

Control of *Aspergillus flavus* in wheat grains using *Cymbopogon flexuosus* essential oil

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Abstract

Fungi are one of the main food spoilage agents. Numerous species when subjected to stress conditions produce secondary metabolites known as mycotoxins, which are mutagenic and carcinogenic substances. The fungus *Aspergillus flavus* is one of the main contaminants of grains and is known to produce Aflatoxin. Pesticides are used in agriculture to contain fungi and other pests, but they harm other species, the environment and the human health, in addition to the development of resistance to these substances in pest species. Natural alternatives have been sought to control these organisms. In this context, essential oils are a viable option against *A. flavus*. The aim of this study was to identify the main components and evaluate the effectiveness of lemongrass essential oil (*Cymbopogon flexuosus*) for controlling the fungus *Aspergillus flavus*. Initially, the effect of essential oil on mycelial growth of the fungus was assessed by *in vitro* tests at the doses: 0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1.6; 3.2; 6.4; and 12.8 $\mu\text{L mL}^{-1}$. The minimum inhibitory concentration (MIC) was 0.8 $\mu\text{L mL}^{-1}$. The *in vivo* test was performed at the following concentrations: 0.6; 0.8; and 1.6 $\mu\text{L mL}^{-1}$. The results showed that the essential oil has fungicidal potential against *A. flavus*. The main component of the essential oil was citral.

Keywords: Citral, fungus, lemongrass, MIC.

Controle do *Aspergillus flavus* em grãos de trigo através do óleo essencial de *Cymbopogon flexuosus*

Resumo

O fungo *Aspergillus flavus* Link (1809) é um dos principais contaminantes de grãos e é conhecido por produzir a aflatoxina. O método para conter fungos e outras pragas na agricultura é com o uso de agrotóxicos, porém estes causam danos a outras espécies, ao meio ambiente e a saúde humana, além das pragas adquirirem resistências a essas substâncias. Tem-se buscado alternativas naturais para controlar esses organismos. Nesse contexto, os óleos essenciais se apresentam como opção. Este estudo identificou os principais componentes e avaliou a eficácia do óleo essencial de *Cymbopogon flexuosus* Stapf (capim limão) para controle do fungo *A. flavus*. O efeito do óleo essencial sobre o crescimento micelial do fungo foi avaliado em testes *in vitro* nas doses entre 0,05 e 12,8 $\mu\text{L mL}^{-1}$. O teste *in vivo* foi realizado nas seguintes concentrações: 0,6; 0,8 e 1,6 $\mu\text{L mL}^{-1}$. O óleo essencial, apresentou composição rica em citral (formado pelos isômeros geranial e neral). A concentração inibitória mínima (CIM) foi de 0,8 $\mu\text{L mL}^{-1}$, apresentou potencial fungicida sobre o *A. flavus*, demonstrando ser promissor na substituição de agrotóxicos, sendo uma alternativa de baixo impacto para a saúde e o meio ambiente.

Palavras-chave: Citral, fungos, capim limão, CIM.

Introduction

A. flavus is one of the main contaminants of grains (Liu, Li, Wu, Xiao, & Xing, 2021), causing large economic losses and potential health issues on humans and animals (Xu *et al.*, 2021) because of the production of mutagenic and carcinogenic secondary metabolites, especially aflatoxin (Tian *et al.*, 2021).

Pesticides are used in crops to fight pests, yet these

substances do not only affect these organisms, they harm other species, the soil, the aquatic ecosystem and human health. Adding to the problem is the fact that field pests, including fungi, become more resistant to pesticides. Within this context, this study has investigated the possibility of replacing these substances by natural alternatives that do not cause environmental damage and harm to species. Essential oils have been presented as an alternative.

Essential oils are extracted from aromatic plants. They are secondary metabolites known for their strong odor, volatility, medicinal, antiseptic, antibacterial, antiviral, and antifungal properties, being widely used in food, health, and pharmaceutical industries (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). The *Cymbopogon* genus has been extensively studied, consisting of around 180 species, including different sub-species, varieties and sub-varieties. Extensive research has been conducted to prove the benefits of the *Cymbopogon* genus, including studies on the extract, essential oils, biological activity of the chemical compounds, and development. Pharmaceutical studies have tested the medical effects of *Cymbopogon* species such as anti-inflammatory, antimicrobial, antioxidant, and anticancer activities (Zahra, Hartati, & Fidrianny, 2020), as well as in the central nervous system (Rojek *et al.*, 2021).

The *C. flexuosus* essential oil has the chemical compound citral as its main component, presenting anti-inflammatory effect on animal and human cells, as well as antimicrobial, antioxidant, and antifungal activities (Han, & Parker, 2017).

This study identified the main components and evaluated the efficacy of *C. flexuosus* essential oils and its major citral compound on the fungus *A. flavus* tested at different doses *in vitro* and *in vivo* experiments, and determined the minimal inhibitory concentration (CIM).

Materials and Methods

C. flexuosus essential oil was purchased from the commercial supplier Ferquima Company[®] and was stored at 4 °C. The tested doses were diluted in distilled water and dimethylsulfoxide 1% (DMSO) to obtain a homogeneous solution for the diffusion test with the substrate and microdilution.

Fungi and spores suspension

The fungus species *A. flavus* (IOC 4102 strain) from the Culture Collection of Filamentous Fungi was kindly provided by the Fundação Oswaldo Cruz. The fungal cultures were grown in potato, dextrose and agar medium (PDA), in petri plates at 30 °C for seven days. To perform the spores collection, 15 mL of sterile distilled water were added to the Petri dishes with PDA medium with 7-day-old fungal colony growth. The fungal suspension was adjusted with sterile distilled water to the final concentration of $4,5 \times 10^6$ spores/mL⁻¹ using a Neubauer chamber (Model: K5-0011, KAVIS, São José do Pinhais, Brasil). The suspension was stored at 4 °C until use.

Microdilution at microplate

The minimum inhibitory concentration (MIC) of the essential oil (EO) on the fungus studied was determined by serial microdilution in 96-well microplates. The doses tested were defined from the results of the *in vitro* test, being evaluated the following essential oil doses: 1.8; 1.6; 1.4; 1.2; 1; 0.9; 0.8; 0.7; 0.6; 0.5; 0.45; 0.4; 0.35; 0.3; 0.25; 0.22; 0.2; 0.17; 0.15 and 0.12 $\mu\text{L mL}^{-1}$.

For each dose tested four replications were performed in PD medium (potato and dextrose) with a solution containing essential oil, DMSO and spores suspension (10^7) and a control treatment without the essential oil. The plates were maintained

in B.O.D (Biochemical Oxygen Demand) chambers (Model: SSBOD 120L, Benfer, São Paulo, Brazil), at 35 °C for 72 hours. After this incubation period the results were analyzed visually. MIC was defined as the lowest essential oil concentration in which no fungal growth had occurred (Pandey, Rai, & Acharya, 2003; Dellavalle *et al.*, 2011).

In vitro test

In the *in vitro* anti-fungal assay, the following *C. flexuosus* essential oil doses were tested: 0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1.6; 3.2; 6.4 and 12.8 $\mu\text{L mL}^{-1}$. Two control treatments without essential oil were performed: one with fungus growing on PDA medium only; and the other with fungus growing in PDA medium added with DMSO to evaluate the influence of the surfactant on fungal growth.

Was added in the cast BDA substrate (45 °C), the essential oil solution was added to liquid PDA medium, diluted in 1% DMSO, to obtain the desired concentration. After homogenization, the substrate was poured to the Petri dishes and incubated with a 7mm mycelial disc of the PDA grown colony of the fungus in study. The control plates, without essential oil, were inoculated following the same process. Afterwards, all the plates were incubated at 30 °C until the fungus covered the entire control plate, that is, between 7 and 10 days, which was considered the end of the incubation time. The experiment was carried out with five replications. The colony diameter was recorded daily with a digital caliper (Model: 150mm, MTX, São Paulo, Brazil). The colony size of each test sample was compared with the size of the control tests. The results were expressed as the diameter size of the microbial growth halo. The percentage of colony inhibition (PI) was calculated according to Billerbeck, Roques, Bessière, Fonvieille and Dargent (2001): $PI = (C - T / C) \times 100$ (Equation 1), where: C = average diameter of the mycelia in the control; T = average diameter of the mycelia in the treatment.

According to the *in vitro* assay results, the MIC test was performed with its major component, the Citral, purchased from the Sigma-Aldrich Company[®]. The doses tested were: 0; 0.2; 0.4; 0.6; 0.8 and 1.6 $\mu\text{L mL}^{-1}$. PI was determined using Equation 1.

Analysis of the control of the fungus by essential oils in infected grains

Seeds of wheat were provided by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) (Wheat/Rio Grande do Sul). The analysis of efficacy of *C. Flexuosus* essential oil on the fungus growth was defined from the doses that showed the largest inhibition percentages (PI) of the mycelial growth in the *in vitro* assay, being 0.6; 0.8 and 1.6 $\mu\text{L mL}^{-1}$ besides the control, which did not contain any treatment with essential oils. These doses were tested in different periods of time (10, 20 and 30 days) in a controlled environment (30°C).

In 1.4 L glass bottles, 200g of wheat grains were autoclaved at 121°C for 15 minutes. Afterwards, to each container, 2mL of the suspension at 10^7 spores/mL⁻¹ of *A. flavus* was inoculated to the grains. The flasks were

incubated in a controlled environment (30 °C) (B.O.D.) for 2 days to promote the fungus growth.

After 48h, filter paper sachets were soaked in different doses of essential oils. The sachets were placed in the bottles with the grains inoculated with the fungus. The control treatment received no dose of the essential oil and was analyzed at the different times that the treatment was applied. Each dose was tested with three replications. These procedures were carried out in a laminar flow cabinet (Model: SP-960/6-HI, SPLABOR, Presidente Prudente, Brazil). Each dose tested was added with the volume of essential oil equivalent to the filter paper sachet: 0.6 $\mu\text{L mL}^{-1}$: 840 μL , 0.8 $\mu\text{L mL}^{-1}$: 1120 μL and 1.6 $\mu\text{L mL}^{-1}$: 2240 μL .

The efficacy of the essential oil was analyzed by collecting samples at each 10 days. Colony Forming Units (CFU) under the effect of each treatment were quantified. At the end of each time, 10g grains of wheat were collected and diluted in 90 mL in saline solution (NaCl 0.9%). Ten-fold serial dilutions (10^{-1} to 10^{-3}) were prepared with this solution. A volume of 0.5 millimeter of each dilution were inoculated to Petri dishes with 20 mL of Sabouraud agar medium. The plates were incubated for 48 h at 30 °C in B.O.D chambers. Plates containing 05 to 250 UFCs were counted and the percentages of inhibition (PI) of growth were calculated according to Tatsadjieu, Yaouba, Nukenine, Ngassoum, & Mbofung (2010): $PI = C_0 - C_t / C_0 \times 100$ (Equation 02), where C_0 = the number of colonies that receive no treatment; C_t = the number of colonies that received the treatment with essential oils.

Essential oil gas chromatography

The analysis of the essential oil constituencies were performed by gas chromatography with mass spectrometry (CG/EM) (Model: GCMS-QP2010, Shimadzu, Quito, Japan) according to the methodology of Lee, Garcia, Martinazzo and de Souza Teodoro (2020). The identification of the compounds were performed by comparing the mass spectrums obtained with the data of the equipment and by the Kovats Retention Index (IK) of each component (Lanças, 2009). The quantitative analysis of the main components of the essential oil, expressed as percentages, were performed by the peak area integration of normalization as described by Zhang, Chen, Wang e Yao, (2006).

Statistical analysis

The results were analyzed using the statistic program SISVAR[®], by analysis of variance and means compared by the Scott-Knott test at 5% significance level. The experiment was arranged in a completely randomized design (Ferreira, 2014).

Results and Discussion

The main component of *C. flexuosus* essential oil used in this study was Citral (72.0%), which is formed by neral isomers (40.0%) and geranial (32.0%; Table 1). The fungitoxic activities of the *C. flexuosus* essential oil on the *A. flavus* mycelial growth inhibition showed a significant difference of the mycelial growth of the fungus in study (Table 2).

Table 1. Main components of the *C. flexuosus* essential oil determined by GC-MS.

Peak	Component	Retention Time (min)	Kovats Index	Other Authors*
02	6-metil-5-hepten-2-ona	8.927	994	992
05	Linalool	13.647	1093	1098
06	Citronella	16.097	1150	1153
09	Neral	20.353	1245	1244
10	Geraniol	20.873	1257	1257
11	Geranial	21.768	1272	1270
12	Geranyl Acetate	26.664	1388	1383
13	Caryophyllene	28.315	1430	1428

* DB-5 Column. Other Authors: all values are based on Adams (2007), except the value of the 13 peak, which is based on Choi (2003).

Table 2. Mean of mycelial growth inhibition, *in vitro*, of the *A. flavus* growth at different doses ($\mu\text{L mL}^{-1}$) of *C. flexuosus* essential oil (EO).

Day	EO dose/ Mycelial growth inhibition (%)					
	0.05	0.1	0.2	0.4	0.6	0.8*
1	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
2	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
3	28.0 ^{cB}	31.0 ^{cB}	46.0 ^{bB}	100 ^{aA}	100 ^{aA}	100 ^{aA}
4	24.0 ^{cB}	26.0 ^{cB}	37.0 ^{bC}	100 ^{aA}	100 ^{aA}	100 ^{aA}
5	24.0 ^{cB}	26.0 ^{cB}	36.0 ^{bC}	93.0 ^{aB}	100 ^{aA}	100 ^{aA}
6	24.0 ^{dB}	26.0 ^{cB}	32.0 ^{cC}	89.0 ^{bB}	100 ^{aA}	100 ^{aA}
7	08.0 ^{dC}	10.0 ^{dC}	18.0 ^{cD}	81.0 ^{bC}	100 ^{aA}	100 ^{aA}
8	0.0 ^{cD}	0.0 ^{cD}	04.0 ^{cE}	70.0 ^{bD}	100 ^{aA}	100 ^{aA}
9	0.0 ^{cD}	0.0 ^{cD}	0.0 ^{cE}	54.0 ^{bE}	100 ^{aA}	100 ^{aA}
10	0.0 ^{cD}	0.0 ^{cD}	0.0 ^{cE}	42.0 ^{bF}	98.0 ^{aA}	100 ^{aA}

Means followed by the same small letter in the row and capital letter in the column are not significantly different by the Scott-Knott's test at 5% significance. *From the 0.8 $\mu\text{L/mL}$ dose the growth inhibition was complete.

The incubation time of *A. flavus* was 10 days, and it showed growth between the 0.05 and 0.6 $\mu\text{L mL}^{-1}$ doses, however the growth was inhibited from the 0.6 $\mu\text{L mL}^{-1}$ dose. At doses below 0.4 $\mu\text{L mL}^{-1}$ the diameter of the fungus inhibition halo ranged between 53 and 92 mm (total diameter of the Petri dish). At the 10th day, a discreet growth was recorded (2 mm) at the 0.6 $\mu\text{L mL}^{-1}$ dose.

Chao, Young and Oberg (2000) evaluated the effects of *C. flexuosus* essential oil on microorganisms and found that neral (43.5%) and geranial (28.8%) were the major components in *C. flexuosus*, with fungitoxic activities against the *Aspergillus* genus.

Cymbopogon species have been reported as having antifungal activity against *A. niger* and *A. fumigatus* (Munda, Dutta, Pandey, Sarma and Lal, 2019) and *A. flavus*. Martinazzo, de Oliveira and de Souza Teodoro (2020) verified inhibition effects of *C. citratus* on *A. flavus* from the 0.6 $\mu\text{L mL}^{-1}$, achieving total control at 1.0 $\mu\text{L mL}^{-1}$. Achar *et al.* (2020) obtained total control at 2000 $\mu\text{L mL}^{-1}$, using the method of diffusion in medium.

The serial microdilution of *C. flexuosus* showed that the MIC of the essential oil on *A. flavus* was 1 $\mu\text{L mL}^{-1}$. Similar results (0.9 $\mu\text{L mL}^{-1}$) were found by Martinazzo, de Oliveira and de Souza Teodoro (2020). However, Munda *et al.* (2019)

found higher values for the MIC of *C. flexuosus* on *A. niger* (> 60 $\mu\text{L mL}^{-1}$) and *A. fumigatus* (10 $\mu\text{L mL}^{-1}$).

Over the time of evaluation, the citral compound was efficient in the complete inhibition of the mycelial growth of *A. flavus* from the dose of 0.8 $\mu\text{L mL}^{-1}$ (Table 3). At lower doses, the maximum growths recorded were: 74 mm (0.05 $\mu\text{L mL}^{-1}$); 65 mm (0.2 $\mu\text{L mL}^{-1}$); 21 mm (0.4 $\mu\text{L mL}^{-1}$) and 05 mm (0.6 $\mu\text{L mL}^{-1}$).

Table 3. Mean of inhibition, *in vitro*, of *A. flavus* growth at different citral dose ($\mu\text{L mL}^{-1}$).

Day	Citral dose/ Mycelial growth inhibition (%)					
	0.05	0.2	0.4	0.6	0.8	1.6
1	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
2	32.0 ^{bB}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
3	31.0 ^{cB}	88.0 ^{bB}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
4	25.0 ^{cC}	62.0 ^{bC}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
5	22.0 ^{cC}	51.0 ^{bD}	100 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
6	19.0 ^{cC}	42.0 ^{bE}	97.0 ^{aA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
7	19.0 ^{dC}	38.0 ^{cE}	92.0 ^{bA}	100 ^{aA}	100 ^{aA}	100 ^{aA}
8	18.0 ^{dC}	32.0 ^{cF}	87.0 ^{bB}	97.0 ^{aA}	100 ^{aA}	100 ^{aA}
9	16.0 ^{dC}	29.0 ^{cF}	80.0 ^{bC}	95.0 ^{aA}	100 ^{aA}	100 ^{aA}
10	16.0 ^{dC}	29.0 ^{cF}	77.0 ^{bC}	94.0 ^{aA}	100 ^{aA}	100 ^{aA}

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

In the same way as with the other results, both for essential oils and citral, the doses at 0.6 $\mu\text{L mL}^{-1}$ showed no significant differences between the control fungus and the doses that had 100% inhibition. Even though the inhibition of the fungus at the end of the incubation times may be a sign of microorganism development over time. Therefore, the dose at 0.8 $\mu\text{L mL}^{-1}$ is recommended as the lowest dose for the control of the *A. flavus* fungus, both for the *C. flexuosus* essential oil and the citral compound.

The *C. flexuosus* essential oil evaluated in this study was also evaluated within its duration of antifungal efficacy over the days. The results of this study show that doses from 0.6 $\mu\text{L mL}^{-1}$ of the *C. flexuosus* essential oil inhibited the fungal growth for at least 30 days, with efficacy of about 99.31% (Table 4).

Table 4. Mean of growth inhibition of *A. flavus* in *T. aestivum* for the different doses ($\mu\text{L mL}^{-1}$) and storage periods of the *C. flexuosus* essential oil (EO).

EO dose	Day/ Fungal growth inhibition over storage (%)		
	10	20	30
0.6	91.78 ^{aA}	99.37 ^{aB}	99.25 ^{aB}
0.8	92.47 ^{aA}	98.90 ^{aB}	99.34 ^{aB}
1.6	87.98 ^{bA}	99.05 ^{aB}	99.34 ^{aB}

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

Luo et al. (2004) investigated the effects of citral on *A. flavus* using broth dilution (MIC = 0.5 $\mu\text{L mL}^{-1}$) and found that citral injured the wall and the membrane of *A. flavus* spore, decreasing its elasticity. After entering the cell, the citral

modified the mitochondrial genetic expression, its reduplication and morphology, also in addition to altering the aggregation of protein-like macromolecules. This led cells, organelles and macromolecules to lose their normal structures and functions, which resulted in the loss of germination capacity of *A. flavus* spores, proving that citral has fungitoxic properties against fungi of the *Aspergillus* genus.

The antimicrobial activity of citral on *A. flavus* was also studied by López-Malo, Alzamora and Palou (2002), in which they found MIC of 1800 $\mu\text{L mL}^{-1}$ (hydrogen potential of 3.5 and 4.5) and verified fungal growth inhibition for 60 days.

Conclusion

The essential oil of *C. flexuosus* showed *in vitro* fungicide effect against the fungus *Aspergillus flavus*, controlling fungal growth from the dose of 0.8 $\mu\text{L mL}^{-1}$. The MIC of the essential oil on *A. flavus* was 1.0 and 0.8 $\mu\text{L mL}^{-1}$.

The major component identified in the essential oil was the citral. The *in vitro* tests with citral showed fungicidal effect against the fungus from the dose 0.6 $\mu\text{L mL}^{-1}$.

The *C. flexuosus* essential oil was proven to be efficient on stored contaminated wheat grains, maintaining the fungicidal capacity even after 30 days of incubation the best control of the fungal *A. flavus* in *T. aestivum*.

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