Original Article

In Vitro Effects of Propolis Mouthwash and Nystatin on *Candida Albicans*: A Comparative Study

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Abstract

Background & Objective: Oral candidiasis is the most common fungal infection of the oral cavity, mainly caused by the overgrowth of *Candida albicans*. One of the topical medications for candidiasis is nystatin, a polyene antifungal agent. Nevertheless, increased resistance to this drug because of overprescription has caused recurrent oral candidiasis. There is a lot of interest today in the use of natural products and compounds due to the side effects of synthetic products. Propolis is a natural resin substance produced by bees through the combination of wax and saliva with resins collected from plants, which has reportedly antibacterial, antifungal and antitumor properties. The purpose of this study was to compare the effects of propolis mouthwash and nystatin on *C. albicans.*

Materials & Methods: The present in vitro study, in which the effects of antifungal agents were investigated on the standard strain of *C. albicans*, was conducted within two groups of 15, nystatin and propolis, using the Kirby-Bauer disc diffusion susceptibility test after preparation and sterilization. The diameter of the zone of inhibition (ZOI) was measured in this assay using the disc diffusion method onto Sabouraud dextrose agar (SDA) medium.

Results: Statistical analysis of data by SPSS software using t-test statistics to evaluate the antifungal effect of the interventions showed a better antifungal effect of nystatin compared to propolis mouthwash against *C. albicans* ($P<0.001$).

Conclusion: The findings of this study demonstrated that the local propolis mouthwash was less potent than nystatin in inhibiting the growth of *C. albicans*, probably due to differences in the concentration and geographical region of collected propolis. Further research on species isolated from oral biofilm is needed to achieve complementary outcomes.

Keywords: propolis mouthwash, nystatin, *Candida albicans*

Introduction

Candida albicans is a fungus as a part of the normal microflora of the oral cavity in 20-50% of the healthy population, which is present in all surfaces of the oral mucosa. The presence of local or systemic predisposing factors causes the pathogenicity of this fungus (1).

C. albicans is known as the most important organism in the pathogenesis of oral candidiasis (2-4). Antifungals can be grouped into several classes depending on their mechanism of action, each of which affects different parts of the fungus, including cell membrane, genetics and cell divisions. Nystatin is an antibiotic of the polyene family that binds to the sterols in the fungal cell membrane, altering the membrane permeability permeability and causing the release

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of essential intracellular contents of the fungus. Nystatin is given as a topical ointment or oral form in the management of mucosal candidiasis, such as infections of the oral cavity and gastrointestinal tract, and the treatment of alopecia areata. At present, the main prescription of nystatin is in the treatment of superficial and cutaneous infections, so that the nystatin ointment is applied directly to the infected site (5). Appropriate clinical responses to treatment are affected by several factors, including the individual's immune system, the susceptibility of the pathogen to the drug, how the drug penetrates and distributes, and the acceptance of the drug by the host, and the presence or absence of an infectious agent. Opportunistic microorganisms will grow and multiply if the host conditions change and the immune systems weaken. Conditions are created for the overgrowth of opportunistic agents under the influence of factors such as the administration of broad-spectrum antibiotics, the use of invasive methods of diagnosis and treatment, immune system defects (such as AIDS and pneumonia) and the use of immunosuppressive drugs in diseases like cancers. Another parameter associated with opportunistic infections is the presence of polymorphism in some of these agents (such as the genus Candida), which are resistant to inhibitory factors (6). Fungal infections caused by *C. albicans*, especially non-albicans Candida (NAC) species, have increased in recent years. Most NAC species have shown greater resistance to antifungals and elevated mortality rates in patients with candidiasis (7). Drug resistance, or antimicrobial resistance, occurs when agents such as bacteria, viruses, parasites, and fungi change in such a way those drugs previously used to treat infections become ineffective. When microorganisms become resistant to most antimicrobials, they can cause death, transmission to others, and high costs to individuals and society (8).

Polyenes target ergosterols, the essential component of fungal cell membranes. The number of yeast species resistant to some polyenes, such as amphotericin B and nystatin, has increased recently (9, 10).

Various antifungals such as nystatin, clotrimazole, amphotericin B are commonly prescribed as the first line of treatment. However, these treatments are associated with side effects such as bitter taste, allergic reactions, adrenal insufficiency, liver necrosis, drug interactions and resistance, which potentially limit their antifungal benefits (11-14). The increasing incidence of clinical resistance to antifungal therapy and the lack of response to it in recent years emphasize the need to introduce new treatments and additional prevention and treatment candidates (15). Accordingly, there is a need to increase general knowledge of natural products; however, the use of these natural products is limited due to scattered research (16-18). Hence, today's global attitude is to reduce the administration of synthetic products and the exploitation of traditional medicine (19). Propolis is a naturally occurring resin substance produced by bees through the combination of wax and saliva with resins collected from plants (20). The propolis has been shown to have antibacterial, antifungal and antitumor properties. The composition of propolis varies in different regions due to differences in plants of various regions, which causes diverse properties of this substance (21, 22). Propolis extract contains a wide range of components such as flavonoids and phenolic acids. The flavonoids in propolis, mainly Pinocembrin, are responsible for the inhibition of Candida (23). Due to the prevalence of fungal diseases, side effects of nystatin administration and differences in propolis compounds (thus its antifungal properties) in different regions, the present in vitro study aimed to compare the antifungal effects of local propolis mouthwash and nystatin on *C. albicans.*

Materials & Methods

The current study was conducted on *C. albicans* prepared from the mycology center of Tehran University of Medical Sciences, Iran (Table 1) (Fig 1-A). Propolis-based mouthwash was native to the city of Mashhad, Iran (Soren Tech Toos, Mashhad, Iran) (Fig1-B). The effective dose of mouthwash was 4 mg/mL. The

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nystatin discs were 100 IU/mL (Padtan Teb, Tehran, Iran) (Table 2) (Fig 1-C). The culture medium was Sabouraud dextrose agar (SDA), prepared according to the instructions on the medium and then autoclaved (at a temperature of 91°C and a pressure of 121 for 15 minutes) (Fig 1-D). After cooling, the sterile medium was distributed in fifteen 10 cm plates and stored in the refrigerator. The therapeutic concentration (without dilution) of propolis mouthwash was then poured into an empty sterile plate. Next, 15 sterile blank paper discs with a diameter of 4.6 mm were taken by sterile forceps and immersed in the mouthwash-containing plate to be completely wet. After draining the excess solution, the plates were placed in an oven at a temperature of 40°C for about 20 minutes to evaporate the excess mouthwash solutions to dry the discs (24) (Fig 1-E). After cooling the medium, the fungal single colony was spread onto the medium by sterile swab under sterile conditions. The dried discs were carefully seeded onto the medium inside the plate (containing fungal culture) via sterile forceps at a distance of 10 to 15 mm from the edge of the plate and gently pressed on the agar surface thus that the whole disc was in contact with the agar. The nystatin discs with a diameter of 4.6 mm were also planted by sterile forceps carefully onto the medium inside the plate (containing fungal culture) at a distance of 10 to 15 mm from the edge of the plate and gently pressed on the agar surface until the whole disc was in contact with the agar (Fig 1-F). Incubation was performed at 37°C for 24 and 48 hours. Finally, the diameter of the zone of inhibition (ZOI) was measured in millimeters by a caliper (with an accuracy of 0.1 mm) and recorded in a pre-prepared form. The diameter of the ZOI appearing around the nystatin and propolis mouthwash discs against *C. albicans* was carefully measured under the desk lamp and compared by analysis of variance (ANOVA) test. The SDA medium was applied to isolate a single colony and the disc diffusion method to determine the susceptibility of fungus to mouthwash and nystatin (25). It is noteworthy that all the above-mentioned steps were carried out aseptically under the hood and in the vicinity of the flame (Fig 1-G).

Table 1. Studied microbial sample properties

Microbial sample name	Common culture medium	Incubation suitable temperature	Incubation time
Candida Albicans	Sabouraud dextrose agar	37º C	24 & 48 hours

Table2. Studied antimicrobial agents

Antimicrobial agent	Manufacturer	Country-city of manufacture	Product Code
Propolis Mouthwash	Soren Tech Toos	Iran, Mashhad	557310
Nystatne disc	Padtan Teb	Iran, Tehran	132464

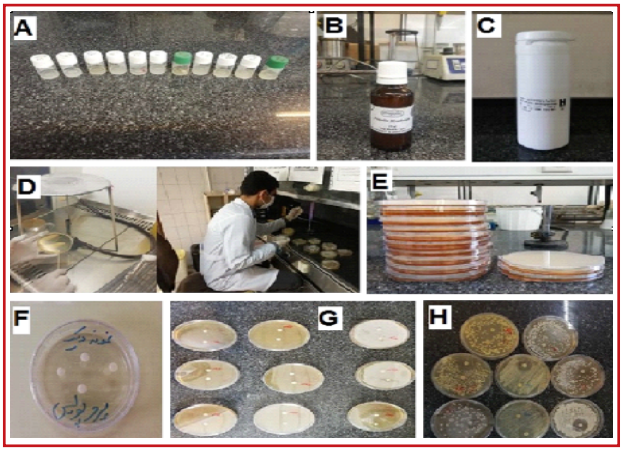

Figure 1. A) Candida Albicans. B) Propolis mouthwash. C) Nystatine discs. D) Prepared culture medium. E) Mouthwash impregnated discs. F) culture mediums containing Nystatine and Propolis. G) working under the hood and in the vicinity of the flame. H) ZOI in culture mediums

Results

In the statistical analysis of the effect of propolis mouthwash and nystatin on *C. albicans*, two different effects were evaluated: 1- the effect of propolis mouthwash on ZOI, and 2- the effect of nystatin on ZOI (Table 3) and (Fig 1-H). The method of data collection in this study was a checklist (Table 4). In data analysis, first, the normal distribution of data was investigated by Chi-square test and the data were expressed as SEM ± Mean and analyzed by unpaired t-test (P<0.05).

According to the relevant variables and the mean ZOI diameter (SEM ± Mean), the results showed that the nystatin was more effective than propolis mouthwash on *C. albicans* and the propolis mouthwash had little effect on *C. albicans* (P<0.001). (Chart 1)

The diameter of the zone of inhibition in the checklist above was measured in millimeters using the caliper.

Table 3. Descriptive Statistics of the effect of the agents on C.albicans

Anti-fungal Agent	Number	Mean	Std.Error Mean	P_value
Nystatine	15	10.53	0.375	0.000
Propolis	15	0.53	0.140	

Table 4. check list of ZOI in millimeters in each of agents

Fungus Anti-Fungal agent	*C. Albicans*					P_value
Nystatine	11	12	12.5	8.5	12	<0.01
	12	10	10.5	10.5	11	
	9	8	10	11	10	
Propolis	2	1	1	0	0.5	
	0.5	1	0.5	0	1	
	0.5	0	0.5	0	0.5	

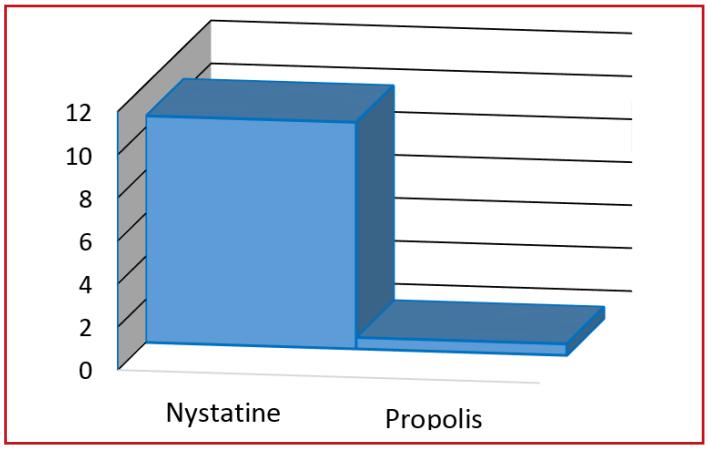

Chart 1. ZOI in millimeter in antifungal agents. (Nytatin: 10.53 mm and Propolis 0.53)

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Discussion

Oral candidiasis is the most common fungal infection of the oral cavity, so it is clear that studies are needed to find a solution to overcome this disease. Following the spread of AIDS and the high prevalence of various cancers, the incidence rate of opportunistic oral infections, such as fungal infections, has increased due to a weakened immune system, the nature of the disease itself or immunosuppressants used in treatment. Finding the right strategy to solve such problems is also necessary in these special cases. Prescribing antiseptics and disinfectants, including mouthwashes, is one of these approaches (26). Various antifungals such as nystatin, clotrimazole, amphotericin B and chlorhexidine are commonly administered as the first line of treatment for candidiasis. However, these treatments are associated with side effects such as bitter taste, allergic reactions, adrenal insufficiency, liver necrosis, drug interactions, and resistance, which potentially limit their antifungal benefits. Despite recent advances and new technologies in the fabrication of health products, there is a great deal of interest in using natural products and compounds due to the side effects of synthetic products, and recent research has focused on investigating the properties and feasibility of their widespread application (16 -18). Propolis is a natural plant-derived resin produced by bees from the flowers, pollen, branches and leaves of plants and used to repair the hive wall and protect the colony against disease (20). Propolis has long been prescribed to heal oral ulcers (27) and has been shown to have antibacterial, antifungal, antiviral, antioxidant, antitumor, and anti-inflammatory properties (28). Propolis extract contains a wide range of components such as flavonoids and phenolic acids. The flavonoids in propolis, mainly pinocembrin, account for the inhibition of Candida. These mouthwashes are better accepted by Iranian patients due to their availability in the country, low cost, acceptable taste and smell, easy use, and non-chemical nature. The fungus studied in this study is part of the microflora of the oral cavity and is involved in the occurrence of oral diseases and is found in all surfaces of the oral mucosa. *C. albicans* is known as the most important organism in the pathogenesis of candidiasis. Therefore, the aim of this in vitro study was to compare the effect of propolis mouthwash and nystatin on *C. albicans* (23).In this study, the ZOI diameter was measured using disc diffusion method onto the SDA medium. This method is based on the drug diffusion in the medium in the vicinity of the organism. In this method, the paper discs impregnated with a specific concentration of the drug were seeded onto SDA medium on which the fungal sample had grown. The results were read after incubation at 37°C for 24 and 48 hours by measuring the ZOI diameter, which is radically formed around the disc, and the susceptibility of the yeasts was evaluated. The results of disc diffusion method and ZOI diameter in this study showed that the efficacy of nystatin was higher than propolis mouthwash on *C. albicans* and propolis mouthwash had little effect on this fungus. The results of this study also revealed that the nystatin had the highest ZOI diameter, or in other words *C. albicans* exhibited the highest sensitivity to the nystatin (Table 3), which can be attributed to the concentration of commercial propolis mouthwash ($P<0.001$). According to the data table, the antifungal effect of nystatin is about 20 times higher than that of propolis mouthwash, and commercial propolis mouthwash has a limited effect on *C. albicans.* Therefore, propolis mouthwash can act against any microorganism in different ways. The difference between the ZOI diameters for nystatin and propolis mouthwash in this method and those found in other studies is probably due to differences in *C. albicans* tolerance or the origin of propolis samples, as the propolis composition depends on the regional vegetation (23). According to the studies of Silva et al. (29), and Prospero et al. (22), the differences in plants of various regions lead to diverse compositions of propolis in different regions, which causes variable properties of the substance. The lower efficiency of propolis mouthwash than nystatin in disc diffusion method on *C. albicans* can be

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due to the constant concentration of mouthwash (with the active ingredient of 4 mg/mL). Therefore, in addition to the cost, if other methods, such as minimum inhibitory concentration (MIC), were performed, fewer efficacies would be seen for this mouthwash compared to nystatin, again due to the presence of a constant concentration of active ingredient. Diba et al. reported a great mean MIC value (30) of alcoholic propolis extract on the growth of Candida and Aspergillus isolates (31). Ganjavi et al. found that the propolis extract with the MIC value of 90 and the minimum fungicidal concentration (MFC) values of 39 and 65µg/mL had the highest antifungal activity compared to other extracts studied, respectively. In addition, nystatin and amphotericin B showed better effects on laboratory fungi than the impact of all extracts studied on *C. albicans* (32). Herrer et al. found that all propolis samples inhibited Candida species; however, there were significant differences in extract concentrations, indicating inhibition of Candida species (33).

Pereira et al. documented evidence of the clinical efficacy of an alcohol-free mouthwash containing 5.0% Brazilian green propolis in the control of plaque and gingivitis (34). Silici et al. concluded that the bee products, especially propolis and pollen, could help control some fluconazole-resistant fungal species (35). According to the results of these studies, the difference in concentrations of propolis extract is one of the effective factors in determining the efficacy and inhibition of microbial species and this extract exerts its effect well at higher concentrations. Based on the studies by Santiago et al. (36), Mohan et al. (37), Acka et al. (38), the results suggest that the propolis mouthwash has stronger antifungal effects, and the reason goes back to the difference in various potential of mouthwashes and the difference in the formulation and properties of propolis studied, because the differences in the region, the season of propolis collection, its contamination with wax and bee species all lead to differences in the properties of propolis. On the other hand, the differences in the microbiological methods studied, including fungal species, cell differentiation phase, culture and time conditions, duration of drug use and study design, are other causes of differences. The present study has advantages over previous studies, such as the use of local propolis; Minimal use of synthetic compounds and alcohol in mouthwash can reduce the effects of long-term use. Many articles have emphasized that geographical area affects the properties of propolis (38, 39).

Conclusion

Considering the results of the study and less efficacy of propolis mouthwash over nystatin, it can be concluded that the treatment with propolis mouthwash can reduce oral diseases, especially fungal infections, although less than antifungals. Due to the stronger antifungal properties and far fewer side effects of herbal mouthwashes than chemical mouthwashes, they may be considered as potent antifungal agents, and since there is limited research on propolis mouthwash and its antimicrobial spectrum is unclear, the manufacturer is suggested increasing the concentration of the active ingredient to enhance the antifungal effect of this mouthwash. Further and extensive studies are needed before recommending the use of this mouthwash as an antifungal agent. According to the results of this study, it is important to note that the susceptibility of the pathogen to mouthwash should be measured in choosing an effective mouthwash to treat the infection, in addition to the type, concentration and antiseptic properties of the mouthwash, because the growth inhibitory effect depends on the type of microorganism, the type of mouthwash and the tested concentrations.

Statistically data analysis

Data were statistically analyzed by SPSS version 2 software. First, the parametric or non-parametric nature of collected data was determined by the Kolmogorov-Smirnov test. Data were reported as Mean ± SEM and analyzed statistically by unpaired t-test student test and $P<0.05$ was considered as a significance level. Finally, the logistic regression test was performed for more accurate analysis. The

project was approved by the Ethics Committee of Aja University of Medical Sciences, Iran, under the code of ethics of IR.AJAUMS. REC.1399.023.

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Conflict of Interest

Authors declare no conflicts of interests associated with the publication of this article.

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