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Evaluation of the antifungal activity of Iranian propolis against *Candida albicans*

Roozbeh Yalfani*, Alireza Khosravi and Behnaz Pirouz

[1]Department of Mycology, Faculty of Veterinary, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Mycology, Faculty of Veterinary, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[3]Food and Drug Laboratory Research Center, Health Ministry, Tehran, Iran.

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Propolis is a resinous substance which has many pharmaceutical and biological effects such as antimicrobial activities. In this study, antifungal aspect of ethanolic solution of propolis was investigated. *Candida albicans* isolates were obtained from patients attending the Mycology Laboratory of Veterinary Faculty of the University of Tehran. BALB/c mice were used for the second part of the study. They were divided into eight groups and infected with different strains of *Candida*. Then, the influence of fluconazole and propolis for prevention of infection was assessed. All of them were treated with different dilutions of fluconazole and propolis samples in microdilution plates. Only two isolates were susceptible to fluconazole. However, they showed significant susceptibility to propolis. Groups of mice which were treated with propolis showed increased resistance to Candidiasis when compared with those that received fluconazole. Results indicate that natural substances like propolis should be used instead of drugs in medicine, because they lack side effects or for its prevention of microbial resistance. Propolis has several chemical constituents, so it seems impossible that microbial strains would be able to resist it.

Key words: Propolis, fluconazole, *Candida albicans*, MIC, mice.

INTRODUCTION

The word “propolis” is derived from the Greek pro (before) and polis (city). Propolis is a natural brownish-green resinous product collected by honey bees and was being used to make the protective shield at the entrance of beehive (Kumar et al., 2008). In general, propolis composition is directly related to bud exudates, collected by bees from various trees: poplar (*Populus spp*.), birch (*Betula alba*), beech (*Fagus stylvatica*), horse chestnut (*Aesculus hippocastanum*), alder (*Alnus glutinosa*) and various conifers (Shalmany and Shivazad, 2006).

It is collected by Apis mellifera bees from various plant sources and mixed with secreted beeswax. It is also used to fill the cracks in the hive, to attach the corners of frames to the grooves in the hive, and also to polish the cells of the honeycomb. The bodies of dead lizards, snakes and mice that die in hives are sealed into the walls with bee glue, thereby protecting the colonies against the unpleasant and bacterial flora of the putrefying corpses (Koo et al., 2002; Kumar et al., 2008).

The combination of temperature, small space and humidity provide condition for bacterial growth in hives. Nevertheless, this does not occur because of the anti-bacterial activity of propolis. That is why in the past, propolis was used in balsam and ointments to treat battle wounds (Ota et al., 2001). The use of propolis as a folk medicine dates back at least to 300 BC.

The chemical substances found in propolis include waxes, resins, balsams, aromatic and ethereal oils, pollen

*Correcponding author. E-mail: yalfani@iauvaramin.ac.ir. Tel: 02166743601. Fax: 02166045587.

and other organic components (Sy et al., 2006). The combination of these substances, probably resulting in a synergic effect, is essential for its biological activity. Its constitutive characteristics, however, can vary according to the bee species, period of the year in which it is collected and, especially botanic origin (Fischer et al., 2007). Chemical analyses revealed that propolis contains more than 300 constituents among them phenolic compounds, including flavonoids as major components (Ansorge et al., 2003). Propolis has several biological properties such as antibacterial, antiviral, fungicidal, antiprotozoan, antitumoral, antioxidant, anti-inflammatory, hepatoprotective, immunostimulating and cytostatic (Sforcin, 2002; Shalmany and Shivazad, 2006). It has been shown to stimulate various enzyme systems, cell metabolism, circulation and collagen formation, as well as it improves the healing of burn wounds. It was reported that propolis stimulated an immune response in mice. It activates immune cells that produce cytokines.

Bee propolis is one of the most promising extracts as antitumor agent (El–Kott and Owayss, 2008). With the increased use of antibiotics and immunosuppressive agents, fungal infections such as Candidiasis are becoming very-very common. Candidiasis is an opportunistic systemic fungal infection caused by fungus *C. albicans*, which occurs primarily when some aspect of the normal host defence is compromised. *C. albicans*, yeast like fungus, is commonly part of the normal flora of the skin, mouth, intestinal tract, and vagina and is present in the oral cavity in 40 to 60% of the population. It is thought to be acquired during passage through the birth canal (Hornby et al., 2001; Rawat et al., 2008). Although candidiasis is usually amenable to therapy with local or systemic antifungal drugs, failures of fluconazole therapy for mucocutaneous infections due to *C. albicans* have been reported (He et al., 1994).

The aim of this study wasthe determination of antifungal properties of Iranian propolis samples in comparison with fluconazole *in vitro* and *in vivo*.

MATERIALS AND METHODS

Propolis samples

Seven propolis samples were collected from Azerbijan and Kerman, different geographical location of Iran. They were kept at -20°C in dark bags till the time of examination. They were marked A to G.

Antifungal agent

Fluconazole (Darupakhsh Pharmaceutical Co., Tehran, Iran) (2 mg/ml) was prepared as stock solution diluted in water (Girmenia, 2000).

Extraction of propolis

An alcoholic extract was obtained by suspending 450 g of natural propolis in 1500 ml of ethanol (Merck, Darmstadt, Germany). This suspension was kept in the dark and was shaken every day for 15 min for a period of 30 days. It was then decanted for about 48 h and subsequently filtered. Then, the lyophilized extract was stocked in a freezer (Ota et al., 2001).

Strains used

The strains used were isolated from patients attending the mycology laboratory of Veterinary Faculty of the University of Tehran. The strains were isolated from urine, mouth, and vagina samples and identified as *C. albicans* using standard microbiological tests (Akortha et al., 2009).

In vitro

Preparation of fungal suspension

Yeast Extract agar (Merck, Darmstadt, Germany) was used for culturing the *C. albicans* strains. Fungal suspensions were prepared by picking up four or five colonies from a 24 h culture plate and suspending them in 5 mL of sterile distilled water. Fungal suspensions were standardized to a 0.5 McFarland turbidity standard, then diluted 1 in 1000 with sterile distilled water to yield an initial inoculum of approximately $1x10^3$ to 5×10^3 cfu/ml (Klepsera and Wolfe, 1998).

Antifungal test

The test was performed in sterilized plastic microplates (Nunclon, Delta, Nunc A/S, Roskilde, Denmark) containing 96 wells organized in eight rows, identified from A to H, with each row containing 12 wells, numbered from one to 12. Each row (A-H) corresponded to one yeast strain. The columns received the serially diluted propolis extract and fluconazole, with each column being twice as diluted as the previous one in YNBG broth (Yeast Nitrogen Base broth, Difco). Aliquots of 0.1 mL of fluconazole and propolis solutions were dispensed into each well. The final concentrations of these, ranged from 0.25 to 128 mg/L for fluconazole and 0.25 to 20 mg/mL for propolis. Then *C. albicans* inocula (100 µL) were added to each well of the microdilution plate. In each plate, negative and positive controls were included. The plates were incubated for 72 h at 35°C with daily monitoring. The colonies were analyzed by visual comparison through reflection on mirror. The MIC was considered as the minimum propolis or fluconazole concentration capable of inhibiting 80% of the growth of each yeast, having as a reference its respecttive positive control. All tests were performed in duplicate (NCCLS, 1997; Oliviera et al., 2006; Girmenia et al., 2000; Sener and Dulger, 2009).

In vivo

BALB/c male mice (6 - 8 weeks and 15 - 20 g weight) were used. After acclimatization (2 weeks), they were allowed food and water *ad libitum*. The temperature and the humidity employed were 23 - 25°C and 50 - 60% respectively and a 12/12 h light/dark cycle was used. Mice were divided at random into nine group (each group consisted of five mice) and examined separately. Animal experiments were performed according to the Principles of Laboratory Animal Care.

They were fed with solutions of fluconazole (200 mg/kg), propolis (150 mg/kg), and normal saline at pre infection stage, a week before examination. *C. albicans* isolates were administered to mice at a dose of 5×10^6 cells per mouse, IV. The mice were treated with solutions at post infection stage, q 48 h (Table 1).

For each group, the period of survival time was recorded (data not shown). For groups 2, 3, 4, and 6, this period lasted longer than the other groups. The mice were treated every other day after

Table 1. Explanation of examination schemes of mice.

Stage	Pre infection			Infection with Isolate			Post infection		
Inoculation with mice	Fluconazole	Propolis	Normal saline	Fluconazole resistant	Fluconazole sensitive	Standard strain*	Fluconazole	Propolis	Normal saline
Group 1	✓	-	-	✓	-	-	✓	-	-
Group 2	✓	-	-	-	✓	-	✓	-	-
Group 3	-	✓	-	✓	-	-	-	✓	-
Group 4	-	✓	-	-	✓	-	-	✓	-
Group 5	✓	-	-	-	-	✓	✓	-	-
Group 6	-	✓	-	-	-	✓	-	✓	-
Group 7	-	-	✓	✓	-	-	-	-	✓
Group 8	-	-	✓	-	✓	-	-	-	✓
Group 9(control)	-	-	✓	-	-	-	-	-	✓

* Standard strain: Candida albicans ATCC 10231.

Table 2. MIC values of isolates against fluconazole (mg/L).

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MIC	128	128	128	128	128	128	128	4	128	1	128	128	128	128	128
Isolate	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
MIC	128	128	128	128	128	128	128	128	128	128	128	128	128	128	128

infection.

Eight weeks after treatment, the remaining animals were exposed to ether resulting in their death. Cultures from liver, kidney, lungs, spleen, and brain of these and previously dead mice were prepared. Briefly, the method was as follows:

The dissection started in the abdomen area and incisions cut down the body from the neck to the lower abdomen. Each organ was removed and 1 g was weighed and homogenized. Then, it was transferred to Yeast Extract Peptone Dextrose Agar (YEPD, Difco) and incubi-ted at 30°C for 48 h. Thereafter, the numbers of colonies were counted to determine the CFU (colony forming unit) for each group. On the other hand, cultures from these organs were accomplished. Briefly, the method was as follows:

1 g from each organ was weighed and homogenized. Then, it was transferred to Yeast Extract Peptone Dextrose Agar (YEPD) and incubated at 30°C for five days. Thereafter, the numbers of colonies were counted to determine the CFU (colony forming unit) for each group.

Statistical analysis

The Student's t-test and one way analysis of variance (one way ANOVA**)** by SPSS 16 was used to assess the statistical significance of the difference. P-values less than 0.05 (P< 0.05) were considered statistically significant.

RESULTS

MIC values of fluconazole and propolis for the isolates are presented in Tables 2 and 3. Table 2 shows the degree of sensitivity of isolates against different dilutions of fluconazole (0.25 to 128 mg/L). There were only two isolates (namely 8 and 10) which showed susceptibility to fluconazole but the others were completely resistant. The MIC values varied from less than 0.25 to 10 mg/mL. But no isolate was identified as a resistant. The consequences of exposure of isolates against ethanolic solution of propolis are shown in Table 4.

The MFC values varied from less than 0.25 to 15 mg/mL. The results of MIC and MFC were summarized for better comparisons of their fungistatic and fungicidal effects (Table 5). There were differences among propolis samples. One sample (C) had the best results, whereas D indicated lesser efficacies than the rest did. The results of the others were between these two samples. In the next step of experiments, the mice were killed and cultures from their different organs prepared. The results of this part are shown in Table 6. The

Table 3. Shows the results of each strain exposed to different dilutions of all samples of propolis entitled A to G, separately.

Isolate	Propolis	MIC (mg/ml)	Isolate	Propolis	MIC (mg/ml)	Isolate	Propolis	MIC (mg/ml)	Isolate	Propolis	MIC (mg/ml)	Isolate	Propolis	MIC (mg/ml)
1	A	4	2	A	4	3	A	5	4	A	5	5	A	5
1	B	5	2	B	5	3	B	5	4	B	5	5	B	5
1	C	5	2	C	5	3	C	5	4	C	5	5	C	2
1	D	10	2	D	10	3	D	10	4	D	10	5	D	10
1	E	4	2	E	5	3	E	5	4	E	5	5	E	2
1	F	10	2	F	10	3	F	10	4	F	10	5	F	5
1	G	5	2	G	5	3	G	5	4	G	5	5	G	5
6	A	5	7	A	1	8	A	0.5	9	A	2	10	A	<0.25
6	B	5	7	B	5	8	B	1	9	B	5	10	B	1
6	C	2	7	C	5	8	C	2	9	C	2	10	C	5
6	D	10	7	D	10	8	D	10	9	D	10	10	D	10
6	E	5	7	E	2	8	E	5	9	E	2	10	E	5
6	F	10	7	F	10	8	F	5	9	F	5	10	F	10
6	G	5	7	G	5	8	G	5	9	G	5	10	G	5
11	A	5	12	A	5	13	A	5	14	A	5	15	A	5
11	B	2	12	B	5	13	B	5	14	B	2	15	B	5
11	C	1	12	C	5	13	C	5	14	C	5	15	C	10
11	D	10	12	D	10	13	D	10	14	D	10	15	D	5
11	E	5	12	E	5	13	E	5	14	E	5	15	E	5
11	F	5	12	F	10	13	F	10	14	F	10	15	F	5
11	G	5	12	G	5	13	G	5	14	G	5	15	G	10
16	A	5	17	A	10	18	A	5	19	A	1	20	A	10
16	B	0.5	17	B	10	18	B	5	19	B	1	20	B	5
16	C	2	17	C	0.5	18	C	5	19	C	1	20	C	5
16	D	4	17	D	10	18	D	10	19	D	1	20	D	10
16	E	4	17	E	10	18	E	10	19	E	1	20	E	10
16	F	5	17	F	5	18	F	5	19	F	0.5	20	F	5
16	G	2	17	G	5	18	G	5	19	G	0.5	20	G	2
21	A	5	22	A	5	23	A	4	24	A	4	25	A	5
21	B	10	22	B	5	23	B	10	24	B	5	25	B	1
21	C	4	22	C	0.5	23	C	5	24	C	0.5	25	C	0.5
21	D	10	22	D	10	23	D	10	24	D	10	25	D	10
21	E	10	22	E	10	23	E	10	24	E	10	25	E	10
21	F	5	22	F	5	23	F	5	24	F	4	25	F	5
21	G	5	22	G	5	23	G	10	24	G	5	25	G	5
26	A	0.5	27	A	10	28	A	10	29	A	5	30	A	5
26	B	10	27	B	5	28	B	10	29	B	10	30	B	5
26	C	1	27	C	10	28	C	10	29	C	1	30	C	5
26	D	10	27	D	10	28	D	10	29	D	10	30	D	10
26	E	10	27	E	10	28	E	10	29	E	10	30	E	10
26	F	2	27	F	5	28	F	5	29	F	5	30	F	5
26	G	5	27	G	10	28	G	5	29	G	2	30	G	5

Table 4. MFC values of isolates against propolis.

Strain	Propolis	MFC	Strain	Propolis	MFC	Strain	Propolis	MFC
1	A	5	2	A	5	3	A	8
1	B	8	2	B	8	3	B	8
1	C	8	2	C	8	3	C	8
1	D	15	2	D	15	3	D	15
1	E	5	2	E	8	3	E	8
1	F	15	2	F	15	3	F	15
1	G	8	2	G	8	3	G	8
4	A	8	5	A	8	6	A	8
4	B	8	5	B	8	6	B	8
4	C	8	5	C	4	6	C	4
4	D	15	5	D	15	6	D	15
4	E	8	5	E	4	6	E	8
4	F	15	5	F	8	6	F	15
4	G	8	5	G	8	6	G	8
7	A	2	8	A	1	9	A	4
7	B	8	8	B	2	9	B	8
7	C	8	8	C	4	9	C	4
7	D	15	8	D	15	9	D	15
7	E	4	8	E	8	9	E	4
7	F	15	8	F	8	9	F	8
7	G	8	8	G	8	9	G	8
10	A	<0.25	11	A	8	12	A	8
10	B	2	11	B	4	12	B	8
10	C	8	11	C	2	12	C	8
10	D	15	11	D	15	12	D	15
10	E	8	11	E	8	12	E	8
10	F	15	11	F	8	12	F	15
10	G	8	11	G	8	12	G	8
13	A	8	14	A	8	15	A	8
13	B	8	14	B	4	15	B	8
13	C	8	14	C	8	15	C	15
13	D	15	14	D	15	15	D	8
13	E	8	14	E	8	15	E	8
13	F	15	14	F	15	15	F	8
13	G	8	14	G	8	15	G	15
16	A	8	A	17	15	18	A	8
16	B	1	B	17	15	18	B	8
16	C	4	C	17	1	18	C	8
16	D	5	D	17	15	18	D	15
16	E	5	E	17	15	18	E	15
16	F	8	F	17	8	18	F	8
16	G	4	G	17	8	18	G	8
19	A	2	A	20	15	21	A	8
19	B	2	B	20	8	21	B	15
19	C	2	C	20	8	21	C	5
19	D	2	D	20	15	21	D	15
19	E	2	E	20	15	21	E	15
19	F	1	F	20	8	21	F	8
19	G	1	G	20	4	21	G	8
22	A	8	A	23	5	24	A	5
22	B	8	B	23	15	24	B	8

Table 4. Contd.

22	C	1	C	23	8	24	C	1
22	D	15	D	23	15	24	D	15
22	E	15	E	23	15	24	E	15
22	F	8	F	23	8	24	F	5
22	G	8	G	23	15	24	G	8
25	A	8	A	26	1	27	A	15
25	B	2	B	26	15	27	B	8
25	C	1	C	26	2	27	C	15
25	D	15	D	26	15	27	D	15
25	E	15	E	26	15	27	E	15
25	F	8	F	26	4	27	F	8
25	G	8	G	26	8	27	G	15
28	A	15	A	29	8	30	A	8
28	B	15	B	29	15	30	B	8
28	C	15	C	29	2	30	C	8
28	D	15	D	29	15	30	D	15
28	E	15	E	29	15	30	E	15
28	F	8	F	29	8	30	F	8
28	G	8	G	29	4	30	G	8

Table 5. The average of MIC and MFC for each sample of propolis.

Sample	MIC (mg/ml)	MFC (mg/ml)
A	4.7 ± 2.66	7.27 ± 3.99
B	5.11 ± 2.94	8.1 ± 4.22
C	3.83 ± 2.78	6.13 ± 4.06
D	9.33 ± 2.10	14 ± 3.15
E	6.66 ± 3.14	10.23 ± 4.50
F	6.38 ± 2.77	9.86 ± 3.98
G	5.05 ± 2.05	8.06 ± 2.89

average of CFU/g number was calculated for each organ.

DISCUSSION

Recently, fluconazole-resistant *C. albicans* strains and intrinsically resistant *Candida* species such as *C. glabrata* and *C. krusei* are emerging in immunocompromised patients treated for therapy or prophylaxis (Casalinuovo et al., 2004). In this study, two isolates (No. 8 and 10; Table 1) showed susceptibility to fluconazole and their MIC were 4 and 1 mg/L respectively, but others (>93.3%) were completely resistant (>64 mg/L). This indicated that drug resistance among *C. albicans* isolates is dramatically widening and it seems treatment of such diseases, especially in immunocompromised patients, will be difficult. To avoid such problems, natural substances like propolis can be used since on the basis of past few years' studies, no resistance among microbial populations was obtained (Herrera et al., 2010; Kosalec et al., 2005). The reason for this is probably because of the many constituents in propolis which act together against microbial cells and generally microbial cells have no chance to resist all of them. On the other hand, these natural products may have side effects slightly. Thus, it seems they are more compatible with humans' and animals' body than chemical products like drugs. In the present study, all of the isolates were fortunately susceptible to propolis with different concentrations.

One propolis sample (C) had high efficacy on isolates growth or death (3.83 and 6.13 for MIC and MFC respectively). Higher quantities (9.33 and 14 respectively) of sample D were required for the same experiments (Table 5).

In this study, we observed that the number of yeast colonies of mice infected with resistant *C. albicans* to whom then offered propolis, were less than those that received fluconazole. Obviously, the former showed fewer number of yeast cells in kidney and lung as compared with mice infected with sensitive *C. albicans* and then treated with fluconazole (Table 6).

Table 6. The results of CFU related to different organs of mice.

Group	Organ				
	Lung	Liver	Kidney	Spleen	Brain
Group 1	1.6×10^2	2.1×10^3	4.8×10^3	175	295
Group 2	260	85	1.2×10^2	25	0
Group 3	22	62	87	12	0
Group 4	11	45	61	4	0
Group 5	29	32	92	23	8
Group 6	12	35	58	21	0
Group 7	1.6×10^3	4×10^4	5.1×10^6	15	306
Group 8	1.2×10^3	4×10^5	4.6×10^5	25	405

Furthermore, fluconazole had no effects on preventing *C. albicans* in mice group 1 because the strains were resistant. In the second, despite the sensitivity of strains to fluconazole, considerable numbers of organisms were detected. Groups 3 and 4 showed similar pattern of distribution of organisms in different organs in respect to the moderate increased number in group 3 (Table 6).

Dimov et al. (1991) showed that the mortality rate of mice with *C. albicans* infection became relatively low when treatment was performed with propolis. They suggested that propolis stimulates macrophages and thus enhances survival. Macrophages are the major factors that prevent bacterial and fungal dissemination (Dimov et al., 1991).

Also, it was shown that macrophages activated with propolis could produce cytokines, such as TNF-α and IL-12, which act on NK cells, increasing its cytotoxic activity (Sforcin et al., 2002).

On the other hand, oxygen intermediate metabolites are related to microbicidal activity of macrophages. It was found that propolis induced NO production in a dose-dependent way, as high concentrations inhibit it and induced a discreet elevation in H_2O_2 release as well. So they suggested that propolis has an important role on the immune system's action on host non-specific immunity by macrophage activation (Orsi et al., 2000).

Also, there was a degree of resemblance between groups 5 and 6 (Table 6). The reason of lesser number of *C. albicans* in groups 3, 4, and 6 was probably as the result of propolis activity. Groups 2 and 5, both received fluconazole as a choice of treatment. But it seems strains which were used for inoculation of mice that belonged to the second group, had higher virulence and caused increased mortality rate. Maybe their recent isolation from patients and consequently their freshness was the reason they were more aggressive than the fifth group. In the absence of fluconazole, both resistant and sensitive isolates represented their pathogenicity in groups 7 and 8.

In general, natural substances such as propolis preferbly can be used as widely as drugs, cosmetics, supplements, etc. Because of increased resistance to drugs and the side effects, it is time to use natural products again, like our ancestors did in the past.

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