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Research paper

Antifungal activity of unifloral and multifloral Pakistani honeys against clinically isolated *Candida* species

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Abstract

Natural honey is utilized worldwide as nutraceutical due to its proven efficacy in treating chronic and acute infections including fungal infections caused by multidrug-resistant microbes. *Candida* species *e.g. Candida albicans, Candida parapsilosis, Candida glabrata* and *Candida tropicalis* are involved in skin infections as well as chronic mucocutaneous candidiasis. Multidrug-resistant candida infections occur frequently in both community and hospitals. To control these devastating fungal infections, identification of new potent antifungal agents is essentially required. Present study was aimed to evaluate antifungal potency of various Pakistani honeys against clinically isolated specimens of *Candida albicans, Candida parapsilosis, Candida glabrata* and *Candida tropicalis* using agar dilution assay. Natural honey samples showed antifungal activity at minimum inhibitory concentration values of 3-10% w/v against all *Candida* species tested except for *Candida glabrata* isolates which showed resistance to honey samples tested.

Key words: Antifungal activity; Honey; *Candida; Candida glabrata*

Introduction:

Honeybees of *Apis* species are the producers of natural honey [1]. Pakistani honeybees are diverse consisting of four A*pis* species i.e. *Apis cerana, Apis dorsata, Apis florea and Apis mellifera.* Honey has been known as a remedy and as a folk medicine around the globe since ancient times. Antibiotic resistance is growing broadly while very few antibiotics are being advanced. Antibiotic-resistant pathogenic microorganisms have been considered a severe risk to human health [2-3]. Hence, combinatorial strategies involving antibacterial, antifungal and antiviral properties should

be there to combat these resistant pathogens. Natural honey has been proved useful in treating chronic and acute infections which do not respond to antibiotic treatment [4]. Honey has been evaluated for its antimicrobial potential by different scientists as well as medical experts for many years [5]. Honey is known to contain antibacterial properties against many diseases [6]. *Penicillium*, *Aspergillus*, *Candida* specie and certain dermatophytes are also sensitive to natural honey [7]. Opportunistic fungi can easily cause mycoses in injured or immuno-compromised hosts [8-9]. These include several *Candida* specie like *C. parapsilosis, C. albicans, C. glabrata* and *C. tropicalis* which can attack and grow on wet body surfaces to cause serious illnesses [10]. Fungal infections with *Candida* spp. are frequent in both the the public and hospitals. *Candida albicans* is known to be the leading causes of oral infections, about half of candidaemia cases [11-12] and more than 90% of vaginal candidiasis [13-14]. Nevertheless, fungal infections involving non-*C. albicans Candida* specie are also clinically important due to their resistance to the typical antifungal treatment based on triazole; other antifungal therapies are not appreciated due to their toxic effects or limited efficacy. The main non-*C. albicans Candida* species which causes blood and vaginal infections is *Candida glabrata* [12,15-16], while *Candida dubliniensis* is found to be involved in oropharyngeal candidiasis [17]. Identification of new potent antifungal agents is essentially required to control fungal infections. The present study was designed to analyze the antifungal potency of several Pakistani honey samples against *Candida albicans*, *Candida parapsilosis*, *Candida glabrata* and *Candida tropicalis* clinical isolates.

Material and methods:

Honey samples:

A variety of Pakistani honey samples were assessed for their antifungal potential including fourteen untreated natural honey samples from various regions of interior Sindh and Khyber Pakhtunkhwa, Pakistan. In the present study, three multifloral wild honeys (codes; SW-1, SAM, and MON) produced by *Apis cerana* specie of honey bee were analyzed. In addition, eleven unifloral honeys produced by *Apis mellifera* foraging on distict plant species like *Acacia modesta* (codes; Kh1, AC-I, and AC-IV), *Trachyspermum* species (codes; Aj-1 and TRI-1), *Citrus* species (codes; CIT-1), *Ziziphus* species (codes; Ziz-1 and SIN) and *Plactranthus* species (codes; Sw2, Sw3 and Sw4) were also analyzed for their antifungal activities [Table 1]. Artificial honey

containing proportions of four major sugars i.e., D-glucose, D-fructose, sucrose and maltose of natural honey, was also analyzed for its antifungal activity [18].

Table 1: Natural honey samples analyzed for their antifungal activity.

No.	**Sample codes**	**Floral source**
1	AC-I	*Acacia modesta*
2	AC-IV	*Acacia modesta*
3	KH-1	*Acacia modesta*
4	SW-2	*Plactranthus* spp
5	SW-3	*Plactranthus* spp
6	SW-4	*Plactranthus* spp
7	ZIZ-1	*Ziziphus* species
8	SIN	*Ziziphus* species
9	AJ-1	*Trachyspermum coptcum*
10	TRI-1	*Trachyspermum coptcum*
11	CIT-1	*Citrus nobili deliciosa*
12	SW-1	Multiforal
13	SAM	Multifloral
14	MON	Multifloral

Isolation and identification of *Candida* species from clinical specimen

We randomly selected three hundred samples including ninty urine, seventy high vaginal swab, sixty blood, fifty tracheal aspirates and thirty wound pus samples from patients in Karachi, Pakistan. To isolate different *Candida* specie, each sample was inoculated in Sabroud's dextrose agar (SDA), Potato dextrose agar and Chocolate agar and incubated at 35ºC for 48 hours. Identification of isolated *Candida* specie was performed using Germ tube test, urea hydrolysis test, Biggy agar, Mycosel agar, Corn Meal Tween-80 agar and API 20 AUX identification system (Biomerieux, France).

Antifungal assay

C. albicans, C. parapsilosis, C. glabrata and *C. tropicalis* were assessed for their susceptibility to different Pakistani honey samples using agar dilution assay. According to the standard protocol, a honey stock solution (60% w/v) was made in pure distilled water. Appropriate quantity of the honey stock solution, double-strength Mueller–Hinton agar and sterile distilled water were used to make agar plates having 3%, 5%, and 10% (w/v) honey concentrations. Five isolated colonies of each of *C. albicans, C. parapsilosis, C. glabrata* and *C. tropicalis* were separately inoculated in sterile saline and shaken for 20 sec. The density of each inoculum suspension was set to the turbidity of a 0.5 McFarland Standard using pure saline followed by a dilution of 1:1000 in phosphate buffer saline (Boukraa et al. 2008). Then 1–3 µl culture was inoculated on agar plates followed by 35 ºC incubation for the whole day. Minimum inhibitory concentrations (MICs) of Pakistani honey samples were then determined [20].

Results:

Isolation and identification of *Candida* species from clinical specimen

During the present study, a total of 300 clinical samples including urine, tracheal aspirate, blood, wound pus and high vaginal swab were collected from infected and immuno-compromised patients. Out of those 300 only 28 samples including 09 urine, 09 high vaginal swab, 03 tracheal aspirates, 04 blood culture and 03 wound pus samples showed the presence of different *Candida* species. The isolated pathogens were identified using different methods (Table 2) and confirmed using API 20 C AUX (Biomerieux, France) as well as through microscopic analysis (Figure 1). *C. albicans, C. parapsilosis, C. glabrata* and *C. tropicalis* were found in 13, 03, 04 and 08 isolates,

respectively, showing *C. albicans* as the most frequent fungal pathogen in these clinical specimens (Figure 2).

Table 2: Identification of different *Candida* specie based on different tests

Organism Identified	Germ Tube Test	Urea hydrolysis	Mycosel Agar	Biggy agar	Microscopic morphology on CMT at 25 C
Candida albicans	+	-	+	Round brown glisring colonies on agar	Pseudo hyphae, cluster of Blastoconidia with Chlamydiospores
Candida tropicalis	-	-	-	Round Brown colonies with Silver sheen	Pseudo hyphae, rabbit ear blastoconidia
Candida parapsilosis	-	-	-	Round creamy colonies with purplish tinge	Pseudo hyphae short curved, giant cell
Candida glabrata	-	-	-	Small round creamy colonies	Only budding yeast cell

Out of 28 fungal infected samples, the presence of *C. albicans* was shown in 05 urine samples, 04 high vaginal swab, 02 wound pus, 01 blood, 01 tracheal aspirate sample. *C. tropicalis* was found in 02 urine samples, 02 blood, 02 high vaginal swab, 01 wound puss and 01 tracheal aspirate sample. *C. parapsilosis* was isolated from 01 urine samples, 01 tracheal aspirate and 01 high vaginal swab. Whereas, 02 high vaginal swab, 01 urine and 01 blood samples were shown to contain *C. glabrata* specie (Table 3).

Antifungal activity of honey:

The susceptibility of *C. albicans, C. glabrata, C. parapsilosis* and *C. tropicalis* to Pakistani natural honey samples is mentioned in table 4. Some natural honey samples (codes; AJ-1, Ac-IV, Ziz-1, SAM and SW-4) showed appreciably higher antifungal activity than other honey samples (codes; SW1, SW-2, SW-3, KH-1, Ac-1, Cit-1, Tri-1, MON, SIN) and artificial honey (AH). Table-4 showed the minimum inhibitory concentration (MIC) values of all honey samples tested. Honey samples codes; AJ-1, Ziz-1, SAM, SW-4 and Ac-IV showed antifungal activity against all *Candida* species except for *C. glabrata,* which showed resistance to almost all honey samples tested. The present work proved the potential role of natural honey in inhibiting *Candida* species growth at MIC values of 3-10% (w/v).

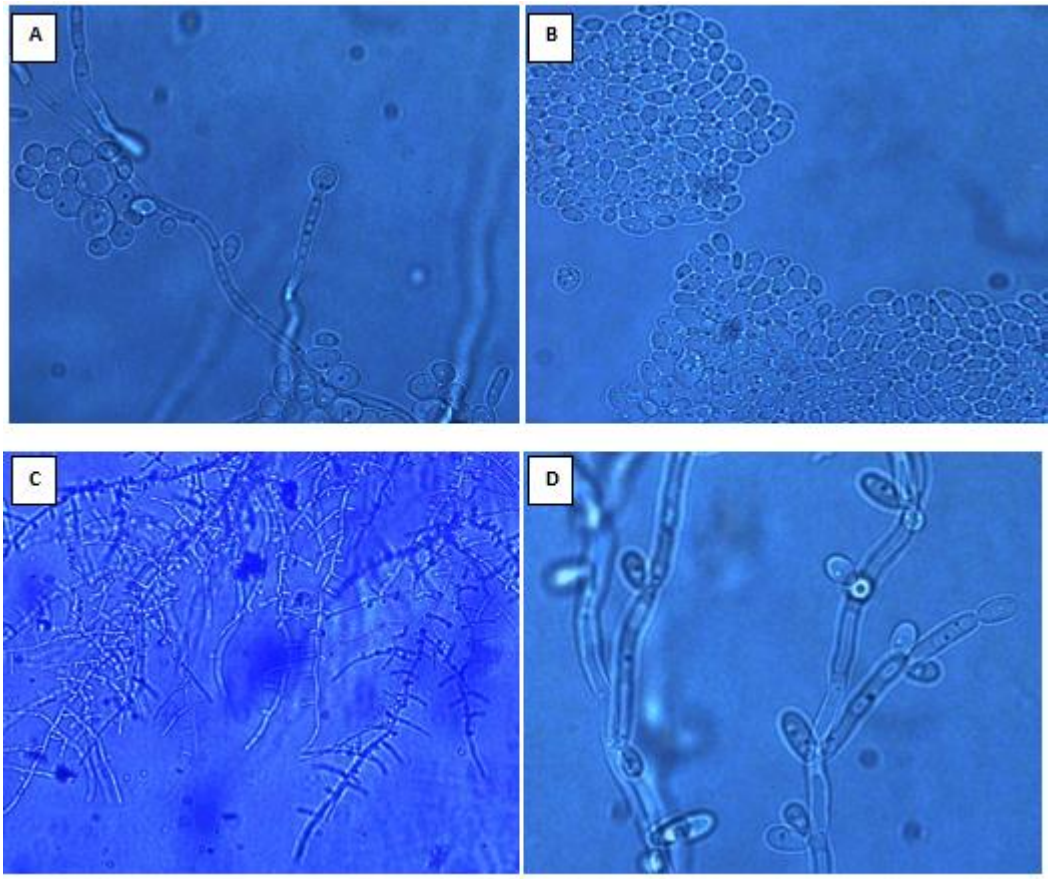

Figure 1: Microscopic analysis of clinically isolated *Candida albicans* (A), *Candida glabrata* (B), *Candida parapsilosis* (C) and *Candida tropicalis* (D).

Table 3: Percentage distribution of different *candida* specie in different clinical specimens

Specimen type	***C. albicans***	***C. tropicalis***	***C. parapsilosis***	***C. glabrata***
Wound pus	7.14%	3.57%	0%	0%
Blood	3.57%	7.14%	0%	3.57%
Urine	17.85%	7.14%	3.57%	3.57%
Tracheal aspirate	3.57%	3.57%	3.57%	0%
High vaginal swab	14.28%	7.14%	3.57%	7.14%

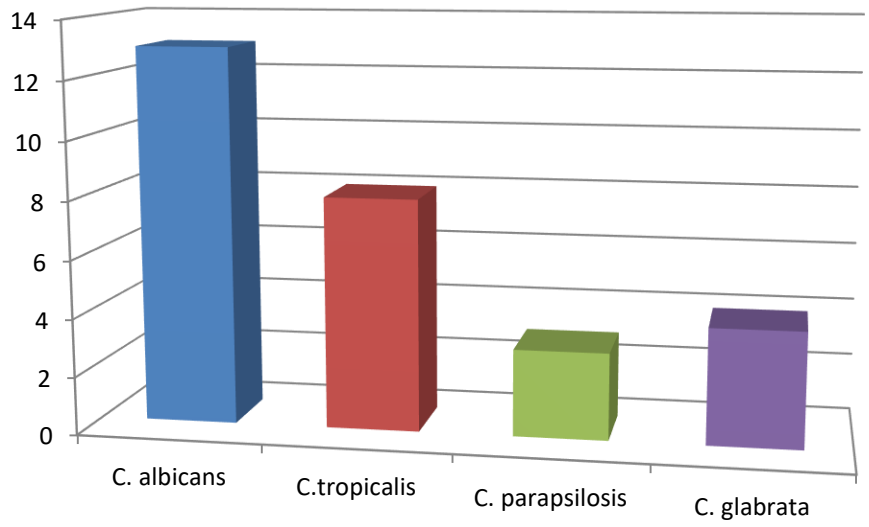

Figure 2: Distribution of four *Candida* species in different clinical specimens. Candida species versus number of positive clinical samples are plotted.

Discussion:

Candida specie are present in our surroundings and are capable of causing a variety of cutaneous, mucocutaneous, subcutaneous candidiasis and/or candidemia. Being opportunistic organisms, *Candida* specie can take the advantage of immunocompromised situation, diabetes mellitus, prolonged antibiotic usage, chemotherapy, invasive surgical procedure and organ transplantation for causing candidiasis [19]. Emerging resistance in *Candida albicans* and non-*Candida albicans* to triazoles have been reported by several studies [21-23]. Considering the continuous emergence of their resistance, the re-assessment of therapeutic potential of natural honey is needed. Being a folk medicine, natural honey has not been reported to have topical side effects so it can be used in wounds, cavities and sinuses to eliminate the infections [19]. Topical action of natural honey has limitation to treat candidaemia but beacuse candidal colonization of external sites for example the oral or vaginal mucosae is the major cause of candidemia [24]. Due to effective topical action, honey may be used as a prophylactic agent to avoid candidaemia. Crude honey has been reported to apply directly around catheters which is equally as effective as 10% povidone iodine or mupirocin in preventing external infection [25-26]. The mechanism behind the antimicrobial activity of honey is still controversial as different factors have been proposed to involve in this phenomenon such as low pH, an osmotic effect, hydrogen peroxide production and certain

phytochemical (non-peroxide) factors [27-29]. Therefore, additional research is required to disclose the exact mechanism behind the antimicrobial potential of honey.

During the course of present study, anti-candidal activity of Pakistani natural honey was evaluated using agar dilution rather than the typical agar diffusion assay as the later has been reported to have several limitations in inspecting honey or other natural antimicrobials [27,30]. Out of 14 different Pakistani honey samples, antifungal activity has been observed in five samples (codes; AJ-1, Ziz-1, SAM, SW-4 and AC-IV) against *C. albicans*, *C. parapsilosis* and *C. tropicalis* whereas C. *glabrata* isolates showed resistance to almost all honey samples uptill 10% (w/v) concentration.

The MIC values for the five varieties of Algerian honey against *C. albicans* ranged between 40% to 45% (v/v) [19], that for Portugalian lavender honey against *C. albicans* was 31.0% (w/v) [31], for Slovenian honey MIC against *C. parapsilosis, C. tropicalis* was found to be greater than 50% (v/v) while *C. albicans* was not inhibited even at higher Slovenian honey concentrations [32]. Moreover, South African Pincushion and Fynbos honeys, Bluegum *E. cladocalyx* honey and New Zealand *L. scoparium (*Manuka) honey showed MIC values of 50% against *C. albicans* [33]. Compared with these previous studies, lower MICs have been observed for certain Pakistani honeys in the present work. Neverthless, anti-candidal activity of stingless bee honey having MICs in the range of 6–8% [34] was found to be comparable with our findings. The results of this study demonstrate the antifungal activity of Pakistani natural honey samples against *Candida* species at different (w/v) concentrations. This activity should be assessed using controlled clinical trials so that natural honey can be established as an antifungal remedy.

Table 4: Minimum inhibitory concentrations (MICs) (% w/v honey) of different Pakistani honey samples against four different *Candidia* species. Isolate codes: *Candida albicans* (CA), *Candida tropicalis* (CT), *Candida parapsilosis (*CP) and *Candida glabrata* (CG).

Codes	SW1	SW2	SW3	KH1	AJ-1	MON	AC-IV	AC-I	CIT-1	ZIZ-1	SAM	SW4	TRI1	SIN	AH
CP-1	>10	5	3	>10	5	>10	3	>10	5	3	3	3	3	>10	>10
CP-2	>10	5	>10	>10	5	>10	3	>10	5	3	3	3	5	>10	>10
CP-3	>10	10	3	>10	5	>10	3	>10	>10	3	3	3	3	>10	>10
CA-1	>10	5	3	>10	5	>10	3	>10	>10	3	>10	3	>10	>10	>10
CA-2	>10	>10	>10	>10	5	>10	3	>10	>10	3	3	3	3	>10	>10
CA-3	>10	5	>10	>10	>10	>10	3	>10	10	3	5	3	>10	>10	>10
CA-4	>10	>10	>10	>10	5	>10	3	>10	>10	3	3	3	>10	>10	>10
CA-5	>10	5	3	>10	5	>10	3	>10	5	3	3	3	>10	>10	>10
CA-6	>10	5	3	>10	10	>10	3	>10	5	3	3	3	>10	>10	>10
CA-7	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
CA-8	>10	10	10	>10	3	>10	3	>10	>10	3	3	3	>10	>10	>10
CA-9	>10	5	3	>10	3	>10	>10	>10	5	3	3	3	>10	>10	>10
CA-10	>10	>10	3	>10	3	>10	3	>10	>10	3	3	>10	>10	>10	>10
CA-11	>10	>10	3	>10	3	>10	3	>10	>10	3	3	3	>10	>10	>10
CA-12	>10	5	3	>10	3	>10	3	>10	10	5	>10	3	>10	>10	>10
CA-13	>10	>10	3	>10	3	>10	3	>10	10	3	3	3	3	>10	>10
CG-1	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
CG-2	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	3	>10	5	>10	>10
CG-3	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
CG-4	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
CT-1	>10	>10	>10	>10	>10	>10	3	>10	>10	>10	3	3	>10	>10	>10
CT-2	>10	>10	>10	>10	10	>10	3	>10	5	5	>10	>10	>10	>10	>10
CT-3	>10	>10	>10	>10	3	>10	3	>10	5	5	>10	>10	>10	>10	>10
CT-4	>10	>10	>10	>10	5	>10	3	>10	5	5	>10	>10	>10	>10	>10
CT-5	>10	10	>10	>10	>10	>10	3	>10	10	3	3	3	5	>10	>10
CT-6	>10	5	3	>10	3	>10	3	>10	5	3	3	3	10	>10	>10
CT-7	>10	>10	3	>10	3	>10	3	>10	5	3	3	3	>10	>10	>10
CT-8	>10	>10	>10	>10	3	>10	3	>10	>10	3	3	3	>10	>10	>10

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