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Antifungal Activity of Turkish Honey against *Candida* spp. and *Trichosporon* spp: an *in vitro* evaluation

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Honey samples from different floral sources were evaluated for their ability to inhibit the growth of 40 yeast strains (*Candida albicans*, *C. krusei*, *C. glabrata* and *Trichosoporon* spp.). Broth microdilution method (CLSI, M27-A2) was used to assess the activity of the honeys against yeasts at different concentrations ranging from 1.25–80% (v/v). All of the yeast strains tested were inhibited by honeys in this study. Broth microdilution assay revealed that inhibition of growth depends on the type and concentration of honey as well as the test pathogen. Little or no antifungal activity was seen at honey concentrations $<2\%$. *Rhododendron* and multifloral honeys have generally more inhibitory effect than eucalyptus and orange honeys ($P<0.05$). Fluconazole-resistant yeast strains were examined for their susceptibility to honeys. This study demonstrated that, *in vitro*, these honeys had antifungal activity at the high concentration of 80% (v/v) in these fluconazole-resistant strains. Further studies are now required to demonstrate if this antifungal activity has any clinical application.

Keywords Honey, *Candida* spp., *Trichosporon* spp., fluconazole, antifungal activity

Introduction

Trichosporon spp. is the causative agents of white piedra, superficial infections and invasive trichosporonosis and has emerged as an opportunistic fungal pathogen [1,2]. Trichosporonosis, the deep-seated infection caused by this fungus, is an often fatal disease affecting mostly immunocompromised hosts [3,4]. Treatment of this infection with the currently available antifungal agents has generally been unrewarding, and clinical failures with amphotericin B (AMB), fluconazole (FLU), and the combination of the two have been reported [1,3,5]. *Candida albicans* once accounted for the most serious nosocomial candidal infections. Over the last decade *Candida* infections, especially candidemia, due to *C. albicans* have declined [6]. Other non-*C. albicans Candida* species (NAC) are now responsible for about half of the candidemias and other *Candida* infections [7,8]. Most NAC infections are caused by *C. glabrata, C. parapsilosis, C. krusei* or *C. tropicalis*, although in total over a dozen NAC have been reported to cause candidemia and other invasive infections [9–11]. AMB, FLU and caspofungin are currently the three antifungal agents most commonly used to treat candidemia and invasive candidiasis. However *in vitro* testing has revealed that there are clear differences among the various NAC in their susceptibility to drugs. The needed long term suppressive therapy with antifungal agents for these infections is widely used, but some of them have been reported as ineffective and even toxic to the host [12]. Most patients who experience clinical failure with FLU will have *Candida* species isolates that show *in vitro* resistance to the antifungal [13,14]. In 1997, the NCCLS Subcommittee for Antifungal Susceptibility Testing proposed tentative breakpoints for antifungal

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agents active against *Candida* species, and these breakpoints were subsequently approved [15]. Isolates with a FLU MIC <8 μg/ml are classified as sensitive, those with 8–32 μg/ml as dose-dependent sensitive, and those with an MIC ≥ 64 μg/ml resistant [15].

Therefore the search for new antifungal agents is necessary and stimulates research on new chemotherapeutic agents in natural products. Honey has been used since ancient times for the treatment of several diseases. The antibacterial activity of honey was reviewed by Molan [16].The high osmolarity and acidity of honey is among the physical characteristics that contribute to its antimicrobial activity. Furthermore hydrogen peroxide and phytochemical components are important factors that provide the antimicrobial activity of honey [16]. Although numerous *in vitro* studies have demonstrated the antibacterial activity of honey [17–19], limited number of investigations have examined the activity of honey against fungi. Sissons *et al*. [20] reported that Manuka honey may be of benefit in oral infections where *C. albicans* is prominent. In a recent study, Boukraa *et al.* [21] found that the MIC of five varieties of honey against *C. albicans* ranged between 40% and 50% (v/v). According to Al-Waili [22], the minimum concentration of honey in nutrient agar media required to inhibit *C. albicans* was 66% (v/v). Irish *et al*. [23] also reported that honey has significant antifungal activity against clinical isolates of *C. albicans*, *C. glabrata* and *C. dubliniensis*. The current study was carried out to assess the *in vitro* antifungal activity of four Turkish honey samples from different botanical origin against *Trichosporon* spp. and *Candida* spp., as well as to determine the activity against FLU-resistant strains with the CLSI M27-A2 method.

Materials and methods

Honey samples

Four honey varieties from different botanical origins were used; rhododendron (*Rhododendron ponticum* L.) from Rize (Black Sea Region), eucalyptus (*Eucalyptus camaldulensis*) and orange trees (*Citrus* spp.) from Mersin (South Anatolia) and multifloral (*Thymus* spp., *Astragalus* spp., *Brassica* spp.) from Erzurum (East Anatolia) which were collected directly from professional beekeepers in Turkey. The extraction of honeys from combs was obtained by centrifugation. The preparation of honey samples for identification of botanical origin followed the standardized method of Louveaux [24]: 10 g of well homogenized honey was dissolved in 20 ml of distilled water and centrifuged for 10 min at 1,350 g at ca. 2,500 rpm [25]. The decanted sediment was washed with 5–10 ml of distilled water. After another centrifugation, the sediment was resuspended in 5 ml of 1:1 glycerin:distilled water mixture and then centrifuged again, decanted, and mounted with glycerin jelly on microscope slides which were sealed with paraffin. The pollen types present in the honey samples were identified, counted, and classified, according to their percentages, as dominant pollen (more than 45% of the total pollen grains counted), secondary pollen (from 16–45%), important minor pollen (from 3–15%), and minor pollen (less than 3%). Counts were expressed as percentages after counting a minimum 1,000 pollen grains on four slides from sample.

Yeast strains and susceptibility testing

Forty strains were isolated from blood, sputum, transtracheal aspirates (TTA), urine, branchalveolar lavage (BAL), and nail specimens in the Microbiology and Clinical Microbiology Department of Gevher Nesibe Hospital of Erciyes University. Isolates were collected over a 6-month period in the Mycology Laboratory. They included ten strains of *C. glabrata*, nine strains of *C. albicans*, eight strains of *C. krusei* and thirteen strains of *Trichosporon* spp. The isolates were identified using standard methods and the API 20C AUX (bioMerieux SA, France) [26], and stored -20°C in tryptic soy broth containing 10% glycerin until used in the study. Prior to use, the yeast were thawed and subcultured at least twice on Sabouraud glucose agar plates. For quality control *C. albicans* ATCC 90028 was also included in the experiments. FLU (Fako Co. Istanbul, Turkey) was obtained from the manufacturers as defined powders. The Clinical and Laboratory Standards Institute (CLSI, M27-A2) microdilution broth method was used to determine the MICs [27]. Stocks and dilutions of honeys and FLU were prepared in sterile distilled water. Final drug concentrations in the microdilution plates ranged from 64–0.125 μg/ml for FLU and from 80–1.25% (v/v) for all of the honeys. The microdilution plates were prepared by using the synthetic medium RPMI 1640 broth medium (Sigma Chemical Company, Madrid, Spain) with l-glutamine but without sodium bicarbonate and buffered at pH 7.0 with 0.165 mol/l of morpholinepropansulfonic acid (MOPS) (Sigma Chemical Company). Yeast suspensions for spectrofotometric measurements were prepared after vortexing and adjusting to a 0.5 Mc Farland standard transmittance at a wavelength of 530 nm for *Trichosporon* spp. after serial dilution of the above described suspensions. The final inoculum yielded was of 0.5×10^3–2.5×10^3 cells/ml, which was confirmed by performing colony counts on SDA plates. MICs were visually determined at 24 h of incubation at 35ºC, and were observed for

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the presence or absence of growth. Each isolate was tested in duplicate and the assays were repeated on a separate day.

Statistical analysis

MIC values (%) were transformed base on Arc sin $\sqrt{Y}$ due to the fact that data do not have normality assumptions. Two-way ANOVA was not performed because of heteroscedasticity. Therefore, Friedman test, a nonparametric test, was performed to test whether there were any differences among the treatments of strains and honeys for MIC values. Then, Dunn's multiple comparison tests were applied to determine any further differences among the groups. All the computational work was performed by means of MINITAB (Minitab V. 13.20, 2000) [28].

Results

The broth microdilution assay enabled screening of honey samples from different botanical sources to assess the inhibition of growth of *Candida* spp. and *Trichosporon* spp. The *in vitro* activity of honey samples and FLU against four species of *Candida* spp. and *Trichosporon* spp. represented by 40 yeast strains are shown in

Table 1 Antifungal activities of honey samples and FLU

			Incubation times (24 h)				
			MIC Values% (v/v)				(μg/ml)
No	Strains	Material	Eucalyptus	Multi-floral	Orange	Rhododendron	FLU
1	*C. albicans*	Blood	80	20	40	40	0.25
2	*C. albicans*	Blood	40	40	40	40	0.25
3	*C. albicans*	Blood	40	40	40	40	0.125
4	*C. albicans*	Blood	40	40	40	40	0.25
5	*C. albicans*	Blood	40	40	80	40	0.25
6	*C. albicans*	Blood	40	40	80	40	0.025
7	*C. albicans*	Blood	40	40	80	40	0.25
8	*C. albicans*	Blood	40	20	80	40	3
9	*C. albicans*	Biopsy	40	40	80	40	0.25
10	*C. glabrata*	Mucus	80	40	80	40	32
11	*C. glabrata*	BAL	80	1.25	40	2.5	8
12	*C. glabrata*	TTA	80	40	40	40	0.5
13	*C. glabrata*	Mucus	80	40	40	40	1.5
14	*C. glabrata*	Mucus	80	40	80	40	0.25
15	*C. glabrata*	Urine	80	80	80	80	64
16	*C. glabrata*	Urine	80	80	80	80	32
17	*C. glabrata*	Mucus	80	80	80	80	96
18	*C. glabrata*	Mucus	80	80	80	80	32
19	*C. glabrata*	Mucus	80	80	80	40	24
20	*C. krusei*	Mucus	40	40	40	40	0.5
21	*C. krusei*	Mucus	80	40	40	40	32
22	*C. krusei*	Mucus	80	40	80	80	32
23	*C. krusei*	Mucus	80	40	80	80	64
24	*C. krusei*	Mucus	80	40	80	80	32
25	*C. krusei*	Mucus	80	20	80	80	32
26	*C. krusei*	Mucus	40	20	40	1.25	32
27	*C. krusei*	BAL	80	20	80	40	32
28	*Trichosporon*	Urine	40	40	40	2.5	2
29	*Trichosporon*	Nail	10	80	40	20	4
30	*Trichosporon*	Urine	40	40	40	40	48
31	*Trichosporon*	Urine	10	10	40	40	1
32	*Trichosporon*	Urine	40	10	40	40	6
33	*Trichosporon*	Urine	40	20	40	40	8
34	*Trichosporon*	Urine	20	40	20	10	2
35	*Trichosporon*	Urine	10	40	40	10	2
36	*Trichosporon*	Urine	10	20	40	10	2
37	*Trichosporon*	Urine	20	40	40	10	4
38	*Trichosporon*	BAL	20	5	10	1.25	4
39	*Trichosporon*	Urine	10	5	20	2.5	0.5
40	*Trichosporon*	Mucus	20	20	40	40	8

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Table 2 Statistical analysis of MIC values of honey samples ($P<0.05$)

	Incubation time (24 h)					
	MIC values% (v/v)					
Strains	Eucalyptus	Multi-floral	Orange	Rhododendron	Mean	SEM
C. albicans	44.44	35.56	62.22	40.00	45.56[b]	2.715
C. glabrata	80.00	56.13	68.00	52.25	64.09[a]	3.694
C. krusei	70.00	32.50	65.00	60.00	56.88[a]	4.027
Trichosporon	22.31	28.46	34.62	24.81	27.55[c]	2.179
Mean	51.25[ab]	37.78[c]	55.25[a]	42.13[bc]		
SEM	4.255	3.430	3.544	3.565		

Different superscript letters (a, b and c) within same line showed significant ($P<0.05$) differences among the group.

Table 1. When the antifungal activity of honeys tested was compared, statistically significant differences were determined among the honeys ($P<0.05$). Multi-floral honey was significantly more active against the three *Candida* species and *Trichosporon* spp. ($P<0.05$) than the other honey samples tested (Table 2). The orange honey exhibited lowest antifungal activity against the yeasts tested ($P<0.05$). The inhibition effect considerably decreased when honey samples were diluted from 40–20% (v/v). In general multifloral and rhododendron honeys showed stronger inhibition of the growth of fungi tested than eucalyptus and orange honeys. *Trichosporon* spp. was the more susceptible than *Candida* spp. ($P<0.05$) and *C. albicans* was the least susceptible of the *Candida* species tested ($P<0.05$).

The results revealed remarkable variations in antifungal activity of the tested honey samples. All of the honey samples had antifungal activity against the tested microorganisms. Although the differences was low, the order of activity in terms of overall activity against all; multifloral > rhododendron > eucalyptus > orange.

It was found that three *Candida* spp. strains (*C. glabrata* 2, *C. krusei* 1), and one of *Trichosporon* spp. failed to respond to FLU (Table 3). In the present study 23 of 40 isolates for which MICs were <8 μg/ml and 13 were dose-dependent strains but 4 of 40 isolates were resistant strains to FLU. MIC ranges of honey samples were between 40–80% (v/v) against FLU resistant strains.

Discussion

This study demonstrates the *in vitro* antifungal activity of locally obtained unprocessed honey samples against *Candida* spp. and *Trichosporon* spp. All honey samples analyzed had antifungal activity which is seen at concentrations as low as 1.25% (v/v); however the greatest

Table 3 Comparison of inhibitory activity of honey samples according to sensitive and resistant to FLU of the strains

	24 h		
Honey concentrations	FLU MIC <8 μg/ml, sensitive strains	FLU 8–32 μg/ml dose-dependent sensitive strains	FLU MIC >32 μg/ml resistant strains
Eucalyptus honey% (v/v)			
≤20	8	1	
40	11	2	1
80	4	10	3
Multifloral honey% (v/v)			
≤20	7	6	
40	15	4	2
80	1	3	2
Orange honey% (v/v)			
≤20	3	–	
40	14	5	1
80	6	8	3
Rhododendron% (v/v)			
≤20	8	2	
40	15	6	1
80	–	5	3
Total strains	23	13	4

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inhibition is seen at high concentrations (40% v/v). This is in agreement with a previous study that the amount of honey present in the honey mixture (50% w/v) inhibited completely the growth of *C. albicans* [22]. It was found that honey concentration ranging from 30–50% inhibited the growth of several pathogenic microorganisms, including *C. albicans* [29]. Additionally Obaseiki-Ebor *et al*. [30] demonstrated the susceptibility of 72 isolates of *C. albicans* to honey. Irish *et al*. [23] reported antifungal efficacy of various honeys against clinical isolates of *C. albicans*, *C. glabrata* and *C. dubliniensis*. Nevertheless, there is no information on antifungal activity of honey against *Trichosporon* species in the literature.

Antifungal activity of honey samples differed according to the botanical source and concentration of honeys and differences were statistically significant ($P < 0.05$). This variation could be attributed to the floral source or geographical origin. The composition of a particular honey sample greatly depends on the composition of nectar, as well as where it originates. The HPLC profile of phenolic components of nectar has also been used to relate nectar to a unifloral honey [31]. Most plants contain an extensive number of polyphenols and flavonoids and each plant tends to have a distinct profile [32]. Antifungal, antiviral and antibacterial activities of flavonoids have been demonstrated [33]. The concentration and type of polyphenolic substances depend on the floral origin of honey and are major factors responsible for biological activities including antimicrobial effects. For example orange honey exhibited lowest antifungal activity in this study. Andrade *et al*. [34] reported that orange honeys are characterized by small amounts of phenolic derivatives. However rhododendron honey is a more effective honey than orange honey against *Candida* and *Trichosporon* spp. ($P < 0.05$). In fact, it was demonstrated in a previous study that rhododendron honey had phenylethyl caffeate, dimethylallyl caffeate, cinnamic acid, chlorogenic acid, m-coumaric acid, o-coumaric acid, p-coumaric acid, vanillic acid, p-hydroxybenzoic acid, caffeic acid, gallic acid and 2,4 dihydroxybenzoic acid [34]. Multifloral honey had the highest antifungal activity and it probably contains the highest total phenolic content.

Honey has a potent antimicrobial activity and is very effective in cleaning infected wounds and protecting them from becoming infected [35]. According to Irish *et al*. [23], honey is limited to topical treatments, and could not be used to treat candidemia, the most serious form of candidiasis. However skin fungal infections contributed to the use of topical preparations that contain antifungal ingredients including flavonoids or antioxidants. Honey may be used to prevent more serious infections and could be incorporated in the therapy of oral and vaginal candidiasis.

The fungal infections have emerged as a major cause of disease and mortality, in part as a consequence of the increase in acquired immunodeficiency syndrome, the long term use of corticosteroids, and even the use of indiscriminate antifungals. Therefore, toxicity concerns, limited spectrum, and the emergence of fungi resistant to currently available agents have created a need for antifungal agents and in particular antifungal agents effective against systemic mycoses caused by *Candida* species. This result allows researchers to adopt different therapeutic options with a large number of species of superficial mycoses and with a high probability of successful results.

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