DOI: 10.17765/2176-9206.2021v14n1.e7727
e-ISSN: 2176-9206

Antifungal and antiaflatoxigenic activities of thymol and carvacrol against Aspergillus flavus

Efeitos antifúngico e antiaflatoxigênico de timol e carvacrol em Aspergillus flavus

Lydiana Pollis Nakasugi [1], Natália Silva Bomfim[2], Jessica Cristina Zoratto Romoli[3], Samuel Botião Nerilo[4], Milena Veronezi Silva[5], Gustavo Henrique Rocha Oliveira [6], Miguel Machinski Jr[7*]

[1] Professor at Ingá University Center - UNINGÁ, Maringá (PR), Brazil. [2] Professor at College ESEFAP/UNIESP, Tupã (SP), Brazil. [3] PhD student at the Postgraduate Program in Health Sciences at the State University of Maringá (UEM), Maringá (PR), Brazil. [4] PhD student at the Postgraduate Program in Food Science at UEM, Maringá (PR), Brazil. [5] PhD student at the Postgraduate Program in Toxicology at the University of São Paulo (USP), São Paulo (SP), Brazil. [6] Permanent professor of the Postgraduate Programs in Health Sciences (PCS) and Food Science (PPC) at UEM, Maringá (PR), Brazil. [7] Permanent professor of the Postgraduate Programs in Health Sciences (PCS) and Food Science (PPC) at UEM, Maringá (PR), Brazil.

***Corresponding author**: Miguel Machinski JR - E-mail: mmjunior@uem.br

ABSTRACT
The aim of this study was to evaluate the effects of thymol and carvacrol upon production of aflatoxins by Aspergillus flavus and upon its growth. Minimal inhibitory concentration (MIC), fungitoxic spectrum and mycotoxin inhibition were assessed. Results showed thymol and carvacrol exhibited fungicidal action, as determined by MIC values of 2500 and 30 μg mL^{-1}, respectively. Both thymol and carvacrol significantly inhibited growth of A. flavus ($p<0.05$) at concentrations of 600 and 15 μg mL^{-1}, respectively. Fungal biomass, as estimated by determination of ergosterol concentration, was significantly reduced ($p<0.05$) at thymol concentrations of 2500 μg mL^{-1} and at carvacrol concentrations of 250 μg mL^{-1}. Thymol and carvacrol exhibited antiaflatoxigenic effects at concentrations of 600 and 125 μg mL^{-1}, respectively. While both thymol and carvacrol showed possessing antifungal activities, neither were highly antiaflatoxigenic. Carvacrol and thymol might be considered for use as potential antifungal natural compounds against A. flavus.
Keywords: Anti-infective agents. Aflatoxins. Mycotoxins. Natural products. Terpenes.

RESUMO
O objetivo deste estudo foi avaliar os efeitos do timol e carvacrol contra Aspergillus flavus e a produção de aflatoxinas. Foram determinadas a concentração inibitória mínima (CIM), o espectro fungitóxico e a atividade inibidora de micotoxinas pelo timol e carvacrol. Os resultados mostraram que timol e carvacrol exibiram ação fungicida de acordo com a CIM de 2500 e 30 μg mL^{-1}, respectivamente. Tanto o timol quanto o carvacrol inibiram significativamente o crescimento de A. flavus ($p<0,05$) a partir de 600 e 15 μg mL^{-1}, respectivamente. A biomassa fúngica, estimada pela determinação da concentração de ergosterol, foi significativamente reduzida ($p<0,05$) em 2500 μg mL^{-1} de timol e 250 μg mL^{-1} de carvacrol. O timol e o carvacrol exibiram efeitos antiaflatoxigênicos em 600 e 125 μg mL^{-1}, respectivamente. O timol e o carvacrol exibiram atividade antifúngica, mas não exibiram alta atividade antiaflatoxigênica. Carvacrol e timol podem ser considerados como potentes compostos naturais antifúngicos contra A. flavus.
Palavras-chave: Aflatoxinas. Agentes antimicrobianos. Micotoxinas. Produtos naturais. Terpenos.

Received in: October 01, 2019
Accepted on: Julhy 07, 2020

INTRODUCTION

Aflatoxins (AFs) are highly toxic, mutagenic and carcinogenic secondary metabolites produced by species of Aspergillus. Species from this genus are widespread in natural environments, posing a risk to both human and animal health. Aflatoxins are among the most potent mutagenic and carcinogenic substances known, based on studies carried on experimentation animals and on human epidemiological studies; hepatitis B virus (HBV) is a critical contributor to the potential of aflatoxins in inducing liver cancer.[1] The International Agency for Research on Cancer (IARC) classifies AFs as group I carcinogens, and thus carcinogenic to humans.[2] Numerous food products can be contaminated by AFs, such as cereals, oilseeds, fine spices, cotton seeds, milk, meat and dried fruits; however, corn, peanuts and rice are the most commonly affected items.[1]

For years, synthetic chemical fungicides were deemed enough to control fungal growth on food produce. However, these products pose potential hazards to human health and contribute to environmental degradation. Furthermore, the continued use of these synthetic compounds has led in many cases to proliferation of resistant biotypes of fungal pathogens. Therefore, new approaches for controlling postharvest diseases have shifted towards prioritizing alternatives to synthetic fungicides.[3]

Essential oils (EOs) are natural substances of antibacterial, antifungal and antioxidant properties, commonly used in the food industry; they are classified as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration (FDA).[4] Many chemicals components found in EOs have also been accepted by the European Commission as flavoring agents for use in foods such as linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene.[5] The chemical composition of EOs varies depending on plant genetics, timing of harvest, climatic and geographic conditions, luminosity, seasonal changes and extraction methods.[6] Indeed, scientific literature describes variations in the chemical profiling of several EOs. Hence, the chemical composition of an EO needs to be standardized before it can be fully assessed and recommended as a food preservative since any variation in its composition would systematically modify its antifungal activity.[7] Such antifungal activity is due to presence of major compounds or to synergistic interactions between different minor compounds. Therefore, the use of isolated compounds from EOs might represent an alternative not only to the use of synthetic fungicides, but also to the use of EOs in natura due to the variation of their chemical profiling.[3,5,8]

Several authors have demonstrated and/or compared the antifungal action of whole EOs or of their isolated compounds. Abbaszadeh et al.[8] demonstrated the antifungal efficacy of thymol, carvacrol, eugenol and menthol on control of food-relevant fungi. Thymol was associated with the inhibition of growth of Cladosporium spp. and carvacrol had significantly fungicidal activity upon Aspergillus spp. Authors suggest the role of minor compounds acting synergistically with major ones (α-pinene, 1.8-cineol and camphor) enhance the antifungal activity of intact Rosmarinus officinalis, Salvia officinalis, Lavandula dentata and Laurus nobilis EOs upon A. flavus and A. carbonarius.[7,9] Eugenol and nerol have potential inhibitory effects upon Aspergillus strains.[10] Wang et al.[11] demonstrated antifungal effects of cinnamaldehyde, citral, geraniol and carvacrol upon A. flavus, A. carbonarius and Penicillium viridicatum. Lasram et al.[12] also reported antifungal and antiaflatoxigenic effects of carvone upon A. flavus.

Carvacrol and thymol are the main chemical compounds found in some EOs, such as those obtained from oregano (Oreganum sp.) and thyme (Thymus sp.). These active compounds have been shown as efficient in reducing microbial contamination of foods, and have also been shown to improve overall quality of stored fruits, preserving their organoleptic, nutritive and functional properties for longer.[13] Therefore, the aim of this study was to evaluate the effects of thymol and carvacrol upon Aspergillus flavus growth and production of aflatoxins.

METHODOLOGY

Standard solutions of thymol and carvacrol: Thymol and carvacrol standards were acquired from Sigma-Aldrich (Saint Louis, MO, USA). Standards were dissolved separately in aqueous solutions containing

Tween 80 (0.1%) (Vetec, Rio de Janeiro, Brazil) in order to obtain working standard solutions at concentrations of 250 to 20,000 μg mL^{-1} of thymol and carvacrol, respectively. Solutions were then stored at 4 °C until further use.

Microorganism culturing conditions: the AF42 A. flavus strain, isolated from stored groundnuts and previously identified as high producer of aflatoxins B_1 (AFB_1) and B_2 (AFB_2), was selected for this investigation. The fungi strain was kept in glycerol (15%) at -20°C in the bank of isolated fungi of the State University of Maringa (UEM), Maringa, Brazil. New microorganism cultures were grown in tubes containing Potato Dextrose Agar (PDA) (Neogen® Co., Lansing, MI, USA), and kept for 7 days at 25°C under dark conditions in a BOD incubator (model 411 – FPD 335, Ethik Technology, Vargem Grande Paulista, Brazil) until the following experiments were carried out: determination of minimal inhibitory concentration (MIC), determination of minimal fungicidal concentration (MFC), evaluation of mycelial growth and assessment of thymol and carvacrol effects on aflatoxin and ergosterol production.

MIC and MFC determination: in order to determine MIC and MFC of thymol and carvacrol, experiments were carried out according to document M38-A2 from the National Committee for Clinical Laboratory Standards,[14] with due adaptations to broth macrodilutions for filamentous fungi. Thymol and carvacrol were dissolved in Tween 80 (0.1%) so concentrations ranging from 600 to 10,000 μg mL^{-1} for thymol and from 15 to 250 μg mL^{-1} for carvacrol would be obtained. A suspension containing fungal conidia of A. flavus at a concentration of 10^4 CFU mL^{-1} was prepared in synthetic RPMI-1640 medium (0.5 mL) using a Neubauer chamber. A tube containing the conidia suspension only was also prepared as a control. The tubes were incubated at 35°C for 72 h. MIC was defined as the lowest concentration of the tested compounds that inhibited visual growth of A. flavus. Subsequently, aliquots were taken from the tubes that exhibited no visual fungal growth and inoculated in Petri dishes containing Sabouraud agar. The plates were incubated at 25°C for 72 h. MFC was determined according to absence of fungal growth on the plates.

Effects of thymol and carvacrol on A. flavus growth: Thymol and carvacrol were diluted in a sterile Tween 80 (0.1%) solution according to the established MIC and MFC concentrations. Two concentrations higher and two concentrations lower than the MFC were tested. Different solutions containing the test compounds were added to Erlenmeyer flasks containing 24 mL of yeast extract-sucrose liquid medium (YES). Aliquots of 10^6 A. flavus conidia mL^{-1} were added to each flask containing culture medium and test compounds. The flasks were incubated at 25°C in the dark for 7 days. One flask containing nystatin at a concentration of 1,000 μg mL^{-1} was also prepared as a positive control (PC). Another flask containing only conidia in culture medium was prepared as a fungal control (FC).

After the incubation period, the contents of each flask were filtered through standard filter paper. The mycelial biomass separated from the liquid content was weighed and subsequently used for extraction of ergosterol, while the filtered contents were later used for extraction of aflatoxins.[15]

Determination of mycelia wet mass: mycelia retained in the filter paper were collected in previously weighed tubes, which were then reweighed; the difference was determined as the values for wet mycelial weight.[16] The percentage of mycelial growth inhibition was determined using the formulae: [Pc – Pt)/Pc] x 100, where Pc stands for average weight of mycelia obtained from the negative control flasks, while Pt stands for the average weight of mycelia obtained from the test flasks.

Extraction of ergosterol: fungal mycelia were transferred to tubes containing each 20 mL of methanol, 5 mL of ethanol and 2 g of potassium hydroxide (Merck, Darmstadt, Germany). Each tube containing this solution was homogenized for 5 minutes in tube agitators (KMC 1300V, Bucheon, Korea) and left in a water bath EV 015 (Evlab, Londrina, Brazil) at 70 °C for 40 min. After cooling at room temperature, 5 mL of distilled water were added to each tube which were then centrifuged at 1735 g for 20 min (Universal 320R, Hettich, Tuttingen, Germany). Supernatants were removed and n-hexane (FMaia, Cotia, Brazil) was added in equal volume to each tube. After 2 minutes of agitation, the organic fraction was collected and placed in an amber

glass flask. The solvent in each tube was evaporated (White Martins, Rio de Janeiro, Brazil). The residue obtained was stored at -20°C until further analysis.[15]

Chromatographic parameters for detection and quantification of ergosterol: samples were resuspended in 1 mL of ethanol (Panreac, Barcelona, Spain) and filtered through Millex-LCR syringe filters (0.45 μm x 13 mm, modified PTFE) (EMD Millipore Corporation, Billerica, MA, USA). Ergosterol standard was acquired from Sigma Chemical (St. Louis, MO, USA) and prepared according to the manufacturer's instructions. The high-performance liquid chromatography (HPLC) equipment used was a Finnigan Surveyor Plus (Thermo Scientific, San Jose, CA, USA) possessing an UV/VIS detection system. The chromatographic column used was a reverse phase C_{18} analytical column Spherisorb® (150 x 4.6 mm, 5 μm, Waters, Wexford, Ireland). Chromatographic conditions were mobile phase methanol (100%), analysis time of 10 min, injection volume of 100 μL, flow rate of 1.5 mL min^{-1} and wavelength of 282 nm. All reagents were of HPLC grade. A calibration curve of ergosterol standard ranging from 10 to 100 μg mL^{-1} was constructed to determine ergosterol concentrations. The retention time was of 4.6 minutes. Detection and quantification limits were 0.15 and 10 mg mL^{-1}, respectively. Recovery was 77.4 ± 7.2%.[15]

Evaluation of antiaflatoxigenic effects of thymol and carvacrol:

Extraction of aflatoxins: the filtrate obtained from the samples was subjected to extraction processes.[15] From the filtered solution, for each sample, 20 mL were transferred to separation funnels (125 mL) to which 10 mL of n-hexane were then added. After 1 minute of manual agitation, the aqueous phase was separated from the organic phase, which was discarded. The aqueous phase was again placed in the separation funnel, to which 10 mL of chloroform were added (F. Maia Indústria e Comércio Ltda., Cotia, Brazil). The separation funnel was agitated for 3 minutes, and the contents allowed to rest for 8 minutes. The chloroform fraction was then filtered through standard filter paper with the aid of anhydrous sodium sulfate and collected in amber glass flasks. Another 10 mL of chloroform were added to the aqueous phase and extraction and filtration processes were repeated. Flasks were then placed in a water bath at 65°C for evaporation of solvent. The residue thus obtained was stored at -18°C until further analysis.

Determination of aflatoxins by HPLC: residues obtained after aflatoxin extraction were resuspended in 1 mL of methanol-water (50:50 v/v) and then filtered through a 0.45 μm membrane. Aflatoxin standards were acquired from Sigma-Aldrich (St. Louis, USA) and working and stock solutions were prepared according to the Manual of Official Methods of Analysis, from the Association of Official Agricultural Chemistry International.[17] Concentrations of aflatoxins B_1 and B_2 were assessed using the same equipment described above, but with a reverse phase C_{18} chromatographic column (250 x 4.6 mm, 5 μm, Pickering Laboratories Inc., Mountain View, CA, USA). Post-column derivatization was carried out using a Kobra Cell® derivatization cell (Biopharm Rhone Ltda., Glasgow, Scotland). Chromatographic conditions were: mobile phase methanol-water-acetonitrile (20:60:20 v/v/v/) containing 119 mg L^{-1} of potassium bromide (Quimex SA, Productos y Insumos Quimicos, Lima, Peru) acidified with 350 μL of nitric acid 4 mol L^{-1} (Labsynthy®, Diadema, SP, Brazil); injection volume of 100 μL; flow rate of 1 mL min^{-1}; fluorescence detection at excitation and emission wavelengths of 362 and 425 nm, respectively. All reagents were of HPLC grade. A calibration curve ranging from 1 to 20 ng mL^{-1} was constructed using different solutions containing AFB_1 and ABF_2 standards at concentrations 1, 2.5, 5, 7.5, 10, 15 and 20 ng mL^{-1} to determine aflatoxins concentration. The run time was of 25 minutes. The retention times were of 11 min for AFB_2 and of 13 min for AFB_1. The average recovery and precision for AFB_1 and AFB_2 were 93.1 and 7.9%, 103 and 6%, respectively. The limit of detection was 0.05 and 0.04 ng mL^{-1}, and the limit of quantification was 0.5 and 0.4 ng mL^{-1} for AFB_1 and AFB_2, respectively. The percentage of AFB_1 and AFB_2 inhibition of production under each thymol and carvacrol concentrations was calculated in comparison to the fungal control.

Statistical analysis: all experiments were carried out in quadruplicates. Results were analyzed using GraphPad Prism 5.0 software (GraphPad Software,

Inc.). In order to determine differences for single factors with multiple comparisons, one-way analysis of variance (ANOVA) tests followed by Tukey post hoc tests were performed. Results were expressed as mean ± standard-deviation, and differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

MIC AND MFC

MIC and MFC for thymol were both determined at a concentration of 2500 μg mL^{-1}, at which it was verified thymol possesses fungicidal actions. When tested upon Candida albicans, MIC for thymol was 125 μg mL^{-1} and when tested upon Penicillium digitatum and P. italicum, MIC was 500 μg mL^{-1}, which evidences the fungicidal action of thymol depends on the fungal species.[18,19] Abbaszadeh et al.[8] determined the MIC for thymol ranged between 100 and 500 μg mL^{-1}, depending on different fungal isolates, corroborating data here shown. The most efficient growth inhibition was verified when thymol was tested on Cladosporium spp., followed by Aspergillus spp., Fusarium oxysporum, Botrytis cinerea, Penicillium spp., Alternaria alternata and Rhizopus oryzea.

As for carvacrol, MIC was determined at a concentration of 30 μg mL^{-1}, while MFC was determined at a concentration of 60 μg mL^{-1}. In the present study, unlike thymol, carvacrol exhibited fungistatic action. These results corroborate the data by Abbaszadeh et al.,[8] who reported carvacrol possesses fungicidal actions against the same fungus, MIC being of 100 μg mL^{-1} and MFC of 125 μg mL^{-1}.

EFFECTS OF THYMOL AND CARVACROL ON A. flavus GROWTH

Thymol (Table 1) significantly reduced fungal mycelial weight (wet weight) at all tested concentrations in comparison to the fungal control (FC). The same was observed in the positive control sample (PC). Carvacrol (Table 1) significantly reduced fungal mycelial weight at all concentrations assessed, inhibition percentages ranging from 38.40 to 48.80%. Complete inhibition was observed only in the sample corresponding to the positive control (PC).

Table 1. Inhibitory effects of thymol and carvacrol on mycelial growth of Aspergillus flavus

Thymol (μg/mL)	**Mycelial weight (g)**	**Growth inhibition[a] (%)**
FC	12.5	-
600	5.73*	54.4±0.30*
1250	4.63*	63.2±0.15*
2500	0.23*	98.4±0.05*
5000	-	100.0±0.0*
PC	-	100.0±0.0*
Carvacrol (μg/mL)	**Mycelial weight (g)**	**Growth inhibition[b] (%)**
FC	12.5	-
15	7.83*	38.4±0.40*
30	7.23*	42.4±0.78*
60	7.56*	40.0±0.64*
125	7.70*	38.4±0.52*
250	6.39*	48.8±0.62*
PC	-	100.0±0.0*

*significant difference ($p<0.05$) compared to fungal control.

Values expressed as mean ± standard deviation in the growth inhibition.

FC – fungal control, inoculum.

PC – positive control, inoculum treated with 1000 μg mL^{-1} nystatin.

Similarly, Gandomi et al.[20] evaluated the effects of oil obtained from Zataria multiflora Boiss (a thyme-like plant which grows only in Iran, Pakistan and Afghanistan) on A. flavus. The authors reported the oil compound found at the highest concentration was carvacrol (71.12%), and that all concentrations tested (50 to 1,000 μg mL^{-1}) were capable of preventing fungal growth, especially at concentrations higher than 400 μg mL^{-1}, where growth inhibition was complete. Carvacrol, the main phenolic compound found in the essential oil of Z. multiflora, was indicated as the compound most likely responsible for the antifungal effects.

EFFECTS OF THYMOL AND CARVACROL ON ERGOSTEROL BIOSYNTHESIS

Ergosterol is responsible for maintaining cell integrity and functionality. A reduction in ergosterol content usually leads to loss of membrane stability and leads to osmotic disturbances and compromises fungal cell growth and proliferation.[21] Therefore, the amount of ergosterol produced by A. flavus exposed to different concentrations of thymol and carvacrol was quantified, allowing for quantification of fungal biomass in turn. The mechanism of action of thymol and carvacrol which leads to inhibition of mycelial growth of toxigenic fungi is unclear; apparently, these compounds form hydrogen bonds at active sites of key enzymes. Our results confirm such inferences, as the amount of ergosterol in the fungal cell decreased when the A. flavus strain was exposed to thymol and carvacrol at all concentrations (Fig. 1 and 2).

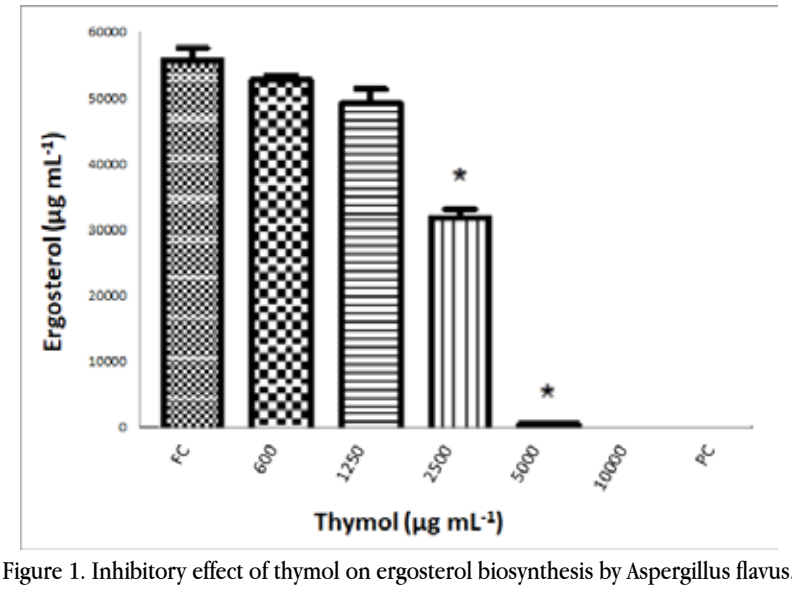

Figure 1. Inhibitory effect of thymol on ergosterol biosynthesis by Aspergillus flavus.
(FC) – Fungal control with no treatment. (PC) – positive control, nystatin (1000 μg mL^{-1}). Test tubes were incubated for 7 days at 25°C in YES liquid medium (n=4). *p<0.05.

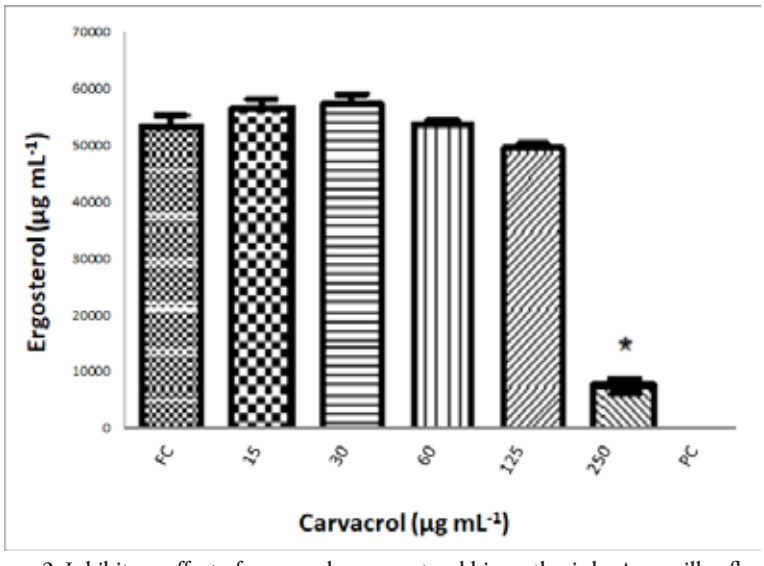

Figure 2. Inhibitory effect of carvacrol on ergosterol biosynthesis by Aspergillus flavus. (FC) – Fungal control with no treatment. (PC) – positive control, nystatin (1000 μg mL^{-1}). Test tubes were incubated for 7 days at 25°C in YES liquid medium (n=4). *p<0.05.

Figure 1 shows the inhibition of ergosterol production by A. flavus after treatment with thymol. Results show a significant reduction in ergosterol amounts at concentrations of 2500 and 5000 μg mL^{-1} compared to the fungal control (FC). Inhibition at these concentrations is also significantly more marked compared to the results obtained at lower concentrations. Ochoa-Velasco et al.[3] reported similar results when testing the effects of thymol on F. verticillioides.

The results shown in Figure 2 demonstrate the inhibitory action of carvacrol on production of ergosterol by A. flavus. There is a significant inhibition of ergosterol production at a concentration of 250 μg mL^{-1} not only compared to the fungal control (FC), but also relative to the other tested concentrations. A study carried out by other authors showed that thymol and carvacrol, when tested on C. albicans, reduced ergosterol production by up to 98%, depending on the concentration tested (100 μg mL^{-1}).[22] Ahmad et al.[22] reported inhibition of ergosterol production at concentrations above the values established for MIC, similar to the results found in the present study.

Some antifungal compounds can affect mycelial development and ergosterol production due to biochemical tension. These compounds are able to activate a compensation mechanism which generates an adaptive response by the fungi, causing reprogramming of the expression of genes responsible for translation of cell wall proteins.[23] The immediate result was a non-significant increase (p>0.05) in ergosterol biosynthesis, as observed in Figure 2 with the use of carvacrol at concentrations of 15, 30 and 60 μg mL^{-1}. Other authors observed that at low concentrations, essential oils and their compounds can damage fungal cell wall and cause an increase in ergosterol production, resulting in increased fungal biomass.[24]

ANTIAFLATOXIGENIC EFFECTS OF THYMOL AND CARVACROL

Table 2 shows the results for the effects of thymol on the production of aflatoxins B_1 and B_2. Thymol exhibited antiaflatoxigenic effects at concentrations above 600 μg mL^{-1}, as it reduced AFB_1 and AFB_2 production. Aflatoxin production increased, but not significantly (p> 0.05) at concentration of 1250 μg mL^{-1} of thymol. Lower concentrations of inhibitory compounds may cause an adaptive response to protect the fungus, resulting in epigenetic changes causing increased toxin production.[24]

Table 2. Inhibitory effects of thymol on production of aflatoxins by Aspergillus flavus

Thymol (μg mL^{-1})	Aflatoxin B_1		Aflatoxin B_2	
	Concentration (ng mL^{-1})	Inhibition (%)	Concentration (ng mL^{-1})	Inhibition (%)
FC	133301 ± 3971	-	7095 ± 345.9	-
600	64054 ± 84420*	51.94	3576 ± 4747*	49.59
1250	146395 ± 27246	-	8558 ± 828.3	-
2500	93949 ± 68918*	29.52	4626 ± 3145*	34.79
5000	ND*,**	100.00	ND*,**	100.00
PC	ND*,**	100.00	ND*,**	100.00

ND – No detection

* significant difference compared to negative control ($p<0.05$).

** no significant difference between the compared group (PC).

Values of concentration obtained by HPLC analysis expressed as mean ± standard deviation.

FC – fungal control, inoculum.

PC – positive control, inoculum treated with 1000 μg mL^{-1} nystatin.

Table 3 shows the results for the effects of carvacrol on production of aflatoxin by A. flavus. A significant reduction of aflatoxin production can be observed only at a carvacrol concentration of 125 μg mL^{-1}, being of only 39.68 and 19.15% for AFB_1 and AFB_2, respectively. Nystatin, the reference antifungal used as the PC, completely inhibited synthesis of AFB_1 and AFB_2 by A. flavus. Corroborating with our data, other authors reported that carvacrol inhibited aflatoxin production on A. parasiticus by 92.64% at a concentration of 1320 μg mL^{-1}, evidencing this compound indeed possesses antiaflatoxigenic effects.[26]

Table 3. Inhibitory effects of carvacrol on production of aflatoxins by Aspergillus flavus

Carvacrol (μg mL^{-1})	Aflatoxin B_1		Aflatoxin B_2	
	Concentration (ng mL^{-1})	Inhibition (%)	Concentration (ng mL^{-1})	Inhibition (%)
FC	133301 ± 3971	-	7095 ± 345.9	-
15	127702 ± 6193*	4.20	7570 ± 172*	-
30	127549 ± 10089*	4.31	7355 ±160.9*	-
60	118349 ± 7555*	11.21	6832 ±151.8*	3.70
125	80404 ± 20334*,**	39.68	5736 ±553.4*,**	19.15
PC	ND*,**	100.00	ND*,**	100.00

ND – No detection

*significant difference compared to negative control ($p<0.05$).

**no significant difference between the compared group (PC).

Values of concentration obtained by HPLC analysis expressed as mean ± standard deviation.

FC – fungal control, inoculum.

PC – positive control, inoculum treated with 1000 μg mL^{-1} nystatin.

Other authors when analyzing effects of the essential oil of Z. multiflora on A. flavus, found that it exhibited antiaflatoxigenic action at concentrations of 50 and 150 μg mL^{-1}.[20] They showed that carvacrol, one of the main compounds of this essential oil, can be considered an inhibitor of toxin production. However, the association with other compounds found in the essential oil must also be taken into consideration. Prevention of mycelia and conidia formation by toxigenic fungi is one of the characteristics of aflatoxin synthesis inhibition.

CONCLUSIONS

The present investigation demonstrates that carvacrol and thymol could directly inhibit mycelia growth of A. flavus. Thymol and carvacrol also appear to target ergosterol biosynthesis by A. flavus, leading to severe membrane disruption. Thymol and carvacrol both exhibited antifungal activity but did not exhibit high antiaflatoxigenic activity. Carvacrol and thymol, which are regarded as safe (GRAS) natural plant compounds, can be considered as potential antifungal natural compounds to be used against A. flavus. Further studies should focus on expanding on the application of carvacrol and thymol in agricultural practices as natural fungicides that could partially substitute synthetic fungicides.

ACKNOWLEDGEMENTS

This paper is derived from a master's thesis presented by the first author and funded by the Coordination for the Improvement of Higher Education – CAPES (028054/2009). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, as well as to CNPq due to their concession of scientific initiation scholarship to the third author.

REFERENCES

1. Joint FAO/WHO Expert Committee on Food Additives (JECFA). Evaluation of certain contaminants in food: eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: WHO Technical Report Series, N. 1002; 2017.

2. Rushing BR, Selim MI. Aflatoxin B_1: A review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. Food Chem Toxicol. 2019; 124:81-100.

3. Ochoa-Velasco CE, Navarro-Cruz AR, Vera-López O, Palou E, Avila-Sosa R. Growth modeling to control (in vitro) Fusarium verticillioides and Rhizopus stolonifer with thymol and carvacrol. Rev Argent Microbiol. 2018; 50:70-74.

4. Food and Drug Administration (FDA). Foodborne Pathogenic Microorganisms and Natural Toxins. 2nd ed. Silver Spring: U.S. Department of Health and Human Services, 2012.

5. An P, Yang X, Yu J, Qi J, Ren X, Kong Q. -Terpineol and terpene-4-ol, the critical components of tea tree oil, exert antifungal activities in vitro and in vivo against Aspergillus niger in grapes by inducing morphous damage and metabolic changes of fungus. Food Control. 2019;98:42-53.

6. Bomfim NS, Nakassugi LP, Oliveira JFP, Kohiyama CY, Mossini SAG, Grespan R, Nerilo SB, Mallmann CA, Abreu Filho BA, Machinski Jr M. Antifungal activity and inhibition of fumonisin production by Rosmarinus officinalis L. essential oil in Fusarium verticillioides (Sacc.) Nirenberg. Food Chem. 2015; 166:330-6.

7. Dammak I, Hamdi Z, El Euch SK, Zemni H, Mliki A, Hassouna M, Lasram S. Evaluation of antifungal and anti-ochratoxigenic activities of Salvia officinalis, Lavandula dentata and Laurus nobilis essential oils and a major monoterpene constituent 1,8-cineole against Aspergillus carbonarius. Ind Crops Prod. 2019; 128:85-93.

8. Abbaszadeh S, Aharifzadeh A, Ahokri H, Khosravi AR, Abbasdeh A. Antifungal efficacy of thymol, carvacrol, eugenol and menthol as

alternative agents to control the growth of food relevant fungi. J Mycol Med. 2014; 24:51-6.

9. Prakash B, Kedia A, Mishra PK, Dwivedy AK, Dubey NK. Assessment of chemically characterized Rosmarinus officinalis L. essential oil and its major compounds as plant-based preservative in food system based on their efficacy against food-borne moulds and aflatoxin secretion and as antioxidant. Int J Food Sci Technol. 2015; 50:1792-98.

10. Wang Y, Zeng X, Zhou Z, Xing K, Tessema A, Zeng H, Tian J. Inhibitory effect of nerol against Aspergillus niger on grapes through a membrane lesion mechanism. Food Control. 2015; 55:54-61.

11. Wang H, Yang Z, Ying G, Yang M, Nian Y, Wei F. Antifungal evaluation of plant essential oils and their major components against toxigenic fungi. Ind Crops Prod. 2018; 120:180-6.

12. Lasram S, Zemni H, Hamdi Z, Chenenaoui S, Houissa H, Tounsi MS, Ghorbel A. Antifungal and antiaflatoxigenic activities of Carum carvi L., Coriandrum sativum L. seed essential oils and their major terpene component against Aspergillus flavus. Ind Crops Prod. 2019; 134:11-8.

13. Palou L, Ali A, Fallik E, Romanazzi G. GRAS, plant- and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest disease of fresh horticultural produce. Postharvest Biol Technol. 2016; 122:41-52.

14. Clinical and Laboratory Standards Institute (CLSI). Reference Method for broth dilution antifungal susceptibility testing of conidium--forming filamentous fungi. NCCLS document M38-A2. Wayne: National Committee for Clinical Laboratory Standards; 2008.

15. Kohiyama CY, Ribeiro MMY, Mossini SAG, Bando E, Bomfim NS, Nerilo SB, Rocha GHO, Grespan R, Mikcha JMG, Machinski Jr M. Antifungal properties and inhibitory effects upon aflatoxin production of Thymus vulgaris L. by Aspergillus flavus Link. Food Chem. 2015; 173:1006-10.

16. Tariq S, Wani S, Rasool W, Shafi K, Bhat MA, Prabhakar A, Shalla AH, Rather MA. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. Microb Pathog. 2019; 134(1-20):103580

17. Association of Official Agricultural Chemists International (AOAC). Natural Toxins. In: AOAC. Official Methods of Analysis of the Association of Official Analytical Chemists. 20th ed. Arlington: The Association of Official Analytical Chemists; 2016. p. 2-46.

18. Braga PC, Culini M, Alfieri M, Dal Sasso M. Thymol inhibits Candida albincans biofilm formation and mature biofilm. Int J Antimicrob Agents. 2008; 31:472-7.

19. Pérez-Alfonso CO, Martínez-Romero D, Zapata PJ, Serrano M, Valero D, Castilho S. The effects of essential oils carvacrol and thymol on growth of Penicillium digitatum and Penicillium italicum involved in lemon decay. Int J Antimicrob Chemother. 2012; 158:101-6.

20. Gandomi H, Misaghi A, Basti AA, Bokaei S, Khosravi A, Abbasifar A, Javan AJ. Effect of Zataria multiflora Boiss essential oil on growth and aflatoxin formation by Aspergillus flavus in culture media and cheese. Food Chem Toxicol. 2009; 47:2397-2400.

21. Prakash B, Dubey NK, Kedia A, Singh A, Singh P. Efficacy of essential oil combination of Curcuma longa L. and Zingiber officinale Rosc. as a postharvest fungitoxicant, aflatoxin inhibitor and antioxidant agent. J Food Saf. 2012; 32:279-88.

22. Ahmad A, Khan A, Akhtar F, Yousuf S, Xess I, Kham LA, Manzoor N. Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against Candida. Eur J Clin Microbiol Infect Dis. 2011; 30:41-50.

23. Prakash B, Kedia A, Mishra PK, Dwivedy AK, Dubey NK. Assessment of chemically characterized Rosmarinus officinalis L. essential oil and its major compounds as plant-based preservative in food system based on their efficacy against

food-borne moulds and aflatoxin secretion and as antioxidant. Int J Food Sci Technol. 2015; 50:1792-98.

24. Nerilo SB, Rocha GHO, Tomoike C, Mossini SAG, Grespan R, Mikcha JMG, Machinski Jr M. Antifungal properties and inhibitory effects upon aflatoxin production by Zingiber officinale essential oil in Aspergillus flavus. Int J Food Sci Technol. 2016; 51:286-92.

25. Razzaghi-Abyanch M, Shams-Ghahfarokh M, Yoshinari T, Mohammad-Bagher R, Jaimand K, Nagasawa H, Sakuda S. Inhibitory effects of Satureja hortensis L. essential oil on growth and aflatoxin production by Aspergillus parasiticus. Int J Food Microbiol. 2008; 123:228-33.