Original Article

The Effect of *Echinacea Purpurea* L. (Eastern Purple Coneflower) Essential Oil on Hematological Parameters and Gut Microbial Population of Zebrafish (*Danio Rerio*) With Aflatoxicosis

Tina Hasankhani[1, 2], Donya Nikaein[1, 2*], Alireza Khosravi[1, 2], Hooman Rahmati-holasoo[3], Mona Hasankhany[4]

1. Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
2. Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
3. Department of Aquatic Animals Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
4. Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

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ABSTRACT

Background: Aflatoxin is one of the most important fungal toxins with documented hepatotoxic, teratogenic, and immunosuppressive properties. This mycotoxin is mainly produced by species of the genus *Aspergillus* in feed. Therefore, the application of compounds, which prevent complications of aflatoxins without losing feed quality, is highly beneficial.

Objectives: This study aimed to determine the effect of the *Echinacea purpurea* L. essential oils on the regulation of the microbial population of the gastrointestinal tract and some blood factors of aflatoxin-fed zebrafish.

Methods: Zebrafish were divided into four groups of 45 fish in three replicates: control (G1); G2, fish fed with feed containing 500 µg/kg *E. purpurea* L. essential oils; G3, fish fed with feed containing 500 µg/kg *E. purpurea* L. essential oil and 3 ppm aflatoxin B1(AFB1); and G4, fish fed with feed containing 3 ppm AFB1. The fish were fed with diets for 60 days. After this period, they were euthanized, blood was collected from the tail vein, and blood smears were prepared. Fish hepatopancreas were used to measure alanine aminotransferase, aspartate transaminase, and alkaline phosphatase enzymes with an auto-analyzer. Also, intestinal contents were cultured to evaluate microbial population.

Results: Results showed that liver enzymes increased in the aflatoxin group ($P<0.05$), and concurrent use of the essential oil along with AFB1 reduced the liver enzymes compared with the AFB1-treated group. Moreover, AFB1 could convert the microbial population to pathogens. Differential blood counts in the G2 and G3 groups showed an increase in the percentages of neutrophils and thrombocytes.

Conclusion: According to the results of this study, *E. purpurea* L. essential oils could reduce the adverse effects of chronic contamination with AFB1 in zebrafish. Nevertheless, more studies are needed to better understand the immunological function of *E. purpurea* L. in zebrafish and its mechanism of action against AFB1.

Keywords: Aflatoxin B1, *Echinacea purpure*a L. essential oils, Intestinal microbes, Liver enzymes, Zebrafish (*Danio rerio*), Blood cells

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*** Corresponding Author:**
Donya Nikaein, Assistant Professor.
Address: *Department of microbiology and immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.*
Phone: *+98 (21) 61117144*
E-mail: *dnikaein@ut.ac.ir*

1. Introduction

Mycotoxins are toxic secondary fungal metabolites produced by three main fungal genera: *Aspergillus*, *Fusarium*, and *Penicillium* spp. They contaminate food and feedstuff (Gonçalves et al., 2020). Mycotoxin contamination in food usually results in health deterioration and reduced fish performance, affecting feed nutrient bioavailability (Tasa et al., 2020). Approximately 25%-50% of cereal products are estimated to be contaminated with mycotoxins worldwide (Wang et al., 2018).

Aflatoxins are one of the most important mycotoxins. They are predominantly produced by *Aspergillus flavus* and *A. parasiticus*. Among 18 types of aflatoxins, aflatoxin B1 (AFB1) is the most toxic and prevalent. It is a natural hepatocarcinogenic compound (Imani et al., 2017). Moreover, Aflatoxin B1 consumption results in poor growth, decreased immune system responsiveness, anemia, blood clotting deficiency, liver, and other organ damage, and increased susceptibility to infectious diseases and mortality (Marijani et al., 2019; Tacon, 1992). Recent studies showed that aflatoxin also affects the diversity of gut microbiota (Voth-Gaeddert et al., 2019; Wang et al., 2016). Studies have demonstrated the adverse effects of aflatoxin B1 in aquaculture (Tasa et al., 2020); however, there are still gaps in the characterizations of its impact, especially in aquaculture.

Regarding the effects of aflatoxins in aquaculture, finding reliable strategies to eliminate and or modulate the adverse effects of aflatoxins is important (Imani et al., 2017). Medicinal herbs and their byproducts have been used to treat many diseases, from autoimmune disorders to cancer, for centuries (Yamada et al., 2011). Many of these herbs have shown efficacy against mycotoxins (Tasa et al., 2020). *Echinacea purpurea* L. (the purple coneflower) is a medicinal herb known for improving the immune system with its antimicrobial and anti-inflammatory properties (Hill et al., 2006). Studies have been done to evaluate its antifungal and anti-mycotoxin production effects in feed (Dhanapal et al., 2015; Ibrahim et al., 2020; Nasir and Grashorn, 2010). However, little is known about its effects on mycotoxicosis *in vivo*.

Zebrafish is a unique animal model; this species is widely used in both adult and embryo stages in toxicological studies. Its rapid developmental stages allow easy monitoring and evaluation of defects, and in addition, it has 87% similarity to the human genome. Zebrafish has also been an effective model for studying aflatoxin toxicity (Chen et al., 2017; Zuberi et al., 2019). In this study, we investigated the effect of *E. purpurea* L. essential oil on gut microbial populations and hematological factors of zebrafish (*Danio rerio*) fed with a diet containing low-dose AFB1.

2. Materials and Methods

Zebrafish

Laboratory strain adult male zebrafish (*D. rerio*) with a mean weight of 5±0.25 g were obtained from the Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Iran. Zebrafish were maintained in several separate static 50-L aquariums under a water circulating system at 28°C and 14 h light/10 h dark cycle. Tanks were equipped with an outside biological filter. Fish were fed with commercial pellets twice daily at 3% body weight. Ten fish were examined for infection before the experiment to reject any apparent disease (Alavinia et al., 2018).

E. purpurea L. essential oil

E. purpurea L. essential oil was extracted using Clevenger apparatus under a distillation procedure and was in the form of suspension. In brief, 100 g dried *E. purpurea* plant was submitted to hydrodistillation at 100°C for 5 h. The essential oil was collected and dried over anhydrous sodium sulfate. It was kept in the dark glass tubes at 4°C before use (Gandomi et al., 2014).

Determination of sublethal dose of aflatoxin

AFB1 was purchased from Sigma-Aldrich Company, UK (20 µg/mL). It was diluted to gain a concentration of 3 ppm. Then, it was stored at -20°C until used (Ahmadi et al., 2021). A total of 45 zebrafish were divided into 3 groups with three replicas to determine the sublethal dose of aflatoxin for chronic aflatoxicosis. Three doses of AFB1 (1.5, 3, and 4.5 ppm) were added to the zebrafish diet for 4 weeks, and body weight and feed consumption of fish were monitored. The concentration in which no mortality was observed but affected growth and feed intake parameters was chosen for the experiment (Dalvi & McGowan, 1984; Zychowski et al., 2013) (data not shown).

Diet preparation and experimental design

Diets were prepared according to Sanden et al. (2012) with some modifications. In brief, the selected concentrations of materials were dissolved in 0.09% gelatin

suspension and mixed with fish feed until a homogenous composition was obtained. The feed was dried at 40°C temperature.

Zebrafish were divided randomly into four groups containing 45 fish in three replicas. Fish were fed one of the following diets: control (G1), 500 µg/kg *E. purpurea* L. essential oil (G2) (Borges et al., 2018); 500 µg/kg *E. purpurea* L. essential oil plus 3 ppm AFB1 (G3); and 3 ppm AFB1 (G4). Feeding was continued for 60 days.

Blood sampling

Fish were anesthetized using Tricaine methane sulfonate (MS222). Blood was collected according to Deebani et al. (2019) study with some modifications. In brief, zebrafish were laid on paper towels, and the caudal artery was clipped using dissecting scissors. Blood was collected into microtubes containing 0.5 µL sodium citrate and mixed properly. Fish were then euthanized and used for further experiments.

Hematological parameters

After clipping the caudal artery, a thin layer of blood smears was provided, air dried, fixed in methanol and stained with Giemsa (ratio of 1:9). Slides were examined under light microscopy with oil immersion (1000×magnification). Blood cells were identified based on morphological appearances and descriptions of teleost blood cells (Jagadeeswaran et al., 1999).

Measurement of liver enzymes

The hepatopancreas tissues of zebrafish were homogenized and put in microtubes containing phosphate buffer saline (PBS) (Dong et al., 2013; Nikaein et al., 2018). Liver enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), were measured by an automatic chemical analyzer (DANA 1700).

Identification of culturable gut microbes

The sampling procedure was done according to Cantas et al. (2012) with some modifications. In brief, the whole gut was removed from each fish and transferred into separate microtubes containing 200 µL sterile PBS. Samples were vortexed for 30 s, cultured on 5% sheep blood agar, Brocalin agar, and YGC agar (yeast extract, glucose, chloramphenicol agar), and incubated at 28°C for up to 30 days under aerobic and anaerobic conditions.

Bacterial isolates

Isolated pure bacterial colonies, subcultured on separate blood agars, were examined under a light microscope using the Gram-staining method to study their morphology. Biochemical tests such as motility, catalase, oxidase, and coagulase activity, IMViC (indole test, methyl red test, Voges-Proskauer test, and citrate utilization test) reactions, ability to use citrate, H_2S production, sugar fermentations, β-galactosidase, etc. were done according to our previous study (Erfanmanesh et al., 2019). Isolates were biochemically identified, comparing our results with a practical identification manual (De Smet & Blust, 2001). The species of bacteria was confirmed using 16s rDNA analysis (Cantas et al., 2012).

Fungal isolates

Molds

Molds were identified at genus and or species level using morphological characteristics under a light microscope (staining with lactophenol cotton blue), slide culture mounts, colony morphology, and investigating growth at different temperatures (Freitas et al., 2020).

Yeasts

Identification of yeasts was made using colony morphology, germ tube test, CHROM agar medium, urease test, sugar fermentation, and assimilation tests using RapID yeast plus system (Remel Inc., Lenexa, KS, USA).

Statistical analysis

The obtained data were analyzed with a one-way ANOVA test using SPSS software, version 21. Tukey post hoc test was performed for statistical comparison between groups. A $P<0.05$ was considered statistically significant.

3. Results

Fish survival rate

The mean survival rate in different groups was about 93.33%, with no significant differences ($P>0.05$).

Hematological parameters

Figure 1 shows differential blood counts done on hematological smears. Figure 2 shows the percentage of white blood cells in different treatment groups. The G4

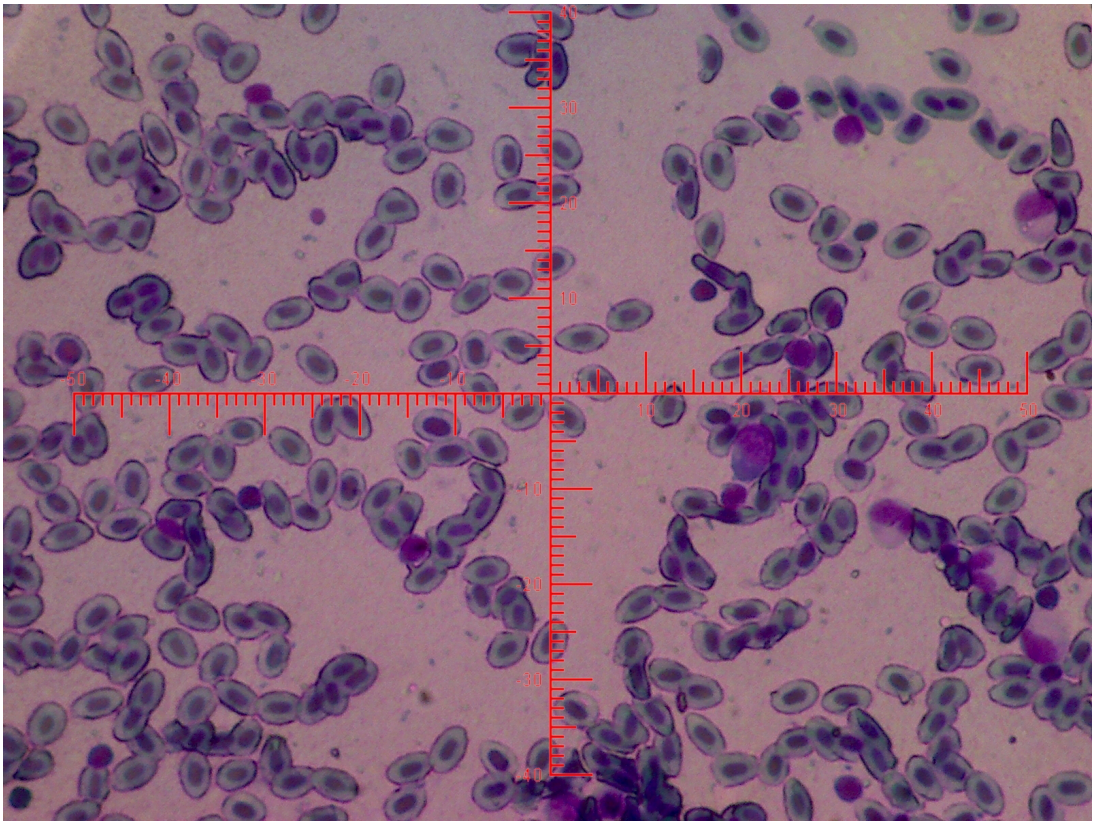

Figure 1. Differential blood count of zebrafish blood smear (1000x magnification, wright-giemsa staining)

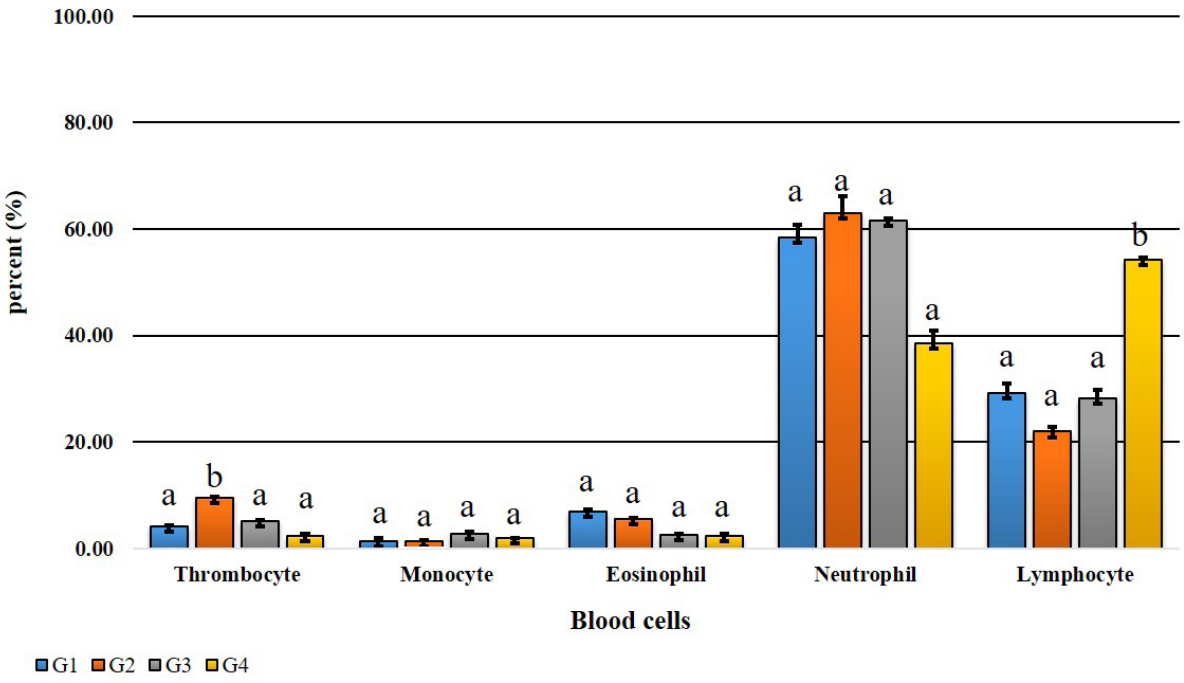

Figure 2. Percentages of different blood cells in differential blood count

Note: G1: Control group; G2: Zebrafish fed with 500 µg/kg *E. purpurea* L. essential oil; G3: Zebrafish fed with 500 µg/kg *E. purpurea* L. essential oil and 3 ppm Aflatoxin B1; G4: Zebrafish fed with 3 ppm aflatoxin B1.

group, fed with AFB1, had significantly higher percentages of lymphocytes (P<0.05). The G3 group (zebrafish fed with essential oils and aflatoxin simultaneously) had the highest counts of monocytes. However, this increase was not significant compared with other groups (P>0.05). Regarding other blood cells, there was an increase in neutrophils and thrombocytes in the G2 group; however, this increase was only significant in thrombocytes (P<0.05). Other hematological factors did not show any differences between the studied groups.

Measurement of liver enzymes

The results of measuring three major liver enzymes (ALT, AST, and ALP) are presented in Table 1. G2 and G4 had the highest amounts of ALT, which was significant compared with the control group (P<0.05). There was no significant difference between levels of ALT in G3 and control (P>0.05). The highest amount of AST belonged to the G4 and the lowest to the G2 group; these differences were significant compared to the control group (P<0.05). G3 also had significantly elevated AST amount (P<0.05). ALP enzyme levels showed no significant differences between the AFB1 alone and control groups (P>0.05). However, it was significantly lower in *E. purpurea* and *E. purpure*a plus AFB1-fed zebrafish (P<0.05).

Identification of Culturable *Gut Microbiota*

Table 2 presents the results of the identification of gut microbiota. A total of 7 bacterial isolates were identified in different groups. No difference was seen between aerobic and anaerobic conditions; the control and G2 groups had the same isolates, including, *Enterococcus faecium*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Staphylococcus epidermis* (P>0.05). No Gram-positive bacteria were isolated from the G3 group. *Citrobacter freundii*, a pathogenic bacteria in aquaculture, was isolated from the G3 and G4 groups, and *Streptococcus iniae*, a well-known pathogen in aquatic species, was identified in the G4 group. No significant differences were observed between molds in treatment groups (P>0.05). Among fungal isolates, in the control group (G1), *Rhodotorula rubra* and *Cladosporium* spp. had the highest and lowest prevalence, respectively, while in the G4 group, *Cladosporium* spp. was the most prevalent fungal isolate, and *R. rubra* was not identified. *Trichosporon beigelii*, an opportunistic yeast, was found in the G4 group.

4. Discussion

Contamination of feed with aflatoxins results in both acute and chronic toxicity and carcinogenicity. However, little is known about the aflatoxin toxicity mechanisms (Ghafarifarsani et al., 2021). Since controlling the production of aflatoxins in feed is still challenging, it is important to look for methods to prevent their adverse effects on animals and humans. In the present study, we tried to understand the anti-aflatoxin B1 potential of *E. purpure*a L. essential oils.

In the present study, ALT and AST had a significant increase in zebrafish fed with the AFB1 diet. The addition of *E. purpurea* L. essential oils to feed prevented this elevation (P<0.05). In a study, the effects of sublethal doses of *Euphorbia turcomanica* extract on liver enzymes in *Zebra Aphanius* were evaluated (Zare et al., 2014). They observed a significant increase in AST and a decrease in ALT after 30 days of treatment and no sig-

Table 1. Measurement of zebrafish liver enzymes using autoanalyzer (IU/L)

Groups	Liver enzymes (IU/L)		
	ALT**	AST	ALP
G1	1251.33±185.32^{a}	3134.67±57.98^{a}	1753.33±117.28^{a}
G2	3546.67±263.40^{b}	2078.33±51.34^{b}	1238±62.45^{b}
G3	1213.33±6.67^{a}	3869.67±81.49^{c}	1135.67±39.88^{b}
G4	3413.33±54.57^{b}	6753.33±340.41^{d}	1702±48.35^{a}

Data are presented as Mean±SE, Different letters indicate statistically significant differences between groups.

Note: G1: Control group; G2: Zebrafish fed with 500 µg/kg *E. purpurea* L. essential oil; G3: Zebrafish fed with 500 µg/kg *E. purpurea* L. essential oil and 3 ppm aflatoxin B1; G4: Zebrafish fed with 3 ppm aflatoxin B1.

Abbreviations: ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.

Table 2. Culturable microbes isolated from zebrafish gut in different treatment groups under aerobic and anaerobic conditions (frequencies are shown as Mean±SE)

Groups	Fungal Isolates		Bacterial Isolates
	Mold	Yeast	
G1*	*Cladosporium* spp. (10±1.4) *Penicillium* spp. (10±0)	*R. rubra* (22±0.05)	*E. faecium* (8±0.6) *A. hydrophila* (6±1.2) *P. aeruginosa* (12±3.2) *S. epidermis* (14±0.02)
G2	*Cladosporium* spp. (9.5±0.2) *Aspergillus niger* (7±0.22) *Penicillium* spp. (11±0.05)	*R. rubra* (15±4.5)	*E. faecium* (7±2.6) *A. hydrophila* (5.4±0.33) *P. aeruginosa* (11.5±7.2) *S. epidermis* (12.4±1.05)
G3	*Penicillium* spp. (8.6±3.33) *Cladosporium* spp. (11±1.55)	*R. rubra* (18±3.12)	*E. faecium* (7.5±1.5) *Escherichia coli* (12±0.04) *C. freundii* (9.2±0.45) *A. hydrophila* (4.2±0.04)
G4	*Cladosporium* spp. (13.6±0.4) *Penicillium* spp. (10±5.23)	*T. beigelii* (13±1.05)	*E. faecium* (10±0.04) *E. coli* (15.2±3.2) *C. freundii* (8.4±1.33) *A. hydrophila* (5.3±0.45) *S. iniae* (14±1.6)

Note: G1: Control group; G2: Zebrafish fed with 500 µg/kg *E. purpurea* L. essential oil; G3: Zebrafish fed with 500 µg/kg *E. purpurea* L. essential oil and 3 ppm aflatoxin B1; G4: Zebrafish fed with 3 ppm aflatoxin B1.

nificant changes in ALP levels. In our study, *E. purpurea* L. addition to fish feed significantly increased ALT; however, AST and ALP were significantly decreased ($P<0.05$). These enzymes are an indicator of liver function, and it is believed that elevation in liver enzymes shows the liver injury. However, more experiments should be done to understand if *E. purpurea* L. affects liver function. In another study, Sancho et al. (2010) investigated the toxic effects of the fungicide tebuconazole on zebrafish. They reported significant increase in liver enzymes, ALT, AST, and ALP after 7 and 14 days of exposure (Sancho et al., 2010). We observed an increase in liver enzymes in the AFB1 group as well. They also researched AST and ALT elevation in the gills, kidneys, and liver of *Cyprinus carpio* after exposure to cadmium (De Smet & Blust, 2001). Both enzymes are important in protein synthesis and energy production during stress conditions. AFB1 is a mycotoxin with immunosuppressive effects, although studies have shown that short-time usage and low doses of this toxin could indicate immune-stimulative effects (Valtchev et al., 2015). In our study, differential blood count showed an increase in the percentage of lymphocytes in the aflatoxin B1 fed group; this could result from sublethal AFB1 doses and long-term application of AFB1.

E. purpurea L. is known for its immunomodulatory properties (De Rosa et al., 2019; Yang et al., 2017). We demonstrated a slight increase in neutrophils and a significant increase in thrombocyte counts in groups fed with *E. purpurea* L. It has been documented that fish thrombocytes could act as a link between innate and adoptive immunity (Passantino et al., 2005). So, it is recommended to analyze the effect of *E. purpurea* L. essential oil on innate and, to some extent, adoptive immunity in response to AFB1 exposure in zebrafish in further works.

In research performed on fish gut microbiota, some bacteria, including *E. coli* (most prevalent), *E. faecium*, *Aeromonas*, *Yersinia*, and *Streptococcus* spp., have been isolated frequently (López Nadal et al., 2020; Wang, al., 2018; Xia et al., 2018). Our study investigated the culturable microbes and similar bacterial species that were isolated. Interestingly, it was shown that AFB1 could shift the microbial flora to Gram-negative and more pathogenic bacteria. *C. freundii* was isolated in both groups fed with AFB1, and *E. purpurea* L. did not affect the microbial population. We isolated *S. iniae*, a well-known pathogen, from the AFB1-fed group; it might result from lab contamination with this bacterium. In other studies, changes in the microbiota have been seen after AFB1 exposure (Wang et al., 2021; Wang et al., 2018).

There are few studies on molds isolated from fish intestines. However, in a review article on yeasts living in the fish gut, *Rhodotorula*, *Candida*, *Cryptococcus*, *Trichosporon*, and *Debaryomyces* species have been reported (Gatesoupe, 2007). In the present study, we isolated two yeast species, including *R. rubra* and *T. beigelii*, that are compatible with the species documented in this review, and *Rhodotorula* had the highest prevalence. However, in our study, *T. beigelii* was only isolated from AFB1-fed zebrafish. This fungus is known as an opportunistic pathogen and can cause intestinal disorders. According to our results, it is suggested that feed manipulation could increase the occurrence of molds in intestines so that in our treatment groups, *Cladosporium* and *Penicillium* had the highest frequency. But since these fungi are environmental contaminants, more studies with metagenomics methods are needed to better interpret changes in gut microbiota after exposure to AFB1 and treatment with *E. purpurea* L. essential oils.

5. Conclusion

Aflatoxicosis affect the physiological activities of zebrafish in different organs. The present study evaluated the neutralizing effects of *E. purpurea* L. essential oil in fish involved with aflatoxicosis. According to our results, *E. purpurea* L. essential oil could reduce the adverse effects of chronic contamination with AFB1 in zebrafish in the liver and help maintain the microbial gut population. Nevertheless, more studies, for example, molecular experiments on potentially affected signaling pathways, are needed.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Deputy of Research and Technology, Faculty of Veterinary Medicine, University of Tehran (Ethical Code 30792/6/1). All ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, and redundancy) have been completely observed by the authors.

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Authors' contributions

Conceptualization and supervision: Donya Nikaein, Alireza khosravi and Hooman Rahmati-Holasoo; Methodology: Tina Hasankhani; Investigation, writing – original draft, and Writing – review & editing: All authors;Data collection: Tina Hasankhani and Mona Hasankhany; Data analysis: Donya Nikaein and Hooman Rahmati-Holasoo.

Conflict of interest

The authors declare no conflicts of interest.

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مقاله پژوهشی

ارزیابی اثر اسانس اکیناسه آ پورپورا (گیاه سرخارگل) بر فراسنجههای هماتولوژیکی و جمعیت میکروبی دستگاه گوارش ماهی زبرا مبتلا به آفلاتوکسیکوزیس مزمن

تینا حسنخانی[1,2]، *دنیا نیک آئین[1,2]، علیرضا خسروی[1,2]، هومن رحمتی هولاسو[3]، مونا حسنخانی[4]

۱. گروه میکروبیولوژی و ایمونولوژی، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.
۲. مرکز تحقیقات قارچ شناسی، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.
۳. گروه بهداشت و بیماریهای آبزیان، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.
۴. دانشکده شیلات و محیط زیست، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران.

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چکیده

زمینه مطالعه: آفلاتوکسین یکی از مهم ترین سموم قارچی با خواص سمی کبدی، تراتوژنیک و سرکوب کننده سیستم ایمنی است. این مایکوتوکسین عمدتاً توسط گونه هایی از جنس آسپرژیلوس در خوراک تولید می شود. بنابراین، استفاده از ترکیباتی که می تواند از عوارض متنوع آفلاتوکسین ها جلوگیری کند و هیچ گونه عوارض جانبی بر کیفیت خوراک نداشته باشد، از اهمیت بالایی برخوردار است.

هدف: هدف از این مطالعه تعیین اثر اسانس سرخارگل بر تنظیم جمعیت میکروبی دستگاه گوارش و برخی فاکتورهای خونی ماهی زبرا تغذیه شده با آفلاتوکسین بود.

روش کار: بدین منظور، ماهی زبرا در سه تکرار به چهار گروه ۴۵ تایی شامل شاهد (G1)، ماهی تغذیه شده با خوراک حاوی ۵۰۰ میکروگرم بر کیلوگرم اسانس اکیناسهآ پورپورا (G2)، ماهی تغذیه شده با خوراک حاوی ۵۰۰ میکروگرم بر کیلوگرم اسانس اکیناسهآ پورپورا و ppm3 آفلاتوکسین ب۱ (G3) و ماهیان تغذیه شده با خوراک حاوی ppm3 آفلاتوکسین ب۱ (G4) تقسیم شدند. ماهیان با جیره به مدت ۶۰ روز تغذیه شدند. پس از این مدت، ماهیان معدوم شده و از ورید دمی خون گرفته و اسمیر خون تهیه شد. آنزیم‌های آلانین آمینوترانسفراز، آسپارتات ترانس آمیناز و آلکالین فسفاتاز از هپاتوپانکراس ماهی با استفاده از اتوآنالایزر اندازه‌گیری و محتویات روده‌ای برای ارزیابی جمعیت میکروبی کشت داده شد.

نتایج: نتایج نشان داد که آنزیم های کبدی در گروه آفلاتوکسین افزایش یافت ($p<0/05$) و مصرف همزمان اسانس به همراه AFB1 توانست آنزیم های مذکور را در مقایسه با گروه تیمار شده با AFB1 کاهش دهد. علاوه بر این، AFB1 می تواند جمعیت میکروبی را به سمت پاتوژن ها منحرف کند. شمارش افتراقی خون در گروه های G2 و G3 افزایش درصد نوتروفیل ها و ترومبوسیت ها را نشان داد.

نتیجه‌گیری نهایی: با توجه به نتایج این مطالعه می توان نتیجه گرفت که اسانس سرخارگل می تواند اثرات نامطلوب آلودگی مزمن به AFB1 در ماهی زبرا را کاهش دهد. با این وجود، مطالعات بیشتری برای درک بهتر عملکرد ایمونولوژیک اکیناسهآ پورپورا در ماهی زبرا و مکانیسم اثر آن در برابر AFB1 مورد نیاز است.

کلیدواژه‌ها: آفلاتوکسین ب۱، آنزیمهای کبدی، اسانس اکیناسهآ پورپورا، جمعت میکروبی دستگاه گوارش، ماهی زبرا، گلبولهای خونی

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* **نویسنده مسئول:**
دنیا نیک آئین
نشانی: تهران، دانشگاه تهران، دانشکده دامپزشکی، مرکز تحقیقات قارچ شناسی.
تلفن: ۶۱۱۱۷۱۴۴ (۲۱) ۹۸+
رایانامه: dnikaein@ut.ac.ir