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Litsea cubeba ESSENTIAL OIL AND β-CYCLODEXTRIN: MOLECULAR INCLUSION, CHARACTERIZATION AND BIOACTIVITY

GISELI CRISTINA PANTE

Maringá

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Tese apresentada ao programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de doutora em Ciência de Alimentos.

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GISELI CRISTINA PANTE

"*Litsea cubeba* Essential oil and β-Cyclodextrin: Molecular Inclusion, Characterization and Bioactivity"

Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

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Maringá – 2021

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BIOGRAFIA

Possui graduação em Tecnologia em Alimentos pela Universidade Tecnológica Federal do Paraná e mestrado em Ciência de Alimentos pela Universidade Estadual de Maringá. Tem experiência nas áreas de microbiologia e toxicologia de alimentos, atuando principalmente nos seguintes temas: controle de qualidade na indústria alimentícia, análises microbiológicas de alimentos, cromatografia e micotoxinas.

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APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de dois artigos científicos.

1. Giseli Cristina Pante, Juliana Cristina Castro, Renata Sano Lini, Jéssica Cristina Zoratto Romoli, Rafaela Takako Ribeiro de Almeida, Francielle Pelegrin Garcia, Celso Vataru Nakamura, Eduardo Jorge Pilau, Benício Alves de Abreu Filho, Miguel Machinski Junior. *Litsea cubeba* essential oil: chemical profile, antioxidant activity, cytotoxicity, effect against *Fusarium verticillioides* and fumonisins production. Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes. https://doi.org/10.1080/03601234.2021.1890519

2. Giseli Cristina Pante, Juliana Cristina Castro, Renata Sano Lini, Jéssica Cristina Zoratto Romoli, Thiago Yoshioka Pires, Francielle Pelegrin Garcia, Celso Vataru Nakamura, Ana Claúdia Nogueira Mulati, Graciette Matioli, Miguel Machinski Junior. Inclusion complexes of *Litsea cubeba* essential oil into β-cyclodextrin: Preparation, physicochemical characterization, cytotoxicity and antifungal activity. Industrial Crops and Products.

GENERAL ABSTRACT

INTRODUCTION. Essential oils (EOs) are a natural mixture of volatile chemical compounds. They are known worldwide for their potent biological properties, which are attributed to the chemical constituents, mainly terpenoids and phenolic compounds. *Litsea cubeba* is an important medicinal plant, which is distributed in China, Japan and Southeast Asian countries. Several researchers have reported the bioactivities of *L. cubeba* essential oil (LCEO), including antioxidant, antimicrobial, antifungal, anti-inflammatory, insecticidal and anticancer properties. However, the applications of EOs, are limited due to its unstable to light, oxygen and temperature. Thus, an improvement in the stability of EOs is necessary, in order to expand the application fields in food, cosmetic and medicine industries. In this context, alternative strategies have been developed, such as inclusion of EOs into macromolecules. Among them, cyclodextrins are cyclic oligosaccharides with a truncated-cone shape containing glucopyranose units. The most used is β-cyclodextrin (β-CD), which contain 7 glucose units, hydrophobic cavity and hydrophilic external surface

AINS. Investigate the bioactivity of LCEO *in vitro,* describing its chemical profile, and assessing its potential antioxidant, cytotoxicity, antifungal and antimycotoxigenic activities against *Fusarium verticillioides*. Moreover, to prepare inclusion complexes with β-CD by different methods and to characterize them in terms of physicochemical properties, as well as cytotoxic effect and antifungal activity.

MATERIAL AND METHODS. To evaluate the bioactivity of LCEO, we carried out the analysis of chemical profile by gas chromatography-mass spectrometry (GC-MS), antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) methods, cytotoxicity by MTT assay against HT-29 and HeLa cancer cells and antifungal and antimycotoxigenic activities against *F. verticillioides*. The inclusion complexes of LCEO and β-CD were prepared using physical mixture, kneading (KN) and co-precipitation (CP) methods. Moreover, the complexation efficiency and the physicochemical properties of the inclusion complexes using ATR-FTIR, FT-Raman, DSC and TG were evaluated. As well as cytotoxicity against human colorectal and cervical cancer cells and antifungal activity against *Aspergillus flavus* and *F. verticillioides*.

RESULTS AND DISCUSSION. Most of the compounds observed in the EO were neral (32.75%) and geranial (37.67%). The radical scavenging capacity of DPPH and ABTS was 104.4 and 56.4 mmol Trolox mg^{-1}, respectively, indicating good antioxidant activity. The EO studied by us revealed cytotoxic effect against HT-29 and HeLa cancer cells. The Minimum Inhibitory and Minimum Fungicidal Concentrations against *F. verticillioides* were both 125 μg mL^{-1}. Morphological investigation showed that hyphae and microconidia structures underwent changes after treatment with the EO. Analyses performed with the EO strongly reduced the mycelial development of *F. verticillioides* and the synthesis of fumonisins B_1 and B_2 in dose-dependence effect compared ($p<0.01$) with the fungal control (10^5 conidia mL^{-1}) and positive control (fludioxonil + metalaxyl-M). The complexation efficiency results presented significant evidence of LCEO:β-CD inclusion complex formation, being KN (83%) and CP (73%) the best methods used in this study. All tested LCEO:β-CD inclusion complexes exhibited toxicity to HT-29 cells. Although, cytotoxic effect was less pronounced in HeLa tumor cell, LCEO-KN was more active against Hela than

non-tumor cell. LCEO-KN and LCEO-CP inclusion complexes were efficient for both toxigenic fungi.
CONCLUSION. The results obtained *in vitro* suggest that LCEO has excellent antioxidant, fungicide and antimycotoxigenic effects. Moreover, the molecular inclusion of LCEO into β-CD was successful, as well as the preliminary biological results, evidencing that β-CD inclusion process may be a viable alternative to facilitate and increase future applications of this EO. Additional studies, *in situ* and *in vivo*, still need to be carried out to prove the effectiveness of LCEO as an alternative biofungicide, as well as its therapeutic potential.

KEYWORDS: *Litsea cubeba* essential oil; biological activities; biofungicide; molecular inclusion; β-cyclodextrin.

RESUMO GERAL

INTRODUÇÃO. Os óleos essenciais (OE) são formados por uma mistura natural de compostos químicos voláteis. São conhecidos mundialmente por suas potentes propriedades biológicas, atribuídas aos constituintes químicos, principalmente terpenoides e compostos fenólicos. A *Litsea cubeba* é uma importante planta medicinal, distribuída na China, Japão e países do Sudeste Asiático. Vários pesquisadores relataram as bioatividades do óleo essencial de *L. cubeba* (OELC), incluindo propriedades antioxidantes, antimicrobianas, antifúngicas, anti-inflamatórias, inseticidas e anticâncer. No entanto, as aplicações de OEs são limitadas devido à sua instabilidade à luz, oxigênio e temperatura. Dessa forma, é necessária uma melhoria na estabilidade dos OEs para expandir os campos de aplicação nas indústrias de alimentos, cosméticos e medicamentos. Nesse contexto, estratégias alternativas têm sido desenvolvidas, como a inclusão de OE em macromoléculas. Dentre eles, as ciclodextrinas são oligossacarídeos cíclicos com formato de cone truncado contendo unidades de glicopiranose. A mais utilizada é a β-ciclodextrina (β-CD), que contém 7 unidades de glicose, cavidade hidrofóbica e superfície externa hidrofílica.

OBJETIVOS. Investigar a bioatividade do OELC *in vitro*, descrevendo seu perfil químico, e avaliando seu potencial antioxidante, citotoxicidade, atividades antifúngicas e antimicotoxigênicas contra *Fusarium verticillioides*. Além disso, preparar complexos de inclusão com β-CD por diferentes métodos e caracterizá-los em termos de propriedades físico-químicas, bem como efeito citotóxico e atividade antifúngica.

MATERIAL E MÉTODOS. Para avaliar a bioatividade do OELC, realizamos a análise do perfil químico por CG-EM, atividade antioxidante pelos métodos DPPH e ABTS, citotoxicidade pelo ensaio do MTT contra células cancerosas HT-29 e HeLa e atividades antifúngica e antimicotoxigênica contra *F. verticillioides*. Os complexos de inclusão entre OELC e β-CD foram preparados utilizando métodos de mistura física, amassamento (AM) e co-precipitação (CP). Além disso, a eficiência da complexação e as propriedades físico-químicas dos complexos de inclusão usando ATR-FTIR, FT-Raman, DSC e TG foram avaliadas. Bem como citotoxicidade contra células humanas de câncer colo retal e cervical e atividade antifúngica contra *Aspergillus flavus* e *F. verticillioides*.

RESULTADOS E DISCUSSÃO. A maioria dos compostos observados no OE foram neral (32,75%) e geranial (37,67%). A capacidade de eliminação de radicais de ABTS e DPPH foi de 104,4 e 56,4 mmol de Trolox mg^{-1}, respectivamente, indicando boa atividade antioxidante. O OE estudado por nós revelou efeito citotóxico contra células cancerígenas HT-29 e HeLa. As Concentrações Inibitórias Mínimas e Fungicidas Mínimas contra *F. verticillioides* foram ambas 125 μg mL^{-1}. A investigação morfológica mostrou que as hifas e as estruturas dos microconídios sofreram alterações após o tratamento com o OE. As análises realizadas com o EO reduziram fortemente o desenvolvimento micelial de *F. verticillioides* e a síntese das fumonisinas B_1 e B_2 no efeito dose-dependência em comparação ($p<0,01$) com o controle fúngico (10^5 conídios mL^{-1}) e o controle positivo (fludioxonil + metalaxil-M). Os resultados de eficiência da complexação apresentaram evidências significativas de formação do complexo de inclusão OELC:β-CD, sendo AM (83%) e CP (73%) os melhores métodos utilizados neste estudo. Todos os complexos de inclusão OELC:β-CD testados exibiram toxicidade para células HT-29. Embora o efeito

citotóxico tenha sido menos pronunciado nas células tumorais HeLa, o OELC-AM foi mais ativo contra Hela do que as células não tumorais. Os complexos de inclusão OELC-AM e OELC-CP foram eficientes para ambos os fungos toxigênicos.
CONCLUSÃO. Os resultados obtidos *in vitro* sugerem que o OELC possui excelentes efeitos antioxidantes, fungicidas e antimicotoxigênicos. Além disso, a inclusão molecular de OELC em β-CD foi bem sucedida, assim como os resultados biológicos preliminares, evidenciando que o processo de inclusão de β-CD pode ser uma alternativa viável para facilitar e aumentar futuras aplicações deste OE. Estudos adicionais, *in situ* e *in vivo*, ainda precisam ser realizados para comprovar a eficácia do OELC como biofungicida alternativo, bem como seu potencial terapêutico.

PALAVRAS CHAVE: Óleo essencial de *Litsea cubeba*; atividades biológicas; biofungicida; inclusão molecular; β-ciclodextrina.

ARTIGO 1 – ACEITO

Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes

***Litsea cubeba* essential oil: chemical profile, antioxidant activity, cytotoxicity, effect against *Fusarium verticillioides* and fumonisins production**

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ABSTRACT

The purpose of this study was to determine the chemical profile of *Litsea cubeba* essential oil, carry out an *in vitro* evaluation of its antioxidant potential and its cytotoxicity, as well as its antifungal and antimicotoxigenic activities against *Fusarium verticillioides*. Most of the compounds observed in the EO were neral (32.75%) and geranial (37.67%). The radical scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid was 104.4 and 56.4 mmol Trolox mg^{-1}, respectively, indicating good antioxidant activity. The EO studied by us revealed cytotoxic effect against HT-29 and HeLa cancer cells. The Minimum Inhibitory and Minimum Fungicidal Concentrations against *F. verticillioides* were both 125 µg mL^{-1}. Morphological investigation, performed by fluorescence microscopy and scanning electron microscopy, showed that hyphae and microconidia structures underwent changes after treatment with the EO. Analyses performed with the EO strongly reduced the mycelial development of *F. verticillioides* and the synthesis of fumonisins B_1 and B_2 in dose-dependence effect compared ($p<0.01$) with the fungal control (10^5 conidia mL^{-1}) and positive control (fludioxonil + metalaxyl-M). Thus, the results obtained *in vitro* suggest that *L. cubeba* EO has excellent antioxidant, fungicide and antimycotoxigenic effects. Additional studies, *in situ* and *in vivo*, still need to be carried out to prove the effectiveness of *L. cubeba* EO as an alternative biofungicide, as well as its therapeutic potential.

KEYWORDS: *Litsea cubeba*; essential oil; biological activities; biofungicide; environmental science.

1. INTRODUCTION

Fungal contamination is responsible for the spoilage of agri-commodities worldwide, and it can cause numerous safety problems.[1] Toxigenic fungi are reported as disease-causing in agriculture. Therefore, they affect productivity, global economy and public health.[2] *Fusarium verticillioides* is one of the most common toxigenic fungi, for it causes food contamination, mainly when it comes to corn and, consequently, its by-products. It also produces fumonisins, which

are known to be highly hepatotoxigenic, nephrotoxigenic, neurotoxigenic and immunosuppressive.[2] Fumonisins B_1 (FB_1) and B_2 (FB_2) are the most naturally produced secondary metabolites; FB_1 is responsible for 70-80% and FB_2 for 15-25% of food contamination cases.[3] Therefore, ingestion of food contaminated by fumonisins is a risk factor for humans and animals.[2]

The control of this toxigenic fungus and its mycotoxins is generally based on the use of synthetic fungicides. However, the indiscriminate and excessive use of fungicides in crops has been a major cause of the development of resistant pathogens. In addition, this results in consequent damage to the health of humans, animals and the environment, mainly due to the presence of toxic residues in food.[4]

In recent years, several studies have reported that essential oils (EOs) extracted from medicinal and aromatic plants contain bioactive compounds that control the growth of toxigenic fungi and mycotoxin synthesis, and they can be an alternative strategy to the use of synthetic fungicides.[3,5–7] EOs are reported as ecofriendly, renewable and easily biodegradable.[8] Moreover, many EOs belong to the GRAS category (Generally Recognized As Safe), with antioxidant, antimicrobial, antifungal, antimycotoxigenic and antitumoral properties.[3,9–12]

Litsea cubeba is an important medicinal plant, which is distributed in China, Japan and Southeast Asian countries.[13] Traditionally, this plant is used to cure headache, intoxication and inflammation.[1] Its essential oil (EO) is obtained from the fruits or leaves, and has a citrus lemon-like odor.[14] Significant results have been reported for biological activities of *L. cubeba* EO in previous studies. Li et al.[1] described this oil as a natural fumigant against *Aspergillus flavus*. Wang et al.[7] reported its use against *Penicillium viridicatum*, *A. carbonarius* and *A. flavus*. Moreover, this oil exhibited cytotoxic activity against human lung, liver and mouth cancer cells.[14]

As far as we know, antifungal and antimycotoxigenic activities of *L. cubeba* EO against *F. verticillioides* and the cytotoxicity of this oil against human colorectal and cervical cancer cells have not been reported yet. Therefore, the aim of this research was to investigate *L. cubeba* EO *in vitro*, describing its chemical profile, and assessing its potential antioxidant, cytotoxicity, antifungal and antimycotoxigenic activities against *F. verticillioides*.

2. MATERIAL AND METHODS

2.1. Chemicals

Hexane, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Ultrapure water was supplied by a Milli-Q ultrapure water purification system (Millipore, Burlington, MA, USA). Ethanol was provided by Synth (Diadema, Brazil), and formic acid by Panreac (Barcelona, Spain).

Standard mixture of n-alkanes C_8-C_{20}, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), L-glutamine, phosphate-buffered saline (PBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), Tween-80, Roswell Park Memorial Institute medium (RPMI-1640), morpholine propane sulfonic acid (MOPS), Calcofluor White M2R, glutaraldehyde, cacodylate buffer, poly-L-lysine and analytical standards of FB_1 and FB_2 were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Invitrogen (New York, NY, USA). Potato dextrose agar medium (PDA) was purchased from Himedia (Mumbai, India). All reagents were of analytical and HPLC grades.

2.2. Essential oil

L. cubeba EO (By Samia®, Cotia, Brazil) was obtained from the local market in Maringá, Paraná, Brazil, stored at 4ºC and protected from light until the analysis. According to the manufacturer, the EO was extracted by distillation of *L. cubeba* fruits.

2.2.1. Chemical profile of Litsea cubeba essential oil

The chemical profile of *L. cubeba* EO was performed using gas chromatography-mass spectrometry (GC-MS), model FOCUS GC-DSQ II (Thermo Fisher Scientific, Waltham, MA, USA). The GC-MS was equipped with a capillary column (DB-5, Agilent Technologies, Santa Clara, CA, USA, 5% phenyl/95% dimethylsiloxane, 30 m x 0.25 mm x 0.1 µm). Characterization was

performed using a column temperature program that started at 60ºC, followed by a temperature increase of 3ºC min^{-1} to 230ºC. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The injector and detector temperatures were 240ºC, and the total analysis time was 50 min. The aliquot of 2 µL EO was diluted in 1000 µL of hexane, and 1 µL of the sample was injected with a split ratio of 1:10.[11]

Chemical constituents were identified based on retention time and the Kovats index, obtained with a standard mixture of n-alkanes C_8-C_{20}.[15] The compounds were confirmed through mass spectra and expressed as percentages.[16]

2.3. Antioxidant activity of *Litsea cubeba* essential oil

2.3.1. Extract preparation

L. cubeba EO was diluted in methanol, at a concentration of 1 mg mL^{-1}, protected from light.

2.3.2. DPPH method

First, 25 µL of the extract were added to 2 mL of a DPPH• methanolic solution ($6.25x10^{-5}$ mol L^{-1}). After 30 min of incubation in the dark, reading was performed in a spectrophotometer at 517 nm (Genesys 10 UV-Vis, Thermo Spectronic, Virginia, USA) and a standard curve with a Trolox solution was constructed. The results were expressed as millimols of Trolox equivalents per milligram of sample (mmol Trolox mg^{-1}).[10]

2.3.3. ABTS method

30 µL of the extract were added to 3 mL of a diluted $ABTS^{\bullet+}$ solution, and absorbance readings at 734 nm were taken at exactly 6 min after initial mixing. A standard curve with a Trolox solution was constructed and the results were expressed as mmol Trolox mg^{-1}.[10]

2.4. Cytotoxicity of *Litsea cubeba* essential oil

Antitumor activity was performed using the MTT assay, which measured the cytotoxic effects of *L. cubeba* EO in cell lines. Human colorectal

adenocarcinoma (HT-29) and cervical (HeLa) cancer cell lines were used in this study, as well as African green monkey kidney epithelial cells (Vero), which were used as a comparative control. All cell lines were cultured ($2.5x10^5$ cells mL^{-1}) in DMEM medium supplemented with 2 mM L-glutamine and 10% FBS. They were dispensed into a sterile 96-well plate and incubated for 24 h at 37ºC, in a humid atmosphere oven with 5% CO_2 tension.[17]

After incubation, the supernatant was withdrawn and increasing concentrations of *L. cubeba* EO were added (0 to 1000 µg mL^{-1}). After 48 h of incubation under the same culture conditions, the cells were washed with 100 µL of 0.01 M PBS, and 50 µL of MTT at a concentration of 2 mg mL^{-1} were added, which was followed by incubation for 4 h at 37ºC. Formazan crystals were dissolved in DMSO, and absorbance was read at 570 nm in a microplate reader (BioTek Power Wave XS spectrophotometer). Cytotoxic activity was expressed as the concentration of the sample that inhibited 50% of cell growth compared to the control (IC_{50}).[17]

2.5. Antifungal activity of *Litsea cubeba* essential oil

2.5.1. Microorganism

The *F. verticillioides* strain 103-F was obtained from the collection of the Toxicology Laboratory, State University of Maringá, Brazil. This strain was isolated in 1991 from corn residue used in animal feed by Dr. Elisa Yoko Hirooka, from the Department of Food Science and Technology, State University of Londrina. That occurred because this isolate was implicated in poisoning (horses). Thus, this strain was identified at the Science University of Tokyo by Dr. Y. Sugiura, a mycologist, and Dr. Ichinoe, a plant pathologist with experience in the area of *Fusarium* sp, proving the toxigenocity of the isolate.[3] The strain was cultured in PDA medium at 25ºC for 15 days, exposed to blac klight (26W, 3U, 127V) in a biological oxygen demand (BOD) incubator (Ethik Technology, Vargem Grande Paulista, Brazil).

2.5.2. Diffusion disc method

To evaluate the effect of *L. cubeba* EO against *F. verticillioides* hyphal growth, we used the disc diffusion method.[11] For that, plates with PDA were

centrally inoculated with 10 µL of conidia suspension (10^5 conidia mL^{-1}), and incubated at 25ºC for 5 days, exposed to black light in a BOD incubator. Sterile discs (5 mm diameter) were prepared with 10 µL of *L. cubeba* EO at concentrations of 25, 50 and 100% in 1% Tween-80, and control with 1% Tween-80. Therefore, the discs were arranged around the colony on the plate (0.5 cm distance). After 72 h of incubation under the same culture conditions, the inhibition of hyphal growth was visually evaluated and also photographed.

2.5.3. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC was determined using the 96-well microplate microdilution technique, according to the Clinical and Laboratory Standards Institute (CLSI) with a conidia suspension of 10^5 conidia mL^{-1} using a RPMI-1640 medium and L-glutamine without bicarbonate, buffered with 0.165 M of MOPS. *L. cubeba* EO was diluted in 1% Tween-80 and tested at concentrations of 0 to 1000 µg mL^{-1}. The microplate was incubated at 25ºC for 72 h, exposed to black light in a BOD incubator. The MIC was defined as the lowest concentration of the EO that inhibited the visual growth of *F. verticillioides*.[18] As fungal control (FC), we used a medium containing only the conidia suspension (10^5 conidia mL^{-1}). The mixture of fungicides fludioxonil + metalaxyl-M, the most used fungicides for corn crops in Brazil, was used as the positive control (PC).[19] The experiments were performed in triplicate.

To determine the MFC, 10 µL from each well were inoculated to PDA plates, and incubated at 25ºC for 24 h, exposed to black light in a BOD incubator. The MFC was considered the lowest concentration that inhibited fungal growth.[11]

For Hafidh et al. [20], the nature of the antimicrobial effect produced by a substance against a specific microorganism can be classified according to the ratio between its MFC and MIC, where a MFC/MIC ratio between 1:1 and 2:1 indicates a fungicidal chemical, while a MFC/MIC ratio greater than 2:1 suggests fungistatic activity.

2.5.4. Fluorescence microscopy

The MIC and sub-MIC concentrations of *L. cubeba* EO were prepared in 24-well microplates containing cover slips and 500 µL of RPMI-1640 medium. The wells were inoculated with 100 µL of conidia suspension (10^5 conidia mL^{-1}) and the microplate was incubated at 25ºC for 72 h, exposed to black light in a BOD incubator. The cover slips with adhered conidia and hyphae were carefully removed from the microplate, stained with Calcofluor White and mounted on a slide. The slides were observed in an Olympus fluorescent microscope.[21]

2.5.5. Scanning electron microscopy

The inoculum of *F. verticillioides* (10^5 conidia mL^{-1}) was treated with *L. cubeba* EO at the MIC and sub-MIC concentrations and the FC was performed using a medium that contained only the inoculum (10^5 conidia mL^{-1}). After 72 h of incubation at 25ºC, exposed to black light in a BOD incubator, the samples were washed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Subsequently, the samples were fixed with poly-L-lysine, dehydrated in graded ethanol (30-100%), critical-point-dried in CO_2 (CPD-030, Bal-Tec), coated with gold (SCD-050, Bal-Tec) and examined in a scanning electron microscope (Quanta-250, FEI Company, Hillsboro, OR, USA).[21]

2.6. Effect of *Litsea cubeba* essential oil on the production of fumonisins B_1 and B_2 by *Fusarium verticillioides*

2.6.1. Culture conditions

L. cubeba EO and PC (fludioxonil + metalaxyl-M) were dissolved in 1% Tween-80 at the MIC and sub-MIC concentrations, and added to the PDA medium at a temperature of 40-45ºC, then poured into Petri dishes (90 mm). 10 µL of the conidia suspension (10^5 conidia mL^{-1}) were inoculated as soon as the medium was solidified. The FC was prepared similarly by inoculating only conidia suspension (10^5 conidia mL^{-1}). The Petri dishes were placed in a BOD incubator under controlled temperature conditions of 25ºC for 15 days, exposed to black light.

241 *2.6.2. Mycelial development*

242 At the end of the incubation period, fungal growth was determined from
243 the mycelial diameter measured with a ruler.[3,5] The percentage of mycelial
244 growth inhibition (MGI) was calculated according to Equation 1.

245 $$MGI(\%) = \left[\frac{d_c - d_t}{d_c}\right] \times 100 \quad (1)$$

246 Where, d_c (cm) is the mean colony diameter for the control (FC) and d_t
247 (cm) is the mean colony diameter for each group treated with *L. cubeba* EO and
248 the PC (fludioxonil + metalaxyl-M).

249

250 *2.6.3. Extraction of fumonisins B_1 and B_2*

251 After incubation, the contents of the Petri dishes for each treatment were
252 extracted with 20 mL of acetonitrile/water/formic acid (84:16:1, v/v). Extraction
253 was performed by using an orbital shaker (Ethik Technology, Vargem Grande
254 Paulista, Brazil) at 240 rpm for 60 min, followed by ultrasonic bath at 25 kHz for
255 60 min. Then, the mixture was centrifuged (Universal 320R Hettich,Tuttlingen,
256 Germany) at 2040 *g* for 10 min, and the supernatant was filtered through 0.45
257 µm PTFE syringe filters. Extractions were performed in triplicate.

258

259 *2.6.4. Chromatographic determination of fumonisins B_1 and B_2*

260 Chromatographic determination was based on the method described by
261 Avanço et al.[5] 1 µL aliquots of the filtrate from each treatment were injected
262 into an ultra-high performance liquid chromatography system (UHPLC Nexera
263 X2, Shimadzu, Kyoto, Japan). Analytes separation was performed using a 4.6
264 mm x 3.6 µm x 75 mm, Symmetry®-C18 column (Waters, Wexford, Ireland) at a
265 temperature of 40ºC. The gradient elution system used water as mobile phase
266 A and acetonitrile as mobile phase B, both containing 0.1% formic acid. The
267 following gradient was used: 0 min, A/B (95:5); 10 min (50:50); 12 min (5:95);
268 and 17 min (95:5) at a flow rate of 0.8 mL min^{-1}. The analysis time was 20 min,
269 with retention time of 11.5 min for FB_1 and 12.8 min for FB_2.

270 Detection was carried out by a mass spectrometer (MS Q-tof Impact II,
271 Bruker, Germany), with an electrospray ion source operating in positive-ion
272 mode. The following parameters were optimal: capillary voltage set at 4.50 kV,
273 source temperature of 200ºC and desolvation gas flow rate at 8 L min^{-1}. The

three most intense ions of each chromatographic peak were selected for fragmentation. The mass spectra was obtained at the m/z 70-1200 range, with an acquisition rate of 5 Hz (MS and MS/MS), using collision-induced dissociation from a collision energy ramp at the 15-40 eV range.

Identification of FB_1 and FB_2 was performed based on molar mass and retention time, and quantification was carried out by external standardization, with analytical curves (0.25 to 6 μg mL^{-1}).

2.7. Statistical analysis

The data of antioxidant activity, mycelial development and antimycotoxigenic activity were submitted to the one-way analysis of variance (ANOVA), followed by the Tukey test ($p<0.01$), using the statistical program BioEstat 5.3 (Mariraua Institute). The graphic was created by using the SigmaPlot 11.0 Software (Systat Software Inc).

3. RESULTS AND DISCUSSION

3.1. Chemical profile of *Litsea cubeba* essential oil

In total, 12 chemical compounds were identified for *L. cubeba* EO. Retention times, Kovats indexes and relative percentages (%) are listed in Table 1. The dominant compounds were neral and geranial, 32.75% and 37.67%, respectively. Both accounted for 70.42% of the total EO. Note that neral and geranial are isomers of citral.

Several factors influence the chemical composition of EOs, such as plant genetics, geographical region of cultivation, climatic conditions, light, storage period and extraction method.[1,11] That being so, the analysis of the chemical profile of EOs is important to elucidate bioactive properties.

Recent studies have reported variation in the chemical profile of *L. cubeba* EO. Yang et al.[13] described geranial, neral and limonene as major components, with 27.49%, 23.57% and 18.82%, respectively. In addition, Wang et al.[7] reported that geranial and neral amounted to 72.18% of the total identified. These results corroborate our findings. On the other hand, Li et al. [1] found (Z)-limonene oxide, (E)-limonene oxide and (D)-limonene, with 30.14%, 27.92% and 11.86%, respectively.

307 **Table 1.** Chemical profile of *Litsea cubeba* essential oil, as identified by GC-MS.

Compounds	*Litsea cubeba*			
	RT	KI_c	KI_t	%
Methyl heptenone	8.01	985	986	1.65
β-myrcene	8.20	990	990	0.53
Limonene	9.69	1028	1029	10.55
Eucalyptol	9.80	1030	1031	1.78
Linalool	12.49	1099	1097	1.41
Citronellal	14.72	1151	1153	1.68
Verbenol	15.92	1178	1177	0.98
α-terpineol	16.58	1193	1189	1.10
Neral	18.58	1238	1238	32.75
Geraniol	19.03	1248	1253	1.07
Geranial	19.92	1268	1267	37.67
β-caryophyllene	26.21	1414	1419	1.96
Total of identified compounds	93.13 %			

308 RT: Retention time (min). KI_c: Calculated Kovats index. KI_t: Theoretical Kovats index.
309 KI_t on a DB-5 column with reference to n-alkanes.[16]
310

311 ### 3.2. Antioxidant activity of *Litsea cubeba* essential oil

312 The antioxidant activity for *L. cubeba* EO showed different concentrations
313 ($p<0.01$) between the tested methods, DPPH• and ABTS, corresponding to
314 104.4 and 56.4 mmol Trolox mg^{-1}, respectively. Dutra et al.[10] suggested a good
315 antioxidant activity for *Origanum vulgare* EO by DPPH• and ABTS methods
316 (1.142 and 0.363 mmol Trolox mg^{-1}, respectively). These results are lower than
317 that obtained for *L. cubeba* EO, in both analysis methods.

318 It is important to mention that the antioxidant potential of an EO depends
319 on its chemical profile. Furthermore, antioxidant activity may be related with a
320 major proportion of components present in the EOs.[9] Thus, the antioxidant
321 activity obtained for *L. cubeba* EO is related to compounds, such as isomers of
322 citral.

323 Some recent studies have shown antioxidant, antifungal and
324 antimicrobial activities of EOs, which makes it possible to establish a
325 correlation.[5,9,10] Prakash et al.[22] found the relationship between the antifungal
326 and antioxidant activities of *Curcuma longa* and *Zingiber officinale* EOs, which

also inhibited the secretion of aflatoxin from *A. flavus*. Moreover, the antioxidant activity of EOs can also protect agri-commodities from oxidative deterioration.[22]

3.3. Cytotoxicity of *Litsea cubeba* essential oil

L. cubeba EO was tested against tumor (HT-29 and HeLa) and non-tumor (Vero) cell lines to evaluate its cytotoxic effect. As shown in Table 2, the EO presented antitumor activity with a selective effect against tumor cells, since the IC_{50} obtained for these cells were lower than that of the non-tumor cell.

Table 2. Antitumor activity and cytotoxic of *Litsea cubeba* essential oil against cell lines.

Cell lines	IC_{50} (μg mL^{-1})
HT-29	42.3 ± 12.0
HeLa	67.7 ± 20.5
Vero	72.0 ± 9.2

The values are the mean ± standard deviation for triplicates.

IC_{50} = Inhibitory Concentration for 50% of the cell.

Ho et al.[14] evidenced antitumor properties of *L. cubeba* EO against human cancer cell lines, such as lung, liver and mouth cancer. For Elshafie and Camele[23], the general mechanism of the cytotoxic effect of EOs correlates to the presence of phenols, alcohols and monoterpene aldehydes. Chaouki et al.[24] attributed cytotoxic effect on tumor cells to citral and limonene, the same compounds found in *L. cubeba* EO.

Moreover, other researchers have demonstrated that EOs are associated with significant antitumor activity. Döll-Boscardin et al.[25] reported the cytotoxic potential of *Eucalyptus benthami* EO on Jurkat, J774A.1 and HeLa cancer cells lines. Kathirvel and Ravi[26] described an IC_{50} of 90.5 and 96.3 μg mL^{-1} for *Ocimum basilicum* EO against human cervical cancer cell lines (HeLa) and human laryngeal epithelial carcinoma cell lines (HEp-2), respectively. Saab et al.[12] showed that *Laurus nobilis* EO (from the same family as *L. cubeba*, Lauraceae) inhibited the growth of K562 human chronic myelogenous leukemia cells with IC_{50} of 95 μg mL^{-1} to leaf oil and 75 μg mL^{-1} to seed oil. These results are in line with those obtained in our study.

According to the World Health Organization[27], cancer is the second leading cause of death worldwide. Cervical and colorectal cancers are among

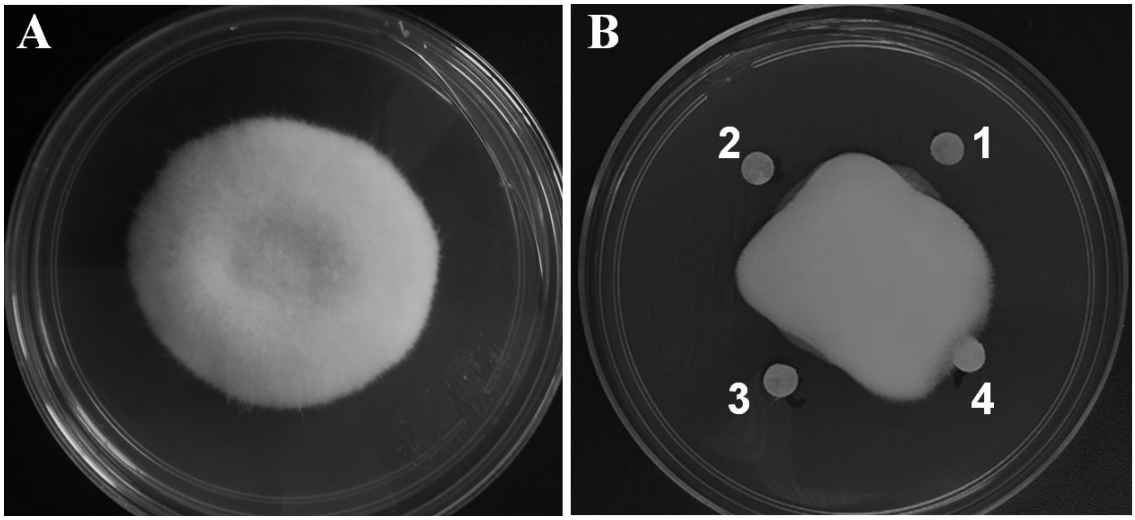
the most common types of cancer. Thus, considering the potential therapeutic applications of *L. cubeba* EO demonstrated in this study, additional *in vitro* and *in vivo* studies are necessary. The use of strategies such as inclusion complexes, encapsulation or the development of emulsions in micro or nanometric scale could be an interesting strategy to provide better stability to the chemical constituents and, consequently, improve selectivity against tumor cells.

3.4. Antifungal activity of *Litsea cubeba* essential oil

3.4.1. Diffusion disc method

Endo et al.[21] described that disc diffusion is a simple method that allows to determine the activity of different substances against microorganisms. Thus, this method was used to visually evaluate the inhibitory capacity of *L. cubeba* EO against *F. verticillioides*. Figure 1 shows inhibition of hyphal growth by *L. cubeba* EO. A refers to *F. verticillioides* control, and B to *F. verticillioides* treated with *L. cubeba* EO. Disc numbers 1, 2, 3 and 4 represent the EO at the concentrations of 100, 50 and 25%, and Tween 80 1%, respectively.

Figure 1. Diffusion disc method for *Litsea cubeba* essential oil in *Fusarium verticillioides*.

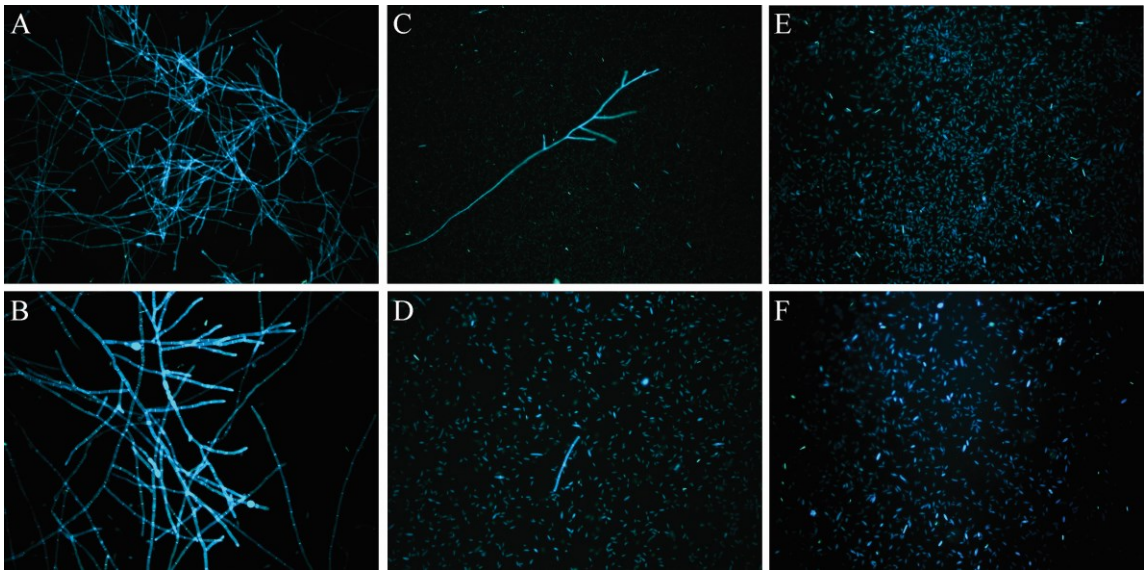


(A) Fusarium verticillioides control. (B) Fusarium verticillioides treated with Litsea cubeba essential oil. Discs numbered 1 (oil pure 100%), 2 (oil 50%), 3 (oil 25%) e 4 (Tween 80 1%).

L. cubeba EO inhibited the growth of the *F. verticillioides* hyphae at all concentrations tested (25, 50 and 100%). Moreover, regarding disc number 4,

383 in which only Tween 80 1% was cultured, there was no inhibition of hyphae
384 growth, as expected.
385
386 *3.4.2. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal*
387 *Concentration (MFC)*
388 Minimum Fungicidal and Minimum Inhibitory concentrations for *L. cubeba*
389 EO were 125 µg mL^{-1}. PC (fludioxonil + metalaxyl-M) also presented the same
390 result of 125 µg mL^{-1}. The MFC/MIC ratio for *F. verticillioides* was 1:1[20],
391 evidencing fungicide action for *L. cubeba* EO, at the same concentration as the
392 synthetic fungicide (fludioxonil + metalaxyl-M). Furthermore, the findings
393 obtained for this oil were in line with the disc diffusion (Fig. 1).
394 Efficacy of EOs against food contaminants has been reported in several
395 studies.[1,6,10] It has been described that the main components, such as citral of
396 *L. cubeba* EO, exhibited strong fungicidal activity against *A. flavus*, *P.*
397 *viridicatum* and *A. carbonarius*.[7] However, as far as we know, no study has
398 tested the activity of *L. cubeba* EO against *F. verticillioides*.
399
400 *3.4.3. Fluorescence microscopy*
401 In Figure 2, microscopy images show hyphal growth inhibition of *F.*
402 *verticillioides*. The untreated cells (Fig. 2A and 2B) presented abundant and
403 preserved hyphae with intense fluorescence. A strong reduction in hyphal
404 growth occurred in *F. verticillioides* treated with the *L. cubeba* EO at sub-MIC
405 concentration of 62.5 µg mL^{-1} (Fig. 2C and 2D). Irregular growth, short hyphae
406 and non-germinated microconidia were observed. Moreover, at the MIC
407 concentration of 125 µg mL^{-1} (Fig. 2E and 2F), there was complete inhibition of
408 hyphal growth and conidial germination.
409 Hyphae and non-germinated microconidia (Fig. 2C to 2F) exhibited less
410 fluorescence than control cells (Fig. 2A and 2B). Calcofluor White is a
411 fluorochrome that binds to chitin in cell walls of fungi. Chitin is synthesized by
412 enzymes present in the plasma membrane, thus, changes in the plasma
413 membrane can affect cell walls chitin structure. Consequently, damage to the
414 cell wall is reflected by a lower intensity of fluorescence compared to a normal
415 cell wall.[21]

Figure 2. Fluorescence microscopy of *Fusarium verticillioides* treated with *Litsea cubeba* essential oil.



(A) and (B) *Fusarium verticillioides* control. (C) and (D) *Fusarium verticillioides* treated with 62.5 µg mL^{-1} of *Litsea cubeba* essential oil. (E) and (F) *Fusarium verticillioides* treated with 125 µg mL^{-1} of *Litsea cubeba* essential oil. Magnifications: 10x and 20x.

3.4.4. Scanning electron microscopy

The morphological and structural characteristics of *F. verticillioides* microconidia that were treated with *L. cubeba* EO are shown in Figure 3. Scanning electron microscopy showed that untreated cells of *F. verticillioides* (Fig. 3A) exhibited healthy microconidia structures, with no visible structural changes.

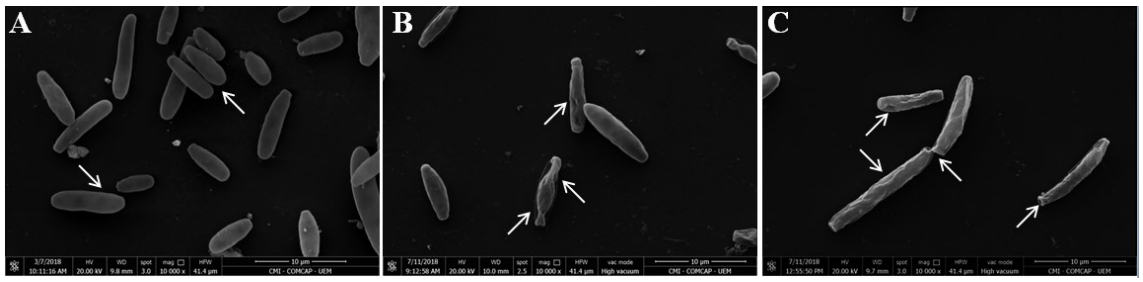
A strong inhibition of microconidia growth and an irregular growth pattern was observed in *F. verticillioides* treated with sub-MIC concentration (Fig. 3B) of *L. cubeba* EO. Also, the appearance of microconidia changed when the MIC concentration (Fig. 3C) of *L. cubeba* EO was used. The microconidia got wrinkled due to the reduction in cytoplasmic and cell extravasation contents.

The antifungal mechanism of action of EOs is not yet completely understood. Terpenes and terpenoids, which are the major components of EOs, due to their lipophilic nature, can modify cell membrane permeability and the functioning of fungal structures.[28]

In previous studies, *Z. officinale* and *Rosmarinus officinalis* EOs effect on *F. verticillioides'* morphology revealed similar damages to our findings.[3,29] Bomfim et al.[3] reported that the antifungal property of *R. officinalis* EO against

F. verticillioides occurred through the loss of membrane integrity and, therefore, the blockage of cell growth.

Figure 3. Scanning electron microscopy of *Fusarium verticillioides* treated with *Litsea cubeba* essential oil.



(A) *Fusarium verticillioides* control. (B) *Fusarium verticillioides* treated with 62.5 μg mL^{-1} of *Litsea cubeba* essential oil. (C) *Fusarium verticillioides* treated with 125 μg mL^{-1} of *Litsea cubeba* essential oil. Magnification: 10.000x.

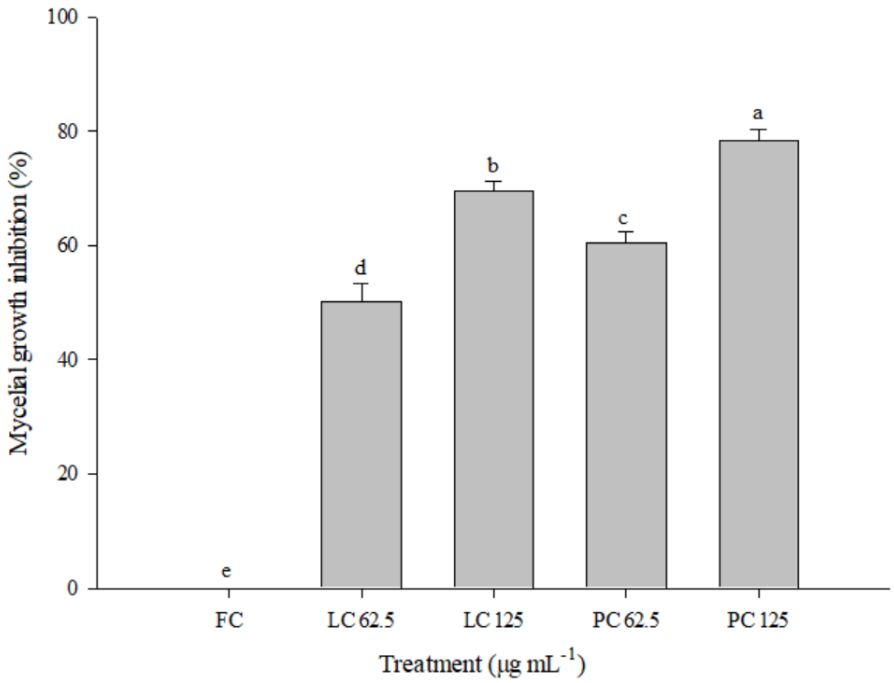
3.5. Effect of *Litsea cubeba* essential oil on production of fumonisins B_1 and B_2 by *Fusarium verticillioides*

3.5.1. Mycelial development

Figure 4 shows the inhibition of the mycelial development of *F. verticillioides* caused by *L. cubeba* EO. According to the percentage of mycelia growth inhibition, *L. cubeba* EO inhibited 50.3% and 69.5% for MIC and sub-MIC concentrations, respectively. Furthermore, the PC showed inhibition of 60.5% for the MIC concentration, and 78.4% for the sub-MIC concentration. These results indicate that all treatments significantly inhibited mycelia growth compared to FC ($p<0.01$) and exhibited high fungicide action.

F. verticillioides growth inhibition has been reported using *R. officinalis* EO at concentrations of 150 μg mL^{-1}, 300 μg mL^{-1} and 600 μg mL^{-1}, with inhibition of 17.0%, 29.7% and 67.0%, respectively.[3] Similarly, Avanço et al.[5] described *F. verticilloides* growth inhibition with the treatment of *C. longa* EO, at concentrations of 73.7 μg mL^{-1} (63.0%), 147.5 μg mL^{-1} (75.0%) and 294.9 μg mL^{-1} (79.3%). In these studies, there is a proportionality between the concentrations of EO and the inhibition of mycelial development. The observations are in agreement with our findings, which depicted an increase in inhibition with increased EO concentrations.

Figure 4. Inhibitory effect of *Litsea cubeba* essential oil on the mycelial growth of *Fusarium verticillioides*.



FC: fungal control (inoculum 10^5 conidia mL^{-1}). LC: *Litsea cubeba* essential oil. PC: positive control (fludioxinil + metaxyl-M).

Different letters between columns refer to significant difference ($p<0.01$) by the Tukey test.

3.5.2. Antimycotoxigenic activity

The antimycotoxigenic effect of *L. cubeba* EO against *F. verticillioides in vitro* is shown in Table 3. The treatments carried out with MIC and sub-MIC concentrations of *L. cubeba* EO significantly inhibited FB_1 and FB_2 production. Our findings indicate that the treatments presented a dose-dependence effect compared with the FC, and showed high antimycotoxigenic activity compared with the PC.

A direct correlation was noticed between the inhibition of mycelial development (Fig. 4) caused by *L. cubeba* EO and the synthesis of FB_1 and FB_2 (Table 3). In the MIC concentration of *L. cubeba* EO, the inhibition of mycelial development was 69.5% and the production of FB_1 and FB_2 was 1.7 and 0.5 µg mL^{-1}, with an inhibition rate of 98.1% and 97.9%, respectively. These results were statistically similar ($p<0.01$) to the MIC concentration of the PC, which demonstrated production of FB_1 and FB_2 equal to 0.4 and 0.2 µg mL^{-1}, with inhibition of 99.5% and 99.4%, respectively. In contrast, the FC showed levels of 87.6 µg mL^{-1} for FB_1 and 25.5 µg mL^{-1} for FB_2.

Table 3. Effect of *Litsea cubeba* essential oil on fumonisins B_1 and B_2 production by *Fusarium verticillioides*, as analyzed by UHPLC-MS.

Treatment (μg mL^{-1})	Fumonisin B_1		Fumonisin B_2	
	Concentration (μg mL^{-1})	Inhibition (%)	Concentration (μg mL^{-1})	Inhibition (%)
FC	87.6^{a} ± 1.2	0.0	25.5^{a} ± 0.6	0.0
LC 62.5	14.0^{b} ± 0.4	84.0	5.0^{b} ± 0.4	80.4
LC 125	1.7^{d} ± 0.3	98.1	0.5^{d} ± 0.1	97.9
PC 62.5	4.8^{c} ± 0.3	94.5	1.9^{c} ± 0.1	92.4
PC 125	0.4^{d} ± 0.4	99.5	0.2^{d} ± 0.1	99.4

The values are the mean ± standard deviation for triplicates.

FC: fungal control (inoculum 10^5 conidia mL^{-1}). LC: *Litsea cubeba* essential oil. PC: positive control (fludioxinil + metaxyl-M).

Different letters between columns refer to significant difference ($p<0.01$) by the Tukey test.

In recent years, many EOs have been reported as potent antifungals, effective in inhibiting mycotoxin synthesis.[5–7,30] Moreover, EOs are described as a safe, ecofriendly, renewable and easily biodegradable option for protection of food products from fungal contamination and subsequent mycotoxin production.[8,31] Therefore, *L. cubeba* EO has the potential to be used as a natural fungicide in the control of *F. verticillioides* and the synthesis of FB_1 and FB_2.

4. CONCLUSION

This study shows that *L. cubeba* EO has strong fungicide action against mycelial development, FB_1 and FB_2 synthesis and morphological and structural alterations for *F. verticillioides in vitro*. In addition, this oil demonstrated antioxidant activity and cytotoxic potential. These findings suggest that *L. cubeba* EO has the potential to be used as an alternative biofungicide, and it may replace synthetic ones. Moreover, the antioxidant activity of *L. cubeba* EO can also prevent oxidation related to agri-commodities spoilage. Given the potential therapeutic applications of *L. cubeba* EO, additional *in vitro* and *in vivo* studies are necessary. Also, *in situ* studies, using crops as their study model, still need to be carried out.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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ARTIGO 2

Industrial Crops and Products

Inclusion complexes of *Litsea cubeba* essential oil into β-cyclodextrin: Preparation, physicochemical characterization, cytotoxicity and antifungal activity

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ABSTRACT: The applications of essential oils (EOs) are limited due to its unstable to light, oxygen and temperature, so the improvement in stability becomes necessary. The aim of this study was to prepare inclusion complexes of *Litsea cubeba* essential (LCEO) with β-cyclodextrin (β-CD) using physical mixture (PM), kneading (KN) and co-precipitation (CP) methods. Moreover, the complexation efficiency and the physicochemical properties of the inclusion complexes using ATR-FTIR, FT-Raman, DSC and TG were evaluated. As well as cytotoxicity against human colorectal and cervical cancer cells and antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides*. The complexation efficiency results presented significant evidence of LCEO:β-CD inclusion complex formation, being KN (83%) and CP (73%) the best methods used in this study. All tested LCEO:β-CD inclusion complexes exhibited toxicity to HT-29 cells. Although, cytotoxic effect was less pronounced in HeLa tumor cell, LCEO-KN was more active against Hela than non-tumor cell. LCEO-KN and LCEO-CP inclusion complexes were efficient for both toxigenic fungi. Therefore, the molecular inclusion of LCEO into β-CD was successful, as well as the preliminary biological results, evidencing that β-CD inclusion process may be a viable alternative to facilitate and increase future applications of this EO as therapeutic medication and natural antifungal.

KEYWORDS: Molecular inclusion, *Litsea cubeba* essential oil, β-cyclodextrin, Antifungal activity, Cytotoxic effect.

1. INTRODUCTION

Essential oils (EOs) are a natural mixture of volatile chemical compounds. They are synthesized by medicinal and aromatic plants as

secondary metabolites. The EOs are known worldwide for their potent biological properties, which are attributed to the chemical constituents, mainly terpenoids and phenolic compounds. Furthermore, the EOs are described as natural, eco-friendly, renewable, safe and easily biodegradable compounds (Falleh et al., 2020).

Litsea cubeba (Lauraceae) is an important medicinal plant, which is distributed in China, Japan and Southeast Asian countries, usually known as May Chang (Kamle et al., 2019). The *L. cubeba* essential oil (LCEO) is extracted from their fresh fruits and has a citrus aroma lemon-like (Yang et al., 2018). The main chemical compounds of LCEO are isomers of citral, neral and geranial (Yang et al., 2018; Kamle et al., 2019). In fact, several researchers have reported the bioactivities of LCEO, including antioxidant, antimicrobial, antifungal, anti-inflammatory, insecticidal and anticancer properties (Ho et al., 2010; Wang and Liu, 2010; Liao et al., 2015; Wang et al., 2016; Zhang et al., 2017; Wang et al., 2018).

The applications of EOs are limited due to its unstable to light, oxygen and temperature (Yang et al., 2018). Thus, an improvement in the stability of EOs is necessary, in order to expand the application fields in food, cosmetic and medicine industries. In this context, alternative strategies have been developed, such as inclusion of EOs into macromolecules. Among them, cyclodextrins are cyclic oligosaccharides with a truncated-cone shape containing glucopyranose units. The most used is β-cyclodextrin (β-CD), which contain 7 glucose units, hydrophobic cavity and hydrophilic external surface (Costa et al., 2015).

Recently, many studies on the complexation of β-CD with EOs have been reported for different purposes. Galvão et al. (2015) reported inclusion complexes of *Citrus sinensis* EO in β-CD on *Aedes aegypti* larvae. Anaya-Castro et al. (2017) related the antimicrobial activity of β-CD inclusion complexes containing *Eugenia caryophyllata* and *Lippia berlandieri* EOs. The *E. brejoensis* EO inclusion complex with β-CD exhibited cytotoxic activity against Hela and J774 cells (Santana et al., 2020).

Furthermore, the biological properties of inclusion complexes between LCEO and β-CD have been studied, such as antibacterial activity against

Staphylococcus aureus (Cui et al., 2019) and antifungal activity on *Penicillium italicum*, *Penicillium digitatum* and *Geotrichum citri-aurantii* (Wang et al., 2020). However, to the best of our knowledge, there are still no studies the effect of LCEO:β-CD inclusion complex on the cytotoxicity against human colorectal and cervical cancer cells, as well as on the antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides*.

Therefore, the aim of this research was to prepare inclusion complexes of LCEO in β-CD by physical mixture, kneading and co-precipitation methods. Moreover, the inclusion complexes were characterized in terms of physicochemical properties, as well as cytotoxic effect and antifungal activity.

2. MATERIAL AND METHODS

2.1. Chemicals

The LCEO (By Samia®, Cotia, Brazil) was acquired in a market located in Maringa City, Parana State, Brazil, in a single lot (number: 217). Ethanol was purchesed from Synth® (Diadema, Brazil) and Potato Dextrose Agar medium (PDA) from Himedia® (Mumbai, India). β-CD, Tween-80, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), L-glutamine, phosphate-buffered saline (PBS) and dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Invitrogen® (New York, NY, USA). All reagents were of analytical grade.

2.2. Preparation of inclusion complexes

The inclusion complexes of LCEO and β-CD were prepared in the molar ratio of 1:1, based on the molecular weight of the citral (152.24 g/mol), using physical mixture, kneading and co-precipitation methods.

The citral chemical compound was the major constituent of LCEO previously determined by gas chromatography coupled with mass spectrometry (GC-MS), accounting 70.42% (32.75% of neral and 37.67% of geranial), followed by limonene with 10.55% of the total EO.

2.2.1. Physical mixture

A physical mixture (PM) was obtained by addition of LCEO to a glass mortar containing β-CD under manual agitation and stored in amber glass containers until the moment of analysis (Galvão et al., 2015).

2.2.2. Kneading

For the kneading (KN), the β-CD and the LCEO were homogenized in a glass mortar. Then, a mixture of distilled water:ethanol (1:1) was added progressively, until the formation of a paste. The resulting material was dried in a desiccator, which was removed by manual trituration and stocked in amber glass containers for further measurements (Galvão et al., 2015).

2.2.3. Co-precipitation

In the co-precipitation (CP) method, the β-CD was solubilized in 40 mL of distilled water in a water-bath at 60 ºC. The solution was cooled to 25 ºC and the LCEO dissolved in ethanol was slowly added. Then, the sample was stirred at 140 rpm and 25 ºC for 60 min and submitted to vacuum filtration. The resulting material was dried in a desiccator and stored in amber glass containers for further analysis (Galvão et al., 2015).

2.3. Complexation efficiency

To determine the LCEO content in the inclusion complexes, 1 mg of EO and complexes were diluted in 2 mL of ethanol and then filtered through 0.45 µm PTFE filters. The absorbances of the samples were determined by UV spectrophotometer (Shimadzu UV 1601 PC, Columbia, SC, USA) at a wavelength of 216 nm. For each measurement, ethanol was used as a reference blank (Mangolim et al., 2014). The complexation efficiency (CE%) was calculated as:

$$CE\% = \left[\frac{L_c}{L_t}\right] * 100 \quad (1)$$

where L_c is the mass of complexed EO and L_t is the total mass of EO added initially.

2.4. Physicochemical properties of inclusion complexes

2.4.1. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of LCEO, β-CD and inclusion complexes (PM, KN and CP) were obtained on a Vertex 70v Spectrometer (Brucker, Germany) with device for attenuated reflectance (Platinum ATR, Bruker, Germany). Spectra were recorded without any sample preparation. The spectral range was 400-4000 cm^{-1} with 128 scans at 4 cm^{-1} resolution (Răileanu et al., 2013; Mangolim et al., 2014).

2.4.2. Fourier transform Raman spectroscopy (FT-Raman)

The FT-Raman spectra of LCEO, β-CD and inclusion complexes (PM, KN and CP) were measured using an infrared Fourier transform Spectrometer (model Vertex 70v with Ram II module, Bruker, Germany) equipped with a liquid nitrogen cooled Germanium detector. Spectra were recorded at wavelength between 400 and 4000 cm^{-1} without any sample preparation. A Nd:YAG laser was used for excitation at 1064 nm with 70 mV. All of the spectra were an average of 128 scans with a 4 cm^{-1} resolution (Mangolim et al., 2014).

2.4.3. Thermal analysis

The differential scanning calorimetry (DSC) and thermogravimetry (TG) analyses were performed on a thermal analyzer (model STA 409 PG LUXX, Netzsch, Selb, Germany) by involving samples of β-CD and inclusion complexes (PM, KN and CP) under a N_2 volumetric flow of 30 mL/min at atmospheric pressure. The temperature varied between 0 ºC and 400 ºC at a heating rate of 10 ºC/min (Valarini Junior et al., 2017).

2.5. Cytotoxicity of inclusion complexes

2.5.1. Cell cultures

We used 3 cell lines: HT-29 as human colorectal adenocarcinoma, HeLa as cervical cancer and Vero as normal cell line (control). The cell cultures were performed (2.5×10^5 cells/mL) using DMEM supplemented with 10% FBS and

2 mM L-glutamine. All cell lines were dispensed into a sterile 96-well plate and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2.

2.5.2. MTT assay

The cytotoxicity was performed using the MTT assay according to Mosmann (1983). After the cell culture period, the supernatant was withdrawn and increasing concentrations of β-CD and inclusion complexes (PM, KN and CP) were added (0 to 1000 µg/mL). After 48 h of incubation under the same culture conditions, the cells were washed with 100 µL of 0.01 M PBS, and 50 µL of MTT at concentration of 2 mg/mL was added, followed by incubation for 4 h at 37 ºC. Formazan crystals were solubilized in DMSO, and the absorbance was evaluated in a spectrophotometer (BioTek Power Wave XS) at 570 nm. The experiment was performed in triplicate and the cytotoxic activity was expressed as the concentration of the sample that inhibited 50% of cell growth compared to the control (IC_{50}).

2.6. Antifungal activity of inclusion complexes

2.6.1. Microorganisms

The strains of *A. flavus* (AF42) and *F. verticillioides* (103F) were obtained from the collection of Toxicology Laboratory, State University of Maringa, Brazil. *A. flavus* was cultivated in PDA medium at 25 ºC for 7 days, without natural or artificial light in a biological oxygen demand (BOD) incubator (Ethik Technology, Brazil). *F. verticillioides* was cultured in PDA medium at 25 ºC for 15 days, exposed to black light (26W, 3U, 127V) in a BOD incubator.

2.6.2. Agar dilution method

The LCEO and inclusion complexes (PM, KN and CP) were dissolved in 1% Tween-80 at the concentration of 1000 µg/mL, and added to the PDA medium at a temperature of 40-45 °C, then poured into Petri dishes (90 mm). The fungi were inoculated as soon as the medium had solidified. Discs mycelial (8 mm) of *A. flavus* and *F. verticillioides*, taken from the edge of 7 and 14-day-old fungal cultures, respectively, were placed at the centre of each Petri dish. The fungal controls were prepared similarly by inoculating only the discs

mycelial. The Petri dishes were placed in BOD incubator under controlled temperature condition of 25 ºC, without light to *A. flavus* and with black light to *F. verticillioides*. The efficacy of treatments was evaluated after 7 days, in triplicate (Moghaddam et al., 2018). The percentage of radial inhibition (RI%) was calculated as:

$$RI\% = \left[\frac{L_c - L_t}{L_c}\right] * 100 \quad (2)$$

where L_c (cm) is the mean of radial growth for the fungal controls and L_t (cm) is the mean of radial growth for each group treated with the LCEO and inclusion complexes.

2.7. Statistical analysis

Data were evaluated using one-way analysis of variance (ANOVA), followed by the Tukey test using BioEstat 5.3 software (Mariraua Institute). The graphics were generated using the softwares SigmaPlot 11.0 (Systat) and Origin 8.0 (Originlab).

3. RESULTS AND DISCUSSION

3.1. Complexation efficiency (CE)

In our study, the CE% showed different percentages ($p<0.01$) for the LCEO:β-CD inclusion complexes. Suggesting that the amount of active substance was entrapped in the inclusion complex (Wang et al., 2020). The LCEO-KN complex had a superior value than the other complexes, corresponding to 83%. The second-best method was LCEO-CP with 73% efficiency, followed by LCEO-PM with 48%. These results indicate good efficiency for the studied inclusion complexes, proving that the LCEO initially added to the process remained in the complexes obtained.

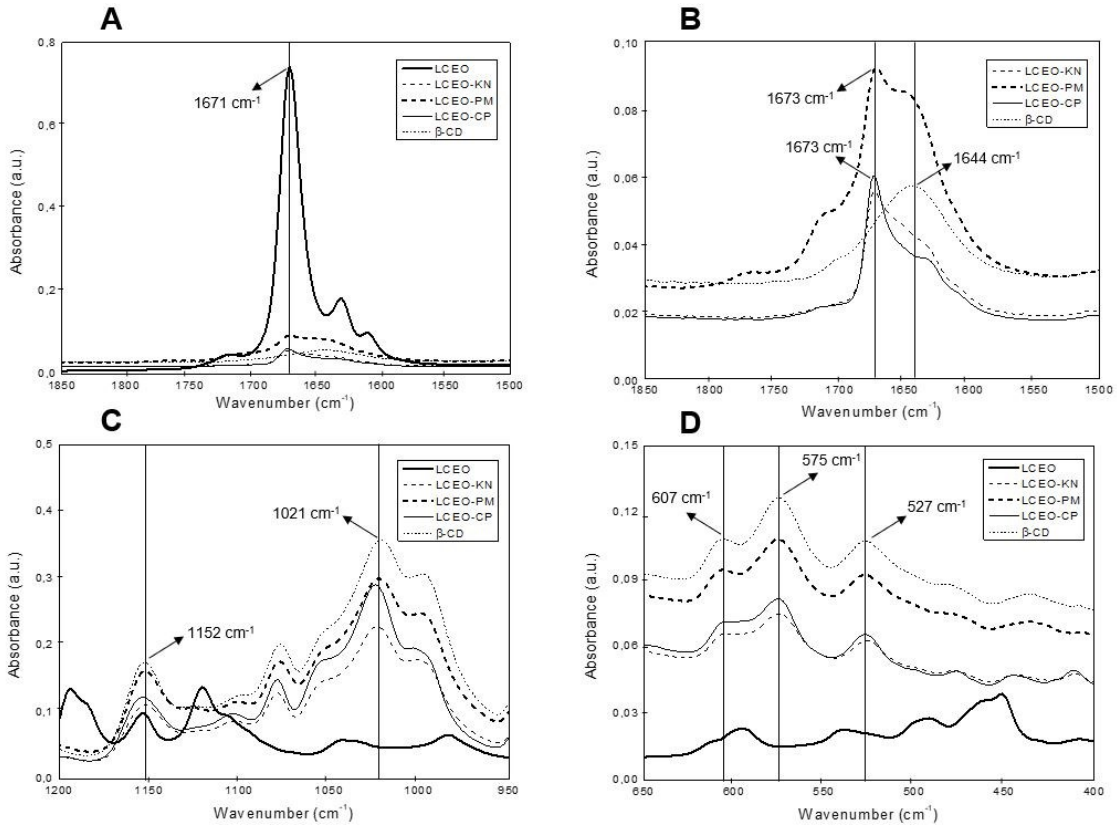
Anaya-Castro et al. (2017) reported CE% for the inclusion complexes of *E. caryophyllata*:β-CD and *L. berlandieri*:β-CD by precipitation method, in the range of 30 to 64% and 57 to 78%, respectively. These results agreed with those obtained in our study. In contrast, Wang et al. (2020) showed lower CE% result for the LCEO:β-CD inclusion complex by saturated aqueous solution method, corresponding to 34%.

3.2. Physicochemical properties of inclusion complexes

3.2.1. ATR-FTIR

The ATR-FTIR and FT-Raman are complementary spectroscopic techniques and were used to characterize and prove the complexation between LCEO and β-CD. The Figure 1 presents the most important regions of ATR-FTIR spectra of β-CD, LCEO and LCEO:β-CD inclusion complexes produced by different methods.

Figure 1. ATR-FTIR spectra of β-cyclodextrin (β-CD), *Litsea cubeba* essential oil (LCEO) and the inclusion complexes of LCEO in β-CD obtained from kneading (KN), co-precipitation (CP) and physical mixture (PM) methods. (A) and (B) Wavenumber from 1850 to 1500 cm^{-1}; (C) Wavenumber from 1200 to 950 cm^{-1}; (D) Wavenumber from 650 to 400 cm^{-1}.



In the ATR-FTIR spectra of β-CD (Fig. 1B-D) several characteristic absorption peaks were observed. The peak at 1644 cm^{-1} (Fig. 1B) represents the deformation mode of crystallization water present in the β-CD cavity (Răileanu et al., 2013). In addition, the absorption peak at 1152 cm^{-1} (Fig. 1C) was related to C-O stretching vibrations and O-H bending, while the band detected at 1021 cm^{-1} was the result of stretching vibration of C-O and C-C

groups (Răileanu et al., 2013). Finally, the peaks at 607 cm^{-1}, 575 cm^{-1} and 527 cm^{-1} (Fig. 1D) represents the out-of-plane bending vibrations of OH (Li et al., 2010).

For LCEO (Fig. 1A), an important band was registrated at 1671 cm^{-1}, which was related to C=O stretching vibration of citral compound, the majoritary constituent of LCEO (Wang et al., 2020). Nevertheless, for all inclusion complexes, this vibration was shifted from 1671 cm^{-1} to 1673 cm^{-1} and the peaks intensity decreased. According to Răileanu et al. (2013), the displacement of bands or change in the intensity of the ATR-FTIR signals are indicative of the interaction between β-CD and EO. This result suggests the synthesis of LCEO:β-CD inclusion complexes.

3.2.2. FT-Raman

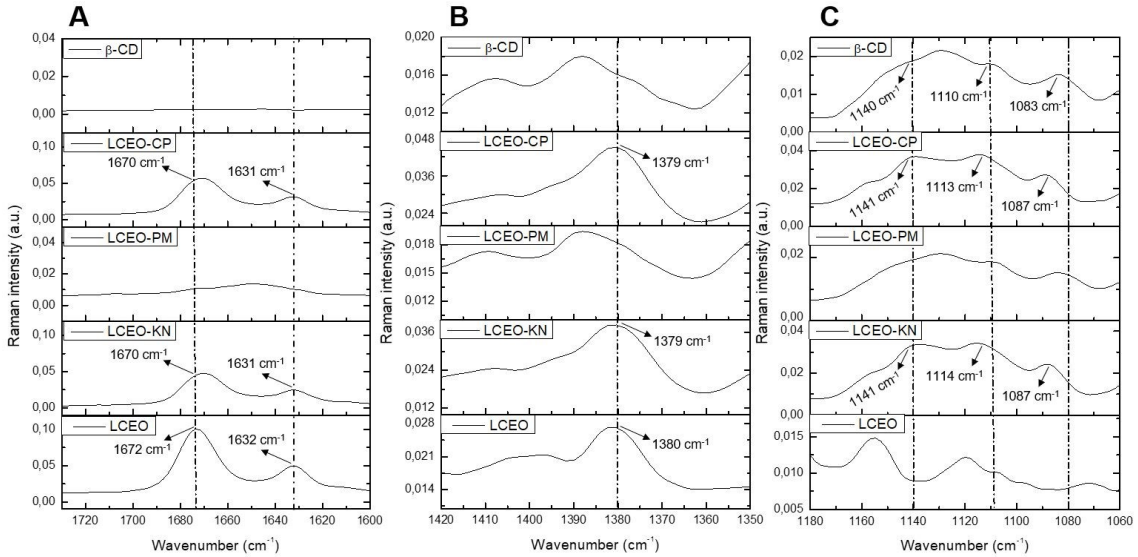
The important regions of Raman spectra for β-CD, LCEO and LCEO:β-CD inclusion complexes produced by different methods are shown in Figure 2. The main chemical compounds previously obtained by GC-MS for LCEO were geranial and neral, with 37.67% and 32.75%, respectively. Both compounds are geometric isomers and aliphatic unsaturated aldehydes, so the expected signals for FT-Raman are related to the C=O and C=C bonds (Jentzsch et al., 2015).

In the Raman spectra of LCEO (Fig. 2A), a strong absorption band appeared at 1672 cm^{-1}, which was assigned to stretching vibration of C=O group in citral compound (Jentzsch et al., 2015). In LCEO-CP and LCEO-KN inclusion complexes, this vibration was shifted from 1672 cm^{-1} to 1670 cm^{-1}. Likewise, the C=C stretching mode was detected at 1632 cm^{-1} for LCEO and shifted to 1631 cm^{-1} in the LCEO-CP and LCEO-KN inclusion complexes. Moreover, the peaks intensity decreased indicating that LCEO was successfully included into the cavity of β-CD.

The band obtained at 1380 cm^{-1} for the LCEO (Fig. 2B) was also observed in LCEO-CP and LCEO-KN inclusion complexes. Vibration was favored after the interaction of the LCEO with β-CD. Meanwhile, LCEO-PM was similar to β-CD. Hanif et al. (2017) attributed the 1378 cm^{-1} wavenumber to the

compound limonene, which is present in the chemical composition of the LCEO with 10.55%.

Figure 2. Raman spectra of β-cyclodextrin (β-CD), *Litsea cubeba* essential oil (LCEO) and the inclusion complexes of LCEO in β-CD obtained from kneading (KN), co-precipitation (CP) and physical mixture (PM) methods. (A) Wavenumber from 1720 to 1600 cm^{-1}; (B) Wavenumber from 1420 to 1350 cm^{-1}; (C) Wavenumber from 1180 to 1060 cm^{-1}.



The FT-Raman spectra of β-CD (Fig. 2C) showed prominent absorption bands at 1083 cm^{-1} for C-O stretching vibration, 1110 cm^{-1} for C-C stretching vibration and 1140 cm^{-1} for C-H scissoring vibration (Li et al., 2010). When comparing the β-CD spectra with the LCEO-CP and LCEO-KN inclusion complexes spectra, we could observe displacement of the β-CD at these wavenumbers, which indicates the formation of the inclusion complexes. On the other hand, the Raman spectra of LCEO-PM (Fig. 2A-C) was similar to β-CD, evidencing little or no interaction between LCEO and β-CD in this inclusion complex.

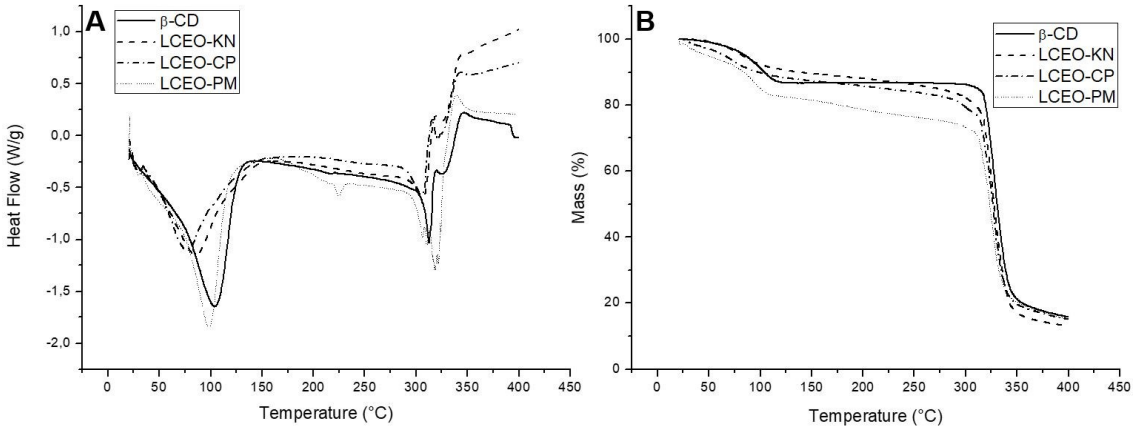
Based on the results of AFT-IR (Fig. 1) and FT-Raman (Fig. 2), we highlight the interaction of the citral functional group in the 1600 cm^{-1} region for both techniques. This same result has been reported by other authors, reinforcing the interaction of this functional group in the hydrophobic cavity of β-CD (Jentzsch et al., 2015; Wang et al., 2020). Thus, it could be inferred that LCEO entered in the hydrophobic cavity of β-CD by co-precipitation (LCEO-CP) and kneading (LCEO-KN) methods.

315 *3.2.3. Thermal analysis*

316 Thermal analyzes were performed to confirm the formation of the
317 inclusion complexes. Figure 3 illustrates the DSC and TG thermograms of β-CD
318 and LCEO:β-CD inclusion complexes produced by different methods.

319

320 **Figure 3.** DSC (A) and TG (B) thermograms in dynamic N_2 atmosphere of β-cyclodextrin (β-CD)
321 and the inclusion complexes of *Litsea cubeba* essential oil (LCEO) in β-CD obtained from
322 kneading (KN), co-precipitation (CP) and physical mixture (PM) methods.



323

324

325 The DSC curve of β-CD (Fig. 3A) showed endothermic peaks at
326 temperatures of 104 ºC and 314 ºC. According to the literature, the first peak
327 indicates the release of water molecules while the second peak correspond to
328 the melting point (Galvão et al., 2015; Rocha Neto et al., 2018). In addition,
329 exothermic peaks were observed around 321 ºC and 346 ºC, attributed to
330 decomposition of β-CD. Likewise, Galvão et al. (2015) reported that peaks
331 above 300 ºC correspond to decomposition and removal of carbonaceous
332 material.

333 The DSC thermogram of LCEO-PM (Fig. 3A) was similar to β-CD,
334 indicating little or no interaction between LCEO and β-CD in this inclusion
335 complex. However, when comparing the DSC curves of LCEO-KN and LCEO-
336 CP inclusion complexes to the β-CD, we could observe variations in both
337 intensities and temperatures. For LCEO-KN (Fig. 3A), the temperatures found
338 were 83 ºC, 307 ºC and 341 ºC and for LCEO-CP (Fig. 3A) were 80 ºC, 305 ºC
339 and 343 ºC. Thus, providing an indication of interaction between LCEO and β-
340 CD in these inclusion complexes.

The TG thermogram of β-CD (Fig. 3B) showed two stages of thermal degradation. The initial mass loss phase occurred at the range of 85-130 ºC, which was possibly caused by water evaporation, while the second weight loss was observed at the range of 310-370 ºC, which is probably attributed to molecular decomposition of β-CD. Our observations were consistent with those found in the literature (Cui et al., 2019; Wang et al., 2020).

For LCEO-PM (Fig. 3B) inclusion complex, the TG curve was similar to β-CD, with initial and secondary mass losses distributed at the range of 90-125 ºC and 300-370 ºC, respectively. Both LCEO-KN and LCEO-CP (Fig. 3B) inclusion complexes presented only one thermal degradation stage, which was relatively slow and stable until the molecular decomposition of β-CD, in the range of 290-370 ºC. In addition, the water loss (85-130 ºC) was lower than β-CD. Wang et al. (2020) attributed this observation to the hydrophobic interaction between EO and β-CD. Therefore, LCEO-KN and LCEO-CP were more thermally stable than LCEO-PM.

3.3. Cytotoxicity of inclusion complexes

The LCEO:β-CD inclusion complexes obtained by different methods were tested against tumor (HT-29 and HeLa) and non-tumor (Vero) cell lines to evaluate its cytotoxic effect. The results for the MTT assay are presented in Table 1. In summary, most of the inclusion complexes of LCEO into β-CD exhibited antitumor activity against the investigated cells. Moreover, cell viability was not affected in cultures treated with β-CD (IC_{50} >1000µg/mL).

Table 1. Antitumor activity and cytotoxicity of β-cyclodextrin (β-CD) and the inclusion complexes of *Litsea cubeba* essential oil (LCEO) in β-CD obtained from kneading (KN), co-precipitation (CP) and physical mixture (PM) methods.

Samples	IC_{50} (µg/mL)		
	HT-29	**HeLa**	**Vero**
β-CD	>1000	>1000	>1000
LCEO-KN	81.7 ± 7.5	88.0 ± 20.8	99.0 ± 11.5
LCEO-CP	74.8 ± 20.9	95.0 ± 21.2	88.0 ± 5.4
LCEO-PM	71.5 ± 5.6	106.3 ± 11.0	90.0 ± 2.6

IC_{50}: Inhibitory Concentration for 50% of the cell.

The values are the mean ± standard deviation for triplicates.

Interestingly, the inclusion complexes showed to be more selective against HT-29 tumor cell (IC_{50}=71.5-81.7 µg/mL), since the IC_{50} obtained for this cell was lower than that obtained for Vero cell (IC_{50}=88.0-99.0 µg/mL). So, all tested LCEO:β-CD inclusion complexes exhibited toxicity to HT-29 cells. Moreover, it was observed that the cytotoxic effect of inclusion complexes was less pronounced in HeLa tumor cell (IC_{50}=88.0-106.3 µg/mL), however LCEO-KN was also more active against Hela than the non-tumor cell.

Previous studies have demonstrated that EOs and their isolated compounds are associated with antitumor activity in different carcinogenic cells, such as *Laurus nobilis* EO on K562 human chronic myelogenous leukemia cells (Saab et al., 2012) and *E. brejoensis* EO against J774 and HeLa tumor cells (Santana et al., 2020), as well as isomers of citral (Bailly, 2020), the same compounds found in LCEO. Furthermore, Santana et al. (2020) described cytotoxicity of the *E. brejoensis* EO into β-CD against J774 and HeLa cell lines.

3.4. Antifungal activity of inclusion complexes

The agar dilution method was used to evaluate the inhibitory capacity of LCEO and its inclusion complexes against *A. flavus* and *F. verticillioides*. The results are shown in the Table 2. In general, the inclusion complexes significantly inhibited radial growth compared to LCEO and FC ($p<0.05$) and exhibited good antifungal activity. In addition, no radial inhibition was observed for the β-CD isolated.

Lower effectiveness of LCEO against *A. flavus* and *F. verticillioides* was observed, followed by LCEO-PM inclusion complex. In contrast, the LCEO-KN and LCEO-CP inclusion complexes were more efficient for both toxigenic fungi evaluated. These findings confirm that the antifungal activity of LCEO was improved after molecular inclusion into β-CD. Thus, the molecular inclusion of LCEO into β-CD has potential application as a natural fungicide in the control of *A. flavus* and *F. verticillioides*.

Our observations were consistent with those found in previous studies (Anaya-Castro et al., 2017; Cui et al., 2019; Wang et al., 2020). Likewise, in these studies the molecular inclusion of EOs improved the antimicrobial and

antifungal activities, possibly due to the greater water solubility of the inclusion complexes compared to EO pure.

Table 1. Antitumor activity and cytotoxicity of β-cyclodextrin (β-CD) and the inclusion complexes of *Litsea cubeba* essential oil (LCEO) in β-CD obtained from kneading (KN), co-precipitation (CP) and physical mixture (PM) methods.

Samples	Radial inhibition (%)	
	Aspergillus flavus	***Fusarium verticillioides***
FC	$0^{c} \pm 0$	$0^{d} \pm 0$
β-CD	$0^{c} \pm 0$	$0^{d} \pm 0$
LCEO	$10.22^{b} \pm 1.88$	$8.55^{c} \pm 2.43$
LCEO-KN	$25.68^{a} \pm 2.44$	$27.41^{a} \pm 2.96$
LCEO-CP	$23.92^{a} \pm 0.80$	$23.04^{ab} \pm 1.61$
LCEO-PM	$11.07^{b} \pm 2.25$	$16.20^{b} \pm 2.16$

FC: fungal control (inoculum 10^5 conidia/mL).
The values are the mean ± standard deviation for triplicates.
Different letters between lines refer to significant difference ($p<0.05$) by the Tukey test.

4. CONCLUSION

The complexation efficiency, ATR-FTIR, FT-Raman, DSC and TG results presented significant evidence of LCEO:β-CD inclusion complex formation. These results indicated that kneading and co-precipitation were the best methods used in this study. Therefore, the molecular inclusion of LCEO into β-CD was successful, as well as the preliminary biological results, evidencing that β-CD inclusion process may be a viable alternative to facilitate and increase future applications of this EO as therapeutic medication and natural antifungal.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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