UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

Guerino Bandeira Junior

POTENCIAL ANTIBACTERIANO DE FITOQUÍMICOS ISOLADOS OU EM COMBINAÇÃO COM ANTIMICROBIANOS FRENTE À BACTÉRIAS PATOGÊNICAS PARA PEIXES

Santa Maria, RS
2018

Guerino Bandeira Junior

POTENCIAL ANTIBACTERIANO DE FITOQUÍMICOS ISOLADOS OU EM COMBINAÇÃO COM ANTIMICROBIANOS FRENTE À BACTÉRIAS PATOGÊNICAS PARA PEIXES

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

Orientador: Bernardo Baldisserotto
Co-orientadora: Agueda Palmira Castagna de Vargas

Santa Maria, RS
2018

Guerino Bandeira Junior

POTENCIAL ANTIBACTERIANO DE FITOQUÍMICOS ISOLADOS OU EM COMBINAÇÃO COM ANTIMICROBIANOS FRENTE À BACTÉRIAS PATOGÊNICAS PARA PEIXES

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do título de **Mestre em Farmacologia**.
A Professora Dra. Fabiana Pilarski participou por videoconferência.

Aprovado em 11 de junho de 2018:

Bernardo Baldisserotto, Dr. (UFSM)
(Presidente/Orientador)

Fabiana Pilarski, Dra. (UNESP)

Roberto Christ Vianna Santos, Dr. (UFSM)

Santa Maria, RS
2018

AGRADECIMENTOS

Agradeço primeiramente à Deus, por me dar saúde para concluir mais uma etapa da minha vida.

Aos meus pais Guerino Bandeira e Irene Judit Bandeira, e às minhas irmãs Denise Bandeira e Danieli Bandeira, pelo apoio incondicional.

À minha namorada Isabel Cristina Markowski Brusque, pela companhia durante minha caminhada.

Ao meu orientador Bernardo Baldisserotto, pelas ideias e pelos conselhos que só um grande pesquisador poderia compartilhar.

À Universidade Federal de Santa Maria (UFSM), pela estrutura e pelas oportunidades ofertadas.

À equipe do Laboratório de Bacteriologia (LABAC) da UFSM, por sempre manter as portas abertas para a realização de pesquisas.

À equipe do Laboratório de Fisiologia de Peixes (LAFIPE) da UFSM, pelo companheirismo durante os anos de convivência.

Enfim, a todas as pessoas que, de alguma forma, contribuíram para a minha formação acadêmica, o meu muito obrigado!

“A educação é a arma mais poderosa que
você pode usar para mudar o mundo.”
(Nelson Mandela)

RESUMO

POTENCIAL ANTIBACTERIANO DE FITOQUÍMICOS ISOLADOS OU EM COMBINAÇÃO COM ANTIMICROBIANOS FRENTE À BACTÉRIAS PATOGÊNICAS PARA PEIXES

AUTOR: GUERINO BANDEIRA JUNIOR
ORIENTADOR: BERNARDO BALDISSEROTTO

A produção aquícola brasileira está intensificando-se, havendo um aumento nos casos de bacterioses, gerando sérios prejuízos aos produtores. Com isso, cresce a utilização de antimicrobianos, muitas vezes de maneira indiscriminada, ocasionando resistência bacteriana aos mesmos. A utilização de concentrações cada vez maiores de antimicrobianos também leva à deposição de seus resíduos no ambiente aquático e na carne de peixe, gerando um problema de saúde pública. Agravando a situação, somente os antimicrobianos florfenicol e oxitetraciclina são legalizados para uso na aquicultura continental, havendo a necessidade da descoberta de novos princípios ativos eficazes e seguros. Óleos essenciais e suas substâncias isoladas (fitoquímicos) têm mostrado potencial antimicrobiano, podendo representar possíveis candidatos à fármacos. *Aeromonas hydrophila*, *A. veronii*, *Citrobacter freundii* e *Raoultella ornithinolytica* são espécies de bactérias encontradas em jundiás (*Rhamdia quelen*) infectados naturalmente, os quais apresentaram elevada mortalidade. No intuito de minimizar o uso de antimicrobianos na piscicultura, este estudo tem como objetivo avaliar a atividade antimicrobiana *in vitro* dos fitoquímicos carvacrol, citral, eugenol, linalol e timol isolados e em combinação com antimicrobianos convencionais (florfenicol e oxitetraciclina), bem como avaliar o potencial de inibição de hemólise e de formação de biofilme de bactérias patogênicas de peixes. Os resultados demonstraram que, quando analisados isoladamente, carvacrol, timol e eugenol apresentaram melhor atividade. A maioria das combinações demonstrou aditividade, sendo que três delas foram sinérgicas: linalol com florfenicol ou oxitetraciclina frente à *A. hydrophila* e citral com oxitetraciclina frente à *C. freundii*. Os fitoquímicos não demonstraram nenhuma interação antagônica com os antimicrobianos convencionais, o que possibilita seu uso combinado, podendo contribuir para a diminuição das concentrações utilizadas dos fármacos convencionais, reduzindo seus resíduos no ambiente aquático. Os cinco fitoquímicos testados demonstraram capacidade de inibir a hemólise de uma cepa de *A. hydrophila* β-hemolítica quando usados em concentrações mais baixas que as necessárias para inibir o crescimento bacteriano. Os fitoquímicos também apresentaram potencial para inibir a formação de biofilme bacteriano, sendo esta inibição possivelmente maior que a provocada pelos antimicrobianos convencionais.

Palavras-chave: sinergismo, formação de biofilme, inibição de hemólise, florfenicol, oxitetraciclina, *Aeromonas* spp.

ABSTRACT

ANTIBACTERIAL POTENTIAL OF PHYTOCHEMICALS ISOLATED OR IN COMBINATION WITH ANTIMICROBIALS AGAINST FISH PATHOGENIC BACTERIA

AUTHOR: GUERINO BANDEIRA JUNIOR
ADVISOR: BERNARDO BALDISSEROTTO

Brazilian aquaculture production is intensifying, leading to an increase of bacterioses, causing economic losses in fish farms. This increases the use of antimicrobials, often indiscriminately, causing bacterial resistance to them. The use of increasing concentrations of antimicrobials also leads to residues deposition in the aquatic environment and fish meat, generating a public health problem. Worsening the situation, only the antimicrobials florfenicol and oxytetracycline are legalized for use in the continental aquaculture, being necessary the discovery of new effective and safe active principles. Essential oils and their isolated substances (phytochemicals) have shown antimicrobial effect, representing potential drug candidates. *Aeromonas hydrophila*, *A. veronii*, *Citrobacter freundii*, and *Raoultella ornithinolytica* are bacteria species found in silver catfish (*Rhamdia quelen*) naturally infected and that presented high mortality. In order to minimize the use of antimicrobials in fish culture, this study aims to evaluate the *in vitro* antimicrobial activity of the phytochemicals carvacrol, citral, eugenol, linalool, and thymol isolated and in combination with conventional antimicrobials (florfenicol and oxytetracycline), as well as to evaluate the inhibition potential of hemolysis and biofilm formation of fish pathogenic bacteria. The results demonstrated that when analyzed isolated, carvacrol, thymol, and eugenol presented better activity. Most of the combinations showed additivity, and three of them were synergistic: linalool with florfenicol or oxytetracycline against *A. hydrophila*, and citral with oxytetracycline against *C. freundii*. Phytochemicals showed no antagonistic interaction with conventional antimicrobials, allowing their combined use, which may contribute to the reduction of the conventional drugs concentrations, reducing their residues in the aquatic environment. The five phytochemicals demonstrated ability to inhibit the hemolysis of a β-hemolytic *A. hydrophila* strain when used at concentrations lower than those required to inhibit bacterial growth. The phytochemicals also demonstrated potential to inhibit the biofilm formation of the bacteria, and this inhibition is possibly greater than that caused by conventional antimicrobials.

Keywords: synergism, biofilm formation, hemolysis inhibition, florfenicol, oxytetracycline, *Aeromonas* spp.

LISTA DE FIGURAS

INTRODUÇÃO

MANUSCRITO

LISTA DE TABELAS

MANUSCRITO

SUMÁRIO

1. INTRODUÇÃO

1.1 AQUICULTURA E ESPÉCIE ESTUDADA

Entende-se por aquicultura a criação de diversos organismos aquáticos, em água doce ou salgada, onde a intervenção ou manejo do processo de criação é imprescindível para o aumento da produção (OLIVEIRA, 2009). A aquicultura é a atividade agropecuária que mais cresce no Brasil e no mundo, sendo o pescado a proteína animal mais produzida no planeta. E essa produção tende a aumentar, pois estima-se que, por volta de 2020, a aquicultura ultrapasse a produção de peixes de captura. Porém, o consumo *per capita* de pescado no Brasil (9,5 kg/hab/ano) ainda é bem abaixo do recomendado (20 kg/hab/ano) (FAO, 2016). No Brasil, a piscicultura (criação de peixes) corresponde a 82% da produção aquícola (SEBRAE, 2015). O país produziu 691700 toneladas de peixes cultivados em 2017, sendo o Paraná o estado líder em produção (112000 toneladas). A espécie de peixe mais produzida no país é a tilápia-do-Nilo (*Oreochromis niloticus*), sendo o Brasil o 4º maior produtor desta espécie no mundo, a qual representa 51,7% da piscicultura brasileira. A região sul é a maior produtora, com 178500 toneladas produzidas em 2017 (PEIXE BR, 2018).

No Rio Grande do Sul, as carpas são as espécies mais produzidas (73%), seguidas pela tilápia-do-Nilo (19%) e pelos peixes nativos (8%), entre os quais destaca-se o jundiá (*Rhamdia quelen*), espécie nativa mais produzida no sul do país (PEIXE BR, 2018) (Figura 1). Neste estudo, as amostras microbiológicas foram coletadas de jundiás naturalmente infectados. Essa espécie localiza-se desde o sudeste do México até a região central da Argentina, sendo comumente encontrada em rios do estado do Rio Grande do Sul. Apresenta boa adaptação à diferentes ambientes e dietas artificiais, resistência ao manejo e boa aceitação comercial (FIGUEREDO et al., 2014).

Figura 1 - Espécime de jundiá (*Rhamdia quelen*). Fonte: autor.

1.2 BACTÉRIAS EMERGENTES

A intensificação na produção piscícola nos últimos anos levou a um aumento na densidade de estocagem de peixes nos viveiros, aumentando o número de disputas territoriais e a matéria orgânica e amônia no tanque e diminuindo o oxigênio dissolvido na água, os quais são estressores para os peixes, e ocasionam a liberação de hormônios como cortisol e catecolaminas, que são imunossupressores (BAKER; GOBUSH e VYNNE, 2013; BALDISSEROTTO et al., 2014; JØRGENSEN et al., 2017), promovendo a instalação de infecções causadas por bactérias oportunistas, gerando sérios prejuízos econômicos ao produtor.

O aumento na prevalência de infecções bacterianas na piscicultura faz com que os produtores utilizem de forma indiscriminada a antibioticoterapia ou a antibioticoprofilaxia. Essas substâncias permanecem no ambiente aquático, exercendo uma pressão seletiva por longos períodos de tempo, resultando no surgimento de bactérias multirresistentes (BELEM-COSTA e CYRINO, 2006). Devido ao surgimento de novas infecções, torna-se necessária a realização de testes de suscetibilidade aos antimicrobianos para se estabelecer o tratamento adequado. O uso indiscriminado de antimicrobianos também gera problemas para a saúde pública, pois aumenta a quantidade de seus resíduos em produtos à base de carne de peixe (CABELLO, 2006). No Brasil, somente duas substâncias antibacterianas são licenciadas para utilização na aquicultura continental, sendo elas o florfenicol e a oxitetraciclina (SINDAN, 2018). Nos Estados Unidos, esse panorama não é muito diferente, pois de acordo com a "Food and Drug Administration" somente três antimicrobianos são licenciados para uso na aquicultura, sendo eles os dois mencionados anteriormente e o sulfadimetoxina/ormetoprim (FDA, 2014). A escassez de antimicrobianos aprovados leva ao uso de substâncias ilegais ou legisladas para outras espécies animais, agravando o problema de saúde pública.

Dentre as bactérias encontradas no ambiente aquático, as mais prevalentes são as do gênero *Aeromonas* spp., sendo que *A. hydrophila* e *A. veronii* são duas espécies amplamente estudadas como causadoras de doenças em peixes, muitas vezes descritas como septicemia hemorrágica, levando também ao aparecimento de úlceras cutâneas (ALYAHYA et al., 2018; JAGODA et al., 2017) (Figura 2). Bactérias desse gênero também podem causar doenças em humanos, sendo a gastroenterite a mais comum delas (GUERRA et al., 2007). Sabe-se também dos sérios efeitos que uma infecção por *Citrobacter freundii* pode causar nos peixes, afetando diversos órgãos desses animais e, muitas vezes, levando à morte (PÁDUA et al.,

2014). *Raoultella ornithinolytica*, por sua vez, é um bacilo Gram-negativo conhecido por causar intoxicação por histamina em humanos pela ingestão de peixes contaminados. Essa bactéria libera uma enzima chamada histidina-descarboxilase, a qual transforma o aminoácido histidina em histamina na musculatura dos peixes (KANKI et al., 2002). Assim, quando um humano ingere um peixe infectado, estará ingerindo histamina e terá sintomas semelhantes aos de uma alergia.

Figura 2 - Necrose de nadadeira e úlcera cutânea em jundiá infectado com *Aeromonas hydrophila*. Fonte: autor.

Bactérias patogênicas para peixes, especialmente as do gênero *Aeromonas* spp., possuem diversos fatores de virulência, sendo que a capacidade de causar hemólise e de produzir biofilme são dois dos mais estudados (MILLEZI et al., 2013; SUTILI et al., 2014). A hemólise é a lise de hemácias causada pela ação de exotoxinas (hemolisinas) produzidas pelas bactérias hemolíticas e que agem induzindo a formação de poros nas membranas das células afetadas (WANG et al., 2003) (Figura 3). Já o biofilme constitui uma estrutura ligada a uma superfície e formada por células microbianas coladas entre si e cercadas por uma matriz polimérica extracelular autoproduzida. A formação de biofilme é considerada uma adaptação dos microrganismos a ambientes hostis e confere maior resistência aos sanitizantes, aos antimicrobianos e às defesas do hospedeiro. A formação e o desenvolvimento típico de biofilme segue alguns estágios: fixação à superfície, formação de microcolônia, desenvolvimento de biofilme jovem, diferenciação em biofilme maduro e dispersão do biofilme maduro (VASUDEVAN, 2014) (Figura 4). Um microrganismo que possua esses

fatores de virulência é mais perigoso e mais difícil de combater, tornando-se necessários estudos a fim de identificar novas substâncias capazes de atenuá-los.

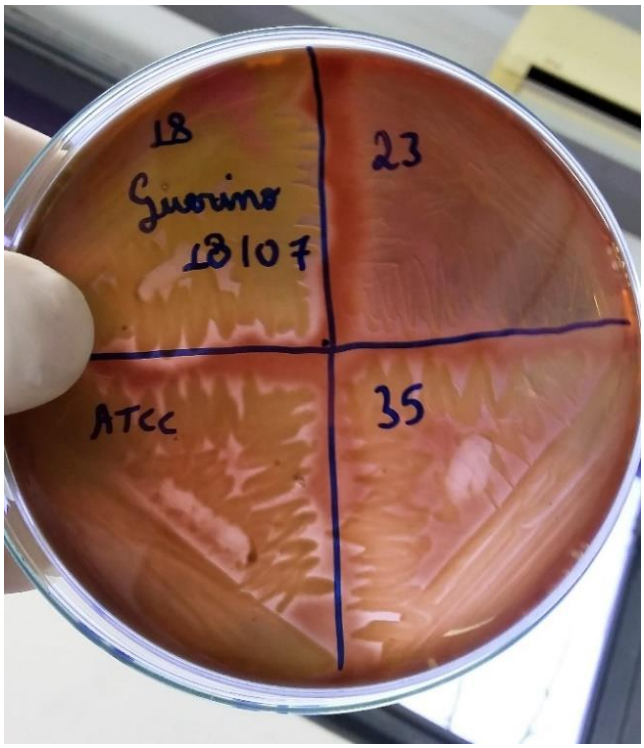


Figura 3 - Hemólise de bactérias isoladas de peixes demonstrada em Ágar Sangue. Fonte: autor.

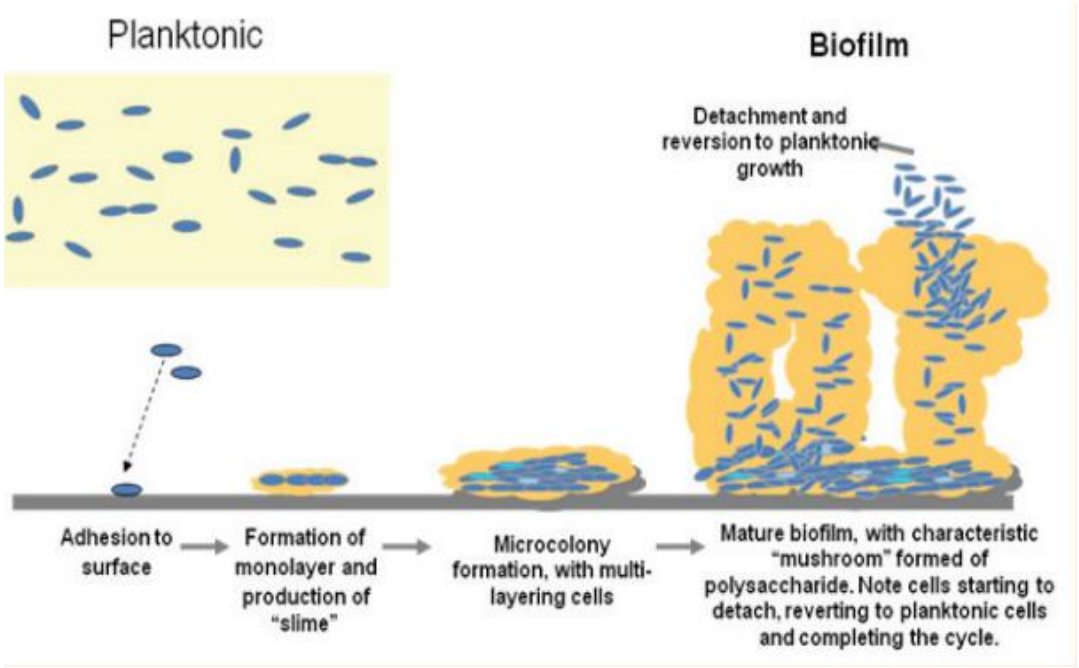


Figura 4 - Estágios de formação e desenvolvimento do biofilme bacteriano. Fonte: Vasudevan (2014).

1.3 COMPOSTOS NATURAIS COMO AGENTES ANTIBACTERIANOS

Compostos derivados de plantas vêm sendo utilizados desde os tempos antigos na medicina tradicional, sendo a maioria deles metabólitos secundários, principalmente fenois. Existem vários benefícios do uso desses compostos, principalmente envolvendo atividades antimicrobianas, antioxidantes, nutricionais e farmacêuticas (LAI e ROY, 2004). Os principais grupos de fitoquímicos responsáveis pela atividade antimicrobiana das plantas incluem fenólicos, ácidos fenólicos, quinonas, saponinas, flavonoides, taninos, cumarinas, terpenoides e alcaloides, sendo que variações na estrutura e composição química desses compostos resultam em diferenças na ação antimicrobiana (GYAWALI e IBRAHIM, 2014).

Os antimicrobianos convencionais têm sido massivamente utilizados no tratamento de doenças infecciosas, mas o surgimento da resistência microbiana vem dificultando este tratamento (DONE; VENKATESAN e HALDEN, 2015). Uma estratégia que pode ser empregada para superar esses mecanismos de resistência é a terapia combinada, em que duas substâncias com efeito sinérgico agem frente a um microrganismo. Aponta-se que combinações de produtos naturais com antimicrobianos convencionais podem ser benéficas no tratamento de infecções bacterianas (HEMAISWARYA; KRUTHIVENTI e DOBLE, 2008). Além de aumentar o sucesso terapêutico, esse tipo de terapia combinada é positiva do ponto de vista ambiental, visto que diminui a quantidade de resíduos de antimicrobianos na natureza. Uma das metodologias mais conhecidas para se testar o efeito antibacteriano de duas substâncias em combinação é o ensaio de “checkerboard”, ou “tabuleiro de xadrez”. Este ensaio é realizado em placas de 96 poços e consiste em colunas contendo alguma quantidade do agente antimicrobiano “A”, diluídas ao longo do eixo x, e linhas contendo o agente antimicrobiano “B”, diluídas ao longo do eixo y (GARCIA e ISENBERG, 2010) (Figura 5).

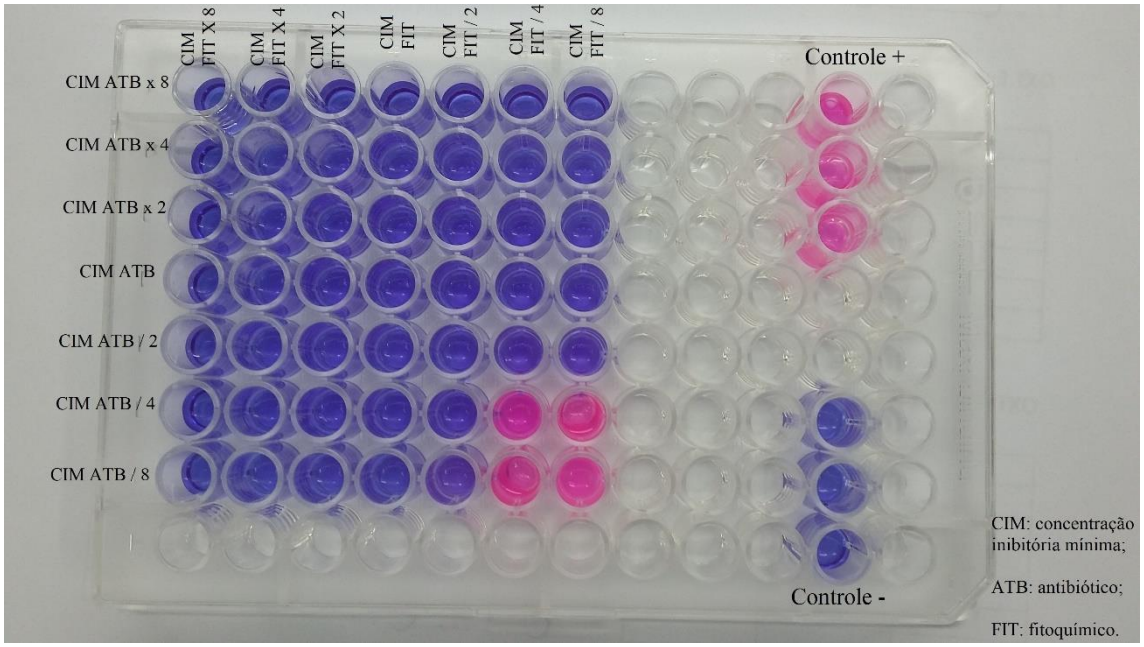


Figura 5 - Exemplo de ensaio de "cherckerboard". Fonte: autor.

1.4 FITOQUÍMICOS EM ESTUDO

Fitoquímicos são compostos químicos produzidos por vegetais, provenientes do metabolismo secundário de plantas e frequentemente associados a propriedades antimicrobianas (KUBMARAWA et al., 2007). No presente estudo, foi avaliada a atividade antimicrobiana de cinco fitoquímicos isolados ou em combinação com os antimicrobianos convencionais florfenicol e oxitetraciclina, além da avaliação da habilidade desses fitoquímicos em inibir a hemólise e a formação de biofilme de bactérias patogênicas para peixes. Os fitoquímicos testados foram os monoterpenoides carvacrol, timol, citral (mistura do *trans*-isômero geranial e do *cis*-isômero neral) e linalol (mistura dos isômeros espaciais (+)-linalol e (-)-linalol), e o fenilpropanoide eugenol (Figura 6). Os isômeros de posição carvacrol e timol foram testados separadamente. Terpenoides são moléculas oxigenadas cuja origem biosintética deriva da condensação de um número variável de unidades isoprênicas (C5). Através deste número é possível classificá-los, sendo que os monoterpenoides possuem dez carbonos (duas unidades isoprênicas) (BAKKALI et al., 2008). Já o nome fenilpropanoide é derivado da junção do grupo fenila (anel aromático) e uma cadeia lateral de três carbonos (grupo propila), o qual é sintetizado a partir do aminoácido fenilalanina na primeira etapa na biossíntese de fenilpropanoides (ZHANG et al., 2015).

1.4.1 Eugenol

Eugenol compreende 70-90% do peso do óleo essencial (OE) de cravo, o qual é derivado do caule, folhas e brotos da árvore *Eugenia caryophyllata*. Porém, OEs de outras plantas também apresentam eugenol em sua constituição (KEENE et al., 1998). Sua eficácia anestésica é comprovada em jundiás (CUNHA et al., 2010), também podendo ser utilizado no tratamento e prevenção de doenças bacterianas nessa espécie de peixe, uma vez que apresentou bons resultados em experimento *in vivo* no controle de *A. hydrophila* (SUTILI et al., 2014). Atividades antioxidante e anti-inflamatória também são atribuídas ao eugenol (LEEM et al., 2011).

1.4.2 Citral

Citral é derivado de diversos OEs, dos quais destacam-se os de *Lippia alba*, *Ocimum americanum* e, especialmente, o de capim-limão (*Cymbopogon citratus*), no qual seu teor varia em torno de 70% (GAUTAM e AGRAWAL, 2017; MONDELLO et al., 2002; VALE et al., 2002). Sabe-se que essa substância possui considerável atividade antimicrobiana frente a bactérias Gram-negativas e Gram-positivas, bem como fungos (LU et al., 2017; SADDIQ e KHAYYAT, 2010). O OE de *Aloysia triphylla* (em torno de 50% citral - mistura de isômeros), quando adicionado na ração de jundiás, é eficaz no controle de *A. hydrophila* nesses animais (DOS SANTOS et al., 2017). O citral também pode ser utilizado com a função de aumentar a vida útil de filé de peixe, devido às suas propriedades antibacterianas (KIM et al., 1995).

1.4.3 Carvacrol

Carvacrol está presente no OE de orégano – *Origanum vulgare* (LAMBERT et al., 2001). Representa em torno de 50% dos componentes do OE de *O. dictamnus* e até 79% dos componentes do OE de *O. majorana* (BASER; KIRIMER e TÜMEN, 1993; LIOLIOS et al., 2009). O OE de orégano possui atividade antioxidante e promotora de crescimento, além de ser eficaz no tratamento de *A. hydrophila*, pois o carvacrol apresenta ação bactericida através da ruptura de membranas (SOUSA et al., 2015; ZHENG et al., 2009).

1.4.4 Timol

Timol corresponde a aproximadamente 38% da constituição do OE de tomilho – *Thymus vulgaris* (POULOSE e CROTEAU, 1978). Também está presente no OE de orégano e representa 56,7% dos constituintes do OE de *Lippia sidoides*, esse último apresentando ação bacteriostática e bactericida frente à *A. hydrophila* (BOTELHO et al., 2007; LAMBERT et al., 2001; MAJOLO et al., 2016). Possui propriedades antioxidantes, promotoras de crescimento e antimicrobianas (AHMADIFAR; FALAHATKAR e AKRAMI, 2011; BOTELHO et al., 2007; YANISHLIEVA et al., 1999).

1.4.5 Linalol

Linalol é constituinte majoritário de diversos OEs de plantas, tais como manjericão (*Ocimum basilicum*), pau rosa (*Aniba rosaeodora*) e erva cidreira brasileira (*Lippia alba*) (AVETISYAN et al., 2017; SAMPAIO et al., 2012; SOUZA et al., 2017). Possui propriedades antibacterianas frente à *A. hydrophila*, sendo o isômero (+)-linalol responsável por essa atividade; além de propriedades sedativas e anestésicas para jundiás, promovidas em maior parte pelo isômero (-)-linalol (DORMAN e DEANS, 2000; HELDWEIN et al., 2014; SILVA et al., 2017).

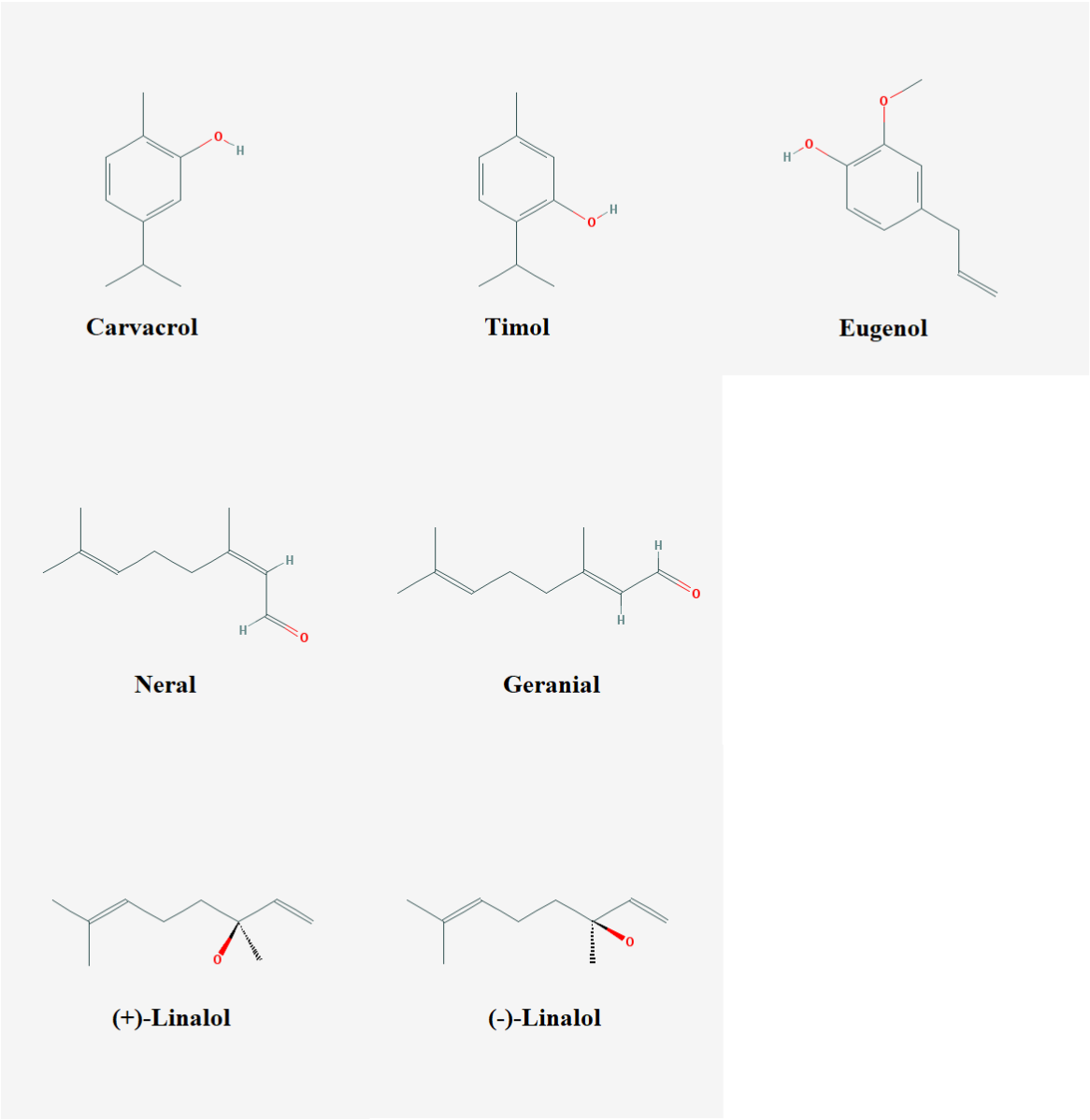


Figura 6 - Estruturas químicas dos fitoquímicos estudados. Fonte: adaptada de NCBI (2018).

Devido ao aumento das infecções bacterianas na piscicultura e ao elevado número de cepas de bactérias isoladas de peixes resistentes a antimicrobianos, faz-se necessário o descobrimento de novas substâncias capazes de combatê-las. Levando em conta as atividades antimicrobianas de fitoquímicos, o presente estudo testou a hipótese de que essas substâncias seriam alternativas viáveis no combate dessas bacterioses, seja utilizadas isoladamente ou em combinação com antimicrobianos convencionais.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Determinar se combinações dos antimicrobianos convencionais florfenicol e oxitetraciclina com cinco fitoquímicos (carvacrol, timol, citral, eugenol e linalol) frente a três bactérias isoladas de jundiás (*A. hydrophila*, *C. freundii* e *R. ornithinolytica*) apresentam efeito sinérgico, antagônico ou aditivo; bem como avaliar o potencial desses fitoquímicos na inibição de hemólise e na formação de biofilme dessas bactérias patogênicas para peixes.

2.2 OBJETIVOS ESPECÍFICOS

- Testar as bactérias *A. hydrophila*, *C. freundii* e *R. ornithinolytica* frente a diversos antimicrobianos, através de teste de disco-difusão em ágar, a fim de determinar seu perfil de suscetibilidade;
- Determinar a Concentração Inibitória Mínima (CIM) e a Concentração Bactericida Mínima (CBM) dos antimicrobianos oxitetraciclina e florfenicol frente a três espécies de bactérias isoladas de jundiás e frente a duas cepas padrões;
- Determinar a CIM e a CBM dos fitoquímicos (eugenol, citral, carvacrol, timol e linalol) frente a três espécies de bactérias isoladas de jundiás e frente a duas cepas padrões;
- Avaliar o efeito antibacteriano de diferentes combinações dos antimicrobianos com os fitoquímicos frente a três espécies de bactérias isoladas de jundiás e frente a uma cepa padrão de *A. hydrophila*, através da técnica de “checkerboard”;
- Determinar o potencial de inibição de hemólise desses fitoquímicos frente a uma cepa de *A. hydrophila* β-hemolítica;
- Determinar a ação desses fitoquímicos e antimicrobianos sobre a formação de biofilme de três isolados clínicos e de uma cepa padrão.

3 MANUSCRITO

O manuscrito intitulado “Antibacterial potential of phytochemicals alone or in combination with antimicrobials against fish pathogenic bacteria” foi aceito para publicação pelo periódico **Journal of Applied Microbiology**.

Antibacterial potential of phytochemicals alone or in combination with antimicrobials against fish pathogenic bacteria

G. Bandeira Junior[1], F.J. Sutili[1], L.T. Gressler[2], V.L. Ely[2], B.P. Silveira[2], C. Tasca[2], M. Reghelin[2], L.B. Matter[2], A.P.C. Vargas[2], B. Baldisserotto[1]

[1]Department of Physiology and Pharmacology, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil. Phone: 55553220-9382.
[2] Department of Preventive Veterinary Medicine, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil. Phone: 55553220-8107.

Correspondence: Bernardo Baldisserotto. Department of Physiology and Pharmacology, Universidade Federal de Santa Maria (UFSM), Av. Roraima, nº 1000, 97105-900, Santa Maria, RS, Brazil. E-mail: bbaldisserotto@hotmail.com. Phone: 55553220-9382.

Running head: Phytochemicals against fish pathogenic bacteria

Abstract

Aims

This study investigated the antibacterial activity of five phytochemicals (carvacrol, citral, eugenol, linalool, and thymol) alone or in combination with florfenicol or oxytetracycline against bacteria isolated from silver catfish (*Rhamdia quelen*). We also analyzed the potential of these compounds to inhibit biofilm formation and hemolysis caused by the bacteria.

Methods and Results

Bacteria were tested with antimicrobials to calculate the multiple antibiotic resistance (MAR). The checkerboard assay was used to evaluate a putative synergy between five phytochemicals and antimicrobials against the strains isolated. The biofilm formation inhibition assay was performed with phytochemicals and antimicrobials, and the hemolysis inhibition assay was performed with the phytochemicals. Carvacrol, eugenol and thymol were the most effective phytochemicals. Three combinations (linalool with florfenicol or oxytetracycline against *Aeromonas hydrophila* and citral with oxytetracycline against *Citrobacter freundii*)

demonstrated synergy in the checkerboard assay. All phytochemicals inhibited biofilm formation and hemolysis activity.

Conclusion

The tested phytochemicals showed satisfactory activity against fish pathogenic bacteria.

Significance and Impact of the Study

The phytochemicals did not present antagonistic interactions with the antimicrobials, allowing their combined use, which may contribute to a decrease in the use of conventional drugs and their residues in aquatic environment.

Keywords: phytochemicals, antimicrobials, biofilm formation, hemolysis activity, synergy

Introduction

Fish are subject to overcrowding with the development and intensification of aquaculture, which leads to stress and, subsequently, the appearance of opportunistic infections caused by bacteria, resulting in economic losses (Baker *et al.* 2013). The increase in the prevalence of bacterial infections in fish farming leads to the indiscriminate use of antimicrobials, which remain in the aquatic environment. Consequently, they exert a selective pressure over long periods, resulting in the emergence of multi-resistant bacteria (Watts *et al.* 2017). The incorrect use of antimicrobials also creates problems for public health through increased amounts of their residues in fish meat products (Cabello 2006). Also, the use of unlicensed drugs is excessive, since only oxytetracycline, florfenicol, and sulfadimethoxine/ormetoprim are antimicrobials approved by the Food and Drug Administration (FDA) for use in aquaculture (FDA 2014).

Aeromonas hydrophila is an important bacterial cause of cutaneous ulcerations and hemorrhagic septicemia in several species of freshwater fish (Ghatak *et al.* 2016). It is a natural inhabitant of organic matter at the bottom of the aquaculture tanks and in the intestinal tract of fish and may cause secondary or even primary infections, depending on the virulence of the strain and the host immune response (Ghatak *et al.* 2016). *Aeromonas veronii* is a highly pathogenic species to fish, presenting several virulence factors (Sun *et al.* 2016). It has been described as species has antibiotic resistance genes and also as responsible for ulcerative syndrome in Chinese longsnout catfish (*Leiocassis longirostris*) (Cai *et al.* 2012; Sreedharan *et al.* 2012). Bacteria of the genus *Aeromonas* are recognized for their capacity of hemolysis

and biofilm formation, factors that interfere in the strain virulence (Millezi *et al.* 2013; Sutili *et al.* 2014). *Citrobacter freundii* is a member of the family Enterobacteriaceae that has been reported causing cutaneous hemorrhagic rash and renal granulomas in moonfish (*Spring springs*), gastroenteritis in fingerlings of rainbow trout (*Oncorhynchus mykiss*), acute hemorrhagic septicemia in common carp (*Cyprinus carpio*), and lesions in the gills and kidneys, severe enteritis, and hemorrhagic septicemia in catfish (*Pseudoplatystoma reticulatum*) (Sato *et al.* 1982; Karunasagar *et al.* 1992; Svetlana *et al.* 2003; Pádua *et al.* 2014). *Raoultella ornithinolytica* is an environmental Gram-negative bacilli that produces histamine, which leads to poisoning by ingestion of contaminated fish (Kanki *et al.* 2002). In humans, it may cause fever-like syndrome, urinary tract infections, cutaneous infections in diabetics, and even septicemia in immunocompromised individuals (Morais *et al.* 2009; Solak *et al.* 2011; Kaya *et al.* 2015; Nakasone *et al.* 2015).

The control of these emergent pathogens is a challenge in aquaculture as well as in human health, and phytochemicals (chemical compounds derived from plants) with antimicrobial properties have been suggested as an alternative treatment to antibiotics. Eugenol is a phenylpropene comprising 70-90% of the weight of clove essential oil (EO), derived from the stem, leaves, and shoots of the *Eugenia caryophyllata* tree (Keene *et al.* 1998). This phenolic compound showed good *in vivo* results in the treatment of bacteriosis caused by *A. hydrophil*a in silver catfish (*Rhamdia quelen*) (Sutili *et al.* 2014). Citral is a monoterpene present in several EOs, such as the EO of *Lippia alba*, *Ocimum americanum*, and *Cymbopogon citratus*, where its content varies around 70% (Mondello *et al.* 2002; Gautam and Agrawal 2017; Souza *et al.* 2017). This compound has considerable antimicrobial activity against Gram-negative and Gram-positive bacteria as well as fungi (Saddiq and Khayyat 2010; Lu *et al.* 2017). Carvacrol is a phenolic monoterpene present in the EO of *Origanum vulgare*, *O. dictamnus* and *O. majorana* (Baser *et al.* 1993; Lambert *et al.* 2001; Liolios *et al.* 2009). The *O. vulgare* EO (carvacrol rich) has been reported to be effective in the treatment of channel catfish (*Ictalurus punctatus*) bacteriosis caused by *A. hydrophila*, probably because it disrupts bacterial membranes (Zheng *et al.* 2009; Sousa *et al.* 2015). Thymol is a colorless aromatic monoterpene and a major component of the *Thymus vulgaris*, *O. vulgare*, and *Lippia sidoides* EOs (Poulose and Croteau 1978; Lambert *et al.* 2001; Botelho *et al.* 2007). This phytochemical has bacteriostatic and bactericidal activity against *A. hydrophila* (Majolo *et al.* 2016). Linalool is a monoterpene and the major component of several plant EOs, such as those of *Ocimum basilicum*, *Aniba rosaeodora*, and

L. alba EOs; it has antibacterial properties against *A. hydrophila* (Dorman and Deans 2000; Sampaio *et al.* 2012; Avetisyan *et al.* 2017; Souza et al. 2017).

Silver catfish is the most farmed native species of fish in South Brazil, presenting a good adaptation to different environments and artificial diets, resistance to handling, and good commercial acceptance (Baldisserotto 2009; Figueredo *et al.* 2014). The majority of the diseases reported in silver catfish farms are related to bacterial pathogens; among these, bacteria of the genus *Aeromonas* are the most frequently reported microorganisms (Baldisserotto and Radünz Neto 2004; Barcellos *et al.* 2008).

In this context, including the need to decrease the environmental impacts of antimicrobials, we tested the hypotheses that the phytochemicals described above (eugenol, citral, carvacrol, thymol and linalool) may have a synergistic effect with two conventional antimicrobials approved for use in aquaculture (florfenicol and oxytetracycline) against emergent pathogenic bacteria for fish in aquaculture (*A. hydrophila*, *C. freundii* and *R. onithinolytica*) through microdilution checkerboard assay. The present study also checked the ability of these phenolic compounds to inhibit both the formation of biofilms and the hemolytic capacity of the bacterial pathogens mentioned. A screening of antibiotic susceptibility and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics and phytochemicals were also performed.

Materials and Methods

Phytochemicals and antimicrobials

The phytochemicals citral and linalool - racemic mixture, carvacrol, and thymol (≥ 96%, ≥ 97%, ≥ 99%, ≥ 99% purity, respectively) and the antimicrobials florfenicol and oxytetracycline (analytical standard) were purchased from Sigma-Aldrich®. Eugenol (≥ 99% purity) was purchased from Biodinâmica®. The antimicrobials (impregnated disks) used for the agar disk diffusion test were purchased from Laborclin®. Nine antimicrobials were used in this test, totalizing eight different antimicrobials groups, as follows: aminoglycosides (gentamicin – GEN 10 µg and streptomycin – STR 10 µg), macrolides (erythromycin – ERY 15 µg), quinolones (norfloxacin – NOR 10 µg), tetracyclines (tetracycline – TET 30 µg), sulfonamides (trimethoprim-sulfamethoxazole – TSU 25 µg), amphenicols (florfenicol – FLOR 30 µg), nitrofurans (nitrofurantoin – NIT 300 µg), and ansamycins (rifampicin – RIF 5 µg).

Clinical isolates

Fifteen strains were isolated from ten naturally infected silver catfish juveniles as follows: *A. hydrophila* (n = 10), *A. veronii* (n = 2), *R. ornithinolytica* (n = 2), and *C. freundii* (n = 1). All animal management procedures were approved by the Comissão de Ética no Uso de Animais (CEUA) of the Universidade Federal de Santa Maria (UFSM) under the protocol number 074/2014. Identification of the strains was performed by biochemical characteristics and confirmed by analysis of the partial sequencing of a triplicate DNA product of approximately 1,500 bp, amplified from the 16S rRNA gene using universal primers (Fredricks and Relman 1998; Buller 2004). The consensus sequence of at least one bacterium for each genus was deposited in GenBank under the ascension numbers MF 565839 (*C. freundii*, fish 3), MF 372511 (*R. ornithinolytica*, fish 8), MF 372509 (*A. hydrophila*, fish 9), MF 372510 (*A. hydrophila*, fish 10), and MF 372508 (*A. veronii*, fish 8).

Agar disk diffusion test

All isolates were subjected to disk diffusion assays in Mueller-Hinton agar - MHA (Himedia Laboratories, Mumbai, India), according to the guidelines of the Clinical and Laboratory Standards Institute - CLSI (CLSI 2014a). The inocula were prepared in Mueller Hinton broth - MHB (Himedia Laboratories, Mumbai, India) at 28°C/24 h, $1x10^8$ colony forming units - CFU ml^{-1}, 0.15 optical density-OD at 600 nm.

Calculation of multiple antibiotic resistance (MAR)

Multiple antibiotic resistance (MAR) was calculated as the ratio between the number of antibiotics to which the microbial culture was resistant and the total number of tested antibiotics. The average MAR values for each bacterial species were also calculated. Values above 0.2 were considered as indicator of antimicrobial resistance (Krumperman 1983). The strains with an intermediate resistance profile were considered resistant for the MAR calculation. In addition, the multidrug-resistance of each strain was determined considering as multidrug-resistant the strains resistant against three or more classes of antimicrobials (Schwarz *et al.* 2010).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

Three clinical isolates (*A. hydrophila* MF 372510, *C. freundii* MF 565839 and *R. ornithinolytica* MF 372511) and two standard strains (*A. hydrophila* ATCC 7966 and *Escherichia coli* ATCC 25922) were selected for the MIC and MBC tests, using the microdilution method in accordance with the guidelines of the CLSI, document VET04-A2 (CLSI 2014b). The phytochemicals were diluted in 96% ethanol and incorporated in MHB at concentrations of 6,400, 3,200, 1,600, 800, 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 µg mL^{-1} (in triplicate). Florfenicol was diluted in 96% ethanol and oxytetracycline was diluted in pH-adjusted water (according to the recommendations of the manufacturer) and both incorporated in MHB at concentrations of 260, 130, 65, 32.5, 16.25, 8.125, 4.06, 2.03, 1.02, 0.51, and 0.25 µg mL^{-1} (in triplicate). The inoculum was prepared in saline solution from cultures grown in MHA (1×10^8 CFU mL^{-1}; 0.15 OD 600 nm) (28ºC/24 h). Ten microliters (1×10^5 CFU) of inoculum were added to each well containing the substances tested. The microplates were incubated at 28ºC for 24 h under aerobic conditions. The same procedure was performed on an ethanol control. Values of MBC were confirmed by re-inoculation of 10 µL of each bacterial culture on MHA (28ºC/24 h), and the lowest concentration of the substances showing no growth was defined as the MBC.

Checkerboard assay

Different combinations of florfenicol and oxytetracycline with each of the five phytochemicals were tested against the three clinical isolates and against the standard strain *A. hydrophila* ATCC 7966, using the checkerboard method (Rand *et al.* 1993). The tests were performed in triplicate. The concentrations tested in combination were below (MIC/8, MIC/4, MIC/2), equal (MIC), or above (MIC x 2, MIC x 4, MIC x 8) the MIC for the microorganisms tested. The checkerboard method consisted of columns containing some amount of antimicrobial agent A, diluted along the x-axis, and lines containing antimicrobial agent B, diluted along the y-axis. The checkerboard synergy testing results were determined through the lowest FIC (fractional inhibitory concentration) index method, as described in the Clinical Microbiology Procedures Handbook (Garcia and Isenberg 2010). The results were analyzed by the calculation of the FIC, as follows: FIC of compound A (FICA) = MIC of compound A in combination/MIC of compound A alone; FIC of compound B (FICB) = MIC of compound B in combination/MIC of compound B alone. The FIC index (FICI) was calculated by FICA + FICB. Synergy effect with antimicrobials can be defined for FICI values less than or equal

to 0.5, additivity for FICI values between 0.5 and 4, and antagonism for FICI values greater than 4 (Souza *et al.* 2014).

Effect on biofilm formation

The effects of different concentrations (MIC/8, MIC/4, MIC/2, MIC, MIC x 2, MIC x 4, and MIC x 8) of the phytochemicals and antimicrobials on the biofilm-forming ability of three clinical isolates and the standard strain *A. hydrophila* ATCC 7966 were tested on polystyrene flat-bottomed microtiter plates (Stepanović *et al.* 2007). Some modifications were made to the usual technique to adapt it for bacteria isolated from fish. Through a previous test, we observed that the bacteria formed more biofilm in tryptic soy broth - TSB (Himedia Laboratories, Mumbai, India) medium without glucose supplementation when compared to TSB supplemented with 1% glucose. Therefore, cultures were grown overnight in 6 ml TSB and diluted to the OD of 0.25 (600 nm). Subsequently, 195 µl of the TSB with the compound to be tested (diluted in ethanol) and 5 µl of the inoculum were added to each well in triplicate for each concentration tested. After incubation for 48 h at 28ºC, each well was washed thrice with sterile distilled water, dried, stained for 5 min with 0.25% gentian violet, and washed again. The stained biofilms were resuspended in 200 µl of alcohol: acetone solution (80:20) and the OD (550 nm) was measured by spectrophotometry using an ELISA reader. Negative controls (without inoculum) corresponding to each compound were added. Biofilm formation was considered weak when the OD of the sample (ODs) increased up to twice as compared to the OD of the negative control (ODnc), moderate when ODs increased between two to four times compared to ODnc, and strong when ODs increased more than four times compared to ODnc (Stepanović *et al.* 2004). In a previous test, without the addition of the compounds, we observed that all strains tested had a strong ability to form biofilms. Therefore, a reduction in the formation of biofilm to moderate or weak may be attributed to the addition of the compounds.

Inhibition of hemolysis

A β-hemolytic strain was selected (*A. hydrophila* MF 372510) for the hemolysis inhibition assay (Sutili *et al.* 2016). This strain was cultured in MHB containing sub-inhibitory concentrations (0, 10, 20, and 40% MIC) of the five phytochemicals tested and incubated at 28°C for 48 h. The bacterial culture was adjusted to an OD of 1.6 (600 nm), centrifuged at 1,250 g for 10 min, and the supernatant was collected. In microtubes, 1 ml of the supernatant of each culture (in triplicate) was mixed with 100 µl of 5% sheep red blood

cells (diluted in phosphate-buffered saline - PBS). Total hemolysis control (1 ml of sterile distilled water and 100 µl of 5% sheep red blood cells) and control without hemolysis (1 ml of sterile MHB and 100 µl of 5% sheep red blood cells) were performed. A control with ethanol was also performed in the highest concentration used to dilute the phytochemicals. Microtubes were incubated at 37°C for 90 minutes and then centrifuged at 450 g for 7 minutes. The hemolytic activity of the supernatant was detected by measuring its OD at 540 nm. The percent hemolysis was calculated by comparison between total hemolysis (100%) and non-hemolysis (0%) controls.

Statistical analysis

For the hemolysis inhibition assay, the homogeneity of variances between groups was tested with the Levene test. When the data were parametrical, comparisons between different groups were made using one-way ANOVA and Tukey's test. In the case of non-parametrical data, Kruskal-Wallis ANOVA and multiple comparisons of mean ranks for all groups were applied (Statistica 7.0, StatSoft Inc., Tulsa, OK, USA).

Results

Agar disk diffusion test

The results of the agar disk diffusion test are presented in Table 1. The tested bacteria showed higher resistance against the antimicrobials streptomycin, erythromycin, and rifampicin. All the strains were sensitive to the antimicrobials norfloxacin, trimethoprim-sulfamethoxazole, and nitrofurantoin. Only one strain was not sensitive to gentamicin (*R. ornithinolytica* was intermediate).

Calculation of multiple antibiotic resistance (MAR)

The MAR was calculated for each bacterial strain and then the mean MAR was calculated for each bacterial species. The MAR index for all bacteria species showed high average values (0.28 for *A. hydrophila*, 0.44 for *A. veronii*, 0.44 for *C. freundii*, and 0.33 for *R. ornithinolytica*). The species *C. freundii*, two strains of *A. hydrophila*, and the *A. veronii* strains presented resistance against four different classes of antimicrobials. Two strains of *A. hydrophila* and one strain of *R. ornithinolytica* showed resistance against three different classes of antimicrobials.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The *A. hydrophila* MF 372510 was the most sensitive of the strains tested, with MIC of 25 µg mL^{-1} for carvacrol, eugenol, and thymol. These three phytochemicals were the most effective of them, with MICs ranging from 25 to 400 µg mL^{-1}, and the highest values were found for the *R. ornithinolytica* MF 372511 strain (Table 2). The MIC values of the antimicrobials found for the standard strains (ATCC strains) are in accordance with the standards established in document VET03/VETO4-S2 of the CLSI, validating the test (CLSI 2014c).

Synergy test (checkerboard)

The FIC and the FICI of phytochemicals, in association with different combinations of the antimicrobials florfenicol and oxytetracycline, are described in Table 3. There was an additive effect for most combinations tested. Linalool and florfenicol or oxytetracycline (against *A. hydrophila* ATCC 7966) and citral and oxytetracycline (against *C. freundii* MF 565839) showed a synergic effect. None of the combinations tested had an antagonist effect, showing promising results with respect to a combination therapy.

Effect on biofilm formation

All phytochemicals presented a potential to inhibit the formation of bacterial biofilms of the four bacterial strains. This potential is exalted in comparison with antimicrobials, since they have shown a low capacity of inhibition of biofilm formation. In several of the tested concentrations, citral, thymol, and linalool were able to totally inhibit the biofilm production. Carvacrol and eugenol were able to reduce biofilm production from strong to weak when used at the some MICs (Table 4).

Inhibition of hemolysis

The phytochemicals carvacrol (A), eugenol (C), and linalool (D) decreased the hemolytic activity of the strain in all concentrations tested (10, 20, and 40% of MIC). The phytochemicals citral (B) and thymol (E) decreased the hemolytic activity only when used at 40% of the MIC concentration (Fig. 1).

Discussion

The phytochemicals carvacrol, citral, eugenol, thymol, and linalool presented antimicrobial activity against fish pathogenic bacteria when used alone or in combination with conventional antimicrobials (florfenicol and oxytetracycline); these combinations resulted in additivity or synergy. These phytochemicals showed the ability to inhibit the formation of bacterial biofilms and the hemolysis of a β-hemolytic strain (*A. hydrophila*). The two tested strains of *A. hydrophila* showed different MIC and MBC results. These strains have different origins, considering that one strain was isolated from fish and another was a standard strain isolated from milk.

The over-prescription of antibiotics to treat human infections and the use of antibiotics in livestock have been linked to the increased ineffectiveness of current antibiotic treatments for both humans and animals. Antimicrobial resistance in traditional fish farming systems has been intensively studied. A high incidence of bacteria resistant to the antimicrobials used in aquaculture as well as of multidrug-resistant bacteria has been found in fish farms and the surrounding aquatic environments (Watts *et al.* 2017). In the present study, multidrug-resistant strains were found, which reinforces the need to carry out antimicrobial susceptibility testing prior to fish treatment.

The susceptibility profile of the bacteria tested in the present study against penicillin G (10IU), ampicillin (10 µg), amoxicillin (10 µg), and oxacillin (1 µg), all antibiotics from the penicillin class, demonstrated a massive resistance in all strains (data not shown). This find may be attributed to the intrinsic resistance of *Aeromonas* bacteria to penicillins, along with data from several studies (Dias *et al.* 2012; Sreedharan *et al.* 2012; Igbinosa 2014; Jagoda *et al.* 2014). Although there is no evidence that *C. freundii* and *R. ornithinolytica* display intrinsic resistance to penicillins, other studies found similar results (Trust and Whitby 1976; Pádua *et al.* 2014; Trivedi *et al.* 2015). Given the possibility of intrinsic resistance, and considering the parameters established by Schwarz *et al.* (2010), we unconsidered the class of penicillins to determine MAR and multidrug-resistance.

The use of inappropriate/unlicensed antimicrobials in aquaculture poses a risk to public health (Watts *et al.* 2017). The combined therapy of conventional antimicrobials with phytochemicals has emerged as an alternative to achieve therapeutic success in cases of MAR. Furthermore, this practice may prevent environmental contamination and bacterial resistance induced by the excessive use of antimicrobials (Bezalwar *et al.* 2017). Taking this

into account, we performed the checkerboard assay to evaluate the interactions between the phytochemicals carvacrol, citral, eugenol, linalool, and thymol with the antimicrobials florfenicol and oxytetracycline (both approved by the FDA for use in aquaculture) against bacteria pathogenic to fish (FDA 2014). Our results indicate that is possible to establish a combination therapy between these substances, since none of the combinations tested had an antagonistic effect. Although most combinations tested had an additive effect, three of them had a synergy effect (linalool with florfenicol or oxytetracycline against *A. hydrophila* and citral with oxytetracycline against *C. freundii*).

Biofilms are complex microbiologic ecosystems embedded in matrixes of organic polymers adhered to a surface (Millezi *et al.* 2013). There is an increase of sessile cell resistance to host defenses, biocides, antibiotics, and various physiochemical agents, leading to persistence and survival even after sanitization processes (Chavant *et al.* 2007). Biofilm formation can be harmful to aquaculture practices, as observed in the high susceptibility of the cage fishing nets to biofilm formation (Ashraf and Edwin 2016). The fact that biofilm-producing bacteria possess greater resistance stimulates studies with substances that might prevent this resistance. Here, all phytochemicals tested inhibited biofilm formation, displaying a superior activity compared to the antimicrobials tested. These results confirm the phytochemical potential against the formation of these extracellular polymer matrixes under the conditions analyzed. In addition, the essential oil of *C. citratus* (with 78% citral) also reduced the *A. hydrophila* biofilm formed on stainless steel surfaces (Millezi *et al.* 2013). Eugenol also has the ability to reduce biofilm formation of *Pseudomonas aeruginosa*, while linalool acts against *Campylobacter* spp. and *Candida albicans*, citral against *Staphylococcus aureus* and *Salmonella* Enteritidis, and thymol and carvacrol against *Streptococcus mutans* (Zhang *et al.* 2014; Duarte *et al.* 2016; Khan *et al.* 2017; Manoharan *et al.*, 2017; Rathinam *et al.* 2017).

Among the several virulence factors of bacteria, the ability to cause hemolysis is one of the most known. The production of exotoxins, known as hemolysins, is a characteristic of bacteria highly pathogenic for fish (Vences *et al.* 2017). For this reason, we evaluated the capability of the phytochemicals to inhibit the hemolysis caused by the β-hemolytic strain isolated from fish. All phytochemicals tested were able to reduce the hemolytic activity of the strain in lower concentrations than MIC. Therefore, these substances do not only have bactericidal/bacteriostatic activities, but are also able to inhibit virulence factors when used in concentrations lower than those that would inhibit bacterial growth. Corroborating our results, it has been reported that eugenol has an ability to inhibit hemolysis caused by *A. hydrophila*

(Sutili *et al.* 2014). There are few studies testing the ability of these phytochemicals to inhibit bacterial hemolysis. Studies investigating the expression of virulence genes from bacteria exposed to these phytochemicals are necessary to confirm these findings.

The phytochemicals showed satisfactory antibacterial activity in all the tests performed. Therefore, these phytochemicals are candidate drugs to be used in the future to combat bacterial diseases in fish. The fact that they do not present antagonistic interactions with the antimicrobials tested allows their combined use, which may contribute to a decrease in the use of conventional drugs and their residues in the aquatic environment. However, *in vivo* studies are needed to confirm our *in vitro* findings.

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) of Brazil.

Conflict of interests

The authors declare that they have no conflicts of interests to disclose.

References

Ashraf, P.M. and Edwin, L. (2016) Nano copper oxide incorporated polyethylene glycol hydrogel: An efficient antifouling coating for cage fishing net. *Int Biodeterior Biodegradation* **115**, 39-48.

Avetisyan, A., Markosian, A., Petrosyan, M., Sahakyan, N., Babayan, A., Aloyan, S. and Trchounian, A. (2017) Chemical composition and some biological activities of the essential oils from basil *Ocimum* different cultivars. *BMC Complement Altern Med* **17**, 1-8.

Baker, M.R., Gobush, K.S. and Vynne, C.H. (2013) Review of factors influencing stress hormones in fish and wildlife. *J Nat Conserv* **21**, 309–318.

Baldisserotto, B. (2009) Freshwater fish culture in Rio Grande do Sul State: actual situation, problems and future perspectives. *Cienc Rural* **39**, 291-299.

Baldisserotto, B. and Radünz Neto, J. (2004) *Criação de jundiá*. Santa Maria: Editora UFSM.

Barcellos, L.J.G., Kreutz, L.C., Rodrigues, L.B., Santos, L.R., Motta, A.C., Ritter, F., Bedin, A.C. and Silva, L.B. (2008) *Aeromonas hydrophila* in *Rhamdia quelen*: macroscopic and microscopic aspect of the lesions and antibiotic resistence profiles. *Bol Inst Pesca* **34**, 355-363.

Baser, K.H.C., Kırımer, N.A. and Tümen, G. (1993) Composition of the essential oil of *Origanum majorana* L. from Turkey. *J Essent Oil Res* **5**, 577-579.

Bezalwar, P.M., Patil, S.P. and Charde, V.N. (2017) Harmony of phytochemicals and antibiotics: a boon in antimicrobial therapy. *Int J Recent Sci Res* **8**, 17930-17938.

Botelho, M.A., Nogueira, N.A.P., Bastos, G.M., Fonseca, S.G.C., Lemos, T.L.G., Matos, F.J.A., Montenegro, D. and Heukelbach, J. (2007) Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res* **40**, 349-356.

Buller, N.B. (2004) *Bacteria from Fish and Other Aquatic Animals*. Cambridge: CABI Publishing.

Cabello, F.C. (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol* **8**, 1137-1144.

Cai, S.H., Wu, Z.H., Jian, J.C., Lu, Y.S. and Tang, J.F. (2012) Characterization of pathogenic *Aeromonas veronii* bv. *veronii* associated with ulcerative syndrome from Chinese longsnout catfish (*Leiocassis longirostris* Günther). *Braz J Microbiol* **43**, 382-388.

Chavant, P., Gaillard-Martinie, B., Talon, R., Hébraud, M. and Bernardi, T. (2007) A new device for rapid evaluation of biofilm formation potential by bacteria. *J Microbiol Methods* **68**, 605-612.

CLSI (2014a) *Antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard*, Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2014b) *Methods for broth dilution susceptibility testing of bacteria isolated from aquatic animals; approved guideline*, Second Ed., Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2014c) *Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals; Second Informational Supplement*, Wayne, PA: Clinical and Laboratory Standards Institute.

Dias, C., Mota, V., Martinez-Murcia, A. and Saavedra, M.J. (2012) Antimicrobial resistance patterns of *Aeromonas* spp. isolated from ornamental fish. *J Aquac Res Dev* **3**, 1-4.

Dorman, H.J.D. and Deans, S.G. (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol* **88**, 308-316.

Duarte, A., Luís, A., Oleastro, M. and Domingues, F.C. (2016) Antioxidant properties of coriander essential oil and linalool and their potential to control *Campylobacter* spp. *Food Control* **61**, 115-122.

FDA (2014) *Approved Drugs;* USA: Food and Drug Administration - Department of Health and Human Services.

Figueredo, A.B., Tancredo, K.R., Hashimoto, G.S.O., Roumbedakis, K., Marchiori, N.C. and Martins, M.L. (2014) Haematological and parasitological assessment of silver catfish *Rhamdia quelen* farmed in Southern Brazil. *Rev Bras Parasitol Vet* **23**, 157-163.

Fredricks, D.N. and Relman, D.A. (1998) Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanetholesulfonate. *J Clin Microbiol* **36**, 2810-2816.

Garcia, L.S. and Isenberg, H.D. (2010) *Clinical Microbiology Procedures Handbook*. (3rd ed.) Washington: ASM Press.

Gautam, M. and Agrawal, M. (2017) Influence of metals on essential oil content and composition of lemongrass (*Cymbopogon citratus* (D.C.) Stapf.) grown under different levels of red mud in sewage sludge amended soil. *Chemosphere* **175**, 315-322.

Ghatak, S., Blom, J., Das, S., Sanjukta, R., Puro, K., Mawlong, M., Shakuntala, I., Sen, A. *et al.* (2016) Pan-genome analysis of *Aeromonas hydrophila*, *Aeromonas veronii* and *Aeromonas caviae* indicates phylogenomic diversity and greater pathogenic potential for *Aeromonas hydrophila*. *Antonie Van Leeuwenhoek* **109**, 945-956.

Igbinosa, I.H. (2014) Antibiogram profiling and pathogenic status of *Aeromonas* species recovered from Chicken. *Saudi J Biol Sci* **21**, 481-485.

Jagoda, S.S., Wijewardana, T.G., Arulkanthan, A., Igarashi, Y., Tan, E., Kinoshita, S., Watabe, S. and Asakawa, S. (2014) Characterization and antimicrobial susceptibility of motile aeromonads isolated from freshwater ornamental fish showing signs of septicaemia. *Dis Aquat Org* **109**, 127-137.

Kanki, M., Yoda, T., Tsukamoto, T. and Shibata, T. (2002) *Klebsiella pneumoniae* produces no histamine: *Raoultella planticola* and *Raoultella omithinolytica* strains are histamine producers. *Appl Environ Microbiol* **68**, 3462-3466.

Karunasagar, I., Karunasagar, I. and Pai, R. (1992) Systemic *Citrobacter freundii* infection in common carp, *Cyprinus carpio* L., fingerlings. *J Fish Dis* **15**, 95-98.

Kaya, S., Bayramoglub, G., Sönmezc, M. and Köksal, I. (2015) *Raoultella ornithinolytica* causing fatal sepsis. *Braz J Infect Dis* **19**, 230-231.

Keene, J.L., Noakes, D.L.G., Moccia, R.D. and Sotoet, C.G. (1998) The efficacy of clove oil as an anaesthetic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquacult Res* **29**, 89-101.

Khan, S.T., Khan, M., Ahmad, J., Wahab, R., Abd-Elkader, O.H., Musarrat, J., Alkhathlan, H.Z. and Al-Kedhairy, A.A. (2017) Thymol and carvacrol induce autolysis, stress, growth inhibition and reduce the biofilm formation by *Streptococcus mutans*. *AMB Express* **7**, 1-11.

Krumperman, P.H. (1983) Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl Environ Microbiol* **46**, 165-170.

Lambert, R.J., Skandamis, P.N., Coote, P.J. and Nychas, G.J. (2001) A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol* **91**, 453-462.

Liolios, C.C., Gortzi, O., Lalas, S., Tsaknis, J. and Chinou, I. (2009) Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. *Food Chem* **112**, 77-83.

Lu, W., Huang, D., Wang, C., Yeh, C., Tsai, J., Huang, Y. and Li, P. (2017) Preparation, characterization, and antimicrobial activity of nanoemulsions incorporating citral essential oil. *J Food Drug Anal* (in press).

Majolo, C., Rocha, S.I.B., Chagas, E.C., Chaves, F.C.M. and Bizzo, H.R. (2016) Chemical composition of *Lippia* spp. essential oil and antimicrobial activity against *Aeromonas hydrophila*. *Aquacult Res* **48**, 2380-2387.

Manoharan, R.K., Lee, J.H., Kim, Y.G., Kim, S.I., Lee, J. (2017) Inhibitory effects of the essential oils α-longipinene and linalool on biofilm formation and hyphal growth of *Candida albicans*. *Biofouling* **33**, 143-155.

Millezi, A.F., Cardoso, M.G., Alves, E. and Piccoli, R.H. (2013) Reduction of *Aeromonas hidrophyla* biofilm on stainless stell surface by essential oils. *Braz J Microbiol* **44**, 73-80.

Mondello, L., Zappia, G., Cotroneo, A., Bonaccorsi, I., Chowdhury, J.U., Yusuf, M. and Dugo, G. (2002) Studies on the essential oil-bearing plants of Bangladesh. Part VIII. Composition of some *Ocimum* oils *O. basilicum* L. var. p*urpurascens*; *O. sanctum* L. green; *O. sanctum* L. purple; *O. americanum* L., citral type; *O. americanum* L., camphor type. *Flavour Frag J* **17**, 335-340.

Morais, V.P., Daporta, M.T., Bao, A.F and Campello, M.G. (2009) Enteric fever-like syndrome caused by *Raoultella ornithinolytica* (*Klebsiella ornithinolytica*). *J Clin Microbiol* **47**, 868-869.

Nakasone, E.S., Kaneshiro, R., Min, K. and Tokeshi, J. (2015) Emergence of *Raoultella ornithinolytica* on O‘ahu: a case of community-acquired *R. ornithinolytica* urinary tract infection. *Hawaii J Med Public Health* **74**, 174-175.

Pádua, S.B., Marques, D.P., Sebastião, F.A., Pilarski, F., Martins, M.L. and Ishikawa, M.M. (2014) Isolation, characterization and pathology of *Citrobacter freundii* infection in native Brazilian catfish *Pseudoplatystoma*. *BJVP* **7**, 151-157.

Poulose, A.J. and Croteau, R. (1978) Biosynthesis of aromatic monoterpenes. *Arch Biochem Biophys* **187**, 307-314.

Rand, K.H., Houck, H.J., Brown, P. and Bennett, D. (1993) Reproducibility of the microdilution checkerboard method for antibiotic synergy. *Antimicrob Agents Chemother* **37**, 613-615.

Rathinam, P., Kumar, H.S.V. and Viswanathan, P. (2017) Eugenol exhibits anti-virulence properties by competitively binding to quorum sensing receptors. *Biofouling* **33**, 624-639.

Saddiq, A.A. and Khayyat, S.A. (2010) Chemical and antimicrobial studies of monoterpene: citral. *Pestic Biochem Physiol* **98**, 89-93.

Sampaio, L.F., Maia, J.G., de Parijós, A.M., de Souza, R.Z. and Barata, L.E. (2012) Linalool from rosewood (*Aniba rosaeodora* Ducke) oil inhibits adenylate cyclase in the retina, contributing to understanding its biological activity. *Phytother Res* **26**, 73-77.

Sato, N., Yamane, N. and Kawamura, T. (1982) Systemic *Citrobacter freundii* infection among Sunfish *Mola* in Matsushima Aquarium. *Bull Jpn Soc Sci Fish* **48**, 1551-1557.

Schwarz, S., Silley, P., Simjee, S., Woodford, N., van Duijkeren, E., Johnson, A.P. and Gaastra, W. (2010) Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *J Antimicrob Chemother* **65**, 601-604.

Solak, Y., Gul, E.E., Atalay, H., Genc, N. and Tonbula, H.Z. (2011) A rare human infection of *Raoultella ornithinolytica* in a diabetic foot lesion. *Ann Saudi Med* **31**, 93-94.

Sousa, J.P., Oliveira, K.Á., Figueiredo, R.C. and Souza, E.L. (2015) Influence of carvacrol and 1,8-cineole on cell viability, membrane integrity, and morphology of *Aeromonas hydrophila* cultivated in a vegetable-based broth. *J Food Prot* **78**, 424-429.

Souza, A.M., Armstrong, L., Merino, F.J.Z., Cogo, L.L., Monteiro, C.L.B., Duarte, M.R., Miguel, O.G. and Miguel, M.D. (2014) In vitro effects of *Eugenia pyriformis* Cambess., Myrtaceae: antimicrobial activity and synergistic interactions with vancomycin and fluconazole. *Afr J Pharm Pharmacol* **8**, 862-867.

Souza, C.F., Baldissera, M.D., Salbego, J., Lopes, J.M., Vaucher, R.A., Mourão, R.H.V., Caron, B.O., Heinzmann, B.M. *et al.* (2017) Physiological responses of *Rhamdia quelen*

(Siluriformes: Heptapteridae) to anesthesia with essential oils from two different chemotypes of *Lippia alba*. *Neotrop Ichthyol* **15**, e160083.

Sreedharan, K., Philip, R. and Singh, I.S.B. (2012) Virulence potential and antibiotic susceptibility pattern of motile aeromonads associated with freshwater ornamental fish culture systems: A possible threat to public health. *Braz J Microbiol* **43**, 754-765.

Stepanović, S., Cirković, I., Ranin, L. and Svabić-Vlahović, M. (2004) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett Appl Microbiol* **38**, 428-432.

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I. and Ruzicka, F. (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS* **115**, 891-899.

Sun, J., Zhang, X., Gao, X., Jiang, Q., Wen, Y. and Lin, L. (2016) Characterization of virulence properties of *Aeromonas veronii* isolated from diseased Gibel Carp (*Carassius gibelio*). *Int J Mol Sci* **17**, 496.

Sutili, F.J., Kreutz, L.C., Noro, M., Gressler, L.T., Heinzmann, B.M., de Vargas, A.C. and Baldisserotto, B. (2014) The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*). *Vet Immunol Immunopathol* **157**, 142-148.

Sutili, F.J., Murari, A.L., Silva, L.L., Gressler, L.T., Heinzmann, B.M., de Vargas, A.C., Schmidt, D. and Baldisserotto, B. (2016) The use of *Ocimum americanum* essential oil against the pathogens *Aeromonas hydrophila* and *Gyrodactylus* sp. in silver catfish (*Rhamdia quelen*). *Lett Appl Microbiol* **63**, 82-88.

Svetlana, J., Dobrila, J.D. and Lj, V. (2003) *Citrobacter freundii* as a cause of disease in fish. *Acta Vet* **53**, 399-410.

Trivedi, M.K., Patil, S., Harish, S., Gangwar, M. and Jana, S. (2015) Biofield treatment: an alternative approach to combat multidrug-resistant susceptibility pattern of *Raoultella ornithinolytica*. *Altern Integr Med* **4**, 1-6.

Trust, T.J. and Whitby, J.L. (1976) Antibiotic resistance of bacteria in water containing ornamental fishes. *Antimicrob Agents Chemother* **10**, 598-603.

Vences, A., Rivas, A.J., Lemos, M.L., Husmann, M. and Osorio, C.R. (2017) Chromosome-encoded hemolysin, phospholipase, and collagenase in plasmidless isolates of *Photobacterium damselae* subsp. *damselae* contribute to virulence for fish. *Appl Environ Microbiol* **83**, e00401-17.

Watts, J.E.M., Schreier, H.J., Lanska, L. and Hale, M.S. (2017) The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. *Mar Drugs* **15**, 1-16.

Zhang, H., Zhou, W., Zhang, W., Yang, A., Liu, Y., Jiang, Y., Huang, S., Su, J. (2014) Inhibitory effects of citral, cinnamaldehyde, and tea polyphenols on mixed biofilm formation by foodborne *Staphylococcus aureus* and *Salmonella* Enteritidis. *J. Food Prot.* **77**, 927-933.

Zheng, Z.L., Tan, J.Y.W., Liu, H.Y., Zhou, X.H., Xiang, X. and Wang, K.Y. (2009) Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*). *Aquaculture* **292**, 214-218.

Table 1 Results of susceptibility testing to antimicrobials and inhibition zone diameter (in parenthesis, in mm) of pathogenic bacteria for fish

Strains fish	**Antimicrobials**								
	GEN	STR	ERY	NOR	TET	TSU	FLOR	NIT	RIF
Ah fish 1	S (19)	S (15)	I (14)	S (27)	S (29)	S (16)	S (27)	S (22)	I (17)
Ah fish 2	S (20)	S (15)	R (12)	S (28)	S (28)	S (17)	S (30)	S (21)	R (14)
Ah fish 3	S (19)	S (15)	R (12)	S (35)	S (29)	S (22)	S (34)	S (27)	R (15)
Cf fish 3	S (17)	I (14)	R (5)	S (31)	S (23)	S (23)	R (13)	S (22)	R (5)
Ah fish 4	S (19)	S (16)	I (17)	S (34)	S (27)	S (21)	S (30)	S (26)	S (23)
Ah fish 5	S (19)	I (13)	R (5)	S (31)	S (24)	S (17)	I (17)	S (20)	R (5)
Ah fish 6	S (20)	S (16)	I (16)	S (31)	S (29)	S (20)	S (30)	S (25)	R (11)
Ah fish 7	S (17)	I (13)	R (13)	S (29)	S (29)	S (20)	S (31)	S (22)	I (17)
Ah fish 8	S (15)	I (12)	I (16)	S (29)	R (11)	S (21)	S (29)	S (21)	R (16)
Av fish 8	S (16)	I (12)	I (16)	S (31)	R (12)	S (21)	S (29)	S (22)	I (17)
Ro fish 8	S (20)	S (17)	R (10)	S (24)	S (23)	S (16)	S (23)	S (21)	R (5)
Ah fish 9	S (17)	I (14)	I (16)	S (25)	S (25)	S (19)	S (30)	S (21)	S (21)
Ah fish 10	S (18)	I (14)	R (12)	S (33)	S (27)	S (25)	S (30)	S (23)	I (19)
Av fish 10	S (15)	R (9)	R (5)	S (26)	R (8)	S (22)	S (24)	S (21)	R (9)
Ro fish 10	I (13)	R (10)	R (10)	S (29)	S (29)	S (26)	S (30)	S (27)	R (14)

Ah, Aeromonas hydrophila; Av, Aeromonas veronii; Cf, Citrobacter freundii; Ro, Raoultella ornithinolytica; GEN, gentamicin; STR, streptomycin; ERY, erythromycin; NOR, norfloxacin; TET, tetracycline; TSU, trimethoprim-sulfamethoxazole; FLOR, florfenicol; NIT, nitrofurantoin; RIF, rifampicin; S, sensitive; I, intermediate; R, resistant.

Table 2 Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of five phytochemicals and two antimicrobials against bacteria isolated from fish and standard strains (ATCC 7966 and ATCC 25922)

	A. hydrophila ATCC 7966		*A. hydrophila* MF 372510		*C. freundii* MF 565839		*R. ornithinolytica* MF 372511		*Escherichia coli* ATCC 25922	
	MIC µg mL^{-1}	MBC µg mL^{-1}	MIC µg mL^{-1}	MBC µg mL^{-1}	MIC µg mL^{-1}	MBC µg mL^{-1}	MIC µg mL^{-1}	MBC µg mL^{-1}	MIC µg mL^{-1}	MBC µg mL^{-1}
Carvacrol	100	200	25	50	100	100	200	400	-	-
Citral	200	200	200	400	3,200	3,200	1,600	1,600	-	-
Eugenol	200	200	25	50	400	800	400	800	-	-
Linalool	3,200	3,200	1,600	1,600	3,200	3,200	1,600	3,200	-	-
Thymol	200	200	25	50	50	50	400	400	-	-
Florfenicol	1.02	130	1.02	4.06	8.13	>260	8.13	>260	4.06	>260
Oxytetracycline	0.51	16.25	0.51	4.06	4.06	260	4.06	260	1.02	130

Table 3 Fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI) of phytochemicals in association with different combinations of the antimicrobials florfenicol (FLF) and oxytetracycline (OXT)

	A. hydrophila ATCC 7966		*A. hydrophila* MF 372510		*C. freundii* MF 565839		*R. ornithinolytica* MF 372511	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
Carvacrol - FLF								
Carvacrol	0.5	1	0.125	1.125	0.5	1	0.25	0.75
FLF	0.5		1		0.5		0.5	
Citral - FLF								
Citral	0.25	0.75	0.125	1.125	0.5	0.625	0.5	1
FLF	0.5		1		0.125		0.5	
Eugenol - FLF								
Eugenol	0.5	1	0.125	1.125	0.5	0.75	0.25	0.75
FLF	0.5		1		0.25		0.5	
Linalool - FLF								
Linalool	0.25	**0.5***	0.125	0.625	0.5	1	0.5	1
FLF	0.25		0.5		0.5		0.5	
Thymol - FLF								
Thymol	0.5	0.625	0.125	1.125	0.125	1.125	0.5	0.625
FLF	0.125		1		1		0.125	
Carvacrol - OXT								
Carvacrol	0.125	1.125	0.125	1.125	0.125	0.625	0.125	0.625
OXT	1		1		0.5		0.5	
Citral - OXT								
Citral	0.125	1.125	0.125	1.125	0.25	**0.5***	0.25	0.75
OXT	1		1		0.25		0.5	
Eugenol - OXT								
Eugenol	0.125	1.125	0.125	1.125	0.125	0.625	0.125	0.625
OXT	1		1		0.5		0.5	
Linalool - OXT								
Linalool	0.25	**0.5***	0.5	1	0.125	0.625	0.125	0.625
OXT	0.25		0.5		0.5		0.5	
Thymol - OXT								
Thymol	0.5	0.625	0.25	1.25	0.125	0.625	0.125	0.625
OXT	0.125		1		0.5		0.5	

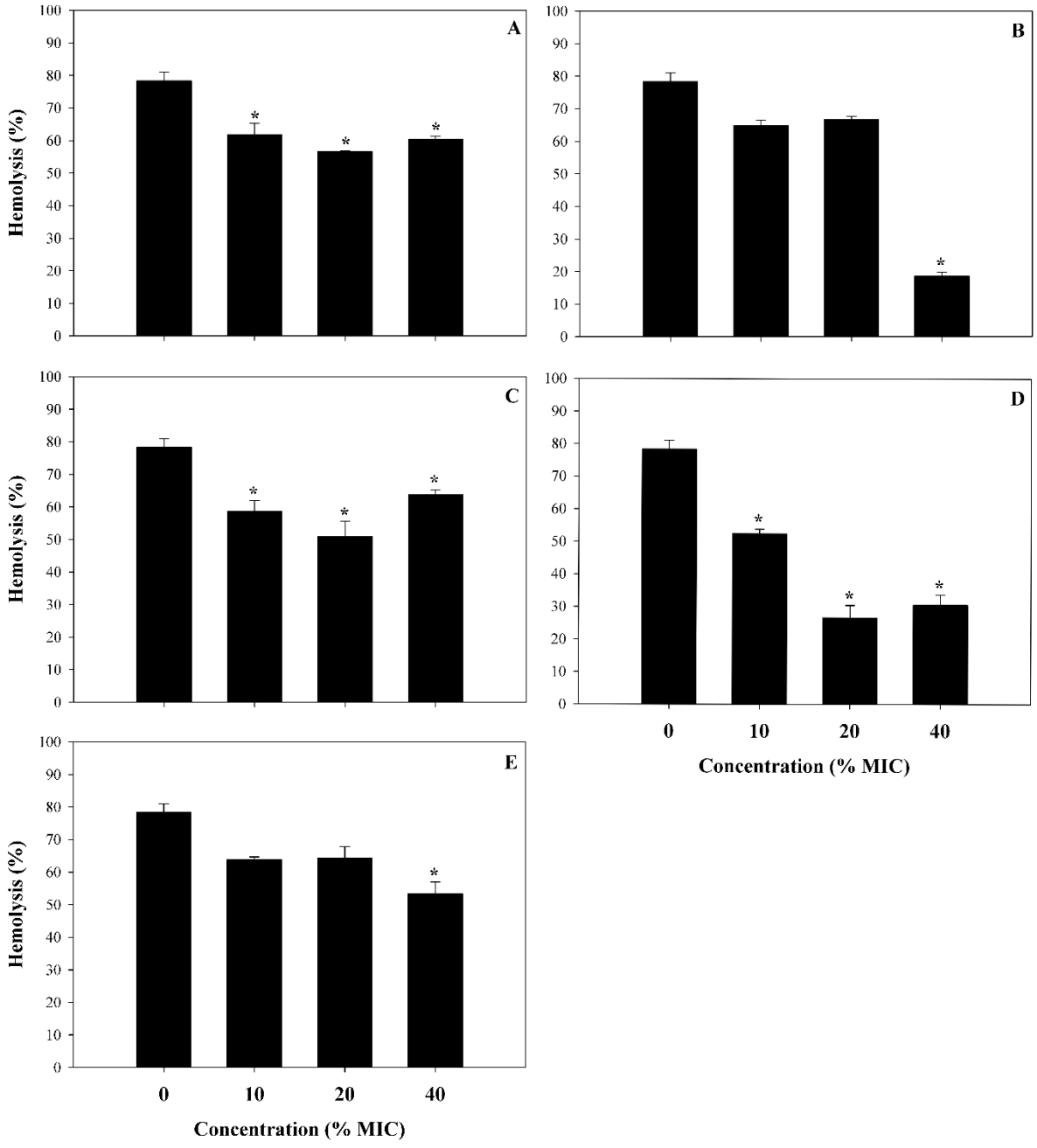
*, asterisk indicates synergic effect.

Table 4 Effects of the phytochemicals carvacrol, citral, eugenol, linalool and thymol, and of the antimicrobials florfenicol and oxytetracycline on the biofilm formation of bacteria pathogenic to fish

	A. hydrophila ATCC 7966							*A. hydrophila* MF 372510							*C. freundii* MF 565839							*R. ornithinolytica* MF 372511						
	Ca	Ci	E	L	T	F	O	Ca	Ci	E	L	T	F	O	Ca	Ci	E	L	T	F	O	Ca	Ci	E	L	T	F	O
MIC x8	0	0	1	1	0	3	0	1	0	1	0	0	3	1	1	0	1	1	1	1	1	1	2	1	0	1	1	1
MIC x4	1	0	1	1	0	3	2	1	1	1	0	2	3	1	1	0	1	1	1	1	1	1	2	1	0	1	1	1
MIC x2	1	1	2	1	0	3	3	1	1	2	0	2	3	3	1	0	1	1	2	3	1	1	2	1	0	1	3	1
MIC	1	1	2	1	0	3	3	2	1	3	0	3	3	3	2	1	2	1	2	3	1	1	2	1	2	1	3	3
MIC /2	2	1	3	1	1	3	3	3	2	3	3	3	3	3	2	1	2	3	2	3	3	2	2	1	2	1	3	3
MIC /4	3	2	3	3	2	3	3	3	3	3	3	3	3	3	2	1	2	3	2	3	3	2	2	1	2	1	3	3
MIC /8	3	2	3	3	2	3	3	3	3	3	3	3	3	3	2	1	2	3	2	3	3	2	2	1	2	1	3	3

MIC, minimum inhibitory concentration; Ca, carvacrol; Ci, citral; E, eugenol; L, linalool; T, thymol; F, florfenicol; O, oxytetracycline; 0, no biofilm production; 1, weak biofilm production; 2, moderate biofilm production; 3, strong biofilm production.

Figure 1 Hemolysis of the *Aeromonas hydrophila* MF 372510 at sub-inhibitory concentrations (10, 20, and 40% MIC) of the phytochemicals carvacrol (A), citral (B), eugenol (C), linalool (D), and thymol (E). *, asterisk represents a significant difference in relation to the control (0%); MIC, minimum inhibitory concentration



4 CONCLUSÕES

- Foram isoladas dos peixes naturalmente infectados cepas de *A. hydrophila*, *A. veronii*, *C. freundii* e *R. ornithinolytica*;

- As espécies bacterianas isoladas são multirresistentes;

- Em relação à MIC e à MBC, carvacrol, timol e eugenol foram os fitoquímicos mais efetivos;

- Em relação ao ensaio de "checkerboard", houve efeito de aditividade na maioria das combinações testadas, sendo que três combinações apresentaram efeito de sinergismo: linalol com florfenicol ou oxitetraciclina frente à *A. hydrophila* e citral com oxitetraciclina frente à *C. freundii*;

- Os fitoquímicos não demonstraram nenhuma interação antagônica com os antimicrobianos convencionais, o que possibilita seu uso combinado, podendo contribuir para a diminuição das concentrações utilizadas dos fármacos convencionais, reduzindo seus resíduos no ambiente aquático;

- Todos os fitoquímicos testados demonstraram capacidade de inibir a hemólise de uma cepa de *A. hydrophila* β-hemolítica quando usados em concentrações mais baixas daquelas necessárias para inibir o crescimento bacteriano;

- Todos os fitoquímicos apresentaram potencial para inibir a formação de biofilmes bacterianos, sendo esta inibição possivelmente maior que a provocada pelos antimicrobianos convencionais testados;

- Os fitoquímicos apresentaram atividade antimicrobiana satisfatória em todos os testes realizados.

REFERÊNCIAS

AHMADIFAR, E.; FALAHATKAR, B.; AKRAMI, R. Effects of dietary thymol-carvacrol on growth performance, hematological parameters and tissue composition of juvenile rainbow trout, *Oncorhynchus mykiss.* **Journal of Applied Ichthyology**: v. 27, n. 4, p. 1057-1060, mai./jul., 2011. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1111/j.1439-0426.2011.01763.x/abstract>. Acesso em: 2 mar. 2018.

ALYAHYA, S. A. et al. Histopathological studies of experimental *Aeromonas hydrophila* infection in blue tilapia, *Oreochromis aureus*. **Saudi Journal of Biological Sciences**: v. 25, n. 1, p. 182-185, jan., 2018. Disponível em: <https://www.sciencedirect.com/science/article/pii/S1319562X17302681>. Acesso em: 27 fev. 2018.

AVETISYAN, A. et al. Chemical composition and some biological activities of the essential oils from basil *Ocimum* different cultivars. **BMC Complementary and Alternative Medicine**: v. 17, n. 60, p. 1-8, jan., 2017. Disponível em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5248495/>. Acesso em: 2 mar. 2018.

BAKER, M. R.; GOBUSH, K. S.; VYNNE, C. H. Review of factors influencing stress hormones in fish and wildlife. **Journal for Nature Conservation**: v. 21, n. 5, p. 309-318, jun./out., 2013. Disponível em: <http://www.sciencedirect.com/science/article/pii/S1617138113000368>. Acesso em: 27 fev. 2018.

BAKKALI, F. et al. Biological effects of essential oils – A review. **Food and Chemical Toxicology**: v. 46, n. 2, p. 446-475, fev., 2008. Disponível em: <https://www.sciencedirect.com/science/article/pii/S0278691507004541>. Acesso em: 1 mar. 2018.

BALDISSEROTTO, B. et al. The effects of ammonia and water hardness on the hormonal, osmoregulatory and metabolic responses of the freshwater silver catfish *Rhamdia quelen*. **Aquatic Toxicology**: v. 152, p. 341-352, abr./jul., 2014. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0166445X14001520>. Acesso em: 27 fev. 2018.

BASER, K. H. C.; KIRIMER, N.; TÜMEN, G. Composition of the essential oil of *Origanum majorana* L. from Turkey. **Journal of Essential Oil Research**: v. 5, n. 5, p. 577-579, nov., 1993. Disponível em: <http://www.tandfonline.com/doi/abs/10.1080/10412905.1993.9698283>. Acesso em: 2 mar. 2018.

BELEM-COSTA, A.; CYRINO, J. E. P. Antibiotic resistence of *Aeromonas hydrophila* isolated from *Piaractus mesopotamicus* (Holmberg, 1887) and *Oreochromis niloticus* (Linnaeus, 1758). **Scientia Agricola**: v. 63, n. 3, p. 281-284, mai./jun., 2006. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0103-90162006000300011>. Acesso em: 27 fev. 2018.

BOTELHO, M. A. et al. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. **Brazilian Journal of Medical and Biological**

Research: Ribeirão Preto, v. 40, n. 3, p. 349-356, mar./jan., 2007. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-879X2007000300010>. Acesso em: 2 mar. 2018.

CABELLO, F. C. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. **Environmental Microbiology**: v. 8, n. 7, p. 1137-1144, abr./mai., 2006. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1111/j.1462-2920.2006.01054.x/abstract;jsessionid=4BD59884EA6103B9ECE6BEDC1E4EDBF3.f03t04>. Acesso em: 27 fev. 2018.

CUNHA, M. A. et al. Anesthesia of silver catfish with eugenol: time of induction, cortisol response and sensory analysis of fillet. **Ciência Rural**: Santa Maria, v. 40, n. 10, p. 2107-2114, jul./out., 2010. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0103-84782010001000009>. Acesso em: 2 mar. 2018.

DONE, H. Y.; VENKATESAN, A. K.; HALDEN, R. U. Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? **The AAPS Journal**: v. 17, n. 3, p. 513-524, mai., 2015. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/25700799>. Acesso em: 28 fev. 2018.

DORMAN, H. J. D.; DEANS, S. G. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. **Journal of Applied Microbiology**: v. 88, n. 2, p. 308-316, set./fev., 2000. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/10736000>. Acesso em: 2 mar. 2018.

DOS SANTOS, A. C. et al. *Aloysia triphylla* essential oil as additive in silver catfish diet: Blood response and resistance against *Aeromonas hydrophila* infection. **Fish and Shellfish Immunology**: v. 62, p. 213-216, mar., 2017. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/28122262>. Acesso em: 2 mar. 2018.

FAO. **The State of World Fisheries and Aquaculture**. Itália: Food and Agriculture Organization of the United Nations, 2016. Disponível em: <http://www.fao.org/3/a-i5555e.pdf>. Acesso em: 17 jun. 2018.

FDA. **Approved Drugs**. Estados Unidos: Food and Drug Administration, 2014.

FIGUEREDO, A. B. et al. Haematological and parasitological assessment of silver catfish *Rhamdia quelen* farmed in Southern Brazil. **Revista Brasileira de Parasitologia Veterinária**: v. 23, n. 2, p. 157-163, abr./jun., 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/25054493>. Acesso em: 26 fev. 2018.

GARCIA, L. S.; ISENBERG, H. D. **Clinical Microbiology Procedures Handbook.** 3. ed. Washington: ASM Press, 2010.

GAUTAM, M.; AGRAWAL, M. Influence of metals on essential oil content and composition of lemongrass (*Cymbopogon citratus* (D.C.) Stapf.) grown under different levels of red mud in sewage sludge amended soil. **Chemosphere**: v. 175, p. 315-322, jun./fev., 2017. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/28235740>. Acesso em: 2 mar. 2018.

GUERRA, I. M. F. et al. *Aeromonas* associated diarrhoeal disease in south Brazil: prevalence, virulence factors and antimicrobial resistance. **Brazilian Journal of Microbiology**: v. 38, n. 4, p. 638-643, out./dez., 2007. Disponível em: <http://www.scielo.br/scielo.php?pid=S1517-83822007000400011&script=sci_arttext&tlng=pt>. Acesso em: 27 fev. 2018.

GYAWALI, R.; IBRAHIM, S. A. Natural products as antimicrobial agents. **Food Control**: v. 46, p. 412-429, dez., 2014. Disponível em: <https://www.sciencedirect.com/science/article/pii/S095671351400320X#bib117>. Acesso em: 28 fev. 2018.

HELDWEIN, C. G. et al. S-(+)-Linalool from *Lippia alba*: sedative and anesthetic for silver catfish (*Rhamdia quelen*). **Veterinary Anaesthesia and Analgesia**: v. 41, n. 6, p. 621-629, mar./nov., 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/24628858>. Acesso em: 2 mar. 2018.

HEMAISWARYA, S.; KRUTHIVENTI, A. K.; DOBLE, M. Synergism between natural products and antibiotics against infectious diseases. **Phytomedicine**: v. 15, n. 2, p. 639-652, ago., 2008. Disponível em: <https://www.sciencedirect.com/science/article/pii/S0944711308001104>. Acesso em: 28 fev. 2018.

JAGODA, S. S. S. S. et al. Genome sequencing and annotation of *Aeromonas veronii* strain Ae52, a multidrug-resistant isolate from septicaemic gold fish (*Carassius auratus*) in Sri Lanka. **Genomics Data**: v. 11, p. 46-48, mar., 2017. Disponível em: <https://www.sciencedirect.com/science/article/pii/S2213596016301556>. Acesso em: 27 fev. 2018.

JØRGENSEN, E. H. et al. Effects of reduced water exchange rate and oxygen saturation on growth and stress indicators of juvenile lumpfish (*Cyclopterus lumpus* L.) in aquaculture. **Aquaculture**: v. 474, p. 26-33, mar./mai., 2017. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0044848617304696>. Acesso em: 27 fev. 2018.

KANKI, M. et al. *Klebsiella pneumoniae* produces no histamine: *Raoultella planticola* and *Raoultella omithinolytica* strains are histamine producers. **Applied and Environmental Microbiology**: v. 68, n. 7, p. 3462–3466, abr./jul., 2002. Disponível em: <http://aem.asm.org/content/68/7/3462>. Acesso em: 27 fev. 2018.

KEENE, J. L. et al. The efficacy of clove oil as an anaesthetic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). **Aquaculture Research**: v. 29, n. 2, p. 89-101, fev., 1998. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2109.1998.00927.x/abstract>. Acesso em: 2 mar. 2018.

KIM, J. M. et al. Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. **Journal of Food Science**: v. 60, n. 6, p. 1364-1368, jul./nov., 1995. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.1995.tb04592.x/abstract>. Acesso em: 2 mar. 2018.

KUBMARAWA, D. et al. Preliminary phytochemical and antimicrobial screening of 50 medicinal plants from Nigeria. **African Journal of Biotechnology**: v. 6, n. 14, p. 1690-1696, jul., 2007. Disponível em: <https://www.ajol.info/index.php/ajb/article/view/57755/46122>. Acesso em: 1 mar. 2018.

LAI, P. K.; ROY, J. Antimicrobial and chemopreventive properties of herbs and spices. **Current Medicinal Chemistry**: v. 11, n. 11, p. 1451-1460, jun., 2004. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/15180577>. Acesso em: 28 fev. 2018.

LAMBERT, R. J. W. et al. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. **Journal of Applied Microbiology**: v. 91, n. 3, p. 453-462, set./dez., 2001. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/11556910>. Acesso em: 2 mar. 2018.

LEEM, H. et al. Antioxidant and anti-inflammatory activities of eugenol and its derivatives from clove (*Eugenia caryophyllata* Thunb.). **Journal of the Korean Society of Food Science and Nutrition**: v. 40, n. 10, p. 1361-1370, out., 2011. Disponível em: <http://agris.fao.org/agris-search/search.do?recordID=KR2012001394>. Acesso em: 2 mar. 2018.

LIOLIOS, C. C. et al. Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and in vitro antimicrobial activity. **Food Chemistry**: v. 112, n. 1, p. 77-83, mai./jan., 2009. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0308814608005980>. Acesso em: 2 mar. 2018.

LU, W. et al. Preparation, characterization, and antimicrobial activity of nanoemulsions incorporating citral essential oil. **Journal of Food and Drug Analysis**: dez./fev., 2017. No prelo. Disponível em: <http://www.sciencedirect.com/science/article/pii/S1021949817300480>. Acesso em: 2 mar. 2018.

MAJOLO, C. et al. Chemical composition of *Lippia* spp. essential oil and antimicrobial activity against *Aeromonas hydrophila.* **Aquaculture Research**: v. 48, n. 5, p. 2380-2387, abr./mai., 2016. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1111/are.13073/abstract>. Acesso em: 2 mar. 2018.

MILLEZI, A. F. et al. Reduction of *Aeromonas hidrophyla* biofilm on stainless stell surface by essential oils. **Brazilian Journal of Microbiology**: v. 44, n. 1, p. 73-80, abr., 2013. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/24159286>. Acesso em: 28 fev. 2018.

MONDELLO, L. et al. Studies on the essential oil-bearing plants of Bangladesh. Part VIII. Composition of some *Ocimum* oils *O. basilicum* L. var. *purpurascens*; *O. sanctum* L. green; *O. sanctum* L. purple; *O. americanum* L., citral type; *O. americanum* L., camphor type. **Flavour and Fragrance Journal**: v. 17, n. 5, p. 335-340, nov./mai., 2002. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1002/ffj.1108/abstract>. Acesso em: 2 mar. 2018.

NCBI. **PubChem Substance Database.** Estados Unidos: National Center for Biotechnology Information, 2018. Disponível em: <https://pubchem.ncbi.nlm.nih.gov/>. Acesso em: 1 mar. 2018.

OLIVEIRA, R. C. O panorama da aquicultura no Brasil: a prática com foco na sustentabilidade. **Revista Intertox de Toxicologia, Risco Ambiental e Sociedade**: v. 2, n. 1, p. 71-89, fev., 2009. Disponível em: <http://www.revistarevinter.com.br/autores/index.php/toxicologia/article/view/18>. Acesso em: 26 fev. 2018.

PÁDUA, S. B. et al. Isolation, characterization and pathology of *Citrobacter freundii* infection in native Brazilian catfish *Pseudoplatystoma*. **Brazilian Journal of Veterinary Pathology**: v. 7, n. 3, p. 151-157, nov., 2014. Disponível em: <http://revistas.bvs-vet.org.br/bjvp/article/view/29816>. Acesso em: 27 fev. 2018.

PEIXE BR. **Anuário PeixeBR da Piscicultura 2018.** Brasil: Associação Brasileira da Piscicultura, 2018. Disponível em: <http://www.peixebr.com.br/anuario-peixebr-2018/>. Acesso em: 17 jun. 2018.

POULOSE, A. J.; CROTEAU, R. Biosynthesis of aromatic monoterpenes. **Archives of Biochemistry and Biophysics**: v. 187, n. 2, p. 307-314, abr., 1978. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/666313>. Acesso em: 2 mar. 2018.

SADDIQ, A. A.; KHAYYAT, S. A. Chemical and antimicrobial studies of monoterpene: citral. **Pesticide Biochemistry and Physiology**: v. 98, n. 1, p. 89-93, jun./set., 2010. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0048357510000829>. Acesso em: 2 mar. 2018.

SAMPAIO, L. F. S. et al. Linalool from rosewood (*Aniba rosaeodora* Ducke) oil inhibits adenylate cyclase in the retina, contributing to understanding its biological activity. **Phytotherapy Research**: v. 26, n. 1, p. 73-77, mai./jan., 2012. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/21544884>. Acesso em: 2 mar. 2018.

SEBRAE. **Aquicultura no Brasil.** Brasília: Serviço Brasileiro de Apoio às Micro e Pequenas Empresas, 2015. 76 p.

SILVA, L. L. et al. *S*-(+)- and *R*-(-)-linalool: a comparison of the *in vitro* anti-*Aeromonas hydrophila* activity and anesthetic properties in fish. **Anais da Academia Brasileira de Ciências**: v. 89, n. 1, p. 203-212, jan./mar., 2017. Disponível em: <http://www.scielo.br/scielo.php?pid=S0001-37652017000100203&script=sci_arttext>. Acesso em: 2 mar. 2018.

SINDAN. **Compêndio de Produtos Veterinários**. Brasil: Sindicato Nacional da Indústria de Produtos para Saúde Animal, 2018. Disponível em: <http://www.cpvs.com.br/cpvs/default.aspx>. Acesso em: 19 jun. 2018.

SOUSA, J. P. et al. Influence of carvacrol and 1,8-cineole on cell viability, membrane integrity, and morphology of *Aeromonas hydrophila* cultivated in a vegetable-based broth.

Journal of Food Protection: v. 78, n. 2, p. 424-429, ago./fev., 2015. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/25710162>. Acesso em: 2 mar. 2018.

SOUZA, C. F. et al. Physiological responses of *Rhamdia quelen* (Siluriformes: Heptapteridae) to anesthesia with essential oils from two different chemotypes of *Lippia alba*. **Neotropical Ichthyology**: v. 15, n. 1, p. 1-10, mar./mar., 2017. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1679-62252017000100203>. Acesso em: 2 mar. 2018.

SUTILI, F. J. et al. The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*). **Veterinary Immunology and Immunopathology**: v. 157, n. 3-4, p. 142-148, fev., 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/24368084>. Acesso em: 28 fev. 2018.

VALE, T. G. et al. Central effects of citral, myrcene and limonene, constituents of essential oil chemotypes from *Lippia alba* (Mill.) N.E. Brown. **Phytomedicine**: v. 9, n. 8, p. 709-714, dez., 2002. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/12587690>. Acesso em: 2 mar. 2018.

VASUDEVAN, R. Biofilms: microbial cities of scientific significance. **Journal of Microbiology & Experimentation**: v. 1, n. 3, p. 1-16, jun., 2014. Disponível em: <http://medcraveonline.com/JMEN/JMEN-01-00014.pdf>. Acesso em: 18 jun. 2018.

WANG, G. et al. Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. **Journal of Clinical Microbiology**: v. 41, n. 3, p. 1048-1054, mar., 2003. Disponível em: <http://jcm.asm.org/content/41/3/1048.full.pdf+html>. Acesso em: 28 fev. 2018.

YANISHLIEVA, N. V. et al. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. **Food Chemistry**: v. 64, n. 1, p. 59-66, nov./jan., 1999. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0308814698000867>. Acesso em: 2 mar. 2018.

ZHANG, Y. et al. Multi-level engineering facilitates the production of phenylpropanoid compounds in tomato. **Nature Communications**: v. 6, p. 1-11, out., 2015. Disponível em: <https://www.nature.com/articles/ncomms9635>. Acesso em: 1 mar. 2018.

ZHENG, Z. I. et al. Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*). **Aquaculture**: v. 292, n. 3-4, p. 214-218, mai./jul., 2009. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0044848609004037>. Acesso em: 2 mar. 2018.