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PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA**

Natacha Cossettin Mori

**CITRAL COMO ADITIVO NA DIETA DE PEIXES ESTUARINOS:
PERFIL REDOX, IMUNIDADE INATA E PARÂMETROS HEPÁTICOS**

Santa Maria, RS, Brasil

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Tese apresentada ao Curso de Doutorado do Programa de Pós-graduação em Farmacologia da Universidade Federal de Santa Maria como requisito para obtenção do grau de **Doutor em Farmacologia**

Orientadores:

Prof. Dra Berta Maria Heizmann

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Santa Maria, RS, Brasil
2019

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“Às vezes penso que sei alguma coisa
De repente percebo que não sei nada;

Às vezes pareço forte

De repente me sinto fraco;

Às vezes tenho certeza que vai dar certo

De repente dá tudo errado

Às vezes acho que Deus me abandonou

De repente sinto ele ao meu lado;

Às vezes penso que é o fim,

De repente é só o começo”.

André Renato

RESUMO

AUTORA: Natacha Cossettin Mori

ORIENTADORA: Berta Maria Heinzmann

O citral é um monoterprenoide que ocorre em óleos essenciais de diferentes espécies de plantas utilizadas na medicina popular, que também apresentaram atividades benéficas quando adicionados à dieta de peixes. Contudo, apesar das inúmeras atividades biológicas já demonstradas para óleos essenciais, estudos “*in vivo*” demonstrando o benefício efetivo do citral são insuficientes, especialmente no que diz respeito ao incremento da produção animal. No Brasil, o crescente interesse pela piscicultura e boas perspectivas de crescimento da produção comercial de peixes resulta na busca de uma dieta mais saudável para a saúde desses animais. Dessa forma, o objetivo deste estudo foi avaliar se o citral adicionado à dieta possui efeitos benéficos sobre o perfil oxidativo e imunidade inata em juvenis de *Centropomus undecimalis* (robalo-flecha) e *Mugil liza* (tainha). Para avaliar a toxicidade hepática do composto testado, foram determinados marcadores bioquímicos (atividade de alanina aminotransferase e aspartato aminotransferase) e histologia hepática (aspecto do tecido e morfometria da veia centro lobular, capilares sinusoides e hepatócitos). O citral foi obtido comercialmente e adicionado à dieta de ambas espécies por 45 dias em diferentes concentrações (0; 0.44; 0.88; 1.76 g kg dieta) originando quatro grupos experimentais. Temperatura, pH e oxigênio dissolvido foram verificados diariamente, enquanto que alcalinidade, amônia e nitrito foram mensurados semanalmente durante todo o período experimental. Ao término do experimento, dez peixes de cada tanque foram anestesiados e eutanasiados por secção da medula espinhal para coleta de sangue e tecidos. Embora estudos “*in vitro*” já tenham demonstrado que o citral apresenta capacidade antioxidante e benefícios com a utilização de óleos essenciais compostos majoritariamente por este monoterprenoide, nossos resultados indicam que para o robalo-flecha a adição de citral na dieta não melhora suas condições de saúde. Apesar do composto em avaliação não promover toxicidade hepática, promoveu lipoperoxidação em brânquias e hiperativação do sistema complemento.

A atividade das enzimas superóxido dismutase, catalase e glutathione-S-transferase aumentou em resposta ao dano oxidativo evidenciado. Contudo, para a tainha a incorporação do citral na alimentação foi benéfica, melhorando parâmetros oxidativos (reduziu lipoperoxidação; aumentou a atividade de superóxido dismutase, glutathione peroxidase e glutathione-S-transferase; aumentou níveis de tióis não proteicos), sem alterar a atividade das enzimas e parâmetros histológicos do fígado. Houve diminuição da atividade de mieloperoxidase nas duas espécies em estudo. Os dados apresentados demonstram que as diferenças fisiológicas entre as duas espécies têm influência direta sobre a atividade do citral, que por sua vez não é indicado como aditivo na dieta para *C. undecimalis*, mas é recomendado para complementar a dieta *M. liza* na concentração de 1.76 g por kg de dieta.

Palavras-chave: aquicultura, *Centropomus undecimalis*, *Mugil Liza*, monoterpenoides, estresse oxidativo, sistema imune, toxicidade hepática.

ABSTRACT

AUTHOR: Natacha Cossetin Mori

ADVISOR: Berta Maria Heinzmann

Citral is a monoterpenoid that occurs in essential oils of different plant species used in folk medicine, which also showed promising effects when added to fish diets. However, in spite of the numerous biological activities demonstrated for essential oils, *in vivo* studies demonstrating the effective benefit of citral are insufficient, especially with regard to the increase of animal production. In Brazil, the growing interest in fish farming and good prospects for growth of commercial fish production increased the search for a healthier diet for the health of these animals. Thus, the objective of this study was to evaluate if the citral added to the diet has beneficial effects on the oxidative profile and innate immunity in juveniles of *Centropomus undecimalis* (common snook) and *Mugil liza* (mullet). Biochemical markers (alanine aminotransferase and aspartate aminotransferase activity) and liver histology (tissue appearance and lobar center vein morphometry, sinusoidal capillaries and hepatocytes) were determined to evaluate the hepatic toxicity of the tested compound. Citral was commercially obtained and added to the diet of both species for 45 days at different concentrations (0, 0.44, 0.88, 1.76mg kg of diet) giving four experimental groups. Temperature, pH and dissolved oxygen were verified daily, while alkalinity, ammonia and nitrite were measured weekly throughout the experimental period. At the end of the experiment, ten fish from each tank were anesthetized and euthanized by section of the spinal cord to collect blood and tissues. Although *in vitro* studies have shown that citral has antioxidant capacity and beneficial effects with the use of essential oils composed mainly of this monoterpenoid, our results indicate that for common snook the dietary addition of citral does not improve its health conditions. Although the compound under evaluation did not promote hepatic toxicity, it promoted lipoperoxidation in gills and hyperactivation of the complement system. The activity of the enzymes superoxide dismutase, catalase and glutathione-S-transferase increased in response to the oxidative damage evidenced. However,

for mullet the incorporation of citral in the diet was beneficial, improving oxidative parameters (reduced lipid peroxidase activity, increased the activity of superoxide dismutase, glutathione peroxidase and glutathione-S-transferase, increased levels of non-protein thiols), without altering the activity of enzymes and histological parameters of the liver. There was a decrease in myeloperoxidase activity in the two species under study. The data presented demonstrated that the physiological differences between the two species have a direct influence on citral activity, which in turn is not indicated as a dietary additive for *C. undecimalis*, but is recommended to supplement the *M. liza* diet at the concentration of 1.76 g per kg of diet.

Keywords: aquaculture, *Centropomus undecimalis*, *Mugil Liza*, monoterpenoids, oxidative stress, immune system, liver toxicity.

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LISTA DE ABREVIATURAS

EROS	Espécies reativas de Oxigênio
DNA	Ácido desoxirribonucleico
SOD	Superóxido dismutase
CAT	Catalase
GPx	Glutathione peroxidase
GST	Glutathione S transferase
GSH	Glutathione reduzida
GSSG	Glutathione oxidada
LPO	Lipoperoxidação
MPO	Mieloperoxidase
OE	Óleo essencial
AST	Aspartato aminotransferase
ALT	Alanina aminotransferase
LOOH	Hidroperóxidos lipídicos
SEM	Erro padrão da média
LPS	Lipopolissacarídeos
TNF-α	Fator de necrose tumoral
IL-6	Interleucina-6
IL-1β	Interleucina-1 β

NF-κB	Fator de necrose κB
PPAR-γ	Receptor Ativado por Proliferadores de Peroxissoma Gama
iNOS	Óxido nítrico sintase induzível
COX-2	Ciclooxigenase 2

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1. APRESENTAÇÃO

1.1 Introdução

O cultivo de organismos aquáticos tem sido intensificado nos últimos anos. Dados publicados pela FAO (Organização das Nações Unidas para a Alimentação e Agricultura) em 2018 a produção global aquícola (incluindo plantas aquáticas) foi de 110,2 milhões de toneladas, estimando-se um valor comercial de primeira venda em 243.500 milhões de dólares. A estimativa é que para 2030 a aquicultura contribua com 60% do pescado para consumo humano e sua produção supere a pesca (54%). Segundo Nadarajah & Flaaten (2017), a intensificação do cultivo e a melhora das condições nutricionais contribuem para aumentar o rendimento desta atividade econômica.

Neste contexto, a composição das dietas administradas aos peixes é de grande relevância para o aumento da produção na aquicultura, uma vez que afeta a saúde e crescimento dos peixes cultivados e está diretamente relacionada à qualidade dos pescados, produto final do processo de cultivo (SANTOS; OBA, 2009). Sendo assim, peixes mais saudáveis refletem no aumento da produtividade e por consequência no incremento do consumo pela população, já que são produzidos peixes de melhor sabor e qualidade. Todos esses fatores beneficiam a atividade econômica.

O citral é um monoterpenoide de sabor agradável e propriedades contra microorganismos patogênicos (SHI et al., 2016), que já foi aprovado pelo órgão americano FDA (Food and Drug Administration) para uso na indústria de alimentos, bebidas e como fragrância. Além disso, já foi relacionado a inúmeras atividades biológicas, tais como: às atividades sedativa (DO VALE et al., 2002), antifúngica (FERREIRA et al., 2009), anti-inflamatória (BACHIEGA; SFORCIN, 2011; PONCE-MONTER et al., 2010), antinociceptiva (ORTIZ et al., 2010) e anestésica (SOUZA et al., 2017). Estudos em *Rhamdia quelen* já demonstraram benefícios da suplementação dietética com óleos essenciais contendo citral em sua composição (BALDISSEROTTO et al., 2015; ZEPPENFELD et al., 2014; ZEPPENFELD et al., 2016; ZEPPENFELD et al., 2017).

Segundo Barroso et al. (2002), dentre outros fatores nutricionais, a palatabilidade das rações é fundamental para a intensificação e sucesso dos cultivos. Frente a isso, avaliamos os efeitos da adição de citral na ração de *Centropomus undecimalis* (robalo-flecha) e *Mugil liza* (tainha) através da análise parâmetros de estresse oxidativo e atividade imunoestimulante por meio da determinação de componentes da imunidade inata. Além disso, visando complementar nosso estudo e evidenciar a segurança do uso deste componente na dieta dos peixes supracitados, avaliamos marcadores bioquímicos e histológicos hepáticos.

A presente tese está fundamentada na apresentação do artigo publicado na revista *Aquaculture*, e outro artigo manuscrito que foi submetido à revista *Aquaculture Nutrition* e encontra-se em fase de avaliação. Sendo assim, consta das seguintes seções: Referencial teórico, objetivos, delineamento metodológico geral, resultados, discussão, conclusão e referências bibliográficas.

1.2 REFERENCIAL TEÓRICO

1.2.1 Aquicultura: panorama atual e desafios

Aquicultura é o cultivo de animais cujo ciclo de vida em condições naturais se dá total ou parcialmente em meio aquático. Nos últimos anos, de acordo com o último relatório da FAO (2018), o crescimento global desta atividade têm sido bastante significativo. A captura de peixes marinhos, no ano de 2016 foi de 79,3 milhões de toneladas, sendo que moluscos com casca (16,9 milhões de toneladas) constituem 58,8% da produção combinada da aquicultura marinha e costeira.

A China destaca-se neste cenário, representando cerca de 60% da produção mundial de pescado, seguida pela Índia e Bangladesh. Já o Brasil ocupa a 13ª colocação no ranking dos principais produtores, com 225.000 toneladas, mobilizando cerca de 800 mil profissionais, entre pescadores e aquicultores, e proporcionando 3,5 milhões de empregos diretos e indiretos (BRASIL, 2014).

A produção de peixes oriunda da pesca é maior nas regiões Nordeste, Norte e Sudeste do Brasil, contudo a aquicultura merece destaque no Centro-Oeste e no Sul do país. A região Sul, em especial, representou em 2011 uma produção aquícola de 172,4 mil toneladas, sendo o mexilhão e a ostra as principais espécies marinhas cultivadas, e tilápia, carpa e jundiá (*Rhamdia quelen*), as espécies que representaram a piscicultura continental (BRABO et al., 2016).

Segundo Routledge et al. (2012) e Kubitzka (2015), o Brasil é considerado um dos países de maior potencial para aquicultura. Essa afirmação pode ser explicada por alguns aspectos: 1) forte mercado doméstico; 2) indústria de rações bem estabelecida; 3) clima tropical; 4) abundância de recursos hídricos (8.500 km de faixa costeira com grandes perspectivas futuras para maricultura); 5) localização estratégica para escoamento da produção para toda a América e Europa e 6) território amplo (8,5 milhões de km²), incluindo grandes áreas favoráveis para a construção de tanques e açudes.

Apesar disso, Kubitzka, Campos, Ono & Istchuk (2012) elencaram inúmeros fatores limitantes para o desenvolvimento da aquicultura brasileira, tais como: 1) dificuldade de regularização ambiental; 2) elevado custo de produção; 3) assistência técnica insuficiente; 4) qualificação dos produtores deficiente; 5) dificuldade de financiamento e de acesso à tecnologia; 6) limitações do mercado regional; e 7) mortalidade de peixes por enfermidades. Por outro lado, Nadarajah & Flaaten (2017) destacam também a influência dos fatores ambientais sobre a produtividade de organismos aquáticos, tais como: as interações entre os animais, o tipo de ração, condições de cultivo e fatores climáticos.

Diante deste cenário, a pesquisa científica destaca-se como uma importante ferramenta para o desenvolvimento da aquicultura brasileira, especialmente em relação à redução dos efeitos ambientais sobre a saúde dos animais. Com relação à esse aspecto, deve-se considerar que o cultivo de peixes mais saudáveis reflete na redução de mortalidade, proporcionando maior regularidade no fornecimento, e também na qualidade dos pescados. Estes fatores favorecem o incremento do consumo de peixes pela população e por consequência da atividade econômica correspondente.

1.2.2 Caracterização das espécies em estudo

1.2.2.1 Robalo-flecha

O robalo-flecha, *Centropomus undecimalis* (Bloch, 1792), representante da família Centropomidae, é uma espécie eurialina, que habita águas marinhas e ambientes estuarinos presentes desde as áreas litorâneas ocidentais do Oceano Atlântico na Flórida, nos Estados Unidos, até Santa Catarina, no Brasil (ALVAREZ-LAJONCHERE; TSUZUKI, 2008). A distribuição do mesmo vai depender de condições ótimas de temperatura e salinidade. Conforme Shafland & Foote (1983) e Howells et al. (1990) a faixa de temperatura entre 25°C e 29°C e salinidade em torno de 34‰ são desejáveis para o melhor desenvolvimento do robalo. O ponto isosmótico encontra-se entre 11‰ e 13‰.

É uma espécie que possui corpo robusto terminando numa cabeça pontuda, nadadeira dorsal com perfil angular e uma linha preta que cruza seu corpo até a nadadeira caudal. Pode chegar a medir cerca de 130 centímetros e pesar entre 20 a 23 kg (Figura 1) (AMSLER, 2000; ALVAREZ-LAJONCHERE & TSUZUKI, 2008).



Figura 1 – Robalo-flecha, *Centropomus undecimalis* (Bloch, 1972). Fonte: Amsler, 2000.

Os robalos são considerados predadores oportunistas, tendo em vista que variam a alimentação de acordo com a disponibilidade de alimento. Seu hábito alimentar preferencial é composto por crustáceos, moluscos e outros peixes, contudo análises do conteúdo estomacal desta espécie encontraram uma grande variedade de itens, que vão desde crustáceos e moluscos até ovos de peixe e insetos (BARROSO et al., 2002; COSTA-FILHO, FABREGAT & ROSA, 2013). Apesar de possuírem um regime alimentar carnívoro, bons resultados vêm

sendo obtidos na engorda de robalos empregando-se rações comerciais (TSUZUKI et al., 2008).

Esta espécie também é protândrica e as fêmeas de robalo-flecha são animais de porte grande o que dificulta a sua captura e manipulação em laboratório, refletindo na permanência em cativeiro. Além disso, alguns estudos demonstraram que este peixe apresentou uma pequena porcentagem de fertilização devido ao pequeno volume de sêmen obtido dos machos. A temperatura pode afetar a maturação de reprodutores em cativeiro enfatizando a importância de novos experimentos para determinar a ação da temperatura e de outros parâmetros ambientais, de forma a se obter melhorias no controle do cultivo de robalos em confinamento (COSTA-FILHO, FABREGAT & ROSA, 2013).

É considerada uma espécie com grande potencial para aquicultura, uma vez que é muito procurada por pescadores esportivos, movimentando um importante segmento de turismo de pesca. Além disso, o interesse pelo cultivo desta espécie pode ser justificado pela rápida taxa de crescimento e grande resistência durante os processos de manejo e boa aceitação do cativeiro. Liebl et al. (2016), ao compararem o desenvolvimento de peixes juvenis das espécies robalo-peva (*Centropomus parallelus*) e robalo-flecha, evidenciaram uma taxa de 0,23% por dia de crescimento para ambas as espécies. No que se refere à resistência ao manejo, apesar dos problemas constatados com o parasitismo e manipulação frequente dos robalos, Souza-Filho & Cerqueira (2003) demonstraram um fácil condicionamento dos juvenis desta espécie às condições de confinamento. Cabe considerar ainda que esta espécie possui uma saborosa carne branca, sendo comercializada a preços elevados (cerca de R\$ 70,00 o kg) (CAVALLI & HAMILTON, 2007; SANCHES, SILVA & RAMOS, 2014).

1.2.2.2 Tainha

A tainha, *Mugil Liza* (Valenciennes, 1938) pertence à família Mugilidae. Trata-se de uma espécie encontrada no Atlântico Sul, Caribe e América do Sul (SICCHA-RAMIREZ et al., 2014). No Brasil ocorre em todo o litoral (SZPILMAN, 2000). Assim como o robalo-flecha, também é eurialina, mas segundo Lisboa et al. (2015) os peixes criados à salinidade 24 ‰ crescem melhor que aqueles mantidos em água doce (salinidade 0 ‰). O ponto isosmótico da espécie é 12 ‰ (LISBOA et al., 2015). Fortunato et al. (2017) explicam que a tainha

apresenta diferentes comportamentos migratórios, parecendo ser uma espécie principalmente costeira, com baixa utilização dos estuários.

Esta espécie possui corpo alongado, fusiforme, com estrias escuras longitudinais alternadas com estrias claras e a ausência de linha lateral. Atinge um comprimento máximo de 80 centímetros, mas o tamanho usual é de 40 centímetros. Possui 5 espinhas dorsais com 8 raios moles dorsais e 3 espinhas anais com 8 raios moles anais. Pode pesar entre 6 e 8 kg (ROBINS, 1986) (Figura 2).



Figura 2 – Tainha, *Mugil liza* (Valenciennes, 1938). Fonte: FAO

A alimentação da tainha é baseada principalmente em detritos e microalgas filamentosas que estão em processo de decomposição microbiana, sendo portanto denominada uma espécie detritívora. De acordo com Galvão et al. (1997) e Ramos et al. (2015) o estômago da tainha possui músculos altamente desenvolvidos, dobras profundas e sem glândulas digestivas que possui a função primária de triturar alimentos, comparável à moela em pássaros. Os machos amadurecem mais cedo que as fêmeas e são menos numerosos (ROBINS, 1986).

Souza, Silva & Steenbock explicam que este peixe é conhecido no Brasil pela importância econômica e sociocultural. A pesca artesanal da tainha no Estado de Santa Catarina foi declarada pela Lei nº 15.922/2012 (SANTA CATARINA, 2012) como integrante do Patrimônio Histórico, Artístico e Cultural do Estado de Santa Catarina. No que se refere ao interesse comercial, a exportação de ovas é o principal objetivo (BRASIL, 2015), mas a carne é bastante apreciada e considerada de boa qualidade pelos mercado interno (SZPILMAN, 2000). Além de sua importância econômica, a tainha também apresenta potencial para a piscicultura por suportar bem as condições de confinamento, aceitar com facilidade a alimentação artificial e resistir às variações amplas de temperatura e de salinidade (VIEIRA & SCALABRIN, 1991).

1.2.3 Fatores que influenciam as condições de saúde dos peixes

Peixes criados em sistemas de aquicultura enfrentam vários tipos de estressores que podem ser classificados em estressores abióticos e bióticos. Os fatores abióticos consistem nas influências que os peixes possam sofrer, derivadas de aspectos físicos, químicos ou físico-químicos do meio ambiente. Já os fatores bióticos dizem respeito à interação dos peixes com outros organismos vivos presentes no meio (SUBRAMANI & MICHAEL, 2017). A Figura 3 exemplifica alguns destes fatores.

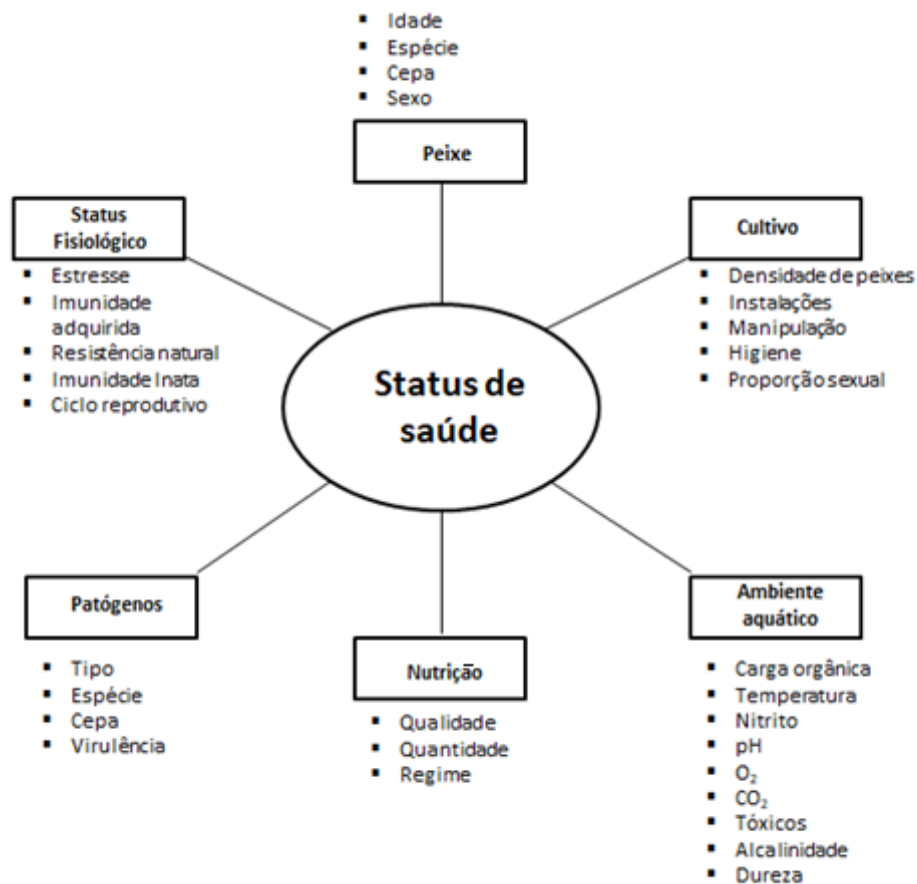


Figura 3 – Fatores que afetam o status de saúde dos peixes. Adaptado de Subramani & Michael (2017).

1.2.3.1 Nutrição

A importância da dieta fornecida aos peixes vai além das perspectivas de crescimento, taxa de conversão alimentar e ausência de síndromes de deficiência. Atualmente, os criadores de peixes adquirem rações para peixes com base em vários aspectos, incluindo saúde normal e resistência a doenças (SUBRAMANI & MICHAEL, 2017).

De acordo com Vallejos-Vidal et. al (2016) compostos com propriedades imunoestimulantes têm sido considerados promissores como aditivos na dieta dos animais. Este tipo de substância, caracterizada como um produto natural ou de natureza química capaz de estimular o sistema imunológico, favorece o ganho de peso, eficiência alimentar e/ou resistência a doenças em peixes cultivados. Todos estes fatores otimizam a produtividade das espécies em cultivo.

Por outro lado, embora ocorra melhora de alguns parâmetros do sistema imunológico, efeitos positivos no resultado final da resposta imune integrada podem não ocorrer, uma vez que a alteração de um único parâmetro pode não causar um impacto detectável na resposta imune dos peixes. Essa afirmação pode ser explicada pela grande diversidade de espécies cultivadas e pela falta de compreensão total sobre o sistema imunológico das diferentes espécies de peixes. Portanto, o estudo da imunonutrição ainda não está totalmente desenvolvido, consistindo num campo bastante amplo para a pesquisa (POHLENZ & GATLIN, 2014).

De acordo com Subramani & Michael (2017) encontrar o efeito de um determinado nutriente no sistema imune dos peixes é desafiador, devido à possível contaminação da água que por si só expõe os peixes à diversos microorganismos e também pela natureza ectotérmica dos animais, tendo em vista que diferentes temperaturas podem gerar resultados diversos.

1.2.3.2 Equilíbrio fisiológico

O cultivo de animais de vida aquática enfrenta dentre outros desafios a manutenção da homeostasia dos peixes, já que os estressores ambientais e de manejo envolvidos resultam em

respostas fisiológicas destes animais, a fim de adaptar seus sistemas biológicos e reorganizar o metabolismo para lidar com o excesso de energia necessária para suportar o estressor (PANAGIOTAKI; MALANDRAKIS, 2018).

O estresse pode ser agudo ou crônico, dependendo da duração e da severidade dos estressores envolvidos. O estresse agudo é um estado que os peixes experimentam por um curto período de tempo. Por outro lado, o estresse crônico do peixe provoca respostas fisiológicas por exposição leve, constante e/ou recorrente. Embora ambos os tipos de estresse apresentem graves efeitos sobre a saúde dos peixes, a resposta ao estresse é um mecanismo de defesa evolutivo (PANAGIOTAKI; MALANDRAKIS, 2018).

Neste contexto, muitos indicadores bioquímicos têm sido propostos como índices de estresse na literatura científica. Dentre eles, os níveis de estresse oxidativo, da atividade do sistema antioxidante e do sistema imune representam indicadores importantes do estado fisiológico que se encontram as espécies em cultivo.

1.2.3.3.1 Estresse oxidativo

O estresse oxidativo (Figura 4) pode ser caracterizado pela elevação nos níveis endógenos de espécies reativas de oxigênio (EROs) em decorrência do excesso de produção das mesmas ou da diminuição dos níveis de antioxidantes celulares (HALLIWELL; GUTTERIDGE, 1999). A mitocôndria é a principal fonte geradora de EROs, cuja toxicidade depende da quantidade, da natureza química, localização celular e tecidual, e das taxas de formação e degradação (SCHMIDT et al., 2015).

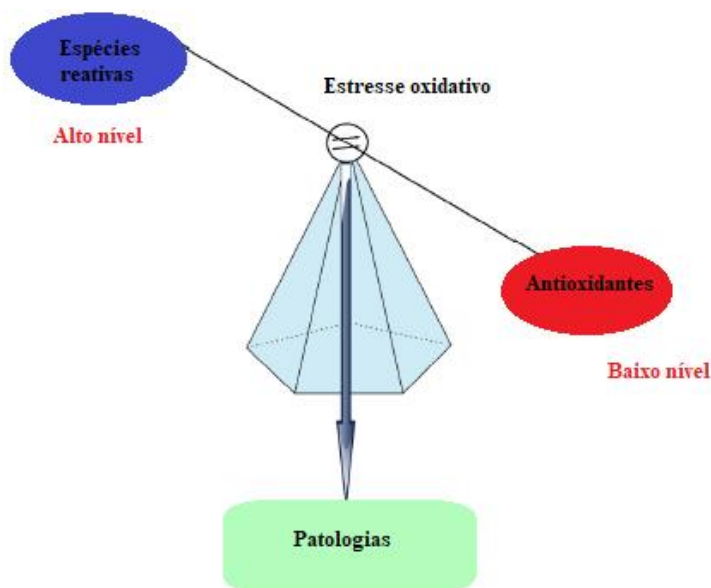


Figura 4 – Estresse oxidativo: Desequilíbrio entre espécies reativas e antioxidantes

Os animais aquáticos estão sujeitos a uma grande quantidade de fatores extrínsecos que podem gerar estresse oxidativo. De acordo com Halliwell & Gutteridge (2015), temperaturas mais altas estão associadas com um aumento na taxa metabólica, o que leva a um aumento do consumo de oxigênio (O_2), gerando estresse oxidativo. O desenvolvimento desta condição também está correlacionada de modo direto com a quantidade de oxigênio dentro de um organismo. Portanto, quanto maiores os níveis de O_2 , maior a probabilidade de ocorrência de oxidação celular. Mudanças na salinidade também induzem estresse oxidativo em peixes, tendo em vista que impõem respostas fisiológicas e comportamentais aos animais. Outro fator importante é a presença de contaminantes e patógenos no meio, pois a ativação do sistema imune é dependente da geração de espécies reativas de oxigênio (EROs) (ASLANI & GHOBADI, 2016; BIRNIE-GAUVIN et al., 2017; LUSHCHAK, 2011).

A excessiva produção de EROs têm por consequência a oxidação de biomoléculas, sobretudo oxidação de lipídeos, proteínas e ácido desoxirribonucleico (DNA) (BARBOSA, 2010; HALLIWELL, WHITEMAN, 2004; MAYNE, 2003; VINCENT; INNES; VINCENT, 2007). Os metabólitos gerados a partir das reações oxidativas podem ser quantificados e, portanto, constituem importantes biomarcadores da ocorrência do estresse oxidativo.

A peroxidação lipídica trata-se de um processo no qual oxidantes atacam lipídios da membrana celular que contém ligações duplas carbono-carbono, especialmente ácidos graxos poli-insaturados. As principais EROs que podem afetar os lipídios são o radical hidroxila ($HO\cdot$)

e hidroperoxila ($\text{H}\cdot\text{OO}\cdot$). Sob taxas de oxidação lipídica fisiológica, as células estimulam sua manutenção através do sistema de defesa antioxidante (AYALA, MUÑOZ & ARGÜELLES, 2014). Todavia, sob condições tóxicas a ocorrência deste processo pode afetar a função celular, a ação de receptores de enzimas e a permeabilidade a alguns íons, desencadeando danos ao organismo dos peixes e, conseqüentemente, diminuindo a produtividade na aquicultura (HALLIWELL; CHIRICO, 1993).

Além dos lipídios, outras biomoléculas que também sofrem alterações oxidativas são as proteínas, especialmente devido a sua abundância nos sistemas biológicos (HOHN; KONIG; GRUNE, 2013). Sua oxidação ocorre em resíduos de metionina, cisteína, prolina, histidina, arginina, lisina, triptofano, tirosina, fenilalanina e valina, e, como consequência, são gerados compostos carbonílicos (OGA; CAMARGO; BATISTUZZO, 2014). A formação de derivados carbonílicos leva a alterações conformacionais e diminuição da atividade catalítica de enzimas, resultando em degradação proteica. Diante disso, a investigação do conteúdo de biomarcadores de oxidação proteica nas células é um parâmetro importante para investigar a influência das condições experimentais em peixes (ALMROTH et al., 2005; CATTANEO et al., 2011; MORAES et al., 2011; PARVEZ; RAISUDDIN, 2005; TONI et al., 2011;).

As EROS podem ainda danificar o DNA. O radical hidroxil em especial, possui a capacidade de adicionar ligações duplas nas bases heterocíclicas de DNA e também de abstrair hidrogênio da base nitrogenada timina e de cada um dos carbonos da desoxirribose (DIZDAROGLU et al., 2002). Tais danos, que podem ser quantificados através da aplicação do teste cometa e determinação de micronúcleo, são frequentemente utilizados para medir o impacto das EROs em peixes expostos aos xenobióticos (VASCONCELOS et al., 2007).

1.2.3.3.2 Sistema antioxidante

Sob condições fisiológicas favoráveis, os antioxidantes endógenos neutralizam o excesso de EROs. Os principais antioxidantes intrínsecos se dividem entre os que agem enzimaticamente, a exemplo da superóxido dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), ou não enzimaticamente, como a glutathione reduzida (GSH) (BARREIROS; DAVID; DAVID, 2006).

As enzimas SOD, CAT e GPx constituem a primeira linha de defesa antioxidante (Figura 5). A primeira enzima a atuar é a SOD. A SOD representa a única enzima que atua de

modo específico sobre o ânion superóxido (O_2^-) catalisando sua dismutação em O_2 e H_2O_2 (LEI et al., 2016), conforme representado na equação (5), a seguir: (5) $O_2^{\bullet-} \rightarrow H_2O_2 + O_2$ Duas isoformas desta enzima já foram identificadas em peixes: (1) MnSOD ou SOD mitocondrial, que possui o manganês como metal cofator em seu sítio ativo; (2) Cu/ZnSOD ou SOD citosólica, cujos cofatores no sítio ativo são cobre e zinco. Tais isoformas, apesar de serem originárias de genes distintos e possuírem localização subcelular, catalisam a mesma reação (FUKAI & USHIO-FUKAI, 2011).

O H_2O_2 resultante da atuação da SOD também constitui uma espécie reativa a ser neutralizada. Neste contexto, evidenciamos a ação de outras duas enzimas, a CAT e a GPx.

A catalase é uma enzima composta por quatro subunidades contendo um grupo heme, com ferro férrico em cada uma das subunidades, encontrada em abundância no fígado, nos rins e nos glóbulos vermelhos (WINTERNITZ; MELOY, 1908). Assim como a GPx, sua principal função é catalisar a dismutação do H_2O_2 em água e oxigênio molecular, conforme representado nas equações (3) e (4), a seguir: (3) $CAT (Porf-Fe^{III}) + H_2O_2 \rightarrow Cpd\ I (Porf^{\bullet+} -Fe^{IV} = O) + H_2O$; (4) $Cpd\ I (Porf^{\bullet+} -Fe^{IV} = O) + H_2O_2 \rightarrow CAT (Porf-Fe^{III}) + H_2O + O_2$. Segundo Valko et al. (2006) existe uma competição entre GPx e CAT para catalisar a hidrólise da espécie reativa supracitada. Durante a reação enzimática que leva à metabolização de H_2O_2 , a catalase é oxidada a um composto intermediário, conhecido como composto I (Cpd I), que é então reduzido de volta, utilizando uma segunda molécula de H_2O_2 (HEINZELMANN, BAUER, 2010; GLORIEUX; CALDERON, 2017). Segundo Krych-Madej & Gebicka (2017), a CAT também é capaz de decompor o peroxinitrito, através da oxidação do óxido nítrico, na presença de H_2O_2 .

Já a GPx, que é uma enzima que existe endogenamente sob duas isoformas (dependente e independente de selênio), fornece a principal proteção contra baixos níveis de dano oxidativo (VALKO et al., 2006). Esta enzima, embora já tenha sido relacionada a inúmeras atividades biológicas, é reconhecida por contribuir com a homeostasia dos níveis de peróxido de hidrogênio (H_2O_2) e hidroperóxidos, que são espécies reativas (ASLANI & GHOBAD, 2016). As reações catalisadas pela GPx ocorrem na presença de GSH e promovem a geração de álcool e água, conforme representado esquematicamente nas equações (1) e (2), a seguir: (1) $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$; (2) $ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$. O principal órgão de síntese desta enzima é o rim, todavia as maiores concentrações teciduais encontram-se no fígado (YOUNG & WOODSIDE, 2001).

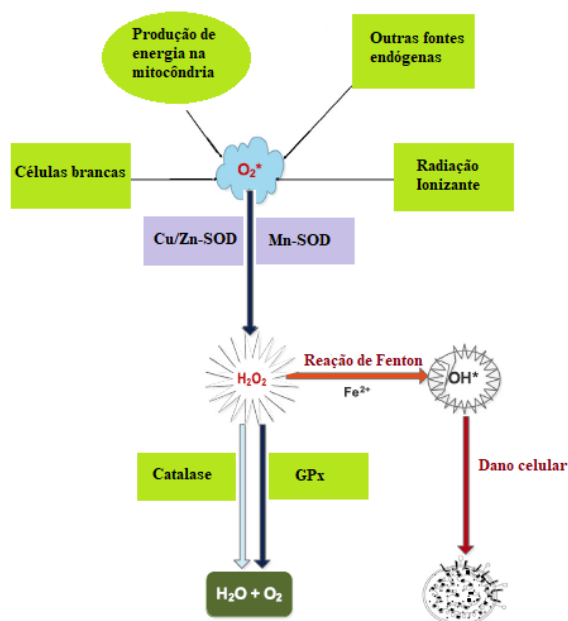


Figura 5 – Primeira linha de defesa antioxidante. Adaptado de Ighodaro & Akinloye (2017)

Outra família de enzimas que funciona catalisando reações de eliminação de xenobióticos, é a das glutathione-S-transferases (GSTs) (JAKOBY, 1978). A atuação desta família de enzimas ocorre através da catalisação da conjugação de GSH a eletrófilos e xenobióticos, facilitando a sua eliminação (SMEYNE & SMEYNE, 2013). Outra função importante, abordada mais recentemente, diz respeito à atuação das GSTs sobre os níveis intracelulares do 4-hidroxinonenal, um produto da peroxidação lipídica que é utilizado como substrato por estas enzimas detoxificantes (AWASTHI et al., 2017). Diante disso, as GSTs representam uma importante linha de defesa para os peixes, visto que estes são naturalmente suscetíveis a xenobióticos presentes na água.

No que se refere à neutralização de EROs através de substâncias não enzimáticas, destaca-se a glutathione reduzida. A GSH é a principal representante do sistema antioxidante não enzimático endógeno. Trata-se de um tiol não proteico que reage com espécies reativas para inibir o estresse oxidativo celular. Além disso, facilita a atividade de enzimas antioxidantes, tais como a GPx e a GST, permitindo a neutralização de compostos oxidantes. A proporção entre os

níveis de GSH e glutathiona oxidada (GSSG) mantém o equilíbrio celular redox (REN et al., 2017).

Cabe salientar ainda a importância de compostos dietéticos no combate às EROs, pois pesquisas têm demonstrado que a adição de antioxidantes naturais à alimentação dos peixes podem contribuir para melhorar o desempenho e a capacidade do sistema antioxidante endógeno (ZHENG et al., 2009; SACCOL et al., 2013). Segundo Birnie-Gauvin et al. (2016), a dieta contendo principalmente antioxidantes lipossolúveis é capaz de beneficiar o *status* oxidativo dos peixes, embora isso possa ocorrer de diferentes maneiras, quando consideramos a espécie e o tipo de dieta utilizada.

Pês et al. (2016) suplementaram a dieta de *Rhamdia quelen* com quercetina e evidenciaram que após 21 dias houve diminuição de LPO em todos os tecidos avaliados e aumento da atividade de SOD (cérebro, brânquias, fígado e músculo), CAT (fígado e rim), GPx (cérebro, brânquias, fígado) e GST (brânquias, fígado, rim e músculo) deste peixe. Em outro estudo conduzido por Saccol et al. (2013), a dieta de *R. quelen* foi suplementada com o óleo essencial de *Lippia alba* e parâmetros de crescimento, metabolismo, sanguíneos e biomarcadores de estresse oxidativo foram avaliados. Tais autores evidenciaram que o aditivo estudado diminui a lipoperoxidação (LPO), aumenta as reservas de glicogênio e lactato e aumenta a resposta antioxidante dos tecidos, sendo portanto recomendada a suplementação da dieta do jundiá com o óleo essencial estudado.

1.2.3.3.4 Sistema imune e a imunidade inata

O sistema imune, apesar de bastante complexo, é definido por Córdova (1994) como um sistema composto por células e moléculas imprescindíveis para a defesa frente a infecções e/ou situações que comprometam a integridade do organismo. Abbas, Lichtman e Pillai (2015) e Levinson (2016) relatam que o sistema imune é responsável pela defesa contra micro-organismos infecciosos e substâncias estranhas não infecciosas. Awad & Awaad (2017) definem o sistema imunológico como um grupo de mecanismos biológicos que protegem organismos vivos de patógenos invasores. Já Rao (2018) explica que apesar de ser conhecido como um sistema de células que ingerem e destroem patógenos, o sistema imunológico desenvolve uma variedade de funções, incluindo (1) a capacidade de discriminar agentes

patogênicos prejudiciais dos antígenos inofensivos; (2) depuração de células e tecidos mortos e (3) a promoção da cicatrização de feridas e reconhecimento de células.

De modo geral, os mecanismos de defesa são mediados pelas reações iniciais da imunidade inata, também denominada imunidade natural ou não-específica e pelas respostas tardias provenientes da imunidade adaptativa ou específica (ABBAS; LICHTMAN; PILLAI, 2015; LEVINSON 2016; SECOMBES & WANG, 2012). As respostas inatas ocorrem em questão de minutos, por meio da atuação de neutrófilos, monócitos/macrófagos, células dendríticas, células “natural killers” e de moléculas como as proteínas do sistema complemento (LE PAGE et al., 2017). Já as defesas específicas demoram dias para o início da atuação e são mediadas pela atuação de linfócitos T, responsáveis pelas defesas celulares e linfócitos B, que atuam através de respostas humorais (anticorpos) (ABBAS; LICHTMAN; PILLAI, 2015; LEVINSON 2016; SECOMBES & WANG, 2012).

Os peixes combinam tanto os mecanismos provenientes da resposta imune inata, que consiste na resposta inicial aos micro-organismos, prevenindo, controlando e eliminando a infecção, quanto os da imunidade adaptativa na defesa contra antígenos ou patógenos (SECOMBES & WANG, 2012). Apesar disso, conforme Bly & Clem (1994) a resposta imune inata desempenha papel de destaque nos peixes, uma vez que nesses animais as defesas específicas respondem de uma forma mais lenta aos agentes agressores quando comparados aos mamíferos (SECOMBES, 1996).

Os componentes da imunidade inata atuam de forma conjunta para limitar a ocorrência de infecções e lesão celular através de dois tipos principais de reações teciduais: (1) inflamação, caracterizada pelo recrutamento de leucócitos e proteínas plasmáticas; (2) defesa antiviral, que consiste em uma reação mediada por citocinas, na qual as células adquirem resistência às infecções virais e também na morte das células infectadas por vírus, através da atuação das células “natural killers” (ABBAS; LICHTMAN; PILLAI, 2015).

As proteínas do sistema complemento constituem um dos principais componentes da imunidade inata humoral, uma vez que participam da remoção dos invasores e ainda da depuração de células auto modificadas, como células apoptóticas e detritos. Esse sistema pode ser ativado por três vias: (1) Via clássica: ativada por um anticorpo ligado ao antígeno alvo ou de forma independente, pela presença de vírus e bactérias Gram-negativas; (2) Via alternativa:

iniciada pela ligação covalente de moléculas C3 com grupos hidroxila ou amina na superfície celular de micro-organismos; (3) Via da lectina: iniciada com a ligação de lectinas ligadoras de manose ou ficolinas a padrões de carboidratos em superfícies microbianas. Após a ativação, as proteínas se depositam na superfície dos micro-organismos invasores e promovem a fagocitose através da opsonização. Além disso, ocorre a recruta, dentre outros, dos mediadores pró-inflamatórios, compostos citolíticos e compostos antimicrobianos prejudiciais aos micro-organismos (SECOMBES & WANG, 2012; ZIFPEL & SHERKA, 2009).

O papel da lisozima na defesa contra micro-organismos também já é amplamente reconhecido. Trata-se de uma proteína básica, composta por 129 resíduos de aminoácidos, presente em vários fluidos do organismo, que atua como um agente bactericida potente. Especialmente contra bactérias Gram-positivas, uma vez que destrói a camada de peptidoglicano presente na parede celular. Para que bactérias Gram-negativas sejam atingidas, é necessário que a barreira de permeabilidade seja superada e a camada de peptidoglicanos interna seja exposta (DI FALCO; CAMMARATA & VIZZINI, 2016; IBRAHIM, AOKI & PELLEGRINI, 2002).

Outro importante componente do sistema imune para o combate às infecções é a mieloperoxidase (MPO). Esta enzima está presente nos neutrófilos e em menor quantidade em monócitos, cuja atuação é fundamental para a fagocitose, pois catalisa a produção de ácido hipocloroso, tóxico aos micro-organismos. Contudo, caso ocorra a difusão desta enzima para os tecidos pode ocorrer dano tecidual e desencadear um processo inflamatório (HOY et al., 2002; KLEBANOFF, 2005; RUSSEL et al., 2017).

Nos últimos anos, estudos que visam identificar compostos imunoestimulantes naturais para prevenir doenças na aquicultura foram intensificados (LEE et al., 2015; SUTILI et al., 2016). Especialmente porque o uso de quimioterápicos e antimicrobianos para controlar doenças nos peixes leva a efeitos desfavoráveis, como o desenvolvimento de cepas resistentes a antimicrobianos e acumulação desses compostos no ambiente e/ou tecido dos peixes que, em última análise, refletem sobre a saúde humana (AWAD & AWAAD, 2017).

Neste contexto, alguns extrativos vegetais têm se mostrado como alternativas na estimulação do sistema imunológico quando adicionados à ração dos peixes, uma vez que são considerados mais seguros e baratos para prevenir e/ou controlar infecções (AWAD &

AWAAD, 2017). A exemplo disso, o extrato de *Origanum vulgare* (1%) melhorou a atividade de explosão respiratória, atividade fagocítica e atividade de lisozima sérica após oito semanas em *Oncorhynchus mykiss* (POURMOGHIM, HAGHIGHI & ROHANI, 2015). A dieta suplementada com a erva chinesa *Sophora flavescens* aumentou significativamente, em todas as doses testadas (0.025%, 0.050%, 0.100%, 0.200%, and 0.400%), dentre outros parâmetros a quantidade de lisozima do soro, a atividade do sistema complemento e da mieloperoxidase na tilápia do Nilo (*Oreochromis niloticus*) desafiada com *Streptococcus agalactiae* (WU et al., 2013).

1.2.4 Antimicrobianos e produtos naturais na aquicultura

Durante as últimas décadas, os antimicrobianos têm sido empregados como principal estratégia para o manejo de doenças de peixes. Contudo, o desenvolvimento e disseminação de bactérias resistentes e genes de resistência, e também a ocorrência de resíduos antimicrobianos em produtos da aquicultura emergem como uma preocupação relacionada à esta vasta utilização (FEČKANINOVÁ et al., 2017). Conforme Gastalho, Da Silva & Ramos (2014) o uso de antimicrobianos em aquicultura apresenta riscos de contaminação do meio ambiente, com possíveis nefastas e imprevisíveis consequências nos ecossistemas aquáticos, assim como impacto na saúde pública.

Outro fator importante, que está relacionado ao desenvolvimento sustentável na indústria da aquicultura, é a necessidade de métodos que minimizem os altos custos do tratamento com antimicrobianos. Nesse sentido, a melhora da resistência dos peixes saudáveis contra patógenos e estresse ambiental associado a baixos custos torna-se fundamental. Para este propósito, o uso de produtos naturais com propriedades antioxidantes, imunoestimulantes ou antimicrobianas tem sido uma alternativa no cultivo de animais aquáticos (ZARGARI; MAZANDARANI & HOSEINI).

Neste cenário, merecem destaque os óleos essenciais (OEs). Estes são líquidos voláteis e aromáticos extraídos das plantas constituídos por uma mistura de compostos orgânicos (KAČÁNIOVÁ et al. 2017). Bajpai, Baek & Kang (2012) & Kačániová et al. (2017) enfatizam que a composição química dos OEs é fundamental para as atividades biológicas demonstradas, uma vez que, dentro da mistura, os constituintes podem apresentar potenciação, adição, sinergia, ou ainda efeitos antagônicos, modificando a atividade quando administrados isoladamente (EFFERTH, KOCH, 2011). Sutili et al. (2017) explicam que o processo de extração, a concentração utilizada, o método de administração e as condições de armazenamento também são determinantes para a atividade biológica dos OEs. A área geográfica de localização da planta e o tempo de colheita também influenciam a composição e por consequência, a atividade dos OEs (DIMA & DIMA, 2015).

1.2.4.1 Citral

O citral, mistura dos diastereoisômeros neral (*Z*-citral ou α -citral) e geranial (*E*-citral ou β -citral) (Figura 6) é um componente de óleos essenciais extraídos de diversas plantas, tais como capim-limão (*Cymbopogon citratus*), melissa (*Melissa officinalis*), verbena (*Verbena officinalis*), erva-cidreira-brasileira (*Lippia alba*), erva-lúisa (*Aloysia triphylla*) entre outros. Na prática é utilizado como aditivo alimentar e como fragrância em cosméticos, devido ao seu agradável aroma de limão (DUDAI et al., 2005).

Biologicamente, este constituinte já foi relacionado às atividades sedativa (DO VALE et al., 2002), antifúngica (FERREIRA et al., 2009) e anti-inflamatória (BACHIEGA; SFORCIN, 2011; PONCE-MONTER et al., 2010), tendo sido detectados também efeitos antinociceptivos (ORTIZ et al., 2010) e mais recentemente um promissor efeito anestésico (SOUSA et al., 2015). Zhang et al. (2014) demonstraram a capacidade do citral de inibição da formação de biofilme de bactérias, indicando que esta substância apresenta potencial de uso como conservante alimentar. Em outro estudo, Silva-Ângulo et al. (2015) reforçaram a hipótese de que o citral possui propriedades antibacterianas, tendo em vista que este monoterprenoide exibiu atividade contra *Listeria innocua* e *Listeria monocytogenes*. O citral também tem sido relacionado a propriedades antioxidantes. Sacchetti et al. (2005) avaliaram *in vitro* as atividades antioxidantes de inúmeros óleos essenciais e encontraram mais de 60% de atividade antioxidante para o óleo essencial de capim-limão, que apresentava um teor de 73,58% de citral em sua composição. Rabbani et al. (2006) testaram em seu estudo a atividade antioxidante do citral *in vitro* e observaram que o monoterprenoide apresenta uma boa atividade de eliminação do radical superóxido. Baschieri et al. (2017) explicaram que alguns terpenoides como o citral reduzem a concentração de radicais peroxila, funcionando como potenciadores de terminação, etapa final da peroxidação lipídica. No tocante aos efeitos sobre o sistema imune, os estudos são escassos.

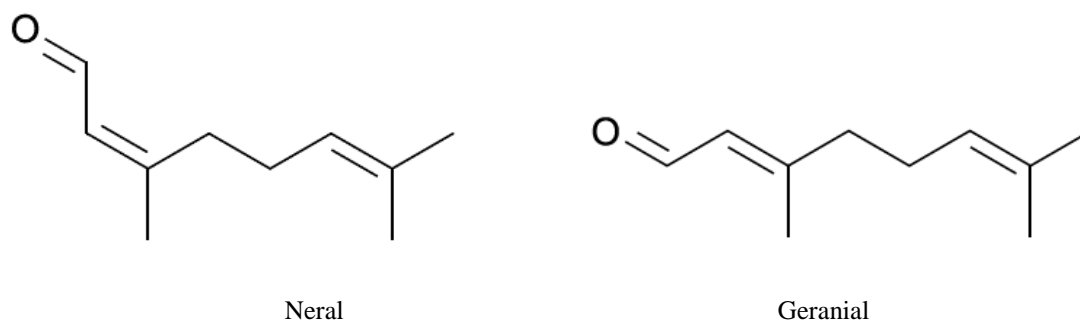


Figura 6 – Estrutura química do neral (α -citral) e geranial (β -citral), isômeros componentes do citral.

No que se refere às espécies aquáticas, alguns estudos também já demonstraram atividade biológica de óleos essenciais compostos majoritariamente pelo citral. Por exemplo, o óleo essencial de *A. triphylla* demonstrou ter a capacidade de estender a vida de prateleira e o tempo de refrigeração do filé de jundiá (*R. quelen*) além de aumentar o crescimento desta espécie, sendo portanto recomendado como um aditivo alimentar (DANIEL et al., 2014; ZEPPENFELD et al., 2015). Kizak et al. (2018), avaliaram a eficácia do OE de *Cymbopogon citratus* como anestésico em duas espécies de peixes ornamentais, *Sciaenochromis fryeri* e *Labidochromis caeruleus*. Neste caso, a concentração de 200 $\mu\text{L/L}$ induziu anestesia profunda, sem promover mortalidade ou efeitos adversos nas duas espécies. Souza et al. (2017), avaliaram o efeito anestésico de OE de dois quimiotipos *Lippia alba* (citral e linalol) em jundiá (*R. quelen*). Para ambas as variedades de plantas o resultado foi promissor, embora aquela que continha o citral como constituinte majoritário (54,26%) tenha induzido maiores níveis de creatinina. Bandeira Junior et al. (2018), avaliaram o efeito anestésico e ansiolítico das folhas de *A. triphylla* (39,91% de α -citral) em jundiá (*R. quelen*) e zebrafish (*Danio rerio*). Para ambas as espécies houve efeito ansiolítico sem alterar padrões locomotores ou indução de comportamento aversivo.

Apesar de numerosos estudos demonstrando o potencial de OEs contendo citral na aquicultura, trabalhos com este constituinte isolado aplicado ao incremento da produção animal são escassos. Assim, considerando o fato de que os peixes não são capazes de sintetizar este monoterprenoide, torna-se importante a investigação dos efeitos da aplicação do mesmo na piscicultura. A complementação dietética e/ou a adição do citral na água poderia ser benéfica tanto para melhorar as condições de saúde, como para a prevenção de doenças, levando a um aumento na produtividade e beneficiando o cultivo de peixes no Brasil.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial do citral para uso como aditivo alimentar na aquicultura em diferentes espécies de peixes

2.2 OBJETIVOS ESPECÍFICOS

- Quantificar os isômeros do citral (α e β -citral = neral e citronelal, respectivamente) presentes na mistura racêmica utilizada no estudo;
- Avaliar o efeito de diferentes concentrações de citral sobre a atividade das enzimas superóxido-dismutase (SOD), catalase (CAT), glutaciona-peroxidase (GPx), glutaciona-S-transferase (GST) em fígado e brânquias de robalo-flecha e tainha;
- Avaliar o efeito de diferentes concentrações de citral sobre os níveis de tióis não protéicos e sobre a formação de hidroperóxidos lipídicos em fígado e brânquias de robalos-flecha e tainha;
- Avaliar o efeito de diferentes concentrações de citral sobre os parâmetros imunológicos de robalos-flecha e tainha: atividade de lisozima, atividade hemolítica do soro e níveis de mieloperoxidase;
- Avaliar o efeito de diferentes concentrações de citral sobre níveis de aspartato aminotransferase (AST) e alanina aminotransferase (ALT) de robalo-flecha e tainha;
- Avaliar o efeito de diferentes concentrações de citral sobre parâmetros histológicos e morfometria do tecido hepático de robalo-flecha e tainha;
- Definir quais as concentrações mais indicadas de citral para incorporação à dieta de robalo-flecha e tainha;

3. DELINEAMENTO METODOLÓGICO GERAL

3.1 Animais e condições de laboratório

Foram utilizados peixes juvenis das espécies *Centropomus undecimalis* e *Mugil liza*, provenientes de reprodução no próprio laboratório, totalizando 40 animais de cada espécie. Os animais foram distribuídos aleatoriamente em quatro sistemas de recirculação de água, utilizando a água da Lagoa da Conceição. Cada sistema consistia em três tanques circulares (150 L). Durante todo o período experimental, a água contida nesses tanques foi removida através de uma tubulação central com filtro de mangas (50 μm), filtro biológico, fracionador de espuma e esterilizador ultravioleta (60 w). Após o tratamento, a água foi devolvida aos tanques experimentais.

No período anterior ao experimento os peixes foram aclimatados às condições experimentais durante quatro dias. Sobras de alimento e fezes foram removidos através de sifonagem e uma média de 25% de água foi renovada diariamente.

Os parâmetros da água foram verificados diariamente (temperatura, pH e oxigênio dissolvido) ou semanalmente (alcalinidade, amônia e nitrito) durante todo o período experimental, conforme descrito por Zeppenfeld et al. (2014).

No experimento com o robalo os parâmetros da água permaneceram estáveis durante todo o período experimental. A temperatura foi mantida em $31,59 \pm 0,08^\circ\text{C}$, pH a $8,18 \pm 0,02$ e oxigênio dissolvido a $4,63 \pm 0,06 \text{ mg L}^{-1}$. Alcalinidade ($102,35 \pm 0,9 \text{ mg CaCO}_3 \text{ L}^{-1}$), nitrito ($0,3 \pm 0,05 \text{ mgL}^{-1}$) e amônia total ($0,3 \pm 0,04 \text{ mg L}^{-1}$) foram mantidos no intervalo desejado.

No experimento com a tainha os parâmetros da água permaneceram estáveis durante todo o período experimental. A temperatura foi mantida a $27,11 \pm 0,11^\circ\text{C}$, pH a $7,58 \pm 0,13$ e oxigênio dissolvido a $5,16 \pm 0,16 \text{ mg L}^{-1}$. Alcalinidade ($103,65 \pm 0,34 \text{ mg CaCO}_3 \text{ L}^{-1}$), nitrito ($0,2 \pm 0,04 \text{ mg L}^{-1}$) e amônia total ($0,2 \pm 0,05 \text{ mg L}^{-1}$) foram mantidos dentro da faixa desejada. A salinidade foi mantida entre 32 e 35 ppt.

3.2 Aspectos éticos

Os experimentos foram realizados no Laboratório de Piscicultura Marinha (LAPMAR) da Universidade Federal de Santa Catarina (UFSC), Florianópolis, Brasil. O estudo foi aprovado pelo Comitê de Ética em Experimentação Animal da UFSC (número de registro PP00861/2013).

3.3 Citral

O Citral foi obtido da Sigma Aldrich[®] (St. Louis, Missouri, EUA). Antes de sua incorporação na dieta, foi realizada a quantificação dos isômeros. A análise foi executada em cromatógrafo a gás Agilent 6890A acoplado a um detector de massas 5973 usando uma coluna capilar quiral HP (30 m x 0,25 mm i.d. x 0,25 μ m espessura do filme) e energia de ionização de 70 eV. O hélio foi usado como gás de arraste em uma vazão de 1,0 mL min⁻¹, temperatura do injetor configurada em 250°C e a temperatura do detector em 280°C. A temperatura do forno foi mantida a 40°C por 4 min e depois elevada para 240 °C a uma taxa de 4 ° C min⁻¹. Soluções de amostra de 1 μ L (2:1000 em hexano, v/v) foram injetadas em modo split. Os índices de retenção de Kovats foram calculados usando uma série homóloga de C8-C40 n-alcanos injetados nas mesmas condições das amostras. Os constituintes foram identificados por comparação dos espectros de massas com a espectroteca do aparelho e do índice de retenção de Kovats com dados da literatura (NIST, 2008). O percentual relativo de compostos foi estimado pela integração da área dos picos obtida do cromatograma (α -Citral = 60,15%; β -Citral = 39,85%).

3.4 Dieta e desenho experimental

Foram preparadas quatro dietas baseadas na formulação inicial contendo diferentes concentrações de citral (0-controle; 0,44; 0,88; 1,76 g por kg de alimento, equivalentes a 0-

controle; 0,5; 1,0; 2,0 mL de citral por kg de dieta). A dieta foi peletizada utilizando uma peneira com tamanho de sedimento de 6 mm. O citral foi previamente misturado com óleo de peixe. Logo, a mistura foi juntada aos demais ingredientes, homogeneizada manualmente e seca a 40 °C durante 24 h. Os peixes receberam as dietas experimentais até a saciedade aparente (Robalo: quatro vezes ao dia; Tainha: duas vezes ao dia) por 45 dias. A alimentação dos animais foi suspensa 24 h antes da amostragem e coleta final do tecido. O delineamento experimental resultou em quatro grupos e todos os ensaios foram realizados em triplicata (BARBUIO, 1999).

A análise da composição e da composição média aproximada da dieta foi realizada em Laboratório de Bromatologia e Nutrição de Ruminantes da Universidade Federal de Santa Maria (UFSM), estado do Rio Grande do Sul (RS), Brasil (Tabela 1).

TABELA 1. Formulação (%) e análise da composição média aproximada (%) da dieta experimental.

Ingredientes	(%)	Composição média aproximada	(%)
Amido	14	Teor de matéria seca	94,32
Lecitina de soja	1	Proteína	53,73
Vitaminas e minerais (pré-mistura) *	0.5	Extrato etéreo	9,19
Lula fresca	12	Matéria Mineral	20,73
Farinha de peixe	70	Fibra em detergente ácido	2,04
Óleo de peixe	2.4	Fibra em detergente neutron	14,31
Vitamina C	0.1	-	-

*Vitaminas e mistura mineral (níveis de segurança por quilograma de produto) - ácido fólico: 250 mg, ácido pantotênico: 5.000 mg, antioxidante: 0,60 g, biotina: 125 mg, cobalto: 25 mg, cobre: 2.000 mg, ferro: 820 mg, iodo: 100 mg, manganês: 3.750 mg, niacina: 5.000 mg, selênio: 75 mg, vitamina A: 1.000.000 UI, vitamina B1: 1.250 mg, vitamina B12: 3.750 mcg, vitamina B2: 2.500 mg, vitamina B6: 2.485 mg, vitamina C: 28.000 mg, vitamina D3: 500.000 UI, vitamina E: 20.000 UI, vitamina K: 500 mg, zinco: 17.500 mg.

3.5 Coleta das amostras

Após 45 dias, dez peixes de cada tanque foram utilizados para coleta de amostras e determinações analíticas. Os peixes foram anestesiados com benzocaína e a coleta de sangue foi realizada a partir da veia caudal com seringas estéreis heparinizadas. Os peixes foram eutanasiados por secção da medula espinhal e os órgãos foram removidos e imediatamente congelados em nitrogênio líquido. Os tecidos foram armazenados a -20°C para posterior análise.

3.6 Determinações analíticas

3.6.1 Parâmetros de estresse oxidativo

Para a mensuração dos biomarcadores de estresse oxidativo, os tecidos foram homogeneizados segundo Azambuja et al. (2011) usando um meio contendo 120 mM KCl e tampão fosfato de sódio 30 mM (pH 7,4). Os homogenatos foram centrifugados a $1000 \times g$ por 10 min. O teor de proteína foi medido usando o método de Lowry et al. (1951) e os resultados são relatados em mg mL^{-1} .

Os níveis de LPO foram estimados utilizando hidroperóxidos lipídicos (LOOH), que podem detectar os produtos primários de peroxidação usando a oxidação de Fe^{2+} por LOOH em um meio ácido com corante laranja de xilenol. As leituras foram realizadas a 560 nm e os resultados são relatados como $\text{nmol mg proteína}^{-1}$ (SÖDERGREN et al., 1998).

Atividade total de superóxido dismutase (SOD), expressa em unidades SOD mg proteína^{-1} , foi baseado na taxa de inibição da geração de adenocromo autocatalítico em 480 nm (MISRA & FRIDOVICH, 1972). A atividade da catalase (CAT) foi avaliada após a diminuição da absorção em 240 nm de H_2O_2 e foi relatado como $\text{pmol min}^{-1} \text{mg de proteína}^{-1}$ (BOVERIS e CHANCE, 1973).

Atividade de glutathione peroxidase (GPx) foi medida com base na oxidação de NADPH, de acordo com o método de Flohé e Gunzler (1984), a 340 nm. Os resultados foram expressos como $\text{nmol min}^{-1} \text{mg de proteína}^{-1}$.

Os níveis de tióis não proteicos (NPSH), uma medida indireta da glutathione reduzida (GSH), foram avaliados após reação com 5,5'-ditiobis (ácido 2-nitrobenzóico) a 412 nm e como $\mu\text{mol mg de proteína}^{-1}$ (ELLMAN, 1959).

A atividade da glutathione S-transferase (GST) foi medida pela taxa de formação de dinitrofenil S-glutathione a 340 nm (HABIG et al., 1974) e os resultados expressos como $\mu\text{mol min}^{-1} \text{mg de proteína}^{-1}$.

3.6.2 Sistema imunológico inato

A atividade hemolítica do sistema complemento foi medida de acordo com Morales-DelaNuez et al. (2009) com as seguintes modificações: eritrócitos de carneiro a 5% (30 μL) foram adicionadas ao plasma (50 μL) e incubados a 25 °C. Solução salina (100 μL) gelada foi adicionada após 45 min, em seguida, as células foram sedimentadas por centrifugação (3800 x g por 10 min) e a absorbância do sobrenadante foi medida em um leitor de microplacas a 540 nm. A percentagem de hemólise foi calculada por comparação entre hemólise total (100%) e não hemólise (0%) da seguinte forma: % hemólise = $[(\text{amostra A540} - \text{A540 não-hemólise}) / (\text{A540 hemólise total} - \text{A540 não-hemólise})] \times 100$.

A atividade da lisozima foi determinada por um ensaio turbidimétrico como descrito por Jørgensen et al. (1993). Resumidamente, uma suspensão (200 μL) de *Micrococcus lysodeikticus* em Solução de PBS (0,2 g / L) a pH 6,2 foi misturada com plasma (10 μL). A atividade de lisozima (unidades/mL) foi calculada usando a seguinte fórmula: $[(\Delta\text{absorbância (4-1min)} / 3) / 0,001] \times 100$. Uma unidade de atividade da lisozima foi definida como a quantidade de enzima que produz diminuição na absorbância de 0,001 / min.

O teor de mieloperoxidase do soro dos peixes foi determinado de acordo com o protocolo de Quade e Roth (1997) com algumas modificações. O soro de peixe (10 μL) foi diluído com 90 μL de Ca^{++} , Mg^{++} e solução de Hank's livre de vermelho de fenol, em placas de fundo plano de 96 poços. Em seguida, 35 μL de OPD (dicloridrato de *o*-fenilenodiamina),

em citrato (0,2 M) e tampão fosfato (0,1 M, pH 5,3) foram adicionados. A reação de peroxidase foi interrompida após 5 min pela adição de 50 μ L de ácido clorídrico (HCl, 3 M). As placas foram lidas em um espectrofotômetro a 450 nm.

3.6.3 Aspartato transaminase (AST) e alanina transaminase (ALT)

A atividade de aspartato transaminase (AST) e alanina transaminase sérica (ALT) foi determinada utilizando kits comerciais (Gold analisa[®]) e expressos em U mL⁻¹.

3.6.4 Parâmetros histológicos do fígado

Fragmentos do fígado foram fixados em líquido de Bouin por 24 h. Posteriormente, foram submetidos à desidratação, diafanização e procedimentos de inclusão na histosina Leica[®]. Seções 4 mm de espessura foram submetidos à coloração com hematoxilina-eosina, segundo McManus (1946), para estudos morfológicos. Além disso, seis campos de alta potência foram selecionados aleatoriamente em cada slide para medir a área da veia central lobular, diâmetro dos capilares sinusoidais e tamanho dos hepatócitos.

3.7 Análise estatística


A distribuição das variáveis foi testada pelo teste de Kolmogorov-Smirnov. Os dados obtidos nos grupos estudados foram submetidos à análise unidirecional de variância, seguida do teste de Tukey-Kramer. Todas as análises foram realizadas com o software GraphPad Prism[®] 5.0. As diferenças foram consideradas significativas quando $p < 0,05$. Todas as determinações biológicas foram realizadas em triplicata e os resultados expressos por média \pm erro padrão da média (SEM).

4. RESULTADOS

Os resultados estão descritos na forma de um artigo científico publicado na revista *Aquaculture* e outro artigo, submetido para revista *Aquaculture Nutrition*, respectivamente.

4.1 Artigo 1

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Citral as a dietary additive for *Centropomus undecimalis* juveniles: Redox, immune innate profiles, liver enzymes and histopathology

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ABSTRACT

The effect of dietary levels of citral in juvenile common snook (*Centropomus undecimalis*) was evaluated by determining biomarkers of oxidative stress and innate immune system, as well as liver parameters. The fish (2.75 ± 0.03 g) were fed diets containing different concentrations of citral (0, 0.44, 0.88, and 1.76 g/kg feed) for 45 days. Results indicate that citral does not cause significant hepatic alterations; nevertheless, this monoterpenoid was able to induce lipoperoxidation in the gills in all concentrations tested. In this context, the altered activity of enzymes such as superoxide dismutase (SOD) in the liver and gills (1.76 g per kg feed), catalase (CAT) in the liver (0.44; 0.88 g per kg feed) and gills (all groups), and glutathione-S-transferase (GST) in the liver (0.44 g per kg feed) and gills (1.76 g per kg feed) occurred in response to the oxidative damage evidenced. Although dietary levels of citral did not influence the lysozyme activity, we verified that the haemolytic activity of the complement system was excessive in the lowest concentration tested and that the myeloperoxidase activity (MPO) decreased in all groups. Thus, although this monoterpenoid is a diet additive which has already been approved for consumption in other animal species, dietary supplementation with citral for common snook has not improved oxidative parameters and innate immunity of this species.

1. Introduction

The cultivation of aquatic animals has emerged as an alternative to fishing and has great development potential in view of population growth and the increasing search for healthy food. According to FAO (2016), global total production of marine fish has shown significant growth, reaching 26.7 million tons in 2014.

Nevertheless, cultivation conditions are a limiting factor to the increase of aquaculture. Among other factors, infection caused by microorganisms, animal handling and transport, water quality parameters, temperature, and diet exert an important influence on fish health conditions, which have a direct effect on productivity and quality of the cultivated species (Wedemeyer, 1996). Profitable production without unduly harming fish health and performance is still a challenge to fish farming.

Plant-based products such as essential oils (EOs) and their isolated constituents have emerged as an alternative to traditional therapies and diet additives (Sutuli et al., 2017). Essential oils are important dietary sources that contain antioxidant potential as they have phytochemicals with this property or produce them in response to stress conditions (Kasote et al., 2015). Additionally, several plant extracts are also alternatives to boost the immune system when added to fish feed (Sutuli et al., 2017). Thus, dietary supplementation with natural additives may favor the functioning of the organism by means of the modulation of oxidative stress and immunostimulation of these animals (Zheng et al., 2009; Saccol et al., 2013, Sutuli et al., 2017).

Citral is a component of EOs extracted from several plants, including *Cymbopogon citratus*, *Melissa officinalis*, *Verbena officinalis*, *Lippia alba*, among others. This monoterpenoid presents sedative (Vale et al., 2002), antifungal (Ferreira et al., 2009), anti-

inflammatory (Ponce-Monter et al., 2010), and antinociceptive effects (Ortiz et al., 2010). Sousa et al. (2015) found a promising anesthetic effect in rats. Zhang et al. (2014) demonstrated that citral is able to inhibit the formation of bacterial biofilms, and Silva-Angulo et al. (2015) demonstrated antibacterial properties, which indicate that this substance presents potential use as a food preservative. Therefore, citral is currently recognized as a safe diet additive by the US Food and Drug Administration (FDA) (GRAS 182.10).

In aquatic species, several studies have demonstrated biological activity of EOs composed mainly of citral. Parodi et al. (2012) described antioxidant properties of *Aloysia triphylla* EO in sub-adult white shrimps (*Litopenaeus vannamei*). *A. triphylla* EO is able to extend shelf life and refrigeration time of silver catfish (*Rhamdia quelen*) fillets, in addition to being capable of increasing the growth of this species (Daniel et al., 2014; Zeppenfeld et al., 2016). In this fish species, *A. triphylla* EO also presented antioxidant capacity and prevented lipoperoxidation (Gressler et al., 2014; Zeppenfeld et al., 2014). Brum et al. (2017) demonstrated that *Zingiber officinale* essential oil (41.1% of citral) improved the growth and present immunomodulatory effect in *Oreochromis niloticus* (Nile tilapia). Other studies have also related citral to the antimicrobial potential (Bandeira Junior et al., 2018) and anesthetic (Souza, et al., 2017). However, despite all these demonstrated activities, possible antioxidant and immunostimulant properties of this monoterpenoid in fish have not been explored yet.

Centropomus undecimalis (Bloch, 1792) (Common snook, Centropomidae) is an euryhaline species found in areas of the west coast of the Atlantic Ocean from Florida, USA, to Santa Catarina, Brazil (Alvarez-Lajonchere & Tsuzuki, 2008). It presents great potential for aquaculture due to its rapid growth rate, great resistance during management processes as well as its high value and market demand (Cavalli & Hamilton, 2007). Therefore, the objective of this study was to evaluate the effects of dietary supplementation with citral on parameters of

oxidative stress and innate immunity in *C. undecimalis*. In addition, the activity of citral on hepatic enzymes and histopathological analysis of liver was determined, aiming to identify possible signs of hepatotoxicity of this monoterpenoid when added to diet.

2. Materials and Methods

2.1 Animals and rearing conditions

The experiment was performed in the Laboratory of Marine Fish Farming (LAPMAR) at the Federal University of Santa Catarina (UFSC), Florianópolis, Southern Brazil. The study was approved by the Ethics Committee on Animal Experimentation of UFSC and was registered under number PP00861/2013.

Juvenile common snook (*Centropomus undecimalis*) (2.75 ± 0.03 g and 7.07 ± 0.04 cm) were randomly distributed into four water recirculation systems with salinity 31.12 ± 2.31 ppt, temperature of $31.59 \pm 0.91^\circ\text{C}$ and photoperiod of 12:12 h LD, as suggested by Michelotti et al. (2018) (n=30 per tank). Each system consisted of three circular tanks (150 L). During the experimental period, the water contained in these tanks was removed through a central pipeline with a bag filter (50 μm), a biological filter, a foam fractionator, and an ultraviolet sterilizer (60w). After the treatment, water returned to the experimental tanks.

Fish were acclimated to the experimental conditions for four days. Remains of food and feces were removed through siphoning and an average of 25% of the water was renewed on a daily basis.

After 45 days, three fish from each tank (n = 9 animals per treatment) were used for sample collection. Also, there was no difference in consumption between the experimental groups.

2.2 Water parameters

Water parameters were checked daily (temperature, pH, and dissolved oxygen) or weekly (alkalinity, total ammonia, and nitrite) throughout the experimental period as described by Zeppenfeld et al. (2014). Water parameters remained stable throughout the experimental period. Temperature was maintained at $31.59 \pm 0.08^\circ\text{C}$, pH at 8.18 ± 0.02 , and dissolved oxygen at $4.63 \pm 0.06 \text{ mg L}^{-1}$. Alkalinity ($102.35 \pm 0.9 \text{ mg CaCO}_3 \text{ L}^{-1}$), nitrite ($0.3 \pm 0.05 \text{ mg L}^{-1}$), and total ammonia ($0.3 \pm 0.04 \text{ mg L}^{-1}$) were maintained within the desired range. The water from Lagoa da Conceição used in the experiments of said laboratory has a total hardness of approximately 6000 CaCO_3 per mL/water.

2.3 Citral

Citral was obtained from Sigma Aldrich[®] (St. Louis, Missouri, USA). Citral density was $0.88 \text{ g}\cdot\text{cm}^{-3}$. Before being incorporated into the diet, isomer quantification was performed. The analysis was carried out with an Agilent 6890A gas chromatograph coupled to an Agilent Mass Selective Detector MSD 5973 with an HP-Chiral capillary GC column (30 m x 0.25 mm i.d. x 0.25 μm film thickness) and operated in electron ionization mode at 70 eV. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} , injector temperature was set at 250 and detector at 280°C . Oven temperature was kept at 40°C for 4 min and then raised to 240°C at a rate of 4°C min^{-1} . Sample solutions of 1 μL (2:1000 in hexane, v/v) were injected in split mode. Kovats retention indices were calculated by means of a homologous series of n-alkanes (C8-C40) that was injected under the same conditions of the samples. The constituents were identified by means of mass spectrometry and Kovats retention index was compared to data from the National Institute of Standards and

Technology Mass Spectral Library (NIST, 2008). The relative percentage of compounds was estimated by under peak area integration obtained from the chromatogram (α -Citral=60.15%; β -Citral =39.85%).

2.4 Diets and Experimental design

2.4 Diets and Experimental design

Citral levels were defined considering previous studies by Zeppenfeld et al (2016) and Zeppenfeld et al. (2017), which used diets enriched with essential oils containing citral as major component. Four diets based on the same initial formulation (Barbuio, 1999) (Table 1) containing different concentrations of citral (0-control; 0.44; 0.88; 1.76 g per kg feed) were pelletized at a 6 mm pellet size. Citral was previously homogenized with fish oil. All ingredients were homogenized manually and dried at 40°C for 24 h. This temperature was chosen to prevent evaporation of citral, since the boiling point of this monoterpenoid is 229 °C (Kang et al., 2007). Fish received the experimental diets until apparent satiation four times a day for 45 days. The feed was suspended 24 h prior to sampling and final collection of the tissues. The experimental design resulted in four groups and all assays were performed in triplicate. The analysis of the composition of the diet was carried out at the Laboratory of Bromatology and Ruminant Nutrition at the Federal University of Santa Maria (UFSM), Rio Grande do Sul (RS), Brazil (Table 2). Dry matter content was determined by oven drying at 105 °C for at least 8 hours (AOAC, 1984). Total nitrogen (N) was determined by the Kjeldahl method (AOAC, 1997). For the conversion of N values to crude protein, the correction factor of 6.25 was used. Determination of ether extract content was performed by AOCS Am5-04 Method (2005). Neutral detergent fiber analysis was based on the procedures described by Mertens (2002) using thermostable α -amylase, except that the samples were weighed into

polyester filter bags (porosity 16 μm) and treated with neutral detergent at 110 ° C for 40 minutes (Senger et al., 2008). Concentrations of acid detergent fiber were analyzed according to AOAC 973.18 Method (1997) except that the samples were weighed into polyester filter bags (16 μm porosity) and treated with autoclave acid detergent at 110 ° C for 40 minutes (Senger et al., 2008). The crude energy was obtained through the total combustion of the sample in calorimetric pump (Model Parr 6400 Oxigen Bomb Calorimeter).

2.5 Sample collection

After 45 days, three fish from each tank (n = 9 animals per treatment) were anesthetized with 50 mg L⁻¹ benzocaine. Blood sampling was performed from the caudal vein with sterile heparin-filled syringes. The fish were euthanized by sectioning the spinal cord and liver and gills were frozen in liquid nitrogen immediately after removal. Tissues were stored at -20°C for 30 days, for further analysis.

2.6 Biological analytical determinations

2.6.1 Oxidative stress parameters

For the measurement of oxidative stress biomarkers, tissues were homogenized according to Azambuja et al. (2011) by means of a medium containing 120 mmol L⁻¹ KCl and 30 mmol L⁻¹ sodium phosphate buffer (pH 7.4). The homogenates were centrifuged at 1000×g for 10 min. Protein content was measured by means of the Lowry protein assay (Lowry et al., 1951) and results were reported in mg mL⁻¹.

Lipid peroxidation levels were estimated using a lipid hydroperoxides (LOOH) technique that detects the primary products of peroxidation through the oxidation of Fe²⁺ by

LOOH in an acidic medium containing xylenol orange dye. The readings were performed at 560 nm and the results were reported in $\text{nmol mg protein}^{-1}$ (Södergren et al., 1998).

Total superoxide dismutase (SOD) activity, which are expressed as SOD units mg protein^{-1} , was based on the inhibition rate of autocatalytic adenochrome generation at 480 nm (Misra and Fridovich, 1972). Catalase (CAT) activity was evaluated by following the decrease in the 240 nm absorption of H_2O_2 , and it was reported in $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ (Boveris and Chance, 1973). Glutathione peroxidase (GPx) activity was measured based on NADPH oxidation at 340 nm, according to the method described by Flohé and Gunzler (1984). Results were expressed in $\text{nmol min}^{-1} \text{mg protein}^{-1}$. Non-protein thiol (NPSH) levels, an indirect measure of reduced glutathione (GSH), was evaluated after reacting with 5,5'-dithiobis (2-nitrobenzoic acid) at 412 nm and reported in $\mu\text{mol mg protein}^{-1}$ (Ellman, 1959). Glutathione S-transferase (GST) activity was measured by the rate of dinitrophenyl S-glutathione formation at 340 nm (Habig et al., 1974) and the results expressed in $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$.

2.6.2 Immunological parameters

2.6.2.1 Lysozyme activity assay

Lysozyme activity was determined by a turbidimetric assay as described by Jørgensen et al. (1993). Briefly, a suspension (200 μL) of *Micrococcus lysodeikticus* in PBS solution (0.2 g L^{-1}) at pH 6.2 was mixed with plasma (10 μL). Lysozyme activity (units/mL) was calculated using the following formula: $[(\Delta_{\text{absorbance}(4-1\text{min})} / 3) / 0.001] \times 100$. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min.

2.6.2.2 Haemolytic activity of complement system

Haemolytic activity of complement system (HACS) was measured according to a method described by Morales-DelaNuez et al. (2009) with the following modifications: washed sheep red blood cells at 5% (30 μ L) were added to the plasma (50 μ L) and incubated at 25 °C. Ice-cold saline (100 μ L) was added after 45 min, cells were pelleted by centrifugation (3800 x *g* for 10 min), and the absorbance of the supernatant was measured using a microplate reader at 540 nm. Hemolysis percentage was calculated by comparison between total hemolysis (100%) and no-hemolysis (0%) controls as follows: % hemolysis = $[(A_{540 \text{ sample}} - A_{540 \text{ no-hemolysis}}) / (A_{540 \text{ total hemolysis}} - A_{540 \text{ no-hemolysis}})] \times 100$.

2.6.2.3 Myeloperoxidase assay (MPO)

The peroxidase content of fish plasma was determined according to the protocol of Quade and Roth (1997) with some modifications. Fish plasma (10 μ L) was diluted with 90 μ L of Ca^{2+} , Mg^{2+} and phenol red-free Hank's solution in flat-bottomed 96-well assay plates. Then, 35 μ L of OPD (*o*-phenylenediamine dihydrochloride) in citrate (0.2 M) and phosphate buffer (0.1 M, pH 5.3) were added. The peroxidase reaction was stopped after 5 min by adding 50 μ L of hydrochloric acid (HCl, 3 M). Plates were read in a spectrophotometer at 450 nm.

2.6.3 Biochemical parameters

Aspartate transaminase (AST) and alanine transaminase (ALT) levels in plasma were determined using commercial kits (Gold Analisa[®]) and expressed in U/L (One is defined as micromoles of substrate transformed per minute per one litre).

2.6.4 Histopathological analysis

Fragments of the liver were fixed in Bouin liquid for 24 hours. Subsequently, they were submitted to dehydration, diaphanization, and inclusion procedures in Leica[®] historesin. Sections 4 µm thick were submitted to hematoxylin-eosin staining, according to McManus (1946), for morphological studies. In addition, six high-power fields were randomly selected on each slide to measure lobular center vein area, sinusoidal capillaries diameter, and hepatocytes size.

2.7 Statistical analysis

The biological determinations of all samples were performed in triplicate and the results expressed as mean \pm standard error of the mean (SEM). The distribution of the variables was tested using the Kolmogorov-Smirnov test. Data obtained from the groups, for the same parameter, were submitted to one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. All analyses were performed using GraphPad Prism[®] Software 5.0 and differences were considered significant at $p < 0.05$.

3. Results

3.1 Oxidative stress parameters

Levels of LPO in the liver did not differ from the control group. However, fish fed with citral 0.44 and 0.88 g per kg feed were different from each other. LPO significantly increased in the gills of fish that received diets containing citral supplementation (0.44; 0.88; 1.76 g per kg feed) when compared to control (Fig. 1a and 2a).

The activity of SOD significantly increased in the liver and gills of fish fed a diet supplemented with citral at a concentration of 1.76 g per kg feed when compared to the fish fed control diet (Fig. 1b and 2b). On the other hand, CAT activity was reduced in the liver (0.44; 0.88 g per kg feed) and gills (0.44; 0.88; 1.76 g per kg feed) of animals treated with diets containing citral when compared to control (Fig. 1c and 2c). GPx activity in the liver and gills was not significantly affected by citral-containing dietary supplementation (Fig. 1d and 2d).

Levels of NPSH decreased in the liver of fish fed diet containing citral at 0.44 g per kg feed when compared to the control fish (Fig 1e and 2e). GST activity in the liver did not differ from the control group. Nevertheless, fish fed with citral 0.44 and 0.88 g per kg feed differed from each other. There was a significant increase of GST activity in the gills of fish fed citral at 1.76 g per kg feed when compared to control (Fig. 1f and 2f).

3.2 Immunological parameters

Diets containing different citral concentrations did not exert any significant effect Lysozyme activity. Complement activity increased in the group fed diet containing citral at

0.44 g per kg feed (Fig. 3b). There was a significant decrease in myeloperoxidase levels in all treated groups when compared to the control group (Fig. 3c).

3.3 AST and ALT

Diets containing different citral concentrations did not exert any significant effect on plasma AST and ALT activity (Table 3).

3.4 Histopathological analysis

The histopathological analysis did not show the presence of any cellular alterations. Despite this, the presence of lipid vacuoles was identified in all groups (Fig.4). The hepatic morphometric analysis presented in Table 4 shows significant differences in the size of the sinusoidal capillaries of the groups fed with citral 0.88 and 1.76 g per kg feed compared to the group 0.44 g citral per kg feed and control group.

4. Discussion

The search for nutritional alternatives that optimize natural defense mechanisms in fish plays an important role in producing robust juveniles and adults (Sweetman et al., 2010). The positive effect of different secondary plant metabolites as EOs in fish antioxidant defenses (Azambuja et al., 2011; Zeppenfeld et al., 2014) has stimulated their evaluation as food additives in aquaculture, aiming to develop possible strategies for health management in commercial aquaculture facilities (Saccol et al., 2013).

Zeppenfeld et al. (2017) recently reported the beneficial effects of a diet supplemented with the EO of *A. triphylla* on oxidative status in *R. quelen*, whose major components were α -citral (29.41%) and β -citral (20.78%) (Zeppenfeld et al., 2016). Since the chemical composition of EOs is influenced by different factors, such as plant chemotype, collection season, temperature, soil composition, extraction method, rainfall, wind regime, among others (Gobbo-Neto and Lopes, 2007), the evaluation of dietary supplementation with the isolated major component of the EO of *A. triphylla* was proposed.

Oxidative stress, which is due to excessive production of ROS or a decline in antioxidant activity, may trigger lipid peroxidation of the cell membrane as well as damage proteins, enzymes, carbohydrates and DNA (Birnie-Gauvin et al., 2016). Nevertheless, some studies with vertebrates have considered that oxidative stress could be a link in the activity of the immune system, whose action is extremely relevant to the defense against pathogenic microorganisms (Biller-Takahashi et al., 2015; Tort, 2011).

According to Li et al. (2015), the liver is the main target organ of oxidative processes; however, extrahepatic damage, such as cerebral, renal, and respiratory deficiency may also occur. Thus, to verify if citral could prevent LPO in the membrane, levels of lipid hydroperoxides in the liver and gills of *C. undecimalis* were analyzed. No alterations were observed in the liver, although contrary to our hypothesis, citral increased lipoperoxidation in the gills at 0.44, 0.88 and 1.76 g per kg feed. According to Tamir et al. (1984), compounds containing carbonyl groups, such as citral, are highly reactive and can induce cellular injury by membrane lipoperoxidation.

As a counterpoint, the endogenous cellular antioxidant system is able to repair these oxidative damages by restoring oxidized lipids to phospholipases, peroxidases or

acyltransferases or by neutralizing ROS (Hitchon & El-Gabalawy, 2004). For example, the superoxide anion may be transformed by enzymes belonging to the superoxide dismutase (SOD) family, producing O_2 (molecular oxygen) and H_2O_2 (hydrogen peroxide) (Pisoschi and Pop, 2015). The elevation of SOD activity in the liver and gills of the group fed with 1.76 g/kg of citral-enriched diet may suggest that the observed LPO may have occurred due to increased superoxide anion production since a high activity of this enzyme indicates an attempt to neutralize the aforementioned ROS.

However, it is important to note that H_2O_2 , which results from the action of SOD, is also highly reactive. According to Kohen & Nyska (2002), its action involves attacking proteins, enzymatic inactivation and oxidation of DNA, lipids, sulfhydryl groups, and ketoacids. In this sense, the performance of the CAT and GPx is fundamental since they are able to neutralize H_2O_2 . Citral as a dietary supplementation for *C. undecimalis* did not alter GPx activity, although it reduced CAT activity in both the liver (0.44; 0.88 g per kg feed) and gills in all groups fed this monoterpenoid. In light of this evidence, the observed reduction in CAT activity could justify the occurrence of LPO in the gills of this group, since the neutralization of H_2O_2 was probably deficient and consequently allowing the attack to the cell membrane lipids by ROS.

GST family of enzymes facilitates the elimination of xenobiotics by catalyzing thioether bonds between GSH and small electrophilic centers (Jakobsson et al., 1999; Tew et al., 2015). The activity of this enzyme increased in the gills in the group that received the highest concentrations of citral. This result may be explained by the fact that the enzymatic Glutathione S-transferase/ GST reactions are an important step in intracellular metabolism of citral (Kapur et al., 2016). Besides, Nakamura et al. (2003) demonstrated that GST activity was induced approximately 1.6-fold when hepatocytes were exposed to geraniol for 24 h and

that the inducing activity of citral was dose-dependent, justifying the response only at the highest concentration tested in this study.

The measurement of non-protein thiol levels is an indirect measure of GSH levels, which is considered to be the main non-enzymatic endogenous antioxidant. GSH participates in numerous physiological functions, such as the elimination of ROS by detoxification and consequently protection against oxidative damage as well as cellular immunity (Deponte, 2013). Fish that received the diet enriched with citral at 0.44 g per kg feed had lower levels of non-protein thiols in the liver. Citral may combine with sulfhydryl groups of the enzymes involved in GSH synthesis and thus cause a decline in non-enzymatic antioxidant levels within the cell. Furthermore, this monoterpenoid can have interfered with the activity of the enzyme glutathione reductase (GR), decreasing GSH synthesis in this group (Couto et al., 2016). In several situations the effect of natural products (as essential oils and their isolated compounds) do not present a dose-effect relationship, justifying the observation of this result for this concentration only (Liao et al., 2015).

The lysosomal oxidative enzyme MPO plays a primordial role in the phagocytic activity of fish and thus contributes to the functioning of fish immune system (Russel et al., 2017). The presence of citral in the diet reduced MPO activity, suggesting that citral impairs the ability fish have to respond to infections (Kreutz et al., 2011). Sinha et al. (2014) tested the effect of citral on cell viability of a human lymphocyte line and demonstrated that this monoterpenoid reduces the viability of these defense cells to 75.69%. In this context, neutrophil viability may also be affected by this monoterpenoid, justifying the decrease in MPO levels. An alternative explanation is that this result may indicate anti-inflammatory activity for the compound tested since the release of MPO is related to tissue damage (Pitanga et al., 2014). Liao et al. (2015) demonstrated anti-inflammatory activity of citral isomers.

Neral, in particular, has been more efficient in inhibiting interleukin 1 β , nitric oxide synthase, and cyclooxygenase 2 expression in murine macrophages stimulated by lipopolysaccharides.

The complement system constitutes another primordial defense mechanism in fish since it participates in the opsonization and consequent phagocytosis of pathogenic microorganisms (Magnadóttir, 2006). Boshra et al. (2006) argue that this system is likely to be more evolved in fish since it is necessary for them to overcome survival requirements imposed by the aquatic environment and management conditions. In the present study, citral induced the highest activity of the complement system at the lowest concentration tested. Although this finding indicates that clearance of possible infectious diseases would be more efficient in this group (Zipfel & Skerka, 2009), the excessive activity of the complement system may be detrimental to cell membrane due to excessive production of ROS according to Shen et al. (1997) and Secombes & Wang (2012). Thus, the increase in this biomarker in the group that received the diet containing citral at 0.44 mg per kg feed may also have contributed to the occurrence of LPO in the fish gills.

Lysozyme, which is a vital component of the innate immune system (Nilojan et al., 2017), is present in phagocytic cells and recognized for its bactericidal action, especially in Gram-positive bacteria (Klockars & Roberts, 1976). Also, this enzyme is able to activate the complement system, acting as an opsonin (Jolles and Jolles, 1984). However, citral no altered lysozyme levels when added to the diet of *C. undecimalis* in any of the evaluated concentrations. This result does not explain the increase in complement system haemolytic activity in fish treated with citral at 0.44 g per kg feed.

The activities of AST and ALT can be used as biomarkers for cellular damage in fish since they participate in transamination reactions, which predominantly occur in the liver, cardiac cells, and striated muscle tissue (Vutukuru et al., 2007). In addition, changes in

hepatic structure may be significant to evaluate possible toxic effects of citral, since the liver is fundamental for the metabolism of xenobiotics (Wolf and Weeler, 2018). In the present study, alterations in AST and ALT activity of citral-treated animals were not observed, as expected. This finding corroborates the results of Li et al. (2017), in which the activities of the above-mentioned enzymes in plasma of rats fed citral showed no significant differences when to the control group. In addition, no cellular damage was found in the liver of the fish, since the presence of the hepatic vacuoles is considered a normal finding, because teleosts can have the liver parenchyma rich in lipids and glycogen (Welsch and Storch, 1973). On the other hand, the difference in the size of sinusoidal capillaries may also have elapsed from a normal adrenergic response, since no other changes were observed in liver (Barton, 2011).

All fish fed with citral presented increased LPO and decreased catalase activity in the gills. In addition, in the liver there was also a decrease in catalase activity (0.44, 0.88 g per kg feed) and non-protein thiol levels (0.44 g per kg feed). These oxidizing effects may have activated physiological antioxidant responses evidenced by the significant increase in SOD and GST activity in the groups receiving the highest concentration tested in this study. Regarding the innate immune system, we showed that the presence of citral in the diet decreased the activity of myeloperoxidase and hyperactivated the complement system (0.44 g per kg feed), damaging the fish defense system. Therefore, although hepatic enzyme activity and histological markers were considered normal, the addition of citral in the diet of common snook is not recommended.

These results were unexpected and may suggest that biological activity of complex mixtures, such as EOs, may be due to the action of minor components. The principles governing phytotherapy, in which pharmacological effects result from complex interactions between different components with additive, synergistic, and even antagonistic effects, must

also be considered (Efferth and Koch, 2011). Another fundamental aspect is the physiological divergences between fish species, which can promote different responses to the same therapeutic agent (Gioda et al., 2017). Therefore, future studies should focus on evaluating the effects of citral on other fish species and on testing some isolated minor components from *A. triphylla* EO, the cytotoxicity attributed to this monoterpenoid against fish pathogenic microorganisms or a possible anti-inflammatory activity of monoterpenoid.

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TABLES

Table 1

Formulation (%) of the experimental diet

Ingredients	(%)
Starch	14
Soy lecithin	1
Vitamins and minerals (premix)*	0.5
Fresh squid	12
Fish meal	70
Fish oil	2.4
Vitamin C	0.1

*Vitamin and mineral mixture (security levels per kilogram of product) — folic acid: 250 mg, pantothenic acid: 5.000 mg, antioxidant: 0.60 g, biotin: 125 mg, cobalt: 25 mg, copper: 2.000 mg, iron: 820 mg, iodo: 100 mg, manganese: 3.750 mg, niacin: 5.000 mg, selenium: 75 mg, vitamin A: 1.000.000 UI, vitamin B1: 1.250 mg, vitamin B12: 3.750 mcg, vitamin B2: 2.500 mg, vitamin B6: 2.485 mg, vitamin C: 28.000 mg, vitamin D3: 500.000 UI, vitamin E: 20.000 UI, vitamin K: 500 mg, zinc: 17.500 mg.

Table 2

Composition (%) of the experimental diet

	Groups (g citral)			
	0	0.44	0.88	1.76
Dry matter content	94.21	94.42	94.21	94.44
Protein	53.32	53.96	54.29	53.35
Ether extract	9.37	9.75	9.33	9.77
Mineral matter	20.35	21.48	20.22	20.87
Acid detergent fiber	1.79	2.03	1.98	2.05
Neutral detergent fiber	15.17	12.78	14.18	13.96

Table 3

Hepatic enzymes in plasma of *Centropomus undecimalis* fed with diets containing different concentrations of citral

	<i>Control</i>	<i>0.44</i>	<i>0.88</i>	<i>1.76</i>
<i>AST</i>	33.38 ± 10.39^a	43.76 ± 14.42^a	51.58 ± 15.71^a	33.12 ± 10.30^a
<i>ALT</i>	68.90 ± 13.25^a	52.89 ± 2.32^a	57.23 ± 17.60^a	54.73 ± 4.23^a

AST – Aspartate aminotransferase; ALT – Alanine aminotransferase.

Values are mean \pm SEM (n= 4). Different lowercase letters indicate significant difference between the treatments using one-way ANOVA and Tukey's test ($p < 0.05$)

Table 4 Liver histology parameters (μm) in *Centropomus undecimalis* fed with diets containing different concentrations of citral.

	<i>Control</i>	<i>0.5</i>	<i>1.0</i>	<i>2.0</i>
<i>Lobular center vein</i>	1539 ± 274.4^a	1376 ± 132.1^a	1501 ± 136.8^a	1937 ± 433.5^a
<i>Sinusoidal capillaries</i>	5.84 ± 0.20^a	5.31 ± 0.12^a	4.31 ± 0.28^b	4.03 ± 0.20^b
<i>Hepatocytes</i>	118.0 ± 4.77^a	108.2 ± 3.28^a	110.1 ± 3.76^a	112.4 ± 3.46^a

All the values are expressed as mean \pm SEM (n= 4). Different lowercase letters indicate significant difference between the treatments using one-way ANOVA and Tukey's test ($p < 0.05$).

FIGURES

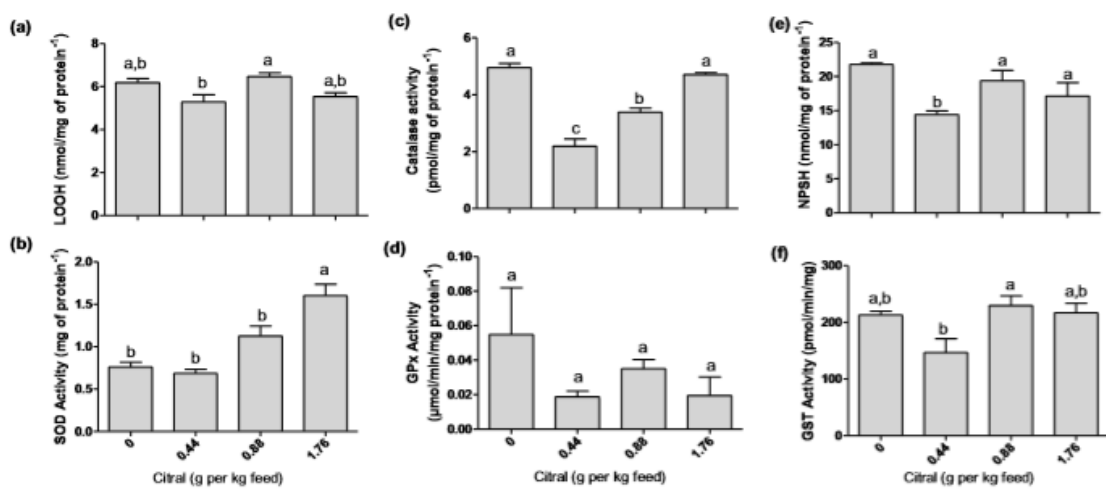


Fig. 1. (a) Lipid hydroperoxides levels (LOOH), (b) superoxide dismutase (SOD) activity, (c) catalase (CAT) activity, (d) glutathione peroxidase (GPx) activity, (e) non-protein thiol (NPSH) levels, and (f) glutathione S-transferase (GST) activity in the liver of *Centropomus undecimalis* fed diets containing different concentrations of citral. All values are expressed as mean \pm SEM (n = 4). Different lowercase letters indicate significant difference between treatments (p < .05).

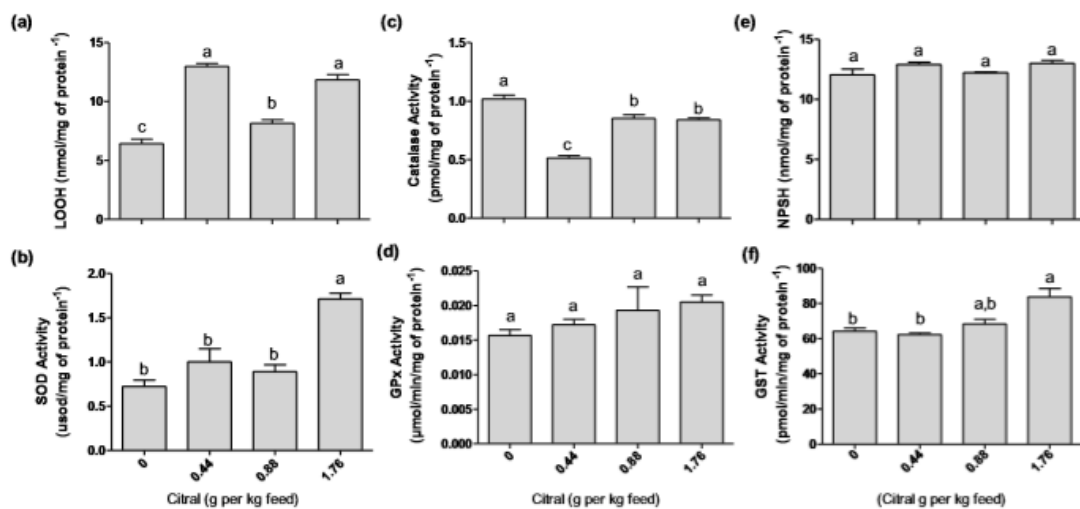


Fig. 2. (a) Lipid hydroperoxides (LOOH) levels, (b) superoxide dismutase (SOD) activity, (c) catalase (CAT) activity, (d) glutathione peroxidase (GPx) activity, (e) non-protein thiol (NPSH) levels, and (f) glutathione S-transferase (GST) activity in the gills of *Centropomus undecimalis* fed diets containing different concentrations of citral. All values are expressed as mean \pm SEM (n = 4). Different lowercase letters indicate significant difference between the treatments (p < .05).

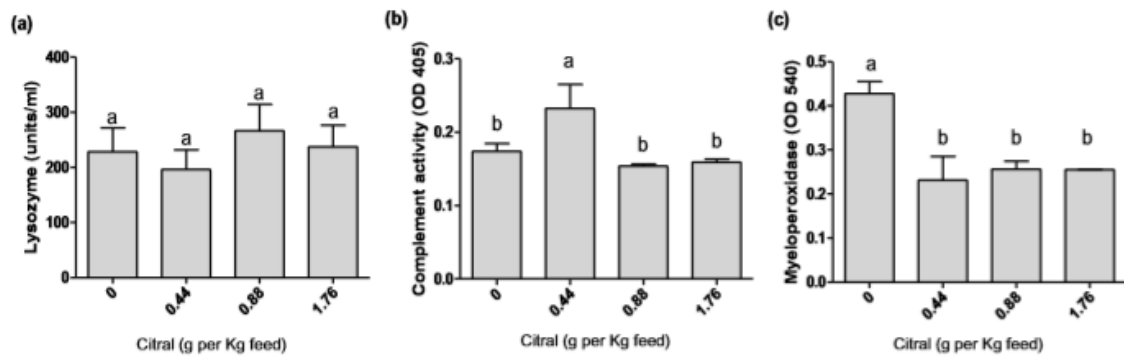


Fig. 3. (a) Lysozyme activity (units/ml), (b) haemolytic activity of complement system (%), and (c) Myeloperoxidase levels (OD540) in plasma obtained from *Centropomus undecimalis* fed diets containing different concentrations of citral. All values are expressed as mean \pm SEM (n = 4). Different lowercase letters indicate significant difference between the treatments (p < .05).

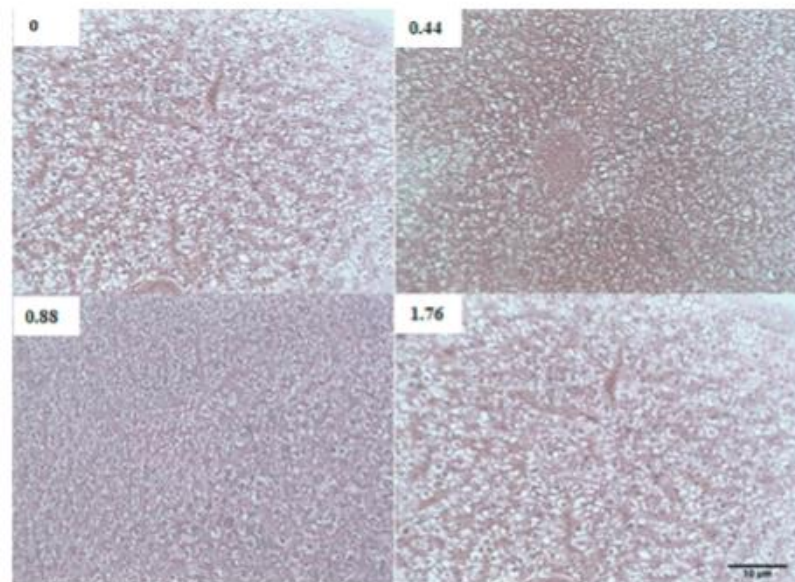


Fig. 4. Histological characteristics of liver from *Centropomus undecimalis* fed with diets containing different concentrations of citral (g per Kg feed).

4.2 Artigo 2

Redox profile, innate immune system, and hepatic parameters in *Mugil liza* juveniles after the dietary addition of citral

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Running title: Citral as a dietary additive for *Mugil liza*

Key words: monoterpenoids, fish, diet, oxidative stress, innate immune system, hepatic biomarkers.

ABSTRACT: Different citral concentrations (0, 0.44, 0.88, and 1.76 g per kg feed) were added to the diet of *Mugil liza* juveniles for 45 days and their effect on oxidative stress biomarkers, innate immune system, and liver damage were evaluated. The presence of citral reduced lipoperoxidation in the liver, improved superoxide dismutase activity, glutathione peroxidase, glutathione-S-transferase in the liver, gills, and brain, in addition to increasing non-protein thiol levels in the gills and brain, especially at higher concentrations. Myeloperoxidase levels were lower in groups that consumed 0.88 and 1.76 g citral per kg feed. These effects occurred without promoting hepatic injury, since histological parameters and serum aspartate aminotransferase (and alanine aminotransferase) as well as histological parameters from treated fish did not differ from the control group. The use of citral at 1.76 g per kg feed improved the health of *Mugil liza*; therefore, it is recommended for the increased productivity of this species.

1. Introduction

Animals grown in aquatic environments are naturally predisposed to stress development. This is because the handling and transport of animals, changes in water quality, and presence of chemicals, contaminants, and pathogens are common in aquaculture. These alterations reflect directly on the productivity of cultivated species, generating a negative economic impact on this activity (Tort, 2011).

The stress of managing these animals in such environments also induces responses in the oxidative system. In general, there is an imbalance between antioxidants and oxidants in favor of the latter, leading to the disruption of redox signaling and to molecular control and/or damage (Sies, 2015). According to Birnie-Gauvin et al. (2017), antioxidant defenses minimize oxidative stress and are associated with fertility and survival, while oxidative stress negatively affects fish reproduction and growth. This occurs because reactive oxygen species (ROS)

which are generated under oxidative stress attack biomolecules, impairing their function (Gebicki, 2016). Thus, a redox profile can be used to evaluate health of *Mugil liza*.

The immune system is particularly sensitive to oxidative stress because (1) ROS generation is necessary for the microbicidal activity of phagocytic cells; (2) polyunsaturated fatty acids from the plasma membrane of the immune cells are susceptible to external ROS and (3) antioxidants from the immune cells preserve the integrity and function of macromolecules as well as control signal transduction of gene expression (De La Fuente, 2002; Aslani and Ghobadi, 2016). Thus, according to Tort (2011), stress responses may affect immune system efficiency, consequently compromising immune defences and resistance to pathogens in fish.

Therefore, compounds that may minimize oxidative stress and supplementation of immunostimulant diets to improve weight gain, feed efficiency, and/or disease resistance in cultured fish have been employed to minimize the impact of managing conditions in fish productivity (Vallejos-Vidal et al., 2016). Hence, natural compounds have shown important advantages in aquaculture, considering they have expressive biological activity and low occurrence of adverse events (Sutili et al., 2017).

Citral, which is a monoterpene aldehyde composed of the geometric isomers neral and geranial, is found in a wide variety of plants (Saddiq and Kayyat, 2010) and has already been linked to benefits in some fish species. Brum et al., (2017) reported that *Zingiber officinale* essential oil (41.1% citral) supplementation improved growth and presented immunomodulatory effects on Nile tilapia (*Oreochromis niloticus*) after the challenge performed with *Streptococcus agalactiae*. Dietary supplementation with *Aloysia triphylla* essential oil (50.2% citral) also stimulated growth, antioxidant capacity, prevented lipoperoxidation, and decreased cortisol release in *Rhamdia quelen* (Zeppenfeld et al., 2017).

In vitro studies have also linked citral to anti-inflammatory properties. For example, Liao et al. (2015) demonstrated that neral promoted inhibition of cytokine secretion and inflammatory molecule expression of lipopolysaccharide-stimulated macrophages. Mori et al. (2018) detected decreased myeloperoxidase activity after the addition of citral in the diet of *Centropomus undecimalis*. Although numerous studies with essential oil (EO) containing citral have shown good results in fish, the use of citral in aquaculture is still conflicting. *Mugil liza* is a marine fish of the Mugilidae family. It inhabits coastal regions from the Caribbean Sea to northern Patagonia in Argentina and estuarine environments (Fortunato et al., 2017). According to Lisboa et al. (2015), the cultivation of this species has attracted the attention of fish farmers due to its low position in the food chain and ease of handling. Therefore, in order to increase the possibilities of chemical and biochemical interventions to improve the health and productivity of this species, the possible benefits of citral in the diet of *Mugil liza* redox profile and innate immune system were investigated. Furthermore, this substance was also tested for hepatic toxicity in this fish species.

2. Materials and methods

2.1 Fish conditioning and water quality

This experiment was performed at the Laboratory of Marine Fish Farming (LAPMAR) of Federal University of Santa Catarina (UFSC), Florianópolis, Southern Brazil. The study was approved by the Ethics Committee on Animal Experimentation of UFSC (registration number PP00861/2013).

Mugil liza juveniles (6.69 ± 0.06 g and 8.56 ± 0.01 cm) were randomly distributed into four water recirculation systems with salinity 32.19 ± 0.18 ppt. Each system consisted of three circular tanks (150 L). During the experimental period, the water contained in these tanks was removed through a central pipeline with a bag filter (50 μ m), biological filter, foam fractionator, and ultraviolet sterilizer (60w). After the treatment, the water was returned to the experimental tanks. Fish were acclimated to experimental conditions for four days. Food and feces were removed through siphoning and an average of 25% of water was renewed on a daily basis.

Water parameters were checked daily (temperature, pH, and dissolved oxygen) or weekly (alkalinity, ammonia, and nitrite) throughout the experimental period as described by Zeppenfeld et al. (2014). Water parameters remained stable during the whole of the experimental period. The temperature was maintained at 27.11 ± 0.11 °C, pH at 7.58 ± 0.13 , and dissolved oxygen at 5.16 ± 0.16 mg L⁻¹. Alkalinity (103.65 ± 0.34 mg CaCO₃ L⁻¹), nitrite (0.2 ± 0.04 mg L⁻¹), and total ammonia (0.2 ± 0.05 mg L⁻¹) were maintained within the desired range.

2.2 Citral

Citral was obtained from Sigma Aldrich® (St. Louis, Missouri, USA). Isomer quantification was performed before incorporating citral into the diet. Composition analyses are reported in Mori et al. (2018). The relative percentage of compounds was estimated by under peak area integration obtained from chromatogram (α -Citral, syn geranial = 60.15%; β -Citral, syn neral = 39.85%).

2.3 Diets and experimental design

Four diets based on the same initial formulation (Barbuio, 1999) (Table 1) containing different concentrations of citral (0-control; 0.44; 0.88; 1.76 g per kg feed equivalent to 0-control; 0.5; 1.0; 2.0 mL per kg feed) were pelletized using a 6 mm pellet size. Citral was previously mixed with fish oil. All ingredients were homogenized manually and dried at 40 °C for 24 h. Fish received the experimental diets until apparent satiety, four times a day for 45 days. The feed was suspended 24 h prior to sampling and final tissue collection. The experimental design resulted in four groups and all assays were performed in triplicate. Diet composition analysis was carried out as described by Mori et al. (2018) (Table 1).

2.4 Sample collection

After 45 days, ten fish from each tank were used for sample collection and analytical determination. Blood sampling was performed from the caudal vein with sterile heparin-filled syringes. The fish were anesthetized with 50 mg L⁻¹ benzocaine and euthanized by sectioning the spinal cord and liver. The gills and brain were frozen in liquid nitrogen immediately after removal. Tissues were stored at -20 °C for further analysis.

2.5 Prooxidant and antioxidant analyses

Tissues were homogenized according to Azambuja et al. (2011) using a medium containing 120 mM KCl and 30 mM sodium phosphate buffer (pH 7.4). Protein content was measured using the method of Lowry et al. (1951), and results are reported as mg mL⁻¹.

Lipoperoxidation (LPO) levels were estimated according to the method described by Södergren et al. (1998). This technique can detect lipid hydroperoxides (LOOH) through Fe²⁺

oxidation by primary products of peroxidation in an acidic medium with xylenol orange dye. The readings were performed at 560 nm and the results were reported as $\text{nmol mg protein}^{-1}$. Total superoxide dismutase (SOD) activity was performed according to Misra and Fridovich (1972) and based on the inhibition rate of autocatalytic adenochrome generation. Readings were performed at 480 nm and the results were reported as $\text{SOD units/mg protein}^{-1}$. Catalase (CAT) activity was evaluated as described by Boveris and Chance (1973). Results were reported as $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ and readings performed at 240 nm. Glutathione peroxidase (GPx) activity was measured according to the method by Flohé and Gunzler (1984), which is based on NADPH oxidation at 340 nm. Results were expressed as $\text{nmol min}^{-1} \text{mg protein}^{-1}$.

Non-protein thiol (NPSH) content, which is an indirect measure of reduced glutathione (GSH), was evaluated after reacting with 5,5'-dithiobis (2-nitrobenzoic acid) at 412 nm and reported as $\mu\text{mol mg protein}^{-1}$ (Ellman, 1959). Glutathione S-transferase (GST) activity was measured by the rate of dinitrophenyl S-glutathione formation at 340 nm (Habig et al., 1974) and the results were expressed as $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$.

2.6. Innate immune system measurements

Hemolytic activity of the complement system (HACS) was measured according to Morales-DelaNuez et al. (2009) and with the modifications described in detail by Mori et al. (2019). The percent of hemolysis was calculated by comparing total hemolysis (100%) and no-hemolysis (0%) controls as follows: $\% \text{ hemolysis} = \frac{[(A_{540 \text{ sample}} - A_{540 \text{ no-hemolysis}})]}{(A_{540 \text{ total hemolysis}} - A_{540 \text{ no-hemolysis}})} \times 100$.

Lysozyme activity was determined by a turbidimetric assay as described by Jørgensen et. al (1993). A suspension (200 μ L) of *Micrococcus lysodeikticus* in PBS solution (0.2 g/L) at pH 6.2 was mixed with plasma (10 μ L). Lysozyme activity (units/mL) was calculated using $[(\Delta_{\text{absorbance}(4-1\text{min})} / 3) / 0.001] \times 100$. A lysozyme activity unit was defined as the number of enzymes decreasing in absorbance of 0.001/min.

The peroxidase content of fish serum was determined according to the protocol of Quade and Roth (1997) with some modifications, as described in detail by Mori et al. (2019).

2.7 Aspartate transaminase (AST) and Alanine transaminase (ALT) levels

Hepatic enzyme levels in serum were determined using commercial kits (Gold analisa[®]) and expressed as U mL⁻¹.

2.8 Liver morphometry

Liver fragments were fixed in Bouin liquid for 24 h and subsequently submitted to dehydration, diaphanization, and inclusion procedures in Leica[®] historesin. Moreover, 4- μ m thick sections were submitted to hematoxylin-eosin staining for morphological analysis according to McManus (1946).

2.9 Statistical analysis

Variable distribution was tested using the Kolmogorov-Smirnov test. Data obtained from the studied groups, for the same parameter, were submitted to one-way analysis of variance followed by the Tukey-Kramer test. All analyses were performed with GraphPad Prism[®] Software 5.0. Differences were considered significant at $p < 0.05$. All biological

determinations were performed in triplicate and results expressed by means \pm standard error of the mean (SEM).

3. Results

3.1 Prooxidant and antioxidant analyses

At 45 days of supplementation, hepatic LOOH significantly decreased in fish that received citral at 0.44 and 1.76 g per kg feed. In addition, the LOOH of the gills of fish fed citral concentration of 0.88 g per kg feed decreased (Table 2). All fish that received citral had lower levels of LOOH in the brain compared to the control group.

Regarding antioxidant enzymes, SOD activity was higher in liver from the group fed 0.44 and 0.88 g citral per kg feed and in gills from the group fed 0.88 and 1.76 g citral per kg feed compared to the control group. Superoxide dismutase had also higher activity in the brain of fish fed 0.44 g citral per kg feed. The group fed 0.88 g citral per kg feed showed lower SOD activity than the other two groups fed citral supplementation; however, it did not differ from the control. Nevertheless, CAT activity did not differ among the experimental groups in all tissues. Glutathione peroxidase activity was significantly higher in the liver and brain in all groups that received citral supplementation compared to the control group. Additionally, the activity of this enzyme in the gills was the highest in fish fed 1.76 g citral per kg feed. GST activity increased significantly in the liver, gills and brain of *Mugil liza* supplemented with 0.88 and 1.76 g citral per kg feed compared to the control group. The highest concentrations tested showed higher GST activity compared to the group fed citral 0.44 g per kg feed in liver and gills (Table 2).

Non-protein thiol levels did not differ among the experimental groups in liver, but increased significantly in the gills and brain of fish fed 1.76 g citral per kg feed compared to the control fish and fish that received citral 0.44 and 0.88 g per kg feed in liver and gills (Table 2).

3.2 *Innate immune system measurements*

Lysozyme and complement activity were not affected significantly by the treatments. Nevertheless, myeloperoxidase activity significantly decreased in fish fed 0.88 and 1.76 g citral per kg feed compared to the control group. The group fed 0.88 g citral per kg feed showed lower myeloperoxidase activity compared to the fish fed 1.76 g citral per kg feed (Fig. 1).

3.3 *Aspartate transaminase (AST) and Alanine transaminase (ALT) levels*

No significant differences were observed in hepatic enzymes among treatments (Table 3).

3.4 *Liver histology parameters*

Hepatocytes of all groups were rounded with basophilic spheroidal nuclei located in the centrobasal portion of the cell and with the presence of vacuoles in the cytoplasm (Fig. 2). In morphometric analysis, *Mugil liza* liver samples showed similar lobular center vein, sinusoidal capillaries, and hepatocytes size in all groups (Table 4).

4. Discussion

The occurrence of oxidative stress has been related to increased susceptibility to different environmental or biotic stress and development of different types of pathologies in distinct animal species (Blier, 2014). Oxidative profile has been considered an important tool to evaluate metabolic and general health of fish populations.

Lipid peroxidation (LPO) can be described as a process in which oxidant species attack lipids from the cellular membrane, which results in lipid peroxy radicals and hydroperoxides (Yin et al., 2014). Fish fed the diets containing citral presented lower levels of lipid peroxidation in the liver, gills, and brain. This corroborates the lower LPO levels in the liver and muscle of silver catfish transported in water containing *A. triphylla* essential oil (27 and 36 mg/L) in relation to the control fish (Zeppenfeld et al., 2014). The dietary supplementation with the *A. triphylla* essential oil at 1.76 g per kg feed also decreased LPO levels (Zeppenfeld et al., 2017). Our results suggest that citral may be the most notable factor in the beneficial effects observed in *M. liza*.

To protect themselves against potentially damaging ROS, organisms have developed systems to prevent, eliminate or repair the effects of oxidative stress. The first line of antioxidant defense is composed of enzymes that act extremely fast to neutralize ROS or any ROS-inducing molecules (Ighodaro and Akinloye, 2017).

The enzyme SOD is responsible for anion superoxide dismutation into hydrogen peroxide (H₂O₂) and water (Birnie-Gauvin et al., 2017). In the present study, SOD activity was higher in the gills (1.76 g per kg feed) and brain (0.44 g per kg feed) of fish fed citral, indicating that the presence of this monoterpenoid may increase superoxide neutralization.

To neutralize H_2O_2 produced independently or by SOD action, fish rely on CAT and GPx activities. Catalase is highly efficient, since it can break down millions of hydrogen peroxide molecules to water and molecular oxygen in one second (Glorieux and Calderon, 2017; Ighodaro and Akinloye, 2017). In the current study, CAT activity was low in all tissues, which may have contributed to the lack of citral activity in this enzyme. According to Vélez-Alavez et al. (2015), low or no CAT activity is a common feature in marine teleosts. An alternative explanation is that H_2O_2 concentrations in this species is not high enough to induce the antioxidant activity of this enzyme (Powers and Jackson, 2008). On the other hand, GPx utilizes reducing GSH equivalents to reduce peroxides (Lei et al., 2016). The presence of citral in the diet of *M. liza* increased significantly the GPx activity in the liver, gills, and brain. According to Urso and Clarkson (2003), GPx has a much higher affinity to hydrogen peroxide than CAT, and it may detoxify peroxides. Therefore, the present results indicate that neutralizing H_2O_2 was more efficient in fish that received dietary citral supplementation.

Reduced glutathione (GSH) is the dominant antioxidant in cells that react non-enzymatically with oxidants to inhibit oxidative stress. In addition, GSH reacts enzymatically with GPx and GST, contributing to their action (Ren et al., 2017). The highest concentration of dietary citral tested increased NPSH levels in the gills and brain of *M. liza*, which is an indirect measure of GSH. Dietary supplementation with *Cymbopogon citratus* essential oil (α -citral=31.8%; β -citral=27.75%) increased GSH levels in Nile tilapia (Al-Sagheer et al., 2017). Data regarding NPSH determination is an important result, since according to Ming et al. (2015), increasing GSH improves parameters such as growth, survival rate, non-specific immunity, and antioxidant capacity in fish.

Glutathione-S-transferases (GSTs) are the main phase II detoxification enzymes and are responsible for catalyzing the conjugation of a wide variety of structurally dissimilar

compounds containing electrophilic carbon, nitrogen or sulfur atoms with GSH. They play a role in eliminating xenobiotics and protection against oxidative damage (Singhal et al., 2015). In the present study, the influence of dietary citral on GST levels was evident, since the highest citral concentration increased this parameter in all tissues analyzed. Nakamura et al. (2003) reported that, by studying the *in vitro* GST activation by citral in an epithelial cell line obtained from normal rat livers, the *E*-isomer geranial is the main contributor to GST induction. Moreover, the same authors postulated that the α,β -unsaturated carbonyl group with a *trans*-double bond is an essential structural factor for GST induction by citral.

Overall, the dietary supplementation with citral in *M. liza* induced beneficial effects in oxidative parameters. Moreover, the results here corroborate studies performed with essential oils rich in citral (Zeppenfeld et al., 2017), which also reduced oxidative stress in fish when used for diet supplementation. However, these data can not be generalized since similar dietary citral supplementation for common snook caused oxidative stress, and thus not being recommended for dietary supplementation (Mori et al., 2018).

In addition to benefit redox homeostasis, nutritional strategies may also contribute to modulate immune responses, which are fundamental for the survival of captive-bred animals (Sutili et al., 2017). Although the addition of citral in the diet of *M. liza* did not modulate lysozyme activity and the complement system, which are two important weapons against microorganism infection, it was able to decrease myeloperoxidase levels. This suggests that this monoterpenoid may present antiinflammatory properties (Pitanga et al., 2014). Mori et al. (2018) also demonstrated that citral decreased MPO levels when added to the diet of common snook. Katsukawa et al. (2010) showed that citral is able to activate peroxisome proliferator-activated receptors (PPAR α and γ) and suppress COX-2 expression, which plays a key role in inflammation.

Natural compounds such as essential oils and their main constituents are of interest primarily due to their potential therapeutic effects. However, the investigation of cytotoxic effects is of fundamental importance in order for biological activities to be successfully used in practice (Sharifi-Rad et al., 2017). Thus, considering the hepatic metabolism of citral (Kohlert et al., 2000), its effect on the activity of liver enzymes and liver histology was evaluated. The activity of AST and ALT, as well as histological parameters were not different from the control group and among the the experimental groups , demonstrating that citral did not promote hepatic injury for the mullet in the proposed experimental conditions. The presence of hepatic vacuoles in cells is not astonishing, since different species of teleosts can have the liver parenchyma rich in lipids and glycogen (Welsch and Storch, 1973). These results corroborated Mori et al. (2018), in which AST and ALT levels in common snook were also not affected by dietary citral supplementation.

In conclusion, citral contributed to neutralizing and eliminating ROS, consequently decreasing oxidative stress without causing hepatic toxicity in mullets. Furthermore, the monoterpenoid tested appears to exhibit anti-inflammatory properties in this species. Thus, citral can be considered a potential antioxidant and anti-inflammatory agent with the recommended dietary addition of 1.76 g citral per kg feed for *M. Liza*.

Conflicts of interest

The authors declare no conflicts of interest.

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FIGURE CAPTIONS

Fig. 1. (a) Lysozyme activity (units/mL), (b) Hemolytic activity of the complement system (%), and (c) Myeloperoxidase levels (OD540) in plasma of *Mugil liza* fed diets containing different citral concentrations. All values are expressed as mean \pm SEM (n= 3). Different lowercase letters indicate significant difference between treatments ($p < 0.05$).

Fig. 2. Histological characteristics of the liver of *Mugil liza* fed diets containing different citral concentrations (g per kg feed). Letters: A= Lobular center vein; B= Sinusoidal capillaries; C= Hepatocytes; D= hepatic vacuoles.

Table 1

Formulation (g/Kg) and analyzed proximate average composition (g/Kg) of the experimental diet.

Ingredients	(g/Kg)	Proximate average composition	(g/Kg)
Starch	140	Dry matter content	943.2
Soy lecithin	10	Protein	537.3
Vitamins and minerals (premix)*	5	Ether extract	91.9
Fresh squid	120	Mineral matter	207.3
Fish meal	700	Acid detergent fiber	20.4
Fish oil	24	Neutral detergent Fiber	143.1
Vitamin C	1	-	-

*Vitamin and mineral mixture (security levels per kilogram of product) — folic acid: 250 mg, pantothenic acid: 5.000 mg, antioxidant: 0.60 g, biotin: 125 mg, cobalt: 25 mg, copper: 2.000 mg, iron: 820 mg, iodine: 100 mg, manganese: 3.750 mg, niacin: 5.000 mg, selenium: 75 mg, vitamin A: 1.000.000 UI, vitamin B1: 1.250 mg, vitamin B12: 3.750 mcg, vitamin B2: 2.500 mg, vitamin B6: 2.485 mg, vitamin C: 28.000 mg, vitamin D3: 500.000 UI, vitamin E: 20.000 UI, vitamin K: 500 mg, zinc: 17.500 mg.

Table 2

Oxidative stress biomarkers in tissues of *Mugil liza* fed diets containing different citral concentrations. Values are mean \pm SEM (n= 3). Different lowercase letters indicate significant difference among treatments using one-way ANOVA and Tukey's test (p< 0.05).

	Citral (g per kg feed)			
	0.00	0.44	0.88	1.76
Liver				
LOOH	4.60 \pm 0.02 ^a	2.19 \pm 0.18 ^b	4.61 \pm 0.30 ^a	1.98 \pm 0.13 ^b
SOD	2.02 \pm 0.02 ^a	2.49 \pm 0.04 ^b	2.6 \pm 0.11 ^b	2.31 \pm 0.09 ^{ab}
CAT	3.18 \pm 0.06 ^a	2.67 \pm 0.39 ^a	3.52 \pm 0.19 ^a	2.78 \pm 0.008 ^a
GPx	1.96 \pm 0.22 ^c	9.79 \pm 0.59 ^b	10.06 \pm 0.34 ^b	16.22 \pm 0.65 ^a
NPSH	4.15 \pm 0.10 ^a	3.73 \pm 0.20 ^a	3.90 \pm 0.08 ^a	3.94 \pm 0.04 ^a
GST	113.8 \pm 2.64 ^b	95.42 \pm 4.20 ^b	163.56 \pm 5.83 ^a	173.42 \pm 12.79 ^a
Gills				
LOOH	7.64 \pm 0.29 ^a	5.84 \pm 0.36 ^a	3.68 \pm 0.35 ^b	6.76 \pm 0.68 ^a
SOD	1.24 \pm 0.11 ^{bc}	1.02 \pm 0.11 ^c	1.98 \pm 0.11 ^a	2.32 \pm 0.08 ^a
CAT	0.41 \pm 0.13 ^a	0.47 \pm 0.01 ^a	0.53 \pm 0.004 ^a	0.64 \pm 0.09 ^a
GPx	4.71 \pm 0.19 ^b	4.91 \pm 0.44 ^b	4.50 \pm 0.09 ^b	9.79 \pm 0.20 ^a
NPSH	7.79 \pm 0.09 ^b	7.33 \pm 0.17 ^b	7.22 \pm 0.13 ^b	12.07 \pm 0.45 ^a
GST	7.23 \pm 0.82 ^b	7.71 \pm 0.06 ^b	14.10 \pm 0.48 ^a	14.20 \pm 0.04 ^a

Brain

LOOH	7.50 ± 0.04^a	3.44 ± 0.24^b	3.22 ± 0.001^b	3.52 ± 0.09^b
SOD	1.27 ± 0.06^{bc}	2.59 ± 0.08^a	0.67 ± 0.02^b	1.69 ± 0.06^c
CAT	0.04 ± 0.02^a	0.03 ± 0.00^a	0.03 ± 0.001^a	0.05 ± 0.001^a
GPx	16.01 ± 0.69^b	36.73 ± 1.19^a	30.59 ± 0.89^c	27.99 ± 0.14^c
NPSH	11.26 ± 0.07^b	11.32 ± 0.12^b	8.60 ± 1.53^b	15.42 ± 0.30^a
GST	129.16 ± 5.45^b	140.98 ± 5.98^{bc}	150.39 ± 4.03^{ac}	213.35 ± 1.20^a

LOOH— lipid hydroperoxides (nmol/mg protein), SOD — superoxide dismutase (units/mg protein), CAT — catalase (pmol/mg protein), GPx — glutathione peroxidase (nmol/min/mg protein), NPSH — non-protein thiols (μmol/mg protein), GST — glutathione S-transferase. (μmol/min/mg protein).

Table 3

Hepatic enzymes in plasma of *Mugil liza* fed diets containing different citral concentrations. Values are mean \pm SEM (n= 3). Different lowercase letters indicate significant difference among treatments using one-way ANOVA and Tukey's test ($p < 0.05$).

Citral (g per kg feed)	AST	ALT
0.00	55.15 \pm 13.28	7.89 \pm 0.47
0.44	68.72 \pm 4.84	11.28 \pm 1.64
0.88	47.04 \pm 4.60	7.61 \pm 1.65
1.76	58.65 \pm 11.40	10.39 \pm 1.53

AST – Aspartate aminotransferase; ALT – Alanine aminotransferase.

Table 4

Liver histology parameters (μm) of *Mugil liza* fed diets containing different citral concentrations. Values are mean \pm SEM (n= 3). Different lowercase letters indicate significant difference among treatments using one-way ANOVA and Tukey's test (p< 0.05).

	Citral (g per kg feed)			
	0.00	0.44	0.88	1.76
<i>Lobular center vein</i>	1.469 \pm 421.03	2.079 \pm 449.3	2.362 \pm 218.68	2.005 \pm 458.9
<i>Sinusoidal capillaries</i>	4.28 \pm 0.15	4.74 \pm 0.04	3.90 \pm 0.55	5.03 \pm 0.04
<i>Hepatocytes</i>	67.33 \pm 3.51	65.63 \pm 3.51	70.69 \pm 1.49	63.34 \pm 0.05

FIGURES

Fig. 1

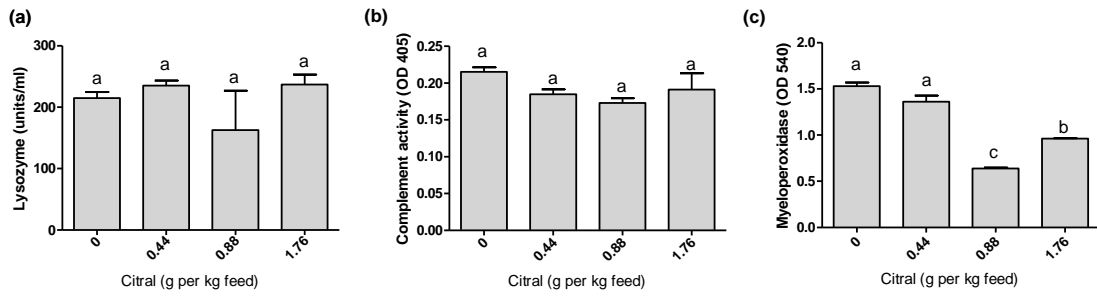
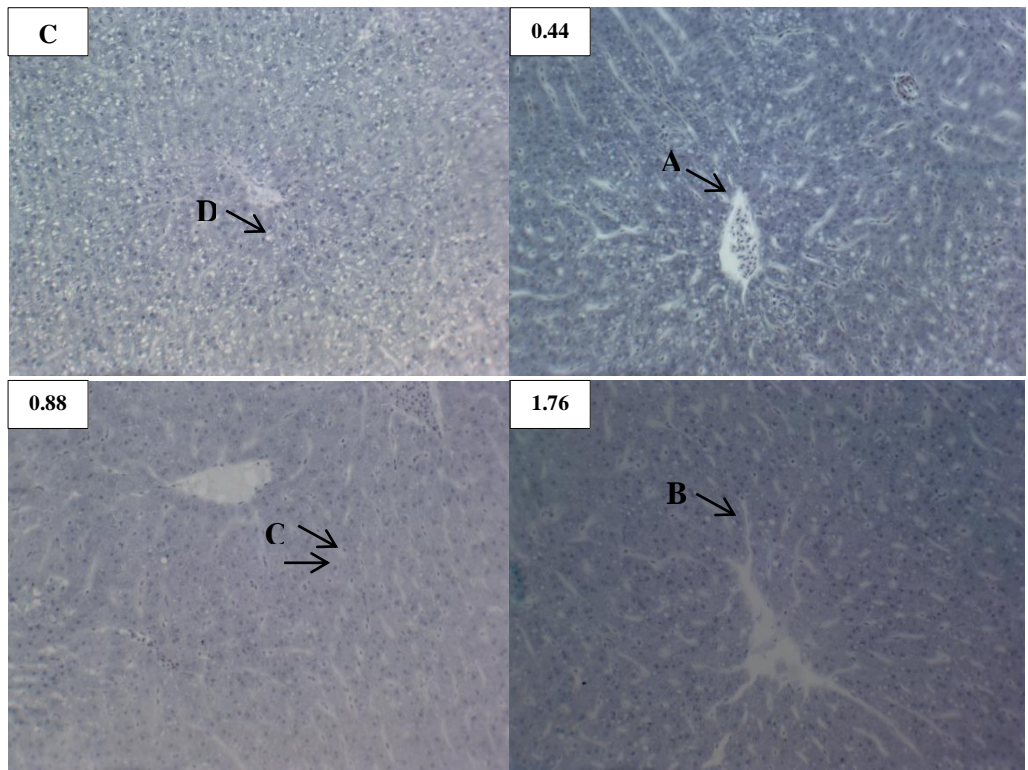


Fig. 2



5. DISCUSSÃO

A utilização de constituintes vegetais aplicados à aquicultura tem sido proposta para promover a saúde e o bem-estar de peixes criados em sistemas de cultivo, especialmente porque os antimicrobianos comumente usados como agentes profiláticos e terapêuticos têm sido relacionados à disseminação de resistência bacteriana (PÉREZ-SANCHÉZ, MORA-SANCHÉS & BALCÁZAR, 2018). Outra vantagem do uso de fitoaditivos na aquicultura é o fato dos mesmos serem substâncias naturais e, na maioria das vezes, não representarem ameaça para o peixe, o homem ou o meio ambiente (GABOR, SARA & BARBU, 2010).

Nesse sentido, são bem vindos produtos naturais que promovam um equilíbrio adequado entre oxidantes e antioxidantes no organismo animal, já que danos oxidativos às macromoléculas podem acumular-se em órgãos vitais e, ao longo do tempo, participar na etiologia de vários distúrbios crônicos e degenerativos. Em adição à isso, elevados níveis de EROs podem afetar negativamente tanto as células do sistema imunológico, bem como o seu mecanismo de defesa, favorecendo o desenvolvimento de doenças dos animais (ASLANI & GHOBADI, 2016).

Em relação à isso, Biller-Takahashi et. al (2017) confirmaram a existência de uma correlação positiva entre a melhora do status antioxidante e a produção de componentes do sistema imune inato, como a atividade da lisozima, evidenciando uma estreita relação entre o equilíbrio oxidativo e a atividade do sistema imune. Tais autores investigaram o efeito da suplementação da dieta do pacu (*Piaractus mesopotamicus*) com selênio orgânico através de indicadores de estresse oxidativo (CAT, GST, GPx e GSH), perfil hematológico e parâmetros do sistema imune inato (explosão respiratória de leucócitos, atividade hemolítica do sistema complemento sérico e atividade da lisozima).

De acordo com Aslani & Ghobadi (2016), a ingestão adequada de nutrientes antioxidantes pode ser uma estratégia útil para melhorar mecanismo de defesa antioxidante, bem como por desencadear respostas imunes mais eficientes, preservando a saúde e prevenindo vários distúrbios. Já Birnie-Gauvin (2017) destacam que os antioxidantes solúveis

na gordura podem ser especialmente benéficos quando adicionados à dieta dos peixes, pois não podem ser sintetizados pelos animais. Consequentemente, podemos esperar que a composição da dieta dos peixes promova respostas fisiológicas distintas.

Sutili et al (2017) explicam que, apesar de ser evidente que os compostos naturais afetam funções fisiológicas, os efeitos “*in vivo*” do tratamento dietético são controversos. O efeito biológico de determinado extrativo depende de muitos fatores, tais como: (1) composição química do extrativo; (2) processo de extração; (3) concentração da(s) substância(s) ativa(s); (4) método de administração; (5) condições de armazenamento da alimentação. No caso da administração de uma substância isolada como no caso do citral, os primeiros três fatores acima citados podem ser desconsiderados. Além disso, em nosso estudo fornecemos a mesma dieta para diferentes espécies de peixes estuarinos. Neste caso, eliminamos a influência de todos os fatores supracitados, pretendendo observar se as diferenças fisiológicas e de hábitos alimentares (carnívoro e detritívoro) entre as espécies testadas seriam determinantes para a atividade do citral quando administrado como aditivo alimentar.

Os nossos resultados mostram que apesar do citral não ter sido capaz de melhorar a atuação do sistema antioxidante e ainda promover lipoperoxidação nas brânquias do robalo-flecha, para a tainha a presença do citral na dieta permitiu a redução da lipoperoxidação no fígado, melhorou a atividade do sistema antioxidante no fígado, brânquias e cérebro e aumentou os níveis de tióis não proteicos nas brânquias e cérebro. No tocante à imunidade inata, apenas no robalo-flecha observamos uma superativação das proteínas do sistema complemento. Contudo, houve diminuição da atividade da mieloperoxidase nas duas espécies. Os biomarcadores hepáticos não foram diferentes do grupo controle e entre as espécies.

Estudos realizados até o momento usando aditivos dietéticos têm mostrado resultados diferentes entre as espécies e até dentro das espécies (DALMO E BOGWALD, 2008). Por exemplo, Misra et al. (2006) administraram β -glucana em diferentes doses na dieta de alevinos *Labeo rohita* desafiados com *Aeromonas hydrophila* e *Edwardsiella tarda*. Quatro dietas diferentes, com 0, 100, 250 ou 500 mg de β -glucana kg^{-1} foram administradas por 56 dias. Após este período parâmetros imunológicos e zootécnicos melhoraram significativamente e portanto, os autores recomendaram a administração de 250 mg de β -glucana kg^{-1} para o aumento da imunidade, crescimento e sobrevivência de alevinos de *L.*

rohita. Por outro lado, Whittington, Lim & Klesius (2005) suplementaram a dieta de tilápia do Nilo imunizada e não imunizada com *Streptococcus iniae* com β -glucana por 14 semanas e observaram que não houve efeito na estimulação da resposta imune e resistência da tilápia do Nilo contra a infecção por *S. iniae*.

Outro exemplo consiste na comparação entre os trabalhos realizados por Brum et al. (2017) e Nya & Austin (2009). Para os primeiros autores a suplementação das dietas de *Oreochromis niloticus* infectados com *Streptococcus agalactiae* com óleo essencial de gengibre (*Zinziber officinale*), cuja principal substância ativa é o citral, obteve resultados negativos com a maior concentração testada (1,5%) para ganho de peso, taxa de crescimento específico e conversão alimentar. Já no trabalho realizado pelos segundos autores supracitados, a suplementação com pó de gengibre em concentrações entre 0,1% e 1,0% promoveu melhorias no ganho de peso, taxa de crescimento específico e taxa de conversão alimentar em truta arco-íris (*Oncorhynchus mykiss*) infectadas com *Aeromonas hydrophila*.

Sendo assim, podemos levantar algumas hipóteses sobre os resultados dos nossos estudos. A primeira delas é que o citral possa ter afetado negativamente a microbiota intestinal saprófita apenas do robalo-flecha, já que é sabido que a diversidade dos hábitos alimentares tem por consequência a colonização por diferentes comunidades bacterianas no TGI dos peixes. Duas cepas de bactérias ácido-lácticas, *Lactobacillus plantarum* CPQBA 007-07 e *Lactococcus* sp., que foram isolados do trato intestinal de juvenis de robalo-peva, possuem a capacidade de inibir o crescimento da população de *Vibrio* spp., que embora também faça parte da microbiota de peixes marinhos, são agentes causadores de doenças e mortalidade maciça em sistemas de cultivo (SOUZA et al., 2010). Nesse sentido, considerando o reconhecido potencial antimicrobiano do citral, é possível que através da destruição da flora saprófita tenha favorecido o desequilíbrio da homeostase intestinal. Apesar de não terem sido observados sinais clínicos de infecção, a superativação do sistema complemento no grupo que recebeu a concentração mais baixa de citral também poderia ser um indicativo da atuação de micro-organismos patogênicos. Essa hipótese não se aplica a concentrações maiores, pois não observamos a hipermodulação do sistema complemento nestes grupos (SADDIQ & KHAYYAT, 2010; SILVA-ÂNGULO et al., 2015; YANG et al., 2016).

As diferenças fisiológicas entre as espécies também poderiam explicar as respostas opostas à presença do citral na dieta. O robalo-flecha apresenta estômago com maior volume e

intestino mais curto, o que pode ter influência direta sobre a superfície de absorção do monoterpenoide. Através dos nossos resultados também é possível observar que o efeito do citral não é dose-dependente em nenhuma das espécies, portanto não podemos excluir o fato de que concentrações extremamente baixas tenham efeitos prejudiciais à saúde do robalo-flecha.

Outra hipótese seria de que o citral tenha sido biotransformado em metabólitos tóxicos apenas ao *C. undecimalis* pela acidez estomacal (pH= 2,0 a 6,0), o que não ocorreu com *M. liza*, que possui uma digestão reportada como alcalina (CARDONA, 2015; DIMA & DIMA, 2015; ZHANG et al. 2016). Neste caso a encapsulação do citral poderia ser uma alternativa, uma vez que este composto seria protegido da degradação estomacal.

Uma explicação adicional estaria relacionada à diferentes respostas bioquímicas entre as espécies (ROCHA et al., 2009). Quintaneiro, Ranville & Nogueira (2015) evidenciaram diferentes respostas bioquímicas entre duas espécies detritívoras (*Atyaephyra desmarestii* e *Echinogammarus meridionalis*) expostas por 48 h ao cobre e zinco. O zinco induziu o sistema de defesa em *E. meridionalis* sem evidência de dano oxidativo. No entanto, em *A. desmarestii* exposto ao zinco foi observado dano oxidativo. Além disso, apenas o zinco reduziu significativamente a taxa de ingestão e apenas para *E. meridionalis*. O nosso estudo é pioneiro na comparação entre *C. undecimalis* e *M. liza*, portanto o comportamento metabólico destas espécies ainda é um campo a ser explorado.

Por outro lado, tanto *C. undecimalis* quanto *M. liza* apresentaram diminuição na atividade de mieloperoxidase plasmática após a ingestão do citral. Este achado pode ser interpretado de maneira controversa. Por um lado, pode representar a diminuição da capacidade fagocítica, pois as células brancas utilizam a mieloperoxidase para este fim (KREUTZ et al., 2011). No entanto, por se tratar do citral, acreditamos que a redução na atividade enzimática possa estar relacionada com a atividade anti-inflamatória do monoterpenoide estudado (LIAO, 2015).

Inúmeros estudos, embora não tenham sido conduzidos com peixes, têm demonstrado o potencial do citral no combate à inflamação. Shen, Sun & Guo (2015) investigaram os efeitos do citral na lesão pulmonar aguda induzida por lipopolissacarídeos (LPS) em camundongos. Os resultados mostraram que o pré-tratamento com citral notavelmente

atenuou o edema pulmonar e a produção dos marcadores inflamatórios TNF- α (Fator de necrose tumoral α), IL-6 (Interleucina-6) e IL-1 β (Interleucina-1 β). A ativação do NF- κ B (Fator de necrose κ B) induzida por LPS também foi inibida pelo citral. Além disso, os autores propuseram que o citral teria ação anti-inflamatória devido a ativação do PPAR- γ (Receptor Ativado por Proliferadores de Peroxissoma Gama). Bachiega & Sforcin (2011) também analisaram o efeito anti-inflamatório do citral em diferentes concentrações “*in vitro*” utilizando macrófagos peritoneais de camundongos BALB. Os resultados mostraram que o monoterpene inibiu a liberação de IL-1 β , IL-6 e IL-10. Liao et al. (2015) investigaram as respostas inflamatórias de neral e geranial, isômeros que compõem o citral, em macrófagos de camundongos. Embora os dois compostos tenham inibido a expressão gênica de marcadores inflamatórios, o neral mostrou maior inibição de TNF- α e IL-6 e melhor eficácia na inibição da expressão dos mediadores pró inflamatórios IL-1 β , iNOS (Óxido nítrico sintase induzível), COX-2 (ciclo-oxigenase 2) e inflamassoma NLRP-3 do que o geranial.

Da mesma forma que a atividade da mieloperoxidase, os parâmetros hepáticos avaliados em nosso estudo responderam na mesma direção nas duas espécies testadas, sem demonstrar diferença significativa do grupo controle. Uchida et al. (2017) avaliaram os efeitos do citral em um modelo murino de hepatotoxicidade utilizando o monoterpene como pré-tratamento para evidenciar seus efeitos protetores do fígado. Os resultados mostraram que o citral diminuiu significativamente os níveis de atividade de ALT, AST, fosfatase alcalina e gama-glutamyltransferase e ainda, melhorou lesões hepáticas evidenciadas na análise histopatológica. Outro estudo realizado por Zeppenfeld et al. (2017) determinou os níveis de AST e ALT em *R. quelen* alimentados com óleo essencial de *A. tryphylloides* (α -citral 294.1 g kg⁻¹; β -citral 207.8 g kg⁻¹). Neste caso, a atividade das enzimas hepáticas não foi diferente do grupo controle, corroborando com os resultados do nosso trabalho.

6 CONCLUSÕES

- O citral possui potencial oxidativo quando administrado na dieta de *C. undecimalis*, tendo em vista que promoveu LPO nas brânquias dos animais experimentais. No entanto, quando fornecido na dieta de *M. liza* demonstrou atividade antioxidante, já que diminuiu LPO no fígado, brânquias e encéfalo;
- A atividade enzimática da SOD foi elevada no fígado e brânquias de *C. undecimalis* e também em brânquias e encéfalo de *M. liza* alimentadas com citral. Porém, houve redução na atividade da CAT em fígado e brânquias de *C. undecimalis*. Não houve modulação da atividade desta enzima em *M. liza*. A adição dietética de citral não afetou a atividade de GPx de *C. undecimalis*, contudo elevou a atividade desta enzima em fígado, brânquias e encéfalo de *M. liza*. Citral reduziu níveis de NPSH no fígado de *C. undecimalis*, porém elevou os níveis deste marcador de *M. liza* no fígado, brânquias e encéfalo. Citral aumentou a atividade de GST nas brânquias de *C. undecimalis*. Esta atividade também foi elevada em fígado, brânquias e encéfalo de *M. liza*;
- O citral reduziu a atividade da enzima MPO em todas as concentrações testadas e ainda promoveu uma hiperativação do sistema complemento quando administrado a *C. undecimalis*. Quando administrado à *M. liza* também reduziu a atividade de MPO;
- A presença de citral na dieta não alterou a atividade de AST e ALT e parâmetros histológicos hepáticos de *C. undecimalis* e de *M. liza*;
- A adição dietética de citral não é indicada para *C. undecimalis*. Porém, para *M. liza* a adição de citral 1.76 g por kg dieta melhora parâmetros de estresse oxidativo sem causar danos hepáticos, sendo portanto recomendada para esta espécie;
- Os resultados sugerem que o citral possa apresentar potencial anti-inflamatório para as espécies de peixes estudadas, portanto sugerimos a realização de estudos futuros que investiguem a atuação do monoterpenoide na terapêutica de processos inflamatórios.

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ANEXOS

ANEXO 1 – Normas Revista Aquaculture Nutrition

Sections

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2. AIMS AND SCOPE

Aquaculture Nutrition provides a global perspective on the nutrition of all cultivated aquatic animals. Topics range from extensive aquaculture to laboratory studies of nutritional biochemistry and physiology.

Aquaculture Nutrition publishes papers which strive to:

- increase basic knowledge of the nutrition of aquacultured species and elevate the standards of published aquaculture nutrition research
- improve understanding of the relationships between nutrition and the environmental impact of aquaculture
- increase understanding of the relationships between nutrition and processing, product quality, and the consumer.
- help aquaculturalists improve their management and understanding of the complex discipline of nutrition
- help the aquaculture feed industry by providing a focus for relevant information, techniques, tools and concepts.

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