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Research Paper

The potential effects of *Zataria multiflora Boiss* essential oil on growth, aflatoxin production and transcription of aflatoxin biosynthesis pathway genes of toxigenic *Aspergillus parasiticus*

R. Yahyaraeyat[1], A.R. Khosravi[1], D. Shahbazzadeh[2], V. Khalaj[2]

[1]Mycology Research Centre, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
[2]Department of Biotechnology, Pasteur Institute of Iran, Tehran, Iran.

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Abstract

This study aims at evaluating the effects of *Zataria multiflora* (*Z. multiflora*) essential oil (EO) on growth, aflatoxin production and transcription of aflatoxin biosynthesis pathway genes. Total RNAs of *Aspergillus parasiticus* (*A.parasiticus*) ATCC56775 grown in yeast extract sucrose (YES) broth medium treated with *Z. multiflora* EO were subjected to reverse transcription- polymerase chain reaction (RT-PCR). Specific primers of nor-1, ver-1, omt-A and aflR genes were used. In parallel mycelial dry weight of samples were measured and all the media were assayed by high-pressure liquid chromatography (HPLC) for aflatoxinB1 (AFB1), aflatoxinB2 (AFB2), aflatoxinG1 (AFG1), aflatoxinG2 (AFG2) and aflatoxin total (AFTotal) production. The results showed that mycelial dry weight and aflatoxin production reduce in the presence of *Z. multiflora* EO (100 ppm) on day 5 of growth. It was found that the expression of nor-1, ver-1, omt-A and aflR genes was correlated with the ability of fungus to produce aflatoxins on day 5 in YES medium. RT-PCR showed that in the presence of *Z.multiflora* EO (100 ppm) nor-1, ver-1 and omtA genes expression was reduced. It seems that toxin production inhibitory effects of *Z. multiflora* EO on day 5 may be at the transcription level and this herb may cause reduction in aflatoxin biosynthesis pathway genes activity.

Key words: *Zataria multiflora*, reverse transcription-polymerase chain reaction, aflatoxin, *Aspergillus parasiticus*.

Introduction

Aflatoxins are polyketide secondary metabolites produced by the important food and feed contaminating species *Aspergillus flavus* (*A.flavus)* and *A. parasiticus*. All the major aflatoxins, AFB_1, AFB_2, AFG_1 and AFG_2 are difuranocoumarin derivatives and have been known as potent liver carcinogens for a wide variety of animal species, including humans. Biochemical pathways and genetic regulation of aflatoxin biosynthesis have been well characterized. Some overviews on the genetic background of the aflatoxin biosynthetic pathway have already been published (Brown *et al.*, 1999; Woloshuk and Prieto, 1998). Twenty five genes involved in the aflatoxin biosynthetic pathway form a 70-kb cluster (Yu *et al.*, 2004), Most of which appear to be co- regulated by the DNA binding protein AflR, coded by the aflR gene (Chang, 2003; Ehrlich *et al.*, 2003).

Comparative genome sequencing data on two aflatoxin producing strains, *A. flavus* and *A. parasiticus* has confirmed a high level of homology (98%) between these two species (Yu *et al.*, 1995). Different PCR systems based on nor-1, ver-1, omt-A and aflR genes have been described for the rapid detection of aflatoxigenic fungi (Criseo *et al.*, 2001; Geisen, 1996; Konietzny and Greiner, 2003; Shapira *et al.*, 1996).

These PCR systems have also been used to detect *A. flavus* in wheat (Shapira *et al.*, 1996) and figs (Farber *et al.*, 1997).

Previous studies have shown that the detection of aflatoxin gene-specific DNA fragments in a PCR-based diag-

Send correspondence to A.R. Khosravi. Mycology Research Centre, Faculty of Veterinary Medicine, University of Tehran, Azadi Street, P.O. Box 14155-6453, Tehran, Iran. E-mail: khosravi@ut.ac.ir.

nostic assay for aflatoxigenic aspergilla is only indicating the presence of genes and does not necessarily point to the mycotoxicological safety of the product (Criseo *et al.*, 2001).

Mycotoxin biosynthetic genes may be active or inactive, depending on the environmental conditions. A more reliable approach is to detect a specific aflatoxin-related mRNA. If this mRNA is detected, it is ensured that the aflatoxin biosynthetic gene is actively transcribed and it can be assumed that aflatoxin will be produced under the conditions analyzed, Then a reliable rapid test like RT-PCR is necessary to monitor the activity of the genes. There is clear evidence that fungal development and aflatoxin biosynthesis are linked and may share regulatory elements. Variants of *A. flavus* and *A. parasiticus* with altered morphology and reduced sporulation did not produce detectable levels of aflatoxin intermediates and lacked *aflR*, *nor1* and *omtA* transcripts (Payne and Brown, 1998).

Natural compounds from plants have been used traditionally to preserve foods and feeds in some countries .The extracts and powders of some local plants have the ability of reducing the growth and toxin production of toxigenic fungi in synthetic media (Thanaboripat *et al.*, 1989, 2000). Many EOs have been reported as effective inhibitors of fungal growth and aflatoxin production. Hence, a considerable interest has been developed during recent years on the preservation of grains by the use of EOs as safer and more effective substitutes than synthetic antimicrobial agents and fungicides. These products effectively retard or inhibit fungal growth and mycotoxin production (Bullerman *et al.*, 1977; Mahmoud, 1994).

Zataria multiflora is a spice and medicinal plant belonging to the Laminaceae family that geographically grows only in Iran, Pakistan, and Afghanistan (Ali *et al.*, 2000).This plant known as Avishan-e-Shirazi (in Iran) is used as a flavour agent in a variety of foodstuffs in Iran and traditionally has different effects such as antiseptic, anaesthetic, antispasmodic, antioxidant, antibacterial and immunomodulation (Fazeli *et al.*, 2007; Hitokoto *et al.*, 2000; Khosravi *et al.*, 2007; Misaghi and AkhondzadehBasti, 2007; Sharififar *et al.*, 2007).

The main constituents of the EO of this plant are phenolic compounds such as carvacrol, thymol and eugenol (Basti *et al.*, 2007; Ebrahimzadeh *et al.*, 2003; Shaffiee and Javadnia, 1997). Previous studies have shown that this herb has inhibitory effects on fungal growth and toxin production of some fungi but there is no any study regarding the effects of *Z. multiflora* on aflatoxin biosynthesis pathway genes activity (Gandomi *et al.*, 2009).

This study aims at evaluating the inhibitory effects of *Z. multiflora* EO on growth, aflatoxin production and transcription of aflatoxin pathway structural and regulatory genes o f toxigenic *A.parasiticus*.

Materials and Methods

Fungal strains

Wild-type aflatoxigenic isolate of *A.parasiticus* ATCC56775 was obtained from the collection of Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Essential oil

Purified *Z. multiflora* EO was obtained from Baridge Essence Company (Kashan, Iran).

Extraction and determination of EO composition

Essential oil composition of *Z. multiflora* was identified by gas chromatography (GC) and gas chromatography-mass (GC-MS) spectrometry. Air-dried aerial parts of the *Z. multiflora* plant were subjected to steam distillation for 2 h using Clevenger-type apparatus. The EO obtained from the air-dried material was analyzed by GC (Thermoquest 2000, UK). The chromatograph was equipped with DB5 capillary column (30 x 0.25 mm ID x 0.25 μm film thicknesses) and the data were collected under the following conditions: initial temperature 50 °C; program rate 2.5 °C; final temperature 265 °C and injector temperature 250 °C.

The carrier gas was helium and the split ratio was 120. The EO was also analyzed by GC-MS (Termoques Finningan, UK) using the same capillary column and analytical conditions aforementioned. The MS was run in the electron ionization mode, using ionization energy of 70 eV (Khosravi *et al.*, 2009).

Antifungal assays

Fresh spores of *A. parasiticus* were harvested from a 7-day-old culture of the strain grown on Potato Dextrose Agar slant(Merck, Germany).Suspension of the conidia was prepared in tween 80 solution and the number of conidia in the suspension was adjusted to approximately 10^8 conidia/mL. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were assessed according to a broth dilution method as follows: 50 μL from each of various dilutions of the EO (0, 25, 50, 75, 100, 125, 150, 175 ,200 , 225, 250, 275, 300, 325, 350, 375, 400, 425, 475, 500 ppm) were added to 5 mL of YES broth tubes containing 10^7 spores/mL and incubated on an incubator shaker to evenly disperse the EO throughout the broth in the tubes. Incubation temperature was 30 °C. After 48 h, the highest dilution (lowest concentration), showing no visible growth, was regarded as MIC. Cells from the tubes showing no growth were subcultured on potato dextrose agar plates to determine if the inhibition was reversible or permanent. The plates were incubated at 30 °C until growth was seen in the growth control subculture. The MFC was the lowest drug concentration that showed either

no growth or fewer than three colonies (approximately 99 to 99.5% killing activity) (RazzaghiAbyaneh *et al.*, 2008).

Culture and growth conditions

YES broth medium was used as the basal medium for aflatoxin production. In stationary cultures, 0.5 mL of the spore suspension (10^8 conidia/mL) was added to 50 mL YES medium (2% Yeast extract - 15% Sucrose) in a 250 mL Erlenmeyer flask. *Z. multiflora* EO was diluted to 1% in methanol and then appropriate amounts of the essence (1%) were added to the culture medium to reach concentrations of: 0, 100 and 200 ppm. The cultures were incubated in the dark at 25 °C under stationary conditions. The strains were grown for 5 days and 9 days under conditions of continuous shaking (180 rpm). Triplicate cultures were assayed on day 5 and 9 for aflatoxin production by HPLC analysis, mycelial dry weight determination and total RNA isolation concurrently.

Aflatoxin quantification

The effect of *Z. multiflora* EO on the production of aflatoxins by *A. parasiticus* was evaluated in 5-day-old and 9-day-old cultures by the HPLC method (AOAC 2000). Direct extraction of YES-culture filtrates with methanol and subsequent analysis by HPLC allowed the detection and identification of aflatoxins AFB1, AFB2, AFG1, AFG2 and AFTotal. To evaluate the aflatoxin formation, *Z. multiflora* EO at concentrations lowers than MIC (0%, 50% and 100% of MIC equal to 0, 100 and 200 ppm) was used. Inoculation and incubation conditions were carried out as described above. Following the incubation period, the cultures were autoclaved and the biomasses were collected through filtration and were dried.

Determination of aflatoxins (AFB1, AFB2, AFG1, and AFG2) was performed by RP-HPLC (Waters, USA) according to AOAC (2000). Aflatoxin standards were purchased from Sigma (St. Louis, MO, USA).The limit of detection of the HPLC method was 0.5 ng /mL for all aflatoxins and the recovery rates were found to be 78.5%, 82.7%, 83%, and 80.9% for AFB1, AFB2, AFG1, and AFG2, respectively. The inhibition percentage of aflatoxin production was calculated by the following equation:

$$\text{Inhibition of aflatoxin production}\,\% = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

where Ac is the amount of aflatoxin in the control sample, As is the amount of aflatoxin in treated sample.

DNA and RNA manipulations

Fungal DNA was prepared as described before (Möller *et al.*, 1992). RNA samples were purified using a commercial kit (Qiagen, RNA easy® plant Mini kit, USA). For PCR amplification of target fragments, the forward and reverse primers were designed based on the published sequences of three structural genes (nor-1, ver-1, omt-A) and one regulatory gene (aflR) of aflatoxin biosynthesis pathway (Table 1). For RT-PCR reactions, cDNA was synthesized using RevertAidTM (Fermentas, USA). In all cDNA synthesis reactions, 1 µg of total RNA (adjusted with Nanodrop 1000) was used. PCR amplification of cDNAs was performed in a two-step cycle program as follows: 10 cycles of 95 °C for 1 min, 62 °C for 30 s and 72 °C for 45 s followed by 25 cycles as before except a 5 s increase in each cycle for the extension step at 72 °C. ß-tubulin (Sweeny *et al.*, 2000) fragment was amplified as the control during RT-PCR programs.

Results

Chemical composition of *Z. multiflora* EO

The chemical components of *Z. multiflora* EO (as determined by GC and GC-MS) have been given in Table 2. As it is shown, the main components of the EO were carvacrol (61.29%) and thymol (25.18%).

MIC, MFC, Mycelial dry weight assay and toxin analysis

The MIC and MFC assays were employed to assess the fungistatic and fungicidal properties of the EO and were determined 48 h after culturing. The fungistatic and fungi-

Table 1 - Primers used in this study, target gene, sequence, and expected PCR/RT-PCR product size.

RT-PCR product size (bp)	PCR product size(bp)	Primer sequence	Annealing temperature	Gene	Primer code
485	537	5'-GCCGCAGGCCGCGGAGAAAGTGGT-3' 5'-GGGGATATACTCCCGCGACACAGCC-3'	71.3 °C	ver-1	ver-1 ver-2
741	794	5'-GTGGACGGACCTAGTCCGACATCAC-3' 5'-GTCGGCGCCACGCACTGGGTTGGGG-3'	67.9 °C	omt-A	omt-1 omt-2
1032	1032	5'-TATCTCCCCCCGGGCATCTCCCGG-3' 5'-CCGTCAGACAGCCACTGGACACGG-3'	71.3 °C	aflR	aflR-1 arlR-2
985	1300	5'-GGTAACCAAATAGGTGCCGCT-3' 5'-TAGGTCTGGTTCTTGCTCTGGATG-3'	62 °C	ß-tubulin	tubF tubR
228	281	5'-CTACGCCATGCCGGGATAGA-3' 5'-GGCATCAGTTTCCGAGTCGC-3'	61.4 °C	nor-1	norexonF norexonR

Table 2 - EO composition of *Z. multiflora* identified by gas chromatography and gas chromatography-mass spectrometry.

Component	Component(%)
Carvacrol	61.29
Thymol	25.18
Linalool	1.96
ρ-Cymene	1.90
β-Caryophyllene	1.82
α-Pinene	0.34
α-Thujene	0.10
Myrcene	0.27
α-Terpinene	0.76
Thymul methyl ether	0.95
Carvacrol methyl ether	0.95
Camphene	0.01
1-Octen-3-ol	0.02
3-Octanone	0.05
β-Penene	0.09
α-Phellandrene	0.02
Limonene	0.21
Linalool oxide trans	0.07
Linalool oxide cis	0.06
4-Terpineol	0.30
α-Terpineol	0.54
Bornyl acetate	0.01
Carvacerol acetate	0.02
β-Phellandrene	1.82
Aromadendrene	0.23
Alloaromadendrene	0.11
α-Humulene	0.02
Valencene	0.14
Spathulenol	0.37
Widdrol	0.64
Total	99.09

cidal effects were revealed at 200 and 400 ppm of EO, respectively. Table 3 represents the results of biomass measurements and aflatoxin quantification for *A. parasiticus* grown in YES broth medium containing different concentrations of *Z. multiflora* EO.

Z. multiflora EO significantly reduced the fungal growth and mycelial dry weight on day 5 in the cultures containing 100 ppm of *Z. multiflora* EO (62.91%), whereas this reduction was only 6.52% on day 9. The amounts of aflatoxins AFB1, AFB2, AFG1, AFG2 and AFTotal were reduced on day 5 and increased on day 9 in the flasks containing 100 ppm of *Z. multiflora* EO.

In the flasks containing 200 ppm of the essence, the amounts of biomass and aflatoxin production were trace and not significant.

RT-PCR

In the present study, RT-PCR confirmed the expression of aflatoxin pathway genes in the presence or absence of *Z. multiflora* EO (Figure 1). However, the level of aflatoxin production was found to be correlated with toxin related genes' transcription only on day 5 when compared with tubulin level.

Discussion

Essential oils and their phenolic compounds have been used as natural inhibitors of fungal growth and mycotoxin production during recent decades. Many of the spices and herbal essential oils which have been tested have an antagonistic effect against aflatoxigenic Aspergillus strains. The thymus essences, which mainly consist of thymol, have previously been shown to inhibit both the growth and aflatoxin production in *A. flavus* and *A. parasiticus* (Rasooli and RazzaghiAbyaneh, 2003; Soliman and Badeaa, 2002).

Although the main component of *Z. multiflora* EO is carvacrol, the inhibitory effect of this EO on aflatoxin production was obvious. The EOs of Labiateae family members have inhibitory effects on the aflatoxigenic fungi and

Table 3 - Effects of *Z. multiflora* EO on mycelial growth and aflatoxin formation by *A. ParasiticusATCC* 56775 in YES broth Medium.

Z. multiflora EO concentration (ppm)	Day	Mycelial dry weight (g)	Aflatoxin concentration (ppb)				
			B1	B2	G1	G2	Total
0	5th	0.6908(0)	4181.87	323.4	8852.5	360.2	13717.88
	9th	1.0155(0)	10442.72	560.45	10814.55	457.58	22283.3
100	5th	0.2562(62.91)	2211.09	56.09	1136.4	29.64	3433.22
	9th	0.9492(6.52)	20818.87	1150.29	45268.11	1586.74	68824.02
200	5th	0.0410(94.06)	2.5	ND**	0.59	ND	3.09
	9th	0.1515(94.06)	5.11	ND	2.14	ND	7.25

*Values in parentheses indicate percentage of inhibition against control sample.
**ND: Non-detectable.

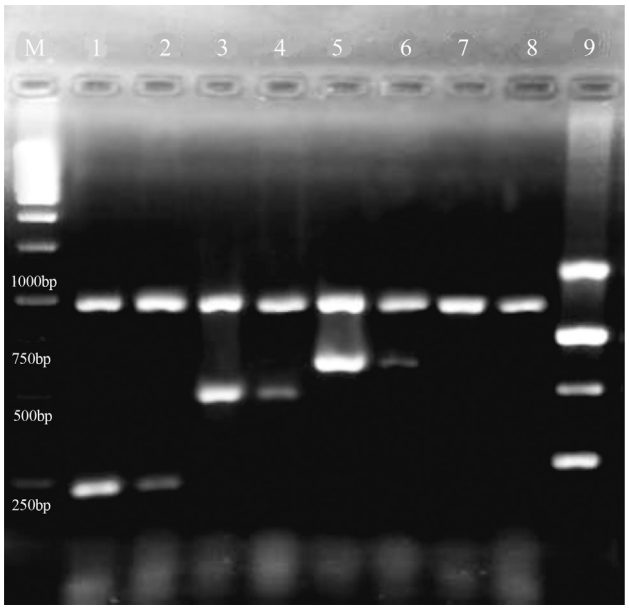

Figure 1 - Agarose gel analysis of RT-PCR products from *A. parasiticus* ATCC 56775 on day5 *Z.multiflora* EO (100 ppm). Lane M, DNA molecular size marker, Lanes 1, 2, primer norexonF, norexonR and tubF, tubR (non treated with EO and treated with 100 ppm EO respectively), lanes 3, 4, primer ver-1, ver-2 and tubF, tubR (non treated with EO and treated with 100 ppm EO respectively), Lanes 5, 6, primer omt-1, omt-2 and tubF, tubR (non treated with EO and treated with 100 ppm EO respectively), lanes 7, 8 primer aflR-1, aflR-2 and tubf,tubR, Lane 9, genomic DNA PCR product primers nor-exonF, nor-exonR, ver-1,v er-2, omt-1, omt-2 and tubF, tubR.

the level of inhibition is well-correlated with the essence concentration used (Soliman and Badeaa, 2002).

In the present study, as it was shown in Table 3, *Z. multiflora* EO significantly reduced the fungal growth and mycelial dry weight on day 5 in the cultures containing 100 ppm of *Z. multiflora* EO (62.91%), whereas this reduction was only 6.52% on day 9. The amounts of aflatoxins AFB1, AFB2, AFG1, AFG2 and AFTotal were reduced on day 5 and increased on day 9 in the flasks containing 100 ppm of *Z. multiflora* EO.

In the flasks containing 200 ppm of the essence, the amounts of biomass and then aflatoxin production were trace and not significant.

In the other study Oregano essential oil, thymol or carvacrol at concentrations of 0.025% and 0.05% completely inhibited the growth of Foodborne fungi (Akgul and Kivanc, 1988). Hitokoto *et al.* (1980) showed that Eugenol extracted from cloves and thymol from thyme caused complete inhibition of the growth of both *Aspergillus flavus* and *Aspergillus versicolor* at 0.4 mg/mL or less. It was shown by several investigators that chemical compounds of the essences may cause reduction or stimulation in the toxin production and the antitoxigenic effects of the essences are not necessarily related to their antifungal activity. Values for growth inhibition were calculated as 0.79 and 0.86 mM for carvacrol and thymol, while for AFB1 and AFG1, it was reported as 0.50 and 0.06 mM for carvacrol and 0.69 and 0.55 mM for thymol (RazzaghiAbyaneh *et al.*, 2008). It was shown by wright *et al.* (2000) that aflatoxin production by the fungus was reduced by *n*-decyl aldehyde and hexanal, but was stimulated by octanal. Their results indicated that all three volatile compounds reduced radial growth but only *n*-decyl aldehyde significantly inhibits aflatoxin biosynthesis in *A. parasiticus*.

Difference in antifungal and aflatoxin inhibition efficacy of *Thymus* and *Zataria* essential oils in different studies may be attributable to the oil compositions. Two major components of thyme and *Zataria* are thymol and carvacrol. *Thyme* contains higher thymol than *Zataria* and *Zataria* contains more carvacrol. The other factors are also important for causing these differences such as differences in Culture media used, culture conditions, temperature, pH and durations of culture.

According to the results of the present study, it seems that the inhibitory effect of the essence has been decreased on day 9 which can be as a result of the volatility of the essence or the increased production of toxin by the fungus in undesirable conditions.

The RT-PCR approach outlined here is a rapid, sensitive and highly specific technique for the detection of *nor-1*, *ver-1* and *omt-A* expression in *A. parasiticus*. Based on aflatoxin biosynthesis pathway genes of *A. parasiticus* , different RT-PCR systems have been described which are applicable for another aflatoxigenic strain, *A. flavu*s (Payne and Brown, 1998). In addition to *nor-1*, *ver-1* and *omt-A*, other genes like *aflD, aflO*, and *aflP* have been used for the detection of aflatoxin producing strains (Chang *et al.*, 1992; Scherm *et al.*, 2005).There are limited studies on the using of essences associated with molecular assays and usually mycological and toxicological assays have been conducted in this regard.

According to the results obtained in the present study, it seems that the inhibitory effect of *Z. multiflora* EO may be at transcriptional level and more study is needed to prove this claim. According to the results it seems that EO is able to present antifungal and antitoxigenic activity in the specific time, dosage and conditions of using.

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