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Antifungal properties and inhibitory effects upon aflatoxin production of *Thymus vulgaris* L. by *Aspergillus flavus* Link

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ABSTRACT

The antifungal and antiaflatoxigenic properties of *Thymus vulgaris* essential oil (TEO) were evaluated upon *Aspergillus flavus* "*in vitro*". Suspension containing 10^6 of *A. flavus* were cultivated with TEO in concentrations ranging from 50 to 500 μg/mL. TEO reached minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) at 250 μg/mL. Inhibition of ergosterol biosynthesis was detected at a concentration of 100 μg/mL of TEO. Morphological evaluation performed by both light microscopy and scanning electron microscopy showed that antifungal activity of TEO could be detected starting at a concentration of 50 μg/mL and the fungicide effect at a concentration of 250 μg/mL. TEO completely inhibited production of both B_1 and B_2 aflatoxins (AFB_1 and AFB_2) at a concentration of 150 μg/mL. This way, fungal biomass development and aflatoxin production were dependent on TEO concentration. Therefore, TEO was capable of controlling the growth of *A. flavus* and its production of aflatoxins.

1. Introduction

Aflatoxins are produced by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* and are widely distributed in nature. Such mycotoxins are toxic metabolites, difuran coumarin derivates, responsible for carcinogenic, mutagenic and teratogenic effects and represent a risk to human and animal health (Abdel-Wahhab et al., 2010; Yazar & Omurtag, 2008).

Essential oils, present in many plant organs, contain compounds related to several functions necessary to plant survival. About 60% of essential oils show antifungal properties and 35% show antibacterial properties (Lima, Oliveira, Lima, Porto Farias, & Souza, 2006). Studies performed with essential oils and different aromatic plant extracts showed "*in vitro*" antifungal activity upon species of *Fusarium* sp. and *Aspergillus* sp. (Bomfim et al., 2015; Ferreira et al., 2013; Omidbeygi, Barzegar, Hamidi, & Naghdibadi, 2007; Yamamoto-Ribeiro et al., 2013). Within this context, essential oils have been studied as an alternative method for controlling of microorganism proliferation in foods and grains stored for longs periods of time.

Thyme (*Thymus vulgaris* L.), a plant species native to the western Mediterranean area, a member of the Lamiaceae family, is an aromatic/medicinal plant of increasing economic importance for North America, Europe, North Africa and Asia is now widely cultivated as a spice throughout temperate climes (Alçiçek, 2011; Letchamo & Gosselin, 1996). *T. vulgaris* has antioxidant properties, is widely used in folk medicine for its expectorant, antitussive, antibronchiolitic, antispasmodic, anthelmintic, carminative and diuretic properties. Additionally antimicrobial and anti-inflammatory effects have been shown (ESCOP, 2003). This way, the objective of this study was to study the antifungal properties and the inhibitory effects upon aflatoxin production of thyme essential oil (*T. vulgaris*) in *A. flavus* cultures.

2. Materials and methods

2.1. Extraction and characterisation of T. vulgaris essential oil

Fresh aerial parts of thyme were harvested from open areas of South Brazil during the blooming season (late December, 2012).

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The plants age ranged from 6 months to 3 years. The leafs were then dried under ambient conditions (30–40 °C) for 3 days on a large screened tray and commercialised by Nuticrok Alimentos Ltda, Maringa, Brazil (*T. vulgaris* L. – allotment: 3003/50 W). Thyme essential oil was obtained from dried thyme leafs by hydrodistillation using a Clevenger apparatus, as described by the European Pharmacopea (Council of Europe, 1997). The oil obtained was stored at 4 °C and protected from light prior to chemical analysis and use.

Essential oil compounds were determined by gas chromatography coupled to mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) spectroscopy. Analysis by GC–MS was performed using a quadrupole mass spectrometer (Thermo Electron Corporation. modelo DSQ II, San Jose, CA, EUA), under the following conditions: a DB-5 capillary column (30 m × 0.32 mm × 0.50 mm); temperature programing: 60–180 °C (3 °C/min); injector temperature: 220 °C; detector temperature: 220 °C; carrier gas: helium (White Martins, Rio de Janeiro, Brazil) adjusted to 1.0 mL/min speed; split ratio 1:10; injected volume: 1 μL diluted in acetone 1:10 (Merck, São Paulo, Brazil); ionising energy: 70 eV. Identification of individual compounds was based on comparison of their retention indexes in non-polar columns and on comparison of their mass spectres against authentic standards acquired from Sigma Aldrich (St. Louis, MO, EUA) literary database (Adams, 2001). For the NMR analysis, the ^{1}H (300.06 MHz) and ^{13}C NMR (75.45 MHz) spectra were obtained using a deuterated chloroform ($CDCl_3$) solution using a Mercury-300BB spectrometer with the δ (ppm) value and the spectra referred to those of $CDCl_3$ (δ: 7.27 for ^{1}H and 77.00 for ^{13}C) as an internal standard.

2.2. Microorganism and culture conditions

A. flavus (AF42) was obtained from the isolated fungi storage bank of the Toxicology Laboratory in the State University of Maringa. For conidia production, the fungus was cultivated in Potato Dextrose Agar – PDA (Neogen® Co. Lansing, MI, EUA) for 7 days, in the dark and in a BOD incubator (FANEM model 347G, São Paulo, Brazil) (Agarwal, Walia, Dhingra, & Khambay, 2001; Betina, 1984; Smith & Onions, 1983). 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 4×10^5 or 10^6.

2.3. Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

Determination of MIC was performed using the broth microdilution method, following the norm M38-A of the *National Committee for Clinical Laboratory Standard* (NCCLS, 2002). TEO was diluted in a sterile solution of Tween 80 at 0.001% (Vetec, Rio de Janeiro, Brazil) and evaluated in final concentrations that ranged from 6.25 to 4000 μg/mL. At each different TEO concentration, 100 μL of a suspension containing 4×10^5 CFU/mL of *A. flavus* were added to synthetic medium RPMI-1640 (0.1 mL). The microplates were incubated at 35 °C for 72 h. MIC was determined according to the lowest concentration of TEO that was capable of inhibiting the visual growth of *A. flavus*. The positive control was performed in the medium containing only the conidia suspension. For MFC determination of the essential oil, samples were taken from the wells that did not show any visible fungal growth and re-inoculated in plates containing Sabourand Agar. These plates were incubated at 25 °C for 72 h. The lowest concentration of TEO that was capable of inhibiting fungal growth was considered to be the MFC. At the other experiments, the methodology proposed by Shukla, Singh, Prakash, and Dubey (2012) was utilised with some modifications, being utilised three concentrations below and one above the MIC and MFC.

2.4. Effect of essential oil on A. flavus

TEO was previously diluted in a sterile Tween 80 solution at 0.001% (Vetec, Rio de Janeiro, Brazil) and added to 25 mL of liquid medium Yeast Extract Sucrose (YES) (Smith & Onions, 1983), containing yeast extract (BD®, New Jersey, EUA) and sucrose (Labsynth, Diadema, Brazil) in order to obtain final concentrations of 50, 100, 150, 250 and 500 μg/mL. In each medium, 10^6 of *A. flavus* were inoculated (Taniwaki, Fonseca, & Pizzirani-Kleiner, 1993); the media were then incubated at 25 °C for 7 days. Controlling of fungal growth and of aflatoxin production was performed in the medium containing the inoculate absent of TEO. After the incubation period, the contents of each flask were filtered in common filter paper. Mycelial biomass was weighted and utilised for ergosterol determination, while the filtrated flask contents were utilised for aflatoxin determination. Both control and tests were performed in triplicates.

2.4.1. Ergosterol and aflatoxin standards

Ergosterol (Sigma Chemical, St. Louis, MO, EUA) was dissolved in pure ethanol (Merck, São Paulo, Brazil) so that the working concentration of 1000 μg/mL could be obtained. From this solution, a calibration curve was constructed in the range of 10–100 μg/mL.

The aflatoxin standards (Sigma–Aldrich, St. Louis, EUA) and their respective solutions were prepared following AOAC's "Manual of Official Methods and Analyses" (1995). Concentrations of aflatoxins B_1 (AFB_1) and B_2 (AFB_2) standards in their respective individual solutions were determined by UV spectrophotometry utilising a Shimadzu UV-1601 spectrophotometer (Shimadzu, Tokyo, Japan) at 350 nm. The solutions remained stored at −20 °C until use.

2.4.2. Analytical determination of ergosterol

Ergosterol extraction was performed according to Silva, Corso, and Matheus (2010). 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 homogenised for 5 min in tube agitators (KMC 1300 V, Bucheon, Korea) and left in a water bath EV 015 (Evlab, Londrina, Brazil) at 70 °C/40 min. After cooling at room temperature, 5 mL of distilled water were added to each tube and they were then centrifuged at 1735g/24 min (Universal 320R, Hettich, Tuttlingen, Germany). Supernatants were removed and n-hexane (FMaia, Cotia, Brazil) was added in equal volume to each tube. After 2 min agitation, the organic fraction was collected and put in an ambar glass flask. The solvent in each tube was evaporated (White Martins, Rio de Janeiro, Brazil). The obtained residue was stocked at −18 °C until analysis was performed.

Ergosterol quantification was performed according to Salmanowicz, Nylund, and Wallander (1990) with modifications. A high proficiency liquid chromatographer (HPLC) Finnigan Surveyor Plus (Thermo Scientific, San Jose, CA, EUA) was used with a UV/VIS Finnigan Surveyor detection system at a wavelength of 282 nm. The extracts were resuspended with 1 mL of pure ethanol, and 100 μL from each solution were injected into the chromatographic system. The mobile phase was HPLC grade methanol, at a 1.5 mL/min flow. The chromatographic column was a C_{18} Spherisorb® 5 μm (150 × 4.6 mm, Waters, Weford, Ireland). Retention time was 4.6 min. The detection limit and quantification limit were 0.15 and 10 μg/mL, respectively. The percentage of ergosterol recovered was 77.4 ± 7.2 with the used method of extraction.

2.4.3. Aflatoxin extraction and its determination by HPLC

The aflatoxin extraction process was performed according to the method of Taniwaki et al. (1993), with modifications. A total of 20 mL of each of the previously filtered solutions were manually agitated for 1 min in a separating funnel (125 mL) with 10 mL of

hexane (F. Maia Indústria e Comércio Ltda, Cotia, SP, Brazil). The organic fraction was discarded and 10 mL of chloroform (F. Maia Indústria e Comércio Ltda, Cotia, SP, Brazil) were added to each aqueous phase, followed by manual agitation for 1 min and then an 8 min rest. The chloroform fractions were recovered and filtered with anhydrous sodium sulphate. The solvent was evaporated in a sample concentrator TE-0194 (Tecnal, Piracicaba, SP, Brazil) at 65 °C. The obtained residue was stocked at −18 °C until analysis was performed.

The aflatoxin analyses were performed in a Finnigan Surveyor Plus HPLC system (Thermo Scientific®, San Jose, CA, EUA), equipped with a Finnigan Surveyor fluorescence detection system. Emission wavelength was 430 nm and excitation wavelength was 365 nm. Residues were re-suspended with 1 mL of methanol:water (1:1), and 100 μL of each solution were injected in the chromatographic system. The mobile phase was water:acetonitrile (65:35 v/v) at a 1.0 mL/min flow. The chromatographic column was a C_{18} Pickering 5 μm (250 × 4.6 mm, Pickering Laboratories®, Mountain View, CA, EUA). Retention time was 10 and 13 min for AFB_2 and AFB_1, respectively. The limits of detection and quantification of the samples were 333 and 1000 ng/mL, respectively. The average recovery rate was 85.5% for aflatoxins.

2.4.4. Scanning electron microscopy (SEM)

From the cultured *A. flavus* strain (AF42) in PDA, an 8 mm diameter disk was inoculated in a plate containing Yeast Extract Sucrose (YES) medium, to which 2% agar was also added. TEO was added to the medium in order to obtain the final concentrations ranging from 50 to 500 μg/mL. Plates were incubated at 25 °C for 7 days. Control was done in a plate containing only the inoculated fungi. Test samples and controls were done in duplicates.

Samples were prepared according to Endo, Cortez, Ueda-Nakamura, Nakamura, and Dias-Filho (2010), pre-fixed with glutaraldehyde 2.5% (Sigma Chemical, St. Louis, MO, EUA) in sodium cacodylate buffer 0.1 M (EM Sciences, Philadelphia, PA, EUA). The pre-fixed material was applied to a poly-L-lysine chip (Sigma–Aldrich, St. Louis, MO, EUA) for 1 h at room temperature. The material was washed with sodium cacodylate buffer 0.1 M and dehydrated with ethanol in continuous concentration increase (30–100%). Samples were submitted to a CO_2 critical point (White Martins, Rio de Janeiro, Brazil) and coated with gold in a metallizer (Shimadzu IC-50, Kyoto, Japan). The morphological characteristics of the fungi were observed by means of SEM (SEM Shimadzu SS 550, Kyoto, Japan) operating at 10.0 kV.

2.5. Statistical analysis

Results were expressed as the mean value ± standard error (SR) and were analysed by one-analysis of variance (ANOVA) for multiple comparisons, followed by Tukey test (Soares & Siqueira, 1999). The software used for analysis was GraphPad Prism 5.0 (GraphPad Software Inc.).

3. Results and discussion

The results of the GC–MS and NMR analysis of the volatile profile of REO are listed in Table 1. Studies demonstrate variations in essential oils chemical composition. In the present study, TEO obtained from dried plants showed borneol was the major compound (40.6%). These findings are similar to the ones described by Jakiemiu et al. (2010), who also used dried plants. In the scientific literature, variations in chemical compounds ratios were found: 17.4–71% of thymol; 12.1–24.5% of carvacrol; 0.2–2.3% of borneol and 2.6–85.5% of limonene (Omidbeygi et al., 2007; Razzaghi-Abyaneh et al., 2008; Sacchetti et al., 2005). However,

Table 1
Chemical composition of *Thymus vulgaris* L. essential oil (TEO).

Compounds	RI[a]	Percentage (%)	Identification[b]
Tricyclene	920	0.7	GC/MS
α-Thujene	924	0.2	GC/MS
α-Pinene	932	6.1	GC/MS, RMN
Camphene	946	12.3	GC/MS, NMR
β-Pinene	974	0.7	GC/MS, NMR
β-Myrcene	988	0.3	GC/MS, NMR
α-Terpinene	1014	0.3	GC/MS, NMR
p-Cymene	1020	2.9	GC/MS, NMR
Limonene	1024	0.8	GC/MS, NMR
γ-Terpinene	1054	1.5	GC/MS, NMR
Terpinolene	1086	0.2	GC/MS, NMR
Linalool	1095	0.9	GC/MS, NMR
Camphor	1141	0.3	GC/MS, NMR
Borneol	1165	40.6	GC/MS, NMR
4-Terpineol	1174	1.1	GC/MS, NMR
α-Terpineol	1186	19.9	GC/MS, NMR
Citronellol	1223	0.3	GC/MS, NMR
Carvacrol methyl ether	1241	0.1	GC/MS, NMR
Isobornyl acetate	1283	1.3	GC/MS
Carvacrol	1298	6.4	GC/MS, NMR
Eugenol	1356	0.9	GC/MS, NMR
β-Caryophyllene	1417	1.2	GC/MS, NMR
γ cadinene	1513	0.1	GC/MS
δ-Cadinene	1521	0.2	GC/MS
Caryophyllene oxide	1582	0.2	GC/MS, NMR
Epi-α-cadinol	1638	0.1	GC/MS
Epi-α-muurolol	1640	0.2	GC/MS
	Total:	99.8	

[a] RI = retention index, obtained with reference to an n-alkyne series C_8H_{18}–$C_{20}H_{42}$ using DB-5 column and the Van den Dool and Kratz equation.
[b] NMR = nuclear magnetic resonance spectroscopy and GC/MS = gas chromatography–mass spectrometry.

TEO obtained from dried plants has been showing as a major compound thymol and carvacrol (Dawidowicz, Rado, Wianowska, Mardarowicz, & Gawdzik, 2008). Such variability in essential oil compounds might be linked to differences in the raw materials used (dried or fresh), to types of soils used for cultivation, to the harvesting time in the year or to differences in oil extraction techniques (Wohlmuth, Smith, Brooks, Myers, & Leach, 2006).

Results obtained from the MIC test were confirmed with further MFC tests, where inhibitory activity of TEO upon *A. flavus* occurred at a concentration of 250 μg/mL. TEO's antifungal activity was demonstrated by reduction of ergosterol production by *A. flavus*. This reduction is due to the interfering upon growth and fungal

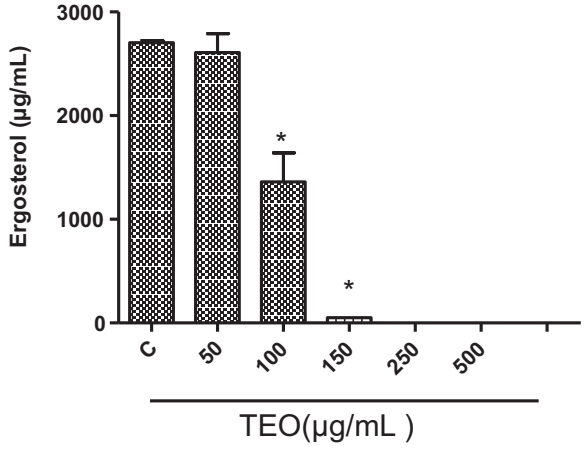

Fig. 1. Effect of *Thymus vulgaris* L. essential oil (TEO) on the production of ergosterol by *Aspergillus flavus*, as determined by HPLC-UV. C – fungal control (inoculum without TEO). *$p < 0.05$.

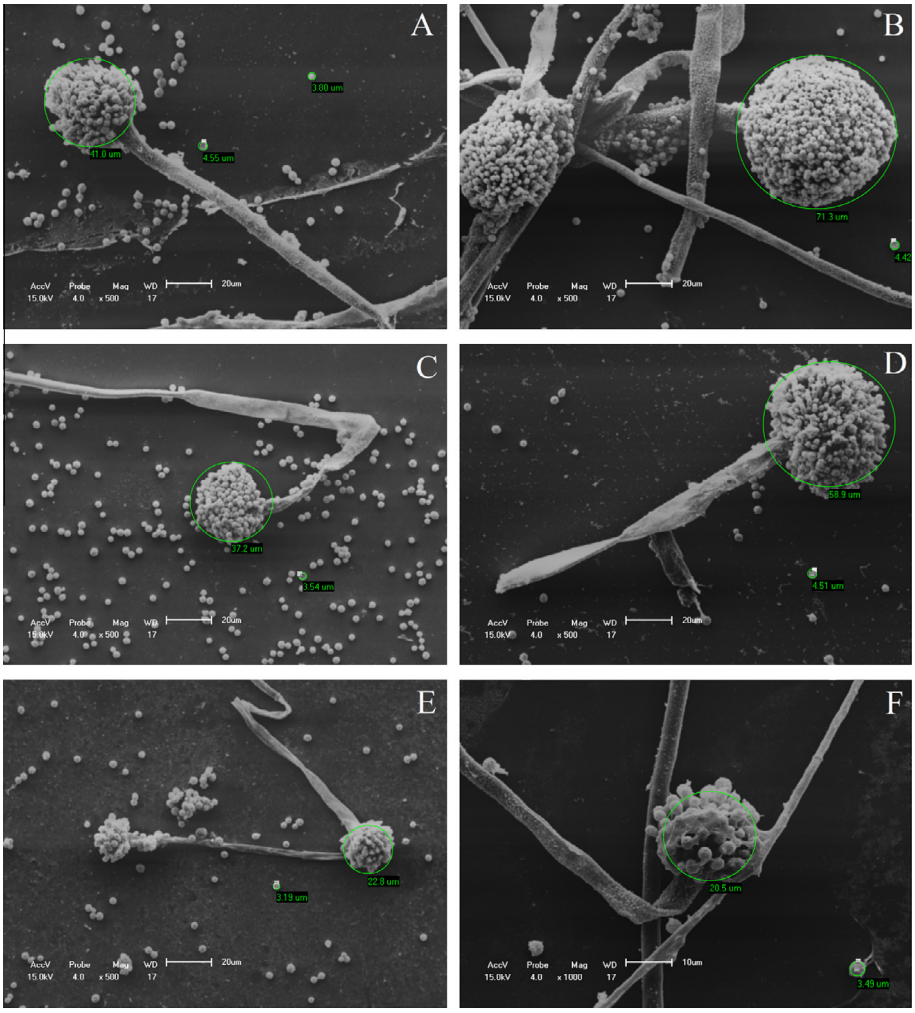

Fig. 2. Thyme essential oil effects (TEO) upon *A. flavus* (AF42). (A) Control, *A. flavus* not treated with TEO; (B, C, D, E and F) *A. flavus* treated with TEO at concentrations 50, 100, 150, 250 and 500 μg/mL, respectively. Images obtained by scanning electron microscopy (SEM) at 500× magnification.

proliferation that essential oils are capable of, which alters protein function and leads to cell death (Nagappan & Deresinski, 2007; Tian et al., 2012). Fig. 1 shows that TEO significantly reduced ergosterol production by *A. flavus* at concentrations 100 and 150 μg/mL ($p < 0.05$), the inhibition being 49.6% and 98.1%, respectively.

Regarding the morphological structure of *A. flavus* analysed by SEM, alterations in conidiophore characteristics were observed. Conidial head size varied ranging from a diameter of 71.3 to 20.5 μm for the samples treated at concentrations ranging from 50 to 500 μg/mL (Fig. 2) and conidia size itself varied ranging from a diameter of 3.2 to 4.6 μm. Also, alterations of hyphae structure were observed as shown in Fig. 2C and F, such as decrease of cytoplasmic content and modifications of membrane integrity. These results were proportional to ergosterol production, which

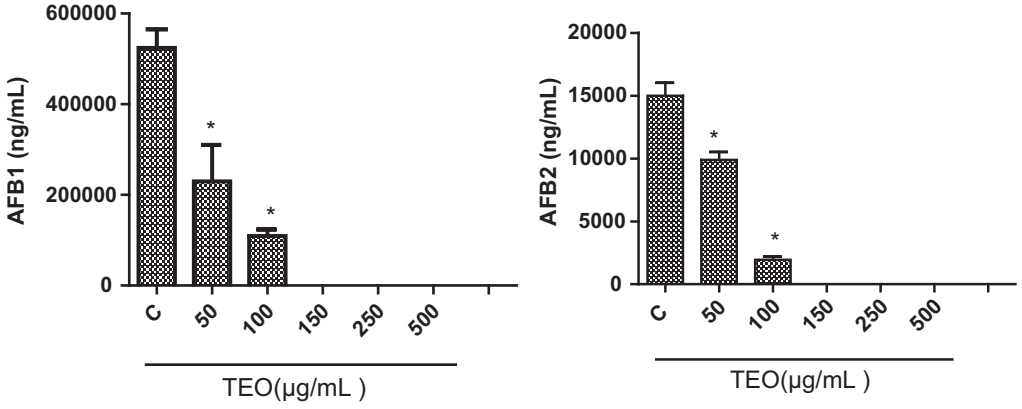

Fig. 3. Effects of different *Thymus vulgaris* essential oil concentrations (50–500 μg/mL) upon AFB_1 and AFB_2 production by *Aspergillus flavus*. The liquid medium by Taniwaki et al. (1993). Cultures were kept in incubation for 7 days at 25 °C ($n = 3$). C – fungal control (inoculum without TEO). Aflatoxin concentrations were determined by HPLC/fluorescence. $^{*}p < 0.05$.

decreased with each oil concentration, as ergosterol is the main sterol present in the cytoplasmic membrane. Such modifications induced by the essential oil might be linked to the interference caused by its components in cell wall synthesis, which affects fungal growth and morphology (Rasooli, Rezaei, & Allameh, 2006), as related by Soylu, Kurt, and Soylu (2010), demonstrating that TEO was responsible for degenerative alterations in hyphae alterations, which appeared degraded or with complete absence of conidia.

TEO's effect upon AFB_1 and AFB_2 production by *A. flavus* showed that inhibition was significant ($p < 0.05$) starting at a 50 μg/mL concentration (Fig. 3). Total inhibition of AFB_1 and AFB_2 production occurred at a 150 μg/mL concentration. TEO showed antiaflatoxigenic actions, as aflatoxin biosynthesis inhibition occurred at a concentration inferior (50 μg/mL) to the concentration required for inhibition of ergosterol production (100 μg/mL) and for morphological alterations of hyphae, conidiophores and conidia (100 μg/mL). This result was similar to the results found in other studies (Kumar, Shukla, Singh, Prasad, & Dubey, 2008; Omidbeygi et al., 2007).

4. Conclusions

Usage of essential oils has been emerging as a possible alternative for the control of toxigenic fungi. The present study demonstrated satisfactory results concerning the effects of *T. vulgaris* essential oil upon *A. flavus*, showing its capabilities of inhibiting fungal development and altering both ergosterol and aflatoxin production. These data suggest that TEO might be used to control *A. flavus* and its toxic metabolites presence. However, future studies must be done to evaluate TEO's application on fungal growth control and on mycotoxins production during planting, harvesting and storage of grains, as well as to assure quality and safety of foods and rations for human and animal consumption treated with TEO.

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