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Comparison of *in vitro* activities of antifungal drugs and propolis against yeasts isolated from patients with superficial mycoses

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Abstract - The *in vitro* susceptibilities of propolis and antifungal drugs were determined against some yeasts isolated from patients with superficial mycoses. The agents tested included fluconazole, itraconazole, ketoconazole, terbinafine and propolis. MICs were determined by the broth microdilution technique following National Committee for Clinical Laboratory Standards document M27-P. For all *Candida albicans* isolates from the patients with superficial mycoses, ketoconazole presented higher ($P < 0.05$) efficiency than that of the other antifungal agents tested. The geometric mean MIC values of antifungal drugs and propolis against the yeasts tested ranged from 0.087 to 12.69 µg/mL and 0.4-0.6 µg/mL, respectively. Propolis also showed an important antifungal activity against the yeasts tested, MIC ranges of the propolis were between 0.01-1.65 µg/mL. Based on these results, propolis requires further investigation as a potential agent for the treatment of superficial mycoses.

Key words: propolis, superficial mycoses, microdilution assay, *Candida* spp.

INTRODUCTION

Superficial mycoses have been known as one of the most common infections in humans. Superficial fungal infections are commonly encountered in some clinical conditions, affecting the skin, hair and nails. The most commonly prescribed modality to treat these infections is the topical therapy (Haris, 2002). The increasing recognition and importance of the fungal infections, some difficulties encountered in their treatment and increase in resistance to antifungals have stimulated the research for the therapeutic alternatives.

Recently, propolis has been attracting the attention of researchers due to its various biological activities and therapeutic properties. Propolis is a resinous material collected by honeybees (*Apis mellifera L.*) from the buds or other parts of plants. The chemical composition of propolis varies according to the plants that can be found in a specific region. In Europe, these resins are mainly collected from trees of the *Populus* species (Greenaway *et al*., 1991). Although propolis samples of different origins have different compositions, they have similar antimicrobial effects (Kujumgiev *et al*., 1999) since this effect has prime importance to the survival of hive. Many authors have studied the antibacterial activity of propolis (Park *et al*., 1998; Kujumgiev *et al*., 1999; Keskin *et al*., 2001) but reports for its activity on yeasts are rare. Holderna and Kedzia (1987) studied the combined action of antimycotic drugs and propolis on *Candida albicans*. Ota *et al*. (2001) reported antifungal activity of propolis against 80 strains of *Candida* yeasts.

In this paper we report the results of comparison of *in vitro* activities of antifungal drugs and propolis against yeasts from the patients with superficial mycoses by using a microdilution assay.

MATERIALS AND METHODS

Origin of propolis. Propolis sample was collected from Kayseri region, in Turkey (Central Anatolia). Hand collected propolis was kept desiccated and in the dark up to their processing. Voucher specimen was deposited in the Department of Microbiology, Faculty of Medicine, University of Erciyes, Kayseri.

A 30 g propolis sample was extracted for a week with 100 mL of 70% ethanol, at room temperature to obtain the extract. After filtration, the extract was evaporated to dry using a vacuum evaporator at 50 ºC.

Test organisms. Fifteen strains were isolated from infected skin and nail in the Microbiology and Clinical Microbiology Department of Gevher Nesibe Hospital of Erciyes University. Isolates were collected over a 6-months period in the Mycology Laboratory. They included 9 strains of *Candida albicans,* 3 strains of *Candida glabrata*, 2 strains of *Trichosporon* spp. and 1 strains of *Rhodotorula* sp. The isolates were identified using standard methods (Waren

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and Hazen, 1995) and stored at –20 ºC in Tryptic Soy broth (Difco, Detroit, MI, USA) containing 10% glycerine. Prior antifungal assays, the yeasts were subcultured at least twice on Sabouraud Glucose agar (Acumedia, USA) plates.

Quality control was performed by testing of *C. albicans* ATCC 90028 according to the recommendations of NCCLS document M27-A (NCCLS, 1995).

Medium. RPMI 1640 broth medium (Sigma Chemical Company, Madrid, Spain) with L-glutamine (0.02 g) and without sodium bicarbonate, buffered at pH 7.0 with 0.165 M morpholinepropansulfonic acid (MOPS) (Sigma, Madrid) was the medium used for the broth microdilution susceptibility testing.

Drugs. Four antifungal drugs, supplied by the manufacturers as powder, were used besides propolis: fluconazole (Fako Co., Ístanbul, Turkey), ketoconazole (Bilim Co., Ístanbul, Turkey), terbinafine (Novartis-Pharma) and itraconazole (Rosco, Denmark). Fluconazole was dissolved in sterile water; ketoconazole, itraconazole and terbinafine were dissolved in 100% dimethylsulfoxide; and the propolis was dissolved in 70% ethanol.

Antifungal susceptibility test. Broth microdilution testing was performed according to NCCLS guidelines by using the spectrophotometric method of inoculum concentration of 0.5×10^3 to 2.5×10^3 cells per mL. The trays were incubated in air at 35 ºC and observed for the presence or absence of the growth at 24 h. Extracts of propolis were weighed under aseptic conditions in sterile volumetric flasks, and dissolved with 70% sterile ethanol to obtain 0·1 mg ml^{-1} extract concentration. These solutions were impregnated on sterile paper discs of 6 mm diameter (20 µl per disc) and discs were left to dry overnight to remove any residual solvent, which might interfere with the determination. The solvent control (ethanol) did not show any antifungal activity. Plates were prepared using 20 ml sterile RPMI-1640 medium. The surface of the plates was inoculated using a sterile swap containing a saline suspension of yeast that was left to dry. Plates were incubated at 25 °C for 24-48 h. All determinations were made in duplicate. Yeast suspensions were adjusted to a concentration 10^6 cells ml^{-1} for the disc diffusion test. Dipping a sterile swab into the cell suspension and streaking it across the surface of the agar inoculated each solidified medium. The plates were dried at room temperature for 15 min before applying the antifungal agent discs. The agar plates were incubated at 25 °C for 24-48 h. Zone diameter end points were read at 80% growth inhibition for ITC. Micro colonies within the diameter were ignored.

Statistics. Geometric mean MIC, MIC range, the MIC at which 50% and 90% of the isolates tested are inhibited (MIC_{50} and MIC_{90}) respectively are provided for all the isolates tested. The significance of differences in mean values was determined by Duncan test. P values < 0.05 were considered statistically different.

RESULTS

The main compounds of propolis sample used in this study were identified by GC-MS in our previous study (Koc *et al*., 2005), and contained chrysin (flavonoid), cinnamyl cinnamate (ester), 9-octadecanoic acid, 2-propenoic acid, caffeic acid (aromatic and fatty), also alcohol, terpene and ketones. Popova *et al*. (2005) stated that propolis samples originating from Central Anatolia (Kayseri) showed high antimicrobial activity and displayed very similar phenolic and flavonoid contents. The composition of Kayseri propolis seems to be directly related to the bud exudates of *Populus* secies.

In vitro activity of the propolis and four antifungal agents against 4 different species of yeasts represented by 15 strains are summarised in Table 1. When all the strains were considered together, geometric means MICs of terbinafine, ketoconazole and itraconazole were apparently lowest than propolis; 0.08 µg/mL, 0.09 µg/mL, 0.184 mg/mL, respectively. However, these results were significantly (P < 0.05) different from those values shown by fluconazole (0.46 µg/mL) and propolis (0.4 µg/mL). Fluconazole had the highest geometric mean MICs; i.e. 12.69 µg/mL and 2.82 µg/mL, in both cases.

Among the strains of the yeasts tested, the most sensitive strain was *Rhodotorula* spp., which showed higher sensitivity than that of the *Candida* species. Both species of *Candida* were shown to be sensitive to the all antifungal drugs and propolis. Among the species of *Candida* tested, *C. albicans* strains were more sensitive than *C. glabrata*. MIC values of antifungal agents were between 0.1 and 4 µg/mL against *C. albicans*.

When *Candida* strains were considered, the geometric mean MICs of ketoconazole, itraconazole and propolis were apparently the lowest, 0.09 µg/mL, 0.184 µg/ml and 0.64 µg/mL, respectively. In contrast, fluconazole had the lowest activity; its geometric mean MICs was 12.69 µg/mL. The MICs of antifungal drugs tested ranged from 0.01 to 4.00 µg/mL. MICs values indicate that propolis was more active (P < 0.05) than that of the rest of the drugs tested.

Propolis was very active against all species and presenting MIC values between 0.01 and 1.65 µg/mL. Propolis showed best activity against *C. glabrata* (0.01 µg/mL -1.65 µg/mL) and *Rhodotorula* spp. (0.01 µg/mL).

DISCUSSION

In recent years, several studies on *in vitro* susceptibility of the superficial mycoses to antifungal drugs have been conducted and the results have shown considerable variation (Jessup *et al*., 2000). This variability may be due to important methodological differences among the laboratories (Fernandez-Torres *et al*., 2000). The NCCLS antifungal collaborative study using the broth macrodilution method (Pfaller *et al*., 1990) and the microdilution method (Espinel-Ingroff *et al*., 1991) evaluated the effect of medium, incubation time (24 versus 48 h) and incubation temperature (30 versus 35 ºC) on intra and interlaboratory variations of MIC endpoints. The highest agreement among laboratories, including the rank order of susceptibility, was obtained with RPMI 1640 medium at 35 ºC and after a 24 h incubation time with antifungal compounds (Elder *et al*., 1996). Therefore we chose the buffered RPMI 1640 medium, the microdilution method and the 24 h incubation time for our susceptibility and comparison study.

Our results were confirmed *in vitro* activity of propolis and four currently available antifungals, terbinafine, keto-

TABLE 1 - Antifungal activity of propolis and antifungal drugs (µg/mL)

Species (tested strains, no.)	Antifungal agents	MIC ranges	MICsGM	MIC_{50}	MIC_{90}
Candida albicans (9)	Flucanozole	0.125-1	0.463	0.5	0.25
	Ketoconazole	0.03-0.45	0.090	0.125	0.125
	Itraconazole	0.125-0.25	0.184	0.25	0.25
	Terbinafine	0.5-4	1.852	2	4
	Propolis	0.1-1.65*	0.550	0.4	1.65
Candida glabrata (3)	Flucanozole	2-64	12.699	16	
	Ketoconazole	1-8.0	3.175	4	
	Itraconazole	0.5-2	0.794	0.5	
	Terbinafine	0.25-8	1.260	0.5	
	Propolis	0.2-1.65*	0.642	0.8	
Trichosporon spp. (2)	Flucanozole	1-8	2.828		
	Ketoconazole	0.5-1	0.707		
	Itraconazole	1-0.125	0.354		
	Terbinafine	0.03-0.25	0.087		
	Propolis	0.4*	0.4		
Rhodotorula spp. (1)	Flucanozole	4-10			
	Ketoconazole	4			
	Itraconazole	1			
	Terbinafine	0.5			
	Propolis	0.01			

(* $P < 0.5$)

conazole, itraconazole and fluconazole. In this study, all the antifungal drugs tested with the exception of fluconazole, displayed excellent activity.

The antifungal activity of propolis was found for all strains of the yeasts tested. Several authors have studied the antibacterial activity of propolis (Pepeljnjak *et al*., 1985; Velikova *et al*., 2000; Kartal *et al*., 2003) but reports about its activity on superficial mycoses are rare. Fernandes Jr. *et al*. (1995) also confirmed propolis action on yeasts with values of 0.40-1.80% (v/v) for *C. tropicalis* and 0.60-3.00% (v/v) for *C. albicans*. Ghisalberti (1979) justifies the antibacterial and antifungal activity of propolis as being due to the presence of poplar compounds, mainly phenols, flavonoids, phenolic acids and esters. The flavonoids in propolis (mainly pinocembrin) have been considered to be responsible for its inhibitory effect on *Candida* (Metzner *et al*., 1979). Popova *et al*. (2005) stated that propolis sample originating from Central Anatolia (Kayseri) showed high antibacterial activity and displayed very similar phenolic and flavonoid content. Propolis sample tested in this study was confirmed to contain the typical poplar flavonoid aglycones (pinocembrin, pinobanksin, pinobanksin-3-O-acetate, chrysin, galangin, kaempferol) phenolic acids (caffeic, ferulic, p-coumaric) and esters (Pentenyl caffeates benzyl and phenethyl esters of caffeic ferulic and p-coumaríc acids). The composition of Kayseri propolis seems to be directly related to the bud exudates of *Populus* species and established antibacterial activity in Kayseri propolis (Silici and Kaftanoglu, 2003).

Overall, our study demonstrated that propolis and other antifungal agents tested are very active against yeasts from patients with superficial mycoses, although these results are clearly species dependent. These results may allow researchers to adopt different therapeutic options for a large number of species of superficial mycoses with a high probability of successful results. However, it will be necessary to obtain more clinical data to confirm if this good in vitro efficacy is predictive for clinical outcome.

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