Original Article

Effects of thiamine on growth, aflatoxin production, and *aflr* gene expression in *A. parasiticus*

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Abstract

Background and Purpose: Mycotoxins are secondary fungal metabolites with a very high diversity that are produced by some species of *Aspergillus* which frequently leads to contaminate food and agricultural products. Recently, elimination of aflatoxin contamination in food and feed has been considered by scientists worldwide. Although, the antibacterial and antifungal effects of vitamins as natural compounds have been proven, the mechanism of vitamins effect on *Aspergillus parasiticus* growth and aflatoxin production is not yet clear. In this study, the effect of thiamine (vitamin B_1) was studied on *Aspergillus parasiticus* growth, aflatoxins production and the *afIR* gene expression.

Materials and Methods: A standard strain of *Aspergillus parasiticus* was applied for performing antifungal susceptibility test in different concentrations of thiamine. Antifungal susceptibility test was performed according to CLSI M38-A2 document. The concentration of aflatoxin was determined by HPLC. Moreover, the quantitative changes in the aflR gene expression were analyzed by Real Time PCR method.

Results: The minimum inhibitory concentration was yielded as > 500 mg/ml. However, HPLC analysis results showed that aflatoxin production reduced in samples treated with 500 mg/ml of thiamine. In addition, the level of *afIR* gene expression was significantly reduced after treating with 500 and 250 mg/ml of vitamin B_1.

Conclusion: Based on the obtained results, thiamine could not inhibit the fungal growth completely. However, the rate of *afIR* gene expression and aflatoxin production was significantly reduced after fungal treating with thiamine. Consequently, using natural compounds such as vitamins may be regarded as potential antitoxic agent in food industry and the industries related to agriculture.

Keywords: Aflatoxin, *AfIR*, *Aspergillus parasiticus*, Gene expression, Thiamine

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Introduction

Aflatoxins, as secondary fungal metabolites, are capable of producing acute toxic, carcinogenic, mutagenic, teratogenic and estrogenic effects in humans and animals exposed to the toxin [1-3]. This toxin is often produced by *Aspergillus flavus* and *Aspergillus parasiticus* [4] and cause loss in a variety of food and stored grains, especially wheat, corn, peanuts, nuts, linseed and cotton seed [1,5] and aflatoxicosis is defined as poisoning and side effects of consuming aflatoxin on food [3]. The toxin can cause harmful effects in different species of animals and humans [1, 2]. Recently, epidemiological studies have indicated that the incidence rates of liver cancer and cirrhosis within the communities are much higher in areas with heavy contamination of food with aflatoxin [3]. *aflR* and *aflJ* genes plays an important regulatory role in the biosynthesis of aflatoxins [7-10]. At least 32 enzymatic reactions involved in the biosynthesis of aflatoxin [7,8]. The protein encoded by *aflR* binds to palindromic sequences located in the promoter of many genes involved in biosynthesis of aflatoxin in *Aspergillus flavus* and *Aspergillus parasiticus* [7-9]. The intervening *afIR* genes group is involved in

transcription and production of aflatoxin protein [7,9,10]. Thus, countering with production of aflatoxin or its removal from food and agricultural products has drawn the attention of many experts during the past years. The antibacterial and antifungal effects of many vitamins have been demonstrated so far and it appears that these natural ingredients can be effective and healthy alternatives for some chemicals and drugs with similar effects. Thiamine is the first vitamin discovered of the "B" group vitamins [11,12]. This water-soluble vitamin contains sulfur and was discovered in1901 by Jansen, Donath, Windhaus, Van veen and Okade [13, 14]. Thiamine has a very important impact on carbohydrates metabolism as well as the nervous system function [13,14]. However, in vitro mechanism of action of thiamine in inhibition of fungal growth and toxin production is not completely understood. According to the above mentioned, exploring factors which are effective on the production of mycotoxin has been always the researchers' focus of attention. Therefore, in this study, the effect of thiamine on the growth of fungi, aflatoxin production and *afIR* gene expression process in *A. parasiticus* was evaluated.

Material and Methods

Strain

The standard strain of *A. parasiticus* (ATCC15517) was cultured on Sabouraud Dextrose agar (SDA) medium (Merck, Germany), and incubated at 30°C for 3 days.

Antifungal susceptibility testing

Antifungal susceptibility test was performed according to recommendations confirmed in the Clinical and Laboratory Standards Institute (CLSI,2008) M38-A2[6]. Although ROMI medium contains thiamin, the concentration is less than effective and does not interfere with the results. Hence using CLSI method is reliable[6].

Vitamin B_1 (thiamin mono-nitrate) was developed as a powder from Osvah pharmaceutical Company. It was dispensed into 96-well microdilution trays at a final concentration of 500-62.5 mg/ml. fungal colonies were suspended in sterile distilled water and adjusted spectrophotometrically at 530 nm wavelengths to an optical density (OD) that ranged from 0.09−0.13. A working suspension was made by a 1:50 dilution of the stock suspension with RPMI medium which resulted in 0.4×10^4 to 5×10^4 CFU/ml. Microdilution plates were incubated at 35°C and examined visually after 24 and 28 h as the concentration of drug that elicited significant inhibition of growth compared with a vitamin-free control. Positive and negative controls were prepared according to the above protocol.

Aflatoxin extraction using High Performance Liquid Chromatography (HPLC) method

The amount of toxin by the fungus was measured by HPLC method when exposing to 500 mg/ml of thiamine. Two samples, one associated with 5 ml of fungal suspension (4×10^4) and the other containing 50 µl of pure aflatoxin were used as positive and the standard sample, respectively.

Negative control (Blank) containing 10 cc of PDB medium alone was also run. All samples were incubated for 10 days at 35 ° C. After 10 days of incubation, the volumes of the PDB medium of the test sample and the positive control were measured. Then, 1.353 g of extra pure NaCl (Merck, Germany) and 67.65 ml of 80% methanol as extraction solvent were added to the test samples. An Amount of 0.982 g of NaCl and 49.1 cc of 80% methanol were added to the positive control. Each of the samples content was added separately into blender at 20,000 rpm for 10 min (2,000 rpm for 30 min) for producing uniformity, the contents of each were separately passed through a funnel containing Whatman filter paper, No. 4 and was poured into the 50 ml Falcon tube. Then, 34 ml of PBS (Phosphate Buffered Saline) was added to a 6 ml of the filtered solution and passed through the glass-fiber filter paper (GFFP).The column temperature was adjusted to the laboratory temperature, and 10 ml of PBS was passed through it. A volume of 10 ml of diluted extract was passed through the column at the velocity of one drop per second. The column was washed with 15 ml of PBS,

and then the column was dried with gentle pressure of air for 10-15 seconds. Aflatoxin exits the column during two stages as follows: A volume of 500 ml of methanol (MeOH - HPLC) was added to the column and the removed solution was collected in a vial. After one minute stop, 750 ml of methanol (MeOH - HPLC) was added to the column and the effluent solution was collected in the same vial. Then, 1750 ml of water (H2O - HPLC) was added to the mentioned vial and mixed with a vortex. The column was then rinsed with 20 ml of PBS, and finally, 200 ml of the extract obtained was injected into the HPLC machine (Scanning fluorescence detector waters TM 474).

Total RNA Extraction and cDNA synthesis:

Total cytoplasmic RNA molecules were isolated from normal as well as thiamin-treated fungal cells (125, 250, 500 mg/ml of vitamin $B_{1)}$ which incubated at 35°C for 72 h by a standard method. Briefly, the harvested mycelial mass was flash-frozen in liquid nitrogen and ground to a fine powder. The mycelial powder was suspended to goanidine Isothiocyanate (GITC 4 M, 2% b-mercaptoethanol, 1% sodium lauroyl sarcosine (SLS) in final concentration) and then hemogenized. Afterward, sodium acetate was added in 1/10 volume of suspension. Phenol and choloroform was then added in equal and 1/5 volume, respectively. Cellular debris was removed by centrifugation at $1200 \times g$ for 5 minutes. Isopropanol was added to supernatant in equal volume and incubated at -20°C for 60 min and RNA was extracted by centrifugation at $1200 \times g$ for 10 minutes. The pellet was then rinsed with cold ethanol and finally dissolved in hot Tris-EDTA (pH=8) buffer. RNA concentration and purity were determined spectrophotometrically (Eppendorf, Germany biophotometer) and equal concentration of RNA (1 µg in 20 µl) were subjected to cDNA synthesis by use of the PrimeScript RT reagent kit (Fermentas, USA).

Primer designing and Real-time PCR:

Primer sequences of "SENSE" and "ANTI SENSE" were designed by specific software (NCBI, Primer 3 plus and Gene Runner) while observing the principles of primers designing according to the target sequence, i.e. the *aflR* gene sequence. The primer sequences for aflR and β-Actin genes are as follow: Sense primer 5'-CggAACAgggACTTC CggCg - 3'; Antisense primer 5'-gggTggCgggggACTCTgAt -3'; Sense primer for βActin gene: 5'-ACgg TAT TTCCA ACTgggACg-3'; Antisense primer for β-Actin gene: 5'-TggAgCTTCggTCAACAAAACTgg-3') To perform Real-time PCR, Power SYBR ® Green Master Mix Kit by Applied Biosystem Company was applied. The βactin gene (endogenous reference gene) was used for gene normalization. The qReal Time PCR method was performed by using the Applied Biosystems StepOne Plus (ABI, USA). The program used for performing quantitative PCR included an initial denaturation step for 30 seconds at 95°C. The original program consisted of heating the materials mixture for 40 cycles, as follows: Denaturation for 5 seconds at 95°C and Annealing & Extension for 30 seconds at 60°C.

Results

Growth inhibitory effect of different concentrations of thiamin on A. parasiticus:

In microscopic examination, wet smears were prepared from all provided dilutions of thiamine, and finally, imaging from the slides was performed to include an average of all the examined chambers. After fungal treatment, the fungal growth was not inhibited completely and the minimum inhibitory concentrations of fungal growth was higher than 500 mg/ml. Fungal mycelia growth was associated with significant reduction compared to the positive

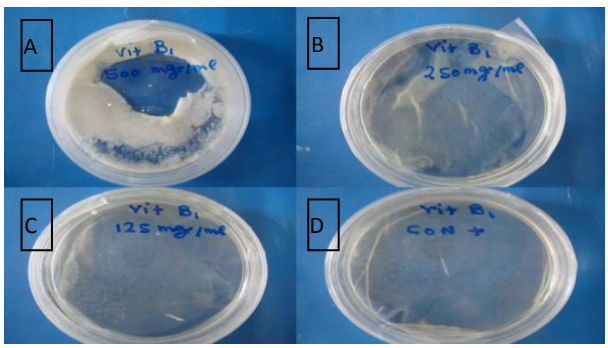

Figure 1. (A) Sample containing 500 mg/ml of vitamin B_1; (B) Sample containing 250 mg/ml of vitamin B_1; (C) Sample containing 125 mg/ml of vitamin B_1; (D) Positive control sample

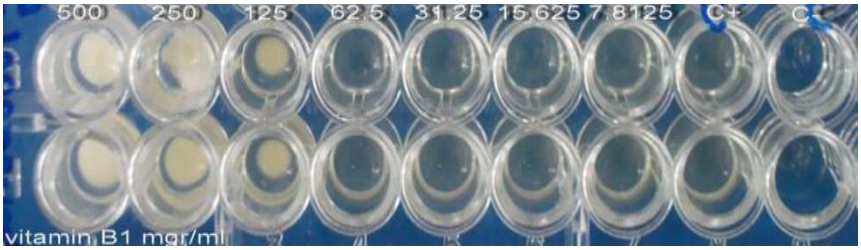

Figure 2. A. parasiticus colonies surface exposed to different concentrations of vitamin B1 after 2 days

Figure 3. Microscopic examination of mycelia growth of *A.parasiticus* after treatment with vitamin B_1; (A) fungal mycelia growth at dilution of 500mg/ml (B) fungal mycelia growth at dilutions of 250 mg/ml (C) Fungal mycelia at dilution of 125 mg/ml; (D) Positive control sample

control sample, while showing increased growth at concentration of 125 mg/ml and at lower dilutions (62.5, 31.25, 15.62, 7.8125 mg/ml) (Figures 1-3).

Effect of different concentrations of thiamine on afIR gene expression

Spectrophotometry method was used for examining and measuring the purity and accurate amount of RNA. The results of [Optical Density: OD] OD260/OD280 were obtained as 1.8-2 for each sample and the positive control, which indicated the high purity of the extracted RNA. Agarose gel electrophoresis experiment confirmed the accuracy and integrity of RNA extraction (Figure 4).

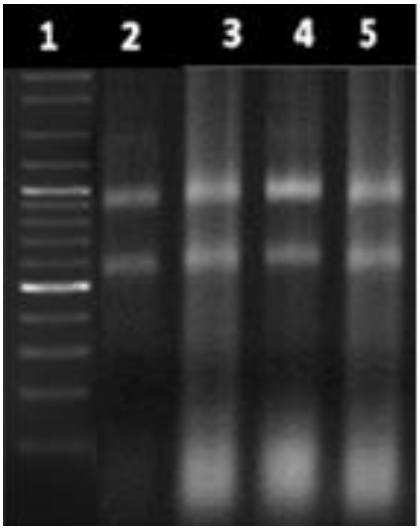

Figure 4. Electrophoresis of extracted RNAs on agarose gel: 1.ladder (100 bp), 2.Vitamin B_1: 500 mg/ml, 3.Vitamin B_1: 250 mg/ml, 4.vitamin B_1 125mg/ml, 5.Control positive

The Real Time PCR was performed using SYBR Green method. The obtained melt curves showed the specificity of the primers (Figure 5). The transcription level of *afIR* gene was significantly affected by 500 mg/ml and 250 mg/ml of vitamin B_1, however, there was a significant increase at a concentration of 125 mg/ml of vitamin B_1 (Figure 6 and Table 1). Amplification curves for control and treated samples are demonstrated in figure 7.

Table1. The *afIR* gene expression rate by Real Time PCR method according to the Rest software

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
Beta Act	REF	1.0	1.000				
Control positive	Control positive	1.0	1.000				
AflR (Vit B500)	TRG	1.0	0.045	0.039 - 0.051	0.035 - 0.051	0.076	DOWN
AflR (Vit B250)	TRG	1.0	0.045	0.039 - 0.053	0.034 - 0.054	0.033	DOWN
AflR (Vit B125)	TRG	1.0	48.044	41.823 - 59.726	34.917 - 61.992	0.000	UP

Legend: *P (H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance. TRG-Target REF Reference*

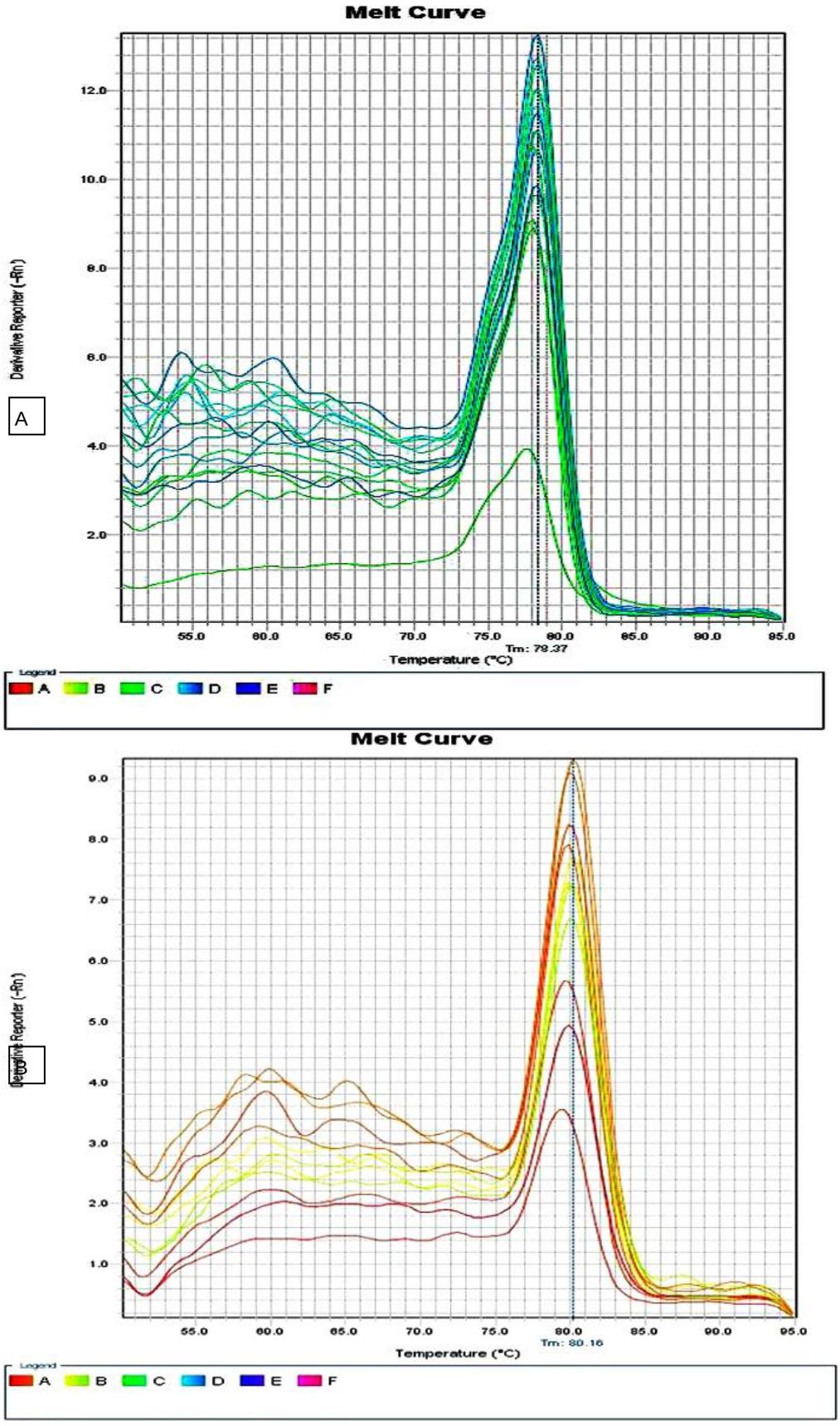

Figure 5. melt curves (A) melt curve of *β-actine* gene,(B)melt curve of *aflR* gene
Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations

Measurement of aflatoxins using HPLC method

No aflatoxins were extracted in sample 1 (negative control). In the sample 2 (standard toxin), a variety of aflatoxins, including G2, G1, B2 & B1 were isolated that the total amount of various aflatoxins was estimated as 6.70 PPM (Parts Per Million), while in sample 3 (positive control), all types of aflatoxins were isolated and the total amount of produced aflatoxins was as 430.90 PPM. Compared with the standard sample, the toxin produced by *A.parasiticus* was much more. In sample 4, which contained500 mg/ml of vitamin B_1, B_1 and G_1 aflatoxins

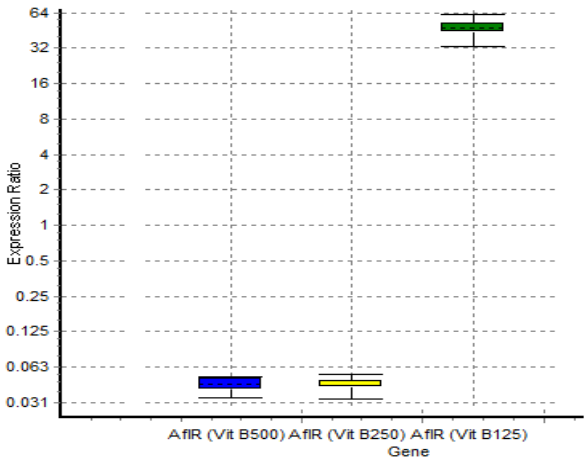

Figure 6. Comparison of aflR gene expression at different dilutions of vitamin B1

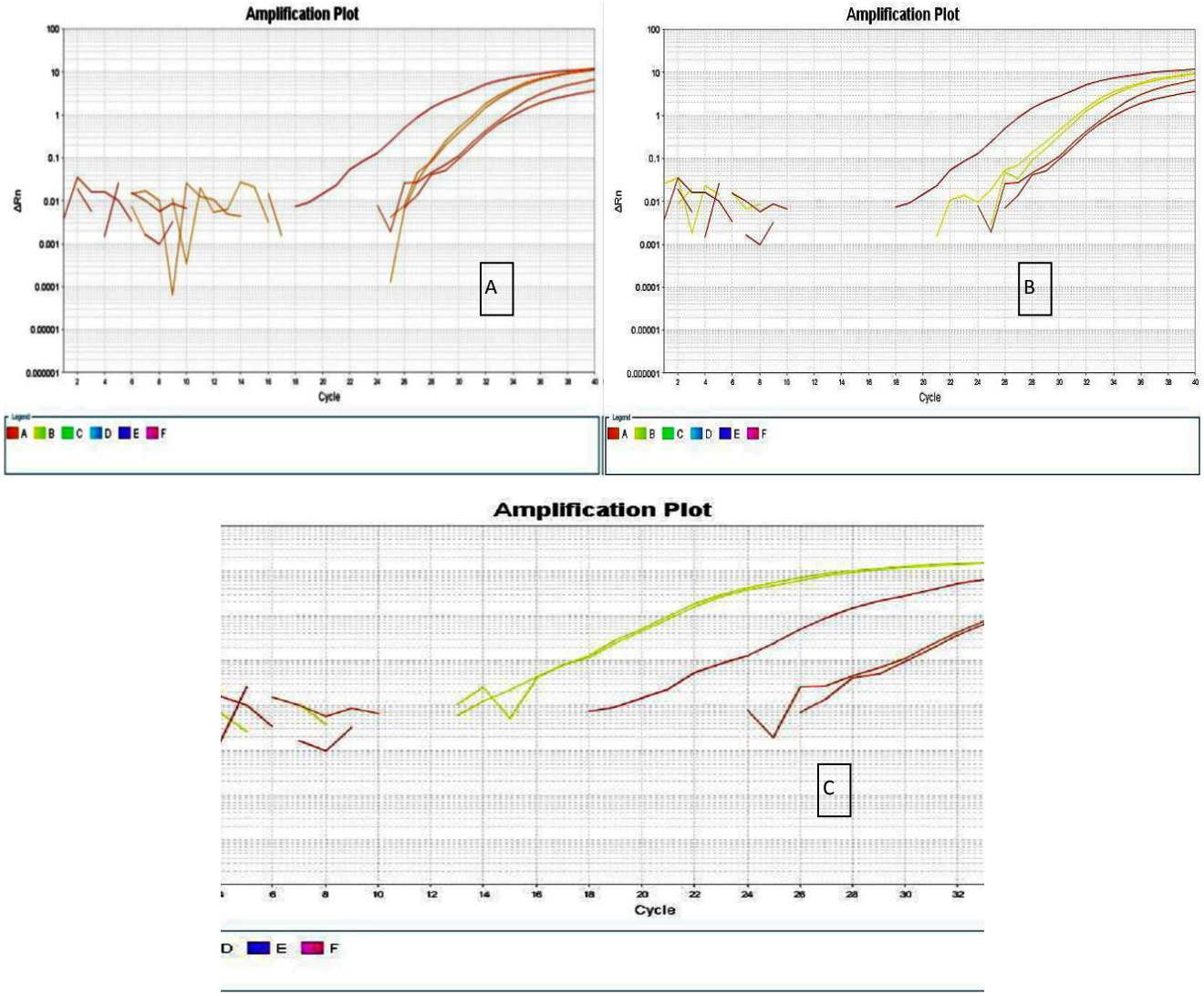

Figure 7. Comparison of *aflR* gene expression at different dilutions of vitamin B_1 with control positive and control negative samples (A)500mg/ml (B)250mg/ml (C)125 mg/ml

Table 2: HPLC Results (1) Negative control (2) Standard toxin sample (3) Positive control (4) Sample containing 500 mg/ml of thiamine

	Aflatoxin B	Aflatoxin B	Aflatoxin G1	Aflatoxin G2	Total
Negative control	00.0	0.00	0.00	0.00	0.00
Standard toxin sample	2.95	0.60	2.61	0.54	6.70
Positive control	267.40	8.10	149.90	5.50	430.90
Sample containing 500 mg/ml of thiamine	0.81	0.00	0.80	0.00	1.61

were isolated and the total amount of produced aflatoxin was as 1.61 PPM that significantly inhibited toxin production compared with the positive control (Table 2). HPLC results analysis in a concentration of 500 mg/ml of vitamin B_1 showed a significant reduction in comparing of aflatoxinin control positive sample. It should be noted that in this study all the experiments were repeated as 3 times.

Discussion

Mycotoxins are secondary fungal metabolites with a very high diversity that are produced by some species of *Aspergillus*, including A. *parasiticus*, *A. flavus* and *A. nomius*, which frequently leads to contaminate food and agricultural products [3]. According to the Food and Agriculture Organization (FAO), 25% of the world's annual food is affected by mycotoxins [1, 3]. The presence of mold in food does not necessarily prove the presence of mycotoxin and its absence does not also implicate the absence of toxin in food, since mycotoxins will remain long after the disappearance of toxigenic molds in food. Although molds can grow and produce toxin in many food in different conditions such as humidity, PH and temperatures, however, they usually grow better in food maintained under hot and humid conditions [3]. Aflatoxins are the most potent cancer-causing agents among the known natural compounds [3]. In most Western countries, the allowed limit of aflatoxins in human food is as 5-20 ppb. The U.S. Food and Drug Administration have determined the allowed levels of AFB1 in many foods as 20 ppb and the allowed levels of AFM1 in milk as 0.5 ppb. In many European countries, the allowed level of aflatoxin in food is as 3-5 ppb [3]. The European Union has determined the rate of AFM1 in milk and infant formula as 0.05 and 0.025, respectively [3]. Thus, inhibition of aflatoxin production by fungi or its removal from food and agricultural crops has drawn the attention of many researchers over the past years. The main objective of this study was to evaluate the effect of vitamin B_1 on the growth of *A. parasiticus* and aflatoxin production by studding the *afIR* gene expression in *A. parasiticus*. In the present study, fungal growth evaluation after treatment with various concentrations of vitamin B_1 showed the minimum inhibitory concentrations of fungal growth higher than 500 mg/ml, and the fungal mycelia had growth in all examined dilutions; however, in dilutions of 500 and 250 mg/ml comparing with the positive control sample, the growth of fungal mycelia was associated with a significant decrease, while it was increased when exposing with 125 mg/ml and lower thiamin concentration. In addition, the *afIR* gene expression rate was investigated in the study following fungal treatment at different concentrations of vitamin B_1. The *afIR* gene is a positive regulatory gene in aflatoxin biosynthesis; in the first stage, in which the norsolorinic acid is formed, the *afIR* functional alleles are required.

This gene is required in duplication practice of most structural genes in *A.flavus*, *A.parasiticus* and *A.nidulans*. It was shown that the *afIR* gene expression rate changes in *A. parasiticus* under the influence of different concentrations of thiamine. Accordingly, the *afIR* gene expression rate showed a 95.5% decrease at concentrations of 500 and 250 mg/ml of vitamin B1. HPLC has been used as a selective method for the determination of aflatoxins. In this study, the production rate of aflatoxin was evaluated in samples of negative control, positive control and samples treated with concentration of 500 mg/ml of vitamin B1 by HPLC method. The analysis of HPLC showed that the aflatoxin production

was reduced as 99.6% compared to the 10 day positive control sample after 10 days of treatment with a concentration of 500 mg/ml of vitamin B1.

In 1996, Hamdy Aly Emara studied the effects of riboflavin and pyridoxine on *A.parasiticus* on Czepek's DOX Broth medium at two concentrations of 5 Mm and 10 Mm. Vitamin B_2 (riboflavin) stimulated the AFB1 growth and production at concentrations of 5 Mm and 10 Mm. Vitamin B_6 (pyridoxine) inhibited the growth and toxin production in a dilution of 5 Mm as 52.8% and 70%, respectively, while the dilution of 10 Mm inhibited growth and toxin production as 46.8% and 42.5%, respectively [4]. In this study, the effect of different concentrations of thiamine in the RPMI medium on *A. parasiticus* was examined where vitamin B_1 (thiamine) in dilutions of 500 and 250 mg/ml inhibited the aflatoxin production as much as 99.6%, while the concentration of 125 mg/ml of vitamin B_1 led to increased fungal growth and significantly strengthened the *afIR* gene expression. In 1997, Hamilton reported that deficiency of vitamins A, D_3 and riboflavin (B_2) in the diet of chickens makes them sensitive to aflatoxin, but thiamine deficiency has a reversed impact and the effects of vitamins K and E are unclear [15]. Our results demonstrated that thiamine can reduces fungal growth, inhibits the production of aflatoxin and the *afIR* gene expression at high concentrations (500 and 205 mg/ml),. In contrast, it may increase the fungal growth and strengthen the *afIR* gene synthesis and at low concentrations. According to the results of the present study, although the concentrations of 500 and 250 mg/ml of vitamin B_1 did not inhibit the fungal growth completely, the fungus growth and aflatoxin production were significantly decreased after fungal treatment with thiamine.

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Authors' Contributions

Sasan Rezaie and Maryam Moazeni designed and managed the research. LadanNazemi performed the tests and wrote the draft manuscript. Maryam Moazeni performed Real Time PCR and analyzed the data. Sassan Rezaie edited the final manuscript. Maryam Akbari Dana, Parivash Kordbacheh and Roshanak Daei Ghazvini were project partners.

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Financial Disclosure

No financial interests related to the material of this manuscript have been declared.

Conflicts of Interests

There is no conflict of interest.

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