Infection and Drug Resistance

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ORIGINAL RESEARCH

N-Butylphthalide Potentiates the Effect of Fluconazole Against Drug-Resistant *Candida glabrata* and *Candida tropicalis*. Evidence for Its Mechanism of Action

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Objective: To define the antifungal activity of n-butylphthalide alone or in combination with fluconazole in *Candida glabrata* and *Candida tropicalis*.

Methods: The antifungal activity of n-butylphthalide alone and in combination with fluconazole was investigated by the classical broth microdilution method and the time-killing curve method. The QRT-PCR method was used to determine gene expressions changes of mitochondrial respiratory chain enzymes, drug efflux pumps and drug target enzymes in *Candida glabrata* and *Candida tropicalis* after n-butylphthalide treatment.

Results: The MIC values of n-butylphthalide against *Candida glabrata* and *Candida tropicalis* ranged from 16 to 64 μg·mL-1. The FICI value of the combination of n-butylphthalide and fluconazole against drug-resistant *Candida glabrata* and *Candida tropicalis* ranged from 0.5001 to 0.5315 with partial synergism. Time-killing curves showed that 256 μg·mL-1 n-butylphthalide significantly inhibited the growth of drug-resistant colonies of *Candida glabrata* and *Candida tropicalis*, and 128 μg·mL-1 n-butylphthalide combined with 1 μg·mL-1 fluconazole had an additive effect. N-butylphthalide could alter the expression of mitochondrial respiratory chain enzymes *COX1, COX2, COX3*, and *CYTB* genes in *Candida glabrata* and *Candida tropicalis* ($P< 0.05$) and downregulate the expression of the drug efflux pump genes *CDR1* and *CDR2* in drug-resistant *Candida glabrata* to 3.36% and 3.65%, respectively ($P<0.001$), but did not affect the drug target enzyme ERG11 in drug-resistant *Candida tropicalis*.

Conclusion: N-butylphthalide had antifungal activity against *Candida glabrata* and *Candida tropicalis*. N-butylphthalide improved the activity of fluconazole against drug-resistant *Candida glabrata* by affecting the expression of mitochondrial respiratory chain enzyme genes and reversing the high expression of drug efflux pump genes CDR1 and CDR2.

Keywords: n-butylphthalide, fluconazole, drug resistance, *Candida glabrata*, *Candida tropicalis*, potentiation

Introduction

With the increasing number of HIV infections, organ transplants, the aging population, and the widespread use of immunosuppressive drugs, the health burden of fungal infections is increasing.[1–4] More than 1 billion people have suffered from fungal infections since 2013, and more than 1.5 million have died from fungal infections.[5] *Candida* spp. is one of the most common pathogenic opportunistic fungi, and invasive infections caused by *Candida* is one of the major causes of human death.[6] The most common *Candida* is *Candida albicans*. The percentage of *non-Candida albicans* has increased in recent years, with *Candida glabrata* and *Candida tropicalis* being the second most frequently isolated after *Candida albicans*. In some studies, invasive *Candida* infections have high mortality rates caused by *Candida glabrata*, and *Candida tropicalis* infection rates can reach 57% to 81.2%.[7,8] The increased mortality and morbidity of these infections is associated with the presence of biofilms on both host and

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abiotic surfaces. Candida can increase pathogenicity and immune evasion by forming biofilms.[9] The biofilm produced by different species of Candida varies greatly, with *Candida tropicalis* showing high biofilm formation ability, followed by *Candida krusei* and *Candida glabrata*.[10] In addition, *Candida tropicalis* also showed higher biofilm production.[11]

The first-line therapeutic agents for *Candida* infections are azoles and echinocandins. Although *Candida albicans* is still highly susceptible to both types of drugs, *Candida glabrata* and *Candida tropicalis* have shown various degrees of resistance to azoles.[12–16] The resistance rates of *Candida glabrata* and *Candida tropicalis* to fluconazole (FLC) ranged from 6.1% to 37% and 4.1% to 39.3%, respectively, and the resistance rates to voriconazole, itraconazole, and posaconazole ranged from 5.7% to 29.1%. Cross-resistance to different azoles has been observed. *Candida* has multiple resistance mechanisms to azoles, including changes in target enzymes and increased drug efflux.[17–19] Combining drugs is a new strategy to enhance antifungal efficacy and reduce drug resistance. Previous studies have confirmed that multiple natural medicinal extracts have antifungal activity and potentiate classical antifungal drugs. Menthol, Acca sellowiana, and carvacrol have sensitization effects on FLC.[20–22]

N-butylphthalide (NBP), also known as butylphthalide, chemically named 3-butyl-1(3H)-isobenzofuranone, is an active monomer isolated from celery seeds. Celery is a plant in the Umbelliferae family, and natural phthalein compounds mainly come from Apiaceae family.[23–25] In the ancient Chinese books "Compendium of Materia Medica", "Materia medica correct" and "The essence of Chinese medicine", it is recorded that a variety of plants rich in phthalides such as *Ligusticum chuanxiong* and *Chinese angelica* can be used to treat infectious diseases such as dysentery and skin diseases caused by microbial infections. In this study, clinical isolates of *Candida glabrata* and *Candida tropicalis* strains were selected to investigate the antifungal activity of NBP and analyze its effect and mechanism in potentiating FLC against drug-resistant *Candida glabrata* and *Candida tropicalis*.

Material

Strains

Twenty-six strains of *Candida glabrata* and eighteen strains of *Candida tropicalis* were used. All were clinical isolates from the Second Hospital of Hebei Medical University. The standard *Candida albicans* ATCC 90028 strain was selected as quality control. The strains were inoculated in liquid strain preservation tubes and stored at −80°C. Before the experiment, the strains were inoculated on Sabouraud dextrose agar (SDA) plates for subculture and recovery.

Drugs and Agents

The following drugs and agents were used: FLC (purity 99.3%, Shanghai Yuanye Biotechnology Co., Ltd.), NBP (purity 98.8%, CSPC Pharmaceutical Group Enbipu Co., Ltd.), RPMI 1640 medium (with glutamine, without bicarbonate, with phenol red as pH indicator, and MOPS as a buffer, Thermo Fisher Scientific, USA), RT First Strand cDNA Synthesis Kit and 2×SYBR Green qPCR Master Mix (None ROX) (Wuhan Servicebio Technology Co.), RT First Strand cDNA Synthesis Kit, and 2 × SYBR Green qPCR Master Mix (None ROX) (Wuhan Servicebio Technology Co.)

Instruments and Consumables

The instruments and consumables used were a carbon dioxide incubator (Model 311, Thermo Fisher Scientific, USA), ultra-clean bench (SW-CJ-1FD, Sujing Antai), quantitative fluorescence PCR (CFX, Bole company), electronic Maclean turbidimeter (21,250, Mérieux, France), and sterile 96-well plates, shaking tubes (Corning, USA), and SDA plates (Wenzhou Kangtai Biotechnology Co., Ltd.).

Methods

Classical Broth Microdilution Method

Minimum inhibitory concentrations (MIC) of NBP alone and in combination with FLC against *Candida* were determined by the classical broth microdilution method.[26] And we declare that these clinical samples were not specifically isolated for this research and they are part of the routine hospital laboratory procedure. FLC was dissolved in sterile water to make stock solutions at 2048 $\mu g \cdot mL^{-1}$ and 1 $mg \cdot mL^{-1}$. NBP was dissolved in absolute alcohol (containing 5% Tween 80) to make a 12.8 $mg \cdot mL^{-1}$ stock solution and stored at −20°C. The FLC stock solution was double diluted to a working

solution with a concentration of 2048–2 μg·mL^{-1}, and the NBP stock solution was double diluted to a working solution with a concentration of 512–8 μg·mL^{-1}. The fungal suspension (2×10^3 CFU·mL^{-1}, 100 μL) was added to each well in the 96-well plate. NBP working solution (50 μL) was added to the NBP control wells. An FLC working solution (50 μL) was added to the FLC control wells. The NBP working solution (50 μL) and the FLC working solution (50 μL) were added to the drug combination wells. Supplement to 200 μL with the RPMI 1640 medium. Control Wells contained only RPMI 1640 medium and fungal solution. The plate was incubated at 35°C for 24 h, and the concentration corresponding to a 50% reduction in colonies was the MICs.

The Spearman coefficient was applied to analyze the correlation between the MICs of NBP and FLC against two *Candida* species. The fractional inhibitory concentration index (FICI) method was used to evaluate the interaction of two drugs in combination with each other.[27,28] FICI is calculated as FICI = $MIC_{FLCcomb}/MIC_{FLC}$ + $MIC_{NBPcomb}/MIC_{NBP}$. Based on FICI values, the interactions were classified as synergism (FICI ≤ 0.5), without interaction (0.5 < FICI<4), and antagonism (FICI>4).

Time-Kill Assay of n-Butylphthalide and Fluconazole

Time-Kill Assay of n-Butylphthalide

500 μL of the fungal suspension and 500 μL of the NBP solution were added to each shaking tube. Dilute to 5 mL with the RPMI 1640 medium. The final concentrations of NBP were 32, 64, 128, and 256 μg·mL^{-1}, and the fungal suspension concentration was 10^5 CFU·mL^{-1}. Control tubes contained only RPMI 1640 medium and fungal solution. The contents were incubated at 35°C (200 rpm), and a 10 μL fungal suspension was taken at 0, 6, 12, 24, 36, and 48 h. The colony counting method was used after multiple dilutions to determine the fungal concentration. A line graph was plotted using logCFU·mL^{-1} against time.[29] The experiment was repeated three times to obtain mean values. The control group did not contain drugs.

Time-Kill Assay of NBP in Combination with FLC

The experiment was divided into four groups: the NBP group (64 μg·mL^{-1}, 128 μg·mL^{-1}), the FLC group (1 μg·mL^{-1}), the drug combination group (64 μg·mL^{-1}NBP +1 μg·mL^{-1}FLC, 128 μg·mL^{-1}NBP+1 μg·mL^{-1}FLC), and the control group. Follow-up was performed as in "Time-kill assay of n-butylphthalide". The interaction was evaluated according to the difference of logCFU·mL^{-1} (ΔlogCFU·mL^{-1}) between the drug combination group and the group with the better antifungal activity.[30] Compared to the single drug group with the best antifungal effect, a synergistic effect was present when the ΔlogCFU·mL^{-1} of the drug combination group decreased >2. In contrast, an antagonistic effect was present when the ΔlogCFU·mL^{-1} of the drug combination group increased >2. If the ΔlogCFU·mL^{-1} decrease was <2 but ≥1, there was an additive effect. No interaction was observed when the ΔlogCFU·mL^{-1} decrease was <1.

QRT-PCR Method to Determine the Expression of the Mitochondrial Respiratory Chain Enzyme Genes

The mitochondrial respiratory chain enzyme complex III is cytochrome B (CYTB), and mitochondrial respiratory chain enzyme complex IV consists of multiple cytochrome C oxidase (COX) subunits, such as COX1, COX2, and COX3.[31,32] The primers are shown in Table 1. The strains used were CG19 and CG20. The experiment was divided into the control and NBP groups. The NBP group contained fungal suspension, drug solution, and the RPMI 1640 medium. The final concentrations were 10^7 CFU·mL^{-1} of the fungal suspension and 128 μg·mL^{-1} of NBP. The growth control group did not contain drugs. The systems were incubated at 35°C, 200rpm for 8h. Then cells were collected and rinsed, then the fungal suspension was digested with lysozyme at room temperature, followed by the addition of TRIzol to disrupt the cells. Then chloroform was added. Take the supernatant after thoroughly mix and centrifugation. After addition of isopropanol and centrifugation, the white precipitate at the bottom of the tube was RNA. Ethanol was added to wash the precipitate, and the upper liquid layer was removed and dried. RNA was dissolved by adding nucleic acid free water and diluted to 200 ng·mL^{-1}. After the reaction solution was added, reverse transcription and quantitative PCR were performed to determine the effect of NBP on the expression of respiratory chain enzyme genes.

The $2^{-\Delta\Delta Ct}$ method was applied to calculate the results.[33] The independent samples *t*-test or Mann–Whitney *U*-test was applied to analyze normally distributed or non-normally distributed data. All statistics were performed using SPSS 25.0. $P<0.05$ was considered statistically significant.

Table 1 Primers Used in This Study

Gene	Primers Sequence (5'-3')
CG-CDR1	F: GAACATACTCGCTATACCACCCA
	R: GACATGGCAAATACTGCAACAA
CG-CDR2	F: CGTGGGTTCGGTGTTGGTAT
	R: GTTGGTCGCATTGGAGGTTAT
CG-CYTB	F: CTCAGCTATTCCTTTCGTTGGT
	R: CATTCCAATTCTATCCATATTACCT
CG-COX1	F: TGATTGGTGCATCAGATATGGC
	R: CACTAGGTCCTGAATGAGCTTGA
CG-COX2	F: TTCAGCTACACCTAATCAAGAAGGT
	R: CATAGCTGGTGAAATAACTTCATCAC
CG-COX3	F: TTCATTCAGCAATGAGTCCAGA
	R: CAGTACCAGCATAGAATACTGATCC
CT-ERG11	F: GCCATTCGGTGGTGGTAGAC
	R: TCCCAAACGATTTCAGCAGGT
CT-CYTB	F: CGTTCCCTTTATTTGAGGTGGT
	R: AATCGGTCGATGTTACCTGTGA
CT-COX1	F: ACCAGCTCCAGTTTCAATGAGT
	R: TTCCTATTTGTTATGCCCTTCC
CT-COX2	F: GCCTTCCCATCATTCATCCTG
	R: TACCTGGCTCAAGCATGTCGT
CT-COX3	F: CGTTCCATTTAGTTGGTCCTTC
	R: AGAATCCCTGATTAAGCCCTCTT

Determination of Drug Resistance-Related Genes by the QRT-PCR Method

Determination of the Expression of the CDR1 and CDR2 Genes

The strains used were CG19 and CG28. The experiment was divided into the susceptible, resistant, and NBP groups, and the follow-up was performed as in "QRT-PCR method to determine the expression of the mitochondrial respiratory chain enzyme genes". The expressions of CDR1 and CDR2 were measured in each group to determine the mechanism of drug resistance in *Candida glabrata* and the effect of NBP on the expression of efflux pump genes.

Determination of the Expression of the ERG11 Gene

The strains used were CT20 and CT39. The experiment was divided into the susceptible group, resistance group, and NBP group, and follow-up was performed as in 2.3. The expression of ERG11 in each group was measured to determine the mechanism of drug resistance in *Candida tropicalis* and the effect of NBP on the expression of ERG11 genes.

Results

MIC of n-Butylphthalide Alone and in Combination with Fluconazole

NBP had antifungal effects on sensitive and resistant *Candida glabrata* and *Candida tropicalis* with MIC values ranging from 4 to 256 $\mu g \cdot mL^{-1}$. Spearman correlation analysis showed that the correlation coefficients of the MIC values of these two drugs against *Candida glabrata* and *Candida tropicalis* were −0.239 and −0.096 ($P > 0.05$), respectively, with no correlation. The results are shown in Table 2.

The combined use of NBP and FLC had a partial synergistic antifungal effect on drug-resistant *Candida glabrata* and *Candida tropicalis*. After the combined use of NBP, the MIC of FLC against drug-resistant *Candida glabrata* could be reduced to 1/512-1/64 of the original value, and the MIC of drug-resistant *Candida tropicalis* could be reduced to 1/1024-1/256 of the initial value. The results are shown in Table 3.

Table 2 MIC of FLC and NBP in Candida Glabrata and Candida Tropicalis

Candida strains[a]	MIC (μg·mL^{-1})		Spearman	
	FLC	NBP	r	P
CG 1	4	128	−0.239	0.240
CG 2	4	4		
CG 3	2	128		
CG 4	2	128		
CG 5	16	256		
CG 6	32	64		
CG 7	2	32		
CG 8	1	128		
CG 9	2	128		
CG 10	2	128		
CG 11	2	128		
CG 12	32	32		
CG 13	1	256		
CG 14	2	64		
CG 15	64	64		
CG 16	64	64		
CG 17	1	64		
CG 18	64	16		
CG 19	512	64		
CG 28	0.5	64		
CG 29	16	32		
CG 30	1	32		
CG 31	2	32		
CG 32	1	32		
CG 33	16	64		
CG 34	16	8		
CT 20	1024	64	−0.096	0.704
CT 21	256	128		
CT 22	128	32		
CT 23	128	32		
CT 24	64	64		
CT 25	64	64		
CT 26	512	64		
CT 27	256	64		
CT 34	256	32		
CT 35	128	16		
CT 36	2	32		
CT 37	1	128		
CT 38	1	16		
CT 39	1	64		
CT 40	256	8		
CT 41	1	64		
CT 42	128	16		
CT 43	1	64		

Abbreviations: [a]CG, Candida glabrata; CT, Candida tropicalis.

Table 3 Static Antifungal Activity of NBP and FLC

Strains[a]	MIC_{FLC} /μg mL^{-1}	MIC_{NBP} /μg mL^{-1}	$MIC_{FLCcomb}$ /μg mL^{-1}	$MIC_{NBPcomb}$ /μg mL^{-1}	FICI[b]	Interpretation
CG15	64	64	1	32	0.5156	Partial synergism
CG16	64	64	2	32	0.5315	Partial synergism
CG18	64	16	1	8	0.5156	Partial synergism
CG19	512	64	1	32	0.5020	Partial synergism
CT20	1024	64	1	32	0.5001	Partial synergism
CT26	512	64	1	32	0.5020	Partial synergism
CT27	256	64	1	32	0.5039	Partial synergism

Abbreviations: [a]CG, Candida glabrata; CT, Candida tropicalis; [b]FICI, fractional inhibitory concentration index, $FICI=MIC_{FLCcomb}/MIC_{FLC} + MIC_{NBPcomb}/MIC_{NBP}$

Time-Kill Assay of NBP and FLC

Compared to the growth control, colony growth was partially inhibited at NBP concentrations of 64 and 128 μg·mL^{-1}. A significant antifungal effect appeared at the NBP concentration of 256 μg·mL^{-1}, which reduced the concentration of fungal suspension to 1/10-1/3 of the original value, indicating that the dynamic antifungal effect of NBP on drug-resistant *Candida glabrata* and *Candida tropicalis* was proportional to NBP concentrations. The result is shown in Figure 1.

Compared to the single-drug group with better antifungal activity, when 64 μg·mL^{-1} NBP combined with 1 μg·mL^{-1} FLC, the logCFU·mL^{-1} of resistant *Candida glabrata* and *Candida tropicalis* decreased by 0.61 and 0.86, respectively, which indicates no interaction. When 128 μg·mL^{-1} NBP was combined with 1 μg·mL^{-1} FLC, the log CFU·mL^{-1} of resistant *Candida glabrata* and *Candida tropicalis* decreased by 1.14 and 1.40, respectively, showing an additive effect. The result is shown in Figure 2.

Effect of n-Butylphthalide on the Gene Expressions of Mitochondrial Respiratory Chain Enzymes

After NBP treatment, the gene expressions of mitochondrial respiratory chain enzymes COX1, COX2, COX3, and CYTB of *Candida glabrata* were inhibited and decreased to 0.50%, 0.12%, 6.41%, and 0.50% of the original levels, respectively. The changes were all statistically significant ($P<0.01$). The results are shown in Figure 3. For *Candida tropicalis*, after treatment with NBP, the gene expressions of mitochondrial respiratory chain enzymes COX3 and CYTB decreased to 8.73% and 12.59% of the original levels, respectively. In contrast, gene expressions of COX1 and COX2 increased to 2.25 times and 5.08 times the initial values, respectively, and the changes were all statistically significant ($P<0.01$). The results are shown in Figure 4.

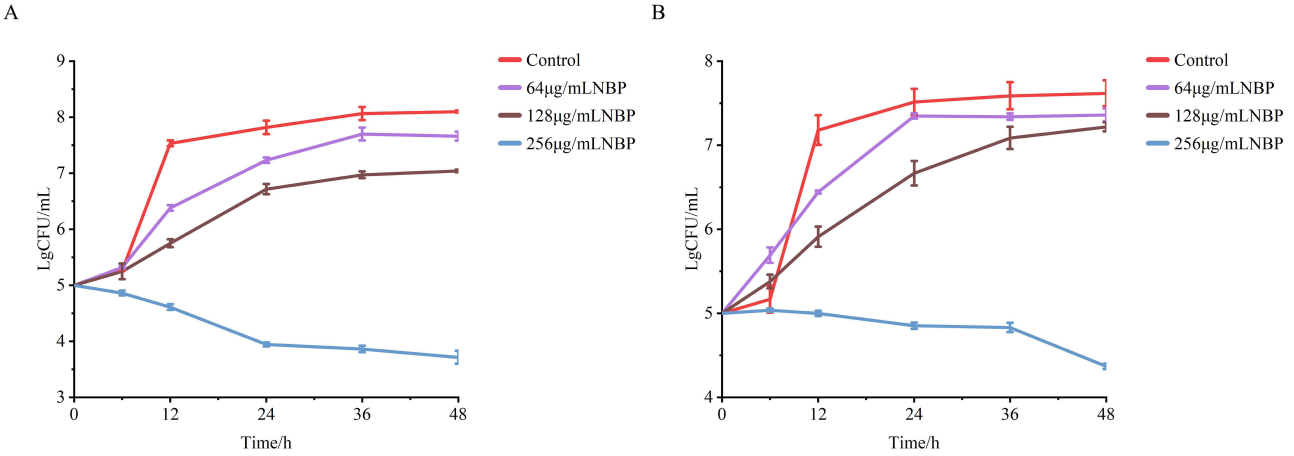

Figure 1 The time-kill curve of NBP on CG19 and CT20((**A**) CG19; (**B**) CT20).

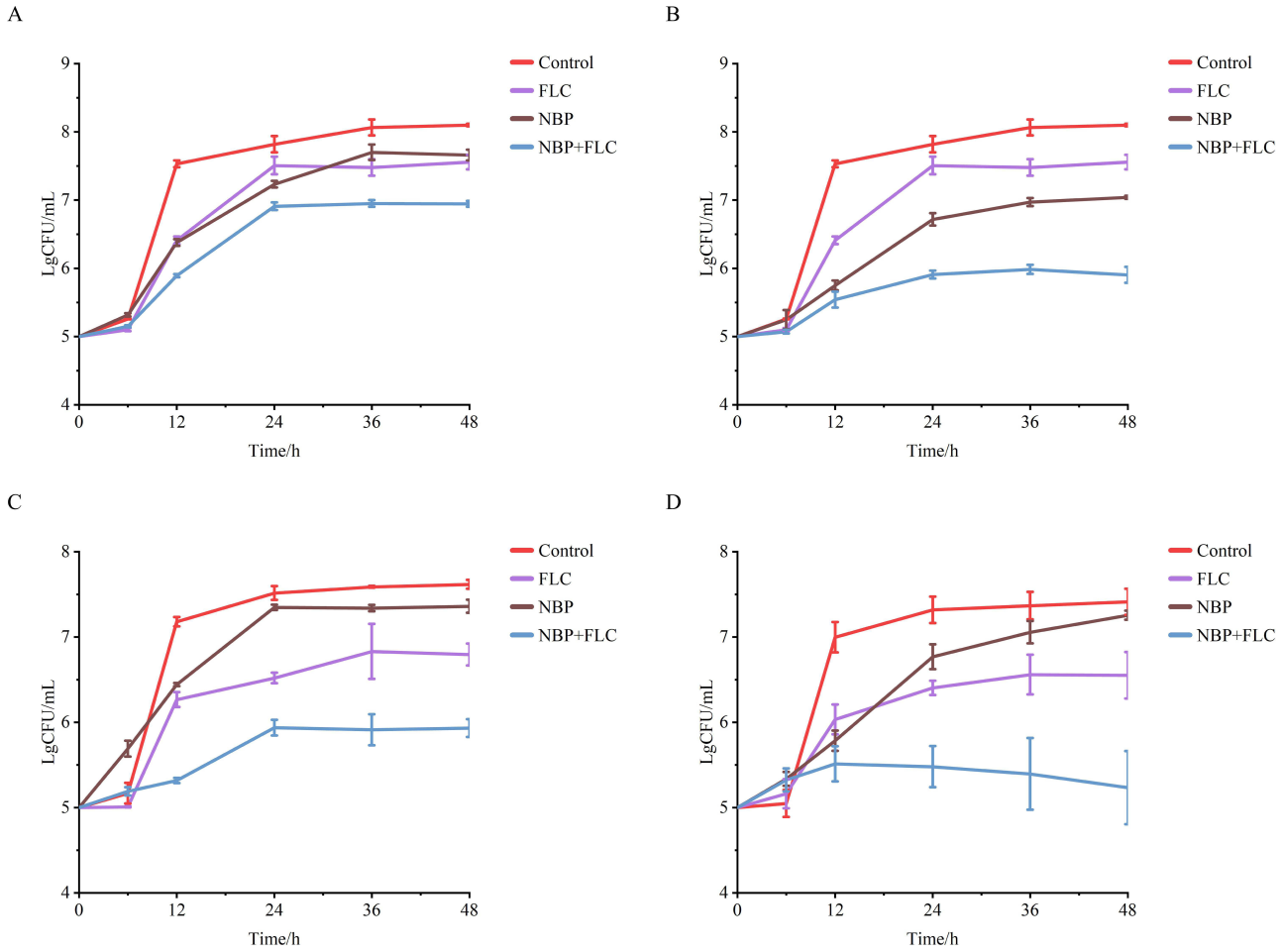

Figure 2 The time-kill curve of NBP and FLC on CG19 and CT20((**A**) 64 μg·mL^{-1} NBP+1 μg·mL^{-1} FLC on CG19; (**B**) 128 μg·mL^{-1} NBP+1 μg·mL^{-1} FLC on CG19; (**C**) 64 μg·mL^{-1} NBP+1 μg·mL^{-1} FLC on CT20; (**D**) 128 μg·mL^{-1} NBP+1 μg·mL^{-1} FLC on CT20).

The Effect of n-Butylphthalide on the Expression of Drug Resistance-Related Genes

The Effect of n-Butylphthalide on the Gene Expressions of CDR1 and CDR2

Compared to the sensitive strain, the expression of efflux pump genes in FLC-resistant *Candida glabrata* was significantly up-regulated (P<0.001), and the expression levels of CDR1 and CDR2 were 4.87 and 11.19 times those of the sensitive strains, respectively. Treatment with NBP could reverse the up-regulation of the efflux pump gene expression, and CDR1 and CDR2 expression levels decreased to 3.36% and 3.65% of the original expression levels (P<0.001) and decreased to 16.36% (P<0.01) and 40.90% (P<0.05) for sensitive strains, respectively. The result is shown in Figure 5.

The Effect n-Butylphthalide on the Gene Expression of ERG11

Compared to the sensitive strain, the gene expression of drug-resistant *Candida tropicalis* ERG11 was significantly up-regulated (P<0.05), and the expression of ERG11 was 1.78 times that of the sensitive strain. There was no significant change after NBP treatment, as shown in Figure 6.

Discussion

Antifungal Effect and the Mechanism of n-Butylphthalide

NBP showed antifungal activity against both sensitive and resistant *Candida*. It positively correlates with drug concentration but not with the strain's resistance to FLC. It may have a different antifungal mechanism than FLC.

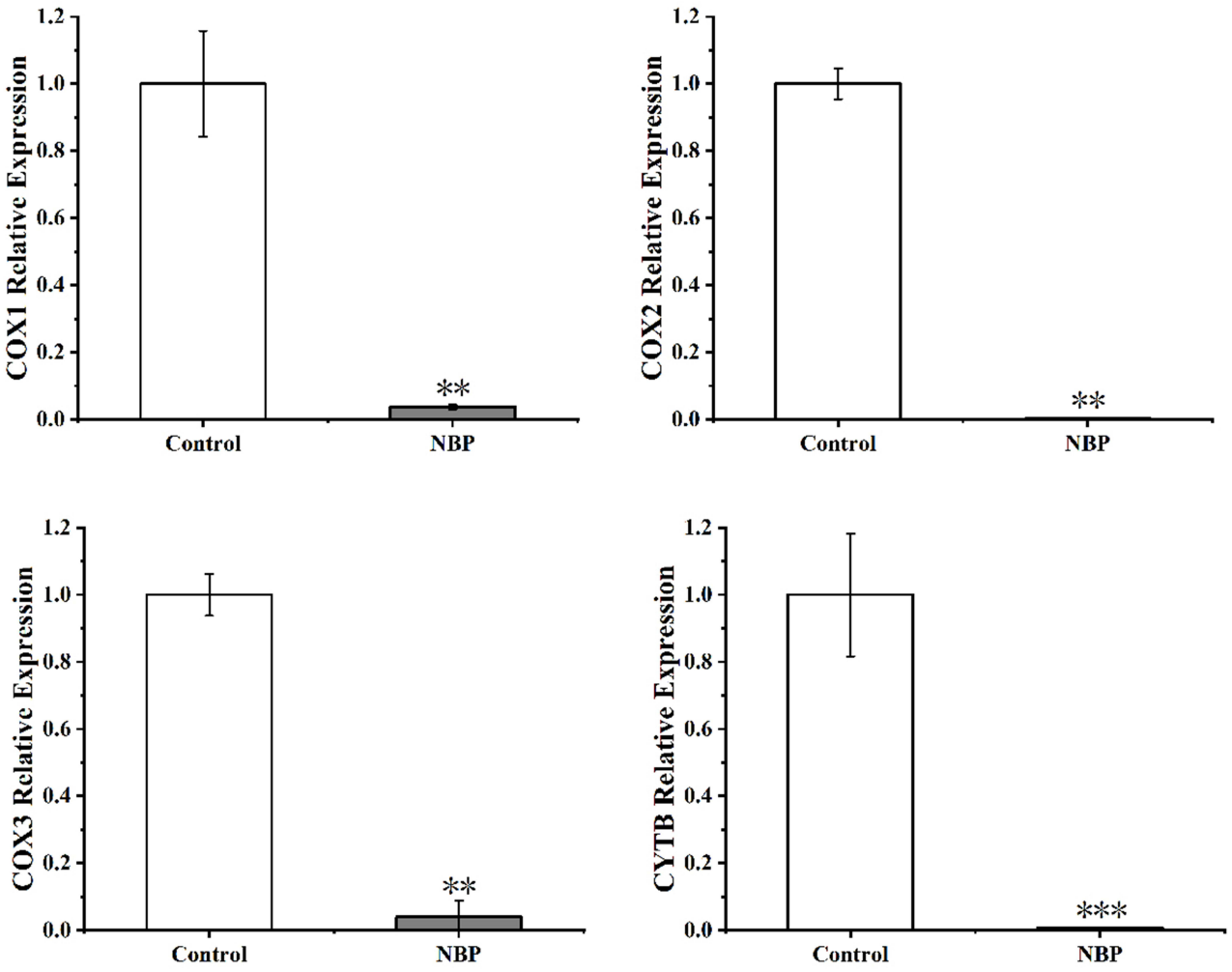

Figure 3 Effect of NBP on the gene expression of the mitochondrial respiratory chain enzyme of CG19(**P<0.01, ***P<0.001, compared to control group).

Various natural drugs can affect *Candida*'s oxidative metabolism and induce the accumulation of reactive oxygen species (ROS) to produce an antifungal effect.[34–36] As byproducts of oxidative metabolism, ROS plays an essential role in cell signal transduction and functional regulation. When their production and elimination are out of balance, they can cause damage to cells and cause many diseases.[37] Reactive oxygen has potent killing activity against bacteria and fungi.[38] Mitochondria is the leading site of active oxygen production in the cell. When there is electron leakage during the transfer of mitochondrial respiratory chain enzymes, the leaked electrons react with oxygen and form superoxide and/or hydrogen peroxide.[39] Mitochondrial respiratory chain enzymes consist of mitochondrial complexes I–IV that play different roles in producing ROS.[40] Inhibition of mitochondrial respiratory chain enzyme complex I and mutations in complex II are the leading causes of increased ROS.[40–42] At the same time, inhibition of complex III causes electron reflux back to complex I and complex II, leading to ROS production.[41] Respiratory chain enzyme complex IV, the regulatory center of mitochondrial oxidative phosphorylation, can prevent the excessive formation of ROS.[42] The presence of its expression can cause an imbalance in intracellular reactive oxygen levels.

This study showed that NBP could inhibit the expression of the mitochondrial respiratory chain enzymes COX1, COX2, COX3, and CYTB in *Candida glabrata*, down-regulate the expression of the mitochondrial respiratory chain enzymes COX3 and CYTB, and up-regulate the expression of COX1 and COX2 in *Candida tropicalis*. COX1, COX2, and COX3 are the constituent subunits of mitochondrial respiratory chain enzyme complex IV. When a specific subunit is overexpressed, the regular assembly of the complex will be affected, and the enzyme's activity will also be affected.[43,44]

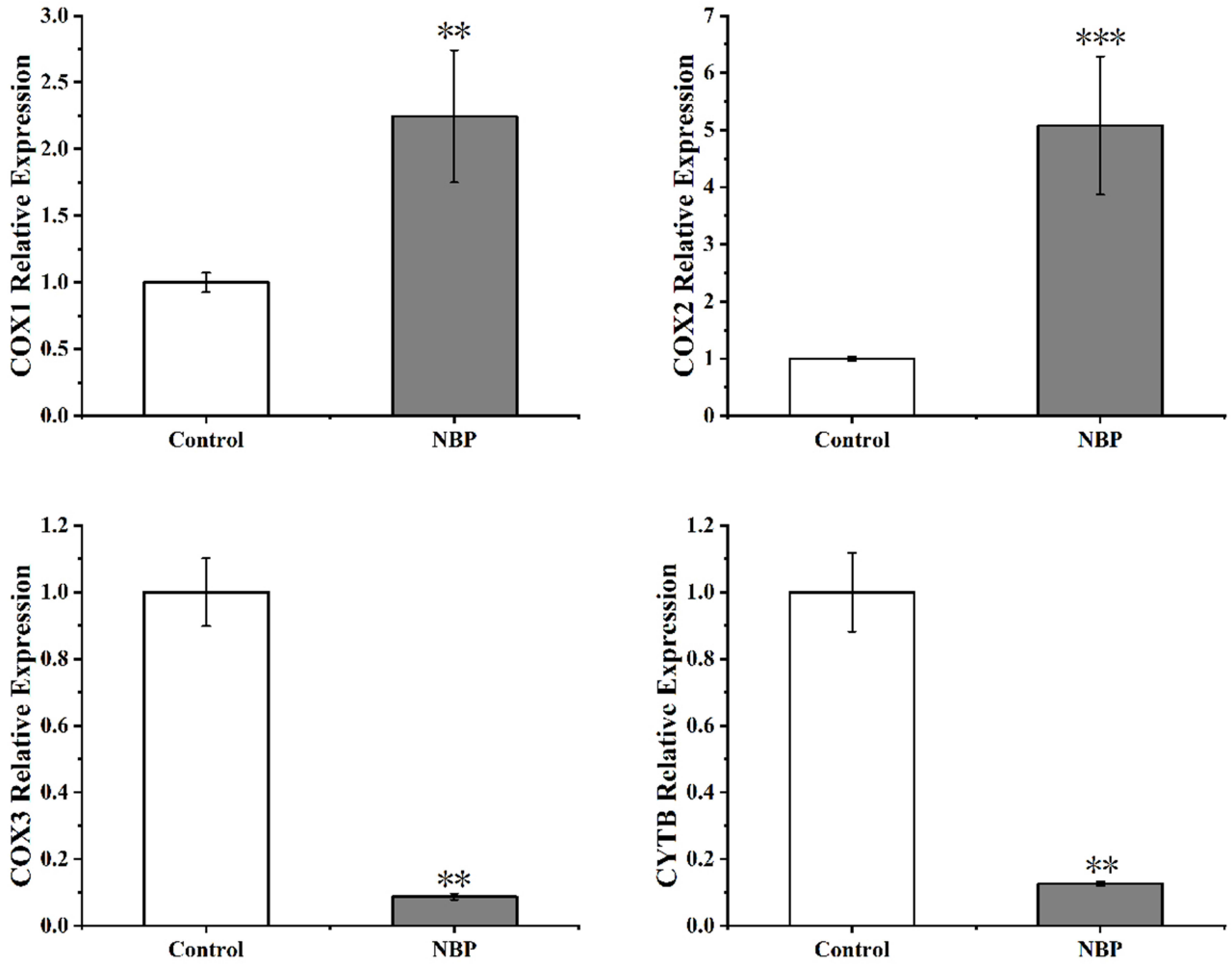

Figure 4 Effect of NBP on the gene expression of the mitochondrial respiratory chain enzyme of CT20(**P<0.01, ***P<0.001, compared to control group).

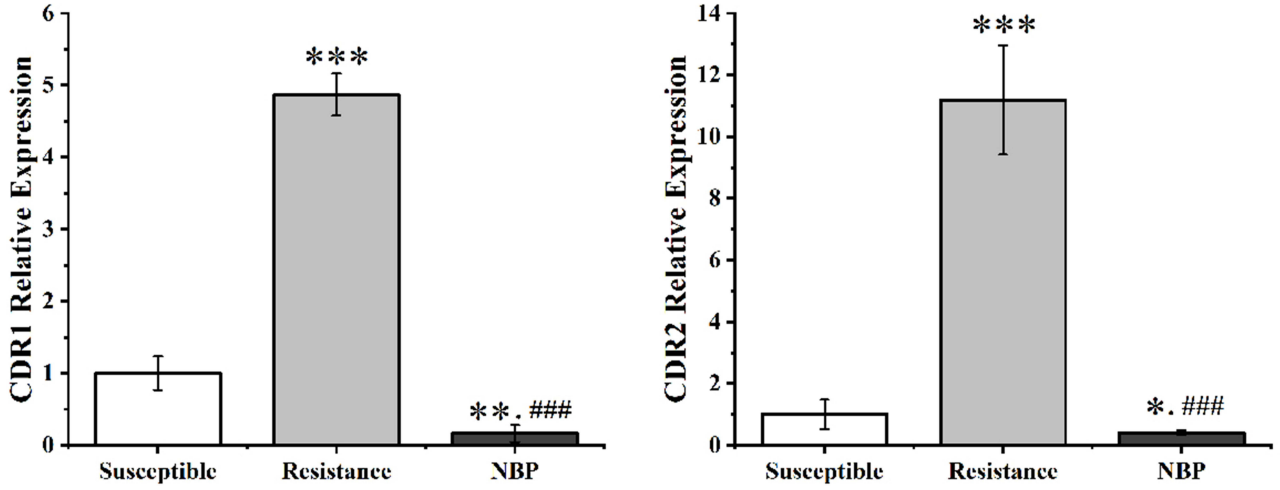

Figure 5 Effect of NBP on the expression of the CDR1 and CDR2 genes of CG19(*P<0.05, **P<0.01, ***P<0.001, compared to the susceptible group; ###P<0.001, compared to the resistance group).

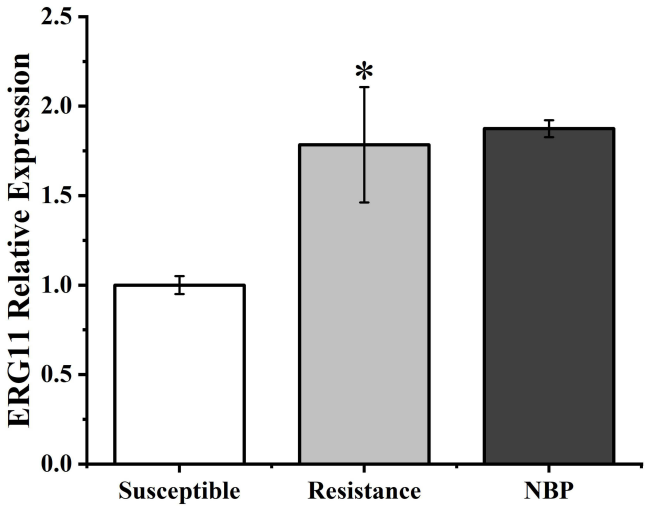

Figure 6 Effect of NBP on the expression of CT20 ERG11 gene (*P<0.05, compared to the susceptible group).

Therefore, it was hypothesized that NBP would increase intracellular ROS production by interfering with normal cellular oxidative metabolism and producing antibacterial effects.

Reversal of Drug Resistance Effects and Mechanisms of NBP

NBP reduced the MIC of FLC by 6–10 dilutions and has a partially synergistic effect on drug-resistant *Candida glabrata* and *Candida tropicalis* when combined with FLC. Compared to previous studies,[34] the potentiation effect of NBP on FLC differed between *Candida* spp., which may have the following reasons.

First, there are some morphological differences between different *Candida. Candida albicans* can produce true hyphae and pseudo-hyphae from the germ tube, while *Candida glabrata* cannot form hyphae. The ability to produce hyphae of *Candida tropicalis* is inferior to that of *Candida albicans*.[45] The inhibitory effect of NBP on biofilm formation and cell conversion from the yeast phase to the mycelial phase may differ in the three *Candida* species.

Moreover, the drug resistance mechanisms of *Candida* spp. are not identical. The drug resistance mechanisms of *Candida* to azoles include alteration of target enzymes and increased drug efflux.[17–19] The target enzyme of azoles refers to cytochrome P450 lanosterol 14α-demethylase (ERG11p) encoded by the EGR11 gene. Mutations in the target enzyme result in altered affinity for azoles. Up-regulation of target enzyme expression requires larger drug doses to produce antimicrobial effects, both of which can lead to drug resistance.[46] *Candida*'s main drug efflux proteins are ATP-binding cassette transporters (ABCT) and the main facilitator superfamily (MFS). The proteins associated with azole drug resistance are CDR1, PDH1 (CDR2), SNQ2 in ABCT, and Mdr1 and TOP3 in MFS. When exocytosis increases, it decreases the intracellular drug concentration, leading to drug resistance. Mechanisms of FLC resistance in *Candida albicans* are mainly due to increased drug efflux and mutation or overexpression of drug target enzymes, in *Candida tropicalis* primarily due to mutation or overexpression of target enzyme genes, and in *Candida glabrata* mainly due to increased drug efflux, with CDR1 and CDR2 as the main efflux proteins.[17–19,47–50] Thus, NBP may have different effects on the reversal of drug resistance in the three species of *Candida*.

This study showed that the gene expressions of CDR1 and CDR2 of drug-resistant *Candida glabrata* (CG19) were up-regulated compared to the sensitive control group. Up-regulation of CDR1 and CDR2 gene expressions in drug-resistant *Candida glabrata* was reversed after NBP treatment, and the gene expressions were lower than those in sensitive strains. NBP enhances the antifungal effect of FLC against drug-resistant *Candida glabrata* by inhibiting drug efflux. Compared to sensitive controls, the ERG11 gene expression of resistant *Candida tropicalis* (CT20) was up-regulated, and there was no significant change after NBP treatment. It is speculated that NBP does not reverse drug resistance by downregulating the expression of drug target enzymes. Further research is required to verify whether NBP affects the target enzyme mutations.

Limitations

NBP has antifungal activity against resistant and susceptible *Candida*. However, its use in vivo may be limited due to the large MIC values. Thus, its potential benefit against invasive *Candida* infection in clinical practice may be limited. However, for superficial candidiasis, which accounts for approximately 1% of dermatology outpatients and 7% of hospitalized patients, improper treatment can lead to invasive candidiasis.[51,52] NBP, as a natural plant extract, may have potential development value as an in vitro treatment. NBP, as a long-standing drug in clinical practice, exhibits few adverse reactions and good safety profile. Moreover, it also demonstrates certain anti-inflammatory and immunomodulatory effects, probably through the mitigation of oxidative stress and modulation of the Nuclear factor-kappa light chain enhancer of activated B cells (NF-κB) pathway.[53,54]

This study showed that the effect of NBP on *Candida* mitochondrial respiratory chain enzymes was opposite to its protective effect on neuronal cells.[55] *Candida* can use hexose for aerobic oxidation and anaerobic glycolysis. It can also use two-carbon compounds for the glyoxylate cycle and gluconeogenesis. The two pathways interact,[56,57] and the effect of NBP on *Candida* may be related to this. Relevant experiments should be conducted to investigate the relationship described above, and a subsequent collection of drug-resistant strains can verify individual differences between strains.

Conclusion

In this study, static and dynamic antifungal effects were investigated. NBP was found to have antifungal effects against sensitive and resistant *Candida glabrata* and *Candida tropicalis* and partially reversed resistance to FLC in *Candida glabrata* and *Candida tropicalis*. Gene expression studies found that NBP could inhibit the gene expression of the *Candida* mitochondrial respiratory chain enzyme and reverse the overexpression of the drug efflux pump gene of resistant *Candida glabrata*. The targets for the action of NBP for interfering with mitochondrial function were found to be mitochondrial respiratory chain enzyme complexes III and IV, providing ideas for future studies and new possible targets for drug development.

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Disclosure

The authors declared that they have no conflicts of interest in this work.

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