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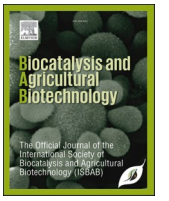

Antifungal, antibiofilm and anti-resistance activities of Brazilian monofloral honeys against *Candida* spp.

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ABSTRACT

Candida is a fungus commonly involved in diseases of the skin and mucous membranes and has a high resistance to antifungal drugs. Moreover, there are few therapeutic options to treat these infections. Honey is a popular product that is easily accessible and has a diverse chemical composition, such as phenolic compounds that can have an antimicrobial effect in various metabolic pathways. Therefore, in this study, we aimed to characterize the antifungal activity of tree popular Brazilians honey against *Candida* species. Aroeira, Eucalyptus and Assa-Peixe honey was characterized by total phenolics and its antifungal activity on *Candida* species was tested by the microdilution method, antibiofilm activity, yeast to hypha (Y–H) transition, combination assays, multistage resistance study, and antibi-scanning electron microscopy of the fungi. Aroeira honey shown potent antifungal activity *in vitro* against planktonic cells of *C. albicans* (MIC 20–30% w/v) and non-*albicans* (MIC 30–40% w/v) and also breaks down mature biofilms formed by these yeasts at 40% w/v. Urethral catheters sensitized with 30% Aroeira honey reduced adhesion and biofilm formation by *C. albicans*. Aroeira honey did not induce resistance in *C. albicans* during the 21-day exposure. Its ability to alter fungal membrane structure contributes to the synergistic effects of Aroeira honey with commercial antifungal agents (miconazole, ketoconazole, itraconazole). Aroeira honey inhibits yeast-to-alpha transition in C. albicans, and its prior exposure for 1 h reverses the azole resistance profile of a highly resistant strain of *C. albicans*. Taken together, these results indicate that Aroeira honey is a promising therapeutic agent against superficial candidiasis.

1. Introduction

In apitherapy, bee products such as honey, propolis, wax, pollen, bee venom, and royal jelly are used to prevent cure or treat diseases (Trumbeckaite et al., 2015). Apitherapy products have been used as natural remedies since ancient Egypt (Fratellone et al.,

Abbreviations: VVC, Vulvovaginal candidiasis; ATCC, American Type Culture Collection; FIOCRUZ, Oswaldo Cruz Foundation; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CLSI, Clinical Laboratory Standard Institute; CFU, Colony-forming units; MIC, Minimum inhibitory concentration; MFC, Minimum fungicidal concentration.

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2016). More recently, bee products have been incorporated into medical practice with an emphasis on wound healing and treatment of skin or mucous membrane infections (Fratellone et al., 2016; Fratini et al., 2016; Trumbeckaite et al., 2015; Wehbe et al., 2019). Among apitherapy products, honey is most commonly used to treat superficial infections. In fact, the use of honey *in natura* has shown promising effects in the preventive, clinical and symptomatic treatment of labial and genital herpes (Al-Waili, 2004; Corey et al., 1982), gynecological bacterial infections after vulvectomy (Cavanagh et al., 1970) and bacterial growth in diabetic foot ulcers (Gurusamy et al., 2013; Imran et al., 2015; Moghazy et al., 2010).

In addition, honey-based products have also been successfully used to treat superficial fungal infections. Al-Waili (2004) showed that a mixture of honey, olive oil, and beeswax (1:1:1) applied three times daily for a maximum period of 4 weeks produced mycological healing in patients with pityriasis versicolor, tinea cruris, tinea corporis, and tinea faciei. In another study, application of diluted raw honey (90% honey in warm water) every other day for 20 min on the hair of patients with dandruff resulted in improvement of itching, scaling, hair loss and lesions after 2 weeks (Al-Wailli, 2001). In addition, Banaeian and coworkers (2017) demonstrated that the use of a vaginal cream containing 70% honey applied at night for seven days reduced inflammation, vaginal discharge, and irritation in women with vulvovaginal candidiasis (VVC) to a similar extent as positive control (Clotrimazole 1% cream; (Banaeian et al., 2017). Since superficial fungal infections are very common worldwide (Magill et al., 2012) and antifungal drug resistance has become an increasing problem (Centers for Disease Control and Prevention, 2020), these clinical studies show that honey is a promising antifungal agent. Therefore, a pharmaceutical formulation containing honey or a honey derivate may help to expand the limited therapeutic arsenal currently available against superficial fungal infections.

Honey is a complex apitherapeutic product that may contain sugars, proteins, amino acids, vitamins, minerals, pollen, wax and phenolic compounds. The phenolic compounds have for antimicrobial, antifungal, anti-inflammatory and antioxidant effects (Bogdanov, 1997; Meda et al., 2005). However, the doses and composition of these phenols vary depending on geographic distribution, season, and botanical origin (Bogdanov, 1997; Calaça et al., 2018; Miguel Alvarez-Suarez et al., 2009).

Properties such as low cost, limited toxicity, low ability to develop resistance, and potent antifungal activity make honey a potential source for the development of new antifungal therapeutic strategies (Fernandes et al., 2021; Samarghandian et al., 2017). In this study, the antifungal activity of tree monofloral honeys were evaluated against reference and clinical isolates of *Candida* species commonly involved in superficial infections. To this end, we seek to elucidate the effect of honey against key fungal virulence factors (i.e., biofilm and morphological transition), its interaction with commercial antifungal agents, its ability to reverse fungal resistance, and its ability to induce resistance *in vitro*.

2. Material and methods

2.1. Reagents

Amphotericin B (Inlab, São Paulo, SP, Brazil), miconazole (Pharma Nostra, Rio de Janeiro, RJ, Brazil), nystatin (Fragon, São Paulo, SP, Brazil), crystal violet, 95% ethanol, dimethyl sulphoxide (DMSO), sodium chloride (NaCl), sodium carbonate (Synth, São Paulo, SP, Brazil), fetal bovine serum (Gibco, ThermoFisher Scientific, Frankfurt, HE, Germany), glucose (Inlab, São Paulo, SP, Brazil), Folin-Ciocalteu, gallic acid, sucrose, maltose, glucose, fructose (Sigma-Aldrich, San Francisco, CA, USA) were purchased from commercial suppliers and used without additional purification. Sabouraud-Dextrose broth (SDB) and Sabouraud-Dextrose agar (SDA) were purchased from Kasvi (São José do Pinhais, PR, Brazil).

Three different types of honey from *A. mellifera* collected in 2020 were used: (i) Aroeira, from Janaúba-MG (Brazil), unifloral source (*Astronium urundeuva* (M. Allemão) Engl.); (ii) Eucalyptus, from Betim-MG (Brazil), unifloral source (*Eucalyptus globulus* Labill.), and (iii) Assa-Peixe, from Betim-MG (Brazil), unifloral source (*Vernonia polyanthes* Less). Honey samples (250 g) were purchased from a commercial supplier. The samples were packed and sealed in amber glass bottles and stored at 4 °C in the dark until processing. The botanical origin of the honey was confirmed by traditional qualitative microscopic analysis and determination of the frequency of pollen grain classes in the samples. Before the analyses were performed, all samples were stored overnight at room temperature. The artificial honey, which was used to simulate the high sugar content, was prepared *in-house*. For this purpose, 7.5 g sucrose, 37.5 g maltose, 167.5 g glucose and 202.5 g fructose were dissolved in 85 mL sterile water. The sugar composition of the solution also corresponded to natural honey: fructose (40.5%), glucose (33.5%), maltose (7.5%) and sucrose (1.5%). The honey was mixed with a magnetic stirrer for 30 min until it was homogenous (Yung Yung An et al., 2016).

2.2. Microorganisms

The microorganisms used in this study were from the American Type Culture Collection (ATCC) and were kindly provided by the Reference Microorganisms Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ; Rio de Janeiro, RJ, Brazil). Antifungal activity was evaluated against *C. albicans* ATCC 10231, *C. albicans* ATCC 18804, *C. glabrata* ATCC 2001, *C. krusei* ATCC 34135, and *C. tropicalis* ATCC 28707. According to ATCC specifications, *C. albicans* 10231 is resistant to anidulafungin, voriconazole, itraconazole, and fluconazole. *C. albicans* ATCC 18804, *C. glabrata* ATCC 2001, and *C. tropicalis* ATCC 2870 are susceptible to all azoles, echinocandin, and polyenes. In turn *C. krusei* ATCC 34135 is inherently resistant to azoles but susceptible to echinocandins and polyenes (American Type Culture Collection, 2021).

Antifungal activity was also determined against three clinical isolates of *C. albicans* derived from vaginal secretions of women with VVC. All clinical isolates were kindly provided by Dr. Jaqueline M. S. Ferreira (Laboratory of Medical Microbiology, Universidade Federal de São João Del Rei, Divinopolis, MG, Brazil) and were identified by biochemical, phenotypic, and morphological tests (Lima et al., 2019a). In addition, all isolates were confirmed to the species level by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method using a Microflex LT spectrometer (BrukerDaltonics, MA, USA).

2.3. Antifungal activity

The antifungal activity of monofloral honeys against *Candida* spp. was evaluated using the broth microdilution method described in Clinical Laboratory Standard Institute (CLSI) document M27 (2017) with slight modifications (Lima et al., 2019a). Honey solutions were prepared by sonication in sterile water and then diluted in microplates with SDB in the concentration range of 5–40% w/v. Honey solutions were sterilized by filtration in a 0.22 μM filter (Millipore Merck; Darmstadt, Germany). Then, 100 μL of a *Candida* inoculum containing 10^3 colony-forming units per milliliter (CFU)/mL, prepared from a 48 h culture in appropriate medium, was added to each well. The plates were incubated at 37 °C for 48 h, and the minimum inhibitory concentration (MIC) was determined visually as the lowest concentration at which no visible growth was observed. In addition, the minimum fungicidal concentration (MFC) was determined by plating 10 μL of the optically growth-free wells in the MIC assay on SDA. After incubation for 48 h at 37 °C, the MFC was defined as the lowest concentration of compounds that killed at least 99.9% of the original inoculum compared to the untreated control.

Artificial honey was used as a control to the detriment of commercial antifungals. This is because the activity of commercial antifungals is usually expressed in μg/mL and the activity of honey is expressed in % w/v (or v/v). Therefore, it is difficult to compare these amounts. To overcome this limitation, many authors suggest the use of artificial honey (Anand et al., 2019; Anyanwu, 2012; Fernandes et al., 2021; Koc et al., 2009), which was also used in this study.

2.4. Dosage of total phenolics of honey samples

The total phenolic compound of all honeys samples was quantified using the commercial Folin-Ciocalteu reagent (Singleton et al., 1999). Honey was weighed (1 g), diluted in 10 mL of Milli-Q water, and filtered through a 45 μm filter paper. Then, 0.5 mL of the honey solution and 2.5 mL of 0.2 N Folin & Ciocalteu's phenol solution were added to test tubes and homogenized for 5–8 min. Then, 2 mL of sodium carbonate solution 75 $g.L^{-1}$ was added to the samples, which were homogenized and left to rest for 2 h. The absorbance was measured at 760 nm using a UV-VIS spectrophotometer (ShimadzuUV-1650PC, Japan, Kyoto), and ultrapure water was used as blank. The calibration curve was prepared using gallic acid (GA), and the total phenolic content of the samples was determined using a straight equation and expressed as mg of GA per 100 g honey.

2.5. Yeast-to-hypha (Y–H) transition

The ability of all honey samples to inhibit yeast-to-hypha (Y–H) transition was evaluated as described by Lima et al. (2019b). *C. albicans* ATCC 10231 and a clinical isolate of *C. albicans* were seeded at 10^3 cells/well in heat-inactivated fetal bovine serum (FBS). Then, cells were treated with honey at different concentrations (10%, 20%, and 40% w/v) and incubated at 37 °C for 48 h. *Candida* morphology was then visualized using a Nikon TE 2000-U Eclipse microscope equipped with a DC300F Digital Imaging System at 400 × magnification (Leica Microsystems, Germany). Artificial honey at 20% and 40% w/v was used as a control.

2.6. Antibiofilm activity

Mature biofilm assay: The effect of different honeys on *Candida* preformed biofilm was evaluated according to the methodology described by Andrade et al. (2021). *Candida* cells (10^4 CFU/mL) were cultured for 48 h at 37 °C in SDB fortified with 100 mM glucose to allow biofilm adhesion. Then, cells were washed, treated with honey (10%, 20% and 40% w/v) and incubated at 37 °C for 48 h. Then the cells were washed and crystal violet 0.1% was added to the biofilms for 30 min at room temperature. Finally, the microtiter plates were washed to remove the excess crystal violet, dried at room temperature and, incubated with ethanol 95% for 15 min. Biofilms were quantified by measuring optical density at 570 nm ($OD_{570\ nm}$) in a microplate reader (Bio-Tek Instruments, New York, NY, USA), and results were plotted graphically as a function of the percentage of reduction of mature biofilm versus honey concentration.

Anti-adhesive propeties: Biofilm formation on sterile catheter segments sensitized with Aroeira honey was evaluated according to de Sousa et al. (2019). One-centimeter sterile urethral catheter segments were directly immersed in a stirred solution of Aroeira honey at three concentrations (15%, 30%, and 60% w/v) for 90 min. The segments were dried overnight and rinsed with sterile distilled water to remove unbound substances. Then, segments were transferred to sterile tubes containing 5 ml of SDB and 100 mM glucose and inoculated with 100 μL of a suspension containing 10^6 cells of *C. albicans* ATCC 10231 (determined spectrophotometrically). After 48 h incubation, the fungal culture was discarded and the catheter segments were immersed in 5 ml of sterile saline for 30 min with shaking. The segments were removed with sterilized forceps, transferred to another tube containing 5 ml of sterile saline and sonicated at 40 KHz for 5 min (Unique, Indaiatuba, SP, Brazil). After sonication, samples were homogenized, serially diluted in sterile saline (10^{-1}-10^{-3}), and 100 μl were spread on SDA. Plates were incubated at 37 °C for 48 h for posterior colonies (CFU/cm catheter). Non-sensitized catheter segments and those sensitized with 60% w/v artificial honey were included as controls.

2.7. Combination assays

Synergism: The effect of interaction of honey samples with azoles (ketoconazole, itraconazole, miconazole, and fluconazole) was evaluated against *Candida* using the checkerboard assay as described by Lima et al. (2019b). Fractional inhibitory concentration (FIC) index (FICI) was determined as the sum of the FIC of the individual honey samples and the FIC of the antifungals (*i.e.*, $FICI = FIC_{honey} + FIC_{antifungal}$, where $FIC = MIC_{honey/antifungal\ in\ combination}/MIC_{honey/antifungal\ alone}$). The effect of the combination is considered synergistic if $FICI \leq 0.5$, additive if $0.5 < FICI \leq 1.0$, indifferent if $1.0 < FICI \leq 4.0$, and antagonistic if $FICI > 4.0$.

Re-sensitization assay: re-sensitization of an azole-resistant *C. albicans* (ATCC 10231) to the antifungal effects of ketoconazole, itraconazole, miconazole, and fluconazole after treatment with Aroeira honey was performed as previously described (Lima et al., 2021). In briefly, ½ × MIC of Aroeira honey was incubated with *C. albicans* ATCC 10231 (1×10^3 CFU/mL) in SDB at room temperature

for 1 h. The incubation was performed as described previously. After incubation, yeast were transferred to 96-well plates and the MIC of each azole was determined. Yest cells untreated with honey and with polyene served as negative and positive controls, respectively. The result was expressed as a fold-change in re-sensitization calculated by the ratio of MIC of antifungal alone and MIC of antifungal agent after previous exposure to ½ × MIC of Aroeira honey or polyene antifungal agents (nystatin and amphotericin B).

Scanning electron microscopy (SEM): 1 mL (1 mL) of *C. albicans* ATCC 10231 (10^6 CFU/mL) suspension grown in SDA was prepared in sterile saline and centrifuged at 12,000×*g* for 5 min. The pellet was resuspended in 1 mL saline, centrifuged again, and treated with 100 μL Aroeira honey at 60% or 80% w/v for 1 h at 37 °C. Pellets treated with artificial honey at 80% served as controls. Then, 15 μL of treated cells were placed in an aluminum stub and humidified at 37 °C for 30 min. The cells were fixed with a solution containing 2.5% (v/v) glutaraldehyde for 3 h at room temperature. The material was then dehydrated in a graded ethanol series (70, 90, and 100% v/v), with the stubs held in each solution for 30 min. Finally, the samples were dried overnight in a desiccator containing silica-gel, and the stubs were coated with a 10 nm thick gold by sputtering and viewed with a scanning electron microscope at 5000 × magnification (Zeiss, Oberkochen, West Germany).

2.8. Multi-step resistance study

The ability of *C. albicans* ATCC 10231 to develop resistance to the most active honey and miconazole was investigated using a multi-step resistance study as previously described (Bogdanovich et al., 2005), but with adaptations. A yeast suspension containing 10^3 CFU/mL in 1 mL of SDB was exposed to two dilutions below the MIC of the respective sample (i.e., most active honey or miconazole) for 21 days. The MIC of the most active honey and the control (miconazole) were determined daily throughout the experimental period, and the results were presented graphically as a fold change in MIC versus time (days).

2.9. Statistical analysis

All tests were performed in triplicate. Normality of the data was assessed with the Shapiro-Wilk test, and all results are expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) was performed, followed by Tukey's *post-hoc* test for multiple comparisons and Dunnett's *post-hoc* test for comparison with the control group. Differences were considered statistically significant if the *p*-value was less than 0.05.

3. Results and discussion

3.1. Antifungal activity

Aroeira honey was active against all *Candida* species tested (Table 1). *C. albicans* (MIC 20–30% w/v) and *C. krusei* (MIC 30% w/v) were very susceptible to the fungistatic activity of Aroeira honey. *C. glabrata* (MIC 40% w/v) and *C. tropicalis* (MIC 40% w/v) were also susceptible to the antifungal activity of this honey. It is important to note that Aroeira honey was active against *C. krusei,* which is naturally resistant to azoles and against *C. albicans* ATCC 10231, which has acquired resistance to azoles and echinocandins (Andrade et al., 2021). This activity profile is important because *C. albicans* is the most importat species found in patients with superficial candidiasis. It is isolated in 52–85% of cases (Millsop and Fazel, 2016; Sobel, 2007).

The results of the antifungal activity of Aroeira honey confirm the previous studies showing that monofloral honey of Eucalyptus (Fernandes et al., 2021), Chestnut (Fernandes et al., 2021), Orange (Fernandes et al., 2021), Rosemary (Fernandes et al., 2021), Heather (Fernandes et al., 2021), Manuka (Anand et al., 2019; Fernandes et al., 2021), Tea tree (Anand et al., 2019), Jelly bush (Anand et al., 2019), Buckwheat (Kolayli et al., 2020), Oak (Kolayli et al., 2020), and Jarrah (Anand et al., 2019) inhibit the growth of *C. albicans, C. glabrata*, *C. parapsilosis* and *Saccharomyces cerevisiae* but do not kill these yeasts. In contrast, Anand et al. (2019), Sayadi et al. (2015), and Anyanwu (2012) have shown that monofloral honey has fungicidal activity against *C. albicans,* and Fernandes et al. (2021) also found that all honey samples tested (i.e., eucalyptus, chestnut, orange, rosemary, heather, and manuka) killed *C. tropicalis* cells. These results indicate that the fungicidal activity depends on the botanical origin of the honey tested and the *Candida* species tested.

Eucalyptus honey, in turn, was active only against *C. albicans* (MIC 40% w/v). In agreement with our results, Fernandes et al.

Table 1
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of honey samples against reference strains and clinical isolates of *Candida* spp.

Microorganisms	Honeys (% w/v)							
	Eucalyptus		Assa-peixe		Aroeira		Artificial	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans ATCC 18804	40	#	#	–	20	#	#	–
Candida albicans ATCC 10231[a]	40	#	#	–	30	#	#	–
Candida albicans CI 1	40	#	#	–	20	#	#	–
Candida albicans CI 2	40	#	#	–	30	#	#	–
Candida albicans CI 3	40	#	#	–	30	#	#	–
Candida glabrata ATCC 2001	#	–	#	–	40	#	#	–
Candida tropicalis ATCC 28707	#	–	#	–	40	#	#	–
Candida krusei ATCC 34135	#	–	#	–	30	#	#	–

#: Inactive; -: not tested

[a] *Candida albicans* ATCC 10231 is resistant to Anidulafungin, Voriconazole, Itraconazole, and Fluconazole.

(2021) showed that the Eucalyptus honey from Portuguese beekeepers had strong activity against planktonic multidrug resistant *C. albicans,* with an MIC of 50% w/v. In addition, Koc et al. (2009) showed that Eucalyptus honey from Turkey was active against eight clinical isolates of *C. albicans* at concentrations ranging from 40 to 80% v/v using the broth microdilution assay. However, although our study showed no activity against the non-*albicans* species tested (i.e., *C. glabrata*, *C. krusei*, and *C. tropicalis*), other authors have demonstrated that eucalyptus honey is also active against *C. krusei* (Koc et al., 2009), *C. tropicalis* (Fernandes et al., 2021), *C. parapsilosis* (Fernandes et al., 2021), and *C. glabrata* (Fernandes et al., 2021; Koc et al., 2009). The difference in the activity profile between the studies can be explained by the origin and the period of collection of the honey studied. Indeed, it has been reported that geographical distribution, seasonal factors, and different floral sources may play an important role in the antimicrobial activity of honey (Mandal and Mandal, 2011).

In turn, Assa-Peixe and artificial honey were inactive and had high MIC values (>40% w/v).

3.2. Dosage of total phenolics of honey samples

Total phenolic concentrations in mg GA/100 g honey were 8.85 ± 2.77 for artificial honey, 43.06 ± 3.63 for Assa-Peixe honey, 73.15 ± 2.16 for Eucalyptus honey and 99.68 ± 4.66 for Aroeira honey. Interestingly, Aroeira honey is the richest in phenolic

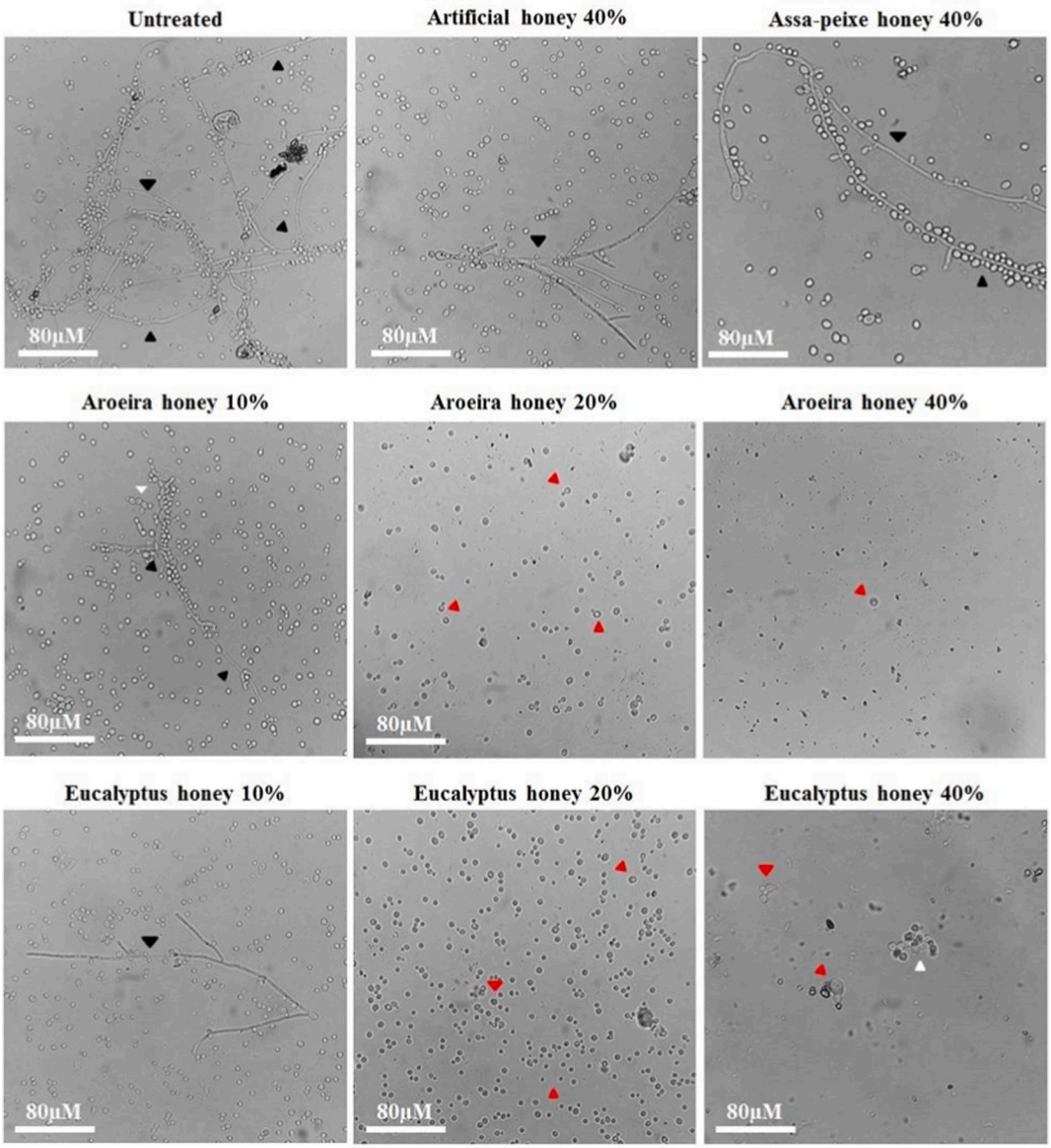

Fig. 1. Hyphal formation of *Candida albicans* ATCC 10231. *Candida albicans* cells were cultured with different types of honeys (Artificial, Assa-Peixe, Eucalyptus, and Aroeira) for 48 h at 37 °C. Experiments were performed in duplicate and in three independent experiments. Representative photomicrographs are shown. The white bar represents a length of 80 µm (magnification × 400).

substances among the different types of honey studied, which is confirmed by its increased antifungal activity. The total phenolic content in honeys from Manuka 163.1 mg/100 g, Chestnut 103.9 mg/100 g, Orange Blossom 34.8 mg/100 g, Rosemary 55.4 mg/100 g and, Heather 179.6 mg/100 g (Fernandes et al., 2021).

The antimicrobial activity of honey is attributed to its high osmolarity, acidity (low pH), hydrogen peroxide (H_2O_2) content, and the presence of bee compounds (e.g., bee defensin-1 and lysozyme) and floral substances (e.g., methylglyoxal, volatile compounds, and phenolic compounds) (Samarghandian et al., 2017). Fungi usually tolerate high osmolarity levels because they have resistant cell walls (Gunde-Cimerman et al., 2009). This justifies the lack of antifungal activity of artificial honey found in this study. In addition, *Candida* produces the enzyme catalase, which degrades H_2O_2, so it can develop in the presence of this substance (Miyasaka et al., 2008). Its ability to survive in acidic pH is also known (Staniszewska, 2020), which makes this pathogen insusceptible to low pH and H_2O_2 in honey. In this context, it is suggested that the antifungal effect of Aroeira and Eucalyptus honey observed in this study may be related to the presence of plant phenolic compounds.

3.3. Yeast-to-hypha (Y–H) transition

Complete inhibition of the Y–H transition was observed after treatment with Aroeira honey at 20% and 40% w/v against azole-resistant *C. albicans* (ATCC 10231) (Fig. 1). In the clinical isolate, all tested concentrations of Aroeira honey inhibited the Y–H transition, with no fungal structures observed under the light microscopy at 40% (Fig. S1, supplementary file). At higher concentrations (40% w/v), Eucalyptus honey inhibited the Y–H transition in the azole-resistant and clinical isolate of *C. albicans*. However, Assa-Peixe and artificial honey did not affect filament formation of *C. albicans*.

Candida species have several virulence factors, such as increased thermotolerance, dimorphism with Y–H transition, enzyme production, and biofilm formation (Staniszewska, 2020). The Y–H transition is the basis for the development of superficial candidiasis, which leads to complications, including development into invasive infections (Andrade et al., 2018; Rodríguez-Cerdeira et al., 2019). The ability of the tested honeys, especially Aroeira honey, to inhibit *C. albicans* filament formation could be related to phenolic substances. Indeed, some phenolics are known to stimulate the synthesis of farnesol, an inhibitor of hyphal formation, via the

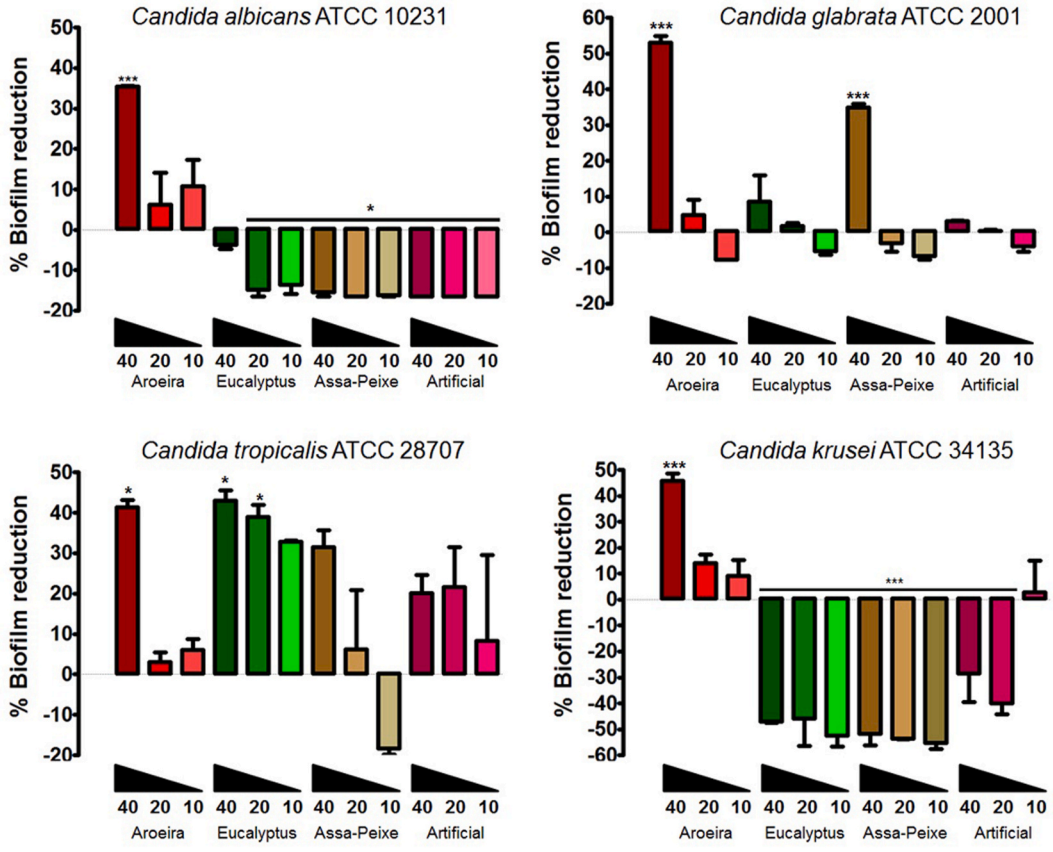

Fig. 2. Anti-biofilm activity. Different honeys were tested at 10%, 20%, and 40% w/v concentrations against mature biofilm of *C. albicans*, *C. krusei*, *C. glabrata*, and *C. tropicalis*. The adherent biofilm was stained with crystal violet, then the dye was extracted with ethanol, the optical density was measured at 595 nm and expressed as the percentage of biofilm reduction compared to untreated cells. All experiments were performed in quadruplicate to ensure statistical significance. One asterisk (*) indicates a statistical difference compared to control with $p < 0.05$. Three asterisks (***) indicates a statistical difference compared to control with $p < 0.0001$. Results were analyzed One-way ANOVA with Dunnett post-hoc. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

upregulation of the *Dpp3* gene (Zhang et al., 2011). In this sense, an anti-hyphae effect was observed in *C. albicans* after treatment with several plant-based phenolic compounds such as bisbibenzyls (Zhang et al., 2011), epigallocatechin-gallate (Han, 2007), licochalcone A, gladribin (Messier and Grenier, 2011), and thymol (Braga et al., 2007). In addition, volatile phenolic compounds present in clove essential oil (e.g., eugenol) (Pinto et al., 2009) and catechol derivatives isolated from onions (metabolites of shikimate pathway) (Ravi et al., 2021) reduce the formation of germ tubes and filaments in *C. albicans*.

3.4. Antibiofilm activity

In addition, the effect of the different honeys on *Candida* biofilms was studied using by the crystal violet test (Fig. 2). Aroeira honey significantly reduced the mature biofilm of all the *Candida* species tested. Aroeira honey reduced biofilm biomass at 40% w/v by 45.89 ± 3.7% in *C. krusei* ($p<0.0001$), 53.08 ± 6.1% in *C. glabrata* ($p<0.0001$), 41.38 ± 1.9% in *C. tropicalis* ($p = 0.03$), and 35.45 ± 0.8% in *C. albicans* ($p<0.0001$), compared to untreated cells. In addition, the Eucalyptus and Assa-Peixe honeys reduced the mature biofilm of *C. tropicalis* (40% w/v: 42.99 ± 11.0%; $p = 0.02$) and *C. glabrata* (40% w/v: 34.90 ± 4.3%; $p<0.0001$). Fernandes et al. (2021) indicated that samples of Heather and Manuka honeys (50% and 75% w/v) also reduce *Candida* species biofilms. In another study, Jujube honey (40% w/v) was found to reduce the size of mature *C. albicans* biofilms and disrupted their structure (Ansari et al., 2013).

Since Aroeira honey has significant antibiofilm activity against azole-resistant *C. albicans* (ATCC 10231), we investigated the ability of this honey to inhibit adhesion and biofilm formation on urethral catheters. Sensitization of the catheter with Aroeira honey at 30% w/v ($p<0.0001$) and 60% w/v ($p = 0.004$) significantly reduced the load of fungal cells adhering to the surface of the catheter (Fig. 3). The fungal load of the untreated catheters was $1.3x10^5$ CFU/cm, while the catheters sensitized with the Aroeira honey had a cell density of $3.2x10^4$ CFU/cm and $2.4x10^4$ CFU/cm at 30% w/v and 60% w/v, respectively. Artificial honey did not inhibit cellular adhesion to the surface of the catheters ($8.3x10^4$ CFU/cm). Although this is the first study to investigate the ability of honey to reduce the adhesion of pathogenic fungi to the surface of medical devices, our study shows that Aroeira honey is a promising source of new compounds to produce active anti-biofilm catheters.

3.5. Combination assays

Aroeira honey acts synergistically with miconazole (FICI 0.03), itraconazole (FICI 0.25), and ketoconazole (FICI 0.32) against azole-resistant *C. albicans*, with miconazole (FICI 0.28) and ketoconazole (FICI 0.50) against *C. tropicalis*, and with miconazole against *C. glabrata* (FICI 0.09) and *C. krusei* (FICI 0.38) (Table 2). Eucalyptus honey interacts synergistically only with ketoconazole against *C. albicans* (FICI 0.32) and with itraconazole (FICI 0.20) and ketoconazole (FICI 0.46) against *C. glabrata*. Assa-Peixe and artificial honeys showed predominantly indifferent effects in the combinations evaluated (Table 2).

Combination therapy is commonly used to treat fungal infections caused mainly by multidrug-resistant microorganisms (Campitelli et al., 2017). The synergistic effect observed in monofloral honey, especially Aroeira honey, can be associated with its complex chemical composition. Each active component of honey has different mechanisms of action that can enhance the effect of commercial antifungal agents (Carvalho et al., 2018). In addition, SEM analysis showed that Aroeira honey structurally alters the fungal membrane (Fig. 4). In cells exposed to this honey at 80% w/v, various changes were observed on the cell surface, including wrinkles, protrusions (red arrow), pores (blue arrow), and vesicles (white arrow) (Fig. 4). Therefore, it is expected that the selective permeability of the fungal membrane is affected, allowing the penetration of larger amounts of antifungal agents, which also helps to justify the observed synergistic effect between azoles and Aroeira honey (Lima et al., 2021; Lima et al., 2021).

Since a strong synergistic effect was observed after combining monofloral honeys with azole antifungals, we investigated whether prior exposure to this bee product reduced the resistance of *C. albicans* ATCC 10231 to conventional antifungals. As shown in Table 3,

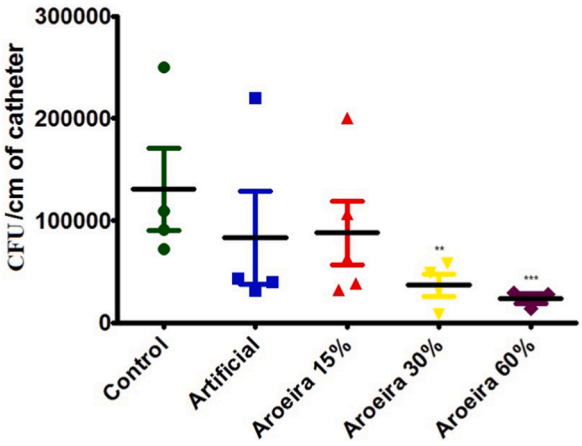

Fig. 3. Anti-adhesive activity of Aroeira honey in urethral catheters. One-centimeter segments of polyethylene urethral catheters were previously sensitized with Aroeira honey at a concentration of 15%, 30% and 60% w/v or with artificial honey at concentration of 60% w/v. They were then challenged with an inoculum of azole-resistant *C. albicans* at 10^4 CFU/mL, and the fungal load of adherent cells was determined after 24 h of incubation. Two asterisks (**) indicate a statistical difference with $p < 0.01$. Three asterisks (***) indicate a statistical difference with $p < 0.0001$. Results were analyzed by One-way ANOVA with Dunnett post-hoc.

Table 2
The fractional inhibitory concentration index (FICI) range of honeys in combination with azoles antifungals against *Candida* species.

Honey	Antifungal	*Candida albicans* ATCC 10231			*Candida glabrata* ATCC 2001			*Candida tropicalis* ATCC 28707			*Candida krusei* ATCC 34135		
		FIC antifungal	FIC honey	∑FICI	FIC antifungal	FIC honey	∑FICI	FIC antifungal	FIC honey	∑FICI	FIC antifungal	FIC honey	∑FICI
Aroeira	Fluconazole	0.50	0.53	1.03 (In)	0.50	0.31	0.81 (Ad)	0.50	0.80	1.30 (In)	1.00	0.53	1.53 (In)
	Ketoconazole	**0.25**	**0.07**	**0.32 (Sn)**	0.25	0.53	0.78 (Ad)	1.00	0.31	1.31 (In)	1.00	0.31	1.31 (In)
	Itraconazole	**0.12**	**0.13**	**0.25 (Sn)**	2.00	0.80	2.8 (In)	**0.50**	**>0.00**	**0.50 (Sn)**	1.00	0.25	1.25 (In)
	Miconazole	**0.03**	**> 0.00**	**0.03 (Sn)**	**0.06**	**0.03**	**0.09 (Sn)**	**0.25**	**0.03**	**0.28 (Sn)**	**0.25**	**0.13**	**0.38 (Sn)**
Eucalyptus	Fluconazole	0.50	0.31	0.81 (Ad)	0.50	0.21	0.71 (Ad)	1.00	0.66	1.66 (In)	1.00	0.66	1.66 (In)
	Ketoconazole	**0.06**	**0.26**	**0.32 (Sn)**	0.50	0.10	0.60 (Ad)	1.00	1.5	2.5 (In)	2.00	1.8	3.8 (In)
	Itraconazole	1.0	1.8	2.8 (In)	**0.03**	**0.17**	**0.20 (Sn)**	1.00	1.7	2.7 (In)	0.5	0.08	0.58 (Ad)
	Miconazole	1.00	2.00	3.00 (In)	**0.25**	**0.21**	**0.46 (Sn)**	0.50	1.0	1.50 (In)	1	2.2	3.2 (In)
Assa-peixe	Fluconazole	0.50	0.10	0.60 (Ad)	0.50	0.21	0.71 (Ad)	**0.12**	**0.08**	**0.13 (Sn)**	0.50	0.21	0.71 (Ad)
	Ketoconazole	0.50	1.40	1.90 (In)	1.00	2.2	3.2 (In)	1.50	1.00	2.50 (In)	0.50	0.10	0.60 (Ad)
	Itraconazole	1.20	1.40	2.60 (In)	2.00	1.30	2.30 (In)	**64**	**4.00**	**68 (An)**	**2.00**	**2.50**	**4.50 (An)**
	Miconazole	1.00	1.70	2.70 (In)	**0.12**	**0.17**	**0.29 (Sn)**	0.50	1.20	1.70 (In)	0.50	0.17	0.67 (Ad)
Artificial	Fluconazole	2.00	1.06	3.06 (In)	1.00	0.80	1.80 (In)	2.00	0.80	2.80 (In)	1.00	2.00	3.00 (In)
	Ketoconazole	1.00	0.53	1.53 (In)	0.25	0.40	0.65 (Ad)	0.50	0.31	0.81 (Ad)	1.00	0.40	1.40 (In)
	Itraconazole	1.00	1.06	2.06 (In)	0.50	0.10	0.60 (Ad)	0.50	0.20	0.70 (Ad)	0.50	0.32	0.82 (Ad)
	Miconazole	1.00	0.32	1.32 (In)	1.00	0.80	1.80 (In)	0.53	0.12	0.65 (Ad)	**0.25**	**0.01**	**0.26 (Sn)**

FIC: Fractional inhibitory concentration; **Σ**FICI: Fractional inhibitory concentration index.
Ad: Additive effect; Sn: Synergic effect; In: Indifferent effect; An: Antagonism
FIC index was interpreted as follows: An FIC index of $\leq$0.5 is considered to demonstrate synergy. Additive was defined as an FIC index between 0.6 and 1. An FIC index between 1.1 and 4 was considered such as indifferent effect. Antagonism was defined as an FIC index of >4.

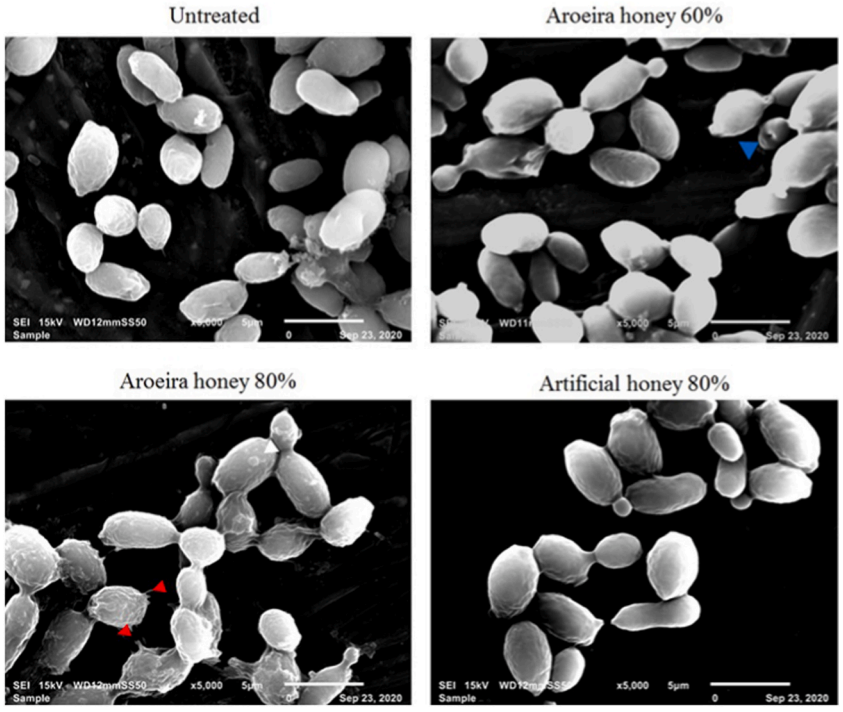

Fig. 4. Evaluation of membrane damaging activity associated with Aroeira honey against azole-resistant *Candida albicans* (ATCC 10231) by scanning electron microscopy (SEM). SEM was carried out with a suspension of untreated *C. albicans* ATCC 10231 or exposed to 60% and 80% w/v Aroeira honey for 1 h at 37 °C. Artificial honey at 80% w/v was also included. It is possible to verify in the Aroeira honey-treated group, it can be seen that the cells exhibited several membrane changes, such as wrinkles, protrusions (red arrow), pores (blue arrow), and vesicles (white arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

treatment of azole-resistant *C. albicans* cells for 1 h with ½ × MIC of Aroeira honey reduced the MICs of fluconazole and ketoconazole by 8- and 16-fold, respectively, compared with unexposed cells. In addition, the MICs of miconazole and itraconazole were reduced 64-fold after exposure to Aroeira honey. For example, the antifungal concentration of miconazole decreased from 8 μg mL^{-1} to 0.125 μg mL^{-1} after treatment with Aroeira honey. Thus, we show for the first time that honey can suppress the resistance of *C. albicans* to conventional antifungal agents. In turn, artificial, Eucalyptus, and Assa-Peixe honeys did not alter the resistance profile of the tested *C. albicans* strain (Table 3). The re-sensitizing effect of Aroeira honey can also be explained by the activity of this honey on the fungal membrane. With the increase in membrane permeability induced by Aroeira honey, the concentration of antifungal agents in fungal cells increases, allowing the pharmacological action of these compounds even in resistant strains (Lima et al., 2021; Lima et al., 2021).

3.6. Multi-step resistance study

MIC values of Aroeira honey (most active in the antifungal assay) remained unchanged during the 21 cell passages (Fig. 5). However, the antifungal concentration of miconazole in a dilution increased on day 21 (from 4 μg mL^{-1} to 8 μg mL^{-1}). Thus, the results of the multi-step resistance study indicate that *C. albicans* cannot develop rapid resistance to Aroeira honey. In fact, natural products with multiple active ingredients (e.g., honey) are an obstacle to the development of resistance. This is because the microorganisms must develop multiple mechanisms to bypass each class of compound present in that mixture in order to become resistant to those agents. Therefore, resistance to natural products requires high cellular cost that can often affect the viability of the pathogen and render it useless (Araújo et al., 2019).

4. Conclusion

In conclusion, the antifungal properties of monofloral honeys presented in this study show that they are an attractive alternative for the treatment of *Candida* infections, especially for topical application on mucous membranes and skin. Moreover, the ability of Aroeira honey to reverse resistance to azoles in less susceptible strains and to interact synergistically with these agents opens a prerogative for its use as combination therapy with commercial antifungal agents. However, in vivo and clinical studies remain to be conducted to confirm the potential of these monofloral honeys as agents for apitherapy against superficial candidiasis.

Declaration of competing interest

The authors report that they do not have any conflicts of interest.

Table 3
Re-sensitization of azole-resistant *C. albicans* (ATCC 10231) to fluconazole, miconazole, itraconazole, and ketoconazole using a sub-inhibitory concentration (½ × MIC) of monofloral honeys.

Antifungals	Fold of re-sensitization			
	Artificial	Aroeira	Eucalyptus	Assa-Peixe
Fluconazole	No sensitization effect	16	No sensitization effect	No sensitization effect
Miconazole	No sensitization effect	64	No sensitization effect	No sensitization effect
Itraconazole	No sensitization effect	64	No sensitization effect	No sensitization effect
Ketoconazole	No sensitization effect	8	No sensitization effect	No sensitization effect

Fold of re-sensitization: It is the ratio of the MIC of antifungal alone divided by the MIC of antifungal after re-sensitization with (½ × MIC) of honeys.

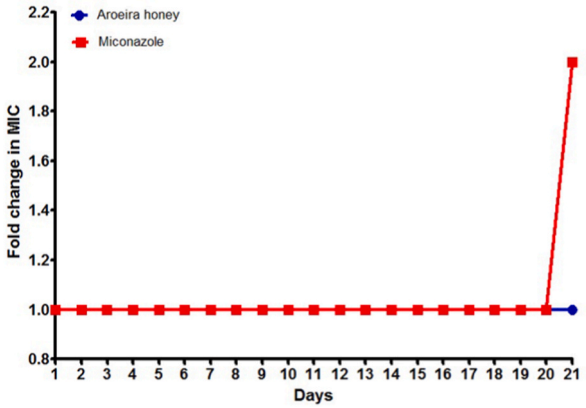

Fig. 5. Multi-step resistance study of Aroeira honey against azole-resistant *C. albicans* (ATCC 10231). Yeast was serially cell passaged over a 21-day period, and the minimum inhibitory concentration (MIC) of Aroeira honey was determined after each cell passage using the broth microdilution assay.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2022.102335.

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