Full Paper

Antifungal Activities of Propolis Collected by Different Races of Honeybees Against Yeasts Isolated From Patients With Superficial Mycoses

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Abstract. Yeasts isolated from patients with superficial mycoses were tested against propolis samples collected from different regions and honeybee races. The minimum inhibitory concentration (MIC) values obtained using the agar dilution methods were compared to the diameters of growth inhibition zones by using the disk diffusion method. The results showed that *Candida albicans*, *C. glabrata*, *Trichosporon* spp., and *Rhodotorula* sp. were susceptible to low concentrations of propolis, the latter showing a higher susceptibility. Relative to the other propolis tested, the propolis sample collected by *Apis mellifera caucasica* possessed the highest antifungal activity against all of the superficial mycoses. In contrast, the propolis samples collected by *A.m. carnica* and *A.m. anatolica* were the least active samples. Also, the propolis sample from the Adana region is more active than samples from other regions. An increase of MIC values was accompanied by a decrease of growth inhibition zone diameters.

Keywords: propolis, *Apis mellifera* L., antifungal activity, superficial mycoses

Introduction

Superficial mycosis refers to the disease of the skin and its appendages caused by fungi. They possess the affinity for parasitizing keratin-rich tissues, produce thermal inflammatory response, and intense itching in addition to a cosmetically poor appearance. Topical or systemic treatments have been empirically administered, when, in general, specific antifungal agents are employed (1, 2). Although such drugs are widely used, some of them have been reported as ineffective and even toxic to the host (1). These factors led to the development and marketing of new drugs, but most of them have the same pharmacologically active groups and the same mechanism of action as those previously commercially available (3). However, some fungi are significantly different regarding susceptibility to such drugs (4, 5).

Recently, propolis has been attracting the attention of researchers due to its various biological activities and therapeutic properties. Propolis is a resinous substance

collected by *Apis mellifera* from various tree buds. Honeybees use propolis to coat hive parts and also to seal cracks and crevices in the hive. The combination of temperature, small space, and humidity provide the hives with good conditions for bacterial growth. Its antimicrobial potency keeps the growth of microbes under control (6). Flavonoids, aromatic acids, diterpenic acids, and phenolic compounds appear to be the principal components that are responsible for the biological activities of propolis samples. The ethanolic extract of propolis has been reported to possess various biological activities such as antibacterial (7–9), antifungal (7, 10–12), antiviral (13, 14), anti-inflammatory (15), local-anesthetic (16) antioxidant (17, 18), immunostimulating (19), and cytostatic (20).

Many authors have studied the antibacterial activity of propolis (7–9), but the reports about its activity on yeast/dermatophyte strains are rare. The aim of this study was to investigate antifungal properties of the ethanol extract of three propolis samples collected by different honeybee races and three samples from different regions of Turkey against 15 strains of yeasts iso-

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lated from patients with superficial mycoses.

Material and Methods

Honeybee races

Honeybee colonies belonging to *A.m. caucasica* (CAU), *A.m. anatolica* (ANA), and *A.m. carnica* (CAR) were located at Atatürk University, The College of Hamza Polat, Research, and The Education Apiary in Erzurum (East Anatolia, Turkey).

Propolis samples

Propolis samples were collected from three different races of honeybee (CAU, ANA, and CAR), which were located in the same apiary (East Anatolia). Other propolis samples were collected from three different localities in Turkey: Artvin (ART, North Anatolia), Kayseri (KAY, Central Anatolia), and Adana (ADA, South Anatolia). The propolis samples were collected by using a plastic propolis trap and before the winter season and were kept desiccated and in the dark up to their processing.

An aliquot of crude propolis (7 g) was dissolved in 80% ethanol by shaking at 50°C for 3 days and protected from light. The aqueous-ethanol extract was filtered through a Whatman 1 paper and concentrated at 50°C. The resin obtained was dissolved in 80% ethanol to a final concentration of 100 mg/ml. This final solution was employed for the antifungal assays.

Isolates

Fifteen strains were isolated from infected skin and nail in the Microbiology and Clinical Microbiology Department of the Gevher Nesibe Hospital of Erciyes University. Isolates were collected over a three months period in the Mycology Laboratory. They included 9 strains of *C. albicans*, 3 strains of *C. glabrata*, 2 strains of *Trichosporon*, and 1 strain of *Rhodotorula*. All isolates were identified by standard methods, which included identification based on the macroscopic and microscopic characteristics of the culture strain (13, 14). The strain *C. albicans* ATCC 90028 was included as a quality control. Itraconazole was used as the comparative antifungal agent.

Medium

RPMI-1640 broth medium (Sigma Chemical Company, Madrid, Spain) with L-glutamine but without sodium bicarbonate buffered at pH 7.0 with 0.165 M morpholinepropane sulfonic acid (MOPS) (Sigma) was the medium used for broth agar dilution susceptibility testing.

Agar formulations used for the disk diffusion test were RPMI-1640 broth supplemented with 1.5% Bacto

agar (Difco Laboratories, Detroit, MI, USA) and 2% glucose, buffered with MOPS (15). The 15-cm diameter petri plates contained RPMI at a depth of 4.0 mm.

Susceptibility testing

Antifungal activity of propolis samples was investigated by the agar dilution and agar diffusion methods, following the National Committee of Clinical Laboratory standard guidelines (21).

Agar dilution in plates: Ethanol solutions of propolis (dissolved within 70% ethanol) were added to sterile RPMI-1640 medium at a temperature of 50°C. It was homogenized, poured into sterile petri plates, and allowed to cool. It contained 0.002–0.3 mg propolis extracts per ml agar. The plates were inoculated using a sterile swab with a saline suspension for each of type of yeast and incubated at 25°C for 24 and 48 h. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of propolis that inhibited the visible growth of yeast on the plates.

Disc diffusion test: All extracts of propolis were weighed under aseptic conditions in sterile volumetric flasks and dissolved with 70% sterile ethanol to obtain 0.1 mg/ml 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. Commercial discs of Itraconazole (8 µg/disc; Rosco, Taastrup, Denmark) were used as positive control. 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 and plates left to dry. Plates were incubated at 25°C 24–48 h. All determinations were made in duplicate. Yeast suspensions were adjusted to a concentration of 10⁶ cells/ml 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 agents discs. The agar plates were incubated at 25°C for 24–48 h. Zone diameter end points were read at 80% growth inhibition for itraconazole. Diffusion of EEP (ethanolic extract of propolis) in agar was good. Micro colonies within the diameter were ignored.

Statistics

SPSS/PC for IBM package (version 9.0) was used for the statistical analysis of data. *P* values of <0.05 were considered statistically significant. The correlation between the microdilution method and disk diffusion

method was analyzed by means of regression analysis. Differences between means were determined using analysis of variance, and group means were compared by Duncan's multiple range test.

Results

The observed MICs of all propolis samples tested showed a broad range of variability against species of *Candida albicans*, *C. glabrata*, *Trichosporon* spp., and *Rhodotorula* sp. All of the extracts succeeded in inhibiting the growth of mycoses at varying concentrations. The value of MICs for the agar dilution test and mean inhibitory zone for the disc diffusion test of propolis

samples and Itraconazole are summarized in Table 1. Among the strains of yeasts, *C. albicans*, *C. glabrata*, *Trichosporon* spp., and *Rhodotorula* spp. were tested, and the most sensitive strain was *Rhodotorula* spp. The most resistant strain was *C. albicans* with the MIC value between 0.2 and 3.75 µg/ml.

There were significant statistical differences between antifungal activities of propolis samples collected by three different honeybee races from same region and also from different regions ($P < 0.01$). Relative to other propolis samples tested, CAU (East Anatolia) and ADA samples (South Anatolia) possessed the highest antifungal activity against superficial mycoses.

The correlation between MIC values and diameter of

Table 1. In vitro activities of propolis samples against superficial mycoses

Species (No. of strains tested)	Samples	MIC ranges (µg/ml)	MICs (GM)	MIC ₅₀ s (µg/ml)	MIC ₉₀ s (µg/ml)	Mean inhibition zone diameter ± S.E.M. (cm)
<i>C. albicans</i> (9)	CAU	0.05 – 3.75	0.43	0.2	3.75	28.4 ± 0.88 ^a
	ANA	3.75	3.75	3.75	3.75	24.8 ± 1.44 ^b
	CAR	0.9 – 7.50	2.66	3.75	3.75	18.4 ± 1.06 ^c
	ADA	0.03 – 1.88	0.42	0.40	1.88	27.0 ± 0.80 ^{a,b}
	KAY	3.75	3.75	3.75	3.75	18.2 ± 0.63 ^c
	ART	0.2 – 3.75	0.57	0.2	1.88	19.9 ± 0.95 ^c
	Itraconazole	0.125 – 0.25	0.20	0.25	0.25	29.20 ± 0.73 ^a
<i>C. glabrata</i> (3)	CAU	0.03 – 3.75				23.33 ± 0.67
	ANA	0.05 – 3.75				20.0 ± 2.31
	CAR	0.03 – 7.5				14.0 ± 2.00
	ADA	0.03 – 1.88				24.0 ± 2.31
	KAY	0.05 – 7.5				18.0 ± 1.15
	ART	0.03 – 7.5				21.13 ± 1.33
	Itraconazole	0.5 – 2.0				16.0 ± 1.00
<i>Trichosporon</i> spp. (2)	CAU	0.1				27.5 ± 0.50
	ANA	0.1 – 0.2				25.2 ± 1.20
	CAR	0.1 – 0.2				23.0 ± 1.00
	ADA	0.20				21.5 ± 0.50
	KAY	0.1 – 0.4				27.5 ± 1.10
	ART	0.1 – 0.4				24.4 ± 0.40
	Itraconazole	0.125 – 1				45.0 ± 5.00
<i>Rhodotorula</i> sp. (1)	CAU	<0.01				28
	ANA	0.01				26
	CAR	0.01				24
	ADA	<0.01				26
	KAY	0.01				24
	ART	0.01				24
	Itraconazole	1.0				20

CAU: propolis collected by *A. mellifera caucasica*, ANA: propolis collected by *A. mellifera anatolica*, CAR: propolis collected by *A. mellifera carnica*, ADA: propolis from Adana (South Anatolia), KAY: propolis from Kayseri (Central Anatolia), ART: propolis from Artvin (North Anatolia). GM: geometric mean. The MICs at which 50% and 90% of the isolates tested were inhibited were determined for each drug (MIC₅₀s and MIC₉₀s, respectively). ^{a,b,c,d} means within the same column with different superscript are significantly different ($P < 0.01$).

inhibition zone was analyzed by means of regression analysis. A favorable correlation was found between MIC and inhibition zone around the disk for the KAY propolis sample and the correlation coefficient was $r = -0.626$ ($P < 0.01$). However, no correlation was seen between the two methods for CAU, ANA, CAR, ADA,

and ART ($r = 0.380, 0.613, 0.412, 0.262$, and 0.114 , respectively). Figures 1 and 2 have been presented to show the samples of propolis, one of which had correlation and the other did not have.

Discussion

In recent years, several studies on the in vitro susceptibility of superficial mycoses to antifungal drugs have been done and the results have shown considerable variation (5). This variability may be due to important methodological differences among the laboratories (22). The NCCLS antifungal collaborative study using the broth macrodilution method (23), and the microdilution method (24) evaluated the effect of medium, incubation time (24 vs 48 h) and incubation temperature (30°C vs 35°C) on intra and inter-laboratory variations of MIC endpoints. The highest agreement among laboratories, including the rank order of susceptibility, was obtained with RPMI-1640 medium at 35°C and after a 24-h incubation time with antifungal compounds (25). Therefore, we chose the buffered RPMI-1640 medium for our susceptibility study.

In this study, the MIC values of the strains varied in a wide range as $<0.01 - 3.75$, $0.01 - 3.75$, $0.01 - 7.50$, $<0.01 - 1.88$, $0.01 - 7.5$, and $0.01 - 7.50 \mu\text{g/ml}$ for CAU, ANA, CAR, ADA, KAY, and ART samples, respectively. Similar results for Brazilian or European propolis were published by other authors (26–29). Ota et al. showed that the fungicidal activity of propolis was found in 80 strains of *Candida* species (10). Lindenfelser found inhibitory activity on 20 strains of 39 fungus species and two of these were shown to be resistant (30). Sforcin et al. showed that *C. tropicalis* and *C. albicans* were susceptible to low concentrations of propolis (Brazilian propolis) and no differences were seen in relation to seasonal effects in the minimal inhibitory concentrations of propolis (9).

Our results confirmed the in vitro antifungal activity of Turkish propolis samples collected from different regions and honeybee races. Relative to the other propolis tested, the propolis sample collected by *Apis mellifera caucasica* possessed the highest antifungal activity against all of the superficial mycoses. In contrast, the propolis samples collected by *A.m. carnica* and *A.m. anatolica* were the least active samples. Also, the propolis sample from the Adana region is more active than samples from other regions. The experiment revealed that there could be minor differences in antifungal activity of propolis extracts depending on the different regions and races of honeybee. Silici and Kutluca also reported the antibacterial activity of Turkish propolis samples collected by different honey

Fig. 1. Kayseri sample disk zone diameters and NCCLS reference method log₁₀MICs. The regression curve is $y = -3.1235 \log_{10}\text{MIC} + 20.033$ (KAY MIC), $r = -0.626$.

Fig. 2. Propolis sample collected by *A.m. caucasica* disk zone diameters and NCCLS reference method log₁₀MICs. The regression curve is $y = 1.378 \log_{10}\text{MIC} + 28.91$ (CAU MIC), $r = 0.145$.

bee races. They reported that propolis collected from *Apis mellifera carnica* hives showed a weaker antibacterial activity than *Apis mellifera anatolica* and *Apis mellifera caucasica* (31).

It is necessary to clarify the quality and quantity of the constituents in propolis in order to evaluate its biological activity (6). Recent studies on Turkish propolis have shown that its main source is poplar bud exudates (32). Popova et al. stated that a detailed chemical study performed by GC-MS showed Kayseri (Central Anatolia) to be of poplar origin and Adana, Artvin, and Erzurum to be of mixed origin (33). Adana (South Anatolia) and Erzurum samples (CAU, ANA, CAR; East Anatolia) were characterized by very low flavones and flavonones concentrations. Honeybee colonies belonging to *A.m. caucasica*, *A.m. carnica*, and *A.m. anatolica* were located in Erzurum (East Anatolia). The presence of most of the chemical compounds in all three samples was explored that all honeybee races were kept at the same apiary and samples were collected in the same season (31).

Antimicrobial testing should be easy to perform and should provide MIC endpoints that are reproducible and readily determined in clinical laboratories. Excellent agreement with MIC results may have been favored by the fact that the same medium was used for both agar dilution and disk diffusion methods. We did not find any correlation between MICs and inhibition zones for all samples except for the KAY sample. The disk diffusion procedure we used differed from the standard method only in the use of RPMI agar in place of Sabouraud agar. Also, zone size limits for propolis have not yet been defined. Selection of agar dilution MIC endpoints presents a similar source of variability that must be controlled, but that is not unique to the test of antifungal agents. Koç et al. advised that the NCCLS M27-A broth microdilution method was superior for the antifungal activity of propolis (34).

However these experimental results indicate that the antifungal activity of propolis was definitely related to the chemical composition of propolis. These differences in propolis composition were due to the collection of propolis from different plants in these local areas by different races of honeybee and different geographical regions.

Moreover, our study suggest that RPMI-1640 medium is a suitable medium for testing yeast to propolis and that the agar dilution and disk diffusion methods of testing give comparable but not identical results. It is important to add, however, that only 15 strains were tested. We therefore caution against extrapolating these results to clinical situations without additional testing of a larger sample of yeasts.

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