

Antifungal Activity of Caffeine in Combination with Fluconazole against *Candida albicans*

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Background: Iranian (Lahijan) black tea caffeine has been previously shown to have antifungal activity against *Candida albicans*. The aim of this study was to investigate whether the combination of caffeine and fluconazole (FLU) has an effective antifungal activity on a FLU-resistant (MIC >64mgL⁻¹) *C. albicans* PTCC5027.

Materials and Methods: Caffeine from Lahijan black tea was extracted and its pharmacological effects against 20 clinical isolates of FLU-sensitive and resistant *C. albicans* was evaluated by Colony Forming Units (CFU) method. Furthermore, the synergistic effect of caffeine and FLU against PTCC-5027 strain was investigated.

Results: Our results indicate the antifungal efficacy of Lahijan black tea caffeine on *C. albicans* isolates and subsequent identification of caffeine in combination with FLU against PTCC-5027 strain. The concentrations of caffeine causing 90% growth inhibition (MIC₉₀) for PTCC-5027 strain, FLU-resistant and -sensitive *C. albicans* isolates were 25mgL⁻¹, 24.4mgL⁻¹ and 37.2mgL⁻¹, respectively. The combination of caffeine with FLU showed stronger antifungal activity against *C. albicans* PTCC5027. The addition of 12.5mgL⁻¹ caffeine to FLU 10-50 mgL⁻¹ (below MIC₉₀) inhibited the growth of *C. albicans* PTCC5027 by 99.3%–99.7%, the concentrations at which neither caffeine nor FLU alone affected the growth.

Conclusion: It can be concluded that caffeine has antifungal effect on *C. albicans* and in combination with FLU can enhance the antifungal activity of FLU against *C. albicans*. The synergism of the combination of caffeine and FLU induces multiple antifungal effects, resulting in the use of lower doses of the FLU. It suggests that this can decrease the side effects of antifungal drugs.

Keywords: Caffeine, fluconazole, antifungal, *Candida albicans*

1. Background

Candida albicans as a part of microbial flora in human beings is a unique opportunistic pathogen causing candidiasis in individuals with an underlying deficient state. Oropharyngeal candidiasis as an initial indicator is common in patients suffering from AIDS. In cancerous patients undergoing chemotherapy or in cases receiving broad-spectrum antibiotics, *C. albicans* causes systemic candidiasis (1).

The widespread emergence of *Candida* infections, paralleling the increasing numbers of immunocompromised patients, can be serious, often resulting in death (2). Azole drugs have emerged as the important drugs used in the treatment of candidal infections. FLU, the most widely used azole in both the treatment and prevention of candidiasis, targets the essential enzyme Erg11, lanosterol 14 α -demethylase, in the ergosterol biosynthetic pathway (3).

However, in recent years, the prevalence of FLU-resistant *C. albicans* has increased due to the prolonged use of FLU (4). One selective pressure contributing to the emergence of FLU-resistance is its fungistatic (inhibiting fungal growth) rather than fungicidal (killing fungi) character (5, 6).

Tea (*Camellia sinensis*) as a natural substance is one of the most popular beverages in the world especially in Asia. Furthermore, tea has excellent pharmaceutical effects; its plants are widely cultivated in Southeast Asia, central African countries and in the northern part of Iran, especially in Lahijan which is distinguished as the tea capital of Iran (7, 8).

Over hundred types of tea are now produced that are generally categorized into three main types (green, oolong and black) differing in processing methods. Health benefits of tea are associated with the presence of polyphenols and caffeine

(9-11). Green tea has more polyphenols and black tea has higher amount of caffeine because of longer fermentation time during processing (12).

Caffeine is an alkaloid that makes tea and coffee valuable to humans. Alkaloids are a class of natural compounds including nitrogen with properties of an organic amine base. Caffeine is mainly acquired from the seeds of the coffee plant, cola nuts and tea leaves (*Camellia sinensis*) (13). In the present study, we focused on the antifungal activity of caffeine. Choosing a method for extraction of a metabolite from its natural source depends on both the properties of the compound and the composition of the source. Since caffeine is soluble in both water and organic solvents, it is possible to extract caffeine from black tea by solid/liquid extraction of hot water. Properties of caffeine imply that it can be separated by water and chloroform. Thus, an effective and safe non-synthetic antifungal agent may be necessary to use for a wide range of *C. albicans*. This aim can be achieved by using combination therapy.

2. Objectives

In this study, we evaluated the antifungal effects of Lahijan black tea caffeine on *C. albicans* alone and in combination with FLU.

3. Materials and Methods

3.1. Extraction of caffeine from Lahijan black tea

To extract caffeine from Lahijan black tea, polar- nonpolar solvent extraction technique was used. As the caffeine is soluble in water (25mgmL⁻¹ at 25°C and 700mgmL⁻¹ at

100°C), 15 grams Lahijan black tea were placed in 300mL distilled water and was boiled for 20 min while stirring, thereafter, the mixture were cooled for 15min. Then, in order to remove all solid particles, vacuum filtration through a Buchner funnel with Watman grade No.1 filter paper was done. Chloroform (100mL) as an organic solvent was added to the filtered solution. As chloroform is insoluble in water and denser, it was allowed to settle to the bottom. Because caffeine is higher soluble in chloroform (150mgmL⁻¹ at 25°C) the chloroform layer was carefully drained into a flask. The chloroform/caffeine solution was filtered using vacuum to filter the chloroform through and trap any water and residue. The organic layer was transferred to a flask. The chloroform solution was placed over the boiling water using a hot water bath at 60°C. The solution was then evaporated and removed from the heat. This process was repeated to evaporate the solution until the concentrated caffeine was obtained. The dry flask was weighted to find the “crude” caffeine weight (14).

3.2. Antifungal activity of caffeine against *C. albicans*

Broth dilution method and calculation of Colony Forming Unit (CFU) was used to examine the susceptibility of *C. albicans* to caffeine. The experimental suspension was prepared by twofold dilution of 200 mgL⁻¹ caffeine with RPMI medium 1640 (Gibco) with shaking (150 strokes/min). The pre-cultured *C. albicans* of 10³ CFUmL⁻¹ was inoculated into 1mL of the media at various concentrations of caffeine. After cultures were shaken at 37°C for 48h, the aliquots of 10-fold dilutions were spread on Sabouraud dextrose agar (SDA) plates in triplicate and were incubated at 37°C for 48h to calculate CFU. The minimum concentration that inhibited the growth of 90% of *C. albicans*, compared with the caffeine-free plates, was accepted as the MIC₉₀ (15). MFC was defined as the minimum fungicidal concentration for the lowest concentration resulting 99.9% or more death of *C. albicans*.

To determine MFCs, the aliquots (0.1mL of the samples) were inoculated on SDA plates in triplicate and incubated at 37°C for 48 h and the CFU were then counted (16-18).

3.3. Measurement of the combined effects of caffeine and FLU

Evaluation of synergistic effects of caffeine and FLU against *C. albicans* PTCC5027 was carried out in a similar manner to that described for the MIC of caffeine alone. Caffeine and FLU were prepared at 12.5-6.25 and 50-10mgL⁻¹ in RPMI respectively. The *C. albicans* (10⁶ CFUmL⁻¹) was adjusted to 10³ CFUmL⁻¹ with RPMI using the 10-fold dilution method for inoculation. One milliliter of these diluted of *C. albicans* was added to 1mL of the mixtures of different concentrations of caffeine and FLU solutions in RPMI. After incubation at 37°C for 48 h with shaking, the cultures were spread on plates in triplicate to calculate the CFU. The percentage inhibition of the growth of *C. albicans* was recorded from the CFU compared with that of caffeine or FLU-free control cultures.

3.4. Statistical analysis

Data were analyzed statistically by calculating means and standard deviation of the means. Student's t-test was used to evaluate the differences in $P < 0.05$ significant level.

4. Results

4.1. Measurement of MIC of caffeine

The MIC₉₀ and MFC of caffeine for 20 clinical isolate of FLU-sensitive and resistant *C. albicans* and PTCC- 5027 strain are shown in Table 1.

Table 1. MIC₉₀ and MFC of caffeine for *C. albicans*.

MIC ₉₀ and MFC (mg/L)		
Clinical isolates of <i>C. albicans</i>	MIC ₉₀	MFC
PTCC-5027	(1) 25	200-100
FLU-resistant isolates	(10) 24.4	200-100
FLU-sensitive isolates	(10) 37.2	300-150

The antifungal effect of caffeine was dependent on the resistance state of isolates to FLU. The MIC₉₀ of caffeine against PTCC-5027 strain was 25mgL⁻¹, nearly similar to the one for FLU-resistant isolates showed the mean of 24.4mgL⁻¹ but the one for the FLU-sensitive isolates was 37.2mgL⁻¹. However the MFC of caffeine against the FLU-resistant isolates and PTCC-5027 strain were 100-200mgL⁻¹ and for FLU-sensitive isolates were 150-300mgL⁻¹. The *C. albicans* PTCC-5027 as a FLU-resistant strain as well as other FLU-resistant isolates were more susceptible to caffeine than the FLU-sensitive ones. The MIC₉₀ of FLU for strain PTCC5027 was 100mgL⁻¹ (data not shown).

4.2. Effect of caffeine on the antifungal activity of FLU

The addition of caffeine to FLU resulted in the enhancement of the antifungal activity of FLU against *C. albicans* PTCC-5027 (Figure 1). FLU at 10 and 50 mgL⁻¹ in the presence of 6.25 mgL⁻¹ caffeine caused, 91.8% and 98.9% inhibition of the growth of PTCC5027 strain, respectively. Stronger growth inhibition was obtained with 12.5mgL⁻¹ caffeine. The effect of the combined use of 10-50mgL⁻¹ FLU and 12.5mgL⁻¹ caffeine was observed to be 99.3% and 99.7% respectively compared with FLU and caffeine alone controls, considering no inhibitory effect of less than FLU 50mgL⁻¹ on PTCC5027 strain.

To confirm the multiple effects of combination therapy with caffeine and FLU, similar tests were performed on FLU-sensitive *C. albicans* isolates using 25mgL⁻¹ caffeine and 0.1-0.2mgL⁻¹ FLU, concentrations at which no FLU alone affected the growth. This combination therapy could inhibit the growth from 98.0% to 99.9% compared with FLU-free growth (data not shown).

5. Discussion

Recently natural products such as herbal extracts were reported to have antimicrobial activity (19, 20). Caffeine has been considered as an antibacterial agent against pathogenic bacteria (21-23). Regarding studies in fungi, the reports of Okubo and colleagues (1991) showed that 2.5% of black tea extract inhibit the growth of filamentous fungi (24) and at more than 10% concentration, inhibit the growth of *C. albicans* (25). One of the main antifungal compounds of black tea extract is caffeine. A normal cup of black tea has a concentration of 30-75mg caffeine, which is the highest amount of caffeine in beverages after coffee (80-125 mg/cup). The caffeine in extracts from green tea is much lesser because of the low fermentation period in green tea processing time (26). Caffeine has also been reported to be involved in antimicrobial acting against *E. coli* acting on DNA and protein synthesis of *E. coli* (27).

In the present study, we found that caffeine against various isolates of *C. albicans* was fungicidal against FLU-sensitive and resistant *C. albicans* isolates slightly stronger in FLU-resistant ones (Table 1). The data also suggests that the antifungal action of caffeine was enhanced by antifungal drug.

Studies of the antifungal activity of caffeine against *Saccharomyces cerevisiae* showed results similar to those against *C. albicans*. the reports indicate that caffeine can enhance the antibacterial effect of some agents such as penic-

illin and tetracycline against *Staphylococcus aureus* (28) and rapamycin against budding yeasts (29). Reinke and colleagues (2006) reported that the mechanism of the fungicidal effects of caffeine involved causing damage to the cytoplasmic membrane of budding yeasts (28).

The current study revealed the synergistic antifungal activity of the combination of caffeine and FLU against *C. albicans*. FLU has antifungal activity against *C. albicans* with strong side effects, even at low doses (30). The combination of caffeine and FLU was tried in an attempt to reduce the effective dose of FLU by using FLU at below MIC, the action resulting fungicidal more than fungistatic.

Since the azoles came on the market as antifungal drugs (31), FLU-resistant *C. albicans* appeared (32). The use of caffeine alone and in combination with FLU was effective against FLU-resistant *C. albicans*. The effective dose of FLU on the growth of PTCC-5027 strain was decreased up to one-tenth (10mgL^{-1}) using 12.5mgL^{-1} caffeine compared with the growth in the presence of FLU alone (Figure 1). The MIC₉₀ of FLU for PTCC-5027 strain was 100mgL^{-1} (data not shown).

C. albicans associated with high FLU resistance, expresses multidrug efflux transporter (MET), which mediates the efflux of a broad range of compounds, including FLU. Cyclosporine, MET inhibitor and FLU showed a significant synergistic effect against *C. albicans* (17). The mechanism of the synergistic effect of the combination of FLU and caffeine is still unknown. The addition of caffeine to FLU induces higher antifungal activity via possible stimulation of multiple functions (Figure 1). The obtained results suggest that FLU-caffeine combination therapy can be better than either FLU or caffeine alone and can produce better treatment outcomes.

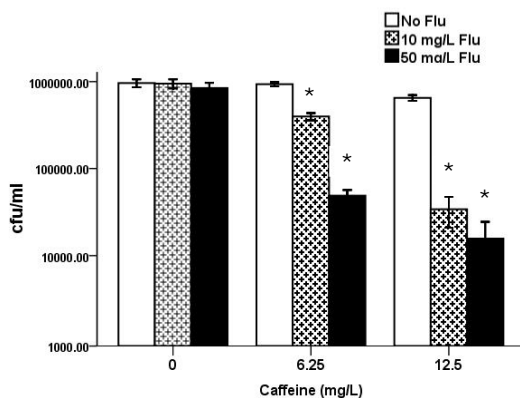


Figure 1. The effect of the combination of FLU with caffeine on the growth of *C. albicans* PTCC5027.

*Values differ significantly ($P < 0.01$) from values without Caffeine.

6. Conclusion

Caffeine combined with FLU and perhaps other antifungal agents may be beneficial as effective in the treatment of candidiasis, such as thrush and intestinal candidiasis. However, in vivo experiments would need to test these possibilities in animals or humans. Available data show a promise for therapy of FLU-resistant candidiasis. However, further studies are required to explore the mechanism of antifungal effect of caffeine in order to develop novel drug candidates.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contributions

Zahra Nasrollahi performed all the laboratory work and Mohammad Hossein Yadegari supervised the process.

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