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The Comparison of Antifungal Effects of Methylene Chloride and Methanol Extracts of Green and Black Tea on Candida Albicans

M. Ashrafpour (PhD)[1], A.R. Ghorbani (DDS)[2], A.A. Sefidgar(PhD)[3], H.H. Kazemi (DDS)[4], A.A. Moghadamnia(PhD)[1], S. Kazemi (MSc)[1], M. Mazleghani (BSc)[3], M. Baradaran (PhD)*[1]

1. Cellular and Molecular Research Center, Babol University of Medical Sciences, Babol, I.R.Iran
2. Student Research Committee, Babol University of Medical Sciences, Babol, I.R.Iran
3. Department of Mycology and Parasitology, Babol University of Medical Sciences, Babol, I.R.Iran
4. Department of Oral Diagnosis, Faculty of Dentistry, Babol University of Medical Sciences, Babol, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: Background: Candidiasis is the most common fungal infection. Nystatin is often used to treating of candidiasis that creates the problem of drug resistance and side effects. Study was performed to determine the antifungal properties of black and green tea extracts against Candida albicans.

METHODS: To do this basic study, at first methylene chloride and methanolic extracts of green and black tea were prepared. Disk diffusion method and measuring the diameter of inhibition zone was used to determine anti-fungal extracts against Candida albicans. The methanolic extract at doses of 2, 4, 6, 8 and 10 mg and methylene chloride extracts with concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg per disk were used and the results compared in 24 and 48 hours.

FINDINGS: Methylene chloride extracts of both type of tea create antifungal activity more than methanol extracts. The maximum antifungal activity 24 hours by a concentration of 1 mg hard methylene chloride extract was obtained and in this concentration with formation of zone inhibition 30.57±4.4 and 34.25±2.7 mm, respectively, for the methylene chloride extract of green tea and black more antifungal effect compared to Nystatin (20±1.06 mm) was established ($p<0.01$).

CONCLUSION: The results have shown that green and black tea leaf extract can create antifungal activity effects against Candida albicans as dose -dependent manner and is more effective in the first 24 hours than the 48 hours.

KEY WORDS: *Candida Albicans, Black tea, Green tea, Methanolic extract, methylene chloride extract*.

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* **Corresponding author: M. Baradaran (PhD)**
Address: Department of Physiology and Pharmacology, Babol University of Medical Sciences, Babol, I.R.Iran
Tel: +98 11 32229591
E-mail: baradaran81@yahoo.com

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Introduction

Candidiasis is the main form of oral fungal infections caused by eight *Candida* species, but *Candida albicans* is the most common causative agent (1). *C. albicans* is a fungus that causes severe opportunistic infections in humans. The fungus colonizes on the surface of the mucous membranes of the mouth and genital or gastrointestinal tract and causes a range of infections in the host depending on the underlying defects. For various reasons, *Candida* infections have increased dramatically in the past two decades (2). In addition, fungal contamination in biofilms is a common problem in patients with valves or adjunctive therapeutic appliances inside their body. *C. albicans* is the most common cause of biofilm infections, and about 40% of patients with intravenous catheters are suffering from a fungal infection that leads to patient mortality in 41% of cases (3). Studies have shown that some plants inhibit mycotoxin-produced fungi. These plants show pharmacological effects through production of a wide range of secondary metabolites such as alkaloids, tannins, glycosides, saponins, flavonoids, and phenolic compounds, and they act as important sources of antimicrobial and antifungal compounds (4, 5).

Use of synthetic drugs is associated with problems such as limited access and high costs. Additionally, synthetic antifungal agents cannot be used to protect food storage due to their toxicity and contamination of the environment. Therefore, it is necessary to identify and introduce alternatives to synthetic antifungal agents. In this regard, studies show that plant extracts have important antibacterial and antifungal properties. Natural antifungal agents, in addition to lower adverse effects on the environment and human health, can be a good alternative to synthetic antifungal agents (6). Clinically, compared to antimicrobial compounds, there is limited access to antifungal drugs. During the previous years, incidence of fungal infections has increased due to immune system suppressive treatments administered for organ transplantation, cancer, or AIDS; this issue, along with antibiotic resistance and toxicity caused by long-term treatment with antifungal agents, necessitate the study of traditional plants to identify new antifungal compounds. Tea is the most important plant of *Camellia* genus, which is known as the second most consumed drink around the globe (7).

In addition to antioxidant and antibacterial effects of tea, recent studies have unmasked the beneficial effects for down-regulation of cholesterol and blood pressure and protection against cardiovascular diseases and cancer (8). Tea contains polyphenols, caffeine, flavonols (quercetin and rutin Camphor), theanine, and aromatic compounds (9, 10). Polyphenols, especially catechin and phenolic acid, play an important role in human health. Catechin in tea is found in various forms including epigallocatechin gallate (EGCG),-epigallocatechin (EGC), -epicatechin gallate (ECG), and -epicatechin, (EC) (11).

EGCG and theaflavins in tea have exhibited antifungal activity against several fungi at different concentrations. The results of some studies have shown that tea catechins have antifungal properties (mostly pH-dependent) against *C. albicans*. In addition, EGCG increases the antifungal activity of amphoterecin-B, and combination therapy with EGCG and fluconazole reduces the resistance of *C. albicans* to fluconazole (12). Due to different processing methods, green and black tea contain different types of active compounds; thus, this study aimed to compare the antifungal effects of methylene chloride and methanol extracts of green and black tea against *C. albicans.*

Methods

In this experimental study, we used the S.Ca-PTCC-265T species of *C. albicans*, which were prepared from Scientific-Industrial Research Center of Iran. In this study, green and black tea were high-quality and free of any essence or additives and were purchased from the Herbatious Sciences Research Center of Red Crescent in Salmanshahr. To obtain the alcoholic extract, maceration method was applied; such that 20 g of each type of tea was separately soaked in 200 ml of 97% methanol for 48 hours. After separating the residue, the resulting solution was passed through a paper filter and then was dried under a hood to obtain dried alcoholic extracts of black and green tea. A 100 mg/ml solution was prepared from dried extract by 97% methanol.

Then, the resulting solution was used to evaluate the antifungal activity at doses of 2, 4, 6, 8, and 10 mg per disc. Similarly, to prepare a methylene chloride extract of green and black tea, 20 g of the samples was boiled gently in 500 ml of distilled water for 20 min. After cooling, it was passed through paper filter. The resulting solution from black and green tea was separately shaken inside a funnel with 70 ml of

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methylene chloride for 5 min. At the end of this phase, two layers were formed in the solution, with the lower one containing the dissolved compounds in methylene chloride. Afterwards, by opening the stopcock of the separating funnel, the lower layer was poured into a separate container. This process was repeated twice for the remaining solution inside the separating funnel. Then, the obtained solutions were centrifuged using Clements 2000 at 3000 rpm for 15 min. To obtain dried methylene chloride extract, the resulting clear solution was dried under the hood for 24 h (13).

The dried extracts were dissolved using 97% methanol at a concentration of 10 mg/ml, and to study the solution, we used doses of 0.2, 0.4, 0.6, 0.8, and 1 mg per each disc. In order to compare the antifungal effects of different concentrations of methanol and methylene chloride extracts, 100 units of nystatin was used as a positive control.

Disc diffusion method was used to evaluate the antifungal properties of different concentrations of green and black tea extracts on standard strains of *C. albicans* (14). To this end, the discs were placed on a container with closed lid and were autoclaved for 15 min at 121°C. Then, a suspension of *C. albicans* was prepared in liquid BHI medium, the turbidity of which was equivalent to 0.5 McFarland. Then, by using a sterile swab, the suspension was extended in contact with surface of the plate containing Sabouraud Dextrose Agar medium and the discs were placed within the plates.

The blank disc and methanol were used as negative control. All the plates were kept at 37°C, and 24 and 48 hours later, by using a ruler, the diameters of growth inhibition zones around the discs were measured as an indicator of antifungal activity. To analyze the data, t-test and One-way ANOVA tests were run, using SPSS version 22. $P<0.05$ was considered statistically significant.

Results

The current results showed acceptable antifungal activity against *Candida* for extracts of green and black tea leaves. Figure 1 (Diagram A and Image B) demonstrate the comparison of the antifungal effect of different concentrations of methylene chloride extracts of black and green tea against *C. albicans* at doses of 0.2, 0.4, 0.6, 0.8, and 1 mg per disc, based on diameter of inhibition zone at 24 hours using the serial dilution method. Methylene chloride extracts of both green and black tea caused comparable antifungal effects with 100 units of nystatin against *C. albicans* (fig 1). At minimum dose of the two extracts (0.2 mg), there was a significant antifungal effect; however, the difference between the extracts and nystatin was not significant in this regard ($p<0.05$).

With increasing the concentrations of both extracts, antifungal activity increased in a dose-dependent manner, which was significantly higher at 0.8 and 1 mg doses of ($p<0.01$ and $p<0.001$, respectively) of green tea, and at 0.6, 0.8, and 1 mg ($p<0.001$) doses of black tea compared to nystatin. The maximum antifungal activity of methylene chloride extracts of green and black tea was obtained at a concentartion of 1 mg per disc, where diameter of inhibition zones of methylene chloride extract of green and black tea were 30.57±4.4 mm and 34.25±2.7 mm, respectively, and stronger higher properties were observed compared to nystatin (20±1.06 mm).

Although at all concentrations, black tea had higher antifungal effect, but this effect was not significant, and a significant difference was observed only at 1 mg ($p<0.01$). It should be noted that in the negative control or blank samples, no inhibition zone was formed. Thus, the comparison of antifungal effects of different concentrations of the extracts with negative control or blank samples was not necessary. Figure 2 (Diagram A and Image B) shows the antifungal effects of methanol extracts of green and black tea at concentrations of 2, 4, 6, 8, and 10 mg doses per disc and 100 units of nystatin on *C. albicans* at the first 24 hours. At all the concentrations, both extracts had antifungal effects. The antifungal effects of different doses of methanol extract of green tea were similar to those of 100 units of nystatin, and no significant difference was observed between nystatin and different concentrations of green tea extract.

As can be noted in Figure 2, the diameter of inhibition zone showed no significant variation with increasing concentration of methanol extract of green tea. Almost at all concentrations, methanol extract of black tea showed higher antifungal effect on *Candida albicans* compared to green tea. The methanol extract of black tea at concentrations of 8 mg and 10 mg per disc showed higher antifungal effects.

At these concentrations, there was a significant difference between 100 units of nystatin and methanol extract of black tea (with inhibition zones of 24.87±2.08 mm and 27.31±1.06 mm, respectively) with regard to antifungal properties ($p<0.001$).

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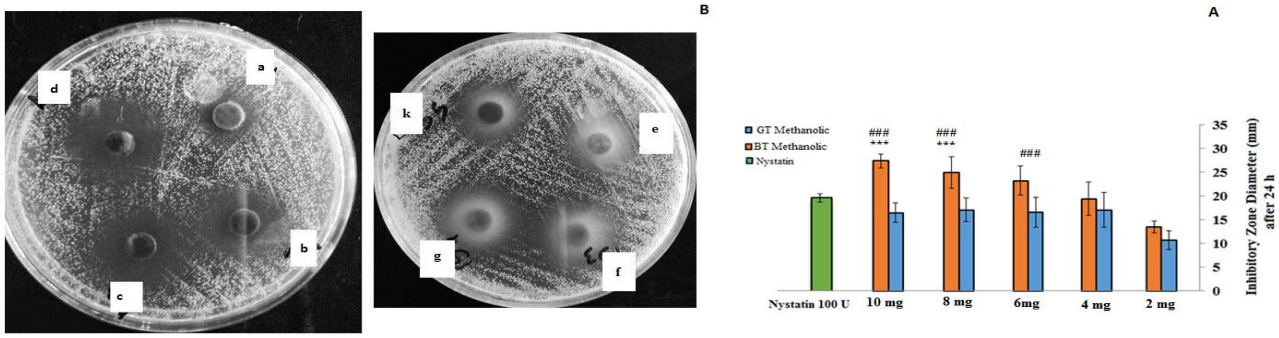


Figure 1. Diagram (A) and Image (B) show the average diameter of inhibition zone of various concentrations of methylene chloride extract of green and black tea leaves against *Candida albicans* after 24 hours. The values of diagram are mean±SD. The number of tests for each concentration was 7. 100 units of nystatin is considered as positive control. * shows significant difference between nystatin and tea extract and # shows significant difference between green and black tea extracts. ** and ## show significance at p<0.01 and * at p<0.001. a and c show 1 mg per disc dose of black and green tea, respectively, and b represents disc containing 100 units of nystatin.**

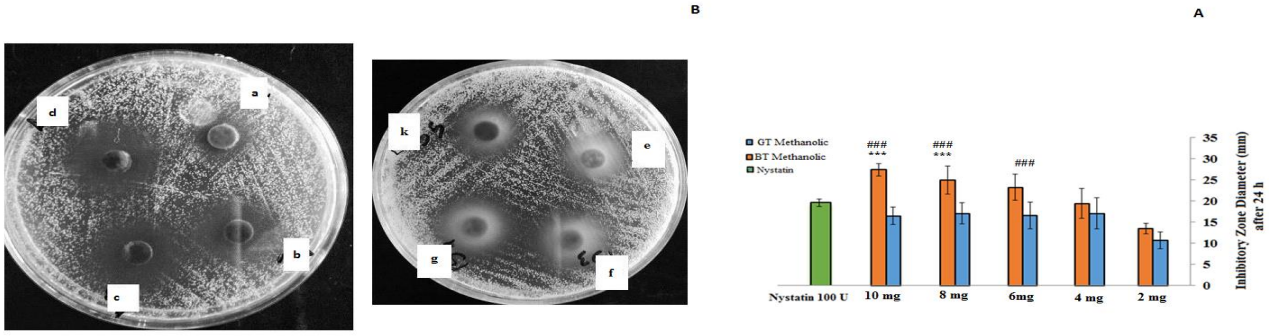


Figure 2. Diagram A and Image B show the average diameter of growth inhibition zones of green and black tea leaf extracts against *Candida albicans* at different concentrations at 24 hours. The values of diagram are mean±SD, and 100 units of nystatin is considered as positive control. * represents significant difference between nystatin and tea and # shows significant difference between extracts of green and black tea. ### and * demonstrate significance level at P<0.001. a, b, c, and d discs contain 4, 6, 8, and 10 mg concentrations of methanol extract of black tea, respectively, and e, f, g, and k are the discs containing of 4, 6, 8 and 10 mg concentrations of methanol extract of green tea, respectively**

However, there was not a statistically significant difference between nystatin and 2, 4, and 6 mg concentrations compared to methanol extract of black tea, indicating the comparable and acceptable antifungal activity of this extract. The comparison of methanol extracts of black and green tea (fig 2) showed that the difference between mean diameters of inhibition zones of the two types of extracts was not significant at 2 and 4 mg concentrations. However, 6, 8, and 10 mg per disc concentrations of methanol extract of black tea showed stronger antifungal activity than methanol extract of green tea, as it created inhibition zones with diameters of 23.18±3.1, 24.87±2.53 and 27.31±2.08 mm, respectively (p<0.001). Our results concerning the antifungal activity of methylene chloride extract of green and black tea at concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg per disc against *C. albicans* at 48 hours are demonstrated in Figure 3. The mean diameter of inhibition zone caused by 100 units of nystatin, as positive control, was 14.12±0.83 mm at 48 hours. Diameter of inhibition zone at 0.2 and 0.4 mg concentrations of methylene chloride of both types of tea was low, but 0.6, 0.8, and 1 mg doses of both types of tea had high antifungal effects. There was not a significant difference between antifungal properties of these concentrations of methylene chloride extracts of green and black tea and 100 units of nystatin (p<0.05). The comparison of methylene chloride extracts of green and black tea at 48 hours at concentrations of 0.6, 0.8, and 1 mg per disc exhibited that the average diameter of inhibition zone for the two extracts were similar, and no statistically significant differences were observed (fig 3). Figure 4 (Diagram A and Image B) exhibits the effects of methanol extracts of green and black tea at concentrations of 2, 4, 6, 8, and 10 mg per disc against *C. albicans* at 48 hours. The methanol extracts of green and black tea at concentration of 2

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mg lacks antifungal properties, as the inhibition zone was low (near zero mm). However, other doses showed significant antifungal activity. Antifungal properties of methanol extract of black tea are dose-dependent. The average diameter of inhibition zone at concentration of 4 mg of methanol extract of black tea was 12.1±1.8 mm, which increased to 19.25±2.5 mm by elevating the concentration up to 10 mg; the difference was significant compared to 4 mg concentration of this extract and100 units of nystatin ($p<0.001$).

Although methanol extract of green tea at 4, 6, 8, and 10 mg concentrations could show significant antifungal properties, but no significant difference was observed in antifungal activity with increasing concentration of the extract. At all the concentrations, methanol extract of green tea showed lower antifungal properties compared to 100 units of nystatin. Figure 4 shows the comparison of antifungal properties of methanol extracts of green and black tea at 48 hours. Although the 4 mg concentration of the two extracts showed equal antifungal activity with regard to the created inhibition zone, the antifungal effects of methanol extract of black tea was stronger than those of green tea at doses of 6, 8, and 10 mg. The average diameter of inhibition zone obtained from methanol extract of black tea at concentrations of 6, 8, and 10 mg were 14.25±1.9, 17.42±2.07, and 19.25±2.5 mm, respectively, which was significantly different from methanol extract of green tea with average inhibition zones of 9.58±1.06, 10.25±0.7, and 11.42±2.1 mm, respectively ($p<0.001$).

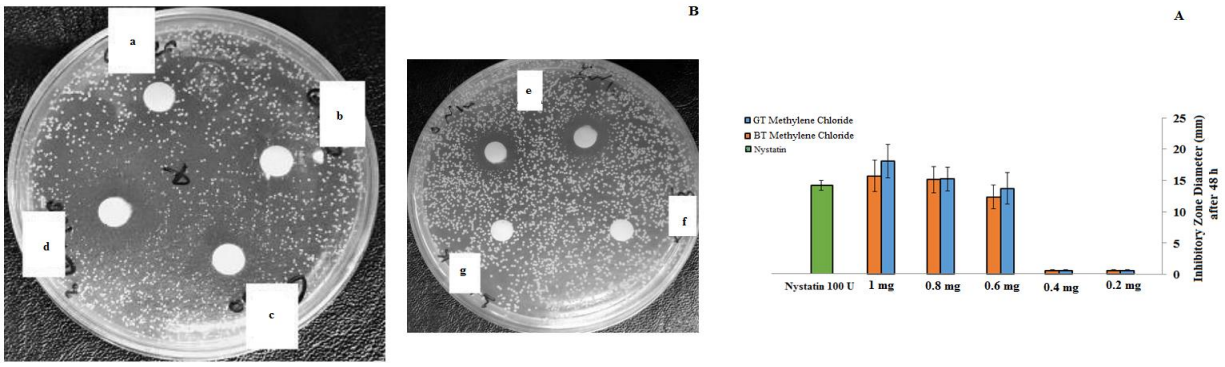


Figure 3. Diagram (A) and Image (B) show the average diameter of the inhibition zone of various concentrations of methylene chloride extract of green and black tea against *Candida albicans* at 48 hours. Values are mean±SD. The number of tests for each concentration was 7, and 100 units of nystatin was considered as positive control; a=0.4 mg of black tea, b=0.4 mg of green tea, c=1 mg of black tea, d=1 mg of green tea, e=100 units of nystatin, f=disk containing solvents, and g=blank disc

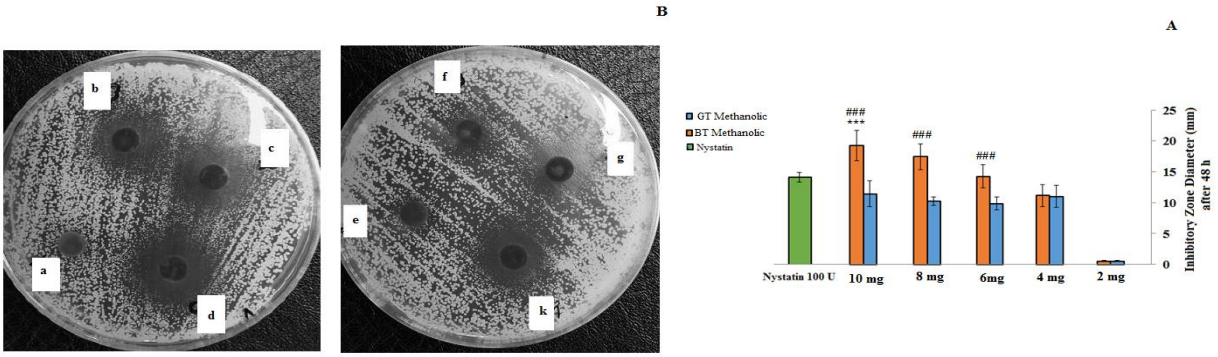


Figure 4. Diagram (A) and Image (B) show the average diameter of the inhibition zones of various concentrations of methanol extracts of green tea and black tea against *Candida albicans* at 48 hours. Values are mean±SD. The number of tests for each concentration was 7. * shows significant difference between nystatin and tea extract, # demonstrates significant difference between green and black tea extracts; ### * indicates significance at the P<0.001 level. a, b, c, and d pertain to disk containing 4, 6, 8, and 10 mg of methanol extract of black tea, respectively, while e, f, g, and k, respectively, show the disks containing 4, 6, 8, and 10 mg of methanol extract of green tea**

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Discussion

Methylene chloride and methanol extracts of green and black tea manifested acceptable antifungal effects at all the concentrations at the first 24 hours compared to nystatin. These results signify the importance of this drink in the prevention and treatment of fungal infections. Moreover, it can be concluded that methylene chloride extracts of both types of tea were more effective, although they were used at a concentration equal to 10% of methanol extract.

The maximum antifungal effects of methylene chloride extract of black tea were achieved at a concentration of 1 mg per disc. This finding implies the importance of extract concentration in antifungal effects. Based on the obtained results, it can be argued that both green and black tea leaves contain active ingredients that can prevent the growth of *C. albicans*, but probably the amount of active ingredients of green and black tea depends on the method of processing their leaves. In addition, the type of solvent used is important in extraction of active ingredients. In this regard, our findings indicate that the quality of natural extracts and their effectiveness depend on not only duration of storage and maintenance, but also the environment and the type of solvent used (16).

Based on our outcomes, it seems that methylene chloride solvent had a better performance in the extraction of active antifungal ingredients of tea. According to phytochemical screening studies, alkaloids, saponins, tannins, catechins, and polyphenols in tea leaves (17) have antimicrobial properties. EGCG in tea can also have antimicrobial activity (18). The current findings confirm the antifungal effects of green and black tea. Moreover, the abovementioned active ingredients of green and black tea contain high amounts of catechin and theaflavin, which provide cumulative and synergistic bioactive effects (19). Methylene chloride can extract higher amounts and possibly a greater variety of active ingredients from tea leaves; thus, stronger antifungal activity can be achieved through the collective impact of these substances. The methanol extract of green tea at all concentrations had the same inhibitory effect on *C. albicans*, while methylene chloride extract of green tea showed a dose-dependent antifungal property.

It may be concluded that by using methylene chloride solvent, higher amounts and more types of antifungal agents can be extracted from green tea leaves due to differences in polarity. By considering the antimicrobial and antifungal effects of green and black tea extracts, conflicting results have been reported so far. Some studies have emphasized on higher antifungal effects of green tea, while some others have reported higher antimicrobial activity for black tea. The findings of a study revealed that aqueous solution of green tea has higher inhibitory effects on *Staphylococcus aureus* resistant to methicillin compared to black tea. (20), but in contrast, other studies have demonstrated that black tea extract has greater antimicrobial effects than green tea on gut flora (21). In another study, black tea was shown as a useful agent acting against pathogenic intestinal bacteria such as *Salmonella typhi* and *Vibrio cholerae* (22). Similarly, our findings showed that black tea has higher inhibitory effects on the growth of the *C. albicans* than green tea, which could be attributed to the presence of higher levels of caffeine in black tea. The amount of caffeine in black tea is reported to be 2-3 times more than green tea. Caffeine is an important component of black and green tea that can be associated with antimicrobial and inhibitory effects (10,23). It is known that caffeine affects through inhibition of the required enzymes of DNA synthesis (24). The higher antifungal activity of black tea, compared to green tea, might be due to chemical instability of the active ingredients of green tea. Therefore, some of the antifungal activities of green tea might be impaired in-vitro. Green tea contains high levels of catechin and polyphenol that can have antimicrobial effects, while they are highly vulnerable to oxidation (25).

Green tea is not fermented while being processed, which helps maintain its enzymes. Whereas, black tea undergoes fermentation before withering, which impairs some of its active compounds (20, 26). Thus, black tea is expected to lose higher amounts of its active ingredients while being processed, which in turn, leads to its lower antifungal properties. Nevertheless, green tea showed lower antifungal properties in the present study, which might be due to its lower solubility while preparing a solution to obtain its different concentrations, this problem was evident while dissolving the green tea extract.

Comparison of findings at 24 and 48 hours showed that antifungal effects of both extracts decreased over time, which might be due to instability or short-term effects of their active compounds. Some of the pharmacological properties of the extracts might be destroyed due to the instability of the chemical compounds in the long term or in-vitro conditions, or it

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might be associated with short half-life of these compounds. However, in humans, especially among people referring for dental care, *C. albicans* is a common fungal infection that can cause many problems for those who are involved. Therefore, in addition to compliance with the provisions and principles of dental care to prevent vertical transmission of infections, we can use effective methods for extraction of natural antifungal agents and production of antifungal solutions using suitable solvents to prevent and manage candidiasis.

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