Effect of the essential oils from *Melaleuca alternifolia*, *Melaleuca quinquenervia* and *Backhousia citriodora* on the synthesis of ochratoxin A by *Aspergillus niger* and *Aspergillus carbonarius* isolated from tropical wine grapes

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Abstract The influence of essential oils (EOs) extracted from the leaves of *Melaleuca alternifolia*, *Melaleuca quinquenervia* and *Backhousia citriodora* on ochratoxin A (OTA) synthesis by fungi was studied. The extraction of EOs was performed by hydrodistillation (Clevenger apparatus) over a 2-h period and subsequently analyzed by GC–MS and GC–FID. The toxigenic activity of the essential oils (31.25; 15.62 and 7.81 $\mu\text{g mL}^{-1}$) was evaluated by inhibiting the production of OTA by *Aspergillus niger* and *Aspergillus carbonarius* in Czapek agar medium culture. The quantification of the toxin was performed by HPLC. The production of OTA was dependent on the fungal species, incubation temperature (15 and 25 °C) and the presence of the essential oils. In tests carried out at 15 °C, the oils caused a reduction in OTA synthesis that ranged from 57.60 to 76.93% and from 54.78 to 98.68% for the fungal species *A. carbonarius* and *A. niger*, respectively. At 25 °C, reductions ranged from the 38.66 to 75.93% and from 17.94 to 71.79% for the respective fungi. The study concluded that natural products could be potential bio-control agents against OTA contamination in food.

Keywords Natural products · Myrtaceae · Fungi · Mycotoxins

Introduction

The development of fungi in food and the subsequent mycotoxin contamination constitute a major public health problem. An alternative method for reducing food contamination by ochratoxin A (OTA) involves the use of essential oils (EOs). Essential oils are secondary metabolites obtained from plants and are constituted mainly of mono- and sesquiterpenes and phenylpropanoids, which are compounds that are responsible for the organoleptic properties of the oils. These oils have an extensive application in the cosmetic and food industries (Bizzo et al. 2009).

The essential oil from *Melaleuca alternifolia*, a species native to Australia, is widely used as an active ingredient in many topical formulations that are employed in the treatment of skin infections because of its antimicrobial properties (Carson et al. 2006). The composition of the essential oil from *Melaleuca quinquenervia* (Cav.) S.T. Blake (Myrtaceae) varies widely (different chemotypes). The principal components listed for this oil are *E*-nerolidol and viridifloral, which have distinct biological properties (Padovan et al. 2010).

Backhousia citriodora, known as murtão-limão, presents antifungal properties that might result from the action of citral, the principal component of the essential oil (80–98%) (Lazar-Baker et al. 2011).

Fungi metabolize and synthesize many organic compounds with maleficent properties during their life cycle. The high degree of food contamination by these microorganisms has generated significant losses to industries because, in addition to deterioration of the food, these microorganisms produce mycotoxins. *Aspergillus carbonarius* and *Aspergillus niger* produce ochratoxin A (OTA) via their secondary metabolism. This mycotoxin has

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been detected in various types of food such as cereals, coffee, beans, dried fruits, grapes, wines and their derivatives. It exhibited nephrotoxic, cancerigenic, immunotoxic, teratogenic and genotoxic activities in test animals (Garcia et al. 2011). Among the abiotic factors, temperature can affect the growth and OTA production of the fungus. These organisms require an optimum temperature for growth, which can be different from the temperature necessary for toxin production (Magan et al. 2011).

The exploitation of the biological activities of the essential oils can represent an alternative form of controlling microorganisms so as to reduce the adverse effects of the use of synthetic additives as preservatives in foods. The objective of the present study was to evaluate the effects of the essential oils from *M. alternifolia*, *M. quinquenervia* and *B. citriodora* on the synthesis of OTA by *A. carbonarius* and *A. niger* under two distinct incubation conditions at 15 and 25 °C.

Materials and methods

Extraction and chemical composition of the essential oils

The *M. alternifolia* and *M. quinquenervia* plants were collected at the Fazenda Guapiara, municipality of Aiuruoca, MG, Brazil. The *B. citriodora* plants were obtained at the Garden of Medicinal Plants of the Universidade Federal de Lavras (UFLA), Lavras, MG, Brazil. The plant material was collected in the morning on a day without precipitation.

The healthy leaves were selected and chopped. The essential oil was isolated by distillation over a 2-h period using a modified Clevenger apparatus (Agência Nacional de Vigilância Sanitária, Anvisa 2010). The hydrolact was centrifuged at 965.36g for 5 min. The essential oils were stored under refrigeration in amber glass containers.

Qualitative analyses were performed on a Shimadzu model QP 5050A gas chromatograph coupled to a mass spectrometer (GC–MS). The chromatograph was equipped with a fused silica (5% phenyl, 95% dimethylpolysiloxane) capillary column (J&W Scientific) (30 m × 0.25 mm i.d.; 0.25 µm film), employing He as the carrier gas at a flow rate of 1.2 mL min^{−1}. The MS employed an ion capture detector operating by electron impact and an impact energy of 70 eV. The comparison of the retention indices with those encountered in the literature was also utilized for identification of the components. The retention indices were calculated using the equation of Van den Dool and Kratz for a homologous series of n-alkanes (nC9–nC18). Two libraries, NIST107 and NIST21, were also used for comparison of the spectral data.

Quantitative analyses were performed using a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector (FID) under the following operational conditions: ZB-5MS fused-silica (5% phenyl, 95% dimethylpolysiloxane) capillary column (30 m × 0.25 mm id; 0.25 µm film), using He as the carrier gas with a flow rate of 1.2 mL min^{−1}. The temperature was maintained at 50 °C for 2 min and was then increased at the rate of 4 °C min^{−1}–200 °C, followed by an increase of 15 °C min^{−1}–300 °C, where the temperature was maintained for 15 min. The injector temperature was 250 °C, and the detector temperature was 280 °C. A 0.5 µL volume of essential oil in hexane was applied to the column (Sigma-Aldrich, ≥98%).

Activity of essential oils in ochratoxin A synthesis

The ochratoxigenic effect of the essential oils was evaluated by the method described by Wang et al. (2012), wherein the production of ochratoxin A by the *A. carbonarius* (CCDCA10408) and *A. niger* (CCDCA10409) fungi in culture medium was inhibited by the addition of the essential oils. To obtain the inoculum, fungal isolates (grapes) obtained from the Microorganism Culture Collection of the Departamento de Ciência de Alimentos (UFLA) were transferred to plates containing the Czapek agar culture medium and incubated at 25 °C for 7 days. A suspension of the spores in sterile distilled water containing 0.5% Tween 80 was prepared. A Neubauer counting chamber was used to determine the final spore concentration (10⁷ spores mL^{−1}).

Ten-microliter aliquots of the spore suspension were inoculated in the center of the plate containing Czapek agar culture medium (CYA) supplemented with different concentrations of the essential oils diluted in dimethylsulfoxide (DMSO). The controls (fungus and fungus + DMSO) and three concentrations of the oils (31.25; 15.62 and 7.81 µg mL^{−1}) were tested. Two concentrations of essential oil were below the minimum inhibitory concentration (MIC) determined in preliminary tests. The plates were incubated in a chamber in the dark at 15 and 25 °C for 10 days.

Extraction of OTA from the fungal cultures

Ochratoxin A was extracted according to the method of Passamani et al. (2014). Initially, three colony plugs were removed from the internal area, the middle and the edge of each colony on the tenth day of incubation. These plugs were weighed and deposited in test tubes wrapped in aluminum foil, and methanol (1 mL) was added. The tubes were stirred vigorously for 5 s and were kept at room temperature for 60 min.

The extracts were filtered through PTFE (polytetrafluoroethylene) filter units (0.22 μm) (Millipore) and analyzed on a Shimadzu HPLC equipped with two model SPD-M20A high pressure pumps, a model DGU 20A3 degasser, a model CBM-20A interface, a model SIL-10AF autosampler and an RF-10 AXL fluorescence detector. An Agilent Zorbax Eclipse XDB-C18 (4.6 mm $\times$ 250 mm, 5 μm) column connected to an Agilent Zorbax Eclipse XDB-C18 4-Pack guard column (4.6 mm $\times$ 12.5 mm, 5 μm) was used.

The wavelengths employed were 332 nm for excitation and emission at 476 nm. The flow rate employed throughout the analysis was 0.8 mL min⁻¹, and the volume of sample and standard injected was 20 μL . The elution was performed in an isocratic system of 35:35:29:1 (methanol: acetonitrile: water: acetic acid).

The quantitative determination of OTA in the samples was performed by external standardization. The limits of detection (LD) and quantification (LQ) were estimated using the parameters obtained from the calibration curve, calculated by their mathematical relationships: LD = 3 DP/m and LQ = 10 DP/m (where SD = estimate of the standard deviation of the regression line and m = slope of the calibration line) (Harris 2008). The values of 0.0004 and 0.0016 $\mu\text{g g}^{-1}$, respectively, were found. All the samples were analyzed in duplicate, and the OTA standard solutions were injected in triplicate.

Statistical analysis

The Scott-Knott test at 5% significance was used to evaluate the differences in the production of OTA (Ferreira 2011).

Results and discussion

The greatest decrease in the production of OTA was observed at 25 °C for both fungi. There was a reduction of approximately 8.77 (15 °C) to 2.09 $\mu\text{g g}^{-1}$ (25 °C) and 0.300 (15 °C) to 0.039 $\mu\text{g g}^{-1}$ (25 °C) for the *A. carbonarius* and *A. niger* fungi, respectively (Fig. 1). Generally, the OTA production by *A. carbonarius* is greater than that of *A. niger*. This fact is justified by the specific behavior of the isolates tested.

According to Magan et al. (2011), the most important factors influencing the growth, sporulation and toxin production by fungi are temperature and water activity. Pasamani and collaborators (2014) evaluated the effects of abiotic factors on the production of OTA and observed higher toxin concentrations for *A. carbonarius* (8–10 $\mu\text{g g}^{-1}$) under stress conditions (15 °C), and the levels were lower at 25 °C (1–3 $\mu\text{g g}^{-1}$). For *A. niger*, the

greatest synthesis of toxin (6–7 $\mu\text{g g}^{-1}$) occurred at 15 °C and *A. niger* did not produce toxin at 20–25 °C. These observations were similar to the results obtained in the present study.

EOs caused a decrease in the production of OTA at 15 °C that ranged from 57.60 to 76.93% and from 54.78 to 98.68% for *A. carbonarius* and *A. niger*, respectively. In the tests at 25 °C, the reductions ranged from 38.66 to 75.93% and from 17.94 to 71.79% for the respective fungi (Table 1).

The principal constituents in the essential oil from *M. alternifolia* have previously been found were terpinen-4-ol (56.80%), γ -terpinene (19.30%) and *p*-cymene (9.84%) (Table 2). The principal compounds found in the oil from *M. quinquenervia* were α -pinene (61.37%) and linalool (22.08%); whereas the oil extracted from the leaves of *B. citriodora* was composed principally of citral (91.19%). In general, although the oils exhibited distinct chemical compositions, they presented effects on OTA synthesis (Fig. 1; Table 1).

Jersey et al. (2014) studied the activity of the EOs obtained from thyme (*Origanum vulgare* L.), peppermint (*Mentha piperita* L.), fennel (*Foeniculum vulgare* Mill.) and pine (*Abies alba* Mill.) They also noted the importance of these products in reducing the concentration of OTA produced by *Penicillium verrucosum*. They argued that the combination of mono- and sesquiterpenes, classes of compounds found in the EOs obtained in this study, inhibits the growth and toxin production by the fungi.

Studies by Sonker et al. (2014) confirmed that the essential oil from *Cymbopogon citratus* (DC.), consisting of 92.28% citral, also acts to reduce mycotoxin production by *A. niger*, *A. flavus* and *A. ochraceus*. Terzi et al. (2007) investigated the effect of the essential oil from *M. alternifolia* and its major constituents—terpinen-4-ol, γ -terpinene and 1,8-cineole—on *Pyrenophora graminea*, *Fusarium graminearum* and *Fusarium culmorum* and observed that terpinen-4-ol was the most efficient for reducing in mycelial growth of the fungal species. This fact suggests that the compounds citral and terpinen-4-ol might be responsible for the antiocrotogenic activity of *B. citriodora* and *M. alternifolia*, respectively.

According to Sokolic-Mihalak et al. (2012), the mechanisms of action of essential oils on the fungi have not yet been fully elucidated. Some antifungal agents operate in the ergosterol biosynthesis in fungal hyphae (fungal lipid component); the absence of this component induces the collapse of the fungal cells (mycelium) and the interruption of mycelial growth (fungal body) (Hua et al. 2014).

Yamamoto-Ribeiro et al. (2013) studied the effect of the EO from *Zingiber officinale* (ginger) against the *Fusarium verticillioides* fungus and observed an increase in the production of ergosterol when a concentration of 1000 $\mu\text{g mL}^{-1}$ of the EO was used and 57 and 100%

Fig. 1 Effects of the essential oils on the production of ochratoxin A by *A. carbonarius* (a) and *A. niger* (b).

M.a = *Melaleuca alternifolia*;
M.q = *Melaleuca*

quinquenervia;

B.k = *Backhousia citriodora*;

Control = Fungo and DMSO.

Concentrations:

(1) = $31.25 \mu\text{g mL}^{-1}$;

(2) = $15.62 \mu\text{g mL}^{-1}$ e

(3) = $7.81 \mu\text{g mL}^{-1}$. Different

upper-case letters represent the comparison between the concentrations of each treatment

and different lower-case letters represent the comparison within each treatment according to the

Scott-Knott test

Table 1 Percent reduction (%) of ochratoxin production with the use of essential oils

Treatments		Fungus			
		<i>A. carbonarius</i> (%)		<i>A. niger</i> (%)	
		15 °C	25 °C	15 °C	25 °C
<i>M. alternifolia</i> ($\mu\text{g mL}^{-1}$)	DMSO	0.00	0.00	0.00	0.00
	31.25	73.83	75.93	84.16	71.79
	15.62	57.60	61.05	54.78	17.94
	7.81	70.23	0.00	97.68	56.41
<i>M. quinquenervia</i> ($\mu\text{g mL}^{-1}$)	31.25	60.32	55.93	83.49	0.00
	15.62	66.80	57.22	92.41	35.89
	7.81	68.12	59.95	83.16	0.00
<i>B. citriodora</i> ($\mu\text{g mL}^{-1}$)	31.25	68.57	71.53	98.68	0.00
	15.62	76.93	73.20	87.79	0.00
	7.81	68.16	59.67	89.43	0.00

Percent decrease calculated relative to the control, which was considered to be 100%. Means followed by the same letter in the same column do not differ significantly at 5% of probability by the Scott-Knott test

inhibitions at concentrations of 4000 and 5000 $\mu\text{g mL}^{-1}$, respectively. According to Lucini et al. (2006), some compounds may cause lipid peroxidation in fungi, which

induces an adaptive response, reprogramming of gene expression to protect the cell wall structure, and, hence, an increase in ergosterol biosynthesis.

Table 2 Composition of the essential oils from *B. citriodora*, *M. alternifolia* and *M. quinquenervia*

Compound	RI _{CAL}	RI _{LIT}	BK	M.a	M.q
Isobutyl isobutyrate	904	908	–	–	0.90
α -Pinene	929	932	–	2.72	61.37
β -Pinene	970	974	–	–	1.53
α -Phelandrene	996	1002	–	–	1.59
α -2-Carene	1009	1001	–	5.47	–
ρ -Cymene	1017	1020	–	9.84	3.24
Limonene	1021	1024	–	–	–
1,8-Cincole	1024	1026	–	–	0.05
1,8-Cineol	1026	1025	–	–	–
Linalool	1095	1092	–	–	22.08
<i>endo</i> -Fenchol	1107	1114	–	–	–
<i>exo</i> -Fenchol	1108	1118	–	–	0.46
6-Camphenol	1119	1111	–	–	–
<i>trans</i> -Pinocarveol	1134	1135	–	–	1.40
γ -Terpinene	1052	1054	–	19.30	–
Pinocarvona	1158	1160	–	–	1.00
Borneol	1161	1165	–	–	–
Terpinen-4-ol	1177	1174	–	56.80	–
α -Terpineol	1186	1188	–	2.58	–
Mirtenol	1191	1194	–	–	–
Neral	1236	1235	39.82	–	–
Geranial	1266	1264	51.37	–	–
Total (%)			91.19	96.71	93.62

Items in bold represent the principal components in the oil

RI_{cal} calculated retention index, RI_{lit} retention index obtained from the literature, BK essential oil from the leaves of *B. citriodora*, M.a essential oil from the leaves of *M. alternifolia*, M.q essential oil from the leaves of *M. quinquenervia*

Hua et al. (2014) evaluated the effect of essential oils and some compounds on the growth of *Aspergillus ochraceus* and the production of OTA and found that citral did not inhibit the synthesis of ergosterol at concentrations of 25–150 $\mu\text{g mL}^{-1}$. However, the biosynthesis was influenced at the concentration of 200 $\mu\text{g mL}^{-1}$. Total inhibition was observed in the production of OTA for monoterpene concentrations of 75–200 $\mu\text{g mL}^{-1}$, a result that differs from the results obtained in this study.

However, the concentrations used by the authors were higher than those used in the present EO tests, and different fungal species were used in this study. Moreover, the essential oil from *B. citriodora*, for example, consists mainly of citral, with the presence of minor components that might influence the activity of the oil. In fact, it was suggested that the activity of citral found by Hua et al. (2014) in reducing the production of mycotoxin was not due to the decrease in fungal mass, but may be due to suppression of transcription of the genes for OTA biosynthesis, such as the polyketide synthase gene (*pks*),

responsible for the synthesis of dihydroisocoumarin and involved the first steps of the pathway of ochratoxin A formation in the metabolism of *A. carbonarius* (Gallo et al. 2014) and *A. niger* (Castellá et al. 2015).

Thus, we suggest that essential oils be used in food products susceptible to contamination by OTA-producing fungi. In vivo trials are needed to confirm the effectiveness of these oils, and they should be evaluated toxicologically to certify the safety of their use for human consumption. In addition, analysis of food products will furnish information on possible changes in organoleptic properties caused by the addition of the oils.

Conclusion

The essential oils from *M. alternifolia*, *M. quinquenervia* and *B. citriodora* influenced the production of OTA by *A. carbonarius* and *A. niger* fungi. The oils caused a decrease in OTA reduction, ranging from 57.60 to 76.93% and from 54.78 to 98.68% for *A. carbonarius* and *A. niger*, respectively, in tests performed at 15 °C. At 25 °C, reductions ranged from the 38.66 to 75.93% and from 17.94 to 71.79% for the respective fungi.

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