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MICROBIOLOGY

Association of the essential oil of *Cymbopogon citratus* (DC) Stapf with nystatin against oral cavity yeasts

LUIZ FRANCISLEY DE PAIVA, ANA BEATRIZ A. TEIXEIRA-LOYOLA, TAYLOR B. SCHNAIDER, ANGÉLICA CRISTINA DE SOUZA, LIDIANY M. ZACARONI LIMA & DISNEY R. DIAS

Abstract: Oral thrush or candidiasis is a fairly common infectious fungal disease that is mainly caused by species of the genus *Candida* spp. In this work, we evaluated the antifungal activity of the essential oil of *Cymbopogon citratus* (DC) Stapf (lemongrass) and its combination with nystatin against oral cavity yeasts. The oil was extracted by the steam distillation method, and its constituents were quantified. The yeasts were identified using MALDI-TOF MS. The sensitivity to the essential oil and its association with nystatin was verified by the minimum inhibitory concentration (MIC) and checkerboard methods. In the essential oil samples, there were 84.53% of citral. A total of 64.77% of strains were identified as *Candida albicans*. On susceptibility tests, 83.55% of the yeast isolates were inhibited in concentrations of ≥16 µg/mL of nystatin. *C. citratus*'s oil was capable of inhibiting and killing all the isolates tested with concentrations that varied from 0.137 to 2.2 mg/mL. The association of oil and nystatin had an additive effect on more than 78% of the strains. The association of this herbal drug with nystatin potentialized the antifungal effect on yeast samples isolated from the oral cavity of oncologic patients.

Key words: infectious diseases, medicinal plants, Nystatin, oral Candidiasis, pharmacological synergism.

INTRODUCTION

Oral thrush or oral candidiasis is a very common oropharyngeal fungal infectious disease (Mycosis) (Sholapurkar et al. 2009). It is mostly caused by the species *Candida albicans*. However, other species such as *Candida tropicalis*, *Candida Krusei*, *Candida parapsilosis* and *Candida guilliermondii* may rarely be found in the lesions (Wille et al. 2013). The clinical aspects of the lesions caused by those fungi are varied, as they may cause cutaneous or mucocutaneous lesions, and even septicemic or visceral diseases.

The oral form of thrush is more common, and most of the patients who suffer from this problem have a certain degree of immunosuppression (Sahand et al. 2009). In AIDS patients, the development of oropharyngeal candidiasis occurs in over 90% of the cases as the disease progresses (Li et al. 2012). The incidence of oral thrush in ongoing chemotherapy patients is 20 to 40%, depending on the dose, duration, and type of treatment, in which higher doses and longer treatment may have severe consequences from this infection (Diaz et al. 2019).

Among the species of *Candida* spp., *C. albican* is the one most reported in clinical

cases, followed by *C. parapsilosis*, *C. glabrata*, *C. tropicalis,* and *C. krusei*. These are considered the main species with clinical interest, together with *C. guilliermondii* and *C. lusitaniae*, even though with fewer occurrences. There are still emerging species that have often been described, such as *C. dubliniensis*, *C. kefyr*, *C. rugosa*, *C. famata*, *C. utilis*, *C. lipolytica*, *C. norvegensis*, *C. inconspicua,* and *C. auris* (Lopez et al. 2005, Panizo et al. 2009, Meis & Chowdhary 2018).

The treatment for oral thrush is antifungal medication, such as nystatin and miconazole, which are the most used topically. Concerning the systemic treatment, fluconazole and itraconazole are usually the first-choice (Quindos et al. 2019).

The azoles are less toxic and develop their effect slower than polyenes because its action against the fungal membranes is more selective. The presence of side effects in fluconazole are low, in which nausea, vomit, headaches, skin rash, stomachache, and diarrhea are the most frequent (Quindos et al. 2019).

The indiscriminate or unregulated use of the antifungal drugs available today on the market contributes to a larger quantity of resistant pathogens, which leads to disease recurrence. In addition, there are many reports by the patients of undesirable side effects (Simões et al. 2013).

A larger number of strains from several species of *Candida* spp. are becoming resistant to the available antifungal drugs (Hazen et al. 2003). When it comes to antifungal resistance, the non-*albicans* species of *Candida* show greater etiological importance in infections, increasing the preocupations concenrning the emergence of multiresistant strains to conventional drugs (Abrantes et al. 2013).

The plant *Cymbopogon citratus* (DC) Stapf is known for its antimicrobial, anti-inflammatory, and antineoplastic effects, as well as in the healing of wounds. These properties make *C. citratus* a potentially beneficial species to be used in human health (Boukhatem et al. 2014). Among all the pharmacological effects of this essential oil, its antifungal activity stands out. Citral, the plant's odoriferous active ingredient present in its leaves, is the molecule responsible for the antimicrobial activity (Lorenzi & Matos 2002). The components of the essential oil may interfere in the biosynthesis of fungal cell walls (Pierce et al. 2013, Sookto et al. 2013). Studies suggest that terpenes are the constituents responsible for that activity, which citral is the main component in the species *Cymbopogon citratus* with 80% of the oil's total volume (Lucena et al. 2015, Leite et al. 2014).

Phytotherapy represents an efficient method to increase therapeutic options. It is an essential source of innovation in human health and may strengthen even more the production and exploration of the abundant Brazilian biodiversity (Hasenclever et al. 2017).

The herbal drug industry, whose prices for its products are usually more accessible, may be an excellent alternative to ensure access for the general population to safe, efficient, good quality, and less expensive medications. Therefore, it represents a source of innovation and improvement in health and social inclusion offered to users of the Unique Health System (SUS) in Brazil (Hasenclever et al. 2017). With the insertion of phytotherapy in the SUS, it became evident the need to study those substances to enrich the knowledge of health-care workers, as well as make sure of their efficiency (Bettega et al. 2011).

This study aimed to study the antifungal activity of *Cymbopogon citratus* (DC) Stapf's essential oil and its combination with nystatin against oral fungal infections.

MATERIAL AND METHODS

Design and place of the study

This work was an *in vitro*, observational, analytic, transversal study with a qualitative and quantitative approach. The ethics data were evaluated by the Research Ethics Committee (CEP), that emitted a formal opinion in a document number 2.400.440. The document authorized the use of the microorganisms. The tests were conducted after a favorable opinion.

The tests were conducted in the Laboratory of Basic Research of Vale do Sapucaí University (UINVÁS), as well as in the Laboratory of Molecular Biology of Food Science and Laboratory of Organic Chemistry – Essential oils of the Department of Chemistry at the University of Lavras (UFLA).

We studied 292 strains of pre-identified fungus that belong to the genus *Candida* deposited in the collection of microorganisms of the Laboratory of Basic Research. These strains come from previous studies, in which they were isolated from the oral cavity of oncological patients on chemotherapy at the Hospital of Clinics Samuel Libânio (HCSL) in Pouso Alegre – MG. We also used six standard strains: *Candida albicans* ATCC 90028, *Candida dubliniensis* CBS 7987, *Candida parapsilosis* ATCC 22019, *Candida glabrata* MYA 2950, *Candida krusei* ATCC 6258, and *Candida utilis* ATCC 9950.

Essential oil extraction

The leaves of *Cymbopogon citratus* (DC) Stapf were collected from adult plants grown in a plant nursery in the Biology laboratory at UNIVÁS (22.219737S and 45.914720W GRW) in the mornings throughout September 2018. The plant material was identified in the herbarium of UNIVÁS, by Professor MSc. F.S. Braz, according to the criteria established by Farmacopéia Brasileira V (Brasil 2010) and the testimony material was deposited in the form of exsiccata, under the registration number: UNIVAS - 002.

Right after the harvest, three repetitions of a 25g fresh foliar sample were used to determine the humidity, using the gravimetric method proposed by Rocha et al. (2012). The samples were weighed before and after the incubation in an oven at 103 ± 2°C. The incubation time was interrupted right after the sample had a weight variation no more than 0.0001g in analytical scales. Humidity content (H) was calculated using the formula $H = \frac{FBM - DBM}{FBM} x100$, where H is the humidity content (%), FBM is the fresh biomass, and DBM is the dry biomass.

The essential oil was obtained by the hydrodistillation of the leaves at UNIVÁS's Laboratory of Phytotherapy, using the steam distillation method in a modified version of the Clevenger apparatus, according to Farmacopéia Brasileira V (Brasil 2010).

Two kilograms of *C. citratus* leaves were dried at room temperature for seven days. Afterwards, the dried material was ground in a PH900 Turbo Philco food processor to obtain small dehydrated fragments. These allow the vapor stream to efficiently drag the volatile substances stored in the cells, even when compared to the fresh green material (Martins et al. 2002).

After the dehydration process, 600g of the dry plant material was soaked in water (in the proportion of 1:10) in a modified Clevenger apparatus. The time for the extraction was of four hours. The volume of the essential oil extracted was directly measured in the volumetric scale of the separator tube. The oil was stored in a sterilized amber recipient at 4 to 8°C, in a place without direct light incidence.

Essential oil yielding was calculated on a dry matter basis, according to Santos et al. (2004), using the formula: $OY = \frac{OV}{PB - \left(\frac{PB x H}{100}\right)} x100$, where OY

is the oil yield; OV is oil volume; PB is plant biomass; H is humidity content.

Physical-chemical analyses and gas chromatography

Physical-chemical analyses of the essential oil were performed according to Farmacopéia Brasileira V (Brasil 2010). The relative density, refraction index, solubility in ethanol, color, and appearance were determined.

The identification of the chemical substances was conducted by a gas chromatography technic coupled to mass spectrometry (GC-MS), using a Shimadzu GCMSQP2010 Plus equipment connected to an AOC 5000 autoinjector. The chromatographic column used was the SBLTM-5MS type with a 0.25 µm caliber, 30 m of length, and 0.25 mm of internal diameter (bonded phase; 5% diphenyl, 95% dimethyl polysiloxane). The carrier gas was helium in a flux of 1.18 mL/min. The oven's initial temperature was 60°C for two minutes, programmed to increase 3°C every minute up to the maximum temperature of 240°C. After that, it increased 10°C/min up to 300°C, staying in this temperature for seven minutes.

The other parameters were: injection temperature, 220°C; injection mode, SPLIT 1:100; temperature of ions source, 200°C; interface temperature, 240°C; solvent cut, 2.4 minutes; and m/z values analyzed, 45-500Da.

The mass spectrums obtained were compared to Wiley's 8.0 equipment database. The compound retention index was calculated after the previous analysis of a homologous series of alkenes and compared to literature data (Adams 2007).

Reactivation and identification of fungal strains

The strains were reactivated by sowing them on a Sabouraud Dextrose Agar supplemented with Chloramphenicol. The plates were incubated at 37 °C. After the growth of the colonies, a new stocking of strains was performed in a slant culture of Corn Meal Agar in order to renew and maintain the collection specimens.

The tests to identify the species were performed in a MALDI-TOF Mass Spectrophotometry in the Department of Food Science - UFLA. The samples were sown and grown on Sabouraud Dextrose Agar for 24 hours at 37 °C. After this period, a process of protein extraction was realized with formic acid and acetonitrile.

Using a disposable inoculating loop calibrated at 10 µL, portions of the cells were transferred to Eppendorf® tubes and suspended with 300 µL of HPLC grade distilled water. The material was homogenized on a vortex mixer for 30 seconds. Afterwards, 900 µL of anhydrous ethyl alcohol were added and the solution was homogenized again. After this process, the samples were centrifuged for two minutes at 13,000 rpm. The supernatant was removed, and the precipitate resuspended with 50 µL of 70% formic acid. Then, an aliquot of 50mL of 100% acetonitrile was added to the tubes and the mixture homogenized once again. Lastly, the mixture was centrifugated at 13.000 rpm and the supernatant transferred to another tube, which was identified and stored in a freezer at -20°C.

The tests were conducted by pouring 1 µL of the extraction-obtained supernatant on Bruker's 96-well microplate. The samples were partially dried and covered with 1 µL of the α-cyano-4-hydroxycinnamic acid (CHCA) matrix solubilized in 33.3% ethanol, 33.3% of acetonitrile, and 33.3% of trifluoracetic acid 10%. The microplates were dried in room temperature and the tests realized in triplicates.

The analyses were performed in a MALDI-TOF mass spectrometer Microflex LT Bruker and measured using the system MALDI Biotyper

(Bruker Daltonics, Bremen, Germany). The identification at the level of species was confirmed when the results obtained had a score higher than two.

Nystatin sensitivity profile

The susceptibility to nystatin profile was determined by the minimum inhibitory concentration technic (MIC), according to the broth microdilution method described in the normative "M27-A2 - Método de Referência para Testes de Diluição em Caldo para a Determinação da Sensibilidade de Leveduras à Terapia Antifúngica" and published by the *Clinical Laboratory Standards Institute* (CLSI 2002).

The standard antifungal solution was prepared with dimethyl sulfoxide (DMSO) at a concentration of 1600 µg/mL. The different dilutions were acquired in concentrations 100 times higher than the concentration used. Therefore, the drug was diluted 10 times, and then again diluted to 1:5 with the medium RPMI-1640 (*Roswell Park Memorial Institute)* to achieve a concentration twice the one needed for the microdilution test. The concentration range was 0.0313 to 16µg/mL.

From a 24-hour culture grown at 37 °C in a Sabouraud Dextrose Agar, a 5 mL yeast suspension was prepared using sterile distilled water in a test tube. After homogenizing the material on a vortex mixer, the suspension turbidity was adjusted to 0.5 according to the McFarland's scale, using a spectrophotometer (CELM E-225 D) at 625nm, to obtain standard yeast suspensions with $1x10^6$ to $5x10^6$ CFC/mL (CFC stands for colony formation cell). The standard suspension was homogenized once again in a vortex for 15 seconds, diluted to 1:50 and then to 1:20 with the culture medium RPMI-1640. The twice-concentrated inoculum obtained was used in the test (from $1x10^3$ to $5x10^3$ CFC/mL). Finally, the inoculum was diluted to 1:1 when the wells were inoculated, achieving the final desired concentration of $0.5x10^3$ to $2.5x10^3$ CFC/mL.

The microdilution test was conducted on sterile microplates containing 96 flat-bottomed wells. The twice-concentrated material was placed on rows from one to ten in a volume of 100 µL per well. The first row contained the largest drug concentration (16µg/mL), and the last row had the smallest (0.03 µg/mL). On row eleven, we placed the medium without the drug, which was considered as the positive control. Row twelve was filled with 200 µL of the medium with no drug and used as the sterility control, as well as the blank.

It was inoculated 100 µL of the yeast suspension twice-concentrated in each microplate well, which takes the diluted drug and inoculum to the concentrations mentioned before. The plates were sealed with Contact® adhesive paper and incubated for 48 hours at 35° C.

The readings were conducted on a microplate reader (Polaris® - Celer Biotecnologia S/A) at 405nm. The absorbance values obtained for the wells were subtracted from the values obtained on row-twelve wells (Blank). A counterproof was performed by adding 20µL of 2% tetrazolium triphenyl chloride (TTC) at in every well and re-incubating the plates for two more hours. The staining promotes the coloring of the colonies without compromising their viability. The wells that present fungal activity are colorless, while the wells where there is microbial activity stays red. The MIC value was defined as the smallest concentration in which we observe a reduction of 100% of turbidity of the wells in comparison to the wells on row eleven (growth control), which demonstrated no visible microbial growth.

Antifungal susceptibility to the essential oil of *Cymbopogon citratus* (DC) Stapf

The dilution preparation of the essential oil followed a model adopted by Lima et al. (2006). In a sterile glass tube, we added 0.8 mL of the essential oil, 0.05 mL of Tween-80, and 4.2 mL of the medium RPMI-1640, which corresponded to a concentration of 140.8 mg/mL. The tube was agitated for five minutes in a vortex mixer. Afterwards, by using a seriated dilution procedure (1:1) in the culture medium, we obtained the other concentrations that varied from 0.06875 to 35.2 mg/mL, which presented twice the concentrations needed for the microdilution test.

The essential oil microdilution test was performed on sterile 96-well microplates. The methodology of inoculation, incubation, and measurement followed the same protocol used in sensitivity tests to the antifungal drug, described previously.

After reading the MIC but before adding the 2% TTC, as a form to determine the minimum fungicide concentration (MFC), 10 µL of inoculum was inoculated in Sabouraud Dextrose Agar in each well and incubated for 48 hours at 35°C. The MFC is the antimicrobial drug concentration that causes the death of a microorganism and was defined as the lowest oil concentration capable of killing all of the cells (Cantón et al. 2003).

Synergistic assay of the essential oil of *Cymbopogon citratus* (DC) Stapf associated with nystatin - the Checkerboard Method

The combined effect of two substances (essential oil + nystatin) was determined by the checkerboard microdilution technic, according to Fernandez-Cuenca et al. (2003), for the derivation of the fractional inhibitory concentration (FIC) index.

The test was performed using sterile flat-bottomed 96-wells microplates. Four combinations of the essential oil (0.137 – 0.275 – 0.55, and 1.1 mg/mL) with ten nystatin concentrations (ranging from 0.0313 to 16µg/mL) were tested. Each plate received one concentration of the essential oil added to the ten nystatin concentrations. The rows eleven and twelve were used as a positive control for growth (medium without oil or drug) and negative/blank (medium with oil but no inoculum), respectively.

The inoculation, incubation, and measurement method followed the same protocol used for the antifungal susceptibility test, described previously.

The minimum inhibitory concentration combined (MICC) is defined as the smallest concentration in which it is observed a reduction of 100% of turbidity in the well when compared to row-eleven wells (growth control).

The FIC index was calculated as the sum of FIC_A + FIC_B, where "$_A$" represents the essential oil and "$_B$" the nystatin. The FIC_A, by its turn, is calculated through the ratio $MICC_A/MIC_A$, whereas FIC_B is equal to $MICC_B/MIC_B$. The FIC index is therefore interpreted as synergism (<0.5), additivity (from 0.5 to 1.0), indifferent (>1 and <4), or antagonism (>4) (Fernandez-Cuenca et al. 2003, Santos et al. 2009).

Statistical Analyses

The data were organized, and the statistical analyses were conducted using the software SPSS® Statistic version 17. The tests of descriptive frequency and Pearson's chi-squared were conducted at $P<0.05$.

RESULTS

Analysis of *C. citratus*'s essential oil

The physical-chemical parameters found in the evaluation of the essential oil and the percentage

of extraction yield are displayed in Table I.
After the essential oil gas chromatography analyses and the comparison of their results with the database, it was possible to identify the most prevailing volatile compounds in the oil, as seen in Table II.

Identification of yeasts

In this study, the 298 yeast strains from the oral cavity that are deposited in the microbiological collection from the Laboratory of Basic Research at UNIVÁS were identified at the level of species. Their diversity are demonstrated in Table III. A total of 64.76% of the strains were identified as being *Candida albicans*. Therefore, it is the most frequent microorganism found. We also found the species *Candida dubliniensis*, which is a microorganism phenotypically similar to *C. albicans*.

Profile of the susceptibility to nystatin

In this study, 33.2% of the isolates had a MIC > 16 µg and 50.3% with 16 µg. In the isolates from *Candida albicans*, the interval was from 2.0 to >16.0 µg, whereas the non-*albicans* species of *Candida* had 4 to >16 µg. When compared to the sensitivity profile between the species, there were no significant statistical differences at P=0.074

Table I. Physical-chemical parameters and yield of essential oil extraction content from *Cymbopogon citratus* (DC) Stapf.

Parameters	Essential Oil
Refraction index	1.4872
Density (g/mL) (20°C)	0.881
Solubility in ethanol 100%	1:1
Solubility in ethanol 80% (v/v)	1:5
Color	Yellow
Transparency	Limpid
Yielding (%)	0.94%

Table II. Constituents of essential oil of *Cymbopogon citratus* (DC) Stapf.

[1]Compound name	[2]RT (min)	[3]RI_C	[4]A (%)
6,7-Diazabicyclo[3.2.2]nona-3,6-diene, 2-methylene	4.905	868	0.64
Myrcene	8.090	988	13.76
Cyclohexaneacetaldehyde, 2-methylene	15.664	1179	1.07
Neral	18.257	1239	37.18
Geraniol	19.565	1268	47.35
Total			100

1: Compounds identified by their mass spectrum and retention index according to the Adam's library (2007); compounds with area ≥ 0.1%; 2. Retention time (RT) of the compound in the chromatographic column in minutes; 3: Retention index (RI) calculated in relation to various alkenes; 4: Percentual area (A) of each peak in relation to the whole chromatogram.

Yeast antifungal susceptibility to the essential oil

In this study, the inhibitory action of the essential oil occurred in all tested yeast strains. In *Candida albicans* isolates, the range was from 0.275 to 2.2 mg / mL, whereas non-*albicans Candida* species it ranged from 0.137 to 2.2 mg / mL. The MIC evaluation showed values equal and inferior to 2.2 mg / mL for the isolates evaluated, with the MIC_{50} having a value of 0.55 mg/mL and MIC_{90} of 1.1 mg/mL.

The fungicidal action of the essential oil also occurred in all strains of yeast tested at the same range of the aforementioned concentrations. As for the fungicidal activity, the results were the same, MFC_{50} and MFC_{90} of 0.55 and 1.1 mg/mL, respectively.

Evaluation of the association of the essential oil with nystatin against yeasts -checkerboard method

Through the checkerboard method, it was observed a positive interaction when we compare the MIC of nystatin alone to the $MICC_B$ of nystatin combined with the essential oil, as seen in Table IV. The correlation is statistically significant, with

Table III. Diversity of yeasts from the microbiological collection of the Laboratory of Basic Research from the oral cavity of oncological patients.

Species	Frequency	%	Accumulated %
Candida albicans	193	64.77	64.77
Candida glabrata	49	16.44	81.21
Candida tropicalis	27	9.06	90.27
Candida parapsilosis	8	2.68	92.95
Candida dubliniensis	5	1.68	94.63
Clavispora lusitaniae	3	1.01	95.64
Issatchenkia orientalis	3	1.01	96.65
Meyerozyma guilliermondii	2	0.67	97.32
Pichia norvegensis	2	0.67	97.99
Candida inconspicua	1	0.34	98.33
Candida metapsilosis	1	0.34	98.67
Candida pararugosa	1	0.34	99.01
Cyberlindnera jadinii	1	0.34	99.35
Kluyveromyces marxianus	1	0.34	99.69
Saccharomyces cerevisiae	1	0.34	100
Total	**298**	**100**	**100**

P<0.000. Concerning the essential oil, there was also a positive interaction when the MICC of the association is compared to the MIC of the essential oil alone, as seen in Table V. The correlation is statistically significant at P<0.000.

The effects of the essential oil of *Cymbopogon citratus* (DC) Stapf with nystatin are displayed in Table VI, where we can observe that 82.3% of yeast isolates were more susceptible to the combination of agents than the agents alone. It was verified that there is no antagonistic effect between the associated agents.

DISCUSSION

In the present study, the extraction yield of the essential oil was between the values found by Santos et al. (2009), which varied from 0.465 to 1.18%. The relative density and the refraction index of the essential oil were also similar to the study, with values of 0.957 g/mL and 1.4815, respectively. The major components found in the essential oil in this study were citral (84.53%) and myrcene (13.76%). According to Boukhatem et al. (2014), citral is composed of the *cis*-isomer geranial and *trans*-isomer neral. Wilson et al. (2002) observed that citral might be present in concentrations that vary from 65 to 85%. By studying the action of citral upon *Candida albicans*, Leite et al. (2014) concluded that it had significant antifungal activity against that species and discovered that the concentrations that inhibit its growth is the same that causes its death. By studying the effects of six terpenoids upon species of *Candida* spp., Zore et al. (2011) concluded that all of them displayed excellent antifungal activity, but linalool and citral were the two most effective.

The fungi diversity found in this study evidenced a higher frequency of *C. albicans* over the others. Various species may be etiological agents of oral candidiasis. Nonetheless, *C. albicans* is the most common, followed by *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *Pichia kudriavzevii*. Those five species represent over 95% of fungal infections. However, many other species may also be isolated and naturally occur in humans (Kidd et al. 2016).

The concentration range of nystatin tested in this study was 0.031 – 16 µg/mL. However, the values of MIC found are between 2 – 16 µg/

Table IV. Fractioned inhibitory concentration of antifungal nystatin against yeasts.

Concentration (µg/mL)	MIC Alone		MICC Combined	
	Frequency	%	Frequency	%
0.03	0.0	0.0	212	71.14
0.06	0.0	0.0	10	3.36
0.125	0.0	0.0	6	2.01
0.25	0.0	0.0	7	2.35
2	4	1.3	1	0.34
4	9	3.0	3	1.01
8	40	13.4	9	3.02
16	143	48.0	50	16.78
> 16	102	34.2	0	0
Total	**298.0**	**100.0**	**298**	**100.0**

Chi-squared test P<0,000.

mL. These finds are higher than those from the literature. In a study with 558 specimens of *Candida* spp. isolated from patients with oropharyngeal candidiasis, Yu et al. (2019) found a range that varied from 1 – 4 µg/mL. Godoy et al. (2012), studying oral candidiasis in patients with chronic kidney insufficiency that are on hemodialysis treatment, found MICs ranging from 0.125 – 8 µg/mL for *C. albicans* and 0.125 – 4 µg/mL for the non-*albicans* species of *Candida*. Kuriyama et al. (2005) used in their study a cut value for antifungal resistance of ≥ 16 µg/mL (MIC), based on the results from other studies. If the present study were compared to those data, 83.56% of nystatin-resistant yeast would be found.

Nowadays, there are various antifungal agents to treat candidiasis. However, there is increasing resistance to conventional drugs by these opportunistic fungi, besides their diverse side effects. Therefore, it is highly necessary alternative antimicrobial drugs on the market, which motivates researchers to search for new treatment options, and herbal therapy is one of them.

The essential oil of *C. citratus* has already been used in other works as a potential antifungal substance. Tyagi & Malik (2010), analyzing oils from *Eucalyptus globulus*, *Mentha piperita*, and *Cymbopogon citratus* against *Candida albicans*, concluded that lemongrass's essential oil is better and highly effective in the volatile phase against *C. albicans*, leading to deleterious morphological changes in its cell structures. The MIC and MFC found by those authors were 0.288 mg/mL and 0.567 mg/mL, respectively.

Due to toxicological complications of the treatment for fungal infections, and the fact that the microorganisms are increasingly resistant to drugs, one of the strategies to overcome the lack of therapeutic alternatives is the evaluation of antifungal medication combined with natural substances. In this work, by associating sub-inhibitory concentrations of the essential oil of *C. citratus* with nystatin (in the same

Table V. Fractioned inhibitory concentration of essential oil against the yeasts.

Concentration (mg/mL)	MIC Alone		MICC Combined	
	Frequency	%	Frequency	%
0.137	3	1.0	14	4.7
0.275	29	9.7	58	19.5
0.55	171	57.4	196	65.8
1.1	87	29.2	30	10.1
2.2	8	2.7	0.0	0.0
Total	**298**	**100**	**298**	**100**

Chi-squared test P<0,000.

concentrations used for susceptibility tests), we found a synergistic effect in 3.4% and a partially-synergistic effect in 78.9% of the samples tested. Synergism is a positive interaction, in which the combined effect of antimicrobial drugs is significantly greater than their effect when administrated separately.

No antagonistic cases were found in this study. This result reinforces the idea of a positive interaction between the essential oil of *C. citratus* and nystatin, once antagonism is a negative interaction in which the effect of combined substances é significantly smaller than when administrated separately. The indifference percentage was 17.8%, which means that there was no significant interaction between the associated antimicrobial drugs.

The results observed in the present study suggest that there is a positive interaction between those compounds. According to Sookto et al. (2013), the components of the essential oils interfere in the biosynthesis of fungal cell walls, and terpenoids interact with the lipidic components in cell structure, causing an increase in the membrane permeability and electrolytic unbalance. However, Leite et al. (2014) affirm that the mechanism of action of the isolated compound citral does not involve the cell wall or ergosterol. Concerning nystatin, Santos et al. (2017) highlighted that ergosterol is one of the key factors in explaining its antifungal activity. Nonetheless, other membrane proprieties must be considered when approaching the molecular mechanism of action and cytotoxicity of the drug. According to Johnson et al. (2004), it is possible to increase the penetration of the antifungal agent as a result of the antifungal activity either on the cell wall or membrane. Therefore, the interaction of the compounds in this study is probably caused by oil penetration when aided by the nystatin action on the microorganism's cell membrane, or the transport interaction between the compounds, which facilitates the

Table VI. Index of the fractioned inhibitory concentration of essential oil of *Cymbopogon citratus* (DC) Stapf combined with nystatin.

FIC Index	Frequency	%
Antagonism	0	0
Indifferent	53	17.8
Additivity	235	78.9
Synergism	10	3.4
Total	**298**	**100.0**

access of both compounds to the cytoplasmic membrane. Nevertheless, these hypotheses demand more studies to be confirmed.

The essential oil of the plant *Cymbopogon citratus* (DC) Stapf shows fungistatic and fungicidal activity against the yeast from oral cavities. The association of this herbal drug to nystatin potentialized the antifungal effect on isolated samples from oncological patients. The use of this phytotherapic formulation by patients that are already in treatment with nystatin may benefit the procedure, potentializing the antimicrobial power of this drug.

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REFERENCES

ABRANTES MR, LIMA EO, MEDEIROS MAP, MENEZES CP, GUERRA FQS & MILAN EP. 2013. Atividade antifúngica de óleos essenciais sobre leveduras *Candida* não *albicans*. Rev Bras Farm 94: 227-233.

ADAMS RP. 2007. Identification of Essential oil Components by Gas Chromatography/Mass Spectrometry. 4th ed., Carol Stream, Illinois: Allured Publishing Corporation, 804 p.

BETTEGA PVC, CZLUSNIAK GR, PIVA R, NAMBA EL, RIBAS CR, GRÉGIO AMT & ROSA EAR. 2011. Fitoterapia: dos canteiros ao balcão da farmácia. Arch Oral Res 7: 89-97.

BOUKHATEM MN, FERHAT MA, KAMELI A, SAIDI F & KEBIR HT. 2014. Lemon grass (*Cymbopogon citratus*) essential oil as a potent anti-inflammatory and antifungal drugs. Libyan J Med 9: 25431.

BRASIL. 2010. Farmacopéia Brasileira 5ª ed., Agência Nacional de Vigilância Sanitária. Brasília, DF: Anvisa, 899 p.

CANTÓN E, PEMÁN J, VIUDES A, QUINDÓS G, GOBERNADO M & ESPINEL-INGROFF A. 2003. Minimum fungicidal concentrations of amphotericin B for bloodstream *Candida* species. Diagn Microbiol Infect Dis 45: 203-206.

CLSI. 2002. M27-A2. Método de Referência para Testes de Diluição em Caldo para Determinação da Sensibilidade de Leveduras à Terapia Antifúngica: Norma Aprovada 2ª ed., Wayne, Pennsylvania: Clinical Laboratory Standards Institute, 51 p.

DIAZ PI ET AL. 2019. Integrated Analysis of Clinical and Microbiome Risk Factors Associated with the Development of Oral Candidiasis during Cancer Chemotherapy. J Fungi 5: 49.

FERNÁNDEZ-CUENCA F, MARTÍNEZ-MARTÍNEZ L, PASCUAL A & PEREA EJ. 2003. In vitro Activity of Azithromycin in Combination with Amikacin, Ceftazidime, Ciprofloxacin or Imipenem against Clinical Isolates of Acinetobacter baumannii. Chemotherapy 49: 24-26.

GODOY JSR, BONFIM-MENDONÇA PS, NAKAMURA SS, YAMADA SS, SHINOBU-MESQUITA C, PIERALISI N, FIORINI A & SVIDZINSKI TIE. 2012. Colonization of the oral cavity by yeasts in patients with chronic renal failure undergoing hemodialysis. J Oral Pathol Med 42: 229-234.

HASENCLEVER L, PARANHOS J, COSTA CR, CUNHA G & VIEIRA D. 2017. A indústria de fitoterápicos brasileira: desafios e oportunidades. Cien Saude Colet 22: 2559-2569.

HAZEN KC, BARON EJ, COLOMBO AL, GIRMENIA C, SANCHEZ-SOUSA A, DEL PALACIO A, BEDOUT C, GIBBS, DL & THE GLOBAL ANTIFUNGAL SURVEILLANCE GROUP. 2003. Comparison of the Susceptibilities of *Candida* spp. to Fluconazole and Voriconazole in a 4-Year Global Evaluation Using Disk Diffusion. J Clin Microbiol 41: 5623-5632.

JOHNSON MD, MACDOUGALL C, OSTROSKY-ZEICHNER L, PERFECT JR & REX JH. 2004. Combination Antifungal Therapy. Antimicrob Agents Chemother 48: 693-715.

KIDD S, HALLIDAY C, ALEXIOU H & ELLIS D. 2016. Descriptions of Medical Fungi. 3ª ed. Canberra, Austrália: The National Library of Australia, 278 p.

KURIYAMA T, WILLIAMS DW, BAGG J, COULTER WA, READY D & LEWIS MAO. 2005. *In vitro* susceptibility of oral *Candida* to seven antifungal agents. Oral Microbiol Immunol 20: 349-353.

LEITE MCA, BEZERRA APB, SOUSA JP, GUERRA FQS & LIMA EO. 2014. Evaluation of Antifungal Activity and Mechanism of Action of Citral against *Candida albicans*. Evid Based Complement Alternat Med 11: 1-9.

LI X, LEI L, TAN D, JIANG L, ZENG X, DAN H, LIAO G & CHEN Q. 2012. Oropharyngeal *Candida* colonization in human immunodeficiency virus infected patients. APMIS 121: 375-402.

LIMA IO, OLIVEIRA RAG, LIMA EO, FARIAS NMP & SOUZA EL. 2006. Atividade antifúngica de óleos essenciais sobre espécies de *Candida*. Rev Bras Farmacogn 16: 197-201.

LOPEZ C, GIRO L, RAMOS L, RAMADÁN S & BULACIO L. 2005. Comparación de diferentes métodos para la identificación de espécies del género *Candida*. Rev Argent Microbiol 37: 16-21.

LORENZI H & MATOS FJA. 2002. Plantas Medicinais do Brasil: nativas e exóticas cultivadas, Nova Odessa, SP: Instituto Plantarum, 576 p.

LUCENA BFF ET AL. 2015. Avaliação da atividade antibacteriana e moduladora de aminoglicosídeos do óleo essencial de *Cymbopogon citratus* (DC.) Stapf. Acta Biol Colomb 20: 39-45.

MARTINS PM, MELO EC, BARBOSA LCAB, SANTOS RHS & MACHADO MC. 2002. Influência da temperatura e velocidade do ar de secagem no teor e composição química do óleo essencial de capim-limão. Acta Hortic 155-160.

MEIS JF & CHOWDHARY A. 2018. *Candida auris*: a global fungal public health threat. Lancet Infect Dis 18: 1298-1299.

PANIZO MM, REVIÁKINA V, DOLANDE M & SELGRAD S. 2009. *Candida* spp. *in vitro* susceptibility profile to four antifungal agents. Resistance surveillance study in Venezuelan strains. Med Mycol 47: 137-143.

PIERCE CG, SRINIVASAN A, UPPULURI P, RAMASUBRAMANIAN AK & LÓPEZ-RIBOT JL. 2013. Antifungal therapy with an emphasis on biofilms. Curr Opin Pharmacol 13: 726-730.

QUINDOS G, GIL-ALONSO S, MARCOS-ARIAS C, SEVILLANO E, MATEO E, JAUREGIZAR N & ERASO E. 2019. Therapeutic tools for oral candidiasis: Current and new antifungal drugs. Med Oral Patol Oral Cir Bucal 1;24: e172-180.

ROCHA RP, MELO EC, DEMUNER AJ, RADÜNZ LL & BRAUN H. 2012. Effect of drying air velocity on the quality of essential oil From Lemon grass. Gl Sci Technol 5: 23-31.

SAHAND IH, MAZA JL, ERASO E, MONTEJO M, MORAGUES MD, AGUIRRE JM, QUINDÓS G & PONTON J. 2009. Evaluation of CHROM-Pal medium for the isolation and direct identification of *Candida dubliniensis* in primary cultures from the oral cavity. J Med Microbiol 58: 1437-1442.

SANTOS A, PADUAN RH, GAZIN ZC, JACOMASSI ED, OLIVEIRA PS, CORTEZ DAG & CORTEZ LER. 2009. Determinação do rendimento e atividade antimicrobiana do óleo essencial de *Cymbopogon citratus* (DC). Rev Bras Farmacogn 19: 436-441.

SANTOS AG, MARQUÊS JT, CARREIRA AC, CASTRO IR, VIANA AS, MINGEOT-LECLERCQ M-P, ALMEIDA RFM & SILVA LC. 2017. The molecular mechanism of Nystatin action is dependent on the membrane biophysical properties and lipid composition. Phys Chem Chem Phys. 19: 30078-30088.

SANTOS AS, ALVES SM, FIGUEIREDO FJC & ROCHA NETO OG. 2004. Descrição de sistema e métodos de extração de óleos essenciais e determinação de umidade de biomassa em laboratório. Comunicado Técnico-Embrapa 99. 1-6.

SIMÕES RJ, FONSECA P & FIGUEIRA MH. 2013. Infecções por *Candida* spp. na cavidade oral. Odontol Clín-Cient 12: 19-22.

SHOLAPURKAR A, PAI K & RAO S. 2009. Comparison of efficacy of fluconazole mouthrinse and clotrimazole mouthpaint in the treatment of oral candidiasis. Aust Dent . 54: 341-346.

SOOKTO T, SRITHAVAJ T, THAWEBOON S, THAWEBOON B & SHRESTHA B. 2013. *In vitro* effects of *Salvia officinalis* L. essential oil on *Candida albicans*. Asian Pac J Trop Biomed 3: 376-380.

TYAGI AK & MALIK A. 2010. Liquid and vapour-phase antifungal activities of selected essential oils against *Candida albicans*: microscopic observations and chemical characterization of *Cymbopogon citratus*. BMC Complement Altern Med 10: 1-11.

WILLE MP, GUIMARAES T, FURTADO GHC & COLOMBO AL. 2013. Historical trends in the epidemiology of candidaemia: analysis of an 11-year period in a tertiary care hospital in Brazil. Mem Inst Oswaldo Cruz 108: 288-292.

WILSON ND, IVANOVA MS, WATT RA & MOFFAT AC. 2002. The quantification of citral in lemongrass and lemon oils by near-infrared spectroscopy. J Pharm Pharmacol. 54: 1257-1263.

YU S-Y ET AL. 2019. *Candida* isolates causing refractory or recurrent oropharyngeal candidiasis in 11 hospitals in China. Infect Drug Resist 12: 865-875.

ZORE GB, THAKRE AD, JADHAV S & KARUPPAYIL SM. 2011. Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. Phytomedicine 18: 1181-1190.

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LUIZ FRANCISLEY DE PAIVA[1]
https://orcid.org/0000-0001-6497-7468

ANA BEATRIZ A. TEIXEIRA-LOYOLA[1]
https://orcid.org/0000-0002-9089-7012

TAYLOR B. SCHNAIDER[1]
https://orcid.org/0000-0002-2784-9659

ANGÉLICA CRISTINA DE SOUZA[2]
https://orcid.org/0000-0002-4013-6354

LIDIANY M. ZACARONI LIMA[3]
https://orcid.org/0000-0001-5785-5526

DISNEY R. DIAS[4]
https://orcid.org/0000-0002-1010-1484

[1]University of the Vale do Sapucaí, Laboratory of Basic Research, Av: Alfredo Custódio de Paula, 320, Centro, 37553-068 Pouso Alegre, MG, Brazil

[2]Federal University of Lavras, Department of Food Microbiology, Aquenta Sol, 37200-900 Lavras, MG, Brazil

[3]Federal University of Lavras, Department of Chemistry, Aquenta Sol, 37200-900 Lavras, MG, Brazil

[4] Federal University of Lavras, Department of Food Science, Aquenta Sol, 37200-900 Lavras, MG, Brazil

Correspondence to: **Luiz Francisley de Paiva**
E-mail: francisleybiologo@yahoo.com.br

Author contribution

LFP: conceptual ideas, methodology, investigation, formal analyses, writing the manuscript. ABATL: conceptual ideas, methodology, supervision, writing the manuscript. TBS: conceptual ideas, methodology, supervision, writing the manuscript. ACS: methodology, investigation, writing the manuscript. LMZL: methodology, investigation, writing the manuscript. DRD: supervision, writing the manuscript. All authors read and approved the final version of the article.