



## Evaluation of Antifungal Activities of the Crude Leaf Extracts of *Mitracarpus vilosus*

Asogwa SI<sup>1</sup>, Mbah CC<sup>2\*</sup>, Attama AA<sup>1</sup>, Okore VC<sup>1</sup>

<sup>1</sup>Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

<sup>2</sup>Department of Pharmaceutical Technology and Raw Materials Development, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

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### ABSTRACT

The antifungal activities of fractions of the leaf extract of *Mitracarpus vilosus* were evaluated in comparison with some standard antifungal drugs (ketoconazole, clotrimazole, fluconazole, nystatin and tioconazole), against certain isolates of moulds and yeasts. Air-dried pulverized leaves of *Mitracarpus vilosus* (500 g) were extracted successively with methanol, chloroform and ethyl acetate, using the cold maceration method. The susceptibilities of solutions of the fractions in DMSO (well diluted with sterile distilled water), as well as the standard antibiotics, to some fungal isolates were tested using agar broth dilution method. Subsequently, the minimal inhibitory concentrations (MICs) were determined using the agar broth dilution method. Their synergism were determined using the Checker-board method. All the fractions and their combinations with standard drugs showed significant antifungal activities against the fungal and yeast isolates used, with the chloroform fraction being the most active against majority of the organisms. The IZDs against *A. niger* were 15, 35, 27, and 9 mm for the methanolic extract, chloroform, methanolic and ethyl acetate fractions, respectively. The MICs obtained for the chloroform fraction were: 1.56, 6.25, 3.13, 6.25, 1.56, 3.13 and 1.56 mg/ml for the *A. niger*, *Penicillium spp*, *M. furfur*, *A. fumigatus*, *C. albicans* 1, 2 and 3 isolates, respectively. At FIC index of 0.21, the combination of the chloroform fraction with ketoconazole (at a ratio 9:1) against *Penicillium spp*, the highest synergism of all the combinations was observed. However, ketoconazole alone showed the highest activity against *A. niger*, *Penicillium spp.*, *A. fumigatus* and all the candida isolates compared to the extracts/fractions, while clotrimazole was the most active against *M. furfur*. Based on this study, crude extract and fractions of *M. vilosus* leaves, especially the chloroform fraction, can be used alone or in combination with some standard antifungal drugs for the treatment of superficial mycosis and candidiasis caused by susceptible species of these organisms.

**KEYWORDS:** *Mitracarpus vilosus*, leaf extracts and fractions, antifungal activities, synergy of combinations.

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### INTRODUCTION

Plants serve not only as food for man but also as sources of remedy for ailments. Recognition of plants as sources of medicinal substances dates back to antiquity. In African ethnomedical practices, therapeutic agents can be obtained from plants, minerals and animals. The World Health Organization (WHO) estimates that 80 per cent of the world's population uses herbal medicines for some aspects of primary health care [1]. This apparent reliance on herbal medicines is easily attributed to the widely held belief that industrially manufactured pharmaceuticals are expensive, inaccessible or unsuitable. So plant materials, which have been found to be effective against resistant

microorganisms, are used as medicines in traditional, and some aspects of conventional health care practices.

*Mitracarpus vilosus* is a plant of the family *Rubiaceae*. The common names of *M. vilosus* in Nigeria are *obuobwa*, *ogwu-ugwa*, *gududal* and *irawo-ile*. The family has about 500 genera and 6,000 species distributed all over the world. It is a perennial herb with rough leaves and green flora. Traditionally, an oral formulation of the leaf extract is used in the treatment of venereal diseases and leprosy [2]. Also a topical preparation is available for the treatment of tinea infections, lice, itching, and for dressing of wounds or ulcers. Empirically, leaf



extracts of *M. vilosus* have been shown to exhibit broad antibacterial and antimycotic activities [3]. Pentalogin isolated from fresh aerial parts of *M. vilosus* showed antifungal activities against *Candida albicans* and *Trichophyton sudanense* [4]. Extracts of *M. vilosus* leaves have been formulated into pleasant oral dosage forms for the treatment of some throat and upper respiratory tract diseases [4].

Fungal infections are often opportunistic and are usually caused by some endogenous organisms, such as *C. albicans* and *Pityriasis versicolor* [5]. However, candidiasis may be sexually transmitted if it affects the urogenital system. Dermatophytes are superficial or cutaneous diseases caused by dermatophytes such as the *Tinea* spp. Pityriasis is also a superficial mycotic infection caused by an over-growth of *Tinea versicolor* or the dimorphic yeast, *Malassezia furfur*. *M. furfur* has sometimes been implicated in seborrhoeic dermatitis, folliculitis, neonatal pastulosus and blepharitis [6].

In many situations, where the facilities for purification of potent plant extracts are not readily available, the crude extracts may be used to initiate treatment. The present study, therefore, seeks to evaluate the antifungal potencies of fractions of crude leaf extracts of *M. vilosus*, as well as their combinations with some standard antifungal drugs, against common infectious fungi, moulds and yeasts. The fungal pathogens of interest are those associated with increasing incidence of resistance to available drugs.

## MATERIALS AND METHODS

### Materials

The following antifungal drugs were obtained as gifts from the named companies: clotrimazole powder (Fidson Drugs Nigeria PLC), ketoconazole powder (Janssen Nigeria PLC), tioconazole powder (Neimeth International Pharmaceuticals PLC), fluconazole powder (Medreich Nigeria Ltd.), and nystatin powder (Janssen-Cilag Nigeria PLC). Sabouraud's dextrose agar (SDA) and nutrient agar (Fluka) were employed in the various tests. Clinical isolates of *C. albicans*, *Aspergillus niger*, *Aspergillus fumigatus*, *Malassezia furfur* and *Penicillium* spp. were used in the study. Methanol, chloroform, ethyl acetate and n-hexane (Sigma – Aldrich, Germany) and distilled water were used for either extraction or fractionation. Dimethylsulfoxide (DMSO) and potassium hydroxide (Merck, Germany) were also employed at various stages. All other reagents were of analytical standard and were used without further purification.

## Methods

### Collection and preparation of plant material

Leaves of *M. vilosus* were collected from Nsukka, Enugu State, Nigeria on January 15, 2010, and were authenticated by Mr. A. Ozioko, a taxonomist in the Department of Botany and Plant Science, University of Nigeria, Nsukka. The leaves were air-dried, pulverized and subjected to series of extractions by the cold maceration method using water and methanol, respectively [7]. The methanol filtrates were exposed to air, until the solvent evaporated to dryness. The dried residue was successively fractionated with chloroform, ethyl acetate and n-hexane. The extracts and fractions were packaged in air-tight containers and stored in a refrigerator until used in the studies.

### Susceptibility studies of different strains of yeasts and moulds

The sensitivities of the organisms to the extracts and fractions of *M. vilosus* and the standard antifungal agents were determined using agar dilution method [8]. Weighted quantity of the extract or fraction was dissolved in 2 ml dimethyl sulfoxide (DMSO) and later diluted to concentration of 100 mg/ml using sterile distilled water. A 20 ml volume each of molten Sabouraud dextrose agar (SDA) was seeded separately with 0.1 ml of standardized cultures of fungi in Petri dishes. A total of 5 wells of 8 mm in diameter were bored in the seeded agar plate using a sterile cork borer. Two drops of 0.4 ml each of the solutions of extract or fraction were carefully placed into each well using Pasteur pipettes, while for control, two drops of 2-fold diluted DMSO were put in the centre. The plates were left for 1 h at room temperature for pre-diffusion, after which they were incubated at 25 °C for 24 h for *C. albicans* and *M. furfur* and 25 °C for 7 days for some moulds as the case may be. The inhibition zone diameters (IZDs) were then measured.

### Determination of minimal inhibitory concentration

The agar dilution method was adopted. Stock solutions of the extract, fractions and standard drugs were prepared using DMSO. Two-fold serial dilutions of the solutions were made with sterile distilled water. Thereafter, 10 ml of each of the solutions were mixed with an equal volume of double strength molten Sabouraud dextrose agar in sterile Petri dishes and allowed to solidify after which the standardized inoculums of yeasts and moulds were streaked on designated segments of

the agar medium. The Petri dishes were then incubated at 25 °C for 24 h for *C. albicans* and *M. furfur* and at 25 °C for 7 days for other moulds, and then observed for the presence or absence of growth. The MICs were deduced as the minimal concentration of drug or extract/fraction allowing no growth.

#### Determination of minimal fungicidal concentration

This is usually an extension of the MIC determination. A reaction mixture prepared from the regions that showed no growth in the MIC test was used to inoculate fresh sterile molten SDA plates. The plates were incubated for 48 h at 25 °C for *C. albicans* and *M. furfur* and 25 °C for 14 days for other moulds. The plates were then observed for presence or absence of growth of the organisms. A control not containing the plant extract or drug was also included. The minimal concentration of the extracts or standard drugs that showed no growth was taken as the minimal fungicidal concentration.

#### Test for combined activity of the extracts with standard antibiotics

This was done to evaluate the combined activity of the crude extracts or fractions and the standard broad spectrum antifungal agents (clotrimazole, ketoconazole, tioconazole or fluconazole) against the test yeasts and moulds using the Checker-board method. Stock solutions containing the MIC of each extract or standard drug were prepared by dissolving a weighed amount in a minimal volume (3 – 5 ml) of DMSO and made up to 10 ml with sterile distilled water. The solutions of any two combined

agents were then mixed in different complimentary ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Each of these mixtures was diluted 2-fold serially up to the 12<sup>th</sup> tube; with sterile distilled water. Each dilution was placed, by means of an adsorbent paper disc, on a designated segment of an SDA plate containing the organism. The plates were then incubated at 28 °C for 24 h for *C. albicans* and *M. furfur* and 7 days at 25 °C for moulds, after which they were observed for evidence of growth inhibition. The fractional inhibitory concentration of

$$\text{FIC of Agent} = \frac{\text{MIC of agent in combination}}{\text{MIC of agent used alone}} \quad \text{Eqn. 1}$$

each agent was calculated from the lowest combined concentrations that produced growth inhibition thus [9]:

$$\text{FIC Index of Combination} = \text{FIC of Agent I} + \text{FIC of Agent II}$$

## RESULTS AND DISCUSSION

### Susceptibility studies of different streams of yeasts and moulds

Table 1 shows results obtained from the susceptibility studies. Except for the n-hexane fraction and the water extract which showed no sign of inhibition, the methanol extract and all the other fractions as well as the standard drugs showed relative levels of activity against the various strains of organisms studied as indicated by the IZDs measured. The extract, fractions and standard drugs that passed the susceptibility test were then selected and used for further tests.

**Table 1: Inhibition zone diameters (IZDs) (mm) of extracts and fractions of *M. vilosus* and standard drugs against the different strains of yeasts and moulds**

Organism/Strain	ME	CF	MF	EF	nHF	WE	K	C	F	N	T
<i>A. niger</i>	15.0	35.0	27.0	9.0	nil	nil	18.0	35.0	25.0	20.0	17.0
<i>A. fumigates</i>	17.0	40.0	19.0	8.0	nil	nil	18.0	39.0	30.0	21.0	15.0
<i>P. lilacium</i>	16.0	50.0	24.0	8.0	nil	nil	20.0	25.0	32.0	15.0	9.0
<i>Fusarium spp</i>	9.0	40.0	24.0	10.0	nil	nil	20.0	28.0	19.0	11.0	15.0
<i>Cladosporium spp</i>	15.0	40.0	21.0	11.0	nil	nil	38.0	28.0	27.0	15.0	11.0
<i>T. soudanense</i>	11.0	45.0	20.0	10.0	nil	nil	35.0	22.0	24.0	13.0	16.0
<i>T. mentagrophytes</i>	16.0	33.0	19.0	12.0	nil	nil	17.0	27.0	27.0	30.0	17.0
<i>M. furfur</i>	14.0	37.0	21.0	13.0	nil	nil	25.0	19.0	22.0	9.0	14.0
<i>C. albicans</i> 1	17.0	33.0	25.0	13.0	nil	nil	29.0	25.0	17.0	18.0	15.0
<i>C. albicans</i> 2	15.0	55.0	16.0	7.0	nil	nil	18.0	11.0	15.0	22.0	14.0
<i>C. albicans</i> 3	13.0	53.0	20.0	6.0	nil	nil	20.0	30.0	16.0	15.0	13.0
<i>C. albicans</i> 4	14.0	51.0	22.0	7.0	nil	nil	22.0	28.0	19.0	18.0	12.0
<i>C. albicans</i> 5	12.0	49.0	18.0	9.0	nil	nil	17.0	25.0	14.0	18.0	13.0

ME = methanol extract, CF = chloroform fraction, MF = methanol fraction, EF = ethylacetate fraction, nHF = n-hexane fraction, WE = water extract, K = ketoconazole, C = clotrimazole, F = fluconazole, N = nystatin, T = tioconazole; **Concentration of extracts and fractions were expressed in mg/ml and standard drugs in µg/ml.**

### Test for combined activity of the extracts with standard antibiotics combinations of chloroform fraction and clotrimazole

The combined effect of the chloroform fraction and clotrimazole against different isolates of moulds and yeasts are shown in the histogram in Fig. 1. In the evaluation of interactions between antimicrobial agents by the Checker-board method, values of FIC index less than one indicate synergism, values equal to one imply additive effect and values above one indicate indifference while values more than two indicate antagonism [10]. Very often, varied ratios of the combinations of two agents manifest different types of synergy. This indicates that synergy of antibiotic combinations is not only a function of the nature of antibiotics involved, but also that of their ratios in the combinations [11]. It follows from the histogram that ratio 3:5 exhibited synergism against all the isolates of moulds (*A. niger*, *A. fumigatus* and *P. lilacinum*) and yeasts (*C. albicans* 1, 2, and 3) used, except *M. furfur* for which the ratios exhibited antagonistic effects but synergism in the ratio of 8:2. However, as shown in Fig. 1, ratios 8:2, 1:9 and 5:5 exhibited synergism against all the isolates of *C. albicans*, while ratios 8:2, 4:6, 2:8, 5:5, 3:7 and 1:9 exhibited synergism against only *C. albicans* 2 and 3.

The following ratios were also synergistic against *A. niger*, *P. lilacinum* and *A. fumigatus*: 4:6, 2:8, 5:5, 3:7 and 8:2. The ratios of 5:5, 3:7 and 1:9 exhibited antagonistic effects against *M. furfur*, while 4:6 and 2:8 were also antagonistic against *C. albicans* 1. Similarly, combination ratio of 4:6 was antagonistic against *C. albicans* 3, while 9:1 was antagonistic against *A. niger*. All other combinations presented indifferent effects. From the results of the combinations of chloroform fraction and clotrimazole against the isolates of fungi, the ratio of 5:5 gave the best index of synergistic output except in the case of *M. furfur* for which combination in the ratio of 8:2 gave the best synergistic effect.

### Combinations of chloroform fraction and ketoconazole against isolates of moulds and yeasts

Results obtained from combinations of chloroform fraction and ketoconazole against the isolates are presented in Fig. 2. From the results, it is obvious that ratios of 9:1, 8:2, 6:4, 5:5, 4:6, 3:7 and 1:9 were synergistic against *M. furfur*, while 9:1 and 7:3 exhibited synergism against *P. lilacinum* and *C. albicans* 3.

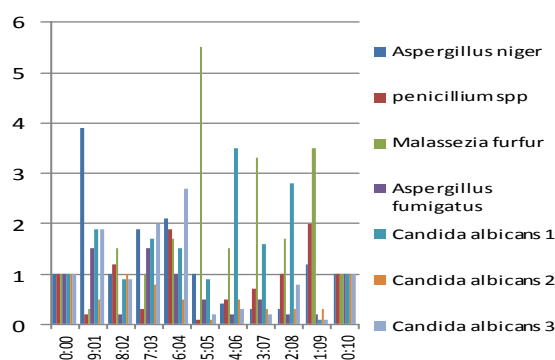


Fig. 1: FIC index of the combination ratio of chloroform fraction and clotrimazole against isolates of moulds and yeasts

Ratios of 9:1, 8:2, 7:3 and 6:4 were synergistic against *C. albicans* 2, while 4:6 exhibited synergism against *A. fumigatus*. All the combinations exhibited antagonism against *A. niger* except 3:7, which was indifferent with the organism. The ratio 5:5 was antagonistic against all the *C. albicans* strains tested whereas 9:1, 7:3, 5:5, 3:7 and 1:9 ratios were specifically antagonistic against *C. albicans* 1. Combinations 6:4, 5:5, 4:6 and 3:7 were antagonistic against *C. albicans* 3. All the combination ratios were indifferent against *A. fumigatus* except ratio 4:6 which was synergistic against it. The ratio 2:8 was indifferent against all the *C. albicans* whereas ratios 8:2, 6:4, 4:6 and 2:8, were specifically indifferent to *C. albicans* 1, while 3:7, 2:8, and 1:9 were indifferent against *C. albicans* 3. Finally combinations ratio 3:7 was additive against all the isolates. From these results it can be deduced that combinations of ketoconazole and chloroform fractions at all ratios (except perhaps 2:8) would bring about synergism against *M. furfur*.

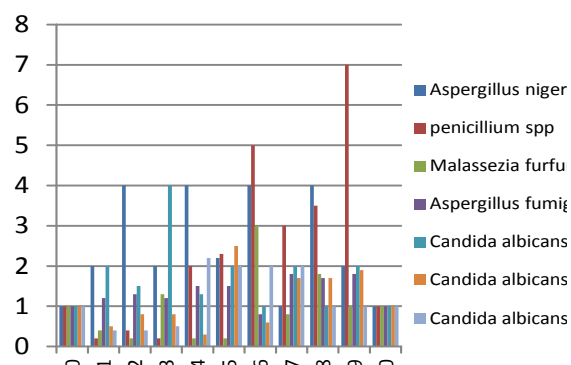
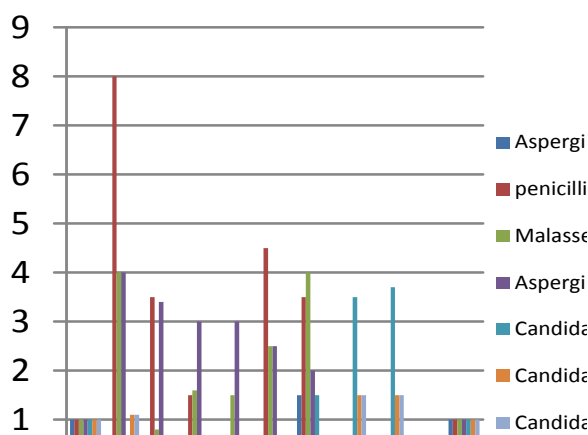


Fig. 2: FIC index of the combination ratio of chloroform fraction and ketoconazole against isolates of moulds and yeasts

**Combinations of methanol fraction and tioconazole against isolates of moulds and yeasts**

The results of combinations of methanol fraction and tioconazole against the test isolates are presented in Fig. 3. The results showed that ratios 9:1, 8:2, 7:3, 6:4 and 5:5 were synergistic against *A. niger* and *C. albicans* 1, while 8:2, 7:3, 6:4, 5:5 and 4:6 exhibited synergism against *C. albicans* 2 and 3. Ratio 8:2 was synergistic only against *M. furfur* whereas ratio 6:4 was synergistic only against *P. lilacum*. Also some combinations showed indifferent effects such as 9:1 and 3:7 ratios, which were indifferent against *C. albicans* 2 and 3. Ratios 7:3 and 6:4 were indifferent against *M. furfur*, with 7:3 ratios being indifferent against *P. lilacum* and 4:6 was in different against *A. niger*. All the combinations exhibited antagonism against *A. fumigatus* except for the ratios of 3:7, 2:8 and 1:9. In the ratios of 9:1, 8:2, 5:5 and 4:6, the combinations were antagonistic against *C. albicans* 1, whereas combinations in the ratios of 5:5 and 4:6 were antagonistic against *M. furfur*. In this study, combination ratios of 3:7 and 2:8 had no activity against the moulds and ratio 1:9 had no activity against both moulds and *C. albicans* which means that these ratios should not be adopted for clinical purposes.

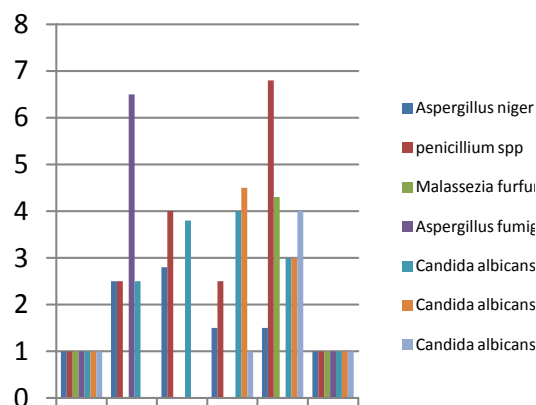


**Fig 3 FIC index of the combination ratio of chloroform fraction and tioconazole against isolates of moulds and yeasts**

**Combinations of ethylacetate fraction and fluconazole against isolates of moulds and yeasts**

The results of the interaction studies between ethylacetate fraction and fluconazole against the isolates of moulds and yeasts are shown at Fig. 4.

None of the combination ratios exhibited synergism. Most of the ratios showed growth of the isolates of the organisms used indicating absence of antimicrobial activities of the combinations. Ratio 2:3 exhibited indifference against *A. niger* and *C. albicans* 3. These results showed that the combination of ethylacetate fractions and fluconazole would not be of any therapeutic value.



**Fig. 4: FIC index of combinations of ethylacetate fraction and fluconazole against isolates of moulds and yeasts**

Antibiotic combinations represent a therapeutic option in treatment of infections. As a result of the increasing appearance of multi-drug resistant microorganisms in various clinical settings, combination therapy has become a valuable approach in reducing the emergence of microbial resistance to infectious agents. Combinations are justified when they enhance the potency of individual antimicrobial agents by means of synergistic interactions. This enhancement has been very useful in clinical practice involving treatment of chronic infections and in situations of decreased susceptibility [12]. Results of this study have demonstrated that methanol extract of the leaves of *M. vilosus*, as well as the chloroform, methanol and ethylacetate fractions have potent antifungal activities against the isolates of moulds and yeast used in the study.

**CONCLUSION**

Based on the results of the MIC determination, the chloroform fraction was the most active of the plant extract and fractions against all the organisms tested. For the standard drugs, ketoconazole was the most active against *A. niger*, *Penicillium* spp, *A. fumigatus* and all the candida isolates. Clotrimazole was the most active against *M. furfur*. These results of combination studies based on the Checker-board technique showed that synergism could be

achieved against the test fungi with a wide variety of combinations of the extracts or fractions of *M. vilosus* and some available antifungal drugs, with the expectations that resistance would be brought under control.

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