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Article

Colombian essential oil of *Ruta graveolens* against *Candida* sp. isolated from the oral cavity of patients with head and neck cancer

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Abstract: The problem of drug resistance in terms of antifungal therapy, unknown until a few years ago, is assuming increasing importance. Particularly in immunosuppressed patients and subject to chemotherapy and radiotherapy. In the last years the use of essential oils as approach to improving the effectiveness of antifungal agents and reducing the antibiotic resistant has been proposed. Our research aimed to evaluate the antifungal activity of Colombian essential oil of *Ruta graveolens* (REO) against clinical strains of *Candida albicans, Candida parapsilopsis, C. glabrata* and *Candida tropicalis*. The data obtained showed that *Candida tropicalis* and *Candida albicans* were most sensible strains showing minimum inhibitory concentrations (MIC) of 0.5 and 1.0 μg/ml of REO. The Time Kill Kinetics assay demonstrated that REO showed fungicide effect against *C. tropicalis* and fungistatic effect against *C. albicans*. In addition, the 40% of the biofilm formed by *C. albicans* was eradicated using 1% of REO after 1 hour of exposure.

Keywords: *Candida* sp.; head - neck tumor; innovative antifungals; azole-resistant; *Ruta graveolens*

1. Introduction

Oral candidiasis (OC) is a common fungal disease caused by *Candida* spp. It presents with the appearance of white lesions that generally affect oral mucosa or oropharynx [1]. Although the progress in retroviral therapy, the OC remains the most common cause of infections in immunocompromised patients with diseases as the human immunodeficiency virus (HIV) [2]. Reports indicated that during the progression of their condition, more than 90% of people infected with HIV develop debilitating infections as oropharyngeal and esophageal candidiasis when they do not receive highly active antiretroviral therapy [3]. Furthermore, Candida species are the most common pathogen isolated in patients in the critical care setting. It is commonly found in elderly subjects, diabetic patients, and solid organ transplant recipients and is also an etiological agent of urinary and vaginal tract infections [4].

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Although *Candida albicans* is the predominant pathogenic fungus responsible for the OC [5], non-*albicans Candida* (NCAC) species has begun as frequently isolated of *Candida* infections. The incidence of species such as *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* has been reported widely in the last ten-year [6]. In particular, *C. glabrata* and *C. parapsilosis* is frequently isolated in North and Central Europe and North America, and *C. tropicalis* in South America and Asia [7]. Besides, it has documented the potential of these species to exhibit resistance and cross-resistance to azole drugs which could led to the failure of therapeutic strategies [8].

In order to find new classes of antifungal, the use of essential oils (EOs) has been highlighted, and many studies have focused on studying their properties and their application in fungal control [9]. Several works have reported the efficacy of EOs as a strategy against different preharvest and postharvest pathogens of fruits [10] and in human invasive fungal infection [11]. It was used in some medieval rites to protect the house against negativity. *Ruta graveolens* is a plant, which is used in traditional and herbal medicines. Folk medicine has used Rue to treat coughs, diphtheria laryngitis, colic, headaches and as an antidote in case of mushroom poisoning, snake bites and insect bites, in addition it has been used as stimulating, stomachic, emmenagogue consumed as an infusion and to treat headache, muscular and joint pain, and anti-inflammatory applying its oil or extract [12]. In the Middle Ages, however, the Ruta was used to ward off the plague: its smell is in fact very strong and pungent. Nowadays, in some Latin American countries it is used as fungicide and pesticide in organic agriculture [13,14]. In this regards previous studies, have been demonstrated the effectiveness of the *Ruta graveolens* essential oil (REO) *in vitro* against phytopathogens as *Colletotrichum gloeosporioides* [15,16], *Cladosporium herbarum, Aspergillus fumigatus, Fusarium oxysporum, Aspergillus flavus,* and *Alternaria alternata* [17]. Besides, studies *in-situ* in guava [15], papaya [16], gooseberries [18], tomato [19] and pear [20], have been demonstrated a remarkable reduction of the fruit decay and conserving their physicochemical properties. Although there are data in scientific literature of the effect of REO in phytopathogens, scarce are studies of REO activity in human pathogens.

The present study aimed to clarify the antifungal activity of *Ruta graveolens* against species of multi-resistant *Candida* spp. of clinical origin evaluating the time-kill kinetics and the capacity to reduce the biofilm formation, in order to find new alternatives to help overcome drug resistance of *Candida* spp.

2. Materials and Methods

2.1. Strains

A collection of 24 clinical isolates belonging to 4 different species of Candida spp. was selected for this study: *C. albicans* (6), *C. parapsilosis* (6), *C. tropicalis* (6), *C. glabrata* (6). The isolates were cultured from specimens of hospitalized patients isolated from the oral cavity of patients with head and neck cancer at the Otolaryngology Clinic of the Department of Medical, Surgical and Experimental Sciences of the University of Sassari. All microorganisms were identified by standard methods and stored on Sabouraud dextrose agar plates until the study was performed. All microorganisms were identified by standard methods.

2.2. Reagents

Fluconazole (FLC) was obtained from Sigma-Aldrich. Stock solutions of FLC were prepared in dimethyl sulfoxide. The final concentration of DMSO was not higher than 0.14%. What's more, RPMI 1640 (Thermo Fisher Scientific), were used in this study. Rue essential oil (REO) was obtained from Kräuter SAS (Bogotá- Colombia).

2.3. Antifungal susceptibility testing

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The minimum inhibitory concentrations (MICs) of antifungal agents (REO and FLC) against the Candida spp. strains were determined according to the broth microdilution assay in 96-well microtitration plates as described by the method M27-A3 of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [21]. Two fold serial dilutions in RPMI 1640 medium were performed to obtain the final concentrations ranged from 0.0035% to 16% V/V for REO and from 0.125 to 512 µg/mL for FLC. The test was carried out in a final volume of 200 µl total per well: 100 µl of the culture medium and 100 µl of fungal inoculum at a concentration of 10^6 CFU / mL. Each strain was tested in duplicate and a positive growth control (the strain under test without REO) and a negative one (medium only) were included in each test. The plate was incubated at 37 ° C and the minimal fungicide concentration (MFC) was determined by taking 10 µl from each well and spreading them on Sabouraud-Dextrose agar. The plates were incubated at 37 C ° for 24/48 h and checked to detect microbial growth. MFC is considered the lowest concentration capable of inhibiting 99% fungal growth. Three independent experiments were performed.

2.4. Determination of Minimum Fungicidal Concentration (MFC).

In order to establish the MFC of *Candida* species, the broth dilution method was used, as recommended by the Clinical and Laboratory Standard Institute (Clinical and Laboratory Standards Institute. 2008). Yeasts were cultivated at 37 °C on Sabouraud-Dextrose Agar plates (Microbiol, Cagliari, Italy) for 24 hours. The inoculum was prepared by a dilution of the colonies in salt solution, at a concentration of 0.5 McFarland and confirming the concentration by a spectrophotometric reading at a wavelength of 530 nm. The sensitivity test was carried out in RPMI 1640, using 96-well plates. Oil concentrations were prepared by serial one to two dilutions from 16% (v/v) to 0,125% (v/v). After shaking, 100 µl of each oil dilution and 100 µl of yeast suspension at a concentration of 10^6 CFU/mL, were added to each well and then incubated at 37 °C for 48 hours.

In order to determine the MFC value, 10 µL were seeded on Sabouraud-dextrose medium, the plates were incubated for 24-48 h at the temperature of 37°C. Minimal Fungicidal Concentration (MFC) was considered as the lowest concentration inhibiting fungal growth. Moreover, each yeast strain included in the study was tested for its sensitivity to fluconazole, voriconazole, amphotericin B. Each experiment was performed in duplicate and repeated three times.

2.5. Time kill kinetics

In order to evaluate further the REO effect, the time-kill assay in two *C. albicans* and two *C. tropicalis* strains was performed following the method proposed by Chaves-López et al., [22] with some modifications. A cell suspension was prepared for each strain, starting with an inoculum of 4.5 ± 0.5 and 5.0 ± 0.5 log CFU/mL and inoculating it into an emulsion of REO 1% in Yeast Potato Dextrose broth (YPD). The suspension was incubated at 37 °C for 48 h, and an aliquot of 1 mL was taken in the times 0, 1, 2, 3, 4, 5, 24, 36, and 48 h to prepare a series of 10-fold dilutions for after to inoculate 0.1 ml in Petri dishes with YPD agar. After incubation at 37 °C for 48 h, the colonies were counted for each dilution. All microbiological tests were repeated in two different experiments. Each experiment was performed in triplicate.

In order to determine the character fungicidal or fungistatic of the REO, a reduction of < 3 log cfu/ml after the treatment in the growth of the starting inoculum was defined as fungistatic activity of REO, and a reduction ≥ 3 log cfu/ml as fungicidal activity according to the proposed by Scorneaux et al., [23]. Subsequently, the time necessary to achieve 50, 90, and 99% of reduction in growth from starting inoculum was determined.

2.6. Quantitative assessment of biofilm formation

Samples standardized of four Candida strains were evaluated to quantitate the reduction of biofilm in the presence of REO in 96- well polystyrene microplates according to the methodology reported by Rossi et al., and Chaves-López et al., [24,25]. To biofilm formation, 200 µl of samples were cultured in each well in YPD broth and incubated at 37 °C for 48 h. Then, the YPD broth was removed from the microplate, and 200 µl REO 1%-YPD emulsion was added, with an incubation a 37 °C for one hour. After, the floating cells were removed, and the biofilm at the bottom of the wells was washed with deionized water three times. Six replicates were dispensed of each sample, and cultures without REO were taken as control. The reduction of biofilm was quantified stained the wells with 0.1% crystal violet (Sigma-Aldrich, Italy) for 20 min at room temperature. Samples were rewashed with deionized water until to remove the excess of dye. Finally, the samples were soaked in 250 µl of 30% glacial acetic acid (Carlo Erba reagents, Italy).

The absorbances values at 590 nm (OD_{590}) for each strain were measured using a Biolog MicroStation system (Biolog Inc., Hayward, USA), and it was grouped the biofilm productions into: OD_{590} < 0.1 = non-producers (NP), OD_{590} 0.1–1.0 = weak producers (WP), OD_{590} 1.1–3.0 = moderate producers (MP), and OD_{590} > 3.0 = strong producers (SP). The biofilm reduction was calculated using the following equation

$$\%\ Biofilm\ reduction = \frac{Abs_{CO} - Abs_{REO}}{Abs_{CO}} \times 100$$

Where Abs_{CO} = absorbance sample control, Abs_{REO} = absorbance sample treated with REO.

2.7. Data analysis

Experimental results were expressed as means ± standard deviations, and the data were evaluated by analysis of variance (ANOVA), and compared by 95% Tukey's HSD test, using Statistica 13.5 software (TIBCO, Tulsa, US).

3. Results

3.1. Oil characterization

Data regarding the characterization by mass spectrometry-gas chromatography (MS-GC) of the REO were reported in our previous work [15] (Supplementary Materials, Table S1). REO present a content predominant of aliphatic ketones where 2- undecanone is the majority component in the oil.

3.2. Antifungal susceptibility testing

The antifungal activities of REO and FLC alone was determined by the broth microdilution assay. Among the 16 isolates of *Candida* species tested, 8 isolates were resistant to FLC with MIC values ranging from 8 to 256µg/mL, and 8 isolates were sensitive to FLC with MICs ranging from 1 to 4µg/mL. The MICs of REO were in a range of 0,005–16 % (V/V) against all the *Candida* spp. isolates (Table 1).

Table 1. Inhibition effect of *Ruta graveolens* L. against different *Candida* species.

Fungi (n)	Ruta graveolens L.	Fluconazole
	Range (% V/V)	Range
	MIC	(µg/mL)
C. albicans (4)	1.0± 0.5	0.25-1
C. parapsilosis (4)	2.0± 0.5	0.5-2
C. tropicalis (4)	0.5 ± 0.25	0.5-2
C. glabrata (4)	16 ± 0.5	8-256

3.3. Determination of Minimum Fungicidal Concentration (MFC).

The MFC data for clinical Candida species show that after 24 hours the values obtained with REO essential oil were 1µg / ml, 16-8 µg / ml, 0.5 µg / ml, 2 µg / ml and 0, 5-2

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µg / ml for *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* respectively. After 48 hours the MFCs obtained were 1-1.5 µg / ml for *C. albicans*, 16 for *C. glabrata*, 1 µg / ml for *C. tropicalis* and 2 µg / ml for *C. parapsilosis*. Furthermore, *C. glabrata* and *C. parapsilosis* were resistant to fluconazole (MFC: 128 and 256 µg / ml after 24 hours and 2 and 4 µg / ml after 48 hours, respectively) and *C. glabrata* also resistant to voriconazole (MFC: 2 µg / ml after 24 hours and 4 µg / ml after 48 hours). The anti-fungal effect of REO was therefore highlighted against *C. albicans* and *C. tropicalis* also with respect to synthetic drugs such as Amphotericin B and fluconazole.

Table 2. *In vitro* susceptibility of *Candida* spp. isolates to *Ruta graveolens* oil and antifungal drugs.

Strains	*Ruta graveolens (REO)* (µg/ml) MFC		Amphotericin B (µg/ml) MFC		Fluconazole (µg/ml) MFC		Voriconazole (µg/ml) MFC	
	24 h	**48 h**	**24 h**	48 h	24 h	48 h	24 h	48 h
C. albicans ORL02	1 ± 0.5	1.5 ± 0.5	0.5 ± 0.25	0.5 ± 0.25	0.5 ± 0.25	1 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. albicans ORL03	1 ± 0.5	1 ± 0.5	1 ± 0.5	0.5 ± 0.25	0.5 ± 0.25	1 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. albicans ORL05	1 ± 0.5	1.5 ± 0.5	0.5 ± 0.25	1 ± 0.5	0.5 ± 0.25	1 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. albicans ORL07	1 ± 0.5	1 ± 0.5	1 ± 0.5	1.5± 0.5	0.5 ± 0.25	1 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. albicans ORL08	1 ± 0.5	1.5 ± 0.5	0.5 ± 0.25	0.5 ± 0.25	1 ± 0.5	1.5 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. albicans ORL09	1 ± 0.5	1 ± 0.5	0.5 ± 0.25	0.5 ± 0.25	1 ± 0.5	1.5 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. glabrata ORL02	16 ± 1	16 ± 1	2 ± 0.5	1 ± 0.5	128 ± 2	128 ± 2	2 ± 0.5	4 ± 0.5
C. glabrata ORL11	16 ± 1	16 ± 1	2 ± 0.5	1 ± 0.5	128 ± 2	256 ± 2	2 ± 0.5	4 ± 0.5
C. glabrata ORL15	8 ± 1	16 ± 1	1 ± 0.5	1 ± 0.5	128 ± 2	128 ± 2	1 ± 0.5	2 ± 0.5
C. glabrata ORL20	16 ± 1	16 ± 1	2 ± 0.5	1 ± 0.5	128 ± 2	256 ± 2	2 ± 0.5	4 ± 0.5
C. glabrata ORL22	8 ± 1	16 ± 1	1 ± 0.5	1 ± 0.5	128 ± 2	256 ± 2	1 ± 0.5	4 ± 0.5
C. glabrata ORL13	16 ± 1	16 ± 1	2 ± 0.5	1 ± 0.5	128 ± 2	256 ± 2	2 ± 0.5	4 ± 0.5
C. tropicalis ORL18	0.5 ± 0.25	1 ± 0.5	0.5 ± 0.25	1 ± 0.5	1 ± 0.5	2 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. tropicalis ORL19	1 ± 0.5	1 ± 0.5	1 ± 0.5	1 ± 0.5	1 ± 0.5	2 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. tropicalis ORL20	0.5 ± 0.25	1 ± 0.5	0.5 ± 0.25	1 ± 0.5	1 ± 0.5	2 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. tropicalis ORL21	0.5 ± 0.25	1 ± 0.5	1 ± 0.5	1 ± 0.5	1 ± 0.5	2 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. tropicalis ORL22	0.5 ± 0.25	1 ± 0.5	0.5 ± 0.25	1 ± 0.5	1 ± 0.5	2 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. tropicalis ORL23	0.5 ± 0.25	1 ± 0.5	0.5 ± 0.25	1 ± 0.5	2 ± 0.5	2 ± 0.5	0.03 ± 0.005	0.03 ± 0.005
C. parapsilosis ORL25	2 ± 0.5	2 ± 0.5	0.5 ± 0.25	1 ± 0.5	2 ± 0.5	4 ± 0.5	0.125 ± 0.05	0.250 ± 0.05
C. parapsilosis ORL25	2 ± 0.5	2 ± 0.5	1 ± 0.5	1 ± 0.5	2 ± 0.5	4 ± 0.5	0.125 ± 0.05	0.250 ± 0.05
C. parapsilosis ORL27	2 ± 0.5	2 ± 0.5	0.5 ± 0.25	1 ± 0.5	2 ± 0.5	4 ± 0.5	0.125 ± 0.05	0.250 ± 0.05
C. parapsilosis ORL28	2 ± 0.5	2 ± 0.5	0.5 ± 0.25	1 ± 0.5	2 ± 0.5	4 ± 0.5	0.125 ± 0.05	0.250 ± 0.05
C. parapsilosis ORL29	2 ± 0.5	2 ± 0.5	0.5 ± 0.25	1 ± 0.5	2 ± 0.5	4 ± 0.5	0.125 ± 0.05	0.250 ± 0.05
C. parapsilosis ORL30	2 ± 0.5	2 ± 0.5	0.5 ± 0.25	1 ± 0.5	2 ± 0.5	4 ± 0.5	0.125 ± 0.05	0.250 ± 0.05

3.4. Time kill kinetics (TKK)

For the TKK assay we take in consideration two representative strains of *C. albicans* and *C. tropicalis* which showed high sensibility to REO.

The mean time–kill graphs and standard deviations of the REO and Fluconazole against the four *Candida* strains tested are depicted in Figure 1. The kinetics of inactivation monitored over 48 h evidenced the activity of REO against the strains tested with a fungicidal and fungistatic behavior. confirming that the *C. tropicalis* strains were more sensitives to the treatment than *C. albicans*. In addition, differences between the strains of the same specie was also revealed. In fact, 15 min after treatment the cell count was reduced of about 1.4 log cfu/ml. and 1.0 respectively for C. *tropicalis* ORL21 and *C. tropicalis* ORL20. After 2 hours of treatment there was a further decrease of about 1.7- 1.5 log cfu/ml and for both strains. With the increase of the exposure time both strains were reduced further respectively reaching values of 1.5 log cfu/ml and 1.15 log cfu/ml. thus evidencing a fungicidal activity (a kill of ≥ 3 log cfu/ml).

The effect of REO was more reduced in *C. albicans* showing only 0.98 log cfu/ml after 2 h of exposure in *C. albicans* ORL08 and 0.25 log cfu/ml in *C. albicans* ORL03. Also, in this case there was a further reduction of the yeast population achieving counts of 3.58 and

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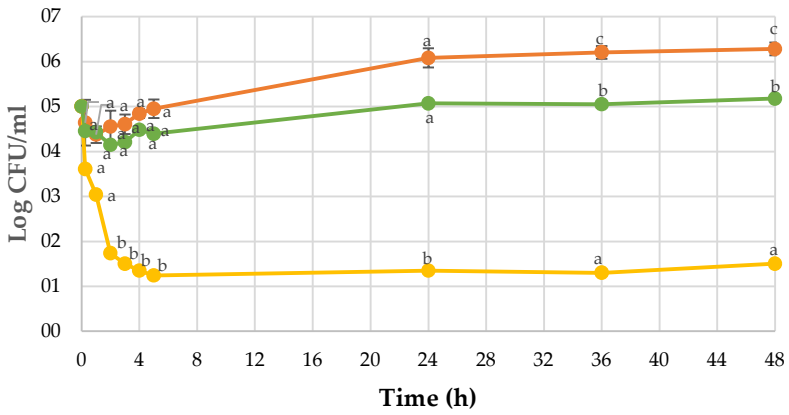
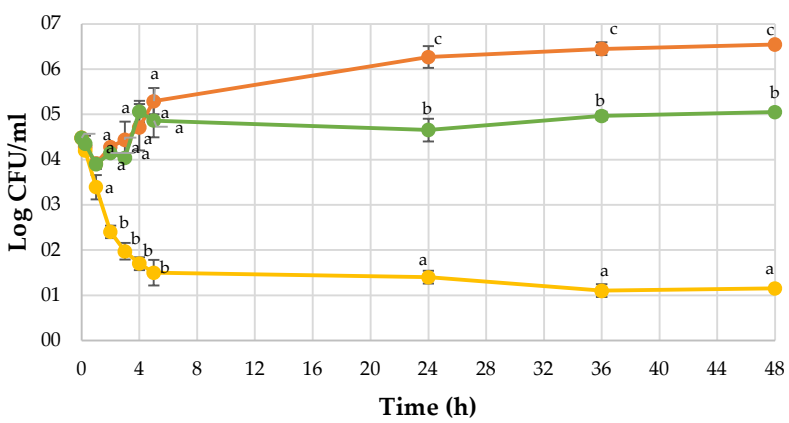
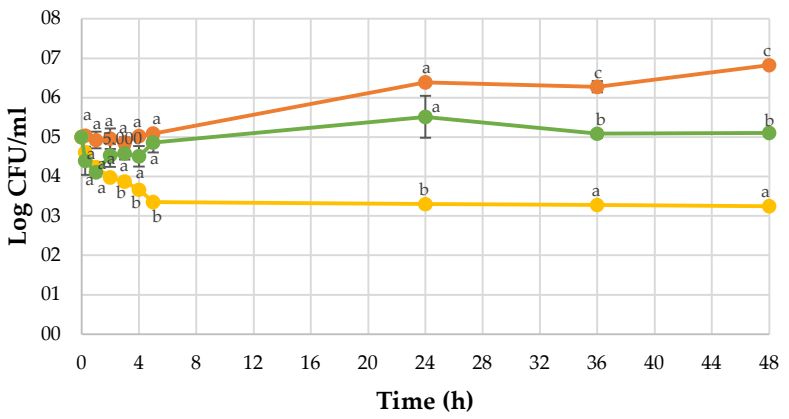
2.92 log cfu/ml for *Candida albicans ORL08* and *ORL03*. respectively reflecting a fungistatic activity. Additionally. it was found that concurrent time-kill experiments on isolates with fluconazole failed to show reductions in starting inoculum.







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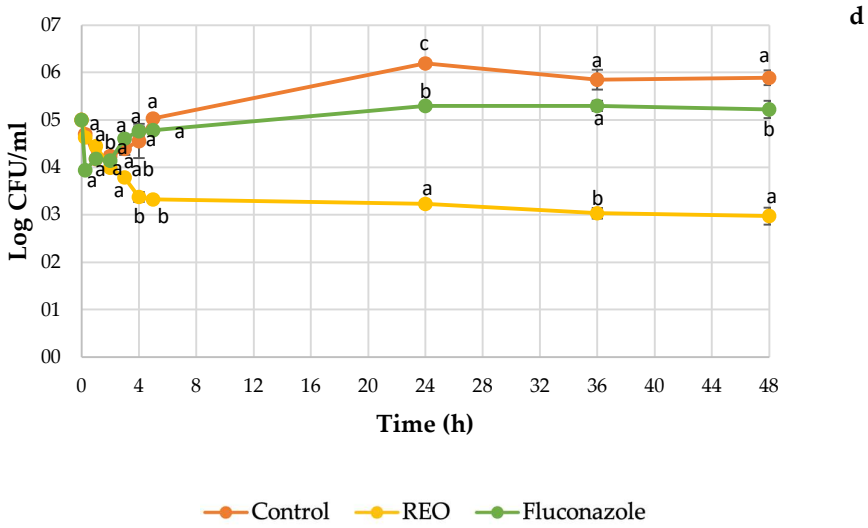

Figure 1. Time kill kinetics for REO 1% and Fluconazole against *Candida tropicalis ORL21* (a). *Candida tropicalis ORL20* (b). *Candida albicans ORL08* (c). and *Candida albicans ORL03* (d) at 37 °C. According to the ANOVA test. mean values and intervals of Tukey test for treatments with 95%. Different lowercase letters (a. b. c) indicate significant differences between treatments according to the Tukey test in a confidence interval of 95%.

The time required by REO to achieve a reduction of growth of the starting inoculum was determined for each strain (Table 3). For *C. tropicalis*. the time needed to reach 50% of the reduction was less than an hour. but it presented differences between ORL20 and ORL21 strains. being this last the more sensitive to REO with 0.39 h. After about 1.5 h. a 90% reduction in the growth was evidenced to both strains. reaching 99.9% at 1.8 and 2.9 h for *C. tropicalis* ORL21 and ORL20. respectively. Concerning *C. albicans*. the reduction of 50% of growth with respect to the starting inoculum was reached in a range of 3.6 and 4.5 h. No was achieved a reduction in the CFU of 90% and 99.9 %

Table 3. Time for REO 1% to reach 50%, 90%, and 99.9% growth reduction concerning start inoculum to *C. tropicalis* and *C. albicans* strains.

Strain	Growth reduction	REO 1%[1]
Candida tropicalis ORL21	50 %	0.39
	90 %	1.57
	99.9 %	1.79
Candida tropicalis ORL20	50 %	0.88
	90 %	1.65
	99.9 %	2.93
Candida albicans ORL08	50 %	4.51
	90 %	N.A
	99.9 %	N.A
Candida albicans ORL03	50 %	3.63
	90 %	N.A
	99.9 %	N.A

N.A.: not achieved.
[1] Treated at 37 °C for 48h.

3.5. Biofilm reduction

All the strains tested produced biofilms on polystyrene microplates after 48 h of incubation at 37 °C, as shown in Table 1. The two *C. tropicalis* strains and *C. albicans*

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ORL03 presented a strong production of the biofilm, while *C. albicans ORL08* have a weak production.

REO had considerable anti-biofilm-forming effects on *C. albicans*, as reflected in significant differences compared with the control. The percentage of biofilm eradication after 1 h of the REO 1%, exposure was 41.2 and 36.1 % for *ORL08* and *ORL03* strain, respectively. No reduction of the biofilm formed by *C. tropicalis* strain.

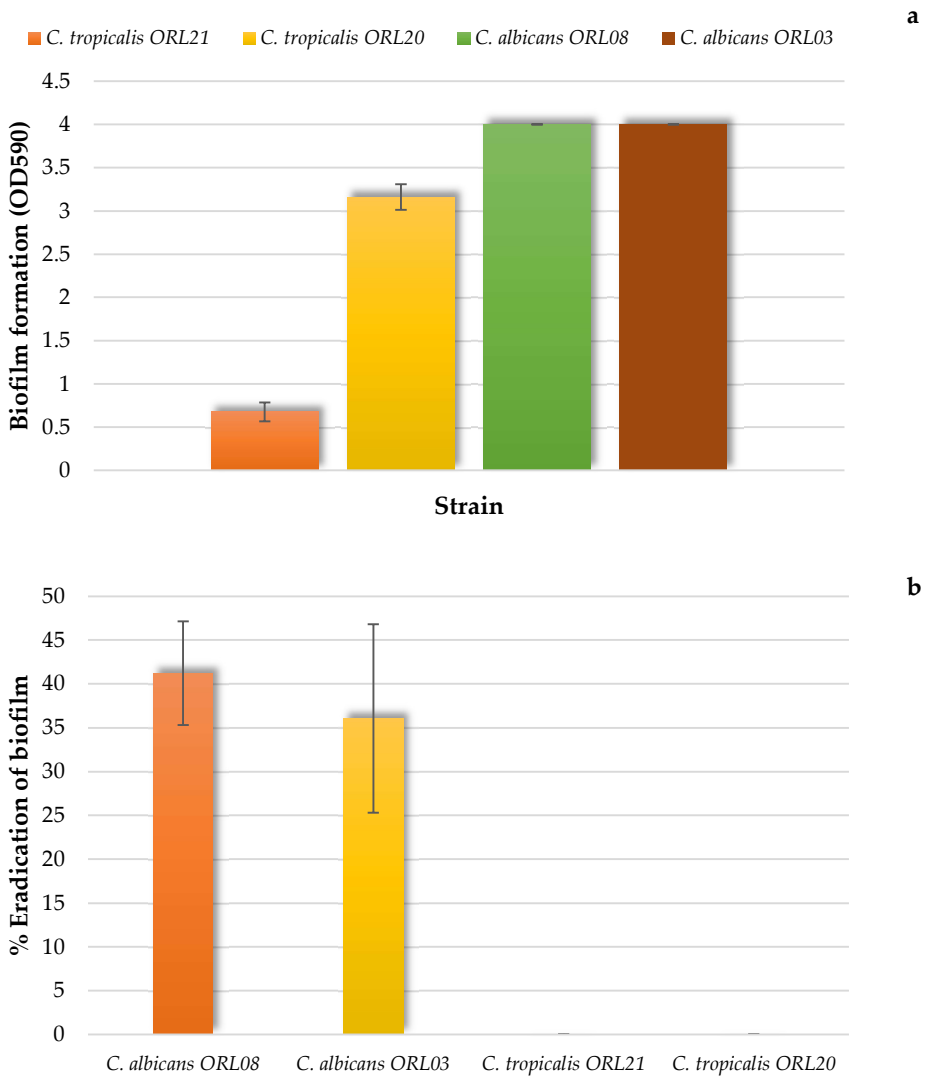

Figure 2. Effect of REO on biofilm of *C. tropicalis* and *C. albicans*. Formation of biofilm at 37 °C for 48h, where OD590 < 0.1 = non-producers (NP), OD590 0.1–1.0 = weak producers (WP), OD590 1.1–3.0 = moderate producers (MP), and OD590 > 3.0 = strong producers (SP) (a), percentage of eradication of biofilm after 1 h of REO 1% treatment (b).

4. Discussion

Resistance may be due to an altered intracellular accumulation of the drug, an altered composition of membrane sterols, an alteration of ERG11 (the gene that encodes the enzyme lanosterol-14α-demethylase, the target of these drugs), or to an alteration of the functionality of the efflux pumps [26]. These last two mechanisms are the most frequently called into question. The alteration of the target enzyme can be linked both to an upregulation of the gene that encodes it and to mutations of the gene itself. In the first case the

need is created for a higher intracellular concentration of azoles to be able to complex all the enzymatic molecules present in the cell, while in the second case there is the production of a modified enzyme for which the drug has a reduced affinity. These mechanisms have been described in isolates of *C. albicans*, *C. neoformans* and *Malassezia sp.* [27, 28]. The intrinsic resistance to azole antifungals in *C. albicans* seems to be due precisely to a reduced susceptibility of the target enzyme. The other mechanism of resistance to azoles may occur due to the inability of antifungal agents to accumulate in the cell due to a high outflow of the drug in turn due to an alteration of the functionality of transporters present on the membrane of the fungus. Two types of transporters mediate this mechanism: the "ABC transporters", encoded by the CDR genes, and the Major Facilitators, encoded by the MDR genes. These mechanisms have been described in isolates of *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* [29,30].

In this study, we demonstrated that *Ruta graveolens* essential oil tested had good antifungal activity against *C. tropicalis and C. albicans* associated with oral candidiasis. Preliminary studies have been demonstrated that the antifungal activity of this oil is due overall to the main component 2-nonanol and 2-undecanone, which exhibited the most potent antifungal effect [16]. On the other hand, Reddy et al., [31] tested REO against six diverse fungi species, evidenced the most significant antifungal activity against *C. albicans* with 86 % of growth reduction in comparison to the control positive (Amphotericin B); the authors showed that the antifungal activity is related to the abundance of ketones and alcohols in the REO. In addition, Attia et al., [32] also founded antifungal activity against two *C. albicans* clinical strains, a *C. glabrata* with MIC of 1.14–2.5 μg/ml, besides was observed morphological changes including cell surface deformation, disruption and prevented the germ tube production, additionally was demonstrated a direct correlation between the percentage of ketones and the antimicrobial activity.

The time-kill kinetics demonstrated that 1% of REO had an effect against the four strains of *C. albicans* and *C. tropicalis* tested. REO evidenced the highest levels of killing against *C. tropicalis* strains showing antifungal activity. The time to achieve a 50% reduction of starting inoculum growth was less than an hour with a decrease in growth concerning control of 76.1 % for *C. tropicalis ORL21* and 82% for *C. tropicalis ORL20* after 48 h of treatment. However, the REO effect was not so fast with *C. albicans* strains with just a 50% reduction after 3.5 hours, a final reduction of 48% for *C. albicans ORL08*, and 50% for *C. albicans ORL03*, indicating fungistatic activity of the REO. These results suggest that the REO effect depends on the *Candida* species. Similar results have been reported with other essential oils; they present fungicidal or fungistatic activity according to the *Candida* species. The *Ocimum gratissimum* L. essential oil was fungicide against *C. tropicalis* but showed fungistatic activity against *C. albicans* [33]. Some authors have reported the fungicidal effect of REO treated with salicylic acid in *C. glabrata* and *C. albicans* with a time of above 1.5 h to reach a 50% of reduction of growth [32].

Candida biofilm is a well-organized formed by planktonic and mycelial yeast form, surrounded by extracellular polymeric substances; this structure is effective microbial protection and can be generated the already known drug resistances [34]. REO demonstrated an anti-biofilm action to the two *C. albicans* strains evaluated; this activity was detected with only one hour of the treatment. Biofilm was reduced more in *C. albicans* ORL08 than *C. albicans* ORL03 in 5%. Studies have reported anti-biofilm *Candida* activity of different EOs such as Peppermint, Eucalyptus, Ginger grass, Clove, and Thyme essential oils in ranges into 28- 85%[33,34]. Some authors suggested that the anti-biofilm capacity of EOs is related to the inhibition of filamentation and germ tube formation, and interference with the cell membrane of planktonic and sessile cells of *Candida albicans*, also the hydrophobic character of EOs may increase the absorption through of charged extra-cellular polymers, producing that oil has greater contact and permeation in the membrane to the cells [35-37]. REO has demonstrated a similar action mechanism in *C. gloesporioides*, where it is observed compromise of the membrane after one hour of exposure. No anti-biofilm activity was observed with REO in *C. tropicalis* strains. Al-Fattani

et al., [38] reported that biofilms of *C. tropicalis* are mainly constituted by hexosamine matrix in comparison with a glucose-rich matrix of the *C. albicans* biofilm; this structure from the last one permits more rapidly drug penetration. Besides, one study with several candida species measuring drug diffusion rates was reported that the slowest rates of penetrations were presented with *C. tropicalis* [39]. Taking into account the above we could suggest that is necessary the exposure to REO for more time to observe biofilm reductions in *C. tropicalis* strains.

In summary, our results demonstrated the efficacy of REO in growth reduction and biofilm eradication of *C. albicans* and *C. tropicalis* after 1 hour of exposure. For the first time we reported the potential of REO to exert antifungal activity against non-albicans Candida (NCAC) species. Further studies will be address to study the mechanisms of action of REO.

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