Appl Biol Chem (2018) 61(5):489–497
https://doi.org/10.1007/s13765-018-0382-4

Online ISSN 2468-0842
Print ISSN 2468-0834

ARTICLE

p-Cymene and its derivatives exhibit antiaflatoxigenic activities against *Aspergillus flavus* through multiple modes of action

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Received: 3 May 2018 / Accepted: 21 June 2018 / Published online: 28 June 2018

Abstract Three monoterpenes, 1-methyl-4-(1-methylethyl)-benzene, and its derivatives, carvacrol and thymol, were tested for their antifungal and antiaflatoxigenic activities against *Aspergillus flavus*, and their potential in vitro mechanisms were evaluated. The monoterpenes significantly inhibited mycelial growth, spore production, and aflatoxin production in a dose-dependent manner. Furthermore, their antifungal effects were related to the suppression of fungal development regulatory genes (*brlA*, *abaA*, and *wetA*) and inhibition of ergosterol synthesis. Additionally, the down-regulation of the relative expression of genes related to aflatoxin biosynthesis (*aflD*, *aflK*, *aflQ*, and *aflR*) revealed an antiaflatoxigenic mechanism of the monoterpenes. These observations suggest that the three monoterpenes exhibit antiaflatoxigenic activities through multiple modes of action and may be useful for controlling aflatoxin contamination in food.

Keywords Antiaflatoxigenic · Antifungal · Carvacrol · 1-methyl-4-(1-methylethyl)-benzene · Gene expression · Membrane · Sporulation · Thymol

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13765-018-0382-4) contains supplementary material, which is available to authorized users.

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Introduction

Aflatoxins are secondary metabolites produced by certain toxigenic strains of *Aspergillus* species and are well known for their potent toxicity, immunosuppressive activity, mutagenicity, teratogenicity, and carcinogenicity. They are classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC) [1]. Contamination of agricultural products with aflatoxins poses a serious threat to human health and food security [2]. Most traditional control methods, which involve the application of synthetic chemicals, are not always successful. More importantly, the use of synthetic chemicals to control food spoilage fungi is restricted because of their long degradation period, effects on food, and side effects on humans which includes carcinogenicity, teratogenicity, and residual toxicity [3]. For example, pentachlorophenol and 2,4,6-trichlorophenol have been widely used as fungicides, but their production and use are currently restricted because carcinogenicity was observed in experimental animals. Exposure to pentachlorophenol and 2,4,6-trichlorophenol was reported to be associated with an increased risk of non-Hodgkin lymphoma and hepatocellular tumors, respectively, and thus were categorized as group 1 and group 2b carcinogens by the IARC in 2016 [4]. Thus, alternative constituents for controlling and reducing the growth of aflatoxigenic fungi and aflatoxin production are required.

Monoterpenes, such as borneol, bornylacetate, camphor, carvacrol, 1-methyl-4-(1-methylethyl)-benzene (*p*-cymene), eucalyptol, γ-terpinene, and thymol, are the most important constituents of extracts and essential oils of various plants and possess important antifungal and antiaflatoxigenic properties [5–7]. For example, Reddy et al. [8] reported that plant extracts from *Syzygium aromaticum*,

Curcuma longa, *Allium sativum*, and *Ocimum sanctum* effectively inhibit growth and aflatoxin production of *Aspergillus flavus* grown in rice [8]. The extracts of *Azadirachta indica* and *Thymus vulgaris* were found to be good inhibitors of both the growth and aflatoxin production of *A. flavus* and *A. parasiticus* in vitro [9–11]. In contrast, some authors also observed stimulation of aflatoxin biosynthesis when plants extracts or essential oils were applied to aflatoxigenic fungi [12, 13]. The mechanism of action requires detailed analysis to determine why some constituents possess strong antifungal and antiaflatoxigenic efficacies, while others do not.

p-Cymene is a monoterpene found in over 100 plant species such as thyme (*Thymus vulgaris*) and origanum (*Origanum vulgare*) and is used for medicinal and culinary purposes [14]. *p*-Cymene and its derivatives, carvacrol and thymol, show a range of biological activities, including antioxidant, anti-inflammatory, anxiolytic, anticancer, and antimicrobial effects [15]. While *p*-cymene, carvacrol, and thymol are the major constituents of extracts and essential oils with high antifungal and antiaflatoxigenic activities, knowledge of their mechanism of action is limited and further studies are required to before *p*-cymene, carvacrol, and thymol can be recommended as promising candidates to fight *Aspergillus* app. and aflatoxin contamination.

The main objective of this study was to characterize the in vitro antiaflatoxigenic activities of *p*-cymene and its derivatives, carvacrol and thymol, on *A. flavus* and explore their mechanism of action by determining the effects on (1) fungal growth, (2) sporulation, (3) fungal membrane, (4) aflatoxin production, and (5) expression of related genes.

Materials and methods

Microorganism and culture conditions

Aspergillus flavus KCCM 60330 was obtained from the Korea Culture Center of Microorganisms (KCCM, Seoul, Korea). All cultures were grown on potato dextrose agar medium (PDA, BD Difco™, Detroit, MI, USA) in a thermostatic incubator (Sejong Scientific Co., Gyeonggi-do, Korea) at 28 °C in the dark.

Reagents and standards

p-Cymene, carvacrol, and thymol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All monoterpenes were diluted with dimethylsulfoxide to a final concentration of 500 mg/L and stored at 4 °C until use.

Standards of aflatoxins were purchased from Sigma-Aldrich and prepared according to the manual of official methods of analysis [16]. All reagents, fine chemicals, and solvents for high-performance liquid chromatography (HPLC) were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA).

Determination of fungal radial growth

To determine the effects of the three monoterpenes on the radial growth of *A. flavus*, PDA was prepared by adding *p*-cymene, carvacrol, and thymol (20, 40, and 80 mg/L) to the medium before solidification. PDA without the three monoterpenes was used as a control medium. Each medium was inoculated with 20 µL of conidia suspension and incubated at 28 °C in the dark for 7 days. Colony growth diameter was measured after incubation. All treatments were replicated three times. Growth inhibition of treatment against the control was calculated as a percentage using the following formula:

$$\text{Inhibition rate } (\%) = (C - T)/C$$

where C is an average of hyphal extension (mm) in the control and T is an average of hyphal extension (mm) of plates treated with an individual monoterpene.

Determination of fungal spore production

To evaluate spore production, 3 agar plugs (8 mm in diameter) were taken from each PDA plate and added to 10 mL of sterile saline solution. The solution was mixed for 10 min using a vortex mixer and then diluted several times, after which the spores were counted using a hemocytometer.

Inspection of fungal conidial head

After incubation, mycelia of *A. flavus* KCCM 60330 were collected from the control and test groups for microscopic observation. All samples were fixed in 95% (v/v) ethanol for 30 min. The mycelia samples were washed and resuspended in phosphate-buffered saline (pH 7.5). The fungal conidial head was visualized and photographed with a microscope (Nikon, Inc., Tokyo, Japan).

Determination of ergosterol content

Fungal mycelia harvested from each group were washed twice with distilled water and dried at 60 °C for 3 h. One hundred milligrams of dry mycelia were ground into a powder, mixed with 8 mL of 3 M KOH (in ethanol), and incubated at 80 °C on a shaker in a water bath for 4 h. The solutions were then centrifuged for 15 min at 4000 rpm, and the supernatants were transferred into new tubes and diluted with 2 mL of distilled water. Ergosterol was then extracted from the solutions by two successive applications

of 5 mL *n*-hexane. The organic fractions were evaporated under N2 gas and resuspended in 1 mL of methanol. The concentration of ergosterol was determined with HPLC using an Agilent 1200 LC system (Agilent Technologies, Santa Clara, CA, USA). The ergosterol in the injected 100 μL samples was separated with a reversed phase C18 column (250 mm × 4.6 mm, 5 μm; Waters, Milford, MA, USA) using 100% methanol as mobile phase. The mobile phase was pumped at a constant flow rate of 1.5 mL/min for 20 min. The retention time of ergosterol was approximately 11.18 min. The amount of ergosterol was quantified using a UV detector (at 280 nm). Ergosterol concentration was calculated based on a standard calibration curve. The organic fractions were evaporated under nitrogen, resuspended in 1 mL of methanol, and tested with a HPLC system with UV detection at 280 nm. The ergosterol biosynthesis inhibition rate was calculated using the following equation:

$$\text{Inhibition rate } (\%) = (Ec - Et)/Ec$$

where *Ec* is the average mycelial ergosterol content (mg/mg mycelium) in the control and *Et* is the average mycelia ergosterol content (mg/mg mycelium) in samples treated with an individual monoterpene.

Aflatoxin production analysis

Aflatoxin B1 was determined as described previously [17] with minor modifications. For aflatoxin extraction, 3 agar plugs were collected from each PDA plate and added to test tubes. Aflatoxins were extracted three times with 1 mL methanol and isolated with an immunoaffinity column (VICAM, Nixa, MO, USA) according to the manufacturer's instructions. The eluate was directly purged using N_2 gas, and the dried residue was redissolved in 1 mL of a mixture of 0.1% acetic acid/acetonitrile/methyl alcohol (59:14:29, v/v/v, mobile phase). The solution was filtered through a 0.2 μm syringe filter and stored at − 20 °C before analysis. HPLC analysis was performed using an Agilent 1200 LC system (Agilent Technologies, Santa Clara, CA, USA). The aflatoxin B1 in the injected 50 μL samples was separated with a reversed phase C18 column (150 mm × 4.6 mm, 3.5 μm; Waters, Milford, MA, USA). The mobile phase was pumped at a constant flow rate of 0.5 mL/min for 20 min. The retention time of aflatoxin B1 was approximately 10.92 min. The amount of aflatoxin B1 was quantified using a fluorescence detector (excitation at 360 nm and emission at 440 nm). Aflatoxin B1 concentration was calculated based on a standard calibration curve. The aflatoxin production inhibition rate was calculated using the following equation:

$$\text{Inhibition rate } (\%) = (Ac - At)/Ac$$

where *Ac* is the average aflatoxin B1 content in the control and *At* is the average aflatoxin B1 content in samples treated with an individual monoterpene.

Gene expression analysis

Fungal mycelia on the PDA medium were harvested and placed in a mortar and ground to a fine powder with an appropriate amount of liquid nitrogen. Total RNA was extracted from the mycelia using QIAzol Lysis reagent (Qiagen, Hilden, Germany). The RNA extracts were quantified by determining the absorbance at both 260 and 280 nm using a μDrop plate system (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and RNA was then evaluated qualitatively by agarose gel electrophoresis (1%, v/w) with ethidium bromide. cDNA was prepared using a Maxima first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.). A Rotor-Gene SYBR Green PCR Kit (Qiagen) was used for real-time PCR analysis.

Specific primers synthesized by Genotech (Daejeon, Korea) were used in this study to test the effects of the three monoterpenes (Table 1). Amplification was performed as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 20 s, and elongation at 72 °C for

Table 1 List of qRT-PCR primers

Target genes	Sequences
Conidial head development regulation	
brlA	F: 5′-GATCTCACCTCGAGCGAAAC-3′
	R: 5′-ATAGTCTGGGAGGGGCATCT-3′
abaA	F: 5′-ATCTGCAGGTCCTCGACTCT-3′
	R: 5′-TCGTTCTAATGCTGGCTCAC-3′
wetA	F: 5′-TCGTTCTAATGCTGGCTCAC-3′
	R: 5′-TCGTTCTAATGCTGGCTCAC-3′
Aflatoxin production	
aflD	F: 5′-TCCAGGCACACATGATGGTC-3′
	R: 5′-TGTGGATAACGAAGTGCCCC-3′
aflK	F: 5′-GAACTGCTTCAGTTGCCGTG-3′
	R: 5′-ACGAGGGTTCGTTTCTGGAC-3′
aflQ	F: 5′-TTAAGGCAGCGGAATACAAG-3′
	R: 5′-GACGCCCAAAGCCGAACACAAA-3′
aflR	F: 5′-GCACCCTGTCTTCCCTAACA-3′
	R: 5′-ACGACCATGCTCAGCAAGTA-3′
Ergosterol biosynthesis	
erg28	F: 5′-TTGCCTCCTTTTGAGGGCTT-3′
	R: 5′-CACAGGGGTCGTGATGTTGT-3′
Internal standard	
β-tubulin	F: 5′-TCCAAGGTTTCCAGATCACC-3′
	R: 5′-GAACTCCTCACGGATCTTGG-3′

30 s. Forty rounds of amplification were conducted according to the thermal cycling procedure with a post-cycling step at 95 °C for 5 min. Real-time PCR was performed three times for each sample in a CFX96 system (Bio-Rad, Hercules, CA, USA). Differences in gene expression were calculated using the ΔCt method.

Statistical analysis

Experiments were performed three times, and the data were expressed as the mean ± standard deviation (SD). Statistically significant differences between experimental groups were determined by one-way analysis of variance with Duncan's multiple range test. Differences between experimental groups were considered significant at $p < 0.05$. Statistical analyses were performed using SPSS Version 19 (SPSS, Inc., Chicago, IL, USA).

Results

Effect of three monoterpenes on growth of *A. flavus*

p-Cymene, thymol, and carvacrol effectively inhibited the growth of *A. flavus* in a concentration-dependent manner (Table 2). All three tested monoterpenes completely blocked the growth of *A. flavus* at a concentration of 80 mg/L. Thymol and carvacrol showed slightly stronger inhibition activities than *p*-cymene. Notably, the observed decrease in fungal growth was accompanied by a loss of mycelial pigmentation (Fig. S1).

Effect of three monoterpenes on sporulation of *A. flavus*

The three monoterpenes showed different levels of inhibition of sporulation of *A flavus*. Thymol exhibited the highest inhibition rate (32% at 20 mg/L and 67% at 40 mg/L), followed by carvacrol (46% at 20 mg/L and 59% at 40 mg/L). *p*-Cymene did not significantly inhibit spore production (Fig. 1).

Microscopic observation revealed that thymol had the most marked effects on the morphology of the fungal conidial head. Upon thymol treatment, decreased conidial head size and lack of sporulation were observed (Fig. 2D). Treatment with *A. flavus* with carvacrol had a similar effect (Fig. 2C). However, treatment with *p*-cymene resulted in no obvious morphological difference or changes in sporulation compared to in the control (Fig. 2B).

Figure 3 shows the results of qRT-PCR experiments for *brlA*, *abaA*, and *wetA* in *A. flavus*. Compared to the control, all three genes were downregulated following treatment with the three monoterpenes. Carvacrol treatment resulted in the most significant inhibition, followed by *p*-cymene, and thymol treatment. These results suggest that the three tested monoterpenes affect the regulation of fungal development at the transcription level.

Table 2 Effect of *p*-cymene, carvacrol, and thymol on growth and aflatoxin B1 production by *Aspergillus flavus*

Monoterpenes	Concentration (mg/L)	Fungal growth		Aflatoxin B1 production[2]	
		Colony diameter (mm)[1]	Inhibition (%)	Aflatoxin B1 (ng)[1]	Inhibition (%)
p-Cymene	0	83.5 ± 0.5[a]	–	860.75 ± 3.05[a]	–
	20	57.2 ± 4.6[b]	31.5 ± 5.5	1.93 ± 0.25[b]	99.8 ± 0.03
	40	41.2 ± 1.8[c]	50.7 ± 2.1	0.96 ± 0.12[c]	99.9 ± 0.01
	80	ND	100	ND	100
Carvacrol	0	83.5 ± 0.5[a]	–	860.75 ± 3.05[a]	–
	20	53.6 ± 2.3[b]	35.8 ± 2.7	22.85 ± 2.31[b]	97.4 ± 0.27
	40	29.7 ± 3.4[c]	64.4 ± 4.1	1.03 ± 0.37[c]	99.9 ± 0.04
	80	ND	100	ND	100
Thymol	0	83.5 ± 0.5[a]	–	860.75 ± 3.05[a]	–
	20	53.6 ± 2.3[b]	35.8 ± 2.7	242.67 ± 23.75[b]	71.8 ± 2.76
	40	30.9 ± 2.3[c]	63.0 ± 2.7	15.89 ± 0.68[c]	98.2 ± 0.08
	80	ND	100	ND	100

[1]Values given are mean ± SD of three separate experiments. Values followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$

[2]Aflatoxin B1 production was represented by the aflatoxin B1 quantity in three agar plus taken from each group as described in section Materials and methods

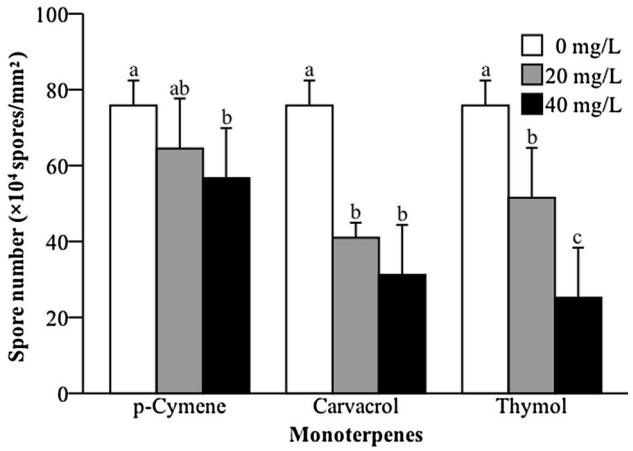

Fig. 1 Effect of *p*-cymene, carvacrol, and thymol on sporulation of *A. flavus*. Fungal colonies were cultured at 28 °C in the dark for 7 days on PDA medium supplemented with 0 (control), 20, and 40 mg/L of *p*-cymene, carvacrol, and thymol. Bars represented standard deviations of the means. Values followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$

Effect of three monoterpenes on ergosterol biosynthesis

Ergosterol levels were dose-dependently decreased in fungal cells after treatment with the three monoterpenes at 0, 20, and 40 mg/L (Table 3). *p*-Cymene and carvacrol exhibited stronger inhibition against ergosterol production than did thymol.

qRT-PCR analysis of *erg28*, an essential biosynthesis gene for ergosterol production, showed that both carvacrol and *p*-cymene effectively interfered with the ergosterol biosynthesis pathway. Thymol showed no obvious effects at the lower concentration (20 mg/L) (Fig. 4).

Effect of three monoterpenes on aflatoxin B_1 production by *A. flavus*

Table 2 shows the effect of the three monoterpenes on aflatoxin B_1 production. There was a significant decrease in aflatoxin production by *A. flavus* treated with the tested monoterpenes. Treating *A. flavus* with monoterpenes resulted in great reductions in aflatoxin B_1 production. At 20 mg/L, *p*-cymene, carvacrol, and thymol decreased aflatoxin production by 99.8, 97.4, and 71.8%, while decreasing fungal growth by 31.5, 35.8, and 35.8%, respectively. Upon treatment at 40 mg/L, aflatoxin production was reduced by 99.9, 98.2, and 99.9% and fungal growth reduced by 50.7, 63.0, and 64.4%, respectively.

Figure 5 shows the changes in *aflD*, *aflK*, *aflQ*, and *aflR* mRNA expression by *A. flavus* upon treatment with the three monoterpenes. Treatment with *p*-cymene and carvacrol significantly affected aflatoxin B_1 production, similar to treatment with thymol at a higher concentration (40 mg/L). However, expression of aflatoxin production-related genes was not affected by treatment with thymol at a lower concentration (20 mg/L). Unexpectedly, the levels of these mRNAs were significantly increased.

Discussion

Monoterpenes from different plants or their essential oils have always been of great interest to researchers because of their potential to inhibit pathogens and their bioactivities [18]. The antimicrobial properties of a few monoterpenes have been reported, but their mechanisms of action are not well understood [18, 19].

The results of the fungal radial growth experiments suggested that *p*-cymene and its derivatives, carvacrol and thymol, exhibited extensive antifungal activity against *A. flavus*. Both thymol and carvacrol significantly inhibited conidial production and hyphal growth of *A. flavus*. However, *p*-cymene inhibited fungal growth but did not affect spore production. This effect was confirmed by microscopy inspection, which indicated that the size and shape of the *A. flavus* conidial head was not affected by *p*-cymene treatment. This disparity suggests a different mode of action among *p*-cymene and its derivatives, carvacrol and thymol.

Treatment with *p*-cymene and its derivatives, carvacrol and thymol, caused a significant loss in *A. flavus* colony pigmentation. This suggests that these three monoterpenes block the biosynthesis of fungal melanins, a high-molecular weight hydrophobic pigment synthesized via the polyketide synthase-dependent pathway [20, 21], in *A. flavus* fungal cells. It has been suggested that blocking melanin biosynthesis in pathogenic fungi can decrease their pathogenicity and increase their sensibility to various biotic and abiotic stresses [22, 23]. Aflatoxins are produced also via the polyketide synthase pathway. The first stable intermediate in the aflatoxin biosynthesis pathway, norsolorinic acid, has a very similar structure to the pigments of *A. flavus* spores [24], suggesting that the aflatoxin and melanin biosynthetic pathways have common initial stages.

As the major sterol constituent in the fungal membrane, ergosterol plays an important role in maintaining the integrity and function of the plasma membrane and localization of membrane proteins [25]. Therefore, ergosterol has been used as a classical drug target to combat fungal infection. In this study, our results showed that treatment with *p*-cymene and its derivatives, carvacrol and thymol, significantly decreased the ergosterol content in *A. flavus*. Similar results were reported for other fungal species [26]. The down-regulation of *erg28* expression induced by the tested monoterpenes suggests that they disrupt ergosterol biosynthesis at the gene expression level.

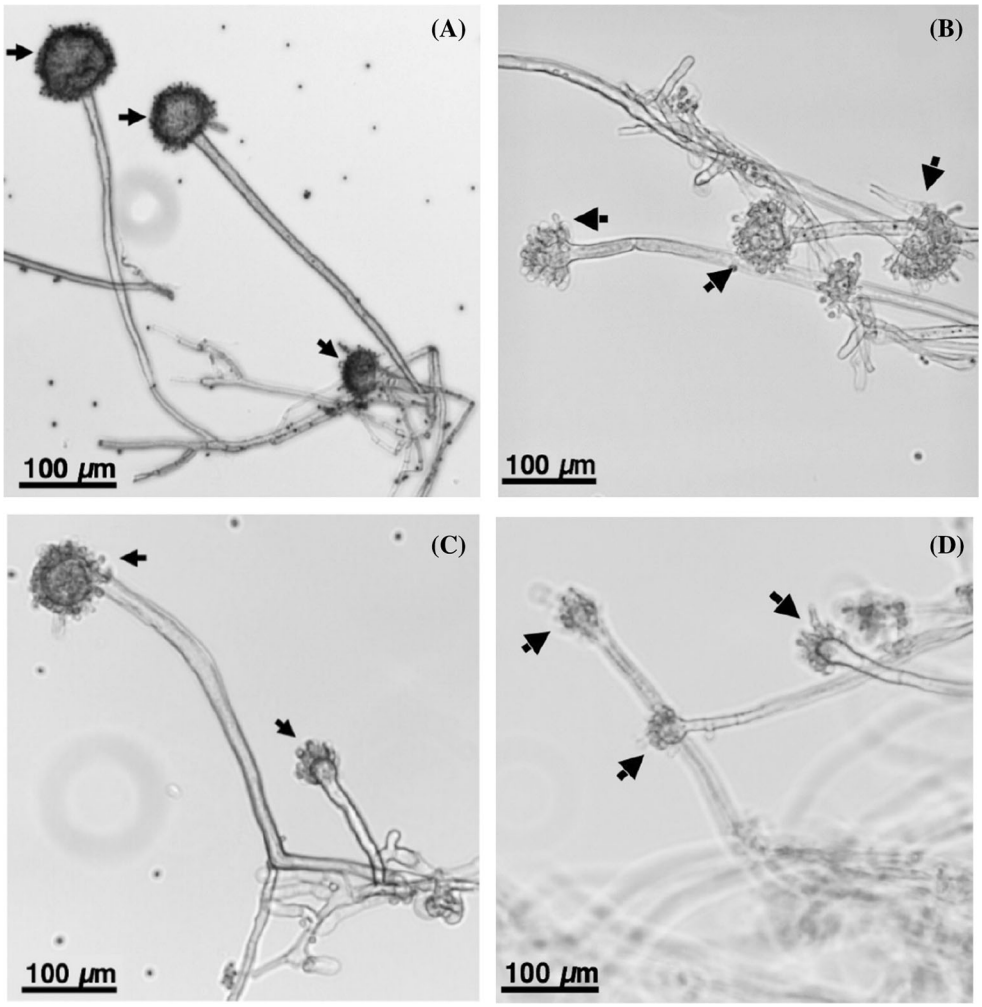

Fig. 2 Microscopic inspection of *A. flavus* conidial head. Conidial heads were taken from fungal colony cultured on PDA (**A**) and PDA supplemented with (**B**) *p*-cymene, (**C**) carvacrol, and (**D**) thymol

All three tested monoterpenes exhibited much higher activity against aflatoxin production than against fungal growth, suggesting that the antiaflatoxigenic activity of *p*-cymene and its derivatives, carvacrol and thymol, is not a secondary effect of fungal growth inhibition. *p*-Cymene had the weakest effect on fungal growth; however, its inhibition of aflatoxin production was the strongest among the three monoterpenes. Carvacrol showed higher anti-aflatoxigenic activity than thymol at the same concentration, although a similar growth inhibition rate was observed. The tested monoterpenes inhibited both aflatoxin production and conidia production of *A. flavus*. It has been suggested that there is a relationship between conidiation and aflatoxin production in *Aspergillus* spp. [27]. Many conidiation-inhibiting constituents also inhibit aflatoxin production. For example, Yoshinari et al. [28] found that dioctatin A and 1,4-diamino-2-butanone inhibit aflatoxin production and conidiation of *A. parasiticus*. The effects of *p*-cymene, carvacrol, and thymol on the key regulatory (*aflR*) and structural genes (*aflD*, *aflK*, and *aflQ*) involved in aflatoxin production were also analyzed. The results suggested that the tested monoterpenes strongly inhibited the expression of genes related to aflatoxin biosynthesis. Because *p*-cymene, carvacrol, and thymol reduced both the mRNA levels of aflatoxin biosynthesis genes and fungal development regulatory genes (*brlA*, *abaA*, and *wetA*), they may affect a regulatory system that controls both aflatoxin production and conidiation.

This study demonstrated that three monoterpenes, *p*-cymene, carvacrol, and thymol, have antifungal and

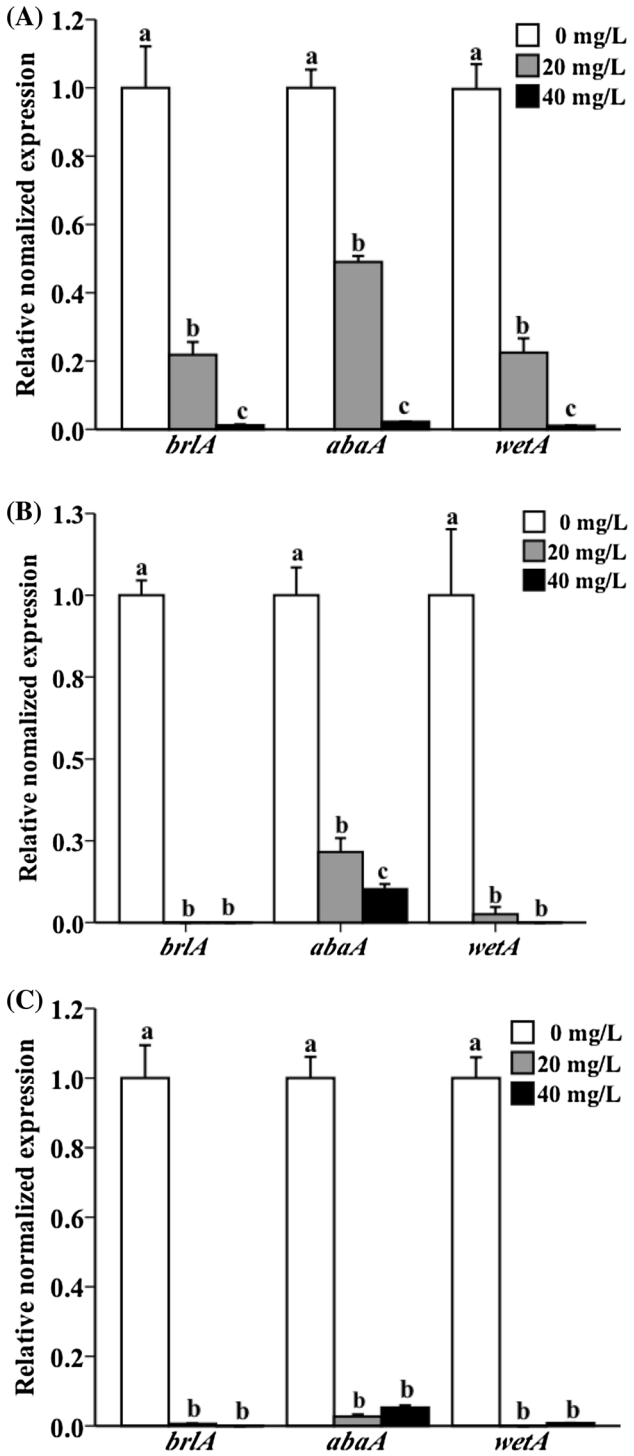

Fig. 3 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of the expression of *brlA*, *abaA*, and *wetA* in *A. flavus* growth under (**A**) *p*-cymene, (**B**) carvacrol, and (**C**) thymol treatment. Bars represented standard deviations of the means. Values followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$

Table 3 Effect of *p*-cymene, carvacrol, and thymol on ergosterol content of mycelia of *A. flavus*

Compounds	Concentration (mg/L)	Ergosterol content (mg/g)[1]
Control	0	3.99 ± 0.03^{a}
p-Cymene	20	3.22 ± 0.06^{b}
	40	2.37 ± 0.05^{f}
Thymol	20	3.27 ± 0.07^{b}
	40	2.75 ± 0.01^{d}
Carvacrol	20	3.05 ± 0.07^{c}
	40	2.58 ± 0.24^{e}

[1]Values given are mean ± SD of three separate experiments. Values followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$

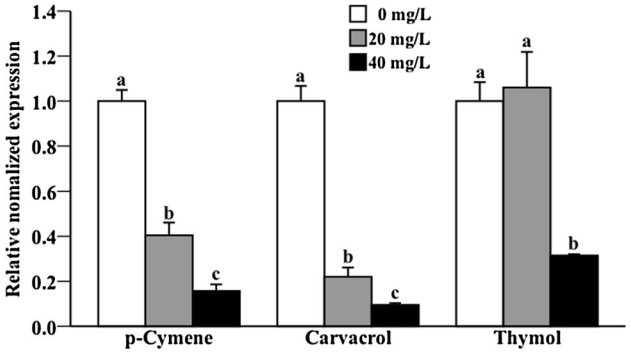

Fig. 4 Relative expression levels of the gene (*erg28*) responsible for ergosterol biosynthesis in fungal cells growth on a medium supplemented with *p*-cymene, carvacrol, and thymol. Bars represent standard deviations of the means. Values followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$

antiaflatoxigenic activities through multiple modes of action. Their activities may be attributed to their ability to (1) inhibit conidia production by interrupting fungal development regulatory factors; (2) cause cell membrane function disruption by inhibiting ergosterol biosynthesis, and (3) block the transcription of regulatory and structural genes involved in aflatoxin production. Additionally, because the three monoterpenes used in this study have been listed by the U.S. FDA as "Generally Recognized as Safe (GRAS)" substances, they also hold potential for application against *Aspergillus* app. and aflatoxin contamination in food.

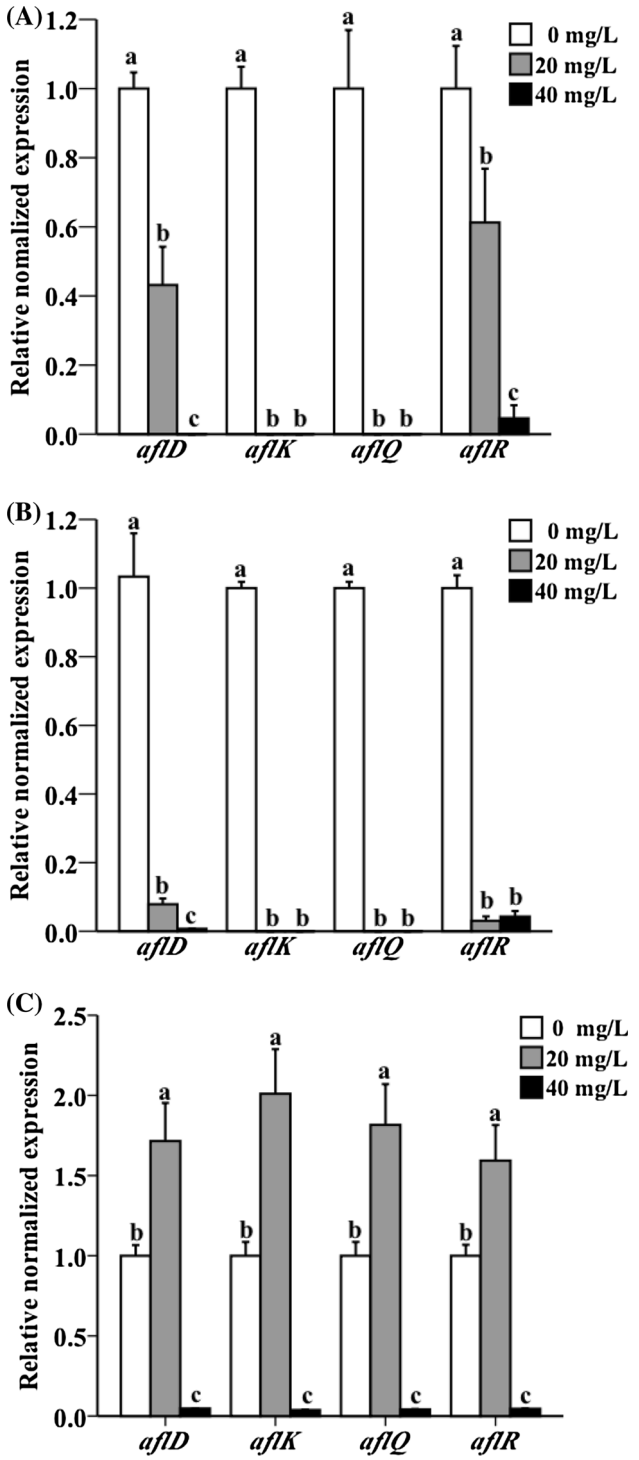

Fig. 5 Relative expression levels of genes (*aflD*, *aflK*, *aflQ*, *aflR*) responsible for aflatoxin biosynthesis in fungal cells growth on medium supplemented with (**A**) *p*-cymene, (**B**) carvacrol, and (**C**) thymol. Bars represent standard deviations of the means. Values followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$

Acknowledgments This work was supported by the Bio-Synergy Research Project (NRF-2013M3A9C4078156) of the Ministry of Science, the Chung-Ang University Graduate Research Scholarship, Chung-Ang University Young Scientist Scholarship (CAYSS), and BK21 Plus (Brain Korea 21 Program for Leading Universities & Students) Scholarship, Republic of Korea.

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