Antifungal Activities of Pair Combinations of Extracts from *Morinda lucida* Benth by Decimal Additive Assay

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Abstract

Interactions between the root, leaf and stem-bark extracts of *Morinda lucida* were investigated for their antifungal activities against *Candida albicans* by using pair combinations in an in vitro decimal additive assay based on disk diffusion. Additive interactions were observed in each pair of the combinations. Neither synergistic nor antagonistic interactions were recorded. Phytochemical analysis suggests the presence of similar phytochemicals in all the extracts.

Introduction

Candida albicans is an oval budding yeast that produces a pseudomycelium in culture, tissues and exudates. It is an opportunistic fungal pathogen that causes candidiasis in persons with an underlying pathological process or deficiency state (Sobel, 1986). The fungicidal polyene compounds such as nystatin and amphotericin B provided the most effective treatment for candidiasis (despite their potential toxicity on humans) until recently with the emergence of the azoles antifungal compounds whose antifungal activities also are rather fungi static than fungicidal (Tariq et al 1995). These shortcomings among others have ignited interest in medicinal plant research aimed at discovering better treatment options in managing fungal infections at large and candidiasis in particular. In one of such studies in our laboratory, we identified *Morinda lucida* root,leaf and stem-bark extracts as having substantial antifungal activities especially on *Candida albicans.*(Nweze et al,2004). Improvements in the efficacy of antifungal drug therapy may be achieved by using combinations of drugs (Polak, 1990). To date however, there have been few investigations between antifungal agents (Tariq et al 1995,Odds et al 1986) especially with natural products. The aim of this study is to demonstrate additivity in pair wise combinations of *Morinda lucida* plant extracts against *Candida albicans* in vitro.

Materials and Methods

Isolate and test compounds: The isolate of *Candida albicans* used in the study was collected from the Mycology section of the Department of Microbiology, University of Nigeria, Nsukka, Nigeria. The isolate was maintained at 4 C on slants of sabouraud dextrose agar (SDA).*Morinda Lucida* extracts were obtained by placing separately 100g each of leaf, root and stem-bark in ground form into glass flasks containing 500ml of analytical grade Methanol. The mixture is allowed to stand for 48 hr at room temperature 28+1 C. At the end of this period, the extract from each flask was filtered and the filtrate concentrated using rotary evaporator. The dried extracts were then stored at 4+1 C and used later for the tests.

Standard dose-response curves: Samples (10ml) of sabauraud dextrose broth (SDB) were inoculated with *Candida albicans* and incubated overnight at 30 ^{0}C. The resulting spore suspension were diluted with SDB to provide a final concentration of 2x 10 cells per ml (estimated with a haemocytometer). A 500ul aliquot of the fungal suspension was transferred to a plate containing 20ml of SDA and the suspension was transferred to a plate containing 20ml of SDA and the suspension was distributed evenly over the surface of the medium. Crude extracts assay disks were prepared on the day of use by applying 20ul of an appropriate dilution of the test solution to 6mm diameter disks (Whatman International

Ltd, Maidstone, UK, No.1 filter paper) before placing them on the surfaces of the inoculated plates. The plate was then incubated at 30 C for 48 hr after which time the diameters of zones of inhibition surrounding the disks were measured (Miller et al 1984). Disk diffusion assays were initially performed for each extract on its own over a range of concentration (four replicate disks per concentration) in order to obtain a standard dose response curve by linear regression analysis.

Decimal additive assay (DAA) interactions between pairs of *M. lucida* extracts were investigated. For each pair of extracts to be tested, a target size of the zone of inhibition was selected from the midrange of the standard dose response curve for each of the pair of compounds so that increase or decrease in zone size due to compound interaction could be detected. The mass of each extract required to attain this zone size was calculated by using the linear regression equation for the standard dose response curve. Each mass defined as the biologic equivalence factor (BEF) represents the mass of each of the two compounds, which produced the same size of zone of inhibition. With the BEF calculated, a series of eleven decimal mixtures of the two extracts to be examined in combination was prepared and 20ul of each mixture was applied to four assay disks, which were then placed unto the surfaces of *Candida albicans*, inoculated plates. The plates were then incubated at 30*C for 18hr after which time, the diameters of the zones of inhibition surrounding the disks were measured; each of the eleven decimal mixtures should have generated the target size for the zone of inhibition (ie exhibited additivity). The results obtained with the combination had to lie outside the range of those obtained with the individual components on their own in order to be indicative of a synergistic or antagonistic interaction (Nweze, 2000).

Statistical analysis: Data were analyzed by analysis of variance to determine whether differences in the mean diameters of the zones of inhibition were statistically significant (Daniel, 1987).

Phytochemical analysis: phytochemical analysis was done by established methods as described by Iwu and Chiori (1984).

Results and Discussion

Table 1 shows the dose range of each compound required to obtain a linear relationship between log dose (in micrograms) and the zone of inhibition in ml for *Candida albicans* in disc diffusion assays.

Table 1: Dose range of each extract from *Morinda lucida* required to obtain a linear relationship between diameter of inhibition and log10 dose

M. lucida extract	Range of mass of extract (mg/ml)	Range of diameter of inhibition(mm)
Leaf	6.250-200.000	8.000-16.800
Stem-bark	6.250-200.000	6.700-14.600
Root	3.125-100.000	7.500-17.6000

For each compound the biologic equivalence factor (BEF) was calculated by using the linear regression for the standard dose-response curve as shown in Figure 1.

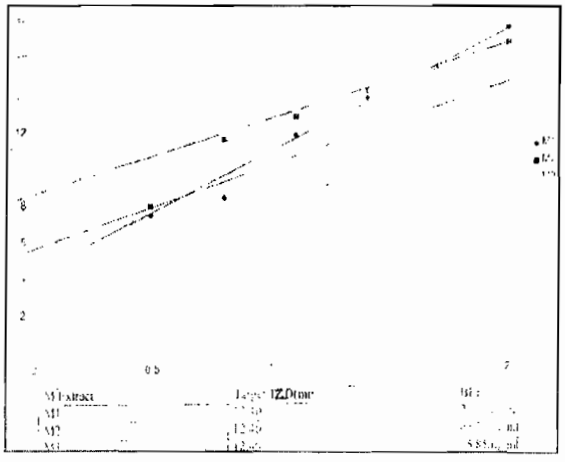

Fig. 1: Graph of Mean Zone Diameter (mm) Vs Log10 (mass) for *M. lucida* leaf extract (M1). Mean Zone Diameter (mm) Vs log10 (mass) for *M. lucida* stem bark extract (M2). Mean Zone Diameter (mm) Vs log10 (mass) for M. lucida root extract (M3)

Decimal mixtures of pairs of extracts from *Morinda lucida* and the corresponding inhibition zone diameter obtained for the different combinations are shown in Tables 2 - 4. For each pair of compounds tested,

Table 2: Decimal additive assay for Morinda lucida leaf (M1) and stem-bark extracts (M2)

Decimal mixtures			Decimal portion of biologic equivalence Factor (BEF)			Inhibition zone diameter (IZD)
M1	+	M2	M1	+	M2	mm
10 (M1)	+	0 (M2)	20.00	+	0.00	14.20
9 (M1)	+	1 (M2)	18.00	+	6.31	13.80
8 (M1)	+	2 (M2)	16.00	+	12.62	14..30
7 (M1)	+	3 (M2)	14.00	+	18.93	14.50
6 (M1)	+	4 (M2)	12.00	+	25.24	12.40
5 (M1)	+	5 (M2)	10.00	+	31..55	14.90
4 (M1)	+	6 (M2)	8.00	+	37.86	15.10
3 (M1)	+	7 (M2)	6.00	+	44.17	14.70
2 (M1)	+	8 (M2)	4.00	+	50.48	14.10
1 (M1)	+	9 (M2)	2.00	+	56.79	13.50
0 (M1)	+	10(M2)	0.00	+	63.10	12.80

Table 3: Decimal additive assay for *Morinda lucida leaf (MI) and root (M3) extracts*

Decimal mixtures	Decimal portion of biologic equivalence factor (BEF) MI + M3	Inhibition zone diameter (IZD) mm
10 (MI) + 0(M3)	20.00 + 0.00	14.30
9 (MI) + 1(M3)	18.00 + 1.59	14.10
8(MI) + 2(M3)	16.00 + 3.17	13.90
7(MI) + 3(M3)	14.00 + 4.76	14.30
6(MI) + 4(M3)	12.00 + 6.34	14.95
5(MI) + 5(M3)	10.00 + 7.93	15.40
4(MI) + 6(M3)	8.00 + 9.51	15.20
3(MI) + 7(M3)	6.00 + 11.10	15.30
2(MI) + 8(M3)	4.00 + 12.68	15.60
1(MI) + 9(M3)	2.00 + 14.27	15.90
0(MI) + 10(M3)	0.00 + 15.85	15.00

the selected target size for zones of inhibition, the mass of each extract required to achieve this target zone i.e. the BEF, and the mean diameters of the zones of inhibition (with 95% confidence intervals) actually obtained as shown in Table5. Only additive interactions were observed among the pairs of *Morinda lucida* extracts tested ($p > 0.5$). In testing antimicrobial drug interactions, Sanders et al (1993) developed the decimal additive assay to overcome some of the limitations due to methodology associated to checkerboard titration and time-kill techniques (Sanders et al 1993). In the time survival studies, a dose response relationship is rarely determined while synergy and antagonism are difficult to define, while in checkerboard titration method, interpretation of fractional inhibitory indices leaves little scope for the interpretation of true additive interaction. In addition, the complete inhibition of growth is a qualitative measure rather than a quantitative parameter that can be predicted on a dose -response basis. Conversly, decimal additive assay was designed to have a quantitative endpoint, which varied in a predictable dose-response manner. It has a precisely defined point for additivity and a range of drug ratios used provides a more complete description of the interactions obtained. Tariq et al (1995) also suggested that the use of decimal additive assay helps to identify the drug ratio at which the interaction is maximal. Sanders et al (1993) suggested that comparing the amounts of each crude extract required to produce the interaction could help to predict whether the interaction may occur at clinically attainable concentration. It is possible that in some tests significant interactions may be detected in some but not all decimal mixtures for example in antagonistic interactions. For this reason, it is important that all datum points are plotted, since it may be appropriate to select data from only

Table 4: Decimal additive assay for *Morinda lucida* leaf (M2 and stem-bark extracts (M3)

Decimal mixtures	Decimal portion of biologic equivalence factor (BEF) M2 + M3	Inhibition zone diameter (IZD) mm
10 (M2) + 0(M3)	63.10 + 0.00	12.60
9 (M2) + 1(M3)	56.76 + 1.59	11.80
8(M2) + 2(M3)	50.48 + 3.17	12.90
7(M2) + 3(M3)	44.17 + 4.76	13.70
6(M2) + 4(M3)	37.86 + 6.34	16.00
5(M2) + 5(M3)	31.55 + 7.93	15.10
4(M2) + 6(M3)	25.24 + 9.51	14.80
3(M2) + 7(M3)	18.93 + 11.10	13.90
2(M2) + 8(M3)	12.63 + 12.68	14.60
1(M2) + 9(M3)	6.31 + 14.27	15.20
0(M2) + 10(M3)	0.00 + 15.85	14.80

Table 5: Interactions between pairs *of Morinda lucida* extracts in the inhibition of Candida albicans

Extract	Diameter of target Zone of inhibition	Biologic equivalence factor (BEF) (mg/ml)	Mean diameter of inhibition attained (mm)	Type of interaction
Leaf	12.40	20.00	14.40+0.20	Additive
stem-bark	12.40	63.10	12.90+0.20	(p.0.05)
Leaf + stem-bark			14.90+0.02	
leaf	12.40	20.00	14.20+0.10	Additive
root	12.40	15.85	15.00+0.10	(p>0.05)
Leaf + root			15.40+0.20	
stem-bark	12.40	63.10	12.70+0.20	Additive
root	12.4	15.85	14.80+0.20	(p>0.05)
stem-bark + root	15.10+0.20			

some decimal mixtures in order to calculate the mean diameter of zone of inhibition attained by the *Morinda lucida* crude extracts combination (Sanders et al 1993).

The decimal additive assay though originally developed for use in assessments of antibacterial activity, proved to be equally effective in distinguishing interactions between antifungal drugs during the inhibition of *C. albicans* invitro. In this study combination of crude plant extracts from leaf, stem-bark and root of *Morinda Lucida* yielded an additive response. The results seems to agree with the views of Tariq et al that pairs of compounds closely related to one another behaved in an additive manner when combined in decimal mixtures. However in the aforementioned study, synthetic substances were used rather than crude plant extracts used in our own study. We believe that the use of such drug/ crude extract therapy may broaden the antifungal spectrum, attain fungicidal activity and lower the risk of resistance (Polak et al, 1990).

The results of phytochemical analysis are shown in Table 6. It reveals similar phytochemical constituents in the various extracts of *Morinda lucida* used in the study. This seems to agree with the antifungal activities of the various extracts.

Table 6: Phytochemical analyses of the different parts of *Morinda lucida* extracts.

Tested for	Leaf	Stem-bark	Root
Alkaloids	+	+	+
Tannins	+	+	+
Saponins	+	+	+
Protein	+	+	+
Steriodal aglycone	+	+	+
Cardiac glycosides	+	+	+
Anthracene glycosides	+	+	+
Cyanogenic glycosides	+	+	+

Although we could not determine the quantitative values of each of the phytochemicals tested, nor the specific phytochemicals with anticandida activities we predict that such figures if obtained in future research studies will not likely be significant amongst themselves. This is because the various extracts exhibited similar antifungal activities on *Candida albicans* in vitro.

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