/2014).

Essential oil (EO) extraction and S-(+)-linalool isolation

S-(+)-linalool was isolated from the EO of *Lippia alba*, which was cultivated in the UFSM campus in Frederico Westphalen, Brazil (Santa Maria Departamento de Biologia voucher no. 10050, Department of Biology, UFSM). The EO extraction was performed by steam distillation using fresh leaves, using a modified Clevenger apparatus for 3 h, according to the method described by the European Pharmacopoeia (17). The EO was stored in a sealed amber glass vial at -4°C until the isolation process. The isolation of S-(+)-linalool from EO was performed using column chromatography (CC). Compound identification and purity was evaluated by gas chromatography-mass spectrometry (GC-MS), as described by Heldwein et al. (11).

Commercially obtained drugs

The thymol and carvacrol isomers (≥ 99.0 purity) used for the anesthetic induction tests were purchased from Sigma-Aldrich, Brazil. Picrotoxin (Sigma-Aldrich, Brazil), a blocker of the $GABA_A$ receptor type, and propofol (Propotil®, Biochimico, Brazil), a $GABA_A$ agonist anesthetic, were included in the protocol in order to evaluate the sedative and anesthetic effects of the studied compounds on the $GABA_A$ receptor type. Diazepam (Uni-Diazepax®, Chemical Union, Brazil) and flumazenil (Flamazil®, Cristália Ltda., Brazil), selective agonist and antagonist, respectively, of the benzodiazepine site of the $GABA_A$ receptor, were used to evaluate the anesthetic effect of thymol on benzodiazepine receptors (18). Prior to the experiments, S-(+)-linalool, carvacrol and thymol were diluted in 95% ethanol (1:10), and picrotoxin was diluted in Tween 80 (0.033% in water). Propofol, diazepam and flumazenil have good solubility in water, and did not require solubilization prior to the experiments.

Experiment 1: Induction of anesthesia and recovery

Silver catfish juveniles (n = 6 for each concentration and compound; 12.84 ± 0.34 g and 10.5 ± 0.22 cm) were exposed to 25, 50, 75 or 100 mg/L thymol or carvacrol in an aquarium filled with 1 L water. The anesthetic induction stages were evaluated as described by Gomes et al. (19): stage (S) 2, sedation characterized by the loss of response to external stimuli, determined by hitting the bottom of the aquarium with a glass rod; S3a, characterized by a total loss of balance, with fish able to maintain their swimming ability; S3b, total loss of equilibrium and swimming ability, with fish still responsive to application of pressure on the

caudal peduncle using a glass rod; and S4, deep anesthesia, in which fish do not respond to any external stimuli. Evaluation was carried out until the animals reached the anesthesia stage, or after a maximum period of 30 min. Fish were then placed in anesthetic-free aquaria until complete recovery, or a maximum period of 30 min. The animals were considered recovered once they had demonstrated normal swimming capacity and equilibrium, and also responded to stimulation from a glass rod hitting the aquarium bottom. Survival of the animals was assessed 48 h after the experiment.

Experiment 2: Acetylcholinesterase (AChE) activity

As muscle contractions were observed in experiment 1, it was necessary to evaluate the AChE activity in the brain and muscle of silver catfish exposed to monoterpenoid with anesthetic properties used in the studies.

Silver catfish juveniles (n = 6 each concentration and compound; 9.51 ± 0.32 g and 10.0 ± 0.25 cm) were exposed to 50 or 100 mg/L of thymol or carvacrol and 153 mg/L of S-(+)-linalool for 10 min (mean time for anesthesia). These fish were then euthanized by spinal cord section to collect the brain and muscle. Samples from the control group (exposed to water only) and from fish exposed to the highest ethanol concentration (1377 mg/L; used to dilute the compounds) were also collected. Enzymatic activity and the protein concentration were measured as described by Golombieski et al. (20). The enzyme activity was expressed as μmol of acetylthiocholine (ASCh) hydrolyzed per milligram of protein per minute.

Experiment 3: Evaluation of activity on $GABA_A$ receptors

Juvenile silver catfish (8.86 ± 0.42 g and 9.47 ± 0.17 cm) were exposed to S-(+)-linalool, thymol or propofol (n = 16 per group) at their respective anesthetic (S4) concentrations (153, 50 and 2.5 mg/L). The mechanism of action of carvacrol was not investigated because it did not show good performance as an anesthetic (see results). After induction of anesthesia, the animals were transferred to aquaria with anesthetic-free water (n = 8) or to aquaria containing 100 mg/L picrotoxin (n = 8) to measure their partial recovery (response of fish to pressure stimulus on the caudal peduncle with a glass rod) and total recovery (normal swimming with response to external stimulus) time. The maximum observation time was 30 min. The concentrations of propofol and S-(+)-linalool used in this experiment were selected according to Gressler et al. (10) and Heldwein et al. (11), respectively, and the choice of thymol and picrotoxin concentrations were in accordance with preliminary tests.

To ensure that picrotoxin did not itself present a stimulatory effect, silver catfish (n = 4) were exposed to picrotoxin (100 mg/L) or water for 30 min. No distinct behavioral differences were observed between groups.

Experiment 4: Evaluation of activity on the benzodiazepine site of $GABA_A$ receptors

This experiment evaluated the involvement of the benzodiazepine site on $GABA_A$ receptors in the anesthetic effect of thymol. Fish (11.63 ± 0.42 g and 9.85 ± 0.10 cm) were anesthetized with 50 mg/L thymol or 42 mg/L diazepam (n = 16 per group). Fish were then placed in an anesthetic-free aquarium (n = 8) or in an aquarium containing 3.0 mg/L flumazenil (n = 8) to recover. The partial and total recovery times for each animal were evaluated as described in experiment 3. The concentrations of diazepam and flumazenil used in this experiment were selected according to Garlet et al. (25).

Statistical analysis

The data were submitted to Levene's test to determine homogeneity of variances, then one- or two-way ANOVA was performed, and where appropriate, followed by Tukey's post hoc test. Kruskal-Wallis ANOVA by ranks was used for nonparametric data obtained for brain AChE activity (thymol 50 and 100 mg/L). The tests were performed using Statistica software (version 11.0), and the minimum significance level for all analyses was 95% ($p < 0.05$). Data are reported as mean ± SEM.

Results

Experiment 1: Induction of anesthesia and recovery

No mortality was detected 48 h after exposure to thymol, whereas carvacrol caused 50, 33, 33 and 16% mortality during the first 24 h after exposure to 25, 50, 75 and 100 mg/L, respectively. Involuntary muscle contractions were observed in animals exposed to all concentrations of carvacrol. The same was observed for exposure to all concentrations of thymol, but with a reduced frequency and intensity when compared to carvacrol.

Regression analysis showed a concentration-response relationship for thymol and carvacrol for all stages of anesthetic induction, but not for recovery (Table 1). Carvacrol induced S2 (all concentrations), S3a and S3b (25 and 50 mg/L) stages faster than thymol. However, exposure to thymol at 100 mg/L induced deep anesthesia (S4) significantly faster than carvacrol. Only fish exposed to 50 mg/L carvacrol recovered within 30 min, and the

recovery time was significantly longer than recovery from anesthesia with the same concentration of thymol. On the other hand, fish anesthetized with 100 mg/L thymol did not completely recover within 30 min (Table 1).

Experiment 2: Acetylcholinesterase (AChE) activity

Exposure to ethanol and S-(+)-linalool (153 mg/L) did not significantly alter AChE activity compared to the control group for the analyzed tissues (brain and muscle). Fish anesthetized with 50 mg/L carvacrol and 100 mg/L thymol showed significantly higher brain AChE activity compared to the control group. In the muscle, only 100 mg/L carvacrol decreased AChE activity compared to the control group (Figure 2).

Experiment 3: Evaluation of activity on $GABA_A$ receptors

Partial and total recovery of animals anesthetized with propofol was significantly faster with picrotoxin than water, validating the protocol used. The same was observed for the partial and total recovery of fish exposed to thymol. Picrotoxin did not alter the recovery of fish anesthetized with S-(+)-linalool when compared to water control group (Figure 3).

Experiment 4: Evaluation of activity on the benzodiazepine site of $GABA_A$ receptors

Fish anesthetized with diazepam showed significantly faster partial and total recovery in the flumazenil recovery bath when compared with the water control group. However, the recovery of fish anesthetized with thymol was not affected by flumazenil (Figure 4).

Discussion

Anesthesia

Thymol and carvacrol were found to be sedatives (25 mg/L) and anesthetics (50–100 mg/L) in silver catfish. These results were expected, as the EOs of *L. sidoides* from the thymol and carvacrol chemotypes (containing 68.40% and 67.89% of these compounds, respectively) have been previously reported to induce anesthesia in silver catfish (8). In addition, Brito and Brito (21) reported the popular use of the EO of *L. sidoides* thymol chemotype as a local anesthetic and sedative. Moreover, recent studies with rodents have reported the anxiolytic and antidepressant action of carvacrol (22,23) and anticonvulsant activity of thymol (24).

Anesthesia was induced in silver catfish with 50 mg/L thymol and carvacrol, and the time of induction and recovery was lower when compared to other terpenoids, such as (+)-dehydrofukinone (50 mg/L) and (+)-spathulenol (51.2 mg/L), as evaluated in the same species (2, 25). Thymol and carvacrol were found to be more potent than S-(+)-linalool, as only 50 mg/L of both compounds was required to induce the S4 stage, whereas fish exposed to 51 mg/L S-(+)-linalool had previously been reported to only reach the S3a stage (11). In common carp (*Cyprinus carpio*), terpenoids such as menthol, myrcene and linalool (enantiomeric form not specified) were also reported to act as anesthetics at concentrations ≥ 50 mg/L (50, 150 and 200 mg/L, respectively) (3,26).

Exposure to 25 mg/L thymol for 30 minutes only induced sedation, indicating that this concentration (or lower) may be suitable for use in fish transport. The sedation of fish prior to transportation is carried out to reduce stress and physiological responses that are detrimental to animal welfare (27). For anesthesia, 100 mg/L thymol has been reported to induce anesthesia in less than 3 minutes (the optimal time for anesthesia according to Gilderhus and Marking (28)), however, the total recovery exceeded 30 minutes. Prolonged recovery has also been reported for other anesthetics (25), and may be related to slow clearance. Therefore, intermediate concentrations (50 and 75 mg/L) have a higher cost-benefit as they are associated with faster recovery.

Compared to thymol, carvacrol induced sedation in a shorter time, especially at concentrations of 25 and 50 mg/L. However, the side effects of carvacrol observed during (strong muscular contractions) and after anesthesia (mortality) and long recovery time prevent its application as an anesthetic. These results corroborate those of Silva et al. (8) in silver catfish anesthetized with the EO of *L. sidoides* containing thymol and carvacrol. However, the muscle contractions occurred independent of the concentration used, and therefore, they are not a determining factor for carvacrol mortality. Furthermore, there was no mortality associated with thymol exposure, despite fish presenting mild contractions.

Interestingly, the mortality induced by carvacrol exposure decreased with increasing concentrations. At lower concentrations, fish required a longer period of carvacrol exposure to reach the desired stage, which may have contributed to the increased mortality observed. The long exposure period, combined with the high lipid solubility of anesthetics, facilitates accumulation in lipophilic compartments such as biological membranes and adipose tissue, thereby hindering their elimination (1).

4.2 Acetylcholinesterase (AChE) activity

The observation of muscle contractions in fish is attributed to the anticholinesterase activity of some compounds (15). However, in this study only 100 mg/L carvacrol was found to reduce AChE activity in muscle, contrary to what was expected, as muscle contractions were observed during anesthesia at all concentrations tested. Thymol, which also caused muscle contractions, but with reduced intensity and frequency than carvacrol, did not inhibit AChE activity at any concentration tested. Therefore, it appears that the muscle contractions induced by thymol and carvacrol involve a mechanism unrelated to AChE inhibition. Studies in the literature have reported inhibition of AChE activity *in vitro* by thymol and carvacrol, however, the activity of carvacrol was found to be 10-fold more potent than thymol (29). This difference in potency for inhibition of the enzyme could explain why thymol did not inhibit AChE activity *in vivo* at the concentrations tested. Consequently, thymol may be a safer anesthetic than carvacrol. Similarly, our observation that exposure to S-(+)-linalool at an anesthetic concentration did not alter AChE activity emphasizes its efficacy as an anesthetic.

Unlike the muscle, AChE activity in the brain was increased in silver catfish exposed to 50 and 100 mg/L of carvacrol and thymol, respectively. This result is rather curious, although similar results have been reported for other compounds. A study by López et al. (14) showed that *in vitro* administration of compounds in the same class, such as ɣ-terminene, geraniol and camphor, activated AChE at a lower concentration (0.04 mM), whereas a higher concentration (5 mM) caused inhibition. Therefore, we can infer that the influence of monoterpenoids on AChE activity is likely to be concentration- and tissue-dependent.

4.3 Mechanism of action for thymol and S-(+)-linalool

$GABA_A$ receptors are ligand-regulated ion channels responsible for mediating rapid inhibitory synapses. Activation of $GABA_A$ produces CNS depression, involved in the mechanism of action of anxiolytics, sedative-hypnotics, anticonvulsants and anesthetics (18). The interaction of thymol and S-(+)-linalool with $GABA_A$ receptors was assessed in silver catfish by placing them in water containing picrotoxin ($GABA_A$ receptor channel blocker) in order to evaluate recovery from sedation and/or anesthesia. Picrotoxin has convulsant properties, so it is only used for research purposes to induce seizures or antagonize the effect of $GABA_A$ agonists (30).

Silver catfish that were anesthetized with thymol recovered faster in a picrotoxin bath when compared to the water control group. Likewise, the group anesthetized with propofol (positive control) also showed a reduced recovery time when placed in a picrotoxin bath.

However, recovery from anesthesia induced by S-(+)-linalool was not altered by picrotoxin. The antagonistic effect of picrotoxin following thymol-induced anesthesia supports the proposed interaction of thymol with $GABA_A$ receptors, but does not indicate a specific site of action within this receptor. The results observed for thymol in the current study corroborate that previously reported in *in vitro* studies (31,32).

Interaction with the $GABA_A$ receptor can also occur through the $GABA_A$/benzodiazepine binding site (18). These binding sites are known to modulate the affinity of the $GABA_A$ receptor for GABA, increasing Cl^- influx through the channel (5). Interactions with the $GABA_A$/benzodiazepine site have been previously evaluated for EOs and their isolated compounds with sedative and anesthetic activities in silver catfish (13,25). To assess the affinity of thymol for the $GABA_A$/benzodiazepine binding site, we evaluated the effect of flumazenil, a competitive antagonist of this binding site (18). We did not observe any interaction between thymol and the $GABA_A$/benzodiazepine site, as recovery from thymol-induced anesthesia was unaffected by flumazenil. This is in agreement with results from a study that evaluated human $GABA_A$ receptors expressed in *Xenopus laevis* oocytes, in which the behavior of thymol was similar to a flumazenil-insensitive positive allosteric modulator at the $GABA_A$/benzodiazepine site (33). Thymol also showed direct action (Cl^- currents induced in the absence of GABA) on the $GABA_A$ receptors of HEK 293 cells, similar to the effect of propofol (34).

In contrast to results previously observed for thymol, our results did not indicate an interaction of S-(+)-linalool with $GABA_A$ receptors. The interaction of S-(+)-linalool with the $GABA_A$/benzodiazepine site in silver catfish had been disregarded in a previous study (11). Our results are similar to those reported by Silva Brum et al. (35), who suggested that the anticonvulsant activity of the racemic mixture of linalool did not result from interaction with $GABA_A$ receptors. Instead, this compound is believed to interact with *N*-metil-D-aspartate (NMDA) receptors, although other mechanisms related to GABA release and absorption cannot be disregarded. Additionally, the antinociceptive activity of R-(-)-linalool in rodents has been attributed to its action on opioid, cholinergic (36), dopaminergic and glutamatergic systems (37). According to Leal-Cardoso et al. (38) the main mechanism by which linalool (enantiomeric form not specified) affects neuronal excitability is through the inhibition of voltage-regulated sodium channels and consequently blocking the action potentials.

The biological effects of linalool are related to different mechanisms of action, however, only Heldwein et al. (11) has reported the mechanism of action involved in the sedative and anesthetic activity for the enantiomer S-(+)-linalool to date. Other studies have

described the effects of the racemic mixture, R-(-)-linalool, or did not specify the enantiomer used (39). It is noteworthy that the affinity and interaction with receptors, as well as the intensity of the biological effect, depends on the specificity of binding of the molecule to the receptor. Enantiomers may differ from each other in their pharmacodynamic and pharmacokinetic processes, therefore, the correct identification of these compounds is important (40).

In conclusion, thymol and carvacrol induced sedative and anesthetic activities in silver catfish at the same concentrations, however, carvacrol is not recommended as an anesthetic for fish due to the high rates of mortality after exposure. Thymol interacts with $GABA_A$ receptors, but not with the $GABA_A$/benzodiazepine site. In contrast, S-(+)-linalool does not appear to interact with the $GABA_A$ receptors in fish.

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Figure captions

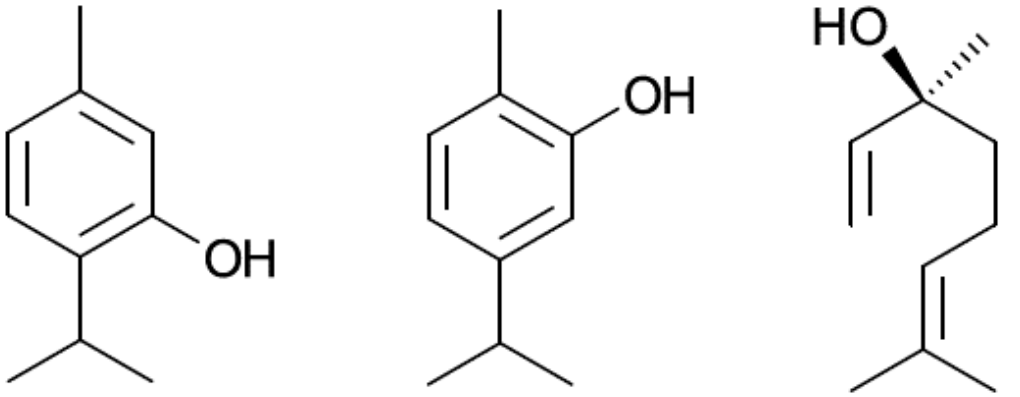
Figure 1. Chemical structures of the evaluated monoterpenoids.

Figure 2. Acetylcholinesterase (AChE) activity in the brain and muscle of juvenile silver catfish (*Rhamdia quelen*) anesthetized with thymol and carvacrol (50 and 100 mg/L) and S-(+)-linalool (153 mg/L). * Significantly different compared to the control (water) group in the same tissue, as determined by one-way ANOVA and Tukey's test or Kruskal-Wallis ANOVA by ranks ($p < 0.05$; $n = 6$).

Figure 3. Time required for recovery (partial and total) in water and picrotoxin (100 mg/L) of silver catfish juveniles (*Rhamdia quelen*) anesthetized with propofol (2.5 mg/L), S-(+)-linalool (153 mg/L) and thymol (50 mg/L). * Significantly different compared to the group that recovered in water, as determined by two-way ANOVA and Tukey's test ($p < 0.05$; $n = 8$).

Figure 4. Time required for recovery (partial and total) in water and flumazenil (3 mg/L) of silver catfish juveniles (*Rhamdia quelen*) anesthetized with diazepam (42 mg/L) and thymol (50 mg/L). * Significantly different compared to the group that recovered in water, as determined by two-way ANOVA and Tukey's test ($p < 0.05$; $n = 8$).

Figure 1



Thymol Carvacrol S-(+)-linalool

Figure 2

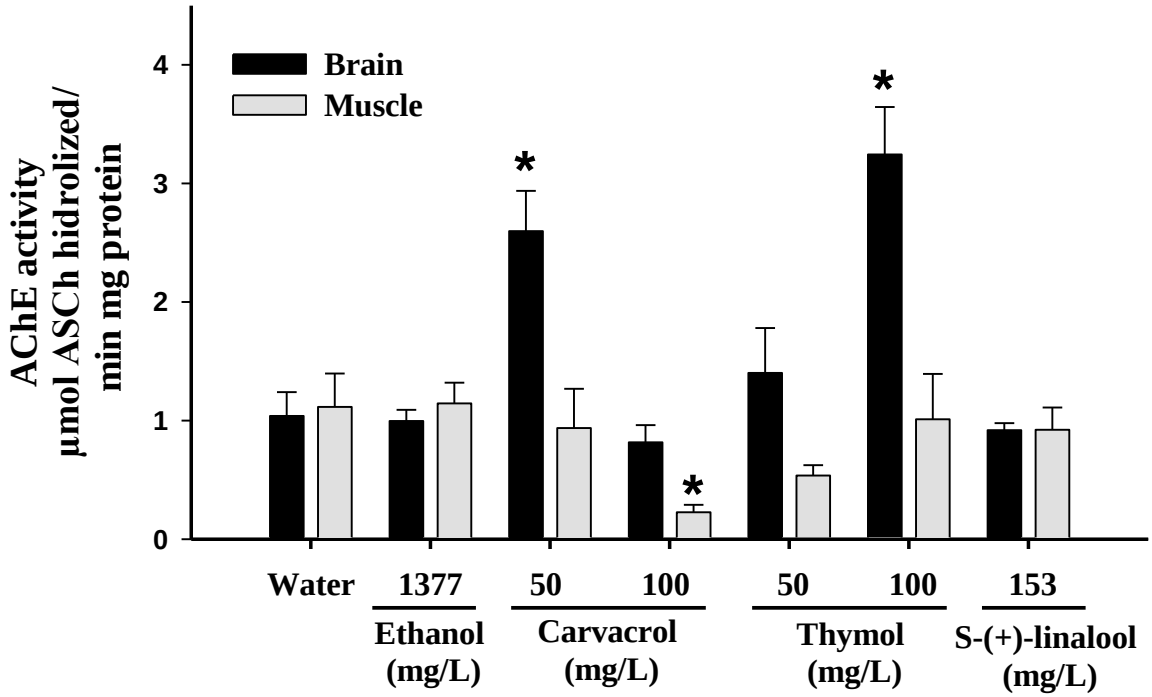


Figure 3

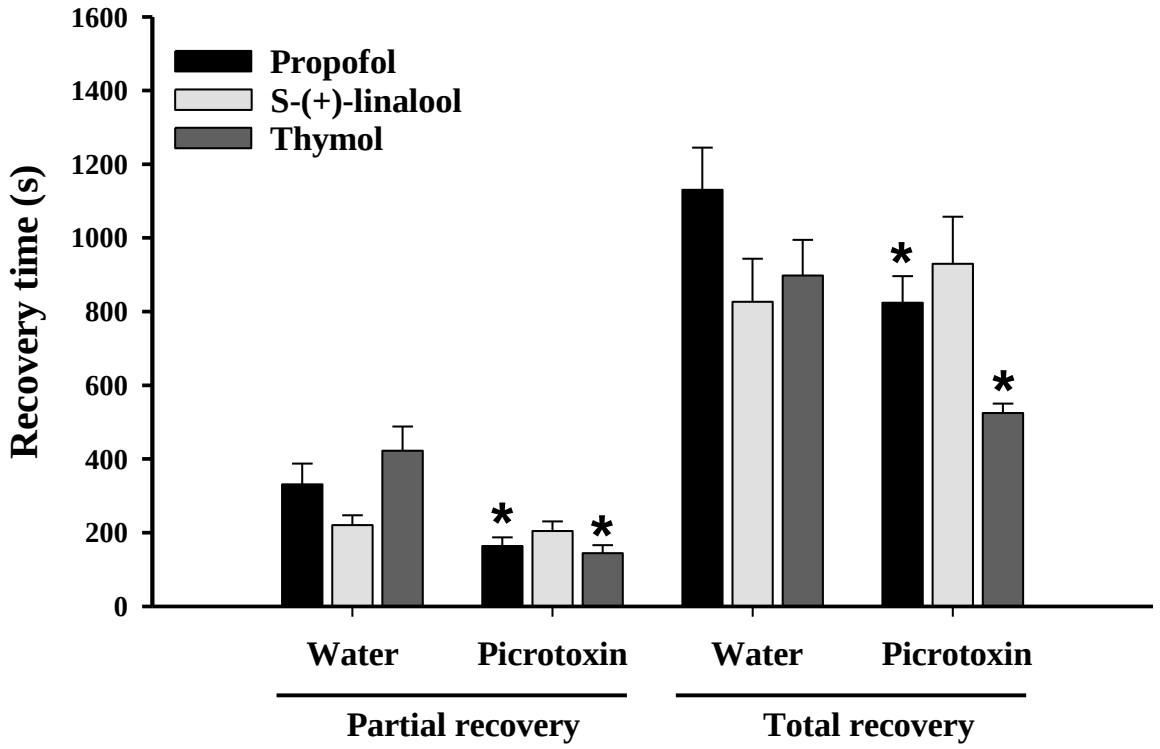


Figure 4

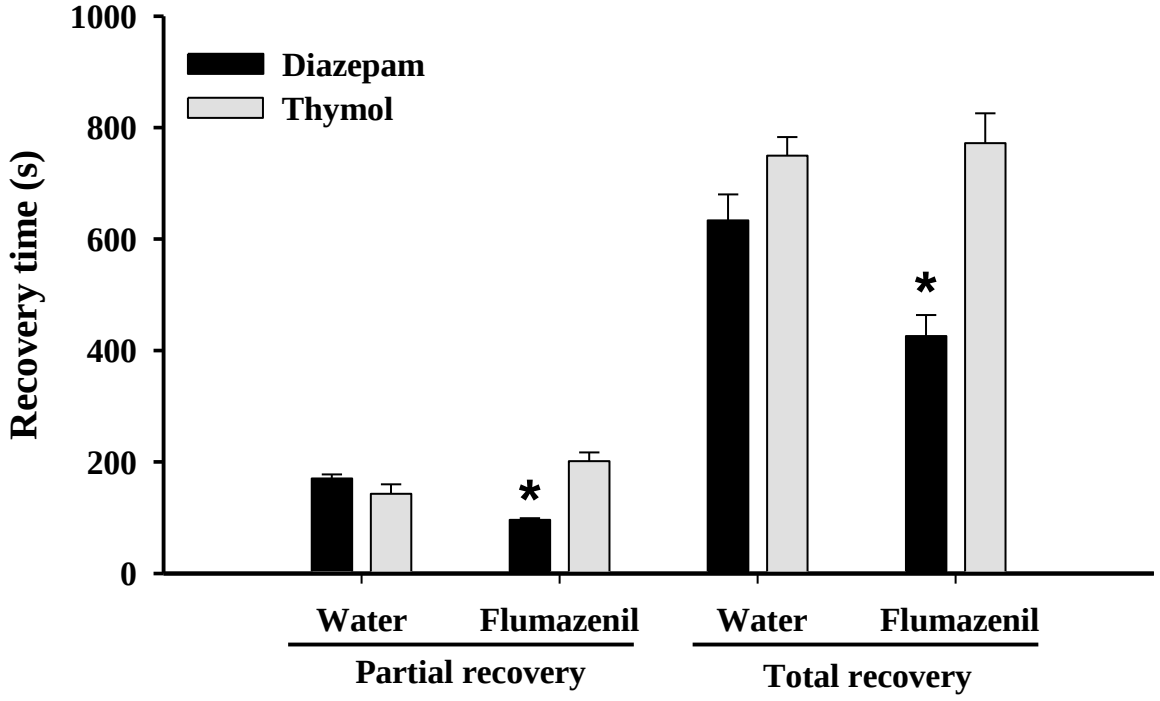


Table 1. Time required for induction and recovery from anesthesia with thymol and carvacrol in juvenile silver catfish (*Rhamdia quelen*).

Concentration (mg/L)	Stages (time until induction, s)				
	S2	S3a	S3b	S4	Recovery
Thymol					
25	109.66 ± 2.23	319.66 ± 46.23	611.00 ± 37.65	> 1800	1649.00 ± 47.78
50	55.66 ± 3.68	86.67 ± 6.78	284.00 ± 23.96	491.50 ± 10.49	919.67 ± 122.55
75	20.00 ± 1.41	40.83 ± 5.13	175.00 ± 14.68	373.33 ± 15.85	1375.67 ± 150.86
100	14.33 ± 2.40	28.50 ± 2.84	55.80 ± 7.16	170.00 ± 11.87	> 1800
Equation	$y = 190.75 - 3.70x + 0.0193x^2$ $R^2 = 0.98$	$y = 624.58 - 14.71x + 0.0883x^2$ $R^2 = 0.82$	$y = 1384.20 - 42.89x + 0.5395x^2 - 0.0024x^3$ $R^2 = 0.94$	$y = 472.33 + 3.79x - 0.0681x^2$ $R^2 = 0.95$	
Carvacrol					
25	58.83 ± 1.85*	74.00 ± 1.73 *	352.33 ± 22.56*	> 1800	> 1800
50	33.50 ± 2.86*	51.33 ± 3.29 *	76.75 ± 2.72*	524.67 ± 36.09	1580.83 ± 81.69*
75	12.33 ± 0.88*	30.83 ± 1.94	195.00 ± 11.05	386.33 ± 9.26	> 1800
100	8.67 ± 0.88*	24.67 ± 1.33	70.17 ± 3.61	299.17 ± 13.42*	> 1800
Equation	$y = 98.33 - 1.77x + 0.0087x^2$ $R^2 = 0.95$	$y = 75.00 - 0.29x - 0.018x^2 + 0.0001x^3$ $R^2 = 0.96$	$y = 1655.16 - 81.14x + 1.33x^2 - 0.0068x^3$ $R^2 = 0.95$	$y = 954.83 - 10.65x + 0.0409x^2$ $R^2 = 0.77$	

* Significantly different from thymol at the same concentration at the same induction stage, as determined by one-way ANOVA and Tukey's test ($p < 0.05$; $n = 6$). In the equations, x represents the concentration of the compound (mg/L), and y represents the time taken to reach the stage of induction or recovery from anesthesia (s).

4 CONCLUSÕES

- Timol e carvacrol possuem atividade sedativa (25 mg/L) e anestésica (50, 75, 100 mg/L) em jundiás, mas devido à mortalidade causada pelo carvacrol em todas as concentrações testadas, seu uso como anestésico em peixes é desaconselhado;

- A mortalidade causada por carvacrol parece estar relacionada com o maior tempo de exposição e não com o aumento da concentração;

- Timol e carvacrol causaram contrações musculares involuntárias durante a indução e recuperação da anestesia. Contudo as contrações causadas por carvacrol foram mais pronunciadas;

- S-(+)-linalol não altera a atividade enzimática da AChE no músculo e cérebro de jundiás anestesiados com 153 mg/L. Timol (100 mg/L) e carvacrol (50 mg/L) aumentaram a atividade da AChE no cérebro e carvacrol (100 mg/L) diminuiu a atividade da AChE no músculo. Desta forma a concentração de timol 50 mg/L é mais indicada para anestesia em jundiás;

- A inibição da atividade da AChE no músculo e a ativação no cérebro foi concentração-dependente, enquanto as contrações musculares involuntárias não.

- O mecanismo de ação anestésica de S-(+)-linalol em jundiás não envolve a interação com receptores $GABA_A$;

- Timol apresentou interação com receptores $GABA_A$ na anestesia de jundiás, porém essa interação não ocorre através do sítio benzodiazepínico.

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