Susceptibilities of *Candida albicans* Mouth Isolates to Antifungal Agents, Essentials Oils and Mouth Rinses

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Abstract Forty *Candida albicans* strains isolated from patient's mouth with fixed orthodontic appliances were analyzed to their susceptibilities to antifungal agents, mouth rinses and essential oils. Susceptibility to fluconazole, econazole, miconazole and ketoconazole, amphotericin B and nystatin was assessed by the disk diffusion (DD) method based on the Clinical and Laboratory Standards Institute M44-A protocol, and by Etest (fluconazole and amphotericin B). The susceptibilities to mouth rinses and essential oils were also determined by the DD technique. All isolates tested were susceptible (S) to amphotericin B, nystatin and fluconazole. The overall concordance between the DD and the Etest was 100% for amphotericin and fluconazole. One isolate was resistant to econazole (2.5%) and the other to ketoconazole (2.5%). Econazole and ketoconazole had the highest percentages of susceptible dose dependent (SDD), 55 and 95%, respectively. Regarding to the susceptibility isolates profile, seven phenotypes were detected, and the 3 more represented (90% of the isolates) of them were SDD to one, two or three azoles. The study of mouth rinses showed a high variability of efficacy against *C. albicans*. The results showed that the isolates susceptibility to essential oils differed ($P < 0.05$). The profile activity was: cinnamon > laurel > mint > eucalyptus > rosemary > lemon > myrrh > tangerine. The main finding was that the susceptibility to cinnamon and laurel varied among the three more representative antifungal phenotypes ($P < 0.05$). The susceptibility of econazole-SDD isolates to cinnamon and lemon was higher than those of the econazole-S yeasts ($P < 0.05$). In contrast, econazole-SDD isolates were less affected by laurel than econazole-S counterparts ($P < 0.05$).

Keywords *Candida albicans* · Susceptibility · Antifungal · Essential oils · Mouth rinses

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Introduction

The yeast *Candida albicans* is a commensal organism frequently found in the oral cavity [1] that can cause opportunistic infections when some predisposing

factors are present among the immunodeficiency, endocrine disorders, age extremes, radiotherapy, antibiotic therapies, transplants, malignant diseases and the use of orthodontic appliances [2–5]. The most common treatment is the use of antifungal agents, such as azoles (fluconazole, itraconazole, miconazole and ketoconazole) and polyenes (amphotericin B or nystatin). The control of the infections caused by *Candida* faces several problems, including the limited number of effective antifungal agents, their high toxicity and costs, the recurrence of the infection and, mainly, the increasing resistance to them [6, 7].

In general, oral *C. albicans* isolates have high levels of susceptibilities to a range of antifungal agents [8], but some studies reported high levels of azoles resistance in *C. albicans* strains isolated from the throat and mouth [9, 10]. The use of some mouth wash solutions to control microbial mouth growth might represent a valid alternative to topical use of antifungal substances. In vitro studies provided evidence that chlorhexidine digluconate (CHX) was fungicidal [11, 12]. Additionally, mouth rinses may contain alcohol or other compounds that could significantly affect their antimicrobial action [13]. Among those compounds, plant extracts and essential oils are used. Due to their antibiotic properties, essential oils use in the pharmaceutical and food industry had been generalized, constituting an alternative to the use of antimicrobials [14]. Also, the growing resistance of *C. albicans* to antifungal agents stimulated the research of new therapeutic alternatives, like the use of essential oils [15–17].

This study aims to test the susceptibility of *C. albicans* isolates from patients with orthodontic appliances, to (1) different antifungals (fluconazole, miconazole, econazole, ketoconazole, nystatin and amphotericin B); (2) mouth rinses and essential oils (lemon, eucalyptus, myrrh, cinnamon, laurel, mint, rosemary and tangerine) efficacies against the same isolates; and (3) to search for possible relations among the isolates susceptibilities to antifungal drugs, essential oils and mouth rinses.

Materials and Methods

Origin of *C. albicans* Isolates

Forty isolates of *C. albicans* used in this study were obtained from 25 patients using fixed orthodontic appliances who had attended a dental clinic, using the medium CHROMagar™ Candida. The green colonies, presumptively identified as *C. albicans*, were purified and cryo-preserved (−80°C). The identification of the isolates were confirmed based on phenotypic features, such as their macro- and micromorphology, fermentation of D-glucose, assimilation of carbohydrates D-galactose, maltose, sucrose, cellobiose, trehalose, raffinose, melezitose, soluble starch, L-arabinose and D-glucosamine, formation of hyphae/pseudohyphae [18], chlamydospore production [19] and germ tube formation [20].

Antifungal Susceptibility Tests

Disk diffusion (DD) testing of amphotericin B, nystatin, ketoconazole, econazole, fluconazole and miconazole was performed as described by CLSI guidelines (M44-A protocol [21]) and [22] except the use of methylene blue at 0.5 μg/ml. Amphotericin B (10 μg), nystatin (50 μg), ketoconazole (15 μg), econazole (10 μg), fluconazole (25 μg) and miconazole (10 μg) were from Neo-sensitabs™ ROSCO®. Agar plates (90-mm diameter) containing Mueller–Hinton (MH) agar (Difco Laboratories) supplemented with 2% glucose were inoculated with yeast cells, previously suspended in a saline solution (0.85%) with the turbidity 0.5 in a McFarland scale. The plates were incubated at 36 ± 1°C for 24 h. After 24 h cultivation, the inhibitory diameter zone (dz) was measured [23]. The interpretative criteria for fluconazole according to CLSI guidelines [21] were: susceptible (S) $\geq$19 mm; susceptible dose dependent (SDD) 18–15 mm and resistant (R) $\leq$14 mm. For the other antifungal agents, we followed the manufacture's interpretation: ketoconazole (S $\geq$ 28 mm; SDD 27–21 mm and R $\leq$ 20 mm), econazole (S $\geq$ 20 mm; SDD 19–12 mm and R $\leq$ 11 mm), miconazole (S $\geq$ 20 mm; SDD 19–12 mm and R $\leq$ 11 mm), amphotericin B (S $\geq$ 15 mm; SDD 14–10 mm and R $<$ 10 mm) and nystatin (S $\geq$ 15 mm; SDD 14–10 mm and R $\leq$ no zone).

The minimum inhibitory concentration (MIC) for fluconazole and amphotericin B was also determined by the Etest method (ET), using the ET strips (AB BIODISK) with the concentration range from 0.002 to 32 μg/ml for amphotericin B and 0.016–256 μg/ml for fluconazole. We used the MH agar to perform the test, with 2% glucose. The inoculated suspension was treated as for DD testing. The interpretative MIC

breakpoints were recommended by the manufacture: fluconazole (S $\leq$ 8 µg/ml and R $\geq$ 64 µg/ml) and amphotericin (S $\leq$ 1 µg/ml and R $\geq$ 4 µg/ml).

Quality Control

Quality control (QC) for DD and ET was performed by using *C. albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 [22]. These species were included in all runs, and the results were within published limits.

Essential Oils and Mouthwashes Susceptibility Tests

All essential oils were obtained from one standard commercial supplier and derived from plants whose extracts or active substances are frequently found in toothpastes. The plants belongs to 5 families: Lauraceae, *Cinnamomum zeylanicum* Blume (cinnamon) and *Laurus nobilis* L. (laurel); Rutaceae, *Citrus limonum* L. (lemon) and *Citrus reticulata* Blanco (tangerine/mandarin); Lamiaceae, *Mentha piperita* L. (mint) and *Rosmarinus officinalis* L. (rosemary); Burseraceae, *Commiphora myrrha* (Nees) Engl. (myrrh); and Myrtaceae, *Eucalyptus globulus* Labill (blue gum eucalyptus). The rosemary, mint, laurel and eucalyptus essential oils were obtained from the leaves of their plants, the lemon and tangerine oils from the fruits peel, myrrh oil from the plant resin and cinnamon essential oil from bark and leaves. For the susceptibility tests, 15 µl of each essential oil was put on blank disks (6-mm diameter), allowed to dry and placed in a MH plate, previously inoculated with a 0.5 McFarland yeast suspension. The plates were incubated at 36 $\pm$ 1°C for 24–48 h, and dz (mm) read.

The seven commercial mouthwashes tested varied in their composition, namely in the main active compounds (chlorhexidine digluconate-CHX, alcohol or hexetidine-HEX) and their concentration (Table 1): five had CHX with ($n = 2$) or without alcohol ($n = 3$), one had HEX and the last only alcohol. They also differ in the type of the excipients. For the susceptibility test, the same procedure was followed for essential oils. Additionally to *C. albicans* isolates ($n = 40$), the two ATCC yeasts were also tested.

Data Analysis

To test whether mouthwashes or essential oils affect yeast growth (dz), we used the Wilcoxon matched pair test. The phenotypes and the susceptibility classification of the isolates to the antifungals were compared against each essential oil and mouthwash, by the nonparametric Kruskal–Wallis, for multiple independent groups, or Mann–Whitney's, for two independent groups, tests. All analyses were computed by STATISTICA version 9.1. [24].

Table 1 Main composition of the mouthwashes tested, regarding to their active compounds and excipients

Mouthwash	Active compounds	Excipients
A	Chlorhexidine digluconate (0.2%) Alcohol (14%)	Water, sorbitol, castor oil, flavor, dyes
B	Chlorhexidine digluconate (0.2%) Alcohol (7%)	Water, castor oil, sorbitol, peppermint essence
C	Hexetidine (0.1%)	Water, sodium saccharin
D	Alcohol (no mention)	Water, sorbitol, Poloxamer 407, benzoic acid, methyl salicylate, flavor, sodium saccharin, sodium benzoate, thymol, eucalyptol, menthol, dyes
E	Chlorhexidine digluconate (0.12%)	Water, xylitol, glycerine, propylene glycol, castor oil, sodium saccharin, Poloxamer 407, acesulfame potassium, lactic acid, methyl salicylate, Neohesperidin dihydrochalcone, flavor, menthol, D-limonene, dyes
F	Chlorhexidine digluconate (0.2%)	Water, sorbitol, glycerine, castor oil, flavor, citric acid, sodium methylparaben, methyl salicylate, sodium saccharin, menthol, eugenol, cinnamal, D-limonene
G	Chlorhexidine digluconate (0.12%)	Water, propylene glycol, glycerine, castor oil, flavor, methylparaben, propylparaben, acesulfame potassium, dye, D-limonene

Results

Morphological and biochemical tests done to yeasts confirmed that all were C. *albicans* isolates. According to the antibiogram results (Table 2), the isolates were classified as susceptible (S), resistant (R) and intermediate resistant or susceptible dose dependent (SDD), according to CLSI guidelines. The polyenes nystatin and amphotericin B and the azole fluconazole were efficient against all tested *C. albicans*. Regarding the other azoles, the SDD group was the most represented among isolates against ketoconazole (95%) and econazole (55%). The majority of the isolates (65%) were susceptible or SDD (35%) to miconazole. None of the isolates were resistant to miconazole, while 2.5% of them were resistant to econazole or ketoconazole.

Etest interpreted with the CLSI breakpoints identified all fluconazole-S isolates as susceptible. Also, the manufacture breakpoints to amphotericin B showed an excellent overall agreement (100%) between DD test and Etest susceptibilities. The isolates MIC range for amphotericin B was 0.064–0.19 μg/ml and for fluconazole was 0.064–0.5 μg/ml.

Table 3 showed the seven different phenotypes, based on the tested antifungal substances, and respective percentages. The phenotypes differ among them in their response to econazole, miconazole and ketoconazole. The phenotype I was the most frequent (37.5%), followed by the phenotypes II (27.5%) and III (25.5%). Each of remaining four phenotypes found was represented by one isolate, and overall represent 10% of the isolates. The three most represented phenotypes were econazole-S, miconazole-S and ketoconazole-SDD, econazole-SDD, miconazole-S and ketoconazole-SDD, and econazole-SDD, miconazole-SDD and ketoconazole-SDD.

Figure 1a summarizes the in vitro efficacy of the mouthwashes in growth control of the isolates. To evaluate whether the differences between the mouthwashes were significant, we used the non-parametric Wilcoxon matched pair test. All mouthwashes differed ($P < 0.05$) among each other. It is possible to consider five groups of mouthwashes: efficient (mean dz > 20 mm) that includes A and B mixtures; median efficient (mean $16 < $ dz $ < 18$ mm) that groups E, F and G solutions; low efficient (mean dz < 9 mm) represented by C; and inefficient (mean dz $= 6$ mm) by D. The susceptibilities to the mouthwashes in *C. albicans* ATCC 90028 were in the range of those obtained to *C. albicans* oral isolates, except for solutions C and E. In general, *C. parapsilosis* ATCC 22019 was more susceptible to mouthwashes than the isolates of *C. albicans*.

The results of susceptibility to essential oils are presented in Fig. 1b, and clearly cinnamon was the most efficient essential oil in inhibiting *C. albicans* growth. Based on the mean of dz, essential oil activities followed the profile cinnamon > laurel > mint > eucalyptus > rosemary > lemon > myrrh > tangerine. The non-parametric Wilcoxon matched pair test found differences ($P < 0.05$) between almost oils, except between the pairs laurel/mint, rosemary/lemon and rosemary/eucalyptus ($P > 0.05$). The less efficient essential oils in controlling the isolates growth were myrrh and tangerine. In average, *C. albicans* oral isolates exhibited similar susceptibilities to essential oils (in the standard deviation range) to the *C. albicans* ATCC strain, except for myrrh and laurel.

For the comparison of yeast phenotypes susceptibilities to essential oils, we performed the Kruskal–Wallis test, after removing the phenotypes IV, V, VI and VII, all with 1 isolate. The susceptibility responses of phenotypes I, III and II to essential oils were significantly different to laurel ($H = 10.56$; $P = 0.0059$) and cinnamon ($H = 7.78$; $P = 0.0206$). The differences were between the phenotypes I and III,

Table 2 Classification of *C. albicans* isolates ($N = 40$) based on the interpretative standards of the tested antifungal, using the DD method

Classification	Econazole	Miconazole	Fluconazole	Ketoconazole	Amphotericin B	Nystatin
S	17 (42.5%)	26 (65.0%)	40 (100%)	1 (2.5%)	40 (100%)	40 (100%)
SDD	22 (55.0%)	14 (35.0%)	0	38 (95.0%)	0	0
R	1 (2.5%)	0	0	1 (2.5%)	0	0

For each antifungal total number and the percentage are represented

S Susceptible; *SDD* susceptible dose dependent; *R* resistant

Table 3 Percentage of the seven different phenotypes

ECO	MIC	FLC	KTC	AMB	NYT	Phenotype	Isolates (N)	%
S	S	S	SDD	S	S	I	15	37.5
SDD	SDD	S	SDD	S	S	II	11	27.5
SDD	S	S	SDD	S	S	III	10	25.0
S	SDD	S	SDD	S	S	IV	1	2.5
S	S	S	S	S	S	V	1	2.5
R	SDD	S	SDD	S	S	VI	1	2.5
SDD	SDD	S	R	S	S	VII	1	2.5

ECO Econazole; *MIC* miconazole; *FLC* fluconazole; *KTC* ketoconazole; *AMB* amphotericin B; *NYT* nystatin; *S* susceptible; *SDD* susceptible dose dependent; *R* resistant
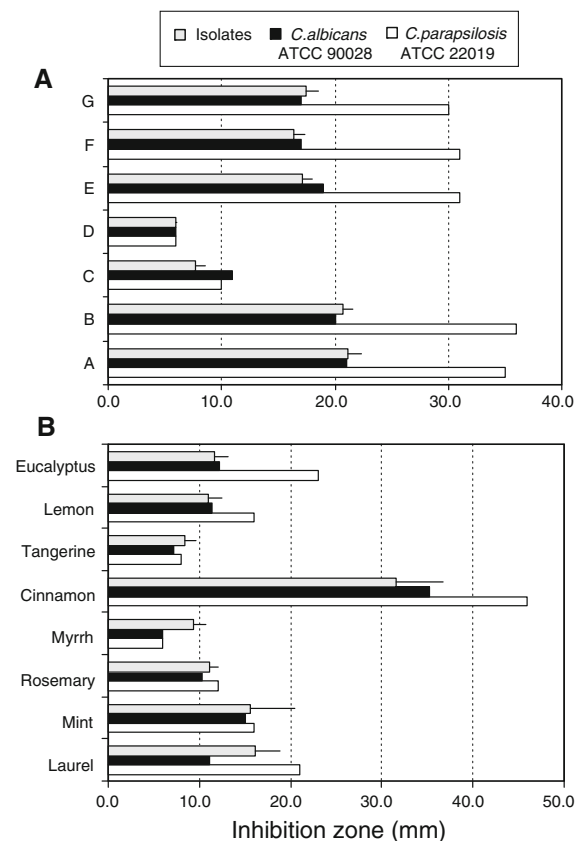

Fig. 1 Average diameter ($n = 40$) and standard error of the inhibition zone (mm) obtained in *C. albicans* isolates, tested with commercial mouthwashes (**a**) and with essential oils (**b**). The two type *Candida* culture (*C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028) were used for control

which differ from each other only in their response to econazole susceptibility groups (S or SDD). When econazole-S and -SDD had their susceptibility to laurel, cinnamon and lemon compared by the Mann–Whitney *U*-test, the (S) group was significantly less susceptible to cinnamon ($P = 0.0058$) and lemon ($P = 0.033$) than the (SDD) group. Contrary, the (S) group was significantly more susceptible to laurel ($P = 0.010$) than the (SDD) group. No differences in the susceptibilities to mouthwashes were found among the phenotypes bases on the interpretative criteria to antifungal agents.

Discussion

Orthodontic devices and other oral appliances seems to alter the oral ecological environment, hence may tip the balance to favor the *C. albicans* presence. Amphotericin B is an effective treatment, but often causes adverse effects such as kidney injury and hypokalemia [25, 26].

Yeast azole resistance may be intrinsic or acquired (see Niimi and collaborators for a review [27]). To overcome the problem of antifungal resistance, plants have been extensively studied as alternative treatments [17, 25, 26]. Because some essential oils have been reported to have antifungal effects, we investigated and compared mouthwashes and antifungal drugs and various essential oils.

The results of DD test to amphotericin B and nystatin were in line with the distribution profiles in yeasts isolated from dentures [28], from inside the patient's mouth with dental stomatitis [29], and from biological fluids [30]. During a surveillance study of antifungal susceptibility by oral *C. albicans* isolates, only 0.3% of them were resistant to fluconazole [8],

and Dorocka-Bobkowsha et al. [29] reported that 88.7% of *C. albicans* isolates were susceptible to fluconazole. Regarding the four azoles tested, the isolates were all susceptible to fluconazole (results obtain by DD and E tests), a similar result to Arendrup et al. [30]. The isolates varied only among the susceptibility profile to the 3 azoles econazole, miconazole and ketoconazole. Also, 2.5% of them exhibited resistance either to ketoconazole or econazole, a result that contrasts with those obtained by Kuriyama et al. [8], who reported that all mouth isolates were sensible to ketoconazole. None of the isolates were resistant to miconazole, although Paniagua et al. [9] reported that 45% of *C. albicans* isolated from the throat were resistant to miconazole. Despite the wide availability and use of miconazole, primary a topical antifungal [31], we did not found any isolate resistant to this drug, in spite of 35% of the isolates were miconazole-SDD. The high percentage of the SDD profile to two (econazole and ketoconazole) and three (econazole, miconazole and ketoconazole) azoles is the source of concern due to the potential cross-resistance among them [32].

All mouthwashes differ in their ability to inhibit yeast growth ($P < 0.05$). The most efficient solutions (A and B) had both CHX (0.2%) and alcohol (14 or 7%), while the solutions that exhibited an intermediate efficacy (E, F and G) had either similar CHX concentration or lower (0.12%), but without ethanol. The two mouth rinses (C and D) had the smallest inhibition zones, 7.9 and 6.0 mm, respectively. The former (C) has in its composition HEX (0.1%), while the latter (D) has only alcohol and excipients. The ability of CHX to inhibit *C. albicans* growth is well documented [1, 12, 33–35]. It seems that CHX associated with alcohol is an effective combination in this species control. Contrary, HEX did not show activity against the isolates, a result also reported previously [12].

This work evaluated the anti-candidial activity of eight plant essential oils usually presents on mouthwashes and toothpastes, and with their antimicrobial properties recognized [15, 36–40]. Our results showed that the isolates susceptibilities to oils differed among them, except between laurel and mint ($P > 0.05$). In this study, cinnamon was the most efficient oil. Previous studies recognize that cinnamon oil is an efficient antifungal or anti-candidal agent [36, 37, 40–42] and have interest as a source of natural products

for potential use as alternative drugs to heal many infectious diseases [43–45]. Besides cinnamon, other essential oils exhibited high efficacy, as laurel and mint. Both plants have high concentrations of 1,8-cineole, in association with other substances, for instance, menthol in the case of peppermint oil, which has been indicated as an antimicrobial agent [39, 40]. Ezzat [38], who investigated the antifungal ability of several essential oils by the DD method, found that *Mentha piperita* had activity against *C. albicans*, a similar result obtained in our work. However, different results were expressed in other studies. Higher inhibition zones for mint oil were obtained by Abdel-Mallek and collaborators [37], but others [36] obtained lower values. The rosemary, eucalyptus and lemon essential oils exhibited a moderate anti-candidal activity, results corroborate by others [36, 46]. The tangerine and myrrh oils presented a weak anti-candidal activity. Hammer and colleagues [15] tested twenty plant oils and extracts and reported that myrrh and mandarin oils had low or no antimycotic activity. Although a different method was used (microdilution method), these findings were confirmed in the present investigation.

Plants belonging to the same family in general have similar inhibitory abilities, probably due to the proximity of the chemical composition of their oils [47]. Nevertheless, cinnamon had a higher anti-candidal activity than laurel, both from Lauraceae family. Also, the Rutaceae (lemon and tangerine), and the Lamiaceae (rosemary and mint) members exhibited distinct efficacies. Even in the same species, the essential oil composition can vary greatly, depend on the geographical and environmental conditions, existing numerous chemotypes [44]. One of the factors that difficult the comparison of the results is the absence of standardization of antimicrobial activity assays with essential oils [45].

The phenotypes II and IV showed differences in the susceptibilities to cinnamon ($P < 0.05$) and to laurel ($P < 0.001$). These phenotypes differ between them in the econazole susceptibility response: econazole-S isolates were less susceptible to cinnamon ($P < 0.01$) and lemon ($P < 0.05$) oils than econazole-SDD isolates. Pozzatti et al. [45] had reported that fluconazole-resistant *C. albicans* susceptibility to cinnamon and other essential oils were lower than their fluconazole-susceptible counterparts. In contrast, laurel essential oil had higher ($P < 0.05$) antifungal properties against econazole-S yeasts, than against econazole-SDD.

The essential oil extracts used in this work demonstrated antifungal activity, but, in general, to a lesser extent than the antifungal drugs and the mouthwashes. Despite the fact that in vitro studies cannot be directly extrapolated to in vivo effects, the results suggests that the use of essential oils such as cinnamon, laurel and mint against *C. albicans* could be a viable alternative, alone or combined with antifungal agents, for therapeutic and/or preventive purposes against oral candidosis caused by the use of orthodontic devices.

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