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Original Research Article ***In vitro* Antifungal Activity of Ethyl Acetate Fractions of *Mitracarpus villosus* from Abuja, Nigeria.**

ABSTRACT

Introduction: The use of medicinal plants in the treatment of diseases is as old as man. The development of synthetic (orthodox) drugs led to a decline in the use of herbs however in the past few decades, there has been an increase in the use of medicinal plants, especially in developing countries. Several reports have shown that herbal medicines are well tolerated when compared with synthetic drugs. Over eighty percent of the population in Africa, most especially West Africa, has been reported to depend on medicinal plants for the treatment of infections and diseases.

Aims: The main objective of this study is to assess the anti-fungal potentials of the ethyl acetate extract and fractions of the aerial parts of *Mitracarpus villosus* (Sw.) DC from Abuja, Nigeria.

Methods: The powdered plant was extracted successively and exhaustively with hexane, ethyl acetate, ethanol and water. Thirty fractions were obtained from the extract using the bioassay-guided fractionation by means of column chromatographic technique. Antifungal activities of the ethyl acetate extract and fractions of *M. villosus* against clinical isolates of *Candida albicans*, *Candida krusei*, *Trichophyton mentagrophytes*, *Trichophyton verrucosum*, *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 of the extract and fractions ranged between 250 - 4000 µg / ml while 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 promises to hold good potentials for development of novel antifungal drug.

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 recent times, there has been a worldwide increase in the incidence of fungal infections, 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 thus 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].

Mitracarpus villosus (S.W) D.C belongs to the family Rubiaceae. It grows as an erect perennial annual plant with height up to 60cm. It can be found on old and abandoned farmlands where it grows as a weed with a wide distribution ranging from forest to savanna zones in tropical climates. Phytochemical evaluation of the leaves of this plant has been reported to show the presence of alkaloids, tannins, cardiac glycosides, saponins, soluble carbohydrate, flavonoids, reducing sugar, cyanide, glycoside, steroid and terpenoid [6]. In Tropical Africa, fresh extracts of *M. villosus* have been employed in traditional medicine for the treatment of sore throat. In West Africa, natives use extract of the plant for management and cure of several disease conditions which include headaches, toothaches, amenorrhoea, hepatic diseases, gastrointestinal disturbance (like dyspepsia), sexually transmitted diseases, leprosy [7]. The juice from the fresh plant is used to treat skin infections like ringworm and eczema, stoppage of bleeding and as first aid treatment for fresh cuts, wounds and ulcer. The aerial parts of *M. villosus* have also been formulated into lotions and skin ointments for the treatment of skin infections [8]. In Nigeria, the extracts of the juice from the fresh aerial parts of the plant is applied on the skin surface to treat skin diseases and heal wounds [9]. There is limited documentation 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 from Abuja, Nigeria.

2. MATERIAL AND METHODS

Collection and identification of plant material

Fresh aerial parts of *Mitracarpus villosus* was collected between June and September 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.

Drying of plant material

The aerial parts fresh plant was air dried at 25°C for 10 days. The completely dried aerial parts were crushed to coarse powder by grinding with wooden mortar and pestle.

Extraction of plant material

Successive extraction was employed for the extraction of active component of the plant. Simply, the dried powdered aerial parts of the plant was placed in a Soxhlet extractor (Quick Fit- England) and the plant material extracted successively and exhaustively each time with each of the various solvents in the order of increasing polarity i.e. hexane, ethyl acetate and ethanol respectively. Lastly, the marc was macerated in water and all the extract concentrated, dried and weighed [10].

Fractionation of ethyl acetate extract

The fractionation of ethyl acetate extract was done using the bioassay-guided fractionation by employing the accelerated gradient chromatographic (AGC) technique [11]. 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 chromatographic (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 in dimethyl sulfoxide (DMSO, BDH, Germany). Sabouraud dextrose broth was used to dilute the stock solutions to their required concentrations.

Fungi used

The fungi used for the study were clinical isolates of *Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, *Aspergillus niger*, *Trichophyton mentagrophytes* and *Trichophyton verrucosum* obtained from The Department of Microbiology and Biotechnology, National Institute for Pharmaceutical research and Development (NIPRD), Abuja, Nigeria. *Trichophyton verrucosum* was collected from the Dermatophylosis Center, National Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria.

Cultivation and standardization of test fungi

Eighteen-hour culture of the test *Candida spp.* on Sabouraud dextrose agar (SDA) was standardized according to National Committee for Clinical Laboratory Standards [12]. Colonies of the pure culture of the fungi on solid medium was gradually added to normal saline and its turbidity compared to 0.5 McFarland standard of which is approximately 1.5×10^8 cfu/ml. This was finally diluted with SDB to a population of 1.5×10^6 cfu/ml. Fungal spores *Trichophyton spp.* and *Aspergillus spp.*, were harvested from SDA slant cultures (7- 10 day old) by flooding with 10 ml sterile normal saline containing 3 % w/v Tween 80. Sterile glass beads were used to dislodge the spores [13]. Standardization of the fungal spore suspension to 1.0×10^6 spores / ml was carried out using a single-beam spectrophotometer (Spectronic 20D; Milton Roy Company, Pacisa, Madrid, Spain) at a wave length of 530 nm (OD530) and adjusted to 80 – 85 % transmittance (*Aspergillus spp.*) and 70 -72 % (*Trichophyton spp.*). Quantification was done by spreading 100 μ L of suspension on Sabouraud dextrose agar plate and incubated at 37°C for 18 h and 30°C for 72 hours for yeast and moulds respectively [14]. The cultures were checked for purity based on their morphological characteristics on media, morphology on staining and biochemical tests. The fungi were maintained on Sabouraud dextrose agar (SDA) at 4° C until required for use [14].

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

One milliliter of standardized culture of each test organism was spread on Sabouraud dextrose agar (excess aseptically drained) and the plates allowed to dry at 37°C temperature in a sterilized incubator. The susceptibility of the organisms to the plant extracts was carried out using the agar diffusion cup plate method [15]. Simply, a sterile cork borer (6mm) was used to bore holes in the agar plates and the bottoms of the wells sealed with the molten Sabouraud dextrose agar. With the aid of a micropipette, 0.1 ml each of gradient concentrations (mg/ml) of the crude ethyl acetate extracts was dispensed into the holes. Sterile distilled water was used as control. The plates were allowed to stand at room temperature for one hour in order to allow extract diffuse into the agar before incubation at 37°C for 18 hours (yeast) and 30°C for 72 hours up to 5 days (dermatophytes and moulds). The zones of inhibition produced by the extract on the test organisms were measured using a well-calibrated meter ruler to the nearest millimeter. The experiment was carried out in triplicates.

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

129 The minimum inhibitory concentration (MIC) of the extract and fractions was tested using the
130 serial broth micro dilution method [16]. Round bottom 96-well microtitre plates was used for
131 the assay. Fifty microliters of the working solutions of extracts and fractions was added into
132 the wells in row 1 and 2 of each column. The last eleven wells from rows 2 to 12 were filled
133 with 50µl of sterilized Saboraud dextrose broth and an identical two-fold serial dilution were
134 made from wells in rows 2 to the rows 11. The last wells in row 12 served as drug-free
135 controls. Sterile SDB was used as negative control while ketoconazole and fluconazole
136 served as positive controls. Lastly, 50µl of standardized fungal inoculum (10^6 cfu/ml⁻¹) were
137 added in all the wells from column A to H and mixed thoroughly to give final concentrations.
138 Tests were done in triplicates. The inoculated microplates were sealed with parafilm and
139 the plates incubated at 37°C for 18 hours (yeast) and 30°C for 48 hours and up to 5 days
140 (dermatophytes and moulds). MIC was defined as the first well with no visible growth after
141 24 hours.
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143 Determination of minimum fungicidal concentration (MFC) of the extract and 144 fractions.

145 The *in vitro* minimum fungicidal concentration was done by transferring fifty microliters of the
146 wells without any visible growth after MIC determination into fresh wells containing Saboraud
147 dextrose broth and incubated at 37°C for 18 hours (yeast) and 30°C for 48 hours and up to 7
148 days (dermatophytes and moulds). The lowest concentration resulting in no growth on
149 subculture was taken as the minimum fungicidal concentration [17].

150 Statistical analysis

151 The results obtained from the experiment were expressed as mean±standard deviation.
152 One way ANOVA (Smith's Statistical Package version 2.80) at p=0.05 was adopted in the
153 analysis of the findings.
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155 156 3. RESULTS AND DISCUSSION

157 A total of 30 fractions were collected from the elution of the ethyl acetate extract. Identical
158 fractions were combined based on thin layer chromatographic values giving 6 fractions M1 –
159 M6 (Table 1). The result of preliminary antifungal activities showed that the ethyl acetate
160 extract inhibited the growth of the test fungi with varying degree of inhibition which increased
161 with increase in the concentration of the agent (Table 2). The results of the minimum
162 inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) is shown in
163 Tables 3.0 and 4.0 respectively. The effect of ethyl acetate extract and fractions was
164 strongest on *T. verrucosum*. *Trichophyton verrucosum* a zoophilic dermatophyte is known to
165 be the predominant causative agent of cattle ringworm infection. It is also known to affect
166 (with lower prevalence), sheep, goats and other ruminants. *Trichophyton verrucosum* has
167 also been implicated in, several human outbreaks by direct contact with infected animals or
168 indirect contact with infectious propagules in the environment [18].
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170 Plants contain bioactive constituents as protective substances against bacteria, fungi,
171 viruses and pests [19]. The mode of action of ethyl acetate extract could be related to their
172 ability to alter membrane properties leading to cell death [20]. All the test fungi were found to
173 be susceptible to ethyl acetate extracts and fractions of *M. villosus* (Table 2), which is in line

with the works done in the past [21] which reported the potent antifungal activity of *M. villosus*. However, there were differences in the inhibition of growth among the compounds against different strains of fungi. The ethyl acetate extract and fractions were shown to be fungistatic at lower concentrations and fungicidal at higher concentrations. The inhibitory action of *M. villosus* against *Candida spp.* has been linked to the presence of acetophenone derivatives [9]. The alarming increase in the rate of candidiasis infection which has been worsened by the high infection rate of HIV/AIDS, necessitate research into plants with great promise for development into phytomedicine for the treatment of candidiasis in the near future. Recently *Aspergillus fumigatus* have been tagged as the most frequent causative agent of invasive fungal infection in immunocompromised people especially, those receiving immunosuppressive therapy for autoimmune or neoplastic disease, organ transplant recipients, and AIDS patients [22]. With the strong antifungal activity shown by the fractions *M. villosus* against this organism, this plant shows promising characteristics for the use in the treatment and management of invasive fungal infections. Generally, the anti fungal activity of the ethyl acetate extract and fractions of *M. villosus* was strong and comparable to that of ketoconazole and fluconazole, which has been linked to the combined action of the secondary metabolites present in the extract [20].

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

Table 2. Antifungal susceptibility testing of the ethyl acetate extract of *M. 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 ±0.33 ^a	2000.00 ± 0.57 ^b	1000.00± 0.00 ^a	1000.00 ± 0.00 ^a	500.00± 0.57 ^c	2000.00± 0.00 ^b	2000.00 ± 0.00 ^b	25.00± 0.00 ^d	NA
Ca2	1000.00 ± 0.57 ^a	2000.00 ± 0.00 ^b	1000.00± 0.00 ^a	1000.00 ± 0.00 ^a	2000.00 ± 0.33 ^b	500.00± 0.00 ^c	2000.00 ± 0.57 ^b	25.00± 0.33 ^d	NA
Ca3	500.00± 0.00 ^a	1000.00 ± 0.00 ^b	1000.00±0 .33 ^b	2000.00 ± 0.00 ^c	2000.00 ± 0.00 ^c	250.00± 0.00 ^d	1000.00 ± 0.00 ^b	25.00± 0.00 ^e	NA
Ca4	500.00± 0.00 ^a	2000.00 ± 0.00 ^b	500.00± 0.00 ^a	500.00± 0.33 ^a	1000.00 ± 0.33 ^c	250.00± 0.00 ^d	1000.00 ± 0.00 ^c	25.00± 0.33 ^e	NA

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Ck	2000.00 ± 0.33 ^a	4000.00 ± 0.33 ^b	500.00± 0.00 ^c	500.00± 0.00 ^c	1000.00 ± 0.00 ^d	500.00± 0.00 ^c	1000.00 ± 0.33 ^d	200.00 ± 0.00 ^e	NA
AF	2000.00 ± 0.00 ^a	2000.00 ± 0.00 ^a	2000.00±0 .33 ^a	2000.00 ± 0.00 ^a	4000.00 ± 0.00 ^b	4000.00± 0.33 ^b	4000.00 ± 0.00 ^b	NA	25.00 ± 0.33 ^c
AN	1000.00 ± 0.57 ^a	2000.00 ± 0.33 ^b	500.00± 0.00 ^c	1000.00 ± 0.00 ^a	2000.00 ± 0.57 ^b	4000.00± 0.00 ^d	4000.00 ± 0.33 ^d	NA	25.00 ± 0.57 ^e
TM	2000.00 ± 0.00 ^a	1000.00 ± 0.00 ^b	1000.00± 0.00 ^b	1000.00 ± 0.00 ^b	1000.00 ± 0.00 ^b	500.00± 0.00 ^c	500.00± 0.00 ^c	NA	8.00± 0.33 ^d
TV	500.00± 0.33 ^a	250.00± 0.00 ^b	250.00± 0.00 ^b	250.00± 0.00 ^b	250.00± 0.00 ^b	500.00± 0.00 ^a	500.00± 0.57 ^a	NA	4.00± 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	8000.00	1000.00±	4000.00	8000.00	8000.00	4000.00	2000.00	NA

	$\pm 0.57^a$	$\pm 0.33^a$	0.00^b	$\pm 0.00^c$	$\pm 0.00^a$	$\pm 0.00^a$	$\pm 0.57^c$	$\pm 0.00^d$	
AF	4000.00 $\pm 0.33^a$	4000.00 $\pm 0.00^a$	4000.00 $\pm$ 0.33 ^a	4000.00 $\pm 0.00^a$	8000.00 $\pm 0.33^b$	8000.00 $\pm 0.57^b$	8000.00 $\pm 0.00^b$	NA	50.00 $\pm$ 0 .33 ^c
AN	4000.00 $\pm 0.00^a$	4000.00 $\pm 0.33^a$	1000.00 $\pm$ 0.00 ^b	2000.00 $\pm 0.57^c$	4000.00 $\pm 0.00^a$	8000.00 $\pm 0.00^d$	8000.00 $\pm 0.57^d$	NA	50.00 $\pm$ 0 .33 ^e
TM	4000.00 $\pm 0.33^a$	2000.00 $\pm 0.00^b$	2000.00 $\pm$ 0.57 ^b	2000.00 $\pm 0.00^b$	2000.00 $\pm 0.33^b$	1000.00 $\pm 0.33^c$	1000.00 $\pm 0.00^c$	NA	16.00 $\pm$ 0 .33 ^d
TV	2000.00 $\pm 0.00^a$	500.00 $\pm$ 0.00 ^b	500.00 $\pm$ 0.00 ^b	500.00 $\pm$ 0.00 ^b	500.00 $\pm$ 0.00 ^b	1000.00 $\pm 0.00^c$	1000.00 $\pm 0.33^c$	NA	16.00 $\pm$ 0.00 ^d

Values are mean inhibition zone (mm) $\pm$ 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 antifungal activity against a broad spectrum of fungi ranging from yeast to dermatophytes. This medicinal plant holds 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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