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Survey of Expression of Aflatoxin Production Regulator Genes (*AflR* and *Nor1*) in *Aspergillus parasiticus* by *Euphorbia connata* Bioss. and *Pimpinella Anisum* L

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Research Article

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Abstract

Aspergillus species are pathogenic and saprophyticus fungi which is responsible new cases of invasive fungal infection across the world. Aflatoxins (produced by *Aspergillus parasiticus*) are extremely toxic secondary metabolites which contaminate food products. Several investigations have been performed on the removal of aflatoxin using medicinal herbs. In this survey, the effects of *Euphorbia connata* Bioss. and *Pimpinella anisum* L. as natural compounds were examined on *Aspergillus parasiticus* growth, the *aflR* and *Nor1* genes expression. The antifungal susceptibility testing of herbal extracts were performed according to CLSI-M38-A2. Quantitative changes in level of genes expression were evaluated by Real-time PCR method. Results indicated that MIC in the extracts of *E. connata* Bioss and *P. anisum* against *Asp. parasiticus* growth were 31.25mg/ml and 125 mg/ml respectively. The *Nor1* gene was detected at a higher effect than the *aflR* gene in *Asp. parasiticus* after treatment with *E. connata* extracts. There were statistically significant differences in *Nor1* and *aflR* gene expressions after treated with *E. connata* (P=0.001). The *Nor1* mRNA expression levels were down regulated by 75% whereas the *aflR* mRNA expression level was down regulated by 25% (P<0.001) compared to the control group. *E. connata* extract could be a suitable candidate for control of toxin production by *A. parasiticus*. However, the *P. anisum* extract has no effect on gene expression.

Keywords: Aflatoxin; Genes Expression; *Aspergillus Parasiticus*; *Euphorbia connata*; *Pimpinella anisum*

Abbreviations: SDA: Sabouraud Dextrose Agar Media; PDA: Potato Dextrose Agar Media; RPMI: Sterile Roswell Park Memorial Institute; GE: Genes Expression.

Introduction

Aspergillus species (spp.) are pathogenic and saprophyticus fungi which is responsible for annually 200,000 new cases of invasive fungal infection across the world [1,2]. Rapid death, chronic hepatic and pulmonary disease and developmental disorders in children are the most notable complications of *Aspergillus* spp. exposure [3]. Prevalence of complications is so considerable that approximately 3 million people are only infected by chronic pulmonary aspergillosis [4]. Even if we diagnose and start treatment plan, the mortality rate of this fungi is 50% and is 100% in missed cases [1].

A significant part of high mortality trait originates from toxins. Aflatoxin in *Asp. parasiticus* is a life-threatening factor which produced to deteriorate human body structures both directly (inside the body) and indirectly (inside inappropriate food storehouses) [5,6]. At least 32 enzyme and numerous gene profiles participate in aflatoxin synthesis pathway [7]. There are 2 genes in the route called *aflR* and *Nor1 (aflR)*. The former gene is essential for aflatoxin production through inhibition of palindromic sequence in gene promotor [8]. The latter one plays the main role in production of an aflatoxin mediator named norsolorinic acid (Figure 1) [9,10]. Is seems that the invasion of *Asp. parasiticus* might be controlled by gene suppression.

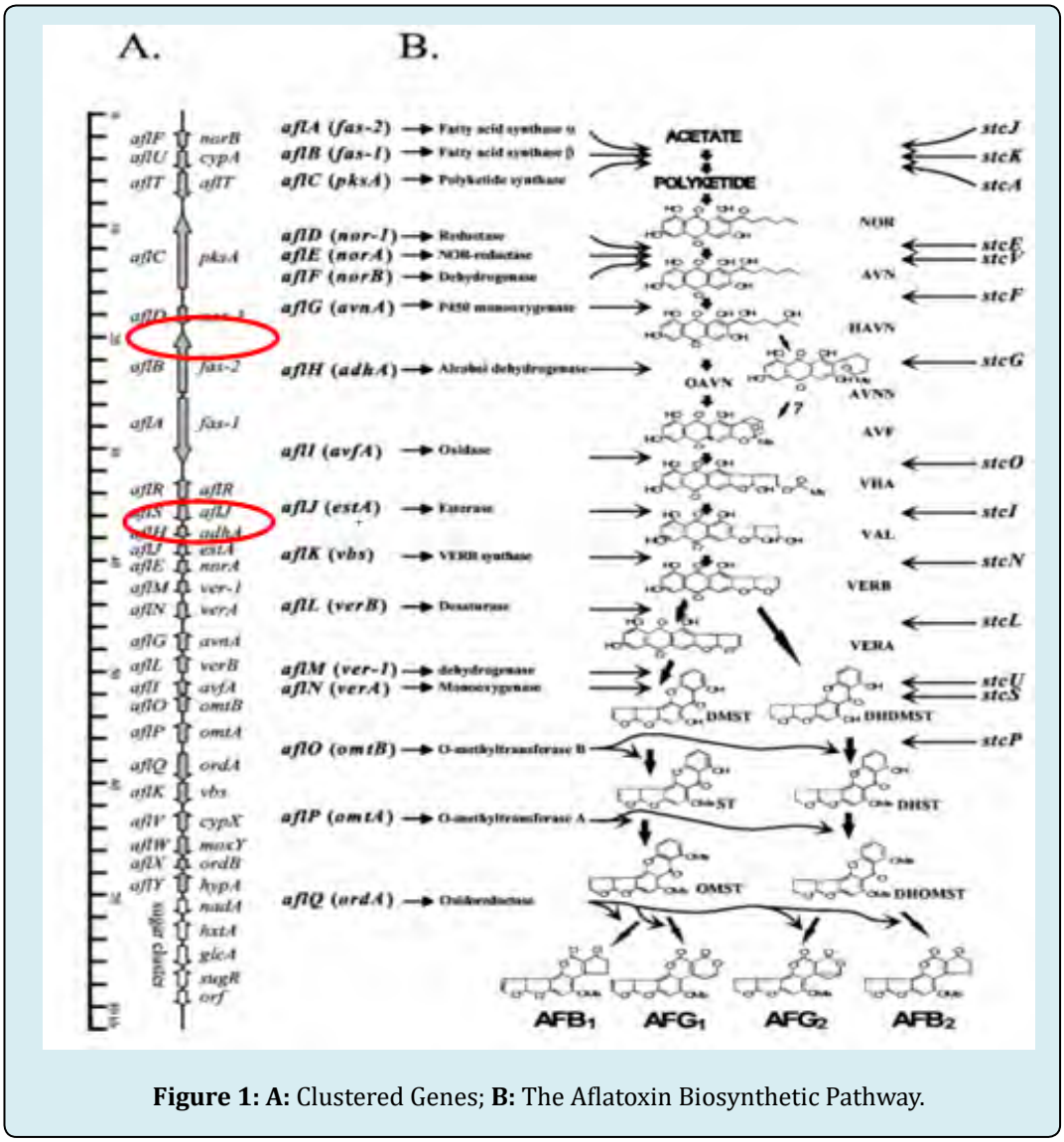

Figure 1: A: Clustered Genes; **B:** The Aflatoxin Biosynthetic Pathway.

Screening of different medicinal potentials of herbals and natural products is one the enormous interest of scientists to discover new and novel lead compounds that they can solve new health problems such as fungi-toxigenic and so on all over the world [11]. This study is intended to screen two different plants from two families: *E. connata* and *P. anisum*.

E. connata from Euphorbiaceae family and in Iran they mainly grow in Kerman, Yazd and Fars provinces [12]. A lot of fractions from Euphorbia have toxic effects and this genus has been used as anti-wart agents in Iranian traditional medicine, diterpenes are major compounds of *E. connata* [13]. The other plant which is used in this research is a grassy annual plant named *Pimpinella anisum* from Umbelliferea family and it cultivated in the Mediterranean region, Mexico, Chile and East of Asia (Iran, India) [13,14]. Various pharmacognostic compounds are determined in both extract and essential oil of this plant and anti-fungal activity is one of the different biological effects of them [15]. The goal of this study is evaluating of the anti-fungal activity of the *E. connata* and *P. anisum* extracts on the growth of fungi and *aflR* and *Nor1* genes expression process in *Asp. parasiticus*.

Material and Methods

Fungal Cell Preparation

Asp. parasiticus strain (ATCC 15517) was incubated for 48h at 30°C on Sabouraud Dextrose Agar media (SDA) (Merck, Germany). Then fresh colony sub cultured on Potato dextrose agar media (PDA) (Merck, Germany) and kept at 30°C for 5-7 days to produce spores. The preparation of the spore suspension was performed according to the previously mentioned procedure. The concentration of spores was calculated using the 0.5Mc Farland turbidity that each well contained 1.5-2 $\times 10^8$ CFUs/mL [16].

Preparation of *E. connata* and *P. anisum* extracts

For collection of plants, fresh stalks, leaves and flowers of *E. connata* and *P. anisum* were collected from wild areas of Iran in Kerman and Tehran provinces, after dried in dark place, the aerial parts were milled.

For extraction procedure, both extracts obtained by maceration technique, to do it, 100g of fine powdered parts of *E. connata* and *P. anisum* respectively, were immersed in 300ml methanol/water (80/20) and ethanol/water (70/30) and they were mixed on a shaker for 24h at room temperature and this procedure was repeated for more 3 times. After filtration, the extracts were concentrated by rotary evaporator at 40°C and they dried in a dry oven at 40°C and then kept in the refrigerator. To make homogeneous concentrations equal to 500mg/ml, each sample was prepared with sterile water/Tween 80 (80/20) solvent.

Determination of Minimal Inhibitory Concentration (MIC)

MIC was performed according to CLSI-document M38-A2 [17]. Sterile Roswell Park Memorial Institute (RPMI) (Sigma chemical Co.) buffered to a pH of 7.0 with 0.165M morpholine propanesulfonic acid MOPS) was used.

750mg of *E. connata* and *P. anisum* powders were dissolved in 1.5ml sterile diluted water to get a concentration of 500mg/mL, and then diluted to the final concentrations of 250-0.97mg/mL in the mentioned medium according to the CLSI protocol.

Microdilution plates (96 U-shaped wells, Roskilde, Denmark) were used for this purpose. First, 200µL each of extract (500µg/mL) was added to the first well. Then 100µL of RPMI was added to the second well, and then 100µL of the extract in the first well was infused to the second well and carried on until the eighth well. The solution in the eighth well was adjusted to give a final concentration of about 0.97µg/mL of each extract. Then for each extract, 100µL of *Asp. parasiticus* suspension was cultured with 1mL extractions in 9mL RPMI medium and incubated at 72h/30C. Controls contained RPMI medium with fungal suspension (positive control) and RPMI medium (Negative control).

All tests were performed in triplicate. The micro dilution plates incubated (48h/35°C). MIC determined on the base of lowest concentrations that could prevent any recognizable growth, optical determination of MIC was based on the lowest concentration that made a 100% inhibition for *E. connata* and *P. anisum* extracts.

For RNA extraction, mycelia mass was collected and frozen in liquid nitrogen. Total RNA molecules were isolated from logarithmic phase of normal fungal cells and fungal cells treated with extracts according to standard protocol [18]. Spectrophotometer (Bio photometer, Eppendorf, Hamburg, Germany) was used for measuring RNA concentration, then equal concentration of RNA (1µg in 20µL) were used to cDNA synthesis according to the kit protocol (Cinnagen co.) by random hexamer primers. A House keeping gene in this protocol was *β-actin* gene (*ACT1*) as normalizer. *aflR*, *Nor1* and *ACT1* primers were designed using the primer 3 software and were Synthesize on the basis of published sequence in NCBI (Table 1).

Step-One-Plus real-time PCR system (Applied Biosystems, Foster city, CA) was used for performing real-time PCR. PCR setup and program have been previously [7].

Gene	Primer Name	Sequence (5'-3')	PCR Product Size (bp)
Nor1	*Nor1- F*	5'-GTCCAAGCAACAGGCCAAGT-3'	66
	Nor1- R	5'-TCG TGCATGTTGGTGATGGT-3'	
aflR	*aflR- F*	5'-CGGAACAGGGACTTCCGGCG-3'	200
	AflR- R	5'-GGGTGGCGGGGGACTCTGAT-3'	
β-actin	β-actin- F	5'-ACGGTATTGTTTCCAACTGGGACG-3'	110
	β-actin- R	5'- TGGAGCTTCGGTCAACAAAACTGG-3'	

Table 1: Primers for Real-Time PCR.

Results

In current study, for evaluation of antifungal activities of *E. connata* and *P. anisum* extracts, broth micro dilution method CLSI document M38-A2 was used.

It is noteworthy, that the inhibitory effect of *E. connata* on *Asp. parasiticus* growth was Significant but *P. anisum* did not reveal any inhibitory effect on the growth. The results demonstrated that the extract of *E. connata*, inhibited *Asp. parasiticus* growth at MIC values of 31.25mg/ml. *P. anisum* which is inhibited *Asp. parasiticus* growth at MIC values of 125mg/ml.

Effect of *E. connata* and *P. anisum* extracts on *aflR* and *Nor1* genes expression showed in Table 2. Real-time quantitative PCR results showing relative quantification of *aflR* and *Nor1* gene expression levels (calculated according to ΔΔCt-method and represented the rate of *aflR* gene expression was significantly decreased after treating the *Asp. parasiticus* with *E. connata* compare to *P. anisum.* Here two scenarios are raise:

a) It seems the plant extracts via direct blocking of the *aflR* gene confer to lowering gene expression
b) May be over expression of other genes which involved the aflatoxin production play as negative regulatory effects of *aflR* gene and resulted to lowering *aflR* gene expression.

The *Nor1* gene was detected at a higher effect than the *aflR* gene in *Asp. parasiticus* after treatment with *E. connata* extracts. *β-actin* gene showed stability in *Asp. parasiticus* in the presence of *E. connata* and *P. anisum*.

There were statistically significant differences in *Nor1* and *aflR* gene expressions after treated with *E. connata* (P=0.001) (Figure 2). The *Nor1* mRNA expression levels were down regulated by 75% whereas the *aflR* mRNA expression level was down regulated by 25% (P<0.001) compared to the control group (Figure 2).

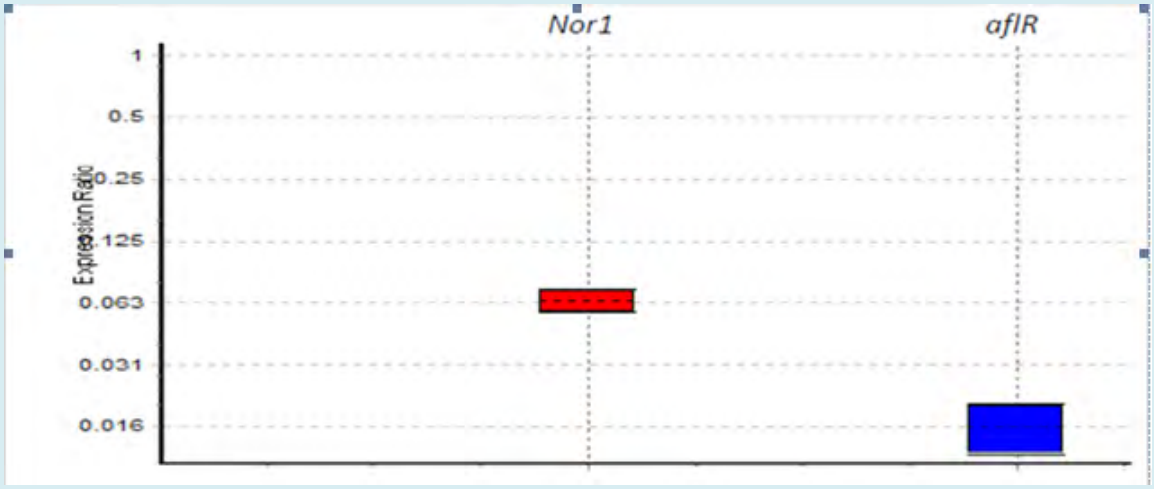

Figure 2: Comparison *aflR* and *Nor1* mRNA Expression Level in *E. connata*.

The results of expression of *aflR* and *Nor1* genes did not show any significant difference after treated with *P. anisum* (Table 2).

Extract	Gene	Type	Reaction Efficiency	Expression	Std Error	95% CI	Result
E. connata	*aflR*	TRG	1	0.015	0.011-0.020	0.011-0.020	Down expression
	Nor1	TRG	1	0.064	0.056-0.073	0.056-0.073	Down expression
P. anisum	*aflR*	TRG	1	0.109	0.091-0.121	0.084-0.132	Sample group is not different to control group
	Nor1	TRG	1	0.108	0.082-0.129	0.071-0.151	Sample group is not different to control group
	β-actin	REF	1	1	-	-	-

Table 2: Relative expression of *aflR* and *Nor1* genes using Real-Time PCR analysis.

aflR and *Nor1* genes expression were normalized to the housekeeping gene, *β-actin* and analyzed by using REST© software (2008, v.2.0.7). The software uses the comparative Ct method (ΔΔCt) to analyse the data. A sensitive strain (positive control) of *Asp. parasiticus* was included in each run of the experiment as a positive control.

Discussion

Aflatoxins are the most important mycotoxins produced by *Aspergillus* species (*Asp. flavus, Asp. parasiticus, Asp. sojae*, and *Asp. oryzae*) [19]. Aflatoxin B1 has been identified as the most toxic aflatoxin by the International Agency for Research on Cancer [20].

More than 20 different enzymes are involved in aflatoxin synthesis pathways, and most genes associated with aflatoxin production are located in a 75 kb region of the fungal genome [21-23].

The *aflR* gene has been implicated in the regulation of aflatoxin biosynthesis [24,25]. The biosynthesis is aflatoxin [26]. The *aflR* gene encodes the *AflR* protein, which has a zinc-finger motif of the GAL4 type and activates most of the structural genes of the aflatoxin production pathway such as *Nor-1* [27]. Research has shown that the absence of the *aflR* gene or the presence of an abnormal form of this gene blocks the expression of other genes in the aflatoxin production pathway [28,29]. *Aspergillus flavus* and *Aspergillus parasiticus* are able to grow on a variety of substrates and toxin production under humid conditions [30,31]. High levels of aflatoxin as a carcinogen in the liver can cause acute liver necrosis, cirrhosis or hepatocellular carcinoma. It can also cause bleeding, edema, altered digestion, altered absorption or metabolism of foods, adverse effects on the lungs, heart and kidneys [32,33]. Because aflatoxins are resistant to normal food processing conditions and are not degraded [31,34,35], therefore, an appropriate method is needed to break down aflatoxins while maintaining the quality and quality of the food. Available Aflatoxin degradation methods are costly and reduce the quality of food [36-38].

Reduction of the expression of *aflR* considered as an important target. As it has already been mentioned, Anti-fungal activity of methanol extract of *E. connata* against an aflatoxin-producing *Asp. parasiticus* have not evaluated yet. Most of scientific studies have been done on the essential oil of *P. anisum* and few articles showed this plant's extract biological activities; According to Mehmet Musa Ozcan, et al. anti-fungal activity of essential oil of fruit of *P. anisum* was evaluated against *Alternaria alternata*, *Asp. niger* and *Asp. parasiticus*. Results of study showed that anise essential oil has anti-fungal activity and can use in food preparations [15]. In the other study, Bluma RV, et al. the application of essential oils of some plants such as: *P. anisum* and their impact on *Aspergillus* growth parameters and aflatoxin accumulation were assessed. The result showed that this essential oil has a significant inhibitory effect on growth rate and aflatoxin accumulation [39]. In 2016, another study was done by Alsalhi M, et al. [40] who published their study in 2016, referred to efficacy of anti-microbial and cytotoxicity of nanoparticles using *P. anisum* seeds. These nanoparticles have effect on selected pathogens: *Staphylococcus pyogenes*, *Acinetobacter baumannii, Klebsiella pneumoniae*, *Salmonella typhi* and *Pseudomonas aeruginosa*.

The main compounds of genus *Euphorbia* are a complex mixture of macro-cyclic diterpenoids that they have different profiles of the acylation of their polyol core [41]. According to various applications of terpenes such as antibiotics effects [42], it can be said that likely anti-fungal activity of methanol extract of *E. connata* has a correlation with these components.

The plant *P. anisum* contain many secondary bioactive substances such as: terpenes and flavonoids [13], these two main compounds are responsible for the most biological effects of these two genera.

Conclusion

The results by real-Time PCR provided insights into the patterns of the two genes' mRNA expression levels in exposure with two extracts. In this study, the expression rates of *aflR*

and *Nor1* genes were significantly different (P=0.003 and P=0.001, respectively) after treated with *E. connata* extract, according to results, 25% and 75% down regulation in the *aflR* and *Nor1* genes were shown, respectively. So this extract could be a suitable candidate for control of toxin production by *Asp. parasiticus*. The data showed that the difference was not significant between two genes' mRNA expression (*aflR* and *Nor1*) after treated with *P. anisum* (P=1).

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