

Improved in vitro and in vivo Anti-*Candida albicans* Activity of *Cymbopogon nardus* Essential Oil by Its Incorporation into a Microemulsion System

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Purpose: Vulvovaginal candidiasis (VVC) is an opportunistic fungal infection that adversely affects a woman's health, due to unpleasant symptoms, therapeutic challenges, and the emergence of resistant strains. The association of natural products and nanotechnology is important to improve the antifungal potential of medicinal plants. We aimed to evaluate the in vitro and in vivo anti-*Candida albicans* activity of unloaded (EO) and loaded (ME+EO) essential oil of *Cymbopogon nardus* in the microemulsion (ME).

Methods: The chemical analysis of the EO was performed by gas chromatography-mass spectrometry. The ME and ME+EO were characterized by scattering, zeta potential, polarized light microscopy, rheological assays, mucoadhesiveness and transmission electronic microscopy. The in vitro antifungal activity of the EO and ME+EO were evaluated by microdilution technique. The toxicity of EO and ME+EO was analyzed on human cell line HaCat and using alternative model assay with *Artemia salina*. The experimental in vivo VVC was performed in female mice (C57BL/6).

Results: The main compounds of the EO were found to be citronellal, geraniol, geraniol, citronellol, and neral. The formulations exhibited suitable size, homogeneity, negative charge, isotropic behavior, highly organized structure, and pseudoplastic behavior, for vaginal application. TEM photomicrographs showed possible EO droplets inside the spherical structures. The EO, when loaded into the ME, exhibited an improvement in its antifungal action against *C. albicans*. The EO was not toxic against brine shrimp nauplii. An in vivo VVC assay showed that the use of the ME significantly improved the action of the EO, since only the ME+EO promoted the eradication of the fungal vaginal infection on the third day of treatment.

Conclusion: The EO and ME+EO are promising alternatives for the control of fungal infections caused by *C. albicans*, once the use of nanotechnology significantly improved the antifungal action of the EO, especially in an in vivo model of VVC.

Keywords: nanostructured lipid system, citronella, vulvovaginal candidiasis, therapeutic treatment

Introduction

Vulvovaginal candidiasis (VVC) is the second most common cause of vaginitis after bacterial vaginosis, which highlights it as an important women's health issue.¹ According to Denning et al, 2018, 75% of women have had VVC at least once during their lives and recurrent episodes may occur, leading to recurrent vulvovaginal candidiasis (RVVC). Although VVC is not a lethal disease, quality of life is drastically affected, both socially and economically, especially in cases of RVVC, which is considered the chronic phase of the disease.³

Candida albicans is the species responsible for over 90% of VVC cases, followed by *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. lusitanae*.¹ VVC is common in women following antibiotic treatment, in pregnant women, and in women undergoing hormone replacement.² The symptoms of VVC include pain, vulvar erythema, abrasions, pruritus, and a “curd-like” or watery vaginal discharge.⁴

The limitations of antifungal therapy, such as high cost, toxicity, drug interactions, and low drug bioavailability, are factors that contribute to the inefficiency of conventional antifungals, as represented by azole, polyenic, and echinocandin compounds.⁵ In addition, the emergence of drug-resistant strains has stimulated the search for new antifungal agents with different mechanisms of action.⁶

Plants are an important therapeutic possibility for treating fungal infections. The essential oil (EO) of the leaves of *Cymbopogon nardus* (L.) Rendle (citronella) is applied in cosmetic and perfumery industries and is used as an insect repellent. Geraniol, citral, citronellal and citronellol, the main chemical compounds from this EO, have various biological activities.⁷⁻⁹ Toledo et al (2016) reported the antifungal activity of the EO of *C. nardus* against standard and clinical strains of *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. Moreover, the EO was able to inhibit the main virulence factors, such as hyphal formation, of *C. albicans* and the mature biofilm of *Candida* species. The advantages presented by plant extracts have stimulated research on the application of alternative methods to improve the antifungal activity of plant derivatives, such as the use of nanotechnology, which allows the enhancement of biological activity, the control of drug release, and a reduction of side effects.^{11,12}

Microemulsions (MEs) are potential nanostructured drug delivery systems. MEs present characteristics such as transparent emulsions, in which an oil is dispersed in an aqueous medium (or vice versa), containing a surfactant associated with an appropriate co-surfactant. The system is a thermodynamically stable system and shows internal phase with droplets of nanometer order, thus improving drug solubilization.^{12,13}

Studies involving the use of natural products and nanotechnology in in vivo VVC models have been employed due to economic reasons, accessibility, and the anatomical similarity of the animal to humans. These studies have shown promising antifungal potential of the tested products, with remarkable results.^{14,15}

The antifungal potential of *C. nardus* EO shown in previous study¹⁰ and the advantages of using nanostructured

drug delivery systems, such as MEs, has stimulated the development of alternative antifungal agents. Furthermore, citronella EO production occurs on a large scale, which facilitates its use as a raw material for the production of herbal medicines. Furthermore, EO extraction by hydrodistillation shows advantages over the use of organic solvents for the production of plant extracts, as it takes into consideration green chemistry principles,^{16,17} reducing the environmental impact and the cost of the process.^{18,19}

In this context, the aim of this study was to evaluate the possible improvement of the anti-*C. albicans* activity of the EO of *C. nardus* when loaded in a microemulsion (ME+EO). The mode of action was assessed by analyzing sorbitol and ergosterol interaction. The human cell line, HaCat, and an alternative model of *Artemia salina* were used to evaluate toxicity. Furthermore, in vivo VVC treatment was assessed in mice, in order to investigate the efficacy of vaginal administration of EO and ME+EO for the control of vaginal fungal infection.

Materials and Methods

Plant Material

The leaves of *C. nardus* (L.) Rendle were collected from April to June 2016, in the morning, in the Garden of Toxic and Medicinal Plants: “Profa. Dra. Célia Cebrian de Araújo Reis” (São Paulo State University [UNESP], Araraquara, São Paulo, Brazil). A voucher specimen (HRCB-60752) was deposited at Herbarium Rioclarense of the Institute of Biosciences (UNESP, Rio Claro, São Paulo, Brazil). This work was approved by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under license numbers A2B917A and AF35617/CNPJ 48.031.918/0001-24.

Extraction of Essential Oil from *C. nardus*

EO was extracted from fresh leaves of *C. nardus* (150 g) by hydrodistillation, using a Clevenger-type apparatus attached to a round bottom flask (3 L) with 1500 mL of deionized water (4 h). Residual water in the EO was separated from the sample by freezing. The yield of the EO was 0.7% (w/w). EO samples were stored at -20°C until used for chemical analysis and biological assays.

Gas Chromatographic Analysis of Essential Oil from *C. nardus*

Gas Chromatography-Mass Spectrometry

Chemical analysis of EO from *C. nardus* was performed by gas chromatography-mass spectrometry (GC-MS),

using a Shimadzu[®] GCMS-QP2010S gas chromatograph (mass detector: electron impact ionization; mass quadrupole analyzer), fitted with an Rtx-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm ; Shimadzu[®], Kyoto, Japan). Helium was used as the carrier gas at a flow rate of 1.00 mL/min (58.8 kPa) and a linear velocity of 36.8 cm/s. The oven temperature program was 60–246°C at 3°C/min for 62 min, the injector temperature was 240°C, the injection volume was 1 μL , the splitting ratio was 1:20, the ionization energy was 50 eV (280°C), and the transfer line temperature was 250°C. The EO was solubilized in hexane (chromatographic grade) at a ratio of 1:100 (v/v) and standard solutions were injected at concentrations of 5.0, 4.0, 3.0, 2.0, 1.0, 0.5, and 0.25 mg/mL. EO compounds were identified by comparing acquired mass spectra (from chromatogram peaks) with reference spectra of the National Institute of Standards and Technology mass-spectral library version 2.0 (2012) and data from the literature. Arithmetic retention indices²⁰ were calculated as previously described (Adams, 2007), by linear interpolation relative to the retention times (t_R) of a series of *n*-alkanes (C8–C20). These values were compared with a published retention indices.²¹ Relative amounts of EO components were calculated using the chromatogram peak area normalization method. The quantification of major EO compounds was performed using an analytical curve (external standard method), considering the commercial purity of each of the following standards (Sigma-Aldrich, St Louis, MO, USA): citronellal (95.0%), citronellol (95.0%), and geraniol (95.0%).

Microemulsion: Phase Diagram Development

MEs were prepared according to the method of Bonifácio et al²² (2015). MEs consisted of grape seed oil as the oil phase (OP), polyoxyethylene (23) lauryl ether (Brij35[®]) + soy phosphatidylcholine (2:1) as the surfactant (S), and phosphate-buffered saline (PBS) as the aqueous phase (AP).

The phase diagram development generated 36 formulations, mixing different concentrations of the constituents, but maintaining constant oil phase and surfactant proportions. The mixtures were sonicated (Q500; QSonica, Newtown, CT, USA) with a potency of 500 watts and a 20% amplitude, in discontinuous mode for 7 min, with an interval of 30 s in an ice bath every 59 s. The EO (2000 $\mu\text{g/mL}$) was loaded to the ME (ME+EO) by sonication for 30 s.

Microemulsion Characterization

Mean Diameter and Polydispersity Index

The mean diameter of the ME was performed by photon correlation using a dynamic laser scattering instrument (Brookhaven Instruments, Holtsville, NY, USA). The hydrodynamic radius of the colloidal particles of the Einstein-Stokes equation (Eq. [1]) was calculated as follows:

$$D = \frac{K.T}{6.\pi.\eta.Rh} \quad (1)$$

where D is the diffusion coefficient of the particles, K is the Boltzmann's constant ($1.3807 \times 10^{-23} \text{ NmK}^{-1}$), T is the absolute temperature (293.15 K), $\pi = 3.141592$, η is the viscosity ($1.002 \times 10^{-3} \text{ NM}^{-2\text{s}}$), and Rh is the hydrodynamic radius.

The ME (without EO) and ME+EO were analyzed in the analysis chamber with a laser beam crossing throughout the dispersion. The system was maintained at 25°C, with a laser wavelength of 532 nm and the refractive index for each sample was analyzed according to the index observed. Ten determinations of the mean diameter and the polydispersity index (PDI) of droplets of each sample were performed ($n = 3$). This assay was performed after 24 h (T0) and 3 months (T3) of samples preparation in order to verify stability of formulations and possible alterations in particle diameter.

Zeta Potential

Zeta potential (ZP) was determined by assessing electrophoretic mobility using a Zetasizer Nano NS instrument (Malvern Instruments, Malvern, UK). Analyses were performed 24 h after ME preparation. The samples were diluted 1:10 in ultra-purified water, placed in the electrophoretic cell, and 3 determinations of surface potential were performed for each sample (ME and ME+EO). The mean and standard deviation were calculated. Zeta potential analyses were carried out after 24 h (T0) and 3 months (T3) of samples preparation in order to verify stability of formulations.

Polarized Light Microscopy

ME and ME+EO were placed on a glass slide, covered with a cover slip, and analyzed by polarized light microscopy (PLM; BX41 coupled with QColor3 Section; Olympus, Tokyo, Japan) at room temperature ($25 \pm 0.5^\circ\text{C}$).

Continuous Shear Rheology

An AR2000 pressure-controlled rheometer (TA Instruments, New Castle, DE, USA) was used for rheometry assays. Analyses were performed in triplicate, using cone/plate (40 mm diameter at a 52 μm gap) and plate/plate (40 mm diameter at a 200 μm gap) geometry at $37.0 \pm 0.1^\circ\text{C}$. A controlled shear rate was used, ranging from 0 to 100 s^{-1} , during 120 s (each step) with 10 s interval between curves. The shear was minimal and equilibrated 1 min prior to each analysis and the formulations were applied to the bottom plate. The consistency index and the flow index were determined using eq 2 for a quantitative analysis of flow behavior.

$$\tau = \kappa \cdot \dot{\gamma}^n \quad (2)$$

where τ is the shear stress, $\dot{\gamma}$ is the shear rate, κ is the consistency index, and n is the flow index.²³

Oscillatory Rheology

Oscillatory rheometry was performed using the same parameters (rheometer, geometries, and temperature, $37.0 \pm 0.1^\circ\text{C}$, to simulate the in vivo environment) as described for continuous shear rheometry. To establish the viscoelastic region of the formulations, a stress scan was performed, resulting in an ideal frequency condition ranging from 1 to 10 Hz. To perform a quantitative analysis of the dependence of G' on the frequency, the exponent n and S were calculated using eq 3, which indicates the structure of the systems obtained.

$$G' = S \cdot \omega^n \quad (3)$$

where G' is the storage modulus, ω is the oscillatory frequency, S is the formulation strength, and n is the viscoelastic exponent.²³

Mucoadhesion Force

A TA.XTplus texture analyzer (Stable Micro Systems, Surrey, UK) was used, in the adhesion test mode, to evaluate the detachment force between porcine vaginal mucosa and the formulations tested (ME; ME+EO). Two mm thickness of porcine vaginal mucosa was fixed to the cylindrical probe (10 mm diameter) of the equipment. The temperature during the analyses was controlled at $37 \pm 0.5^\circ\text{C}$. ME or ME+EO samples were added to small containers below the probe (same direction). The probe was lowered (constant rate, 1 mm/s) until the mucosa had direct contact (60 s) with the formulations (ME; ME+EO), without force employed during this period.

At a constant velocity (0.5 mm/s), the probe was lifted until the mucosa detached from the formulations. The force required to detach the formulation from the mucosa was calculated from the force versus time curve. Five replicates were performed.²⁴

Transmission Electron Microscopy

The morphology and structure of the ME and ME+EO were visualized by transmission electron microscopy (TEM), using a JEM-100CX2 transmission electron microscope (JEOL, Tokyo, Japan), with an acceleration of 100 kV. The samples were diluted in water (1:10 v/v), coated on a copper grid (200 mesh), and dried at room temperature ($25 \pm 0.5^\circ\text{C}$). Images were acquired at 50,000 \times magnification.²⁵

Antifungal Activity Fungal Strains

Candida albicans ATCC 10231 (CA-ATCC) and one clinical isolate (CAV – human vaginal origin) were used in this study. The clinical strain was donated from the Microbiology Laboratory of the Medicine School in Sao Jose do Rio Preto (FAMERP) for the purpose of scientific research after written consent from the donors. The Human Research Ethics Committee of FAMERP approved the use of this strain (project identification code 152/20066, December 2006).

Determination of Minimal Inhibitory Concentration

The minimal inhibitory concentrations (MICs) of EO and ME+EO were determined using a microplate dilution technique, according to protocol M27-A3,²⁶ with some modifications. Roswell Park Memorial Institute (RPMI) 1640 medium (0.1 mL) was added to a 96-well microplate and was inoculated with 0.1 mL of a fungal suspension containing 2.5×10^3 colony-forming units (CFU)/mL. EO and ME+EO were tested at concentrations ranging from 7.8 to 500 $\mu\text{g}/\text{mL}$. Amphotericin B (AmB, Sigma-Aldrich) and fluconazole (Sigma-Aldrich) were used as positive controls. Culture medium, yeast growth, EO, ME+EO, ME, and solvent were used as additional (sterility) controls. Microplates were incubated at 37°C for 48 h. After incubation, 20 μL of an aqueous solution of 2% 2,3,5-triphenyltetrazolium chloride was added and the plates were incubated at 37°C for 2 h.²⁷ All tests were performed in triplicate.

Determination of Minimal Fungicidal Concentration

To determine the minimum fungicidal concentration (MFC), an aliquot from each well that showed antifungal activity was plated on a Petri dish containing Sabouraud

dextrose agar (DIFCO; BD Biosciences, Franklin Lakes, NJ, USA). The tests were carried out in triplicate. MFC was defined as the lowest concentration of EO and ME+EO that prevented visible growth on the solid medium.¹⁴

Ergosterol Assay

The binding of EO and ME+EO to membrane ergosterol in *C. albicans* was evaluated according to the method described by Peixoto et al²⁸ (2017). Microplates were prepared according to protocol M27-A3,²⁶ with some modifications. EO and ME+EO were evaluated at concentrations ranging from 5000 µg/mL to 2.44 µg/mL. *C. albicans* (ATCC 102331 and CAV) suspensions (10³ cells/mL) were prepared (100 µL) in RPMI 1640 medium supplemented with 200 µg/mL exogenous ergosterol. Medium without exogenous ergosterol was used as a control. Microplates were incubated at 35°C and readings were performed after 2 d.

Sorbitol Assay

To evaluate the interactions of EO and ME+EO with the fungal cell wall, microplates were prepared as described in the MIC determination experiments, according to protocol M27-A3,²⁶ with some modifications. EO and ME+EO were added to microplates at concentrations ranging from 1,000 µg/mL to 0.48 µg/mL. One hundred microliters of *C. albicans* (ATCC 102331 and CAV) suspensions (10³ cells/mL), prepared in RPMI 1640 medium supplemented with 0.8 M sorbitol, were added. Medium without sorbitol was used as a control. Microplates were incubated at 35°C and readings were performed after 2 and 7 d.²⁸

Toxicity Assays

HaCat Cell Line

Cytotoxicity assays were performed in the HaCat human keratinocyte cell line obtained from Banco de células do Rio de Janeiro (BCRJ: 0341), according to a previously described protocol²⁹ using the resazurin reduction method. Cells (2.5 × 10⁴ cells/mL) were added to a 96-well microplate (Costar, Cambridge, MA, USA) for 24 h. After this process, cells were treated with EO (from 7.8 to 1000 µg/mL) or ME+EO (from 0.1 to 30 µg/mL). After 24 h of incubation, the medium was removed and 50 µL of resazurin, diluted to 0.01% in DMEM, was added. The microplates were then incubated at 37°C for 3 h. Fluorescence was measured using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Untreated cells (viable cells) were used as negative controls, cells treated with 5 µg/mL doxorubicin (Sigma-Aldrich) were used as positive controls (dead cells), and 1% DMSO was used as the vehicle control. Assays were performed in triplicate. IC₅₀ values, which represent the concentrations required to inhibit 50% of cells, were calculated by regression analysis from a calibration curve.

Artemia Salina Model

Brine shrimp cysts of *Artemia salina*, for use in the conventional Artemia test, were acquired from *Artemia salina* of RN, Natal, Brazil, produced by Flagner Soares de Souza. In vitro toxicity was assessed using an *Artemia salina* (brine shrimp) model, according to a previously described method (Parra et al³⁰, 2001), with some modifications. Briefly, 25 mg of *A. salina* cysts was incubated for 24 h in artificial saline water consisting of 23 g of NaCl, 11 g of MgCl₂.6H₂O, 4 g of Na₂SO₄, 1.3 g of CaCl₂.H₂O, and 0.7 g of KCl dissolved in 1000 mL of distilled water. An oxygen pump was placed in the saline water and a light was coupled on top of the container to keep the temperature at 25°C during the experiment. After 24 h of incubation, the nauplii were fed with 0.6 g of *Saccharomyces cerevisiae* and incubated for another 24 h.

The next day, a suspension of nauplii (10–15 organisms/well) in a volume of 100 µL of saline water was added to a 96-well microplate. In a separate microplate, a serial dilution of the compounds was prepared with concentrations of EO and ME+EO ranging from 0.2 to 1000 µg/mL. The controls consisted of untreated nauplii (negative control), 2% DMSO (vehicle control), and potassium dichromate (positive control). After 24 h of treatment, the plate was examined under a binocular stereoscopic microscope and the number of dead nauplii in each well was counted to calculate the median lethal concentration (LC₅₀). Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation.

In vivo VVC Study in a Mouse Model

In vivo VVC assays were performed in a mouse model following the recommended guidelines of the Ethics Principles in Animal Experimentation of the Ethics Committee on the Use of Animals in Research, CEUA School of Pharmaceutical Sciences of Araraquara, UNESP, São Paulo State, Brazil, after approval protocol number 56/2016. Animals were purchased from Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório, CEMIB, Unicamp, Campinas, São Paulo State, Brazil.

Female C57BL/6 mice ($n = 56$) at 8–10 weeks of age, weighing 20–25 g, were used for these experiments. For environmental adaptation, the animals remained in the experimental room for 4 weeks before starting the assay. During the entire experiment, the animals were kept in polypropylene boxes ($50 \times 30 \times 20$ cm), maintained with adequate temperature and ventilation and an alternating 12/12 h light/dark cycle.

The different experimental groups are listed in Table 1. *C. albicans* ATCC 10231 was used in this study. Initially, the pseudoestrus hormonal phase was induced in mice by subcutaneous administration (0.1 mL) of estradiol (Sigma-Aldrich) at a concentration of 2 mg/mL, at 6 and 3 days prior to infection (initial day). On the initial day, 20 μ L of a *C. albicans* suspension containing 2.5×10^8 cells/mL was inoculated into the vaginal environment. At day 1, treatments (20 μ L administered/animal) were initialized (once/day) and additional treatments were administered on days 3, 5, and 7. On days 2, 4, and 6, estradiol administration was continued to maintain the pseudoestrus phase. On days 2, 4, 6, and 8, vaginal lavages were performed with PBS.^{15,31} The infection was monitored by individual vaginal lavage of the mice (70 μ L of sterile PBS), with repeated aspiration, shaking, and subsequent plating on Sabouraud agar + chloramphenicol (Acumedia, Lansing, MI, USA) to determine the number CFUs. After 48 h of incubation of the agar plates at 37°C, the colonies were counted. Colony counts are expressed as log CFU/mL.^{15,31} The animals were euthanized in a CO₂ chamber.

Statistical Analysis

Results are reported as mean \pm standard deviation (SD). Statistical analyses were performed with Prism 7.00 software (GraphPad, San Diego, CA, USA) using a two-way

Table 1 Specification and Treatments Used in the in vivo VVC Study

Specification	Treatments
Negative Infection	Sterile PBS
Positive Infection	Sterile PBS
Positive control	20 μ L of commercial cream (amphotericin-B + tetracycline)
Solvent control	DMSO 10%
EO	20 μ L of EO (2.0 mg/mL)
ME+EO	20 μ L of ME+EO (2.0 mg/mL)
ME control	20 μ L of ME (without EO)

Notes: Treatments were performed with the administration of 20 μ L of each treatment once per day.

Abbreviations: EO, essential oil of *C. nardus*; ME+EO, loaded essential oil in the microemulsion; ME, microemulsion (without EO).

ANOVA, with a post-hoc Tukey's test for multiple comparisons. A Student's *t* test was used to compare means between only two groups. It was considered to be statistically significant if $p < 0.05$.

Results and Discussion

Chemical Composition of *Cymbopogon nardus* Essential Oil

The chemical profile (qualitative and quantitative composition) of the EO from *C. nardus*, as determined by GC-MS, is shown in Table 2. The following oxygen-containing monoterpenes (89.93%) were the major constituents: citronellal (27.34%), geraniol (23.21%), geranial (13.37%), citronellol (12.49%), and neral (10.31%). These monoterpenes are biosynthetically related and are derived from geranyl diphosphate.³²

The citronellal concentration calculated from the analytical curve (area = 5,575,407, $R^2 = 0.997$) was 2.653 mg/mL, which corresponds to a citronellal concentration in the EO of 26.5% (w/w). The citronellol concentration (area = 2,196,889, $R^2 = 0.996$) was 1.116 mg/mL, which can be expressed as 11.1%. The geraniol concentration (area = 4,318,673, $R^2 = 0.997$) was 2.458 mg/mL, which corresponded to 24.6%. These values are consistent with the quantification from the chromatogram peak area normalization method by GC-MS.

EOs from the genus *Cymbopogon* have monoterpenes as their main chemical constituents. Variations in the chemical composition of EOs may occur due to several factors, such as climate, genetics, environment, seasonal variation, geographical conditions, and extraction methods.^{33–36}

This EO chemical profile corroborated the results of a previous study that showed a citronellal concentration of 27.87% in the EO of *C. nardus* (Toledo et al, 2016), which is similar to the concentration found in this study (27.34%) and in a study by Wei and Wee (2013),³⁷ which reported a citronellal concentration of 29.6%. Studies performed by Kandimalla et al³⁸ (2016) in India and Nakahara et al³⁹ (2003) in Japan showed low concentrations of citronellal in the OE of *C. nardus* (6.06% and 5.8%, respectively). Kandimalla et al³⁸ (2016) found that the main constituents were citral (38.75%) and 2,6-octadienal,3,7-dimethyl (31.02%) and Nakahara et al³⁹ (2003) reported geraniol as the main chemical component, with a concentration of 35.7%.

Identification of the chemical compounds in the EO responsible for antimicrobial activity is difficult, since the essential oils are complex mixtures with different chemical

Table 2 Chemical Composition of *C. nardus* Essential Oil

Retention Time	Compound Name	AI ^a (Calculated)	AI ^b (Literature)	Concentration (%)
5.555	5-methyl-5-hexen-2-one	915	895	0.06
7.7	β -myrcene	992	988	0.11
8.105	<i>n</i> -octanal	1005	998	0.11
9.02	D-limonene	1030	1024	3.21
9.295	<i>cis</i> -ocimene	1037	1032	0.24
9.68	<i>trans</i> -ocimene	1048	1044	0.15
9.91	bergamal	1054	1051	0.55
10.605	not identified	-	-	0.33
11.670	β -linalool	1102	1095	0.79
11.93	α -pinene oxide	1108	1099	0.07
12.14	<i>trans</i> -rose oxide	1113	1122	0.12
12.86	oxiranemethanol	1130	1065	0.29
13.605	neo-isopulegol	1148	1144	0.58
13.95	citronellal	1157	1148	27.34
14.075	not identified	-	-	0.31
14.37	phellandral	1076	1148	0.20
15.135	not identified	-	-	0.40
15.62	<i>trans</i> -4-Nonenal	1197	1148	0.05
16.095	decanal	1208	1201	0.52
17.135	β -citronellol	1232	1223	12.49
17.715	neral	1245	1235	10.31
18.335	geraniol	1260	1249	23.21
19.011	geranial	1276	1264	13.37
22.42	citronellol acetate	1356	1350	0.30
23.725	geranyl acetate	1387	1379	0.20
25.25	β -caryophyllene	1424	1417	1.07
26.64	α -humulene	1458	1452	0.09
29.09	germacrene-D	1519	1513	1.24
29.455	δ -cadinene	1528	1522	0.22
29.57	citronellyl butyrate	1531	1530	0.21
30.47	elemol	1554	1563	0.17
30.845	geraniol butyrate	1564	1548	0.15
31.820	caryophyllene oxide	1589	1582	0.59
34.035	<i>trans</i> -cadinol	1648	1638	0.12
34.5	α -muurolol	1661	1644	0.29
	Monoterpene hydrocarbons			3.73
	Oxygen containing monoterpenes			89.93
	Sesquiterpene hydrocarbons			3.33
	Oxygen containing sesquiterpenes			0.91
	Other compounds			0.56
	Total identified			98.46

Notes: ^aArithmetic retention indices²⁰ relative to C8-C20 *n*-alkanes calculated.²¹; ^bArithmetic retention indices.^{20,21}

compounds that can act synergistically.^{40,41} A previous study has shown that the EO of *C. nardus* has greater antifungal activity against planktonic cells of *Candida* species than citronellal, which is its main chemical compound. This indicates a possible synergic action among the chemical compounds present in the EO.¹⁰

Microemulsion Formation

A phase diagram (Figure 1) was constructed to demonstrate the 36 points of formulations obtained from the mixtures of different constituents and a visual classification was evaluated for each formulation. The formulations were visually classified as a transparent liquid system

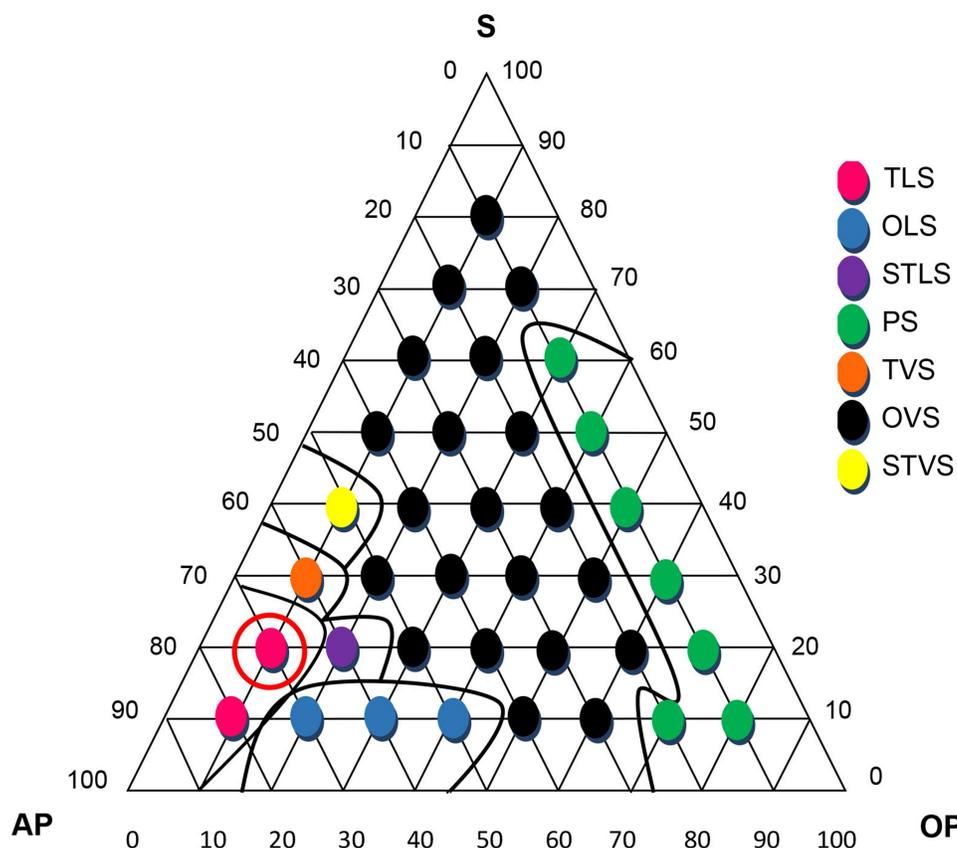


Figure 1 Phase diagram for formulations containing PBS (AP), Brij 35[®] + soy phosphatidylcholine and grape seed oil (OP).

Abbreviations: (S), grape seed oil (OP); AP, aqueous phase (PBS); S, surfactant (Brij 35[®]); OP, oil phase (grape seed oil); TLS, transparent liquid system; OLS, opaque liquid system; STLS, semi-transparent liquid system; PS, phase separation; TVS, transparent viscous system; OVS, opaque viscous system; STVS, semi-transparent viscous system.

(TLS), an opaque liquid system (OLS), a semi-transparent liquid system (STLS), a phase separation (PS), a transparent viscous system (TVS), an opaque viscous system (OVS), or a semi-transparent viscous system (STVS). Therefore, different regions of the phase diagram were delineated and the best formulation for loading the essential oil in the ME was selected. The ME with the TLS characteristic (circled red point – Point 9) was chosen for use in in vitro and in vivo assays. It was composed of 70% PBS, 20% Brij35 + soy phosphatidylcholine (2:1), and

10% grape seed oil. The EO (2000 µg/mL) was loaded in the ME (point 9, ME+EO).

Microemulsion Characterization

Mean Diameter, Polydispersity Index, and Zeta Potential

The mean values and standard deviation of particle size and PDI for the ME and ME+EO are demonstrated in Table 3. According to Formariz et al⁴² (2010) the particle size of MEs range from 10 to 250 nm. The results obtained

Table 3 Mean Diameter of the Particle Size, Polydispersity (PDI) and Zeta Potential for ME and ME+EO After 24 h (T0) and 3 Months (T3) of Samples Preparation

Samples	Mean Diameter ± SD (nm)*		Mean PDI ± SD (nm)*		Zeta Potential ± SD (mV)	
	T0	T3	T0	T3	T0	T3
ME	55.2±0.291	57.3±0.191	0.35±0.04	0.32±0.020	-2.8±0.929	-2.9±0.072
ME+EO	70.1±0.848	72.2±0.032	0.39±0.006	0.35±0.053	-2.8±0.349	-2.9±0.092

Notes: *Values expressed in nanometer (mean diameter and PDI) and millivolts (ZP) by determining of mean and standard deviation from three different assays.

Abbreviations: ME, microemulsion; ME+EO, loaded essential oil in the microemulsion.

in this study corroborate these data, since the particles sizes were 55.23 and 70.14 nm for the ME and ME+EO, respectively. Small differences in particle diameter size were observed between the ME and ME+EO, which suggested the incorporation of the EO in the ME. PDI is an index that evaluates the relative homogeneity of the particle sizes distributed in the sample.^{43,44} Therefore, these results demonstrated that the ME and ME+EO were homogeneous. These data confirmed the results of a previous study of an ME containing orange oil, with varying oil concentrations of 20%, 25%, and 30%, which exhibited a particle size of 70 nm and an IPD of 0.22 nm.⁴⁵

An analysis of the ZP of the ME and ME+EO showed a negative charge, which is likely due to the presence of soy phosphatidylcholine in the system that may favor the negative potential because of free esters present in the lipid structure.⁴⁶ However, phosphatidylcholine provides a phosphate group (negative charge) and a choline group (positive charge), which can cancel out each other, generating a zero charge at neutral pH. Thus, the hydration layers, lipid headgroups, and water polarization are factors that can be considered as the cause of the negative ZP.^{47,48} No significant changes were observed between time 0 and time 3 months, in relation to the particle diameter, IPD and zeta potential, which indicates stability of ME and ME+EO.

PLM

The ME and ME+EO were analyzed by PLM to evaluate the structure of the formulations. Figure 2 shows the PLM photomicrographs of the ME (A) and ME+EO (B). Photomicrography A (10% oil phase) showed isotropic behavior (dark field), which, in the plane of polarized

light, did not deviate light, suggesting the formation of a microemulsified system. Photomicrography B (10% oil phase), after the incorporation of the EO in the ME, also showed a dark field and therefore, the lipid system containing the active ingredient maintains the characteristics of a microemulsified system.

Oscillatory Rheology

The viscoelastic behavior of the formulations (ME and ME+EO) was evaluated by oscillatory measurements. Rheological analyses are relevant, as they provide parameters for the characterization of the formulations, including structure and viscosity, which are important for the development of formulations for topical use.⁴⁹ G' represents the storage modulus of the energy of stress, which can be restored when the stress is released and G'' represents the flow resistance of the sample.⁵⁰

Figure 3 shows the elastic modulus (G') and viscous modulus (G'') of the formulations. The ME and ME+EO showed elastic behavior ($G' > G''$), indicating a highly organized structure. These results demonstrated that the incorporation of EO in the system did not alter the viscoelastic behavior of the formulations. Viscoelasticity is important for vaginal application, due to the ease of administration and suitable scattering in the vaginal environment.⁵¹

Continuous Shear Rheology

Continuous shear rheology data for the ME and ME+EO are shown in Figure 4. The ascending (filled symbol) and descending curves (empty symbol) indicate the flow behavior of the formulations. Table 4 shows the properties related to flow behavior (n) and consistency of the formulations (k). The ME

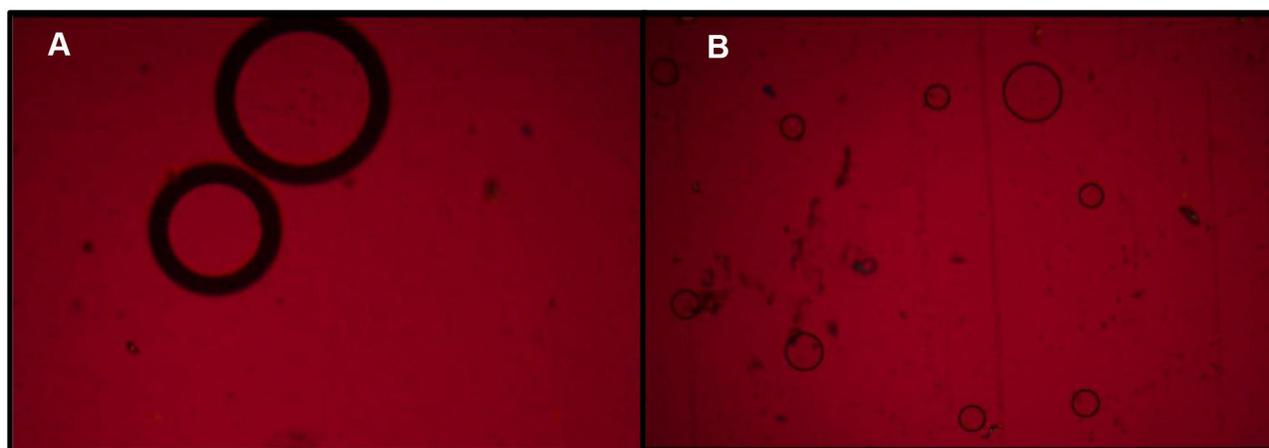


Figure 2 PLM photomicrographs of ME (A) and ME+EO (B) at magnification 20 \times .

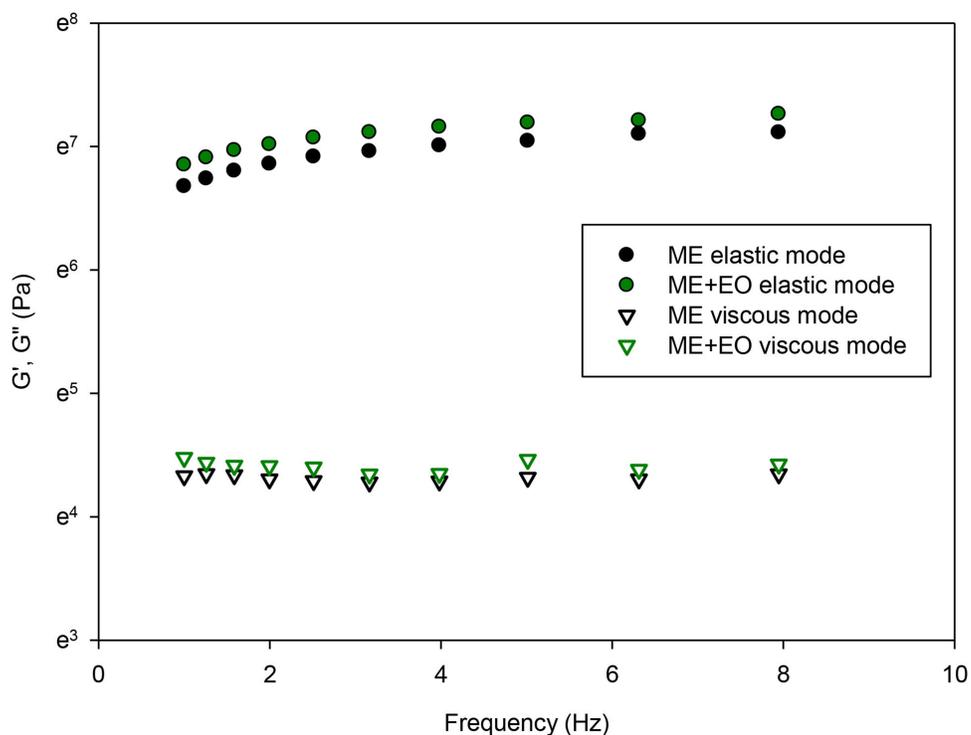


Figure 3 Rheological behavior in oscillatory flow conditions of ME and ME+EO.

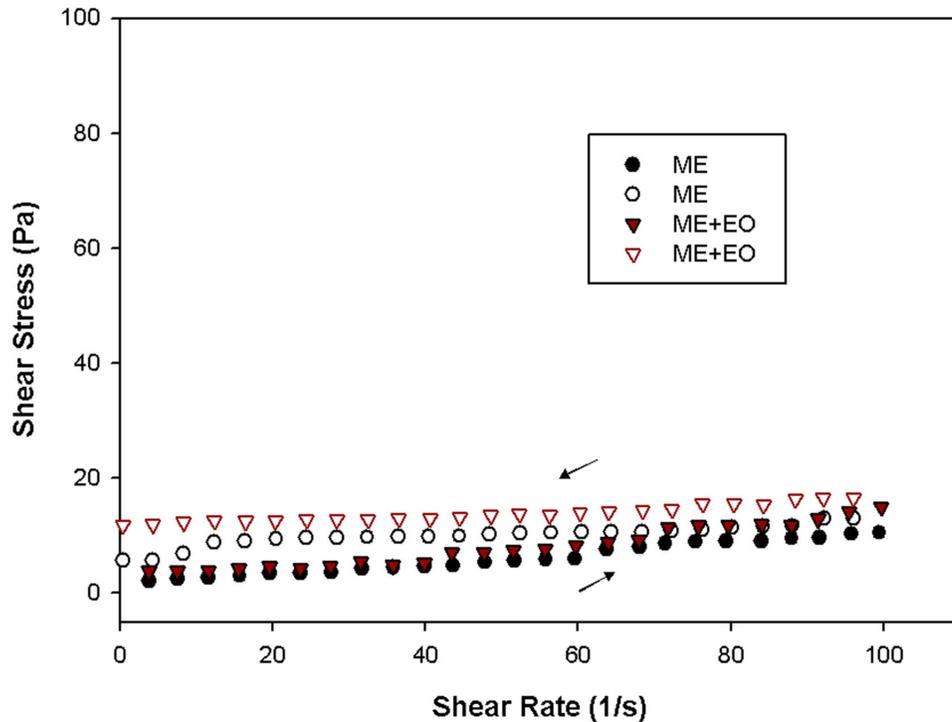


Figure 4 Continuous shear rheogram of ME and ME+EO. Filled symbol represents the ascending curve and empty symbol indicates the descending curve; up arrow: ascending; down arrow: descending.

Table 4 Flow Behavior (n) and Consistency Index (k) of ME and ME+EO by Continuous Flow Rheology

Formulations	n	K
ME	0.7543±0.04	0.3218±0.06
ME+EO	0.7846±0.06	0.3688±0.10

Abbreviations: ME, free formulation; ME+EO, loaded essential oil in the ME.

and ME+EO demonstrated non-Newtonian characteristics, with pseudoplastic behavior ($n < 1$). This result is relevant for the appropriate application of pseudoplastic formulations in the vaginal environment, since an increase in viscosity occurs under the application of shear stress; however, when the force is removed, the system structure returns to its initial properties.⁵²

These results are in agreement with the work of Ramos et al²⁴ (2019), who showed that a methanolic extract of *Syngonanthus nitens* loaded in a nanoemulsion showed pseudoplastic behavior and allowed effective application for vulvovaginal candidiasis treatment. Another study demonstrated the pseudoplastic behavior of multiple emulsions loaded with clotrimazole as an adequate topical (vaginal) delivery system, showing effective antifungal activity when compared to clotrimazole, a commercial antimycotic drug.⁵³

Mucoadhesion Properties

Suitable mucoadhesion characteristics are relevant for the attachment of a formulation to vaginal mucosal membranes, in order to promote extended contact between the formulation and the vaginal cavity, thus allowing an increased residence time and bioavailability of the drug.

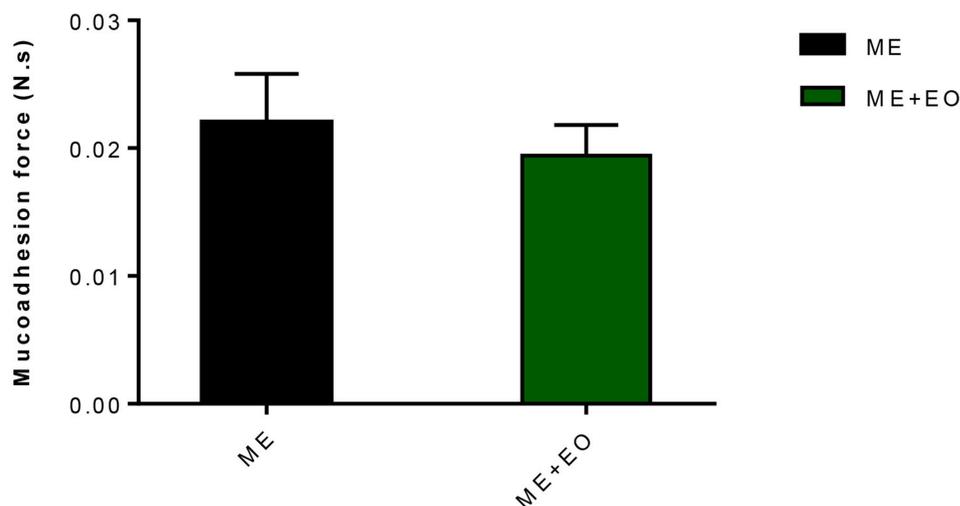


Figure 5 Peak of mucoadhesion (N.s) of ME and ME+EO. Each value represents the mean (\pm standard deviation) of at least five replicates.

Moreover, adequate mucoadhesion avoids drainage of the formulation outside the vagina, which is an unfortunate condition for users.⁵⁴

The peaks of mucoadhesion of the ME and ME+EO are shown in Figure 5. There was no significant difference ($p > 0.05$) in mucoadhesion between the ME and ME+EO, indicating that, after incorporation of the EO in the ME, mucoadhesion remained stable. The formulations showed adequate mucoadhesion for use in an in vivo VVC assay, which was in agreement with the results of studies by Bonifácio et al²² (2015) and Ramos et al²⁴ (2019), in which the mucoadhesivity of loaded vegetal extracts in nanoemulsions were suitable for application in in vivo vulvovaginal candidiasis studies.

Transmission Electron Microscopy

Figure 6 shows TEM images of the ME and ME+EO at a magnification of 50,000 \times . The photomicrographs of the ME and ME+EO showed spherical structures; however, ME+EO images showed possible EO droplets inside the spherical structures, which suggested incorporation of the EO.⁵⁵ The particle sizes of the formulations obtained from DLS measurements were confirmed by TEM photomicrographs, which showed particles ranging from 50 to 200 nm.

Antifungal Activity

MIC and MFC of EO and ME+EO

The MIC and MFC results for EO and ME+EO are shown in Table 5. The EO showed antifungal activity with MIC values of 500 $\mu\text{g/mL}$ and $>500 \mu\text{g/mL}$ against *C. albicans* ATCC and CAV, respectively. ME+EO showed the lowest MIC

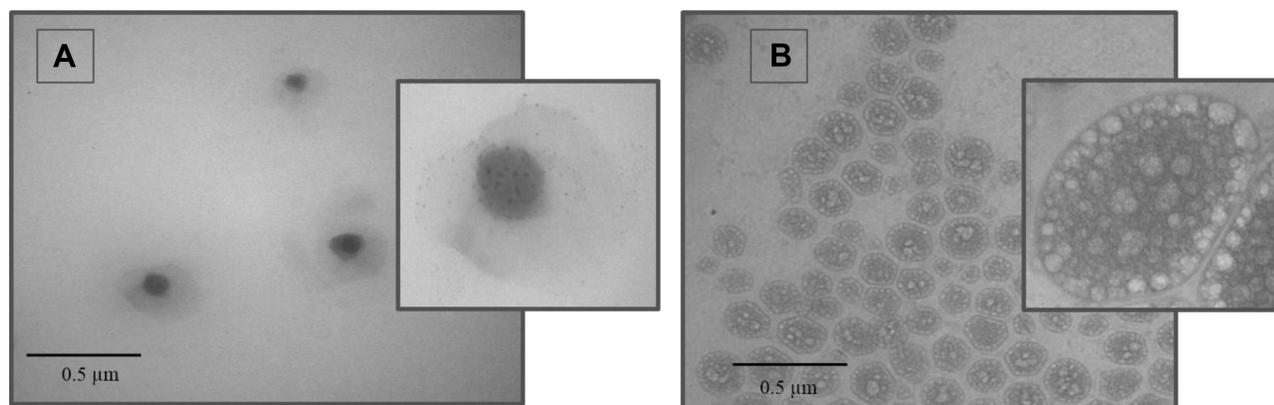


Figure 6 TEM photomicrographs of the ME (A) and ME+EO (B) at magnification of 50,000 \times .

(31.2 $\mu\text{g/mL}$) against CAATCC and CAV (62.5 $\mu\text{g/mL}$). Thus, the use of an ME considerably improved the action of the EO. The growth control showed satisfactory results, whereas the solvent and ME controls did not show any antifungal activity.

Several studies have demonstrated the anti-*Candida* activity of EOs.^{56–59} The genus *Cymbopogon* has been shown to have effective antifungal activity against pathogenic fungi. A previous study demonstrated the antifungal activity of the EO of *Cymbopogon citratus* against different *Candida* species, showing MIC values ranging from 125 to 175 $\mu\text{g/mL}$.⁶⁰ Another study demonstrated the anti-*Candida* action of the EO from *C. nardus* against *C. albicans*, with MIC values ranging from 32 to 64 $\mu\text{g/mL}$. Furthermore, the EO of *C. nardus* inhibits the adherence of *C. albicans* to dental implants and cover screws.⁶¹

The use of nanotechnology as an alternative to improving the antifungal activity of natural products has been used with promising results. In an in vivo study of the prophylactic action of a methanolic extract of *Syngonanthus nitens* (Bong.) Rhulland loaded into a drug delivery system, the prevention of VVC development caused by *C. krusei* was demonstrated.⁶² Bonifácio et al²² (2015) studied the antifungal activity of an ethanolic extract from *Astronium*

urundeuva (Engl.), unloaded or loaded in an ME, against *C. albicans* and showed an improvement in the inhibitory capacity of the extract after incorporation into the nanostructured system.

The incorporation of oils⁶³ and chemical compounds from EOs⁶⁴ in nanostructured delivery systems has been used to combat microorganisms. A previous study showed greater antifungal activity against *Candida* species using clotrimazole loaded into lipid nanoparticles containing EOs compared to prolonged in vitro delivery of clotrimazole. Moreover, increased anti-*Candida* action was observed for loaded clotrimazole nanoparticles prepared with EOs of *Lavandula* or *Rosmarinus*.⁶⁵

The use of MEs is important to enhance the solubilization of the EO, which improves the safety and stability of the system and consequently, may enhance the antifungal activity of the EO.⁶⁶

Ergosterol and Sorbitol

Table 6 shows MIC values for EO, ME+EO, and AmB, in the absence and presence of exogenous ergosterol, against *C. albicans* ATCC 10231 and CAV. The MIC values of EO

Table 6 MIC Values of EO, ME+EO and Amphotericin-B (AMB) in Absence (A) and Presence (P) of Exogenous Ergosterol Against *C. albicans* ATCC 10231 and CAV

<i>C. albicans</i>	MIC ($\mu\text{g/mL}$)					
	EO		ME+EO		AMB	
	(A)	(P)	(A)	(P)	(A)	(P)
CA-ATCC 10231	500	2500	31.2	625	0.12	4
CAV	>500	5000	62.5	>5000	0.12	4

Abbreviations: EO, essential oil; ME+EO, loaded essential oil in the microemulsion; AMB, amphotericin-B; A, absence; P, presence; CA-ATCC 10231, standard strain of *C. albicans*; CVV, clinical strain of *C. albicans*.

Table 5 MIC and MFC of EO and ME+EO Against *C. albicans*

<i>C. albicans</i>	MIC/MFC* MIC*			
	EO	ME+EO	AMB	FLU
CA-ATCC 10231	500/500	31.2/>500	0.12	R
CAV	>500/>500	62.5/>500	0.12	R

Note: *Values expressed in $\mu\text{g/mL}$.

Abbreviations: EO, essential oil; ME+EO, loaded essential oil in the microemulsion; AMB, amphotericin-B; FLU, fluconazole; CA-ATCC 10231, standard strain of *C. albicans*; CVV, clinical strain of *C. albicans*.

and ME+EO increased 5-fold and 20-fold, respectively, in the presence of exogenous ergosterol. These results suggested that EO and ME+EO bound to ergosterol in the fungal membrane, which required higher concentrations of EO and ME+EO to promote fungal cell death, demonstrating action on the ionic permeability of the fungal cell membrane. The positive control (AmB) showed the same mode of action. The antifungal activities of EO and ME+EO are not related to the biosynthetic pathways of the cell wall, since the MIC values were maintained in the presence of sorbitol.

A previous study investigated the mode of action of *Thyme vulgaris* EO, against *Rhizopus oryzae*, via interaction with ergosterol, and verified that this EO can interact with the fungal membrane, as evidenced by increasing MIC values in the presence of ergosterol.⁶⁷ Another study demonstrated that *Laurus nobilis* EO may interfere have two mechanisms of action against *C. albicans*, (cell wall biosynthesis and ionic permeability of the fungal cell membrane), considering that increased MIC values for this EO are observed in the presence of ergosterol and sorbitol.²⁸

Toxicity Studies

HaCat Cell Line

Table 7 shows the cytotoxic activity (IC₅₀) of the EO, ME+EO, and ME against HaCat cells. The EO showed lower cytotoxicity than ME+EO and ME. Koba et al⁶⁸ (2009) compared the cytotoxicity of EOs from the genus *Cymbopogon* (*C. nardus* and *C. citratus*) against HaCat cells and showed that the *C. nardus* EO had lower cytotoxicity than the *C. citratus* EO, with IC₅₀ values of 450 µL/mL and 150 µL/mL, respectively.

The IC₅₀ values of the ME+EO and ME were similar to conventional drugs used in the clinical practice, such as AmB (IC₅₀ = 4.0 µg/mL).²⁴ Topical administration is proposed for the formulations in this study. The route of administration is important for toxicity parameters, since topical administration exhibits lower toxicity than oral administration.⁶⁹

Table 7 Cytotoxic Activity (IC₅₀) of EO, ME+EO and ME Against HaCat Cell Line

Samples	HaCat (IC ₅₀)*
EO	235
ME+EO	5.5
ME	4.5

Note: *Values expressed in µg/mL.

Abbreviations: EO, essential oil; ME+EO, loaded essential oil in the microemulsion; ME, microemulsion.

Brine Shrimp Lethality

Table 8 shows the lethal concentration (LD₅₀) of the EO, ME+EO, and ME against brine shrimp (*A. salina*) nauplii. Several studies have described the toxicity of EOs against brine shrimp nauplii.^{70–72} However, the results of this study showed that EO was not toxic against brine shrimp nauplii, with LD₅₀ values higher than 1000 µg/mL. Moreover, brine shrimp nauplii treated with ME+EO and ME were found to be viable, with LD₅₀ values >30 µg/mL, which was the highest tested concentration of these formulations in this assay.

Parra et al³⁰ (2001) used the *A. salina* model to determine the toxicity of 20 plant extracts. The authors found a good correlation between in vivo and in vitro tests, concluding that brine shrimp lethality studies are a useful alternative to predict the acute oral toxicity of plant extracts. Moreover, the *A. salina* model is a useful tool for toxicity studies, due to the benefits of the method, which include quickness, cost-effectiveness, and reproducibility.⁷³

In vivo VVC Assay

Table 9 shows the results of fungal burden (CFU) from vaginal lavages collected at days 2, 4, 6, and 8. The EO of *C. nardus* was found to have significant therapeutic activity against VVC caused by *C. albicans* in mice. All animals treated with ME+EO were cured on the third day of treatment (experimental day 5). Moreover, incorporation of the EO into an ME enhanced the antifungal action of the EO (free), which was able to eradicate fungal infection in 50% of the animals. In the positive infection, solvent, and ME control experimental groups, the infection remained constant on all days of treatment, with a high fungal load present in 100% of the animals.

These data are significant, since the ME+EO demonstrated superior antifungal action compared with both free EO and a commercial cream used in clinical therapy (AmB + tetracycline), thus demonstrating the effectiveness

Table 8 Lethal Concentration (LD₅₀) of EO, ME+EO and ME Against Brine Shrimp Nauplii

Samples	Artemia salina (LD ₅₀)*
EO	>1000
ME+EO	>30
ME	>30

Note: *Values expressed in µg/mL.

Abbreviations: EO, essential oil; ME+EO, loaded essential oil in the microemulsion; ME, microemulsion.

Table 9 Fungal Burden (CFU) from the Vaginal Lavages Collected at Days 2, 4, 6 and 8, During Treatment Period

Experimental Groups	Days/CFU/mL*							
	IA	2	IA	4	IA	6	IAA	8
Negative infection	0/8	0±0.00	0/8	0±0.00	0/8	0±0.00	0/8	0±0.00
Positive Infection	8/8	3±0.03	8/8	3.2±0.00	8/8	4.4±0.00**	8/8	4.5±0.00**
Positive control	8/8	2.6±0.05	5/8	3±0.01	5/8	3.6±0.00 [#]	5/8	3.8±0.00 [#]
Solvent control	8/8	2.6±0.05	8/8	3±0.01	8/8	4.4±0.00	8/8	4.5±0.01
EO	8/8	2.5±0.09	4/8	3±0.00	4/8	3.5±0.00 [#]	4/8	3.7±0.00 [#]
ME+EO	8/8	2.4±0.04	3/8	2.4±0.3	0/8	0±0.00 ^{a###}	0/8	0±0.00 ^{a###}
ME control	8/8	2.9±0.02	8/8	3.1±0.01	8/8	4.2±0.00	8/8	4.3±0.00

Notes: *Values expressed as mean ± standard deviation of CFU/mL (log) obtained from vaginal lavage cultures. ^aStatistically significant difference between the treatments on the days 6 and 8; [#]significant difference (p < 0.05); ^{**}very significant difference (p < 0.01).

Abbreviations: EO, essential oil; ME+EO, loaded essential oil in the microemulsion; ME, microemulsion; IA, number of infected animals.

of the nanotechnology application as an alternative to improve the antifungal activity of natural products.

Studies using in vivo experimental models of mucosal infections caused by *Candida* are typically performed with mammals (rats, mice) because of economic, ethical, and anatomical reasons, and because these animals are immunologically similar to humans.^{74–76}

Previous studies have shown that plant extracts incorporated into nanostructured lipid systems are effective in the treatment rat models of VVC caused by *C. albicans*. Plant extracts loaded into nanostructured drug delivery systems were shown to be significantly more effective than unloaded plant extracts or the clinical practice drug, AmB, at the complete eradication of the vaginal fungal infection.^{14,22}

Rodero et al¹⁵ (2018) described the use of liquid crystalline systems to improve the action of curcumin in a mouse model of VVC. The authors showed effective antifungal activity of curcumin, loaded in the liquid crystalline system, against *C. albicans*. In addition, the loaded curcumin was effective at modulating the inflammatory response to the infection.

Pietrella et al⁷⁷ (2011) evaluated *Mentha suaveolens* EO in a mouse model of VVC (*C. albicans*). The authors reported that the EO was able to decrease the fungal load of the vaginal infection compared to the saline control, thus accelerating the removal of the infection.

Another study investigated the antifungal activity of clove EO in an emulsion and in a liposomal form in mice with VVC. After 8 d of treatment, a reduction in vaginal fungal load was observed for both forms tested. However, the liposomal form was found to be more effective than the emulsion form of the EO. In addition, the free EO was only able to eliminate the infection after 14 d.

These data corroborate the results of the present study, in which EOs were shown to have improved action when incorporated into a drug delivery system. In addition to improved antimicrobial activity, these drug delivery systems increase the stability and bioavailability of the plant extracts.¹²

Conclusion

The main compounds of the EO of *C. nardus* were found to be the oxygen-containing monoterpenes, citronellal, geranial, geraniol, citronellol, and neral. The ME+EO showed adequate technological properties, providing stability of the system and improvement of solubilization of the EO for incorporation in the ME. The EO and ME+EO exhibited effective in vitro and in vivo antifungal activity against *C. albicans* and demonstrated possible actions on the ionic permeability of the fungal cell membrane. Moreover, the use of nanotechnology significantly improved the antifungal action of the EO, especially in an in vivo model of VVC, in which the ME+EO promoted the eradication of the fungal vaginal infection on the third day of treatment. These encouraging results suggest that the incorporation of the essential oil of *C. nardus* in an ME can considerably enhance the antifungal action of the EO against *C. albicans*. Additionally, EO extraction complies with the principles of green chemistry, being a viable process that facilitates the development and industrial-scale production of antifungal herbal medicines, based on citronella EO.

Abbreviations

AmB, amphotericin-B; AP, aqueous phase; GC/MS, gas chromatography-mass spectrometry; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; EO, essential oil; FLU, fluconazole; ME, microemulsion; ME+EO, loaded essential oil in the microemulsion; OP, oil phase; PBS, phosphate-buffered saline; RPMI, Roswell

Park Memorial Institute; S, surfactant SDA, Sabouraud Dextrose Agar; SDB, Sabouraud Dextrose broth; SisGen, National System for the Management of Genetic Heritage and Associated Traditional Knowledge; VVC, vulvovaginal candidiasis.

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Disclosure

The authors report no conflicts of interest in this work.

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