Original Article

Survey of Expression of Aflatoxin Production Regulator Gene (*aflR*) in *Aspergillus Parasiticus* by *Alpinia Galanga L* and *Dorema Aucheri*

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Received: 25 October, 2017; Accepted: 21 December, 2017

Abstract

Background: Aflatoxins are one of the highly toxic secondary metabolites, which are mainly produced by Aspergillus parasiticus. This species frequently cause of food and agricultural products contamination including cereals, peanuts, and crops in the field. During recent years, researchers have considered research on elimination of aflatoxin and antifungal effects of medicinal herbals, such as Alpinia galanga L and Dorema aucheri. In this study, the effect of A.galanga L and D.aucheri a natural compound was examined on Aspergillus parasiticus growth, aflatoxins production and the aflR gene expression.

Materials and Methods: Antifungal susceptibility *A.galanga L* and *D.aucheri* was performed according to CLSI document M38-A2. Quantitative changes in *aflR* gene level of expression were analyzed by Real-time PCR method.

Results: Our result obtained that the MIC of extracts on *A. parasiticus* growth 250 mg/mL for *D.aucheri* and 800 mg/mL for *A.galanga L*. *D.aucheri* has antitoxic properties as well as its effective ability to decrease aflatoxin production. The level of *aflR* gene expression was decreased significantly after the exposure of fungal cell to *D.aucheri* extract, but *A.galanga L* didn't have significant effect.

Conclusion: This research indicated that *D.aucheri* has antifungal effects more than *A.galanga L.* Due to our obtained result we can suggest that *D.aucheri* herbal extract may have antifungal potential in medicine or agriculture.

Keywords: Aflatoxin, *Aspergillus parasiticus*, *Alpinia galanga L*, *Dorema aucheri*

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Please cite this article as: Arab-Mazar Z, Lotfali E, Ghadjari A, Aghaei Gharehbolagh S, Mohammadi R, Nouripour-Sisakht S. Survey of expression of aflatoxin production regulator gene (*aflR*) in *Aspergillus parasiticus* by *Alpinia galanga L* and *Dorema aucheri*. Novel Biomed. 2018;6(1):29-34.

Introduction

One of the most common contaminants in the worldwide is fungi toxigenic. Some fungi annually that cause a significant economics problem[1,2], affected approximately 25% of the world crops. Mycotoxins are well known as a secondary fungal metabolism with very high diversity and they have hazardous effects for animal and humans[3,4]. Aflatoxins are types of mycotoxins that are most toxic for

mammals and it can cause several diseases such as toxic hepatitis, hemorrhage edema, and hepatic carcinoma[5]. At least 14 various types of aflatoxin are produced in nature including B1, B2, G1, and G2, among them the most potent hepatoxic and carcinogenic one is AFB1[6,7]. One of the *Aspergillus* sp. that is able to produce aflatoxin is *Aspergillus parasiticus*[8]. Aflatoxins could find in wide range of food such as cereal grains, oil seeds, milk, cheese, nut products and numerous other agriculture products[9].

In the biosynthesis of aflatoxins, *aflR* and *aflJ* genes play an important regulatory role[10,11]. In the biosynthesis of aflatoxin at least, 32 enzymatic reactions involved and the protein which is encoded by *aflR* binds to palindromic sequences located in promoter of genes in *Aspergillus flavus* and *Aspergillus parasiticus*[12].

During last decade, many studies have been done about natural therapies. For a long time, plants as natural products have been note as a worthy source for treatment and human health. According to WHO and result of many researches medical plants would be the best source for obtaining veracity of drugs[13,14]. Some studies reports antifungal activity of some botanical extracts such as *Alpinia galanga L* and *Dorema aucheri*. *Dorema aucheri* is one of the endemic plant species which is growing in central Zagros Mountains of the southwest of Iran in early spring[15, 16]. Various member of *Dorema* are used as a green vegetable or as a medicine for treatment of many human diseases such as microbial and fungal infections[17, 18]. Moreover, *D. aucheri* have some anti-bacterial ingredient such as terpene, flavonoid and phenolic compounds[19].

Alpinia galanga (Zingiberaceae) is widely cultivated in India, China and Southeast Asian countries[20] and its well known for its root extract substances, which are resistance to bacteria and fungi[21]. The aim of this study was evaluating antifungal effect of the *Alpinia galanga L* and *Dorema aucheri* extracts on the growth of fungi and *afIR* gene expression process in *A. parasiticus*.

Methods

Fungal cell preparation: In the current study, *Aspergillus parasitius* strain (American type culture collection (ATCC) 15517) was incubated at 30°C for 2 days on Sabouraud Dextrose Agar (SDA) (Merck, Germany).

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 0.5 McFarland turbidity and then adjusted in such a way that each test well contained 5×10^4 CFUs/ mL (7).

Preparation of *Alpinia galanga L* and *Dorema aucheri* extracts: *Alpinia galanga L* and *Dorema aucheri* extracts were obtained from medicinal plants research center, Yasuj University of Medical Sciences, Yasuj, Iran. Five grams of the powder was prepared in 200 mL ethanol then 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 determination of MIC was performed according to Clinical and Laboratory Standard Institute (CLSI) document M38-A2 with some modification. (CLSI. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: approved standard. 2008)

Roswell Park Memorial Institute (RPMI) (Sigma chemical Co.) 1640 medium 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 was applied. *Alpinia galanga L* and *Dorema aucheri* 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 mentioned medium according to the standard CLSI protocol. All tests were done in duplicate. Microdilution plates (96 U-shaped wells, Nunc, Roskilde, Denmark) were used. Negative (only RPMI 1640 medium) and positive control (fungal suspension with RPMI medium without licorice extract) were also run alongside each experiment. The plates were incubated for 48 h at 35°C. MIC endpoints were determined on the base of lowest concentrations that could prevent any recognizable growth, mostly based

on antifungal capacity of the extraction.

***A. parasiticus* cultivated in the *Alpinia galanga L* and *Dorema aucheri*:** For determination of the effect of *A. galanga L and D. aucheri* on expression of *aflR* 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 30C for 3 days.

RNA extraction and real-time PCR assay: Real-time PCR assay were used for analyzing of changing in expression of the *aflR* gene. After incubation at 30°C for 3days, the mycelia mass was harvested and frozen in liquid nitrogen, total cytoplasmic RNA molecules were isolated from normal and *A.* galanga *L and D. aucheri* treated fungal cells by a standard method[22].

For measuring of purity and RNA concentration spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany) was used and equal concentration of RNA which is 1 µg in 20 µL were used to cDNA synthesis by random hexamer primers, according to the kit protocol (Cinnagen co.). *β-actin* gene as a House keeping gene was used in order to compare gene expression. *aflR* and *β-actin* gene (*ACT1*) primers were design on the basis of published sequence 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 PCR setup and program have been previously described[7].

Results

Determination of MIC: In current study, for evaluation of antifungal activities of extracts, broth microduilution method was used (CLSI document M38-A2). *Dorema aucheri* had significant inhibitory effect on *Aspergillus parasiticus* growth, but *Alpinia galanga L* had not revealed any efficiency to inhibit the growth of this fungus.

Effect of *Alpinia galanga L and Dorema aucheri* extracts on *aflR* gene expression: Our result demonstrated that the extract of *D. aucheri* which is inhibited *A. parasiticus* growth at MIC values of 250 mg/ml. According to quantitative real-time PCR results, the rate of *aflR* gene expression was significantly decreased after treating the *A. parasiticus* with *D. aucheri compare to A. galanga*. The result of an analysis of relative quantification of the *aflR* gene after treatment with *A. galanga L* and *D. aucheri* are shown in table 2. *β-actin* gene showed staibility in *A. parasiticus* in the presence of *A. galanga L* and *D. aucheri*.

The highest inhibition was observed in MIC=250 mg/mL of *D. aucheri*; however, the minimum inhibitory effect was related to MIC=800 mg/mL of *A. galanga L*. (Fig1).

Discussion

During recent years, one of the serious concerns in food crops industry is contamination by different types of toxic fungi. Aflatoxins are a group of mycotoxins which are secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, and *A. ochraceoroseus*. Thses mycotoxins are toxic, carcinogenic and mutagenic[23].

Since ancient times, humans used plants and herbals in the treatments of different diseases and infections. Nowadays in modern medicine, natural therapy has high priority due to high interest and effectiveness. Since some chemical and synthesis drug has adverse effect, herbal medicine popularity increased globally[24]. The antimicrobial and antifungal effects of many herbals have been studied and it seems that natural ingredients can be effective and healthy alternatives for some chemicals and drugs.

In the current study, for the first time antifungal activities of *A. galanga L* and *D. aucheri* was evaluated against an aflatoxin-producing *A.parasiticus* in relation to the reduction of the expression of *aflR* which is important key gene of aflatoxin. The result of antifungal activity of *A. galanga L* and *D. aucheri* extracts were showed that *D. aucheri* extract in 250 mg/mL had significant inhibitory effect in growth as well as *aflR* gene down exprerssion in *A. parasiticus* but *Alpinia galanga L* extract is not able to prevent the growth of fungi on culture. *Dorema aucheri* is genus of plants that its active ingredient was reported Senosionine, flavonoid and alkaloid compounds with antioxidant properties[25].

Table 1: Primers for Real-Time PCR analyzed.

Gene	Primer name	Sequence (5'-3')	PCR product size (bp)	Gene Bank
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'		

Table 1: Relative expression of *aflR* gene using Real-Time PCR analysis.

Extract	Gene	Type	Reaction efficiency	Expression	Std Error	95% CI	**P(H1)**	Result
	β-actin	REF	1.0	1.000	-	-	-	-
Dorema aucheri	*aflR* (250 mg/ml)	TRG	1.0	0.108	0.093 - 0.129	0.084 - 0.141	0.000	Down
Alpinia galanga	*aflR* (800 mg/mL)	TRG	1.0	0.109	0.085 - 0.144	0.074 - 0.163	0.182	-sample group is not different to control group

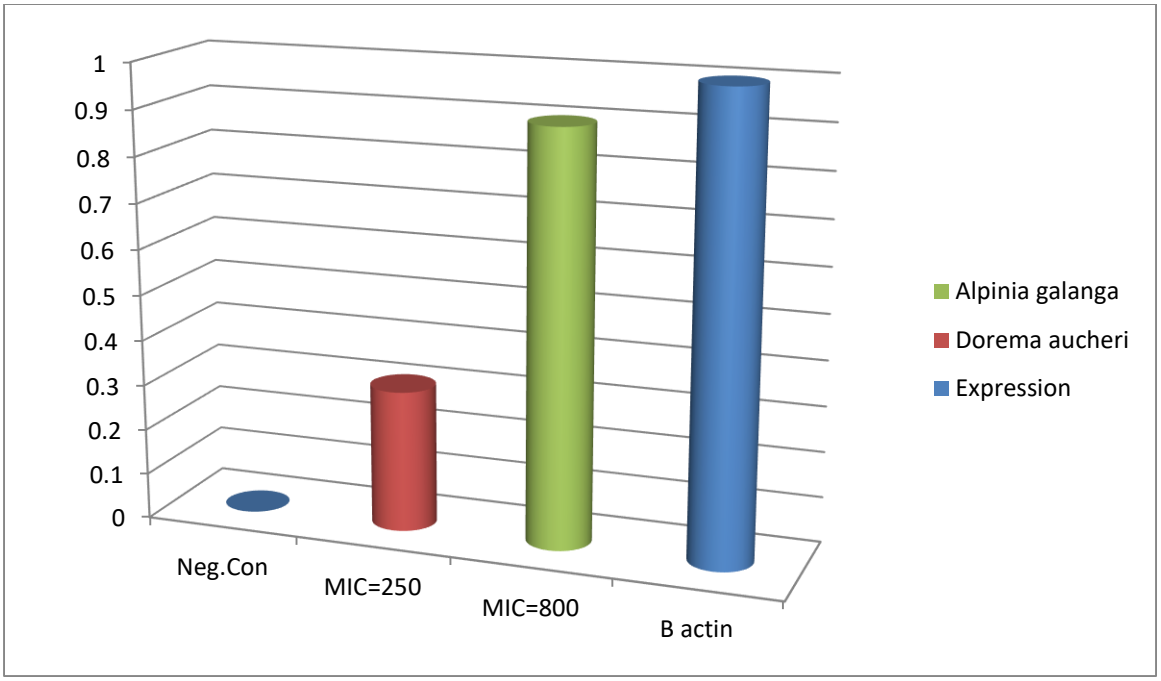

Figure 1. Effects of *Alpinia galanga L* and *Dorema aucheri* on *aflR* Gene Expression (MIC=mg/ml).

According to result of our study, it could be recommended as a source of antifungal instead of synthetic chemicals used for this purpose. Our results in relation to antifungal properties of herbal extracts on *A. parasiticus* are confirmed previous investigations.

Ghesari et al. compare antifungal activity of *Cichorium intyus*, *Dorma Aucheri* and *Prangos ferulacea* extracts against some food borne pathogen. The studied extracts showed vary degree of antimicrobial activities. The extract of *D. Aucheri* showed the best of activity [17]. In the other study,

Minabadi et al evaluated antimicrobial and anti-oxidative effects of methanolic extracts of *D. aucheri.* The result of their study demonstrated that leaf, stem, and flower extracts had inhibitory activity against bacteria[26].

Khodavandi et al in 2013, have tested antifungal activity of *Rhizome coptidis* and *Alpinia galanga L* against Candida species. They investigated *A. galanga L* was able to inhibit the growth of *Candida tropicalis* and *Candida glabrata* and the MIC value of *A. galanga L* was 64 µg/ml for both Candida tested[27]. In another study, Avasthi et al. were investigated fractions of methanolic extract of *A. galanga* for their antibacterial, antifungal and antioxidant potential. They demonstrated that all the fractions of *A. galanga* has significant antifungal activity against *Fusarium moniliforme* and *Candida albicans*[28].

Conclusion

Aflatoxin genes expression analysis by Real-Time PCR showed inhibitory effects of *A. galanga L* and *D. aucheri* on expression of *aflR* gene. *D. aucheri* extract reduce aflotoxin production; as a result it could be a good candidate for controlling of *A. parasiticus.* Further studies on these extracts confirmed the result of this research.

Acknowledgment

The authors would like to thank personals of Medicinal Plants Research Center in Yasuj University of Medical Sciences, for helping in this research.

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