

Full Length Research Paper

***In vitro* evaluation of antimicrobial activity of combinations of nystatin and *Euphorbia hirta* leaf extract against *Candida albicans* by the checkerboard method**

Clement Jackson^{1*}, Akeem Agboke¹ and Victor Nwoke²

¹Department of Pharmaceutics and Pharmaceutical Technology, University of Uyo, Nigeria.

²University Teaching Hospitals, Port Harcourt, Nigeria.

Accepted 30 June, 2009

The *in vitro* combined effects of Nystatin and methanol extract of leaves of *Euphorbia hirta* against clinical isolates of *Candida albicans* were investigated using the Checkerboard technique. The organism was susceptible to the extract with MIC of 25 mg/ml, while Nystatin had MIC of 0.165 mg/ml against *C. albicans*. Upon combination, some ratios showed synergistic while others indicated indifferent activities against the isolates. These results indicate that some combinations of the extract with nystatin could be synergistic in activity for some ratio combinations and indifferent for some others.

Key words: *In vitro*, nystatin, *Euphorbia hirta*, *Candida albicans*, checkerboard

INTRODUCTION

Simultaneous use of two or more antimicrobial agents has certain rationale and is recommended in specifically defined situations (Esimone et al., 2006a; Esimone et al., 2006 b).

Several reasons have been advanced to justify the use of combination of two or more antibiotic treatment (Esimone et al., 2006 b; Ibezim et al., 2006). For many years now; combination of two or more antibiotics has been recognized as an important method for, at least, delaying the emergence of bacterial resistance (Chambers, 2006). Besides, antibiotic combinations may also produce desirable synergistic effects in the treatment of bacterial infections (Zinner et al., 1981).

However, the selection of an appropriate combination requires an understanding of the potential for interaction between the antimicrobial agents.

Accordingly, methods have been developed to quantify the effect of antimicrobial combinations on bacterial growth *in vitro*. Two very distinct traditional methods of testing *in vitro* antibiotic interaction are the checkerboard technique and the time killing curve method (Eliopoulos

et al., 1988).

Chinwuba et al., 1991 developed a technique known as Overlay Inoculum Susceptibility Disc (OLISD) method, which is essentially a modification of the Disc agar diffusion method. Sanders et al also went ahead to describe a new *in vitro* test for antimicrobial agents used in combination (Sanders et al., 1993). This new method, the Decimal Assay for Additivity {DAA} was based on Disc diffusion assay and was designed to have a precisely defined end point for additivity so that interactions greater or less than additivity could be respectively defined as synergism and antagonism respectively.

Nystatin is a tetraene macrolide produced by *Streptomyces noursei*. Its mechanism of action involves binding to ergosterol of sensitive fungi. This action forms pores or channels which result in increased permeability of the fungal membrane allowing leakage of intracellular contents. Other mechanisms of actions include oxidative damage to fungal cells. Nystatin is basically used for candidiasis and is supplied in preparations intended for cutaneous, vaginal, or oral administration for this purpose (Goodman and Gilman, 2001).

The leaves of *Euphorbia hirta* are found to contain triterpenoids, sterols, alkaloids, glycosides and tannin (Anozie, 1991).

*Corresponding author. E-mail: clementjackson2008@yahoo.com. Tel: 2348035499427, 23485660597.

The plant has a reputation as remedy for bronchitis, asthma, eczema, laryngeal spasm and cough (in liquid extract or tincture form). Other uses include lactation, as tonic, anthelmintic, anticonvulsant, mild sedative and antimicrobial agent and in the treatment of wounds and tumors.

In this study, the interaction between nystatin and methanol extract of *E. hirta* leaves has been investigated using Checkerboard method. The results of this research could provide rational basis for the use of standardized herbal drugs in combination therapy of prevailing diseases

MATERIALS AND METHODS

Culture media

The media employed for the study are: Nutrient agar and Sabouraud dextrose agar containing 0.4% Chloramphenicol.

Test microorganisms

Clinical isolates of *Candida albicans* were obtained from the Department of Pharmaceutical Microbiology, University of Nigeria, Nsukka.

Plant materials

The leaves of *E. hirta* were collected from Nsukka, Enugu state, Nigeria. The plant was authenticated by Mr. A. O. Ozioko of Bioresources Development and Conservation Programme, Nsukka and stored in Department of Pharmacognosy, University of Nigeria with Voucher Number; E. h cco; 002

Extraction of *Euphorbia hirta* leaves

The dried leaves were extracted with appropriate quantity of methanol. The solution was allowed to dry under the atmosphere to concentrate the extract and stored in the refrigerator.

Sterilization of materials

The Petri dishes and pipettes packed into metal canisters were appropriately sterilized in the hot air oven (Ov-335, Hareus) at 170°C for 1 h at each occasion. Solution of the extract and culture media were autoclaved at 121°C for 15 min.

Preparation of culture media

All culture media were formulated according to manufacturers' specification. Basically for nutrient agar, this involves appropriate weighing of nutrient agar, distributing into bijou bottles (in 20 ml) and then sterilization using autoclave at 121°C, 151 b/sq. inch for 15 min; then allowed to cool to 45°C before pouring into the agar plate. The pH of the agar medium was maintained at 7.4.

Sabouraud Agar Media equally was homogenized in distilled water with the aid of heat adding 0.4% Chloramphenicol dissolved in ethanol and distributed in bijou bottles and sterilized with autoclave as above.

Maintenance and standardization of test organisms

The organism (*Candida albicans*) was maintained by weekly sub culturing on sabouraud agar slant. Before each experiment, the organism was activated by successive sub culturing and incubation. Standardization of the test microorganism was done according to previously reported method (Chinwuba et al.,; Esimone et al., 1999).

Preparation of drug stock solution

The stock solution of *E. hirta* leaf extract was prepared on each occasion by careful weighing and dissolving in suitable volume of Dimethylsulphoxide (DMSO) to get a concentration of 50 mg/ml. A tablet of Nystatin was dissolved in appropriate volume of water to get 16.67 mg/ml of stock solution.

Sensitivity of test microorganism

The sensitivity of the test microorganism to the methanol extract and nystatin was evaluated by determining the minimum inhibitory concentration (MIC) of both using the two fold broth dilution technique previously described.

Evaluation of combined effects of *Euphorbia hirta* methanol extract and Nystatin

Stock solutions of *E. hirta* (50 mg/ml) and nystatin (16.67 mg/ml) prepared in double-strength nutrient broth and autoclaved at 121°C for 15 min were employed. Varying proportions of the Extract (E) and Nystatin (N) were prepared according to the continuous variation Checkerboard method previously described by NCCLS, 1990.

Each proportion of the herbal extract/ nystatin combination was serially diluted (2 fold) , inoculated with 0.1 ml of 10^6 cfu/ ml culture of test microorganism and then incubated for 24 h at 37°C. Interaction was assessed algebraically by determining the fractional inhibitory concentration (FIC) indices according to the equations below:

$$FIC_{\text{index}} = FIC_{\text{Extract}} + FIC_{\text{Nystatin}} \quad \text{Eqn 1}$$

$$FIC_{\text{Extract}} \text{ (fractional inhibitory concentration of Extract)} \\ = \frac{\text{MIC of Extract in combination with Nystatin}}{\text{MIC of Extract alone}} \quad \text{Eqn 2}$$

$$FIC_{\text{Nystatin}} \text{ (fractional inhibitory concentration of Nystatin)} \\ = \frac{\text{MIC of Nystatin in combination with Extract}}{\text{MIC of Nystatin alone}} \quad \text{Eqn 3}$$

RESULTS

The combined effects of *Euphorbia hirta* leaf extract and nystatin against *Candida albicans* are presented in Table 1.

DISCUSSION

Combined drug use is occasionally recommended to

Table 1. Combined Activity of *E. hirta* Leaf extract and nystatin against *Candida albicans*.

Ratio of Drug Combination Nys : Euph.	MIC Nys (mg/ml)	MIC Euph. (mg/ml)	FIC Nys	FIC Euph.	FIC Index	Inference
10 : 0	0.165	-	-	-	-	-
9 : 1	0.148	1.25	0.9	0.05	0.95	Syn
8 : 2	0.132	2.5	0.8	0.1	0.9	Syn
7 : 3	0.116	3.75	0.7	0.15	0.85	Syn
6 : 4	0.099	5.0	0.6	0.2	0.8	Syn
5 : 5	0.165	12.5	1.0	0.5	1.5	IND
4 : 6	0.132	15.0	0.8	0.6	1.4	IND
3 : 7	0.099	17.5	0.6	0.7	1.3	IND
2 : 8	0.066	20.0	0.4	0.8	1.2	IND
1 : 9	0.033	22.5	0.2	0.9	1.1	IND
0 : 10	-	25	-	-	-	-

Key:

MIC = Minimum Inhibitory Concentration.

Nys = Nystatin.

Euph = *Euphorbia hirta*.

FIC = Fractional Inhibitory Concentration.

Syn = Synergism.

IND = Indifference.

prevent resistance emerging during treatment and to achieve higher efficacy in the treatment of infections and diseases. The combination is hoped to achieve a desirable synergistic effect in this study. Results of the systematic and scientific evaluation of the *in vitro* effects of *E. hirta* leaf extract and Nystatin have been presented in this paper.

FIC_{index} values < 1 were considered as synergy and the degree of synergy increases as the value tends towards zero. FIC_{index} values of 1 indicate additivity, values greater than 1, but less than 2 represent indifference while values greater than 2 show antagonism (Chinwuba et al.,; Esimone et al., 1999).

Based on these, synergistic effect was obtained by combination of Nystatin and *E. hirta* against *C. albicans* in the ratios (9:1, 8:2, 7:3 and 6:4) while others(5:5, 4:6, 3:7, 2:8,1:9) were indifference.

The results reveal that *E. hirta* extract has antifungal properties. A plausible mechanism of action could be suggested that the *E. hirta* leaf extract potentiated the activity of Nystatin, giving rise to synergism.

The result of these *in vitro* tests indicate that the combination of *E. hirta* leaf extract and Nystatin at a given ratio has a possible clinical significance in the treatment of fungal infection caused by *Candida albicans*. Unguided and indiscriminate combination may result to an effect or outcome which has no clinical significance.

Moreover, this herbal extract is widely available, cheap and quite safe. It also has mild side effects of nausea and vomiting.

In conclusion, it may be stated that there is a favorable interaction between *E. hirta* leaf extract and Nystatin against *Candida albicans* in some given combination

ratios.

ACKNOWLEDGEMENTS

We appreciate the members of technical staff of Department of Pharmaceutics, University of Nigeria, Nsukka for their assistance. We also acknowledge the staff of God's Glory Computers Institute, Uyo, Nigeria, especially Miss Grace Edet for typing the article for free

REFERENCES

- Anozie VC (1991). God's healing power in Plants. Clin. Pharm. Herb. Medium. 7: 23-25.
- Chamber HF (2006). General Principles of antimicrobial therapy, in Goodman and Gilman's Pharmacological Basis of therapeutics, Bruton LL (Ed), 11th Ed McGraw Hill; USA p. 1102-1104.
- Chinwuba GN, Chiori GO, Ghobashy AA, Okoro VC (1994). Determination of synergy of Antibiotic combination by overlay inoculum susceptibility disc method, Arzneimittel for schung/drug research 41: 148-150.
- De chene M, Leying H, Cullman W (1990). Role of the outer membrane for Quinolone Resistance in enterobacteria. Chemotherapy 36: 13-23.
- Eliopoulos GM, Eliopons CT (1988). Antibiotic combinations: Should be tested? Clin. Microbial. Rev. 1: 139-156.
- Esimone CO, Adikwu MU, Uzuegbu DB, Udeogaranya PO (1999). The effect of ethylenediaminetetraacetic acid on the antimicrobial properties of Benzoic acid and cetrime J. Pharm. Res. Dev. 4(1): 1-8.
- Esimone CO, Iroha IR, Ibezim EC, Okeh CO, Okpana EM (2006). An *in vitro* Evaluation of the interaction between tea extracts and penicillin G against *staphylococcus aureus* by the decimal assay for additivity (DAA) method. Afr. J. Biotechnol. 5(11): 1082-1086.
- Esimone CO, Iroha IR, Ude IG, Adikwu MU (2006). *In vitro* interaction of ampicillin with ciprofloxacin or spiramycin as determined by the

- Decimal assay for additivity technique. Nigerian J. Health Biomed. Sci. 5(1): 12 – 16.
- Goodman, Gilman's (2001). The Pharmacological basis of therapeutics. Tenth Edition Library of Congress Cataloging-in-Publication Limited pp. 1298-1310.
- Ibezim EC, Esimore CO, Obodo CE, Nnamani PO Brown SA, Onyishi IV (2006). .A study of the *in vitro* interaction of cotrimoxazole and ampicillin using the checker Board method. Afr. J. Biotechnol. 5(13): 1284-1288.
- National Committee for Clinical Laboratory standards (1990). Performance standards For Antimicrobial Disc susceptibility test 4th ed. Approved Document M2 – A4 (NCCLS) Villanova pa.
- Sanders CC, Sanders WE (Jr), Moland S (1993). Decimal assay for additivity of drugs p Permits delineation of synergy and antagonism. Antimicrob. Agents chemother 37: 260-264.
- Zinner SN, Klastersky J, Gaya BC, Riff JC (1981). *In vivo* and *in vitro* studies of three antibiotic combinations against Negative bacteria and Staphylococcus aureus. Antimicrob. Agents chemother. 20: 463 – 469.