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In vitro activity of Propolis alcoholic extract on opportunistic pathogenic fungi

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Abstract

***Background & Aims*:** Several studies have shown that Propolis has antibacterial, antifungal, antiviral, and antiparasitic activities. Furthermore, Propolis has been described to have therapeutic effects in some fungal infections. Our aim is to study the inhibitory effects of alcoholic extract of Propolis on Candida and Aspergillus spp.

***Material & Methods*:** To determine inhibitory and fungicide dose of Propolis extract, we prepared serial dilution of the extract including 1/20, 1/40, 1/80, 1/160, 1/320, and 1/640 in 1 ml of liquid medium Sabouraud dextrose broth. Given the numbers of Candida, yeasts in 1 ml were added to the above dilution tubes. Candida and Aspergillus cultures were incubated at 30°C and 25°C, respectively, for 24-72 h.

***Results*:** The concentration of 0.25 g/dl of Propolis extract showed an inhibitory and cidal effect on more than 50% of the clinical isolates. But, there were no inhibition and killing at concentrations 0.0312 g/dl and 0.0625 g/dl on the Candida isolates. Our findings showed that 0.0312 g/dl of the extract was partially active against Aspergillus fumigatus and dilution of 0.125 g/dl was active on Aspergillus Niger. In the agar dilution method, some changes were observed in morphological features (depending on the extract dilution) and quantitative effects of the dilution of the extract were found on the colonies.

***Conclusion*:** We realized that the alcoholic extract of Propolis had prominent antifungal activity and inhibitory effect on Candida and Aspergillus isolates.

***Key words*:** Propolis, Candida, Aspergillus, Inhibitory

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Introduction

Propolis is a natural product which is derived from plant resins gathered by bees. The substance made by bees is used in the inner walls of the hive and is responsible for protecting bees and wax (1). More than 300 different combinations such as polyphenols, phenolic aldehydes, monoterpene, amino acids, steroids, and other inorganic compounds have been found in Propolis structure (2). The amount of these compounds depends on the location and time of collection of plant resources and the wax which is used by bees. The biological activity of Propolis mainly depends on phenolic compounds such as flavonoids.

Propolis has been used as a traditional medicine in the treatment of human diseases since very ancient times. It can also have anti-bacterial, anti-tumor, anti-virus, and anti-inflammatory properties (3). Propolis is a solid, pasty, and viscous greenish brown to dark brown substance under normal conditions and depends on the origin of the resin components and its duration and storage conditions. Propolis and its extracts are used in the treatment of rheumatism, diabetes, cancerous tumors, allergies, asthma, heart/stroke disease, pneumonia, influenza, peptic ulcer, and chronic vaginitis. Propolis has stimulating activity on both humoral and cell mediated immunity, potency for antioxidant, anti-inflammatory, non-reinforced soft tissue, and inhibition of hydrolase activity. Also it is used for storing foods (3). Numerous studies have been done on the effects of Propolis in the prevention and treatment of many different diseases and complications, such as effects of Propolis on prostate cancer cells (4), treatment of chronic recurrent vulvovaginitis (5,6), and enhancement of human immunity system. However, the role of Propolis has been proven in the treatment of periodontal and oral infections. The recent studies have shown anti-bacterial, anti-protozoa, anti-fungal, and anti-viral activities of Propolis (7-9). Its inhibitory effects on the growth of at least 21 species of bacteria, protozoa, 9 fungal species, and 3 species of viruses (such as herpes and influenza) were also detected (10).In Kuk's study, antifungal effect of Propolis ethanol extract was studied on dermatophytes: *Trichophyton rubrum* and *Trichophyton mentagrophytes*, compared with other anti-fungal drugs and Propolis was introduced as a substance with antifungal activity. Also, the influence of different alcohol concentrations of Brazilian propolison *Candida albicans* and *Candida tropicalis* has been well studied by determining the minimum inhibitory concentrations (MICs) (11, 12). Along with the increasing abundance of fungal diseases and incidence of drug resistance, the necessity of introducing new and drugs is necessary. Therefore, extensive research must be done in this regard. The most important fungi that have been prevalent recently as several agents in the creation of multiple opportunistic infections in humans are bisexual species of Candida and Aspergillus. There are rare similar studies carried out in Iran on determining the exact effects of inhibitory and fungicidal activity of Propolis. Therefore, this study deals with evaluating the anti-growth properties of Propolis on Candida and Aspergillus species as the most important opportunistic fungi.

Materials & Methods

This study was performed from autumn to winter 2007 in Public Health Research Center of Tehran University of Medical Sciences.

A. Crude extract preparation: 500 g primary raw material or Propolis was purchased from aromatic pharmaceutical companies in Isfahan and was ordered for extraction to the same center; only 10 g raw ethanol extract was prepared by suckslet extraction. For the preparation of micro-samples of Propolis, 10 g was accurately weighed and poured in a 250 ml flask; then, the sample got to 100 ml by 96% ethanol and the mixture was well homogenized; this process was repeated twice a day for three days and then the mixture was kept in a warm (above 30°C) and dark place for 1-2 weeks. Afterwards, it was purified and the product was kept in the refrigerator at 1-4°C for one day. The dilution was filtered and the obtained extract was stored in a dark and gated glass.

B. Case studies of strains: The strains were selected according to the research objectives of Candida and Aspergillus species. The organisms were collected from the clinical specimens referring to Department of Mycology, Faculty of Public Health, and Institute of Health Research. The species were identified by cultivation specificities, microscopic morphology, and some biochemical properties. In addition, two standard strains including *Candida albicans* ATCC10261 and *Aspergillus fumigatus* TIMM 2920 were purchased from Global Bank of Standard Strain of Japan JCM.

C. Preparing fungal suspension: In order to obtain uniform or homogeneous suspensions from fungal concentration, a turbidimetric criterion called McFarland standard 0.5, which contained mixture of barium chloride and sulfuric acid. The prepared suspension was diluted with the turbidity equivalent to the standard McFarland and estimated for the Candida species which contained approximately 10^5 cells and also for Aspergillus with 10^3 cells.

D. Preparing serial dilution from crude alcoholic extract of Propolis:

1. Serial tube dilution method**:** The decreasing dilutions were selected to hold a range of different amounts of fungal elements as1/20, 1/40, 1/80, 1/160, 1/320, and 1/640**.** To obtain these concentrations, five medium sterile covered tubes were prepared, which contained 1ml cultivation liquid of Sabouraud broth. Then, the initial dilution of 1/10 was created by weighing 10 g concentrated extract of Propolis in 100 ml of distilled water (one gram per deciliter). One ml of the initial dilution was added to the first series tubes of each test, so the concentrations of Propolis were about 1/20 or%5 g/dl on the tubes. Likewise a decreasing serial dilution provided in tubes by transferring 1 ml of each series tube into the next tubes and discarding 1 ml of last tubes.

2. Serial dilutions on agar: In this method, 1 ml of each MIC tubes transferred to the SGA 4% media to make a serial decreasing plate dilutions included; reducing serial dilution including 1/20, 1/40, 1/80, 1/160, 1/320, 1/640.

E. Adjacency of prepared fungal suspensions by extract dilutions:

1) Serial tube dilution method: Aseptically, 1ml of fungal suspension with the turbidity of 0.5 Mc Farland standard was added to every tube containing dilutions of various extracts and also the positive and negative controls were respectively made by adding 2 ml of the extract dilution. Serial tube dilution method was used for yeast.

2) Serial agar dilution method: This method was used just for Aspergillus isolates; thus, one loop of a spore suspension was inoculated into each plate in 5 separate places. The negative control plates received no cell inoculation and positive control plates had no treatment. Incubation conditions for *Candida* and *Aspergillus* isolates included 24-48 h at 30˚C and up to 7 days at 25˚C, respectively.

3) Measuring minimum inhibitory concentrations (MICs): The tubes were examined for the turbidity growth after 24 h of incubation. The standard turbidity was considered 0.5 McFarland solution dilutions. Thus, the fungal growth in the dilution tubes was compared with the turbidity of McFarland. Lack of turbidity could shown inhibitory effect from Propolis extract.

F) Determining minimum fungicidal concentration: After determining minimum inhibitory concentration (MIC), a small volume of tubes transferred to the plates containing SGA 4% medium. The growth checked after 24-72 hours for determination of the minimum fungicidal concentration (MFC**). T**he MICs and MFCs for all Candida and Aspergillus isolates measured.

Results

The growth inhibitory and fungicidal effects of the alcoholic extract of Propolis were studied on 40 isolated *Candida albicans* and 18 isolated Aspergillus including: *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus Niger*. The result of dilution effects of 1/20 to 1/64 on the yeast suspensions with the turbidity of McFarland standard 0.5 is shown in Table 2. As observed, more than half of the strains were affected by the inhibitory dilution extracts of 1/40. Also, the minimum fungicidal concentration of the extract was determined in more than half of the isolates (MFC 0/25g/dl). It should be noted that fewer strains were affected by higher fungicidal effect than inhibitory effect. Our observations indicated that the dilution 1/640 and 1/320 of Propolis extract did not have inhibitory and fungicidal effects on the isolates. The dilution 1/20 of the extract had inhibitory effects on all the strains (MIC 0/5 g/dl); with the exception of one case, this dilution had fungicidal effects on all the strains. Dilutions

of the extract on the formation of Aspergillus species using agar dilution method had different results in terms of changes in the species. More than half of *Aspergillus flavus* was used as a side effect of the complete dilution of 125/0 and the other half was used as the relative dilution ratio of 065/0 extracts, i.e. it did not completely stop the growth and it was observed like small colonies with slow growth. *Aspergillus fumigatus*, even in the pitiful extract of 0/0313, was relatively effective. Finally, the effect of the extract on *Aspergillus niger* was relatively observed by dilution of 0/0625 (Table 1). In serial agar dilution method, in addition to quantifying the effect of the extract on colony growth, morphological changes in colony color, colony diameter, and sporulation rates were observed, which depended on the dilution of the extract (Table 1). However, the morphological changes of the microscopic fungi were not considered.

Figure 1 a: Morphological evolutions of Aspergillus flavus (color colonies and spore formation) diluted from 1/20 to 1/320 of Propolis extract. As can be observed, the size of the colonies and the colorimetric and spore formation were decreased with increasing concentration. **B:** Morphological changes of *Aspergillus fumigatus* with the dilution of 1/20 to 1/320 extract of D. The changes of rapid growth could be observed at high concentrations of the extract if lower concentrations only include morphological changes. C: comparing morphological characteristics of *Aspergillus fumigatus* with *Aspergillus niger* in dilutions, 1/20 to 1/320 of the Propolis extract shows in addition to the morphological characteristics, growth and colony formation were also affected.

Among the tested Aspergillus species, *Aspergillus fumigatus*, *Aspergillus flavus,* and *Aspergillus niger* and also *Candida albicans* as the most common species isolated from patients were tested by this method. Strains of Candida albicans which were obtained from the samples of the vagina, skin, nails, and body, respectively, and tested in four groups of ten according to their source.

Table 1: Mean values of MFC Propolis extract for different *Aspergillus species*

Species	Number of isolates	Mean of minimal bactericidal concentration(MFC)
Aspergillus flavus	11	0/0625 g/dl
Aspergillus Niger	2	0/125 g/dl
Aspergillus fumigatus	5	0/0313 g/dl

Table 2: Values of MFC and MIC Propolis extract different strains of *Candida albicans*

Isolates of Candida (40 isolates)	Mean of minimum inhibitory concentration (MIC)	Mean of minimal bactericidal concentration (MFC)
Vagina-Group 1 (1-10)	0/0625 g/dl	0/25 g/dl
Skin- Group 2 (11-20)	0/25 g/dl	0/25 g/dl
Nail- Group3 (21-30)	0/25 g/dl	0/25 g/dl
Phlegm-group 4 (31-40)	0/25 g/dl	0/5 g/dl

Discussion

The antifungal activity of Propolis against Candida species in the treatment of patients with onychomycosis (14) and vulvovaginal candidiasis has been studied (15). The effect of Brazilian Propolis extracts has been well studied at different concentrations on *Candida albicans* and *Candida tropicalis* with the identification of (MIC) (16). In the present study, the growth inhibitory effect of

Propolis crude extract was studied against a group of yeasts and mold fungi mostly, included *Candida albicans*. According to the results, the mean of minimum inhibitory concentration of the extract against *Candida albicans* isolates was 0/25g/dl and reflected the fact that this material was relatively resistant strains of *Candida albicans*. These findings were confirmed by previous studies[18]. The minimum mean inhibitory concentration of Propolis extract was determined on isolates of *Aspergillus flavus* 0.0625 g/dl, *Aspergillus niger* 0.125 g/dl, and *Aspergillus fumigtus* 0/0313 g/dl, which showed a significant difference in sensitivity to Propolis. The activity of Propolis was substantial against more pathogen Aspergillus species (*Aspergillus fumigatus*) considered as the main cause of invasive aspergillosis (IA) due to the ability of this species to grow, withstand at higher temperature, potency to invide the blood vessels and some other virulence factors. That is an important point that Aspergillus spores disseminate in hospital indoor including surgical wards, intensive care units, and surgery rooms, especially during the constructions and renovations so that the exposure of the patients to aerosols is inevitable. There has been no considerable studies on the Propolis antifungal activities especially on aspergillus isolates of HAI cases. Our experimental findings showed significant macroscopic changes in the colony production of Aspergillus, including color and diameter, sporulation rate depending on dilution titers of the Propolis alcoholic extract (Table 1). Against that, the microscopic characteristics were not considerable. Observable differences in the inhibition capacity of Propolis alcoholic extract in the same laboratory conditions may indicate the differences between physical and cellular structures. Overall contents of the cell wall of fungi include glucan, chitin, chitosan, mannan, and galactan. This structure differences are considerable among fungal genera and species. The most important difference could be observed between yeasts and molds. However, our results indicated that, in addition to the inhibitory effects of different species and genus levels, inter-species differences could be seen in response to the inhibitory effect of Propolis extract; for example, all of the isolates belonging to the same species do not show growth inhibition of *Candida albicans* in the presence of extracts (Table 2). On the other hand, we can see the same effect of the Propolis crude extract on the growth of clinical Aspergillus isolates in the level of species, thus, it can be said that the inhibitory effect of Propolis do not undergo little structural changes among the species. Fungicidal activity has been described for the inhibitory activity of phospholipase (5, 8). The nature of the inhibitory effect of Propolis extract is not completely clear some factors such as differences in physiological and structural differences within species and acquisition resistance of individual genetic and experimental errors are considered**.** As a conclusion, the ethanol extract of Propolis shows absolute inhibitory and deforming activities on the growth of Candida and Aspergillus clinical isolates respectively.

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