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Inhibitory effect of the essential oil of *Curcuma longa* L. and curcumin on aflatoxin production by *Aspergillus flavus* Link

Flavio Dias Ferreira [a], Carlos Kemmelmeier [b], Carla Cristina Arrotéia [b], Christiane Luciana da Costa [b], Carlos Augusto Mallmann [c], Vanderly Janeiro [d], Francine Maery Dias Ferreira [a], Simone Aparecida Galerani Mossini [a], Expedito Leite Silva [e], Miguel Machinski Jr. [a,*]

[a] *Department of Basic Health Sciences, State University of Maringá, Maringá 87020-900, Brazil*
[b] *Department of Biochemistry, State University of Maringá, Maringá 87020-900, Brazil*
[c] *Laboratory of Mycotoxicological Analyses, Federal University of Santa Maria, Santa Maria 97105-900, Brazil*
[d] *Department of Statistics, State University of Maringá, Maringá 87020-900, Brazil*
[e] *Department of Chemistry, State University of Maringá, Maringá 87020-900, Brazil*

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ABSTRACT

Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic mycotoxins. Consumption of aflatoxin-contaminated food and commodities poses serious hazards to the health of humans and animals. Turmeric, *Curcuma longa* L., is a native plant of Southeast Asia and has antimicrobial, antioxidant and antifungal properties. This paper reports the antiaflatoxigenic activities of the essential oil of *C. longa* and curcumin. The medium tests were prepared with the oil of *C. longa*, and the curcumin standard at concentrations varied from 0.01% to 5.0%. All doses of the essential oil of the plant and the curcumin standard interfered with mycotoxin production. Both the essential oil and curcumin significantly inhibited the production of aflatoxins; the 0.5% level had a greater than 96% inhibitory effect. The levels of aflatoxin B_1 (AFB_1) production were 1.0 and 42.7 μg/mL, respectively, for the samples treated with the essential oil of *C. longa* L. and curcumin at a concentration of 0.5%.

1. Introduction

Mycotoxins are toxic contaminants produced by fungi via their secondary metabolism. Despite efforts to control fungal contamination, toxigenic fungi are ubiquitous in nature and occur worldwide in food supplies due to mould infestation of susceptible agricultural products, such as cereal grains, nuts and fruits. The natural fungal flora associated with foods is dominated by the *Aspergillus*, *Fusarium*, and *Penicillium* species (Murphy, Hendrich, Landgren, & Bryant, 2006). Aflatoxins (AFs) are potent mycotoxins produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. These fungal metabolites induce mutagenic, teratogenic and carcinogenic effects (Rustom, 1997). Aflatoxins may contaminate many crops, including peanuts, corn, cottonseed, pistachios and other nuts, with widespread contamination in hot and humid regions of the world (Murphy et al., 2006). Aflatoxin B_1 (AFB_1) is the most toxic for mammals and induces cell injury, free radical liberation and lipid peroxidation. AFB_1 is metabolised by the liver through the cytochrome P450 enzyme system to generate the major carcinogenic metabolite AFB_1-8,9-epoxide or other less mutagenic forms (Biehl & Buck, 1987; Murphy et al., 2006).

During the last decades, control of plant diseases has been accomplished through the use of synthetic fungicides. However, decreasing efficacy and increasing concern over the adverse environmental effects of these chemicals have brought about a need for the development of alternative crop protection methods, with or without reduced use of conventional fungicides. Essential oils may be an alternative to common chemical control agents because they constitute a rich source of bioactive compounds (Burt, 2004). Several authors have demonstrated the antifungal activity of plant extracts and their ability to inhibit mycotoxin production. In addition, they have attempted to elucidate the effect of bioactive chemicals on growth and morphological features and on primary and secondary fungal metabolism (Arrotéia, Kemmelmeier, & Machinski, 2007; Mossini, Oliveira, & Kemmelmeier, 2004).

Turmeric, *Curcuma longa* L. (family *Zingiberaceae*) is native to Southeast Asia and has a long history of therapeutic uses and a variety of important antimicrobial, antifungal, insecticidal, anti-inflammatory and antioxidant properties (Apisariyakul, Vanittanakom, & Buddhasukh, 1995; Khattak, Rehman, Shah, Ahmad,

* Corresponding author. Address: Laboratory of Toxicology, Department of Basic Health Sciences, State University of Maringá, Avenue Colombo 5790, 87020-900 Maringá, PR, Brazil. Tel./fax: +55 44 3011 4489.
E-mail address: mmjunior@uem.br (M. Machinski Jr.).

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& Ahmad, 2005). Potent fungicidal activities against phytopathogenic fungi have been demonstrated in greenhouse settings (Kim, Choi, & Lee, 2003). Turmeric has been found effective for controlling mycelial growth of *Fusarium oxysporum* (Singh, Singh, & Maurya, 2002), using isolates of *A. flavus*, *A. parasiticum*, *Fusarium moniliforme* and *Penicillium digitatum* (Jayaprakasha, Jagan, Rao, & Sakariah, 2005) and isolates of *Alternaria dianthi* and *Curvularia trifolii* (Babu, Shanmugan, Ravindranath, & Joshi, 2007). The essential oil of turmeric rhizomes showed toxicity to fungi that were involved in the deterioration of stored agricultural commodities (Dhingra, Jham, Barcelos, Mendonca, & Ghiviriga, 2007). Therefore, the aim of this study was to investigate the effect of essential oil of *C. longa* L. and curcumin on the production of AFB_1 and AFB_2 by *A. flavus*.

2. Material and methods

2.1. Microorganism and culture conditions

The aflatoxin-producing (AFB_1 and AFB_2) isolate of *A. flavus* Link (AF42) was obtained from the culture collection of the Laboratory of Chemistry and Physiology of Microorganisms (Department of Biochemistry, State University of Maringá, Brazil). All *A. flavus* cultures were grown on potato dextrose agar medium (PDA). Conidia were harvested from plates that were incubated at 25 °C/7 d (FANEM Model 347G, São Paulo, Brazil). Later, conidia were placed in 10 mL of a 1:1 mixture consisting of a sterilised solution NaCl (0.89%, w/v) and Tween 80 (0.1%, v/v), counted in a Neubauer chamber and diluted to a concentration of 10^6 conidia/mL. The broth medium employed was Yeast Extract Sucrose (YES). It was prepared for the experiments by adding the essential oil of *C. longa* and the curcumin standard. YES without oil or standard was used as the control medium. Tests were conducted four times, and the essential oil (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.5 and 5.0% v/v) and curcumin standard (0.01, 0.1, 0.25 and 0.5% v/v) were added to the YES medium before inoculation. The YES cultures were incubated at 27 °C/7 d (FANEM – Model 347 G, São Paulo, Brazil). Inoculate containing 10^6 conidia of *A. flavus* was added to the YES medium control and tests.

2.2. Preparation of the essential oil of C. longa

Ground curcumin was obtained from the Açafrão Co-operative in Mara Rosa, Brazil (2009 crop). The essential oil of *C. longa* was obtained by hydrodistillation using a Clevenger-type apparatus in accordance with the method recommended by the European Pharmacopoeia, as described by Péret-Almeida, Naghetini, Nunan, Junqueira, and Glória (2008). The oil obtained was stored at 4 °C and protected from light for subsequent use and chemical analysis.

2.2.1. Analysis and identification of essential oil

2.2.1.1. Gas chromatography–mass spectrometry analysis (GC–MS). A Focus GC system (Thermo Electron Corporation®, San Jose, CA, EUA) was used. It was equipped with a packed column DB-5 (30 m × 0.32 mm, 0.50 mm). The column temperature was maintained at 60 °C/min and then increased to 180 °C/min; the oven temperature was 3 °C/min. Injector port and detector temperatures were both 220 °C. Helium was the carrier gas at a flow rate of 1 mL/min. The split ratio was 1:25, and the ionisation voltage was 70 eV. The sample was diluted with acetone (1:1), and 1 μL of the sample was injected. Characterisation of isolated compounds was based on the comparison of their retention index with mass spectrometry standards from Sigma–Aldrich (St. Louis, MO, EUA) (Adams, 2001).

2.2.1.2. Nuclear magnetic resonance (NMR). Spectrums of (300.06 MHz) and ^{13}C (75.45 MHz) were analytically obtained using the Varian Mercury-300BB spectrometer system (Santa Clara, CA, EUA) with δ (ppm) and then compared with $CDCl_3$ (δ 7.27 by ^1H and 77.00 to ^{13}C) as the internal standard.

2.3. Reagents and standards

Standards of curcumin and aflatoxins were purchased from Sigma–Aldrich (St. Louis, MO, EUA). Stock solutions of each aflatoxin (AFB_1, AFG_1, AFB_2 and AFG_2) were prepared according to the Manual of Official Methods of Analysis (AOAC, 1995) in benzene-acetonitrile and a mixed solution obtained containing each aflatoxin: AFB_1 and AFG_1 at 5 μg/mL and AFB_2 and AFG_2 at 1.5 μg/mL. All reagents, fine chemicals and solvents were obtained from Honeywell Burdick & Jackson (Muskegon, MI, EUA), Fisher Scientific (Fair Lawn, NJ, EUA), Mallinckrodt Baker (Xalostoc, Mexico), FMaia (Cotia, Brazil), Merck (Darmstadt, Germany) and Labsynth (Diadema, Brazil).

2.4. Aflatoxin extraction

For extraction, detection and quantification of aflatoxins, the culture media ($n = 52$) were filtered and a 20 mL aliquot was partitioned twice with 30 mL chloroform (Taniwaki, Fonseca, & Pizzirani-Kleiner, 1993). The extracts were treated with anhydrous sodium sulphate, filtered and evaporated to dryness (Fisatom Modelo 803, São Paulo, Brazil). The residues were re-suspended in benzene: acetonitrile (98:2 v/v), separated into two equal portions and used for the detection and quantification of mycotoxins using thin layer chromatography and a high-performance liquid chromatography system with fluorescence detection.

2.5. Aflatoxin identification and quantification

2.5.1. Thin layer chromatography (TLC)

Extracts were submitted to thin layer chromatography (TLC) on silica gel plates (Silica gel 60, Merck Co., Whitehouse Station, EUA). The plates were developed in a saturated chamber with toluene-ethyl acetate-chloroform-formic acid (70:50:50:20 v/v/v/v). Aflatoxin spots were observed under long-wave ultraviolet light (365 nm) and determined by visual comparison with prepared AFB_1, AFB_2, AFG_1 and AFG_2 standards. Confirmatory tests for aflatoxins were performed using trifluoroacetic acid.

2.5.2. HPLC parameters

The Agilent HP 1200 HPLC system (Agilent, Santa Clara, CA, EUA) was used with a fluorescence detector that was set at 362 nm excitation and 455 nm emission for aflatoxins G_1 and G_2 and 425 nm emission for AFB_1 and AFB_2. To perform aflatoxin derivatisation, 700 μL of trifluoroacetic-glacial acetic acid–water (2:1:7 v/v/v) was added to extracts and the mixture heated at 65 °C for 10 min. The extracts were re-suspended in 200 μL of benzene and cleaned up with a C18 Spherisorb® 5 μm (250 × 4.6 mm, Waters®, Wexford, Ireland) column maintained at 50 °C. The mobile phase was 0.2% acetic acid–acetonitrile–methanol (78:12:10 v/v/v) with a flow rate of 1 mL/min. A standard comprising a mix of the AFB_1, AFB_2, AFG_1 and AFG_2 was used to construct a five-point calibration curve of peak areas versus concentration. The injection volume was 50 μL for both the standard solution and sample extracts. The detection limit of the method was 1 μg/kg, and the recovery rate was 85.5%.

Table 1
Effects of the essential oil of *Curcuma longa* L. on aflatoxin production by *Aspergillus flavus* L. *in vitro*.

Essential oil (%)	Aflatoxin B_1		Aflatoxin B_2	
	Inhibition (%)	Concentration (μg/mL)[c]	Inhibition (%)	Concentration (μg/mL)[c]
0 (Control)	–	1075.2	–	256.5
0.01	36.4	684.2	58.2	107.2[a]
0.1	53.8	496.4[a]	78.3	55.6[a]
0.2	66.0	365.8[a]	87.0	33.4[a]
0.3	80.3	212.3[a]	89.3	27.4[a]
0.4	84.6	165.5[a]	91.5	21.8[a]
0.5	99.9	1.0[a,b]	99.6	1.0[a,b]
1.0	99.9	1.2[a,b]	99.6	1.0[a,b]
2.5	99.9	1.6[a,b]	99.6	1.0[a,b]
5.0	99.7	3.1[a,b]	99.6	1.0[a,b]

The fungus exposed to different concentrations of plant essential oil as described in "Materials and Methods".
Results are the means obtained from four separate experiments.
[a] Statistically significant differences compared to the control.
[b] Do not differ between results.
[c] Values were obtained from HPLC analyses and are expressed as the means of four replicates.

Table 2
Effects of curcumin on aflatoxin production by *Aspergillus flavus* L. *in vitro*.

Curcumin (%)	AFB_1		AFB_2	
	Inhibition (%)	Concentration (μg/mL)[c]	Inhibition (%)	Concentration (μg/mL)[c]
0 (Control)	–	1075.15	–	256.5
0.01	35.0	699.0	57.3	109.4[a]
0.10	49.2	546.4[a]	58.5	106.6[a]
0.25	83.3	179.4[a,b]	94.8	13.3[a,b]
0.50	96.0	42.7[a,b]	98.6	3.6[a,b]

The fungus exposed to different concentrations of plant essential oil as described in "Section 2".
Results are the means obtained from four separate experiments.
[a] Statistically significant differences compared to the control.
[b] Do not differ between results.
[c] Values were obtained from HPLC analyses and are expressed as the means of four replicates.

2.6. Statistical analysis

All experiments were repeated four times. Treatment results were statistically evaluated using the Kruskal–Wallis test (nonparametric single-factor ANOVA) followed by multiple comparisons for pairs of treatments with a 5% significance level (Conover, 1999). The data were analysed using the statistical programme R (R Development Core Team, 2006).

3. Results and discussion

The results showed that the essential oil of *C. longa* and curcumin inhibited *A. flavus* (AF42) aflatoxin production (Tables 1 and 2). All treatments consisting of 0.01–5% (v/v) of the essential oil and curcumin standard prevented aflatoxin production. There was no statistically significant difference up to a concentration of 0.5% (v/v) for oil and curcumin. The inhibitory property developed according to increases in concentration ($p = 1.455 \times 10^{-6}$ for AFB_1 and 2.007×10^{-6} for AFB_2). Thus, 0.5% of the essential oil of *C. longa* reached 99.9% and 99.6% inhibition and 0.5% of the curcumin standard reached 96.0% and 98.6% inhibition for AFB_1 and AFB_2 production, respectively, in *A. flavus* (Table 1, Fig. 1). Similar results were obtained by Gowda, Malathi, and Suganthi (2004) and Reddy, Reddy, and Muralidharan (2009); they showed 77% and 72.2% reduction of AFB_1 and AFB_2 production, respectively, using 0.5% (5 g/kg) of ground *C. longa*. Soni, Rajan, and Kuttan (1992) showed a 90% reduction of aflatoxin production in concentrations ranging from 5 to 10 mg/mL (0.05–0.1%) using curcumin rhizome. However, Sindhu, Chempakam, Leela, and Bhai (2011) demonstrated that turmeric leaf oil exhibited 100% inhibition of aflatoxin production at 1.5%.

AFB_1 production was observed at 1.0 and 42.7 μg/mL following treatment with the essential oil of *C. longa* and curcumin at 0.5%, respectively (Tables 1 and 2). Statistical analysis showed that the essential oil exhibited a higher antiaflatoxigenic activity compared to curcumin in AFB_1 and AFB_2-producing *A. flavus* (AFB_1 = 45.75 and 35.75 μg/mL for essential oil and curcumin, respectively; AFB_2 = 45.5 μg/mL and 34.5 μg/mL for essential oil and curcumin, respectively).

Table 3 and Fig. 2 show the major components of essential oil: ar-turmerone (33.2%), α-turmerone (23.5%) and β-turmerone (22.7%). Similar results were obtained by Singh et al. (2002), Jayaprakasha et al. (2005) and Péret-Almeida et al. (2008). Dhingra et al. (2007) showed that ar-turmerone, which is one of the main components of the oil, mediates the fungitoxic component of essential oil. Gill, Delaquis, Russo, and Holley (2002) showed that essential oils, as a whole, exhibit higher antimicrobial activity than their major compounds individually. This finding suggested that minor compounds might have an important additive or synergistic role. Therefore, additional studies on the components of the essential

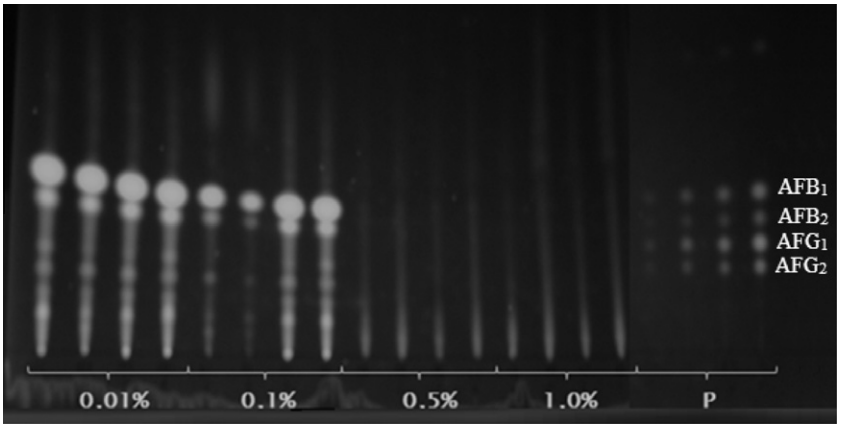

Fig. 1. Thin layer chromatography of aflatoxin production by *Aspergillus flavus* L. isolates that were cultured with the essential oil of *Curcuma longa* L. (0.01%, 0.1%, 0.5% and 1.0%) and AFB_1, AFB_2, AFG_1 and AFG_2 standards (P), which were prepared as described in "Materials and Methods". Ultraviolet light visualisation.

Table 3
Major compounds of *Curcuma longa* L. essential oil determined using GC/MS and NMR techniques.

Retention index (RI)	Compound	(%)
6.96	α-Pinene	0.6
7.43	Unidentified	0.2
7.80	Vinyl propionate	1.7
8.29	Unidentified	0.6
8.71	Unidentified	0.5
9.49	p-Cymene	0.8
10.28	1.8-cineole	0.7
14.93	Camphor	0.1
16.88	α-Terpineol	0.2
21.04	Unidentified	0.1
24.14	Unidentified	0.1
26.11	Unidentified	0.1
26.70	β-Caryophyllene	0.4
27.74	γ-Curcumene	0.5
29.27	ar-Curcumene	2.6
29.76	α-Zingiberene	1.0
30.90	β-Sesquiphellandrene	2.4
33.08	ar-Turmerol	1.5
35.16	α-Cadinol	1.3
36.32	ar-Turmerone	33.2
36.44	α-Turmerone	23.5
37.64	β-Turmerone	22.7
39.23	Unidentified	0.8
40.21	(*6R*, *7R*)-Bisabolone	3.1
41.51	(E)-α-Atlantone	1.4

% – Percentage content of each constituent in total essential oil.

oil of *C. longa* will be necessary to elucidate its antiaflatoxigenic action.

The mechanism of inhibition effect of the essential oil of *C. longa* L. and curcumin for aflatoxins production may be related to inhibition of the ternary steps of aflatoxins biosynthesis involving lipid peroxidation and oxygenation. Hua, Grosjean, and Baker (1999) showed that phenolic compounds exhibit inhibitory activity on AFB_1 biosynthesis by *A. flavus*. It is clear that phenolic compounds have inhibited one or more early, rather than late, steps in the aflatoxins biosynthesis pathway. According to Farag, Daw, Hewedi, and El-Baroty (1989) the presence of phenolic groups to form hydrogen bonds with the active sites of target enzymes was capable of increasing antimycotoxigenic activity. Jayashree and Subramanyam (1999) reported that phenolic compounds inhibited aflatoxin production without any significant effect on growth of the organism.

Turmeric (*C. longa*) has many biological activities, such as the antioxidant properties demonstrated by Zaeoung, Anuchit, and Niwat (2005) and Singh et al. (2010). Zjalic et al. (2006) and Kim et al. (2008) showed that aflatoxins biosynthesis is related to oxidative stress and peroxidation of fungal cells. These studies showed an ability of phenolic compounds to inhibit lipid peroxidation without changes in growth or primary metabolism. Thus, the antioxidant property of *C. longa* could be important to the inhibition of aflatoxin production.

Because essential oils are easily acquired and employed, are cheap to produce, lack the problems that are inherent in synthetic chemical products, have low mammalian toxicity and are biodegradable and non-persistent in the environment, developing them for use in crop protection may be an attractive option. However, further studies need to be conducted to evaluate the cost and efficacy of these essential oils for fungal control programs.

4. Conclusion

Natural plant compounds have also been shown to inhibit mycotoxins production and may be an alternative to synthetic chemical agents. Vegetal oils have been used as inhibitors of toxigenic mould and may be safer for consumption. This paper describes the antiaflatoxigenic effects of the essential oil of *C. longa*. The current study demonstrated that 0.5% of the essential oil of *C. longa* inhibited 99.9% and 99.6% AFB_1 and AFB_2 production by *A. flavus*, respectively. Additional research is needed to determine its potential usefulness in fungal control programs.

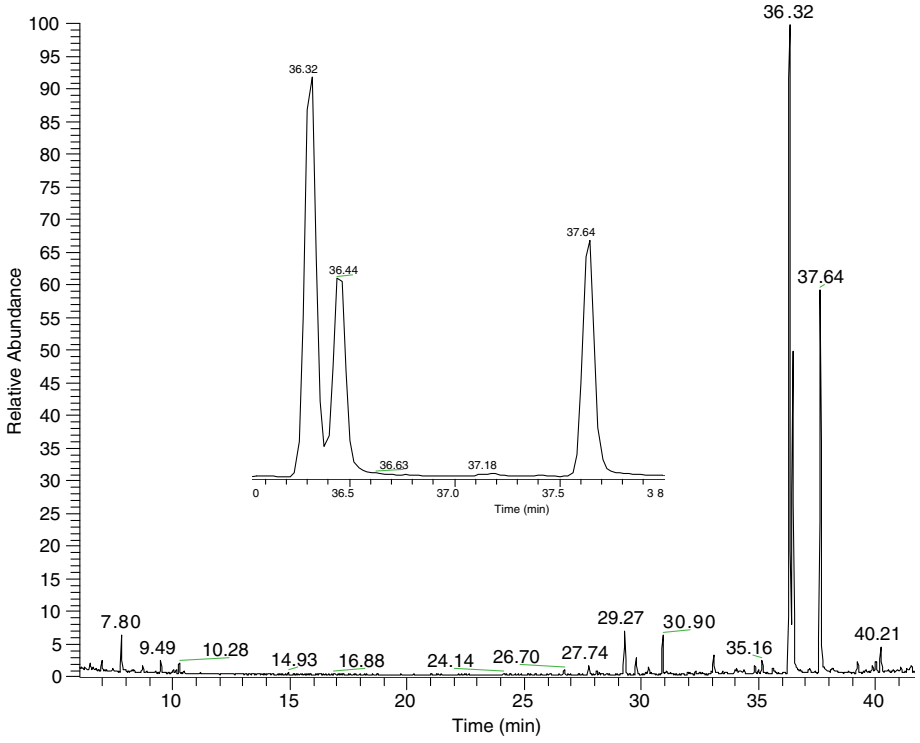

Fig. 2. Chromatogram of the essential oil of *Curcuma longa* L.

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