CIÊNCIA e NATURA

UFSM

Ci. e Nat., Santa Maria v.41, e20, p. 01-08, 2019
DOI:10.5902/2179460X36055
ISSN 2179-460X

Química

Submissão: 06/01/2019 Aprovação: 12/03/2019 Publicação: 16/07/2019

Artigo Original

Atividade antifúngica do óleo essencial de *Cymbopogon citratus no controle do Aspergillus flavus*

Antifungal activity of *Cymbopogon citratus* essential oil against *Aspergillus flavus*

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Resumo

A busca por alternativas de controle da contaminação microbiológica em alimentos têm sido objeto de estudo em diferentes áreas científicas. O presente trabalho objetivou avaliar a eficiência do óleo essencial de capim-limão (Cymbopogon citratus) no desenvolvimento do fungo Aspergillus flavus em três formas de análise: Primeiro por testes in vitro, nas doses de óleo essencial entre 0,2 a 1,0 µL/mL. Segundo por microdiluição seriada para determinação da concentração mínima inibitória, em doses entre 0,1 a 1,2 µL/mL. Por último pela inibição do crescimento fúngico em grãos de milho contaminados com a aplicação de doses de óleo essencial de 0,4; 0,7 e 1,0 µL/mL, em diferentes períodos de incubação: 14, 28 e 42 dias. Os resultados demonstraram que o óleo essencial apresentou, nos testes in vitro, controle sobre o fungo, em doses a partir de 0,6 µL/mL, sendo, porém, obtido 100% de controle do crescimento até o oitavo dia de incubação na dose de 1,0 µL/mL a partir do qual decresce. Pela análise da microdiluição, a dose mínima inibitória foi 0,9 µL/mL. Na avaliação em grãos de milho, para todas as doses de óleo essencial e períodos testados, houve inibição de 100% no crescimento fúngico.

Palavras-chave: Microrganismo; Armazenagem; Micotoxina

Abstract

The search for alternatives for the control of microbiological contamination in foods has been the object of study in different scientific areas. This study aimed to evaluate the efficiency of lemon grass (*Cymbopogon citratus*) essential oil in controlling the growth of the fungus *Aspergillus flavus* in three types of analysis: first, by *in vitro* tests, in essential oil doses between 0.2 and 1.0 µL/ml; second, by serial microdilution to determine the minimum inhibitory concentration, in doses between 0.1 and 1.2 µL/mL; and third, by inhibition of fungal growth in corn kernels contaminated using essential oil doses of 0.4, 0.7, and 1.0 µL/mL, in the incubation times of 14, 28, and 42 days. The *in vitro* tests showed that the essential oil controlled the fungus from doses of 0.6 µL/mL, but the dose of 1.0 µL/mL controlled 100% growth until day eight of incubation, from which it decreased. The minimum inhibitory concentration for the microdilution analysis was 0.9 µL/mL. The evaluation of the corn kernels for all doses of essential oil and times tested showed 100% inhibition of the fungal growth.

Keywords: Microorganism; Storage; Mycotoxin

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1 Introduction

To cope with the population growth, a search for technologies is required to increase food production. Because of this search for higher productivity, third generation chemical fungicides have been widely used. Depending on the active ingredient and amounts improperly used, harm is caused to the consumers and the environment, as well as the development of microorganism resistance, which reduces product effectiveness (HEMALANTHA et al., 2016; OUEDGHIRI, et al., 2016).

As an alternative to synthetic products, studies on the biological activity of secondary compounds of plants have demonstrated that that they can be effective to control stored grain pests. Studies from different regions of the world and guided by the popular use of native species seek to prove that extracts and essential oils of medicinal plants are efficient in controlling the growth of a wide variety of microorganisms such as filamentous fungi, yeasts, and bacteria (JANSEN et al., 1987, CHAO and YOUNG, 2000, SCHWAN-ESTRADA, 2009). These natural products generate ecologically clean products and without health risks

Fungi of the genus *Aspergillus* occur worldwide in different habitats and are known for food deterioration, as well as the production of mycotoxins and frequent intoxication of animals and humans (SAMSON et al., 2014). The species *Aspergillus flavus* is pathogenic and grows well in stored products such as corn, peanuts, cotton, and walnuts (AMAIKE and KELLER, 2011). Intake of aflatoxin has been shown to reduce fish and bird weight gain, and there is evidence of carcinogenic effects in humans (SELIM, El-HOFY, KHALIL, 2014, LEE and RYU, 2015, RUYCK et al. JAHANIAN, et al., 2016).

Researchers have sought alternative approaches to inhibiting fungal growth, and among them, the use of essential oils (ABDULAZEEZ; ABDULLAHI; JAMES, 2015; DONSI e FERRARI, 2016; SALEM et al., 2016; DWIVEDY, et al., 2016; SANCHEZ-RUBIO et al, 2016; DAVARI e EZAZI, 2017; EL OUADI et al., 2017; XIE et al., 2017). In addition to fungus control, studies have shown that essential oils have insecticidal (DERMIRCI et al., 2016, POLATOĞLU et al, 2016, AMBROSIO et al., 2017, KIRAN et al., 2017) and antibacterial properties (DENG et al., 2016; LEU et al., 2017; LOU et al., 2017).

Essential oils are present in different plant parts in different amounts and composition, which can be changed by various environmental and farming factors. The International Standart Organization (ISO) defines essential oil as an aromatic product derived from plant species, which is obtained through distillation, mechanical compression, or aqueous phase separation by physical processes (SIMONES et al., 2003; MARTINAZZO, 2006; TISSERAND and YOUNG, 2013).

Cymbopogon is a genus with approximately 180 species, subspecies, varieties, and sub-varieties, native to regions with tropical temperatures of the old world and Oceania. The species *Cymbopogon citratus* (DC.) Stapf is native to India and produces essential oil. Known popularly as lemon grass, it was introduced in Brazil in the colonial time and is currently found and cultivated in all Brazilian territory. The composition of its essential oil as a plant drug is at least of 0.5% volatile oil, with 60% citral, its main component, a mixture of the isomers neral and geranial (LEWINSOHN et al., 1998; CASTRO and RAMOS, 2003, SILVA Jr., 2003, AKHILA, 2010, BRAZIL, 2010, AJAYI, SADIMENKO, AFOLAYAN, 2016).

The objectives of this study were to analyze the *in vitro* antifungal activity of the essential oil of *Cymbopogon citratus* in the control of *Aspergillus flavus* and in contaminated corn (*Zea mays*) kernels, as well as to identify the main components of this essential oil.

2 Materials and Methods

Chromatographic Analysis of Essential Oil

The essential oil of *Cymbopogon citratus* was purchased from the cosmetics industry and trade company Argila Indústria & Comércio de Cosméticos®, Juiz de Fora, MG.

Analysis of essential oil constituents was performed by gas chromatography-mass spectrometry (GC/MS). The compounds were separated in a fused-silica capillary column with DB-5 stationary phase (30 m long x 0.25 mm internal diameter x 0.25 uM inner film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL min^{-1}. The temperature of the injector was hold at 220 °C and the detector at 240 °C. The initial oven temperature was maintained at 60 °C for 2 min and programmed with a heating rate of 3 °C min^{-1} to 240 °C and held for 30 min, in a total analysis time of 91 minutes. The split ratio was 1:20 and the solvent cut-off time was 5 minutes. The sample injection volume was 1 µL, at a concentration of 10,000 ppm, using hexane as solvent.

Compounds were identified by comparing the mass spectra obtained with those of the apparatus database and by the Kovats Retention Index (IK) of each component (LANÇAS, 1993). The quantitative analysis of the main components of the essential oil, expressed as a percentage, was performed by the peak area integration normalization method, as described by ZHANG et al. (2006).

Biological Material

Aspergillus flavus strains were kindly provided by the Oswaldo Cruz Foundation – FIOCRUZ, from the collection of filamentous fungi. Cultures were grown in BDA medium (potato, dextrose and agar) in Petri dishes at 30 ºC for seven days. For spore collection, the plates were flooded with 15 mL sterile distilled, and conidia were harvested with a pipette. The spore suspension was adjusted with sterile distilled water to give the final concentration of 4.5 x 106 spores mL-1 using a Neubauer chamber. The suspension was stored at 4 ºC until use.

In vitro test: plate assay

For the in vitro assay, 20 mL of BDA culture medium were poured into Petri dishes previously sterilized at

121 °C for fifteen minutes in autoclave, containing *C. citratus* essential oil concentrations of six treatments, 0; 0.2; 0.4; 0.6; 0.8, and 1 µl/ml diluted in 1% DMSO (dimethyl sulfoxide).

Petri dishes were incubated with 7 mm mycelial discs of both species in the center of the plate. Four replicates were used for each treatment.

Two control treatments without essential oil were performed: one with fungus growing on BDA medium only; and the other with fungus growing in BDA medium added with DMSO to evaluate the influence of the surfactant on fungal growth. Because no influence was detected, the controls (dose 0) were used with DMSO, in a completely randomized design.

The plates were incubated in BOD at 30 °C until the mycelial growth in the control treatments covered the entire Petri dish, with 92 mm diameter, which was considered the end of the incubation time. The colony diameter was recorded daily with a digital caliper.

The percentage of colony inhibition (PI) was calculated with the following equation (TATSADJIEU et al., 2009):

$$PI = \frac{D_c - D_o}{D_c} \cdot 100 \qquad \text{(Equation 01)}$$

where: D_c - diameter of colonies without treatment; D_o - diameter of colonies treated with essential oil.

In vitro test: microdilution

The minimum inhibitory concentration (MIC) of the essential oil (EO) on the fungi studied was determined by serial microdilution in microplate. The doses tested were defined from the results of the *in vitro* test and the following EO doses were tested: 0.2; 0.3; 0.4; 0.5; 0.6; 0.8; 0.9; 1.0; 1.2 µL/mL.

Each dose tested had four replicates in BD medium (potato and dextrose) with the solution containing essential oil, DMSO, and spore suspension (10^7), and a control treatment without the essential oil. The plates were kept in a BOD chamber at 35 ºC for 72 H.

After the incubation time, the results were analyzed visually. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of essential oil in which no fungal growth occurred (PANDEY, RAI, ACHARYA, 2003, DELLAVALE et al., 2011).

Evaluation of infected corn kernels

For the experiment of growth inhibition of the fungus on corn kernels by the essential oil of *C. citratus*, 1.5 L bottles containing 200 g of kernels were autoclaved at 121 °C for twenty minutes. After cooling, 2.0 ml of *Aspergillus flavus* spore suspension at the concentration of 1.7 x 10^7 spores/ml was inoculated to the kernels.

The material was incubated in a BOD at a constant temperature of 30 °C. After 48 hours, the concentrations of 0; 0.4; 0.7, and 1.0 µL/mL of the essential oil were applied to the kernels stored. The storage times evaluated were 14, 28, and 42 days. At the end of each time, three samples were randomly collected from each treatment and diluted in 0.9% saline solution. A volume of 0.1 mL of the prepared dilution was inoculated into Petri dishes containing approximately 20 mL of Sabouraud agar medium. The plates were incubated at 28 °C for 72 h in a BOD. Plates containing from 05 to 250 CFU (Colony Forming Unit) were counted and the percent inhibition of growth (PI) was calculated according to Equation 01.

Statistical analysis

The experiment was arranged in a completely randomized design. The results were analyzed by analysis of variance and means compared by the Scott-Knott test at 5% significance level. Data analysis was performed using SISVAR®.

3 Results and Discussions

Chromatographic analysis of *Cymbopogon citratus* essential oil

Figure 01 shows the chromatogram obtained in the identification of the components of the *Cymbopogon citratus* essential oil.

Table 1 presents the mean retention time and the Kovats index of the components identified by the chromatogram shown in Figure 01.

Data from this table and data from Figure 01 indicate that the main component of the lemon grass essential oil used in this study is citral, a mixture of neral and geranial. The concentration of citral was 79% of the oil composition, with 44% geranial and 35%

Figure 1- Chromatogram of the *Cymbopogon citratus* essential oil used in the experiment.

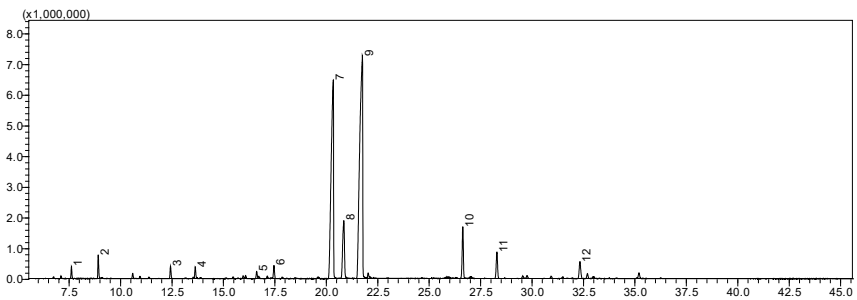

Table 1- Main components of *Cymbopogon citratus* essential oil determined by GC-MS.

Peak	Component	Retention Time* (min)	Kovats Index		
			Present study	Adams (1995)	Choi (2003)
02	6-methyl-5-hepten-2-one	8.914	994	992	
04	Linalol	13.630	1094	1098	
07	Neral	20.338	1244	1244	
08	Geraniol	20.850	1257	1257	
09	Geranial	21.754	1276	1275	
10	Geranyl Acetate	26.634	1389	1383	
11	Caryophyllene	28.291	1429	-	1428 (1)

DB-5 column.

neral, corroborating the reports of other authors for the same species (HANAA et al., 2012; MACHADO et al., 2012; MARTINAZZO et al., 2013; BOUKHATEM et al., 2014; BOSSOU et al., 2015). According to Bakkali et al. (2008), essential oils are characterized by two or three major components in high concentrations (20 - 70%) compared to other components in lower concentrations.

In vitro antifungal activity of lemon grass essential oil (*Cymbopogon citratus*) against the fungus *Aspergillus flavus*

Figure 2 illustrates the inhibitory effect obtained with the essential oil of lemon grass on the mycelial growth of fungus *A. flavus* at the different doses tested during the incubation time.

From the data in Figure 02, it is apparent that in the first 24 hours, the fungal growth at dose zero (control without EO) was 3.8 mm, while at the other doses of the essential oil there was no growth. From 0.2 to 0.8 µL/mL, the mean diameter of fungal growth ranged from 40 to 80 mm at the end of the incubation time. For 1.0 µL/mL, the final mean diameter was 16 mm, which only started to grow from the day eight onwards, demonstrating that it was the dose with the highest control.

To evaluate the effect of the EO doses, the Percentage of Inhibition (Equation 01) of each treatment on the fungus was calculated and the statistical analysis was performed. The analysis of variance showed a significant effect of the different doses of *C. citratus* essential oil (D) for the incubation time (t) and for the interaction (D x t), indicating that the growth inhibition of *A. flavus* by the essential oil depends on the interaction between the dose applied and the time of microorganism incubation. Therefore, the unfolding of the interaction was performed to study the behavior of microorganism control within each factor as described in Table 02.

Also in Table 02, the coefficient of variation (CV) of 5.55% provides information about the precision of the experiment. According to Pimentel-Gomes (1987), the lower the coefficient of variation, the greater the experimental precision of the results.

Table 2 shows that in the first 24 h, all EO doses provided total inhibition of fungal growth. From 48 h, the inhibitory effect of the doses 0.2 and 0.4 started to decrease, with significant difference from each other and from the other doses. The doses 0.6 and 0.8 µL/mL

Figure 02 - Effect of different concentrations of *Cymbopogon citratus* essential oil on the mycelial growth of the fungus *Aspergillus flavus*.

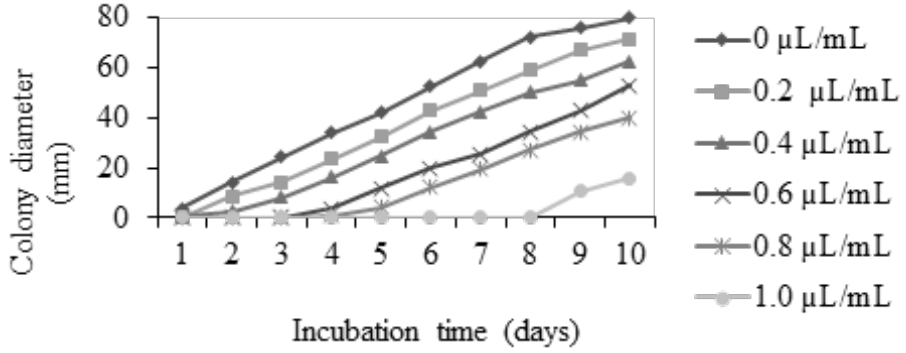

Table 2 - Percentage inhibition of mycelial growth of *Aspergillus flavus* at different doses (µL/mL) of *Cymbopogon citratus* essential oil in different times of incubation.

Essential oil dose (µL/mL)	Incubation time (days)									
	1	2	3	4	5	6	7	8	9	10
0.2	100^{Aa}	47^{Cb}	42^{Cb}	31^{Dc}	23^{Ed}	19^{Ed}	19^{Ed}	18^{Ed}	11^{Ee}	11^{Ee}
0.4	100^{Aa}	82^{Bb}	67^{Bc}	52^{Cd}	41^{De}	35^{Df}	33^{Df}	31^{Df}	28^{Dg}	22^{Dg}
0.6	100^{Aa}	100^{Aa}	100^{Aa}	89^{Bb}	72^{Cc}	62^{Cd}	59^{Cd}	53^{Ce}	44^{Cf}	34^{Cg}
0.8	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	90^{Bb}	71^{Bc}	69^{Bd}	63^{Be}	55^{Bf}	50^{Bf}
1.0	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	86^{Ab}	80^{Ac}
CV = 5.55%										

Means followed by the same capital letter in the column and small letter in the row are not significantly different by the Scott-Knott's test at 5% significance.

provided total control of the fungus up to days three and four, respectively. Similar results were obtained by Mishra and Dubey (1994), who found that at day seven of incubation, the dose 0.5 µL/mL inhibited 52% of the fungal mycelial growth, which was close to the result we found at the dose 0.6 µL/mL, with 59% percent inhibition.

The dose 1.0 µL/mL EO provided, without significant statistical difference, control of mycelial growth during the eight days of observation and, from onwards, the inhibition was reduced. Tatsadjieu et al. (2009) evaluated the percentage inhibition of *A. flavus* growth on agricultural products with essential oils and found a reduction in the inhibition over time. The authors suggested that this may be caused by the evaporation, during some time, of some volatile components of the oils, reducing their concentration and their effect on the microorganism.

The present finding is consistent with findings of past studies by Paranagama et al. (2003), who found that the concentration 1.0 µL/mL of *Cymbopogon citratus* EO completely inhibited *A. flavus*.

On the other hand, Helal et al. (2007) in a study carried out in Egypt, found that the 100% inhibition of *A. flavus* required the concentration of 2.0 µL/mL of *C. citratus* essential oil. A possible explanation for the differences between the results obtained by these authors and ours may be the amount of citral present in the essential oils, which were 68.4% for the authors and 79% for the present study.

Studies by Onawunmi; Yisak and Ogunlana (1984), Rice and Coats (1994), Garcia et al. (2008) demonstrated the antibacterial, antifungal, and insecticidal properties of citral. Lee (2017), when evaluating the *in vitro* fungitoxic activity of citral (0.2, 0.4, 0.6, 0.8, and 1.6 µL/mL) on the development of *A. brasiliensis* and *A flavus*, reported effect of this component on fungi starting from the dose of 0.6 µL/mL.

Analysis of antifungal activity by microdilution of the *Cymbopogon citratus* essential oil on *Aspergillus flavus*

The minimum inhibitory concentration (MIC) of the essential oil on fungal growth was determined by serial microdilution in microplate. The results are presented in Table 03. The screened concentrations were based on the *in vitro* test results.

Table 03 - *Aspergillus flavus* growth in serial microdilution at different doses (µL/mL) of *Cymbopogon citratus* essential oil.

Essential oil dose (µL/mL)	Fungal growth
1.2	-
1.0	-
0.9	-
0.8	+
0.6	+
0.5	+
0.4	+
0.3	+
0.2	+
0.1	+

(+) indicates fungal growth and (-) indicates growth inhibited.

From the data in Table 03, it is apparent that from the dose of 0.9 µL/mL of essential oil there was no fungal growth. Considering the minimal differences between doses, the results are close to that obtained in the *in vitro* test for the dose that provided the highest control (1.0 µL/mL).

Inhibition of *A. flavus* growth on corn kernels by the essential oil of *C. citratus*

The analysis of variance of the effects of lemon grass essential oil and incubation time on fungal mycelial growth of corn kernels found no significant effect of the different EO doses (D), the incubation time (t), or the interaction (D x t). This results indicate that the inhibition of *A. flavus* growth on corn by the lemon grass essential oil is independent of the variation of these factors. All

essential oil doses (0.4, 0.7, and 1.0 µL/mL) and incubation times (14, 28, and 42 days) tested provided 100% of fungal growth inhibition.

Tullio et al. (2006) observed that, in general, the fumigation of essential oil requires lower concentrations than its application in the liquid state to inhibit microbiological growth. This explains why for all doses and times tested there was 100% of fungal growth inhibition and demonstrates the efficacy of the *C. citratus* essential oil in the control of *A. flavus* in corn kernels as substrate.

Similarly, Boukaew; Prasertsan; Sattayasamitsathit (2017) found that the fungal species *Aspergillus flavus* was completely inhibited in corn kernels over a 22-day storage time by 0.5 µL/mL of *Melissa officinalis* essential oil and, as in the present work, having citral as its main component.

4 Conclusions

The results of this investigation demonstrate the decrease in the growth of Aspergillus flavus with the application of the essential oil of Cymbopogon citratus. This study has identified citral as the main component of the essential oil, corresponding to 79% of its chemical composition. The most efficient dose of the essential oil, among those tested, was 1.0 µL/mL, with a minimum inhibitory concentration of 0.9 µL/mL. The analysis of the effect of essential oil on mycelial growth on corn kernels showed 100% inhibition of fungal development was observed at all doses (0.4, 0.7, and 1.0 µL/mL) and incubation times (14, 28, and 42 days) tested.

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