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In vitro and *in vivo* fungicidal and anti-ochratoxigenic activities of the essential oils of *Backhousia citriodora* and *Lippia origanoides* Kunth. in coffee fruits

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Abstract: The fungicidal and anti-ochratoxigenic potentials of the essential oils (EO's) from *Backhousia citriodora* and *Lippia origanoides* were studied through in vitro and in vivo tests on coffee fruits. The EO's were extracted by hydrodistillation and characterized by gas chromatography using mass spectrometer (GC-MS) and flame ionization detectors (GC-FID) The in vitro antifungal activity of EOs against *A. carbonarius* and *A. ochraceus* was determined by measuring the percentage of inhibition of mycelial growth (MFC). The in vivo antifungal potential was evaluated by plating fractions of the pericarp and coffee beans (*Coffea arabica* L. and Catuaí) inoculated with spore suspensions and sprayed with essential oils (Blotter Test). The inhibition of OTA synthesis in vivo was quantified by High Performance Liquid Chromatography (HPLC). The geranial (57.55%) and neral (42.44%) isomers were the principal compounds from the *B. citriodora* EO, and carvacrol (65.53%), p-cymene (12.13%) and thymol (7.05%) were found in the EO from *L. origanoides*. The EO's from *L. origanoides* and *B. citriodora* inhibited the growth of *A. carbonarius* at a minimum fungicidal concentration (MFC) of 250 µL L^{-1} and 500 µL L^{-1}, respectively, and they inhibited the growth of *A. ochraceus* at a MFC of 500 µL L^{-1} and 250 µL L^{-1}, respectively. The EO's presented an antitoxigenic effect on coffee fruits and can be promising for improving the quality and maintaining the safety of the coffee beans as well as the beverage.

Keywords: *Coffea arabica*; ochratoxin A; lemon myrtle; rosemary d'angola; antifungal; coffee beans.
Abbreviations: BOD_biochemical oxygen demand; EO('s)_essential oil(s); GC-FID_gas chromatography-flame ionization detector; GC-MS_gas chromatographer-mass spectrometer; HPLC_high-performance liquid chromatography; IARC_international cancer research agency; LD_detection limit; LQ_quantification limit; MA_malt agar; MAPA_Ministério da Agricultura e Pecuária; MFC_minimum fungicidal concentration; NRPS_nonribosomal peptide-synthetase; OTA_ochratoxin A; PBS_phosphate buffered saline; PKS_Polyketide synthases; RPM_revolution per minute.

Introduction

Coffee is one of the most widely consumed beverages in the world, having spread to different cultures and peoples. It is used in different ways and yields a wide variety of by-products that are produced and exported by more than 47 countries (ICO, 2019). The economic and commercial importance of coffee is immeasurable, especially in countries where the economy is directly linked to the coffee industry, such as Brazil, Vietnam and Colombia (Debastiani et al., 2019; dos Santos, Alvarenga and Boffo, 2020). Brazil stands out as the largest coffee exporter, with a yearly production of 40.6 million bags. This production represents great socioeconomic importance for the country's development, ensures the generation of jobs and taxes and contributes to the country's revenue (FAO, 2019). The world consumption of coffee is increasing every year, along with the concern of consumers with the quality of the beverage, and the coffee industry needs new technologies to meet a greater demand without decreasing the quality and sustainability of the production chain (dos Santos et al., 2020).

One of the factors that affect the quality and causes major economic impacts is the susceptibility of coffee fruits to the action of microorganisms throughout the entire production chain and especially in the post-harvest period. The filamentous fungi of the *Aspergillus, Penicillium* and *Fusarium* genus compromise the visual aspect of the grains and the quality of the taste and aroma of the beverage (Iamanaka et al., 2014). In addition, some species of the *Aspergillus* genus produce secondary metabolites, such as ochratoxin A (OTA), during their growth and development. This mycotoxin is toxic to animals and humans, and it is considered to be nephrotoxic, cytotoxic, carcinogenic, teratogenic and immunosuppressive (Chalfoun et al., 2018). The International Cancer Research Agency (IARC) classifies ochratoxin A in group 2B as being possibly carcinogenic in humans (IARC, 1993). According to Silva et al. (2020), regulatory authorities established that the maximum level of this substance permitted in coffee is 5 and 10 µg kg^{-1} of processed coffee in the European Union and Brazil, respectively. The contamination of coffee fruits by fungi that produce ochratoxin A occurs mainly during the process of drying on coffee terraces, a time that covers the grace period for synthetic antifungals used in the previous stages of the production chain (Vitoratos et al., 2013).

The effects caused by the presence of fungi and the synthesis of

Table 1. Chemical composition of the essential oils of *Backousia citriodo*ra and *Lippia oiganoides* Kunth.

Percentage of constituent in the essential oil		
Constituent	***B. citriodora***	***L. origanoides***
Carvacrol	-	65.5307
Geranial	57.55	-
Myrcene	-	1.3542
Neral	42.44	-
Caryophylene oxide	-	1.5621
p-Cymene	-	12.1347
Terpine-4-ol	-	0.4647
Thymol	-	7.0471
Trans-β-Caryophylene	-	4.0466
α-Phelandrene	-	0.3046
α-Pinene	-	0.1753
α-Terpinene	-	0.8335
α-Tujene	-	0.6346
Total percent	**99.99**	**99.6677**
Compound groups		
Monoterpene hydrocarbons	-	21.0165
Oxygenated monoterpenes	99.99	73.0425
Sesquiterpene hydrocarbons	-	4.0466
Oxygenated sesquiterpenos	-	1.5621

*The quantity of each chemical constituent in the essential oils is expressed as a percentage. (-) unidentified chemical constituent.

OTA in coffee fruits can be minimized or controlled with the use of products of natural origin or their derivatives. Secondary metabolites produced by plants act as potent antimicrobials, and they are capable of inhibiting the growth of various fungi, including toxigenic fungi (Stierle and Stierle, 2015). Essential oils have been explored in the control of the growth of fungi as well as in the production of OTA in fruits during the post-harvest period because they are composed of a complex mixture of terpenes and phenylpropanoids (Vitoratos et al., 2013; Kapetanakou et al., 2019). The aromatic species *Backhousia citriodora* and *Lippia origanoides* Kunth., respectively known as lemon myrtle and rosemary d'angola, contain essential oils that are rich in non-aromatic and aromatic monoterpenes such as citral and carvacrol, which exhibit a high toxicity to microorganisms and can act as fungicides during the post-harvest period and processing of coffee fruits (Sarrazin et al., 2015; Teixeira, 2019). The objectives of this work were to chemically characterize the essential oils from the leaves of *Backhousia citriodora* and *Lippia origanoides* Kunth., to evaluate the fungicidal potential against *A. carbonarius* and *A. ochraceus*, and to determine the anti-ochratoxigenic activity through in vivo tests on coffee fruits.

Results and Discussion

Chemical characterization of essential oils

The chemical constituents present in the essential oils extracted from the leaves of *B. citriodora* and *L. origanoides* are shown in Table 1. Citral, consisting of the mixture of the geranial (57.55%) and neral (42.44%) isomers, was the principal constituent of the essential oil from *B. citriodora*. Southwell et al. (2003) studied the chemical composition of the oil of lemon myrtle leaves obtained from commercial plantations in Australia and found the oxygenated monoterpenes, neral and geranial, to be the principal components. The compounds myrcene, linalool and citronellal were also identified, although in low concentrations. The results found by Southwell et al. (2003) partially corroborate those found in this study.

The principal compounds in the essential oil from *L. origanoides* were carvacrol (65.53%), *p*-cymene (12.13%) and thymol (7.05%). The results partially corroborate those found by Sarrazin et al. (2015), who analyzed the composition of the essential oil from *L. origanoides* Kunth. collected in the state of Pará, Brazil. They determined that carvacrol (47.2%), thymol (12.8%), *p*-cymene (9.7%) and *p*-methoxythymol (7.4%) were the principal components. Because essential oils are composed of volatile compounds and act in the defense system of the plants, qualitative and quantitative differences in their components as a result of intrinsic, extrinsic and abiotic factors can occur (Dhouioui et al., 2016).

In vitro antifungal activity

A remarkable exponential antifungal activity against the microorganisms under study (Figure 1) was observed for the essential oils. The essential oil from *L. origanoides* inhibited the proliferation of the *A. carbonarius* and *A. ochraceus* fungi with minimum fungicidal concentrations (MFC) of 250 µL L^{-1} and 500 µL L^{-1}, respectively. The mycelial growth of *A. carbonarius* and *A. ochraceus* was completely inhibited by the essential oil from *B. citriodora*, with MFCs of 500 µL L^{-1} and 250 µL L^{-1}, respectively.

Essential oils act on fungi by several mechanisms. One of the mechanisms reported in the literature involves the ability of essential oils to alter the permeability and integrity of fungal cell membranes to affect their growth (Guo et al., 2017; Adelakun et al., 2016). These mechanisms are related to the lipophilic characteristic and the low molecular weight of the constituents present in essential oils. These substances interact easily with the fungal cell membrane and alter its functions (Pawar and Thaker, 2006).

For An et al. (2019), highly lipophilic oxygenated monoterpenes can exert antifungal activities through the rupture of the cell wall, membrane and cytoplasm. Consequently, they block the synthesis of components of cell walls, cytomembranes, cytoplasm and organelles and cause modifications in the growth and morphology of mycelia and spores. In addition, monoterpenes can negatively influence metabolic and energetic pathways, thereby interfering in the development of fungi.

Antifungal activity in Coffea arabica fruits

The values referring to the plating of coffee pericarps after treatments against *A. carbonarius* and *A. ochraceus* with the essential oils from *B. citriodora* and *L. origanoides* are shown in Figure 2, where the antifungal activity was evaluated by spore counts for *A. carbonarius* (Figure 2A) and with respect to the occurrence index for *A. ochraceus* (Figure 2B). During the plating of the grains, the presence of the inoculated fungi in the fruits was not observed because there was no rest period, a step that favors the penetration of microorganisms present in the pericarp of the green grain. Thus, the rest period was observed to contribute to the contamination of grains by pathogens present in the fruits.

The application of the essential oils resulted in an exponential decrease in pathogens in the pericarps, reducing the number of *A. carbonarius* spores by 71.43% and 69.57% for treatments with

Table 2. Effects of essential oils from *B. citriodora* and *L. origanoides* Kunth. on the synthesis of ochratoxin A by *A. carbonarius* in coffee pericarp.

Treatment	Concentration	Production of OTA (µg Kg^{-1})	Value – p^2			
CF	-	1.64	CF	CN	500	1000
CN	10%	1.60	0.876	-	-	-
	500 µL L^{-1}	1.48	**0.014**	0.088	-	-
B. citriodora	1000 µL L^{-1}	1.42	**0.001**	**0.007**	0.653	-
	2000 µL L^{-1}	1.42	**0.001**	**0.006**	0.638	1.000
CF	-	1.64	CF	CN	250	500
CN	10%	1.60	0.907	-	-	-
	250 µL L^{-1}	1.65	1.000	0.834	-	-
L. origanoides	500 µL L^{-1}	1.61	0.982	0.999	0.951	-
	1000 µL L^{-1}	1.47	**0.015**	0.079	0.011	0.045

Values of p^2 <0.05 differ by the Tukey test at the level of 5% probability; CF = Fungal control: only containing inoculum; CN = Negative control: 10% powdered milk solution.

Table 3. Effects of the essential oils from *B. citriodora* and *L. origanoides* Kunth. on the synthesis of ochratoxin A by *A. ochraceus in* coffee pericarp.

Treatment	Concentration	Production of OTA (µg Kg^{-1})	Value – p^2			
CF	-	1.16	CF	CN	500	1000
CN	10%	1.17	1.000	-	-	-
	250 µL L^{-1}	1.12	0.962	0.950	-	-
B. citriodora	500 µL L^{-1}	1.12	0.935	0.919	1.000	-
	1000 µL L^{-1}	1.04	0.197	0.182	0.554	0.620
CF	-	1.16	CF	CN	250	500
CN	10%	1.17	1.000		-	-
	500 µL L^{-1}	1.11	0.160	0.131	-	-
L. origanoides	1000 µL L^{-1}	1.06	0.005	0.004	0.344	-
	2000 µL L^{-1}	0.99	**0.000**	**0.000**	**0.002**	0.059

Values of p^2 <0.05 differ by the Tukey test at the level of 5% probability; CF = Fungal control: only inoculum; CN = Negative control: 10% powdered milk solution.

the highest concentrations of the essential oils from *B. citriodora* and *L. origanoides*, respectively. The incidence of *A. ochraceus* also decreased by 74.86% and 51.33% in treatments with the highest concentrations of these essential oils.

The antifungal activity was observed at higher concentrations in vivo than was observed in vitro, probably because of the volatility of the essential oils. The coffee fruits were exposed to solar radiation and a variation in temperature during drying. The fact that the complexity of the investigated system was greater in in vivo tests was also a factor.

There are no reports in the literature of studies in which the biological potential of essential oils from *B. citriodora* and *L. origanoides* was evaluated against *A. carbonarius* and *A. ochraceus* in fruits of *Coffea arabica*. However, authors such as Sonker, Pandey and Singh (2015) evaluated the antifungal effect of the essential oil from *Artemisia nilagirica* on deteriorating fungi such as *A. ochraceus* in grapes. They observed that the essential oil, rich in monoterpenes, inhibited fungal growth, and the useful life of the grapes increased without modification of sensory properties of the fruits or phytotoxic effects. The results found by the authors and in the present study are in agreement, confirming that essential oils rich in monoterpenes, aromatic or not, are effective in microbiological control, both in vitro and in vivo.

Ochratoxin A in pericarps and Coffea arabica grains

The production of ochratoxin A in coffee pericarps infected with *A. carbonarius* and *A. ochraceus* (fungal controls) and the influence of the treatment with essential oils on the production of the toxin are shown in Tables 2 and 3, respectively. The OTA production in the coffee pericarp by the controls was 1.64 and 1.16 µg Kg^{-1} for *A. carbonarius* and *A. ochraceus*, respectively. In general, the values obtained are below the limits established by European and Brazilian legislation of 5 µg Kg^{-1} and 10 µg Kg^{-1}, respectively (EC, 2006; ANVISA, 2011). However, the production of ochratoxin A was slightly lower in treatments with both essential oils, which showed a dose-dependent effect.

The presence of ochratoxin A could not be determined in coffee beans whose fruits were infected with *A. ochraceus*. Although the proliferation of fungi in coffee beans whose fruits were infected with *A. carbonarius* was not observed, contamination by OTA (1.16 µg Kg^{-1}) was observed. The level was 29.27% lower than that found in the pericarps. The application of both essential oils resulted in a decrease in OTA production by 18.68% relative to the fungal control (Table 4).

The data obtained in this work corroborate the results obtained by Aldred et al. (2008), who investigated the effectiveness of essential oils from cinnamon, cloves, and laurel and that of the antioxidant resveratrol against the production of OTA by *Penicillium verrucosum* and *A. westerdijkiae* in wheat grains. Wang et al. (2018) stated that the reduction in OTA production caused by the principal compound in the essential oil from cinnamon, cinnamaldehyde, is a consequence of the disregulation in the expression of genes (PKS, NRPS, VeA, LaeA and VelB) responsible for OTA biosynthesis. The authors studied the antifungal and antitoxigenic effects of cinnamaldehyde at the morphological and structural levels, together with the inhibition of OTA synthesis at the transcriptional level, and determined that cinnamaldehyde can act as a safe and effective natural agent against OTA contamination.

Research by Hua et al. (2014) showed that citral, the only compound present in the essential oil under study (*B. citriodora*), is capable of suppressing the transcription of the PKS genes responsible for OTA biosynthesis. Previously, Tian et al. (2012) suggested that compounds rich in terpenes and terpenoids can easily cross cell membranes to induce biological responses. This principle can help us to understand the effect of aromatic monoterpenes, such as carvacrol, the principal component in the essential oil from *L. origanoides*, on toxigenic fungi. The phenolic hydroxyl of the molecule can form hydrogen bonds with enzymes to modify their activities and, as a consequence, it can

Table 4. Effects of essential oils from *B. citriodora* and *L. origanoides* Kunth. on the synthesis of ochratoxin A by *A. carbonarius* in coffee beans.

Treatment	Concentration	Production of OTA (µg Kg^{-1})	Value – p^2			
CF	-	0.91	CF	CN	500	1000
CN	10%	0.90	0.989	-	-	-
	500 µL L^{-1}	0.86	0.025	0.068	-	-
B. citriodora	1000 µL L^{-1}	0.76	**0.000**	**0.000**	**0.000**	-
	2000 µL L^{-1}	0.74	**0.000**	**0.000**	**0.000**	0.510
CF	-	0.91	CF	CN	250	500
CN	10%	0.90	0.996	-	-	-
	250 µL L^{-1}	0.81	**0.000**	**0.001**	-	-
L. origanoides	500 µL L^{-1}	0.76	**0.000**	**0.000**	0.071	-
	1000 µL L^{-1}	0.74	**0.000**	**0.000**	0.013	0.898

Values of p^2 <0.05 differ by the Tukey test at the level of 5% probability; CF = Fungal control: only inoculum; CN = Negative control: 10% powdered milk solution.

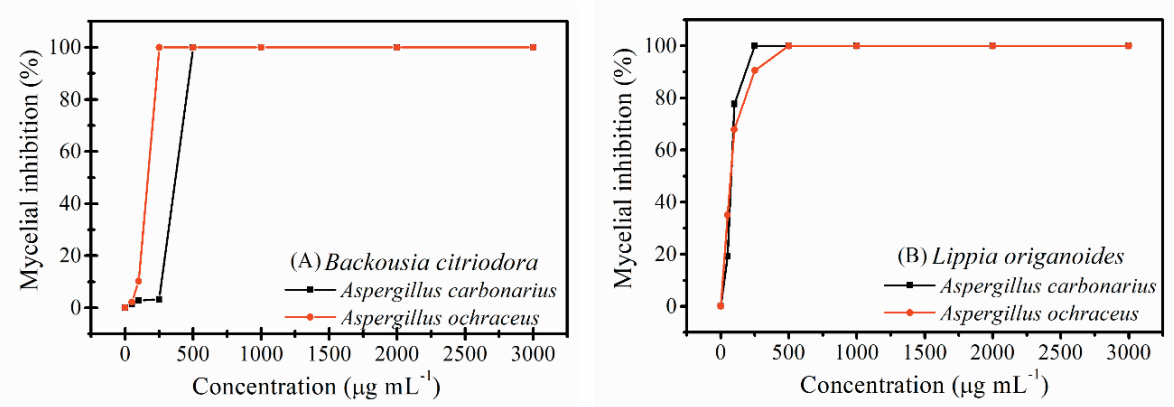

Fig 1. Inhibition of mycelial growth of *Aspergillus carbonarius* and *Aspergillus ochraceus* by the essential oils from *Backousia citriodora* and *Lippia origanoides* Kunth.

cause changes in the behavior of the microorganism and in the production of secondary metabolites, such as toxins (Goncalves and Romano, 2017).

In conclusion, neral (42.44%) and geranial (57.55%) were the principal constituents in the essential oil from *B. citriodora*, and carvacrol (65.63%), *p*- cymene (12.13%) and thymol (7.04%) were found to be the principal constituents in the essential oil from *L. origanoides*. The essential oils were effective in inhibiting the mycelial growth of *A. carbonarius* and *A. ochraceus* in vitro and exerted antifungal and anti-ochratoxigenic effects on fungi in pericarps and coffee beans. Essential oils reduced the formation of *A. carbonarius* spores and the rate of occurrence of *A. ochraceus* in coffee pericarps. For the first time, the influence of essential oils on the proliferation of toxigenic fungi and on the inhibition of ochratoxin A biosynthesis in coffee fruits was demonstrated. The essential oils from *B. citriodora* and *L. origanoides* could be favorable for the environment. They generate a minimum amount of residues in the protection of coffee and other foods contaminated by toxigenic fungi because the oils are volatile. Thus, they are promising for the development of new natural antifungals to guarantee food quality and safety.

Material and Methods

Collection of plant material and extraction of essential oils

The *B. citriodora* and *L. origanoides* species were collected at the Horto de Plantas Medicinais, Universidade Federal de Lavras (UFLA) (21° 13' south latitude and 44° 58' west longitude) in March 2018. The exsiccata of *B. citriodora* and *L. origanoides* were deposited in the ESAL Herbarium under registration numbers 30300 and 23660, respectively. The essential oils were extracted by hydrodistillation using the Clevenger apparatus modified in accordance with the procedure described by Anvisa (2010).

Chemical characterization of essential oils

The compounds comprising the essential oils were identified by gas chromatography coupled to mass spectrometry (GC/MS, Shimadzu Corporation, model 110 QP 2010 Plus, Kyoto, Japan), and the quantification of each constituent was obtained by normalizing areas (%) of peaks obtained by gas chromatography with a flame ionization detector (GC/FID, Shimadzu GC-17A) as described by Brandão et al. (2020).

The Van den Dool and Kratz (1963) equation was used to calculate the retention index, and the standards used were the homologous series of n-alkanes (nC8-nC18). The compounds identified yielded mass spectra and retention indices with up to 95% similarity with those of the equipment libraries (FFNSC 1.2, NIST 107 and NIST 21) and with the literature retention indices of Adams (2017).

Isolation of fungi and preparation of the spore suspension

The fungal isolates of the *Aspergillus* genus were obtained by direct plating on coffee beans by the Blotter Test method (Tempe, 1963). The identification of the species of the selected isolates proceeded according to the isolation of the microorganisms in Czapeck Yeast Agar (CYA, HiMedia Laboratories Pvt. Ltd., Munbai, India) after incubation at 25 °C and 37 °C for a period of 7 days according to the identification keys proposed by Samson et al. (2014).

The isolates that presented the set of favorable characteristics were selected, that is, greater speed of mycelial growth and high potential for the production of ochratoxin A. The selected isolates were identified as *Aspergillus carbonarius* and *Aspergillus ochraceus*. The fungi were incubated in a manner similar to a procedure described elsewhere (Brandão et al., 2020).

In vitro antifungal activity of essential oils

The antifungal activities of the essential oils were evaluated in vitro according to a modification of the method described by

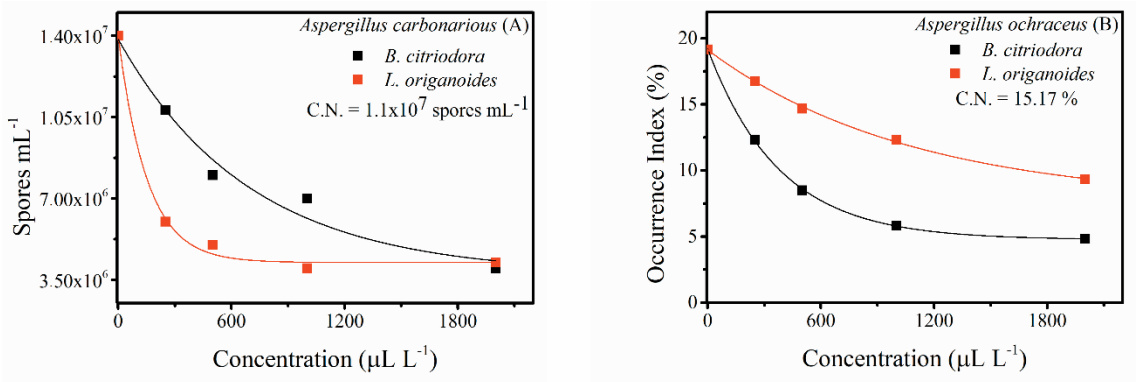

Fig 2. Antifungal activity of essential oils from *B. citriodora* and *L. origanoides* against contamination of *Coffea arabica* L. beans. Influence of the action of essential oils on: (A) spore count of *A. carbonarius* and (B) index of occurrence of *A. ochraceus*. C.N.- Negative control: 10% powdered milk solution.

Brandão et al. (2020). The isolates (*A. carbonarius* and *A. ochraceus*) were reactivated in Petri dishes containing Malt Agar culture medium (MA, HiMedia Laboratories Pvt. Ltd., Munbai, India) at 25 °C for 10 days. After this period, the plating was performed by replicating the fungus in the center of the plate containing 50, 100 250, 500, 1000, 2000 and 3000 µL L^{-1} concentrations the essential oils (*B. citriodora* and *L. origanoides*) in 20 mL of MA medium. Petri dishes containing just the culture medium and the microorganism were used as the fungal control. All the treatments were incubated in BOD in the dark for 15 days at 25 °C, and orthogonal measurements of the diameter of the mycelial growth were performed on the last day of incubation. These analyses were performed in triplicate. The percentage of inhibition of mycelial growth by the essential oils was calculated according to Brandão et al. (2020).

Preparation of coffee beans for the in vivo test

The in vivo experiments were performed on coffee beans of the species *Coffea arabica* L. cv Catuaí Amarelo, 2017/2018 crop, harvested at the berry maturation stage. The fruits were disinfected with a 1% hypochlorite solution and washed in running water. At the semi-dry state, in which the berries contained 18-20% moisture, the spore suspensions (10^6 spores mL^{-1}) of the fungi (*A. carbonarius* and *A. ochraceus*) were added to the coffee beans and incubated. The treatments were accomplished by spraying the essential oils from *B. citriodora* and *L. origanoides*, diluted in a 10% powdered milk solution in the concentrations of 250, 500, 1000 and 2000 µL L^{-1}, over the *A. carbonarius* and *A. ochraceus* cultures. Fungal control (fungal spore suspension only) and negative control (fungal spore suspension and 10% powdered milk solution) were used. The fruits, when reaching 12% moisture, were submitted to mechanical processing to remove the pericarp. Mechanical processing was performed by collecting pericarps and grains separately.

Antifungal activity of essential oils on Coffea arabica fruits

The antifungal potentials of the essential oils were evaluated by plating fractions of pericarp and coffee beans using the Blotter Test method (Tempe, 1963; Angélico and Lima, 2012). The pericarps were plated without disinfection, whereas disinfestation of the grains was performed to observe the microbiota present inside. The disinfestation of the grains was achieved by treatment with 70% alcohol for one minute, followed by treating with 1% sodium hypochlorite for 30 seconds and washing three times with sterile distilled water.

Petri dishes (15 cm in diameter) containing two sheets of sterile filter paper moistened with 10 mL of sterile distilled water were used. For each repetition of in vivo treatments (triplicate), 25 fractions of pericarp or grain were plated per plate and incubated in a BOD at 25 °C with a photoperiod of 12 hours for 10 days.

The results for *A. carbonarius* (pericarps and grains) were expressed by counting spores mL^{-1} in a Newbauer chamber and adding 10 mL of distilled water containing 1% Tween 80 to the plate. The results for *A. ochraceus* (pericarps and grains) were expressed by identifying the main fungal genera from the samples with the aid of a Phoenix CP 608 stereoscopic microscope. The incidence of *A. ochraceus* was determined by the Mckinney equation (Mckinney and Davis, 1925), usually used in the calculation of the occurrence index:

$$\text{OI (\%)} = \frac{(f \times n_0) + (f \times n_1) + (f \times n_2) + (f \times n_3) + (f \times n_4)}{FN100}$$

where f is the number of individuals for each grade; F is 25 (total number of individuals); N is 4 (maximum attributable score). Notes (0, 1, 2, 3, 4) were estimated according to the area occupied in the pericarp or grain by the fungus and represented by the weighted average of the notes attributed to this occurrence, where $n_0 = 0$ (note for the absence of fungus in the region); $n_1 = 1$ (note for 1% to 25% of the region occupied by the fungus); $n_2 = 2$ (note for 25% to 50% of the region occupied by the fungus); $n_3 = 3$ (note for 50% to 75% of the region occupied by the fungus); $n_4 = 4$ (score for 75% to 100% of the region occupied by the fungus).

Anti-ochratoxigenic activity in pericarps and Coffea arabica beans

The anti-ochratoxigenic activity of essential oils on samples of pericarps and grains contaminated by *A. carbonarius* and *A. ochraceus* was analyzed according to the official method of the MAPA (Ministério da Agricultura e Pecuária), published in the Diário Oficial da União, through the Normative Instruction SDA Nº. 09, of March 24, 2000 (DOU, 2000). The concentrations evaluated were the MFC and two concentrations greater than the MFC that was determined in the in vitro test.

A representative homogeneous sampling of pericarps and beans was obtained among the treatments and controls. The samples were ground separately in their entirety to obtain a particle size of 20 mesh. Then, 25.00 g of the powder was subjected to extraction of OTA. The samples were stirred for 10 minutes in 200 mL of 3% methanol:sodium bicarbonate solution (1:1, v/v), at a speed of 254 rpm. The extract was vacuum filtered, and 10-mL aliquots of the filtrate were diluted to 100 mL with PBS buffer. The diluted sample was passed through an immunoaffinity column (OchratestTM WB - Vicam, Watertown. USA) at a flow rate of 2 to 3 mL per minute using a 60 mL syringe. Four mL of methanol (Merck, Darmstadt, Germany, PA grade) was added to the immunoaffinity column, and the methanol was left in contact with the antibodies for three minutes. The OTA present in the sample was eluted from the immunoaffinity column using a flow rate of 2 to 3 mL min^{-1}. The solvent was evaporated on a water bath, the residue was re-dissolved with 400 µL of the methanol: acetonitrile: water: acetic acid (35:35:29:1) mobile phase, and 20 µL of each filtrate was injected directly into the HPLC system (HPLC, Shimatzu, Kyoto, Japan). The HPLC was equipped with two high-pressure pumps (SPD-M20A), a degasser (DGU 20A3), an interface

(CBM-20A), an automatic injector (SIL-10AF) and a fluorescence detector (RF-10 AXL). Separations were performed using an Agilent-Zorbax Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm) connected to an Agilent-Zorbax Eclipse XDB-C18 4-Pack column (4.6 x 12.5 mm, 5 µm). Twenty microliters of the samples and standard were injected, and the solutes were eluted with an isocratic system of methanol:acetonitrile:water:acetic acid (35:35:29:1) having a flow rate of 0.8 mL min^{-1}. External standardization was used to quantify OTA (Sigma-Aldrich®, São Paulo, Brazil) in the samples, performed using the analytical curve (y = 3799.6 x -3149, with a determination coefficient (r2) of 0.999. The limits of detection (LD) and quantification (LQ) were 0.24 and 0.8 µg Kg^{-1}, respectively. All OTA analyses were performed in triplicate.

Statistical analysis

The ANOVA test was used to compare the concentrations in each essential oil under study (*B. citriodora* and *L. origanoides*) in the in vivo anti-ochratoxigenic test (Montgomery, Peck and Vining 2020), whereas the Tukey's test (Tukey, 1949) was employed for multiple comparisons. The software used in the analyses was R (version 3.5.1).

Conflict of interest

There are no conflicts of interest to declare.

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