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Original Article

Drug interaction of traditional Chinese medicines with fluconazole against fluconazole resistant strains of *Candida albicans*

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Abstract: Introduction: Wide use of fluconazole has led to the development of its resistant strains, which necessitates the introduction of agents that can be used in combination with fluconazole to increase its sensitivity. In this study, we evaluated the antifungal effect of two Chinese herbs Panax Notoginseng Saponins (PNS) and Ginseng Stem Leave Saponins (GSLS) and checked its interaction with fluconazole (FLC). Materials and methods: we performed in vitro drug susceptibility tests for PNS, GSLS and FLC alone and FLC combined with PNS or GSLS against fluconazole resistant 19 strains of *Candida albicans (C. albicans)* following Clinical and Laboratory Standard Institute (CLSI) M27-A3 guidelines. Drug interactions were evaluated by checkerboard method and results of interactions were assessed by fractional inhibitory concentration index (FICI) model. Results: Minimum inhibitory concentration (MIC) at which 50% fungal growth was inhibited (MIC_{50}) ranged from 64 to 128 μg/ml and 32 to 256 μg/ml for PNS and GSLS respectively. However, when combined with fluconazole, their MIC_{50} significantly decreased and ranged from 8 to 32 μg/ml and 2 to 32 μg/ml for PNS and GSLS respectively. All the 19 strains tested showed synergistic effect for both the combinations (PNS plus FLC and GSLS plus FLC). Conclusions: fluconazole used in combination with PNS or GSLS is effective against resistant strains of *C. albicans* and this combination can be considered an alternative therapeutic option for resistant strains. However, there remains a need for further screening of such combinations before it can be widely used in clinics.

Keywords: Minimum inhibitory concentration, fractional inhibitory concentration index, checkerboard method

Introduction

Fluconazole belongs to a group of first generation triazole-based antifungal drugs [1]. It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system [2]. Fluconazole is indicated for the treatment and prophylaxis in a number of fungal infections where other antifungals have failed or are not tolerated (e.g., due to adverse effects) [3]. It can also be used as a first-line drug in a number of conditions like coccidioidomycosis, cryptococcosis, histoplasmosis and prophylaxis of candidiasis in immunocompromised people [3]. Despite all these advantages, its use has been limited because of the development of wide range of resistant strains. This requires an urgent need of alternative agents, which are as effective and as safety as fluconazole. In recent years, drug combinations have been tried as an effort to overcome the emergence of resistant fungi. However, high costs and serious side effects have put limitations on the combinations of antifungal drugs [4, 5]. This may be the cause of attempts being made to develop stable and safe antifungal agents from natural products including Chinese herbs. In preliminary studies, previously it have shown that some Traditional Chinese Medicinal (TCM) herbs possess interesting antifungal properties [6, 7]. PNS and GSLS are Chinese herbs that has been traditionally used in China since decades and is believed to be beneficial for prevention and treatment of various diseases, such as cardio- and cerebrovascular diseases, pains, and bleeding [8]. However, antifungal property of these herbs has yet not been

known. In the present study, we aim to evaluate the antifungal effect of these herbs. Either effective or not solely, we further aim to study its effect in combination and its interaction with commonly used antifungal drug, fluconazole.

Materials and methods

Experimental strains

Clinical strains of *C. albicans* were collected from the department of dermatology, Shanghai Tongji Hospital affiliated to Tongji University (Shanghai, China). A drug susceptibility testing was performed for fluconazole and 19 resistant strains were selected for further study. Among these 19 strains (indicated by the number 1, 32, 77, 119, 137, 140, 163, 176, 189, 198, 224, 262, 271, 277, 299, 307, 322, 359 and 362) tested, 7 were isolated from sputum, 5 from vaginal secretion, 4 from feces, and 3 from urine samples. The strains were subcultured onto Sabouraud dextrose agar and the incubation temperature throughout was 35°C. Quality control was ensured by testing the CLSI-recommended strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258.

Experimental agents: Fluconazole used in this experiment was purchased from Shanghai Sunve Pharmaceuticals Co., Ltd., Shanghai, China. Panax Notoginseng Saponins and Ginseng Stem Leave Saponins were purchased from China Institute of Pharmaceutical and Biological Product, Beijing, China. These agents were obtained as powders and stored at -60°C after preparing stock solution. FLC was dissolved in sterile water and PNS and GSLS were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solution.

Drug susceptibility testing

Susceptibility testing was performed for each of the three drugs FLC, PNS and GSLS following CLSI M27-A3 guidelines [9]. Twofold dilutions of each drug was prepared so as to make a final concentration ranging from 64~0.125 μg/ml for FLC and 256~0.5 μg/ml for PNS and GSLS. The reason for preparing PNS and GSLS at such a high concentration was because at lower concentration it failed to inhibit fungal growth.

The inoculum was prepared by suspending fungal colonies in sterile saline solution. The cell density was adjusted with a spectrophotometer to produce a transmittance as produced by a 0.5 McFarland standard at 530 nm wavelength. The resulted stock suspension was diluted in Rosewell Park Memorial Institute (RPMI) 1640 broth medium to yield a working suspension of 1×10^3~5×10^3 CFU/ml.

For combination drug susceptibility test, the final concentration of drugs ranged from 32~0.0312 μg/ml for FLC and 128~2 μg/ml for PNS and GSLS. The combinations tested were FLC plus PNS and FLC plus GSLS. Each combination was tested in duplicate. 50 μl of each dilution of FLC was added to the 96-well microtiter plates in the vertical direction, while 50 μl of each dilution of PNS or GSLS was added in the horizontal direction, so that various combinations of FLC and PNS or GSLS could be achieved. Also, 100 μl of inoculum (1×10^3~5×10^3 CFU/ml) was added to each well. After adding various concentrations of drugs and inoculum to a 96-well plate, the plate was incubated at 35°C for 48 hours. MIC values for all drugs alone and in combination were determined as the drug concentration at which 50% fungal growth was inhibited (MIC_{50}).

Synergy testing

The method used to calculate fractional inhibitory concentration index (FICI) in this experiment is based on the Loewe additivity theory, which is one of the several common reference models used for measuring the effects of drug combinations. Loewe additivity is based on the idea that an agent should not have synergistic interaction with itself or similar agents. The nonparametric approach is based on FICI, which is expressed by the equation:

$$\sum FIC = FIC_A + FIC_B = C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$$

Where MIC_A^{alone} and MIC_B^{alone} are the MICs of drugs A and B when acting alone and C_A^{comb} and C_B^{comb} are the concentrations of drugs A and B at isoeffective combinations, respectively [10]. Among all the ∑FICs calculated for each data set, the FICI was determined as the ∑FICmin (the lowest ∑FIC) when the ∑FICmax (the highest ∑FIC) was less than 4; otherwise, the FICI was determined as the ∑FICmax [10].

The interpretation of the FICI was as follows: [11, 12].

Table 1. MIC of FCZ and PNS alone and in combination and interpretation of their interaction

Fungal strain	MIC of FLC (μg/ml)		MIC of PNS (μg/ml)		FLC plus PNS (FICI)	Interaction
	Alone	Combined	Alone	Combined		
1	64	4	128	32	0.312	Synergism
32	64	4	128	32	0.313	Synergism
77	64	8	128	32	0.375	Synergism
119	64	2	64	16	0.281	Synergism
137	64	2	128	32	0.281	Synergism
140	64	4	64	8	0.188	Synergism
163	64	4	128	16	0.187	Synergism
176	64	4	128	16	0.188	Synergism
189	64	4	128	32	0.313	Synergism
198	64	8	128	32	0.375	Synergism
224	64	2	128	16	0.156	Synergism
262	64	4	128	16	0.187	Synergism
271	64	4	128	32	0.313	Synergism
277	64	8	128	32	0.375	Synergism
299	64	4	128	16	0.188	Synergism
307	64	2	128	8	0.09	Synergism
322	64	4	128	32	0.313	Synergism
359	64	4	128	16	0.188	Synergism
362	64	2	64	16	0.281	Synergism

Table 2. MIC of FCZ and GSLS alone and in combination and interpretation of their interaction

Fungal strain	MIC of FLC (μg/ml)		MIC of GSLS (μg/ml)		FLC plus GSLS (FICI)	Interaction
	Alone	Combined	Alone	Combined		
1	64	8	64	16	0.375	Synergism
32	64	8	128	8	0.185	Synergism
77	64	8	128	8	0.185	Synergism
119	64	4	64	16	0.313	Synergism
137	64	4	64	16	0.313	Synergism
140	64	4	128	32	0.313	Synergism
163	64	4	64	16	0.313	Synergism
176	64	8	128	32	0.375	Synergism
189	64	8	128	8	0.185	Synergism
198	64	4	128	32	0.312	Synergism
224	64	8	32	2	0.185	Synergism
262	64	8	32	2	0.185	Synergism
271	64	8	128	32	0.375	Synergism
277	64	4	128	4	0.09	Synergism
299	64	8	256	8	0.155	Synergism
307	64	4	64	8	0.188	Synergism
322	64	16	256	8	0.28	Synergism
359	64	2	128	16	0.156	Synergism
362	64	4	128	4	0.09	Synergism

FICI ≤ 0.5 indicates synergistic effect; 0.5 < FICI ≤ 4 indicates indifference, and FICI > 4 indicates antagonistic effect.

Statistical analysis

SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis, using a paired t-test and the geometric mean. *P* value < 0.05 was considered to indicate a statistically significant result.

Results

MIC values of FLC and PNS when used alone and in combination are shown in **Table 1**. Similarly, MIC values of FLC and GSLS when used alone and in combination are shown in **Table 2**. We can see that MIC values of FLC, PNS and GSLS are significantly reduced when used in combination compared to those when used alone. Notably, while PNS and GSLS alone showed no significant antifungal effects on *C. albicans*, their MIC values were significantly reduced when used in combination with FLC. We further analyzed the effect of combinations of drugs by calculating FICI (**Tables 1** and **2**). The FICI values for all the 19 strains tested for both the combinations were < 0.5 suggesting that the effects of FLC plus PNS and FLC plus GSLS are synergistic. As shown in **Table 3**, while there is variation in the MIC ranges (MIC_R) of FLC, PNS and GSLS alone and in combination use, the range of FICI values ($FICI_R$) was similar for both the combinations tested. Additionally, geometric mean of FICI for FLC plus PNS and FLC plus GSLS combinations is 0.243 and 0.221 respectively with a difference of only 2.2% suggesting that both PNS and GSLS are effective in combination with FLC. Similarly, if we compare the geometric mean of MIC val-

Table 3. Range of all the MIC values, FICI values and their geometric mean when all the strains are considered together

Drugs used alone and in combination	MIC_R	MIC_{GM}	$FICI_R$	$FICI_{GM}$
FLC_{alone}	64	64	-	-
PNS_{alone}	64~128	114.73	-	-
$GSLS_{alone}$	32~256	99.15	-	-
FLC plus	2~8	3.72	0.09~	0.243
PNS	8~32	20.66	0.375	
FLC plus	2~16	5.76	0.09~	0.221
GSLS	2~32	10.33	0.375	

MIC_R: range of MIC values, MIC_{GM}: geometric mean of MIC values, $FICI_R$: range of FICI values, $FICI_{GM}$: geometric mean of FICI.

ues (MIC_{GM}) of FLC in combination with PNS or GSLS, it is seen that MIC of FLC is decreased by 94.19% and 91% respectively, revealing a difference of only 3.19%. Thus it is suggested from this experiment that both the Chinese herbs PNS and GSLS are worth considering important agents to be used in combination with FLC.

Discussion

The polymorphic yeast *Candida albicans* is a commensal opportunistic human pathogen that is estimated to colonize more than 70% of the human population without causing any symptoms of disease [13, 14]. Its ability of morphogenetic transition between the yeast, pseudohyphal and hyphal cells plays a key role in colonization, invasion and dissemination in host tissues [15, 16].

It is also the most common opportunistic fungal pathogen of humans causing from benign infections such as oral and vaginal candidiasis to fatal, systematic diseases in immune compromised or critically ill patients. In the past years, these infections were successfully being treated with azole group of drugs among which fluconazole was considered most safe and effective. Fluconazole works by inhibiting the fungal cytochrome P450 enzyme 14α-demethylase. But the fungistatic nature and the development of resistance in fungi have restricted the use of fluconazole [17, 18]. Therefore, there is an urgent need for new therapeutic options for efficient management of Candidal infections. Medicinal plants used in TCM (traditional Chinese medicine) could be one potent source for such exploration. Moreover, increasing impact of fungal infections, incidence of drug-resistant pathogens and toxicity of available antifungal drugs, at least in part, have become a main encouraging factors leading to the development of interest in studying natural products as an alternative therapeutic option in treating fungal infections. Thus, a variety of natural products like Farnesol [19], Berberine [20], Catechin [21], and Pomegranate peels [22], etc. have been studied in the past for their antifungal activity and their synergism with antimycotic agents and have shown to reduce the MIC of various antifungal agents when used in combination.

In this study we evaluated the in vitro efficacy of two TCM herbs (PNS and GSLS) for their antifungal activity against fluconazole resistant strains of *Candida albicans*. PNS has been previously known to be effective for various medical conditions such as, inflammation [23], cerebrovascular diseases [24], oxidative stress [25], and malignancy [26], etc. Similarly, GSLS is seen to have a broad range of biological activities including, anti-inflammatory activity, antioxidant, anti-tumor effects, as well as adjuvant property with low hemolytic activity [27]. However, as per our best knowledge, antifungal activity of these two herbs is not yet reported. The mechanism by which PNS and GSLS could synergize with fluconazole is also uncertain. We wish to continue to study the synergistic effect of these two combinations (FLC plus PNS and FLC plus GSLS) with large number of fungal strains in future. Studying the mechanism of these natural products and to see what role they have in the balance of the sterol biosynthetic pathway and how it interferes with cell viability would also be in consideration in our future studies. In present study, when used alone, the two Chinese herbs (PNS and GSLS) showed no effect as an antifungal evidenced by high MIC ranging from 64 to 256 μg/ml, but when combined with fluconazole, MIC was significantly decreased ranging from 2 to 32 μg/ml. Compared to PNS or GSLS alone, when combined with fluconazole, produced stronger antifungal activity. In addition, their combination with fluconazole showed synergistic effects against all the 19 strains tested, which is suggested by FICI value < 0.5 (**Tables 1** and **2**).

Furthermore, the MIC of fluconazole when used alone was 64 μg/ml for all the 19 strains but when combined with PNS or GSLS, the MIC was markedly reduced ranging from 2 μg/ml to 16 μg/ml (**Tables 1** and **2**).

A study done by Hirasawa and Takada [21] to look for multiple effects of green tea Catechin on the antifungal activity of antimycotics against *Candida albicans* demonstrated similar results concluding that combined treatment of antimycotic with catechin allows the use of lower doses of antimycotics and induces multiple antifungal effects. The study resulted that the combined use of 12.5 mg/L Epigallocatechin gallate and fluconazole 10-50 mg/L (below MIC) inhibited the growth of fluconazole-resistant *C. albicans* by 98.5%-99.7%. In respect to these views, the combinations of antifungal with natural products are worth considering a therapeutic option in treating fungal infections. However, there remains a need for further screening of such combinations before it can be widely used in clinics. Many hundreds of plants worldwide have traditionally been used as treatments for microbial infections and some of these have also been subjected to in vitro screening, but the efficacy of such herbal medicines in combination therapy need to be tested in rigorous clinical trials. In addition, ahead of clinical application, safety of these compounds must be firmly established.

Conclusion

Fluconazole used in combination with PNS or GSLS is effective against resistant strains of *C. albicans* and this combination can be considered an alternative therapeutic option for resistant strains. However, there remains a need for further screening of such combinations before it can be widely used in clinics.

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Disclosure of conflict of interest

None.

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