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The Effect of *Echinacea purpurea L.* (eastern purple coneflower) Essential Oil On Hematological Parameters And Gut Microbial Population Of Zebrafish (*Danio rerio*) With Aflatoxicosis

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Running title: Echinacea and aflatoxicosis in zebrafish

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, application of compounds, which can prevent diverse complications of aflatoxins and no side effects on feed quality, is highly significant.

Objectives: The aim of this study was to determine the effect of the *Echinacea purpurea (E. 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 fishes in three replicates including 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 the diets for 60 days. After this period, fish were euthanized and blood was collected from the tail vein and blood smears were prepared. Alanine Aminotransferase, Aspartate Transaminase and Alkaline Phosphatase enzymes were measured from hepatopancreas of fish by auto-analyzer and intestinal contents were cultured to evaluate microbial population.

Results: Results showed that liver enzymes increased in aflatoxin group ($P<0.05$) and concurrent use of the essential oil along with AFB1 was able to reduce mentioned enzymes in comparison with AFB1 treated group. Moreover, AFB1 could divert microbial population to

pathogens. Differential blood count in G2 and G3 groups showed an increase in the percentage of neutrophils and thrombocytes.

Conclusions: According to the results of this study, it can be concluded that *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 immunological function of *E. purpurea* L. in zebrafish and its mechanism of action against AFB1.

Keywords: Aflatoxin B1, *Echinacea purpurea L.* essential oils, Intestinal microbes, Liver enzymes, zebrafish (*Danio rerio*), blood cells

Introduction

Mycotoxins are toxic secondary fungal metabolites produced by three main fungal genera including *Aspergillus*, *Fusarium* and *Penicillium* spp. They contaminate food and feedstuff (Gonçalves *et al*., 2020). Mycotoxin contamination in food usually result in health deterioration and reduction of fish performance affecting bioavailability of feed nutrients (Tasa *et al*., 2020a). It is estimated that approximately 25-50% of cereal products are contaminated with mycotoxins worldwide (Y. Wang, Zhao, *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 one. It is considered as 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 organs' damage, increase susceptibility to infectious diseases and mortality (Marijani *et al*., 2019; Tacon, 1992).

Recent studies showed that aflatoxin affect the diversity of gut microbiota as well (Voth-Gaeddert *et al*., 2019; J. Wang *et al*., 2016). Studies have demonstrated the adverse effects of aflatoxin B1 in aquaculture (Tasa *et al*., 2020b), however in spite of these researches, there are still gaps in characterization of its effects especially in aquaculture.

According to the effects of aflatoxins in aquaculture, finding reliable strategies to eliminate and/or modulate adverse effects of aflatoxins is significant (Imani *et al*., 2017). Medicinal herbs and their byproducts have been used in treatment of 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*., 2020b). *Echinacea purpurea L.* (commonly known as purple cone flower) is a medicinal herb known for its abilities to improve immune system, along with antimicrobial and anti-inflammatory properties (Hill *et al*., 2006). Studies have done to evaluate its antifungal and anti-mycotoxin production effects in feed (Dhanapal *et al*., 2015; Ibrahim *et al*., 2020; Nasir & Grashorn, 2010). However, little is known about its effects on mycotoxicosis *in vivo*.

Zebrafish is a unique model organism; this species is widely used in both adult and embryo stages in toxicological studies. Its rapid developmental stages allows easy monitoring and evaluation of defects, and in addition, it has 87% similarity to the human genome. zebrafish has also been an effective model to study aflatoxin toxicity (Chen *et al*., 2017; Zuberi *et al*., 2019), in this study, we investigated the effect of *Echinacea purpurea L.* essential oil on gut microbial populations and hematological factors of zebrafish (*Danio rerio*) fed with diet containing low dose aflatoxin B1.

Materials and methods

Zebrafish

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

Echinacea purpurea L. essential oil

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

Determination of sub-lethal dose of Aflatoxin

AFB1 was purchased from Sigma-Aldrich Company, UK (20 µg/ml). it was diluted to gain a concentration of 3 ppm; it was stored at -20°C until used (Ahmadi *et al*., 2021). To determine the sub-lethal dose of Aflatoxin for chronic Aflatoxicosis, 45 zebrafish were divided into 3 groups with three replicas. Three doses of Aflatoxin B1 1.5, 3 and 4.5 ppm were added to 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 then dried at 40°C temperature (Sanden *et al*., 2012).

Zebrafish were divided randomly into four groups each containing 45 fish in three replicas. Fish were fed one of the following diets: control (G1), 500 µg/kg *Echinacea purpurea L.* essential oil (G2) (Borges *et al*., 2018), 500 µg/kg *Echinacea purpurea L.* essential oil plus 3 ppm AFB1 group (G3), and 3 ppm AFB1 (G4). Feeding was continued for 60 days. Experiments were done according to the national standards established for animal ethics (ethical code number: 30792/6/1).

Blood sampling

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

Hematological parameters

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

Measurement of liver enzymes

Hepatopancreas tissue 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

Sampling procedure was done according to Cantas *et al* (2012) with some modifications. In brief, whole gut was removed from each fish and transferred into separate microtubes containing 200µl sterile PBS. Samples were vortexed for 30s and 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 (Cantas *et al*., 2012).

Bacterial isolates

Isolated pure bacterial colonies which were subcultured on separate blood agars were examined under light microscope using gram stain method to study their morphology. Biochemical tests such as motility, catalase, oxidase and coagulase activity, IMViC 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 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 done 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

Data were analyzed using SPSS software version 21 and one-way ANOVA test. Tukey post hoc test was performed for statistical comparison between groups. A p value of less than 0.05 was considered statistically significant ($P<0.05$).

Results

Fish survival rate

Mean survival rate in different groups was about 93.33% and no significant differences was observed ($P>0.05$).

Hematological parameters

Differential blood count was done on hematological smears (Figure1). Figure 2 shows the percent of white blood cells in different treatment groups. G4 group which were fed with AFB1 had a significant increase in percent of lymphocytes (P<0.05). G3 (zebrafish fed with essential oils and aflatoxin simultaneously) had the highest counts of monocytes however this increase was not significant in comparison with other groups (P>0.05). Regarding other blood cells, there was an increase in neutrophils and thrombocytes' percent in G2 group however this increase was only significant in thrombocytes (P<0.05). Other hematological factors did not show any changes between studied groups.

Measurement of liver enzymes

The results on measuring three major liver enzymes (ALT, AST and ALP) are shown in table 1. G2 and G4 had the highest amounts of ALT which was significant in comparison with control group (P<0.05). There was no significant differences between levels of ALT in G3 and control (P>0.05). The highest amount of AST belonged to G4 and the lowest to G2, these differences were significant in comparison to control group (P<0.05). G3 had also a significant elevation in AST amount (P<0.05). ALP enzyme levels showed no significant differences between groups AFB1 alone and control (P>0.05) however it had a significant decrease in *E. purpurea* and *E. purpurea* plus AFB1 fed zebrafish (P<0.05).

Identification of culturable gut microbiota

Table 2 demonstrates the results of identification of gut microbiota. A total of seven bacterial isolates were identified in different groups. No difference was seen between aerobic and

anaerobic conditions, control and G2 had the same isolates including, *Enterococcus faecium*, *Aeromonas hydrophila, Pseudomonas auroginosa* and *Staphylococcus epidermis* ($p>0.05$). No gram positive bacteria were isolated from G3 group. *Citrobacter freundii* a pathogenic bacteria in aquaculture was isolated from G3 and G4 groups and *Streptococcus iniae* a well-known pathogen in aquatic species was identified in G4. No significant differences was observed between molds in treatment groups ($p>0.05$). Among fungal isolates, in control group (G1) *Rhodotorula rubra* and *Cladosporium* spp had the highest and lowest prevalence respectively while in G4 *Cladosporium spp* was the most prevalent fungal isolate and *R. rubra* was not identified. *T. beigelii* an opportunistic yeast was found in G4.

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 production of aflatoxins in feed is still a challenge, it is considerable to look for methods to prevent their adverse effects in animals and human. In the present study, we tried to understand the anti-aflatoxin B1 potential of *Echinacea purpurea L.* essential oils.

In the present study, ALT and AST had a significant increase in zebrafish fed with AFB1 diet and addition of *Echinacea purpurea L.* essential oils to feed showed inhibition of this elevation ($P<0.05$). In a study on 2014, the effects of sub-lethal doses of *Euphorbia turcomanica* extract on liver enzymes in zebra Aphanius was evaluated (Zare *et al*., 2014). They observed a significant increase in AST and decrease in ALT after 30 days of treatment and no significant 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 for liver function, it is believed that elevation in liver enzymes liver shows liver defect, 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 fungicide tebuconazole on zebrafish, they reported significant increases in liver enzymes, ALT, AST and ALP after 7 and 14 days exposure (Sancho *et al*., 2010), we observed an increase in liver enzymes in AFB1 group as well. They are also researches on AST and ALT elevation in gills, kidney and liver of *Cyprinus carpio* after exposure to cadmium (De Smet & Blust, 2001). Both enzymes play an important role 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 present study, differential blood count showed an increase in percent of lymphocytes in aflatoxin B1 fed group; this could be as a result of sub-lethal AFB1 doses and long term application of AFB1.

Echinacea 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 significant increase in thrombocytes 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 *Echinacea purpurea L.* essential oil on innate and in some extent adoptive immunity in response to AFB1 exposure in zebrafish in further works.

In researches performed on fish gut microbiota, bacteria including *E. coli* (most prevalent), *E. faecium*, *Aeromonas*, *Yersinia* and *Streptococcus* spp. have been isolated frequently (López

Nadal *et al*., 2020; Y. Wang, Wang, *et al*., 2018; Xia *et al*., 2018). In our study, we investigated the culturable microbes and similar bacterial species were isolated, interestingly, it was shown that AFB1 can shift the microbial flora to gram negatives and more pathogenic bacteria. *C. freundi* was isolated in both groups fed with AFB1 and *E. purpurpurea L.* hadn't an effect on microbial population. We isolated *S. iniae* a very well-known pathogen from AFB1 fed group, it might be as a result of lab contamination with this bacterium. In other studies changes in variation of microbiota has seen after AFB1 exposure (Y. Wang *et al*., 2021; Y. Wang, Wang, *et al*., 2018).

There are few studies on molds isolated from fish intestines. However, in a review article on yeasts living in fish gut *Rhodotorula*, *Candida*, *Cryptococcus*, *Trichosporun*, and *Debaryomyces* species have been reported (Gatesoupe, 2007). In the present study, we isolated two yeast species including *R. rubra* and *T. beigelii* that is 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 fungi 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 *Cladospoium* and Penicillium had the highest frequency. But since these fungi are environmental contaminant as well more studies with metagenomics methods are needed to have a better interpretation of changes in gut microbiota after exposure with AFB1 and treatment with *Echinacea purpurea L.* essential oils.

Conclusion

Aflatoxicosis affect 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 liver and helped in maintenance of microbial gut population. Nevertheless more mechanism studies, for example molecular experiments on potential affected signaling pathways are needed.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data availability

The datasets generated and/or analyzed in the current study are available from the corresponding author on reasonable request.

Ethical approval

This study was approved by 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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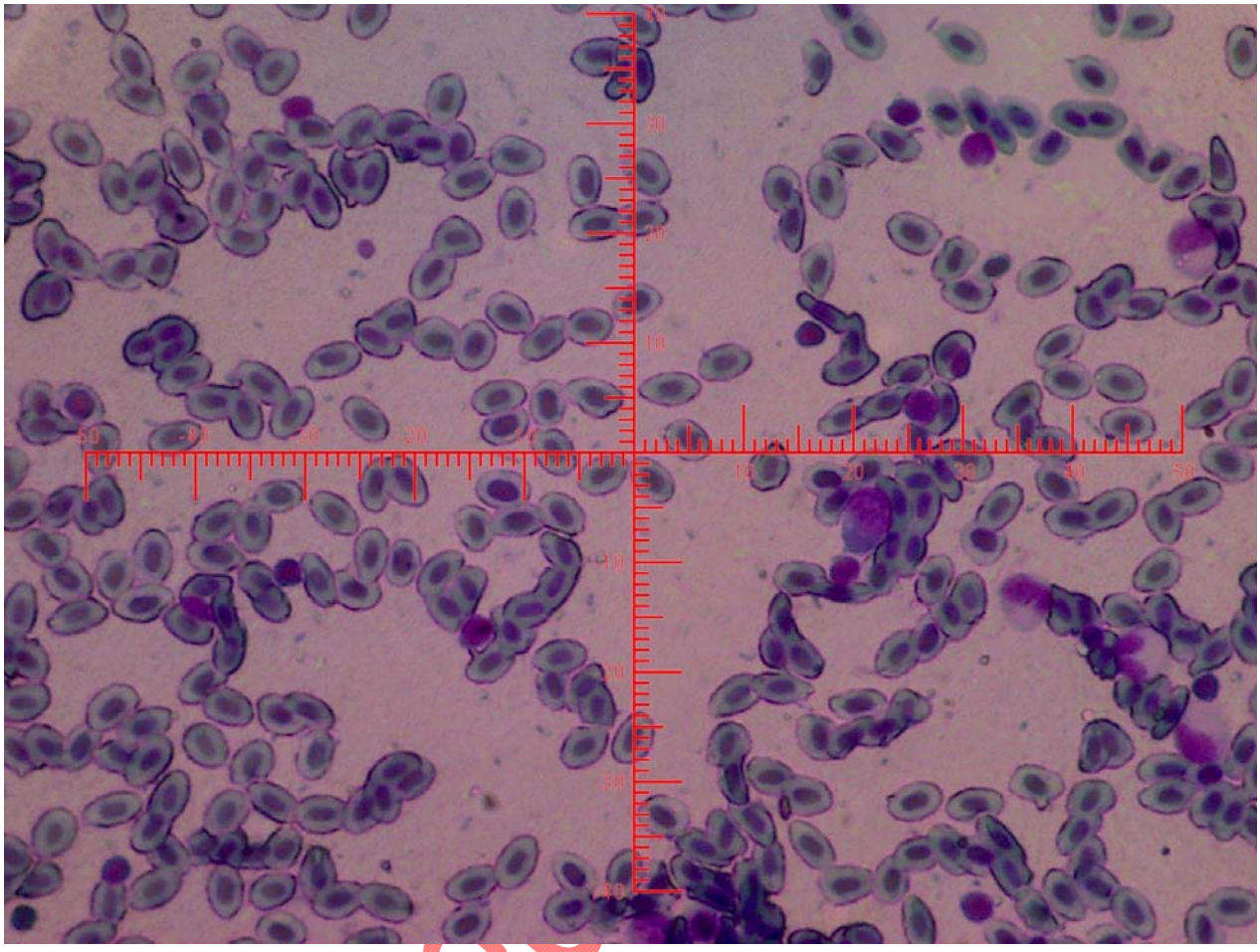

405 Figure 1- differential blood count of zebrafish blood smear (×1000 magnification, wright giemsa staining)

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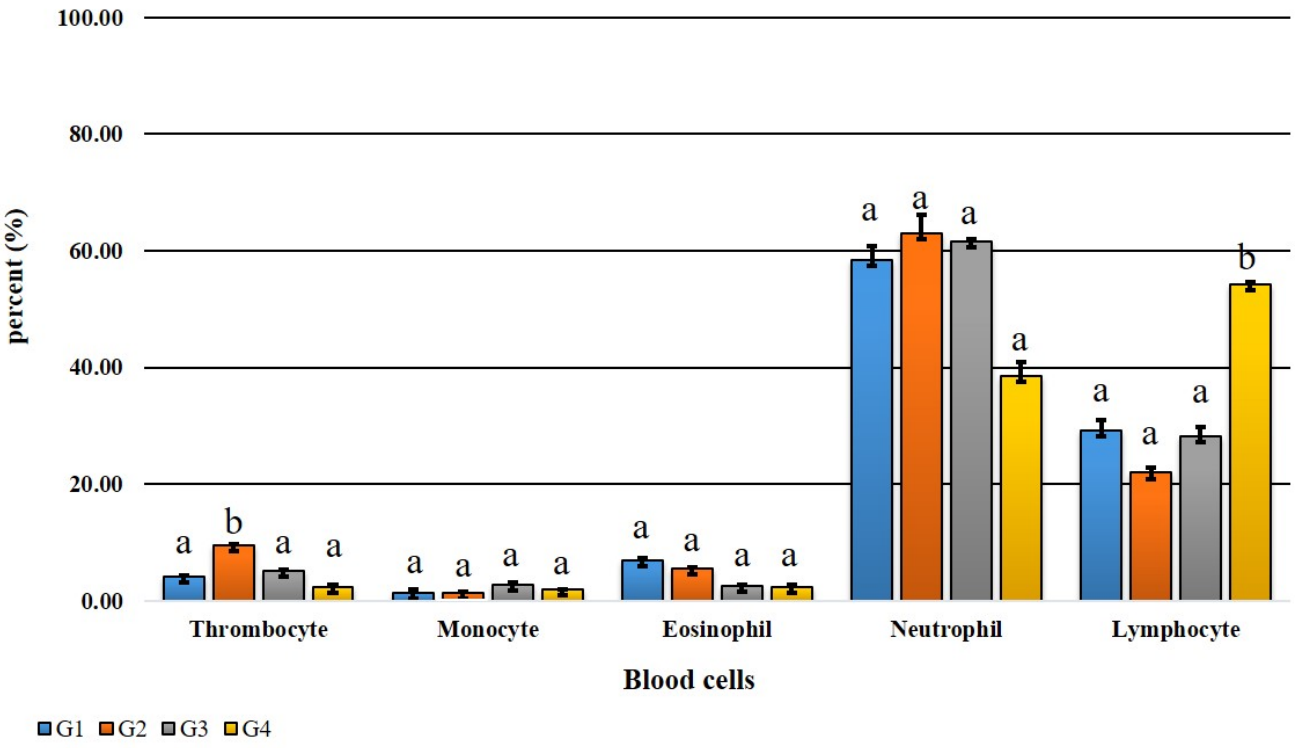

Figure 2- percent of different blood cells in differential blood count.

415 G1. Control group/ G2. Zebrafish fed with 500µg/kg *Echinacea purpurea L.* essential oil/ G3. Zebrafish fed with 500µg/kg *Echinacea purpurea L.* essential oil and 3 ppm Aflatoxin B1/ G4. Zebrafish fed with 3 ppm Aflatoxin B1.

Table1- Measurement of zebrafish liver enzymes using autoanalyzer (IU/L). Data are shown as mean±SE

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]

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

** ALT. Alanine aminotransferase/ AST. Aspartate aminotransferase/ ALP. Alkaline phosphatase

***Different characters show significant differences

Table2- 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)	*Rhodotorula rubra* (22±0.05)	*Enterococcus faecium* (8±0.6) *Aeromonas hydrophila* (6±1.2) *Pseudomonas auroginosa* (12±3.2) *Staphilococcus epidermis* (14±0.02)
G2	*Cladosporium spp* (9.5±0.2) *Aspergillus niger* (7±0.22) *Penicillium spp* (11±0.05)	*Rhodotorula rubra* (15±4.5)	*Enterococcus faecium* (7±2.6) *Aeromonas hydrophila* (5.4±0.33) *Pseudomonas auroginosa* (11.5±7.2) *Staphilococcus epidermis* (12.4±1.05)
G3	*Penicillium spp* (8.6±3.33) *Cladosporium spp*(11±1.55)	*Rhodotorula rubra* (18±3.12)	*Enterococcus faecium* (7.5±1.5) *Escherichia coli* (12±0.04) *Citrobacter freundii* (9.2±0.45) *Aeromonas hydrophila* (4.2±0.04)
G4	*Cladosporium spp* (13.6±0.4) *Penicillium spp* (10±5.23)	*Trichosporon beigelii* (13±1.05)	*Enterococcus faecium* (10±0.04) *Escherichia coli* (15.2±3.2) *Citrobacter freundii* (8.4±1.33) *Aeromonas hydrophila* (5.3±0.45) *Streptococcus iniae* (14±1.6)

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

430

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

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خلاصه

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.

اهداف: 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 45 تایی شامل شاهد (G1)، ماهی تغذیه شده با خوراک حاوی 500 میکروگرم بر کیلوگرم اسانس *اکیناسهآ پورپورا* (G2)، ماهی تغذیه شده با خوراک حاوی 500 میکروگرم بر کیلوگرم اسانس *اکیناسهآ پورپورا* و 3ppm آفلاتوکسین ب1 (G3) و ماهیان تغذیه شده با خوراک حاوی 3ppm آفلاتوکسین ب1 (G4) تقسیم شدند. ماهیان با جیره به مدت 60 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 AFB1توانست آنزیم های مذکور را در مقایسه با گروه تیمار شده با AFB1 کاهش دهد. علاوه بر این، AFB1می 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: 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.

کلمات کلیدی: آفلاتوکسین ب1، آنزیم‌های کبدی، اسانس *اکیناسهآ پورپورا*، جمعت میکروبی دستگاه گوارش، ماهی زبرا، گلبول-های خونی