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Original Article

ANTIMYCOBACTERIAL, ANTIMICROBIAL AND ANTIFUNGAL ACTIVITIES OF GERANIUM OIL-LOADED NANO CAPSULES

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ABSTRACT

Objective: The aim of this study was to perform the first ever investigation of the effect of activities in the nano capsules containing Geranium oil (NC1) against different species of pathogens such as *Mycobacterium genus* (both fast growing and slow growing), bacterial, and yeasts.

Methods: The GO was analyzed by GC and GC/MS. Nano capsule suspensions (NC) were prepared by interfacial deposition of a preformed polymer method and the MICs were determined for the antimycobacterial, antimicrobial, and antifungal activities.

Results: GO-loaded nano capsules (NC1) presented nano metric mean diameters (188 nm), polydispersity indices below 0.149, pH (5.5), and zeta potentials (about-10.8 mV). The MICs were determined for the antimycobacterial, antimicrobial, and antifungal activities. The NC1 was effective to *Mycobacterium smegmatis* (149.7 µg/ml), *M. abscessos* (35.9 µg/ml), *M. massiliense* (35.9 µg/ml), *M. avium (*71.8 µg/ml), *Enterococcus faecalis*, *Streptococcus sp.* (149.7 µg/ml) and *Listeria monocytogenes* (35.9 µg/ml). The NC1 was able to significantly reduce the number of cells of *C. albicans* (by approximately 5 log), 4 log the number of cells of *C. dublinensis*, *C. glabrata,* and *C. krusei,* and 2 log the number of cells of *C. parapsilosis* compared to the control group.

Conclusion: Our study showed that the geranium oil-loaded nano capsules have antimycobacterial activities similar to free oil. The GO was effective in inhibiting the formation of germ tubes of *Candida albicans*, yet the nano capsule containing GO failed to inhibit the formation of this important virulence factor.

Keywords: Nano capsule**,** Geranium oil, Antimycobacterial, Antimicrobial, Antifungal, Nanotechnology.

INTRODUCTION

Recently, the clinical use of essential oils has expanded worldwide to include therapy against various kinds of diseases, as leishmaniasis, malaria, Chagas, skin disorders, respiratory diseases among other. The antimicrobial and antifungal properties of essential oils have been documented and have acquired greater importance. Essential oils can be effective in the treatment or prevention of parasitic, bacterial and fungal diseases due to properties, such as low density and rapid diffusion across cell membranes [1-7].

The oil *Pelargonium graveolens*, also known as geranium oil or mauve is extracted from the tree *Pelargonium odorantissimum* originating from South Africa. The *Pelargonium genus* (Geraniaceae) is represented by many essential oil producing species: *P. graveolens*, *P. odoranissimum*, *P. zonale*, *and P. roseum*. Geranium oil (GO) is obtained from the leaves, flowers, and stalks using steam or hydrodistillation. The GO has historically been used in the treatment of dysentery, hemorrhoids, inflammation, heavy menstrual flows, and even cancer [8]. The French medicinal community currently treats diabetes, diarrhea, gallbladder problems, gastric ulcers, liver problems, sterility, and urinary stones with GO. The main constituents responsible for biological activity are citronellol, geraniol, linalool, isomenthone, nerol, and citronellyl formate [9]. However, because of their chemical complexity, susceptibility to degradation, and volatility and insolubility in water, it is necessary to employ procedures to improve the oil's stability, contributing to the product's effectiveness.

Nanostructured systems appear as a potential system for asset management with lipophilic character. An important advantage of these systems is their small size (below 1 mm). In addition, further advantages are the possibility of increasing the effectiveness and stability of formulations or active substances, as well as their gradual release in adequate doses. Thus, one of the most promising areas for the use of nano capsules is the vectorization of essential oils with antimicrobials activities [10-12].

Little is known about the biological activities of GO. The search for new antifungal and antimicrobial agents is an important field. The prevalence of resistance among key microbial pathogens is increasing at an alarming rate worldwide [13]. The antibacterial activity of essential oils depends on their chemical composition, climate, season, geographical conditions, harvest period, and distillation technique. Bacteria have a genetic ability for transmitting and acquiring resistance to drugs. Recently, antimycobacterial activity of oil *Melaleuca alternifolia* and nanoparticles across the different strains of mycobacteria were evidenced [14].

The fungi, like *Candida*, are opportunistic etiological agents. This means that the infection and the expansion occur only in the event of a predisposition of the host organism [15-18]. Studies indicate that geraniol, the major constituent of GO, shows activity against gram-negative bacteria and some *Candida* species [19]. Studies have revealed that nanostructures could be a delivery system to enhance the stability and water solubility of essential oils [20,21]. The advantages compared with conventional drug-delivery systems

include improved efficacy, reduced toxicity, protection of active compounds, and enhanced biocompatibility [22].

Szweda et al. [23] investigated the in vitro antifungal activity of selected essential oils, ethanolic extracts of propolis and silver on TiO2 nanoparticles dropped against azole-resistant C. albicans, C. glabrata and C. krusei clinical isolates. Aiming to expand the utilization of GO and seeking to increase the use of this kind of medical form and take advantage of proven pharmacological actions of the essential oil, we evaluated the effect of GO-loaded nano capsules (NC1) against different species of pathogens, such as Mycobacterium genus (both fast growing as slow growing), bacterial, and yeasts for the first time.

MATERIALS AND METHODS

Acquisition of GO and reagents

The geranium essential oil (Lot STD1012) was purchased from Seiva Brázilis Ativos Naturais Ltd, São Paulo, Brazil; Dimethyl sulfoxide (DMSO) was used to dilute GO. Amphotericin B, for an antifungal activity, and Amikacin, for antimicrobial activity, was used as controls in the experiments.

Geranium oil analysis

Oil composition and yield were analyzed using the gas chromatography (GC) carried out using an Agilent Technologies 6890N GC-FID system, equipped with a DB-5 capillary column (30 m x 0.25 mm x 2.5 mm film thickness) and connected to a flame ionization detector (FID). The injector and detector temperatures were set to 250 °C. The carrier gas was helium with a flow rate of 1.3 ml/min. The thermal programmer was 100-280 °C at a rate of 10 °C/min. Two replicates of samples were processed in the same way. The injection volume of the GO was 1 µl [24]. GC-Mass Spectroscopy (GC-MS) analyses were performed on an Agilent Technologies Auto System XL GC-MS operating in the EI mode at 70 eV, equipped with a split/split less injector (250 °C). The transfer line temperature was 280 °C. Helium was used as a carrier gas (1.5 ml/min) and the capillary columns used were an HP 5MS (30 m x 0.25 mm x 2.5 µm film thickness) and an HP Innowax (30 m x 0.32 mm i.d., film thickness 0.50 µm). The temperature programmed was the same as that used for the GC analyses. The injected volume was 1 µl of the essential oil.

Identifying the constituents of GO was performed on the basis of retention index (RI), determined with reference to the homologous series of n-alkanes C7-C30, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature. The relative amounts of individual components were calculated based on the CG peak area (FID response).

Preparation of the formulation

Nano capsule suspensions (NC) were prepared (n = 3) by interfacial deposition of the preformed polymer method. Briefly, an organic phase composed of GO (0.9 g), sorbitan mono oleate (0.192 g), poly (e-caprolactone) (0.25 g), and acetone (67.0 ml) was added to an aqueous solution (133.0 ml) containing polysorbate 80 (0.192 g) and kept under moderate magnetic stirring for 10 minutes. The Nano capsule containing GO was labeled NC1 and a control Nano capsule (NC2) containing the same constituents of NC1 was produced but without GO. This Nano capsule was added to the (0.9 g) medium-chain triglycerides (MCT). The organic solvent was then eliminated from both NC1 and NC2 in a rotary evaporator (Fisatom, São Paulo, Brazil) at 60 rpm and 30-35 °C temperature. The final volume of the formulations was fixed in 25 ml to obtain a concentration of 1% of oil (10 mg/ml).

The particle sizes and polydispersity index (n=3) were measured by photon correlation spectroscopy (Malvern Zetasizer/Nanosizer®) and zeta potential values were measured by electrophoretic mobility, after dilution of 20 µl samples in 20 ml of NaCl (1 mM). The pH value of the nano capsules was analyzed by Digimed direct readings potentiometer (São Paulo, Brazil) at room temperature.

Dilution of GO and nano particles

The density of the GO (0.92 g/ml) was determined, and the same dilution (1: 1) was performed in DMSO to reach a concentration of 460 mg/ml(Solution I). Afterwards, dilution was made at 1:100 in a Middlebrook 7H9 base medium (antimycobacterial activity) or a Mueller-Hinton broth (antimicrobial activity) to yield a concentration of 4.600 µg/ml(Solution II). Then, 50 µl (antimycobacterial activity) or 200 µl (antimicrobial activity) of solution II were added to the first well of the microplates and, after homogenization, were moved to the same volume and so on, yielding final concentrations of: 2.300, 1.150, 575, 287.5, 149.7, 71.8, 35.9, 17.9, 8.9, 4.4 µg/ml. NC1 (575 µg/ml) and NC2 were added to the first well and after homogenization were transferred to the second, and so on to give final concentrations of 287.5, 149.7, 71.8, 35.9, 17.9, 8.9, 4.4 µg/ml, respectively.

Antimycobacterial activity

For the antimycobacterial activity of GO and NC1, four strains of genus *Mycobacterium*, three fast growing strains (*Mycobacterium smegmatis* ATCC 700084, *M. abscessus* ATCC 19977 and *M. massiliense* ATCC 48898) and one slow growing strains (*M. avium* LR541CDC) were used in the study. The mycobacterial strains were thawed, picked to Lowenstein-Jensen medium, and kept in an incubator until visible growth of the colonies. Subsequently, colonies were suspended in a Middlebrook 7H9 base medium supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) (Difco Laboratories, Detriod, Michigan) and 0.2% glycerol (MD7H9) then incubated for 3 to 7 days at 35±2 °C in a tube containing glass beads. This suspension was then homogenized in a vortex shaker and standardized to 0.5 on the Macfarland scale; the fast-growing mycobacteria suspension was further diluted in MD7H9 to a concentration of 1 x 10^5 UFC/ml. From this bacterial suspension, the assay was performed based on the protocol M7-A6 [25]. The assay was performed in microtiter plates of 96 wells in triplicate. Serial dilutions were performed GO, NC1, and NC2 as described above. A volume of 50 µl of each dilution was added to the well along with 50 µl of each bacterial suspension. Were also carried out controls of the medium, the microorganism, GO, NC1, and NC2. The plates were sealed with parafilm before being sterilely capped to prevent contamination occurred and volatilized oil. Subsequently, the plates were incubated at 35±2 °C for 5 to 7 days in a humid chamber. The results were observed by the formation of bacteria dotted at the bottom of the wells.

Antimicrobial activity

To evaluate the antimicrobial activity of GO and NC1, the following microorganisms were used: *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644, *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 e *Pseudomonas aeroginosa* ATCC 340. Clinical isolates of *Streptococcus sp*, *Staphylococcus aureus*, *Klebsiella pneumoniae* KPC+(HCPA), *Klebsiella pneumoniae* KPC+(USP), *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Enterococcus sp,* and *Shigella flexneri.* The determination of the minimum inhibitory concentration was performed based on the protocol M7-A7 [26]. The bacterial suspension was prepared in saline with a turbidity equivalent to tube 0.5 of the MacFarland scale (1 x 10^8 UFC/ml). Then, this suspension was diluted at 1:100 in a Mueller-Hinton broth, yielding as inoculum 1 x 10^6 UFC/ml. This suspension was inoculated with 10 µl (1 × 10^4 UFC) into each well already containing 200 µl of different concentrations of the GO, NC1, and NC2 as described above. The microplates were incubated at 35±2 °C for 24 hours, under aerobic conditions. The MIC (Minimal Inhibitory Concentrations) was defined as the lowest concentration of compounds that inhibits bacterial growth. This test was performed in triplicate on separate occasions. The 2,3,5-triphenyltetrazolium chloride was used as an indicator of bacterial growth.

Antifungal activity

The fungal isolates used in the study included one strain of each species: the yeasts *Candida albicans* ATCC 14053, *Candida tropicalis* ATCC 66029, *Candida glabrata* ATCC 66032, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Candida geochares* ATCC 36852, *Candida magnoliae* ATCC 201379, *Candida kefyr* ATCC 66028, *Candida guilliermondii* ATCC 6260, *Candida catenulata* 10565, *Candida membranaefaciens* ATCC 201377, *Candida lusitaneae* ATCC

42720, *Candida dublinienesis* CBS 7987, and *Malassezia furfur* ATCC 14521. All strains were inoculated on sabouraud dextrose agar and incubated at 35±2 °C for 24 hours before the tests. For *M. furfur* was added in sabouraud agar olive oil. Subsequently, five colonies were picked with a diameter of approximately 1 mm, which were suspended in 5 ml of sterile 0.85% saline. The resulting suspension was placed on a vortex mixer for 15 seconds, and the cell density was adjusted using a spectrophotometer, adding sufficient saline to obtain equivalent transmittance to that of a standard solution in 0.5 McFarland scale at 530 wavelength. This procedure provided a standard yeast suspension containing 1 x 10^6 to 5 x 10^6 cells per ml. The suspension was produced making a 1:50 dilution followed by a 1:20 dilution of the standard suspension in RPMI 1640 medium supplemented with L-glutamine (Sigma Chemical Co., St Louis, Missouri, USA), buffered with MOPS [acid 3-(N-morpholino-propane sulfonic acid)] (0.165 mol/l) (Sigma), pH 7.0, to give the inoculum 2-fold concentrated used in the test (1 x $10^3$to 5 x 10^3 UFC/ml). After 100 µl of each concentration of GO, NC1, and NC2 (as described above) was diluted in RPMI, each was then transferred to a well and added to 100 µl of inoculum. The final concentration after inoculation test was 0.5 x 10^3 the 2.5 x 10^3 UFC/ml, as recommended by the document M27-A3 [27]. The microplates were incubated at 37 °C for 48 hours in triplicate. The MIC was determined based on the lowest concentration of oil which completely inhibited the growth of yeasts. For a better understanding of the antifungal activity of the NC1 and NC2 front, the yeasts were held in the broth macro dilution method [28,29]. Briefly, the NC1 and NC2 were diluted to a final concentration of 50%, directly in RPMI 1640 supplemented with L-glutamine (Sigma Chemical Co., St Louis, Missouri, USA), buffered with 0.165 mol/l MOPS [acid 3-(N-morpholino-propane sulfonic acid)] (0.165 mol/l) (Sigma), pH 7.0. The method also follows the recommendations of the protocol M27-A (CLSI), but with modifications: sterile 11 x 70 mm tubes were used and the final volume in each tube was 1 ml; the incubation time with the NC1 and NC2 was increased to 72 hours and the quantification of the number of colonies present was held at the beginning of treatment and at the end of it. For this, the fungi were inoculated on Sabouraud dextrose agar and incubated at 35±2 °C for 24 hours before the tests, the results were expressed as mean±SD of log/UFC/ml. All experiments were performed in triplicate, being used as growth control only for the suspension of each fungus.

Inhibition of germ tube formation

The suspension of *C. albicans* ATCC 14053 was prepared from colonies growing on Sabouraud agar for 24h dispersed in 0.85% saline. The suspension was standardized by spectrophotometer at 530 nm resulting in a concentration of yeast cells of 1 x 10^6–5 x 10^6 UFC/ml. A volume of 100 µl of citrated human plasma was added to each well of a sterile microplate. Then, 100 µl of GO at concentrations of 4.4, 8.9 e 17.9 µg/ml (MIC/2, MIC, MIC 2x), NC1 with 287.5 µg/ml and NC2 were added to the wells. Subsequently, 10 µl of the yeast suspension were added to each well. Control of germ tube formation was performed only with citrated human plasma and a yeast suspension. To verify the inhibition of germ tube formation, Amphotericin B (50 µg/ml) was used. The plate was incubated at 35 °C±2 for 2 hours. Inhibition of germ tube formation was estimated directly in a Neubauer chamber, and the results were expressed as percentage (%). All samples were tested in triplicate in two independent experiments.

Statistical analysis

Results were subjected to analysis of variance (ANOVA) and Tukey´s test to verify the accuracy of the data. Values $p<0.05$ were considered statistically different. In the comparisons between two variables, we used the nonparametric Wilcoxon Test; when comparisons involved three or more variables, we used the nonparametric Kruskal-Wallis.

RESULTS AND DISCUSSION

GC analysis

The main components were citronellol (31.37%) and geraniol (10.34%). The chemical composition of the oil was similar to other previously studied species. Our study showed that the citronellol (31.37%), geraniol (10.34%), citronellyl formate (6.51%), and α-guaiene (5.13%) were the major compounds in the oil, with minor quantities of geranyl tiglate (2.07%) and geranial (2.18%). Other constituents were found in smaller amounts (<2%). The results of chemical analysis of the GO are presented in table 1.

Table 1: Composition of the geranium essential oil

Compounds	RI[a]	RI[b]	Geranium oils
			Area (%)
Linalool	1098	1098	3.46
Isomenthone	1164	1159	4.21
Citronellol	1228	1228	31.37
Geraniol	1255	1253	10.34
Citronellyl formate	1275	1275	6.15
α-Guaiene	1439	1443	5.13
6,9-Guaiadiene	1465	1465	5.09

Relative proportions of the essential oil constituents were expressed as percentages. [a]Retention indices from literature [24]. [b]Retention indices experimental (based on homologous series of *n*-alkane C_7-C_{30}).

The rose geranium oil consisted mainly of oxygenated monoterpenes and oxygenated sesquiterpenes. The data presented here are consistent with previous reports of Boukhatem *et al.* [30], which demonstrated that geranium oils are characterized by citronellol (29.13%) and geraniol (12.6%). Some differences can be observed in the chemical composition of geranium oil; this is due to a number of factors including differences in local climatic and geographical conditions, season at the collection, and fertilization [31, 32].

The essential oils are composed of specific cells found in the leaves, flowers, seeds, stems and roots. The complex mixtures of volatile substances such as alcohols, esters, aldehydes, ketones, phenols, among others, are important properties and some of these hydrophobic components are responsible for antimicrobial and antifungal activities. The main constituents responsible for the biological activity of GO are citronellol, geraniol, linalool, isomenthone, nerol and citronellyl formate. Due to these components, the essential oil has a strong and antibacterial effect [33, 34].

Physicochemical properties of nano capsules

The NC containing essential GO (NC1) and NC containing MCT (NC2) appeared macroscopically homogeneous and opalescent. The physicochemical characteristics of the formulations are presented in table 2.

GO-loaded nano capsules presented nanometric mean diameters (188 nm) as well as polydispersity indices below 0.149 indicating an adequate homogeneity of these systems. The formulation showed acid pH (5.5) and negative zeta potentials (about-10.8 mV). The negative zeta potential values presented by the samples are related to the presence of polysorbate 80, presenting a negative surface density of charge due to the presence of oxygen atoms in the molecules. GO, as well as other essential oils, has a pronounced odor that sometimes should be masked in formulations. This way, we analyzed the odor of our formulation, comparing the intensity of their odor with the pure essential oil. It is important to point out that the incorporation of GO in nano capsules allows for a considerable reduction in the odor of the oil. This result is in agreement with the ability of polymeric nano capsules to mask physicochemical properties of some substances [35].

Antimycobacterial and antimicrobial activity

Evaluation of the activity against *Mycobacterium genus* strains was analyzed. NC1 and GO showed to be active against *M. abscessos, M. massiliense, M. Smegmatis* and *M. avium.* The antimicrobial activity was also determined against different bacteria. The MIC demonstrated that NC1 and GO were able to inhibit bacterial growth in small concentrations for these strains. These results are shown in table 3.

Table 2: Physicochemical properties

Formulation	Particle size (nm)	PDI*	Zeta potential (mV)	pH
NC1	188±0.025	0.149±0.009	-10.8±0.08	5.5±0.1
NC2	233.3±0.030	0.185±0.011	-10.7±0.09	5.8±0.09

* PDI: polydispersity index.

Table 3: Antimycobacterial and antimicrobial activity (MIC µg/ml) of geranium essential oil, nanostructures using microdilution method

Microorganism	Geranium Oil	NC1	NC2
	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)
M. abscessus ATCC19977	35.9	35.9	ND
M. smegmatis ATCC 700084	35.9	149.7	ND
M. massiliense ATCC 48898	35.9	35.9	ND
M. avium LR541CDC	17.9	71.8	ND
Enterococcus faecalis ATCC 29212	149.7	149.7	ND
Streptococcus sp-IC	149.7	149.7	ND
Sthaphylococcus aureus-IC	149.7	ND	ND
Listeria monocytogenes ATCC 7644	149.7	35.9	ND
Pseudomonas aeroginosa ATCC 340	149.7	ND	ND
Salmonella enteritidis-IC	149.7	ND	ND

ND: not detected**.**

After evaluating the antimycobacterial activity, it was observed that GO had activity against *M. abscessos, M. massiliense, M. Smegmatis,* and *M. avium* with low MIC values (17.9–35.9 µg/ml). The antimicrobial activity was also evaluated and observed for *S. aureus*, *Streptococcus, Sthaphylococcus, Listeria monocytogenes, Pseudomonas aeruginosa, and Salmonella enteritidis* (149.7 µg/ml). The obtained results for antimicrobial activity are also in accordance with the literature, showing that geranium oil has antimicrobial properties against all tested strains. The GO obtained from *Pelargonium graveolens* shows a very strong activity against the standard strain *S. aureus* (ATCC 433000) and also against the examined strains *S. aureus* obtained from the clinical materials. The values of MIC against clinical *S. aureus* strains ranged from 0.25 µg/ml to 2.5 µg/ml [36]. Prabuseenivasan *et al.* [37] reported that the oil obtained from *Pelargonium graveolens* was used at concentrations higher than 12.8 mg/ml inhibited the growth of the *S. aureus* ATCC 25923. In another study, the aim was to determine the antimicrobial activity of GO against Gram-negative bacterial clinical strains. The microdilution broth method was used to check the inhibition of microbial growth at various concentrations of GO. The tested geranium oil was efficacious against Gram-negative pathogens [38].

In our investigation, it has been found that NC1 is effective against *M. abscessos, M. massiliense, M. Smegmatis,* and *M. avium* with low MIC values (35.9–149.7 µg/ml) and *E. faecalis* ATCC29212, *Streptococcus sp*-IC and *L. monocytogenes* ATCC 7644. The NC1 showed no activity against other strains tested. Recently, Souza *et al.* [14] reported antimycobacterial activity of *Melaleuca alternifolia* nanoparticles with MICs ranged from 0.002 to 2.5%. To date, there are no reported studies using nano capsules containing GO with antimycobacterial activity for comparison.

Antifungal activity

The determination of the Minimal Inhibitory Concentration (MIC) was measured after dilution of GO following the M27A3 protocol; the results can be found in table 4**.**

ND: not detected

One can show that the GO showed a similar MIC (8.9 µg/ml) for the strains of *C. albicans, C. kefyr, C. dubliniensis, C. glabrata,* and *C. lusitaneae.* Interestingly, it also showed the same MIC to *Malassezia furfur*. *C. krusei* was observed to have a MIC of 17.9 mg l and *C. guilliermondii* an MIC of 149.7 mg l. This MIC value is observed for all the yeasts studied after contact with NC1. Because of this, the broth macrodilution method was used to evaluate the activity of NC1. The antifungal activity by the macrodilution method showed a reduction in the number of colony forming units (CFU/ml) between different species of *Candida* tested within 72 hours. The NC1 was able to significantly reduce the number of cells of *C. albicans* (CA) by approximately 5 log, 4 log the number of cells of *C. dublinensis* (CD), *C. glabrata* (CG) and *C. krusei* (CK) and 2 log the number of cells of *C. parapsilosis* (CP) compared to control (fig. 1).

Table 4: Antifungal activity (MIC µg/ml) of geranium essential oil, nanostructures using the micro dilution method

Microorganism	Geranium Oil	NC1	NC2
	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)
C. tropicalis ATCC 66029	575	>149.7	ND
C. geochares ATCC 36852	1150	>149.7	ND
C. albicans ATCC 14053	8.9	>149.7	ND
C. kefyr ATCC 66028	8.9	>149.7	ND
C. parapsilosis ATCC 22019	1150	>149.7	ND
C. guilliermondii ATCC 6260	149.7	>149.7	ND
C. dublinienesis CBS 7987	8.9	>149.7	ND
C. glabrata ATCC 66032	8.9	>149.7	ND
C. krusei ATCC 6258	17.9	>149.7	ND
C. lusitaneae ATCC 42720	8.9	>149.7	ND
C. membranafaciens ATCC 201377	2300	>149.7	ND
Malassezia furfur ATCC 14521	8.9	>149.7	ND

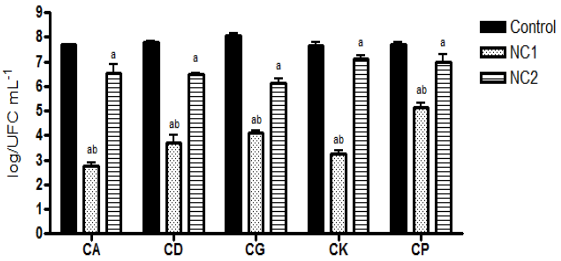

Fig. 1: Antifungal activity of the nano capsules containing Geranium oil after 72 hours incubation performed method by the macrodilution against different *Candida* species. *Candida albicans* (CA), *Candida dublinensis* (CD), *Candida glabrata* (CG), *Candida krusei* (CK) *and Candida parapsilosis* (CP). Values were statistically significant at p<0.05 when compared to [a](growth control with NC1 and NC2) and [b](NC1 with NC2). Data are expressed as means±SD of log/UFC/ml of at three independent experiments

Fluconazole (FLC) susceptibility of isolates of *Candida spp*., (n = 42) and efficacy as well as the mechanism of anti-Candida activity of three constituents of geranium oil were evaluated. No fluconazole resistance was observed among the clinical isolates tested. Geraniol and geranyl acetate were equally effective; fungicidal at 0.064% v/v concentrations, i.e., MICs (561 µg/ml and 584 µg/ml, respectively) and killed 99.9% inoculum within 15 and 30 min of exposures respectively [16]. Oliveira *et al.* [39] investigated the activity of the essential oil of *Cymbopogon winterianus* against fifteen strains of *C. albicans* by MIC. The MIC was determined by the microdilution method. The phytochemical analysis of the oil showed the presence of citronellal (23.59%), geraniol (18.81%) and citronellol (11.74%). The GO showed antifungal activity, and the concentrations 625 µg/ml and 1.250 µg/ml inhibited the growth of all strains tested and it was fungicidal, respectively. These results corroborate with the ours, where the GO showed high antifungal activity against *C. albicans* ATCC 14053, *C. kefyr* ATCC 66028, *C. dubliniensis* CBS 7987, *C. glabrata* ATCC 66032, *C. lusitaneae* ATCC 6258 *and Malassezia furfur* ATCC 14521. Recently, Szweda *et al.* [23] revealed that essential oils, propolis and silver nanoparticles represent a high potential for controlling and prevention candidiasis.

Inhibition of germ tube formation

The effect of GO, NC1, and NC2 for germ tube formation was evaluated using a suspension of yeast in human plasm. GO values relating to the determined MIC microdilution test, half the MIC, and two times the MIC were employed. As for the NC1 and NC2, the same values as for the macrodilution tests were used. As a positive control, Amphotericin B (50 µg/ml) was used. After the incubation period of 2 hours, the treated yeast was quantified microscopically in a Neubauer chamber. It was observed that the concentration of GO related to MIC and twice the MIC could significantly inhibit germ tube formation in *C. albicans*. In NC1, NC2, and the negative control, it was possible to observe the formation of characteristic structures of the germ tube indicating no inhibition of their formation (fig. 2).

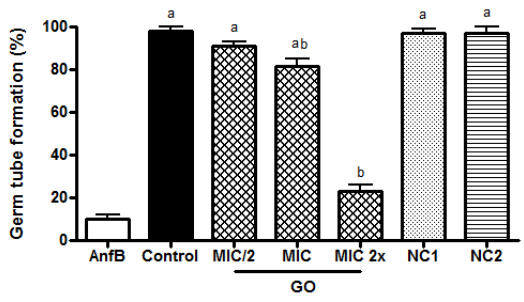

Fig. 2: Inhibition of germ tube formation (%). Values were statistically significant at p<0.05 when compared to [a](AnfB with growth control, GO, NC1 and NC2) and [b](growth control, GO, NC1 and NC2). Data are expressed as means±SD of at three independent experiments

C. albicans is a polymorphic fungus that can present various morphologies for better adaptation to the environment. The formation of hyphae, pseudohyphae, and chlamydospores are important in their persistence in the site of infection and resistance to antifungal drugs [40]. The germ tube is the passage from the yeast form to the filamentous form of the fungus, where this process helps the yeast to penetrate and adhere more easily in cells. In our study, it was possible to observe that the GO was able to inhibit germ tube formation significantly when the concentration used was MIC/2 and MIC; however, such inhibition became more significant when the MIC value was doubled.

Budzyńska *et al.*[41] proved that GO showed an inhibitory effect on germ tube formation in 95–100% at a concentration of 0.097% (v/v) of the cells compared with the control using RPMI supplemented with fetal bovine serum. In another study, the oil of *Lavandula luisieri* showed an inhibition of 95% more than the tube formation [42]. However, NC1 has not inhibited germ tube formation of *C. albicans*. This may be due to the short exposure of the NC1, since this test was realized within two hours. To solve this problem, an alternative would be the use of nano emulsions because they are systems in which the GO could be more easily released. Thus, the contact of GO against the fungus would be faster and would possibly exert its effect. Nano capsules are used to increase the solubilization and absorption of lipophilic drugs [43]. These systems function as carriers, releasing substances of low water solubility that could be associated with oil droplets nanometers in size and/or system interface [44].

CONCLUSION

The analysis of the geranium oil presented the citronellol and geraniol as major components. To prevent degradation of these compounds and increase their stability, nano capsules containing the oil were successfully produced. The obtained particles showed spherical conditions and mean diameters smaller than 200 nm. The physicochemical characteristics showed homogenous formulation, with polydispersity index and zeta potential suitability. Our study showed for the first time that the geranium oil-loaded nano capsules have antimycobacterial and antimicrobial activities similar to free oil. However, NC1 was not effective in inhibiting the formation of germ tubes of *Candida albicans*.

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CONFLICT OF INTERESTS

Declared None

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