



Effect of Extracts of *Capparis spinosa*, *Withania somnifera* and *Peganum harmala* on *Aspergillus flavus* Growth and Expression of Major Genes in Aflatoxin Biosynthetic Pathway

Ahmad Mehraban¹, Javad Abkhoo^{2,*} and Ebrahim Dahmardeh²

¹Department of Agronomy, Zahedan Branch, Islamic Azad University, Zahedan, Iran

²Agricultural Research Institute, University of Zabol, Zabol, Iran

*Corresponding author: Agricultural Research Institute, University of Zabol, Zabol, Iran. Email: javad.abkhoo@yahoo.com

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Abstract

Background: Aflatoxins, a polyketide group derived from furanocoumarins, are highly toxic carcinogens produced by *Aspergillus flavus* and other closely related species.

Objectives: In this research, the effects of *Capparis spinosa*, *Withania somnifera*, and *Peganum harmala* extracts on *A. flavus* growth and the expression of some major genes in aflatoxin biosynthetic pathway were investigated.

Methods: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were measured and the expression of *aflQ*, *aflO*, *aflS*, and *aflR* genes was evaluated by using quantitative real-time polymerase chain reaction (qRT-PCR) technique.

Results: *Capparis spinosa* extract had the lowest MIC value (200 mg/mL) and the extracts of *W. somnifera* and *P. harmala* had the highest MIC value (400 mg/mL). The extracts of *W. somnifera* and *P. harmala* had no fungicidal activity, and the *C. spinosa* extract had the lowest MFC value (400 mg/mL) and had the highest fungicidal effect on *A. flavus*. The expression of *aflQ*, *aflO*, *aflS*, and *aflR* genes was significantly decreased by the *C. spinosa* extract.

Conclusions: In general, the *C. spinosa* extract could have fungicidal and inhibitory effects on *A. flavus* and reduce expression of major genes in the aflatoxin biosynthetic pathway.

Keywords: Antifungal Activity, Aflatoxin, *Aspergillus flavus*, *Capparis spinosa*, *Withania somnifera*, *Peganum harmala*

1. Background

Aflatoxins, a polyketide group derived from furanocoumarins, are highly toxic carcinogens produced by *Aspergillus flavus* and other closely related species (1). The four major types of aflatoxins are B1, B2, G1, and G2. Aflatoxin B1 is more important because it is more toxic and more carcinogenic (2). Aflatoxin contaminated foods can lead to acute hepatitis, immune suppression, liver cancer, and neutropenia (3). A collection of 25 genes within a 75-kb region of genome *A. flavus* are involved in aflatoxin production (1, 4). Studies have shown that there is a high correlation between the production of aflatoxin and the expression of *aflQ*, *aflO*, *aflD*, *aflS*, and *aflR* genes in *A. flavus* (5, 6).

Biological and chemical control of pistachio *A. flavus* is restricted or prohibited due to international rules and regulations (7). Therefore, studies have focused on the use of natural compounds, especially essential oils and plant extracts, to prevent the growth of these fungi and the production of aflatoxin (8-15). Pasture plants, *Capparis spinosa*, *Withania somnifera*, and *Peganum harmala* have been proved to have antifungal effects. Studies have

shown that *W. somnifera* extracts have antifungal properties against *Fusarium oxysporum* and *Colletotrichum capsici* (16, 17), and methanolic extract of *W. somnifera* has fungicidal properties against *A. flavus* and *A. niger* (18, 19). Furthermore, the ethanolic extract of *W. somnifera* has fungicidal properties against *Candida albicans* (20). *Capparis spinosa* has antifungal properties (21-23). Studies have shown that *P. harmala* extract has fungicidal properties (24-27).

2. Objectives

The purpose of this study was to investigate the effect of extracts of some pasture plants on *A. flavus* growth and expression of some major genes in aflatoxin biosynthetic pathway.

3. Methods

The present experimental study was conducted at the agricultural research institute, University of Zabol and approved by the ethical committee of the Islamic Azad University of Zahedan.

3.1. Plant Material and Preparation of Plant Extracts

Plants *C. spinosa*, *W. somnifera*, and *P. harmala* were collected from Sistan and Balochestan province, Iran. The collected plants were identified at the department of Medicinal plants of agricultural research institute, University of Zabol. The seeds of *P. harmala* and leaves of *C. spinosa* and *W. somnifera* were ground using a mortar and pestle. Ten grams of grinded powder was soaked in 100 mL of 96% ethanol for one day. The supernatant was filtered through Whatman No. 1 filter paper. The dried extracts were preserved at 4°C for further use (28).

3.2. Minimal Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC was determined by a microplate method (29). The MFC values of plant extracts were determined using the method described by Espinel-Ingroff et al. (30).

3.3. Effect of *C. spinosa* Extract on Aflatoxin Biosynthesis-Related Genes

The standard isolate of *A. flavus* PFCC 116 was cultured in potato dextrose broth (PDB, Sigma-Aldrich, Germany) (as control) and PDB medium containing 200 mg mL⁻¹ of *C. spinosa* extract and incubated at 35°C for three days. After three days of incubation, the fungal mycelia was harvested. Total RNA was extracted using the *Top* plant and fungi RNA purification kit (TOPAZ GENE RESEARCH, Iran), based on the method described in the kit's manufacturer's instructions. The first strand of cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, Massachusetts, U.S.A.). Expression of genes was evaluated using the quantitative real-time PCR (qRT-PCR) technique with Eva Green fluorescent dye and their specific primers. The qRT-PCR was performed with a Rotor-Gene 3000 (Corbett Robotics, Australia). The process was performed with 20 µL of reaction mixture consisting of 1 × HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Riia, Estonia), 1 µL cDNA, and 10 µM of each forward and reverse primer. The following protocol was applied: initial polymerase activation: 15 minutes at 95°C; 40 cycles of 30 seconds at 95°C, 30 seconds at 61°C, and 45 seconds at 72°C. The characteristics of the primers used for qRT-PCR are listed in Table 1. The relative changes in the expression of target genes were normalized to the β -tubulin housekeeping gene (31). Analysis of relative gene expression data was carried out using the Relative Expression Software Tool (REST) 2009 software version 2.0.13 (Qiagen, Hilden, Germany) (32).

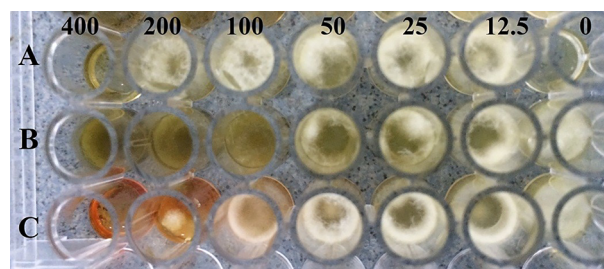


Figure 1. The level of *Aspergillus flavus* colonies in different concentrations of extracts of *Withania somnifera* (A) *Capparis spinosa* (B), *Peganum harmala* (C), and control

3.4. Statistical Analysis

In this experiment, three replicates were considered for each treatment and the meanings were compared with t-test using SPSS statistics version 19 (IBM, U.S.A). The differences with $P < 0.05$ were considered significant.

4. Results

4.1. Antifungal Activity

The results of the effects of different concentrations of *C. spinosa*, *W. somnifera*, and *P. harmala* extracts on *A. flavus* PFCC 116 growth two days after incubation at 35°C is shown in Figure 1. *Aspergillus flavus* covered the entire surface of the control microplate and in other microplates, the fungal growth rate decreased with increasing concentration of extracts, and in comparison with other microplates, the control microplate had the ability to inhibit growth of *A. flavus* by the extracts. The MICs of *C. spinosa*, *W. somnifera* and *P. harmala* extracts were 200, 400, and 400 mg/mL, respectively (Table 2). The MIC values ranged from 200 to 400 mg/mL, and the *C. spinosa* extract had the lowest MIC value (200 mg/mL), and *W. somnifera* and *P. harmala* extracts had the highest MIC value (400 mg/mL).

Fungicidal effects of different concentrations of *C. spinosa*, *W. somnifera*, and *P. harmala* extracts on *A. flavus* is shown in Table 2. The MFC of *C. spinosa* extract was 400 mg/mL and the extracts of *W. somnifera* and *P. harmala* had no fungicidal activity, and the *C. spinosa* extract had the lowest MFC value (400 mg/mL) and had the highest fungicidal effect on *A. flavus*.

4.2. Effect of *C. spinosa* Extract on Expression of Major Genes in aflatoxin Biosynthetic Pathway

Results of expression of *aflR* gene in samples treated with *C. spinosa* extract (200 mg/mL) and non-treated (control) *A. flavus* PFCC 116 are shown in Figure 2. After treatment with *C. spinosa* extract, a strong reduction (98.8%) was

Table 1. Nucleotide Sequences of Primers Used in This Study

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Amplicon Length, bp	Tm, °C	Accession Number
<i>aflR</i>	CTCAAGGTGCTGGCATGGTA	CAGTGCCACTGTGTGGTTTC	86	61	XM-002379905
<i>aflQ</i>	GTGTCGCGAGTGTCTAGCTT	GCTCAAAGGTCCGAGAGTA	115	61	XM-002379890
<i>aflO</i>	GGGACGATTTGATGGAGCAG	TTAGGTCTCTTGGCTACAGCA	101	61	XM-002379892
<i>aflS</i>	AAGCTAAGGCCGAGTCTGG	TGCTCGTAGTTTCAAGGCA	75	61	XM-002379904
β -tubulin	CTTGTTGACCAGGTTGTGGAT	GTCGCAGCCCTCAGCCT	51	61	

Table 2. Minimal Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Crude Extracts from Plant Species Against *Aspergillus flavus*

Plant Species	MIC	MFC
<i>Withania somnifera</i>	400	-
<i>Capparis spinosa</i>	200	400
<i>Peganum harmala</i>	400	-

observed at the transcriptional level of *aflR* gene. The results showed significant differences between the expression of *aflR* gene expression in treated and non-treated samples at 5% level. Results of determination of expression of *aflQ* gene in samples treated with *C. spinosa* extract (200 mg/mL) and non-treated (control) *A. flavus* PFCC 116 are shown in Figure 2. After treatment with the *C. spinosa* extract, a strong reduction (99.9%) was observed at the transcriptional level of the *aflQ* gene. The results showed significant differences between the expression of *aflQ* gene expression in treated and non-treated samples at the 5% level. Results of determination of expression of *aflO* gene in samples treated with *C. spinosa* extract (200 mg/mL) and non-treated (control) *A. flavus* PFCC 116 are shown in Figure 2. After treatment with the *C. spinosa* extract, a strong reduction (96.9%) was observed at the transcriptional level of the *aflO* gene. The results showed significant differences between the expression of *aflO* gene expression in treated and non-treated samples at the 5% level. Results of determination of expression of *aflS* gene in samples treated with *C. spinosa* extract (200 mg/mL) and non-treated (control) *A. flavus* PFCC 116 are shown in Figure 2. After treatment with the *C. spinosa* extract, a strong reduction (98.4%) was observed at the transcriptional level of the *aflS* gene. The results showed significant differences between the expression of *aflS* gene expression in treated and non-treated samples at the 5% level.

5. Discussion

In this research, the antifungal properties of the ethanol extract of *C. spinosa* were investigated against a toxinogen isolate of *A. flavus* and its effect on the reduction

of the expression of the main genes in aflatoxin biosynthetic pathway, *aflR*, *aflQ*, *aflO* and *aflS* genes. According to several reports, *C. spinosa* had antifungal activity at certain concentrations. Mahboubi and Mahboubi (21) investigated antifungal activity of ethanolic extract of root and fruit of *C. spinosa* against *A. niger*, *A. parasiticus*, and *A. flavus*. The ethanolic extract of root and fruit of *C. spinosa* showed fungicidal and inhibitory effects against *A. niger*, *A. parasiticus* and *A. flavus*, which was similar to the results of the current research. Ali-Shtayeh and Abu Ghdeib (22) examined the antifungal properties of aqueous extract of *C. spinosa* against *Microsporum canis*, *Trichophyton mentagrophytes* and *Trichophyton violaceum*. The aqueous extract of *C. spinosa* completely prevented the growth of *M. canis* and *T. violaceum*. Al-Askar (23) examined the antifungal properties of ethanolic extract of *C. spinosa* in vitro against *Alternaria alternata*, *F. oxysporum*, *Phoma destructiva*, *Rhizoctonia solani* and *Sclerotium rolfsii*. The *C. spinosa* extract had different degrees of antifungal properties against the tested fungi.

In this study, the *C. spinosa* extract reduced expression of *aflR*, *aflQ*, *aflO* and *aflS* genes. The *aflR* gene is required for transcriptional activation of most structural genes in *A. flavus* (1). The *aflS* gene enhances the transcription of toxigenic genes in *A. flavus* and the *aflO* and *aflQ* genes, producing O-Methyltransferase B and Cytochrome P450 monooxygenase enzymes, are needed for biosynthesis of aflatoxins (1). Verheecke et al. (33) examined the expression of aflatoxin genes during the interaction of *Streptomyces Aspergillus*. Expression of *aflR* and *aflS* was generally repressed in *A. flavus* and *A. parasiticus*. Al-Saad et al. (34) investigated the effect of four bacteria antagonists against *A. flavus* to control the production of aflatoxin B₁. These studies showed that some of the bacterial species could significantly inhibit the relative expression of the *aflD* and *aflR* genes. Mohseni et al. (12) studied the effect of *Glycyrrhiza glabra* extract on *aflR* gene expression and aflatoxin production in *A. parasiticus*. Five hundred mg/mL concentration of *G. glabra* extract inhibited *aflR* gene expression and consequently the aflatoxin production efficiently in *A. parasiticus*. Jahanshiri et al. (35) studied Eugenol effect on

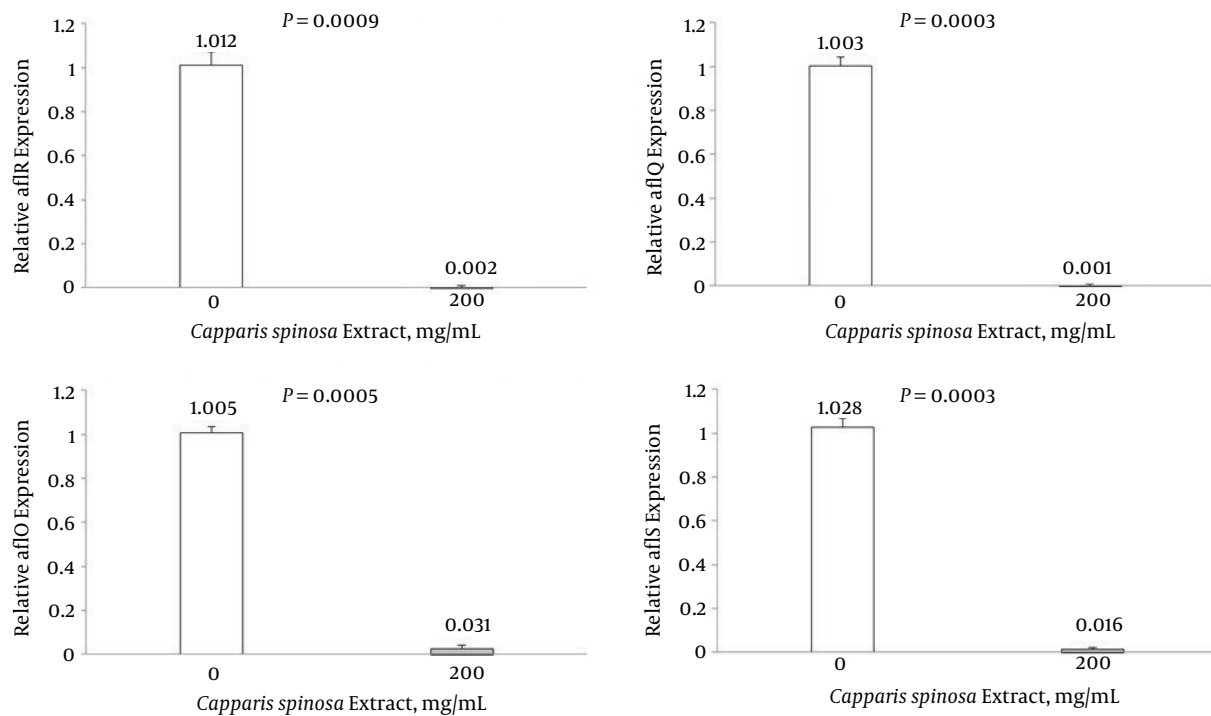


Figure 2. Level of mRNA expression of *aflR*, *aflQ*, *aflO* and *aflS* in the treated (200 mg mL⁻¹) and the non-treated (control) samples in *Aspergillus flavus*. Each sample is normalized for the amount of the template to the levels of β -tubulin. Significant differences were observed for mRNA levels for each of gene between the treated and the non-treated fungal cell ($P < 0.05$). The bars represent the mean \pm SD values.

A. parasiticus growth and aflatoxin production and the expression of major genes involved in the aflatoxin biosynthesis pathway. Eugenol (62.5 and 125 μ g mL⁻¹) significantly suppressed the expressions of *ver-1*, *nor-1*, *pksA*, *omtA*, and *aflR* genes. Jahanshiri et al. (36) examined the effect of curcumin on fungal growth and aflatoxin production in *A. parasiticus* NRRL 2999. The curcumin significantly inhibited the expression of the *aflR* gene.

5.1. Conclusion

In general, the *C. spinosa* extract can have fungicidal and inhibitory effects on *A. flavus* and reduce expression of major genes in aflatoxin biosynthetic pathway and further studies are proposed because this study is the first major study on the effect of the *C. spinosa* extract on expression of major genes in aflatoxin biosynthetic pathway.

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Footnotes

Authors' Contribution: All authors had an equal role in design and manuscript writing.

Conflict of Interests: All authors declare that they had no conflict of interest.

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