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EFFECTS OF ANTIFUNGAL DRUG INTERACTION PATTERNS ON THE GROWTH OF SOME CANDIDA SPECIES

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ABSTRACT

Background: Fungal pathogen especially opportunistic mycoses of Candida origin has been on a significant increase nowadays. Finding more on the way commonly available antifungal drugs interact could be of interest in maximizing treatment outcomes of patients suffering from fungal infections. **Objective:** To evaluate the effects of antifungal drug associations on the growth of some Candida species. **Method:** Fluconazole and clotrimazole (azole) and nystatin and amphotericin B (polyene) were bought from licensed pharmacies. Six fungal clinical isolates were used in this study. Broth microdilution was used to determine the MIC of the different combinations of the antifungal drugs at various concentrations from 512 to 0.0625 µg/ml. Checker board method and fractional inhibitory concentration index (FICI) were used to determine the interaction patterns when azoles were associated with polyenes. Fungal growth kinetics and effects of various test combinations on total fungal protein levels were evaluated. Data was analyzed using Statistical Package for Social Science. **Results:** It was observed that interaction patterns for a given combination varied across the studied fungi. FLU+NYS gave s synergistic effect on *C. albicans* CPC2091 and *C. glabbrata* but had no interaction the other fungi. FLU+NYS exhibited mostly antagonistic effects on 4/6 of the fungi no interaction on 2/6. The time-kill curves further elucidated synergistic combinations of FLU+NYS and CLO+NYS. The combined effect fluconazole with nystatin on *C. albicans* CPC2091 increased fungal proteins at all concentrations on like clotrimazole and nystatin on *C. dubliniensis*. **Conclusion:** The antifungal drug interactions studied in this paper showed varying synergistic, no interaction and antagonistic patterns on the studied fungi based on types of drugs associated. Use of fungal drug combination therapy warrants proper diagnosis of disease agent and knowledge of drug association effects.

KEYWORDS: Antifungal drugs, interaction patterns, growth kinetics, proteins, Candida species.

INTRODUCTION

There has been a significant increase in the rate fungal pathogen especially opportunistic mycoses of Candida origin.[1] The treatment of fungal infections necessitate use of antifungal drugs like azoles, echinocandins, flucytosine, and amphotericin B which belong to different pharmacological classes and possess distinct mechanisms of action.[2] Finding more on the way these drugs interact could be of interest in maximizing treatment outcomes of patients suffering from fungal infections.

The mechanism of action of polyenes, such as nystatin and amphotericin B, is by interfering with ergosterol. They bind to ergosterol thereby causing cell membrane leakage. Amphotericin B is used for the treatment of severe, potentially life threatening fungal infections even though very toxic.[3] Other antifungals like echinocandins (caspofungin) inhibit the synthesis of beta-1,3-glucan while flucytosine inhibits fungal RNA and DNA synthesis.[4,5]

Some studies on the combinations of polyene and azole have revealed that the azole compound is largely the beneficiary. For example, amphotericin B plus triazole; but these effects have usually not been superior to the results seen with amphotericin B alone. Better overall efficacies are sometimes obtained with lower dose requirements for an azole (amphotericin B).[6,7] Other researchers have reported a more of indifference

response than synergy when amphotericin B and fluconazole were combined.[8,9]

The search for new strategies from drug combinations to treat candidiasis is worth giving a try. Some studies have reported on possible combinations of antifungal drugs, but these research have been reported as being poorly evaluated in medical mycology.[10]

The aim of this study was to investigate the *in vitro* interactions among fluconazole, clotrimazole, nystatin and amphotericin B in double combinations against clinical isolates of *Candida albicans*.

MATERIALS AND METHODS

Drugs

The drugs to be tested below were chosen from the classification of antifungal drugs and two drug groups were available in the pharmacies.

Fluconazole and Clotrimazole

They belonging to the azole group which inhibits lanosterol-14α-demethylase the enzyme required to convert lanosterol into ergosterol (inhibition of ergosterol synthesis).

Nystatin and Amphotericin B

These ones belong to the polyene group which disrupts the fungal cell membrane by binding to ergosterol, the main sterol in the membrane, resulting in pore formation and leakage of cellular cations and anions leading to fungal cell death.

These drugs (Table 1) were obtained from licensed pharmacies in Mbouda and Dschang, West Region of Cameroon.

Table 1: Information on drugs used in the study.

Drug Name	Brand Name	Manufacturer	Batch/Reg Number	Active Ingredient	Reference Dates	Pharmaceuti-cal Form
Fluconazole 100mg	Flugen	Generic healthcare PUT.LTD INDIA	GS170229	FluconazoleUSP 100mg	MFG: 02/2017 EXP:01/2020	Tablet
Cotrimazole	Paucotrim	PAUCO pharmaceutical IND-LTD Nigeria	Bat:09 Reg number: A4-0055	Sulphamethoxazole B.P 400mg Trimethoprim B.P 80mg	Mfg:09/17 Exp:05/2021	Tablet
Amphotericin B 10g	Fungizone	Haupt pharma wolfratshausen GMBH plaffenriender strasse 5 Allemagne	Lot:AI01	Amphotericin B 10g	Exp 05/2020	Syrup
Nystatine	Nystop	Pharm- inter sprl Bruxelles Belgique	LOT:171122	Nystatine 500.000UI	Mfg :11/2017 Exp :11/2020	Tablet

Microorganisms

A total of six fungal clinical isolates were used in this study. *Candida albicans*, coded CPC2091 was gotten from Centre Pasteur Cameroon while the other *Candida albican* and the rest of the isolates- *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida dubliniensis* were obtained from the Regional Hospital Bamenda. These isolates came from vagina smears of women who complained of symptoms similar to those of vagina yeast infections. Samples were confirmed by smear culture and microscopic observation.

Positive samples were differentiated by culture on CHROM agar. The different strains were then sub-cultured several times and conserved in glycerol and Sabouraud dextrose broth 50% each in the refrigerator at -2 degree Celsius.

Growth and Maintenance of Fungi

SDA and SDB (Liofilchem srl- Italy) were used for the isolation, culture and maintenance of fungi. Ciprofloxacin was added as a broad spectrum antimicrobial to inhibit the growth of a wide range of gram positive and gram negative bacteria after the medium was prepared and sterilized in the autoclave.[11]

Preparation of Drug Test Solution

The stock solutions were prepared by measuring the mass of the active principle in relation to each pharmaceutical dosage form. A tablet was completely dissolved in 4 ml of sterile distilled water in small bottles (penicillin bottles) and the concentration of the active principle calculated. Amphotericin B sirup was used directly.

Preparation of Fungal Inocula

Inocula were prepared from 18-24-hour old culture on SDA plates. With the aid of a flame sterilized wire loop a small amount of fungal strain on an agar plate culture was transferred to a tube containing 10 ml of sterile saline (0.9%) water. This was well mixed using the Heidolph top-mix 94323 shaker. It was then compared to 0.5 of Mac Farland solution to have a concentration of 1.5×10^6 cfu/ml.[12] An amount of 133.33 µl from this fungal suspension was further diluted in 10 ml broth to give a final concentration of 2.0×104 cfu/ml serving as fungal inocula.

Determination of Minimum Inhibitory Concentrations (MIC)

For the determination of MIC, Sabouraud dextrose broth was prepared according to the specification provided by manufacturer. It was then sterilized in the autoclave and allowed to cold. Ciprofloxacine was then added to prevent bacterial growth. A total amount of 9.5 ml of prepared culture broth was then transferred to the number of 96 well plates needed, 100 µl per well.

After preliminary work, a final concentration of 2048 µg/ml was chosen to make a first-line (line A) concentration of 1024 µg/ml then two fold serial dilutions were made to the rest of the well. After dilution at the last well, the content in the pipette tip was discarded giving a final volume of 100 µl of broth and drug in each well for all plates. Then 100 µl of prepared fungal inocula was pipette and added to all the wells. Two plates were used per drug to obtain drug test concentration range as 512, 256, 128, 64, 32, 16, 6, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg/ml. Sterility control wells had only the test fungal drug and broth but no inocula. The plates were then labelled, sealed and incubated for 48 hrs and the MIC was read thereafter. The MIC was read by observing the under plates with the naked eyes. The lowest drug concentration having a clear well as compared to the control was considered as the MIC.[13]

Determination of the Effects of Antifungal Drugs Associations on the Growth of Candida spp

Drugs were tested single and in combinations of two at various fractions of MIC values from 512 to 0.25µg/ml using the micro dilution checkerboard method. These drugs were tested on the six fungal isolates. The different antifungal combinations: fluconazole + nystatin (FLU+NYS); fluconazole + amphotericin B (FLU+AMP); clotrimazole + nystatin (CLO+NYS); and clotrimazole + amphotericin B (CLO+AMP).

Isolates were activated the previous day and the day of the work SDB was prepared and sterilized and ciprofloxacin added. To each well in the 96 well plates 100 µl of broth was added. Later 100 µl of one drug e.g: drug A was added and serial dilutions done. The concentration of this first drug increases vertically. Then different concentrations (two-fold dilutions) of the second drug (drug B) were prepared in small flasks and 50 µl of each concentration set added to the corresponding column of the first drug already in the plates. An amount of 50 µl of prepared inoculum in broth was finally added. Four wells were left as negative control and four wells were added inoculum without drugs as the positive control. Each well had a final volume of 200 µl content. The first column line of wells and the second to the last row were used for the single drugs alone without combination.[14] The plates were then labelled, sealed and incubated for 48 hrs. Tests were repeated 3 times. By observing the under plates with the naked eyes, the effects of antifungal drugs associations on the growth of fungi was sorted. The fractional inhibitory concentration index (FICI) for each drug combination per fungus was calculated as the sum of the MIC of each drug when used in combination divided by the MIC of the drug when used alone.[15,16] FICI values ≤0.5 represent synergy (S); >0.5 to 4 represent no interaction (NI); and >4 represent antagonism (A).

Determination of Kinetics for Drugs Combination

Candida dubliniensis (CLO+NYS), Candida albicans strain 1 (FLU+NYS), and Candida glabrata (CLO+NYS) which showed more synergistic effects were chosen among those that had a synergistic effect. They were then tested using the chequerboard method repeated with 6 plates (each plate corresponds to 8, 16, 24, 32, 40 and 48 hrs different) per combination of isolate done. After every 8hrs one plate was removed from each group up to 48 hrs when the sixth plate was removed from the incubator. From these plates 200 µl was removed from each well with growth, put into small labelled tubes and solubilised with sterile distilled water. These tubes were then centrifuged, supernatant discarded and the pellets resolubilised in sterile distilled water. The optical density was read at 420 nm using the Genesy 20 spectrophotometer.[14]

Determination of Drug Combination Effects on Fungal Proteins

Two isolates from above *Candida albicans* CPC2091 (FLU+NYS) and *Candida dubliniensis* (CLO+NYS) were used to determine the effects of drug combination on the quantity of proteins. For the combined drugs, one was kept at a constant concentration while the other varied over three concentrations for each fungus. Then the latter was kept constant and matched with various concentrations of the former.

Briefly, 20 µl of each microbial inocula was introduced into each tube containing 2980 µl of single or combined drugs in broth making a final volume of three millilitres. The tubes were then covered with cotton and incubated at 30 degrees Celsius for 48 hrs on a plate shaker. After 48 hrs the tubes were centrifuged, the supernatant discarded and the pellet suspended in 950 µl of sterile distilled water. Fifty microlitres of concentrated sulphuric acid was added to the 950µl solution in small bottles. This was meant to lyse the cells. After 30 minutes these different solutions in small bottles were transferred to labelled Eppendorf tubes and centrifuged. The pellet with cell debris discarded and the supernatant collected into a new set of labelled Eppendorf tubes. Their optical densities were then measured at 260 nm (to determine DNA and RNA contaminations) and 280 nm (for protein quantification) and recorded. The protein concentration in mg/ml was then calculated as: Protein = (1.55×A280) - (0.76×A260).[17,18] Each assay for single drugs or in combinations was repeated 3 times.

Statistical Analysis

Statistical evaluation of the data generated was carried out using Statistical Package for Social Science (SPSS) version 20. Multiple comparisons were done using Student-Newman-Keuls at $p<0.05$ where appropriate.

Ethical Issues

This work was done under the administrative authorization from the North West Regional Delegation of Public Health (N^0.54/ATT/NWR/RDPH/2019).

RESULTS

Effects of Various Drug Combinations on Fungal Growth

The results for drug combinations as presented using fractional inhibitory concentration index (FICI) showed various interaction patterns on fungal growth (Table 2). FICI was defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug when used alone. This interaction pattern was considered as: FICI of ≤0.5 represented synergy (S), >0.5 to 4 represented no interaction (NI), and >4 represented antagonism (A). It was observed that the interaction pattern for a given combination varied across the studied fungi. A combination of FLU+NYS gave synergistic effect on *C. albicans* CPC2091 and *C. glabbrata* but had no interaction the other fungi. A combination of FLU+NYS exhibited mostly antagonistic effects on 4/6 of the fungi with no interaction on 2/6. Also combining CLO + AMP gave a lone synergistic effect on *C. krusei*. The interaction of FLU and the two polyenes was however not similar on the two *C. albicans* isolates.

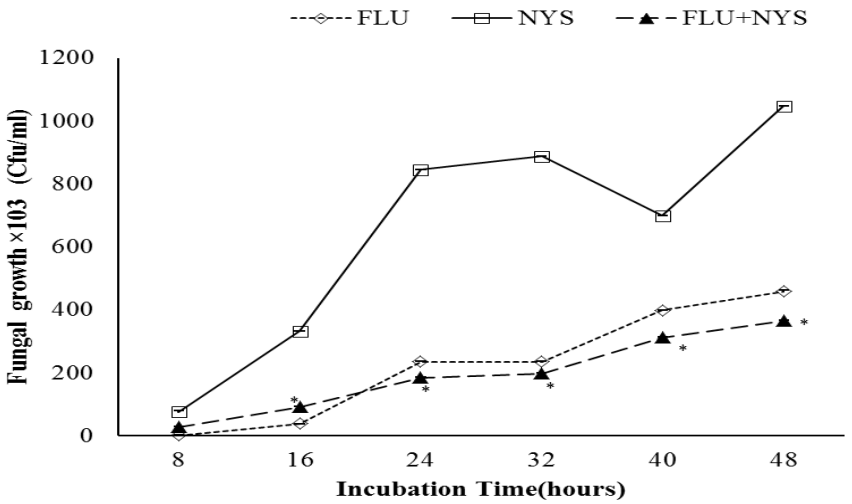
Table 2: Interaction patterns of combination drug effects on fungal growth.

Fungi	Drug 1	MIC (µg/ml)	Drug 2	MIC (µg/ml)	Drug 1 + drug 2	MIC (µg/ml)	FICI	Interaction pattern
Candida albicans CPC2091	FLU	512	NYS	512	FLU/NYS	16/64	0.15	S
	FLU	512	AMP	0.5	FLU/AMP	4/0.25	0.51	NI
	CLO	512	NYS	512	CLO/NYS	512/512	2	NI
	CLO	512	AMP	0.5	CLO/AMP	512/0.5	2	NI
Candida albicans	FLU	512	NYS	512	FLU/NYS	512/512	2	NI
	FLU	512	AMP	0.5	FLU/AMP	512/8	5	A
	CLO	512	NYS	512	CLO/NYS	512/512	2	NI
	CLO	512	AMP	0.5	CLO/AMP	512/0.5	2	NI
Candida krusei	FLU	512	NYS	512	FLU/NYS	64/512	1.12	NI
	FLU	512	AMP	4	FLU/AMP	64/64	16.13	A
	CLO	512	NYS	512	CLO/NYS	256/256	1	NI
	CLO	512	AMP	4	CLO/AMP	128/1	0.5	S
Candida glabbrata	FLU	512	NYS	512	FLU/NYS	64/64	0.25	S
	FLU	512	AMP	2	FLU/AMP	4/0.5	4	NI
	CLO	512	NYS	512	CLO/NYS	8/64	0.14	S
	CLO	512	AMP	2	CLO/AMP	4/0.5	4	NI
Candida parapsilosis	FLU	512	NYS	512	FLU/NYS	512/512	2	NI
	FLU	512	AMP	1	FLU/AMP	128/8	8.25	A
	CLO	512	NYS	512	CLO/NYS	512/512	2	NI
	CLO	512	AMP	1	CLO/AMP	512/1	2	NI
Candida dubliniensis	FLU	8	NYS	512	FLU/NYS	2/512	1.25	NI
	NYS	512	AMP	1	FLU/AMP	512/1	65	A
	AMP	1	NYS	512	CLO/NYS	128/32	0.31	S
	CLO	512	AMP	1	CLO/AMP	512/2	3	NI

FLU= fluconazole, CLO= clotrimazole, NYS= nystatin, AMP= amphotericin B, S, synergism; A, antagonism; NI, no interaction; FICI, fractional inhibitory concentration index.

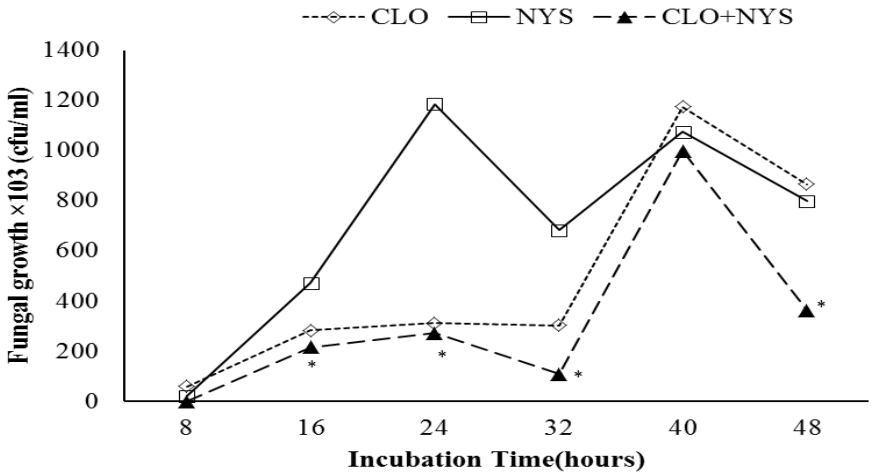
Effects of Drug Combinations on Fungal Growth Kinetics

The time-kill curves technique, which measures the fungicidal activity of the tested drugs single/and in combinations were used to provide a dynamic picture of interaction over time for the most synergistic combinations. The results are displayed in colony forming units (CFU/ml) of viable yeast cells per incubation time. From these curves, (Fig. 1- FLU+NYS on *C. albicans* CPC2091; Fig. 2- CLO+NYS on *C. dubliniensis*; Fig. 3- CLO+NYS on *C. glabbrata*) the synergistic interaction showed the greatest killing effect translated here as lowest viable fungal cells at the growth intervals considered.



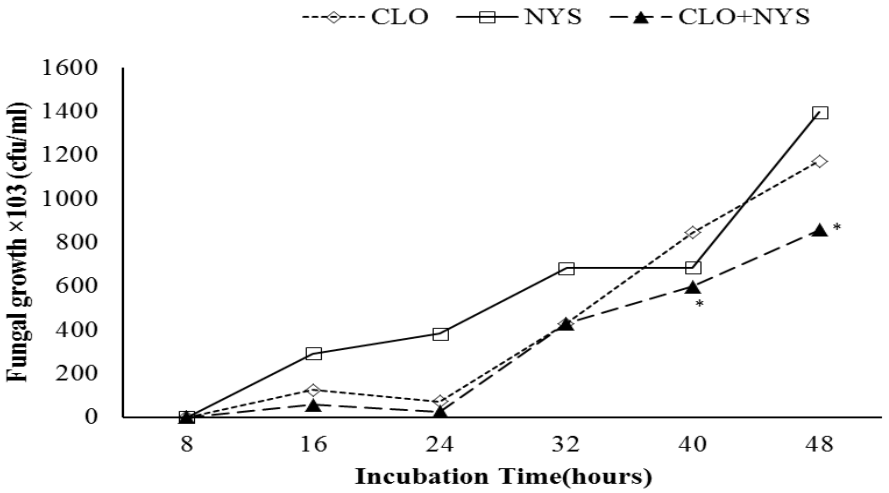
FLU=fluconazole, NYS=nystatin, FLU+NYS=fluconazole plus nystatin

Figure 1: *C. albicans* CPC2091 growth rate (viable cells) in the presence of single and combined drugs.



CLO=clotrimazole, NYS=nystatin, CLO+NYS=clotrimazole plus nystatin

Figure 2: Evolution of *C. dubliniensis* viable cells per time in the presence of drugs.

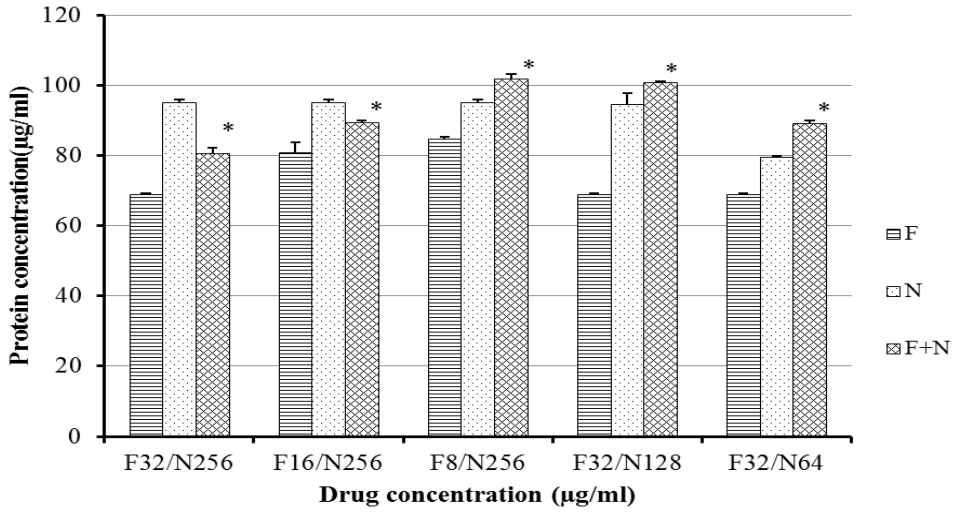


CLO=clotrimazole, NYS=nystatin, CLO+NYS=clotrimazole plus nystatin

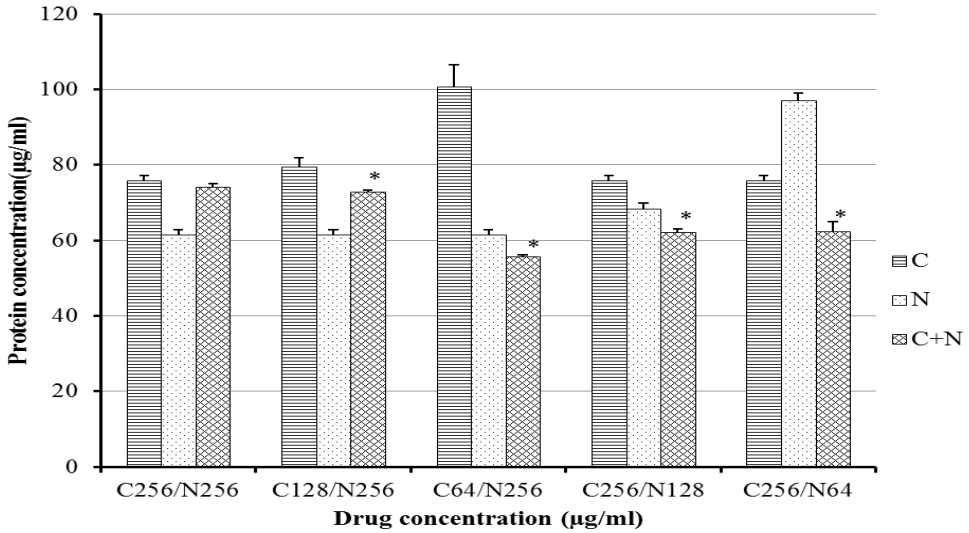
Figure 3: Evolution of Candida glabbrata viable cells per time in the presence of drugs.

Variation of Fungal Protein Levels at Various Drug Combination Concentrations

The leel of proteins for single and combined drugs were studied at concentrations below their MIC values. The results of fluconazole at 32, 16 and 8 µg/ml single and in combination with a fixed MIC/2 (256 µg/ml) of nystatin action on *C. albicans* CPC2091 (Fig. 4) showed a general increase in fungal proteins for all the combined concentrations compared to single drug effects. However the protein levels were observed to decrease generally when clotrimazole and nystatin were combined at various concentrations and tested on *C. dubliniensis* (Fig. 5).



F-Fluconazole at concentrations of 32, 16 and 8µg/ml; N- Nystatin at 256, 128 and 64 µg/ml
Figure 4: Effects of fluconazole and nystatin combinations on *C. albicans* CPC2091 protein levels.



C-Clotrimazole at concentrations of 256,128 and 64µg/ml; N-Nystatin at 256, 128 and 64 µg/ml.
Figure 5: Effects of clotrimazole and nystatin combinations on *C. dubliniensis* protein levels.

DISCUSSION

Combining drugs having different modes of action could be vital in treating invasive fungal infections. These drugs, acting at different target site could reinforce each other, allowing a decrease in doses and thus reducing side effects for patients.[19] In this study we focused on azoles and polyenes as membrane-active drugs such as against Candida because of somewhat limited data addressing the relationship.[20,21]

The results for drug combinations as presented using fractional inhibitory concentration index (FICI) showed various interaction patterns on fungal growth. FICI of ≤0.5 represented synergy (S), >0.5 to 4 represented no interaction (NI), and >4 represented antagonism (A). Combining FLU with NYS gave synergistic effect on *C. albicans* CPC2091 and *C. glabbrata* but had no interaction the other fungi. Though the mode of action of each of these drugs is well elucidated, their combination may result to some kind of interactions/reactions producing other unknown substances susceptible to

influence the overall drug activity vis-à-vis the microorganism under study.[22,23,24]

A combination of FLU+NYS exhibited mostly antagonistic effects on 4/6 of the fungi no interaction on 2/6. Also combining CLO + AMP gave a lone synergistic effect on *C. krusei*. The interaction of FLU and the two polyenes was however not similar on the two *C. albicans* isolates. It has been reported that the effects of drug combinations on fungal growth *in vitro* heavily depend upon the ratios and concentrations of the drugs employed, as well as the fungal strains tested. Drug combinations can have a synergistic, additive, antagonistic, or indifferent effect, depending upon the nature and relative concentrations of the drugs used.[25]

Most of the drug interactions on these fungi were antagonistic or no interaction. Probably some inherited characteristics regarding drug metabolism may lead to such situations. Mostly antagonistic/no interactions have been reported for *C. krusei* at various drug combinations.[12,16,19,26,27]

Drug combinations that exerted synergy with very small FICI values ≤0.5 (FLU+NYS and CLO+NYS) were used for growth kinetics study. Fungal growth kinetics under the fungicidal activity of single and combined drugs were expressed as evolution of viable cells for the study duration. The synergistic interaction showed the greatest killing effect translated here as lowest viable fungal cells at the growth intervals considered for *C. albicans* CPC2091, *C. dubliniensis* and *C. glabbrata*). The nature of interactions depicted by time-kill curves is similar to those reported by other researchers.[28,29]

In this study, we observed a general increase in fungal proteins for all the combined concentrations compared to single drug effects for fluconazole and nystatin action on *C. albicans* CPC2091. This observation was however different for *C. dubliniensis* when subjected to the combined effects of clotrimazole and nystatin as the protein levels decreased generally at various combined concentrations. Normally the combined drugs in this assay showed synergistic interactions, so it is expected that less growth of fungal cells due to action combined antifungal agents would lead to lower protein levels like the case of *C. dubliniensis* in presence of CLO+NYS.

In the case of *C. albicans* CPC2091 treated with FLU+NYS probably a mechanism was put in place to increase fungal protein as observed in the results. The results from growth kinetics indicated a reduction in viable fungal cells when treated with FLU+NYS. One could think that this fungus increased its protein level in a way to minimize the harmful effects of these drugs. It has been reported that some fungi have developed resistance mechanisms, such as over expression of efflux pump proteins.[30] Moreover, some proteins like Heat shock protein 90 (Hsp90) is known to be synthesized as an adaptive response to noxious conditions in order to enhance survival of pathogenic microorganisms.[31]

CONCLUSION

The antifungal drug interactions studied in this paper showed varying synergistic, no interaction and antagonistic patterns on the studied fungi based on types of drugs associated. Use of fungal drug combination therapy warrants proper diagnosis of disease agent and knowledge of drug association effects. Since it cannot be assumed that the use of two or more effective drugs with distinct mechanisms of action would produce an improved outcome compared to using a single compound.

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