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ÓLEOS ESSENCIAIS DE PLANTAS COMO ALTERNATIVA AOS FÁRMACOS CONVENCIONAIS NA PRODUÇÃO DE PEIXES

Santa Maria, RS
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Tese de Doutorado apresentada ao Curso de Doutorado do 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 **Doutor em Farmacologia**

Orientador: Prof. Dr. Bernardo Baldisserotto

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RESUMO

ÓLEOS ESSENCIAIS DE PLANTAS COMO ALTERNATIVA AOS FÁRMACOS CONVENCIONAIS NA PRODUÇÃO DE PEIXES

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Os óleos essenciais (OE) obtidos de plantas são uma grande fonte de novas moléculas e vêm sendo estudados como alternativas aos fármacos convencionais no controle de enfermidades, como moduladores da resposta imune e como novos agentes promotores de crescimento em peixes. O objetivo deste estudo foi avaliar o potencial de aplicação de diferentes OEs, provenientes das plantas *Ocimum americanum* (OA), *Ocimum gratissimum* (OG), *Hesperozygis ringens* (HR), *Cymbopogon flexuosus* (CF) e *Melaleuca alternifolia* (MA) no controle de doenças e como imunomoduladores e promotores de crescimento em peixes das espécies *Rhamdia quelen* e *Sciaenops ocellatus*. Os OEs de OA, OG e HR apresentaram atividade fraca a moderada *in vitro* contra a bactéria *A. hydrophila*, no entanto, inibiram significativamente a atividade hemolítica provocada pela bactéria em eritrócitos de peixes. O OE de OA também apresentou atividade antiparasitária *in vitro* e *in vivo* contra o parasito monogenético *Gyrodactylus* sp. *In vitro*, os OEs de OA, CF e MA estimularam a produção de ânion superóxido (O_2^-) em leucócitos de *S. ocellatus* (macrófagos renais e/ou leucócitos do sangue). O uso *in vivo* dos OEs de OA e HR promoveu a sobrevivência de *R. quelen* infectados por *A. hydrophila*, bem como, apresentou potencial modulador da resposta imune inata em ambas as espécies de peixes estudadas: após exposição por meio de banhos (*R. quelen*) ou adicionado à dieta (OA em *S. ocellatus*). Fitoterápicos tais como os OEs se mostram como alternativas seguras na produção de peixes por serem produtos naturais e biodegradáveis contribuindo na redução da utilização de fármacos convencionais e pesticidas.

Palavras-chave: Fitoterápico. Bactéria. Parasito. Imunidade. Crescimento. Piscicultura.

ABSTRACT

PLANT ESSENTIAL OILS AS AN ALTERNATIVE TO CONVENTIONAL DRUGS IN FISH PRODUCTION

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The essential oils (EO) obtained from plants are a major source of new molecules and have been studied as alternatives to conventional drugs in the treatment of diseases, as immune response modulators and as new growth promoters in fish. The aim of this study was to evaluate the potential use of different EOs from the plants *Ocimum americanum* (OA), *Ocimum gratissimum* (OG), *Hesperozygis ringens* (HR), *Cymbopogon flexuosus* (CF) and *Melaleuca alternifolia* (MA) in the control of diseases, as immunomodulators and as growth promoters in the fish species *Rhamdia quelen* and *Sciaenops ocellatus*. The EOs from OA, OG and HR showed weak to moderate activity *in vitro* against the bacterium *A. hydrophila*, however, they significantly inhibited the hemolytic activity caused by this bacterium in fish erythrocytes. The EO of OA also presented antiparasitic activity *in vitro* and *in vivo* against the monogenean parasite *Gyrodactylus* sp. The EOs from OA, CF and MA were capable of triggering (*in vitro*) superoxide anion (O_2^-) production in *S. ocellatus* leukocytes (head-kidney macrophages and/or blood leukocytes). The use of the OA and HR EOs *in vivo* promoted survival of *R. quelen* infected with *A. hydrophila*, as well as showed potential as modulator of the innate immune response in both studied fish species: after exposure through baths (*R. quelen*) or added to the diet (OA in *S. ocellatus*). Herbal products such as EOs appear as safe alternatives in fish production because they are natural and biodegradable products, contributing to the reduced use of conventional drugs and pesticides.

Keywords: Phytotherapic. Bacteria. Parasite. Immunity. Growth. Fish Culture.

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1. INTRODUÇÃO

1.1. ÓLEOS ESSENCIAIS

Os óleos essenciais (OE) são compostos naturais, voláteis e complexos caracterizados por um forte odor, sendo sintetizados por plantas aromáticas durante o metabolismo secundário. Apresentam-se na forma líquida, de coloração límpida, solúveis em lipídeos e solventes orgânicos e geralmente apresentam densidade inferior à da água. Eles podem ser sintetizados por todas as partes da planta, ou seja, botões, flores, folhas, caules, ramos, sementes, frutas, raízes, casca e madeira. São armazenados em células secretoras, cavidades, canais, células epidérmicas ou tricomas glandulares (BAKKALI et al., 2008). Como os compostos ativos presentes nos OEs são originados a partir dos metabólitos das plantas, a sua composição química pode variar conforme a parte da planta, o grau de desenvolvimento, o horário do dia ou época do ano e o ambiente onde tais plantas se encontram (BARROS et al., 2009).

Os OEs são misturas naturais muito complexas que podem conter dezenas de componentes em diferentes concentrações. Normalmente os OEs são caracterizados por dois ou três constituintes principais em concentrações relativamente elevadas (20-70%) em comparação com outros componentes presentes em quantidades vestigiais (BAKKALI et al., 2008). Geralmente, estes constituintes principais determinam as propriedades biológicas dos OEs. No entanto, devido à diversidade de moléculas, a bioatividade destes produtos pode ser resultado do sinergismo ou efeitos aditivos das moléculas constituintes. As moléculas que compõem os OEs incluem dois grupos de origem biosintética distinta; o grupo principal é composto por terpenos e terpenóides e o outro por constituintes aromáticos e alifáticos, todos caracterizados por baixo peso molecular (CARSON; HAMMER, 2011). Devido a sua grande disponibilidade e diversidade química, muitos OEs têm sido relatados como alternativas eficazes como complementos de compostos sintéticos na indústria química, para aplicação em saúde humana, agricultura e meio ambiente (CARSON; RILEY, 2003). Estes produtos apresentam grande potencial de utilização na aquicultura devido a uma série de efeitos biológicos benéficos já descritos em várias espécies de animais aquáticos, tais como a promoção do crescimento e estimulação do apetite, imunomodulação, propriedades antibacterianas, antiparasitárias, anestésicas e antiestresse (CHAKRABORTY et al., 2014; CITARASU, 2010; HARIKRISHNAN et al., 2011; SILVA et al., 2013).

1.2. APLICAÇÃO DOS ÓLEOS ESSENCIAIS NA PRODUÇÃO DE PEIXES

Vários fármacos e produtos veterinários são frequentemente utilizados no cultivo de peixes no intuito de evitar perdas econômicas relacionadas a problemas sanitários. Antimicrobianos, pesticidas e outros medicamentos são usados como profiláticos, na terapêutica ou como promotores de crescimento em peixes, administrados regularmente como aditivos em alimentos ou por meio de banhos e injeções (RICO et al., 2013). No entanto, a indústria aquícola está sob pressão para diminuir o uso destes produtos, devido ao risco causado aos seres humanos, resíduos em alimentos e ocorrência de resistência microbiana. A utilização de medicamentos veterinários é cada vez mais restrita, uma vez que eles apresentam numerosos efeitos secundários e nocivos para o ambiente e a segurança da saúde animal e humana (BULFON et al., 2015; REVERTER et al., 2014).

Considerando os potenciais danos de tratamentos com medicamentos veterinários na aquicultura e, em alguns casos, sua eficácia limitada, a gestão sanitária deve concentrar-se em métodos menos nocivos, preventivos e duradouros (REVERTER et al., 2014). Consequentemente, esforços estão sendo feitos no intuito de explorar os fitoterápicos, como por exemplo os OEs e/ou seus componentes isolados como possíveis alternativas aos fármacos convencionais. Especial atenção é dada às informações sobre os efeitos destes extrativos vegetais no crescimento dos peixes, perfis hematológicos, às respostas imunológicas e tratamento de doenças (BULFON et al., 2015; CHAKRABORTY et al., 2014; HARIKRISHNAN et al., 2011).

1.3. ESPÉCIES ESTUDADAS

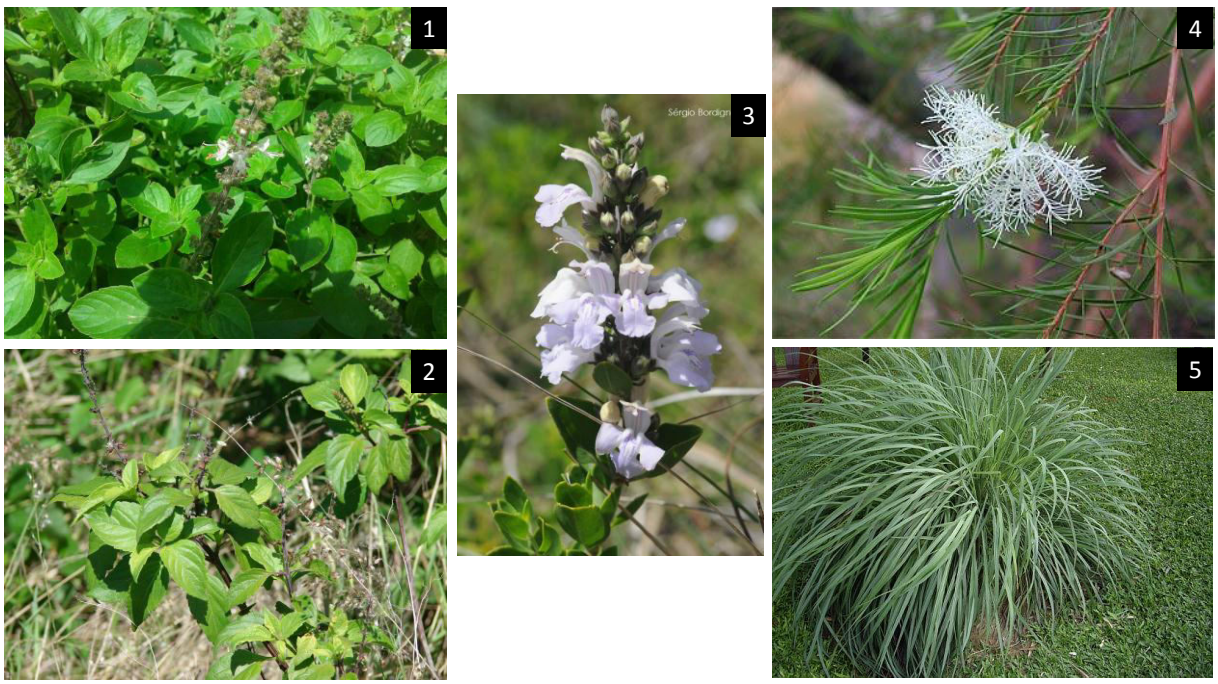
1.3.1. Espécies vegetais

Ocimum americanum (Fig. 1-1), conhecida popularmente como manjericão-branco ou alfavaca-do-campo, é uma planta nativa da África e da Ásia amplamente distribuída em regiões tropicais e subtropicais, muitas vezes utilizada como uma planta medicinal (PATON et al., 1999). Atividades antifúngica e antibacteriana foram relatadas para o OE desta planta (CIMANGA et al., 2002; THAWEBOON; THAWEBOON, 2009), bem como atividade repelente de insetos (SEYOUM et al., 2002). *Ocimum gratissimum* (Fig. 1-2), conhecida popularmente como alfavaca-cravo ou simplesmente manjericão, é uma planta amplamente utilizada na medicina tradicional e como tempero na culinária. Seu OE apresenta atividade

anti-helmíntica, antifúngica e antibacteriana (PESSOA et al., 2002; PRABHU et al., 2009). Atividade anestésica em peixes foi relatada para o OE de ambas as espécies de *Ocimum* estudadas (SILVA et al., 2012; 2015).

Hesperozygis ringens (Fig.1-3) (nome popular: "espanta-pulga") é uma planta lenhosa endêmica no sul do Brasil, conhecida por suas propriedades antiparasitárias e inseticidas (RIBEIRO et al., 2010; VON POSER et al., 1996). Atividade anestésica e sedativa em peixes também foi relatada para o OE desta planta (SILVA et al., 2013; TONI et al., 2014, 2015). *Melaleuca alternifolia* (Fig. 1-4), conhecida como "Tea tree" ou árvore-do-chá, é uma planta nativa da Austrália empregada como medicamento para várias doenças, em grande parte devido às suas propriedades antimicrobianas. Seu OE é incorporado como ingrediente ativo em muitas formulações tópicas utilizadas para o tratamento de infecções cutâneas (CARSON et al., 2006). *Cymbopogon flexuosus* (Fig. 1-5) popularmente conhecida como capim-limão, é uma gramínea tropical cultivada principalmente para extração do seu OE (AKHILA, 2009), empregado nas indústrias médica e cosmética. Seu OE apresenta propriedades biológicas importantes tais como atividade anti-cancerígena e antimicrobiana (ADUKWU et al., 2012; SHARMA et al., 2009).

Figura 1 – Espécies vegetais estudadas: *Ocimum americanum* (1), *Ocimum gratissimum* (2), *Hesperozygis ringens* (3), *Melaleuca alternifolia* (4) e *Cymbopogon flexuosus* (5).



Fonte: 1 - o autor, 2 - disponível em: http://www.onlyfoods.net, 3 - Sérgio Bordignon, disponível em: http://www.ufrgs.br/fitoecologia, 4 - disponível em: http://www.terapiacomaromas.com.br, 5 - disponível em: http://www.hydroponics.eu.

1.3.2. Espécies de peixes

Jundiá, *Rhamdia quelen* (Fig. 2-1), é um peixe de água doce de grande importância econômica no Sul do Brasil. É uma espécie nativa que responde facilmente à reprodução induzida e está bem adaptada para o cultivo, com alta taxa de fertilização, mostrando um crescimento rápido, com boa aceitação no mercado consumidor (BALDISSEROTTO; RADÜNZ NETO, 2004). "Red drum", *Sciaenops ocellatus* (Fig. 2-2), é um peixe marinho encontrado na costa da América do Norte, do golfo do México ao norte dos Estados Unidos. É uma espécie economicamente importante no sudeste dos Estados Unidos, apresentando características desejáveis, como a carne saborosa e crescimento relativamente rápido, sendo também importante na pesca esportiva (CHENG et al., 2011; DAVIS, 1990).

Figura 2 – Espécies de peixes: *Rhamdia quelen* (1), *Sciaenops ocellatus* (2).

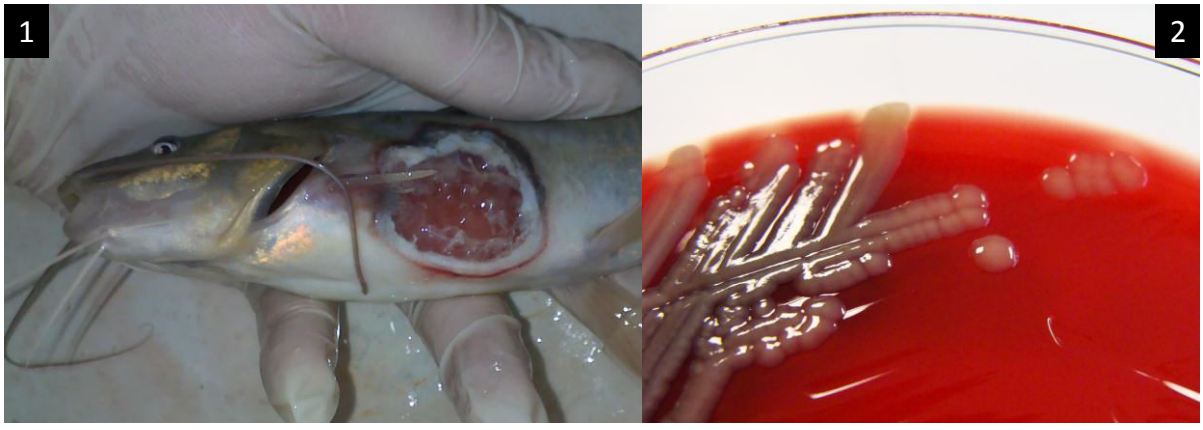
Fonte: 1 - o autor, 2 - Leonard Lovshin, disponível em http://www.fishbase.org.

1.3.3. Espécie bacteriana: *Aeromonas hydrophila*

O gênero *Aeromonas* é essencialmente onipresente na biosfera microbiana, podendo ser isolado a partir de praticamente todos os nichos ambientais em ecossistemas bacterianos existentes. Estes incluem habitats aquáticos, peixes, alimentos, animais domésticos, espécies de invertebrados, aves, insetos, carrapatos, e os solos naturais. O gênero é considerado não só como um importante patógeno causador de doenças de peixes e outras espécies ectotérmicas, mas também como o agente etiológico responsável por uma variedade de complicações infecciosas, como em pessoas imunocompetentes e imunocomprometidas (JANDA; ABBOTT, 2010).

Normalmente considerado um agente patogênico secundário, *A. hydrophila* (Fig. 3) também pode agir como um agente patogênico primário em alguns ambientes, causando elevada mortalidade em explorações piscícolas (NIELSEN et al., 2001). No cultivo de peixes os surtos causados por *A. hydrophila* estão associados a mudanças nas condições ambientais e/ou fatores de estresse, como infecções parasitárias (BARCELLOS et al., 2008). Sua patogênese é multifatorial e depende da secreção de inúmeros fatores extracelulares que influenciam na virulência bacteriana (YU et al., 2004). Hemolisinas, amilases, quitinases, elastases, aerolisinas, nucleases, gelatinases, lecitinases, lipases e proteases são produtos extracelulares biologicamente ativos de espécies de *Aeromonas* que promovem sua virulência (PEMBERTON et al., 1997).

Figura 3 – Jundiá apresentando lesão causada por *A. hydrophila* (1), Cultivo de *A. hydrophila* em meio ágar sangue (2).

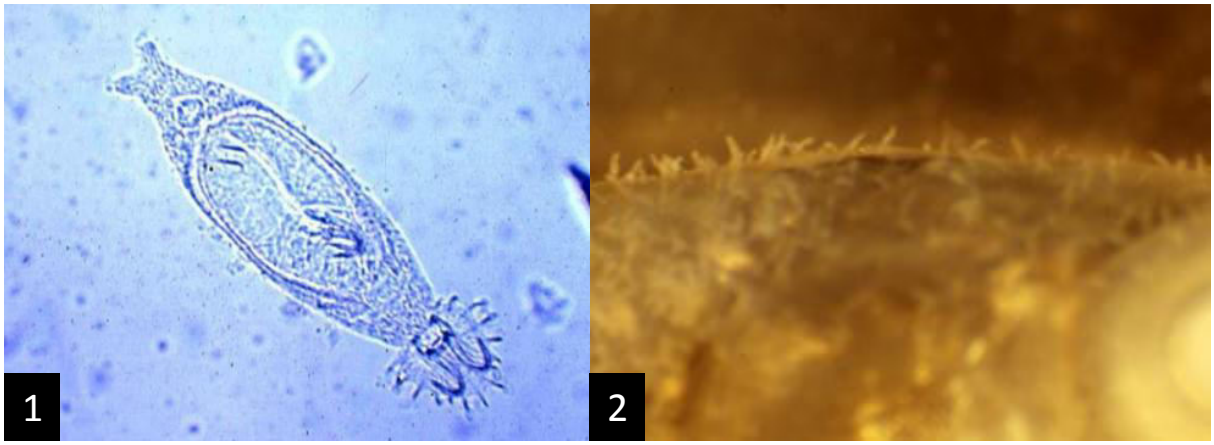


Fonte: 1 - o autor, 2 - Nathan Reading, disponível em http://www.flickr.com.

1.3.4. Parasito: *Gyrodactylus* sp.

As infecções causadas por helmintos monogenéticos, principalmente os pertencentes às famílias Dactylogyridae e Gyrodactylidae, são comuns em fazendas de peixes e estão entre as parasitoses mais importantes para a aquicultura. Monogenéticos são parasitos com alta especificidade de hospedeiro e comumente encontrados na pele e brânquias de peixes marinhos e de água doce (BOEGER; VIANNA, 2006). *Gyrodactylus* (Fig. 4) é um gênero diverso com centenas de espécies descritas. Estes parasitos se alimentam principalmente de muco e células do hospedeiro e ocasionalmente de sangue (CONE, 1995). Em altos níveis de infecção podem causar a morte do hospedeiro. Em casos de baixa carga parasitária podem atuar como um vetor mecânico de outros patógenos, como vírus e bactérias, levando a infecções secundárias e/ou problemas osmóticos associados com os ferimentos provocados durante a fixação e a alimentação (PAVANELLI et al., 2008).

Figura 4 – Parasito adulto: *Gyrodactylus* sp. (1), *Gyrodactylus* sp. parasitando uma carpa (2).



Fonte: 1 - Craig Banner, disponível em: http://fishpathogens.net, 2 - disponível em: http://www.vetbook.org.

1.4. ÓLEOS ESSENCIAIS COMO ANTIBACTERIANOS E ANTIPARASITÁRIOS

Óleos essenciais têm uma aplicação potencial como antibacterianos e antiparasitários na cultura de organismos aquáticos, principalmente pela fácil obtenção, relativo baixo custo, por agirem contra um amplo espectro de patógenos e por comumente apresentarem mais de um modo de ação (GALINA et al., 2009; NAZZARO et al., 2013). As moléculas encontradas em OEs podem atuar diretamente sobre a bactéria, causando a lise da célula, potencializar a

atividade antibacteriana de outra substância e inibir mecanismos de resistência e fatores de virulência bacterianos (HARRIS, 2002; STAVRI et al., 2007), como por exemplo, bloqueando a síntese de proteínas e de DNA, inibindo secreções de enzimas e interferindo no mecanismo bacteriano de sinalização via *quorum sensing* (CITARASU, 2010). No entanto, a maior parte dos efeitos descritos envolve interações com membranas biológicas, tais como alterações morfológicas e ruptura de membrana com extravasamento citoplasmático (DEVI et al., 2010; SIKKEMA et al., 1995; XU et al., 2008). OEs e seus componentes podem alterar a atividade de bombas de efluxo, proteínas da membrana bacteriana que facilitam o bombeamento rápido do fármaco para fora da célula muito antes deste atingir a concentração desejada e exercer seus efeitos (WALSH, 2000), apresentando assim, potencial no desenvolvimento de novos fármacos contra estirpes multirresistentes (Fig. 5) (STAVRI et al., 2007, YAP et al., 2014).

Vários estudos, principalmente ensaios *in vitro,* têm mostrado o potencial de uso dos OEs de plantas contra bactérias importantes na aquicultura. O OE de *Cymbopogon nardus* apresentou potencial de uso contra *Edwardsiella* spp., *Vibrio* spp., *Aeromonas* spp., *Escherichia coli*, *Salmonella* spp., *Flavobacterium* spp., *Pseudomonas* spp. e *Streptococcus* spp. isolados a partir de órgãos internos de 10 diferentes espécies de animais aquáticos (WEI; WEE, 2013). STARLIPER et al. (2015) mostraram que diferentes espécies de *Aeromonas* (*A. salmonicida, A. hydrophila* e *A. veronii*), agentes patogênicos comuns em peixes, foram sensíveis aos OEs de *Cinnamomum cassia*, *Origanum vulgare* e *Cymbopogon citratus in vitro*. OE de *Zataria multiflora* em concentrações subinibitórias suprimiu a expressão dos genes responsáveis pela codificação de proteínas, fibronectina e hemolisina, fatores de virulência conhecidos de *Lactococcus garviae* (SOLTANI et al., 2015). Os OEs de *Rosmarinus officinalis* e *Z. multiflora* foram eficazes na redução da expressão do gene da estreptolisina, importante fator de virulência do *Streptococcus iniae*, isolados a partir de truta arco-íris, *Oncorhynchus mykiss*, além de diminuir a hemólise provocada pela bactéria (SOLTANI et al., 2014). O OE de *Thymus vulgaris* reduziu significativamente a formação de biofilme em *A. hydrophila* (MILLEZI et al., 2013).

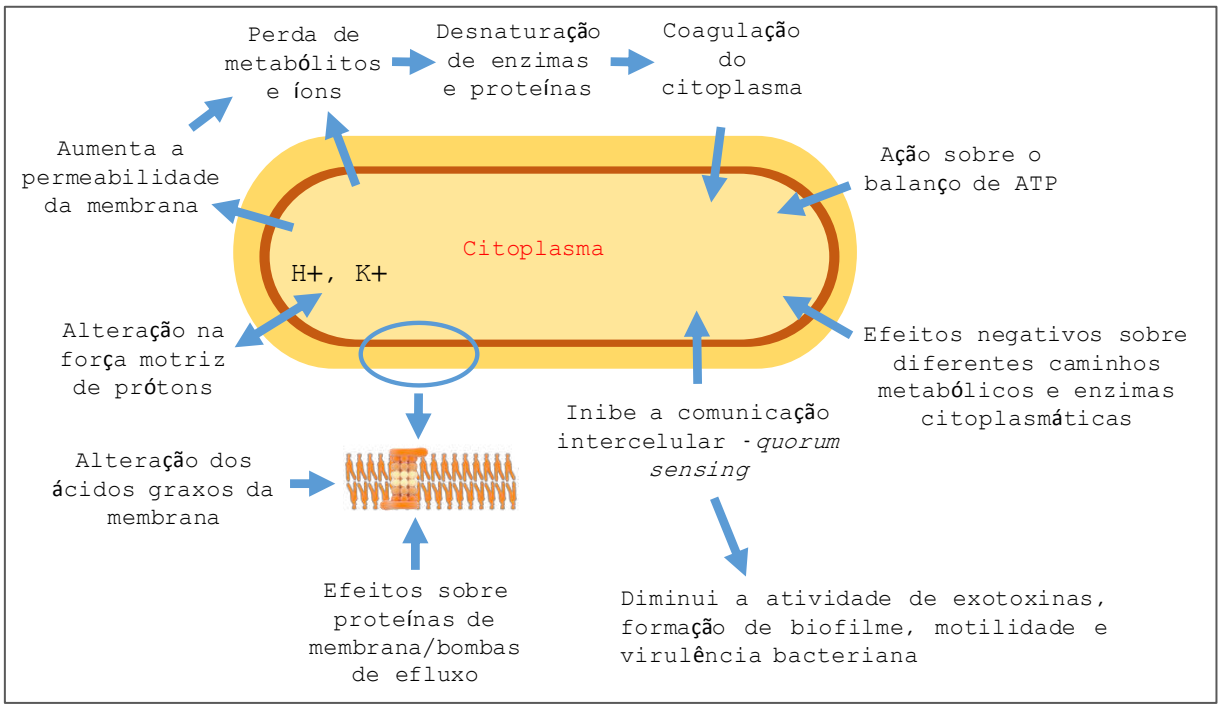
In vivo, a aplicação de OEs e/ou seus compostos isolados como medida profilática ou terapêutica no combate a doenças bacterianas em organismos aquáticos pode ser realizada, principalmente, de duas formas: através de banhos ou por incorporação na ração (SACCOL et al., 2013; SUTILI et al., 2014, 2015). OE de cravo (*Syzygium aromaticum*) adicionado à dieta (3%) promoveu a sobrevivência de tilápias nilóticas (*Oreochromis niloticus*) experimentalmente infectadas com *Lactococcus garviae*. Peixes tratados com o OE

apresentaram sobrevivência similar ao grupo tratado com o antibiótico oxitetraciclina (RATTANACHAIKUNSOPON; PHUMKHACHORN, 2009). Tratamento com banhos diários (1h/5dias) com eugenol (10 mg/L), principal composto do óleo de cravo, promoveu a sobrevivência de jundiás infectados experimentalmente com *A. hydrophila* (SUTILI et al., 2014). Óleo essencial de *Lippia alba* adicionado à água nas concentrações 16 e 40 mg/L promoveu a sobrevivência de jundiás naturalmente infectados com *Aeromonas* sp. (SUTILI et al., 2015). Em ambos os trabalhos a sobrevivência dos peixes nas concentrações citadas não diferiram dos respectivos controles tratados com o antibiótico gentamicina (10 mg/L).

Além do potencial de uso como antibacterianos o uso de OEs é uma alternativa no controle de parasitas na aquicultura. Há um número crescente de estudos publicados destacando o potencial de aplicação dos OEs e seus componentes no tratamento de doenças parasitárias de peixes, apesar de ainda serem poucos os estudos sobre o tratamento de parasitas monogenéticos. No entanto, os resultados são promissores, mostrando que OEs e seus componentes são fortes alternativas aos quimioterápicos e pesticidas convencionais, como por exemplo a formalina. Ao contrário do que ocorre com produtos químicos e fármacos sintéticos, que geralmente causam aumento da resistência dos parasitas, e têm um elevado tempo de permanência no ambiente, acredita-se que os extrativos vegetais como os OEs possam causar um desenvolvimento lento de resistência, diminuir amplamente a emissão de resíduos (biodegradáveis) e, consequentemente, serem inócuos ao ambiente (CHAGAS, 2004).

Banhos de 1 h com eugenol a 10 mg/L foram eficazes no tratamento de parasitose por monogenéticos branquiais em tambaquis (*Colosssoma macropomum*), diminuindo sua contagem em 80% (BOIJINK et al., 2015). De acordo com STEVERDING et al. (2005) o OE de *Melaleuca alternifolia* mostrou ser eficaz no tratamento de *Gyrodactylus* spp. em peixes da espécie *Gasterosteus aculeatus*. HASHIMOTO et al. (2016) demonstraram a eficácia de banhos terapêuticos usando OE de *Lippia sidoides* e *Mentha piperita* contra diferentes parasitos monogenéticos em brânquias de tilápia do Nilo (*Oreochromis niloticus*). O OE de *L. alba* apresentou eficácia *in vitro* contra monogenéticos de tambaquis (*Anacanthorus spathulatus, Notozothecium janauachensis and Mymarothecium boegeri*) (SOARES et al., 2016).

Figura 5 – Mecanismos e sítios de ação dos óleos essenciais na célula bacteriana.



Fonte: elaborado pelo autor.

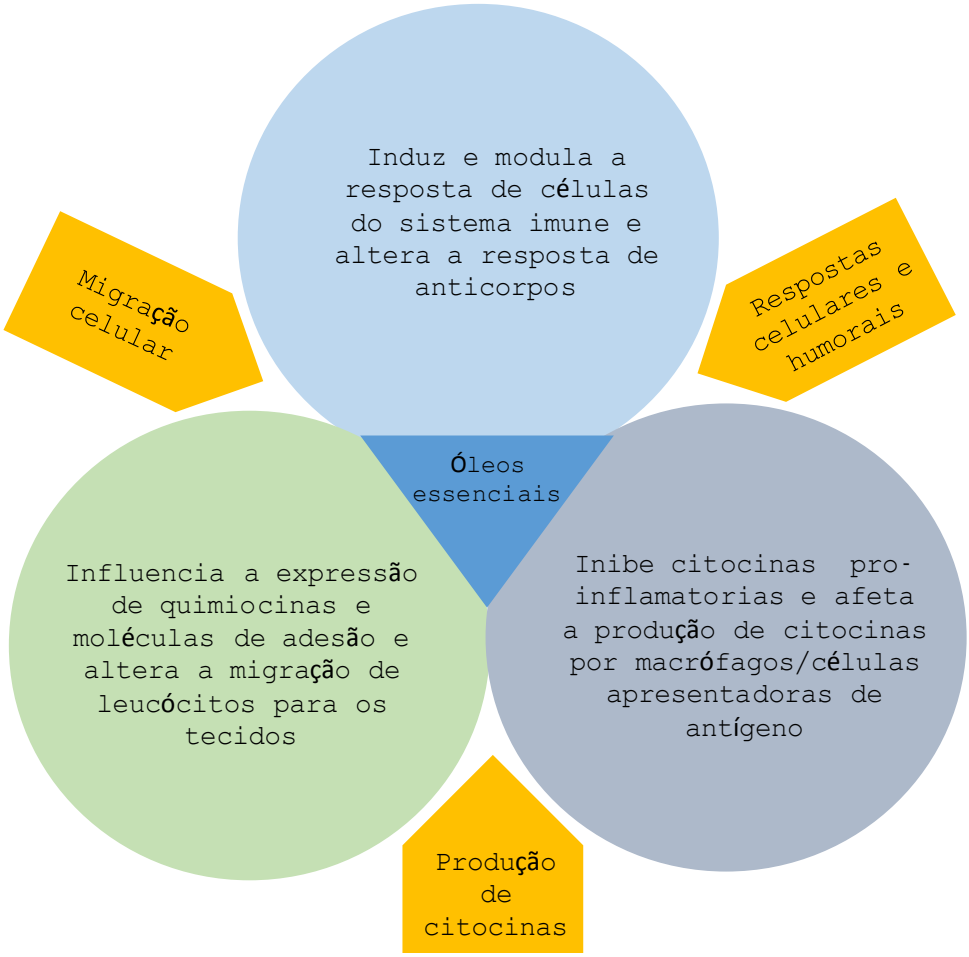
1.5. ÓLEOS ESSENCIAIS COMO MODULADORES DA RESPOSTA IMUNE INATA

Nos casos em que os surtos de doenças são cíclicos e/ou podem ser previstos, imunoestimulantes podem ser utilizados em antecipação dos eventos para melhorar o mecanismo de defesa não específico, e, portanto, evitar perdas por doenças. No entanto, deve-se tomar cuidado, uma vez que imunoestimulantes potentes podem suprimir ou alterar negativamente certos caminhos biológicos se forem utilizados de forma inadequada (CITARASU, 2010). As moléculas encontradas nos OEs de plantas podem inibir ou ativar os componentes do sistema imunitário dos peixes (Fig. 6), facilitando essencialmente a função de células fagocíticas, aumentando a sua atividade bactericida, estimulando células NK ("natural killer"), atividade do sistema complemento, atividade da lisozima e respostas de anticorpos que conferem maior proteção contra doenças infecciosas (HARIKRISHNAN et al., 2011).

Peixes da espécie *Sparidentax hasta* anestesiados com OE de tomilho *Thymus vulgaris* apresentaram atividade da lisozima plasmática aumentada quando comparados a peixes não expostos ao OE (AZAD et al., 2014). Anestesia causada por óleo de cravo (*Eugenia*

aromatica) em truta arco-íris (*Oncorhynchus mykiss*) não alterou a atividade hemolítica do complemento, mas reduziu a explosão respiratória produzida por leucócitos sanguíneos 24 h após a anestesia (KANANI et al., 2013). Linfopenia, neutrofilia e aumento da atividade da lisozima foram observados em truta arco-íris 24 h após anestesia com óleo de cravo (KANANI et al., 2011). Carpas (*Cyprinus carpio*) alimentadas com dietas contendo OE de *Z. multiflora* apresentaram um incremento no número de leucócitos no sangue (SOLTANI et al., 2010). Aumento na atividade da lisozima plasmática foi observado em bagre de canal (*Ictalurus punctatus*) alimentados com uma dieta contendo OE de *Origanum heracleoticum* (ZHENG et al., 2009). OE de laranja *Citrus sinensis* adicionados à dieta aumentaram a atividade da lisozima e mieloperoxidase em tilápias moçambicanas (*Oreochromis mossambicus*) (ACAR et al., 2015). Desta forma, a utilização destes produtos naturais principalmente durante o manejo dos peixes pode melhorar a defesa inata dos animais favorecendo a resistência a patógenos durante períodos de estresse elevado, tais como classificação, reprodução, transferência e vacinação.

Figura 6 – Principais efeitos dos óleos essenciais sobre a resposta imune.



Fonte: elaborado pelo autor.

1.6. ÓLEOS ESSENCIAIS COMO PROMOTORES DE CRESCIMENTO

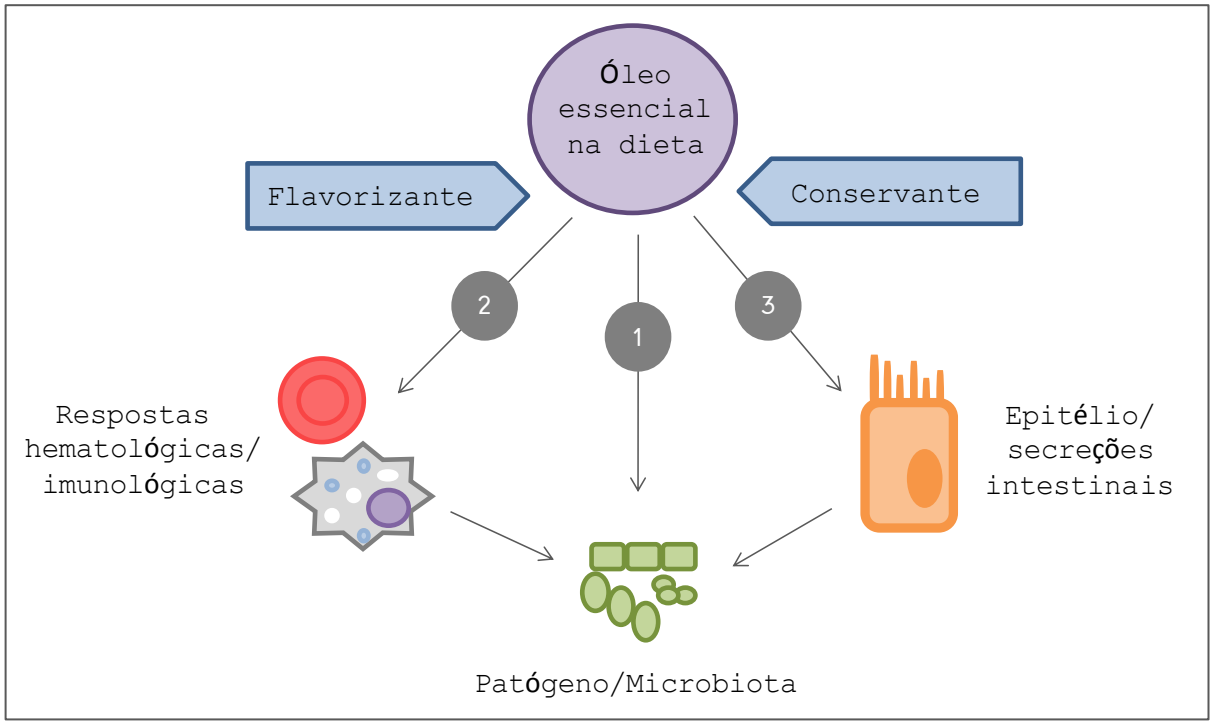
Na nutrição animal os OEs essenciais podem ser utilizados como flavorizantes e/ou conservantes (antioxidante) quando adicionados à dieta (FRANZ et al., 2010). Além das propriedades antimicrobianas e imunoestimulantes os OEs podem induzir alterações positivas na morfologia intestinal do animal, apresentar atividade anti-inflamatória, antioxidante, influenciar a quantidade e tipo de secreções produzidas pela mucosa intestinal e alterar as propriedades físicas e químicas do ambiente intestinal (Fig. 7) (CITARASU, 2010; FRANZ et al., 2010; HARIKRISHNAN et al., 2011; GIANNENAS et al., 2012; BENTO et al., 2013; SACCOL et al., 2013; CHAKRABORTY et al., 2014; REVERTER et al., 2014; ZENG et al., 2015). Os OEs podem estimular as secreções do intestino, permitindo a microbiota modular e melhorar a digestão e absorção de nutrientes, proporcionando assim uma maior variedade de aminoácidos para a síntese de proteínas e, assim, aumentar o teor de proteína corporal (FRECCIA et al., 2014). Como resultado destas alterações pode-se observar um reforço na resistência a doenças e no crescimento dos animais.

Vários estudos têm discutido o uso destes produtos como aditivos na ração de peixes, uma vez que o somatório dessas atividades biológicas faz dos fitoterápicos uma das principais alternativas aos antibióticos e/ou fármacos sintéticos utilizados como promotores de crescimento na produção animal (CHAKRABORTY et al., 2014; CITARASU, 2010; HARIKRISHNAN et al., 2011). Desta forma, os efeitos dos OEs sobre a população bacteriana do intestino também podem ser indiretos, em contraste com os efeitos observados quando se utiliza antibióticos convencionais como promotores de crescimento, onde os mecanismos são principalmente relacionados com a atividade diretamente sobre a bactéria (BUTAYE et al., 2003; LIN et al., 2011; YANG et al., 2015).

A adição do OE de *L. alba* na dieta de jundiás diminuiu a peroxidação lipídica, aumentou as reservas de glicogênio e lactato e aumentou a resposta antioxidante tecidual (SACCOL et al., 2013). Jundiás apresentaram maior crescimento e ganho de peso após 60 dias de alimentação com dietas contendo OE de *Aloysia triphylla* (ZEPPENFELD et al., 2016). OE extraído da planta *O. heracleoticum* (orégano), contendo carvacrol e timol como principais compostos, promoveu o crescimento de bagre de canal após 8 semanas. Observou-se maior ganho de peso, além de melhor taxa de eficiência proteica, fator de condição e taxa de conversão alimentar (ZHENG et al., 2009). Os autores especularam que a razão para este efeito pode ser ligada às propriedades antimicrobianas conhecidas do OE de orégano que poderiam controlar a microflora intestinal e influenciar positivamente o desempenho dos

peixes. GIANNENAS et al. (2012) demonstraram que a adição dos compostos isolados carvacrol e timol na dieta modificaram a microbiota intestinal de truta arco-íris. Contagens totais de bactérias anaeróbias foram menores nos peixes alimentados com dietas contendo ambos compostos. Os autores não observaram diferenças significativas no peso final e ganho de peso dos peixes, no entanto, o grupo alimentado com dietas contendo timol apresentou melhor taxa de conversão alimentar. Tilápias moçambicanas alimentadas com dietas contendo OE extraído da casca de laranja doce (*Citrus sinensis*) apresentaram maior peso final, ganho de peso e taxa de conversão alimentar após 90 dias de tratamento (ACAR et al., 2015). FERREIRA et al. (2014) concluíram que OE de orégano (*O. vulgare*) promoveu o crescimento e melhorou a composição da carcaça de *Astyanax altiparanae*. Com o aumento dos níveis do OE na dieta houve redução dos lipídios e um aumento do teor de proteína da carcaça.

Figura 7 – Óleos essenciais como promotores de crescimento em peixes.



1 - Os óleos essenciais podem atuar como promotores do crescimento agindo diretamente sobre os patógenos e/ou a microbiota intestinal. 2 - Modular respostas hemato-imunológicas e fisiológicas, apresentando atividade anti-inflamatória, imunoestimulante e antioxidante. 3 - Podem atuar induzindo alterações positivas na morfologia intestinal, influenciando a quantidade e tipo de secreções produzidas pela mucosa, alterando as propriedades físicas e químicas do ambiente intestinal, modificando a população microbiana e assim modular a digestão e a absorção de nutrientes. Fonte: elaborado pelo autor.

1.7. OBJETIVOS

1.7.1. Objetivo geral

Este estudo teve por objetivo avaliar o potencial de uso de diferentes OEs (*Ocimum americanum, Ocimum gratissimum, Hesperozygis ringens, Cymbopogon flexuosus* e *Melaleuca alternifolia*) no tratamento e prevenção de doenças, e como imunomoduladores e promotores de crescimento em *Rhamdia quelen* e *Sciaenops ocellatus*.

1.7.2. Objetivos específicos

- Avaliar a atividade antibacteriana *in vitro* dos OEs obtidos a partir das folhas de *H. ringens*, *O. gratissimum* e *O. americanum* contra a bactéria *A. hydrophila*, bem como, avaliar o potencial de uso *in vivo*, por meio de banhos terapêuticos e preventivos, em jundiás infectados.

- Avaliar a atividade antibacteriana e antiparasitária (*in vitro* e *in vivo*) do OE de *O. americanum*, obtido a partir das inflorescências, frente à bactéria *A. hydrophila* e o parasito monogenético *Gyrodactylus* sp.

- Verificar o efeito *in vitro* dos OEs de *O. americanum*, *C. flexuosus* e *M. alternifolia* sobre parâmetros de imunidade não-específica e fragilidade osmótica eritrocitária de "red drum", a fim de avaliar o seu potencial de uso *in vivo*.

- Avaliar o efeito da adição do OE de *O. americanum* na dieta de "red drum" sobre o desempenho produtivo, respostas imunológicas não-específicas e população microbiana do trato digestivo.

2. ARTIGOS

2.1. ARTIGO 1

O **Artigo 1** relata a atividade antibacteriana *in vitro* e *in vivo* dos OEs de *H. ringens, O. gratissimum* e *O. americanum* obtidos a partir de folhas das plantas contra *A. hydrophila*. O potencial de uso *in vivo* dos óleos foi avaliado através de banhos terapêuticos e preventivos em jundiás infectados experimentalmente por *A. hydrophila*, onde foram avaliados sobrevivência e parâmetros de imunidade inata. Este artigo foi publicado no periódico **Journal of Applied Microbiology**.

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ORIGINAL ARTICLE

Plant essential oils against *Aeromonas hydrophila*: *in vitro* activity and their use in experimentally infected fish

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Keywords
cortisol, *Hesperozygis ringens*, innate immune system, *Ocimum americanum*, *Ocimum gratissimum*, *Rhamdia quelen*.

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Abstract

Aims: The aims of this study were to investigate the *in vitro* antibacterial activity of the essential oils (EOs) of *Hesperozygis ringens* (HREO), popularly known as 'espanta-pulga' and two different species of basil, *Ocimum gratissimum* (OGEO) and *Ocimum americanum* (OAEO), as well as, the potential of these products to be used in silver catfish (*Rhamdia quelen*) infected with *Aeromonas hydrophila*.

Methods and Results: OGEO and HREO showed better antibacterial activity *in vitro*. Subinhibitory concentrations of all EOs inhibited haemolysis caused by *Aer. hydrophila* in fish erythrocytes (100% reduction for OAEO at 100 μg ml^{-1} and more than 90% for HREO and OGEO at 150 μg ml^{-1}). However, OAEO and HREO showed the best survival results (75 and 70% respectively) after their use as treatment (therapeutic baths—1 h daily/5 days) in silver catfish experimentally infected with *Aer. hydrophila*. A second *in vivo* assay using healthy fish was conducted to verify the potential of the EOs (preventive baths—1 h daily/5 days) to promote fish survival. Fish exposed to HREO and OAEO and their diluent (ethanol) showed significant lower haematocrit values and higher complement system activity compared to control. Plasma cortisol level was significantly higher in the groups exposed to both EOs. There was no significant difference in survival of silver catfish challenged with *Aer. hydrophila* after preventive baths with HREO, OAEO and control group.

Conclusions: All tested EOs showed *in vitro* antibacterial properties against *Aer. hydrophila* and HREO and OAEO showed potential to be used in the treatment of infected fish.

Significance and Impact of the Study: These products can be used in aquaculture as therapeutic and prophylactic agents against fish pathogens, with antimicrobial and/or immunostimulant properties.

Introduction

Plant-derived products with antimicrobial and/or immunostimulant properties can be used in fish culture as therapeutic and/or prophylactic agents against several fish pathogens. These products can act on several important bacteria in fish culture, since they can disrupt the bacterial cell wall, block the synthesis of proteins and DNA, inhibit secretion of enzymes and interfere with bacterial signalling mechanism via *quorum sensing* (Citarasu 2010). The immunostimulants molecules found in plant-derived products can inhibit or activate components of the fish

immune system, mainly facilitating the function of phagocytic cells, increasing their bactericidal activities, and stimulating the natural killer cells, complement system, lysozyme activity and antibody responses which confer enhanced protection from infectious diseases (Harikrishnan *et al.* 2011).

Hesperozygis ringens is an endemic woody herb native to rocky fields of southeastern Rio Grande do Sul, south Brazil. The plant is known by the vernacular name 'espanta-pulga' (literally 'to keep fleas away') and is employed for its antiparasitic activity (Ribeiro *et al.* 2010). *Ocimum gratissimum*, commonly known as alfavaca or tree basil, is widely used as food condiment and in traditional medicine. Antifungal and bactericidal properties were reported for the essential oil (EO) of this plant species (Prabhu *et al.* 2009). *Ocimum americanum,* also known as basil, is frequently used as a medicinal plant (Vieira *et al.* 2003). Its EO presents antimicrobial potential against fungi and bacteria (Cimanga *et al.* 2002; Thaweboon and Thaweboon 2009).

Essential oils obtained from plants have been widely tested as an alternative to conventional drugs due to the potential of their bioactive principles. EOs contain a rich mixture of highly functional molecules and, compared to pesticides and synthetic drugs, such molecules have low environmental toxicity (Park *et al.* 2011). In fish culture, EOs have been given to aquatic organisms via baths or incorporation in the feed (Saccol *et al.* 2013; Sutili *et al.* 2014). Compounds with biological properties produced by plants and found in EOs can be used for synthesis of new drugs or as adjuvants or substitutes for synthetic active substances (Ahmad and Beg 2001). They may also potentiate the activity of other substances by inhibiting mechanisms of resistance or virulence factors developed by pathogens (Harris 2002; Stavri *et al.* 2007).

Usually considered a secondary pathogen, bacteria of the *Aeromonas* genus can also act as a primary pathogen in some environments. These bacteria comprise important pathogens that cause septicaemia in humans and are frequently recovered from several animal species or food (Janda and Abbott 2010). In fish farms, *Aeromonas hydrophila* often causes outbreaks with high mortality and significant economic losses (Nielsen *et al.* 2001). Considering the importance of this bacterium as a lethal pathogen in fish farms and the characteristics of the mentioned plants, the present study reports the *in vitro* activity of *H. ringens* EO (HREO), *O. gratissimum* EO (OGEO) and *O. americanum* EO (OAEO) against *Aer. hydrophila* as well as their *in vivo* activity in infected silver catfish (*Rhamdia quelen*).

Materials and methods

Essential oils obtention and major constituents

Leaves of *Hesperozygis ringens*, *Ocimum gratissimum* and *Ocimum americanum* were, respectively, collected in São Francisco de Assis, Santa Maria and Encantado municipalities (Rio Grande do Sul, Brazil). Voucher specimens (n°. SMDB 13427, 11167 and 13163 respectively) were identified by Drs. Solon Jonas Longhi, Adelino Alvares Filho and Sérgio Augusto de Loreto Bordignon and deposited in the Herbarium of the Department of Biology, Universidade Federal de Santa Maria. The EOs were extracted by hydrodistillation using a Clevenger type apparatus for 2 h (*H. ringens* and *O. americanum*) and 3 h (*O. gratissimum*) (European Pharmacopoeia 2007). The obtained samples were stored at −4°C in amber glass bottles until analysis by gas chromatography coupled with mass spectrometry (GC-MS) and biological tests. GC-MS TIC analysis was performed using an Agilent-7890 gas chromatograph coupled with an Agilent 59753 mass selective detector using an HP5-MS column (5% phenyl, 95% methylsiloxane, 30 m × 0·25 mm i. d. × 0·25 μm) (Silva *et al.* 2012). The constituents of the EOs were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library (NIST 2002), and literature data (Adams 2001). Major constituents of *H. ringens*, *O. gratissimum* and *O. americanum* EO are shown in Table 1.

Antibacterial assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the EOs for *Aer. hydrophila* were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute, document M31-A3 (CLSI 2008). Fourteen strains of *Aer. hydrophila*, which were isolated from infected fish obtained from local fish farms, and *Aer. hydrophila* ATCC 7966 were used. The EOs were diluted in 96% ethanol, incorporated in a Müller-Hinton broth (MHB) (Himedia Laboratories, Mumbai, India) and tested at 3200, 1600, 800, 400, 200, 100 μg ml^{-1} (in triplicate). The inoculum was prepared in saline solution from cultures grown in Müller-Hinton agar (Himedia Laboratories) (1×10^8 colony forming units (CFU) ml^{-1}; 0·15 OD 600 nm) (30 °C/24 h). Ten microlitres (1×10^5 CFU) of inoculum was added to each well containing EO. The microplates were incubated at 30°C for 24 h under aerobic conditions. The MBCs were confirmed by reinoculation of 10 μl of each bacterial culture on Müller-Hinton agar incubated at 30°C for 24 h and the lowest concentration of each EO showing

Table 1 Chemical major composition of the essential oils obtained from the leaves of *Hesperozygis ringens*, *Ocimum gratissimum* and *Ocimum americanum*

	Composition (%)	RI (experimental)	RI (literature)*
H. ringens			
Pulegone	96·63	1243	1239
Limonene	1·16	1025	1022
Linalool	0·92	1099	1100
2·3-Epoxy-geranylacetate	0·88	1406	1393
α-Pinene	0·50	930	933
O. gratissimum			
Eugenol	91·47	1373	1370
Z-β-ocimene	5·93	1038	1037
Germacrene D	1·22	1482	1485
β-caryophyllene	0·38	1420	1419
E-β-ocimene	0·14	1048	1050
O. americanum			
1·8-cineole	21·0	1031	1031
β-linalool	20·18	1105	1101
Eugenol	17·17	1362	1364
Camphor	11·96	1145	1144
Germacrene D	3·49	1481	1480

RI, retention índex.
*Adams (2001); NIST/EPA/NIH (2002).

no growth was defined as the MBC. The same procedure was performed with an ethanol control.

Haemolysis assay

The haemolysis assay was performed according to the methodology described by Sutili *et al.* (2014). A β-haemolytic strain was selected from investigated strains during the MIC-test. This strain was cultured in MHB in subinhibitory concentrations of the EOs, which were previously diluted in ethanol (0-control, 100, 150 and 200 μg ml^{-1}) (concentrations with no bactericidal activity against this strain for all EOs), at 30°C for 24 h, and prepared at a concentration of $2{\cdot}1 \times 10^9$ CFU ml^{-1} (1·75 OD 600 nm). Bacterial cultures were spun (5500 ***g***, 4°C, 1 min), the supernatant was collected, and the residual cells were removed using a 0·2 μm filter. Prior to the addition of 100 μl of defibrinated fish blood, a 200 μl volume of culture supernatant was diluted to a volume of 1000 μl through the addition of phosphate-buffered saline (PBS) in triplicate. After incubation for 90 min at 37°C, the unlysed blood cells were pelleted by centrifugation (5500 ***g***, room temperature, 1 min). The haemolytic activity of the supernatant was detected by measuring its optical density at 540 nm. The per cent haemolysis was calculated by comparison between total haemolysis (100%) and no-haemolysis (0%) controls. The same procedure was performed with an ethanol control.

Survival assays and water quality

Two experiments were conducted to evaluate survival of fish experimentally infected with *Aer. hydrophila* and exposed to the EOs. The silver catfish from the same spawning were transferred from a local fish culture to the laboratory, where they were acclimated for 7 days in continuously aerated tanks under controlled water parameters (21·0–23·0°C, pH: 7·0–8·0, dissolved oxygen levels: 5·0–7·0 mg l^{-1}). During the experiments dissolved oxygen and temperature were measured with an YSI oxygen meter (Model Y5512). The pH was verified with a DMPH-2 pH meter (Digimed, São Paulo, Brazil). Total ammonia levels were determined according to Verdouw *et al.* (1978) and un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). The mean water quality parameters were: temperature 21·5 ± 0·2°C, dissolved oxygen level 6·2 ± 0·2 mg l^{-1}, pH 7·7 ± 0·06, total ammonia 1·92 ± 0·8 mg l^{-1} and nonionized ammonia 0·04 ± 0·016 mg l^{-1}.

In both experiments fish were fed once a day to satiation with commercial feed, and uneaten food, other residues and faeces were removed 30 min after feeding. Dead fish were also removed daily. Bacterial re-isolation was made from lesions and kidneys of fish. Morphological and biochemical characteristics were analysed according to Quinn (1994) for bacterial identification and characterization.

Experiment I

Two hundred and sixteen silver catfish fingerlings (10·0 ± 1·0 g) were distributed in 27 plastic boxes (30 l). They were anesthetized with 50 mg l^{-1} eugenol (Cunha *et al.* 2010) and 100 μl of *Aer. hydrophila* solution (ATCC 7966; $1{\cdot}5 \times 10^9$ CFU; 1·2 OD 600 nm) was inoculated intramuscularly in the latero-dorsal right side. The groups were (in triplicate, $n = 8$): control (silver catfish inoculated with 100 μl sterile saline), negative control (untreated infected fish), gentamycin 10 mg l^{-1} (Belem-Costa and Cyrino 2006), HREO (20 and 40 mg l^{-1}), OGEO (5 and 10 mg l^{-1}) and OAEO (10 and 20 mg l^{-1}). These EOs concentrations have no sedative effect in silver catfish (Silva *et al.* 2012, 2013), and were chosen to avoid behavioural side effects during the long exposure time of the baths protocol. Exposure to the EOs was carried out through daily baths (1 h) for 5 days. Mortality caused by *Aer. hydrophila* was observed in each group for two additional days. The EOs were previously diluted 1 : 10 in 96% ethanol before addition to the bath water.

Experiment II

In this experiment the possible prophylactic effect of treatment with the EOs was evaluated. Ninety six silver

catfish (25·0 ± 1·8 g) were distributed in twelve 30 l plastic boxes (in triplicate, $n = 8$) in four groups: control (water only), ethanol (at the concentration used for the dilution of the EOs), HREO (20 mg l^{-1}) and OAEO (20 mg l^{-1}) (treatments with the best survival results in the previous assay). The bath protocol was the same as in Experiment I. After exposure, two silver catfish from each box (six fish per group) were anesthetized (eugenol 50 mg l^{-1}) for blood collection from the caudal vein using sterile heparinized syringes. Haematocrit, plasma cortisol and nonspecific immune responses (complement, agglutination and bactericidal activity of plasma) were verified. The remaining fish (18 fish per group) were challenged with *Aer. hydrophila* as mentioned in Experiment I. Mortality in each group was observed for 7 days.

Haematocrit, cortisol and nonspecific immune assays

The haematocrit was assessed by the microhaematocrit method. Plasma cortisol levels were determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Dorchester, Ontario, Canada). Absorbance was estimated spectrophotometrically at 450 nm. The inter- and intra-assay variation coefficients were 5·15 ± 0·53% and 4·13 ± 0·67% respectively.

The complement system activity was determined according to Castro *et al.* (2008) using fresh plasma (without freezing) from each fish. Rabbit red blood cells were added to normal plasma and incubated at 20°C. Ice-cold saline was added 1 h after to stop the complement activity, then cells were pelleted by centrifugation and the absorbance of the supernatant was measured in a microplate reader at 405 nm. The per cent haemolysis was calculated by comparison between total haemolysis (100%) and no-haemolysis (0%) controls.

The natural agglutination activity of fish plasma was investigated using 'U'-shaped 96-well plates. Plasma was diluted two-fold in PBS (pH 7·4, Ca^{+2} and Mg^{+2}), and an equal volume of washed *Aer. hydrophila* (ATCC 7966) in suspension (0·4 OD 600 nm) was added to each well. The plates were incubated for 2 h at 25°C and then overnight at 4°C; the titre was defined as the logarithm of the highest dilution of the plasma that caused complete agglutination of the bacterial cell (Sutili *et al.* 2014).

Plasma bactericidal activity was assessed against *Aer. hydrophila* (ATCC 7966) by the method of diffusion in agar using paper disk (5 mm diameter). The bacteria were seeded in Petri dishes (solid medium Müller-Hinton) (McFarland 0·5 × 10^8 cells ml^{-1}) and plasma was soaked on the discs (20 μl). The plates were incubated at 30°C for 24 h. The diameter of the halo formed around the discs was assessed (Dotta *et al.* 2014).

The methodology of these experiments was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process n° 046/2010).

Statistical analysis

The homogeneity of variances between groups was tested with the Levene test. Comparisons between different groups were made using one-way ANOVA and Tukey's test (STATISTICA 7.0, StatSoft Inc., Tulsa, OK, USA). Fish survival was compared using Kaplan–Meier survival analysis with the Logrank test (SIGMA PLOT 10.0, Systat Software, San Jose, CA, USA). The minimum significance level was set at $P \leq 0·05$.

Results

Antibacterial and haemolysis assays

The MIC and MBC values for HREO against *Aer. hydrophila* ranged from 800 to 3200 μg ml^{-1}. For OGEO MIC values ranged from 200 to 1600 μg ml^{-1} and MBC values ranged from 400 to 1600 μg ml^{-1} (Fig. 1a). The MIC values for OAEO were higher than the highest concentration tested. In the haemolysis inhibition assay *Aeromonas* cells cultured without EOs presented 23% haemolysis of fish erythrocytes. Ethanol control showed similar percentage of haemolysis. When *Aer. hydrophila* was treated with HREO, OGEO and OAEO at 100 μg ml^{-1}, the haemolysis reductions were 71, 77% and 100% respectively. The haemolysis reduction was more than 90% for HREO and OGEO at 150 μg ml^{-1} compared to control group. The percentage of haemolysis for OAEO at 150 and 200 mg l^{-1} was similar to control. Haemolysis at 200 μg ml^{-1} for HREO and OGEO were higher than in control (Fig. 1b).

Experiment I

In the first *in vivo* assay there was 100% survival in the noninfected group. In the infected fish group treated with the antibiotic gentamycin (10 mg l^{-1}), survival was near 80%. Fish exposed to 20 and 40 mg l^{-1} HREO and 20 mg l^{-1} OAEO presented survival of 70, 66 and 75%, respectively, and did not differ statistically from the group treated with the antibiotic. The infected fish group that did not receive any treatment had near 40% survival. In the group exposed to OAEO (10 mg l^{-1}) fish survival was 50%. Fish exposed to 5 and 10 mg l^{-1} OGEO presented 30 and 37% survival respectively. These groups did not differ statistically from control group (untreated infected fish) (Fig. 1c).

Experiment II

In the second *in vivo* assay fish exposed to ethanol and both EOs showed significant lower haematocrit values

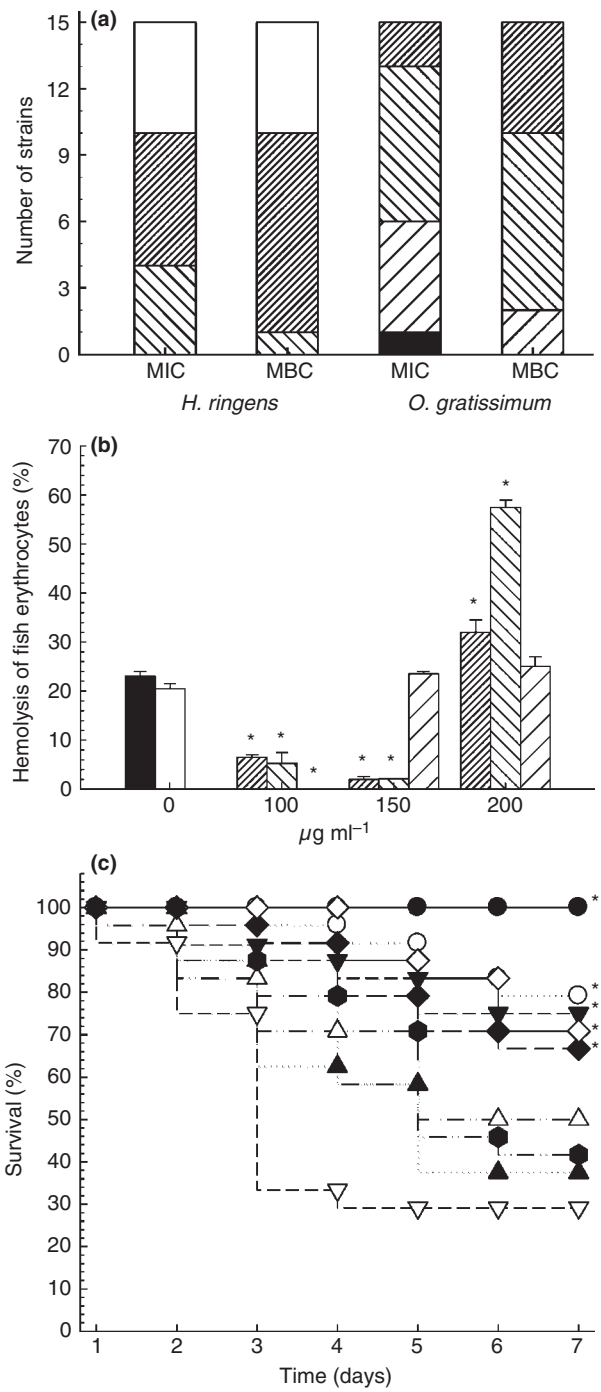

Figure 1 (a) Essential oils (EOs) activity against *Aeromonas hydrophila*. Minimum inhibitory concentration (MIC). Minimum bactericidal concentration (MBC). ■ 200, ▨ 400, ▧ 800, ▨ 1600, □ 3200 μg ml^{-1}. (b) Hemolytic activity of *Aer. hydrophila* culture supernatant treated with subinhibitory concentrations of EOs. (*) indicates significant difference relative to control (0 μg ml^{-1}). One-way ANOVA and Tukey's test ($P \leq 0{\cdot}05$). ■ Control, □ Ethanol control, ▨ *Hesperozygis ringens*, ▧ *Ocimum gratissimum*, ▨ *Ocimum americanum*. (c) Survival of silver catfish infected with *Aer. hydrophila* after therapeutic baths (1 h daily/5 days). (*) indicates significant difference relative to negative control (untreated infected fish). Kaplan–Meier survival analysis with Logrank test ($P \leq 0{\cdot}05$). (-●- Control—fish inoculated with sterile saline), (-○- fish inoculated with *Aer. hydrophila* and treated with antibiotic gentamycin 10 mg l^{-1}), (*O. americanum* -▼- 20 and -△- 10 mg l^{-1}), (*H. ringens* -◇- 20 and -◆- 40 mg l^{-1}), (*O. gratissimum* -▽- 5 and -▲- 10 mg l^{-1}), (-●- Negative control—untreated infected fish).

(Fig. 2a); however, the complement system activity was significantly higher in these groups compared to control (Fig. 2b). Plasma cortisol level was significantly higher in the groups exposed to both EOs (Fig. 2c). There was no significant difference in fish survival among the groups (Fig. 2d). Titre and halo formation were not verified in the plasma agglutination and plasma bactericidal assays respectively. *Aeromonas* cells were re-isolated from the lesions and kidneys of dead fish in both survival assays.

Discussion

Ilory *et al.* (1996) reported antibacterial activity for leaf extracts of *O. gratissimum*, which was investigated by disc diffusion and tube dilution methods against *Aeromonas sobria*. The observed MIC value was 16 000 μg ml^{-1}. Junaid *et al.* (2006) found MIC values of 50 000 and 100 000 μg ml^{-1} for *O. gratissimum* cold H_2O extract and hexane extract of fresh leaves, respectively, against *Aer. hydrophila*. The MICs found in the present study for *O. gratissimum* EO are below the reported values. However, comparisons are not possible due to methodological differences in the determination of MICs and/or because the mentioned assays used extracts. Sutili *et al.* (2014) evaluated the antibacterial activity of eugenol (the major compound of OGEO) against *Aer. hydrophila* and found MIC values (1600–3200 μg ml^{-1}) similar to those observed in this study for OGEO and HREO. To the best of our knowledge, no data are available about the specific activity of HREO and OAEO against the *Aeromonas* genus.

The pathogenesis of *Aer. hydrophila* is multifactorial and depends upon the secretion of numerous extracellular factors that influence virulence (Yu *et al.* 2005). An alternative strategy for the treatment of bacterial infections is to target bacterial virulence (Cegelski *et al.* 2008). Research shows that subinibitory concentrations of EOs or isolated compounds from plants decrease or inhibit the production of certain virulence factors in bacteria. Essential oils from plants can inhibit biofilm formation or the production of exotoxins such as haemolysins, which cause erythrocytes haemolysis, thus reducing the bacteria pathogenicity (Smith-Palmer *et al.* 2004).

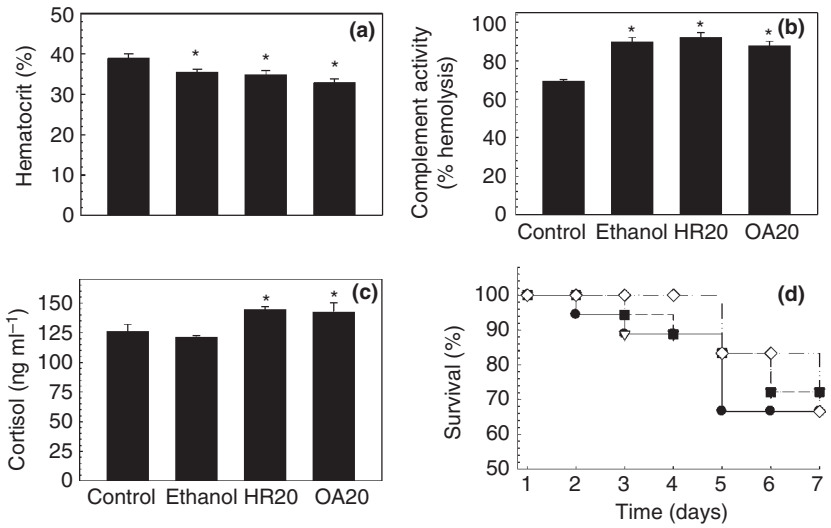

Figure 2 (a, b and c) Silver catfish responses after preventive essential oils (EOs) baths (1 h daily/5 days). HR20—*H. ringens* 20 mg l^{-1} and OA20—*O. americanum* 20 mg l^{-1}. (*) indicates significant difference relative to control. One-way ANOVA and Tukey's test ($P \leq 0{\cdot}05$). (d) Survival of silver catfish infected with *Aer. hydrophila* after preventive EOs baths (1 h daily/5 days). (–●– Control), (–▽– Ethanol), (–■– *H. ringens* 20 mg l^{-1}), (–◇– *O. americanum* 20 mg l^{-1}). Kaplan–Meier survival analysis with Logrank test ($P \leq 0{\cdot}05$).

Ocimum gratissimum EO inhibited extracellular protease activity and expression of cell wall lipopolysaccharide in multi-resistant strains of *Shigella* (Iwalokun *et al.* 2003). Subinibitory concentration of eugenol (50 μg ml^{-1}) significantly reduced (35%) the haemolytic activity of *Aer. hydrophila* supernatant in fish erythrocytes (Sutili *et al.* 2014). *Thymus vulgaris* EO (1·8-cineole chemotype) significantly reduced biofilm formation in *Aer. hydrophila* (Millezi *et al.* 2013). In the present study, subinhibitory concentrations of all tested EOs were efficient in reducing the haemolysis produced by *Aer. hydrophila* in fish erythrocytes. Nonetheless, haemolysis increased at the highest concentration tested (200 μg ml^{-1}), which may have resulted from the presence of the EO in the bacterial culture supernatant and its cytotoxicity against the erythrocyte membrane. Most of the effects described for EOs involve interactions with biological membranes, such as morphological changes with rupture of membrane and cytoplasm leakage (Sikkema *et al.* 1995; Devi *et al.* 2010).

Eugenol (the major compound found in OGEO) (10 mg l^{-1}) promoted 66% survival in silver catfish infected with *Aer. hydrophila* using the same bath protocol as the one employed in the present study (Sutili *et al.* 2014). Addition of 16 and 40 mg l^{-1} *Lippia alba* EO to the water promoted survival of silver catfish naturally infected with *Aeromonas* sp. (Sutili *et al.* 2015). In the first *in vivo* assay HREO and OAEO promoted survival in fish infected with *Aer. hydrophila*. Although OGEO presented antibacterial and haemolysis inhibition activity *in vitro*, this EO did not increase silver catfish survival.

In the second *in vivo* assay, healthy fish were exposed to HREO and OAEO to evaluate the possible preventive effect of these EOs. The activity of the complement system and the haematocrit cannot define if the EOs alone could alter these parameters. Sutili *et al.* (2014) reported no significant changes in the haematological parameters and complement serum activity in silver catfish after 5 days of baths with eugenol. Only fish exposed to ethanol showed significantly higher haematocrit compared to control. Based on these data, both EOs and ethanol seem to interfere with silver catfish responses. Despite the differences observed for the haematocrit among the groups, all treatments presented values within the basal range previously reported for the species (Tavares-Dias *et al.* 2002; Barcellos *et al.* 2004; Sutili *et al.* 2014).

Elevation in plasma cortisol concentration is recognized as the main hormonal response to stressors and is widely used as a stress response indicator (Barton and Iwama 1991). In this study there was a significant increase in plasma cortisol levels in the groups exposed to both EOs (HREO and OAEO) compared to control and ethanol groups. Nevertheless, the values found for all groups are similar to those reported for silver catfish by Barcellos *et al.* (2004) after acute stress induced by handling (transfer between tanks), a procedure which is similar to that used throughout the groups in this study. Therefore, the EOs appear to act as an additional stressor during baths, besides handling. The lower mortality observed in the second experiment was probably due to the difference in fish size, since the concentration of *Aeromonas* inoculated in both experiments was the same.

All tested EOs presented antibacterial properties against *Aer. hydrophila*, and HREO and OAEO showed potential to be used in infected fish. Furthermore, ethanol can interfere with haematological and immunological responses of fish. The effect of these EOs in the first survival assay was probably not due to their bactericidal

activity; instead, the subinhibitory concentrations of the EOs may have decreased *Aer. hydrophila* virulence, thus facilitating the self-healing of fish. These results support the therapeutic application of such products against bacterial diseases, in addition to their use as sedatives and anaesthetics.

Acknowledgements

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Conflict of Interest

The authors have no conflict of interest.

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2.2. ARTIGO 2

O **Artigo 2** relata a atividade *in vitro e in vivo* do OE de *O. americanum* (inflorescências) contra os patógenos de peixes *A. hydrophila e Gyrodactylus* sp., bem como o potencial desse óleo e de seu componente majoritário (linalol) em inibir a hemólise causada pela bactéria em eritrócitos de peixes. Este artigo foi publicado no periódico **Letters in Applied Microbiology**.

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ORIGINAL ARTICLE

The use of *Ocimum americanum* essential oil against the pathogens *Aeromonas hydrophila* and *Gyrodactylus* sp. in silver catfish (*Rhamdia quelen*)

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Significance and Impact of the Study: Phytochemicals, such as essential oils (EOs) are a great source of new molecules and have shown potential to be used in aquaculture systems. However, additional studies focused on the *in vivo* efficacy, mode of action and identification of the active compounds are needed. This study determined the potential of *Ocimum americanum* EO for use against two important fish pathogens, *Aeromonas hydrophila* and *Gyrodactylus* sp., as well as providing preliminary information about the role of the main EO compound (linalool) against *Aer. hydrophila* virulence.

Keywords
haemolysis, linalool, monogenea, plant, virulence.

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Abstract

The bactericidal activity (minimum inhibitory concentration (MIC)-test) of *Ocimum americanum* (inflorescences) essential oil (OAEO) against *Aeromonas hydrophila* was determined in this study. Also investigated was the potential of OAEO and the main compound found in the oil (linalool) at subinhibitory concentrations to be inhibitors of haemolysis caused by *Aer. hydrophila* in fish erythrocytes. An *in vivo* experiment was conducted to evaluate the survival of fish (*Rhamdia quelen*) experimentally infected with *Aer. hydrophila* and exposed to OAEO. A second experiment was conducted to evaluate the *in vitro* and *in vivo* activity of OAEO (mix from inflorescences and leaves) against the parasite *Gyrodactylus* sp. The OAEO showed weak *in vitro* activity against *Aer. hydrophila* (6400 μg ml^{-1}). Subinhibitory concentrations of OAEO (100 μg ml^{-1}) inhibited haemolysis (90%) caused by *Aer. hydrophila* in fish erythrocytes, however, linalool did not inhibit haemolysis activity. At the low concentrations (10 and 20 mg l^{-1}) added to the water, OAEO promoted the survival of fish experimentally infected with *Aer. hydrophila*. Lastly, the OAEO mix (50 mg l^{-1}) was effective against *Gyrodactylus* sp., significantly reducing (60%) the number of parasites in the fish.

Introduction

Aeromonas hydrophila is a zoonotic bacterium that affects fresh water fish farming worldwide, is responsible for high fish mortality and is considered as a significant economic problem (Belem-Costa and Cyrino 2006). Antibiotic therapy is the main treatment used to control bacterial diseases in fish farms, however, inadequate dosage levels and overdosing can lead to drug resistance in the bacteria (Saavedra *et al.* 2004). Monogeneans are a diverse group of parasites with a high specificity to the host and are commonly found on the skin and gills of marine and freshwater fish (Boeger and Vianna 2006). Infections caused by monogenean helminths, mainly Dactylogyridae and Gyrodactylidae families, are common in fish farms and are among the most important for aquaculture, as

these parasites feed on blood and host tissues and may act as a mechanical vector of other pathogens, such as viruses and bacteria (Cone 1995).

Essential oils (EOs) and their molecules are among the most promising alternatives studied to improve the growth and health of fish. These products can be used in aquaculture as therapeutic and/or prophylactic agents against bacterial and parasite pathogens promoting the survival of aquatic animals (Reverter *et al.* 2014). Plants of the *Ocimum* genus are collectively called basil and are used as a source of essential oils, spices, ornamentals and medicines. Antimicrobial activity against fungi and bacteria (Thaweboon and Thaweboon 2009), insect repellent activity (Seyoum *et al.* 2002) as well as *Aedes aegypt* larvicidal activity (Cavalcanti *et al.* 2004) have been reported for *Ocimum americanum* derived products.

Given the above, the aims of this study were to verify the *in vitro* and *in vivo* activity of *O. americanum* EO (OAEO) against *Aer. hydrophila* and the monogenean fish parasite *Gyrodactylus* sp. Also investigated was the potential of the main compound found in the oil *R*-(−)-linalool, as well as, its isomeric form *S*-(+)-linalool at subinhibitory concentrations to inhibit haemolysis caused by *Aer. hydrophila* in fish erythrocytes.

Results and discussion

Aeromonas hydrophila

For *Aer. hydrophila* ATCC 7966 and one of the clinical isolates the OAEO-MIC values were higher than the highest concentration tested; the other two isolates were inhibited at the highest OAEO concentration (6400 μg ml^{-1}). In the haemolysis inhibition assay *Aeromonas* cells cultured without OAEO presented 23% haemolysis of fish erythrocytes. When *Aer. hydrophila* was treated with OAEO at 100 μg ml^{-1} the haemolysis reduction was more than 90% (Fig. 1a). All the tested concentrations for both *S*-(+)- and *R*-(−)-linalool did not show haemolysis inhibition activity (Fig. 1b). In the *in vivo* assay there was 100% survival in the non-infected group (Fig. 1c). Fish exposed to 10 and 20 mg l^{-1} OAEO presented survival of 66 and 70%, respectively, and did not differ statistically from the group treated with the antibiotic (80%). The infected fish group that did not receive any treatment had a near 40% survival (Fig. 1c).

With regard to their biological properties, it is important to know that essential oils and extracts are complex mixtures of different molecules and their biological effects may result from a synergy of these molecules, or from the action of the major components. In a previous study Silva (2014) determined the *in vitro* antibacterial activity of *S*-(+)- and *R*-(−)-linalool against the same *Aer. hydrophila*

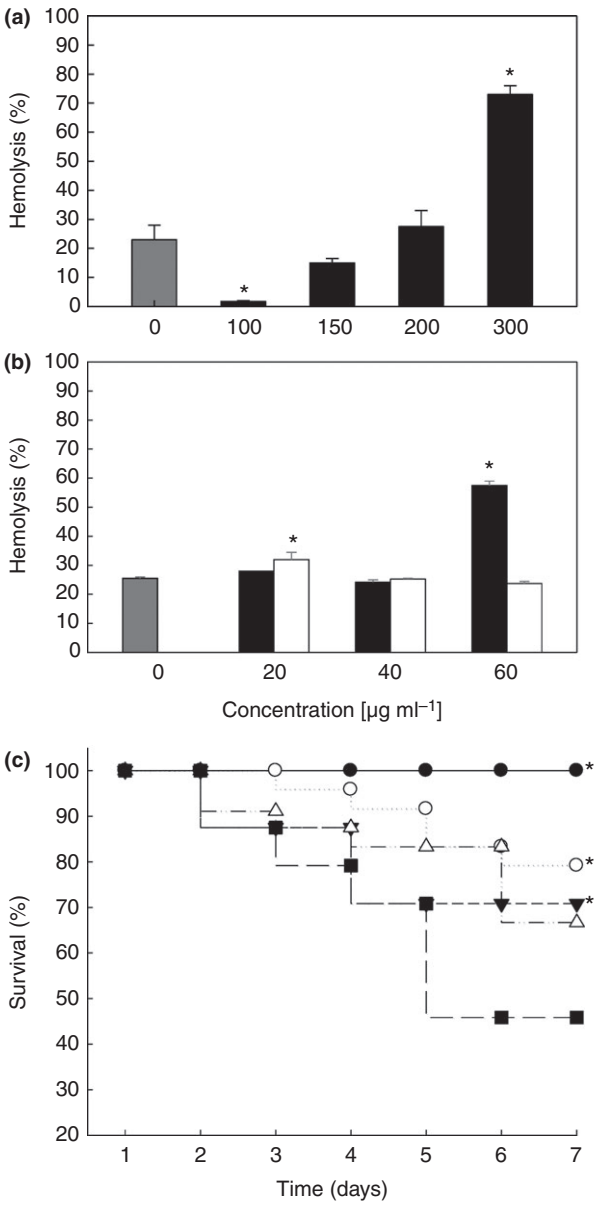

Figure 1 (a) Haemolytic activity of *Aeromonas hydrophila* culture supernatant treated with subinhibitory concentrations of *Ocimum americanum* essential oil (black bars) and control group (grey bar). (b) Haemolytic activity of *Aer. hydrophila* culture supernatant treated with the main compound found in the essential oil (*R*-(−)-linalool) (black bars) and its isomeric form (*S*-(+)-linalool) (white bars); control group (grey bar). *Significant difference relative to control (0 μg ml^{-1}). One-way ANOVA and Tukey's test ($P \leq 0{\cdot}05$). (c) Survival of silver catfish infected with *Aer. hydrophila* after therapeutic essential oil baths (1 h daily/5 days). —●— control – fish inoculated with sterile saline, ·····○····· fish inoculated with *Aer. hydrophila* and treated with antibiotic gentamycin 10 mg l^{-1}, – –△– – fish inoculated with *Aer. hydrophila* and treated with essential oils 10 and –·–▼–·– 20 mg l^{-1}, — —■— — negative control – fish inoculated with *Aer. hydrophila* and untreated. *Significant difference relative to negative control (untreated infected fish). Kaplan–Meier survival analysis with Logrank test ($P \leq 0{\cdot}05$).

strains as used in this study. However, only the *S*-(+)-isomer showed antibacterial activity against *Aer. hydrophila* ATCC 7966 and a clinical isolate at 3200 μg ml^{-1}, but it did not act against the other two clinical isolates tested. No activity was detected for the *R*-(−)-form *in vitro*. Considering the values found in this study for OAEO, and the *S*-(+)- and *R*-(−)-linalool values found by Silva (2014), these products presented weak inhibition activity against *Aer. hydrophila* (Ríos and Recio 2005).

It is recognized that the production of bacterial toxins can be modulated by natural antimicrobials, especially essential oils, even at subinhibitory concentrations (Smith-Palmer *et al.* 2004; Nazzaro *et al.* 2013; Soltani *et al.* 2014). *R*-(−)-Linalool alone does not seem to be responsible for the OAEO haemolysis inhibition, otherwise, *R*-(−)-linalool at 40 μg ml^{-1} should present some inhibitory activity, since it is approximately the same amount present (46%) as in the effective concentration of OAEO. Nonetheless, haemolysis increased at the highest concentration of OAEO tested (300 μg ml^{-1}) and *R*-(−)-linalool (60 μg ml^{-1}) (Fig. 1a,b), which may have resulted from the presence of the product in the bacterial culture supernatant and its cytotoxicity to the erythrocyte membrane. Most of the effects described for essential oils involve interactions with biological membranes, such as morphological changes with rupture of the membrane and cytoplasm leakage (Nazzaro *et al.* 2013).

While the antibacterial activity of essential oils and its constituents is increasingly being determined *in vitro*, *in vivo* studies are scarce. However, different plant essential oils have been given to aquatic organisms via baths or by incorporation in the feed, presenting promising results as a treatment in experimentally and naturally infected fish (Rattanachaikunsopon and Phumkhachorn 2009; Sutili *et al.* 2014b, 2015a,b). Identifying the mode of action of the essential oils requires a study of raw materials, since the mechanisms of action of essential oils are dependent on its composition and/or chemical structure of the constituent molecules. Furthermore, the mode of action must also be studied in various bacterial species. In addition, it should be borne in mind that plant essential oils contain many active compounds known to have inhibitory or stimulatory effects on the fish immune system (Harikrishnan *et al.* 2011).

Gyrodactylus sp

In the *in vitro* test formalin killed all the parasites after 1 h of exposure. In the groups exposed to 10 and 50 mg l^{-1} of the OAEO mix the parasite mortality was 75 and 98%, respectively. Both OAEO mix groups differed significantly from their respective control at 0 h. In the ethanol and water control groups, parasite survival was approx. 95% in both groups after 1 h (Fig. 2a). After 1 h in the *in vivo* assay all parasites dropped off the fish in the group exposed to formalin (Fig. 2b). There was a significant reduction of attached parasites (60%) in the group exposed to 50 mg l^{-1} of the OAEO mix. At 10 mg l^{-1} the OAEO mix did not significantly reduce the intensity (13%) of the parasite infection. In the control group there was no significant difference in the number of parasites between 0 and 1 h (Fig. 2b).

Existing methods for controlling monogenean parasites rely heavily on synthetic and toxic anthelmintics that are toxic to the fish, the handler, the environment and the consumer, if grace periods are not respected. The most effective indicated treatment against monogenean parasites in fish consists of baths with formalin (1 : 4000/1 h) (Pavanelli *et al.* 2002), a chemical product classified as carcinogenic to humans (WHO 2006). Therefore, the use of phytochemicals as anthelmintic drugs for fish is receiving increased attention as a sustainable and environmentally acceptable alternative (Hao *et al.* 2012).

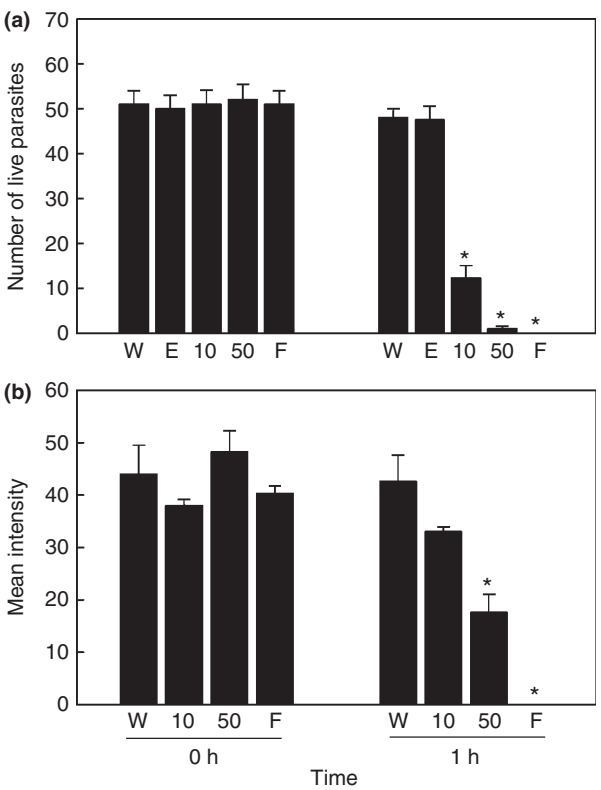

Figure 2 (a) Number of live parasites (*in vitro* test) and (b) mean intensity ± SEM of *Gyrodactylus* sp. in silver catfish following 1 h exposure to *Ocimum americanum* essential oil mix. W, water control; E, ethanol control; F, formalin 1 : 4000; 10 and 50 *O. americanum* essential oils mix at 10 and 50 mg l^{-1}. *Significant difference relative to respective group at 0 h. One-way ANOVA and Tukey's test ($P \leq 0{\cdot}05$).

Studies showed that different plant essential oils and their constituents have significant killing effects *in vitro* and *in vivo* on various monogenean species. Eugenol, an extremely versatile phenylpropanoid, found in several plant essential oils, used and recommended as an alternative anaesthetic for several fish species, was effective *in vitro* (5 and 10 mg l^{-1}) against *Gyrodactylus* sp. isolated from silver catfish (Sutili *et al.* 2014a). Bathing with 10 mg l^{-1} eugenol solution for 1 h was efficient against monogean gill infection in tambaquis (*Colosssoma macropomum*), decreasing their count by 80% (Boijink *et al.* 2015).

According to Steverding *et al.* (2005), Australian tea tree (*Melaleuca alternifolia*) oil in combination with tween 80 as a diluent was shown to be effective as a treatment for *Gyrodactylus* spp. infection in fish. Hashimoto *et al.* (2016) determined that therapeutic baths containing *Lippia sidoides* and *Mentha piperita* essential oils were effective in controlling different species of monogenean parasites in *Oreochromis niloticus*. *Lippia alba* EO showed an *in vitro* effect against monogenean gill parasites in tambaquis (*C. macropomum*). However, because of the low concentrations of essential oil (100 and 150 mg l^{-1}) tolerated by the fish the efficacy *in vivo* was low (Soares *et al.* 2016).

The mainly antiparasitic action mode of small lipophilic secondary metabolites, such as terpenoids or phenylpropanoids found in the essential oils of many plants, is to dissolve biomembranes and to disturb their fluidity and the function of membrane proteins (Wink 2008). These products may cause a slow development of resistance, and are easily biodegradeable and harmless to the environment (Liu *et al.* 2011; Wink 2012) showing high potential for use in aquaculture systems.

In conclusion, the results demonstrate that OAEO obtained from inflorescences has a weak bactericidal activity *in vitro* against *Aer. hydrophila* (minimum inhibitory concentration (MIC) test). At subinhibitory concentrations, OAEO (100 μg ml^{-1}) inhibited haemolysis caused by *Aer. hydrophila* in fish erythrocytes; however, the main EO component (linalool) alone did not seem to be responsible for the inhibition of haemolysis. In addition, the EO added to water at lower concentrations than the sedative concentration promoted survival in fish infected with the same bacterium. Lastly, the OAEO mix (leaves and inflorescences) at 50 mg l^{-1} showed antiparasitic activity against the fish monogenean parasite *Gyrodactilus* sp. Further studies should be conducted to verify this activity against other pathogens of interest in aquaculture and to verify the possible mode of action or the fish immune response involvement, as well as to identify the molecules responsible for the biological activities.

Material and methods

Essential oil and major constituents

Ocimum americanum was collected in Encantado-RS, Brazil. The species was identified by Dr Sérgio Augusto de Loreto Bordignon, and a voucher specimen (no. SMDB 13163) was deposited in the herbarium of the Department of Biology, Federal University of Santa Maria. The OAEO extraction (inflorescences and leaves) and gas chromatography/mass spectrometry (GC-MS) analysis were performed as described by Sutili *et al.* (2015b). The constituents of OAEO were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library (NIST/EPA/NIH 2002) and literature data (Adams 2001). The major OAEO constituents from inflorescences and a mix from leaves and inflorescences are represented in Table 1.

In vitro antibacterial assays

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards

Table 1 Major constituents of the *Ocimum americanum* essential oils obtained from inflorescences and mix (1 : 1) of leaves and inflorescences

Composition	%	RI (experimental)	RI (literature)*
Inflorescences			
β-Linalool	46·61	1105·3	1101
Camphor	9·5	1144·8	1144
1.8-Cineole	8·43	1031·4	1031
Germacrene D	4·76	1481·2	1480
Fenchone	3·59	1085·7	1087
β-Cariofilene	3·27	1418·9	1418
Eugenol	3·22	1362·5	1364
τ-Cadinole	3·09	1643·1	1642
β-Elemene	2·08	1391·8	1391
α-Terpineole	1·79	1190·4	1190
Inflorescences and leaves (mix)			
β-Linalool	32·43	1105	1101
1.8-Cineole	16·13	1031	1031
Camphor	11·23	1148	1144
Eugenol	9·67	1362	1364
β-Myrcene	3·52	989	992
β-Cariofilene	2·84	1423	1418
τ-Cadinole	2·76	1646	1642
Fenchone	2·58	1087	1087
α-Terpineole	2·30	1191	1190
β-Elemene	1·72	1394	1391

RI, retention index.
*Adams (2001), NIST/EPA/NIH (2002).

Institute; document M31-A3 (CLSI 2008) as described by Sutili *et al.* (2015b). Briefly, three strains of *Aer. hydrophila*, which were isolated from infected fish and *Aer. hydrophila* ATCC 7966 were used. The OAEO from inflorescences diluted in 96% ethanol and incorporated in a Mueller-Hinton broth (MHB) (Himedia Laboratories, Mumbai, India) at concentrations of 6400, 3200, 1600, 800, 400, 200, 100 μg ml^{-1} (in triplicate) was tested. The inoculum was prepared in saline solution from cultures grown in Mueller-Hinton agar (Himedia Laboratories; 1 × 10^8 colony forming units (CFU) ml^{-1}; 0·15 OD600nm; 30°C/24 h). Ten microlitres (1 × 10^5 CFU) of inoculum was added to each well containing OAEO. The microplates were incubated at 30°C for 24 h under aerobic conditions. The same procedure was performed on an ethanol (EO-diluent) control (final concentration 0·34%). The MBCs were confirmed by reinoculation of 10 μl of each bacterial culture on Mueller-Hinton agar (Himedia Laboratories; 30°C/24 h) and the lowest concentration of the OAEO showing no growth was defined as the MBC.

The haemolysis assay was performed according to the methodology described by Sutili *et al.* (2015b). A β-haemolytic strain was selected from the investigated strains during the MIC test. This strain was cultured at subinhibitory OAEO concentrations (0-control, 100, 150, 200 and 300 μg ml^{-1}) (30°C/24 h), prepared at a concentration of 2·1 × 10^9 CFU ml^{-1} (1·75 OD600nm) and then centrifuged (5500 ***g***, 4°C, 1 min). Prior to the addition of 100 μl of defibrinated fish blood, a 200 μl volume of culture supernatant was diluted to a volume of 1000 μl through the addition of phosphate-buffered saline (PBS) in triplicate. After incubation for 90 min/37°C, the unlysed blood cells were pelleted by centrifugation (5500 ***g***, room temperature, 1 min). The haemolytic activity of the supernatant was detected by measuring its optical density at 540 nm. The same assay was conducted for the main compound found in OAEO (*R*-(−)-linalool) and its isomeric form (*S*-(+)-linalool) at subinhibitory concentrations (0-control, 20, 40 and 60 μg ml^{-1}). These compounds were previously isolated from the essential oils of *O. americanum* and *L. alba* as described by Silva (2014). The percent haemolysis was calculated by comparison between total haemolysis (100%) and no-haemolysis (0%) controls. The same procedure was performed on an ethanol (EO-diluent) control.

In vivo assay – *Aeromonas hydrophila*

Fish were transferred from a local fish farm to the Fish Physiology Laboratory-UFSM. They were maintained and acclimated for 7 days in continuously aerated tanks, with controlled water parameters (22·0 ± 1·0°C, pH: 7·5 ± 0·5, dissolved oxygen levels: 6·0 ± 1·0 mg l^{-1}). One hundred and twenty healthy silver catfish fingerlings (10 ± 2 g and 10 ± 1 cm) were distributed in 15 plastic boxes (30 l). They were anaesthetized (50 mg l^{-1} eugenol) and 100 μl of *Aer. hydrophila* solution (ATCC 7966; 1·5 × 10^9 CFU ml^{-1}; 1·2 OD600nm) was inoculated intramuscularly into the latero-dorsal right side of the fish. The groups were (in triplicate, n = 8 per box): control (silver catfish inoculated with 100 μl sterile saline), negative control (infected fish untreated), gentamycin 10 mg l^{-1} (Belem-Costa and Cyrino 2006) and OAEO from inflorescences (10 and 20 mg l^{-1}). These EOs concentrations have no sedative effect in silver catfish and were chosen to avoid behavioural side effects during the long exposure time of the bath protocol. The OAEO and antibiotic exposure was carried out through daily baths (1 h) for 5 days. Fish from the control groups were exposed to the same bath protocol. Mortality caused by *Aer. hydrophila* was observed in each group for two additional days (total 7 days). The OAEO was previously diluted 1 : 10 in 95% ethanol and added to the bath water. During the experiment the temperature, pH and dissolved oxygen values were similar to the values of the acclimatization period. Total and non-ionized ammonia levels were: 1·9 ± 0·7 and 0·024 ± 0·01 mg l^{-1}, respectively. Fish were fed once a day to satiation with commercial feed and uneaten food, other residues and faeces were removed 30 min after feeding. Dead fish were also removed daily. The bacterial re-isolation was made from lesions and kidneys of the fish. Morphological and biochemical characteristics were analysed according to Quinn (1994) for bacterial identification and characterization.

In vitro antiparasitic assay

The parasite was identified by Dr Walter Boeger (Universidade Federal do Paraná, Brazil). Four silver catfish (12 ± 1 g) highly parasitized by monogenean parasites were selected and the assay was conducted as described by Sutili *et al.* (2014a). Following euthanasia of the fish by spinal cord section, the maxillary barbels were removed. Polystyrene plates with 12 wells and flat bottoms were utilized for the assay. The parasitized barbels were divided into smaller fractions (3·0 mm), and the barbel fragments (±50 parasites) and 2 ml of the treatment solution were added to each well. The EO was diluted 1 : 10 in 96% ethanol for better solubilization in water. The following treatments were tested in triplicate: OAEO mix (10 and 50 mg l^{-1}); formalin 1 : 4000 (Pavanelli *et al.* 2002); ethanol (at the same concentration used to dilute the highest concentration of EO) and water control. Parasite mortality was evaluated after a 1 h exposure with the assistance of a stereomicroscope (10× total magnification). The parasite was considered dead if it did not exhibit any movement after 2 min of observation.

In vivo assay – *Gyrodactylus* sp

The efficacy of 1 h bath treatments using the OAEO mix was tested as described by Fridman *et al.* (2014) with slight modifications. Naturally parasitized silver catfish (12 ± 1 g) with a low number of parasites were selected and individually placed in 1 l plastic boxes equipped with aeration ($n = 5$ per treatment). To determine the infection rate at time 0 h, every fish was separately placed in a glass container and the parasites were counted in the dorsal region above the lateral line. Fish were exposed to the treatments (water control, formalin 1 : 4000 and OAEO mix at 10 and 50 mg l^{-1}) for 1 h and then removed; the parasite load was measured as before. The mean intensity was calculated as the mean number of parasites found in the infected hosts (Bush *et al.* 1997).

The methodology of these experiments was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process no. 046/2010).

Statistical analysis

The homogeneity of variances between the groups was tested with the Levene test. Comparisons between different groups were made using one-way ANOVA and Tukey's (STATISTICA 7.0; StatSoft Inc., Tulsa, OK). Fish survival was compared using Kaplan–Meier survival analysis with the Logrank test (SIGMA PLOT 10.0; Systat Software, San Jose, CA). The minimum significance level was set at $P \leq 0{\cdot}05$.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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2.3. ARTIGO 3

O **Artigo 3** reporta o efeito *in vitro* dos OE das plantas *O. americanum, C. flexuosus* e *M. alternifolia* em parâmetros de imunidade não específica e fragilidade osmótica dos eritrócitos de red drum, a fim de avaliar a sua potencial utilização *in vivo*. O artigo foi publicado no periódico **Journal of Animal Physiology and Animal Nutrition.**

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ORIGINAL ARTICLE

In vitro effects of plant essential oils on non-specific immune parameters of red drum, *Sciaenops ocellatus* L.

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Summary

Phytochemicals such as plant essential oils (EOs) have been reported to favour various activities in the innate immune system of fish. Thus, the aim of this study was to verify the *in vitro* effect of three different plant EOs (*Ocimum americanum, Cymbopogon flexuosus* and *Melaleuca alternifolia*) on non-specific immune parameters and erythrocyte osmotic fragility of red drum, *Sciaenops ocellatus*. Concentrations of each plant EO evaluated in preparations of head-kidney macrophages, blood leucocytes and blood plasma were as follows: 0.0 (control), 1.0, 2.0, 4.0, 8.0, and 16.0 μg/ml. Red drum head-kidney macrophages significantly increased extracellular superoxide anion production when exposed (20 h) to *O. americanum* EO (1.0-8.0 μg/ml) and *C. flexuosus* EO (2.0 and 4.0 μg/ml). The respiratory burst of blood leucocytes (NBT test) significantly increased in all concentrations when compared to the respective control group, for all EOs. At the highest concentration (16.0 μg/ml), *C. flexuosus* EO significantly inhibited the haemolytic activity of complement system in red drum blood after 1 h exposure. None of the tested concentrations significantly altered plasma lysozyme activity or erythrocyte osmotic fragility after exposing (1 h) red drum whole blood to each EO. This study demonstrated that these plant EOs are capable of triggering superoxide anion production in red drum leucocytes (head-kidney macrophages and/or blood leucocytes). *In vivo* studies are warranted to address their potential as immunostimulants in the diet of red drum and other aquacultured species.

Keywords complement system, erythrocyte osmotic fragility, lysozyme, phytochemical, superoxide anion

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Introduction

According to Sakai (1999), an immunostimulant is defined as a chemical, drug, stressor or action that enhances the non-specific immune response by directly interacting and activating immune cells. Fish rely on both specific and non-specific mechanisms to protect themselves against invading pathogens, mobilizing cellular and humoral non-specific defences. The major components that make up the non-specific defence mechanisms in fish are macrophages, monocytes, granulocytes and humoral elements, such as lysozymes or the complement system. In addition, phagocytic function and its mediators comprise one of the main defence mechanisms of non-specific immunity to pathogens including bacteria, viruses and parasites in fish (Dügenci et al., 2003; Galina et al., 2009). In recent years, due to strengthened regulations on the use of drugs for disease control in fish, studies aimed at identifying immunostimulants as a preventive measure against disease outbreaks in aquaculture operations have been intensified (Lee et al., 2015).

Phytochemicals such as essential oils (EO) seem to represent a promising source of bioactive molecules that can be used, mainly, as additive in diets or through baths to enhance immune functions in fish. The immune system of fish can be triggered by EO components, and these molecules may have different targets and effects on the organism, showing inhibitory and/or stimulatory activity. Thus, these products can be used for the prevention of diseases improving the health status of the fish, to promote survival and growth and against monogenean parasites (Galina et al., 2009; Harikrishnan et al., 2011; Bulfon et al.,

2013; Chakraborty et al., 2014; Hashimoto et al., 2016).

Plants such as *Ocimum americanum*, *Cymbopogon flexuosus* and *Melaleuca alternifolia* are mainly known for their potential as modulators of the inflammatory process and their antimicrobial activity (Carson et al., 2006; Mahajan et al., 2013; Avoseh et al., 2015). However, despite their pharmacological properties, information on their potential as immunostimulants for aquacultured species is limited. Therefore, the objective of this study was to verify the *in vitro* effect of these three different plant EOs on non-specific immune parameters and erythrocyte osmotic fragility of red drum (*Sciaenops ocellatus*) in order to assess their potential use *in vivo*.

Material and methods

Essential oils

Ocimum americanum was cultivated at Federal University of Santa Maria/CESNORS, Frederico Westphalen city, Rio Grande do Sul, Brazil (voucher specimen n°. SMDB13163). The EO from *O. americanum* was obtained from fresh leaves by steam distillation using a Clevenger-type apparatus for 3 h (Bayala et al., 2014). The EO of *C. flexuosus* was provided by Deonise Mrozinski Irgang; National Registry of Legal Entities: 02087725/0001-94, Três Passos city (Rio Grande do Sul, Brazil). These EOs were analysed as described by Silva et al. (2012), and the components were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library (NIST, 2010) and literature data (Adams, 2007) and quantified by GC-FID analysis in triplicate. The major components of the EOs were determined to be (%): linalool (33.5), eugenol (18.2), 1.8-cineole (12.8) and camphor (12.5) for *O. americanum* EO and α-citral (48.9) and β-citral (37.4) for *C. flexuosus* EO. Lastly, EO of *M. alternifolia* (100% Australian tea tree oil, terpinen-4-ol chemotype) was obtained commercially (Humco Holding Group, Inc., Texarkana, TX, USA).

Essential oil concentrations

The EO concentrations evaluated were as follows: 0.0 – control, 1.0, 2.0, 4.0, 8.0 and 16.0 μg/ml. These concentrations were chosen based on literature data (Manosroi et al., 2006; de Almeida et al., 2007; Kumar et al., 2008; Santin et al., 2009; Kpoviessi et al., 2014; Nogueira et al., 2014; Low et al., 2015) and preliminary cytotoxicity studies of the EOs on red drum head-kidney macrophages by trypan blue exclusion test (Valadares et al., 2007), as well as by examining haemolytic activity of the EOs against red drum red blood cells as described by Rodrigues et al. (2013).

Blood samples, phagocytic cell isolation and attachment

Fish (approximately 200 g) were anesthetized (MS-222) for blood collection and subsequently euthanized. Approximately 1.5 ml of blood from each fish was collected from the haemal arch in the caudal peduncle using 3-ml heparinized syringes. Head-kidney macrophages were collected from euthanized fish following the procedures of Sealey and Gatlin (2002). Briefly, using sterile techniques, head-kidneys were dissected and pushed through a 100-μm screen tissue homogenizer with cold (4 °C) L-15 medium (Sigma, St. Louis, MO, USA) containing 2% foetal calf serum (FCS; Sigma) and 100 units (U)/ml penicillin. The resultant suspension was layered on to a 34/51% Percoll gradient and centrifuged at 400 *g* for 30 min at 4 °C to enrich for macrophage isolation. The band of cells lying at the interface was collected by pipette and washed twice (1000 *g*; 10 min; 4 °C) in Hanks Balanced Salt Solution (HBSS; Sigma) to remove Percoll, then re-suspended to approximately 2×10^7 cells/ml with L-15 media supplemented with 0.1% FCS and 100 U/ml penicillin. Cell viability was assessed using trypan blue (0.4%) exclusion and determined to be greater than 90%. In 96-well plates, a 100 μl aliquot of the cell suspension was added to each well. These plates were then kept undisturbed for 1 h at room temperature for cell attachment.

Culture conditions and incubation

The culture conditions and incubation of head-kidney macrophages were performed following the procedures described by Sealey and Gatlin (2002) and Li et al. (2009), with modifications. An initial concentrated solution of each plant EO was prepared by weighing an aliquot of each respective product into a volumetric flask and bringing to volume with L-15 culture medium. An aliquot of this solution was pipetted into another volumetric flask and brought to its final volume with culture medium (L-15 medium, 10% FCS, 100 U/ml penicillin) to achieve the highest EO concentration. This solution was then serially diluted into individual volumetric flasks whose volumes were completed with culture medium. The sixth volumetric flask was the control solution (0.0 μg EO/ml) containing only culture medium. All EO solutions were prepared such that each solution when added to the wells (100 μl) reached the desired final EO

concentration at a final well volume of 200 μl. Plates were incubated with gentle agitation for 20 h at room temperature after which supernatants were removed and attached cells were washed twice with warm (18–20 °C) L-15 media and excess solution was removed.

The influence of EOs on respiratory burst of whole blood, lysozyme activity and complement system activity of red drum plasma was performed according to the procedure described by Zanuzzo et al. (2012). Blood samples were mixed (1:1) with each EO solutions prepared with phosphate-buffered saline (PBS). The mixture was incubated for 1 h, at room temperature. The control solution was only PBS. After the incubation period, the mixtures with whole blood and EO solutions were used for the respiratory burst assay. Lysozyme activity and haemolytic activity of total complement system were determined using the supernatant of the mixture separated by centrifugation (3800 *g* for 12 min at 5 °C). After the centrifugation process, the remaining red blood cells were resuspended in PBS and the erythrocyte osmotic fragility assay was performed.

Extracellular superoxide anion production

Extracellular superoxide anion (O_2^-) production (ECSA) was performed as described by Secombes (1990). Head-kidney macrophage layers, prepared and cultured as described above, were covered with 100 μl of 2 mg/ml ferricytochrome c with 1 μg/ml of phorbol myristate acetate (PMA) in phenol red-free HBSS. Superoxide dismutase (SOD) was used to block the reaction and verify specificity. Plates were then read immediately on a multiscan spectrophotometer at 550 nm every 15 min for 1 h. The amount of ECSA was calculated according to Pick and Mizel (1981) using the following formula: nmol of ECSA/well = $[(\Delta_{\text{absorbance after 60 min}} \times 100)/6.3]$.

Respiratory burst assay

Respiratory burst activity of blood leucocytes was determined as described by Siwicki et al. (1994). Whole blood and EO solutions mixed as described above (100 μl) was added to 100 μl of nitro blue tetrazolium (NBT) solution (0.2%) and incubated for 30 min at 25 °C. Next, 50 μl of this mixture was added to 1 ml of *N,N* dietilmethylformamide (Sigma) and centrifuged. The supernatant was collected, and its optical density was determined (OD 545 nm). Absorbance was converted to NBT, mg/ml blood-EO, based on the standard curve of NBT diformazan solution: $40 \times (A_{\text{absorbance545}} - 0.0245)/5.8564$.

Haemolytic activity of complement system

Haemolytic activity of complement system (HACS) was measured according to the procedure of MoralesdelaNuez et al. (2009) with the following modifications. Washed equine red blood cells at 5% (20 μl) were added to the blood-EO solution supernatants (100 μl) and incubated at 25 °C. Ice-cold saline (100 μl) was added after 1 h, then cells were pelleted by centrifugation (3800 *g* for 10 min) and the absorbance of the supernatant was measured in a microplate reader at 405 nm. The per cent haemolysis was calculated by comparison between total haemolysis (100%) and no-haemolysis (0%) controls as follows: % haemolysis = $[(A_{405\text{sample}} - A_{405\text{no-haemolysis}})/(A_{405\text{ total haemolysis}} - A_{405\text{ no-haemolysis}})] \times 100$.

Lysozyme activity assay

Lysozyme activity was determined by a turbidimetric assay as described by Jørgensen et al. (1993). Briefly, a suspension (200 μl) of *Micrococcus lysodeikticus* in PBS solution (0.2 g/l) at pH 5.2, previously determined to be optimum for red drum lysozyme activity was mixed with blood-EO solution supernatant (10 μl). Lysozyme activity (units/ml) was calculated using the following formula: $[(\Delta_{\text{absorbance(4–1 min)}}/3)/0.001] \times 100$. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min.

Erythrocyte osmotic fragility

Erythrocyte osmotic fragility (EOF) was performed as described by Meamarbashi and Rajabi (2013) with modifications. Briefly, whole blood and EO solutions were mixed at 1:1 proportion (250:250 μl). As described above, after 1 h the mixtures were centrifuged to collect the supernatant and the remaining red blood cells were resuspended to 0.5 ml PBS. A 10 μl aliquot of the erythrocyte suspension was then added to 1 ml salt solution (0.45% NaCl) in microtubes. A total haemolysis control (distilled water, 0.0% NaCl solution) and no-haemolysis control (0.9% NaCl solution) were performed individually. After incubation at room temperature for 15 min, the tubes were centrifuged at 3000 *g* for 3 min. The supernatant (200 μl) was transferred to 96-well microtitre plates, and the absorbance of the samples was read at 415 nm. The haemolysis (%) at 0.45% saline solution for each sample was calculated relative to the respective total haemolysis.

The experimental procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Statistical analysis

Resulting data from all assays were expressed as the mean ± standard error of the mean (SEM). The homogeneity of variances between groups was tested with the Levene's test. The one-way ANOVA was used to determine significant ($p \leq 0.05$) differences among treatments. When significant differences were detected, the Duncan's multiple range test was used for the separation of treatment means. All statistical analyses were performed using the STATISTICA® software 7.0 (StatSoft, Tulsa, OK, USA).

Results

Red drum head-kidney macrophages significantly increased ECSA when exposed (20 h) to *O. americanum* (1.0–8.0 μg/ml) and *C. flexuosus* (2.0 and 4.0 μg/ml) EOs compared to the respective control and the highest concentration tested (0.0 and 16.0 μg/ml). Contrastingly, *M. alternifolia* EO did not show any significant effect on ECSA of red drum head-kidney macrophages after 20 h of incubation (Fig. 1a). Despite the differences between EO concentrations tested (1.0–16.0 μg/ml), the respiratory burst of blood leucocytes (NBT test) significantly increased in all concentrations when compared to respective control group, for all EOs (Fig. 1b). HACS was not affected when red drum blood was incubated (1 h) with any *O. americanum* or *M. alternifolia* EO concentrations. However, *C. flexuosus* EO at the highest tested concentration (16.0 μg/ml) significantly inhibited the HACS in red drum measured in the EO-blood solution supernatant (Fig. 1c). None of the tested EO significantly altered plasma lysozyme activity or EOF after 1 h exposure (Table 1).

Discussion

Plant EOs are very complex natural mixtures that may contain dozens of components in different concentrations. The principal plant EOs components include two mainly groups of terpene derived from the same biosynthetic origin, the main group consists of hydrocarbon terpenes/terpenoids and the other group consists of oxygenated derivatives of hydrocarbon terpenes such as alcohols, aldehydes, ketones, acids, phenols, ethers and esters. In EOs may also occur phenylpropanoid derivatives, which have different biosynthetic origin (Carson and Hammer, 2011). These molecules may directly initiate activation of the innate defence mechanisms acting on receptors of cell

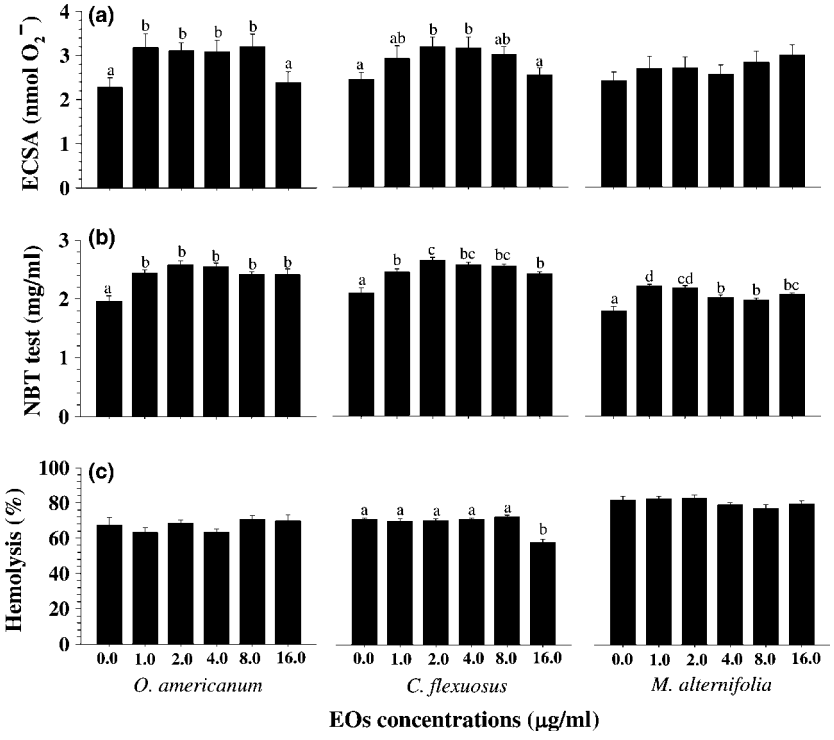

Fig. 1 (a) Extracellular superoxide anion production (ECSA) of head-kidney macrophages of red drum after 20-h incubation with different concentrations of *Ocimum ameri canum*, *Cymbopogon flexuosus* and *Melaleuca alternifolia* EOs ($n = 10$/concentration). (b) Respiratory burst activity of blood leucocytes (NBT test) after 1-h incubation with different concentrations of the EOs ($n = 9$/concen tration). (c) Haemolytic activity of complement system in the EO-blood solutions supernatant after 1 h of incubation of whole blood with different concentrations of the EOs ($n = 6$/concentration). Data are reported as the mean ± SEM. Different letters indicate significant differences between concentrations (ANOVA and Duncan's multiple range test, $p \leq 0.05$).

Table 1 Lysozyme activity (Lyz) measured in the blood-EO supernatant and erythrocyte osmotic fragility (EOF) after red drum blood exposure (1 h) to different plant essential oils

	Ocimum americanum		*Cymbopogon flexuosus*		*Melaleuca alternifolia*	
EO μg/ml	Lyz	EOF	Lyz	EOF	Lyz	EOF
0.0	211.1 ± 42.1	12.1 ± 0.5	374.0 ± 43.8	9.2 ± 1.2	385.4 ± 48.0	11.5 ± 1.3
1.0	211.1 ± 47.6	10.5 ± 0.6	301.6 ± 47.7	7.5 ± 0.4	307.6 ± 38.7	10.3 ± 0.4
2.0	269.0 ± 49.6	9.1 ± 0.6	381.6 ± 50.5	9.2 ± 1.5	346.7 ± 36.1	10.9 ± 0.4
4.0	284.8 ± 63.6	11.2 ± 0.8	264.3 ± 25.4	8.1 ± 0.7	343.7 ± 43.1	10.4 ± 1.1
8.0	263.3 ± 43.1	9.7 ± 0.9	256.3 ± 24.6	8.8 ± 1.0	295.0 ± 47.0	9.8 ± 1.0
16.0	286.1 ± 58.9	10.0 ± 1.0	337.5 ± 50.9	10.0 ± 1.0	295.0 ± 48.3	10.2 ± 0.8
p-Value	0.800	0.150	0.191	0.580	0.729	0.822

Data are reported as the mean ± SEM (n = 6/concentration). Lyz – unit/ml, EOF – % haemolysis at 0.45% saline solution.

surface, triggering intracellular gene activation and/or cell mediators that may result in production of antimicrobial molecules (Bricknell and Dalmo, 2005). The resulting increase in immunity components confers enhanced protection from infectious diseases by facilitating the function of phagocytic cells, increasing their bactericidal activities, and stimulating the natural killer cells, complement system, lysozyme activity and antibody responses (Sakai, 1999; Dügenci et al., 2003; Harikrishnan et al., 2011).

Inhibitory and/or stimulatory effects on non-specific immune parameters as found in this study have been reported in other *in vitro* studies with different plant EOs and isolated compounds. Pérez-Rosés et al. (2015) investigated the *in vitro* activity of fifteen different EOs (*Myristica fragrans, Melaleuca quinquenervia, M. alternifolia, Melaleuca cajuputi, Syzygium aromaticum, Artemisia dracunculus, Coriandrum sativum, Juniperus communis, Zingiber officinale, Rosmarinus officinalis, Laurus nobilis, Cymbopogon martini, Citrus limon, Thymus zygis, Coridothymus capitatus*), pure compounds (eugenol, carvacrol and thymol) and EO fractions on phagocytosis by human neutrophils and on the complement system. Most of the products tested showed mild-to-moderate inhibition of phagocytosis and the classical pathway of complement system, whereas all products were found inactive in altering the activity of the alternative pathway of the complement system. However, in other *in vitro* experiments, it was found that eugenol, found in the EO of *O. americanum*, was able to recover phagocytic capacity previously reduced in murine peritoneal macrophage (Mahapatra et al., 2011) and the terpene D-limonene increased nitric oxide (NO) production in peritoneal macrophages obtained from tumour-bearing mice (Del Toro-Arreola et al., 2005).

Essential oils and their isolated compounds present different potential uses in fish culture (e.g., alternative anaesthetics, growth promoters and/or to treat fish diseases) showing immunomodulatory properties. Then, the majority of studies evaluating the effects of EOs in non-specific immune parameters of fish comprise *in vivo* studies. Anaesthesia caused by clove oil (*Eugenia aromatica*) in rainbow trout (*Oncorhynchus mykiss*) did not alter the haemolytic activity of the alternative complement, but it depressed respiratory burst (Kanani et al., 2013) and increased lysozyme activity (Kanani et al., 2011) 24 h after anaesthesia. Preventive baths (1 h) with eugenol (5 and 10 mg/l) for 5 days did not alter phagocytic index, respiratory burst and myeloperoxidase activity and HACS in silver catfish (*Rhamdia quelen*) (Sutili et al., 2014). Silver catfish exposed to *Hesperozygis ringens* and *O. americanum* EOs (20 mg/l) diluted in ethanol (baths-1 h daily/5 days) increased the HACS and cortisol levels in plasma (Sutili et al., 2015). Dietary supplementation of 3 g/kg of carvacrol in commercial diets improved lysozyme and myeloperoxidase activity in rainbow trout (Yilmaz et al., 2015). Lysozyme activity was also high in channel catfish fed a diet with *Origanum vulgare* EO (Zheng et al., 2009).

Fish have several types of phagocytic leucocytes, which are present in blood, the peritoneal cavity, and a variety of tissue locations. Phagocytosis and the production of oxygen free radicals via the respiratory burst are important events in bactericidal pathways in fish (Dügenci et al., 2003). Phagocytosis of bacteria is accompanied by a dramatic increase in oxygen consumption resulting in the formation of O_2^- to neutralize the invading organisms (Miguel, 2010). Molecules found in plant EOs can act in advance inducing reactive oxygen species production in different cells due their intrinsic pro-inflammatory potential. Furthermore, besides the role in the host defences against infections, these free radicals have a crucial role in inflammatory conditions as well as in tissue repair and

regeneration (Koko et al., 2008; Cosentino et al., 2014). In the present *in vitro* study, *O. americanum* and *C. flexuosus* EOs were capable of triggering and increase O_2^- production in red drum head-kidney macrophages. In addition, all EOs also increased the respiratory burst of blood leucocytes.

The use of *in vitro* assays to evaluate the immunostimulant potential or toxicological effects of candidate products to be used in fish culture offers many advantages relative to *in vivo* evaluations. These include the need for fewer animals, lower cost and time to be performed, aid in the definition of effective doses, enhancement of *in vivo* predictability and evaluation of specific mechanisms of action (Ouedraogo et al., 2012). Overall, the results of the present study reinforced the applicability of *in vitro* assays in the evaluation of potential immunostimulants for fish.

Conclusions

Our findings show that the *O. americanum* and *C. flexuosus* EOs can stimulate O_2^- production in red drum head-kidney macrophages as well as in blood leucocytes. Both EOs are good candidates to be evaluated *in vivo* as modulators of innate immune responses in fish.

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2.4. ARTIGO 4

O **Artigo 4** relata o uso do OE de *O. americanum*, obtido a partir das folhas da planta, como aditivo na dieta de red drum. Foram avaliados crescimento, composição centesimal, pH do trato gastrointestinal, parâmetros de imunidade não específica e composição da microbiota intestinal. Este artigo foi publicado no periódico **Fish and Shellfish Immunology**.

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Full length article

Evaluation of *Ocimum americanum* essential oil as an additive in red drum (*Sciaenops ocellatus*) diets

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ABSTRACT

This study evaluated productive parameters, whole-body composition, non-specific immune responses and pH and microbiota of digestive tract contents of red drum (*Sciaenops ocellatus*) fed diets supplemented with *Ocimum americanum* essential oil (OAEO) (0 – control, 0.25, 0.5, 1.0 and 2.0 g/kg diet). After 7 weeks no significant differences in productive parameters and whole-body composition were observed. Plasma and intestinal lysozyme measurements and pH of the stomach and intestine (6 h after feeding) did not show significant differences among groups. Intestinal microbial community in fish fed the basal and OAEO diets (all concentrations) were identical. However, red drum fed the diet with OAEO at 1.0 g/kg had significantly increased intraperitoneal fat deposition and stomach pH (2 h after feeding) and decreased superoxide ion production (NBT-test) compared to the control group. Hemolytic activity of the complement system increased in fish fed diets containing OAEO. Red blood cells from fish fed the lowest OAEO concentration (0.25 g/kg) showed significant lower fragility in erythrocyte osmotic fragility assay, but fish fed 0.5 and 1.0 g/kg showed significant higher erythrocyte fragility. Lysozyme measurement in the supernatant of stomach content was significantly higher in fish fed the diet supplemented at 0.5 g/kg. Based on these various results, OAEO at different supplementation levels did not influence growth performance and intestinal microbial community; however, the EO added to the diet showed effects on immunological responses of red drum.

1. Introduction

Natural products derived from plants are among the most interesting options for the replacement of antibiotic growth promoters and synthetic drugs in animal production [1–5]. These products are commonly recognized as safe for animals, consumers and the environment [6], showing high potential to be used in aquaculture systems. Several studies have discussed the potential use of plant extracts, essential oils (EOs) and plant-isolated compounds in fish production [4,7,8]. These studies show that the use of these products as additive in fish diets or through baths can result in an improvement of productive parameters and health status of various fish species.

Essential oils obtained from plants are a natural blend of organic substances characterized by a strong fragrance, synthesized by aromatic plants during secondary metabolism. They contain a rich mixture of highly functional molecules with a wide spectrum of biological activity. Studies have demonstrated that EOs added to diets can activate the fish antioxidant defense system, enhance immune functions, change the intestinal morphology and microbiota, as well as increase digestibility and nutrient absorption [7–10].

Plants of the genus *Ocimum* are collectively called basil and are used as a source of EOs, spices, medicinal and ornamental plants [11]. Basil has long been used by people to treat digestive problems and as an antiseptic. In addition, it is known to have strong antioxidant properties, especially the extract and EO [12]. *Ocimum americanum* (Lamiaceae) is a plant species native to Africa and Asia, widely distributed in the tropics and subtropics and found growing

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spontaneously in tropical America [13]. Antifungal and antibacterial properties [14–16], as well as anesthetic activity of the EO in fish [17] have been reported as biological activities for this plant. Therefore, the present study assessed the effect of different levels of dietary addition of the *O. americanum* EO (OAEO) on red drum (*Sciaenops ocellatus*) productive performance, non-specific immune responses, as well as pH and microbiota composition of the digestive tract.

2. Materials and methods

2.1. Essential oil and major constituents

Ocimum americanum was cultivated at Federal University of Santa Maria/CESNORS, Frederico Westphalen city, Rio Grande do Sul, Brazil (voucher specimen nº. SMDB13163). The EO was obtained from fresh leaves by steam distillation using a Clevenger-type apparatus for 3 h [18]. The EO was analyzed using an Agilent-7890A gas chromatograph coupled with an Agilent 5975C mass selective detector (GC-MS) equipped with a capillary column HP5-MS (5% phenyl, 95% methylsiloxane, 30 m × 0.25 mm i.d. x 0.25 μm), following the analysis program described by Silva et al. [19]. Constituents of the EO were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library [20] and literature data [21]. The components of the EO were quantified by gas chromatography with flame ionization detection (GC/FID) on an Agilent-7890A. Major constituents of the OAEO are shown in Table 1.

2.2. Experimental design and diet

Two hundred and forty healthy red drum juveniles (17.75 ± 0.1 g) were obtained from the Texas Parks and Wildlife Department (Lake Jackson, TX) and were maintained at the Texas A&M University Aquacultural Research and Teaching Facility. Fish were acclimated to experimental conditions for 1 week prior to the feeding trial, and then, they were randomly distributed as groups of 12 fish (five treatments and four replicates) into 20 glass aquaria (110 L each) with individual aeration, operated as a closed recirculating system (1 L/min) with biological/mechanical filtration. Water temperature remained at 26 °C throughout the trial by conditioning ambient air. Salinity was maintained at about 8 g/L by mixing well water and synthetic sea salt (Fritz Industries Inc., Dallas, TX). During the experimental period, pH was 7.5–8.0, total ammonia-nitrogen was lower than 0.5 mg/L, and dissolved oxygen was not less than 6.0 mg/L.

A basal diet was formulated and analyzed to contain, on a dry-matter (DM) basis, 40% crude protein from menhaden fishmeal and soybean protein concentrate, 10% total lipid from menhaden fish oil, and dextrin as the soluble carbohydrate to provide approximately 3.1 kcal digestible energy/g diet. The pH of the diet was measured as described by Castillo et al. [22] (Table 2). All ingredients were weighed and mixed until homogeneous. Different OAEO levels (0-control, 0.25, 0.5, 1.0 or 2.0 g/kg diet) were added to the diet ingredient mixture together with fish oil. Water was then added to the diets, and pellets were produced by passing the mash through a 3-mm die plate at the end of a meat grinder. After pelleting, a drying process was performed by forcing ambient air (room temperature) over the pellets for 24 h. Finally, the pellets were broken, sieved to an appropriate size for the fish and stored (−18 °C) until used. The fish received the experimental diets twice a day (8 a.m. and 4 p.m.). The feeding regime for the trial was 4% of body weight during weeks 1–3, 3% of body weight during weeks 4–6, and 2% of body weight during the seventh week. The biomass of fish in each replicate tank was determined weekly to adjust the feed quantities.

2.3. Productive parameters and whole-body composition

Productive performance was calculated as follows: Relative weight gain (RWG%)=(final weight, g − initial weight, g)/(initial weight, g)×100; Feed efficiency (FE)=(weight gain, g)/(feed consumed, g); Condition factor (CF)=(final weight, g)/(body length, cm)^3; Hepatosomatic index (HSI%) and Intraperitoneal fat ratio (IPF %)=(liver or IPF weight, g)/(final weight, g)×100; Fillet yield (FY %)=(fillet weight, g)/(final weight, g)×100; Survival rate (%)=(initial number of fish)/(final number of fish)×100. The proximate composition of whole-body tissues was determined on pooled samples of two fish per aquarium (eight fish/treatment) using standard procedures as described by Webb and Gatlin [23].

Table 1
Chemical composition of the essential oil obtained from leaves of *Ocimum americanum*.

RI experimental	RI literature*	Constituent	(%) Composition
991	984	β-Pinene	0.59
1028	1030	Limonene	0.95
1030	1030	1,8-Cineole	12.89
1087	1086	Fenchone	1.16
1100	1097	Linalool	33.54
1143	1143	Camphor	12.52
1190	1189	α-Terpineol	2.76
1358	1356	Eugenol	18.23
1422	1420	β-Caryophyllene	1.29
1438	1440	α-Bergamotene	1.89
1484	1481	Germacrene D	3.48
1509	1513	α-Bulnesene	0.66
1517	1517	δ-Amorphene	0.84
1644	1642	τ-Cadinol	2.50
Identified constituents			93.3

RI = Retention index; * [20,21].

Table 2
Composition of the experimental diets containing *O. americanum* EO (OAEO) on a dry-weight basis.

Ingredient	OAEO levels in experimental diets (g/kg)				
	0.0	0.25	0.5	1.0	2.0
Menhaden meal[a]	290.0	290.0	290.0	290.0	290.0
Soy protein concentrate[b]	277.0	277.0	277.0	277.0	277.0
Dextrin[c]	140.0	140.0	140.0	140.0	140.0
Menhaden oil[a]	60.0	59.75	59.5	59.0	58.0
Essential oil	0.0	0.25	0.5	1.0	2.0
Vitamin premix[d]	30.0	30.0	30.0	30.0	30.0
Mineral premix[d]	40.0	40.0	40.0	40.0	40.0
Carboxymethyl cellulose[c]	20.0	20.0	20.0	20.0	20.0
Cellulose[c]	127.4	127.4	127.4	127.4	127.4
L-Lysine HCl[c]	2.1	2.1	2.1	2.1	2.1
DL-Methionine[c]	3.5	3.5	3.5	3.5	3.5
Glycine[c]	10.0	10.0	10.0	10.0	10.0
Diet pH	5.96	5.97	5.95	5.96	5.96
Proximate composition (%)					
Moisture	6.92	6.44	6.39	6.73	7.13
Protein	40.7	40.8	40.5	40.7	40.8
Lipid	9.4	10.3	9.5	9.8	10.4
Ash	6.7	6.7	6.6	6.6	6.5

[a] Omega Protein Corp., Houston, TX, USA.
[b] Solae LLC, St. Louis, MO, USA.
[c] US Biochemical Corp., Cleveland, OH, USA.
[d] Same as Moon and Gatlin, 1991.

2.4. Blood sample collection, immunological assays and erythrocyte osmotic fragility

At the end of the trial, fish were anesthetized with tricaine methane sulfonate (MS-222), and blood was collected from the caudal vasculature (four fish per aquarium) using heparinized syringes. Plasma was obtained by centrifugation (3800 × *g* for 12 min at 5 °C). Respiratory burst activity of blood leukocytes (NBT-test) was determined spectrophotometrically by the means of nitroblue tetrazolium (NBT) reduction, as described by Siwicki et al. [24]. Hemolytic activity of the complement system (HACS) was determined according to Morales-delaNuez et al. [25] using bovine and channel catfish (*Ictalurus punctatus*) red blood cells as targets. Lysozyme activity was determined by a turbidimetric assay as described by Jørgensen et al. [26] at pH 5.2, which was previously determined to be optimum for red drum lysozyme activity. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min. Erythrocyte osmotic fragility (EOF) was performed as described by Meamarbashi and Rajabi [27] with some modifications. Briefly, 10 μL of blood from each fish was added to 1.5 mL salt (0.4% NaCl solution) in microtubes. A total hemolysis control (distilled water, 0.0% NaCl solution) and no-hemolysis control (0.8% NaCl solution) were performed individually. After incubation at room temperature for 35 min, the tubes were centrifuged at 3000 × *g* for 5 min. Supernatant (200 μL) was transferred to 96-well microtiter plates and the absorbance of the samples was read at 415 nm. The hemolysis (%) at 0.4% saline solution for each sample was calculated relative to the respective total hemolysis.

2.5. pH and lysozyme measurement of digestive tract contents

At the end of the feeding trial, two fish from each aquarium were randomly selected, euthanized and their digestive tracts were aseptically dissected. Stomach contents were collected at 2 h and 6 h after feeding, and intestinal contents were collected at 6 h after feeding. The pH of digestive tract contents was measured as described by Castillo et al. [22]. The stomach and intestine were gently scratched and the contents (6 h after feeding) were centrifuged twice at 15000 × *g* for 10 min at 5 °C and the supernatant was collected. The supernatant was used as a crude enzyme solution. Lysozyme activity of the gastric content supernatant was determined by the same method used to measure plasma lysozyme activity.

2.6. DGGE analysis

Intestinal microbiota profiles were determined using DGGE analysis (denaturing gradient gel electrophoresis). Six hours after the final feeding, the intestinal tracts of two additional fish per aquarium were aseptically removed, and the contents were expressed into a sterile microcentrifuge tube. The pooled intestinal samples were frozen at −80 °C until DGGE analysis could be performed. Genomic DNA was isolated from the intestinal contents (from 0.2 to 0.5 ml) of each pooled sample with the Bio-Rad Aqua Pure DNA Isolation Kit (Bio-Rad, Hercules, CA, USA) using the method supplied by the manufacturer with some modifications as described by Burr et al. [28]. The isolated DNA was quantified, and concentrations were adjusted for PCR analysis. For DGGE analysis, equal portions (5 μL) of PCR mixtures from samples in each treatment group were pooled and analyzed by DGGE. Visualization of bands was accomplished using an imager (Alpha Innotech, San Leandro, CA). PCR and DGGE procedures, as well as band pattern relatedness (percentage similarity coefficient) were determined according to the methods of Hume et al. [29] as described by Burr et al. [28].

The experimental procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee.

2.7. Statistical analysis

The results are expressed as the means ± standard error of the mean (SEM). The homogeneity of variances between groups was tested with Levene's test. Comparisons between different groups were made using one-way ANOVA and Duncan's test or Kruskal-Wallis test when appropriate (Statistica 7.0, StatSoft Inc., Tulsa, OK, USA). The minimum significance level was set at $P \leq 0.05$.

3. Results

3.1. Productive parameters and whole-body composition

There were no significant differences in productive parameters (final weight, RWG, FE, CF, HSI, FY and survival) of red drum fed diets containing OAEO when compared to the control group. However, fish fed the diet supplemented with OAEO at 1.0 g/kg had significantly increased IPF deposition compared to the control group. Whole-body composition was not affected by OAEO addition to the diet, as crude protein, lipid, moisture and ash were similar among fish fed all treatments (Table 3).

3.2. Immunological parameters and erythrocyte osmotic fragility

Respiratory burst activity of blood leukocytes decreased at the two highest OAEO concentrations tested, and at 1.0 g/kg the NBT values were significantly lower compared to those of fish in the control group (Fig. 1A). Fish fed diets containing OAEO showed an increase in HACS. When bovine red blood cells were used as the target, the HACS was significantly high in the groups fed OAEO at 0.5 and 2.0 g/kg. When channel catfish red blood cells were used as the target, the hemolysis values were lower compared to bovine cells; nevertheless, HACS was higher in all groups fed OAEO when compared to the control group (Fig. 1B). There were no differences among treatments in plasma lysozyme activity (Fig. 1C). Red blood cells from the group fed the lowest OAEO concentration (0.25 g/kg) showed significant lower fragility in the EOF assay compared to the control group. However, fish fed diets with OAEO supplemented at 0.5 and 1.0 g/kg diet showed significant higher erythrocyte fragility (Fig. 1D).

3.3. pH, lysozyme and microbiota of digestive tract contents

Stomach pH was significantly higher at 2 h after feeding in fish that received the diet supplemented with 1.0 g/kg of OAEO compared to the control group. At 6 h after feeding, stomach and intestinal pH were not significantly different among the groups. Lysozyme measurement in the supernatant of stomach content was significantly higher in fish fed the diet supplemented with OAEO at 0.5 g/kg. Despite the numerical difference between the control and fish fed OAEO-supplemented diets, intestinal lysozyme activity was not significantly different among the treatments (Table 4). The intestinal microbial community of fish fed the basal and OAEO diets (all concentrations) were identical.

4. Discussion

4.1. Productive parameters and whole-body composition

Some studies have shown that products derived from plants of the genus *Ocimum* when added to fish diets can improve productive

Table 3
Productive parameters and whole-body composition of red drum fed diets containing different concentrations of *O. americanum* essential oil (OAEO) for 7 weeks.

Variable	OAEO levels in experimental diets (g/kg)				
	0.0	0.25	0.5	1.0	2.0
Final weight (g)	72.69 ± 2.32	80.51 ± 3.64	78.50 ± 3.98	81.80 ± 5.14	81.14 ± 3.95
Relative weight gain (%)	309.7 ± 13.3	352.5 ± 20.0	342.1 ± 22.3	361.1 ± 28.7	356.8 ± 22.0
Feed efficiency ratio (g/g)	0.85 ± 0.03	0.90 ± 0.02	0.86 ± 0.02	0.88 ± 0.02	0.89 ± 0.03
Condition factor (g/cm^3)	1.02 ± 0.01	1.03 ± 0.02	1.03 ± 0.02	1.05 ± 0.02	1.03 ± 0.03
Hepatosomatic index (%)	2.36 ± 0.19	2.46 ± 0.17	2.48 ± 0.09	2.44 ± 0.17	2.43 ± 0.15
Intraperitoneal fat ratio (%)	0.14 ± 0.02^a	0.18 ± 0.02^{ab}	0.18 ± 0.02^{ab}	0.22 ± 0.02^b	0.20 ± 0.02^{ab}
Fillet yield (%)	35.2 ± 0.27	35.7 ± 0.31	35.8 ± 0.60	36.0 ± 0.70	36.0 ± 0.52
Survival (%)	89.5 ± 5.2	89.5 ± 2.1	87.5 ± 2.4	79.1 ± 7.9	85.4 ± 4.0
Whole-body composition (%)					
Moisture	76.3 ± 0.2	76.5 ± 0.3	74.8 ± 0.5	75.6 ± 0.4	76.5 ± 0.8
Crude protein	16.6 ± 0.2	16.3 ± 0.2	17.0 ± 0.2	16.5 ± 0.2	16.5 ± 0.5
Lipid	3.65 ± 0.4	3.46 ± 0.2	3.88 ± 0.5	5.0 ± 0.6	4.4 ± 0.6
Ash	4.7 ± 0.4	4.4 ± 0.6	4.6 ± 0.4	4.4 ± 0.3	5.5 ± 1.1

Data are reported as means ± SEM (standard error) of four replicate tanks. Values within the same row having different superscripts are significantly different (ANOVA and Duncan's test, $P \leq 0.05$).

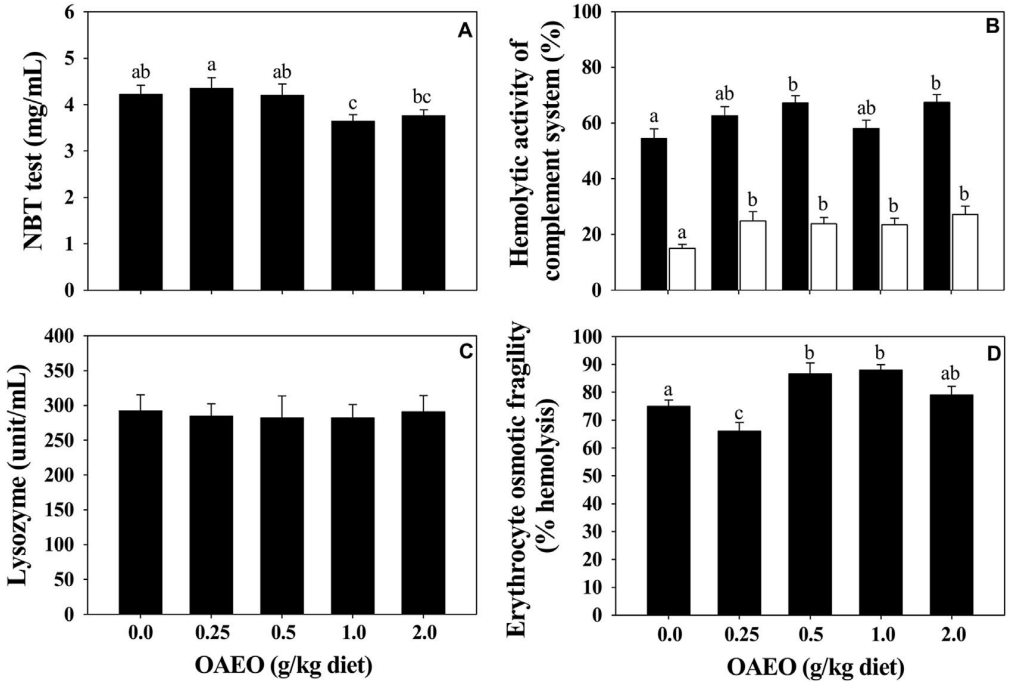

Fig. 1. Non-specific immune responses and erythrocyte osmotic fragility of red drum fed diet containing different concentrations of *O. americanum* essential oil (OAEO) for 7 weeks. (A) Respiratory burst activity of blood leukocytes, (B) Hemolytic activity of complement system – bovine (dark bars) and channel catfish (white bars) red blood target-cells, (C) Plasma lysozyme activity, (D) Erythrocyte osmotic fragility-hemolysis (%) at 0.4% saline solution. Data are reported as the mean ± SEM (standard error) (16 fish/treatment). Error bars with different superscripts are significantly different (ANOVA and Duncan's test, $P \leq 0.05$).

Table 4
pH and lysozyme activity of digestive tract contents from red drum fed diet containing different concentrations of *O. americanum* essential oil (OAEO) for 7 weeks.

Variables	OAEO levels in experimental diets (g/kg)				
	0.0	0.25	0.5	1.0	2.0
Stomach pH (2 h)	5.06 ± 0.10^a	5.27 ± 0.03^{ab}	5.16 ± 0.14^{ab}	5.60 ± 0.03^b	5.23 ± 0.15^{ab}
Stomach pH (6 h)	4.68 ± 0.11	4.47 ± 0.17	4.62 ± 0.18	4.70 ± 0.20	4.50 ± 0.16
Intestine pH (6 h)	8.01 ± 0.08	8.27 ± 0.09	8.18 ± 0.06	8.04 ± 0.07	8.25 ± 0.08
Stomach lysozyme (unit/mL)	208.3 ± 24.4^a	237.5 ± 34.9^a	377.0 ± 42.0^b	206.1 ± 26.1^a	176.6 ± 32.9^a
Intestine lysozyme (unit/mL)	402.4 ± 67.7	280.9 ± 52.2	269.0 ± 36.0	321.4 ± 56.4	271.1 ± 83.5

Data are reported as means ± SEM (standard error) of two fish in each of four replicate tanks (8 fish/treatment). Values within the same row having different superscripts are significantly different (ANOVA and Duncan's test or Kruskal-Wallis test, $P \leq 0.05$).

parameters. Inclusion of dried basil leaves (plant species not mentioned) up to 20 g/kg in hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) feeds gave a positive response to growth, feed and protein conversion, as well as improved digestibility of protein and energy although the digestion of lipid and carbohydrates declined [30]. The highest weight gain values were observed in *Cyprinus carpio* fed supplemented diets with extracts obtained from *Ocimum basilicum* (400 mg/kg) [31]. El-Dakar et al. [32] found a significant improvement in growth, feed utilization and digestive enzyme (amylase and lipase) activities by the administration of basil (*O. basilicum*) seeds to sea bream (*Sparus aurata*) diets.

The addition of OAEO in the diets did not interfere in the productive parameters of red drum; however, intraperitoneal fat index was high in fish fed the diet supplemented at 1.0 g/kg. Whole-body composition of red drum also was not influenced by inclusion of OAEO in diet. *Clarias gariepinus* fed diets containing different levels of *Ocimum gratissimum* leaf meal showed less crude protein and higher lipid concentrations in the carcass [33], as well as lower weight gain [34]. Similar to results of this study, Saccol et al. [10] did not find any influence of *Lippia alba* EO (linalool-chemotype) addition to the diet on silver catfish (*Rhamdia quelen*) growth. Linalool is also the major compound found in OAEO. Nevertheless, in the same study, *L. alba* EO increased the antioxidant status in different silver catfish tissues.

4.2. Immunological parameters and erythrocyte osmotic fragility

Plant products such as EOs contain many active compounds known to have immunomodulatory properties in fish. Sometimes they are described as a stimulant based on their effect on some immunological parameters, and at other times, they are described as inhibitors of certain immunological events [8]. As described in the literature, inhibitory (NBT) and stimulatory (HACS) effects on non-specific immune parameters were observed in this study with red drum fed supplemented diet with OAEO.

The same inhibitory and/or stimulatory effects have been reported in different fish species exposed to many different products derived from plants [7,8]. *Labeo rohita* fed diets containing *Ocimum sanctum* extract (0.05–0.2%) showed enhanced superoxide anion production and lysozyme activity; however, at the two highest inclusion levels (0.5% and 1%) the values were similar to that of the control [35]. Anesthesia caused by clove oil (*Eugenia aromatica*) in rainbow trout (*Oncorhynchus mykiss*) did not alter the hemolytic activity of the alternative complement, but it depressed respiratory burst activity 24 h after anesthesia [36]. Rainbow trout fed with ginger (*Zingiber officinale*) extract had significantly higher extracellular activity of phagocytic cells in blood [37]. On the other hand, *Scutellaria radix* extract in the diet significantly inhibited extracellular superoxide anion production in tilapia (*O. niloticus*) [38].

Decreased NBT values at the highest OAEO concentrations in the red drum diet may be correlated to the antioxidant properties of the OAEO. Essential oil compounds have antioxidant and immunomodulatory activities protecting the organism against damage from reactive oxygen species (ROS) production [8,39]. Silver catfish fed diets containing *L. alba* EO (linalool-chemotype) exhibited higher superoxide dismutase (SOD) and other antioxidant enzymes activity in different tissues. SOD is a key antioxidant enzyme in the metabolism of ROS, removing superoxide (O_2^-) and preventing the formation of other ROS [10].

The lowest OAEO concentration in the red drum diet seemed to increase erythrocyte resistance to osmotic stress/lysis in the EOF test, and thus reduce membrane fragility. EOF is said to be an indirect method of assessing oxidative stress. The use of this test to evaluate the effect of varied concentrations of different phytochemicals on erythrocyte membranes reveal appreciable membrane protective effects from these products and an inhibitory action on hemolysis of red blood cells [27,40]. An improvement in erythrocyte membrane resistance associated with immunomodulatory effects may influence the fish's health status, mainly in cases of bacterial diseases where the hemolytic activity of the bacterium is present. Examples of such infections include *Aeromonas hydrophila* and *Streptococcus iniae* [41,42].

At high concentrations, however, most of the effects described for EOs involve interactions with biological membranes, such as morphological changes with rupture of membranes and cytoplasm leakage [43,44]. Therefore, these plant products can show dual actions, both protective and disruptive, on red blood cells, depending on their specific chemical structure and whether an exogenous stress is present [45].

4.3. pH, lysozyme and microbiota of digestive tract contents

According to Reyes-Chilpa et al. [46] phytochemicals can interact with the H^+,K^+-ATPase pump, which is the last step involved in gastric acid secretion. Generally, compounds obtained from plants exert their effects by various mechanisms. These include either stimulating the protective factors of the gastric mucosa due to increased synthesis of prostaglandins, which stimulate the secretion of mucus and bicarbonate, inhibiting acid secretion by interacting with different receptors, or by regulating enzymes or hormones involved in the secretory process [47].

In mammals, *O. sanctum* extract (100 mg/kg diet) significantly inhibited the offensive acid-pepsin secretion and lipid peroxidation as well as increased the gastric defensive factors like mucin secretion, cellular mucus and life span of mucosal cells [48]. Aqueous leave extracts of *O. gratissimum* decrease gastric acid secretion and ulceration; it also produced an increase in the gastric mucus secretion [49]. These changes in pH and in the mucus layer can lead to a villi-related protective effect and reduce the chances of epithelial microbial adhesion, thereby hypothetically changing the gastric microbial population [1]. Besides, to survive passage through the digestive tract, bacteria must be able to resist pH changes, digestive enzymes, the effects of lysozyme and immunoglobulins in gut mucus, and possibly anaerobic conditions in some regions [50].

The EO components can act directly on fish because they have immunomodulatory properties in fish through inhibitory or stimulatory effects on the innate immune system [8]. Besides, EOs have antibacterial properties [44] and can also directly interfere with growth and proliferation of gastric microbial populations. Thus, the EOs can indirectly influence lysozyme release, because lysozyme concentration, and therefore activity, is greatest at the digestive tract mucosa–lumen interface and their functions largely limit microbial attachment and invasion into the mucosa [51]. Despite the observed changes in stomach pH (1.0 g/kg) and lysozyme (0.5 g/kg) of red drum, in the present study, differences in these parameters were not verified in the intestine.

In addition, no interference of the OAEO in the microbial population of red drum intestine was observed. As found in this study, *Thymus vulgaris* EO (thymol-chemotype) at 0, 5, 10 and 20 mg/kg added to the diet did not significantly alter the bacterial population of rainbow trout intestine after 5-week period [52]. However, in another study [9] a commercial product (encapsulated) containing carvacrol or thymol modulated intestinal microbial communities of rainbow trout when added to the diet. The protection by the encapsulation process may explain the action of the product on intestinal microbiota. EOs are composed of unstable and volatile molecules, susceptible to reactions to environmental factors, food ingredients and the gastrointestinal tract of the fish, before reaching their optimum site of action.

5. Conclusions

In conclusion, OAEO added to red drum diet did not alter growth parameters, whole-body composition and intestinal microbiota population. However, the addition of OAEO up to 1.0 g/kg in the diet altered IPF deposition, pH of the stomach, and influenced the membrane fragility of red blood cells, besides enhancing non-specific immunomodulatory responses in red drum.

Conflict of interest

The authors declare that there are no conflicts of interest.

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3. CONCLUSÕES E CONSIDERAÇÕES FINAIS

Melhorar a eficiência bio-econômica da aquicultura depende de avanços na biologia, nutrição, gestão ambiental, e tecnologias de produção. Paralelamente ao crescimento contínuo da indústria aquícola global está a intensificação dos sistemas de cultivo, a fim de maximizar o lucro e o controle nas operações. No entanto, as condições estressantes dos sistemas de alta densidade frequentemente levam a surtos de doenças e consequentemente ao uso de diferentes fármacos e produtos. Estes produtos incluem antibióticos, antiparasitários, anestésicos, sedativos, imunomoduladores, prebioticos dentre outros; a maioria dos quais pode acabar na água e ambiente. Os potenciais abusos destas drogas na profilaxia e tratamento de organismos aquáticos e outras espécies animais têm sido associados ao aumento da ineficácia dos atuais tratamentos (principalmente antibacterianos e antiparasitários), resistência microbiana e problemas ambientais, aumentando assim a consciência da necessidade de alternativas mais adequadas.

Os OEs avaliados nestes estudos apresentaram atividade antibacteriana contra *A. hydrophila* (*O. americanum*, *O. gratissimum* e *H. ringens*), antiparasitária contra *Gyrodactylus* sp. (*O. americanum*) e atividade moduladora da resposta imune inata *in vitro* e *in vivo* (*O. americanum*, *H. ringens, C. flexuosus* e *M. alternifolia*). Devido às estas propriedades reportadas, estes óleos podem constituir alternativas aos agentes profiláticos e terapêuticos utilizados na aquicultura. Muitos fatores têm contribuído para o aumento do uso destes produtos naturais na produção animal, incluindo o alto custo dos medicamentos convencionais e a tendência de usar produtos "ambientalmente amigáveis". Além do custo relativamente baixo, facilidade de uso – via banhos ou incorporados na dieta – e os efeitos colaterais limitados em animais.

Apesar dos resultados promissores na avaliação dos OEs e seus componentes como alternativas aos fármacos convencionais, os efeitos *in vivo* sobre a saúde dos peixes são ainda controversos. A sobrevivência dos peixes nos ensaios antibacterianos *in vivo* provavelmente não se justifica pela atividade bactericida dos OEs contra *A. hydrophila.* Esses resultados preliminares indicam que os OEs em concentrações subinibitórias (utilizadas nos ensaios *in vivo*), além do potencial imunomodulador, podem ter diminuído a virulência bacteriana, facilitando assim a auto-cura do peixe. Novos estudos se fazem necessários para esclarecer o possível modo de ação (bactericida ou inibidor dos fatores de virulência bacterianos) e/ou identificar as moléculas responsáveis pelas atividades biológicas dos OEs. Além disso, devido à diversidade química e as possíveis interações entre as moléculas, deve se ter em mente que a

utilização de OEs ou misturas podem ter um efeito maior que uma única molécula isolada devido à diversidade de sítios de ação.

Os possíveis efeitos dos OEs nos peixes dependem muito de uma variedade de fatores, desde o processo de extração até sua utilização, principalmente quando se trata do uso como aditivo alimentar. Estudos são necessários para se ter mais conhecimento e compreensão dos mecanismos e locais de ação dos OEs sobre o organismo do peixe, os microrganismos patogênicos e da flora normal e avaliar o seu impacto sobre o meio ambiente. Consequentemente, qualquer processo capaz de proteger os OEs (ex.: encapsulamento) pode ser uma alternativa viável para melhorar sua estabilidade, proteger os OEs das interações com o hospedeiro (permitindo que atinja o local de ação desejado), evitar interações com os ingredientes alimentares e ambiente e, potencialmente, assegurar sua atividade como promotores da saúde e aumentar a fiabilidade dos resultados.

BILIOGRAFIA GERAL

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