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Carine de Freitas Souza

ADIÇÃO DO ÓLEO DE *Melaleuca alternifolia* NA DIETA DE PEIXES MINIMIZA EFEITOS TÓXICOS CAUSADOS PELO CONSUMO DIÁRIO DE AFLATOXINA

Orientador: Aleksandro Schafer da Silva
Co-orientador: Bernardo Baldisserotto

Santa Maria, RS, 2020

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Tese apresentada ao Curso de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**

Orientador: Prof. Dr. Aleksandro Schafer da Silva
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Santa Maria, RS, Brasil
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Aprovado em 9 de abril de 2020:

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Aleksandro Schafer da Silva, Dr. (UDESC/UFSM)
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Santa Maria, RS
2020

DEDICATÓRIA

Dedico esta tese aos meus pais.
Que sempre me apoiam nos momentos mais críticos,
me acalentam o coração mesmo que a distância e
que nunca mediram esforços para investir nos meus estudos.
Sem vocês, jamais teria chegado até aqui!

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Por fim, agradeço imensamente a todos que se envolveram mesmo que indiretamente para que esta tese fosse concretizada, afinal nada se faz sozinho.

"Não conheço ninguém que conseguiu realizar seu sonho, sem sacrificar feriados e domingos pelo menos uma centena de vezes. Da mesma forma, se você quiser construir uma relação amiga com seus filhos, terá que se dedicar a isso, superar o cansaço, arrumar tempo para ficar com eles, deixar de lado o orgulho e o comodismo. Se quiser um casamento gratificante, terá que investir tempo, energia e sentimentos nesse objetivo.

O sucesso é construído à noite!

Durante o dia você faz o que todos fazem. Mas, para obter um resultado diferente da maioria, você tem que ser especial. Se fizer igual a todo mundo, obterá os mesmos resultados. Não se compare à maioria, pois, infelizmente ela não é modelo de sucesso. Se você quiser atingir uma meta especial, terá que estudar no horário em que os outros estão tomando chope com batatas fritas. Terá de planejar, enquanto os outros permanecem à frente da televisão.
Terá de trabalhar enquanto os outros tomam sol à beira da piscina. A realização de um sonho depende de dedicação, há muita gente que espera que o sonho se realize por mágica,
mas toda mágica é ilusão, e a ilusão não tira ninguém de onde está, em verdade a ilusão é combustível dos perdedores pois...

Quem quer fazer alguma coisa, encontra um MEIO.
Quem não quer fazer nada, encontra uma DESCULPA."

ROBERTO SHINYASHIKI

RESUMO

ADIÇÃO DO ÓLEO DE *Melaleuca alternifolia* NA DIETA DE PEIXES MINIMIZA EFEITOS TÓXICOS CAUSADOS PELO CONSUMO DIÁRIO DE AFLATOXINA

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CO-ORIENTADOR: Bernardo Baldisserotto

Micotoxinas são toxinas naturais produzidas através do metabolismo secundário de fungos toxigênicos. Mais de 400 classes de micotoxinas são conhecidas, no entanto as aflatoxinas (AFs), produzidas principalmente pelas espécies de *Aspergillus*, estão entre as classes toxigênicas, consideradas inclusive como mais carcinogênicas. Quando ingerida, essa micotoxina causa hepatotoxicidade, prejudica o equilíbrio antioxidante/pró-oxidante e danifica moléculas biológicas, incluindo lipídios, proteínas e DNA. Neste sentido, a combinação dessas manifestações leva ao estresse oxidativo que prejudica o funcionamento do fígado, que é o principal órgão desintoxicante do corpo. Neste contexto, estudos recentes têm sugerido que o uso de extrativos vegetais, ricos em compostos fenólicos e flavonóides possam auxiliar na absorção das micotoxinas, aliviando seus efeitos tóxicos em animais. Sendo assim, o objetivo deste estudo foi investigar se os efeitos antioxidantes e anti-inflamatórios do óleo essencial (OE) de *Melaleuca alternifolia* – tea tree oil (TTO) é capaz de amenizar ou evitar os danos causados pela aflatoxicose em tecido hepático e cerebral, quando adicionado a dieta. Este trabalho foi realizado em três etapas: (I) foi investigado as respostas do sistema de defesa antioxidante de jundiás (*Rhamdia quelen*) frente a ingestão de AFB_1, onde foi determinado que a adição de 1177 $\mu g\ kg^{-1}$ de AFB_1 na dieta causa estresse oxidativo no fígado. Foi observado que a intoxicação com aflatoxina causa um aumento de espécies reativas ao oxigênio (ERO) e ao nitrogênio (NOx) e consequente danos a lipídeos e proteínas. Na outra etapa, (II) foi avaliado se a adição do OE de TTO, adicionado a dieta dos peixes, seria capaz de reduzir os efeitos tóxicos causados pela aflatoxina. Nesta etapa os peixes foram alimentados com uma ração contento 1mL kg^{-1} de OE de TTO juntamente da dieta contaminada com aflatoxina (1177 $\mu g\ kg^{-1}$). Observou-se que a adição do OE de TTO na dieta contaminada com aflatoxina mostrou um efeito protetor, evitando o aumento dos níveis de ROS, e danos aos lipídios e proteínas no plasma e fígado. Na etapa seguinte, (III) foram analisados os efeitos tóxicos da aflatoxina no sistema purinérgico e colinérgico no cérebro dos peixes, além de avaliar se o OE de TTO seria capaz de minimizar os efeitos tóxicos causados pela aflatoxicose. Notou-se que aflatoxina causa aumento na atividade de acetilcolinesterase (AChE) e adenosina desaminase (ADA). Além disso, foi observado um aumento na expressão do receptor p2y11, provavelmente na tentativa de modular a inflamação causada pela aflatoxicose. Esses dados em conjunto corroboram para o entendimento dos mecanismos de toxicidade da aflatoxina e permitem concluir que a suplementação do OE de TTO na concentração de 1 mL kg^{-1} na dieta pode ser usada para aumentar o status antioxidante e reduzir efetivamente os efeitos negativos causados pela aflatoxicose, além de reduzir o dano ao tecido cerebral associado à aflatoxina.

Palavras-chave: aflatoxicose, tea tree oil, estresse oxidativo, sistema purinérgico, aflatoxina B_1

ABSTRACT

ADDITION OF *Melaleuca Alternifolia* OIL IN DIET FOR FISH MINIMIZES TOXIC EFFECTS CAUSED BY DAILY AFLATOXIN CONSUMPTION

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Mycotoxins are natural toxins produced through the secondary metabolism of toxigenic fungi. More than 400 classes of mycotoxins are found, however, as aflatoxins (AFs), produced mainly by *Aspergillus* species, including toxigenic and more carcinogens species. When ingested, this mycotoxin causes hepatotoxicity, impairs the antioxidant / pro-oxidant balance and damages biological molecules, including lipids, proteins and DNA. In this sense, a combination of these manifestations leads to oxidative stress and initiates the malfunction of the liver, which is the body's main detoxifying organ. In this context, recent studies have suggested the use of plant extracts, rich in phenolic and flavonoids compounds, which can assist in the absorption of mycotoxins that alleviate their toxic effects in animals. Therefore, the objective of this study was to investigate the antioxidant and anti-inflammatory effects of the essential oil (EO) of the *Melaleuca alternifolia* - tea tree oil (TTO) is able to abrogates or prevent the damage caused by aflatoxicosis in the hepatic and cerebral tissues, when added to the diet. This work was carried out in three stages: (I) it was investigated as responses of the antioxidant defense system of silver catfish (*Rhamdia quelen*) against ingestion of AF_1 (AFB_1), where it was determined that the addition of 1177 μg kg^{-1} of AFB_1 in the practice of oxidative physical activity in the liver. It was observed that intoxication with aflatoxin causes an increase in reactive species to oxygen (ROS) and nitrogen (NOx) and consequent damage to lipids and proteins. In step (II) was evaluated if EO of TTO, added to the fish diet, it would be possible to reduce the toxic effects caused by aflatoxin. In this step, the fish were fed with a concentration of 1mL kg^{-1} of EO of TTO associated with a diet contaminated with aflatoxin (1177 μg kg^{-1}). It was noted that the addition of EO of TTO to the aflatoxin-contaminated diet showed a protective effect, reduced the increase in ROS levels, and damaged lipids and proteins in the plasma and liver. In step (III), the toxic effects of aflatoxin on the purinergic and cholinergic system in the fish brain were analyzed, in addition to assessing the EO of TTO would be able to reduce the toxic effects caused by aflatoxicosis. It is not known that aflatoxin causes increased activity of acetylcholinesterase (AChE) and adenosine deaminase (ADA). In addition, the expression of the p2y11 receptor was observed, probably in an attempt to deal with an inflammation caused by aflatoxicosis. Taken together, as three stages of the study, corroborate the understanding of the mechanisms of toxicity of aflatoxin and that EO of TTO added in the concentration of 1 mL kg^{-1} in the diet can be used to increase the antioxidant status and effectively reduce the effects impaired by aflatoxicosis, in addition to reducing the damage in brain tissue associated with aflatoxin.

Keywords: aflatoxicosis, tea tree oil, oxidative stress, purinergic system, aflatoxin B_1

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ABREVIAÇÕES

AChE – Acetilcolinesterase

ADA – Adenosina desaminase

ADP – Adenosina difosfato

AFB_1 – Aflatoxina B_1

AFB_2 – Aflatoxina B_2

AFBO – AFB_1-8, 9-epóxido

AFG_1 – Aflatoxina G_1

AFG_2 – Aflatoxina G_2

AFs – Aflatoxinas

AMP – Adenosina monofosfato

ATP – Trifosfato de Adenosina

BChE – Butirilcolinesterase

CAT – Catalase

CHC – Carcinoma hepatocelular

DAMPs – Padrões moleculares associados a danos

DL50 – Dose letal média

DON – Desoxinivalenol

ERN – Espécies reativas ao nitrogênio

ERO – Espécies reativas ao oxigênio

GPx – Glutationa peroxidase

GST – Glutationa-*S*-transferase

HRO2● - Hidroperoxil

IARC – International Agency for Research on Cancer

MFO – Oxidações de funções mistas

NO● – Óxido nítrico

NO2● – Dióxido de nitrogênio

NO_2Cl – Cloreto de nitrila

Nrf2 –Nuclear eritróide 2 – relacionado ao fator 2

NTPDase – Nucleosídeo trifosfato difosfohidrolase

O2●- – Ânion radical superóxido

OE – Óleo essencial

OH● – Radical hidroxil

ONOO- – Peroxinitrito

OTA – Ocratoxina A

PAS – Sítio aniônico periférico

PUFAS – Ácidos graxos poli-insaturados

RO2.● – Peroxil

SOD – Superóxido dismutase

TTO – *Tea tree oil* (óleo da árvore do chá)

UDP – Difosfato de uridina

ZEN – Zearalenona

1 INTRODUÇÃO

1.1 O IMPACTO DAS MICOTOXINAS

Há muito tempo tem se observado a ocorrência de micotoxinas e de seus impactos toxicológicos e econômicos em escala mundial, sendo considerado um grande problema, uma vez que a contaminação pode ocorrer em toda a cadeia alimentar. A ingestão de alimentos contaminados pode causar intoxicações alimentares agudas e crônicas que podem induzir a uma variedade de efeitos adversos à saúde tanto em humanos quanto em animais, tornando as micotoxinas tóxicos naturais (ESKOLA et al., 2019). Além disso, a grande estabilidade química que as micotoxinas apresentam, permite a sua permanência no alimento mesmo após a remoção dos fungos pelos processos normais de industrialização (TRISTAN, 2002).

Micotoxinas são compostos com baixo peso molecular (0,3-0,7 kDa), que são secretados como metabólitos secundários produzidos por fungos filamentosos que podem contaminar produtos alimentares em seu cultivo agrícola e durante o armazenamento, transporte e processamento de alimentos, afetando à saúde humana e animal, além da indústria de alimentos e do comércio internacional (WIELOGORSKA et al., 2016). As principais espécies de micotoxinas pertencem aos gêneros *Fusarium, Claviceps, Alternaria, Aspergillus e Penicillium* (MARIN et al., 2013). Essas espécies precisam de condições ecofisiológicas específicas, como temperatura e umidade, para crescer e sintetizar esses metabólitos secundários que têm efeitos adversos na saúde animal e humana. Os principais substratos ou culturas com capacidade para apoiar o crescimento e o acúmulo dessas toxinas são cereais, nozes, oleaginosas, frutas secas, café e especiarias e seus derivados.

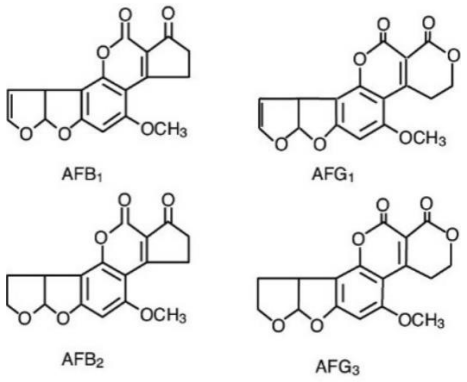
De acordo com a literatura, dentre os mais de 300 compostos secundários identificados, desoxinivalenol (DON), ocratoxina A (OTA), zearalenona (ZEN) e aflatoxinas (AFs) foram apontados como as micotoxinas mais estudadas (GRATZ et al., 2017; GAMBACORTA et al., 2018; KHANEGHAH et al., 2018). As micotoxinas podem apresentar várias manifestações toxicológicas. Algumas apresentam efeitos sobre o sistema imunológico, enquanto que outras são consideradas teratogênicas, mutagênicas e/ou carcinogênicas além de estarem também associadas a várias doenças crônicas e agudas (LOPES et al., 2005). No entanto, as aflatoxinas são as micotoxinas mais tóxicas produzidas por fungos e resultam em inevitável contaminação de alimentos e rações em concentrações muito baixas. Além disso, AFs foram listadas como agentes cancerígenos de Classe I, pela Organização Mundial da Saúde (MØLLER; HESELTINE; VAINIO, 1995).

1.2 AFLATOXINAS

As aflatoxinas constituem um grupo de metabólitos secundários de hidrocarbonetos aromáticos heterocíclicos, de ocorrência natural, produzidas como metabólitos secundários pelo fungo *Aspergillus flavus, A. parasiticus* e *A. nominus* (JAIMEZ et al., 2000; GARDEN; STRACHAN, 2001; WILD; TURNER, 2002; KHLANGWISET; SHEPHARD; WU; XIE; XU 2018;). São comumente encontradas em diversos alimentos, especialmente em cereais como milho, trigo, sorgo e arroz, em subprodutos de cereais, rações para animais de criação e em uma série de alimentos para humanos, tais como: produtos de salsicharia, vinhos, leguminosas, frutas, leites e derivados (FERREIRA, et al., 2006; GIMENO; MARTINS, 2011).

O nome “aflatoxina” deriva da primeira letra em *Aspergillus* e das três primeiras letras em *flavus* (SCHOENTAL, 1967). Aproximadamente 20 aflatoxinas foram identificadas e quatro delas ocorrem naturalmente, incluindo aflatoxina B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) e G_2 (AFG_2) (DUTTON, 1988; YABE; ANDO; HAMASAKI, 1988) (Figura 1). As AFs são muito estáveis a altas temperaturas, sendo que as iniciais B e G devem-se ao fato destas apresentarem fluorescência azulada (blue) e esverdeada (green), respectivamente, quando observadas sob luz ultravioleta (LEESON et al., 1995). Entre estas, a AFB_1 é a mais tóxica, devido suas propriedades hepatotóxicas, imunotóxicas, mutagênicas, carcinogênicas e teratogênicas em humanos e animais experimentais e de criação (MEISSONNIER et al., 2008; KENSLER et al., 2011; SUN et al., 2014; 2016; THEUMER et al., 2018).

Figura 1 - Estruturas de aflatoxinas de ocorrência natural.

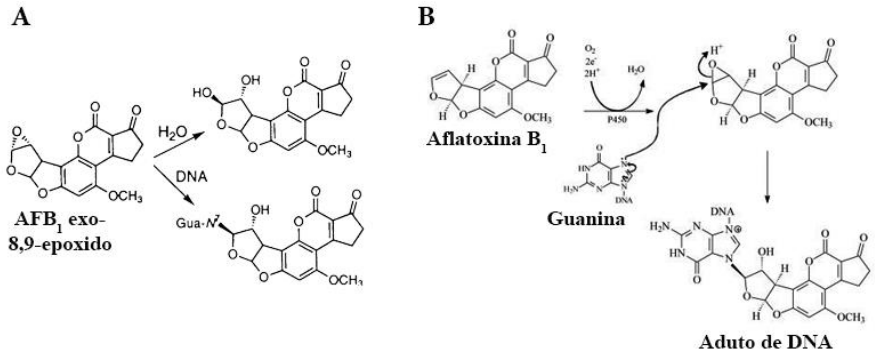


Fonte: Leeson; Diaz; Summers (1995)

1.2.1 Biotransformação das aflatoxinas

A exposição à aflatoxina B_1 (AFB_1) é um fator responsável por desencadear toxicidade aguda e crônica e gerar danos cancerígenos, mutagênicos, hepatotóxicos e imunossupressores (FERREIRA et al., 2006; ŁAZICKA; ORZECHOWSKI, 2010). AFB_1 é ativado enzimaticamente pelo citocromo P450 3A4 a um epóxido muito reativo (BUSBY; WOGAN, 1985; UENG et al., 1995). O *exo*-8,9-epóxido de AFB_1 é o isômero genotóxico e reage eficientemente com o DNA na posição N7 de guanina, após intercalação (IYER et al., 1994). A aflatoxina B_1 torna-se altamente carcinogênica quando inicia o processo de formação de seu epóxido. Em seguida, o nitrogênio (nucleófilo) da base guanina do DNA ataca o epóxido (eletrófilo) formando o aduto de DNA (JARDIM; CALDAS, 2009) (Figura 2).

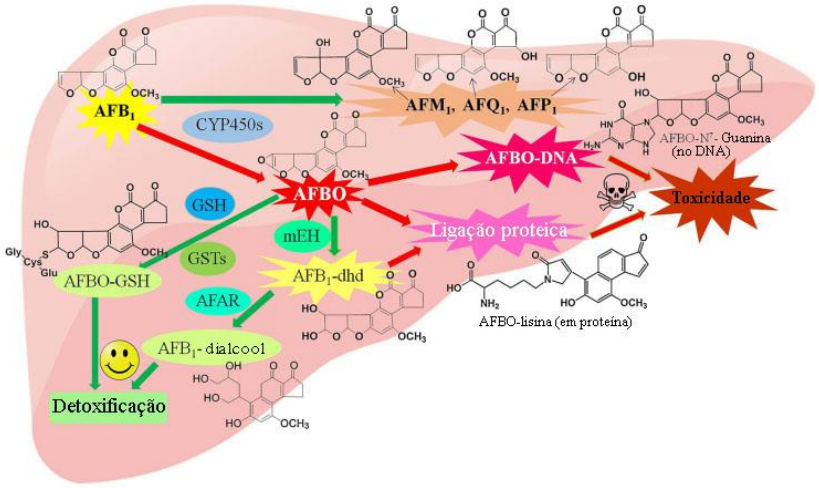
Figura 2 - Reação de *exo*-8,9-epóxido de AFB_1 com H_2O e DNA (A) e reação da aflatoxina B_1 catalisada pela enzima mono-oxigenase do complexo citocromo P450 na formação do aduto de DNA (B).



Fonte: Johnson; Harris; Guengerich (1996); Jardim; Caldas; (2009)

A toxicidade do AFB_1 depende das vias de bioativação e desintoxicação (Figura 3). A via metabólica do AFB_1 é caracterizada principalmente por duas vias com dois principais sistemas catalisadores: (1) a Fase I, ou fase de ativação, mediada pelas oxidases de função mista (MFO) dependentes do citocromo P450 e (2) Fase II, ou fase de desintoxicação, compreendendo os dois desintoxicantes mais importantes, a difosfato de uridina (UDP) glucuronil-transferase e, em menor grau, a glutationa (GSH) e glutationa-*S*-transferase (GST) (LIVINGSTONE 1998).

Figura 3 – Visão geral das vias metabólicas do AFB_1. AFB_1, aflatoxina B_1; AFBO, *exo*-AFB1-8,9-epóxido; AFB_1-dhd, AFB_1-8,9-di-hidrodiol; AFAR, aflatoxina-aldeído redutase; CYP450 s, citocromo P450 s; GSH, glutationa; GSTs, glutationa-*S*-transferases; mEH, epóxido hidrolase microssômica.



Fonte: Adaptado de Deng et al. 2018

1.3 EFEITOS TOXICOLÓGICOS DA AFLATOXINA B_1 (AFB_1)

Aflatoxicose é o nome dado a intoxicação resultante da ingestão da aflatoxina em alimentos e rações contaminadas. Neste sentido, a aflatoxicose além de afetar seriamente a saúde dos seres humanos e de animais de produção, pode causar perdas econômicas significativas para os produtores (DOHLMAN, 2003; RAWAL et al., 2010; WILLIAMS et al., 2011; WU; GUCLU, 2012). O impacto negativo de AFB_1 sobre a saúde é muito significativo, seja de maneira direta, afetando os órgãos envolvidos nos processos de digestão e absorção de nutrientes ou, de maneira indireta, atuando sobre o sistema imunológico, tornando os animais menos resistentes a doenças (WALTE et al., 2016). Além disso, após a avaliação de estudos epidemiológicos publicados, os quais demonstraram um forte vínculo entre o consumo de AFB_1 e a ocorrência de câncer, a *International Agency for Research on Cancer* (IARC) concluiu que haviam evidências suficientes para classificar as aflatoxinas como cancerígenas em humanos. Desta forma, AFB_1 é considerada a mais cancerígena das aflatoxinas e está bem documentada como um agente causador de carcinoma hepatocelular (CHC), bem como a supressão do crescimento, da modulação do sistema imunológico, condição aguda de cirrose hepática, e desnutrição

(IARC, 2012), dependendo da dose e do tempo de exposição (WILLIAMS et al., 2004).

A toxicidade do AFB_1 em diferentes espécies varia de acordo com a idade, sexo, sensibilidade à AFB_1 específica da espécie, dosagem e duração da exposição a toxinas (DHANASEKARAN et al., 2011). De acordo com a American Public Health Association (APHA, 1995), nenhuma espécie animal é resistente aos efeitos tóxicos da aflatoxina, assumindo-se que humanos possam ser igualmente afetados. Uma grande variação nos valores da dose letal média (DL50) tem sido obtida em espécies animais, isto é, de 0,5 a 1,0 mg kg^{-1} corpóreo.

1.3.1 Estresse oxidativo

O estresse oxidativo, é uma condição biológica em que ocorre desequilíbrio entre a produção de espécies reativas de oxigênio (ERO) e a sua remoção através de sistemas (enzimáticos ou não enzimáticos) que as removam ou reparem os danos por elas causados (BARBOSA et al., 2010).

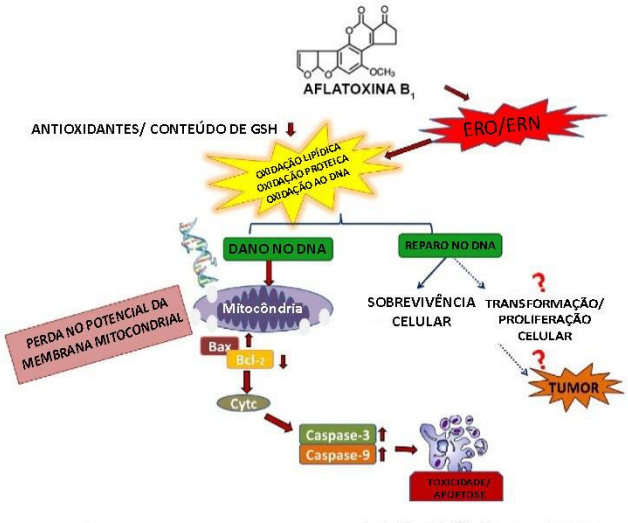
As espécies reativas de oxigênio (ERO) incluem radicais livres como o ânion superóxido (O_2●-), peroxil (RO_2.●), hidroperoxil (HRO2●) e o radical hidroxila (OH●), este último é considerado o mais reativo por combinar-se rapidamente com metais, podendo causar danos como mutação ou inativação do DNA celular, além de iniciar a oxidação dos ácidos graxos poli-insaturados das membranas celulares (lipoperoxidação) (STOHS, 1995; HALLIWELL, 2000). As espécies não radicalares, apesar de não possuírem elétrons desemparelhados, são muito instáveis como, por exemplo, o peróxido de hidrogênio (H_2O_2). Uma das causas da toxicidade induzida pela AFB_s é o estresse oxidativo, que leva à geração ERO e ao dano oxidativo do DNA. Durante a via metabólica de AFB_1, ocorre um aumento na produção de ERO e peróxidos lipídicos, que finalmente leva a danos celulares (SHEN et al., 1996).

De acordo com Marin e Taranu (2012), AFB_1 é associada ao aumento de ERO, que supera a capacidade dos mecanismos antioxidantes de defesa, deixando as células vulneráveis a ácidos nucleicos, proteínas ou oxidação de lipídios. Neste contexto, a lesão celular mediada por AFB_s pode estar associada à liberação dessas espécies reativas, e assim iniciam a peroxidação lipídica e um processo prejudicial nos sistemas biológicos, uma vez que todas as membranas celulares contêm os ácidos graxos poliinsaturados (PUFAs), que são substratos para essa reação. Em adição, a secreção extracelular de ERO por leucócitos e macrófagos evoca uma resposta imune contra bactérias, vírus, células degeneradas e outras substâncias estranhas, como as aflatoxinas.

A secreção intracelular destas espécies reativas estimula as vias de sinalização celular e desencadeia a resposta de defesa do estresse oxidativo, bem como a apoptose (Figura 4) (SIMKÓ et al., 2011).

O aumento de ERO pode induzir ao aumento espécies reativas ao nitrogênio (ERN) que também podem ativar fatores de transcrição ou agir diretamente em proteínas, lipídeos e DNA alterando as suas funções (CALCUTT et al., 2009). São chamadas de espécies reativas ao nitrogênio (ERN), oxidantes contendo nitrogênio, como óxido nítrico (NO●), peroxinitrito ($ONOO^-$), dióxido de nitrogênio (NO_2●) e cloreto de nitrila (NO_2Cl) (BEDARD e KRAUSE, 2007). Deste modo, as ERN atuam junto com as ERO danificando as células, causando o estresse nitrosativo. Por conseguinte, estas duas espécies são frequentemente referidas coletivamente como ERO/ERN.

Figura 4 - Modo de ação mediado pelo estresse oxidativo/nitrosativo proposto para AFB_1.



Fonte: Adaptado de Mishra e Das 2003

Na tentativa de evitar um quadro de estresse oxidativo, organismos animais sintetizam enzimas que refutam eficientemente as ERO. Superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) são enzimas antioxidantes intracelulares que convertem os substratos potenciais (radicais ânion superóxido e peróxido de hidrogênio) a formas menos reativas no corpo (REMACLE, 1992; FINAUD et al., 2006; AFONSO et al., 2007). Dentre as enzimas antioxidantes, destaca-se a glutationa-*S*-transferase (GST), cujo papel crucial na detoxificação da AFB_1 foi anteriormente reportado por Saad-Hussein et al. (2014). Este autor

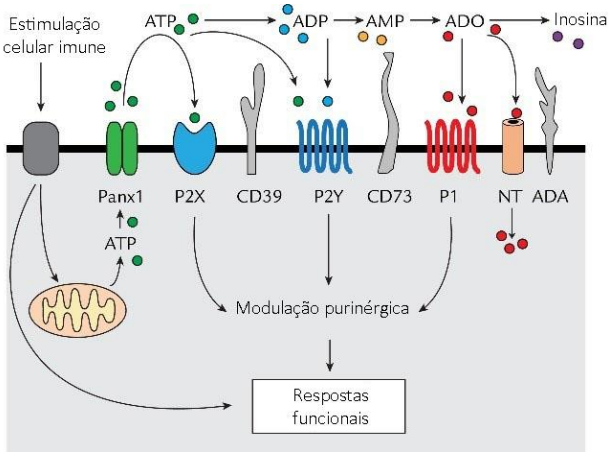
relatou que a GST, juntamente com a SOD foram detectadas como mecanismo na linha de frente na defesa contra os efeitos tóxicos oxidativos da AFB_1 no fígado; além disso, os papéis significativos do zinco e da vitamina C na ativação desses processos também foram detectados. Além disso, muitos estudos anteriores indicaram que o GST desempenha um papel fundamental na proteção das células da toxicidade do AFB_1, porque a conjugação do AFB1-8,9-epóxido eletrofílico com GSH é um destino alternativo à ligação aos centros nucleofílicos nas macromoléculas celulares (FARIAS et al., 2012). Geralmente, é um mecanismo adaptativo ou compensatório para aumentar a resistência celular e proteger os tecidos dos efeitos deletérios causados pelas aflatoxinas.

1.3.2 Sinalização purinérgica

Além de desencadear a cascata do estresse oxidativo, um estudo recente demonstrou que a sinalização purinérgica também pode ser uma via envolvida no comprometimento das respostas imunes e inflamatórias causadas por AFB_1 (BALDISSERA et al., 2018a). Desta maneira, vários mediadores estão relacionados à ativação de células inflamatórias. Esses mediadores inflamatórios incluem óxido nítrico e padrões moleculares associados a danos (DAMPs), incluindo adenosina trifosfato (ATP), considerado o mais poderoso e onipresente sinal de estresse ou dano celular (PATEL, 2018; MIHM, 2018).

O ATP é conhecido há muito tempo como a principal fonte de energia nas células vivas. Sob condições fisiológicas, o ATP é quase exclusivamente presente dentro da célula; por outro lado, seus níveis são praticamente insignificantes no ambiente extracelular (BURNSTOCK; KNIGHT, 2018). Em resposta à liberação de ATP no meio extracelular, a sinalização purinérgica pode atuar como uma via anti-ou pró-inflamatória, dependendo de sua regulação enzimática de nucleotídeos e nucleosídeos. Esta regulação inicia-se com a hidrólise de ATP pela nucleosídeo trifosfato-hidrolase (NTPDase) em adenosina difosfato (ADP) e adenosina monofosfato (AMP). O AMP é posteriormente hidrolisado em adenosina (Ado) pela atividade da 5'-nucleotidase e, finalmente, a Ado é desaminada pela adenosina desaminase (ADA) em inosina (ZIMMERMMAN, 2000) (Figura 5). Assim, esta via pode contribuir para o comprometimento da resposta imune e consequente patogênese da aflatoxicose.

Figura 5 - Elementos dos mecanismos de sinalização purinérgica autócrina nas células.



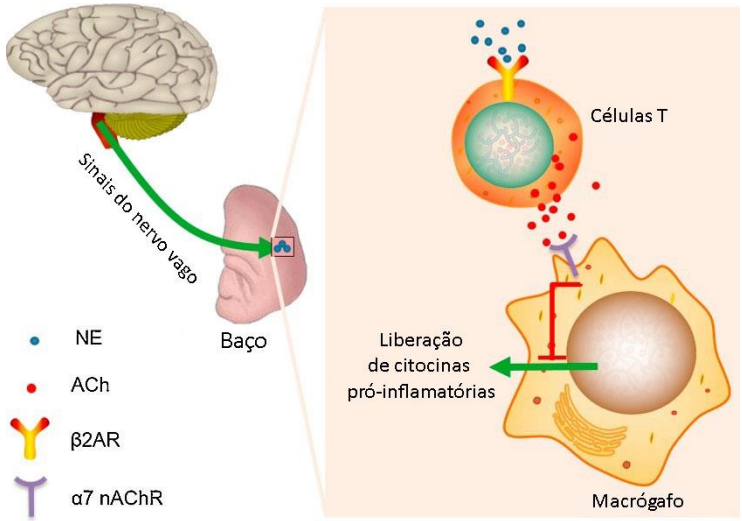
Fonte: Adaptado de Ledderose et al. 2016

Embora a sinalização purinérgica exerça um papel significativo no controle das respostas imunes e inflamatórias durante o processo inflamatório causado por agentes patogênicos, um estudo recente realizado por Savio et al. (2018) demonstrou que a inibição da atividade da NTPDase e, consequentemente, a liberação de grandes quantidades de ATP, resulta em um ciclo pró-inflamatório auto-sustentado devido a uma ativação constante do purineceptor P2X, ou seja, a sinalização purinérgica pode contribuir para a intensificação do processo inflamatório e, portanto, dano celular. Ainda que a via exata envolvida nos efeitos tóxicos das aflatoxinas ainda seja pouco estudada, um recente estudo conduzido por Baldissera et al. (2018b) demostrou que o sistema purinérgico é uma via diretamente envolvida nos efeitos imunotóxicos da AFB_1 em jundiás alimentados com dietas contaminadas, através da redução na atividade da NTPDase (ATP como substrato) e do aumento da atividade ADA no baço e linfócitos esplênicos dos peixes.

1.3.3 Sinalização colinérgica

Desde sua descoberta em 2000, a via anti-inflamatória colinérgica tem sido extensivamente estudada devido ao seu papel na modulação da resposta imune. Esse caminho depende de uma interação neuro-imune robusta na qual os nervos periféricos se comunicam e podem alterar a atividade do sistema imunológico. O mecanismo proposto postula que, em resposta a infecção ou lesão, o nervo vago parassimpático transmite sinais do cérebro para o nervo esplênico adrenérgico, que interage com as células imunes esplênicas (HAN; LI; HAO, 2017) (Figura 6).

Figura 6 - Ilustração esquemática do efeito anti-inflamatório colinérgico nas células imunes.



Fonte: Adaptado de Han; Li; Hao 2017

O sistema colinérgico está envolvido em reações anti-inflamatórias, pois a acetilcolinesterase (AChE) é a enzima responsável pela a hidrólise do neurotransmissor acetilcolina (ACh) em colina e acetato, uma molécula com propriedades anti-inflamatórias, porque inibe o processo inflamatório (DAS, 2007). Foi relatado que o tratamento agudo de AFB_1 em animais experimentais causa uma diminuição das enzimas regionais da acetilcolinesterase cerebral que podem afetar as funções cognitivas, bem como a memória e o aprendizado do indivíduo, enquanto a exposição crônica aumenta a atividade da AChE adeno-hipofisária (COULOMBE Jr., 1994).

Embora poucos estudos relacionem os efeitos das micotoxinas com o sistema colinérgico em animais, já se sabe que a AFB_1 é um inibidor da atividade biológica da acetilcolinesterase (AChE) (STEPURSKA et al., 2015) que pode ser encontrado no sistema nervoso central e, portanto, também pode atuar como um composto neurotóxico (EGNIBUKE; IKEGWUONU, 1984). Estudos experimentais in vitro mostraram que a AFB_1 inibe a atividade da AChE de maneira mista reversível seletiva (POHANKA; MUSILEK; KUCA, 2010) (ARDUINI et al., 2007), por inibição competitiva e não competitiva (POHANKA, MUSILEK E KUCA, 2010), com mais afinidade pela ligação no sítio aniônico periférico (PAS) de AChE (POHANKA, 2014). Além disso, de acordo com Cometa et al., (2005), as interações da AFB_1 com a AChE estão relacionadas a um mecanismo adicional responsável pela carcinogenicidade da AFB_1 em

humanos. Em adição, um recente estudo demonstrou que há uma inibição na atividade da AChE inclusive em tecido esplênico de jundiás 14 e 21 após a alimentação com AFB_1 (BALDISSERA et al., 2019).

1.4 PRODUTOS NATURAIS COMO ADITIVOS ANTI-MICOTOXINAS

Os extratos vegetais incluem várias moléculas antioxidantes como fenóis, tocoferóis, flavonóides e outras moléculas ativas. Entre eles, os óleos essenciais (OE) ganharam grande popularidade nas aplicações biomédicas, farmacêuticas, cosméticas, alimentícias, agrícolas e veterinárias. Além de atuar como alternativa natural às preparações sintéticas, esses compostos podem ter propriedades antimicrobianas e controlar fungos micotoxigênicos em alimentos e rações, sendo uma alternativa "eco-amigável", reduzindo o uso de produtos químicos sintéticos (FIRENZUOLI, et al., 2014).

Na última década, estudos sobre agentes antifúngicos naturais foram registrados por inúmeros pesquisadores. Vários extratos de plantas brutas eram ricos em polifenóis e alcaloides, relatando uma eficiência antifúngica e antiflatoxigênica (QUIROGA et al., 2009; RAMADAN et al., 2014; BADR et al., 2017). Além disso, a adição de produtos naturais como aditivos em dietas, fornece uma grande quantidade de fitoquímicos com propriedades antioxidantes que conferem proteção contra danos celulares (SINGH et al., 2009).

Recentemente, alguns estudos têm se proposto a estudar a eficácia de plantas contra a toxicidade das aflatoxinas. Ponzilacqua et al. (2019) em um estudo *in vitro*, mostrou que o extrato de alecrim (*Rosmarinus officinalis*) causou degradação de ~60% de AFB_1 após 48 h de exposição. Recentemente, Souza et al. (2020) demonstraram que a adição de biocolina vegetal (800 e 1200 mg kg^{-1}) na dieta contaminada com AFB_1 teve um efeito protetor significativo, evitando o aumento dos níveis de ERO, peroxidação lipídica e carbonilação das proteínas no fígado de tilápias que receberam ração contaminada por aflatoxina.

1.4.1 *Melaleuca alternifolia*

O óleo da árvore do chá (tea tree oil – TTO) é um OE extraído das folhas da *Melaleuca alternifolia* (HAMMER, 2005). O TTO é geralmente composto de terpenos, particularmente monoterpenos e sesquiterpenos, que são formados por muitos compostos orgânicos derivados de metabólitos secundários (LANGENHEIM, 2003).

Devido as suas vastas atividades biológicas, esta planta tem atraído a atenção de inúmeros pesquisadores nas últimas décadas (YADAV et al., 2017), destacando-se atividades anti-inflamatórias, antioxidantes, antifúngicas e antitumorais (BAKKALI et al., 2008; ASSMANN et al., 2018). Além disso, estudos recentes demonstraram que o TTO é capaz de ativar o fator nuclear eritróide 2 relacionado ao fator 2 (Nrf2) em roedores, uma via de sinalização antioxidante essencial (LEE et al., 2017) e também melhora o status antioxidante e evitando o dano oxidativo em peixes (SOUZA et al., 2018).

Estudos anteriores relataram atividades antifúngicas do TTO contra vários fungos patogênicos, incluindo cepas produtoras de micotoxinas (DE ANDRADE SANTIAGO et al., 2017). Além disso, recentemente, nosso grupo de pesquisa vem realizando inúmeros trabalhos utilizando o OE de *M. alternifolia*, e descobriu várias propriedades medicinais da mesma para *R. quelen*, podendo ser utilizada como antibacteriano, anti-inflamatório, antioxidante (SOUZA et al., 2016; BALDISSERA et al., 2017a, BALDISSERA et al., 2017b; BALDISSERA et al., 2017c). Neste sentido, esperamos que a adição de TTO na ração dos peixes expostos a AFB_1, possa evitar e/ou amenizar os efeitos danosos causados pela aflatoxicose, através de sua ação antioxidante e anti-inflamatória, uma vez que, uma das maneiras fundamentais de desintoxicar o AFB_1 é através da conjugação de AFB1-8,9-epóxido (AFBO) com GSH/GST.

1.5 MODELO EXPERIMENTAL

O jundiá, *Rhamdia quelen* (QUOY; GAIMARD, 1824) é um peixe da família Heptapteridae, nativo da América do Sul, ocorrendo desde o sul do México ao centro da Argentina (SILFVERGRIP, 1996), amplamente cultivada devido à boa adaptação climática e rápido crescimento (BOCHI et al., 2008) (Figura 6). Esta espécie de peixe provou ser um modelo animal promissor em várias áreas, como análise do comportamento, atividade sedativa / anestésica, doenças microbianas, toxicidade, inclusive como modelo para o estudo dos efeitos de micotoxinas (GARLET et al., 2016; BALDISSERA et al., 2018b; SOUZA et al., 2019). Além disso, é importante ressaltar que mundialmente, a demanda por pescado tem sido impulsionada nas últimas décadas, principalmente em função do crescimento populacional e da busca dos consumidores por alimentos mais saudáveis, sendo o pescado uma excelente fonte de proteínas de alto valor biológico, ácidos graxos insaturados e vitaminas, com baixo teor de colesterol, constituindo uma opção de consumo mais saudável do que as outras carnes

(GONÇALVES, 2011; REVERTER et al., 2014). Desta forma, evitar ou minimizar a contaminação com aflatoxinas torna-se inevitável.

Figura 7. Exemplar de jundiá, *Rhamdia quelen.*

Fonte: Construção do autor

1.6 JUSTIFICATIVA

As micotoxinas constituem-se em um perigo elevado a saúde animal e humana. Neste sentido, podem entrar na cadeia alimentar humana de maneira direta ou indiretamente, seja através do consumo dos cereais, oleaginosas e derivados ou através da ingestão de produtos obtidos de animais que se alimentam com rações contaminadas que consequentemente, podem excretar micotoxinas no leite, carne e ovos, e consequentemente, constituem-se uma fonte de contaminação indireta para os humanos. Outrossim, a contaminação com micotoxinas é uma preocupação mundial, uma vez que a aflatoxicose em especial, pode provocar sérios danos à saúde de animais e humanos, incluindo imunossupressão, cirrose hepática, necrose aguda e até o surgimento de câncer.

Uma solução eficiente e cientificamente “amigáveis” para controlar e/ou minimizar a contaminação por micotoxinas, consiste no uso de estratégias nutricionais focadas no uso de produtos naturais com propriedades antifúngicas e imunoestimulantes. Nas últimas décadas, vários estudos relataram resultados positivos do uso de óleos essenciais (OE) de plantas como aditivo dietético em várias espécies de organismos animais quando comparados a dieta controle,

servindo de preventivo contra infecções bacterianas, como promotor de crescimento e ainda melhorando o status antioxidante.

Diante do exposto, acreditamos que o óleo essencial de *M. alternifolia*, um óleo com propriedades antifúngicas, antioxidantes e anti-inflamatórias, poderia ser adicionado na dieta dos peixes com o intuito de prevenir e/ou reduzir os efeitos causados pela contaminação por micotoxinas em peixes alimentados com uma ração contaminada por AFB_1.

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar se consumo de aflatoxina causa estresse oxidativo em peixes e se a adição do óleo essencial de *M. alternifolia* na dieta é capaz de prevenir e/ou evitar os efeitos deletérios causados pela contaminação por micotoxinas.

2.2 OBJETIVOS ESPECÍFICOS

Em peixes *(R. quelen*) alimentados com uma ração contaminada por AFB_1 e submetidos a uma dieta com óleo essencial de *M. alternifolia* será investigado:

- O conteúdo de Vitamina C, metabólitos do óxido nítrico, níveis de peroxidação lipídica e carbonilação proteica, capacidade antioxidante e conteúdo de GSH. Também serão avaliadas as atividades da GPx, da CAT e da SOD no fígado e soro, bem como a análise histopatológica do fígado.
- Os níveis de peroxidação lipídica, de proteína carbonil e capacidade antioxidante, bem como atividade da SOD, da GST e da GPx no fígado e plasma.
- A atividade das enzimas NTPDase, 5´nucleotidase, adenosina desaminase e AChE, bem como a análise histopatológica no cérebro.
- A regulação da transcrição gênica de receptores purinérgicos e adenosinérgicos no cérebro.

3 DESENVOLVIMENTO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos. Os itens Resumo, Introdução, Materiais e métodos, Resultados, Discussão e Referências encontram-se nos próprios artigos. Os artigos estão dispostos conforme as normas das revistas científicas que foram enviados.

3.1 ARTIGO I

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Serum and hepatic oxidative damage induced by a diet contaminated with fungal mycotoxin in freshwater silver catfish *Rhamdia quelen:* Involvement on disease pathogenesis

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Serum and hepatic oxidative damage induced by a diet contaminated with fungal mycotoxin in freshwater silver catfish Rhamdia quelen: Involvement on disease pathogenesis

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ABSTRACT

It has been recognized that oxidative stress is implicated in the initiation and progression of diseases due to the excessive formation of free radicals and impairment of the antioxidant defense system, contributing to the mortality of affected animals. The occurrence of a disequilibrium between the antioxidant/oxidant status in serum and liver of freshwater fish fed with aflatoxin B1 (AFB_1) remains poorly understood and limited to only a few oxidant variables. Thus, the aim of this study was to evaluate whether an AFB1-contaminated diet causes disturbance on the antioxidant and oxidant status in silver catfish (*Rhamdia quelen*) of freshwater. Serum and hepatic reactive oxygen species (ROS), metabolites of nitric oxide (NOx), and lipid hydroperoxide increased on days 14 and 21 post-feeding in animals that received AFB_1 contaminated diet compared to the control group (basal diet), while protein carbonylation levels increased on day 21 post-feeding. On the other hand, serum and hepatic antioxidant capacity against peroxyl radical and vitamin C levels, as well as glutathione peroxidase and catalase activities were lower on days 14 and 21 post-feeding in animals that received AFB1 contaminated diet compared to the control group. No difference was observed between groups regarding the superoxide dismutase activity and glutathione levels. Based on these evidences, an AFB_1-contaminated diet causes a disturbance on serum and hepatic antioxidant/oxidant system due to lipid and protein damage elicited by excessive ROS and NOx production. Also, the antioxidant defense system was unable to avoid or minimize ROS and NOx deleterious effects, and consequently, the oxidative damage. In summary, this disturbance can contribute to understand the pathophysiology and mortality of fish after the consumption of AFB_1-contaminated diets.

Keywords: antioxidant system; damage liver; mycotoxin; pathogenesis; ROS

1. INTRODUCTION

Feed is considered vital to fish production and factors that affect its quality and safety are significant impediment to aquaculture, as contamination with aflatoxin B1 (AFB_1), which is the most occurring aflatoxin found in fish feed [1,2]. The occurrence of AFB_1 in fish feed has significantly increased due to the extensive use of plant-based ingredients to minimize production costs, since a high percentage of worldwide crops are contaminated by aflatoxins before or post-harvest [3]. AFB_1 is a secondary toxic metabolite produced by certain fungi belonging to the genus *Aspergillus flavus* and *Aspergillus parasiticus* that occur as natural

contaminants of fish food [4], being associated with significant negative impact in fish health, such as decreased growth and weight gain in Nile tilapia (*Oreochromis niloticus*) [5], hepatic damage in sea bass (*Dicentrarchus labrax*) [6], behavioral abnormalities in silver catfish (*Rhamdia quelen*) [7], and some alterations linked to antioxidant/oxidant status in serum of *O. niloticus* [8], but the occurrence of disturbances in the antioxidant/oxidant status of *R. quelen* fed with AFB_1-contaminated diet remains unknown. Oxidative stress is defined as a disturbance of antioxidant/oxidant status in favor of the later, which happens when the production of free radicals is faster than they are scavenged by antioxidant mechanisms [9]. This imbalance contributes to lesions of macromolecules and impairment of physiological metabolism [10]. In order to avoid or reduce the production of free radicals, such as reactive oxygen species (ROS), fish may activate several antioxidant mechanisms as a primary antioxidant defense system composed by the enzymes catalase (CAT) and superoxide dismutase (SOD). A secondary antioxidant defense system formed by glutathione peroxidase (GPx) and glutathione reductase may also be activated. Both mechanisms are needed to limit the prooxidant activity of ROS [11]. Although some alterations of antioxidant/oxidant status have already been reported in fish fed with AFB1, a complete and expressive evaluation of antioxidant/oxidant system remains unknown, since so far only few parameters such as lipid peroxidation and CAT activity in serum and liver of Nile tilapia [8] and common carp (*Cyprinus carpio*) [12] were determined. Also, it is important to emphasize that these studies did not evaluate the direct effect of AFB_1 because a mixture of aflatoxins (AFB_1 and AFB_2) was provided in fish diet. Thus, our hypothesis is that AFB_1 can cause an increase of free radicals and activation and/or inhibition of antioxidant defenses of silver catfish fed with a contaminated diet. In this context, the aim of this study was to evaluate whether an AFB_1-contaminated diet could cause disturbances in the antioxidant/oxidant status of freshwater silver catfish.

2. MATERIALS AND METHODS

2.1. AFB_1 and fish maintenance

AFB_1 (molecular weight: 312.27 g/mol; from Aspergillus flavus) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and used to contaminate fish feed. Healthy fish for experimental purposes were collected from a fish farm located in Southern Brazil, and water quality parameters (dissolved oxygen, temperature, total ammonia, and nonionized ammonia levels) were recently published in details by Baldissera et al. [7].

2.2. Diet and the experimental study

A basal diet was formulated as established in details by Baldissera et al. [7], and was experimentally contaminated with AFB_1 (1177 ppb kg/feed), as preconized by Lopes et al. [13]. A total of thirty-six juvenile silver catfish (90.32 ± 7.54 g; 25 ± 3.5 cm) were used as the experimental model to evaluate parameters linked to the oxidative stress. The animals were divided into two groups named: control (C) and aflatoxin (A) with 18 animals each, and subdivided into six subgroups with 6 animals each (C1, C2, and C3; A1, A2, and A3). The control subgroups (C1, C2, and C3) received a basal diet, while the aflatoxin groups (A1, A2, and A3) were fed with an AFB_1 contaminated diet. Fish received the experimental diet twice a day (9 a.m. and 5 p.m.) at a proportion of 5% of total biomass for 7 days (subgroups C1 and A1), 14 days (subgroups C2 and A2), and 21 days (subgroups C3 and A3).

2.3. Sample collection and tissue preparation

Total blood samples were collected in tubes without anticoagulant on days 7 (subgroups C1 and A1), 14 (subgroups C2 and A2), and 21 (subgroups C3 and A3) after the use of a natural anesthetic (eugenol 30 mg/L), followed by spinal cord section according to the Ethics Committee recommendations. Thereafter, liver tissue was removed and dissected in a glass dish over ice to evaluate the oxidant/antioxidant status. Blood samples were centrifuged at 1000× g for 15 min at 4 °C to obtain serum that was stored at −20 °C until measurement of oxidant/ antioxidant parameters. Liver was homogenized (1:10 w/v) in a glass Potter tube with Tris-HCl buffer (10 mM, pH 7.4) and centrifuged at 2000× g for 10 min, and the supernatant used to evaluate the oxidative parameters.

2.4. Serum and hepatic oxidant parameters

2.4.1. Reactive oxygen species (ROS) levels

Serum and hepatic ROS levels were determined by the DCFH oxidation method described by LeBel et al. [14], recently published in details by Biazus et al. [15] using excitation and emission wavelengths of 485 and 538 nm, respectively, and results were expressed as U DCF/mg of protein.

2.4.2. Metabolites of nitric oxide (NOx) levels

Serum and hepatic NOx levels were measured indirectly by nitrite/ nitrate (NOx) quantification according to the Griess method as previously described by Tatsch et al. [16], with modifications. Briefly, 40 μL of sample was pipetted into the reaction cuvette and 80 μL of vanadium (III) chloride (VCl3) combined with 80 μL of Griess reagent was added into the

reaction cuvette. Thus, the mixture sample/VCl3/ Griess reagent was incubated for 12 min and read at 546 nm using the BS-380® (Mindray, Shenzhen, China) automated analyzer. Results were expressed as μmol/L.

2.4.3. Lipid peroxidation (LOOH) levels

For the measurement of lipid peroxidation (LOOH) levels was measured as proposed by Monserrat et al. [17] and reported in details by da Silva Barreto et al. [18]. Serum results were expressed as μmol CHP/mL of serum, while hepatic results were expressed as μmol CHP/g of tissue.

2.4.4. Protein carbonylation levels

The carbonyl protein content was determined in the supernatant as described by Reznick and Packer [19] using a molar extinction coefficient of 22,000 M/cm [20]. Results were expressed as nmol of carbonyls formed per mg of protein

2.5. Serum and hepatic antioxidant parameters

2.5.1. Antioxidant capacity against peroxyl radicals (ACAP) levels

ACAP levels was measured according to the protocol stablished by Amado et al. [21] and reported in details by da Silva Barreto et al. [18] using wavelengths of 485 nm (excitation) and 520 nm (emission) for 40 min at 37 °C. The results were expressed as fluorescence units per mg of protein.

2.5.2. Glutathione peroxidase (GPx) activity

Serum and hepatic GPx activities were measured according to the methodology described by Paglia and Valentine [22], as reported in details by Biazus et al. [15]. The enzymatic activity was expressed as U/ mg of protein.

2.5.3. Catalase (CAT) activity

Serum and hepatic CAT activities were assessed in the supernatants according to Aebi [23], and the specific activity was calculated as μmol of CAT per mg of protein.

2.5.4. Superoxide dismutase (SOD) activity

Serum and hepatic SOD activities were spectrophotometrically evaluated as described by Marklund and Marklund [24], recently published in details by Souza et al. [25]. The enzymatic activity was expressed as SOD units/mg of protein.

2.5.5. Glutathione content

GSH levels were measured according to Browne and Armstrong [26] which is based on the reaction of GSH with the fluorophore ophthaldehyde (OPT) after the sample be

deproteinized with metaphosphoric acid. Results were expressed as nmol of GSH per mg of protein.

2.5.6. Ascorbic acid (vitamin C) content

Serum and hepatic vitamin C content was measured according to Papp et al. [27]. Results were presented as μg per g of protein

2.6. Protein determination

Protein content in serum and liver homogenate was determined by the method of Coomassie blue G dye [28], using serum bovine albumin as the standard.

2.7. Statistical analysis

Normality and homoscedasticity were analyzed through the Shapiro–Wilk and Levene tests, respectively. Therefore, statistical analysis was performed using a bilateral two-way analysis of variance (ANOVA) for independent samples, followed by Tukey post hoc analysis. Significance was set at $p < 0.05$. Thus, F and p values were shown only if $p < 0.05$. The effect of size (r2) was described and scored as follows: ≤ 0.1 (small), > 0.1 to ≤ 0.3 (medium) and ≥0.5 (large).

3. RESULTS

3.1. Performance and fish mortality

No significant difference was observed regarding animal performance (weight and weight gain). Also, no mortality was observed during all experimental period.

3.2. Serum and hepatic oxidant variables

Serum ROS levels increased on days 14 (F 1,30 = 2.44; p = 0.042; r2 = 0.45) and 21 (F 1,30 = 3.12; p = 0.031; r2 = 0.52) post-feeding in animals fed with AFB_1 compared to the control group (Fig. 1A). Moreover, hepatic ROS levels increased on days 14 (F 1,30 = 2.81; p = 0.041; r2 = 0.47) and 21 (F 1,30 = 3.43; p = 0.030; r2 = 0.53) post-feeding in animals fed with AFB_1 compared to control group (Fig. 3A). Serum LOOH levels increased on days 14 (F 1,30 = 3.17; p = 0.033; r2 = 0.51) and 21 (F 1,30 = 4.01; p = 0.024; r2 = 0.61) post-feeding in animals fed with AFB1 compared to the control group (Fig. 1B). Moreover, hepatic LOOH levels increased on days 14 (F 1,30 = 3.02; p = 0.037; r2 = 0.50) and 21 (F 1,30 = 3.31; p = 0.029; r2 = 0.58) post-feeding in animals fed with AFB_1 compared to the control group (Fig. 3B). Serum protein carbonylation levels increased on day 21 (F 1,30 = 3.88; p = 0.020; r2 = 0.57) post-feeding in animals

fed with AFB_1 compared to the control group (Fig. 1C). Moreover, hepatic protein carbonylation levels also increased on day 21 (F 1,30 = 4.11; p = 0.019; r2 = 0.69) post-feeding in animals fed with AFB_1 compared to control group (Fig. 3C). No significant difference was observed over time for the control and aflatoxin groups (Figs. 1 and 3).

3.3. Serum and hepatic antioxidant variables

Serum ACAP levels decreased on days 14 (F 1,30 = 5.77; p = 0.010; r2 = 0.79) and 21 (F 1,30 = 6.62; p = 0.002; r2 = 0.81) post-feeding in animals fed with AFB_1 compared to the control group (Fig. 2A). Moreover, hepatic ACAP levels decreased on days 14 (F 1,30 = 6.14; p = 0.003; r2 = 0.75) and 21 (F 1,30 = 6.95; p = 0.001; r2 = 0.89)
post-feeding in animals fed with AFB_1 compared to the control group (Fig. 4A). Serum GPx activity decreased on days 14 (F 1,30 = 3.12; p = 0.032; r2 = 0.59) and 21 (F 1,30 = 3.67; p = 0.028; r2 = 0.62) post-feeding in animals fed with AFB_1 compared to the control group (Fig. 2B). Moreover, hepatic GPx activity decreased on days 14 (F 1,30 = 3.33; p = 0.029; r2 = 0.56) and 21 (F 1,30 = 3.52; p = 0.027; r2 = 0.62) post-feeding in animals fed with AFB_1 compared to control group (Fig. 4B). Serum CAT activity decreased on days 14 (F 1,30 = 3.99; p = 0.024; r2 = 0.65) and 21 (F 1,30 = 4.15; p = 0.022; r2 = 0.66) post-feeding in animals fed with AFB_1 compared to the control group (Fig. 2C). Moreover, hepatic CAT activity decreased on days 14 (F 1,30 = 3.66; p = 0.028; r2 = 0.61) and 21 (F 1,30 = 3.89; p = 0.026; r2 = 0.63) post-feeding in animals fed with AFB_1 compared to control group (Fig. 4C). No significant difference was observed between groups regarding serum and hepatic SOD activities (Figs. 2D and 4D). Also, no significant difference was observed over time for the control and aflatoxin groups (Figs. 2 and 4).

3.4. Serum and hepatic NOx, GSH and vitamin C levels

Serum NOx levels increased on days 14 (F 1,30 = 4.17; p = 0.012; r2 = 0.77) and 21 (F 1,30 = 5.61; p = 0.0021; r2 = 0.80) post-feeding in animals fed with AFB1 compared to the control group (Fig. 5A), as well as observed for hepatic NOx levels on days 14 (F 1,30 = 4.11; p = 0.011; r2 = 0.77) and 21 (F 1,30 = 5.14; p = 0.0028; r2 = 0.69) (Fig. 6A). Serum vitamin C levels decreased on days 14 (F 1,30 = 4.71; p = 0.011; r2 = 0.77) and 21 (F 1,30 = 5.21; p = 0.0023; r2 = 0.72) post-feeding in animals fed with AFB1 compared to the control group (Fig. 5C), as well as observed for hepatic vitamin C levels on days 14 (F 1,30 = 3.67; p = 0.019; r2

= 0.67) and 21 (F 1,30 = 4.01; p = 0.0029; r2 = 0.67) (Fig. 6C). No difference was observed regarding serum and hepatic GSH levels (Figs. 5B and 6B).

4. DISCUSSION

In the current study, we observed for the first time, that AFB_1- contaminated diet elicited a disturbance of antioxidant/oxidant status in serum and liver of silver catfish after 14 and 21 days post-feeding. Our findings clearly demonstrated damage on important components of cell membranes, as lipids and proteins, as well as an inhibition of the primary and secondary antioxidant defense systems, which were unable to avoid or reduce the oxidative effects of free radicals.

Interestingly, this is the first study that observed a significant increase on serum and hepatic ROS and NOx levels in fish fed with AFB_1, in accordance to Singh et al. [29] while studying the liver of rats intraperitoneally injected with 1 mg/kg of AFB_1. It is important to emphasize that augmentation of ROS and NOx levels may explain the lipid peroxidation observed in fish fed with AFB_1, since excessive ROS and NOx levels enhanced the oxidation of polyunsaturated fatty acids [10]. Abdel-Rahman et al. [8] already reported increase on malondialdehyde levels in the serum of Nile tilapia fed with a diet containing 200 ppm of AFB_1 per kg of feed, similarly as this current study. According to these authors, high levels of malondialdehyde might indicate increased lipid

peroxidation, as observed in the present study, and probably contributes to pathogenesis and mortality of fish fed with AFB_1-contaminated diets. Moreover, we observed a significant increased on serum and hepatic protein carbonylation levels in animals fed with AFB_1-contaminated diet, in accordance to Madhusudhanan et al. [30] in liver, kidney, and brain of rohu carp (*Labeo rohita*) exposed intraperitoneally to 0.1 mg/kg AFB_1 for six days. As expected, serum and hepatic protein carbonylation is a direct effect of excessive ROS and NOx production, which damages principally the amino acids lysine, arginine, proline, and threonine via metal-catalyzed oxidation [31]. According to these authors, protein carbonylation is usually associated with permanent loss of function and can lead to elimination or accumulation of the carbonylated protein, often leading to tissue injury, and eventually cell death by necrosis or apoptosis, contributing to the initiation and progression of diseases. In summary, excessive ROS and NOx levels elicited lipid and protein damage in serum and liver of silver catfish on days 14 and 21 post-feeding, contributing to disease pathogenesis.

In order to avoid or reduce the damage of lipids and proteins elicited by excessive ROS and NOx production, the organism may activate enzymes of the antioxidant defense system, as CAT, SOD, and GPx, which can reduce the levels of lipid hydroperoxide and hydrogen peroxide (H_2O_2) [32], playing a major role in the prevention of oxidative damage. However, we observed a significant reduction of an indicator of antioxidant capacity (ACAP levels) in serum and liver of silver catfish fed with AFB_1, which indicates an impairment of the antioxidant system as an attempt to avoid or minimize the deleterious effects of ROS. This result is in agreement to Abdel-Rahman et al. [8], which observed that the antioxidant defense system of Nile tilapia fed with a mixture of aflatoxins is not modulated in consequence of excessive production of free radicals. Moreover, we observed significant lower activities of specific enzymes of the primary and secondary antioxidant defense system, as CAT and GPx, in serum and liver of silver catfish exposed to a diet containing AFB_1. Similarly, Abdel-Rahman et al. [8] demonstrated significant reduction on serum CAT activity of Nile tilapia exposed to 200 ppm of AFB_1 provided by their diet, concluding that the inhibition of its activity resulted in oxidative damage, evidenced by a reduction on serum total protein, albumin, and globulin levels. On the other hand, a study conducted by Kovesi et al. [12] demonstrated that GPx activity was unaltered in fish fed with diets containing different concentrations of AFB_1 and AFB_2, but gene expression of glutathione peroxidase 4 (gpx4a and gpx4b) were significantly higher, which indicates a stimulation of the antioxidant system as an attempt to reduce the dangerous H_2O_2 into water and oxygen. It is important to highlight that both enzymes are responsible for this reduction and an inhibition of these enzymes would lead to increased levels of H_2O_2, a known cause of cytotoxic and genotoxic effect on DNA, as well as on the ready conversion to reactive hydroxyl radicals ($OH^{\bullet}$) [33]. Also, the content of vitamin C, and important non-enzymatic antioxidant was lower in serum and liver of fish feed with AFB_1, in accordance to observed by Karakilcik et al. [34] in rabbits feed with 0.1 mg/kg AFB_1, indicating an impairment of antioxidant defense system and corroborating the occurrence of oxidative stress. In summary, the primary and secondary antioxidant defense systems are unable to avoid or minimize the excessive ROS formation, and consequently, serum and hepatic oxidative damage.

Based on these evidences, an AFB_1-contaminated diet causes disturbance on serum and hepatic antioxidant/oxidant system evaluated by lipid and protein damage elicited by excessive ROS production. Also, the antioxidant defense system was unable to avoid or minimize ROS

deleterious effects, and consequently, serum and hepatic oxidative damage. In summary, the oxidative stress contributes directly to pathophysiology and mortality of fish fed with AFB_1-contaminated diets.

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Conflicts of interest

All authors declare absence of conflict of interest.

Ethical note

The methodology used in the experiment was approved by the Ethical and Animal Welfare Committee of the Universidade do Estado de Santa Catarina under protocol number 9912090817. All applicable national guidelines for the care and use of animals were followed.

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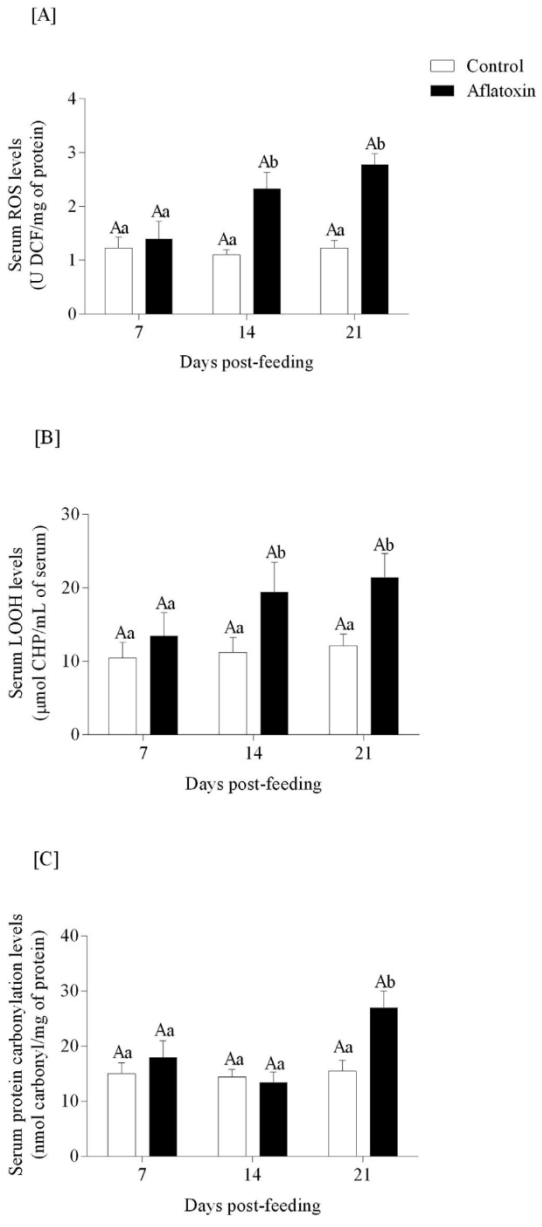
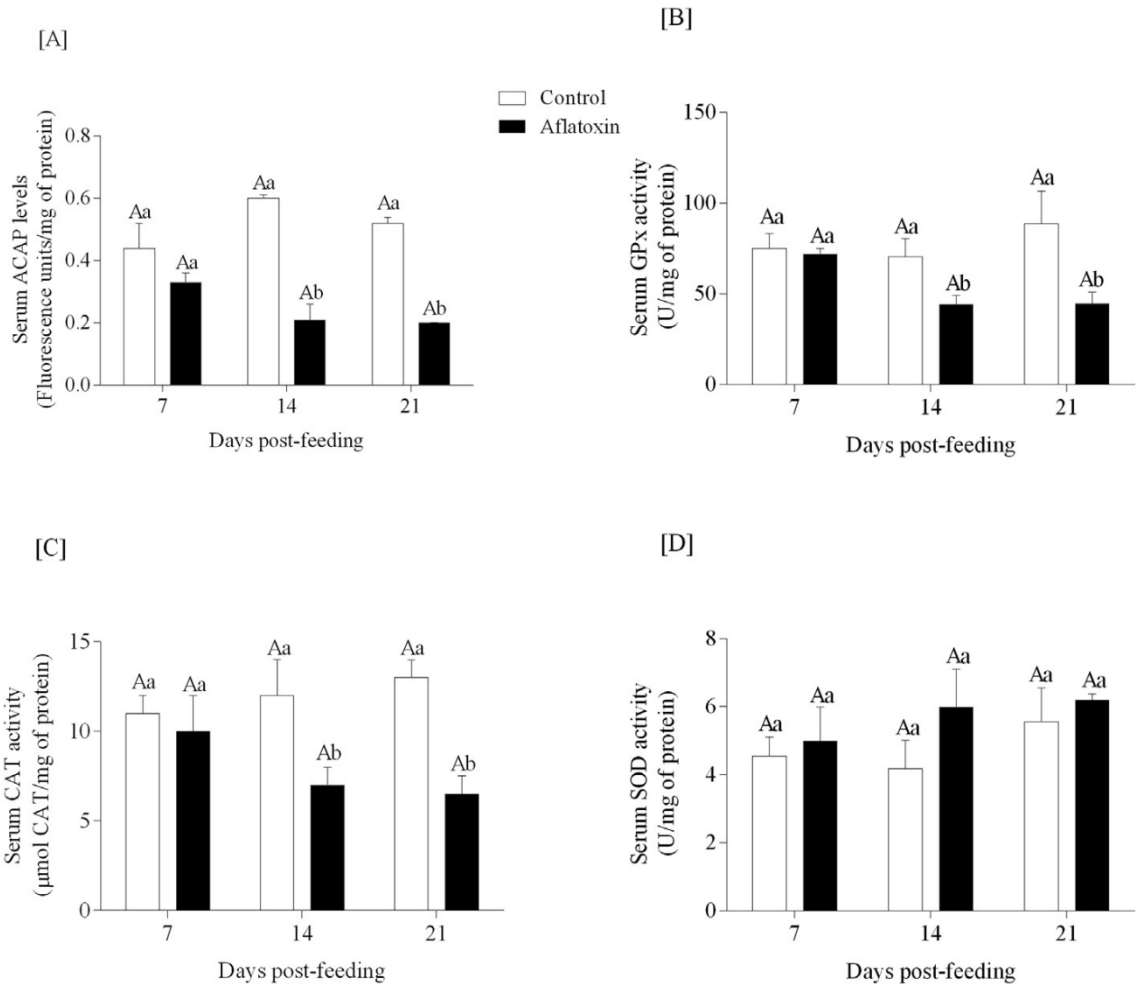
Figure 1: Serum reactive oxygen species (ROS) [A], lipid peroxidation (LOOH) [B] and protein carbonylation levels [C] in silver catfish fed with a diet contaminated by aflatoxin B1 (AFB_1) compared to the control group (basal diet).



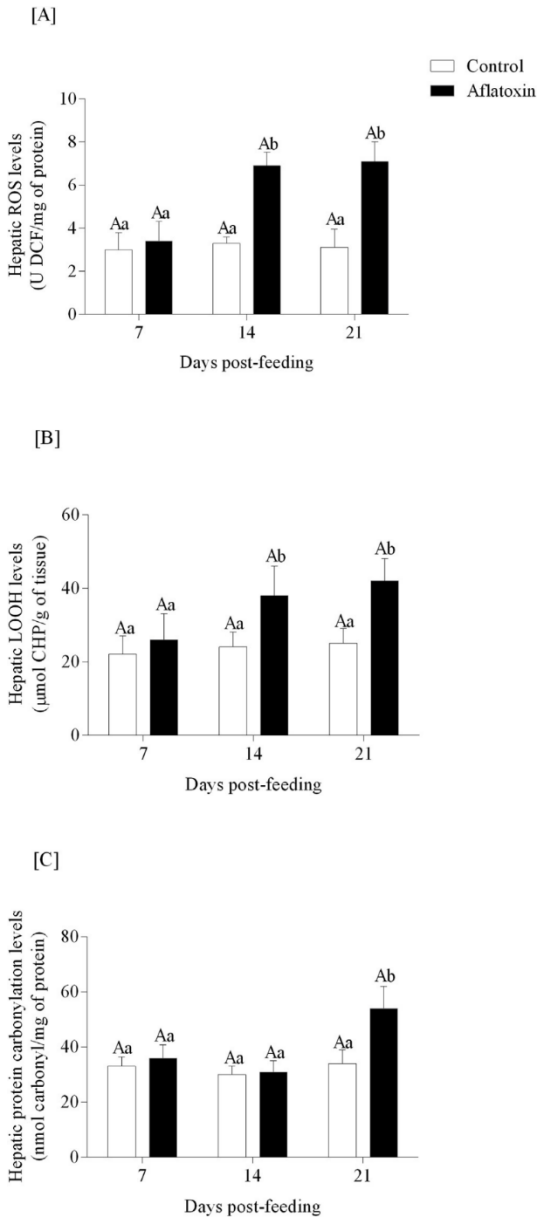
Lowercase letters indicate significant difference between treatment groups at the same time. Capital letters indicate significant difference between times for the same treatment. Bilateral two-way analysis of variance (ANOVA) for independent samples followed by the Tukey post hoc test ($p < 0.05$; $n = 6$ per group).

Figure 2: Serum antioxidant capacity against peroxyl radicals (ACAP) levels [A], glutathione peroxidase (GPx) [B], catalase (CAT) [C] and superoxide dismutase (SOD) [D] activities in silver catfish fed with a diet contaminated by aflatoxin B1 (AFB_1) compared to the control group (basal diet).



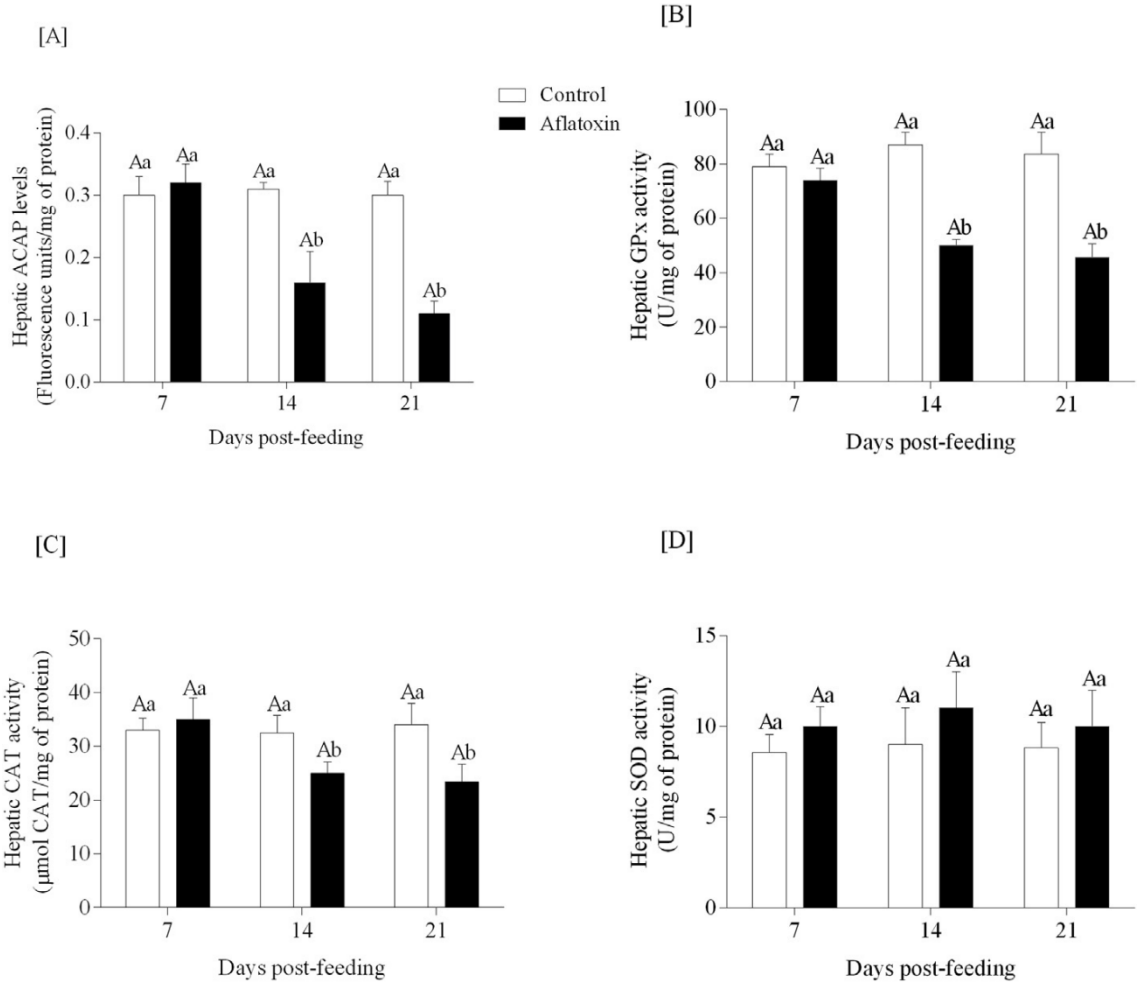
Lowercase letters indicate significant difference between treatment groups at the same time. Capital letters indicate significant difference between times for the same treatment. Bilateral two-way analysis of variance (ANOVA) for independent samples followed by the Tukey post hoc test ($p < 0.05$; $n = 6$ per group).

Figure 3: Hepatic reactive oxygen species (ROS) [A], lipid peroxidation (LOOH) [B] and protein carbonylation levels [C] in silver catfish fed with a diet contaminated by aflatoxin B1 (AFB_1) compared to the control group (basal diet).



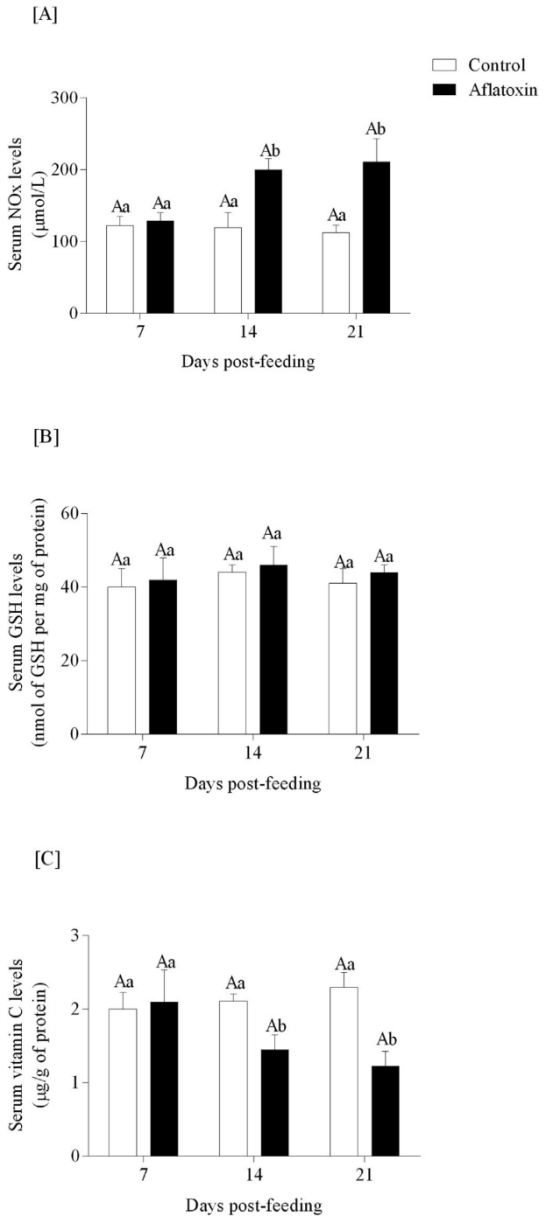
Lowercase letters indicate significant difference between treatment groups at the same time. Capital letters indicate significant difference between times for the same treatment. Bilateral two-way analysis of variance (ANOVA) for independent samples followed by the Tukey post hoc test ($p < 0.05$; $n = 6$ per group).

Figure 4: Hepatic antioxidant capacity against peroxyl radicals (ACAP) levels [A], and glutathione peroxidase (GPx) [B], catalase (CAT) [C] and superoxide dismutase (SOD) [D] activities in silver catfish fed with a diet contaminated by aflatoxin B1 (AFB_1) compared to the control group (basal diet).



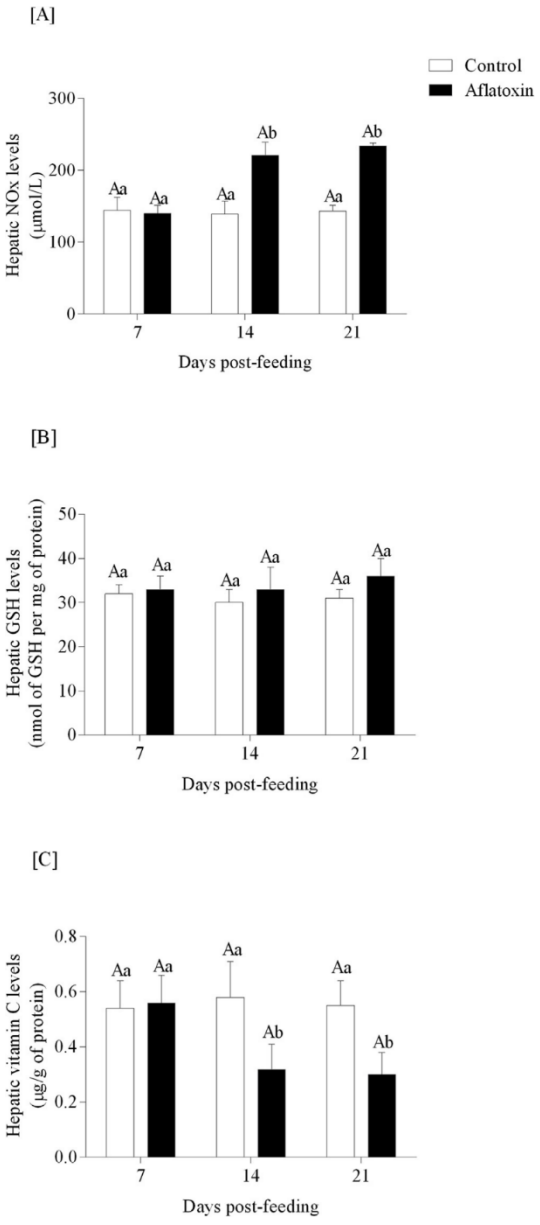
Lowercase letters indicate significant difference between treatment groups at the same time. Capital letters indicate significant difference between times for the same treatment. Bilateral two-way analysis of variance (ANOVA) for independent samples followed by the Tukey post hoc test ($p < 0.05$; $n = 6$ per group).

Figure 5: Serum metabolites of nitric oxide (NOx), glutathione content (GSH) and ascorbic acid (vitamin C) levels in silver catfish fed with a diet contaminated by aflatoxin B1 (AFB_1) compared to the control group (basal diet).



Lowercase letters indicate significant difference between treatment groups at the same time. Capital letters indicate significant difference between times for the same treatment. Bilateral two-way analysis of variance (ANOVA) for independent samples followed by the Tukey post hoc test ($p < 0.05$; $n = 6$ per group).

Figure 6: Hepatic metabolites of nitric oxide (NOx), glutathione content (GSH) and ascorbic acid (vitamin C) levels in silver catfish fed with a diet contaminated by aflatoxin B1 (AFB_1) compared to the control group (basal diet).



Lowercase letters indicate significant difference between treatment groups at the same time. Capital letters indicate significant difference between times for the same treatment. Bilateral two-way analysis of variance (ANOVA) for independent samples followed by the Tukey post hoc test ($p < 0.05$; $n = 6$ per group).

3.2 ARTIGO II

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Melaleuca alternifolia essential oil abrogates hepatic oxidative damage in silver catfish (*Rhamdia quelen*) fed with an aflatoxin-contaminated diet

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Abstract

Mycotoxins are secondary metabolites produced by varieties of fungi that contaminate food and feed resources and are capable of inducing a wide range of toxicity. This problem is extensively aggravated due to the increasing replacement of fish meal by plant-derived proteins. Among the mycotoxins, aflatoxins have received a great deal of attention owing to their great prevalence in plant feedstuffs and to the detrimental effects on animals. The objective of this study was to evaluate whether dietary supplementation with tea tree (*Melaleuca alternifolia*) oil (TTO) would avoid or minimize the negative impacts on silver catfish (*Rhamdia quelen*) fed with aflatoxins-contaminated diets. Four treatments were tested: control (fish fed with a control diet); AFB (fish fed with a mycotoxin-contaminated diet – 1893 µg kg^{-1} of AFB_1 and 52.2 µg kg^{-1} AFB_2); TTO (fish fed with a control diet + 1 mL kg^{-1} of TTO), and TTO+AFB (fish fed with a mycotoxin contaminated diet - 2324 µg kg^{-1} of AFB_1 and 43.5 µg kg^{-1} AFB_2 + 1 mL kg^{-1} of TTO). Diets were tested in three replications and analyzed at days 5 and 10 of dietary intake. Significantly reduced antioxidant enzymes (SOD, GPx, and GST) and increased lipid peroxidation (LOOH) and protein carbonyl (PC) content in plasma and liver, with 16.6 % mortality occurrence, were observed in the group fed aflatoxin-contaminated diet. Furthermore, aflatoxins also significantly increased plasmatic and hepatic ROS levels and decreased hepatic antioxidant capacity against peroxyl radical (ACAP) levels. Plasma cortisol levels were not altered by aflatoxicosis, but the intoxication induced hepatose. Notwithstanding, addition of TTO to the groups receiving aflatoxins showed a protective effect, avoiding the increase of ROS, LOOH, and PC levels in plasma and liver. Moreover, TTO treatment ameliorated the aflatoxin-associated liver damage. Thus, TTO supplementation at concentration of 1 mL kg^{-1} in feed may be used in fish to increase antioxidant status and reduce the negative effects caused by aflatoxins toxicity.

Key words: aflatoxicosis; silver catfish; tea tree oil; oxidative stress

1. Introduction

It is indisputable that fish consumption has been increasing worldwide, mainly due to the availability, access, and price in relation to other sources of meat, such as beef, pork, and poultry (Anater et al., 2016). In order to minimize the costs, aquaculture producers have added vegetable protein sources to fish diets. However, the use of plant-based ingredients such as maize and groundnut in fish feed enhances both the risk of the introduction of mycotoxins (notably aflatoxin B_1 and fumonisin B_1) into the feed at the point of production and/or the production of these mycotoxins during storage of the finished compounded feed (Tedesco et al., 2009; Santos et al., 2010).

Mycotoxins are toxic metabolites produced by fungi mainly of the genera *Aspergillus*, *Fusarium*, and *Penicillium* (Bennett and Klich, 2003). Food and feedstuffs prepared using mycotoxin-containing crops deteriorate nutritional content and represent a potential risk for animal and human health (Hussein and Brasel, 2001). High mycotoxin intake is collectively known as mycotoxicosis and includes organ failure, carcinogenicity, neurotoxicity, immunosuppression, reproductive, and developmental toxicity, among other effects (Kolpin et al., 2014). It is important to emphasize that mycotoxin effects are supported by all participants along the animal production chain, including animal producers, grain handlers and distributors, and crop processors (Rodrigues et al., 2011), but also by consumers. Besides, when exposed for long time, aflatoxin B_1 (AFB_1) accumulates in fish flesh and organs, leading to harmful situations such as human poisoning, liver tumors, and even death resulting from their consumption (Murjani, 2003; Shephard, 2008).

In aquaculture production, the aflatoxins commonly found on feedstuffs are AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Huang et al., 2011; Ottinger and Kaattari 1998; Ottinger and Kaattari 2000; Wang et al., 2016). AFB_1 is one of the most potent carcinogens known, classified as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1993) and highly hepatocarcinogenic (Sharma and Salunkhe, 1991). In fish, this mycotoxin has been reported to produce a range of pathologies such as hepatotoxicity, nephrotoxicity, and immunotoxicity (Manning et al., 2005; Griessler and Encarnacao, 2009; Souza et al., 2018a). According to Anater et al. (2016), an important element to reduce this type of contamination relies on prevention, because several products can be prone

to contamination along the chain of livestock production and it is not easy to identify the contaminated products.

The use of natural plants or their essential oils (EOs) to control mycotoxins has been on the rise (Ozcakmak et al., 2017; Villegas-Rascón et al., 2018), as they have antifungal, anti-aflatoxin and antioxidant activities. EOs have received high acceptance as feed additives as they are safe and biodegradable and present low toxicity (Gupta et al., 2011), and have gained great popularity in biomedical, pharmaceutical, cosmetic, food, agriculture, and veterinary fields due to their many biological properties. In addition, dietary incorporation of Fennel (*Foeniculum vulgare*) essential oil to a feed containing 200 µg of AFB_1 showed positive effects and was able to enhance immunity of Nile tilapia (*Oreochromis niloticus*), mitigating the toxic effects of this contamination. Among this wide array of EOs we can highlight tea tree (*Melaleuca alternifolia* Cheel) oil (TTO), which has been used successfully in the aquaculture field due to its antiparasitic, antibacterial, anesthetic, antioxidant, and hepatoprotective properties (Souza et al., 2016; Valladão et al., 2017; Baldissera et al., 2017a; Baldissera et al., 2017b; Souza et al., 2018b).

In particular, TTO has been reported to have antifungal activity (Carson et al., 2006; Terzi et al., 2007) and is considered a natural product of great medical, veterinary, and agronomic importance (Riccioni and Orzali, 2011; Yadav et al., 2017) thanks to its offer, and low cost, arousing the interest of many researchers to use it as an alternative product to drugs. A recent (*in vitro*) study demonstrated an excellent antifungal action of TTO against ochratoxin A (OTA) produced by *Aspergillus niger* and *A. carbonarius*, indicating that it may be a potential agent against mycotoxin contamination in foods (de Andrade Santiago et al., 2017).

TTO is usually composed of terpenes, particularly monoterpenes and sesquiterpenes, compounds formed by many secondary organic metabolites (Langenheim, 2003). It is important to emphasize that the composition of the TTO can vary with the collection site, crop type, climate, and time of year (Homer et al., 2000), being the terpenes 1,8-cineole and terpinen-4-ol the dominants chemotypes. Therefore, the variation in oil chemical composition can affect the microbiological activities through the different concentrations of the active components and their interaction (Cox et al., 2001; Lee et al., 2002; 2013).

Therefore, the objective of this study was to evaluate whether dietary supplementation with TTO would avoid or minimize the negative impacts on silver catfish fed with an aflatoxin-contaminated diet.

2. Materials and Methods

2.1. Aflatoxin production and analysis

Aflatoxins were produced via fermentation of converted rice by the *Aspergillus flavus* ATCC 13608 strain. Briefly, Erlenmeyer flasks containing 100 g of rice and 40 mL of distilled water were left at least for 2 h at room temperature. Flasks were then sterilized at 121 °C for 30 min and left to reach room temperature again. Subsequently, each flask was inoculated with 2 mL of a suspension of *A. flavus* containing 10^8 spores mL^{-1}. The incubation was carried out in a horizontal shaker with controlled temperature (25 °C) and constant stirring for 21 days. After incubation, fermented material was dried in an oven at 50 °C and grounded. The analyses of the aflatoxin contaminated rice and feed samples were performed by the multi-mycotoxin method developed by Sulyok et al. (2006). The analyses were conducted using a QTrap 5500 LC-MS/MS System equipment (Applied Biosystems, USA) equipped with a Turbo Ion Spray electronspray ionization (ESI) source at atmospheric pressure and an Agilent chromatography system (Agilent, Germany). The chromatographic separation was performed at 25 ºC in a Gemini C18 column, with 150 x 4.6 mm of internal diameter, with particle size of 5 µm (Phenomenex, USA). Eluents A and B were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) or 97:2:1 (eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% within 12 min, followed by a hold-time of 3 min at 100% B and 4-min column reequilibration at 100% A. The flow rate was 1 mL min^{-1}. ESI-MS/MS was performed in the multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte with the following settings: source temperature 550 °C, curtain gas 10 psi (69 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 50 psi (345 kPa of nitrogen), ion source gas 2 (drying gas) 50 psi (345 kPa of nitrogen), ion spray voltage −4,000 V and +4000 V respectively, collision gas (nitrogen) high. The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (diluted in a 1:1 mixture of eluent A and B) into the MS using a 11 Plus syringe pump (Harvard

Apparatus, Holliston, MA, US) at a flow rate of 10 μL min^{-1} (Table 1). Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte. Quantification was performed using an external calibration based on serial dilutions of an aflatoxin stock solution.

2.2. Plant material and essential oil components

Melaleuca alternifolia essential oil was purchased from Importadora Química Delaware Ltda® (Porto Alegre, Brazil). Oil composition and yield was analyzed through gas chromatography (GC) carried out using an Agilent Technologies 6890N GC-FID system, equipped with DB-5 capillary column (30 m × 0.25 mm × 2.5 μm film thickness) connected to a flame ionization detector (FID). The injector and detector temperatures were set to 250 °C. The carrier gas was helium, at a flow rate of 1.3 mL/min. The thermal programmer was 100–280 °C on a rate of 10 °C/min. Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors. The injection volume of the TTO was 1 μL (Hammer et al., 2000; Boligon et al., 2013). GC–mass spectroscopy (GC–MS) analyses were performed on an Agilent Technologies AutoSystem XL GC–MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (250 °C). The transfer line temperature was 280 °C. Helium was used as carrier gas (1.5 mL/min) and the capillary columns used were an HP 5MS (30 m × 0.25 mm × 2.5 μm film thickness) and an HP Innowax (30 m × 0.32 mm i.e., film thickness 0.50 mm). The temperature programed was the same as that used for the GC analyses. Essential oil injected volume was 1 μL.

Identification of TTO components was performed on the basis of retention index (RI), determined with reference of the homologous series of n-alkanes, C7–C30, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature date Adams (1995). The relative amounts of individual components were calculated based on the CG peak area (FID response).

2.3. Animals

Ninety-six juvenile silver catfish (50.32 ± 7.54 g; 23 ± 3.6 cm; mean ± standard deviation) were obtained from a fish farm located in Arroio Grande, district of Santa Maria (Southern Brazil). The animals were evaluated for the absence of ectoparasites or endoparasites in the

gills, skin, and fins, and for the absence of skin lesions compatible to possible bacterial infections that would impact the animal's health. The fish were transported to the Fish Physiology Laboratory at the Universidade Federal de Santa Maria where they were maintained for 10 days in 250-L fiberglass tanks (semi-static renewal system) with continuous aeration under controlled water variables: temperature of 19–21 °C (maintained with an air conditioner), pH of 7.2–7.5, and dissolved oxygen levels of 6.0–7.4 mg L^{-1}. After this period, fish were distributed into 30-L tanks (six fish/tank), in triplicate, in a recirculating aquaculture system. Water quality parameters were verified daily. Dissolved oxygen and temperature were measured with a YSI oxygen meter (model Y5512, Ohio, USA), and pH was measured with a DMPH-2 pH meter (São Paulo, Brazil). Total ammonia levels were determined according to Verdouw et al. (1978), and non-ionized ammonia levels were calculated using a conversion table for fresh water. Water variables (mean ± standard deviation of three replicates) remained stable during all experimental period: temperature (22 ± 0.8 °C), pH (7.1 ± 1.1), dissolved oxygen (6.8 ± 0.5 mg L^{-1}), total ammonia (0.85 ± 0.05 mg L^{-1}), and non-ionized ammonia (0.0033 ± 0.00021 mg L^{-1}).

2.4. Experimental design

The animals were randomly distributed in four groups: control (fish fed with a control diet); AFB (fish fed with a mycotoxin-contaminated diet – 1893 µg kg^{-1} of AFB_1 and 52.2 µg kg^{-1} AFB_2); TTO (fish fed with a control diet + 1 mL kg^{-1} of TTO), and TTO+AFB (fish fed with a mycotoxin contaminated diet - 2324 µg kg^{-1} of AFB_1 and 43.5 µg kg^{-1} AFB_2 + 1 mL kg^{-1} of TTO). The basal diet (Table 2) was formulated based on the study of Zeppenfeld et al. (2016). The addition of AFB_1 followed the theoretical recommendation of Lopes et al. (2005). Fish received the experimental diets until apparent satiation once a day (9 a.m.) for 10 days. Tanks were cleaned 30 min after feeding via siphoning to remove waste (remains of food and feces).

AFG_1 and AFG_2 were not detected. Control and TTO treatments did not show aflatoxin concentrations higher than the limit of quantification (1 µg kg^{-1}).

2.5. Sample collection

Six fish from each treatment were used for sample collection and analysis. Total blood was collected from the caudal vein with heparinized sterile syringes. Plasma was obtained after

centrifugation (3000×g, 10 min, -4 ºC) and subsequently stored at -80 °C for further analyses. The fish were euthanized at days 5 and 10 post-feeding by sectioning of spinal cord, and the livers were removed and stored at -20 ºC until analyses.

2.6.Plasma cortisol

Plasma cortisol levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Canada) following the manufacturer recommendations. Twenty microliters of sample without need of extraction procedures were read in triplicate, and the results were presented as ng/mL.

2.7.Plasma and hepatic oxidant parameters

For the evaluation of oxidant parameters, the liver was homogenized in 10 volumes (1:10, w/v) of 30 mM sodium phosphate buffer, pH 7.4, containing 120 mM KCl. Homogenates were centrifuged at 850×g for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001), and the supernatants utilized for the determination of the parameters.

2.7.1. 2′,7′-Dichlorofluorescein oxidation

Reactive species (RS) production was assessed by determining 2′,7′-Dichlorodihydrofluorescein (DCFH) oxidation (LeBel et al., 1992). DCFH-DA is hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of RS. The DCF fluorescence intensity correlates to the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively, in the SpectraMax i3x (Molecular Devices, USA) microplate reader. Calibration curve was performed with standard DCF (0.1–1 μM), and data were calculated as micromoles DCF formed per mg of protein.

2.7.2. Antioxidant capacity against peroxyl radicals (ACAP)

ACAP was determined according to the method described by Amado et al. (2009) with modification for mammals. This method consists of finding the antioxidant capacity of tissues

using a fluorescent substrate (2′,7′ dichlorofluorescein diacetate - H2DCF-DA) and the production of peroxyl radicals by thermal decomposition of ABAP (2.2′-azobis 2 methylpropionamidine dihydrochloride). The fluorescence was determined using a microplate reader (Spectramax I3), at 37 °C (excitation: 485 nm; emission: 530 nm), with readings at every 5 min, during 30 min. Detection of fluorescence (with and without ABAP) was performed during 40 min at 37 °C and results were expressed by the relative fluorescence area (fluorescence × time) and ACAP was calculated according to the equation: ACAP = 1/[(fluorescence area with ABAP − area without ABAP)/area without ABAP].

2.7.3. Protein carbonyl content

Protein carbonyl formation was examined spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of plasma or liver supernatants were treated with 200 µL of 10 mM 2,4 dinitrophenylhydrazine (DNPH) prepared in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h. Five hundred microliters of 20 % trichloroacetic acid were added to the samples to precipitate the proteins and the tubes centrifuged for 5 min at 9000 g. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and dissolved in 300 µL of 6 M guanidine prepared in 2.5 N HCl at 37 ºC for 5 min. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl formation determined at 365 nm in the SpectraMax i3x (Molecular Devices, USA) microplate reader. The results were calculated as nmol of carbonyl groups mg of protein^{-1}.

2.7.4. Lipid peroxidation (LOOH) levels

LOOH levels were measured as proposed by Monserrat et al. (2003) and reported in detail by Da Silva Barreto et al. (2018), with some adaptations in the volume of reagents for measurement in microplate. Plasmatic results were expressed as µmol CHP mL^{-1} of plasma, while hepatic results were expressed as µmol CHP g^{-1} of tissue (CHP = cumene hydroperoxide).

2.7.5. Glutathione S-transferase (GST) activity

The activity of GST was measured according to Mannervik and Guthenberg (1981), with slight modifications. GST activity was measured by the rate of formation of dinitrophenyl-S-glutathione at 340 nm in a medium containing 50 mM potassium phosphate, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and tissue supernatants

(approximately 0.045 mg of protein). The results were calculated and expressed as U mg protein^{-1}.

2.7.6. Glutathione peroxidase (GPx) activity

GPx activity was measured using tert-butylhydroperoxide as substrate (Wendel, 1981). The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM EDTA, pH 7.7, 2 mM GSH, 0.1 U/mL GR, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide, 0.1 mM NADPH and tissue supernatants. The results were calculated and expressed as U mg protein^{-1}.

2.7.7. Superoxide dismutase (SOD) activity

SOD activity was determined according to the auto-oxidation principle of pyrogallol, which is inhibited in the presence of SOD. The optical density change was determined kinetically for two minutes at 420 nm, at 10-second intervals, according to methodology described by Beutler (1984). Activity was expressed as U mg protein^{-1}.

2.7.8. Total protein content

The protein concentration was determined by the Coomassie Blue method following the methodology described by Read and Northcote (1981), using bovine serum albumin as a standard.

2.8. Histopathological analysis

Animals were euthanized by sectioning the medulla behind the cranial vault. Hepatic tissues were fixed for histopathology using 4% buffered formalin, embedded in paraffin wax, sectioned using a microtome (4-6 μm thickness), and stained with hematoxylin and eosin according to the standard method for histopathological evaluation (Luna, 1968). Two animals were processed per treatment; examining at least five cuts per animal. Images were taken using a Nikon Eclipse 80i photomicrograph at 10x and 40x magnification. Images were analyzed in the Laboratory of Ichthyopathology of the University of Los Llanos-IALL (Instituto de Acuicultura de los Llanos) by a trained veterinarian observer with more than 20 years of experience in fish histopathology.

2.9. Statistical analysis

Normality and homoscedasticity were analyzed through the Shapiro–Wilk and Levene tests, respectively. All treatment groups were compared by using a factorial-ANOVA analysis (treatment x time), (treatment x AFB_1) and (AFB_1 x time) followed by the Tukey's test, or when homogeneity of variances was not obtained, by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test and the Nemenyi test. Fish mortality was evaluated using chi-square test. Analyses were performed using the STATISTICA software, version 5.1 (StatSoft, Tulsa, OK, USA), and the minimum significance level was set at $p < 0.05$. Data are reported as mean ± standard error of the mean (SEM).

3. *Results*

3.1.TTO characterization

Fifteen components representing 95.86% of the total composition were identified in TTO. The results indicated that terpinen-4-ol (41.98%) was the most abundant compound, followed by γ-terpinene (20.15%), α-terpinene (9.85%), 1.8-cineole (6.03%), and terpinolene (4.15%) (Figure 1).

3.2. Performance and fish mortality

No significant difference was observed in animal performance (weight and weight gain). In line with this, fish mortality did not differ among groups, although a mortality rate of 16.6 % was observed in the AFB group at the end of experiment.

3.3. Plasma cortisol

The addition of AFB and TTO to fish diet did not alter plasma cortisol levels. However, a significant decrease was observed in the AFB + TTO group after 10 days of feeding when compared to day 5 of experiment (Figure 2).

3.4. Plasma and hepatic oxidant parameters

Plasma (Fig. 3A) and hepatic (Fig. 4A) ROS levels increased at day 10 post-feeding in animals fed with AFB compared to the control, TTO, and TTO+AFB groups. Plasma LOOH levels decreased at day 10 post-feeding in animals fed with TTO and TTO +AFB groups when

compared to control group (Fig. 3B). In contrast, hepatic LOOH levels decreased at day 10 post-feeding in fish fed with TTO and TTO+AFB diets compared to control and AFB diet (Fig. 4B). Plasma carbonylation levels decreased at day 10 post-feeding with TTO and TTO+AFB diets, compared to control and AFB treatments (Fig. 3C). Hepatic protein carbonylation levels also increased at day 5 post-feeding in fish from AFB group compared to other groups (Fig. 4C).

3.5. Plasma and hepatic antioxidant parameters

Plasma and hepatic ACAP levels decreased at day 5 post-feeding with AFB diet when compared to other groups (Fig. 5A). However, hepatic ACAP levels were decreased at day 5 in fish fed with TTO+AFB diet in comparison to control and TTO groups (Fig. 6A). Plasmatic SOD activity decreased at days 5 and 10 post-feeding in animals fed with AFB when compared to control, TTO, and TTO+AFB groups (Fig. 5B). Hepatic SOD activity decreased on days 5 and 10 post-feeding in animals fed with AFB_1 when compared to other groups. Besides, SOD activity decreased on day 10 post-feeding with TTO+AFB, compared to control and TTO groups (Fig. 6B). Plasma GPx activity decreased only at day 5 post-feeding with AFB_1 and TTO+AFB when compared to control and TTO groups. After day 10 post-feeding plasma. GPx activity did not present any significant difference among groups (Fig. 5C). In line with this, hepatic GPx activity was also not altered (Fig. 6C). Plasma GST activity decreased at days 5 and 10 post-feeding in animals fed with AFB compared to control, TTO, and TTO+AFB groups. However, at day 10, fish fed with TTO diet had an increase in plasma GST activity (Fig. 5D). Conversely, fish fed with AFB diet presented a decrease in hepatic GST activity only at day 5 post-feeding. Moreover, fish fed with TTO and TTO+ AFB showed an increase in hepatic GST activity at day 10 post-feeding compared to control and AFB groups (Fig 6D).

The *p* value for AFB, TTO and its interaction, as well as the interactions with the time is presented in the Table 3.

3.6. Hepatic histopathology

The animals in the control and TTO groups did not present histopathological changes in liver tissue at days 5 and 10 post-feeding (Fig. 7A-C and Fig. 8A-C). However, at day 5 post-feeding with AFB diet, it was observed hepatocyte anisocytosis, degenerate multifocal modifications of the type of moderate fat infiltration, apoptosis and/or multifocal necrosis of

hepatocytes (possibly more apoptosis, considering the low inflammatory reaction that accompanies cell death), moderate nuclear pyknosis common in hepatocytes, characterizing toxic hepatitis (Fig. 7B). At day 10 post-feeding it was possible to observe an increase in the severity of the toxic hepatitis, besides apoptosis and/or multifocal necrosis of hepatocytes, moderate nuclear pyknosis in hepatocytes and reaction by Ito cells in some slightly thickened sinusoids, also with some thickening of biliary canaliculi, characterizing toxic hepatitis and hepatosis (Fig. 8B), On the other hand, the addition of TTO in the diet contaminated with AFB minimized the toxic effects of aflatoxicosis and at days 5 and 10 after feeding it was observed homogenous hepatic parenchyma with slight degenerative changes and possibly of fatty infiltration, light multifocal, considering as a mild hepatosis (Fig 7D and Fig.8D).

4. Discussion

The present study provides insight into the contamination level of aflatoxin in fish feed. The use of aflatoxin-contaminated feed may result in economic losses due to decrease in productivity and higher mortality rates, as documented by Barbosa et al. (2013) and Marijani et al. (2017). In this study, the analysis of the AFB_1 concentration in in the formulated diets showed concentrations higher than the theoretical concentration desired (1117 µg kg^{-1}). Moreover, AFB_2 was also detected, but at low concentrations when compared to AFB_1, which is in line with previous reports (Murphy et al., 2006; Adeyemoet al., 2018). It is an important to emphasize that the occurrence of aflatoxicosis in Nile tilapia has been reported even at the low concentration of 20 µg kg^{-1} of AFB_1 in feed (Mahfouz and Sherif, 2015).

Here it was observed that the AFB-contaminated diet elicited a disturbance of the antioxidant/oxidant status in plasma and liver of silver catfish at days 5 and 10 post-feeding, corroborating the findings of Souza et al. (2018a). In addition, AFB diet also caused a toxic hepatitis and hepatosis. Overall, TTO added to the diet was not toxic to the silver catfish since no significant differences between fish fed with control diet and fish receiving TTO was observed for most of the evaluated parameters.

Interestingly, our findings clearly demonstrated that the addition of TTO minimizes the toxic, histopathological effects of aflatoxin contamination, avoiding or reducing the oxidative effects of free radicals. It is important to emphasize that effects of TTO on mycotoxicosis

remain unknown, but several studies have demonstrated that this EO can exert protective effects on homeostasis by ameliorating the antioxidant system of silver catfish (Souza et al., 2016; Baldissera et al., 2017a; Souza et al., 2018b).

Cortisol is widely known as an important indicator to assess the stress elicited by various factors such as toxicants and environment alterations (Barton, 2000). Nonetheless, this study showed that plasma cortisol levels of *R. quelen* were not altered at days 5 and 10 post-feeding with AFB-contaminated diet. On the other hand, AFB is able to induce reactive oxygen species (ROS) (Adedara et al., 2010; Matur et al., 2011; Souza et al., 2018a), and, as expected, we observed an increase in plasma and hepatic ROS levels and a reduction in total antioxidant capacity (ACAP) in fish feeding AFB diet. Thus, AFB can generate ROS during biotransformation in liver, which leads to lipid peroxidation cytotoxicity and DNA damage (Marin and Taranu, 2012). In the present study, we observed a significant increase on hepatic protein carbonylation and lipid peroxidation levels in animals fed with AFB-contaminated diet. The high level of LOOH indicates increasing in lipid peroxidation or suppression of antioxidant defense. Similar results were also reported in Nile tilapia by El-Barbary and Mohamed (2014) and in silver catfish (Souza et al., 2018a). According to Madhusudhanan et al. (2004), protein carbonylation and lipid peroxidation are usually associated with permanent loss of function and can lead to elimination or accumulation of the carbonylated protein, often leading to tissue injury, and eventually cell death by necrosis or apoptosis, contributing to the initiation and progression of aflatoxicosis.

The imbalance between lipid peroxides and the antioxidant system may result in cell dysfunction and the enrichment of lipid peroxides and free radicals, leading to cell damage (Chen and Chen, 2011). Meanwhile, similar to other vertebrates, antioxidant enzymes in fish could protect them from such damages. The antioxidant defenses in fish ensure low-molecular-weight antioxidants and enzymes such as superoxide dismutase, catalase, and enzymes dependent on glutathione (glutathione peroxidase and glutathione reductase). In the present study, the diet contaminated with aflatoxin caused a decrease in SOD, GPX, and GST activities and an increase in SOD and GST activities in plasma, highlighting the high ability of the fish liver to compensate oxidative stress. This result agrees to Abdel Rahman et al. (2017), which observed that the antioxidant defense system of Nile tilapia fed with a mixture of aflatoxins is not modulated in consequence of excessive production of free radicals.

TTO supplementation in the AFB-contaminated diets resulted in a protective effect for *R. quelen*, which was evident by the significant improvement in many of the tested variables. In addition, TTO has adsorbent effect through binding to AFB_1 and preventing its absorption in the alimentary tract or increasing antioxidant enzymes (Selim et al., 2014). Similar results showing a reduction of MDA with increasing of glutathione and superoxide dismutase activities was found for Nile tilapia receiving diets containing 200 µg kg^{-1} AFB and 1 mL kg^{-1} of Fennel essential oil (Abdel Rahman et al., 2017). Dietary supplementation with TTO for Nile tilapia promotes a rapid activation of the complement system in fish fed for 60 days, revealing that this herbal medicine has great potential to be used as an immunostimulant for fish. The TTO is a potent antioxidant and has been shown to protect most fish tissues against oxidative damage (Baldissera et al., 2017a). Differences in susceptibility to aflatoxins in aquatic organisms appear to correlate with interspecies variations in the efficiency of AFB biotransformation. In some fish species, the metabolic pathways of AFB_1 are mainly characterized by two routes: phase I, or activation phase, mediated by cytochrome P450; and phase II, or detoxification step, involving two enzymes, glucuronyl transferase and, to a lesser extent, glutathione-S-transferase (Santacroce et al., 2008). Therefore, the increase in hepatic GST levels observed in fish fed with AFB+TTO showed an attempt of detoxification, which can be considered one of the mechanisms of action of this natural compound against aflatoxins. In addition to the benefits of using TTO, it is important to note its activity and mechanisms of action against fungi (Hammer et al., 2000, 2002, 2003), which helps to explain the success of the association of the protective effect of TTO against AFB contamination.

The liver is the target organ for aflatoxicosis and aflatoxin metabolites negatively react with various cell proteins, inducing necrosis and tumor or cell death (Santacroce et al., 2008). The histopathological analysis of the present study corroborate this fact, demonstrating that diets contaminated with AFB caused toxic hepatitis from the 5^{th} day post-feeding onwards and hepatosis and necrosis from the 10^{th} day onwards. Hepatocytes necrosis in fish fed aflatoxin-contaminated diet indicates destruction of cell membranes and necrosis of tissues. In addition, aflatoxin-induced damage to liver can disturb homeostasis and physiological balance of fish (Shahafve et al., 2017). Similar histopathological findings were reported in liver of Nile tilapia (Mahfouz and Sherif, 2015), in hybrid sturgeons (Raghavan et al. 2011), in rainbow trout (*Oncorhynchus mykiss*) (Arana et al., 2014), and in and common carp (*Cyprinus carpio*) (Shahafve et a., 2017). In the current study, the diet supplemented with TTO minimized the

severe damage caused by AFB when compared to the group receiving AFB but not treated with TTO. Similar results were reported by Zychowski et al. (2013), where the diet supplemented with NovaSil™ (NS) and calcium montmorillonite clay was able to ameliorate AFB-induced histopathological changes in the liver of red drum (*Sciaenops ocellatus*).

5. Conclusion

Based on our results, AFB causes unfavorable effects on silver catfish and disturbance on plasma and hepatic antioxidant/oxidant system. This was clearly observed by the excessive ROS production, indicating lipid and protein damage, besides causing toxic hepatitis and hepatosis. Dietary incorporation of 1 mL kg^{-1} TTO showed a protective effect and ameliorated these drastic effects, mainly the liver damage, besides enhancement of fish antioxidant status. Therefore, this alternative could be used as a strategy to reduce aflatoxin-related toxicity in fish.

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Conflicts of interest

All authors declare absence of conflict of interest.

Ethical note

The methodology used in the experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria under protocol number 99120908174**.**

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Table 1. Mass spectrometry parameters for MRM transitions in the positive and negative ion mode monitoring.

Analyte	T_R [a] (min.)	Precursor ion m/z	DP[b] (V)	Product ions[c] m/z	Relative intensity[d]	CE[e] (V)[c]	CXP[f] (V)[c]	Period	Dwell Time[c] (ms)
Aflatoxin G1	11.51	329.0 [M+H]+	56	243.2/200.0	0.60	39/59	14/12	+4	60/20
Aflatoxin G2	11.16	331.1 [M+H]+	81	313.2/245.2	0.69	35/43	18/14	+4	60/20
Aflatoxin B1	12.16	313.0 [M+H]+	76	285.2/128.1	0.65	33/91	16/10	+5	60/20
Aflatoxin B2	11.87	315.1 [M+H]+	66	287.2/259.2	0.89	37/43	18/18	+5	60/20

a Retention time
b Declustering potential
c Values are given in the order quantifier ion/qualifier ion
d Intensity of the qualifier transition/intensity of the quantfier transition
e Collision energy

Table 2: Formulation of experimental diet.

Ingredient	**g/kg**
Soybean meal	300
Meat and bone meal	350
Rice brain	120
Corn	150
Canola oil	30
Salt	10
Vitamin and mineral premix[1]	30
Dicalcium phosphate	10

Vitamin and mineral mixture (per kilogram of product): 200 mg folic acid, 5,000 mg pantothenic acid, 0.60 g antioxidant, 125 mg biotin, 25 mg cobalt, 2,000 mg copper, 820 mg iron, 100 mg iodine, 3,750 mg manganese, 5,000 mg niacin, 75 mg selenium, 1,000,000 UI vitamin A, 1,250 mg vitamin B1, 2,500 mg vitamin B2, 2,485 mg vitamin B6, 3,750l mg vitamin B12, 28,000 mg vitamin C, 500,000 UI vitamin D3, 20,000 UI vitamin E, 500 mg vitamin K and 17,500 mg zinc.

Table 3: Effects (*p* value) of aflatoxin (AFB), tea tree oil (TTO) and its interaction, as well as the interactions with time of experiment.

	AFB	**TTO**	**AFB+TTO**	**AFB *x* time**	**TTO *x* time**	**AFB+TTO *x* time**
Plasmatic cortisol	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p = 0.01$
Plasmatic ROS	$p = 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p = 0.0022$	$p < 0.05$
Plasmatic LOOH	$p < 0.05$	$p = 0.0018$	$p = 0.023$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Plasmatic protein carbonylation	$p < 0.05$	$p < 0.05$	$p = 0.011$	$p < 0.05$	$p = 0.038$	$p = 0.20$
Hepatic ROS	$p = 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Hepatic LOOH	$p = 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Hepatic protein carbonylation	$p = 0.001$	$p < 0.05$	$p < 0.05$	$p = 0.01$	$p < 0.05$	$p < 0.05$
Plasmatic ACAP	$p < 0.05$	$p = 0.028$	$p = 0.019$	$p < 0.05$	$p = 0.012$	$p = 0.01$
Plasmatic SOD	$p = 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Plasmatic GPx	$p = 0.0012$	$p < 0.05$	$p = 0.018$	$p = 0.001$	$p = 0.021$	$p = 0.03$
Plasmatic GST	$p = 0.0013$	$p = 0.038$	$p < 0.05$	$p < 0.05$	$p = 0.01$	$p < 0.05$
Hepatic ACAP	$p = 0.0011$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Hepatic SOD	$p = 0.0013$	$p < 0.05$	$p = 0.02$	$p = 0.032$	$p < 0.05$	$p = 0.01$
Hepatic GPx	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Hepatic GST	$p = 0.031$	$p = 0.001$	$p = 0.0001$	$p < 0.05$	$p = 0.001$	$p = 0.0019$

Fig 1. Peaks of compounds present in TTO essential oil: (1) α-Pinene, (2) Sabinene, (3) α-Terpinene, (4) p-Cymene, (5) Limonene, (6) 1.8-Cineole, (7) γ-Terpinene, (8) Terpinen-4-ol, (9) Terpinolene, (10) α-Terpineol, (11) Aromadendrene, (12) Ledene, (13) δ-Cadinene, (14) Globulol, and (15) Viridiflorol quantified by the method of chromatography.

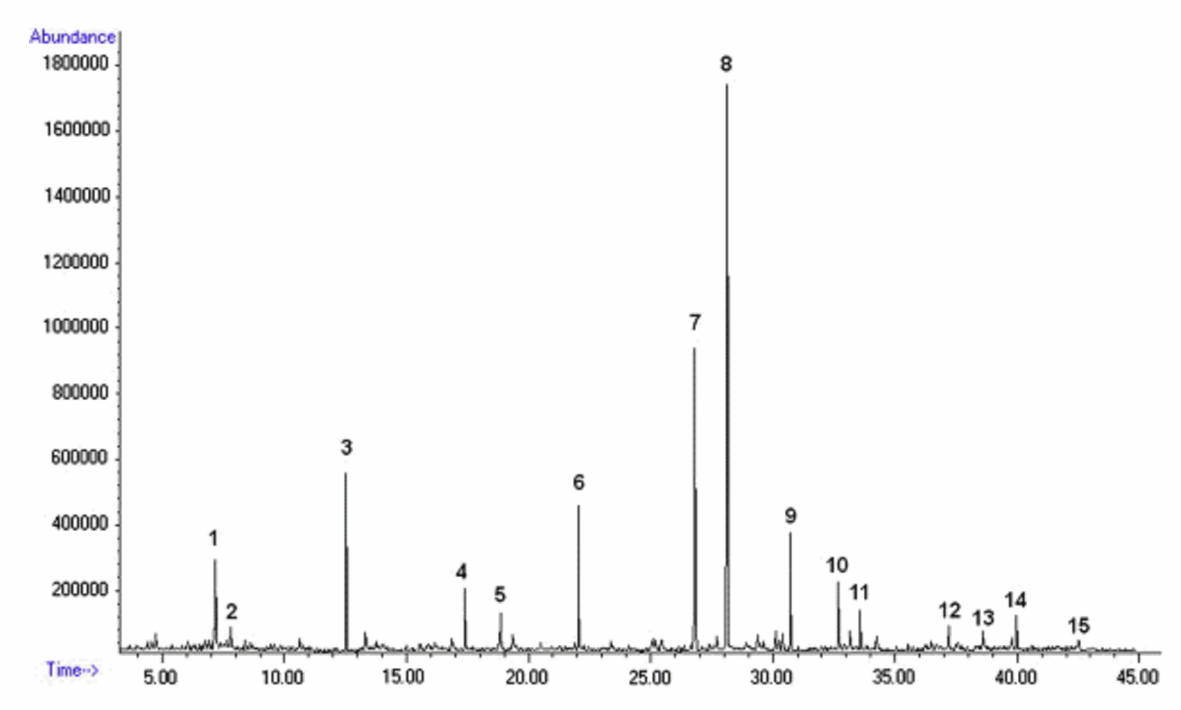
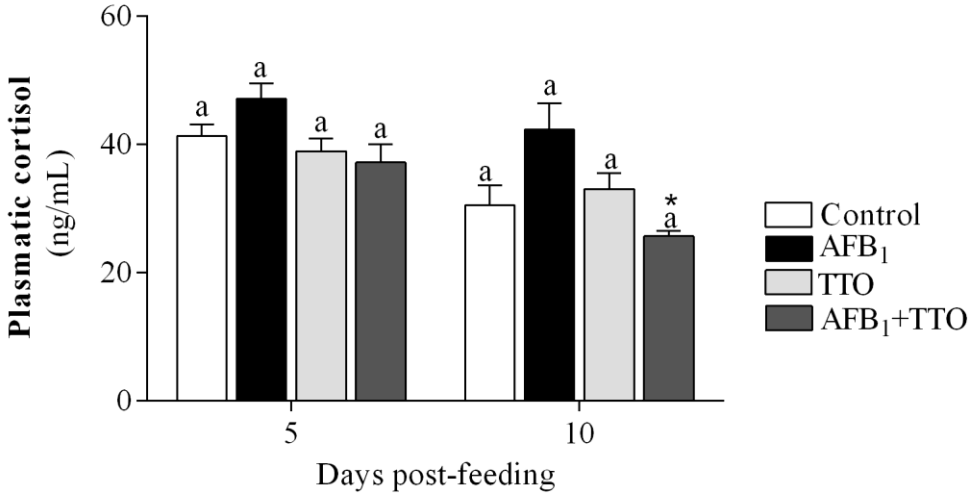
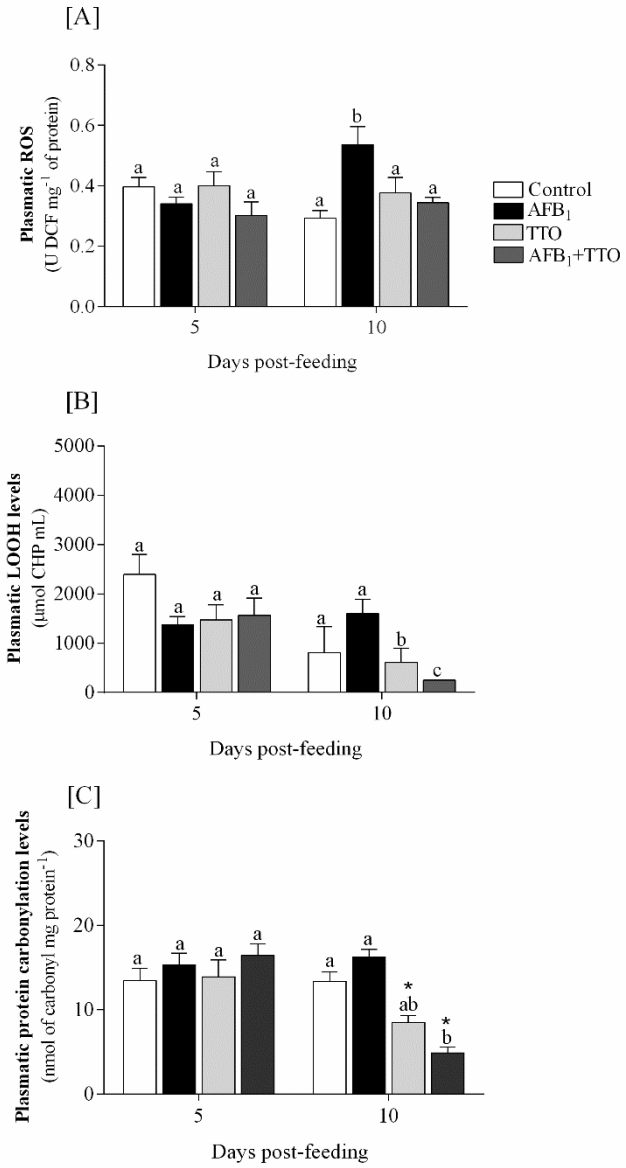


Fig. 2 Effect of aflatoxins and/or tea tree oil (TTO) on plasmatic cortisol levels. Groups within the same time period with no lowercase letters in common are significantly different. Asterisk indicates significant difference between times for the same treatment.



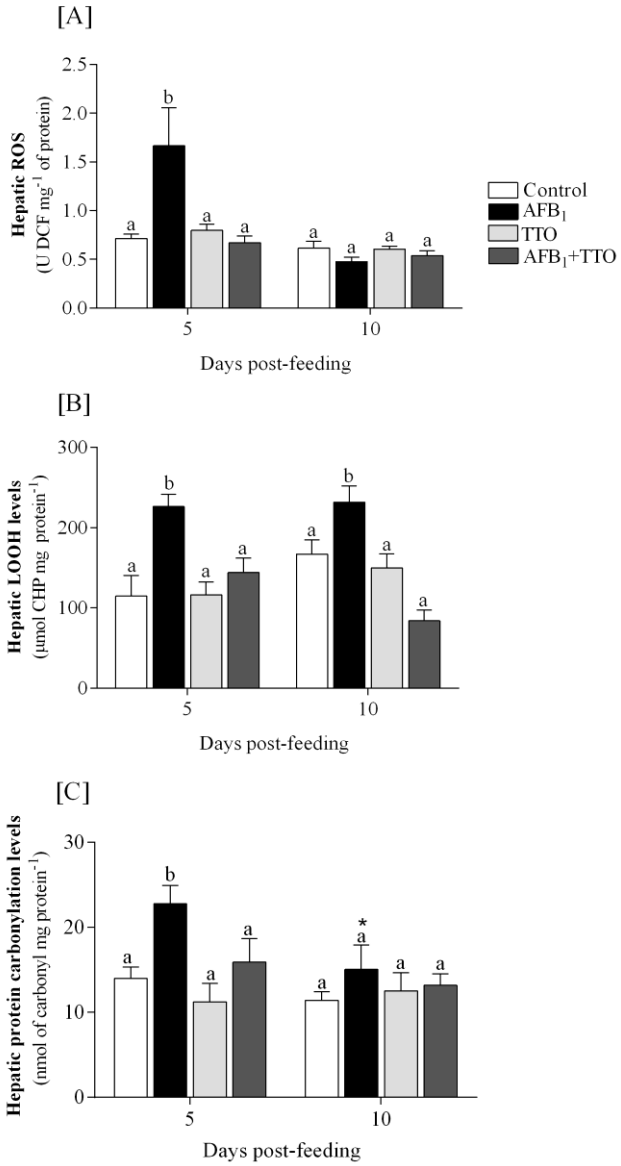
Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$; $n = 6$ per group).

Fig. 3 Effect of aflatoxins and/or tea tree oil (TTO) on plasmatic reactive oxygen species (ROS) [A], lipid peroxidation (LOOH) [B], and protein carbonylation levels [C] in silver catfish.



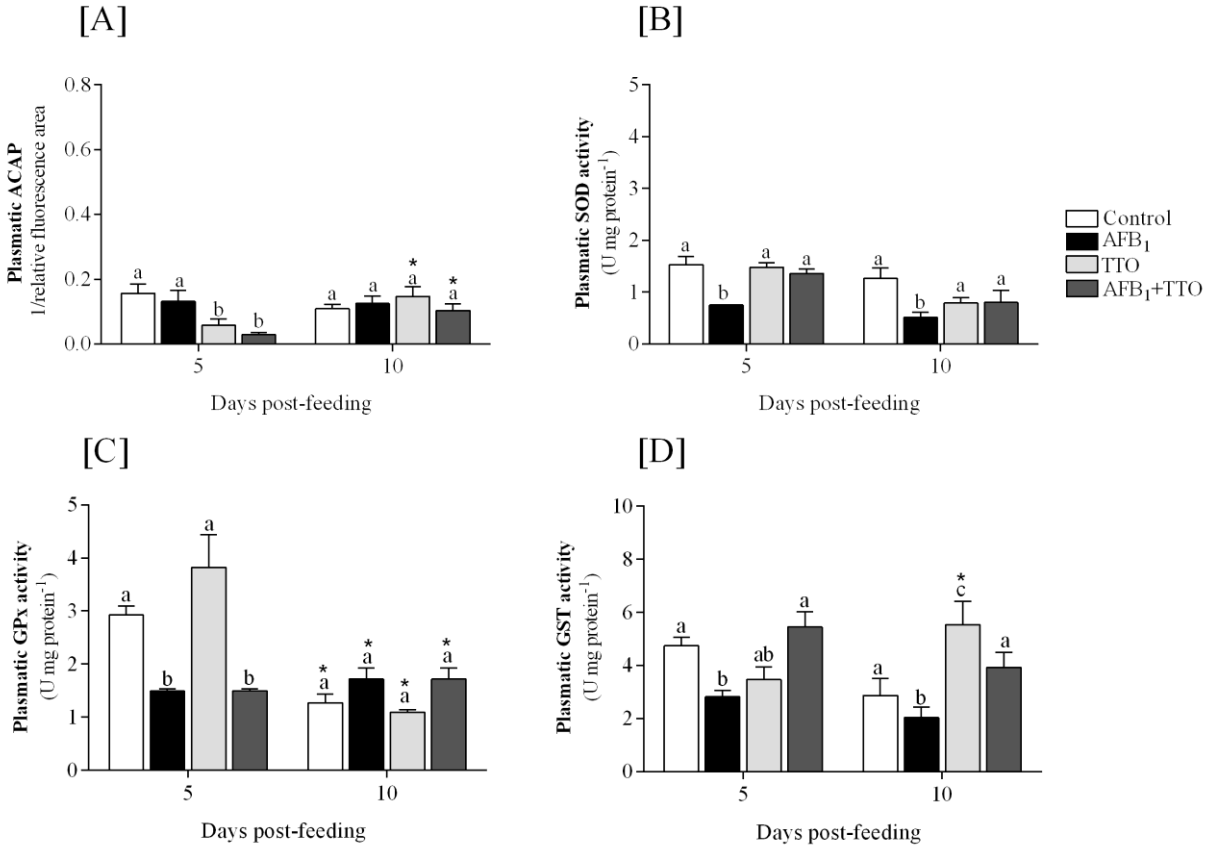
Groups within the same time period with no lowercase letters in common are significantly different. Asterisk indicates significant difference between times for the same treatment. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$; $n = 6$ per group).

Fig. 4 Effect of aflatoxins and/or tea tree oil (TTO) on hepatic reactive oxygen species (ROS) [A], lipid peroxidation (LOOH) [B], and protein carbonylation levels [C] in silver catfish.



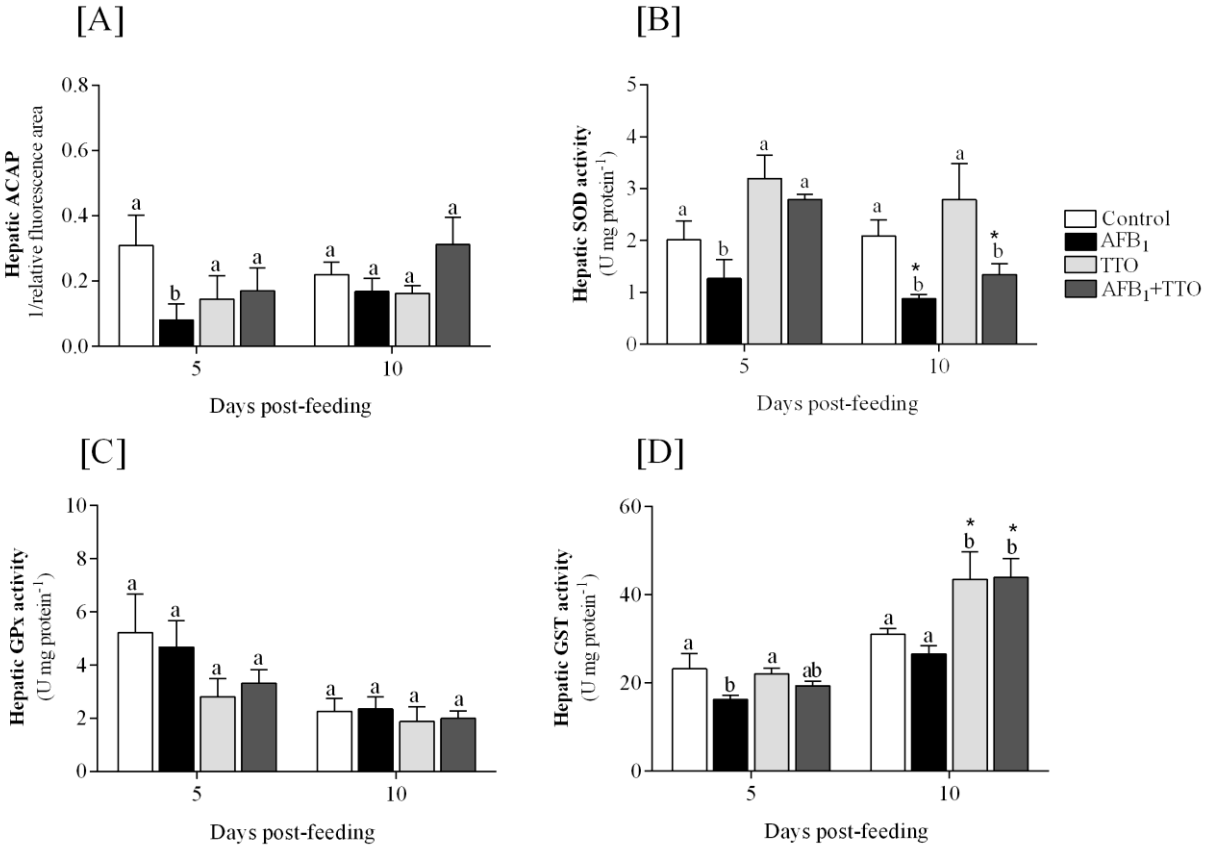
Groups within the same time period with no lowercase letters in common are significantly different. Asterisk indicates significant difference between times for the same treatment. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$; $n = 6$ per group).

Fig. 5 Effect of aflatoxins and/or tea tree oil (TTO) on plasmatic antioxidant capacity against peroxyl radical (ACAP) levels [A], superoxide dismutase (SOD) [B], glutathione peroxidase (GPx) [C], and glutathione-S-transferase (GST) [D] in silver catfish.



Groups within the same time period with no lowercase letters in common are significantly different. Asterisk indicates significant difference between times for the same treatment. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$; $n = 6$ per group).

Fig. 6 Effect of aflatoxins and/or tea tree oil (TTO) on hepatic antioxidant capacity against peroxyl radical (ACAP) levels [A], superoxide dismutase (SOD) [B], glutathione peroxidase (GPx) [C], and glutathione-S-transferase (GST) [D] in silver catfish.



Groups within the same time period with no lowercase letters in common are significantly different. Asterisk indicates significant difference between times for the same treatment. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$; $n = 6$ per group).

Fig. 7 Effect of aflatoxins (AFB) and/or tea tree oil (TTO) on hepatic histopathology of silver catfish at day 5 post-feeding. **A** – control, **B** – AFB, **C** – TTO and **D** – AFB+TTO diets. Fish contaminated with AFB diet showed anisocytosis of hepatocytes, multifocal degenerative changes with moderate fatty infiltration; apoptosis and/or multifocal necrosis of hepatocytes (yellow arrow –left-), moderate nuclear pyknosis in hepatocytes. Toxic hepatitis was also observed (orange arrow -right-). A lesser degree of injuries was observed in the TTO + AFB1 combination with some mild lesions and possible regeneration of hepatocytes by the observation of binucleate cells.

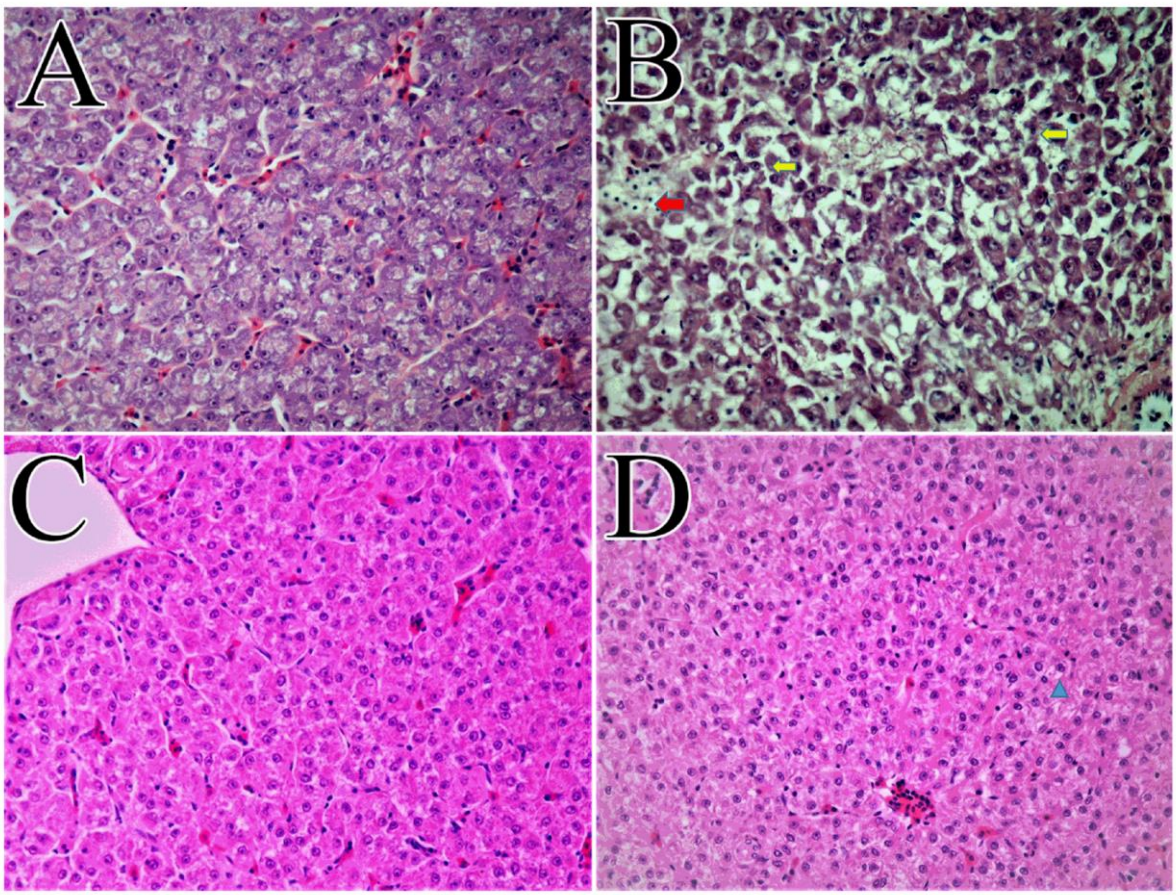
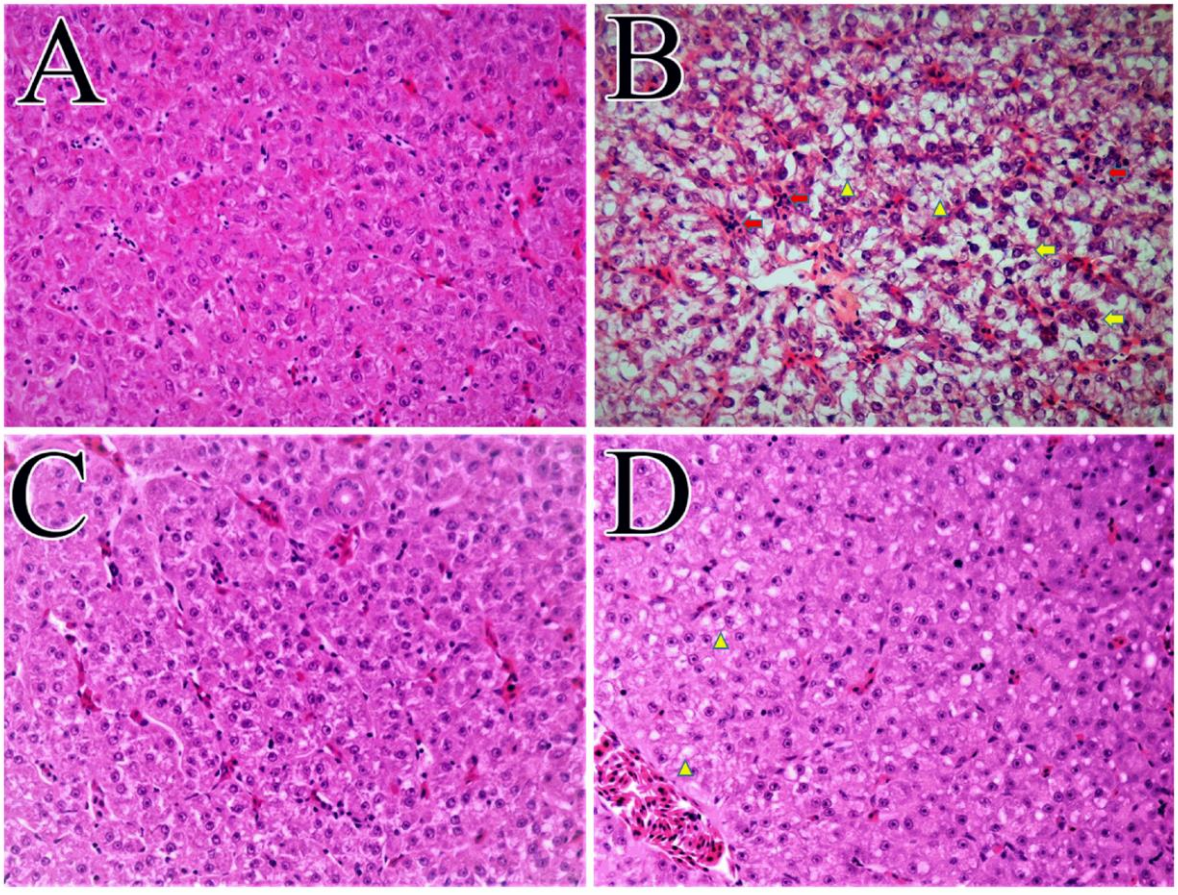


Fig. 8 Effect of aflatoxins (AFB) and / or tea tree oil (TTO) on hepatic histopathology of silver catfish at day 10 post-feeding. A - control, B - AFB, C - TTO and D - AFB + TTO diets. Fish contaminated with AFB diet showed hepatocytes with irregular size, degenerative changes due to fat infiltration, apoptosis and / or multifocal necrosis of hepatocytes (yellow triangles), moderate nuclear pynosis was frequent in hepatocytes, frequent mononuclear (orange arrow) reaction by Ito cells in some slightly thickened sinusoids, also with thickening of bile canaliculi. Hepatosis and toxic hepatitis was observed. A lesser degree of lesions was observed in the TTO + AFB combination with some mild lesions and possible regeneration of hepatocytes by the observation of binucleate cells (yellow arrows).



3.3 ARTIGO III

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Tea tree oil attenuates cerebral damage in silver catfish *(Rhamdia quelen)* fed with an aflatoxin-contaminated diet

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Tea tree oil attenuates cerebral damage in silver catfish *(Rhamdia quele*n) fed with an aflatoxin-contaminated diet

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Abstract

Aflatoxins are fungal metabolites that contaminate foods and feeds, causing adverse health effects in humans and animals. From an economic standpoint, aflatoxins represent one of the most severe problems for the livestock and feed industries. Currently, natural supplements are used to prevent various fungal infections in farmed fish. The aim of the present study was to determine whether dietary supplementation with tea tree oil (TTO: *Melaleuca alternifolia*) would prevent or minimize the effects of aflatoxin (AFB) on silver catfish (*Rhamdia quelen*). Four treatments were tested: control (without AFB); AFB (fish fed with a mycotoxin-contaminated diet – 1893 μg/kg of AFB_1 and 52.2 μg/ kg AFB_2); TTO (fish fed with a control diet + 1 mL/kg of TTO), and TTO + AFB (fish fed with a mycotoxin contaminated diet - 2324 μg/kg of AFB_1 and 43.5 μg/kg AFB_2 + 1 mL/kg of TTO). Treatments were tested in three replications and were analyzed at days 5 and 10 of dietary intake. Increased activities of nucleoside triphosphate diphosphohydrolase (NTPDase) using ADP as substrate and adenosine deaminase (ADA) were observed after 5 day-feeding with AFB_1 diet. Feed contaminated with AFB_1 caused an inhibition of acetylcholinesterase (AChE) activity. Aflatoxicosis caused severe gliosis and subarachnoid edema. The addition of TTO in AFB_1-contaminated feed prevented the inhibition of AChE activity and increased ADA activity. TTO supplementation increased *p2y11* receptor expression, most likely in an attempt to deal with inflammation caused by aflatoxicosis. TTO treatment ameliorated aflatoxin-associated brain tissue damage. These findings suggest that TTO supplementation reduces the effects of aflatoxicosis in silver catfish brain tissue because it prevents most changes caused by AFB_1-contaminated diet. Overall, the results obtained in the present study suggest that TTO could be used as an aflatoxin-detoxifying agent in fish.

Key words: tea tree oil; aflatoxin; purinergic system; acetylcholinesterase; adenosine deaminase

1. Introduction

Aquaculture is a vital part of the global food industry. Fish and fishery products are among the main sources of dietary protein in many parts of the world; they are also rich sources of fatty acids, amino acids, vitamins and minerals essentials for human health (FAO, 2018). Over the past few years, a new trend in aquaculture has been the replacement of expensive animal-derived proteins, including fish meal, with cheaper plant-based protein in commercial fish feed; however, this practice increased the risk of introducing mycotoxins into feed at the point of manufacture and storage, with subsequent transmission of the risk to humans and feedlot animals (Dirican et al., 2015; Osmond and Colombo, 2019).

Aflatoxins are secondary metabolites that are primarily generated by the fungi *Aspergillus flavus* and *A. parasiticus*. These are the most abundant mycotoxins present in fish feeds (Barbosa et al., 2013; Anater et al., 2016). Fish exposed to aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) exhibit weight loss, growth retardation, and lower survival rates (Magouz et al., 2018), as well as hematological and cutaneous alterations (Deng et al., 2010) and immunosuppression (Baldissera et al., 2018a). In the particular interest of study, AFB also affects the fish nervous system via disruption of the blood brain barrier, making the cerebral tissue an important target of its toxic effects (Baldissera et al., 2018b). This phenomenon manifests as abnormal behavior (Deng et al., 2010), loss of equilibrium (El-Sayed and Khalil, 2009), hyperlocomotion associated with impairment of brain neurotransmitters (Baldissera et al., 2018b), and impairment of energetic homeostasis (Baldissera et al., 2018a).

The effects of AFB-contaminated feed on fish brains and their effects on the immune system remain unknown, including the purinergic signaling pathway that serves an essential role in immune and inflammatory responses (OURY et al., 2015).

In recent years, there has been growing interest in the potential of purinergic signaling in mycotoxins contamination because the former plays a key role modulating the inflammatory and immune responses by extracellular nucleotides, principally adenosine triphosphate (ATP) and its derivative, nucleoside adenosine (Ado) (Jia et al., 2011; Baldissera et al., 2018a). Intracellular nucleotides, particularly ATP, function as universal energy sources. However, when released into the extracellular space, ATP acts as a danger-associated molecular pattern (DAMP) that is considered the most powerful and ubiquitous stress or damage signals, eliciting pro-inflammatory effects when present in higher levels in the extracellular milieu (Dou et al., 2018). On the other hand, the nucleoside Ado is thought to be an anti-inflammatory and

immunostimulant molecule that contributes to restriction of inflammatory damage (Di Virgilio and Vuerich, 2015). Levels of these signaling molecules are reduced by a cascade of surface-bound enzymes, including nucleoside triphosphate diphosphohydrolase (NTPDase), that catalyzes the hydrolysis of ATP and ADP into adenosine monophosphate (AMP), which is subsequently converted by 5′-nucleotidase into Ado. Finally, Ado is further cleaved into inosine by adenosine deaminase (ADA) (Bagatini et al., 2018). In the interest of the present study, Baldissera et al. (2018a) suggested that purinergic signaling is a pathway involved in the impairment of the immune and inflammatory responses in the secondary lymphatic organ of silver catfish *Rhamdia quelen* that had been fed with an AFB_1-contaminated diet, contributing to the immunotoxic effects of exposure to AFB_1. The cholinergic system also is notably involved in anti-inflammatory reactions and cholinesterases enzymes, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), serving important functions during immune and inflammatory responses (Baldissera et al., 2016). Acute AFB_1 treatment in experimental animals has been reported to cause a decrease in regional brain acetylcholinesterase enzymes that may affect the cognitive functions as well as memory and learning of the individual while chronic exposure increases adenohypophyseal AChE (Coulombe, 1994). Our hypothesis was that purinergic and cholinergic systems would be involved in the negative effects of AFB related to cerebral tissue.

Recently, there have been studies of dietary supplementation with essential oils to attenuate the toxic effects of AFB in fish (Abdel Rahman et al., 2017; Nazarizadeh et al., 2019), including the use of *Melaleuca alternifolia* essential oil (Souza et al., 2019). *M. alternifolia* essential oil, popularly known as tea tree oil (TTO), is derived from a plant native to Australia and has been used successfully in aquaculture to serve antibacterial (Souza et al., 2016), antiparasitic (Valladão et al., 2016), anti-inflammatory (Baldissera et al., 2017a) and hepatoprotective functions (Baldissera et al., 2017b). In particular interest of study, Souza et al. (2019) found that supplementation with 1.0 ml TTO/kg in feed ameliorated AFB-induced liver damage, concluding that this supplementation can be used to enhance fish antioxidant status and to reduce the negative impacts of AFB on hepatic tissue. Baldissera et al. (2017a) found that TTO used as a prophylactic therapeutic bath improved immune and inflammatory responses via protective effects on the spleen purinergic system of silver catfish experimentally infected with *Aeromonas hydrophila.* Therefore, our hypothesis was that TTO would minimize AFB-mediated neurotoxic effects via protection of cerebral purinergic signaling. The aim of

this study was to determine whether the purinergic signaling pathway and cholinergic system were associated with AFB-mediated neurotoxic effects, as well as to determine whether dietary supplementation with TTO would prevent or minimize these effects.

2. Materials and Methods

2.1. Aflatoxin production and analysis

Aflatoxin production method used in this study was previously detailed by Souza et al. (2019). Basically, aflatoxins were produced by fermentation in converted rice the *Aspergillus flavus* ATCC 13608 under constant stirring and controlled temperature (25 °C) and constant stirring for 21 days. After incubation, fermented material was dried in an oven at 50 °C and grounded, to be later added to the feed. Aflatoxin quantification contained in rice and feed samples were performed by the multi-mycotoxin method by Sulyok et al. (2007). This methodological information can be seen in greater detail in the study recently published by Souza et al., (2019).

2.2. Plant material and essential oil components

Tea tree oil (TTO) was purchased from Importadora Química Delaware Ltda® (Porto Alegre, Brazil). Oil composition and yield was analyzed using gas chromatography (GC) according methodology by Hammer et al., (2000) and Boligon et al., (2013).

2.3. Animals

Juveniles silver catfish (50.32 ± 7.54 g; 23 ± 3.6 cm) purchased from a local fish farm, were transported to the Fish Physiology Laboratory (Federal University of Santa Maria, Brazil), where they were maintained for 10 days in 250-L fiberglass tanks (semi-static renewal system) with temperature controlled for acclimation. Water parameters were controlled as following: dissolved oxygen (7.1 mg/L± 0.06) and temperature (22 ± 0.8 °C) were measured with a YSI oxygen meter (Model Y5512); pH (7.1 ± 1.1) was measured with a DMPH-2 pH meter. Total ammonia levels (0.85 ± 0.05 mg/L) were assessed by the salicylate method (Verdouw et al., 1978). Fish were fed once a day with commercial feed (28 % crude protein) and they were fasted for a 24 h period prior experiments.

2.4. Experimental design

The four diets represent the four treatments provided to the groups juveniles of silver catfish differing in contamination with aflatoxins and in the addition or not of TTO (1 mL/kg) during 10 days of experiment.

The animals were divided in four groups: control, fish fed with a control diet (without experimental contamination); TTO group, fish fed a control diet and 1 mL/ kg of TTO; AFB group, fish fed an experimental diet containing 1893 μg/kg of AFB_1 and 52.2 μg/kg AFB_2; and TTO and AFB group, fish fed an experimental diet containing 2324 μg/kg of AFB_1, 43.5 μg/kg AFB_2 and 1 mL/kg of TTO.

The basal diet was formulated based on the study of Zeppenfeld et al. (2016), and the addition of AFB followed the theoretical recommendation of Souza et al. (2019). Fish received the experimental diets until apparent satiation once a day (9 a.m.) for 10 days.

2.5. Sample collection

Twelve fish from each treatment were used for sample collection and analysis. The fish were euthanized at days 5 and 10 post-feeding by spinal cord sectioning according to Ethics Committee recommendations. The cerebral tissue was collected and six were homogenized (1:10 *w*/*v*) in glass Potter tubes with Tris-HCl buffer (10 mM, pH 7.4). The homogenates were centrifuged at 2000 *x* g for 10 min, and the supernatants were stored at –20 °C for purinergic system analysis. The other six brains were collected and stored in Eppendorf's containing TRIzol® reagent and maintained at –80 °C for gene expression analysis.

2.6. NTPDase and 5′-nucleotidase activities

Cerebral NTPDase and 5′-nucleotidase activities were evaluated using ATP, ADP, or AMP as the substrates according protocol described by Rosemberg et al. (2010) and published in detail by Baldissera et al. (2016). Enzymatic activity was expressed as nmol of inorganic phosphate (Pi) released/min/ mg of protein.

2.7. ADA activity

Cerebral ADA activity were evaluated based on the direct release of ammonia generated when the enzyme acts on Ado (Giusti 1974), as recently published in detail by Baldissera et al. (2016). Enzymatic activity was expressed as U/mg of protein.

2.8 AChE activity

Cerebral AChE enzymatic activity were determined according Ellman et al. (1961). The method is based on increased yellow color of the nitrobenzoate (TNB), ion produced from thiocholine after reaction with 5,5-dithio-bis-acid-nitrobenzoic (DTNB) ion, measured by absorbance at 412 nm and the enzymatic activity was expressed in mmol AcSCh/min mg of protein.

2.9. RNA extraction, cDNA synthesis, and real-time PCR

Total RNA from brain tissue was extracted using TRIzol® as indicated in manufacturer's instructions. Quantification of RNA was performed using a Nano-Drop spectrophotometer, and the RNA purity was measured as the 260/280 nm absorbance ratio (Thermo Scientific, Delaware, USA). RNA was treated with 0.1 U DNase Amplification Grade (Invitrogen) and the Double-stranded complementary DNA (cDNA) and Quantitative polymerase chain reactions (qPCR) protocols were recently published in detail by Souza et al. (2018).

The sequences used to design the primers were according to Baldisserotto et al. (2014) using the Primer Express software 3.3 (Applied Biosystems). Expression levels of mRNA of adenosine deaminase (*ada*), adenosine receptor A1 (a*dora1*) purinergic receptor P2X 3 (*p2rx3*), and purinergic receptor P2Y11 (*p2ry11*) were analyzed (Table 1). Primers were validated using standard curves. Reactions with a coefficient of determination (R^2) higher than 0.98 and efficiencies between 85% to 110% were considered optimized. Samples were run in duplicate and the results are expressed relative to *β-actin* (GenBank acc. No. KC195970) and *ef1a1* (MH107165) levels. Data were then normalized to a calibrator sample using ΔΔCq method as previously described (Pfaffl 2001).

2.10. Cerebral histopathological analysis

Cerebral tissue was fixed for histopathology, with 4% buffered formalin, inclusion of paraffin tissues, sections between 4 and 6 μm, stained with hematoxylin and eosin according to the standard method for histopathological evaluation (Luna, 1968). Methodology of histopathological evaluation was recently published in detail by Souza et al. (2019).

2.10. Statistical analysis

Normality and homoscedasticity were analyzed using Levene test. All treatment groups were compared using a factorial-ANOVA analysis followed by the Tukey's test, or when homogeneity of variances was not obtained, by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test and the Nemenyi test (StatSoft, Tulsa, OK, USA), and the minimum significance level was set at $p < .05$. Data were reported as mean ± standard error of the mean (SEM).

3. Results

3.1. Cerebral NTPDase and 5'-nucleotidase activities

No significant difference was observed between groups or over the time with respect to cerebral NTPDase activity using ATP as substrate (Figure 1A). Cerebral NTPDase activity using ADP as substrate was significantly higher in fish fed with AFB-contaminated feed compared to control group on day 5 post-feeding, while it was lower on day 10 post-feeding. Dietary supplementation with TTO did not significantly affect cerebral NTPDase activity for ADP compared to the control group. Cerebral NTPDase activity for ADP in TTO-supplemented fish was significantly lower than that of fish fed with AFB-contaminated feed, and was similar to that of the control group. Over time, NTPDase activity for ADP reduced from day 5 to 10 post-feed in fish fed with AFB-contaminated diet (Figure 1B).

Cerebral 5'-nucleotidase activity was significantly higher fish receiving AFB-contaminated feed than in the control group on day 5 post-feeding. Supplementation with TTO did not significantly affect cerebral 5'-nucleotidase activity compared to the control group on day 5 post-feeding. Dietary supplementation with TTO did not prevent the augmentation of cerebral 5'-nucleotidase activity compared to fish fed with AFB-contaminated feed on day 5 post-feeding. No significantly differences were observed between groups on day 10 post-feeding, as well as over time (Figure 1C).

3.2. Cerebral ADA activity

Cerebral ADA activity was significantly higher in fish fed with AFB-contaminated when compared to the others treatments on day 10 after feeding (Figure 2).

3.3 Cerebral AChE activity

Cerebral AChE activity was significantly lower in fish fed with AFB-contaminated than in the other treatments on days 5 and 10 after feeding (Figure 3).

3.4. Cerebral expression of purine receptors

No significant difference was observed between groups regarding expression of *p2rx3* purine receptor in both evaluated moments (Figure 4A).

Dietary supplementation with TTO or AFB did not affect the expression of the *p2ry11* purine receptor on days 5 and 10 post-feeding compared to the control group. However, expression of the *p2ry11* purine receptor was upregulated in fish fed with AFB and TTO compared to other groups on days 5 and 10 post-feeding. No significant difference was observed over the time (Figure 4B).

A significant difference was observed in the expression of the *adora1* purine receptor in brains of fish fed with AFB on day 10 after feeding when compared to the other groups (Figure 4C).

3.5 Cerebral histopathology

The animals in the control and TTO groups did not present histopathological changes in cerebral tissue at days 5 post-feeding (Fig. 5A–B). However, at 5 days post-feeding with AFB diet, it was observed as severe gliosis, as well as subarachnoid and peri-neuronal edema. Fish fed with AFT+TTO diet presented with neuropil spongiosis, peri-neuronal edema, and degenerative changes in neurons (Fig. 5C-D).

After day 10 post-feeding, the fish fed with control feed did not present any alterations in cerebral tissue; however, fish supplemented with TTO presented increases in peri-neuronal spaces (Fig. 6A-B). By contrast, we observed increased e severe gliosis, subarachnoid edema and peri-neuronal edema in fish fed with AFB (Fig. 6C). The addition of TTO in the diets contaminated with AFB caused neuropil spongiosis with regenerative changes and slight degenerative changes in neurons (Fig. 6D).

4. Discussion

The high lipid solubility of AFB and its existence in the postmortem brain tissue suggest its ability to cross the blood brain barrier (Oyelami et al., 1995; Qureshi et al., 2015). In the present study, was observed that the AFB-contaminated diet elicited severe gliosis, subarachnoid and peri-neuronal edema in brains of silver catfish on days 5 and 10 post-feeding.

We observed possible regeneration of hepatocytes in fish fed diets containing AFB and TTO (Souza et al., 2019). We observed that brain tissue changes were observed associated with edema (vascular and cerebrospinal fluid changes), as well as mild neuronal degenerative changes in both AFB and AFB + TTO treatment groups. We observed that treatment with TTO was effective in treatment of AFB-mediated neural damage; nevertheless, it may be more difficult to repair neural damage than liver damage.

Several studies have reported that both purinergic (Schetinger et al., 2007; Thomé et al., 2012) and cholinergic systems (Baldissera et al., 2015; 2016) are important in the modulation of the inflammatory and immune responses. The current study demonstrated that fish fed AFB presented decreased cerebral AChE activity, corroborating the findings of Cometa et al. (2005) suggesting that AFBs significantly inhibits AChE activity in the brain and spinal cord, resulting in nerve cell degeneration and neurological transmission, damage to synapse formation and altered neural plasticity (Quinn, 1987; Chen et al., 2014). Our findings also suggest that the effect of AFB_1 on a peripheral AChE binding sites may be an additional mechanism of action responsible for the carcinogenicity of AFB_1, distinct from the known mechanism involving covalent binding of AFB_1 metabolite (derived from 8; 9-epoxide) that forms stable adducts with endogenous proteins and nucleic acids (Hussein and Brasel, 2001).

The addition of TTO in AFB-contaminated feed prevented inhibition of cerebral AChE activity. Pohanka, 2014 found that inhibition of AChE activity displayed an anti-inflammatory profile as an attempt to enhance the immune response and reduce the inflammatory damage through augmentation of levels of ACh, a molecule with anti-inflammatory and immunomodulatory properties (Pohanka, 2014). Treatment with TTO prevents inflammatory processes and this natural product is an excellent immunoprotective agent (Caldefie-Chézet et al., 2006; Shi et al., 2006; Baldissera et al., 2017b). This finding was corroborated by Nogueira et al. (2014), who found that TTO, and especially its major compound terpinen-4-ol, were potent modulators of innate immune responses mediated by the inhibition of excessive production of pro-inflammatory molecules. Caldefie-Chézet et al. found that the anti-inflammatory mechanism of TTO occurred via interference with cytokine secretion by lymphocytes, monocytes and macrophages, decreasing the production of IL-2 and increasing IL-4 and IL-10. We believe that TTO may exert beneficial effects on the immune system, consequently preventing inflammatory processes in fish exposed to aflatoxins.

We also observed that ADA modulates the response to aflatoxin in the brains of animals by increasing its activity, as well as by gene transcription of its *adora1* receptor. This increase in ADA activity in brain tissue and receptor up-regulation observed in fish fed AFB-contaminated feed on day 10 may be considered a pro-inflammatory profile because of the rapid deamination of Ado to inosine. This is because Ado has anti-inflammatory and immunosuppressive properties (Shin et al., 2018). The activation of A1 adenosine receptors promotes inhibitory effects on neurotransmission (Freissmuth et al., 1991).

Because mycotoxins are toxic and harm the health of humans and livestock, research has been focused on the control or elimination of these toxic metabolites in food grains and livestock feeds (Zhou et al., 2017). The therapeutic potential of various plant extracts has already been reported to combat AFB_1-induced toxicity (Abdulmajeed, 2011). Regarding feed containing TTO, our results demonstrated that there were no major changes in brain tissue, and only a significant increase in gene transcription of purinoreceptors p2ry11 was observed at 5 and 10 days of feeding with diets containing AFB_1 + TTO. However, histopathological results revealed that AFB_1 intoxication caused severe gliosis and subarachnoid edema. This was not observed in the brains of fish that consumed diets containing AFB_1 + TTO, demonstrating that TTO has possible anti-inflammatory effects. The anti-inflammatory effect of topical TTO was reported by Koh et al. (2002). Hart et al. (2000) suggested that terpinen-4-ol (42%), alpha-terpineol (3%) and 1,8-cineole (2%) were responsible for this proinflammatory suppression effect, attributed to tea tree oil.

Upregulation of NTPDase activity (ADP as substrate) in animals fed with the AFB_1-only diet may be associated with the organism's attempt to mount an anti-inflammatory response via ADP hydrolysis, possibly causing reductions in ADP content in the extracellular medium. However, suppression of this activity was observed after 10 days of AFB feeding, suggesting a proinflammatory response, showing that brain tissue failed to mount an effective response against aflatoxicosis. According to Cunha and Ribeiro (2000), the decrease in NTPDase activity leads to a reduction in ATP and ADP hydrolysis and an increase in their concentrations. ATP has several functions in the central nervous system as presynaptic neuromodulator and as rapid excitatory neurotransmitter (Cunha and Ribeiro, 2000). ADP also plays an important role in neuronal development and plasticity (Burnstock, 2006).

Recent studies have shown that the p2y11 receptor plays an important role in cellular responses. Tsukimoto et al. (2010) found that extracellular ATP induced IL-6 production

through P2Y receptors, demonstrating an important role in immune activation. (Dreisig and Kornum 2016). In some ways, the addition of TTO to the diet may have increased p2y11 receptor expression as an attempt to induce interleukin production to counteract tissue inflammation caused by aflatoxicosis. In support of this notion, many studies have demonstrated the therapeutic effects of TTO in the reduction or prevention of the inflammatory process during other infectious diseases, included those caused by fungus (Ninomiya et al., 2013).

5. Conclusion

Based on these lines of evidence, we conclude that the purinergic system was altered by aflatoxicosis, an immunomodulatory effect that attempts to counteract brain inflammation by increasing ADP hydrolysis, as evidenced by the histopathological analyses. Inhibition of AChE activity contributes to AFB_1-induced brain damage, acting on synapse formation and plasticity. Addition of TTO prevented the inhibition of AChE and increases of ADA activity, thereby alleviating brain damage. These data suggest that TTO can be used as a food additive in fish diets to mitigate the physiological and morphological injuries caused by aflatoxicosis.

Conflict of interest

The authors declare no conflicts of interest.

Ethical committees

The methodologies used in the experiments were approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (UFSM under protocol number 8700140518/2018.

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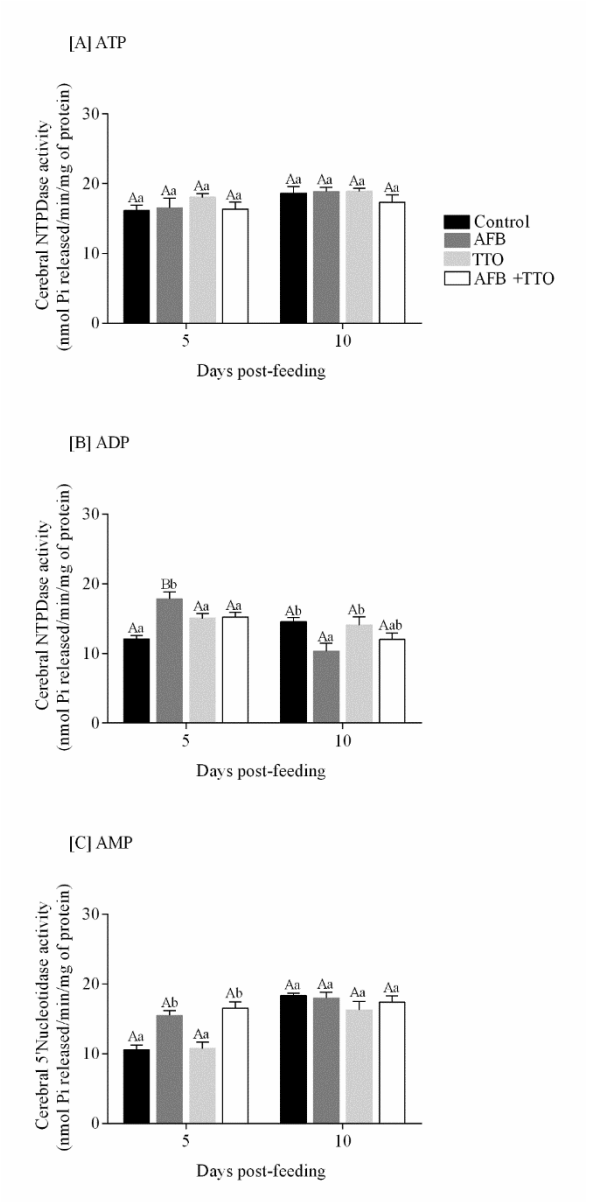
Table 1 - Oligonucleotides used for qPCR quantifications, their position in the sequence, final reaction concentration, amplification efficiency and calibration curve R^2.

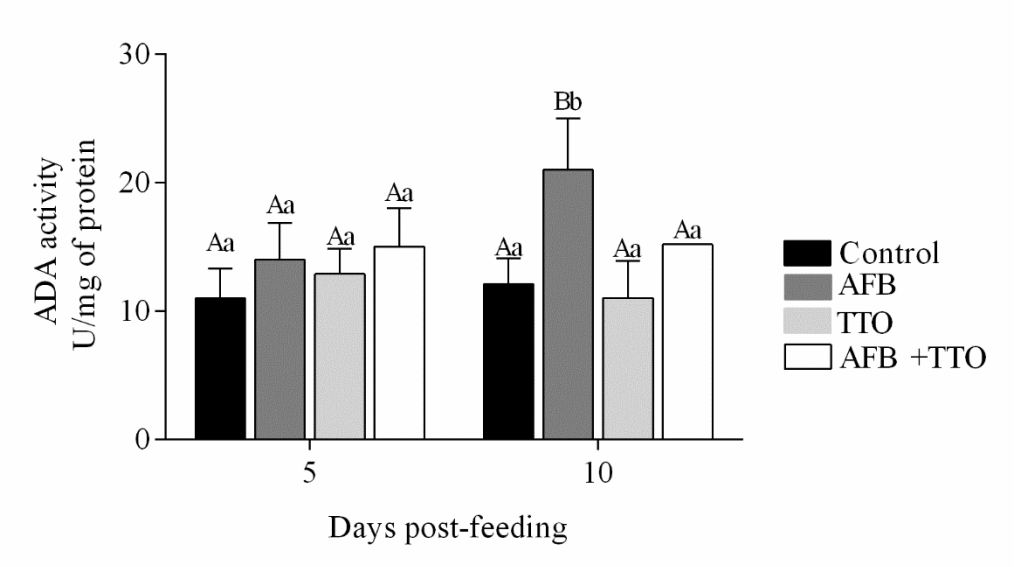
Primer	Direction	Sequence (5′→3′)	Position	Amplicon size (bp)	Primer concentration (nM)	Efficiency	R^2
Q-ADORA	Forward	CCAAGACGAGTGGGATTAGC	1275	114	100	96.90%	0.99
	Reverse	GGAGCCATTTTGTTGCTGAC	1388		100		
Q-P2Y11	Forward	CTGGAACATGGGAGTGGTCT	356	120	100	91.70%	0.992
	Reverse	GCATCACCAAATTCCCACTT	475		100		
Q-P2X3	Forward	GCTTTCCACGCTTCAACTTC	776	205	100	92.1%	0.991
	Reverse	TCACACACCCAGGCTATCTTT	980		100		
Q-ACTB	Forward	GCAATGCCAGGGTACATGGT	847	134	100	100.30%	0.999
	Reverse	CCACCTTCAACTCCATCATGAA	980		100		
Q-EF1A1	Forward	GCTTCCTTGCTCAGGTCATC	1075	127	100	100.10%	0.998
	Reverse	CGGTCGATCTTCTCCTTGAG	1201		100		

Fig. 1 Hydrolysis of ATP (a), ADP (b) and AMP (c) in the brains of fish consuming aflatoxin-contaminated diets and those supplemented with TTO.



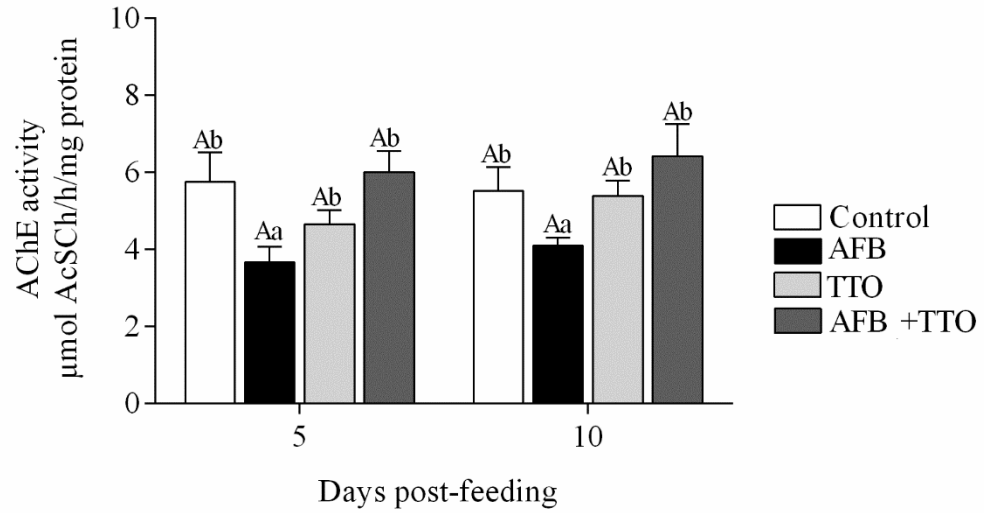
Capital letters indicate significant differences between time points within the same treatment. Lowercase letters indicate significant differences between treatments at the same time point.

Fig. 2 Adenosine deamination by adenosine deaminase in the brains of fish that consumed aflatoxin-contaminated diet and those supplemented with TTO.



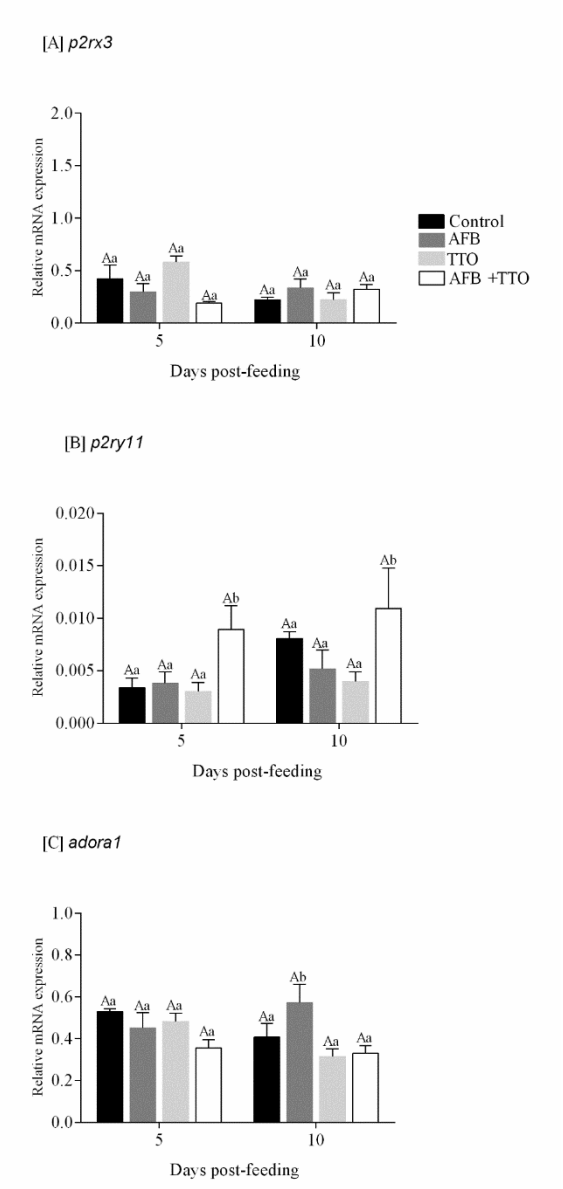
Capital letters indicate significant differences between time points within the same treatment. Lowercase letters indicate significant differences between treatments at the same time point.

Fig. 3 Acetylcholinesterase activity in the brains of fish that consumed aflatoxin-contaminated diets and those supplemented with TTO.



Capital letters indicate significant differences between time points within the same treatment. Lowercase letters indicate significant differences between treatments at the same time point.

Fig. 4. Expression of purinergic system receptors in the brain of fish that consumed aflatoxin-contaminated diet and those supplemented with TTO.



Capital letters indicate significant differences between time points within the same treatment. Lowercase letters indicate significant differences between treatments at the same time point.

Fig. 5 Brain histopathology of fish that consumed aflatoxin-contaminated diet and those supplemented with TTO at day 5 post-diet. (A) fish fed a control diet showed a normal telencephalon, blood vessel (arrow), neurons (N). (B) fish fed a TTO diet exhibited a normal telencephalon, neurons (N). (C) fish fed a AFB diet presented a severe gliosis (Arrow), subarachnoid edema (E), peri-neuronal edema (*). (D) fish fed with AFB+TTO diet showed a neuropil spongiosis (arrow) peri-neuronal edema and degenerative changes in neurons (*)

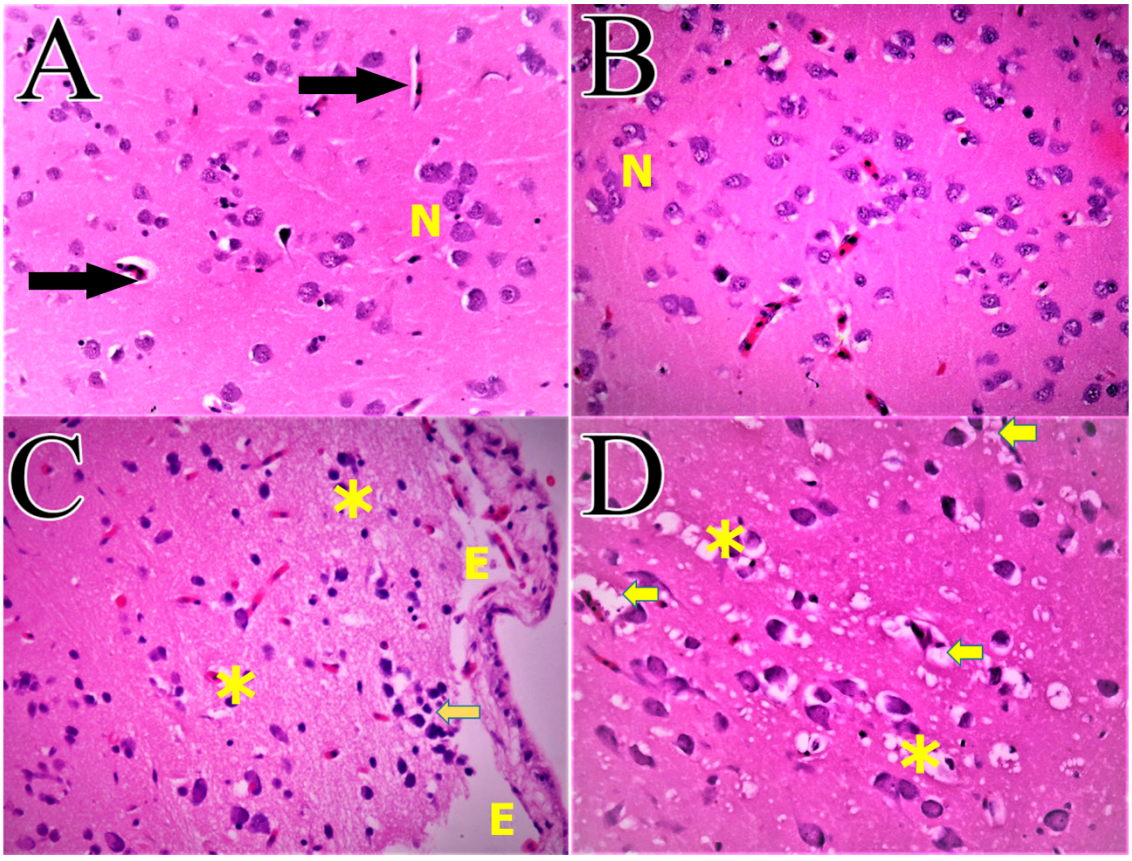
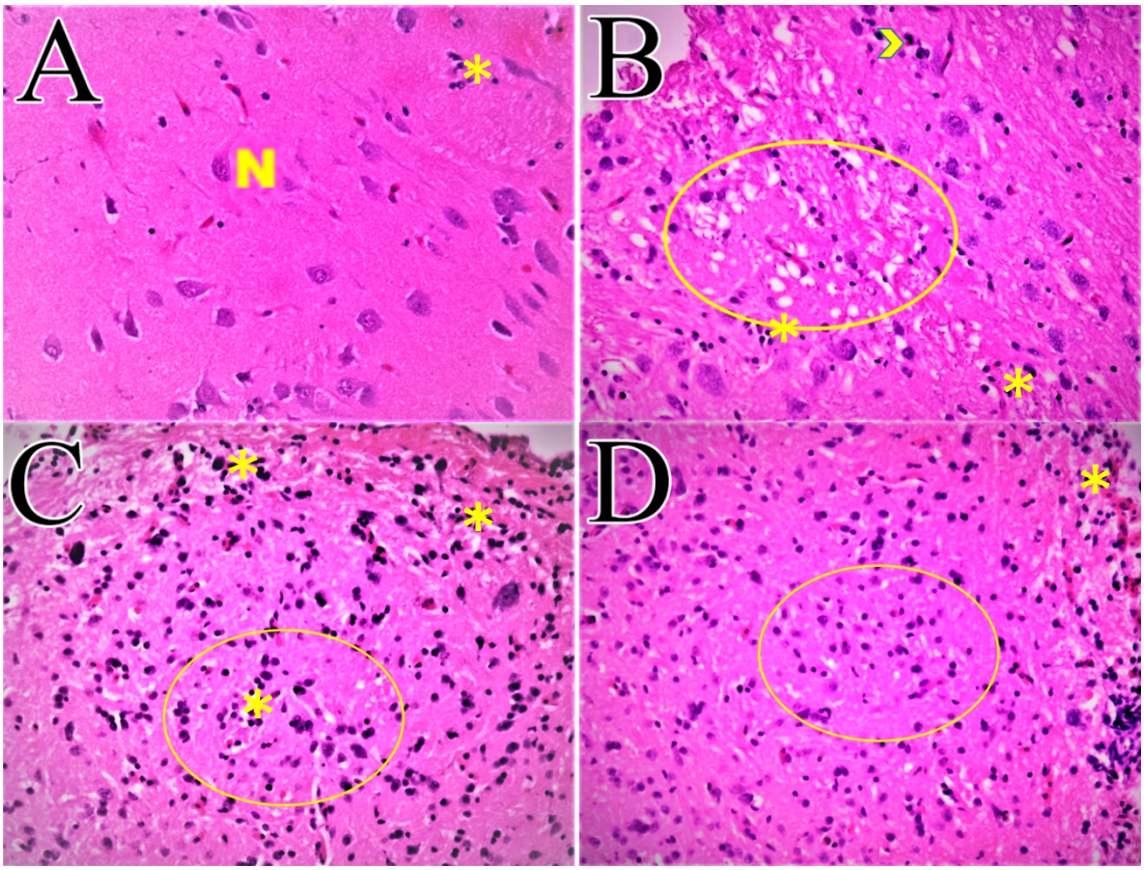


Fig. 6 Brain histopathology of fish that consumed aflatoxin-contaminated diet and those supplemented with TTO at day 10 post-diet (A) fish fed a control diet showed a normal telencephalon, neurons (N), glial cells (*). (B) fish fed a TTO diet exhibited moderate spongiosis: increase of peri-neuronal spaces (oval), mild neuronal degenerative changes (Arrow). (C) fish fed a AFB diet presented a severe gliosis and pyknosis of glial cells and neurons (oval), peri-neuronal edema (*). (D) fish fed with AFB+TTO diet showed a neuropil spongiosis with regenerative changes (oval) and slight degenerative changes in neurons (*)



4 DISCUSSÃO

Micotoxinas são metabólitos secundários, produzidas por algumas espécies de fungos toxigênicos, passíveis de causarem efeitos tóxicos em humanos e animais. A contaminação de alimentos por micotoxinas tem sido relatada no mundo todo, principalmente em alimentos susceptíveis ao crescimento fúngico, como os grãos e cereais (PATRIARCA e PINTO, 2017). A aflatoxina B1 (AFB_1) é a micotoxina tóxica, devido as suas propriedades hepatotóxicas, mutagênicas, carcinogênicas e teratogênicas em muitas espécies de animais, incluindo seres humanos; pois também foi classificada como cancerígena do Grupo I (IARC, 2012; SUN et al., 2014; ZHANG et al., 2016). Na aquicultura, peixes são frequentemente alimentados com uma dieta comercial contendo diferentes ingredientes obtidos de plantas, como farelo de soja e vários grãos de cereais, os quais podem representar uma fonte de micotoxinas (YIANNIKOURIS e JOUANY, 2002). Em peixes, as aflatoxinas podem causar vários efeitos adversos, como crescimento reduzido, perda de apetite (ROYES e YANONG, 2002) e anormalidade tecidual, lesões ou tumores no fígado (HUSSAIN et al., 2017; WU et al., 2009) e ainda induzir um excesso de produção de espécies reativas de oxigênio (ERO) o que pode causar sérios danos às células e tecidos (SOUTO et al., 2018). Por fim, de acordo com estudo conduzido por Tuan et al. (2001), as aflatoxinas são responsáveis por cerca de 60% da mortalidade total de peixes em qualquer sistema de produção. Sendo assim, este estudo buscou investigar os efeitos da adição de aflatoxina na dieta de jundiás (*Rhamdia quelen*) e averiguar o potencial do óleo essencial de *Melaleuca alternifolia* em minimizar os efeitos tóxicos causados pelas aflatoxinas.

No **artigo I** podemos observar que a adição de **AFB (Sigma-Aldrich)** na concentração de 1177 $\mu g\ kg^{-1}$de dieta, provocou estresse oxidativo tanto no soro como no tecido hepático, nos dias 14 e 21 pós-alimentação. Nossos resultados são importantes, uma vez que nossas descobertas demonstraram claramente danos a importantes componentes de membranas celulares, como lipídios e proteínas, bem como uma inibição dos sistemas de defesa antioxidante primários e secundários, incapazes de evitar ou reduzir os efeitos oxidativos das espécies reativas. Além do mais, este é o primeiro estudo que observou um aumento significativo nos níveis séricos e hepáticos de ERO e NOx em peixes alimentados com AFB_1, o que de fato pode explicar o aumento aos danos lipídicos e proteicos no soro e tecido. Abdel-Rahman et al. (2017) observaram a existência de peroxidação lipídica nos níveis séricos de tilápia do Nilo alimentadas com uma dieta contendo 200 ppm de AFB_1 por

kg de ração, da mesma forma que este estudo atual. Para evitar ou reduzir os danos de lipídios e proteínas provocados pela produção excessiva de ERO e NOx, o organismo pode ativar enzimas do sistema de defesa antioxidante, como CAT, SOD e GPx, que pode reduzir os níveis de hidroperóxido lipídico e peróxido de hidrogênio (H_2O_2) (NIMSE E PAL, 2015), desempenhando um papel importante na prevenção de danos oxidativos. No entanto, observamos uma redução significativa de um indicador da capacidade antioxidante (níveis de ACAP) no soro e no fígado de bagres de prata alimentado com AFB_1, o que indica um comprometimento do sistema antioxidante como uma tentativa de evitar ou minimizar os efeitos deletérios da ERO. Além disso, observamos atividades significativamente menores de enzimas específicas da defesa antioxidante primária e secundária como CAT e GPx, no soro e no fígado de jundiás expostos a dieta contendo AFB_1. Neste sentindo, os sistemas de defesa antioxidante foram incapazes de evitar ou minimizar a formação excessiva de EROs e, consequentemente, a oxidação sérica e hepática dos animais alimentados com a dieta contaminada com AFB_1.

Neste sentido, com o intuito de minimizar os efeitos tóxicos causados pela aflatoxicose, foi realizado um segundo experimento (**artigos II e III**), onde uma dieta contendo óleo essencial de *Melaleuca alternifólia* – TTO - (1 mL kg^{-1} de dieta) foi formulada e oferecida para os peixes num período de 10 dias. TTO foi escolhido como composto testado no experimento porque é considerado um produto natural de grande importância médica, veterinária e agronômica (RICCIONI e ORZALI, 2011; YADAV et al., 2017) possuindo atividades antifúngica e antioxidante já comprovadas anteriormente (CARSON et al., 2006; de ANDRADE SANTIAGO et al., 2017). Além disso, é importante ressaltar que neste segundo experimento utilizamos aflatoxina obtida de maneira natural, através da produção da micotoxina pelo fungo *Aspergillus flavus* em cultivo de arroz.

No **artigo II**, a análise da concentração de AFB_1 nas dietas formuladas apresentaram concentrações superiores a concentração teórica desejada (1117 µg $kg^{-}1$). Além disso, o AFB_2 também foi detectado, mas em baixas concentrações quando comparado ao AFB_1, o que também foi observado em estudos anteriores (MURPHY et al., 2006; ADEYEMO et al., 2018). Mesmo com esta pequena diferença na quantificação da aflatoxina do experimento II, observou-se que a dieta contaminada com AFB provocou uma perturbação do estado antioxidante/oxidante no plasma e fígado de jundiás, porém agora já nos dias 5 e 10 após a alimentação, corroborando os achados do **artigo I**.

No **artigo II**, a adição de AFB também causou hepatite e hepatose tóxicas. No geral, o TTO adicionado à dieta não foi tóxico para os peixes. Curiosamente, nossas descobertas demonstraram claramente que a adição de TTO minimiza os efeitos histopatológicos tóxicos da contaminação por aflatoxina, evitando ou reduzindo os efeitos oxidativos das espécies reativas radicais livres. A suplementação de TTO nas dietas contaminadas com AFB resultou em um efeito protetor para *R. quelen*, evidenciado pela melhoria significativa em muitas das variáveis testadas. Além disso, o TTO apresentou um efeito similar a substâncias utilizadas como adsorvente, que agem fazendo ligação a AFB e impedindo sua absorção no trato alimentar ou aumento das enzimas antioxidantes (SELIM et al., 2014). Resultados semelhantes foram encontrados adicionando o óleo essencial de erva-doce em dieta contaminada com AFB_1 para tilápias do Nilo (ABDEL RAHMAN et al., 2017). A suplementação dietética com OE de erva-doce para tilápia do Nilo promove uma rápida ativação do sistema complemento em peixes alimentados por 60 dias, bem como aumento do sistema de defesa antioxidante, revelando que este OE tem um grande potencial para ser usado como um imunoestimulante e antioxidante para peixes.

A contaminação da dieta com aflatoxinas também causou alteração no sistema purinergico e colinérgico no cérebro de jundiás, onde ocorreu um aumento na atividade da nucleosídeo trifosfato difosfo-hidrolase (NTPDase) usando ADP como substrato e adenosina-desaminase (ADA) após 5 dias de alimentação com dieta AFB (**artigo III**). Além disso também foi observado que a contaminação com aflatoxina também provocou a inibição da atividade da acetilcolinesterase (AChE) cerebral. Na literatura essas vias ainda não haviam sido exploradas frente a aflatoxicose. Assim nossos achados representam uma grande importância para o conhecimento dos mecanismos de toxicidade da aflatoxicose, uma vez que estas vias em questão desempenham um papel essencial nas respostas imunes e inflamatórias (OURY et al., 2015). Além disso, foi possível observar no **artigo III**, que a dieta contaminada com AFB provocou gliose grave, edema subaracnóideo e peri-neuronal no cérebro dos peixes nos dias 5 e 10 após a alimentação.

A adição de TTO na dieta contendo AFB, impediu a inibição da atividade cerebral de AChE. De acordo com estudo conduzido por Pohanka (2014) a inibição da atividade da AChE exibe um perfil anti-inflamatório como uma tentativa de melhorar a resposta imune e reduzir o dano inflamatório através do aumento dos níveis de ACh, uma molécula com propriedades anti-inflamatórias e imunomoduladoras. Desta forma, acredita-se que o

tratamento com TTO evita processos inflamatórios e este produto natural é um excelente agente imunoprotetor (CALDEFIE-CHÉZET et al., 2006; BALDISSERA et al., 2017). Esse achado foi corroborado por Nogueira et al. (2014), que descobriram que o TTO, e especialmente seu principal composto terpinen-4-ol, eram moduladores potentes de respostas imunes inatas mediados pela inibição da produção excessiva de moléculas pró-inflamatórias.

No **artigo III**, também observamos que a enzima ADA modula a resposta à aflatoxina no cérebro de animais, aumentando sua atividade, bem como pela transcrição gênica de seu receptor *adora1*. Este aumento na atividade da ADA no tecido cerebral e na regulação positiva de receptores observada em peixes alimentados com alimentos contaminados com AFB no dia 10 pode ser considerado um perfil pró-inflamatório devido à rápida desaminação de Ado em inosina. Isso ocorre porque o Ado possui propriedades anti-inflamatórias e imunossupressoras (SHIN et al., 2018). A ativação dos receptores A1 da adenosina promove efeitos inibitórios na neurotransmissão (FREISSMUTH et al., 1991).

A adição de TTO na dieta contaminada com AFB, causou um aumento significativo na transcrição gênica dos purinorreceptores *p2ry11* nos dias 5 e 10 dias pós dieta. Além disso, os resultados histopatológicos revelaram que a adição de TTO minimiza os danos no tecido cerebral, o que novamente nos leva a crer no efeito de supressão pró-inflamatória deste composto natural (KOH et al. 2002; HART et al. (2000). Além do mais, a adição de TTO na dieta contaminada com AFB, causou uma dimimuição da atividade da NTPDase cerebral. Segundo Cunha e Ribeiro (2000), a diminuição da atividade da NTPDase (ADP como substrato) leva a uma redução na hidrólise de ADP e a um aumento de sua concentração. De acordo com estudo conduzido por Burnstock (2006), o ADP desempenha um papel importante no desenvolvimento neuronal e na plasticidade, corroborando então, uma possível via de mecanismo de supressão pró-inflamatória causadas pela adição de TTO na dieta contendo AFB.

Dentro deste contexto, um estudo recente conduzido por Dreising e Kornum (2016) demostrou que o receptor p2y11 desempenha um papel importante nas respostas celulares. De acordo com Tsukimoto et al. (2010) o ATP extracelular induz a produção de IL-6 por meio de receptores P2Y, demonstrando um papel importante na ativação imune (DREISIG e KORNUM 2016). De certa forma, a adição de TTO à dieta pode ter aumentado a

expressão do receptor p2y11 como uma tentativa de induzir a produção de interleucina para combater a inflamação do tecido causada pela aflatoxicose. Neste sentido, de acordo com estudo conduzido por Ninomiya et al. (2013) o aumento da expressão do receptor p2y11 poderia estar relacionado com redução ou prevenção do processo inflamatório durante a aflatoxicose, o que nos permite reconhecer os efeitos terapêuticos do TTO.

5 CONCLUSÕES

A partir dos resultados deste estudo, podemos concluir que:

- Dieta contaminada com AFB_1 (1177 μg kg^{-1}) causa distúrbios no sistema antioxidante/oxidante no soro e no tecido hepático de jundiás, uma vez que o sistema de defesa antioxidante não é capaz de evitar ou minimizar a formação de espécies reativas ao oxigênio e ao nitrogênio (ERO e ERN) durante a aflatoxicose. Desta maneira, o estresse oxidativo contribui diretamente para a fisiopatologia e mortalidade de peixes alimentados com dietas contaminadas com AFB_1. Além disso, a aflatoxicose causa hepatite tóxica e hepatose no fígado dos peixes, demonstrando que uma dieta contaminada com aflatoxinas é altamente hepatotóxica para os peixes.

- A contaminação com AFBs na dieta de jundiás, altera os sistemas purinérgico e colinérgico, através da inibição da atividade das enzimas AChE e ADA, contribuindo para o dano cerebral.

- A adição de TTO (1 mL kg^{-1}) na dieta dos peixes, mostrou efeito protetor e melhorou esses efeitos tóxicos da aflatoxicose, minimizando os danos ao fígado, além de melhorar o status antioxidante dos peixes. Além disso o uso de TTO adicionado a dieta, apresenta um possível efeito imunomodulador na tentativa de combater a inflamação cerebral, seja aumentando a hidrólise do ADP ou impedindo a inibição da AChE e o aumento da atividade da ADA, aliviando assim os danos cerebrais como evidenciado pelas análises histopatológicas.

Por fim, esses achados sugerem que o TTO pode ser usado como aditivo alimentar em dietas de peixes para minimizar os efeitos tóxicos causados pela aflatoxicose.

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ANEXO A - CARTA DE APROVAÇÃO DA COMISSÃO DE ÉTICA

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Adição do óleo de Melaleuca alternifolia (livre e nanoencapsulado) na redução de efeitos tóxicos causados por dietas contaminada por micotoxinas", protocolada sob o CEUA nº 8700140518, sob a responsabilidade de **Aleksandro Schafer da Silva** *e equipe; Carine de Freitas Souza; Bernardo Baldisserotto; Matheus Dellámea Baldissera* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 21/06/2018.

We certify that the proposal "Addition of essential oil Melaleuca alternifolia (free and nanoencapsulated) in the reduction of toxic effects caused by diets contaminated with mycotoxins", utilizing 324 Fishes (males and females), protocol number CEUA 8700140518, under the responsibility of **Aleksandro Schafer da Silva** *and team; Carine de Freitas Souza; Bernardo Baldisserotto; Matheus Dellámea Baldissera* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 06/21/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 06/2018 a 12/2021 Área: Bioquimica Toxicológica

Origem:	Não aplicável biotério						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	3 a 10 meses	N:	324
Linhagem:	Rhamdia quelen, convencional			Peso:	20 a 50 g		

Resumo: 31/07/2019 Resumo Micotoxinas, são uma preocupação de saúde pública mundial, afetando desde animais de produção até humanos. Estas toxinas são metabolitos secundários tóxicos produzidos por fungos e são capazes de afetar uma largamente cultivo de grãos como trigo, milho e soja. No cultivo de peixes, estes animais são frequentemente alimentados com dieta a base de diferentes grãos como farelo de soja, milho e cereais, que frequentemente, pode apresentar contaminação por diversos tipos de micotoxinas. Além do seu impacto na saúde dos animais, algumas micotoxinas também podem contaminar o tecido dos peixes, representando um risco para a segurança alimentar. Além disso de ser prejudicial para a saúde do organismo, a contaminação por micotoxinas representa sérios desafios para o desenvolvimento da aquicultura. Desta forma, o presente projeto propõe a investigar o uso do óleo essencial de Melaleuca alternifolia na forma livre e nanoencapsulada na dieta de peixes co-contaminados pelas micotoxinas: aflatoxina B1, deoxinivalenol e ocratoxina na tentativa de prevenir e/ou reduzir os seus efeitos tóxicos, utilizando o peixe jundiá (Rhamdia quelen) como modelo experimental.

Local do experimento: Laboratório de Fisiologia de Peixes da Universidade Federal de Santa Maria (Prédio 21, sala 5104)

Santa Maria, 21 de junho de 2018

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria