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Original Article

The Effect of *Chenopodium Album* and *Apium Nodiflorum* on the Expression of the Regulatory Gene (*afl*R) that Produces Aflatoxin in *Aspergillus parasiticus*

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Abstract

Background and Aim: Aflatoxins which are mainly produced by *Aspergillus parasiticus* are among the highly toxic secondary metabolites. This species often contaminate food and agricultural products including cereals, peanuts, and crops in the field. Recently, a number of studies have been carried out on the elimination of aflatoxin, and the antifungal effects of medicinal herbs, such as *Chenopodium album* (36HYU2385) and *Apium nodiflorum* (36HYU2362). In this study, the effects of *C. album* and *A. nodiflorum* as natural compounds were examined on *Aspergillus parasiticus* growth, aflatoxins production and the *afl*R gene expression.

Materials and Methods: The antifungal susceptibility testing of *C. album* and *A. nodiflorum* were performed according to CLSI document M38-A2. Quantitative changes in *afl*R gene level of expression were analyzed by Real-time PCR method.

Results: Results indicated that minimal inhibitory concentration (MIC) in the extracts of *C. album* and *A. nodiflorum* against *A. parasiticus* growth were 100 mg/ml and 120 mg/ml respectively. Extracts of *Chenopodium album* and *Apium nodiflorum* have antitoxic properties. Moreover, they effectively decrease aflatoxin production. The level of *afl*R gene expression was decreased significantly after the exposure of fungal cells to the extracts. The highest inhibition was observed in MIC= 100 mg/ml of *C. album*.

Conclusion: According to the results, it can be suggested that these herbal extracts may have antifungal potential to be used in medicine or agriculture.

Keywords: Aflatoxin, *Aspergillus parasiticus*, *Chenopodium album*, *Apium nodiflorum*

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Introduction

Aflatoxins are capable of generating carcinogenic and mutagenic effects, as well as acute toxics in humans and animals exposed to the toxins (1-3). These secondary metabolites are produced by Aspergillus species especially *Aspergillus flavus* and *Aspergillus parasiticus* (4). These mycotoxins can grow in a wide range of foods, including cereals, spices, dried fruits and stored grains, i.e. wheat, corn, peanuts, maize, cotton seed and crops in the field. They can grow even during storage and postharvest operations (5) which can cause harmful side effects in both animals and humans, especially in the latter (1, 2). At least 14 various types of aflatoxins are produced in nature including B1, B2, G1 and G2 (6).

Among them aflatoxin B1(AFB1) is the most potent hepatotoxic and carcinogenic compound (7). The researches that have been conducted on aflatoxin biosynthesis pathways in *A. flavus, A. parasiticus* and the other Asp species have shown that the *afl*R gene is involved in the regulation of aflatoxin biosynthesis (8-12).

Some synthetic and chemical fungicides have been acquired for the protection of stored grains against mycotoxins; but these compounds have been demonstrated to be harmful for human health. Therefore, it is necessary to use natural materials derived from herbal extracts (7).

In the past years, the removal of aflatoxin from food and agricultural products has been a positive approach in protecting them against fungal infections (3).

The medicinal herbs, *Chenopodium album* (36HYU2385) and *Apium nodiflorum* (36HYU2362), have been traditionally used as therapeutic agents for infections. These two herbs grow around the Zagros Mountains in Iran. *Apium nodiflorum* (Watercress) decreases the oxidative damage in liver and kidney (13). The anticancer activities of this plant have also been reported (14, 15).

Chenopodium album has been used in the treatment of cardiovascular disorders, abdominal pain, eye disease and throat troubles (16).

To our knowledge, very little studies have been documented about the effects of *Chenopodium album and Apium nodiflorum* on the expression of *afl*R gene. In this study, the effects of these herbal extracts were evaluated on the growth and *afl*R gene expression process in *A. parasiticus*.

Materials and Methods

Fungal Cell Preparation

Aspergillus parasitius strain (ATCC 15517) was cultured on Sabouraud Dextrose Agar (SDA) (Merck, Germany) and incubated for 2 days at 30°C.

Preparation of Fungal Inoculums

Potato dextrose agar (PDA) (Merck, Germany) was used for fungal strain subculture and kept at 30 °C for 5 days to allow sporulation. Then the colonies were immersed in 1ml of sterile saline solution, and the culture surface was scraped smoothly with tip and transfer pipette for harvesting the spores. The concentration of fungal spores was calculated using the McFarland Turbidity No. 0. 5, and then adjusted in such a way that each test well contained 5×10^4 CFUs/ml.

Preparation of *Chenopodium album* and *Apium nodiflorum* Extracts

Chenopodium album and *Apium nodiflorum* powders were obtained from Medicinal Plants Research Center, Yasuj University of Medical Sciences, Yasuj, Iran. To prepare their extracts, five grams of the powder was dispersed in 200 ml ethanol. Subsequently, 0.2 gr of alcoholic extract was added to 1ml of distilled water to get a solution with a final concentration of 125µg/ml.

Determination of Minimal Inhibitory Concentration

In vitro antifungal susceptibility testing for the determination of MIC was performed according to Clinical and Laboratory Standard Institute (CLSI) document M38-A2 with some modifications (CLSI 2008). Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma chemical Co.) was buffered to pH 7.0 with 0.165 mol/L MOPS (Sigma) (3-[N-morpholino] propanesulfonic acid), with L-glutamine and phenol red without bicarbonate. *Chenopodium album* and *Apium nodiflorum* extracts were dissolved in sterile diluted water to get a concentration of 1 g/ml, and then diluted to the final concentrations of 15.62–500mg/ml in the RPMI medium according to the standard CLSI protocol. All tests were done in duplicate. Micro dilution plates (96 U-shaped wells, Nunc, Roskilde, Denmark) were used. Negative (only RPMI 1640

Table 1: Primers for Real-Time PCR analyzed.

Gene	Primer name	Sequence (5'-3')	PCR product size (bp)	GeneBank
aflR	F*afl*	5'-CGGAACAGGGACTTCCGGCG-3	200	AF 441438
	R*afl*	5'-GGGTGGCGGGGGACTCTGAT-3'		
β-actin	F*act*	5'-ACGGTATTGTTTCCAACTGGGACG-3'	110	XM 717232.1
	R*act*	5'- TGGAGCTTCGGTCAACAAAACTGG-3'		

medium) and positive controls (fungal suspension with RPMI medium without the extracts) were also run alongside each experiment. The plates were incubated for 48 h at 35°C. MIC endpoints were determined on the base of the lowest concentrations that could prevent any recognizable growth, mostly based on antifungal capacity of the extractions.

A. Parasiticus Cultivated in the *Chenopodium album* and *Apium Nodiflorum*

In order to study, the effects of *C. album* and *A. nodiflorum* on the expression of *afl*R gene in *A. parasiticus*, 100 µL of fungal suspension was cultured with 1mL extraction in 9mL RPMI medium, separately for each extract. Then, these cultures were incubated at 30°C for 3 days.

RNA Extraction and Real-time PCR Assay

In order to analyze the changes in the expression of the *afl*R gene, real-time PCR assay was used. After being incubated at 30°C for 3days, the mycelia mass was harvested and frozen in liquid nitrogen. All cytoplasmic RNA molecules were isolated from normal and *C. album* and *A. nodiflorum* fungal cells were treated by a standard method (17). Purity and RNA concentrations were measured by spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany), and equal concentration of RNA which is 1 µg in 20 µL was used in cDNA synthesis by random hexamer primers, according to the kit protocol (Cinnagen co. Iran). *Afl*R and *β-actin* gene (*ACT1*, as a House keeping gene and an endogenous reference gene) primers were designed on the basis of the published sequences in NCBI (accession no: AF441438) which are shown in Table 1.

StepOnePlus real-time PCR system (Applied Biosystems, Foster city, CA) was used for performing real-time PCR, and SYBER Premix Ex Taq II was used as a reagent specifically designed for intercalator-based real-time PCR.

The program used for performing amplification included an initial duration step for 5 min at 94°C followed by the 35 cycles PCR, as follows: 94C for 45 s and 58C for 45 s, then 72°C for 12min.

Results and Discussion

Determination of MIC

The inhibitory effects of the two extracts, *C. album*

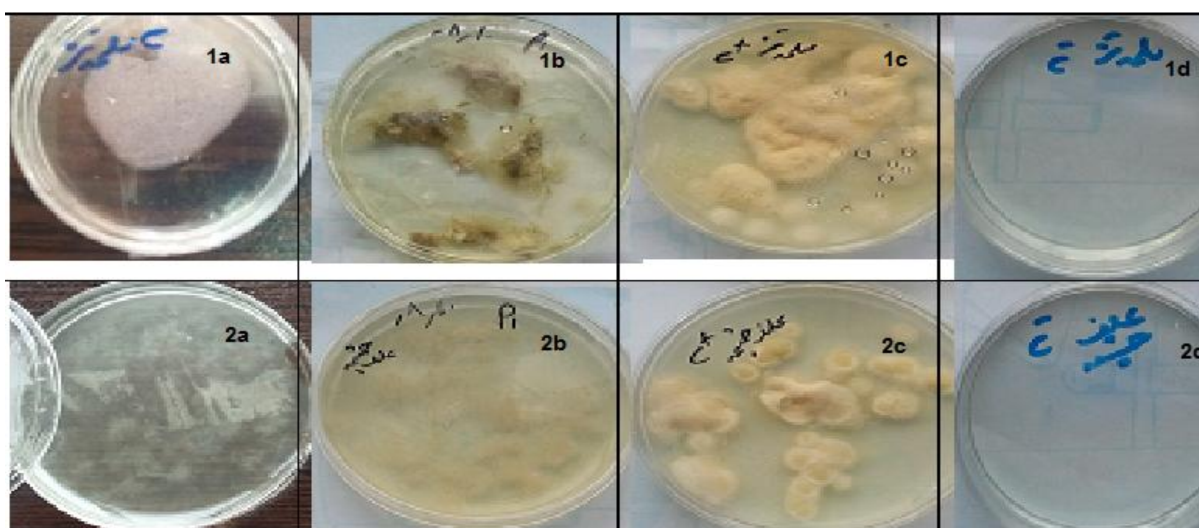

Figure 1. *A. parasiticu*s cultivated in the presence of 1) *Chenopodium album*: a) MIC value of 100 mg/ml; b) MIC value of 50 mg/ml; c) Positive Control; d) Negative Control. 2) *Apium nodiflorum*: a) MIC value of 120 mg/ml; b) MIC value of 60 mg/ml; c) Positive Control; d) Negative Control.

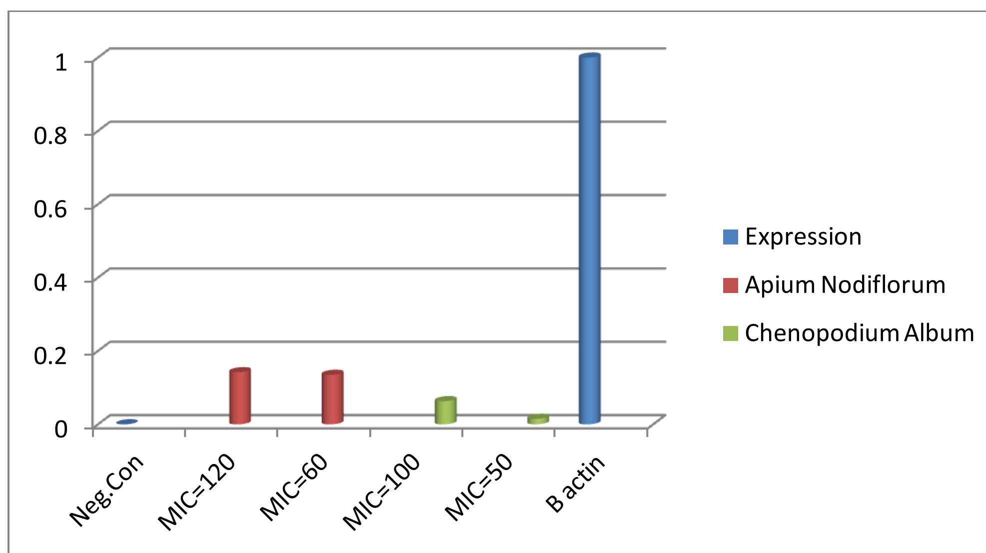

Figure 2. Effects of *Chenopodium album* and *Apium nodiflorum* on *aflR* Gene Expression (mg/ml).

and *A. nodiflorum*, were evaluated. Both extracts had significant inhibitory effects on *Aspergillus parasitius* growth; however, *C. album* reveled greater efficacy to inhibit the growth of this fungus. In the current study, broth microdilution method was used to evaluate the of antifungal activities of *C. album* and *A. nodiflorum* extract (CLSI document M38-A2). Figure 1 shows the inhibitory effect of the extracts on *A. parasiticus* growth at a concentration of 100 mg/ml for *C. album* and 120 mg/ml for *A. nodiflorum.*

Effect of *Chenopodium Album* and *Apium Nodiflorum* Extracts on *Afl*R Gene Expression

The results demonstrated that the extracts of *C. album* and *A. nodiflorum* inhibited *A. parasiticus* growth at MIC values of 100 mg/ml and 120 mg/ml respectively. According to quantitative real-time PCR results, the rate of *afl*R gene expression was significantly decreased after treating the *A. parasiticus* with both extracts. The results of an analysis of relative quantification of the *aflR* gene after treatment with *C. album* and *A. nodiflorum* are shown in Table 2. β-actin gene as a house keeping gene was used in order to compare gene expression. β-actin gene showed stability in *A. parasiticus* in the presence of *C. album* and *A. nodiflorum.*

The highest inhibition was observed in MIC= 100 mg/ml of *C. album*; however, the minimum inhibitory effect was related to MIC= 50 mg/ml of *C. album*. Both concentrations of *A. nodiflorum* showed similar inhibitory effects on *afl*R expression in *A. parasiticus* (Figure 2).

Table 2: Relative expression of *aflR* gene using Real-Time PCR analysis.

Extract	Gene	Type	Reaction efficiency	Expression	Std Error	95% CI	Result
	β-actin	REF	1.0	1.000	-	-	-
C. album	*afl*R (50mg/ml)	TRG	1.0	0.015	0.011 - 0.020	0.011 - 0.020	Down expression
	*afl*R (100mg/ml)	TRG	1.0	0.064	0.056 - 0.073	0.056 - 0.073	Down expression
A.nodiflorum	*afl*R (60mg/ml)	TRG	1.0	0.135	0.123 - 0.149	0.120 - 0.152	Down expression
	*afl*R (120mg/ml)	T1RG	1.0	0.142	0.121 - 0.168	0.111 - 0.181	Down expression

Aflatoxins are extremely toxic substances that are produced by some species of *Aspergillus*, including *A. parasiticus*, *A. flavus* and *A. nominus* which are of high significance in food industry as well as in animal husbandry and medical subjects. These toxins have enormous negative economic impacts. Moreover, aflatoxins are the most potent carcinogenic agents among the known natural compounds (18). During the last decades, several studies have investigated extracts' and plants' compound antibacterial and antifungal activities (19-22). Essential oils are good candidates, as they have been traditionally used for centuries, due to their antifungal properties.

Chenopodium spp. has been cultivated for many years as a leafy vegetable in different parts of the world (23). Currently, some countries, especially European ones, have become more interested in the initiation of researches on Chenopodium spp. These spp. are reach in proteins and free essential amino acids, starch, minerals and oils. They also contain low amounts of several vitamins and antioxidants (24).

Several studies on the plants of the Apiaceae family, to which *Apium nodiflorum* belongs, have reported the chemical composition and biological activities of their extracts and pointed to their significant antifungal activity (25-27).

In the current study, the antifungal activities of *C. album* and *A. nodiflorum* were established for the first time against an aflatoxin-producing *A. parasiticus* in relation to the reduction of the expression of important key gene of aflatoxin which is *afl*R. This research revealed that both of them had inhibitory impacts on the growth as well as down expression of *afl*R gene due to the combined growth of their extracts. The MIC of *C. album* was comparatively lower than *A. nodiflorum*, therefore its potency as an antifungal product was less than *A. nodiflorum*. As *C. album* widely grows in the west of Iran. It is an essential medicinal herb that could be recommended as an easily available antifungal source instead of the synthetic chemicals used for this purpose. Moreover, our study showed that the rate of *afl*R gene expression decreased after treating the fungus with 100 mg/ml of *C. album*.

Our results in relation to the antifungal properties of herbal extracts on *A. parasiticus* confirmed previous investigations. Khodaveisy et al., reported that *P. atlantica* subsp. kurdica can inhibit *A. parasiticus* growth at a concentration of 125 mg/ml (17), and AF production on *A. parasiticus* after exposure of fungal cells to 500g/ml of Licorice extract (7). In another study, the researchers showed that Ephedra major is the potent inhibition of fungal growth and AF production (28). Andrea Maxia et al., who published their study in 2012, referred to the potential of *A. nodiflorum* essential oil in the treatment of dermatophytosis and candidiasis (29). The results obtained by Rajesh Kumar et al., showed the significant effect of *Chenopodium ambrosioides* on *Aspergillus* spp., and indicated that it could be a potential botanical fungi toxicant in the ecofriendly control of food commodities against storage fungi (30). Urszula Gawlik- Dziki et al., have tested the antioxidant and anticancer capacity of *Chenopodium quinoa*. They investigated *C. quinoa* and concluded that because of the high bioavailability of bioactive *C. quinoa* leaves' compounds, they can help in the chemoprevention of cancer and other diseases related to oxidative stress, such as atherosclerosis (31).

Conclusion

Aflatoxin genes expression analysis by real-Time PCR showed the inhibitory effects of *C. album* and *A. nodiflorum* on the expression of *afl*R gene. These extracts reduced aflatoxin production. Consequently, they could be a good candidate for controlling *A. parasiticus*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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