UNIVERSIDADE FEDERAL DE SANTA MARIA

CENTRO DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

Jessyka Arruda da Cunha

EFEITO ANTIBACTERIANO DO ÓLEO ESSENCIAL DE *Origanum majorana* E DE COMPOSTOS ISOLADOS NA FORMA PURA E NANO ENCAPSULADA CONTRA À *Aeromonas hydrophila* EM *Rhamdia quelen*

Santa Maria, RS

2018.

Jessyka Arruda da Cunha

EFEITO ANTIBACTERIANO DO ÓLEO ESSENCIAL DE *Origanum majorana* **E DE COMPOSTOS ISOLADOS NA FORMA PURA E NANO ENCAPSULADA CONTRA À** *Aeromonas hydrophila* **EM** *Rhamdia quelen*

Tese apresentada ao curso de Pós-Graduação em Farmacologia da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do título de **Doutor em Farmacologia.**

Orientador: Prof. Dr. Bernardo Baldisserotto

Santa Maria, RS.

2018

_____

Jessyka Arruda da Cunha

EFEITO ANTIBACTERIANO DO ÓLEO ESSENCIAL DE *Origanum majorana* E DE COMPOSTOS ISOLADOS NA FORMA PURA E NANO ENCAPSULADA CONTRA *Aeromonas hydrophila* EM *Rhamdia quelen*

Tese apresentada ao curso de Pós-Graduação em Farmacologia da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do título de **Doutor em Farmacologia.**
O professor Dr. Maurício Laterça Martins (UFSC) enviou sua avaliação por parecer e a professora Drª Rosa Helena Veras Mourão (UFOPA) participou da avaliação e da decisão por videoconferência.

Aprovado em 07 de dezembro de 2018:

Bernardo Baldisserotto, Dr. (UFSM)

(Presidente/Orientador)

Vânia Lucia Loro, Drª. (UFSM)

Maurício Laterça Martins, Dr. (UFSC)

Ionara Dalcol, Dr. (UFSM)

Rosa Helena Veras Mourão, Drª. (UFOPA)

Santa Maria, RS

2018

RESUMO

EFEITO ANTIBACTERIANO DO ÓLEO ESSENCIAL DE *Origanum majorana* E DE COMPOSTOS ISOLADOS NA FORMA PURA E NANO ENCAPSULADA CONTRA À *Aeromonas hydrophila* EM *Rhamdia quelen*

AUTORA: Jessyka Arruda da Cunha
ORIENTADOR: Bernardo Baldisserotto

O primeiro artigo teve como objetivo fornecer uma visão geral de estudos, *in vivo* e *in vitro*, que abordaram o uso de óleos essenciais (OEs) e seus principais compostos como agentes antimicrobianos em peixes, para identificar os melhores OEs e compostos, além de investigar a viabilidade de aplicação e sugerir possíveis estudos. Até o momento, estudos sugerem que o uso de OEs na prevenção e/ou tratamento de doenças infecciosas em peixes pode ser uma estratégia promissora para reduzir uso de antibióticos convencionais na aquicultura, já que vários EOs reduzem ou evitam os efeitos de infecções bacterianas em peixes. O uso de EOs através de sistemas de distribuição de nanotecnologia, especialmente em experimentos de suplementação dietética, é promissor. Esta forma de aplicação dos OEs permite uma potencialização, visando o efeito desejado dos OEs e também permite a proteção dos constituintes ativos dos EOs. O segundo artigo avaliou a atividade antibacteriana do OE de *Origanum majorana* (EOM) e nanocápsulas deste óleo (NOM) em *Rhamdia quelen*, infectado com *Aeromonas hydrophila*, além de averiguar os possíveis efeitos nos parâmetros metabólicos dos animais. O tratamento foi realizado através de banhos diários de 1h por 5 dias consecutivos. Todos os tratamentos melhoraram a taxa de sobrevivência do peixe infectado, mas sugerimos tratamento de infecções por *A. hydrophila* através de banhos diários com 20 μL L^{-1} EOM ou 5 μL L^{-1} NOM por cinco dias consecutivos. O terceiro artigo teve como objetivo avaliar a resistência de *R. quelen* à infecção por *A. hydrophila* após o tratamento com formas puras e nanoencapsuladas de terpinen-4-ol, timol ou carvacrol e os efeitos desses tratamentos nas respostas metabólicas dos peixes. Após a inoculação com *A. hydrophila*, os peixes foram tratados com banhos diários de 30 min durante seis dias consecutivos. Os peixes tratados com a forma nanoencapsulada dos compostos tiveram uma taxa de sobrevivência elevada, semelhante aos grupos salina e controle negativo. As formas nanoencapsuladas de carvacrol, timol e terpinen-4-ol melhoraram a sobrevivência de jundiás infectados com *A. hydrophila*. Porém, os níveis de glicose e lactato muscular e hepático não são indicados como biomarcadores, pois não apresentaram correlação entre o estado metabólico do peixe e a infecção bacteriana.

Palavras-chave: Carvacrol. Jundiá. Manjerona. Nanotecnologia. Terpinen-4-ol. Timol.

ABSTRACT

ANTIBACTERIAL EFFECT OF ESSENTIAL OIL *Origanum marjorana* AND ISOLATED COMPOUNDS IN THEIR PURE AND NANO ENCAPSULATED FORMS AGAINST *Aeromonas hydrophila* IN *Rhamdia quelen*

AUTHOR: Jessyka Arruda da Cunha
ADVISOR: Bernardo Baldisserotto

The first article aimed to provide a review of the *in vivo* and *in vitro* studies that addressed the use of essential oils (EOs) and their main compounds as antimicrobial agents in fish, to identify the best EOs and compounds, besides investigating feasibility of application and suggest future studies. To date, studies have suggested that the use of EOs in the prevention and/or treatment of infectious diseases in fish may be a promising strategy to reduce the use of conventional antibiotics in aquaculture since several EOs reduce or avoid the effects of bacterial infections in fish. The use of EOs through nanotechnology distribution systems, especially in dietary supplementation experiments, is promising. This form of application of the EOs allows their potentialization, aiming at the desired effect of the EOs and also allows the protection of the active constituents of the EOs. The second article evaluated the antibacterial activity of the EO of *Origanum majorana* (EOM) and nanocapsules of this oil (NOM) in *Rhamdia quelen* infected with *Aeromonas hydrophila*, in addition to investigating the possible effects on metabolic parameters of the animals. The treatment was performed through daily baths of 1h for 5 consecutive days. All treatments improved the survival rate of infected fish, but we suggest treatment of *A. hydrophila* infections by daily baths with 20 μL L^{-1} EOM or 5 μL L^{-1} NOM for five consecutive days. The third article aimed to evaluate the resistance of *R. quelen* to *A. hydrophila* infection after treatment with pure and nanoencapsulated forms of terpinen-4-ol, thymol, or carvacrol and the effects of these treatments on the metabolic responses of fish. After inoculation with *A. hydrophila*, fish were treated with 30 min daily baths for six consecutive days. Fish treated with the nanoencapsulated form of the compounds had a high survival rate, similar to saline and negative control groups. The nanoencapsulated forms of carvacrol, thymol, and terpinen-4-ol improved the survival of silver catfish infected by *A. hydrophila*. However, muscular and hepatic glucose and lactate levels are not indicated as biomarkers, since they did not present a correlation between the metabolic state of the fish and the bacterial infection.

Keywords: Carvacrol. Silver catfish. Marjoram. Nanotechnology. Terpinen-4-ol. Thymol.

LISTA DE ILUSTRAÇÕES

TABELA DE ABREVIATURAS

°C: graus celsius

µl: microlitros

cm: centímetros

DNA: ácido desoxirribonucléico

eV: eletrovolts

g: gramas

GC-MS TIC: espectrofotometria de massas acoplada a ionização de chama

IL: interleucina

Kg: kilograma

L: litros

mg: miligramas

min: minuto

n: número de exemplares

NOM: óleo essencial de *O. majorana* nanoencapsulado

OE: óleo essencial

OEM: óleo essencial de *Origanum majorana*

OEs: óleos essenciais

PGE: prostaglandina E2

rDNA: DNA robossomal

RPM: rotações por minuto

TNF: fator de necrose tumoral

UFC: unidade formadora de colônia

α: alfa

β: beta

γ: gama

SUMÁRIO

1 INTRODUÇÃO

1.1 PISCICULTURA NO BRASIL - *Rhamdia quelen*

O Brasil é um dos países que apresenta grande potencial de produção de pescado, devido ao clima propício, bem como em decorrência da malha hidrográfica, com aproximadamente 12% da água doce disponível no planeta (BRASIL, 2013). Entretanto, alguns fatores que o regime de confinamento proporcionam como a alta densidade dos animais, manejos de cultivo, contaminação da água por produtos tóxicos e falta de saneamento básico podem provocar um estresse crônico nos animais, além de facilitar a disseminação das doenças e dos parasitas (BALDISSEROTTO, 2009; ISHIKAWA et al., 2001).

De maneira geral, esses fatores descritos acima alteram direta e indiretamente a resposta imunológica dos peixes, dificultando a supressão de micro-organismos patogênicos, contribuindo para um aumento da mortalidade, redução na produtividade e perda econômica (KREUTZ et al., 2010). Sendo assim, esses fatores se tornam mais proeminente nas pisciculturas intensivas, pois estes peixes estão muito mais expostos a patologias, principalmente devido ao regime de alimentação, confinamento e densidade populacional, fatores que podem tornar o meio ambiente bastante diferente do natural, embora as doenças também ocorram em peixes de vida livre (CANABARRO et al., 1992).

Dentre as espécies nativas cultivadas no sul do Brasil está o jundiá *Rhamdia quelen* (QUOY & GAIMARD, 1824) (VALLADÃO et al., 2016) (figura 1).

Figura 1 – Jundiá (*Rhamdia quelen*)

Fonte: Arquivo pessoal.

Teleósteo de água doce, membro da família Heptapteridae, é uma das espécies nativas mais comuns de rios e lagoas naturais ou artificiais, tolerando bem os meses frios do inverno, apresentando alta taxa de reprodução e rápido ganho de peso durante os meses mais quentes do ano, mesmo quando criado em tanques artificiais ou misturado com outras espécies de peixes, além de possuir hábito onívoro (GOMES et al., 2000). Devido a estas características, o jundiá tem sido utilizado como um modelo experimental para experimentos de farmacologia aplicada à piscicultura(SALBEGO et al., 2014; SOUZA et al., 2015; SUTILI, et al., 2013, 2015 a,b, 2016; TONI et al., 2014).

Em contrapartida, essa espécie, assim como as demais espécies de teleósteos cultivadas no Brasil, é ameaçada por bactérias pertencentes ao gênero *Aeromonas*. Esse micro-organismo é um dos principais patógenos de peixes e provavelmente a doença bacteriana mais comum em peixes de água doce (BEAZ-HIDALGO E FIGUERAS, 2013; GHATAK, et al., 2016).

1.2 AEROMONAS spp. – *Aeromonas hydrophila*

O gênero *Aeromonas* é formado por bacilos Gram-negativos, oxidase-positivos e aneróbicos facultativos (SUÁREZ E HERRERA, 2012). Apresenta distribuição ubíqua no ambiente, sendo encontrado em vários tipos de produtos animais e vegetais, como peixes, carnes e seus derivados ou qualquer alimento que entre em contato com a água (GHATAK et al., 2016; JANDA E ABBOTT, 1998, 2010; MILLEZI et al*.,* 2013).

Aeromonas hydrophila é mais frequente que as demais espécies de *Aeromonas* no desenvolvimento de doenças nos peixes, além de possuir uma virulência maior (GHATAK et al., 2016). O peixe infectado com essa espécie bacteriana apresenta sinais clínicos que podem variar de lesões de pele à septicemia. Geralmente as lesões de pele apresentam áreas hemorrágicas,

chegando até mesmo a necrose e úlceras que acometem geralmente o tecido muscular (JANDA E ABBOTT, 2010).

Segundo relatos existentes na literatura, a patogênese bacteriana está ligada diretamente aos fatores de virulência. Esses fatores possibilitam a bactéria colonizar e invadir o animal, desencadeando problemas no sistema de defesa, chegando a causar danos no tecido do hospedeiro, podendo provocar hemólise e permitir a invasão das células epiteliais (ALDERMAN E HASTINGS, 1998; VIZZOTTO, 2009). Combater esta doença através do uso de antibióticos não é recomendado desde 2006 pela União Europeia, devido ao risco de criação de cepas resistentes, bioacumulação e poluição ambiental (ENCARNAÇÃO, 2010; HARIKRISHNAN E BALASUNDARAM, 2003; LÜCKSTÄDT, 2006).

Algumas alternativas para minimizar e / ou inibir a ação desses agentes patogênicos nos peixes sem provocar danos ambientais são propostas, como o uso de produtos naturais, vacinas comerciais e estimulação do sistema imune, os quais têm sido particularmente bem sucedidos contra várias doenças bacterianas (CITARASU, 2010; HARIKRISHNAN E BALASUNDARAM, 2003; MILLEZI et al.,2013). As vacinas inativadas estimulam tanto a imunidade inata quanto a humoral, proporcionando mais segurança para o meio ambiente e para os animais, pois minimizam o risco de virulência ao animal (FIGUEREDO E LEAL, 2008; PRIDGEON E KLESIUS, 2010). O uso de fitoterápicos para o tratamento de patologias e demais distúrbios constitui uma importante fonte de pesquisa visando à descoberta de novas substâncias obtidas de plantas com atividades farmacológicas (ALBUQUERQUE E HANAZAKI, 2006; REZENDE E COCCO, 2002).

1.3 ÓLEOS ESSENCIAIS COMO ANTIBACTERIANOS

Entre os produtos naturais empregados em abordagens terapêuticas, os óleos essenciais (OEs), utilizados frequentemente na aromaterapia, são descritos como produtos com grande potencial farmacológico (EDRIS, 2007). Os OEs são compostos naturais, voláteis e complexos extraídos de plantas por

meio de diferentes técnicas. Possuem odor característico e são sintetizados por plantas aromáticas durante o metabolismo secundário (PEREIRA et al., 2008; MACHADO E JUNIOR, 2011).

Pesquisas no ramo da piscicultura têm dado uma atenção especial ao uso de OEs no tratamento de infecção bacteriana desencadeada por *Aeromonas* spp. (DEBBARMA et al., 2012; OLIVEIRA et al., 2009; SUTILI et al.*,* 2015 a,b). Entretanto, apesar de já existirem inúmeras pesquisas *in vivo* com outros EOs sendo utilizados de forma preventiva e/ou curativa e que apresentaram sua eficácia comprovada através da redução ou inibição dos efeitos de infecções bacterianas em peixes, nenhuma pesquisa até o presente momento abordou a utilização do OE de *Origanum majorana* como antibacteriano frente a *A. hydrophila* (CUNHA et al., 2018).

1.4 MANJERONA - *Origanum majorana*

Origanum majorana L. (figura 2), pertence à família Lamiaceae e é conhecida popularmente no Brasil como manjerona. Esta espécie vegetal tem de 15 a 50 cm de altura, possui odor característico, pequenas folhas redondas e esbranquiçadas, flores brancas ou rosa, sementes oblongas, sendo que seu caule e suas folhas contêm taninos, pentoses e minerais (BUCHBAUER, 1993; ERENLER et al., 2015; FONT QUER, 1973).

Figura 2 – *Origanum majorana* L.

Fonte: TROPICOS, ORG, 2016.

Essa planta é comercialmente utilizado como uma especiaria, mas na medicina popular é tradicionalmente utilizado no tratamento da asma, indigestão, dores de cabeça, reumatismo, tonturas, distúrbio gastrointestinal e enxaqueca (ABDEL-MASSIH E ABRAHAM, 2014; MOHAMED E MANSOUR, 2012). O OE de *O. majorana* (OEM) é rico em compostos bioativos de grande interesse para uso em aplicações industriais, como terpinen-4-ol, sabineno, acetato de linalol, γ-terpineno e linalol (FONT QUER, 1973; SELLAMI et al., 2009). Outros compostos como mirceno, α-terpineno, *p*-cimeno, borneol, timol, carvacrol, β-cariofileno, limoneno, α-pineno e β-pineno também podem ser encontrados no OEM (FRATINI et al., 2014; OLIVEIRA et al., 2009; ORHAN et al., 2012; TSERENNADMID et al., 2010). Alguns estudos relatam atividade antibacteriana de *O. majorana* frente às bactérias de importância clínica para humanos, tais como: *Staphylococcus aureus* com concentração inibitória mínima (CIM) de 6.25 a 100 μL mL^{-1}, variando conforme a cepa analisada (MARQUES et al., 2015), inibiu o crescimento de *Salmonella* spp. com halo de inibição de 19 mm, *Bacillus subtilis* com halo de inibição de 25 mm e *Serratia marcencens* com halo de inibição de 20 mm (OMARA et al., 2014). O extrato etanólico obtido por fluido supercrítico de *O. majorana* na concentração de 0,4% apresentou aproximadamente 96% de inibição frente a *Escherichia coli* (VÁGI et al., 2005), já o OE desta espécie vegetal desta espécie vegetal inibiu o crescimento de *Bacillus subtilis* com halo de inibição de 25 mm e *Serratia marcencens* com halo de inibição de 20 mm (TRAJANO et al., 2009).

1.5 TERPINEN-4-OL

Os terpenoides constituem a maior classe encontrada em produtos naturais de plantas, sendo classificados pelo número de carbonos, o qual é resultado do número de moléculas de isopreno (2-metil-1,3-butadieno) presentes em sua estrutura (DUBEY et al., 2003). Nos OEs, os compostos terpênicos mais encontrados são monoterpenos (C10) e sequisterpenos (C15), que têm sido amplamente estudados devido as suas diversas propriedades biológicas apresentadas (DUBEY et al., 2003;VERPOORTE, 2000). Dentre os monoterpenos com ação farmacológica comprovada destaca-se o terpinen-4-ol (figura 3), que pode ser encontrado em diversas espécies vegetais tais como *Alpinia speciosa*, *Alpinia zerumbet*, *Melaleuca alternifolia*, *Origanum majorana*, *Camellia sinensis*, *Myrtus communis*, *Laurus nobilis*, *Croton sonderianus* e *Eucalyptus globulus* (NASCIMENTO et al., 2005).

Figura 3 – Estrutura química do terpinen-4-ol
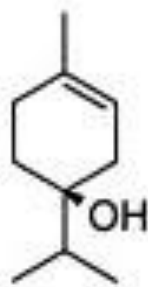
Fonte: FRANK, 1998.

A ação antimicrobiana do composto *in vitro* foi identificada frente as bactérias *Pseudomonas aeruginosa*, *Escherichia coli* e *Staphylococcus aureus* e ao fungo *Candida albicans* (CARSON E RILEY, 2015). Em estudo *in vitro* com o OE de M. alternifolia, que contém 42% de terpinen-4-ol na sua composição, verificou-se a capacidade em suprimir a produção de mediadores pró-inflamatória como fator de necrose tumoral alfa (TNF α), interleucina (IL) -1β, IL-8, IL-10 e prostaglandina E2 (PGE2) (HART et al., 2000). Também se relatou a atividade anestésica e sedativa do terpinen-4-ol nas concentrações de

10 e 3 mg L^{-1}, respectivamente, para exemplares de *R. quelen* (SILVA et al., 2013).

Loughlin et al. (2008) relataram atividade antibacteriana pronunciada do terpinen-4-ol para *S. aureus* resistente à meticilina, além de sua efetividade frente aos isolados de *S. epidermidis*, *S. capitis*, *S. lugdunensis*, *S. hominis*, *S. auriculus*, *S. lentus* e *S. warner* (MONDELLO et al., 2006).

1.6 CARVACROL E TIMOL

Ambos compostos são fenois monoterpenoides biosintetizados em plantas a partir do *γ*-terpineno e *p*-cimeno, sendo estruturalmente muito semelhantes, variando apenas a posição do grupo hidroxila no anel fenólico (BASER E DEMIRCI, 2007; LAMBERT et al,. 2001; NEVEZ, 2009).

O carvacrol é quimicamente denominado 2-metil-5-(1-metiletil)-fenol e possui como fórmula molecular $C_{10}H_{14}O$ (figura 4). Apresenta-se sob forma líquida de coloração amarelo claro, cuja densidade é de 0,975 g mL^{-1}(20°C), possui característica pungente e odor aromático, semelhante ao orégano, e pouca solubilidade em água.

Figura 4 – Estrutura química do carvacrol
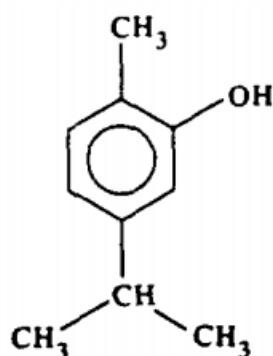
Fonte: AESCHBACH et al., 1994.

O timol foi sintetizado pela primeira vez por Caspar Neumann em 1719 (FRIEDRICH, 2014), e sua nomenclatura química é 5-metil-2-(1-metiletil)-fenol, também possui a fórmula molecular $C_{10}H_{14}O$ (figura 5).

Apresenta-se sob a forma de cristais grandes translúcidos incolores ou brancos, possui odor aromático semelhante ao tomilho, paladar pungente com um leve efeito cáustico sobre os lábios e é uma substância pouco solúvel em água (NEVES, 2009).

Figura 5 – Estrutura química do timol
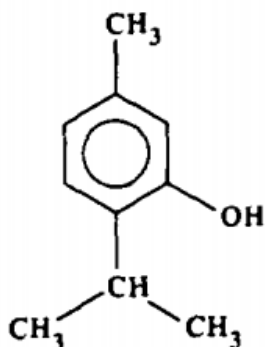
Fonte: AESCHBACH et al.,1994.

Uma gama de propriedades biológicas importantes já foi apontada para ambos compostos, como anti-inflamatório, antioxidante, antibacteriano, antifúngico e anticarcinogênico (ALMEIDA, 2015). Xu et al. (2008) atribuíram a atividade antibacteriana destes compostos as suas capacidades de permeabilizarem e despolarizarem a membrana citoplasmática da *E. coli*. Hammer e heel (2012) observaram por meio da utilização do corante DIOC2 que o carvacrol despolariza a maioria das células de *S. aureus, S. epidermidis* e *Enterococcus* ssp., dependendo do tempo e da concentração. Nostro et al. (2007) demonstraram que o OE de *Origanum vulgare* e seus principais constituintes timol e carvacrol inibiram o crescimento de biofilmes pré-formados de *S. aureus* e *S. epidermidis*.

1.7 NANOTECNOLOGIA E SUA APLICAÇÃO NA AQUICULTURA

A nanotecnologia refere-se ao domínio de partículas e interfaces com dimensões extremamente pequenas, da ordem de 1 a 100 nanômetros. Partículas deste tamanho (nano partículas) diferem das partículas macroscópicas, pois apresentam uma grande área superficial e, geralmente, exibem propriedades mecânicas, ópticas, magnéticas ou químicas distintas, características essas que não seriam apresentadas por essas mesmas substâncias em escala micro ou macro. O estudo dessas propriedades em aplicações tecnológicas forma a base da nanotecnologia, principalmente na ciência de materiais (GRUPO ETC, 2005; QUINA, 2004). Entretanto, por não possuir uma tecnologia específica, a nanotecnologia é uma ciência que apresenta um grupo interdisciplinar, pois abrange áreas na física, química, biologia, engenharias, computação e medicina, permitindo que vários campos façam seu uso (ALVES, 2004).

Dentre estes campos, a indústria farmacêutica mostra-se cada vez mais interessada em estudos e aplicações de nano partículas, pois estas possuem um futuro promissor e sua utilização na vetorização de fármacos está crescendo. A melhora das propriedades farmacológicas e terapêuticas dos fármacos, um controle de liberação sustentado, uma maior seletividade e diminuição de efeitos colaterais são alguns resultados benéficos apresentados devido às características das nanoestruturas (JENA et al., 2013).

Desde o ano 2000, os governos de vários países de primeiro mundo, órgãos institucionais e empresas têm dado grande importância à pesquisa, desenvolvimento e aplicação de nanotecnologias (HUANG et al., 2015). Neste crescimento exponencial estão incluídos nos sistemas coloidais da nanotecnologia as nano emulsões, nano esferas e nano cápsulas, sendo que esses sistemas de nano estruturas têm como objetivo pesquisar novas formas de carrear fármacos para locais específicos com liberação controlada (SCHAFFAZICK et al., 2003). Conceitualmente as nano emulsões consistem em dispersões de óleo estabilizadas por tensoativos (ANTON et al., 2008). As nano cápsulas possuem um núcleo oleoso circundado por um fino invólucro polimérico, e o fármaco encontra-se dissolvido no núcleo, adsorvido ou disperso na parede polimérica (VAUTHIER E BOUCHEMAL, 2009). As nano esferas não apresentam óleo em sua composição, sendo formadas apenas

por uma matriz polimérica, onde o fármaco pode ficar retido ou adsorvido (SCHAFFAZICK et al., 2003).

De acordo com Rather et al. (2011), a indústria de pesca e da aquicultura pode ser revolucionada pelo uso da nanotecnologia como uma nova ferramenta na minimização da rápida proliferação de doenças, reforçando a capacidade dos peixes para absorver drogas como hormônios, vacinas e nutrientes. Além destes benefícios, as nanotecnologias ainda podem ter ampla aplicação na indústria da pesca no que se refere ao tratamento de águas, esterilização de aquários, ração para os animais e controle de doenças aquáticas (HUANG et al., 2015). Embora pesquisas ainda sejam necessárias para aumentar o potencial de uso da nanotecnologia na aquicultura, existem inúmeros vislumbres da futura aplicação desta tecnologia em gestão de saúde para os peixes, tratamento de água e reprodução de animais (RATHER et al., 2011).

2 JUSTIFICATIVA

O aumento de perdas na piscicultura desencadeada pela incidência de infecções por bactérias, juntamente com a resistência que estes patogenos têm desenvolvido aos antimicrobianos, têm levado a uma busca constante por alternativas terapêuticas eficazes que possam oferecer melhores opções de tratamento, principalmente, seguros para o peixe, produtor, consumidor e meio ambiente. Entre as alterativas terapêuticas existentes, podemos dar enfoque ao estudo dos OEs nas formas pura e nanoencapsulada como antibacterianos para peixes. Dentre a gama de OEs disponíveis para testes, escolhemos o OE de *Origanum majorana* pelo fato do mesmo já ter atividade antibacteriana descrita frente a outras bactérias como *S. aureus*, *E. coli*, *Salmonella* spp., *Bacillus subtilis* e *Serratia marcencens* (MARQUES et al., 2015; OMARA et al., 2014; TRAJANO et al., 2009; VÁGI et al., 2005). Os compostos timol, carvacrol e terpinen-4-ol foram escolhidos por fazerem parte da composição química deste OE (FONT QUER, 1973; FRATINI et al., 2014; OLIVEIRA et al., 2009; ORHAN et al., 2012; SELLAMI et al., 2009; TSERENNADMID et al., 2010). Esses estudos são importantes a fim de determinar a viabilidade

dessas substâncias como antimicrobianos eficazes. Além disso, será possível averiguar se o efeito antibacteriano desse OE é devido ao sinergismo dos compostos ou a um ou mais dos compostos principais deste OE e/ou OE nanoencapsulado.

3 OBJETIVOS

3.1 OBJETIVO GERAL

O presente trabalho tem como objetivo utilizar a aplicação do óleo essencial e da nanotecnologia contra à *Aeromonas hydrophila* em *Rhamdia quelen*.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito proveniente da inoculação da bactéria *A. hydrophila* pelo contato com o óleo essencial de *O. majorana* nas formas pura e nano encapsulada

- Analisar o efeito antibacteriano das substâncias isoladas (terpinen-4-ol, carvacrol e timol) nas formas pura e nano encapsulada frente a *Aeromonas hydrophila* através de banhos diários

- Averiguar os efeitos dessas substâncias e/ou bactéria sobre os parâmetros hematológicos e nos níveis de glicose e lactato no músculo e no fígado dos peixes.

4 DESENVOLVIMENTO

4.1 ARTIGO 1

The effects of essential oils and their major compounds on fish bacterial pathogens – a review.

Aceito pelo periodico "Journal of Applied Microbiology", sendo assim esse artigo encontra-se nas normas do mesmo.

Cunha, J.A., Heinzmann, B.M. and Baldisserotto, B. (2018) The effects of essential oils and their major compounds on fish bacterial pathogens-a review.J Appl Microbiol 125,328–344. https://doi.org/10.1111/jam.13911.

THE EFFECTS OF ESSENTIAL OILS AND THEIR MAJOR COMPOUNDS ON FISH BACTERIAL PATHOGENS- A REVIEW

Jessyka A. da Cunha[1], Berta M. Heinzmann[1,2], Bernardo Baldisserotto[1,3]∗

[1]Graduate Program in Pharmacology, Universidade Federal de Santa Maria/ UFSM. 97105-900. Santa Maria. RS. Brazil;

[2]Department of Industrial Pharmacy, UFSM, Santa Maria, RS, Brazil;

[3]Department of Physiology and Pharmacology, UFSM, Santa Maria, RS, Brazil.

Running headline: Review of natural antibacterials.

*Corresponding author: bbaldisserotto@hotmail.com, Phone 55 55 3220-9382; Fax

55 55 3220-8142.

Summary

The increased resistance of fish pathogens to conventional treatments has lead researchers to investigate the antibacterial properties of natural resources, such as essential oils (EOs) of plants, in an effort to find products that are less harmful to the environment. The objective of this review is to provide an overview of the studies, *in vivo* and *in vitro*, that addressed the use of EOs and their major compounds as antimicrobial agents in fish, to identify the best EOs and compounds to investigate considering feasibility of application and suggest possible future studies. To date, studies suggest that the use of EOs in the prevention and/or treatment of infectious diseases in fish may be a promising strategy to reduce the use of conventional antibiotics in aquaculture, since several EOs effectively reduce or avoid the effects of bacterial infections in fish. The use of EOs through nanotechnology delivery systems, especially in dietary supplementation experiments, is promising. This form of application of the EOs allows a potentiation and targeting of the desired effect of the EOs, and also allows the protection of EOs active constituents against enzymatic hydrolysis, deserving further study.

Keywords: alternative treatment; antibacterial; fish-farming; isolated compounds; natural compounds.

Antimicrobial alternatives for fish

According to the Food and Agriculture Organization of the United Nations (FAO), aquaculture is among the fastest growing production sectors and in 2014 world aquaculture produced 49.86 million tons of fish (FAO 2016). Fish farms can

sometimes have stressful conditions, which favor the spread of bacterial and fungal infections (Naylor *et al.* 2000) as well as viral and parasitic diseases (Walker and Winton 2010; Pantoja *et al*. 2012). Immune systems may not be fully capable of avoiding and eliminating a bacterial infection efficiently (Barton and Iwama 1991; Finlay and McFadden 2006). In order to increase fish production and reduce mortality, farmers usually administer antibiotics in the feed or water to treat and prevent bacterial diseases (Markestad and Grave 1997; Cabello 2006). The indiscriminate use of antibiotics is a hazard to the ecosystem because it can lead to the development of bacterial resistance to these drugs (Rhodes *et al.* 2000; Cabello 2006; Acar *et al*. 2009).

Some international organizations such as the World Health Organization (WHO) and FAO have presented some rules to restrict the use of antibiotics in aquaculture to minimize the impact of the indiscriminate use of antimicrobials on human and animal health as well as the appearance and the dissemination of resistant pathogens by the misuse of antibiotics (WHO 2007; FAO 2012). According to FAO (2012), the most commonly used antibiotics worldwide are sulfonamides, oxytetracycline, sarafloxacin, erythromycin, and florfenicol. In Brazil, only florfenicol is allowed for aquaculture practices, and florfenicol, oxytetracycline and sulfadimetoxine + ormetropim are allowed in the USA. This variation in the permission and regulation of the use of each antibiotic depends on the legislation of each country or each legal agreement determined by the country in which the use is made (WHO 2007; FAO 2012).

Consequently, it is important to develop and utilize alternative therapies to treat bacterial infections in fish. Essential oils (EOs) are one of the alternatives being

used as antibiotics and fungicides (Romero *et al*. 2012). EOs are liquid lipophilic mixtures that contain the substances responsible for the aroma of plants and are produced as secondary metabolites (Bakkali *et al.* 2008). It is believed that the primary role of these EOs in plants is to attract pollinators and to avoid pathogens, since they have antibacterial, antifungal, antiviral, and insecticidal effects. The mechanisms of action of EOs depend on their chemical composition (Nazzaro *et al.* 2013), and some plant species may also have different chemotypes, characterized by the major compounds of different EOs, which would change the composition of the EO and its properties (Deering *et al*. 2017). Many EOs contain phenolic compounds that are responsible for their antimicrobial effects (Cosentino *et al.* 1999). The final effect of EOs against a pathogen may result from the synergy of distinct oil constituents or their major components (Sutili *et al.* 2016a). Consequently, the effects of an EO may change with the chemotype; therefore, studies must provide the chemical composition of the EOs analyzed.

The emergence of bacterial resistance to antibiotics in aquaculture and the possible effects of antibiotics on natural microbial communities (Grenni *et al*. in press) makes new alternatives and studies of this subject of great importance. There are reviews regarding the antibacterial properties and applicability of EOs to increase food preservation (Burt 2004; Gómez-Sánchez and López-Malo 2009) and on the effect of EOs as antibacterials against common human pathogens (Reichling *et al*. 2009; Nazzaro *et al*. 2013). Reviews concerning the use of EOs in aquaculture for the control and/or treatment of diseases have already been published (Harikrishnan and Balasundaram 2005; Murthy and Kiran 2013; Bulfon *et al*. 2015) but with a different focus from the present review. Previous reviews presented the advantages of herbal

medicine based on the lack of side effects, being a biodegradable alternative, being available locally, and showing promise in replacing antibiotics to treat diseases. The objective of this review is to provide an overview of the *in vivo* and *in vitro* studies that addressed the use of EOs and their major compounds as antimicrobial agents in fish to identify the best EOs and compounds to investigate considering feasibility of application and suggest possible future studies.

Mechanisms of the antibacterial effects of EOs

The mechanisms through which different EOs are able to damage bacteria depend on their composition. Usually antimicrobial activity is derived not from only a single mechanism of action, but from a cascade of reactions involving the entire bacterial cell because EOs have several chemical structures in their composition and, consequently, several functional groups (Burt 2004; Nazzaro *et al.* 2013).

Overall, gram-positive bacteria are more susceptible to the effects of EOs than gram-negative bacteria (Trombetta *et al.* 2005), due to significant structural differences in the cell wall of these two groups of bacteria. Most of the cell wall of gram-positive bacteria is composed of peptidoglycan, which allows hydrophobic molecules to easily penetrate the cell and act both on the cell wall and within the cytoplasm (Nazzaro *et al.* 2013).

In addition to the peptidoglycan layer, the cell wall of gram-negative bacteria has an outer membrane, which is composed of a double layer of phospholipids linked to the peptidoglycan layer by lipopolysaccharides. Some hydrophobic molecules are able to penetrate the cell, but only through porin proteins that form water-filled

channels distributed throughout the cell wall. Therefore, gram-negative bacteria are more resistant to hydrophobic antibiotics (Nikaido 1994; Nazzaro *et al.* 2013).

EOs are able to affect both the cytoplasm and membrane(s) of bacteria. The mechanisms of action of EOs can include cell wall degradation, damage to the cytoplasmic membrane and membrane proteins, and reduced proton motive force and ATP synthesis. The lipophilic character of EO compounds allows them to penetrate the cell membrane and remain between the phospholipids. In addition, EOs can affect the synthesis of membrane lipids. Both effects can change membrane structure and, consequently, its permeability. EOs can also act on quorum sensing systems (i.e., the bacterial pheromones), which are important to coordinate bacterium-bacterium interactions to regulate virulence factor expression, biofilm formation, sporulation, and mating (Nazzaro *et al.* 2013; Bouyahya *et al*. in press).

Some studies indicate that the mechanism of action of OEs is dependent on their major functional groups. EOs containing mainly terpenes (*p*-cymene, limonene, terpinene, sabinene, and pinenes) and some oxygenated chemical structures (borneol, camphor, 1,8 cineole, *α*-pinene, camphone, verbenonone, and bornyl acetate) have weak or non-existent antibacterial activity, showing more pronounced antimicrobial activity against gram-positive bacteria. The antibacterial effect depends on their final concentration in the solution: at low concentrations, they interfere with enzymes involved in the production of energy, and they are able to cause protein denaturation at higher concentrations (Tiwari *et al*. 2009; Nazzaro *et al.* 2013). Terpenoids (thymol, carvacrol, linalool, menthol, geraniol, linalyl acetate, citronellal, and piperitone) have antibacterial activity mediated by the functional group acting on the outer membrane of the bacterium, thus altering the permeability and/or fluidity of the

membrane as well as affecting membrane proteins and periplasmic enzymes. Phenylpropenoids (eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde) have antimicrobial activity conferred by free hydroxyl groups, and their mechanism of action depends on the type and number of substitutions in the aromatic ring of their structure. Usually the effect of these functional groups is on the membrane, ion transport, ATP production, and alteration of fatty acid and lipid profiles of bacteria; in addition to direct action on some bacterial enzymes such as carboxylase, protease, ATPase, and amylase as well as bacterial growth (Thoroski *et al*. 1989; Wendakoon and Sakaguchi 1995; Nazzaro *et al.* 2013).

In vitro experiments with isolated compounds demonstrated that eugenol exhibits higher antibacterial activity against Gram-negative bacteria (Hyldgaard *et al*. 2012), and 1,8 cineole inhibits quorum sensing (Bouyahya *et al*. in press). *β*-pinene, linalool, cinnamaldehyde, geraniol, *α*-terpinene, *β*-citronellol, and estragole have deleterious effects on the structure and function of the microbial membrane and cell wall (Andrade-Ochoa *et al*. 2015). Linalyl acetate disturbs the lipid fraction, creating leakage of the intracellular bacterial material (Trobetta *et al*. 2005). Citral alters the intracellular pH, membrane integrity, and potential and intracellular ATP concentration (Shi *et al*. 2016) (Figure 1).

In vitro assessment of EOs properties against fish bacterial pathogens

It is important to perform *in vitro* assays to determine if an EO is suitable for *in vivo* antimicrobial tests. According to Ríos and Recio (2005), for an EO to be considered active, it has to kill or inhibit bacterial growth below the concentration of 100 μg ml^{-1}. However, Aligiannis *et al*. (2001) proposed a different classification where a minimum inhibitory concentration (MIC) of 500 μg ml^{-1} or less corresponds

to strong inhibition, moderate inhibition is a MIC between 600 and 1500 μg ml^{-1}, and weak inhibition corresponds to a MIC above 1600 μg ml^{-1}.

According to the MIC test and classification of Ríos and Recio (2005), there are several EOs that present strong inhibition against pathogenic bacteria of fish. Among all EOs tested *in vitro*, the EO of *Syzygium aromaticum* (clove oil) has one of the most promising results, being able to inhibit bacterial growth with a MIC as low as 0.015 μg ml^{-1}. This antibacterial activity evaluated through MIC determined on 96 well microplates with U bottom wells has been shown to be effective against a variety of gram-positive and gram-negative bacteria, including the major pathogens of aquaculture: *Streptococcus agalactiae* (Zhang *et al*. 2013), *Flavobacterium columnare* (Sebastião *et al*. 2013), *Aeromonas hydrophila* (Griffin *et al*. 2013), *Edwardsiella tarda*, and *Edwardsiella ictaluri* (Park *et al*. 2012a; Hawke *et al*. 2013). Other EOs are also effective antibacterial agents against major aquaculture pathogens, such as the EOs of *Zataria multiflora* and *Rosmarinus officinalis*, which decreased the hemolytic activity and down-regulated the transcription of sagA, a streptolysin S gene related to the secretion of virulence factors in *Streptococcus iniae* (Soltani *et al*. 2014). The EO of *Lippia sidoides* was effective against *Aer. hydrophila in vitro* (Majolo *et al*. 2017), and the EO of *Lippia nobilis* against several gram-positive and gram-negative bacterial species isolated from fish and shellfish products (Snuossi *et al*. 2016) (Table 1). The EO of *Melaleuca alternifolia* acts on the membrane of *Pseud. aeruginosa*, causing bilayer expansion, directly interfering with membrane-integrated enzymes and damaging the membrane. This damage results in increased membrane fluidity and release of intracellular components (Cox and Markham 2007).

The diffusion disc test is a qualitative but non-quantitative test. Inhibition is classified as sensitive, when the diameter of the zone of inhibition is at least 3 mm smaller than the diameter found for the positive control; moderately sensitive, when the halo is greater than 2 mm but smaller than the positive control; or resistant, when the diameter of the halo is equal to or less than 2 mm (Karaman *et al.* 2003; Springfield *et al*. 2003). The EOs of *Lavandulae romanae* and *R. officinalis* (chemotype 1,8 cineole) and the nanoemulsion of the EO of *Azadirachta indica* showed a greater inhibition halo against the tested bacteria than the other EOs studied (Table 2).

In vivo assays with EOs as antibacterial agents in fish

The most common method used for the administration of antimicrobials in aquaculture is via the water in which the animals are kept or as a dietary supplement. The first system has the advantage of reaching a large number of animals at the same time; however, it has high costs as it requires a large amount of antimicrobial when applied in tanks, and a known volume of water is required. Farmers can treat the infections by bath to use smaller amounts of water and minimize these disadvantages (Park *et al*. 2012b). Dietary supplementation with EOs to feed fish has increased in recent years, but all experiments were with the use of the dietary supplementation as preventive (Sutili *et al*. 2017). The great advantage of this method is the reduction in waste when compared to application in the water. However, one of the limiting factors of this application may be the need for active feeding of the animals, because infected fish may not feed as well as healthy ones. Further limits are the effectiveness of EOs inclusion in the aquafeed (appropriate technology to be assessed) and the fact that this incorporation can sometimes negatively affect the palatability of feed.

Anyway, a prophylactic medication ration is advised rather than therapeutic use. Other methods such as gavage, injection, and topical application are efficient at the laboratory level; however, in fish farms, it becomes unfeasible due to the labor force necessary and stress on the animals (Park *et al*. 2012b). Another problem is the stability of the EOs through diet preparation, storage, and digestion, because they may lose their biological effects (Sutili *et al*. 2017).

The EOs tested that reduced mortality of infected fish (i.e., used with therapy purpose) through baths are those from *Ocimum americanum* (both chemotypes with R-(-)-β-linalool and 1,8 cineole as major compounds), *Hesperozygis ringens* (Sutili *et al*. 2015a), *L. alba* (chemotype S-(+)-linalool) (Sutili *et al*. 2015b) and *M. alternifolia* (Souza *et al*. 2017a). It is noteworthy that in spite of eugenol being efficient, the EO of *Ocimum gratissimum*, which contains approximately 90% eugenol in its composition, is not as effective as eugenol in bath treatments (Table 3). This indicates that effects of an EO may not be related to its major compound, but rather due to a combination of several of its components.

Most EOs that were evaluated to treat bacterial infections using baths showed effect at concentrations below the MIC observed *in vitro*, even those that have a higher MIC than that recommended by Ríos and Recio (2005) (Table 3, Figure 2A). An analysis of the MIC and the minimum effective bath concentrations or dietary supplementation doses of EOs found in the literature demonstrated that there is no correlation between them (Table 4, Figure 2). This demonstrates that determination of MIC *in vitro* is not a good methodology to predict the *in vivo* effect of EOs.

Previously published studies on this subject indicate that increased resistance to bacterial infections provided by baths with EOs is due not only to bactericidal

effects but also increased extracellular superoxide anion production by head-kidney macrophages and/or blood leucocytes, inhibition of bacterial hemolytic activity (Sutili *et al.* 2015, 2016b), reduction or elimination of biofilm formation (Millezi *et al.* 2013), and altered quorum sensing communication (Olivero-Verbel *et al.* 2014). Preventive baths with the EO of *M. alternifolia* induced a potent anti-inflammatory effect mediated by the adenosinergic pathway and improved the innate immune responses through modulation of the cytokine response during *Aer. hydrophila* infection in *Rhamdia quelen*. This EO also prevents oxidative damage in liver proteins and lipids, as well as maintaining the purinergic system (Baldissera *et al*. 2017b, 2017c). The use of the EOs of *H. ringens* and *O. americanum* in preventive baths increased the complement system activity compared to control, but there was no significant difference in survival of silver catfish challenged with *Aer. hydrophila* (Sutili *et al*. 2015a). Future studies using this methodology with other EOs are warranted, since the antibacterial effects of EOs vary according to composition.

The EOs tested so far that are effective at increasing fish resistance against bacterial infections by dietary supplementation have limonene, thymol, carvacrol, citral, cinnamaldehyde, and 1,8 cineole as major compounds (Table 4). Dietary supplementation with carvacrol (Volpatti *et al.* 2013) and the combination thymol + carvacrol increased resistance to bacterial infection in fish (Zheng *et al*. 2009). Recent work indicates that dietary supplementation with EOs must be carried out several days before infection to improve immune status, enhance resistance, reduce bacterial effects, and prevent outbreaks (Awad and Awaad 2017; Sutili *et al*. 2017).

Nanotechnology and EO interactions in aquaculture

The use of nanotechnology in medicine and veterinary services has increased in recent years. Because the active constituents of EOs can act only on the site of interest, nanotechnology can increase the permanence in the bloodstream, protect the substance against enzymatic hydrolysis (Nair *et al.* 2016), and even enable the transport of the active substances through the blood-brain barrier (Baldissera *et al*. 2017a). Nanotechnology systems include nanoemulsions, which consist of oil dispersions stabilized by surfactants (Anton *et al*. 2008); nanocapsules, which have an oily nucleus surrounded by a thin polymer envelope with the drug dissolved in the core, adsorbed or dispersed in the polymer wall (Vauthier and Bouchemal 2009); and nanospheres, which do not have oil in their composition, formed by a polymer matrix where the drug can be retained or adsorbed (Schaffazick *et al*. 2003).

The application of the EO of *M. alternifolia* through baths had antibacterial activity against *Pseud. aeruginosa* and this effect was improved when this EO was nanoencapsulated (Souza *et al.* 2017a,b). The nanoemulsion of the EO of *Azadirachta indica* promoted 90% survival and avoided the appearance of ulcers typical of *Aer. salmonicida* infection in *Clarias batrachus* (Thomas *et al.* 2013). The nanoemulsion of the EO of *Citrus aurantifolia* reduced histological changes in the liver, skin, and gills of *Oreochromis mossambicus* infected with *Pseud. aeruginosa* (Thomas *et al.* 2014).

Legislation regarding the use of essential oils in aquaculture

Some EOs have achieved the generally recognized as safe (GRAS) label to be used as food additives in the United States, but no clear information existed in the

European legislation (Carocho *et al.* 2015). The Brazilian legislation (interministerial normative instruction N° 28) establishes some standards for organic aquaculture production in the country and includes EOs in the list of permissible substances to be used in the control of pests and diseases (MAPA, 2011).

Concluding remarks

To date, studies suggest that the use of EOs in the prevention and/or treatment of infectious diseases in fish may be a promising strategy to reduce the use of conventional antibiotics in aquaculture, since several EOs are effective to reduce or avoid the effects of bacterial infections in fish. Some studies used several baths to treat bacterial infections; however, this procedure may not be practical. Thus, new studies with one or two baths must be performed. Dietary supplementation with EOs also seems a good alternative to prevent disease outbreaks. The use of EOs through nanotechnology, mainly in dietary supplementation experiments, is promising and deserves further study.

Conflict of Interest

No conflict to declare.

References

Acar, J., Davies, J. and Buckley, M. (2009) *Antibiotic Resistance : An ecological perspective on an old problem*. US American Academy of Microbiology No. 1-32. Washington DC: Government Printing Office

Acar, U., Kesbic, O.S., Yilmaz, S., Gultepe, N. and Turker, A. (2015) Evaluation of the effects of essential oil extracted fromsweet orange peel (*Citrus sinensis*) on growth rate of tilapia (*Oreochromis mossambicus*) and possible disease resistance against *Streptococcus iniae. Aquaculture* **437**, 282-286.

Aligiannis, N., Kalpoutzakis, E., Mitaku, S. and Chinou, I.B. (2001) Composition and antimicrobial activity of the essential oil of two Origanum species. *J Agric Food Chem* **49**, 4168–4170.

Andrade-Ochoa, S., Nevárez-Moorillón, G.V., Sánchez-Torres, L.E., Villanueva-García, M., Sánchez-Ramírez, B.E., Rodríguez-Valdez, L.M. and Rivera-Chavira, B.E. (2015) Quantitative structure-activity relationship of molecules constituent of different essential oils with antimycobacterial activity against *Mycobacterium tuberculosis* and *Mycobacterium bovis. BMC Complement Altern Med* **15**, 2-11.

Anton, N., Benoit, J. P. and Saulnier, P. (2008) Design and production of nanoparticles formulated from nano-emulsion templates – A review. *J Control Release* **128**, 185–199.

Awad, E. and Awaad, A. (2017) Role of medicinal plants on growth performance and immune status in fish. *Fish Shellfish Immunol* **67**, 40-54.

Baba, E., Acar, U., Ontas, C., Kesbiç, O.S. and Ylmaz, S. (2016) Evaluation of *Citrus limon* peels essential oil on growth performance, immune response of Mozambique

tilapia *Oreochromis mossambicus* challenged with *Edwardsiella tarda*. *Aquaculture* **465**, 13-18.

Baba, E., Acar, U., Yılmaz, S., Ontas, C. and Kesbic, O.S. (2017) Pre-challenge and post-challenge haematoimmunological changes in *Oreochromis niloticus* (Linnaeus, 1758) fed argan oil against *Lactococcus garvieae. Aquacult Res* **48**, 4563–4572.

Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. (2008) Biological effects of essential oils - A review. *Food Chem Toxicol* **46**, 446-475.

Baldissera, M.D., Souza, C.F., Boligon, A.A., Grando, T.H., De Sá, M.F., Da Silva, A. S., Stefani, L.M., Baldisserotto, B. and Monteiro, S.G. (2017a) Solving the challenge of the blood–brain barrier to treat infections caused by *Trypanosoma evansi*: evaluation of nerolidol-loaded nanospheres in mice. *Parasitology* **144**, 1543–1550.

Baldissera, M.D., Souza, C.F., Júnior, G.B., de Vargas, A.C., Boligon, A.A., de Campos, M.M.A., Stefani, L.M. and Baldisserotto, B. (2017b) *Melaleuca alternifolia* essential oil enhances the non-specific immune system and prevents oxidative damage in *Rhamdia quelen* experimentally infected by *Aeromonas hydrophila*: effects on cholinergic and purinergic systems in liver tissue. *Fish Shellfish Immunol* **61**, 1–8.

Baldissera, M.D., Souza, C.F., Doleski, P.H., de Vargas, A.C., Duarte, M.M.M.F., Duarte, T., Boligon, A.A., Leal, D.B.R. and Bernardo Baldisserotto (2017c) *Melaleuca alternifolia* essential oil prevents alterations to purinergic enzymes and ameliorates the innate immune response in silver catfish infected with *Aeromonas hydrophila. Microb Pathog* **109**, 61-66.

Bandeira Junior, G., Pês, T.S., Saccol, E.M.H., Sutili, F.J., Rossi, W., Murari, A.L., Heinzmann, B.M., Pavanato, M.A., Vargas, A.C., Silva, L.L. and Baldisserotto, B. (2017). Potential uses of *Ocimum gratissimum* and *Hesperozygis ringens* essential oils in aquaculture. *Ind Crops Prod* **97**,484 -491.

Barton, B. A. and Iwama, G. K. (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Ann Rev Fish Dis* **1**, 3–26.

Bouyahya, A., Bakri, Y., Et-Touys, A., Talbaoui, A., Khouchlaa, A., Charfi, S., Abrini, S. and Dakka, N. (2017) Résistance aux antibiotiques et mécanismes d'action des huiles essentielles contre les bactéries. Phytothérapie **14**, 1-11.

Brum, A., Pereira, S.A., Owatari, M.S., Chagas, E.C., Chaves, F.C.M., Mouriño, J.L.P. and Martins, M.L. (2017) Effect to of dietary essential oils of clove basil and ginger on Nila tilapia (*Oreochromis niloticus*) following challenge with *Streptococcus agalactiae.* Aquaculture **468**, 235-243.

Bulfon, C., Volpatti, D. and Galeotti, M. (2015) Current research on the use of plant-derived products in farmed fish. *Aquacult Res* **46**, 513–551.

Burt, S. (2004) Essential oils: their antibacterial properties and potential applications in foods—A review. *Int J Food Microbiol* **94**, 223–253.

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

Chakraborty, S.B. and Hancz, C. (2011). Application of phytochemicals as

immunostimulant, antipathogenic and antistress agents in finfish culture. Reviews in Aquaculture **3**, 103–119.

Carocho, M., Morales, P. and Ferreira, I.C.F.R. (2015). Natural food additives: Quo vadis? Trends in Food Science & Technology **45**, 284-295.

Cosentino, S., Tuberoso, C.I., Pisano, B., Satta, M., Mascia, V., Arzedi, E. and Palmas, F. (1999) *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Lett Appl Microbiol* **29**, 130–135.

Cox, S.D. and Markham, J.L. (2007) Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. *J Appl Microbiol* **103**, 930–936.

Debbarma, J., Kishore, P., Nayak, B.B., Kannuchamy, N. and Gudipati, N. (2012) Antibacterial activity of ginger, eucalyptus and sweet orange peel essential oils on fish-borne bacteria. *J Food Process Preserv* **37**, 1022–1030.

Deering, K., Nethery, W., Averill, C. and Esposito, E. (2017) Effects of *Thyme* essential oil chemotypes on breast and cervical cancer cell lineages. *Ann Pharmacol Pharmacother* **2**, 1-3.

Diler, O., Gormez, O., Diler, I. and Metin, S. (2016) Effect of oregano (*Origanum onites* L.) essential oil on growth, lysozyme and antioxidant activity and resistance against *Lactococcus garvieae* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquac Nut* **23**, 884-851.

Volpatti, D., Chiara, B., Francesca, T. and Marco, G. (2013) Growth parameters, innate immune response and resistance to *Listonella* (Vibrio) *anguillarum* of *Dicentrarchus labrax* fed carvacrol supplemented diets. *Aquac Research* **45**, 31–44.

Dos Santos, A.C., Sutili, F.J., Heinzmann, B.M., Cunha, M.A., Brusque, I.C.M., Baldisserotto, B. and Zeppenfeld, C.C. (2017) *Aloysia triphylla* essential oil as additive in silver catfish diet: Blood response and resistance against *Aeromonas hydrophila* infection. *Fish Shellfish Immunol* **62**, 213-216

Ekici, S., Diler, O., Didinen, B.I. and Kubilay, A. (2011) Balıklardan ízole edilen bakteriyel patojenlere karşı bazı bitkisel uçucu yağlarının antibakteriyal aktivitesi. *Kafkas Univ Vet Fak Derg* **17**, S47-S54.

Finlay, B.B. and McFadden, G. (2006) Anti-immunology: Evasion of the host immune system by bacterial and viral pathogens. *Cell* **124**, 767–782.

FAO - Food and agriculture organization of the United Nations (2012) *The state of world fisheries and aquaculture* No. 1-209. Rome.

FAO - Food and agriculture organization of the United Nations (2016) *The state of world fisheries and aquaculture* No. 1-223. Rome.

Gómez-Sánchez, A. and López-Malo, A. (2009) Potencial antimicrobiano delos aceites esenciales de orégano (*Origanum vulgare*) y canela (*Cinnamomum zeylanicum*). *T Selec Ingen Alim* **3**, 33–45.

Grenni, P., Ancona, V. and Caracciolo, A.B. (2017) Ecological effects of antibiotics on natural ecosystems: A review. *Microchem J, in press.* Doi:10.1016/j.microc.2017.02.006

Griffin, M. J., Goodwin, A.E., Merry, G.E., Liles, M.R., Williams, M.A., Ware, C. and Waldbieser, G.C. (2013) Rapid quantitative detection of *Aeromonas hydrophila* strains associated with disease outbreaks in catfish aquaculture. *J Vet Diagn Invest* **25**, 473–481.

Harikrishnan, R. and Balasundaram, C. (2005) Modern trends in *Aeromonas hydrophila* disease management with fish. R*ev Fish Sci* **13**, 281–320.

Hawke, J. P., Kent, M., Rogge, M., Baumgartner, W., Wiles, J., Shelley, J., Savolainen, L.C., Wagner, R., Murray, K.and Peterson, T.S. (2013) Edwardsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of zebrafish *Danio rerio.J Aquat Anim Health* **25**, 171–183.

Hyldgaard, M., Mygind, T. and Meyer, R. L. (2012) Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front Microbiol* **3**, 1-24.

Karaman, İ., Şahin, F., Güllüce, M., Öğütçü, H., Şengül, M. and Adigüzel, A. (2003) Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. *J Ethnopharmacol* **85**, 231-235.

Lactococcus garvieae infection in tilapia. *Biosci Biotechnol Biochem* **73**, 2085–2089.

Lee, S., Najiah, M., Wendy, W. and Nadirah, M. (2009) Chemical composition and antimicrobial activity of the essential oil of *Syzygium aromaticum* flower bud (clove)

against fish systemic bacteria isolated from aquaculture sites. *Front Agric China* **3**, 332–336.

Majolo, C., Rocha, S.I.B., Chagas, E.C., Chaves, F.C.M. and Bizzo, H.R. (2017) Chemical composition of *Lippia spp*. essential oil and antimicrobial activity against *Aeromonas hydrophila*. *Aquacul Res* **48**,1–8.

MAPA - Ministry of Agriculture, Livestock and Food Supply (2011) Interministerial regulation instruction No. 28-29. Brasília.

Markestad, A. and Grave, K. (1997) Reduction of antibacterial drug use in Norwegian fish farming due to vaccination. *Dev Biol Stand* **90**, 365–369.

Menanteau-Ledouble, S., Krauss, I., Santos, G., Fibi, S., Weber, B. and El Matbouli, M. (2015) Effect of a phytogenic feed additive on the susceptibility of O*nchorhynchus mykiss* to *Aeromonas salmonicida*. *Dis Aquat Organ* **115**, 57–66

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

Murthy, K. S. and Kiran B. R. (2013) Review on usage of medicinal plants in fish diseases. *Int j pharm bio sci* **4**, 975-986.

Nair, M., Jayant, R.D., Kaushik, A. and Sagar, V. (2016) Getting into the brain: Potential of nanotechnology in the management of NeuroAIDS. *Adv Drug Deliv Rev* **103**, 202–217.

Navarrete, P., Toledo, I., Mardones, P., Opazo, R., Espejo, R. and Romero, J. (2010) Effect of *Thymus vulgaris* essential oil on intestinal bacterial microbiota of rainbow

trout, *Oncorhynchus mykiss* (Walbaum) and bacterial isolates. *Aquacul Res* **41**, 667-678.

Naylor, R. L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.N., Clay, J., Folke, C., Lubchenco, J., Mooney, H. and Troell, M. (2000) Effect of aquaculture on world fish supplies. *Nature* **405**, 1017–1024.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R. and De Feo, V. (2013) Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* **6**, 1451–1474.

Ngugi, C., Oyoo-Okoth, E., Bundi, J., Orina, S., Chemoiwa, E.J. and Aloo, P. (2015) Effects of dietary administration of stinging nettle (*Urtica dioica*) on the growth performance, biochemical, hematological and immunological parameters in juvenile and adult Victoria Labeo (*Labeo victorianus*) challenged with *Aeromonas hydrophila. Fish Shellfish Immunol* **44**,533-541.

Nikaido, H. (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**, 382–388.

Okmen, G., Ugur, A., Sarac, U. and Arslan, T. (2012) *In vivo* and *in vitro* antibacterial activities of some essential oils of lamiaceae species on *Aeromonas salmonicida* isolates from cultured rainbow trout, *Oncorhynchus mykiss. J Anim Vet Adv* **11**, 2762-2768.

Olivero-Verbel, J., Maya, A.B., Sevilla, A.B. and Stashenko, E.E. (2014) Composition, anti-quorum sensing and antimicrobial activity of essential oils from *Lippia alba. Braz J Microbiol* **45**, 759-767.

Ozogul, Y., Kuley, E., Uçar, Y. and Ozogul, F. (2015) Antimicrobial impacts of essential oils on food borne-pathogens. *Recent Pat Food Nutr Agric*. **7**, 53-61.

Pantoja, W. M. F., Neves, L., Dias, M.R.D., Marinho, R.G.B., Montagner, D., Tavares-Dias, M. (2012) Protozoan and metazoan parasites of Nile tilapia *Oreochromis niloticus* cultured in Brazil. *Rev MVZ Cordoba* **17**, 2812-2819.

Park, S., Aoki, T. and Jung, T. (2012a) Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Vet Res* **43**, 67.

Park, Y. H., Hwang, S.Y., Hong, M.K. and Kwon, K.H. (2012b) Use of antimicrobial agents in aquaculture. *Rev sci tech Off int Epiz* **31**, 189-197.

Peterson, B.C., Peatman, E., Ourth, D.D., Waldbieser, G.C. (2015) Effects of a phytogenic feed additive on growth performance, susceptibility of channel catfish to *Edwardsiella ictaluri* and levels of mannose binding lectin. *Fish Shellfish Immunol* **44**, 21-5.

Rather, M.A., Sharma, R., Aklakur, M., Ahmad, S., Kumar, N., Khan, M., Ramya, V.L. (2011) Nanotechnology: A novel tool for aquaculture and fisheries development. A prospective mini-review. *J Fish Aquac* **2011**, FAJ-16.

Rattanachaikunsopon, P. and Phumkhachorn, P. (2009a) Potential of chinese chive oil as a natural antimicrobial for controlling *Flavobacterium columnare* infection in Nile tilapia *Oreochromis niloticus*. *Fisheries sci* **75**, 1431–1437.

Rattanachaikunsopon, P. and Phumkhachorn, P. (2009b) Protective effect of clove oil-supplemented fish diets on experimental *Lactococcus garvieae* infection in tilapia. *Biosci Biotechnol Biochem* **73**, 2085–2089.

Rattanachaikunsopon, P. and Phumkhachorn, P. (2010) Potential of cinnamon (*Cinnamomum verum*) oil to control *Streptococcus iniae* infection in tilapia (*Oreochromis niloticus*). *Fisheries sci* **76**, 287–293.

Reichling, J., Schnitzler, P., Suschke, U. and Saller, R. (2009) Essential oils of aromatic plants with antibacterial, antifungal, antiviral, and cytotoxic properties - An overview. *Forsch Komplementmed* **16**, 79–90.

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P. and Pickup, R.W. (2000) Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: Implication of Tn1721 in dissemination of the tetracycline resistance determinant Tet A. *Appl Environ Microbiol* **66**, 3883–3890.

Ríos, J. L. and Recio, M. C. (2005) Medicinal plants and antimicrobial activity. *J Ethnopharmacol* **100**, 80–84.

Romero, J., Feijoó, C. G. and Navarrete, P. (2012) Antibiotics in aquaculture – Use, abuse and alternatives, health and environment in aquaculture, In Health and Environment in Aquaculture ed. Carvalho, E. D., David, G. S. and Silva, R. J. pp. 159-184. Croatia: InTech Europe.

Schaffazick, S. R., Guterres, S. S., Freitas, L. L. and Pohlmann, A. R. (2003) Physicochemical characterization and stability of the polymeric nanoparticle systems for drug administration. *Quím Nova* **26**, 726-737.

Sebastião, F. de A., Pilarski, F. and Lemos, M. V. (2013) Composition of extracellular polymeric substances (EPS) produced by *Flavobacterium columnare* isolated from tropical fish in Brazil. *Braz J Microbiol* **44**, 861–864.

Shehata, S.A., Mohamed, M.S. and Abd El-Shafi, S. (2013) Antibacterial activity of essential oils and their effects on nile tilapia fingerlings performance. *J Med Sci* **13**, 367-372.

Shi, C., Song, K., Zhang, X., Sun, Y., Sui, Y., Chen, Y., Jia, Z., Sun, H., Sun, Z. and Xia, X. (2016) Antimicrobial activity and possible mechanism of action of citral against *Cronobacter sakazakii. PLoS One* **11**, 1-12.

Silva, L. L., Balconi, L. S., Gressler, L. T., Garlet, Q. I., Fernando J. Sutili, F. J., Vargas, A. P. C., Baldisserotto, B., Morel, A. F. and Heinzmann, B. M. (2017) *S*-(+)- and *R*-(-)-linalool: a comparison of the *in vitro* anti-*Aeromonas hydrophila* activity and anesthetic properties in fish. *An Acad Bras Ciênc* **89** (1), 203-212.

Snuossi, M., Trabelsi, N., Taleb, S.B., Dehmeni, A., Flamini, G., and Feo, V. (2016) *Laurus nobilis*, *Zingiber officinale* and *Anethum graveolens* Essential oils: composition, antioxidant and antibacterial activities against bacteria isolated from fish and shellfish. *Molecules* **21**, 2-10.

Soltani, M., Ghodratnama, M., Ebrahimzadeh-Mosavi, H.A., Nikbakht-Brujeni, G., Mohamadian, S. and Ghasemian, M. (2014) Shirazi thyme (*Zataria multiflora* Boiss) and Rosemary (*Rosmarinus officinalis*) essential oils repress expression of sagA, a streptolysin S-related gene in *Streptococcus iniae*. *Aquaculture* **430**, 248–252.

Soltani, M., Mohamadian, S., Rouholahi, S., Soltani, E. and Rezvani, S. (2015) Shirazi thyme (*Zataria multiflora*) essential oil suppresses the expression of Pava and hly genes in *Lactacoccus garvieae*, the causative agent of Lactacoccosis in farmed fish. *Aquaculture* **442**, 74–77.

Soltani, M., Sheikhzadeh, N., Ebrahimzadeh-Mousavi, H.A. and Zargar, A. (2010) Effects of *Zataria multiflora* essential oil on innate immune responses of common carp (*Cyprinus carpio*). *J Fish Aquat Sci* **5**, 191–199.

Souza, C. F., Baldissera, M.D., Santos, R. C.V., Raffin. R.P. and Baldisserotto, B. (2017a) Nanotechnology improves the therapeutic efficacy of *Melaleuca alternifolia* essential oil in experimentally infected *Rhamdia quelen* with *Pseudomonas aeruginosa. Aquaculture* **473**, 169–171.

Souza, C. F., Baldissera, M.D., Guarda, N.S., Bollick, Y.S., Moresco, R.N., Brusque, I.C.M., Santos, R.C.V. and Baldisserotto, B. (2017b) *Melaleuca alternifolia* essential oil nanoparticles ameliorate the hepatic antioxidant/oxidant status of silver catfish experimentally infected with *Pseudomonas aeruginosa*. *Microb Pathog* **108**, 61–65.

Springfield, E.P., Amabeoku, G., Weitz, F., Mabusela, W. and Johnson, Q. (2003) An assessment of two *Carpobrotus* species extracts as potential antimicrobial agents. *Phytomedicine* **10**, 434-439.

Sutili, F. J., Cunha, M.A., Ziech, R.E., Krewer, C.C., Zeppenfeld, C.C., Heldwein, C.G., Gressler, L.T., Heinzmann, B.M., Vargas, A.C. and Baldisserotto, B. (2015b) *Lippia alba* essential oil promotes survival of silver catfish (*Rhamdia quelen*) infected with *Aeromonas* sp. *An Acad Bras Cienc* **87**, 95–100.

Sutili, F. J., Kreutz, L.C., Noro, M., Gressler, L.T., Heinzmann, B.M., 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., Vargas, A.C., Schmidt, D. and Baldisserotto, B. (2016a) 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.

Sutili, F.J., Gatlin, D.M., Rossi Jr, D., Heinzmann, B.M. and Baldisserotto, B. (2016b) *In vitro* effects of plant essential oils on non-specific imune parameters of red drum, *Sciaenops ocellatus* L. *J Anim Physiol Anim Nutr* **100**, 1113–1120

Sutili, F.J., Gatlin, D.M., Rossi Jr, D., Heinzmann, B.M. and Baldisserotto, B. (2017) Plant essential oils as fish diet additives: benefits on fish health and stability in feed. *Rev Aquacult* **0**, 1-11.

Sutili, F.J., Silva, L.L., Gressler, L.T., Battisti, E.K., Heinzmann, B.M., Vargas, A.P.C. and Baldisserotto, B. (2015a). Plant essential oils against *Aeromonas hydrophila in vitro* activity and their use in experimentally infected fish. *J Appl Microbiol* **119**, 47–54

Thirugnanasampandan, R., Gunasekar, R. and Gogulramnath, M. (2015) Chemical composition analysis, antioxidant and antibacterial activity evaluation of essential oil of *Atalantia monophylla* Correa. *Pharmacognosy Res.* **7**, 52-56.

Thomas, J., Jerobin, J., Seelan, T.S.J., Thanigaivel, S., Vijayakumar, S., Mukherjee, A. and Chandrasekaran, N. (2013) Studies on pathogenicity of *Aeromonas salmonicida* in catfish *Clarias batrachus* and control measure by neem nanoemulsion. *Aquaculture* **396-399**, 71-75.

Thomas, J., Thanigaivel, S., Vijayakumar, S., Acharya, K., Shinge, D., Seelan, T.S.J., Mukherjee, A. and Chandrasekaran, N. (2014) Pathogenecity of *Pseudomonas*

aeruginosa in *Oreochromis mossambicus* and treatment using lime oil nanoemulsion. *Colloids Surf B Biointerfaces* **116**, 372–377.

Thoroski, J., Blank, G. and Biliaderis, C. (1989) Eugenol induced inhibition of extracellular enzyme production by *Bacillus subtilis*. *J Food Prot* **52**, 399–403.

Tiwari, B. K., Valdramidis, V. S., O' Donnell, C.P., Muthukumarappan, K., Bourke, P. and Cullen, P.J. (2009) Application of natural antimicrobials for food preservation. *J Agric Food Chem* **57**, 5987–6000.

Trombetta, D.*,* Castelli, F., Sarpietro, M.G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. and Bisignano, G. (2005) Mechanisms of antibacterial action of three monoterpenes mechanisms of antibacterial action of three monoterpenes. *Antimicrob Agents Chemother* **49**, 2474–2478.

Vauthier, C. and Bouchemal, K. (2009) Methods for the preparation and manufacture of polymeric nanoparticles. *Pharm Res* **26**, 1025–1058.

Walker, P. and Winton, J.R. (2010) Emerging viral diseases of fish and shrimp. *Vet Res* **41**, 1-24.

Wei, L.S. and Wee, W. (2013). Chemical composition and antimicrobial activity of *Cymbopogon nardus* citronella essential oil against systemic bacteria of aquatic animals. *Iran J Microbiol* **5***,* 147-152.

Wendakoon, C. N. and Sakaguchi, M. (1995) Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. *J Food Prot* **58**, 280–283.

WOH - World Organization for Animal Health (2007) *List of antimicrobials of*

veterinary importance. No. 1-9. Paris.

Zhang, D., Li, A., Guo, Y., Zhang, Q., Chen, X. and Gong, X. (2013) Molecular characterization of *Streptococcus agalactiae* in diseased farmed tilapia in China. *Aquaculture* **412–413**, 64–69.

Zheng, Z.L., Tan, J.Y.W., Liu, H.Y., Zhou, X.H. 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.

Zilberg, D., Tal, A., Froyman, N., Abutbul, S., Dudai, N. and Goldhirsh, A.G. (2010) Dried leaves of *Rosmarinus officinalis* as a treatment for streptoccosis in tilapia. *J Fish Dis* **33**, 361-369.

Figures

Figure 1 Mechanisms of action and target sites of isolated EOs compounds in microbial cells. 1: thymol, carvacrol, linalool, menthol, geraniol, linalyl acetate, citronellal, and piperitone; 2: eugenol, isoeugenol, vanillin, safrole and cinnamaldehyde; 3: 1,8 cineole; 4: β-pinene, linalool, cinnamaldehyde, geraniol, α-terpinene, β-citronellol, and estragole; 5: citral; 6: linalyl acetate; 7: p-cymene, limonene, terpinene, sabinene, and pinenes; 8: borneol, camphor, α-pinene, camphone, verbenonone, and bornyl acetate. Adapted from Nazarro *et al*. (2013).
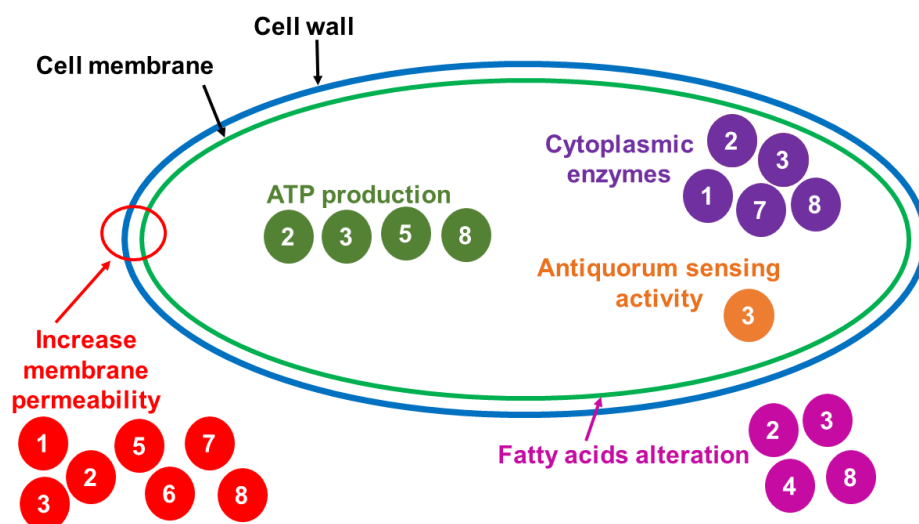
Figure 2 Comparison of *in vitro* and *in vivo* tests with EOs or compounds based on literature data. A: *in vivo* - baths; B: *in vivo* - dietary supplementation; (A) 1: *Lippia alba*, 2: *Hesperozygis ringens*, 3: *Ocimum gratissimum*, 4: eugenol, 5: *Ocimum americanum*. (B) 1: *Zingiber officinale*, 2: *Ocimum gratissimum*, 3: *Rosmarinus officinalis*, 4: *Citrus sinensis*, 5: *Thymus vulgaris*, 6: *Allium tuberosum*, 7: *Sygyzium aromaticum*, 8: *Origanum onites*, 9: *Zataria multiflora*. There is no significant correlation between *in vitro* and *in vivo* tests.
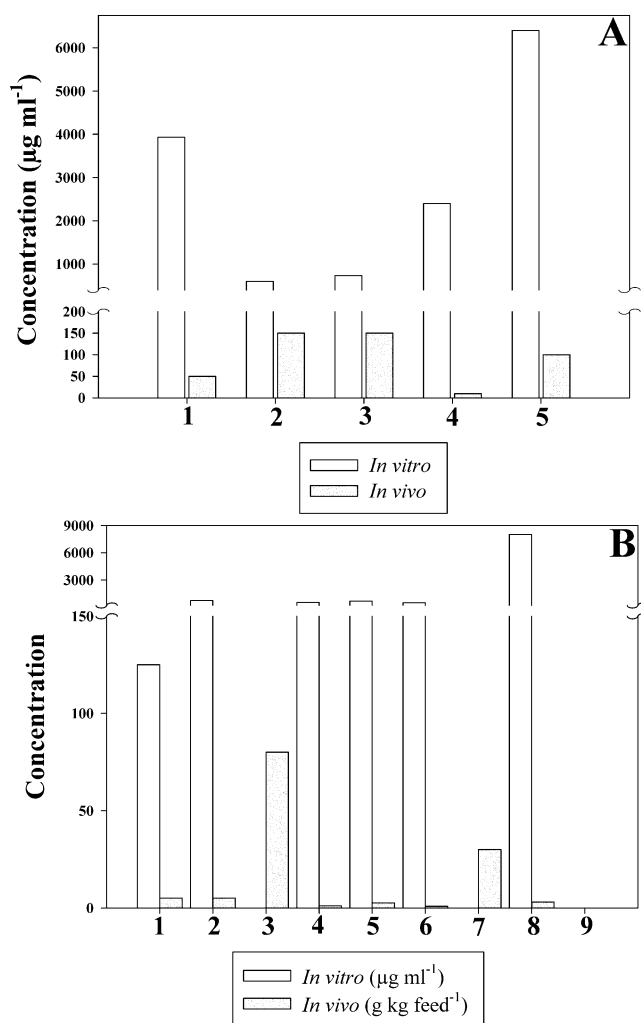
Table 1 *In vitro* assays with essential oils and their antibacterial activity against fish pathogens

Bacterium	Essential oil	Major compounds (%)	MIC ($\mu g\ ml^{-1}$)	MBC ($\mu g\ ml^{-1}$)	Source
Aer. hydrophila *Aer. veronii*	*O. gratissimum*	eugenol (91.47)	400 – 1600‡		Bandeira *et al.* 2017
Aer. hydrophila *Aer. veronii*	*H. ringens*	pulegone (96.63)	400 – 1600‡		Bandeira *et al.* 2017
Cit. freundii *Raoultella ornithinolytica*	*O. gratissimum* *H. ringens*	eugenol (91.47) pulegone (96.63)	1600 - >3200‡		Bandeira *et al.* 2017
Aer. hydrophila	*L. alba*	geranial (25.4)	5000	5000	Majolo *et al.* 2017
	L. origanoides	carvacrol (49.7)	2500	2500	Majolo *et al.* 2017
	L. sidoides	thymol (76.6)	1250	1250	Majolo *et al.* 2017
Aer. hydrophila	*L. alba*	linalool (65.5)	2862	5998	Sutili *et al.* 2015
Aer. hydrophila	*O. americanum*	β-linalool (32.43)	6400	NA†	Sutili *et al.* 2016
Aer. hydrophila	S-(+)- and R-(-)-linalool	-	3200	NA†	Silva *et al.* 2017

Aer. hydrophila	*L. nobilis*	1,8-cineole (56)	50 – 200	6250 - > 25000	Snuossi *et al*. 2016
Ent. cloacae	*L. nobilis*	1,8-cineole (56)	50 – 200	> 25000 - > 50000	Snuossi *et al*. 2016
Kl. ornithinolytica	*L. nobilis*	1,8-cineole (56)	50	6250	Snuossi *et al*. 2016
Kl. oxytoca	*L. nobilis*	1,8-cineole (56)	50	> 12500	Snuossi *et al*. 2016
Staph. lentus	*L. nobilis*	1,8-cineole (56)	50	> 6250	Snuossi *et al*. 2016
Staph. lugdumensis	*L. nobilis*	1,8-cineole (56)	50 – 100	> 12500 - > 25000	Snuossi *et al*. 2016
Ser. odorifera	*L. nobilis*	1,8-cineole (56)	100	> 12500	Snuossi *et al*. 2016
Staph. sciuri	*L. nobilis*	1,8-cineole (56)	50	> 6250 - > 25000	Snuossi *et al*. 2016
Staph. xylosus	*L. nobilis*	1,8-cineole (56)	50	> 6250	Snuossi *et al*. 2016
V. alginolyticus	*L. nobilis*	1,8-cineole (56)	50	> 6250	Snuossi *et al*. 2016
Aer. hydrophila	*Z. officinale*	hydrocarbon monoterpenes (32)	50 – 200	> 780 – 50000	Snuossi *et al*. 2016
Ent. cloacae	*Z. officinale*	hydrocarbon monoterpenes (32)	50 – 100	> 25000	Snuossi *et al*. 2016
Kl. ornithinolytica	*Z. officinale*	hydrocarbon monoterpenes (32)	200	> 25000	Snuossi *et al*. 2016

Kl. oxytoca	*Z. officinale*	hydrocarbon monoterpenes (32)	50	> 12500	Snuossi *et al*. 2016
Staph. lentus	*Z. officinale*	hydrocarbon monoterpenes (32)	50	> 3130	Snuossi *et al*. 2016
Staph. lugdumensis	*Z. officinale*	hydrocarbon monoterpenes (32)	50 – 200	> 12500	Snuossi *et al*. 2016
Ser. odorifera	*Z. officinale*	hydrocarbon monoterpenes (32)	50	> 12500	Snuossi *et al*. 2016
Staph. sciuri	*Z. officinale*	hydrocarbon monoterpenes (32)	50 – 200	1560 - > 25000	Snuossi *et al*. 2016
Staph. xylosus	*Z. officinale*	hydrocarbon monoterpenes (32)	50	> 12500	Snuossi *et al*. 2016
V. alginolyticus	*Z. officinale*	hydrocarbon monoterpenes (32)	50	> 25000	Snuossi *et al*. 2016
Aer. hydrophila	*A. graveolens*	carvone (27)	50 – 200	12500 – 50000	Snuossi *et al*. 2016

Ent. cloacae	*A. graveolens*	carvone (27)	50 – 100	> 25000 – 50000	Snuossi *et al.* 2016
Kl. ornithinolytica	*A. graveolens*	carvone (27)	50	> 25000	Snuossi *et al.* 2016
Kl. oxytoca	*A. graveolens*	carvone (27)	100	50000	Snuossi *et al.* 2016
Staph. lentus	*A. graveolens*	carvone (27)	50	> 25000	Snuossi *et al.* 2016
Staph. lugdumensis	*A. graveolens*	carvone (27)	100 – 390	> 25000 – 50000	Snuossi *et al.* 2016
Ser. odorifera	*A. graveolens*	carvone (27)	100	> 12500	Snuossi *et al.* 2016
Staph. sciuri	*A. graveolens*	carvone (27)	50 – 100	> 12500 - > 50000	Snuossi *et al.* 2016
Staph. xylosus	*A. graveolens*	carvone (27)	100	> 12500	Snuossi *et al.* 2016
V. alginolyticus	*A. graveolens*	carvone (27)	100	50000	Snuossi *et al.* 2016
L. garvieae	*Z. multiflora*	carvacrol (62.82)	0.12*‡		Soltani *et al.* 2015
Aer. hydrophila	*H. ringens*	pulegone (96.63)	800 – 3200‡		Sutili *et al.* 2015
	O. gratissimum	eugenol (91.47)	200 - 1600	400 – 1600	Sutili *et al.* 2015
	O. americanum	1,8-cineole (21)	> than highest concentration tested		Sutili *et al.* 2015
Aer. hydrophila	eugenol	-	800 – 3200	1600 – 3200	Sutili *et al.* 2014
Aer. hydrophila	*A. monophylla*	sabinene (23.81) and isoeugenol-*E* (23.73)[#]	139.32	NA†	Thirugnanasampandan *et al.* 2015

E. coli	*A. monophylla*	sabinene (23.81) and isoeugenol-*E* (23.73)[#]	328.14	NA†	Thirugnanasampandan *et al*. 2015
Kl. pneumoniae	*A. monophylla*	sabinene (23.81) and isoeugenol-*E* (23.73)[#]	516.73	NA†	Thirugnanasampandan *et al*. 2015
Ps. aeruginosa	*A. monophylla*	sabinene (23.81) and isoeugenol-*E* (23.73)[#]	203.84	NA†	Thirugnanasampandan *et al*. 2015
Pr. mirabilis	*A. monophylla*	sabinene (23.81) and isoeugenol-*E* (23.73)[#]	189.57	NA†	Thirugnanasampandan *et al*. 2015
Pr. vulgaris	*A. monophylla*	sabinene (23.81) and isoeugenol-*E* (23.73)[#]	233.73	NA†	Thirugnanasampandan *et al*. 2015
Staph. aureus	*A. monophylla*	sabinene (23.81) and isoeugenol-*E* (23.73)[#]	541.11	NA†	Thirugnanasampandan *et al*. 2015
Aer. hydrophila	*T. vulgaris*	1,8-cineole (53.46)	62	NA†	Millezi *et al*. 2013
	C. citratus	geranial (46.03)	31	NA†	Millezi *et al*. 2013
Strep. iniae	*Z. multiflora*	-	0.06*	0.12-0.25*	Soltani *et al*. 2014

	R. officinalis	-	0.12 –0.25*	0.5 - > 1*	Soltani *et al*. 2014
V. parahaemolyticus	*Z. officinale*	zingiberene (27.40)	31.25*	12.5*	Debbarma *et al*. 2012
	E. camaldulensis		62.50*	12.5*	Debbarma *et al*. 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*‡		Debbarma *et al*. 2012
		limonene (90.94)			
Aer. hydrophila	*Z. officinale*	zingiberene (27.40)	31.25*	62.5*	Debbarma *et al*. 2012
	E. camaldulensis		125*	500*	Debbarma *et al*. 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*‡		Debbarma *et al*. 2012
		limonene (90.94)			
V. vulnificus	*Z. officinale*	zingiberene (27.40)	31.25*	31.25*	Debbarma *et al*. 2012
	E. camaldulensis		31.25*	62.5*	Debbarma *et al*. 2012
	C. sinensis	α-phellandrene (27.52)	125*	250*	Debbarma *et al*. 2012
		limonene (90.94)			
L. monocytogenes	*Z. officinale*	zingiberene (27.40)	15.62*	31.25*	Debbarma *et al*. 2012
	E. camaldulensis		62,500‡		Debbarma *et al*. 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*‡		Debbarma *et al*. 2012

		limonene (90.94)			
E. coli	*Z. officinale*	zingiberene (27.40)	62.5*		Debbarma *et al.* 2012
	E. camaldulensis		31.25*	500*	Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	1,000*	> 1,0000*	Debbarma *et al.* 2012
		limonene (90.94)			
B. subtilis	*Z. officinale*	zingiberene (27.40)	3.9*	15.62*	Debbarma *et al.* 2012
	E. camaldulensis		7.812*	31.25*	Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	500*	> 1,000*	Debbarma *et al.* 2012
		limonene (90.94)			
Salm. typhi	*Z. officinale*	zingiberene (27.40)	31.25*	125*	Debbarma *et al.* 2012
	E. camaldulensis		125*	125*	Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	> 1,0000*‡		Debbarma *et al.* 2012
		limonene (90.94)			
Salm. typhimurium	*Z. officinale*	zingiberene (27.40)	62.5*	125*	Debbarma *et al.* 2012
	E. camaldulensis		125*‡		Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*		Debbarma *et al.* 2012

		limonene (90.94)			
Salm. paratyphi	*Z. officinale*	zingiberene (27.40)	62.5*	125*	Debbarma *et al.* 2012
	E. camaldulensis		125*‡		Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*‡		Debbarma *et al.* 2012
		limonene (90.94)			
Y. enterocolitica	*Z. officinale*	zingiberene (27.40)	31.25*	62.5*	Debbarma *et al.* 2012
	E. camaldulensis		62.5*	125*	Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*‡		Debbarma *et al.* 2012
		limonene (90.94)			
Ps. aeruginosa	*Z. officinale*	zingiberene (27.40)	31.25*	62.5*	Debbarma *et al.* 2012
	E. camaldulensis		125*‡		Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*‡		Debbarma *et al.* 2012
		limonene (90.94)			
S. aureus	*Z. officinale*	zingiberene (27.40)	7.81*	31.25*	Debbarma *et al.* 2012
	E. camaldulensis		31.25*	250*	Debbarma *et al.* 2012
	C. sinensis	α-phellandrene (27.52)	> 1,000*‡		Debbarma *et al.* 2012

limonene (90.94)

Aer. salmonicida	*O. onites*	-	800	NA†	Okmen *et al*. 2012
	Origanum vulgare	-	800	NA†	Okmen *et al*. 2012
	Thymbra spicata	-	800	NA†	Okmen *et al*. 2012
	Satureja thymbra	-	800	NA†	Okmen *et al*. 2012
Strep. iniae	*Cinnamomum verum*	cinnamaldehyde (90.24)	40	NA†	Rattanachaikunsopon and Phumkhachorn 2010
	Citrus hystrix	-	160	NA†	Rattanachaikunsopon and Phumkhachorn 2010
	Cymbopogon citratus	-	320	NA†	Rattanachaikunsopon and Phumkhachorn 2010
	Curcuma longa	-	160	NA†	Rattanachaikunsopon and Phumkhachorn 2010
V. anguillarum	*T. vulgaris*	-	80	NA†	Navarrete *et al*. 2010

Fl. psychrophilum	*T. vulgaris*	-	80 – 1,280	NA†	Navarrete *et al.* 2010
V. ordalii	*T. vulgaris*	-	80 – 1,280	NA†	Navarrete *et al.* 2010
V. parahaemolyticus	*T. vulgaris*	-	80 – 1,280	NA†	Navarrete *et al.* 2010
Fl. columnare	*A. tuberosum*	-	200 – 800	NA†	Rattanachaikunsop and Phumkhachorn 2009
V. spp.	*S. aromaticum*	eugenol (49)	0.015	NA†	Lee *et al.* 2009
Edw. spp.	*S. aromaticum*	eugenol (49)	0.015 - 0.062	NA†	Lee *et al.* 2009
Aer. spp.	*S. aromaticum*	eugenol (49)	0.015 - 0.031	NA†	Lee *et al.* 2009
Fl. spp.	*S. aromaticum*	eugenol (49)	0.031	NA†	Lee *et al.* 2009
E. coli	*S. aromaticum*	eugenol (49)	0.062	NA†	Lee *et al.* 2009
Salm. spp.	*S. aromaticum*	eugenol (49)	0.062	NA†	Lee *et al.* 2009
Strep. spp.	*S. aromaticum*	eugenol (49)	0.062	NA†	Lee *et al.* 2009
Cit. freundii	*S. aromaticum*	eugenol (49)	0.015	NA†	Lee *et al.* 2009
Y. enterocolitica	*S. aromaticum*	eugenol (49)	0.031	NA†	Lee *et al.* 2009
Edw. spp.	*C. nardus*	citronellal (29.6)	0.244 - 0.977	NA†	Wei and Wee 2013
V. spp	*C. nardus*	citronellal (29.6)	0.244- 0.488	NA†	Wei and Wee 2013

Aer. spp.	*C. nardus*	citronellal (29.6)	0.488 – 0.977	NA†	Wei and Wee 2013
E. coli	*C. nardus*	citronellal (29.6)	0.488	NA†	Wei and Wee 2013
Salm. spp.	*C. nardus*	citronellal (29.6)	0.244 – 0.488	NA†	Wei and Wee 2013
Fl. spp.	*C. nardus*	citronellal (29.6)	0.977	NA†	Wei and Wee 2013
Ps. spp.	*C. nardus*	citronellal (29.6)	0.244	NA†	Wei and Wee 2013
Strep. spp.	*C. nardus*	citronellal (29.6)	0.244	NA†	Wei and Wee 2013
Edw. spp.	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013
V. spp	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013
Aer. spp.	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013
E. coli	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013
Salm. spp.	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013
Fl. spp.	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013
Ps. spp.	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013
Strep. spp.	Citronellal	-	0.244 - 0.977	NA†	Wei and Wee 2013

* MIC and MBC are expressed in µl ml^{-1}, because the authors did not report the EO density and for this reason it was not possible to convert the value to µg ml^{-1}. -: Indicates that this type of analysis was not carried out. *Edw.*, *Edwardsiella*; *Ent.*, *Enterobacter* or *Enterococcus*; *Aer.*, *Aeromonas*; *B.*, *Bacillus*; *Cit.*, *Citrobacter*; *E.*, *Escherichia; Fl., Flavobacterium*; *Kl.*, *Klebsiella*; *L.*, *Lactococcus*; *L.*, *Listeria*; *Salm.*, *Salmonella*; *Ser.*, *Serratia*; *Staph.*, *Staphylococcus*; *Strep.*, *Streptococcus*; *Pr., Proteus*; *Ps., Pseudomonas; V.*, *Vibrio*; *Y.*, *Yersinia*. NA†: data not available. [#]: The essential oil of leaves of *A. monophylla* collected in May 2014 had sabinene as major constituent and the essential oil collected in December of 2013 had isoeugenol-*E* as major constituent. ‡: authors indicate this range for both MIC and MBC values.

Table 2 *In vitro* assays of antibacterial activity of essential oils and their compounds with the diffusion disc test.

Bacterial species	Essential oil (EO)	Major compounds of EO	Concentration applied to the disk (µl)	Zone of Inhibition (mm)	Source
Ps. aeruginosa	Nanoemulsion of EO of *C. aurantifolia*	NA†	30	15	Thomas *et al*. 2014
Aer. salmonicida	Nanoemulsion of EO of *A. indica*	NA†	40	30	Thomas *et al*. 2013
Staph. aureus	*Z. officinale*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
Staph. aureus	*N. sativa*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013

Staph. aureus	*T. vulgaris*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
Staph. aureus	*S. aromaticum*	NA†	1000	4.5	Shehata, Mohamed and Abd El-Sha 2013
Staph. aureus	*E. sativa*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
Ps. aeruginosa	*Z. officinale*	NA†	1000	6.7	Shehata, Mohamed and Abd El-Sha 2013
Ps. aeruginosa	*N. sativa*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
Ps. aeruginosa	*T. vulgaris*	NA†	1000	13	Shehata, Mohamed and Abd El-Sha 2013
Ps. aeruginosa	*S. aromaticum*	NA†	1000	2	Shehata, Mohamed and Abd El-Sha 2013
Ps. aeruginosa	*E. sativa*	NA†	1000	10.3	Shehata, Mohamed and Abd El-Sha 2013
E. coli	*Z. officinale*	NA†	1000	8	Shehata, Mohamed and

					Abd El-Sha 2013
E. coli	*N. sativa*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
E. coli	*T. vulgaris*	NA†	1000	5.7	Shehata, Mohamed and Abd El-Sha 2013
E. coli	*S. aromaticum*	NA†	1000	2	Shehata, Mohamed and Abd El-Sha 2013
E. coli	*E. sativa*	NA†	1000	5.3	Shehata, Mohamed and Abd El-Sha 2013
L. monocytogenes	*Z. officinale*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
L. monocytogenes	*N. sativa*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
L. monocytogenes	*T. vulgaris*	NA†	1000	9.5	Shehata, Mohamed and Abd El-Sha 2013
L. monocytogenes	*S. aromaticum*	NA†	1000	6	Shehata, Mohamed and Abd El-Sha 2013

L. monocytogenes	*E. sativa*	NA†	1000	6	Shehata, Mohamed and Abd El-Sha 2013
L. lactis	*Z. officinale*	NA†	1000	5.5	Shehata, Mohamed and Abd El-Sha 2013
L. lactis	*N. sativa*	NA†	1000	7.3	Shehata, Mohamed and Abd El-Sha 2013
L. lactis	*T. vulgaris*	NA†	1000	11.3	Shehata, Mohamed and Abd El-Sha 2013
L. lactis	*S. aromaticum*	NA†	1000	6.3	Shehata, Mohamed and Abd El-Sha 2013
L. lactis	*E. sativa*	NA†	1000	0	Shehata, Mohamed and Abd El-Sha 2013
B. cereus	*Z. officinale*	NA†	1000	6	Shehata, Mohamed and Abd El-Sha 2013
B. cereus	*N. sativa*	NA†	1000	5	Shehata, Mohamed and Abd El-Sha 2013
B. cereus	*T. vulgaris*	NA†	1000	7.5	Shehata, Mohamed and

					Abd El-Sha 2013
B. cereus	*S. aromaticum*	NA†	1000	5.6	Shehata, Mohamed and
					Abd El-Sha 2013
B. cereus	*E. sativa*	NA†	1000	0	Shehata, Mohamed and
					Abd El-Sha 2013
Y. ruckeri	*O. vulgare*	carvacrol (82)	25	8-17.5	Ekici *et al.* 2011
Aer. hydrophila	*M. oleum*	isopropyl myristate (34.4)	25	6.5-23.5	Ekici *et al.* 2011
V. anguillarum	*L. romanae*	isopropyl myristate (92.72)	25	8-32.5	Ekici *et al.* 2011
Fl. psychrophilum	*R. officinalis*	1,8-cineole (30.95)	25	40	Ekici *et al.* 2011
L. garvieae	*Z. officinale*	heneicosane (35.5)	25	6-10	Ekici *et al.* 2011
Strep. agalactiae	*R. officinalis*	carnosic acid (-)	-	17	Zilberg *et al.* 2010
Aer. hydrophila	*P. brutia*	α-pinene (90.18)	50	24.5	Ozogul *et al.* 2015
Aer. hydrophila	*E. globulus*	eucalyptol (59.28)	50	9.5	Ozogul *et al.* 2015
Aer. hydrophila	*T. vulgaris*	carvacrol (71.54)	50	12.5	Ozogul *et al.* 2015
Aer. hydrophila	*S. officinalis*	1,8-cineole (47.51)	50	11	Ozogul *et al.* 2015

Aer. hydrophila	*L. officinalis*	linalool (43.37)	50	7.5	Ozogul *et al*. 2015
Aer. hydrophila	*C. sinensis*	limonene (95.77)	50	11	Ozogul *et al*. 2015
Aer. hydrophila	*C. limonum*	1,8-cineole (29.60)	50	7.7	Ozogul *et al*. 2015
Aer. hydrophila	*M. communis*	limonene (71.77)	50	11.8	Ozogul *et al*. 2015
Aer. hydrophila	*R. officinalis*	1,8-cineole (52.17)	50	11.5	Ozogul *et al*. 2015
Aer. hydrophila	*J. communis*	α-pinene (90.09)	50	6	Ozogul *et al*. 2015
E. coli	*P. brutia*	α-pinene (90.18)	50	5.25	Ozogul *et al*. 2015
E. coli	*E. globulus*	eucalyptol (59.28)	50	2.75	Ozogul *et al*. 2015
E. coli	*T. vulgaris*	carvacrol (71.54)	50	24	Ozogul *et al*. 2015
E. coli	*S. officinalis*	1,8-cineole (47.51)	50	6.25	Ozogul *et al*. 2015
E. coli	*L. officinalis*	linalool (43.37)	50	3	Ozogul *et al*. 2015
E. coli	*C. sinensis*	limonene (95.77)	50	1	Ozogul *et al*. 2015
E. coli	*C. limonum*	1,8-cineole (29.60)	50	5.25	Ozogul *et al*. 2015
E. coli	*M. communis*	limonene (71.77)	50	3	Ozogul *et al*. 2015
E. coli	*R. officinalis*	1,8-cineole (52.17)	50	3	Ozogul *et al*. 2015
E. coli	*J. communis*	α-pinene (90.09)	50	1.5	Ozogul *et al*. 2015
Salm. paratyphi A	*P. brutia*	α-pinene (90.18)	50	6	Ozogul *et al*. 2015

Salm. paratyphi A	*E. globulus*	eucalyptol (59.28)	50	1	Ozogul *et al*. 2015
Salm. paratyphi A	*T. vulgaris*	carvacrol (71.54)	50	27.5	Ozogul *et al*. 2015
Salm. paratyphi A	*S. officinalis*	1,8-cineole (47.51)	50	4.5	Ozogul *et al*. 2015
Salm. paratyphi A	*L. officinalis*	linalool (43.37)	50	3.5	Ozogul *et al*. 2015
Salm. paratyphi A	*C. sinensis*	limonene (95.77)	50	3	Ozogul *et al*. 2015
Salm. paratyphi A	*C. limonum*	1,8-cineole (29.60)	50	6.25	Ozogul *et al*. 2015
Salm. paratyphi A	*M. communis*	limonene (71.77)	50	4.75	Ozogul *et al*. 2015
Salm. paratyphi A	*R. officinalis*	1,8-cineole (52.17)	50	5.5	Ozogul *et al*. 2015
Salm. paratyphi A	*J. communis*	α-pinene (90.09)	50	1.25	Ozogul *et al*. 2015
Kl. pneumonia	*P. brutia*	α-pinene (90.18)	50	20.5	Ozogul *et al*. 2015
Kl. pneumonia	*E. globulus*	eucalyptol (59.28)	50	6.75	Ozogul *et al*. 2015
Kl. pneumonia	*T. vulgaris*	carvacrol (71.54)	50	29.75	Ozogul *et al*. 2015
Kl. pneumonia	*S. officinalis*	1,8-cineole (47.51)	50	4.75	Ozogul *et al*. 2015
Kl. pneumonia	*L. officinalis*	linalool (43.37)	50	6.75	Ozogul *et al*. 2015
Kl. pneumonia	*C. sinensis*	limonene (95.77)	50	16	Ozogul *et al*. 2015
Kl. pneumonia	*C. limonum*	1,8-cineole (29.60)	50	10.75	Ozogul *et al*. 2015
Kl. pneumonia	*M. communis*	limonene (71.77)	50	3.25	Ozogul *et al*. 2015

Kl. pneumonia	*R. officinalis*	1,8-cineole (52.17)	50	4	Ozogul *et al*. 2015
Kl. pneumonia	*J. communis*	α-pinene (90.09)	50	5	Ozogul *et al*. 2015
Y. enterocolitica	*P. brutia*	α-pinene (90.18)	50	15.5	Ozogul *et al*. 2015
Y. enterocolitica	*E. globulus*	eucalyptol (59.28)	50	7.5	Ozogul *et al*. 2015
Y. enterocolitica	*T. vulgaris*	carvacrol (71.54)	50	16	Ozogul *et al*. 2015
Y. enterocolitica	*S. officinalis*	1,8-cineole (47.51)	50	9	Ozogul *et al*. 2015
Y. enterocolitica	*L. officinalis*	linalool (43.37)	50	15	Ozogul *et al*. 2015
Y. enterocolitica	*C. sinensis*	limonene (95.77)	50	9.5	Ozogul *et al*. 2015
Y. enterocolitica	*C. limonum*	1,8-cineole (29.60)	50	6.25	Ozogul *et al*. 2015
Y. enterocolitica	*M. communis*	limonene (71.77)	50	4	Ozogul *et al*. 2015
Y. enterocolitica	*R. officinalis*	1,8-cineole (52.17)	50	3.15	Ozogul *et al*. 2015
Y. enterocolitica	*J. communis*	α-pinene (90.09)	50	5	Ozogul *et al*. 2015
Ps. aeruginosa	*P. brutia*	α-pinene (90.18)	50	15	Ozogul *et al*. 2015
Ps. aeruginosa	*E. globulus*	eucalyptol (59.28)	50	1.5	Ozogul *et al*. 2015
Ps. aeruginosa	*T. vulgaris*	carvacrol (71.54)	50	18.05	Ozogul *et al*. 2015
Ps. aeruginosa	*S. officinalis*	1,8-cineole (47.51)	50	7.95	Ozogul *et al*. 2015
Ps. aeruginosa	*L. officinalis*	linalool (43.37)	50	8	Ozogul *et al*. 2015

Ps. aeruginosa	*C. sinensis*	limonene (95.77)	50	2.5	Ozogul *et al*. 2015
Ps. aeruginosa	*C. limonum*	1,8-cineole (29.60)	50	7.5	Ozogul *et al*. 2015
Ps. aeruginosa	*M. communis*	limonene (71.77)	50	3.75	Ozogul *et al*. 2015
Ps. aeruginosa	*R. officinalis*	1,8-cineole (52.17)	50	11.5	Ozogul *et al*. 2015
Ps. aeruginosa	*J. communis*	α-pinene (90.09)	50	2.5	Ozogul *et al*. 2015
Camp. jejuni	*P. brutia*	α-pinene (90.18)	50	22.25	Ozogul *et al*. 2015
Camp. jejuni	*E. globulus*	eucalyptol (59.28)	50	8.75	Ozogul *et al*. 2015
Camp. jejuni	*T. vulgaris*	carvacrol (71.54)	50	8.75	Ozogul *et al*. 2015
Camp. jejuni	*S. officinalis*	1,8-cineole (47.51)	50	30	Ozogul *et al*. 2015
Camp. jejuni	*L. officinalis*	linalool (43.37)	50	8	Ozogul *et al*. 2015
Camp. jejuni	*C. sinensis*	limonene (95.77)	50	14.25	Ozogul *et al*. 2015
Camp. jejuni	*C. limonum*	1,8-cineole (29.60)	50	12.5	Ozogul *et al*. 2015
Camp. jejuni	*M. communis*	limonene (71.77)	50	9	Ozogul *et al*. 2015
Camp. jejuni	*R. officinalis*	1,8-cineole (52.17)	50	6	Ozogul *et al*. 2015
Camp. jejuni	*J. communis*	α-pinene (90.09)	50	3	Ozogul *et al*. 2015
Ent. faecalis	*P. brutia*	α-pinene (90.18)	50	21.25	Ozogul *et al*. 2015
Ent. faecalis	*E. globulus*	eucalyptol (59.28)	50	12	Ozogul *et al*. 2015

Ent. faecalis	*T. vulgaris*	carvacrol (71.54)	50	15	Ozogul *et al*. 2015
Ent. faecalis	*S. officinalis*	1,8-cineole (47.51)	50	14.5	Ozogul *et al*. 2015
Ent. faecalis	*L. officinalis*	linalool (43.37)	50	17.75	Ozogul *et al*. 2015
Ent. faecalis	*C. sinensis*	limonene (95.77)	50	12	Ozogul *et al*. 2015
Ent. faecalis	*C. limonum*	1,8-cineole (29.60)	50	7.25	Ozogul *et al*. 2015
Ent. faecalis	*M. communis*	limonene (71.77)	50	16.50	Ozogul *et al*. 2015
Ent. faecalis	*R. officinalis*	1,8-cineole (52.17)	50	5.25	Ozogul *et al*. 2015
Ent. faecalis	*J. communis*	α-pinene (90.09)	50	5.25	Ozogul *et al*. 2015
Staph. aureus	*P. brutia*	α-pinene (90.18)	50	28.25	Ozogul *et al*. 2015
Staph. aureus	*E. globulus*	eucalyptol (59.28)	50	18	Ozogul *et al*. 2015
Staph. aureus	*T. vulgaris*	carvacrol (71.54)	50	22.25	Ozogul *et al*. 2015
Staph. aureus	*S. officinalis*	1,8-cineole (47.51)	50	10.75	Ozogul *et al*. 2015
Staph. aureus	*L. officinalis*	linalool (43.37)	50	7.25	Ozogul *et al*. 2015
Staph. aureus	*C. sinensis*	limonene (95.77)	50	10	Ozogul *et al*. 2015
Staph. aureus	*C. limonum*	1,8-cineole (29.60)	50	10	Ozogul *et al*. 2015
Staph. aureus	*M. communis*	limonene (71.77)	50	15	Ozogul *et al*. 2015
Staph. aureus	*R. officinalis*	1,8-cineole (52.17)	50	7.25	Ozogul *et al*. 2015

Staph. aureus	*J. communis*	α-pinene (90.09)	50	4.25	Ozogul *et al*. 2015
V. parahaemolyticus	*Z. officinale*	zingiberene (27.40)	100*	15	Debbarma *et al*. 2012
V. parahaemolyticus	*E. camaldulensis*	α-phellandrene (27.52)	100*	13	Debbarma *et al*. 2012 Debbarma *et al*. 2012
V. parahaemolyticus	*C. sinensis*	limonene (90.94)	100*	0	
Aer. hydrophila	*Z. officinale*	zingiberene (27.40)	100*	10	Debbarma *et al*. 2012
Aer. hydrophila	*E. camaldulensis*	α-phellandrene (27.52)	100*	14.66	Debbarma *et al*. 2012 Debbarma *et al*. 2012
Aer. hydrophila	*C. sinensis*	limonene (90.94)	100*	0	
V. vulnificus	*Z. officinale*	zingiberene (27.40)	100*	16.33	Debbarma *et al*. 2012
V. vulnificus	*E. camaldulensis*	α-phellandrene (27.52)	100*	16.33	Debbarma *et al*. 2012 Debbarma *et al*. 2012
V. vulnificus	*C. sinensis*	limonene (90.94)	100*	7.33	
L. monocytogenes	*Z. officinale*	zingiberene (27.40)	100*	20	Debbarma *et al*. 2012
L. monocytogenes	*E. camaldulensis*	α-phellandrene (27.52)	100*	25	Debbarma *et al*. 2012 Debbarma *et al*. 2012
L. monocytogenes	*C. sinensis*	limonene (90.94)	100*	16.33	
E. coli	*Z. officinale*	zingiberene (27.40)	100*	16.33	Debbarma *et al*. 2012
E. coli	*E. camaldulensis*	α-phellandrene (27.52)	100*	16.66	Debbarma *et al*. 2012 Debbarma *et al*. 2012
E. coli	*C. sinensis*	limonene (90.94)	100*	8	
B. subtilis	*Z. officinale*	zingiberene (27.40)	100*	46	Debbarma *et al*. 2012 Debbarma *et al*. 2012

B. subtilis	*E. camaldulensis*	α-phellandrene (27.52)	100*	29.33	Debbarma *et al.* 2012
B. subtilis	*C. sinensis*	limonene (90.94)	100*	30	
Salm. typhi	*Z. officinale*	zingiberene (27.40)	100*	18.33	Debbarma *et al.* 2012
Salm. typhi	*E. camaldulensis*	α-phellandrene (27.52)	100*	15.33	Debbarma *et al.* 2012 Debbarma *et al.* 2012
Salm. typhi	*C. sinensis*	limonene (90.94)	100*	13	
Salm. typhimurium	*Z. officinale*	zingiberene (27.40)	100*	11.33	Debbarma *et al.* 2012
Salm. typhimurium	*E. camaldulensis*	α-phellandrene (27.52)	100*	15.33	Debbarma *et al.* 2012 Debbarma *et al.* 2012
Salm. typhimurium	*C. sinensis*	limonene (90.94)	100*	8.33	
Salm. paratyphi	*Z. officinale*	zingiberene (27.40)	100*	14.33	Debbarma *et al.* 2012
Salm. paratyphi	*E. camaldulensis*	α-phellandrene (27.52)	100*	18	Debbarma *et al.* 2012
Salm. paratyphi	*C. sinensis*	limonene (90.94)	100*	9.33	Debbarma *et al.* 2012
Y. enterocolitica	*Z. officinale*	zingiberene (27.40)	100*	23.33	Debbarma *et al.* 2012
Y. enterocolitica	*E. camaldulensis*	α-phellandrene (27.52)	100*	22.33	Debbarma *et al.* 2012
Y. enterocolitica	*C. sinensis*	limonene (90.94)	100*	14.66	Debbarma *et al.* 2012
Ps. aeruginosa	*Z. officinale*	zingiberene (27.40)	100*	14.33	Debbarma *et al.* 2012
Ps. aeruginosa	*E. camaldulensis*	α-phellandrene (27.52)	100*	16	Debbarma *et al.* 2012
Ps. aeruginosa	*C. sinensis*	limonene (90.94)	100*	10	Debbarma *et al.* 2012

Fish species	Bacterial species	Essential oil (EO)	Major compounds (%)	Purpose / Concentration / Treatment / Outcome	Source
Staph. aureus	*Z. officinale*	zingiberene (27.40)	100*	24	Debbarma *et al.* 2012
Staph. aureus	*E. camaldulensis*	α-phellandrene (27.52)	100*	25	Debbarma *et al.* 2012
Staph. aureus	*C. sinensis*	limonene (90.94)	100*	24.66	Debbarma *et al.* 2012

The results varied according to the concentration of the EO tested, but, in general, the inhibition halo remained within the range described for each bacterium. *Edw., Edwardsiella; Ent., Enterobacter or Enterococcus; Aer., Aeromonas; B., Bacillus; Camp., Campylobacter; Cit., Citrobacter; E., Escherichia; Fl., Flavobacterium; Kl., Klebsiella; L., Lactococcus; L., Listeria; Salm., Salmonella; Ser., Serratia; Staph., Staphylococcus; Strep., Streptococcus; Ps., Pseudomonas; V., Vibrio; Y., Yersinia.* NA†: data not available; -: not defined. *: This study tested several concentrations (20, 30, 40, 50 and 100 μl), and authors determined the best concentration, this was the concentration chosen to be presented in this table.

Table 3 The use of essential oils by bath as antibacterial agents in fish.

Fish species	Bacterial species	Essential oil (EO)	Major compounds (%)	Purpose / Concentration / Treatment / Outcome	Source
R. quelen	*Ps. aeruginosa* (PA01)	*M. alternifolia* and nanoencapsulated EO of *M. alternifolia*	terpinen-4-ol (41.98) and NA†	Therapeutic / 50 μl l^{-1} / 1 h daily baths - 7 days after, intramuscular inoculation of the bacterium and evaluation after 60 days /	Souza *et al.* 2017a
				Therapeutic / nanoencapsulated EO showed 100% efficacy,	Souza *et al.*

				while the free EO showed 70% efficacy.	2017a
R. quelen	*Aer. hydrophila*	*O. americanum*	β-linalool (46.6)	Therapeutic / 10 and 20 mg l^{-1} / 1h daily baths - 5 days / Increased survival 26-30%	Sutili *et al.* 2016
R. quelen	*Aer. hydrophila*	*H. ringens*	pulegone (96.63)	Therapeutic / *H. ringens*: 20 and 40 mg l^{-1} / 1 h daily baths - 5 days / Increased survival 66-70%	Sutili *et al.* 2015a
R. quelen	*Aer. hydrophila*	*O. gratissimum*	eugenol (91.47)	Therapeutic / *O. gratissimum*: 5 and 10 mg l^{-1} / 1 h daily - 5 days / did not increase fish survival	Sutili *et al.* 2015a
R. quelen	*Aer. hydrophila*	*O. americanum*	1,8-cineole (21)	Therapeutic / *O. americamum*: 10 and 20 mg l^{-1} / 1 h daily baths - 5 days / Increased survival 66-70%	Sutili *et al.* 2015a
R. quelen		eugenol	-	Therapeutic / 5 and 10 mg l^{-1} / 1h daily baths - 5 days / can be used to treat or prevent bacterial diseases	Sutili *et al.* 2014
R. quelen	*Aer. sp.*	*L. alba*	linalool (65.5)	Therapeutic / 20 and 50 µl l^{-1} / 1h daily baths - 10 days / higher survival in fish treated with 50 µl l^{-1}	Sutili *et al.* 2015b

| *O. mykiss* | *Aer. salmonicida* | *S. thymbra* | NA† | Preventive / 100, 200, 400 and 800 mg l^{-1} / The MIC value (800 mg l^{-1}) and its dilutions (400, 200, 100 mg l^{-1}) were injected into experimental fish for *in vivo* studies/ toxic at the effective concentration and without effect at non-toxic concentrations | Okmen *et al.* 2012 |

Edw., Edwardsiella; Ent., Enterobacter or Enterococcus; Aer., Aeromonas; B., Bacillus; Cit., Citrobacter; E., Escherichia; Fl., Flavobacterium; Kl., Klebsiella; L., Lactococcus; L., Listeria; Salm., Salmonella; Ser., Serratia; Staph., Staphylococcus; Strep., Streptococcus; Ps.,Pseudomonas; V., Vibrio; Y., Yersinia. NA†: data not available.

Table 4 Studies of fish fed with dietary essential oils supplementation as preventive against bacterial infections.

Fish	Bacterium	Essential oil (EO)	Major compounds (%)	Doses (g kg feed^{-1}) / feeding time / outcome	Source
O. niloticus	-	*T. vulgaris*	NA†	1, 2.5 and 5 / 90 days / best 2.5	Shehata, Mohamed and El-Shafi 2013
Oreochromis sp.	*Strep. agalactiae*	*R. officinalis*	NA†	80 and 160 / 10 days / lower mortality	Zilberg *et al.* 2010
O. niloticus	*Fl. columnare*	*A. tuberosum*	NA†	0.2, 0.4, 0.6 and 0.8 / 14 days / best 0.8	Rattanachaikunsopon and Phumkhachorn 2009a
O. niloticus	*L. garvieae*	*S. aromaticum*	NA†	5, 10, 20 and 30 / 14 days / no mortality in fish fed diet supplemented with 30	Rattanachaikunsopon and Phumkhachorn 2009b
O. niloticus	*Strep. agalactiae*	*O. gratissimum* and *Z. officinale*	1,8-cineole (40.4) and geranial (24)	0.5, 1.0 and 1.5 / 55 days / improved disease resistance and phagocytic capacity, those treated with 5 had 100% survival	Brum *et al.* 2017

Species	Pathogen	Plant	Compound	Dose/Duration/Effect	Reference
O. mossambicus	*Edw. tarda*	*C. limon*	limonene (54.4)	5, 7.5 and 10 / 60 days / improved non-specific immune parameters and decreased mortality	Baba *et al*. 2016
O. mossambicus	*Strep. iniae*	*C. sinensis*	NA†	0.001, 0.003 and 0.005 / 90 days / reduced mortality	Acar *et al*. 2015
R. quelen	*Aer. hydrophila*	*Aloysia triphylla*	β-citral (20.78)	0.25 and 2 / 21 days / increased resistance to bacterial infection in the dose 2	Santos *et al*. 2017
I. punctatus	*Aer. hydrophila*	*O. heracleoticum*, carvacrol, thymol and Orego-Stim®[1]	NA†	0.5 / 56 days / carvacrol + thymol and OS reduced mortality	Zheng *et al*. 2009
O. niloticus	*Strep. iniae*	*C. verum*	cinnamaldehyde (90.2)	0.001, 0.002, 0.003 and 0.004 / 14 days / no mortality in fish fed with 0.004	Rattanachaikunsopon and Phumkhachorn 2010
I. punctatus	*Edw. ictaluri*	Digestarom®[2]	NA†	0.125, 1.5, 2.5 and 3.0 / 42 days / increased survival	Peterson *et al*. 2015

Oncorhynchus mykiss	*Lactococcus garvieae*	*O. onites*	NA†	0.125, 1.5, 2.5 and 3 / 90 days / increased resistance to bacterial infection with dose 3	Diler *et al*. 2016
Cyprinus carpio	*Aer. hydrophila*	*Z. multiflora*	NA†	0.03, 0.06 and 0.12 / 8 days / immunomodulatory effect in fish fed 0.03	Soltani *et al*. 2010
V. labeo and *L. victorianus*	*Aer. hydrophila*	*U. dioica*	NA†	10, 20 and 50 / 112 days / delayed mortality	Ngugi *et al*. 2015
O. mykiss	*Aer. salmonicida*	Digestarom®[2]	NA†	0.2 / 175 days / increased resistance to bacterial infection and reduced mortality in infected fish from 37 to 18%.	Menanteau-Ledouble *et al*. 2015
O. niloticus	*L. garvieae*	*A. spinosa*	NA†	5, 10 and 20 / 45 days / increased survival in fish receiving doses 10 and 20 and immunomodulatory effect	Baba *et al*. 2017

Edw., Edwardsiella; Ent., Enterobacter or Enterococcus; Aer., Aeromonas; B., Bacillus; Cit., Citrobacter; E., Escherichia; Fl., Flavobacterium; Kl., Klebsiella; L., Lactococcus; L., Listeria; Salm., Salmonella; Ser., Serratia; Staph., Staphylococcus; Strep., Streptococcus; V., Vibrio; Y., Yersinia. [1]commercial product containing natural EO of *Origanum heracleoticum*, [2]commercial product containing carvacrol, thymol, anethol, and limonene. NA†: data not available.

4.2 ARTIGO 2

The antibacterial and physiological effects of pure and nanoencapsulated *Origanum majorana* essential oil on fish infected with *Aeromonas hydrophila*.

The antibacterial and physiological effects of pure and nanoencapsulated *Origanum majorana* essential oil on fish infected with *Aeromonas hydrophila*

Jessyka Arruda da Cunha[a], Cecília de Ávila Scheeren[b], Viviane Pedroso Fausto[c], Larissa Daiane Willrich de Melo[d], Bruno Henneman[d], Clarissa Piccinin Frizzo[d], Rodrigo de Almeida Vaucher[e], Agueda Castagna de Vargas[f], Bernardo Baldisserotto[a,g]*

[a]Graduate Program in Pharmacology, Universidade Federal de Santa Maria (UFSM), 97105-900 Santa Maria, RS, Brazil

[b]Pharmacy Course, UFSM, Santa Maria, RS, Brazil

[c] Graduate Program in Nanoscience, Centro Universitário Franciscano, Santa Maria, RS, Brazil

[d]Center for Analysis and Research Organic, UFSM, Santa Maria, RS, Brazil

[e]Center for Chemical, Pharmaceutical and Food Science, Universidade Federal de Pelotas, Pelotas, RS, Brazil

[f]Department of Preventive Veterinary Medicine, UFSM, Santa Maria, RS, Brazil

[g]Department of Physiology and Pharmacology, UFSM, 97105-900 Santa Maria, RS, Brazil

*Corresponding author. Phone 55 55 3220-9382; fax 55 55 3220-8142.

Email address: bbbaldisserotto@hotmail.com (B. Baldisserotto).

Abstract

This study evaluated the antibacterial activity of *Origanum majorana* essential oil (EOM) and nanocapsules of this oil (NOM) in silver catfish, *Rhamdia quelen*, infected with *Aeromonas hydrophila*, and addressed their effects on silver catfish hematological and metabolic parameters. Fish were inoculated with *A. hydrophila* (360 µL, at a concentration of 1.5×10^9 CFU mL^{-1}) and submitted to 1 h daily baths with EOM (0 (control), 20 or 30 µL L^{-1}), NOM (0 (control), 5 or 10 µL L^{-1}) or a positive control containing florfenicol (30 µL L^{-1}) called group Maxflor® for five consecutive days. All treatments improved the survival rate of the infected fish, but we suggest the treatment of *A. hydrophila* infections through daily baths with 20 µL L^{-1} EOM or 5 µL L^{-1} NOM for five consecutive days as these were the lowest effective concentrations tested. Silver catfish treated with EOM and NOM had higher lymphocyte levels, indicating stimulation of the immune system in these fish. The lowest liver glucose level was found in the group treated with the lowest concentration of NOM, and the lactate values in the liver and muscle of all groups were within the normal values reported for this species. In addition, nanocapsules required much less EOM to elicit effective antibacterial treatment.

Keywords: Marjoram; Nanotechnology; *Rhamdia quelen*; Silver catfish.

1. Introduction

In fish farming, one of the pathogens that causes the most concern belongs to the genus *Aeromonas* [1]. These bacteria can be found in various types of animal and vegetable products such as fish, meat, and meat products or any kind of food that has come into contact with water [1]. *Aeromonas hydrophila* is the most frequent *Aeromonas* species to cause disease in fish. Animals infected by this Gram-negative bacillus have clinical signs that may vary from skin lesions to septicemia [2]. The use of antibiotics to deal with this bacterium has been prohibited by the European Union since 2006 due to the risks of creating resistant strains, bioaccumulation, and environmental pollution [3]. Alternatives that can minimize and/or inhibit the action of these pathogens in fish without causing environmental damage are natural

products like essential oils (EOs), which are a mixture of functionally complex and volatile molecules characterized by a strong odor synthesized during secondary metabolism in plants. They are usually extracted from plants that grow in warm countries, such as in the Mediterranean and the tropics, and represent an important part of the traditional pharmacopoeia [4]. The *Origanum* genus (Lamiaceae) includes 23 plant species known for their aromatic properties, which makes them useful in disinfectants, flavoring agents, and perfumery [5]. It has been popular to use *Origanum majorana* to treat asthma, indigestion, headaches, and rheumatism [6]. Myrcene, *γ*-terpinene, *α*-terpinene, *p*-cymene, borneol, thymol, carvacrol, *β*-caryophyllene, limonene, *α*-pinene, *β*-pinene, linalool, and sabinene are some of the components that may be found in the EO of *O. majorana* – EOM [7]. The EOM has antibacterial [8,9], antifungal [10], insecticide [8], and antioxidant activities [11]. Some studies relate the antimicrobial activity of EOM to its phenolic compounds, like thymol and carvacrol [12]. Other authors attribute the higher antibacterial activity and broad-spectrum activity of EOM to other compounds such as terpinen-4-ol, sabinene, *γ*-terpinene, and linalool [13].

EOs may have limited use in aquaculture due to their low solubility in water, low stability, and volatilization. A solution to these problems could be the application of nanotechnology to EOs [14]. Fish culturing could be revolutionized by the use of nanotechnology as a new prophylactic tool to enhance the ability of fish to absorb drugs, lipophilic bioactive components, antibodies, DNA vaccines, and hormones [15,16]. Among the various vectors available in nanotechnology, this study focuses on the use of nanocapsules containing a compound dissolved in an oily core surrounded by a thin polymeric shell [16]. Nanocapsules have been shown to increase the therapeutic index of several drugs [17]. The use of nanocapsules improved the antioxidant and antimicrobial effects of chitosan and maltodextrin in the conservation of tuna, *Euthynnus affinis* [18], and improved the antioxidant effects of lipoic acid for common carp, *Cyprinus carpio* [19]. The use of nanoparticles potentiated the antimicrobial effect of the EO of *Melaleuca alternifolia* against several *Candida* species through the inhibition of biofilm formation as well as a reduction in the levels of proteins and exopolysaccharides [20].

Therefore, the aim of this study was to evaluate the antibacterial effects of pure EOM and nanocapsules of EO (NOM) through daily bathing of silver catfish, *Rhamdia quelen*, infected with *A. hydrophila*.

2. Materials and methods

2.1. Evaluated drugs

EOM (Vimontti) was obtained commercially. A positive control was performed using the commercial antibiotic Maxflor® (Virbac), which consists of 40% florfenicol.

2.2. Nanocapsule preparation

NOM (n = 3) were prepared by interface deposition on a preformed polymer. Briefly, an organic phase containing EOM (0.9 g), sorbitan monostearate (0.192 g), polycaprolactone (0.25 g), and acetone (67.0 mL) was added to an aqueous solution (133.0 mL) containing polysorbate 80 (0.192 g) and kept under moderate magnetic stirring for 10 min. Medium chain triglycerides (0.9 g) were added to these suspensions. The organic solvent was then removed from both suspensions using a rotary evaporator (Fisatom, São Paulo, Brazil) at 60 rpm at 30–35 °C [21]. The final volume of the formulation was fixed at 25 mL to obtain a concentration of 3.6% EO. Determination of the average particle diameter and the polydispersity index (PDI) was performed using dynamic light scattering (photon correlation spectroscopy) after a 500× dilution in Milli-Q water. The zeta potential was checked by electrophoresis after making a 500× dilution in 10 nM NaCl. All tests were performed in a Malvern Instruments® Zetasizer Nano ZS. The measurements were performed in triplicate on three different lots.

2.3. Animals and water parameters

Juvenile *R. quelen* (voucher UFRGS, 20413) (39.16 ± 9.63 g and 16.30 ± 1.54 cm) were obtained from a local supplier in the city of São João do Polêsine, RS, Brazil. The silver catfish were transferred to the Fish Physiology Laboratory and kept in three continuously aerated 250 L tanks, at a stocking density of 25.2 kg m^{-3}, for 7 days. The animals were fed once daily during the acclimation period with a commercial diet containing 42% crude protein (Supra®, Brazil). The feed was discontinued 24 h prior to testing.

The water quality parameters were measured throughout acclimatization and the trial period. Dissolved oxygen (7.09 ± 0.76 mg L^{-1}) and temperature (22.06 ± 0.06 °C) were measured

with a YSI5512 oxygen meter. The pH (7.64 ± 0.10) was verified with an AT 315 pH meter (Alfakit Ltda, Florianopolis, Brazil). Total ammonia (0.60 ± 0.18 mg L^{-1}) and un-ionized ammonia (0.0031 ± 0.01 mg L^{-1}) were determined as described in previous studies [22,23]. Temperature, pH, and dissolved oxygen were monitored daily and total ammonia was measured every 5 days. The experimental protocols were carried out in accordance with the guidelines approved by the Ethics Committee on Animal Experiments of UFSM (process number 074/2014).

2.4. Aeromonas hydrophila inoculation

Fish were inoculated with *A. hydrophila* (volume 360 μL, inoculum concentration of 1.5 × 10^9 CFU mL^{-1}) and submitted to 1 h daily baths with EOM (20 or 30 μL L^{-1}), NOM (5 or 10 μL L^{-1}), or Maxflor$^®$ (30 μL L^{-1}) for five consecutive days. There was also an infected control group inoculated with *A. hydrophila* and not treated with any compound (each group: n = 9 for each triplicate, total 162 fish). The method of treatment through baths of 1 h for five consecutive days was based on the methodology used by [24]. For all experiments, after inoculation the fish were transferred to 20 L plastic aquaria with a semi-static water recirculation system and UV light. The concentration of the *A. hydrophila* inoculum chosen for this study was based on the lethal concentration previously reported for this species of fish. The inoculum of *A. hydrophila* was confirmed by sequencing of its 16S rRNA gene [25]. The concentration of the *A. hydrophila* inoculum and the period of survival analysis of the animals were chosen based on another study that identified as lethal a concentration eight times lower than the concentration of *A. hydrophila* used in the present study. Survival in the study on which the authors based development of the methodology was evaluated over four days, it being possible to visualize a decrease in the survival of freshwater fish *Arapaima gigas* on the first day after the inoculation; in this way the authors found it better to evaluate survival for 5 days in order to avoid loss of data in the survival analysis [26].

2.5. Blood collection and analysis

Six fish from each treatment group (or the surviving fish) from both experiments were selected randomly on the sixth day of the experiment. The fish were anesthetized with eugenol (50 mg L^{-1}), and blood (0.5-1 mL of each fish) was collected from the caudal vein with heparinized syringes. Analysis of hematological parameters was performed using an XS-800i automatic counter (Symex$^{®}$) [27]. In order to minimize any errors, slides stained with Wright–Giemsa stain were used to count erythrocytes and leukocytes in a Neubauer chamber.

2.6. Determination of metabolites

After collecting blood, fish were euthanized by sectioning the spinal cord. Liver and muscle were collected and frozen at −20 °C. The collected tissues were weighed on a precision scale (25 mg and 50 mg of liver and muscle, respectively) and homogenized with 1 mL 10% trichloroacetic acid using a TURRAX homogenizer (Marconi Equipment for Laboratory Industry Ltda). The homogenates were then centrifuged at 3000 rpm for 5 min and the supernatants were used to determine metabolic parameters. Glucose was measured in the liver and muscle as previously described [28] and lactate was measured as in a previous study [29].

2.7. Analysis of chemical constituents

Analysis by gas chromatography coupled to mass spectrometry total ion chromatogram (TIC) was conducted using an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass selection detector using a DB-5 capillary column (with a film thickness of $30 \times 0.25 \times 2.5$ mm) and ionization energy of mass spectrometry (EI-MS) of 70 eV. The operating conditions were as follows: division input 1:100; temperature program 60–325 °C at 4 °C min^{-1}; the carrier gas, helium, was injected at 1 mL min^{-1}; and the detector temperature was 250 °C. The components of each sample were identified by comparing their mass spectrum to a mass spectra library [30].

2.8. Statistical analysis

The homogeneity of variances between groups was verified using the Levene test. Comparisons between different groups were made using one-way ANOVA and Tukey's post hoc test or a Kruskal–Wallis test when appropriate (Statistica 7.0 software). Fish survival was compared using a Kaplan–Meier analysis with a LogRank post-test (SigmaPlot Software). The minimum significance level was 95% ($P = 0.05$).

3. Results

3.1. Chemical composition

Terpinen-4-ol was identified as the major constituent of the EOM, followed by cis-terpinene and terpineol (Table 1). Identification of the NOM components showed that eudesmol, β-citronellal and 3,7-dimethyl-2,6-octadien-1-ol were the main constituents (Table 2).

Compound	% (relative)	RT (min)
R-Pinene	0.649	5.025
Sabinene	6.958	5.457
Myrcene	2.115	5.566
2-Carene	7.666	5.937
o-Cymene	3.278	6.022
Terpinyl acetate	2.080	6.076
Phellandrene	2.051	6.114
Terpinene	13.136	6.380
Terpineol, cis	4.121	6.525

Terpinolene	2.913	6.682
Linalool	0.947	6.778
Terpineol, trans	12.668	6.874
4-Isopropyl-1-methyl-2-cyclohexen-1-ol	2.474	7.117
Terpinen-4-ol	20.555	7.729
a-Terpineol	4.396	7.847
Linalyl anthranilate	1.141	8.260
β-caryophyllene	2.643	10.054
Elixene	1.852	10.692
Total identified compounds	91.643	

The names of the compounds were suggested by [30]. RT: retention time.

Table 1. Chemical compounds in *Origanum majorana* essential oil (EOM) as quantified by gas chromatography coupled to mass spectrometry (GC-MS).

Compound	% (relative)	Tr (min)
β-citronellal	7.913	8.098
3,7-dimethyl-2,6-octadien-1-ol	6.247	8.374
Germacrene-D	0.412	9.621
β-bourbonene	0.953	9.714
β-caryophyllene	0.648	10.052
1,1,7-Trimethyl-4-methylenedecahydro-1H-cyclopropa[e]azulene	0.385	10.667
Torreyol	0.786	10.825
Geranyl isobutyrate	0.303	11.033
Cedranoxide, 8,14	0.556	11.179
Spathulenol	1.036	11.384
Caryophylene oxide	0.970	11.448
Cubenol	0.791	11.664
Eudesmol	8.437	11.764
Agaruspirol	0.881	11.835
β-eudesmol	1.426	12.011
Widdrol hydroxyether	1.091	12.107
Geranyl tiglate	1.645	12.142
1-Methylene-2b-hydroxymethyl-3,3-dimethyl-4b-(3-methylbut-2-enyl)-cyclohexane	0.268	12.447

9-(1-Methylethylidene)bicyclo[6.1.0]nonane	0.424	13.225
Ethanol, 2-(3,3-dimethylcyclohexylidene)	0.362	13.873
3-[2-(6-Bromo-2-hydroxy-2,5,5,8a-tetramethyldecahydro-1-naphthalenyl)ethyl]-3-butene-1,2-diol	0.327	14.032
14-Isopropyl-3,7,11-trimethyl-2,6,10-cyclotetradecatrien-1-one	0.198	14.448
,3,7,11-Tetramethyltricyclo[5.4.0.0(4,11)]undecan-1-ol	1.161	19.294
1,5,9,9-Tetramethyl-2-oxatricyclo[6.4.0.0(4,8)]dodecane	1.752	21.711
Total identified compounds	38.972	

The names of the compounds were suggested by [30]. RT: retention time.

Table 2. Chemical compounds in nanocapsules of *Origanum majorana* essential oil (NOM) as quantified by gas chromatography coupled to mass spectrometry (GC-MS).

3.2. Survival

Water parameters measured throughout acclimation and the experiments did not show any significant differences. Throughout the experiments, fish from the infected control group did not feed normally. Furthermore, they showed deep and extensive ulcerative lesions arising from the *A. hydrophila* infection. These same manifestations and lesions were not found in groups treated with EOM, NOM and the Maxflor® antibiotic during the evaluation period of these animals 0-96 h (5 days). All fish submitted to daily baths with EOM or NOM showed

significantly higher survival rates than the control fish, and survival rates were similar to those treated with Maxflor® (Fig. 1).
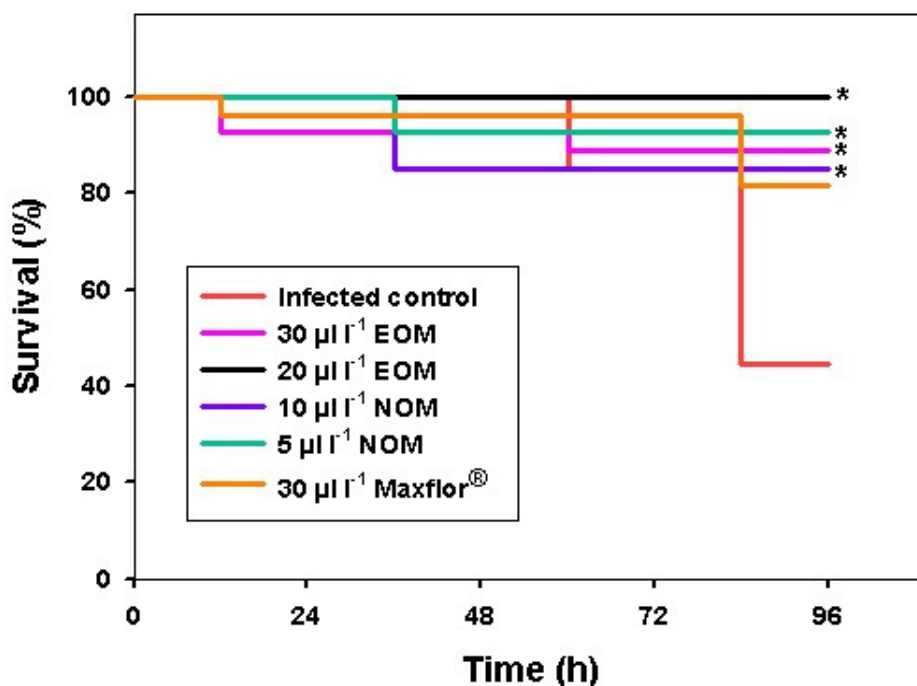
Fig. 1. Survival of *Rhamdia quelen* inoculated with 1.5×10^8 CFU mL^{-1} of *Aeromonas hydrophila* and treated with different concentrations of *Origanum majorana* essential oil (EOM) or nanoencapsulated *Origanum majorana* essential oil (NOM) through 1 h daily baths for five consecutive days. Analysis was carried out through LogRank Kaplan–Meier post-test. *: significant difference (P = 0.05) compared to the control group.

3.3. Hematological parameters

The red blood cell and hemoglobin values in the fish treated in baths with EOM or NOM were similar to those from the Maxflor® group and higher than the levels found in the infected control group. The total number of leucocytes and lymphocytes was higher and heterophils lower in the treated groups compared to the infected control fish. The levels of monocytes

were lower in fish treated with 5 µL NOM, while eosinophils were lower in fish treated with EOM and NOM compared to infected control fish (Table 3).

Parameter	Infected control	EOM (30 µL)	EOM (20 µL)	NOM (10 µL)	NOM (5 µL)	Maxflor® (30 µL)
Erythrocytes $(10^6\ \mu L^{-1})$	0.05 ± 0.01^b	2.41 ± 0.19^a	2.43 ± 1.72^a	3.82 ± 0.24^a	4.00 ± 0.04^a	3.33 ± 0.01^a
Hemoglobin $(g\ dL^{-1})$	1.45 ± 0.27^b	5.08 ± 1.10^a	5.30 ± 3.05^a	8.15 ± 0.60^a	5.23 ± 1.03^a	5.2 ± 0.01^a
Total leukocytes $(10^3\ \mu L^{-1})$	4.67 ± 1.10^c	23.92 ± 10.64^b	43.00 ± 0.23^a	35.49 ± 16.70^b	49.5 ± 4.8^a	34.50 ± 0.02^b
Lymphocytes $(10^3\ \mu L^{-1})$	70.09 ± 0.31^e	88.22 ± 1.3^c	88.66 ± 0.4^c	93.21 ± 0.09^b	97.00 ± 0.29^a	81.95 ± 0.45^d

Heterophils

(10^3 μL^{-1})	15.8 ± 0.92^a	2.25 ± 0.2^b	3.05 ± 2.1^b	2.16 ± 1.30^b	2.01 ± 0.30^b	2.97 ± 0.28^b

Monocytes

(10^3 μL^{-1})	10.06 ± 0.78^{ab}	8.8 ± 3.5^b	7.1 ± 1.9^b	4.02 ± 0.08^{bc}	0.90 ± 0.16^c	13.09 ± 0.12^a

Eosinophils

(10^3 μL^{-1})	3.96 ± 0.21^a	$0.73\pm0.01c$	1.19 ± 0.02^b	0.79 ± 0.01^c	0.09 ± 0.11^d	1.99 ± 0.07^{ab}

Different letters indicate significant differences (P = 0.05) between groups 5 days after inoculation. The results represent the mean ± standard error of the mean (SEM) for each group.

Table 3. Hematological parameters of silver catfish inoculated with 1.5×10^9 CFU mL^{-1} of *Aeromonas hydrophila* and submitted to 1 h daily baths in *Origanum majorana* essential oil (EOM) or nanocapsules of this oil (NOM) for 5 days.

3.4. Metabolites

Silver catfish treated in baths with 20 and 30 µL L^{-1} EOM and 10 µL L^{-1} NOM presented higher glucose levels in the liver compared to untreated (control) fish, while hepatic lactate levels and both parameters in muscle were not significantly affected by the baths when compared to infected control fish (Table 4).

Metabolite	Infected	NOM	NOM	Maxflor®	EOM	EOM

	control	(10 µL)	(5 µL)	(30 µL)	(30 µL)	(20 µL)
Liver						
Glucose	2.56 ± 0.28^{c}	3.55 ± 0.10^{b}	1.58 ± 0.22^{c}	1.46 ± 0.15^{bc}	3.75 ± 0.26^{b}	4.65 ± 0.92^{a}
Lactate	1.02 ± 0.06^{a}	1.08 ± 0.08^{a}	0.98 ± 0.14^{a}	1.00 ± 0.02^{a}	1.25 ± 0.13^{a}	1.44 ± 0.17^{a}
Muscle						
Glucose	4.40 ± 0.52^{ab}	5.67 ± 0.49^{a}	3.43 ± 0.38^{b}	4.62 ± 0.12^{ab}	3.86 ± 0.37^{ab}	3.93 ± 0.56^{ab}
Lactate	3.17 ± 0.19^{ab}	3.09 ± 0.27^{ab}	2.85 ± 0.23^{ab}	2.52 ± 0.08^{b}	2.65 ± 0.05^{b}	3.71 ± 0.32^{a}

The results represent the mean $\pm$ SEM for each group (n = 6). Different letters indicate significant differences (P = 0.05) between groups at 96 h (5 days). Lactate and glucose levels were expressed in µmol g tissue^{-1}.

Table 4. Hepatic and muscle parameters of silver catfish inoculated with 1.5×10^{9} CFU mL^{-1} of *Aeromonas hydrophila* and submitted to 1 h daily baths in *Origanum majorana* essential oil (EOM) or nanocapsules of this oil (NOM) for 5 days.

4. Discussion

Vegetable extracts can be immunostimulatory, antibacterial, and anti-parasitic as they can contain active compounds such as alkaloids, terpenoids, saponins, and flavonoids [31]. In this study, the major compound in EOM was terpinen-4-ol, which is in line with previous studies [32]. This compound is a monoterpene with antibacterial and antifungal activities [33]. Eudesmol, the major component of NOM, is an alcoholic sesquiterpene, which generally protects against bacteria and fungi [34]. This compound was effective *in vitro* against *A. hydrophila*, *Yersinia ruckeri*, and *Streptococcus iniae* [35]. Plant products have been widely used in disease control in aquaculture as an alternative to chemical treatments [31]. However, NOM was more effective than EOM against *A. hydrophila* infection in silver catfish, as lower NOM concentrations in the baths were needed to improve the survival of fish infected with

bacteria. It is important to emphasize that the amount of EOM used to produce NOM was 3.6% of the total NOM composition, demonstrating that the use of nanocapsules gave similar antimicrobial effects to the pure form, but used 50-fold less EOM. This result can be attributed to the slow release of nanoencapsulated compounds [16].

This result is in agreement with *in vivo* experiments demonstrating the improved antimicrobial activity of encapsulated systems [36]. This can be explained by the fact that *A. hydrophila* is a Gram-negative bacterium, i.e. it has a thicker outer membrane layer of hydrophilic lipopolysaccharide, resulting in a lower antimicrobial susceptibility than Gram-positive bacteria. This structural feature creates a barrier to macromolecules and hydrophobic compounds, such as those found in EOs, hindering their antimicrobial activity [37]. The hydrophobic property of EOs is minimized when they are stabilized with surfactants and dispersed in water, such as in nanostructured systems [38]. Some of the active compounds of EOs, such as terpenes, may exhibit low absorption and poor solubility in water, reducing bioavailability and efficacy. Nanostructured systems have the potential to increase or decrease the release of active substances and can assist in the transportation of the molecule to its biological target [39]. Nanocapsules are formed using a polymeric film around an oily nucleus, and the active substance may be dissolved in the nucleus and/or adsorbed in the polymeric wall. These systems have been developed to increase the efficiency of lipophilic substances [17]. Consequently, these nanocapsules easily penetrate the skin of animals, which explains the more effective antibacterial activity of NOM when compared to EOM.

Measurements of hematological parameters are useful to assess fish health. Significantly higher values of heterophils, monocytes, and eosinophils in the untreated infected fish than in the treated fish can be attributed to the bacterial infection. Similar data was reported in animals infected with *Enterococcus* sp. [40]. The remaining infected and non-treated (control) fish in the bath experiment were anemic as expected, because *A. hydrophila* produces proteases that induce erythrocyte hemolysis [40]. All hematological parameters evaluated in the present study were similar to the values of silver catfish experimentally infected with the protozoan *Ichthyophthirius multifiliis* and submitted to 1 h daily baths with EO of *Hyptis mutabilis* or (-) - globulol for four days. The fish treated with the EO of *H. mutabilis* or the compound alone also presented higher values of total leukocytes, hemoglobin, erythrocytes, lymphocytes, heterophils, monocytes, and eosinophils than fish of the control group [27].

Silver catfish treated with EOM and NOM had higher lymphocyte levels, indicating stimulation of the immune system in these animals. The higher survival rate of infected silver catfish treated with the lowest NOM concentration can be attributed to the fact that lymphocyte levels increased and, consequently, defense against infectious agents increased. The immunostimulating capacity of the constituents studied may be linked to the increase in lymphocyte levels and the decrease in heterophil levels, since lymphocytes are cells involved in the production of immunoglobulins and modulation of defense, whereas heterophils are the primary phagocytic leukocytes, which proliferate in the circulation in response to infections, inflammation, and stress [41]. Thus, bathing the fish in 5 µL L^{-1} NOM daily for 5 days may have acted as an immunostimulant and increased the innate immune system response of silver catfish against infection.

The glucose levels of the treated groups did not present significant differences in relation to the control. The infected control group had the lowest levels of glucose in the liver and muscle, which can be attributed to the high bacterial load in these animals. A study of common carp infected with *A. hydrophila* also demonstrated low levels of plasma glucose in the untreated fish [42].

The lowest liver glucose level was found in the group treated with the lowest concentration of NOM. However, the value did not differ from the infected control fish and there are no studies correlating a specific bacterial infection with glucose and lactate levels in the liver and muscle in silver catfish. In general, lactate values in the liver and muscle of all groups were within the normal values reported for this species [43].

5. Conclusion

All treatments improved the survival of infected fish, but we suggest that *A. hydrophila* infections are treated with daily baths containing 20 µL L^{-1} EOM or 5 µL L^{-1} NOM for five consecutive days because these are the lowest effective concentrations tested which did not interfere with the metabolic parameters of the animals. We also emphasize the advantage of the use of nanocapsules because the amount of EOM necessary to obtain effective antibacterial treatment was much lower, besides it being possible to observe an immunostimulatory effect triggered by treatment with the lowest concentration of NOM.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

Funding

References

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

[2] J.M. Janda, S.L. Abbott, The genus *Aeromonas*: taxonomy, pathogenicity, and infection, Clin. Microbiol. Rev. 23 (2010) 35-73.

[3] R. Harikrishnan, C. Balasundaram, Modern trends in *Aeromonas hydrophila* disease management with fish, Rev. Fish. Sci. 13 (2005) 281-320.

[4] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, Biological effects of essential oils – A review, Food Chem. Toxicol. 46 (2008) 446–475.

[5] R. Erenler, O. Sen, H. Aksit, I. Demirtas, A.S. Yaglioglu, M. Elmastas, I. Telci, Isolation and identification of chemical constituents from *Origanum majorana* and investigation of antiproliferative and antioxidant activities, J. Sci. Food Agric. 96 (2015) 822-36.

[6] W.J. Jun, B.K. Han, K.W. Yu, M.S. Kim, I.S. Chang, H.Y. Kim, H.Y. Cho, Antioxidant effects of *Origanum majorana* L. on superoxide anion radicals, Food Chem. 75 (2001) 439–444.

[7] F. Fratini, S. Casella, M. Leonardi, F. Pisseri, V.V. Ebani, L. Pistelli, Pistelli L, Antibacterial activity of essential oils, their blends and mixtures of their main constituents against some strains supporting livestock mastitis, Fitoterapia. 96 (2014) 1–7.

[8] J. M. Freire, M. G. Cardoso, L.R. Batista, M. A. Andrade. Essential oil of *Origanum majorana* L., *Illicium verum* Hook. F. and *Cinnamomum zeylanicum* Blume: chemical and antimicrobial characterization, Rev. Bras. Plantas Med. 13 (2011) 209-214.

[9] C. Valeriano, R.H. Piccoli, M.G. Cardoso, E. Alves. Antimicrobial activity of essential oils in pathogenic food-borne bacteri, Rev. Bras. Plantas Med.14 (2012) 57-67.

[10] R. Santin, C. Giordani, I.M. Madrid, C.B. Matos, R.A. Freitag, M.C.A. Meireles, M.B. Cleff, J.R.B. Mello. Antifungal activity of *Origanum vulgare* essential oil against *Malassezia pachydermatis*, Arq. Bras. Med. Vet. Zootec. 66 (2014) 367-373.

[11] W. J. Jun, B. Han, K. Yu, M. Kim, I. Chang, H. Kim, H. Cho. Antioxidant effects of *Origanum majorana* L. on superoxide anion radicals, Food Chem. 75 (2011) 439-444.

[12] L. Nedorostova, P. Kloucek, L. Kokoska, M. Stolcova, J. Pulkrabek, Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria, Food Control. 20 (2009) 157–160.

[13] I.H. Sellami, E. Maamouri, T. Chahed, W.A. Wannes, M.E. Kchouk, B. Marzouk, Effect of growth stage on the content and composition of the essential oil and phenolic fraction of sweet marjoram (*Origanum majorana L.*), Ind. Crop Prod. 30 (2009) 395-402.

[14] B. Heurtault, P. Saulnier, B. Pech, J.E. Proust, J.P. Benoit, Physico-chemical stability of colloidal lipid particles, Biomaterials 24 (2013) 4283–4300.

[15] M.A. Rather, R. Scharma, M. Aklakur, N. Kumar, M. Khan, V.L. Ramya, Nanotechnology: A novel tool for aquaculture and fisheries development. A prospective mini-review, Int. J. Fish. Aquac. (2011) FAJ-16. Doi: 10.4172/2150-3508.1000016.

[16] C. Vauthier, K. Bouchemal, Methods for the preparation and manufacture of polymeric nanoparticles, Pharmac. Res. 26 (2009) 726-737.

[17] S.R. Schaffazick, S.S. Guterres, L.L. Freitas, A.R. Pohlmann, Characterization and physical-chemical stability of polymer nanoparticulate systems for drug administration, Quím. Nova. 26 (2003).

[18] S. Saloko, P. Darmadji, B. Setiaji, Y. Pranoto, Antioxidative and antimicrobial activities of liquid smoke nanocapsules using chitosan and maltodextrin and its application on tuna fish preservation, Food Biosci.7 (2014) 71–79.

[19] M. Longaray-Garcia, J.A. Flores, I.C. Külkamp-Guerreiro, S.S. Guterres, T.C. Pereira, M.R. Bogo, J.M. Monserrat, Modulation of antioxidant and detoxifying capacity in fish

Cyprinus carpio (Cyprinidae) after treatment with nanocapsules containing lipoic acid, Comp. Biochem. Physiol. 165 (2013) 468–47.

[20] M.E. Souza, L.Q.S. Lopes, P.C. Bonez, A. Gündel, D.S.T. Martinez, M.R. Sagrillo, J.L. Giongo, R.A. Vaucher, R.P. Raffin, A.A. Boligon, R.C.V. Santos, *Melaleuca alternifolia* nanoparticles against Candida species biofilms, Microb. Pathog. 104 (2017) 125-132.

[21] J.L. Giongo, R.A. Vaucher, D. Borin, M.S. Côrre, V.B.D. Santos, R.C.V. Santos, A.A. Boligon, M.L. Athayde, P.C. Bonez, G.G. Rossi, M. Campos, P. Gomes, M. Steppe, Antimycobacterial, antimicrobial and antifungal activities of geranium oil-loaded nano capsules, Int. J. Pharm. Pharm. Sci. 7 (2015) 414-419.

[22] J. Colt, List of spreadsheets prepared as a complement to the book fish hatchery management, in: G.A. Wedemeyer (Ed.), Fish Hatchery Management, American Fisheries Society Publications, Bethesda, 2002.

[23] H. Verdouw, C.J.A. Vanechted, E.M.J. Dekkers, Ammonia determination based on indophenol with sodium salicylate, Water Res. 12 (1978) 399-402.

[24] F.J. Sutili, L.L. Silva, L.T. Gressler, L.T. Gressler, E.K. Battisti, B.M. Heinzmann, A.C Vargas, B. Baldisserotto, Plant essential oils against *Aeromonas hydrophila*: *in vitro* activity and their use in experimentally infected fish, J Appl Microbiol. 119 (2015) 47-54.

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

[26] M.K.R. Dias, L.S. Sampaio, A.A. Proietti-Jr., E.T.O. Yoshioka, D.P. Rodrigues, A.F.R. Rodrigues, R.A. Ribeiro, F.S.E.D.V. Faria, R.O.A. Ozório, M. Tavares-Dias, Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon, Vet. Microbiol. 188 (2016) 12-15.

[27] J.A. Cunha, F.J. Sutili, A.M. Oliveira, L.T. Gressler, C.A. Scheeren, L.L. Silva, R.A. Vaucher, B. Baldisserotto, B.M. Heinzmann, The Essential Oil of *Hyptis mutabilis* in *Ichthyophthirius multifiliis* Infection and its Effect on Hematological, Biochemical, and Immunological Parameters in Silver Catfish, *Rhamdia quelen*, J. Parasitol. 103 (2017) 778-785.

[28] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–358.

[29] J.R. Harrower, C.H. Brown, Blood lactic acid. A micromethod adapted to field collection of microliter samples, J. Appl. Physiol. 32 (1972) 709-711.

[30] NIST, NIST/EPA/NIH, Mass spectral library and search analysis programs, John Wiley and Sons, Hoboken, USA, 2005.

[31] M. Reverter, N. Bontemps, D. Lecchini, B. Banaigs, P. Sasal, Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives, Aquaculture. 433 (2014) 50-61.

[32] E. Vági, B. Simandí, Á. Suhajda, É. Héthelyi, Essential oil composition and antimicrobial activity of *Origanum majorana* L. extracts obtained with ethyl alcohol and supercritical carbon dioxide, Food Res. Int. 38 (2005) 51-57.

[33] R.C.V. Santos, L.Q.S. Lopes, C.F.S. Alves, V.P. Fausto, K. Pizzutti, V. Barboza, M.E. Souza, R.P. Raffin, P. Gomes, D. Takamatsu, Y. Morinaga, A.A. Boligon, M.L. Athayd, C.C. Felippi, R.A. Vaucher, Antimicrobial activity of tea tree oil nanoparticles against American and European foulbrood diseases agents, J. Asian Pac. Entomol. 17 (2014) 343-347.

[34] R. Verpoorte, Pharmacognosy in the new millennium: Lead finding and biotechnology, J. Pharm. Pharmacol. 52 (2000) 253-262.

[35] A. Tukmechi, A. Ownagh, A. Mohebbat, *In vitro* antibacterial activities of ethanol extract of Iranian propolis (EEIP) against fish pathogenic bacteria (*Aeromonas hydrophila, Yersinia ruckeri & Streptococcus iniae*), Braz. J. Microbiol. 41 (2010) 1086-1092.

[36] J.P. Xue, M. Davidson, Q. Zhong, Antimicrobial activity of thyme oil co-nanoemulsified with sodium caseinate and lecithin, Int. J. Food Microbiol. 210 (2015) 1-8.

[37] K. Bhargava, D.S. Conti, S.R. Rocha, Y. Zhang, Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce, Food Microbiol. 47 (2015) 69-73.

[38] R.E. Anton, J.M. Andérez, C. Bracho, F. Vejas, J.L. Salager, Practical surfactant mixing rules based on the attainment of microemulsion–oil–water three-phase behavior systems, Adv. Polym. Sci. 218 (2008) 83-113.

[39] B.V. Bonifácio, P.B. Silva, M.A. Ramos, K.M. Negri, T.M. Bauab, M. Chorilli, Nanotechnology-based drug delivery systems and herbal medicines: A review, Int. J. Nanomed. 9 (2014) 1-15.

[40] M.L. Martins, J.L.P. Mouriño, G.V. Amaral, F.N. Vieira, G. Dotta, A.M.B. Jatobá, F.S. Pedrotti, G.T. Jerônimo, C.C. Buglione-Neto, G. Pereira-Jr, Hematological changes in Nile tilapia experimentally infected with *Enterococcus* sp., Braz. J. Biol. 68 (2008) 631-637.

[41] A.K. Davis, D.L. Maney, J.C Maerz, The use of leukocytes profiles to measure stress in vertebrates: a review for ecologists, Funct. Ecol, 22 (2008) 760-772.

[42] S.A. Brenden, H.W. Huizinga, Pathophysiology of experimental *Aeromonas hydrophila* infection in goldfish, *Carassius auratus* (L.), J. Fish Dis. 9 (1986) 163-167.

[43] R.M. Harikrishnan, N. Rani, C. Balasundaram, Hematological and biochemical parameters in common carp, *Cyprinus carpio,* following herbal treatment for *Aeromonas hydrophila* infection, Aquaculture. 221 (2003) 41-50.

4.3 ARTIGO 3

The survival and metabolic responses of *Rhamdia quelen* inoculated with *Aeromonas hydrophila* and treated with terpinen-4-ol, carvacrol or thymol.

Publicado no periodico "Microbial Pathogenesis", sendo assim esse artigo encontra-se nas normas do mesmo

The survival and metabolic responses of *Rhamdia quelen* inoculated with *Aeromonas hydrophila* and treated with terpinen-4-ol, carvacrol or thymol

Jessyka Arruda da Cunha[1]; Guerino Bandeira Junior[1]; Elisia Gomes da Silva[1]; Cecília de Ávila Scheeren[2]; Viviane Pedroso Fausto[3]; Joseânia Salbego[1]; Rodrigo de Almeida Vaucher[4];Agueda Castagna de Vargas[5]; Bernardo Baldisserotto[1,6*].

[1] Graduate program in Pharmacology, Universidade Federal de Santa Maria/ UFSM. 97105-900. Santa Maria. RS. Brazil; e-mail: jessyka.arruda.c@hotmail.com; guerino.junior@hotmail.com;josalbego2004@yahoo.com.br; elisia.biomedicina@hotmail.com.

[2]Pharmacy course, UFSM, Santa Maria. RS. Brazil; e-mail: ceciliascheeren@hotmail.com.

[3]Graduate program in Nanoscience, Universidade Franciscana. 97010-032. Santa Maria.RS. Brazil; e-mail: vivi.pfausto@gmail.com

[4]Center for Chemical, Pharmaceutical and Food Science, Universidade Federal de Pelotas, Pelotas, RS, Brazil; e-mail: rodvaucher@hotmail.com

[5]Department of Preventive Veterinary Medicine, UFSM, Santa Maria, RS, Brazil; e-mail: agueda.vargas@gmail.com.

[6] Department of Physiology and Pharmacology, UFSM, Santa Maria, RS, Brazil.

*Corresponding author: UFSM. Av. Roraima, 1000. 97105.900, Santa Maria, RS, Brazil. Phone 55 55 3220-9382. Fax 55 55 3220-8142. Email address: bbbaldisserotto@hotmail.com (B. Baldisserotto).

Abstract

The aim of this study was to evaluate the resistance of *Rhamdia quelen* (silver catfish) to *Aeromonas hydrophila* infection after treatment with pure and nanoencapsulated forms either terpinen-4-ol, thymol, or carvacrol and the effects of these treatments on fish metabolic responses. After *A. hydrophila* inoculation, fish were treated with 30 min daily baths for 6 consecutive days with terpinen-4-ol, thymol, or carvacrol in their pure or nanoencapsulated forms at concentrations of 5, 10, 15 or 25 mg L^{-1}. A positive control group, negative control group and saline group were also included. Survival was evaluated at the end of treatment for six consecutive days. Muscle and liver were collected to determine glucose and lactate levels. The fish treated with the nanoencapsulated form of the compounds had a high survival rate, similar to saline group and negative control groups. The carvacrol, thymol and terpinen-4-ol nanoencapsulated forms improved survival of silver catfish infected with *A. hydrophila*. Muscle and liver glucose and lactate levels are not indicated as biomarkers because they did not present any correlation between the metabolic state of the fish and the bacterial infection.

Keywords: Silver catfish; nanotechnology; nanoencapsulated compounds; bacterial infections.

1. Introduction

Bacterial infections triggered by *Aeromonas hydrophila* have been associated with significant mortality on fish farms, becoming a constant concern to producers [1]. Plant-derived products, such as essential oils, can be used in fish farming as antimicrobial in a prophylactic or therapeutic procedure [2,3]. Essential oils of several plants have terpenic compounds, such as *p*-cymene and *γ*-terpinene. Also, when the plant is exposed to stressful factors, *p*-cymene can be transformed to thymol and carvacrol, while *γ*-terpinene can be transformed to terpinen-4-ol [4].

Thymol [5-methyl-2-(1-methylethyl)-phenol] is structurally very similar to carvacrol [2-methyl-5-(1-methylethyl)-phenol], varying only the position of the hydroxyl group in the phenolic ring [5]. The antibacterial activity of these compounds is attributed to their ability to increase the permeability and depolarize the cytoplasmic membrane of bacteria [6]. Carvacrol depolarizes the membrane of most Gram-positive bacteria, including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus*, depending on the time of exposure and concentration [7]. In addition, the essential oil of *Origanum vulgare* and its main constituents, thymol and carvacrol, inhibit the growth of pre-formed biofilms of *S. aureus* and *S. epidermidis* [8].

Terpinen-4-ol is a monoterpene that can be found in several plant species, including *Alpinia speciosa*, *Alpinia zerumbet*, *Melaleuca alternifolia*, *Origanum majorana*, *Camellia sinensis*, *Myrtus communis*, *Laurus nobilis*, *Croton sonderianus*, and *Eucalyptus globulus* [9]. Previous studies have demonstrated the direct action of terpinen-4-ol as an acaricide [10], antimicrobial [11], anti-inflammatory [12] and local anesthetic [13]. The antibacterial activity of terpinen-4-ol against methicillin-resistant *S. aureus* and also in isolates of *S. epidermidis*, *S. capitis*, *S. lugdunensis*, *S. hominis*, *S. auriculus, S. lentus*, and *S. warner* was reported [14].

The activity of a compound can be enhanced by nanoencapsulation because it increases its absorption by the gills, skin, and digestive system, and has a promising applicability because of its distribution, controlled release, high solubility, and the ability of minimize the risk of producing resistant microorganisms and environmental contamination [15]. Various studies have shown that nanoencapsulated forms have increased activity compared to their pure forms. For example, as the antilisterial activity of thymol in milk [16], the activity of thymol against *Escherichia coli* and *Listeria monocytogenes* [15], and the antimicrobial activity of essential oils [17].

Animals infected with *A. hydrophila* have an impaired physiological state and their cellular and humoral defense processes depend on their metabolism to perform properly. Any alteration in the energy levels can trigger a deleterious effect on the fish organism [18]. Some biochemical changes in the body of the fish after a stressful factor can be measured by the lactate and glucose levels. Here we aimed to evaluate the antibacterial activity of thymol, carvacrol, and terpinen-4-ol (pure and nanoencapsulated forms) and their effects on the survival and metabolic responses of silver catfish (*Rhamdia quelen*) infected with *A. hydrophila*.

2. Materials and methods

2.1. Constituents and commercial antibiotic

Terpinen-4-ol (purity ≥97%), carvacrol (purity ≥99%), and thymol (purity ≥99%) were obtained from Sigma-Aldrich Corporation (St. Louis, United States), and a commercial antibiotic that is 40% florfenicol (Virbac, Brazil) was used as a control.

2.2. Animals and water quality parameters

Juveniles of silver catfish (*Rhamdia quelen*, voucher UFRGS 22661) (male and female, body weight 20.00 ± 0.83 g and total length 15.42 ± 0.74 cm) were obtained from a fish farm in São João do Polêsine city, southern Brazil to carry out this experimental design. The fish were kept in three continuously aerated 200 L aquaria at a stocking density of 21.6 kg m^{-3}, for five days. The animals were fed to satiation once daily during the acclimation and trial periods with commercial feed (42% crude protein, Supra$^{®}$, Brazil). The feed was discontinued 24 h prior to testing.

The water quality parameters were measured daily throughout the acclimation and trial periods and were: temperature, $22.5 \pm 0.1°C$; dissolved oxygen, 6.89 ± 0.04 mg L^{-1} (YSI oxygen meter, Model Y5512, YSI Inc., OH, USA); pH (7.60 ± 0.01) (pH meter, microprocessor AT 315, Alfakit, Florianopolis, Brazil); weekly total ammonia levels (1.95 ± 0.03 mg L^{-1}) determined according to [19]; and un-ionized ammonia (0.049 ± 0.001 mg L^{-1}) calculated using a conversion table for fresh water. The experimental protocol was approved by the Ethics Committee on Animal Experiments of UFSM (process number 074/2014).

2.3. Aeromonas hydrophila inoculum

The *A. hydrophila* inoculum was prepared with a bacterium obtained from a clinical isolate of silver catfish in a Müeller Hilton broth (24 h at 28°C) and was confirmed by sequencing its 16S rDNA fragment. The strain used was submitted to a microbes conservation center number MF 372510.The concentration of inoculum was defined based on a previous study [20], equivalent to lethal concentration (LC$_{50}$).

2.4. Fish inoculation

Fish were anesthetized with 50 mg L^{-1} eugenol before inoculation of 100 μL *A. hydrophila* at a concentration of 1.5×10^9 CFU mL^{-1} and then transferred to 20 L aquaria. The solutions were injected intramuscularly, lateral-dorsal, on the right side with the aid of a syringe for all fish.

2.5. Fish treatment

Immediately after inoculation, the fish were treated with 30 min daily baths for 6 consecutive days with carvacrol, thymol, or terpinen-4-ol in their pure or nanoencapsulated forms at concentrations of 5, 10, 15 or 25 mg L^{-1}). A positive control group (no compound added, inoculated with *A. hydrophila*), control negative group (inoculated with *A. hydrophila*, bathed with 50 mg L^{-1} of commercial antibiotic that is 40% florfenicol), and saline group (no compound added, 100 μL of inoculum of saline) were also included. In all groups n=9 each aquaria, three replicates each. Mortality was registered daily for 6 days.

2.6. Determination of metabolites

Six days after injection of the bacterium, fish that did not present symptoms and external lesions typical of the *A. hydrophila* infection were anesthetized with 50 mg L^{-1} eugenol and after euthanized by section of the spinal cord. Liver and muscle were collected and frozen at -20°C. The collected tissues were weighed on a precision scale (25 and 50 mg of liver and muscle, respectively) and homogenized with 1 mL 10% TCA (trichloroacetic acid) using a Turrax type homogenizer (Marconi Equipment). Then, the homogenates were centrifuged at 1,008 g for 5 min, and the supernatants were used for determination of metabolic parameters. Glucose was measured by the method described by [21], and lactate following [22].

2.7. Statistical analysis

The homogeneity of variances between groups was verified by the Levene test. Comparisons between different groups were made using one-way ANOVA and Tukey post-test or Kruskal-Wallis test followed by multiple comparisons of mean ranks when appropriate (Statistica 7.0 software). Survival rates were compared by Kaplan-Meier analysis with Logrank post-test (SigmaPlot Software). The minimum significance level was 95% ($p < 0.05$).

3. Results

The survival rate of silver catfish inoculated with *A. hydrophila* (positive control) was 33.33%. Fish subjected to the baths with all concentrations of thymol and terpinen-4-ol (pure and nanoencapsulated forms) and control negative group showed survival similar to the saline group and higher than the positive control group, whereas carvacrol was only effective as nanocapsules at 5 mg L^{-1} (Table 1).

Glucose levels in the liver were not significantly affected by the inoculation of *A. hydrophila* but increased in the muscle compared to the saline group. The control negative group baths as well as the pure form of the oils tended to reduce glucose levels in the muscle (Fig. 1). Silver catfish submitted to baths with the pure form of terpinen-4-ol showed higher hepatic glucose levels than the saline and positive control groups, but this was not the case for the nanoencapsulated form of terpinen-4-ol (Fig. 1A). However, when compared to the saline and positive control groups, the glucose levels were reduced significantly in the muscle of fish treated with either form of terpinen-4-ol (Fig. 1D).

Compared to the saline and positive control groups, fish treated with the pure form of thymol had significantly higher glucose levels in the liver at 15 mg L^{-1} and in the muscle at 15 and 25 mg L^{-1}. However, with the nanoencapsulated form of thymol, such an increase was observed only in the livers of fish treated with 5, 10 and 15 mg L^{-1}. In the muscle, we noted a reduction in liver glucose levels when using 25 mg L^{-1} of the nanoencapsulated form of thymol (Fig. 1B, E).

Compared to the saline and positive control groups, fish bathed with the pure form of carvacrol had significantly higher hepatic glucose levels at 5 and 25 mg L^{-1}, whereas the nanoencapsulated form did not change this parameter (Fig. 1C). The muscle glucose levels in fish exposed to the pure form of carvacrol (5 mg L^{-1}) were significantly higher than in the saline and positive control groups, but this trend was not observed when using higher carvacrol concentrations. Silver catfish treated with the nanoencapsulated form of carvacrol were similar to the saline group (Fig. 1F).

Lactate levels increased in the liver (but not in the muscle) of silver catfish inoculated with *A. hydrophila* compared to the saline group in the experiment with the pure form of the compounds. However, in the experiment with the nanocapsules, the same inoculation with *A. hydrophila* reduced lactate levels in the liver. Fish inoculated with *A. hydrophila* and bathed in control negative group had the highest liver lactate levels, but not in the muscle in the

experiment with the pure form of the compounds. However, when using the nanoencapsulated forms, lactate levels increased in the muscle (but not in the liver) of fish inoculated with *A. hydrophila* and bathed with control negative group (Fig. 2).

Silver catfish bathed with the pure form of terpinen-4-ol had higher muscle lactate levels than the saline and positive control groups. The liver and muscle lactate levels of the nanoencapsulated terpinen-4-ol treated fish were similar to those of the saline group (except fish bathed with 25 mg L^{-1} nanoencapsulated terpinen-4-ol) (Fig. 2A, D).

Fish treated with the pure form of thymol had significantly higher liver and muscle glucose levels than the saline and positive control groups. Whereas, in the fish treated with the nanoencapsulated form, the lactate levels in both organs were similar to the saline group (except fish bathed with 25 mg L^{-1}) (Fig. 2B, E). Animals bathed with the pure form of carvacrol (but not the nanoencapsulated form) had significantly higher hepatic lactate levels compared to the saline and positive control groups (Fig. 2C). Muscle lactate levels in fish exposed to the pure form of carvacrol at 5 mg L^{-1} were significantly higher than in the saline and positive control groups, but exposure to higher carvacrol concentrations maintained these levels similar to the saline and positive control groups. Silver catfish treated with 5 and 10 mg L^{-1} of the nanoencapsulated form had lower muscle lactate than the positive control fish. The fish bathed in 25 mg L^{-1} of nanoencapsulated carvacrol had lactate values similar to the positive control (Fig. 2F).

4. Discussion

Here we detected an increase in the survival of silver catfish treated with antibacterial baths immediately after infection. The disposal of all compounds in the nanoencapsulated form improved silver catfish survival. To our knowledge, this is the first report of such a finding. This result probably occurred because nanocapsules enhanced ability to absorb these compounds [23,24]. Terpinen-4-ol performed best, which is in line with its known antibacterial activity and broad action [25]. Although the other tested compounds, thymol and carvacrol, have also been reported as having antibacterial activity against *A. hydrophila*, *Escherichia coli*, *Brochothrix thermosphacta*, *Pseudomonas fragi* [26], *Shigella sonnei* and *Shigella flexneri* [27], in our study, thymol was superior to carvacrol.

The essential oils of *Lippia sidoides* (thymol and carvacrol chemotypes) have an anesthetic effect in silver catfish but were not recommended because they killed the fish

(especially the carvacrol chemotype) [28]. Here, although we used a much lower concentration (but longer exposure) of carvacrol than [28], high mortality was again observed. Pacu (*Piaractus mesopotamicus*) has reduced hepatic glycogen and increased blood glucose levels 24 h after *A. hydrophila* inoculation [29], indicating glycogen breakdown to provide glucose as an energy source. It is possible that this also occurs in infected silver catfish.

However, we found that hepatic glucose did not change in silver catfish infected with *A. hydrophila* compared to the saline group. Sepsis in humans may increase glucose and lactate production in skeletal muscle because it impairs mitochondrial function, which favors anaerobic pathways [30]. Infected silver catfish had higher muscle glucose (in the pure form experiment) and lactate (in the nanocapsules experiment) levels than the saline group, demonstrating that these symptoms may also be present in fish infected with *A. hydrophila*. To our knowledge, there are no published data regarding the compounds tested here and glucose and lactate levels in muscle and liver.

5. Conclusions

The terpinen-4-ol, thymol, and carvacrol nanoencapsulated forms promoted a better survival of silver catfish infected with *A. hydrophila* when compared to the results of the compounds applied in the pure form, thus elucidating a potent antibacterial effect of these nanoencapsulated compounds when applied through baths. In the evaluation of the metabolic response the glucose and lactate levels in the muscle and liver are not indicated, since the healthy animals in comparison to the infected animals do not show difference of result in a constant way.

Conflict of interest

The authors declare no competing financial interests.

C.A.S., J.S., G.B.J., and E.G.S. helped with all experiments; V.P.F. assisted with the nanocoating; R.A.V. participated in the interpretation of the data and manuscript corrections; G.B.J., A.C.V., and B.B. reviewed the manuscript. The all authors have none conflict of interest.

Funding

References

[1] E. Goldschmidt-Clermont, T. Wahli, J. Frey, S.E. Burr, Identification of bacteria from the normal flora of perch, *Perca fluviatilis* L., and evaluation of their inhibitory potential towards Aeromonas species, J Fish Diseases, 31 (2008) 353-359. https://doi:10.1111/j.1365-2761.2008.00912.x

[2] R. Harikrishnan, M. Nisha Rani, C. Balasundaram, Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection, Aquaculture, 221 (2003) 41–50. https://doi:10.1016/S0044-8486(03)00023-1

[3] F.J. Sutili, L.L. Silva, L.T. Gressler, L.T. Gressler, E.K. Battisti, B.M Heinzmann, A.C. de Vargas, B. Baldisserotto, Plant essential oils against *Aeromonas hydrophila*: *in vitro* activity and their use in experimentally infected fish, J Appl Microbiol, 119 (2015) 47-54. https://doi:10.1111/jam.12812

[4] K. Hüsnü, K.H.C Baser, F. Demirci, Chemistry of essential oils, In: R.G. Berger (Eds.), Flavours and fragrances: Chemistry, bioprocessing and sustainability, Springer, Berlin, Germany, 2007, pp. 43-86.

[5] R.J.W. Lambert, P. N. Skandamis, P. Coote, G.J. Nychas, A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol, J Appl Microbiol, 91 (2011) 453–462. https://doi:10.1046/j.1365-2672.2001.01428.x

[6] J. Xu, F. Zhou, B.P. Ji, R.S. Pei, N. Xu, The antibacterial mechanism of carvacrol and thymol against *Escherichia coli,* Lett Appl Microbiol., 47 (2008), 174–179. https://doi: 10.1111/j.1472-765X.2008.02407.x

[7] K.A Hammer, K.A. Heel, Use of multiparameter flow cytometry to determine the effects of monoterpenoids and phenylpropanoids on membrane polarity and permeability in staphylococci and enterococci, Int J Antimicrob Agents, 40 (2012) 239-245. https://doi:10.1016/j.ijantimicag.2012.05.01

[8] A. Nostro, A.S. Roccaro, G. Bisignano, A. Marino, A. Cannatelli, F.C. Pizzimenti, P.L. Cioni, F. Procopio, A.R. Blanco, Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms, J Med Microbiol,56 (2007) 519–523. https://doi:10.1099/jmm.0.46804-0

[9] N.R.F. Nascimento, J.H Leal-Cardoso, L.M. Lessa, J.S. Roriz-Filho, K.M. Cunha, M.C. Fonteles, Terpinen-4-ol: mechanisms of relaxation on rabbit duodenum, J Pharm Pharmacol, 57 (2005) 467–474. https://doi: 10.1211/0022357055696

[10] S.F. Walton, M. McKinnon, S. Pizzutto, A. Dougall, E. Williams, B.J. Currie, Acaricidal activity of *Melaleuca alternifolia* (tea tree) oil: *in vitro* sensitivity of *Sarcoptes scabiei* var. *hominis* to terpinen-4-ol, Arch Dermatol, 140 (2004), 563–566. https://doi:10.1001/archderm.140.5.563

[11] C. F. Carson, K.A. Hammer, T.V. Riley, *Melaleuca alternifolia* (tea tree) oil: A review of antimicrobial and other medicinal properties, Clin Microbiol Rev*,* 19 (2006) 50-62. https://doi:10.1128/CMR.19.1.50-62.2006

[12] P. H. Hart, C. Brand, C.F. Carson, T.V. Riley, R.H. Prager, J.J. Finlay-Jones, Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes, Rev. bras. farmacogn., 49 (2000) 619-626. https://doi:/10.1590/S0102-695X2007000300008

[13] C. Ghelardini, N. Galeotti, L.D.C. Mannelli, G. Mazzanti, A. Bartolini, Local anaesthetic activity of betacaryophyllene. II Farmaco, 56 (2001) 387-389. https://doi./10.1016/S0014-827X(01)01092-8

[14] R. Loughlin, B.F. Gilmore, P.A. McCarron, M.M Tunney, Comparison of the cidal activity of tea tree oil and terpinen-4-ol against clinical bacterial skin isolates and human fibroblast cells, Lett Appl Microbiol, 46 (2008) 428–433. https://doi:0.1111/j.1472-765X.2008.02334.x

[15] M. Chen, Q. Wu, J. Zhang, Z. Yan, J. Wang, Prevalence and characterization of *Listeria monocytogenes* isolated from retail-level ready-to-eat foods in South China, Food Control, 38 (2014) 1–7. https://doi:10.1016/j.foodcont.2013.09.061

[16] K. Pan, H.Q. Chen, P.M. Davidson, Q.X. Zhong, Thymol nanoencapsulated by sodium caseinate: physical and antilisterial properties, J Agric Food Chem. 62 (2014) 1649–1657.

[17] I. Liakos, L. Rizzello, D.J. Scurr, P.P Pompa, I.S. Bayer, Athanassiou, All-natural composite wound dressing films of essential oils encapsulated in sodium alginate with antimicrobial properties, Int J Pharm, 463 (2014) 137–145.

https://doi:10.1016/j.ijpharm.2013.10.046

[18] P.H. M. Balm, Immune-endocrine interactions, in: J.K. Iwama, A.D. Pickering, J.P. Sumpter, C.B. Schreck (Eds.), Fish Stress and Health in Aquaculture, Cambridge University Press, Cambridge, UK, 1997, pp. 195-221.

[19] H. Verdouw, C.V. Echteld, E.M.J. Dekkers, Ammonia determination based on indophenol formation with sodium salicylate, Water Res., 12 (1978) 399–402. https://doi:10.1016/0043-1354(78)90107-0

[20] C. L. Boijink, D.A. Brandão, Histologycal and behavioral alterations induced by the inoculation of bacterial suspension (*Aeromonas hydrophila*) in jundiá (*Rhamdia quelen*), Cienc. Rural, 31 (2001) 687–690. https://doi:10.1590/S0103-84782001000400021

[21] M.G. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Roberts, F. Smith, Colorimetric method for determination of sugarsand related substances, Anal. Chem, 28 (1959) 350–356. https://doi:10.1021/ac60111a017

[22] J.R. Harrower, C.H. Brown, Blood lactic acid -A micromethod adapted to field collection of microliter samples, J Appl Physiol., 32 (1972) 709–711.

https://doi:10.1152/jappl.1972.32.5.709

[23] S. R. Schaffazick, S. S. Guterres, L.L. Freitas, A.R. Pohlmann, Physicochemical characterization and stability of the polymeric nanoparticle systemsfor drug administration, Quím Nova, 26 (2003) 726-737.https://doi:10.1590/S0100-40422003000500017

[24] M.A. Rather, R. Sharma, M. Aklakur, S. Ahmad, N. Kumar, M. Khan, V.L. Ramya, Nanotechnology: A novel tool for aquaculture and fisheries development. A prospective mini-review. J Fish Aquaculture, 2011 (2011) FAJ-16. https://doi: 10.1021/jf4055402

[25] I.H. Sellami, E. Maamouri, T. Chahed, W.A. Wannes, M.E. Kchouk, B. Marzouk, Effect of growth stage on the content and composition of the essential oil and phenolic fraction of sweet marjoram (*Origanum majorana L.*), Ind Crops Prod, 30 (2009) 395-402. https://doi:10.1016/j.indcrop.2009.07.010

[26] C.F. Bagamboula, M. Uyttendaele, J. Debevere, Inhibitory effect of thyme and essential oils of basil, carvacrol, thymol, estragol, linalool and *p*-cymene towards *Shigella sonnei* e *Shigella flexneri*, Food Microbiol, 21 (2004) 33-42. https://doi: 10.1016/S0740-0020(03)00046-7

[27] B. Tepe, E. Donmez, M. Unlu, F. Candan, D. Daferera, G. Vardar-Unlu, M. Polissiou, A. Sokmen, Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *S. cryptantha* (Montbret et Aucher ex Benth.) and *S. multicaulis* (Vahl.), Food Chem, 84 (2004) 519-525. https://doi: 10.1016/s0308-8146(03)00267-x

[28] L.L. Silva, Q.I. Garlet, S.C. Benovit, G. Dolci, C.A. Mallmann, M.E. Bürger, B. Baldisserotto, S.J. Longhi, B.M. Heinzmann, Sedative and anesthetic activities of the essential oils of *Hyptis mutabilis* (Rich.) Briq. and their isolated components in silver catfish (*Rhamdia quelen*). Braz J Med Biol Res, 46 (2013) 771–779. https://doi:10.1590/1414-431X20133013

[29] R.Y. Gimbo, G.C. Fávero, L.N. Franco Montoya, E.C. Urbinati, Energy deficit does not affect immune responses of experimentally infected pacu (*Piaractus mesopotamicus*), Fish Shellfish Immunol, 43 (2015) 295-300. https://doi:10.1016/j.fsi.2015.01.005

[30] L. Tappy, R. Chioléro, Substrate utilization in sepsis and multiple organ failure, Crit Care Med, 35 (2007) 531-534. https://doi:10.1097/01.ccm.0000278062.28122.a4

Table 1. Survival of *Rhamdia quelen* inoculated with 1.5×10^9 CFU mL^{-1} of *Aeromonas hydrophila* and treated with 30 min daily baths of terpinen-4-ol, thymol, or carvacrol in their pure or nanocapsulated forms for 6 days. Kaplan-Meier post-test LogRank, $p < 0.05$.

Treatments (mg L^{-1})	Survival (%)
Positive control – 0	33.33
Negative control – 50	100*

Saline	100 *
Pure form	
Terpinen-4ol - 5	100^{a} *
Terpinen-4ol - 10	100^{a} *
Terpinen-4ol - 15	88.88^{a} *
Terpinen-4ol – 25	100^{a} *
Thymol – 5	88.88^{a} *
Thymol - 10	88.88^{a} *
Thymol - 15	88.88^{a} *
Thymol - 25	100^{a} *
Carvacrol - 5	77.77^{b}
Carvacrol - 10	55.55^{b}
Carvacrol - 15	22.22^{b}
Carvacrol - 25	44.44^{b}
Nanocapsule form	
Terpinen-4ol - 5	100^{a} *
Terpinen-4ol - 10	100^{a} *
Terpinen-4ol - 15	100^{a} *
Terpinen-4ol – 25	100^{a} *
Thymol – 5	100^{a} *
Thymol - 10	100^{a} *
Thymol - 15	100^{a} *
Thymol - 25	100^{a} *
Carvacrol - 5	100^{a} *
Carvacrol - 10	55^{b}
Carvacrol - 15	0^{c}
Carvacrol - 25	66.66^{b}

The percentage was calculated from n = 3 replicates corresponds to 100% survival. Different letters in columns indicate statistical difference between groups. * Indicates significant difference compared to the positive control.

Figure captions

Fig. 1. Glucose levels in the liver and muscle of silver catfish inoculated with 1.5×10^{9} UFC mL^{-1} of *Aeromonas hydrophila* or saline and exposed to different concentrations of terpinen-4-ol (A, D), thymol (B, E), or carvacrol (C, F) in either their pure or nanocapsulated forms.

The data are the mean $\pm$ SEM for each group (n=6). Different letters indicate a significant difference between treatments in the same form; ($p < 0.05$). The levels of glucose in the liver are shown in A, B, and C. The levels of glucose in the muscle are shown in D, E, and F.

Fig. 2. Lactate levels in the liver and muscle of silver catfish inoculated with 1.5×10^9 UFC mL^{-1} of *Aeromonas hydrophila* or saline and exposed to different concentrations of terpinen-4-ol (A, D), thymol (B, E), or carvacrol (C, F) in their pure or nanocapsulated forms. The data are the mean $\pm$ SEM for each group (n=6). Different letters indicate a significant difference between treatments in the same form; ($p < 0.05$). Different letters indicate a significant difference between treatments in the same form; ($p < 0.05$). The levels of lactate in the liver are shown in A, B, and C. The levels of lactate in the muscle are shown in D, E, and F.
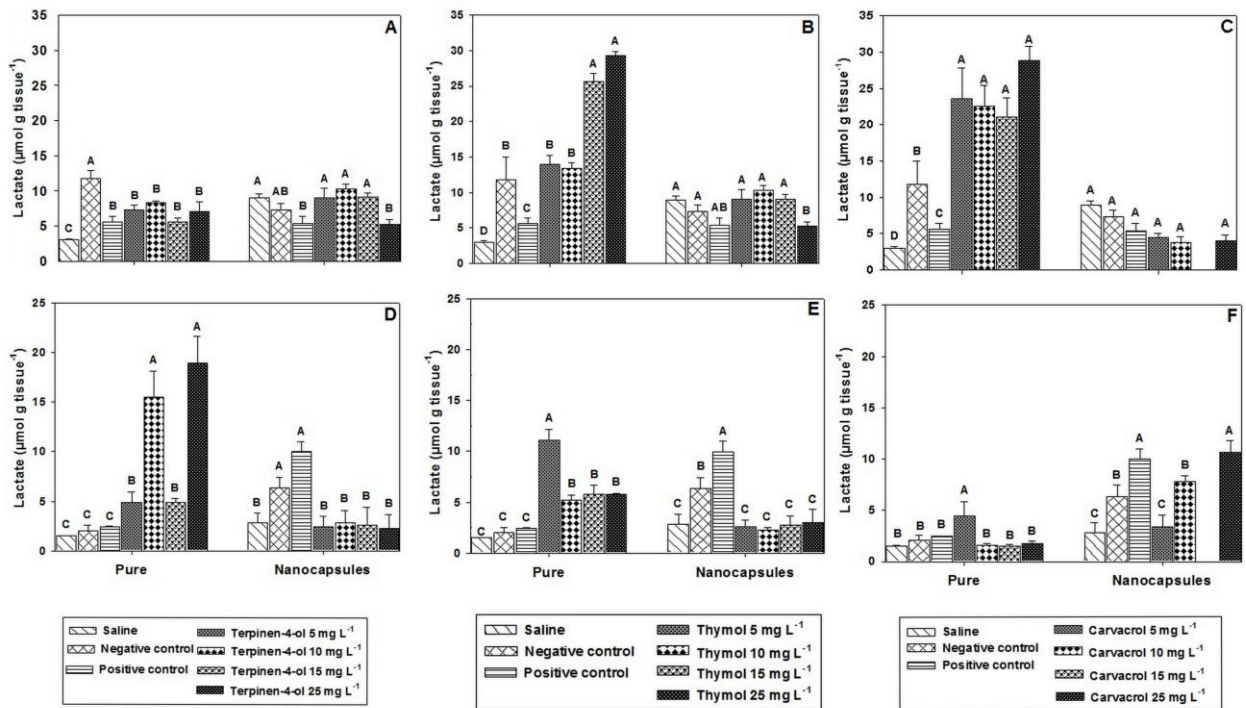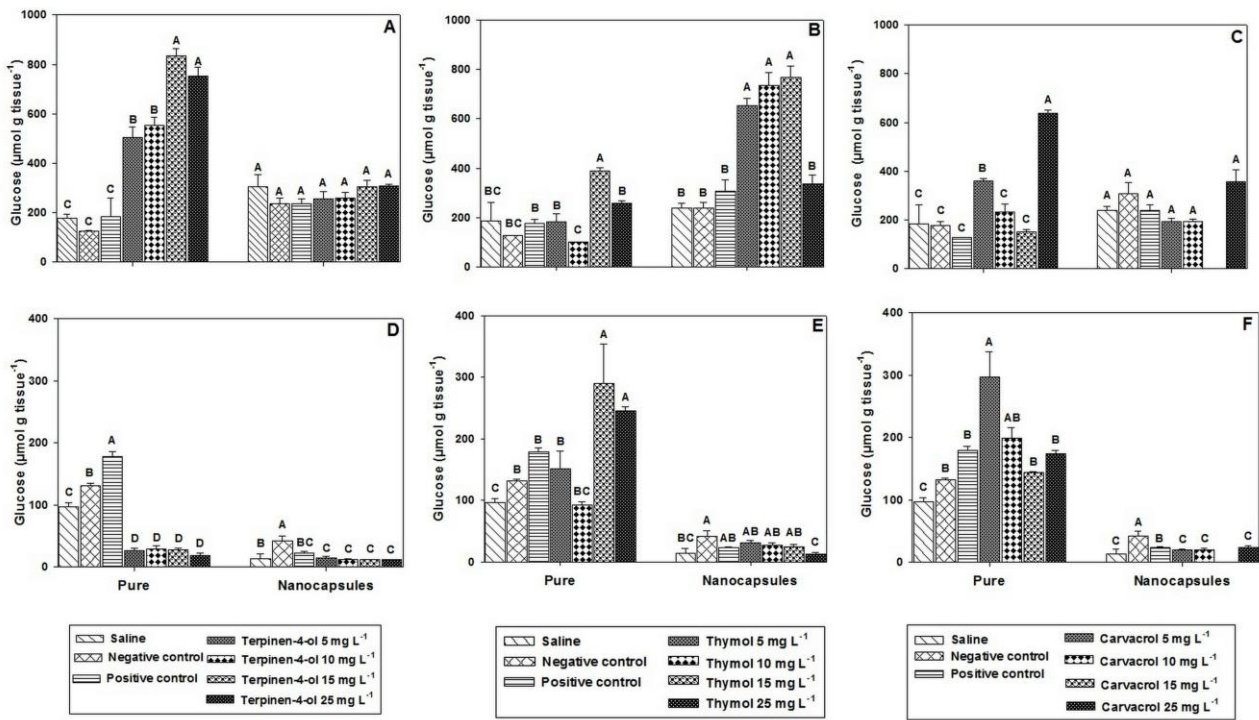
5   DISCUSSÃO GERAL

A utilização insdiscriminada de antibióticos é um perigo para o ecossistema, podendo desencadear uma resistência bacteriana (BUENO et al., 2017; KATLE et al., 2017). Em vista, desse grave problema, uma solução promissora é a utilização de produtos naturais produzidos pelas plantas como metabolismo secundário, os OEs (ROMERO et al. 2012; SHIN et al., 2018).

Entre tantos OEs existentes, o OE proveniente da espécie *O. majorana* tem descritas propriedades antibacterianas (FREIRE et al., 2011; VALERIANO et al., 2012), antifúngicas (SANTIN et al., 2014) e inseticidas (FREIRE et al., 2011). Portanto, o objetivo deste estudo foi avaliar o efeito antibacteriano do OEM através de banhos em *R. quelen* infectado com *A. hydrophila*. Os resultados foram positivos, indicando que esse OE apresenta efeito antibacteriano frente a *A. hydrophila*. Entretanto, apesar do efeito antibacteriano desencadeado pela aplicação do OEM, percebemos que esse efeito poderia ser prejudicado devido a sua baixa solubilidade na água, baixa estabilidade e volatilização. Uma alternativa para eliminar esses problemas poderia ser a aplicação da nanotecnologia (HEURTALT et al., 2013). E tendo em vista os resultados de outras pesquisas que haviam constatado que OEs nanoencapsulados potencializam o efeito antimicrobiano, optou-se por trabalhar com essa tecnologia (SALOKO et al., 2014; SCHAFFAZICK, et al., 2003; SOUZA et al., 2017).

Geralmente a atividade antimicrobiana é derivada não apenas de um único mecanismo de ação, mas a partir de uma cascata de reações envolvendo toda a célula bacteriana. Os EOs possuem várias estruturas químicas em sua composição e, consequentemente,vários grupos funcionais (BURT 2004; NAZZARO et al. 2013), de modo que seu efeito final contra um patógeno pode resultar da sinergia de constituintes distintos do OE ou seus principais componentes (SUTILI et al. 2016). Surgiu então a necessidade de investigarmos se o efeito antibacteriano do EOM era proveniente da sinergia dos compostos presentes na sua composição química ou se poderia ser atribuído a apenas um de seus constituintes, além de averiguar se a utilização da nanotecnologia também iria aumentar a proteção contra os efeitos colaterais existentes em animais infectados. Na identificação da composição química os compostos terpinen-4-ol, timol e carvacrol apresentaram-se como constituintes majoritários e já possuíam registros na literatura de efeitos antibacterianos (CARSON et al., 2006;

HAMMER et al., 2012; LOUGHLIN et al., 2008; XU et al., 2008), por esse motivo avaliamos o efeito antibacteriano desses compostos. A comparação entre os compostos isolados na forma pura e na forma nanoencapsulada também foi realizada de modo a investigar se a nanoencapsulação iria influênciar e/ou potencializar os efeitos antibacterianos dos compostos sem interferir nos parâmetros metabólicos dos animais.

5 CONCLUSÃO

Com os resultados obtidos até o momento podemos inferir que tanto o OEM quanto o NOM exibiram atividade antibacteriana frente a *A. hydrophila*. O uso de OEM e NOM são efetivos no tratamento de doenças infecciosas em peixes, e são estratégias promissoras para reduzir o uso de antibióticos na aquicultura.

Todos os tratamentos melhoraram a sobrevivência dos peixes infectados, mas se compararmos ambos compostos percebe-se que os animais que receberam o NOM nanoencapsulado apresentaram as menores concentrações efetivas testadas que não interferiram com os parâmetros metabólicos dos animais. Nós também enfatizamos a vantagem do uso de nanocápsulas porque a quantidade de OEM necessário para obter tratamento antibacteriano eficaz foi muito menor, além de ser possível observar um efeito imunoestimulador desencadeada por tratamento com a menor concentração de NOM.

As formas nanoencapsuladas de terpinen-4-ol, timol e carvacrol promoveram melhor sobrevivência de jundiás infectados com *A. hydrophila* quando comparados aos resultados dos compostos aplicados na forma pura, elucidando assim um potente efeito antibacteriano desses compostos nanoencapsulados aplicado através de banhos. Na avaliação da resposta metabólica os níveis de glicose e lactato no músculo e fígado não são indicados, uma vez que os animais saudáveis em comparação aos animais infectados não apresentam diferença de resultado de forma constante.

6 REFERÊNCIAS

ABDEL-MASSIH, R. M.; ABRAHAM, A. Extracts of *Rosmarinus officinalis*, *Rheum rhaponticum* and *Origanum majorana* exhibit significant anti-*Staphylococcal* activity. **International Journal of Pharma Sciences and Research**, v. 5, n. 3, p. 819-828, 2014. Disponível em: <http://ijpsr.com/bft-article/extracts-of-rosmarinus-officinalis-rheum-rhaponticum-and-origanum-majorana-exhibit-significant-anti-staphylococcal-activity/>. Acesso em: 7 nov. 2015 . DOI: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(3).819-28.

AESCHBACH, R. et al. Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. **Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association**, v. 32, n. 1, p. 31–6, 1994.Disponível em: <http://ac.els-cdn.com/0278691584900334/1-s2.0-0278691584900334-main.pdf?_tid=d86dc4b8-c436-11e5-92ae-00000aab0f01&acdnat=1453817734_87844e7785b64edfa1e186f2753e6f09>. Acesso em: 25 jan. 2016. DOI: 10.1016/0278-6915(84)90033-4.

ALBUQUERQUE, U. P.; HANAZAKI, N. As pesquisas etnodirigidas na descoberta de novos fármacos de interesse médico e farmacêutico: fragilidades e pespectivas. **Revista Brasileira de Farmacognosia**, v. 16, p. 678–689, 2006. Disponível em: <https://www.academia.edu/1191282/As_pesquisas_etnodirigidas_na_descoberta_de_novos_f%C3%A1rmacos_de_interesse_m%C3%A9dico_e_farmac%C3%AAutico_fragilidades_e_p espectivas>. Acesso em: 5 dez. 2015. DOI: 10.1590/S0102-695X2006000500015.

ALDERMAN, D. J.; HASTINGS, T. S. Antibiotic use in aquaculture: Development of antibiotic resistance - Potential for consumer health risks. **International Journal of Food Science and Technology**, v. 33, n. 2, p. 139–155, 1998. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-0032350961&partnerID=40&md5=b7e11b8dd6fb8d93194bc40cb6d082d0>. Acesso em: 6 dez. 2015. DOI: 10.1046/j.1365-2621.1998.3320139.x.

ALMEIDA, R. R. **Mecanismos de ação dos monoterpenos aromáticos: timol e carvacrol**. Monografia de Trabalho de Conclusão de Curso. Grau Acadêmico Bacharelado. Universidade Federal de São João del-Rei. 2015.

ALVES, O. L. Nanotecnologia, nanociência e nanomateriais: quando a distância entre presente e futuro não é apenas questão de tempo. **Parcerias Estratégicas**, Brasília, n. 18, p. 23-40, 2004. Disponível em: <http://www.cgee.org.br/arquivos/pe_18.pdf >. Acesso em: 25 março 2015.

ANTON, N.; BENOIT, J. P.; SAULNIER, P. Design and production of nanoparticles formulated from nano-emulsion templates – A review. **Journal of Controlled Release**, v. 128, n. 3, p. 185–199, 2008. Disponível em: <http://ac.els-cdn.com/S0168365908001016/1-s2.0-S0168365908001016-main.pdf?_tid=6d5465fc-c11f-11e5-8539-00000aab0f6b&acdnat=1453477822_15c9218314af043a1a9d8710947e50e1>. Acesso em: 3 abr. 2015 . DOI: 10.1016/j.jconrel.2008.02.007.

BALDISSEROTTO, B. Piscicultura continental no Rio Grande do Sul: situação atual, problemas e perspectivas para o futuro. **Ciência Rural**, Santa Maria, v. 39, n. 1, p. 291–299, 2009. Disponível em: <http://www.scielo.br/pdf/cr/v39n1/a46cr443.pdf>. Acesso em: 5 abr. 2015 DOI: 10.1590/S0103-84782008005000046.

BASER, K.H.C.; DEMIRCI, F. Chemistry of Essential Oils. In: Berger RG Ed. Flavours and Fragrances: Chemistry. Bioprospecting and Sustainability. Heidelberg. **Springe**r. p. 43-86, 2007. Disponivel em: <http://link.springer.com/chapter/10.1007%2F978-3-540-49339-6_4#page-1> Acessado em: 26 jan 2016.

BEAZ-HIDALGO, R.; FIGUERAS, M.J. *Aeromonas* spp. whole genomes and virulence factors implicated in fish disease**. Journal of Fish Diseases**, v. 36, p.371–388, 2013.Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/23305319 >. Acesso em: 1 nov 2018.

BUCHBAUER, G. Biological effects of fragrances and essential oils. **Perfumer Flavorist**, v. 21, n. 3, p. 31-36, 1993.

BUENO, I. et al. Impact of point sources on antibiotic resistance genes in the natural environment: a systematic review of the evidence. **Animal Health Research Reviews**, v. 18, p. 112-127, 2017. Disponível: < https://www.ncbi.nlm.nih.gov/pubmed/29231804 >Acesso em: 1 nov. 2018.

BURT, S. Essential oils: their antibacterial properties and potential applications in foods—a review. **International Journal of Food Microbiology**, v.94, p.223–253, 2004. Disponível em: < https://www.sciencedirect.com/science/article/pii/S0168160504001680 >. Acesso em: 25 out. 2018. DOI: https://doi.org/10.1016/j.ijfoodmicro.2004.03.022.

BRASIL. Ministério da Pesca e Aquicultura. **Boletim estatístico de pesca e aquicultura do Brasil 2011**. Brasília, DF. 2013.

CANABARRO, T. F. et al. Isolamentos de bactérias e vírus em peixes de águas do Município de Santa Maria e arredores. **Ciência Rural**, Santa Maria, v. 22, n. 1, p. 93-100, 1992. Disponível em: <http://www.scielo.br/pdf/cr/v22n1/a15v22n1.pdf>. Acesso em: 12 dez. 2015. DOI: 10.1590/S0103-84781992000100015.

CARSON, C. F.; RILEY, T. V. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. **The Journal of applied bacteriology**, v. 78, n. 3, p. 264–269, 2015. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.1995.tb05025.x/epdf>. Acesso em: 20 nov. 2015. DOI: 10.1111/j.1365-2672.1995.tb05025.x.

CARSON, C.F.; HAMMER, K.A.; RILEY, T.V. *Melaleuca alternifolia* (tea tree) oil: A review of antimicrobial and other medicinal properties. **Clinical Microbiology Reviews***,* v.19, p. 50-62, 2006. Disponível em:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1360273/pdf/0029-05.pdf. Acessdo em: 25 out. 2018. DOI:10.1128/CMR.19.1.50-62.2006

CITARASU T. Herbal Biomedicines: a new opportunity for the aquaculture industry. **Aquaculture,** v. 18, p. 403-414, mar./mar. 2010.   Disponivel em: < http://link.springer.com/article/10.1007/s10499-009-9253-7/fulltext.html> Acessado em: 26 jan 2016. DOI: 10.1007/s10499-009-9253-7

DEBBARMA, J. et al.   Antibacterial activity of ginger, eucalyptus and sweet orange peel essential oils on fish-borne bacteria. **Journal of Food Processing and Preservation**,v.37, n. 5, p. 1022–1030, 2013. Disponivel em: < http://onlinelibrary.wiley.com/doi/10.1111/j.1745-4549.2012.00753.x/epdf > Acesso em: 26 jan 2016. DOI: 10.1111/j.1745-4549.2012.00753.x

DUBEY, V. S.; BHALLA, R.; LUTHRA, R. An overview of the non-mevalonate pathway for terpenoid biosynthesis in plants. **Journal of biosciences**, v. 28, n. 5, p. 637–646, 2003. Disponível em: <http://www.ias.ac.in/jbiosci/sep2003/637.pdf>. Acesso em: 18 nov.2015. DOI: 10.1007/BF02703339.

EDRIS, A. E. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. **Phytotherapy Research**, v. 21, n. 4, p. 308–323, 2007. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17199238>. Acesso em: 25 nov. 2015. DOI: 10.1002/ptr.2072.

ENCARNAÇÃO, P. Varied feed additives improve gut, animal health. **Global Aquaculture Advocate**, v. 3 (5/6), p. 41 – 41, 2010. Disponível em: <http://pdf.gaalliance.org/pdf/GAA-Encarnacao-May10.pdf> Acessado em: 26 jan 2016.

ERENLER, R. et al. Isolation and identification of chemical constituents from *Origanum majorana* and investigation of antiproliferative and antioxidant activities. **Journal of the Science of Food and Agriculture**, v. 96, n. 3, p. 822-836, 2015. Disponível em: <http://www.scopus.com/inward/record.url?eid=2-s2.0-84927654557&partnerID=40&md5=c607fb600dd03a20dd3511c848256880>. Acesso em: 25 nov. 2015. DOI: 10.1002/jsfa.7155.

FONT QUER, P. **Plantas medicinales: El Dioscorides Renovado.** Barcelona: Editorial Labor, 1973. 167 p.

FRANK, W. C. Surprising stereoselectivity in the Payne epoxidation ofterpinen-4-ol with acetonitrile/hydrogen peroxide. **Tetrahedron: Asymmetry**, v. 9, p. 3745-3749, 1998. Disponível em: <http://ac.els-cdn.com/S0957416698004078/1-s2.0-S0957416698004078-main.pdf?_tid=b8f963d2-c439-11e5-9820-00000aacb35f&acdnat=1453818970_5880f8be28e91efdea84e65ac5a7d054>. Acesso em: 25 jan. 2016. DOI:10.1016/S0957-4166(98)00407-8.

FRATINI, F. et al. Antibacterial activity of essential oils, their blends and mixtures of their main constituents against some strains supporting livestock mastitis. **Fitoterapia**, v. 96, p. 1–

7, 2014. Disponível em: <http://dx.doi.org/10.1016/j.fitote.2014.04.003>. Acesso em: 18 nov. 2015. DOI: 10.1016/j.fitote.2014.04.003.

FREIRE, J.M. et al. Essential oil of *Origanum majorana* L., *Illicium verum* Hook. F. and *Cinnamomum zeylanicum* Blume: chemical and antimicrobial characterization, **Revista Brasileira de Plantas Medicinais**, v. 13, p. 209–214, 2011.Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-05722011000200013> Acesso em: 25 out. 2018.Doi: doi.org/10.1590/S1516-05722011000200013

FRIEDRICH, C. Pharmacists in German Cultural History. **Anales de la Real Academia Nacional de Farmaci**,v. 80, p,600- 613, 2014. Disponivel em**: <** https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact= 8&ved=0ahUKEwiruJ7r88fKAhUCTSYKHa2aB88QFggcMAA&url=http%3A%2F%2Fww w.analesranf.com%2Findex.php%2Faranf%2Farticle%2Fdownload%2F1519%2F1601&usg= AFQjCNHHKHd2kJmuCw6Wg9aKmczAwZGgBQ&sig2=EV0QWUL3fXllu-5o-FX5gw&bvm=bv.112454388,d.eWE> Acessado em: 26 jan 2016.

GHATAK, S. et al. Pan-genome analysis of *Aeromonas hydrophila*, *Aeromonas veronii* and *Aeromonas caviae* indicates phylogenomic diversity and greater pathogenic potential for *Aeromonas hydrophila*. **Antonie Van Leeuwenhoek**, v.109, p. 945-956, 2016. Disponível em: < https://link.springer.com/article/10.1007%2Fs10482-016-0693-6 > Acessado em : 01 nov 2018.

GOMES, L. D. C. et al. Biologia do jundiá *Rhamdia quelen* (Teleostei, Pimelodidae). **Ciência Rural**, Santa Maria, v. 30, n. 1, p. 179–185, 2000. Disponível em: <http://www.scielo.br/pdf/cr/v30n1/a29v30n1.pdf>. Acesso em: 27 nov. 2015. DOI: 10.1590/S0103-84782000000100029.

GRUPO ETC. **Nanotecnologia: os riscos da tecnologia do futuro: saiba sobre produtos e invisíveis que já estão no nosso dia-a-dia e o seu impacto na alimentação e na agricultura.** Porto Alegre: L & PM, 2005. 197 p.

HAMMER, K.A.; HEEL, K.A. Use of multiparameter flow cytometry to determine the effects of monoterpenoids and phenylpropanoids on membrane polarity and permeability in Staphylococci and Enterococci. **International Journal of Antimicrobial Agents,** v. 40, p. 239-45, 2012. Disponivel em: < http://www.sciencedirect.com/science/article/pii/S0924857912002166> Acessado em: 26 jan 2016. DOI:10.1016/j.ijantimicag.2012.05.015

HARIKRISHNAN, R.; NISHA RANI, M. and BALASUNDARAM, C. Hematological and biochemical parameters in common carp, *Cyprinus carpio,* following herbal treatment for *Aeromonas hydrophila* infection. **Aquaculture,** v. 221, p. 41–50 2003. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0044848603000231> Acessado em: 26 jan 2016. DOI: 10.1016/S0044-8486(03)00023-1

HART, P. H. et al. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. **Inflammation Research**, v. 49, n. 11, p. 619–626, 2000. Disponível em: <https://www.researchgate.net/publication/246994948_Hart_PH_Brand_C_Carson_CF_Riley_TV_Prager_RH_Finlay-Jones_JJ_Terpinen-4-ol_the_main_component_of_the_essential_oil_of_Melaleuca_alternifolia_tea_tree_oil_suppresses_inflammatory_mediator_production_by>. Acesso em: 8 dez. 2015. DOI: 10.1007/s000110050639.

HUANG, S. et al. Nanotechnology in agriculture, livestock, and aquaculture in China. A review. **Agronomy for Sustainable Development**, v. 35, p. 369-400, 2015. Disponível em: <http://link.springer.com/10.1007/s13593-014-0274-x>. Acesso em: 9 dez. 2015. DOI: 10.1007/s13593-014-0274-x.

ISHIKAWA, C.M. et al. Micobacteriose em peixes. **Boletim do Instituto de Pesca**, São Paulo, v.27, n.2, p.231-242, 2001.Disponível em:< https://www.pesca.sp.gov.br/27%5B2%5D-art_13.pdf >. Acesso em: 01 nov. 2018.

JANDA, J. M.; ABBOTT, S. L. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. **Clinical Infectious Diseases**, v. 27, n. 2, p. 332–344, 1998. Disponível em: <http://cid.oxfordjournals.org/content/27/2/332.long>. Acesso em: 9 dez. 2015 . DOI: 10.1086/514652.

JANDA, J. M.; ABBOTT, S. L. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. **Clinical Microbiology Reviews**, v. 23, n. 1, p. 35–73, 2010. Disponível em: <http://pt.scribd.com/doc/48894129/The-genus-Aeromonas-Taxonomy-Pathogenicity-and-infection-Clinical-Microbiology-Reviews-2010#scribd>. Acesso em: 11 dez. 2015 . DOI: 10.1128/CMR.00039-09.

JENA, M. et al. Nanotechnology- future prospect in recent medicine: a review**. International Journal of Basic & Clinical Pharmacology**, v. 2, n. 4, p.353-359, 2013. Disponível em: <http://www.scopemed.org/fulltextpdf.php?mno=37678>. Acesso em: 9 dez. 2015. DOI: 10.5455/2319-2003.ijbcp20130802.

KREUTZ, L. C. et al. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. **Fish & shellfish immunology**, v. 29, n. 4, p. 694–697, 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20685618>. Acesso em: 9 dez. 2015. DOI: 10.1016/j.fsi.2010.06.003.

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.** 91. P. 453–462, 2001. Disponivel em: < http://onlinelibrary.wiley.com/doi/10.1046/j.1365-

2672.2001.01428.x/full> Acessado em: 26 jan 2016. DOI: 10.1046/j.1365-2672.2001.01428.x

LOUGHLIN, R. et al. Comparison of the cidal activity of tea tree oil and terpinen-4-ol against clinical bacterial skin isolates and human fibroblast cells. **Letters in Applied Microbiologyv**, v. 46, n. 4, p. 428–433, 2008. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/18298453>. Acesso em: 10 dez. 2015. DOI: 10.1111/j.1472-765X.2008.02334.x.

MACHADO, B. F. M. T.; JUNIOR, A. F. Óleos essenciais: aspectos gerais e usos em terapias naturais. **Cadernos Acadêmicos**, Tubarão, v. 3, n. 2, p. 105-127, 2011. Disponível em: <http://www.portaldeperiodicos.unisul.br/index.php/Cadernos_Academicos/article/view/718/671#.VqPvySorLIU>. Acesso em: 18 dez. 2015.

MARQUES, J. L. et al. Antimicrobial activity of essential oils of *Origanum vulgare* L. and *Origanum majorana* L. against *Staphylococcus aureus* isolated frompoultry meat. **Industrial Crops and Products**, v. 77, p. 444–450, 2015. Disponível em: <http://ac.els-cdn.com/S0926669015303800/1-s2.0-S0926669015303800-main.pdf?_tid=7e08b804-c218-11e5-81e8-00000aacb35f&acdnat=1453584795_6482646b29a9c8df38f655d1fcb9bcb3>. Acesso em: 18 dez. 2015. DOI: 10.1016/j.indcrop.2015.09.013.

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, 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3804180/>. Acesso em: 18 dez. 2015 . DOI: 10.1590/S1517-83822013005000015.

MOHAMED, H. M. H.; MANSOUR, H. A. Incorporating essential oils of marjoram and rosemary in the formulation of beef patties manufactured with mechanically deboned poultry meat to improve the lipid stability and sensory attributes. **LWT - Food Science and Technology**, v. 45, n. 1, p. 79–87, 2012. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0023643811002325>. Acesso em: 16 dez. 2015 . DOI: 10.1016/j.lwt.2011.07.031.

MONDELLO, F. et al. *In vivo* activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic *Candida* species. **BMC Infectious Diseases**, v. 6, p. 150, 2006.

NASCIMENTO, N. R. F. et al. Terpinen-4-ol: mechanisms of relaxation on rabbit duodenum. **The Journal of Pharmacy and Pharmacology**, v. 57, n. 4, p. 467–474, 2005. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/15831207>. Acesso em: 11 dez. 2015. DOI: 10.1211/0022357055696.

NAZZARO, F. et al. Effect of essential oils on pathogenic bacteria. **Pharmaceuticals**, v.6, p.1451–1474, 2013. Disponível em: < https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3873673/> .Acesso em 25 out. 2018. DOI:10.3390/ph6121451

NEVES, D. P. **Estudo farmacológico Estudo farmacológico do timol e carvacrol sobre a contratilidade da aorta isolada de rato**. Dissertação (Mestrado em Ciências Fisiológicas) - Universidade Estadual do Ceará, Fortaleza, 2009.

NOSTRO, A. et al. Effects of oregano. carvacrol and thymol on Staphylococcus aureus and Staphylococcus epidermidis biofilms. **Journal of Medical Microbiology** v.56, p. 519–523, 2007. Disponivel em: <http://jmm.microbiologyresearch.org/content/journal/jmm/10.1099/jmm.0.46804-0> Acessado em: 26 jan 2016. DOI 10.1099/jmm.0.46804-0.

OLIVEIRA, J. L. T. M. et al. Effectiveness of *Origanum vulgare* L. and *Origanum majorana* L. essential oils in inhibiting the growth of bacterial strains isolated from the patients with conjunctivitis. **Brazilian Archives of Biology and Technology**, v. 52, n. 1, p. 45–50, 2009. Disponível em: <http://www.scielo.br/pdf/babt/v52n1/a06v52n1.pdf>. Acesso em: 30 mai. 2015. DOI: 10.1590/S1516-89132009000100006.

OMARA, S. T.; ABD EL-MOEZ, S. I.; MOHAMED, A. M. Antibacterial Effect of *Origanum majorana* L. (Marjoram) and *Rosmarinus officinalis* L. (Rosemary) Essential Oils on Food Borne Pathogens Isolated from Raw Minced Meat in Egypt. **Global Veterinária**, v. 13, n. 6, p. 1056-1064, 2014. Disponível em: <http://www.idosi.org/gv/gv13(6)14/16.pdf>. Acesso em: 3 jun.2015. DOI: 10.5829/idosi.gv.2014.13.06.9149.

ORHAN, I. E. et al. Antimicrobial and antiviral effects of essential oils from selected *Umbelliferae* and *Labiatae* plants and individual essential oil components. **Turkish Journal of Biology**, v. 36, p. 239–246, 2012. Disponível em: <http://dergipark.ulakbim.gov.tr/tbtkbiology/article/viewFile/5000021371/5000021612>. Acesso em: 5 mai.2015. DOI: 10.3906/biy-0912-30.

PEREIRA, A. A. et al. Caracterização química e efeito inibitório de óleos essenciais sobre o crescimento de *Staphylococcus aureus* e *Escherichia coli*. **Ciência e Agrotecnologia**, Lavras, v. 32, n. 3, p. 887-893, 2008. Disponível em: <http://www.scielo.br/pdf/cagro/v32n3/a28v32n3.pdf>. Acesso em: 3 jul. 2015. DOI: 10.1590/S1413-70542008000300028.

PRIDGEON, J. W.; KLESIUS, P. H. **Fish Vaccines In Aquaculture**: Proactive Treatment Protects Salmon, Catfish, Other Fish. Global Aquaculture Advocate, May/June, 2010 Disponivel em: < http://pdf.gaalliance.org/pdf/GAA-Pridgeon-May10.pdf> Acessado em: 26 jan 2016.

QUINA, F. H. Nanotecnologia e o Meio Ambiente: Perspectivas e Riscos. **Quimica Nova**, v. 27, n. 6, p. 1028-1029, 2004. Disponível em: <http://www.scielo.br/pdf/qn/v27n6/22297.pdf>. Acesso em: 7 mai 2015. DOI: 10.1590/S0100-40422004000600031.

QUOY, J.R; GAIMARD, P. **Zoologie. Poissons**. p. 183-401. In L. de Frecinet. Voyage autour de monde, entrepris par ordre du Roi, exécuté sur les corvettes de S M l'URANIE et la PHYSICIENNE, pendant les années 1817-1820. Paris, 712 p. 1824.

RATHER, M.A. et al. Nanotechnology: A Novel Tool for Aquaculture and Fisheries Development. A Prospective Mini-Review . **Fisheries and Aquaculture Journal**, v. 2011: FAJ-16, 2011. Disponivel em: http://www.omicsonline.com/open-access/nanotechnology-a-novel-tool-for-aquaculture-and-fisheries-development-2150-3508.1000016.pdf?aid=13122> Acessado em: 26 jan 2016.

REZENDE, H. A.; COCCO, M. I. M. A utilização de fitoterapia no cotidiano de uma população rural. **Revista da Escola de Enfermagem da USP**, São Paulo, v. 36, n. 3, p. 282-288, 2002. Disponível em: <http://www.revistas.usp.br/reeusp/article/view/41305/44870>. Acesso em: 10 mai. 2015.

SALBEGO, J. et al. The essential oil from *Lippia alba* induces biochemical stress in the silver catfish (*Rhamdia quelen*) after transportation. **Neotropical Ichthyology**, v. 12, n. 4, 2014. Disponível em: <http://www.scielo.br/pdf/ni/2014nahead/1679-6225-ni-1982-0224-20130178.pdf >. Acesso em: 17 mai. 2015. DOI: 10.1590/1982-0224-20130178.

SALOKO, S. et al. Antioxidative and antimicrobial activities of liquid smoke nanocapsules using chitosan and maltodextrin and its application on tuna fish preservation, **Food Bioscience**, v. 7, p. 71–79, 2014. Disponível em: < https://www.sciencedirect.com/science/article/abs/pii/S2212429214000285 >. Acesso em: 25 out. 2018. DOI: doi.org/10.1016/j.fbio.2014.05.008.

SANTIN, R. et al. Antifungal activity of *Origanum vulgare* essential oil against *Malassezia pachydermatis*, **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 66, p. 367–373, 2014. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-09352014000200007 > Acesso em: 25 out. 2018.Doi: dx.doi.org/10.1590/1678-41626476

SELLAMI, I. H. et al. Effect of growth stage on the content and composition of the essential oil and phenolic fraction of sweet marjoram (*Origanum majorana* L.). **Industrial Crops and Products**, v. 30, n. 3, p. 395–402, 2009. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0926669009001228>. Acesso em: 7 dez. 2015. DOI: 10.1016/j.indcrop.2009.07.010.

SCHAFFAZICK, S.R. et al. Characterization and physical-chemical stability of polymer nanoparticulate systems for drug administration, **Quimica Nova**, v. 26, 2003. Disponível em: <http://www.scielo.br/pdf/qn/v26n5/17209.pdf>. Acesso em: 25 out. 2018. DOI: dx.doi.org/10.1590/S0100-40422003000500017.

SHIN, J.; PRABHAKARAN, V.S.; KIM, K. The multi-faceted potential of plant-derived metabolites as antimicrobial agents against multidrug-resistant pathogens.**Microbial Pathogenesis**, v. 116, p. 209–214, 2018.

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

Journal of Medical and Biological Research, v. 46, n. 9, p. 771–779, 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3854430/>. Acesso em: 5 abr. 2015. DOI: 10.1590/1414-431X20133013.

SOUZA, F. C. et al. Silver catfish submitted to a stressful condition: effect of dietary addition of the essential oil of *Lippia alba* (Mill.) N. E. Brown on metabolism, osmoregulation and endocrinology. **Neotropical Ichthyology**, v.13, n.4, p. 707-714, 2015.Disponível em: < http://dx.doi.org/10.1590/1982-0224-20140153 >.Acesso em : 25 out. 2018

SUÁREZ, W. Q.; HERRERA, F. A. Determination of virulence factors in strains of *Aeromonas spp.*, isolated from fish. **Revista MVZ Córdoba**, v. 17, n. 1, p. 2846-2851, 2012.

SUTILI, F. J. et al. *Lippia alba* essential oil promotes survival of silver catfish (*Rhamdia quelen*) infected with *Aeromonas* spp. **Anais da Academia Brasileira de Ciências**, v. 87, n. 1, p. 95-100, 2015a. Disponível em: <http://www.scielo.br/pdf/aabc/v87n1/0001-3765-aabc-201520130442.pdf>. Acesso em:. DOI: 10.1590/0001-3765201520130442.(a)

SUTILI, F. J. et al. Plant essential oils against *Aeromonas hydrophila*: *in vitro* activity and their use in experimentally infected fish. **Journal of Applied Microbiology**, v. 119, n. 1, p. 47-54, 2015b. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1111/jam.12812/pdf>. Acesso em: 6 dez. 2015. DOI6 dez. 2015: 10.1111/jam.12812.(b)

SUTILI, F. J. et al. The use of nitazoxanide against the pathogens *Ichthyophthirius multifiliis* and *Aeromonas hydrophila* in silver catfish (*Rhamdia quelen*). **Veterinary Parasitology**, v. 197, n. 3-4, p. 522–526, 2013. Disponível em: <http://ac.els-cdn.com/S0304401713003634/1-s2.0-S0304401713003634-main.pdf?_tid=8514382a-c22d-11e5-bdac-00000aacb362&acdnat=1453593826_b5289149f0261c2ca14a057848bd54e3>. Acesso em: 7 jul. 2015. DOI: 10.1016/j.vetpar.2013.06.012.

SUTILI, F.J. et al. The use of *Ocimum americanum* essential oil against the pathogens *Aeromonas hydrophila* and *Gyrodactylus sp*. in silver catfish (*Rhamdia quelen*). **Letter Applied Microbiology**, v.63, p.82–88, 2016. Disponível em: < https://onlinelibrary.wiley.com/doi/abs/10.1111/lam.12602> . Acesso em 25 out. 2018. DOI: 10.1111/lam.12602.

TONI, C. et al. Fish anesthesia: effects of the essential oils of *Hesperozygis ringens* and *Lippia alba* on the biochemistry and physiology of silver catfish (*Rhamdia quelen*). **Fish Physiology and Biochemistry**, v. 40, n. 3, p. 701–714, 2014. Disponível em: <http://link.springer.com/article/10.1007%2Fs10695-013-9877-4>. Acesso em: 16 dez. 2015. DOI: 10.1007/s10695-013-9877-4.

TRAJANO, V. N. et al. Antibacterial property of spice essential oils on food contaminating bacteria. **Ciência e Tecnologia de Alimentos**, v. 29, n. 3, p. 542-545, 2009. Disponível em: <http://www.scielo.br/pdf/cta/v29n3/a14v29n3.pdf>. Acesso em: 26 dez. 2015. DOI: 10.1590/S0101-20612009000300014.

TROPICOS,ORG. **Missouri Botanical Garden**.Saint Louis, 2016. Disponível em: <http://www.tropicos.org/Name/17601585>. Acesso em: 25 jan. 2016.

TSERENNADMID, R. et al. Antibacterial effect of essential oils and interaction with food components. **Central European Journal of Biology**, v. 5, n. 5, p. 641-648, 2010. Disponível em: <http://link.springer.com/article/10.2478%2Fs11535-010-0058-5#/page-1>. Acesso em: 13 dez. 2015. DOI: 10.2478/s11535.010.0058.5.

VÁGI, E. et al. Essential oil composition and antimicrobial activity of *Origanum majorana* L. extracts obtained with ethyl alcohol and supercritical carbon dioxide. **Food Research International**, v. 38, n. 1, p. 51–57, 2005. Disponível em: <http://ac.els-cdn.com/S0963996904001681/1-s2.0-S0963996904001681-main.pdf?_tid=d5e71a4e-c230-11e5-8d46-00000aacb361&acdnat=1453595250_9ba10f29f626a2e9775696aafb489bc2>. Acesso em: 12 dez. 2015. DOI:10.1016/j.foodres.2004.07.006.

VAUTHIER, C.; BOUCHEMAL, K. Methods for the preparation and manufacture of polymeric nanoparticles. **Pharmaceutical Research**, v. 26, n. 5, p. 1025–1058, 2009. Disponível em: <http://link.springer.com/article/10.1007%2Fs11095-008-9800-3>. Acesso em: 5 mar. 2015. DOI: 10.1007/s11095-008-9800-3.

VALLADÃO, G.M.R.; GALLANI, S.U.; PILARSKI, F. South American fish for continental aquaculture. **Reviews in Aquaculture**, v. 10, p. 351- 369, 2016. Disponível em : < https://onlinelibrary.wiley.com/doi/abs/10.1111/raq.12164 >. Acesso em: 1 nov. 2018.

VERPOORTE, R. Secondary metabolism. IN: Verpoorte, R. & Alfermann, A.W. (eds.). Metabolic engineering of plant secondary metabolism, 1ed. Netherlands: Kluwer Academic Publichers, p. 1-29, 2000.

VIZZOTTO, B. S. **Caracterização fenotípica e molecular de estirpes de *Aeromonas* isoladas no Paraná no período de 1999-2009.** 2009. 101 p. Dissertação (Mestrado em Ciências Farmacêuticas) – Universidade Federal do Paraná, Curitiba, 2009.

XU, J. et al. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. **Letters in Applied Microbiology**, v.47, p. 174–179, 2008. Disponivel em: <http://onlinelibrary.wiley.com/doi/10.1111/j.1472-765X.2008.02407.x/epdf> Acessado em: 26 jan 2016. DOI: 10.1111/j.1472-765X.2008.02407.x.