

Original Research Article ***In vitro* Antifungal Activity of Ethyl Acetate Fraction of *Mitracarpus villosus* Found in Abuja, Nigeria.**

ABSTRACT

Aims: To evaluate the anti-fungal activities of the ethyl acetate extract and fractions of the aerial parts of *Mitracarpus villosus* (Sw.) DC obtained in Abuja, Nigeria.

Methods: The powdered plant was extracted successively and exhaustively with hexane, ethyl acetate, ethanol and water. Thirty fractions were elucidated from the extract using the bioassay-guided fractionation by employing the accelerated gradient chromatography (AGC) technique. Antifungal activities of the ethyl acetate extract and fractions of *M. villosus* against clinical isolates of *Candida albicans*, *Candida krusei*, *Trichophyton verrucosum*, *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Aspergillus niger* were investigated using agar diffusion and micro broth dilution methods.

Results: All the fractions showed good antifungal activity against test fungi. The minimum inhibitory concentration and the minimum fungicidal concentration of the ethyl acetate extract and fractions against the test organisms were found to fall between 500 – 16000 µg / ml.

Conclusion: The plant has good potentials for development of new antifungal drugs.

Keywords: antifungal, fractions, minimum inhibitory concentration, minimum fungicidal concentration.

1. INTRODUCTION

Medicinal plants have been a source of wide variety of biologically active compounds for many centuries and used extensively as crude material or as pure compounds for treating various disease conditions [1]. More than 80% of the population in developing countries has been reported to depend on plants for their medical needs [2]. Research have shown that plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties [3].

In the past few decades, a worldwide increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some species of fungi to different fungicidal agents used in medicinal practice [4]. The incidence of fungal infections is increasing at an alarming rate, presenting an enormous challenge to healthcare professionals. This increase is directly related to the growing population of immunocompromised individuals especially children resulting from changes in medical practice such as the use of intensive chemotherapy and immunosuppressive drugs [5].

The plant *Mitracarpus villosus* (S.W) D.C belongs to the family Rubiaceae. It is an erect perennial annual herb up to 60cm high. It grows as a weed on old and abandoned farmlands and is distributed widely from forest to savanna zones of the tropics. In various parts of tropical Africa, it is traditionally used for treatment of sore throat. It has been used in

traditional medicine practices in West Africa for the treatment of headaches, toothaches, amenorrhoea, dyspepsia, hepatic diseases, venereal diseases as well as leprosy [6]. It has also been used to treat ringworm and eczema, fresh cuts, wounds and ulcer. The aerial portion of this plant has also been used to make lotion and skin ointment used for skin diseases and infections [7]. In Nigeria, the extracted juice from aerial parts is topically applied against skin diseases and on wound [8]. Until recently, no work has been documented on the antifungal potential of the ethyl acetate extract of *Mitracarpus villosus*. The main objective of this study is to evaluate the anti-fungal activities of the ethyl acetate extract of the aerial parts of *Mitracarpus villosus* (Sw.) DC obtained in Abuja, Nigeria.

2. MATERIAL AND METHODS

Collection and identification of plant materials

The fresh aerial parts of *Mitracarpus villosus* was collected from the National Institute for Pharmaceutical Research and Development (NIPRD) garden. The plant was identified and authenticated in the herbarium of the Department of Medicinal Plant Research and Traditional Medicine, NIPRD, Abuja, Nigeria. The sample of the plant was deposited in the herbarium for reference purpose with Voucher specimen No. NIPRD/ H/ 4208.

Preparation of crude plant material.

Mitracarpus villosus aerial parts were dried at room temperature for 10 days. The completely dried aerial parts were crushed to coarse powder by grinding with wooden mortar and pestle.

Extraction of plant material

Using a Soxhlet extractor (Quick Fit- England), the powdered aerial parts was extracted successively and exhaustively each time with each of the various solvents starting from the less polar solvent to the more polar solvent. The solvents used were hexane, ethyl acetate and ethanol respectively. Finally the marc was macerated in water. After each extraction, the extract was concentrated, dried and weighed.

Chromatographic analyses of ethyl acetate extract

Ethyl acetate extract was analyzed for chemical composition using the bioassay-guided fractionation by employing the accelerated gradient chromatography (AGC) technique. Silica gel G (E-Merck, Germany) was used as an absorbent. Gradient elution was effected using hexane and ethyl acetate sequentially with increasing polarity. The column was successively eluted with hexane (100%), hexane–ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90%), ethyl acetate (100%), ethyl acetate- ethanol (90:10, 80:20). The fractions were collected and combined on the basis of their thin layer chromatography (TLC) profiles.

Antifungal assay of extracts of *M. villosus*

Preparation of antifungal agents

Stock solutions of fluconazole powder (Sigma Aldrich, Cat No. F8929) and ketoconazole powder (Sigma Aldrich, Cat No. K1003) was prepared by dissolving appropriate quantity of the antifungal agent in dimethyl sulfoxide (DMSO, BDH, Germany) and later diluted to their require concentrations with broth.

Fungi used

The fungi used were clinical isolates from The Department of Microbiology and Biotechnology, NIPRD, Abuja. These include *Candida albicans*, *Candida krusei*, *Aspergillus*

85 *fumigatus*, *Aspergillus niger*, *Trichophyton mentagrophytes* and *Trichophyton verrucosum*.
 86 *Trichophyton verrucosum* was collected from the Dermatophylosis Center, National
 87 Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria.

88 **Cultivation and standardization of test fungi**

89 Eighteen-hour broth culture of the test *Candida spp.* was suspended into sterile Sabouraud
 90 dextrose liquid medium. It was standardized according to National Committee for Clinical
 91 Laboratory Standards [9], by gradually adding normal saline to compare its turbidity to
 92 McFarland standard of 0.5 which is approximately 1.0×10^6 cfu/ml. However, for
 93 *Trichophyton spp.* and *Aspergillus spp.*, fungal spores were harvested from 7- 10 day old
 94 SDA slant cultures by washing with 10 ml sterile normal saline containing 3 % w/v Tween 80
 95 with aid of sterile glass beads to help in dispersing the spores [10]. Thereafter, the spore
 96 suspension was standardized to 1.0×10^6 spores / ml by using a single-beam
 97 spectrophotometer (Spectronic 20D; Milton Roy Company, Pacisa, Madrid, Spain) at 530 nm
 98 (OD530) of the suspensions and adjusted to 80 – 85 % transmittance (*Aspergillus spp.*) and
 99 70 -72 % (*Trichophyton spp.*). All adjusted suspensions were quantified by spreading 100 μ L
 100 on Sabouraud dextrose agar plate and incubated at 37°C for 18 h for yeast and 30°C for 72
 101 hours for dermatophytes and moulds [11]. All cultures were checked for purity (by
 102 morphological growth on media, staining and biochemical tests) and maintained on
 103 Sabouraud dextrose agar (SDA) for the fungal test organisms respectively at 4°C (in the
 104 refrigerator) until required for use.

105 **Susceptibility of the organisms to ethyl acetate extract of *M. villosus***

106 Eighteen hours overnight cultures of *Candida spp.* and inoculum suspensions of the
 107 moulds and dermatophytes prepared from fresh, mature (3- to 5-day-old) cultures (in some
 108 cases an extended incubation was required for proper sporulation of the isolate) in
 109 Sabouraud dextrose liquid medium were standardized to produce inoculum size of 10^6 cfu/ml¹.
 110 One milliliter of the diluted culture of each test organism was used to flood Sabouraud
 111 dextrose agar media and excess aseptically drained. The plates were allowed to dry at 37°C
 112 temperature in a sterilized incubator. Adopting the agar diffusion cup plate method [10], a
 113 sterile cup borer (6mm) was used to bore holes in the agar plates. The bottoms of the wells
 114 (holes) were sealed with the appropriate molten Sabouraud dextrose agar. Using
 115 micropipette, 0.1 ml each of gradient concentrations (mg/ml) of the crude ethyl acetate
 116 extracts were dispensed into the holes. Distilled water was used as control. These were
 117 allowed to diffuse into the agar at room temperature for one hour before incubation at 37°C
 118 for 18 hours (yeast) and 30°C for 72 hours up to 5 days (dermatophytes and moulds). The
 119 zones of inhibition of the test organisms were measured to the nearest millimeter, using a
 120 well-calibrated meter ruler. The experiment was carried out in triplicates.

121 **Determination of minimum inhibitory concentration (MIC) of the extract and fractions**

122 The minimum inhibitory concentration (MIC) of the extract, fractions and reference antibiotics
 123 to the test fungi was determined by using serial broth microdilution method [12]. This assay
 124 was performed using round bottom 96-well clear microtitre plates. The wells in row 1 of each
 125 column were left blank and the last eleven wells from rows 2 to 12 were filled with 50 μ l of
 126 sterilized Sabouraud dextrose broth. Working solution of plant extracts and fractions were
 127 added to the wells in row 1 of each column and wells in row 2 of each column and an
 128 identical two-fold serial dilution were made from wells in rows 2 to the rows 10. The last wells
 129 in row 12 served as drug-free controls. Water was used as negative control. Lastly, 50 μ l of
 130 standardized fungal inoculum (10^6 cfu/ml¹) were added in all the wells from column A to H
 131 and mixed thoroughly to give final concentrations. Tests were done in triplicates. The
 132 cultured microplates were sealed with parafilm. The plates were incubated at 37°C for 18
 133

134 hours (yeast) and 30°C for 48 hours and up to 5 days (dermatophytes and moulds). MIC was
135 defined as the first well with no visible growth after 24 hours.
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137 **Determination of minimum fungicidal concentration (MFC) of the extract and** 138 **fractions.**

139 Fifty microliters of the wells that did not show any visible growth after MIC determination
140 were inoculated in fresh wells containing Sabouraud dextrose broth and incubated at 37°C for
141 18 hours (yeast) and 30°C for 48 hours and up to 7 days (dermatophytes and moulds).
142 Minimum fungicidal concentration (MFC) was determined as the lowest concentration
143 resulting in no growth on subculture [13].

144 **Statistical analysis**

145 Results obtained were expressed as mean±standard deviation and analysed using one way
146 ANOVA (Smith's Statistical Package version 2.80) at p=0.05.
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148 149 **3. RESULTS AND DISCUSSION**

150 A total of 30 fractions were collected from the elution of the ethyl acetate extract. Identical
151 fractions were combined based on thin layer chromatographic values giving 6 fractions M1 –
152 M6 (Table 1). The result of preliminary antifungal activities showed that the ethyl acetate
153 extract and fractions inhibited the growth of the test fungi with varying degree of inhibition
154 which increased with increase in the concentration of the agent (Table 2). The results of the
155 minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) is
156 shown in Tables 3.0 and 4.0 respectively. Generally, the ethyl acetate extract had the
157 strongest antifungal activity on *Trichophyton verrucosum* and *T. mentagrophytes*. *T.*
158 *verrucosum* is the usual zoophilic dermatophyte involved in cattle ringworm. It also affects,
159 but with lower prevalence, sheep, goat and other ruminants. Aside from animal involvement,
160 several human outbreaks of *T. verrucosum* infection have been reported so far by direct
161 contact with infected animals or indirect contact with infectious propagules in the
162 environment [14].
163

164 Plants have been known to possess bioactive constituents as protective substances against
165 bacteria, fungi, viruses and pests [15]. The mode of action of ethyl acetate extract could be
166 related to their ability to alter membrane properties leading to cell death [16]. All the test
167 fungi were found to be susceptible to ethyl acetate extracts and fractions of *M. villosus*
168 (Table 2), which is in line with the works done in the past [17,18] which reported the potent
169 anti fungal activity of *M. villosus*. However, there were differences in the inhibition of growth
170 among the compounds against different strains of fungi. The ethyl acetate extract and
171 fractions were shown to be fungistatic at lower concentrations and fungicidal at higher
172 concentrations. The inhibitory action of *M. villosus* against *Candida spp.* has been linked to
173 the presence of acetophenone derivatives [7]. With the increasing rate of candidiasis
174 infection worsened by the high rate of HIV/AIDS infection, this plant holds great promise for
175 development into phytomedicine for the treatment of candidiasis in the near future. *A.*
176 *fumigatus* is the most frequent cause of invasive fungal infection in immunosuppressed
177 individuals, which include patients receiving immunosuppressive therapy for autoimmune or
178 neoplastic disease, organ transplant recipients, and AIDS patients [19].The anti fungal
179 activity of the ethyl acetate extract and fractions of *M. villosus* is comparable with that of

180 ketoconazole and fluconazole, which has been linked to the combined action of the
181 secondary metabolites present in the extract [20].

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183 **Table 1. Combination of ethyl acetate fractions of *M. villosus***

Sample	Fraction combination
M1	1-6
M2	7 – 9
M3	10 – 12
M4	13 – 14
M5	15 – 25
M6	26 – 30

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188 **Table 2. Antifungal susceptibility testing of crude tannin from *Mitracarpus villosus***

Organisms	Zone of Inhibition (mm)				
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.50 mg/ml
Ca1	27.67±0.33 ^a	26.67±0.33 ^a	22.67±0.33 ^b	22.00±0.00 ^b	21.67±0.33 ^b
Ca2	28.67±0.33 ^a	28.00±0.00 ^a	25.0±0.0 ^b	24.00±0.00 ^b	23.67±0.33 ^c
Ca3	31.33±0.33 ^a	31.00±0.00 ^a	27.00±0.00 ^b	24.67±0.33 ^c	24.00±0.00 ^c
Ca4	31.00±0.00 ^a	30.67±0.33 ^a	27.00±1.00 ^b	24.67±0.33 ^c	23.67±0.33 ^c
CK	28.67±0.33 ^a	26.67±0.33 ^b	26.67±0.33 ^b	21.67±0.33 ^c	21.00±0.00 ^c
AF	24.33±0.33 ^a	22.00±0.00 ^b	20.67±0.33 ^c	19.67±0.33 ^c	19.67±0.33 ^c
AN	25.67±0.33 ^a	24.67±0.33 ^a	21.67±0.33 ^b	20.67±0.33 ^b	20.00±0.00 ^c

TM	27 .67±0.33 ^a	26 .67±0.33 ^a	24 .67±0.33 ^b	23.00±0.00 ^b	22.00±0.00 ^c
TV	28.00±0.00 ^a	26 .67±0.33 ^b	25.00±0.00 ^b	24.00±0.00 ^c	22 .67±0.33 ^d

Values are mean inhibition zone (mm) ± SD of three replicates
Values with different superscripts on the same row are significantly different (*P*=0.05)
Ca, *Candida albicans*; CK, *Candida krusei*; AN, *Aspergillus niger*; AF, *Aspergillus fumigatus*; TM, *Trichophyton mentagrophytes*; TV, *Trichophyton verucossum*

Table 3: Minimum inhibitory concentration of ethyl acetate extract and fractions of *Mitracarpus villosus*

ORG	Minimum Inhibitory Concentration (µg/mL)								
	ETA	M1	M2	M3	M4	M5	M6	FCZ	KCZ
Ca1	1000.00	2000.00	1000.00±	1000.00	500.00±	2000.00±	2000.00	25.00±	NA
	±0.33 ^a	± 0.57 ^b	0.00 ^a	± 0.00 ^a	0.57 ^c	0.00 ^b	± 0.00 ^b	0.00 ^d	
Ca2	1000.00	2000.00	1000.00±	1000.00	2000.00	500.00±	2000.00	25.00±	NA
	± 0.57 ^a	± 0.00 ^b	0.00 ^a	± 0.00 ^a	± 0.33 ^b	0.00 ^c	± 0.57 ^b	0.33 ^d	
Ca3	500.00±	1000.00	1000.00±0	2000.00	2000.00	250.00±	1000.00	25.00±	NA
	0.00 ^a	± 0.00 ^b	.33 ^b	± 0.00 ^c	± 0.00 ^c	0.00 ^d	± 0.00 ^b	0.00 ^e	
Ca4	500.00±	2000.00	500.00±	500.00±	1000.00	250.00±	1000.00	25.00±	NA
	0.00 ^a	± 0.00 ^b	0.00 ^a	0.33 ^a	± 0.33 ^c	0.00 ^d	± 0.00 ^c	0.33 ^e	
Ck	2000.00	4000.00	500.00±	500.00±	1000.00	500.00±	1000.00	200.00	NA
	± 0.33 ^a	±0.33 ^b	0.00 ^c	0.00 ^c	± 0.00 ^d	0.00 ^c	± 0.33 ^d	± 0.00 ^e	
AF	2000.00	2000.00	2000.00±0	2000.00	4000.00	4000.00±	4000.00	NA	25.00
	± 0.00 ^a	± 0.00 ^a	.33 ^a	± 0.00 ^a	± 0.00 ^b	0.33 ^b	± 0.00 ^b		±0.33 ^c
AN	1000.00	2000.00	500.00±	1000.00	2000.00	4000.00±	4000.00	NA	25.00
	± 0.57 ^a	± 0.33 ^b	0.00 ^c	± 0.00 ^a	± 0.57 ^b	0.00 ^d	± 0.33 ^d		±0.57 ^e
TM	2000.00	1000.00	1000.00±	1000.00	1000.00	500.00±	500.00±	NA	8.00±

	± 0.00 ^a	± 0.00 ^b	0.00 ^b	± 0.00 ^b	± 0.00 ^b	0.00 ^c	0.00 ^c		0.33 ^d
TV	500.00±	250.00±	250.00±	250.00±	250.00±	500.00±	500.00±	NA	4.00±
	0.33 ^a	0.00 ^b	0.00 ^b	0.00 ^b	0.00 ^b	0.00 ^a	0.57 ^a		0.00 ^c

Values are mean inhibition zone (mm) ± SD of three replicates
Values with different superscripts on the same row are significantly different (P=0.05)
FCZ, Fluconazole; KTZ, Ketoconazole; NA , Not applicable; ORG, organisms; Ca, *Candida albicans*; CK, *Candida krusei*; AN, *Aspergillus niger*; AF, *Aspergillus fumigatus*; TM, *Trichophyton mentagrophytes*; TV, *Trichophyton verucosum*

Table 4.0: Minimum fungicidal concentration of ethyl acetate extract, secondary metabolites and fractions of *Mitracarpus villosus*

ORG	Minimum Fungicidal Concentration (µg/mL)								
	ETA	M1	M2	M3	M4	M5	M6	FCZ	KCZ
Ca1	4000.00 ± 0.33 ^a	8000.00 ± 0.00 ^b	2000.00± 0.33 ^c	4000.00 ± 0.33 ^a	2000.00 ± 0.00 ^c	4000.00 ± 0.57 ^a	4000.00 ± 0.00 ^a	25.00± 0.33 ^c	NA
Ca2	4000.00 ± 0.00 ^a	4000.00 ± 0.33 ^a	500.00± 0.00 ^b	4000.00 ± 0.00 ^a	4000.00 ± 0.57 ^a	4000.00 ± 0.33 ^a	8000.00 ± 0.33 ^c	25.00± 0.00 ^d	NA
Ca3	4000.00 ± 0.33 ^a	8000.00 ± 0.00 ^b	4000.00± 0.00 ^a	4000.00 ± 0.00 ^a	4000.00 ± 0.00 ^a	1000.00 ± 0.00 ^c	4000.00 ± 0.00 ^a	25.00± 0.57 ^d	NA
Ca4	4000.00 ± 0.00 ^a	8000.00 ± 0.00 ^b	2000.00± 0.33 ^c	4000.00 ± 0.57 ^a	8000.00 ± 0.33 ^b	8000.00 ± 0.57 ^b	16000.0 0± 0.00 ^c	25.00± 0.00 ^d	NA
Ck	8000.00 ± 0.57 ^a	8000.00 ± 0.33 ^a	1000.00± 0.00 ^b	4000.00 ± 0.00 ^c	8000.00 ± 0.00 ^a	8000.00 ± 0.00 ^a	4000.00 ± 0.57 ^c	2000.00 ± 0.00 ^d	NA
AF	4000.00 ± 0.33 ^a	4000.00 ± 0.00 ^a	4000.00± 0.33 ^a	4000.00 ± 0.00 ^a	8000.00 ± 0.33 ^b	8000.00 ± 0.57 ^b	8000.00 ± 0.00 ^b	NA	50.00±0 .33 ^c
AN	4000.00 ± 0.00 ^a	4000.00 ± 0.33 ^a	1000.00± 0.00 ^b	2000.00 ± 0.57 ^c	4000.00 ± 0.00 ^a	8000.00 ± 0.00 ^d	8000.00 ± 0.57 ^d	NA	50.00±0 .33 ^e
TM	4000.00 ± 0.33 ^a	2000.00 ± 0.00 ^b	2000.00± 0.57 ^b	2000.00 ± 0.00 ^b	2000.00 ± 0.33 ^b	1000.00 ± 0.33 ^c	1000.00 ± 0.00 ^c	NA	16.00±0 .33 ^d
TV	2000.00	500.00±	500.00±	500.00±	500.00±	1000.00	1000.00	NA	16.00±

± 0.00^a 0.00^b 0.00^b 0.00^b 0.00^b ± 0.00^c ± 0.33^c 0.00^d

Values are mean inhibition zone (mm)±SD of three replicates

Values with different superscripts on the same row are significantly different ($P=0.05$)

FCZ, Fluconazole; KTZ, Ketoconazole; NA, Not applicable; ORG, organisms; Ca, *Candida albicans*; CK, *Candida krusei*; AN, *Aspergillus niger*; AF, *Aspergillus fumigatus*; TM, *Trichophyton mentagrophytes*; TV, *Trichophyton verucosum*

4. CONCLUSION

The ethyl acetate extract and fractions of *M. villosus* possess anti fungal activity against a broad spectrum of fungi ranging from yeast to dermatophytes. The plants hold great promise for the development of novel antifungal drug.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

CONSENT

Not applicable

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