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Effects of semisynthetic modifications on the antimicrobial activities of ethyl acetate extract of *Mitracarpus villosus* (Sw.) DC aerial part

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Abstract: *Mitracarpus villosus* (Sw.) DC is used for treatment of various ailments including headaches, toothaches, dyspepsia, hepatic diseases, leprosy, ringworm, eczema, fresh cuts, wounds, ulcer, and is taken orally for skin infection and as antidiarrheal and antidysentry. The dried powder of *Mitracarpus villosus* was extracted to obtain the crude ethyl acetate extract (MVET), which was subjected to semisynthetic modifications. Alkaline treatment of MVET yielded the acidic fraction (MVA) and the neutral fraction (MVN). Acetylation of MVET using acetic anhydride yielded the acetylated fraction (MVAC). The crude extract and the various fractions were subjected to thin layer chromatography and antimicrobial analysis. The organisms used for antimicrobial analysis were *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Streptococcus pygenes*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *E. coli*, *Candida albicans*, *Trichophyton rubrum* (ATCC 28188), *Trichophyton mentagrophytes* (ATCC 11621), *Microsporum canis* (ATCC 62903) and *Malassezier furfur* (ATCC 14521). The result of thin layer chromatography analysis showed MVET had six spots with relative retardation factors (Rf) of 0.50, 0.75, 0.88, 1.00, 1.25 and 1.70 respectively relative to β-sitosterol used as external reference standard. MVN had five spots with Rf of 0.75, 0.88, 1.00, 1.25 and 1.70. MVAC showed four spots with Rf of 0.75, 0.88, 1.00 and 1.25. MVA had 2 spots with Rf of 0.50, and 0.75. Results of anti-microbial analysis showed acetylated fraction (MVAC) had improved activity.
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1. Introduction

Medicinal plants have been a source of wide variety of biologically active compounds for many centuries and are used extensively for treating various disease conditions (Aboh *et al*., 2015). Medicinal plants are widely used in non-industrialized societies, mainly because they are readily available and cheaper than modern medicines. In 2017, the potential global market for Botanical extracts and medicines was estimated at several hundred billion dollars (Ahn, 2017). Research has shown that plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, anthraquinones and flavonoids among others, which have been found to have diverse biological and pharmacological activities (Aboh *et al*., 2015).

In Recent times there has been a worldwide increase in the incidence of fungal infections due to the resistance of some species of fungi to different fungicidal agents used in medicinal practices (Aboh *et al*., 2015). This incidence of fungal infections is thus increasing at an alarming rate, presenting an enormous challenge to healthcare professionals and thus, is directly related to the growing population of immune compromised individuals especially children resulting from changes in medical practice such as the use of intensive chemotherapy and immunosuppressive drugs (Aboh *et al*., 2015). The plant *Mitracarpus villosus* [Swartz] DC, formerly known as *Mitracarpus scaber* (Zuccarin), is a perennial annual herb growing to about 30-60cm tall and possesses rough leaves (Adamu *et al*., 2016). It belongs to the Family Rubiaceae and is widely distributed across the South (forest ecological zone) and the North (savanna ecological zone) of Nigeria (Adamu *et al*., 2016).

The plant is usually found in abundance in farmland as weed and is commonly called “Button Weed”, “Harwatsi" and “Yarwatsi" by the Hausa's, “Irawo Ille" by Yorubas, “Obuobwa" by Igbo's and “Gududal" by Sokoto Fulani's (Adamu *et al*., 2016). The seedlings have their cotyledons elliptically shaped at the top and about 7 mm long and 4 mm wide and the Adult Plant is highly branched and measures up to 60cm high, with Swivel roots, the stem is square, the stem is square and covered with fine hairs, the flowers are assembled in clusters consisting of 4 petals and 4 stamen and also possesses a stem that is angled, hairy and sparsely branched (Adamu et al., 2016). The leaves are green in colour when fresh with a mild odour and bitter-peppery taste and the leaf is simple in composition and are lanceolate and sabacute (3.0-6.0 cm long and 0.7-1.5 cm broad), with parallel veination, without leaf stalk that is sessile, having an internode length of 5.2 cm-7.2 cm. The veins are prominent on lower surface with nearly glabrous upper and lower surfaces with hairs on mid-rib regions (Adamu *et al*., 2016). The flowers are white in colour and densely crowded at the nodes, its calyx is lobed and the bracteoles are filiform in shape (Adamu *et al*., 2016).

In various parts of Tropical Africa, *Mitracarpus villosus* (Sw) DC is used for treatment of sore throat and has been used in traditional medicine practices in West Africa for treatment of headaches, toothaches, amenorrhoea, dyspepsia, hepatic diseases, general diseases as well as leprosy (Aboh *et al.*, 2014). In Nigeria, it has been used to treat ringworm, eczema, fresh cuts, wounds and ulcer and has also been taken orally for skin infection and as anti-diarhoea and anti-dysentry (Aboh *et al*, 2014). The extract of the native plant of *Mitracarpus villosus* is used to treat the infection known as “*Dermatophilus congolensis*" of cattle and decoction of its aerial part have been reported to have significant hepatoprotective effect against induced liver injury, both in vivo or vitro (Aboh *et al*, 2014). Various studies have identified the presence of important classes of secondary metabolites in the plant. *Mitracarpus villosus* found in South East and South-Southern Nigeria contained tannins, flavanoids, reducing sugar, cyanide, glycoside, steroid and terpenoid in varying concentrations depending on the environment where the leaves where harvested (Aboh *et al*, 2014). 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 (Dalziel, 1956).

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 (Bisignano *et al*., 2000). 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 (Abere *et al*., 2007). There is limited documentation on the antifungal potential of the ethyl acetate extract of *Mitracarpus villosus*. Hence, aim of this study was to investigate the effects of semisynthetic modifications on the antimicrobial activities of ethyl acetate extract of *Mitracarpus villosus* aerial part.

2. Materials and Methods

Collection and preparation of the plant materials

The fresh aerial parts of *Mitracarpus villosus* were collected from the National Institute of Pharmaceutical Research and Development (NIPRD) garden. The plant was identified in the herbarium of the Department of Medicinal Plant Research and Traditional Medicine, NIPRD, Abuja, Nigeria. The aerial parts were air-dried at room temperature (25 – 30^{o}C) for 10 days. The dried sample was crushed to coarse powder.

Extraction of Plant Material

Using a Soxhlet extractor (Quick Fit, England), the powdered aerial part was extracted successively and exhaustively with hexane and ethyl acetate. The ethyl acetate extract was filtered and concentrated to dryness using rotary evaporator. The dried *Mitracarpus villosus* ethyl acetate extract (MVET) was obtained Aboh *et al*. (2014).

Preparation of Neutral and Acidic Fractions

Two grams of the ethyl acetate extract (MVET) was dissolved in 50 ml of ethyl acetate and extracted with 1 M NaOH (4 x 50 ml) using separating funnel. All the aqueous portions were combined and washed with ethyl acetate (2 x 20 ml). All the ethyl acetate portions were combined, dried with anhydrous sodium sulphate and concentrated to dryness using rotary evaporator to obtain the neutral fraction (MVN). The combined aqueous portion was acidified with concentrated hydrochloric acid drop-wise until the solution became acidic (pH indicator, Macherey Nagel, Düren Germany). The acidified aqueous portion was extracted with ethyl acetate (4 x 50 ml). The combined ethyl acetate portion from the acidified aqueous portion was washed with distilled water until acid-free (pH indicator, Macherey Nagel, Düren Germany). The acid-free ethyl acetate portion was dried with anhydrous sodium sulphate and concentrated to

dryness using rotary evaporator to obtain the acidic fraction (MVA) (Ekong and Okogun, 1967).

Acetylation Reaction

To 2 g of MVET in a 250 ml conical flask was added 40 ml of ethyl acetate, 0.1 g of sodium bicarbonate ($NaHCO_3$) and 20 ml of alkaline treatment (Ac_2O). The conical flask was covered with aluminum foil and the reaction mixture stirred on a magnetic stirrer at room temperature (25 – 30°C) for 24 h. Thereafter, water saturated with sodium bicarbonate was added until the aqueous phase became slightly alkaline (pH indicator, Macherey Nagel, Düren Germany). The phases were separated using rotary evaporator. The organic phase was dried with anhydrous sodium sulphate (Na_2SO_4) and concentrated to dryness to obtain the acetylated product (MVAC) (Fulgentius *et al*., 2013).

Thin Layer Chromatography (TLC) Analysis

The crude ethyl acetate extract (MVET), the neutral fraction (MVN), the acidic fraction (MVA) and the acetylated fraction (MVAc) were spotted on TLC aluminium sheet Silica Gel 60 F_{254} precoated (Merck, Germany), previously activated at 105 °C for 2 hours, and developed with mobile phase solvent system of hexane-ethyl acetate 3:1. Beta-sitosterol (Sigma, Germany) dissolved in ethyl acetate was used as external reference standard and spotted alongside the samples. The TLC plate was dried, sprayed with 10% (v/v) sulphuric acid in ethanol and heated at 105 °C for 10 minutes to aid visualization of the spots. The relative retardation factor (Rr) of each spot relative to beta-sitosterol was calculated using the formulae:
Rr = Retardation factor (Rf) of spot / Retardation factor (Rf) of beta-sitosterol

Antimicrobial Assay

Antibacterial and antifungal activities of the plant extract and semisynthetic modifications were tested using well diffusion method (Gandhiraja *et al*., 2009). 0.1 ml of the overnight culture of the selected strains of bacteria were seeded into molten Muller Hinton media (Difco) and potato dextrose agar for fungi using pour plate method. Wells were made on the agar surface with a 6-mm cork borer. The extracts were poured into the well using sterile syringe. The plates were incubated at 37 ±2°C for 24 h for bacterial and 25 ± 2°C for 48 h for fungal activity. The plates were observed for the zone clearance around the wells. The samples were prepared by dissolving appropriate solvents and distilled water to give a concentration of 16 mg/ml (Oyetayo *et al*., 2007).

Preparation of Antimicrobial Agents

Stock solutions of Gentamycin 10mcg (antibacterial agent) and Terbinafine HCl 100 mcg (antifungal agent) were prepared by dissolving appropriate quantity of the antimicrobial agent in dimethyl sulfoxide (DMSO, BDH, Germany) and later diluted to the required concentration with broth.

Organisms Used for Antimicrobial Analysis

The organisms used included *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Streptococcus pygenes*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *E. coli*, *Candida albicans*, *Trichophyton rubrum* (ATCC 28188), *Trichophyton mentagrophytes* (ATCC 11621), *Microsporum canis* (ATCC 62903), and *Malassezier furfur* (ATCC 14521).

Cultivation and Standardization of Test Fungi

Eighteen-hour broth culture of *Candida albicans* was suspended in sterile Sabouraud dextrose liquid medium. It was standardized according to National Committee for Clinical Laboratory Standards Institute (CLSI, 2002) by gradually adding normal saline to compare its turbidity to McFarland standard of 0.5 which was approximately 1.0 x 10^6 cfu/ml. However, for *Trichophyton* species, fungal spores were harvested from 7 days old SDA slant cultures by washing with 10 mL sterile normal saline containing 3% w/v Tween 80 with the aid of sterile glass beads to help disperse the spores (Olowosulu *et al.*, 2005). Thereafter, the spore suspension was standardized to 1.0 x 10^6 spores/ml using a single-beam spectrophotometer (Spectronic 20D; Milton Roy Company, Pacisa, Madrid, Spain) at 530 nm (OD530) and adjusted to 70-72 % (*Trichophyton* spp.). All adjusted suspensions were quantified by spreading 100 µL on Sabouraud dextrose agar plate and incubated at 37 °C for 18 h for yeast and 30°C for 72 h for dermatophytes (Olowosulu *et al.*, 2005). All cultures were checked for purity (by morphological growth on media, staining and biochemical tests) and maintained on Sabouraud dextrose agar (SDA) for the fungal test organisms respectively at 4°C (in the refrigerator).

Antifungal Screening of Samples

Eighteen hours overnight cultures of *Candida albicans* and innoculum suspensions of the dermatophytes prepared from fresh, mature (3- to 5-day-old) cultures in Sabouraud dextrose liquid medium were standardized to produce inoculum size of 10^6 cfu/mL. One millilitre of the diluted culture of each test organism was used to flood Sabouraud dextrose agar media and the excess was aseptically drained. The plates were allowed to dry at 37°C in a sterilized incubator. Adopting the agar diffusion cup plate method (Olowosulu *et al.*, 2005), a sterile cup borer (6

mm) was used to bore holes in the agar plates. The bottom of the wells (holes) was sealed with the appropriate molten Sabouraud dextrose agar. Using micropipette, 0.1 ml each of 16 mg/ml concentration of MVET, MVN, MVA and MVAC were dispensed into the holes. Distilled water and the solvents used in diluting the extracts were used as control. These were allowed to diffuse into the agar at 25 °C for 1 h before incubation at 37 °C for 18 h (yeast) and 30 °C for 72 h up to 5 days (dermatophytes). The zones of inhibition of the test organisms were measured to the nearest millimeter, using a calibrated meter ruler. The experiment was carried out in triplicates.

3. Results

Table 1: Thin layer chromatography of the crude extract and fractions showing retardation factors and relative retardation factors.

	Crude ethyl acetate extract (MVET)	Neutral fraction (MVN)	Acidic fraction (MVA)	Acetylated fraction (MVAC)
SPOT 1	0.28 (0.50)		0.28 (0.50)	
SPOT 2	0.42 (0.75)	0.42 (0.75)	0.42 (0.75)	0.42 (0.75)
SPOT 3	0.49 (0.88)	0.49 (0.88)		0.49 (0.88)
SPOT 4	0.56 (1.00)	0.56 (1.00)		0.56 (1.00)
SPOT 5	0.70 (1.25)	0.70 (1.25)		0.70 (1.25)
SPOT 6	0.95 (0.70)	0.95 (0.70)		

MVET had six spots with relative retardation factors (Rr) of 0.50, 0.75, 0.88, 1.00, 1.25 and 1.70 respectively relative to β-sitosterol used as external reference standard (spot 4). MVN had five spots with Rr of 0.75, 0.88, 1.00, 1.25 and 1.70. MVAC showed four spots with Rr of 0.75, 0.88, 1.00 and 1.25. MVA had 2 spots with Rr of 0.50, and 0.75.

Table 2: Antimicrobial activity of the crude extracts and modified Fractions at 16mg/ml

Organism	Inhibition Zone Diameter (mm)				
	MVET	MVN	MVAC	MVA	Positive Control
Pseudomonas aeruginosa	12.0	14.0	14.0	13.0	20.0
Bacillus subtilis	12.0	14.0	15.0	13.0	20.0
Streptococcus pygenes	17.0	14.0	15.0	12.0	23.0
Salmonella paratyphi	20.0	19.0	17.0	10.0	25.0
Klebsiella pneumonia	12.0	14.0	13.0	12.0	20.0
Staphylococcus aureus	15.0	16.0	14.0	15.0	27.0
E. coli	14.0	15.0	16.0	14.0	18.0
Candida albicans	14.0	13.0	13.0	11.0	25.0
Trichophyton rubrum **ATCC 28188**	12.0	13.0	11.0	15.0	30.0
Trichophyton mentagrophytes **ATCC 11621**	10.0	12.0	0.0	15.0	30.0
Microsporum canis **ATCC 62903**	12.0	0.0	0.0	0.0	28.0
Malassezier furfur **ATCC 14521**	0.0	12.0	11.0	12.0	30.0

Positive control: Gentamycin 10 µg/ml (antibacterial agent) and Terbinafine HCl 100 µg/ml (antifungal agent). **Bacteria:** *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Streptococcus pygenes*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *E. coli*. **Fungus:** *Candida albicans*, *Trichophyton rubrum* (ATCC 28188), *Trichophyton mentagrophytes* (ATCC 11621), *Microsporum canis* (ATCC 62903), and *Malassezier furfur* (ATCC 14521).

4. Discussion

In the present era, plant and herb resources are abundant, but these resources are dwindling fast due to the onward march of civilization (Vogel, 1991). Although a significant number of studies have been used to obtain purified plant chemical, very few screening programmes have been initiated on crude plant materials. It has also been widely observed and accepted that the medicinal value of plants lies in the bioactive phytocomponents present in the plants (Veeramuthu *et al*., 2008). The result of the extraction and semisynthetic modification of the crude ethyl acetate extract of *Mitracarpus villosus* aerial part yielded the crude ethyl acetate extract of *Mitracarpus villosus* (MVET), the neutral fraction (MVN), the acidic fraction (MVA), and the acetylated product (MVAC). The result of thin layer chromatography of the crude ethyl acetate extract of *Mitracarpus villosus* aerial part (MVET), the neutral fraction (MVN), the acidic fraction (MVA), the

acetylated product (MVAC) and β-sitosterol used as external reference standard is shown in Table 1

Crude ethyl acetate extracts (MVET), which was subjected to semisynthetic modifications. Alkaline treatment of MVET yielded the acidic fraction (MVA) and the neutral fraction (MVN). Acetylation of MVET using acetic anhydride yielded the acetylated fraction (MVAC).

Plants contain bioactive constituents as protective substances against bacteria, fungi, viruses and pests (Aqil *et al*., 2012). The mode of action of ethyl acetate extract could be related to their ability to alter membrane properties leading to cell death (Irobi and Daramola, 1994). All the test fungi were found to be susceptible to ethyl acetate extracts and fractions of *M. villosus* (Table 2), which is in line with the works done in the past (Aboh *et al*, 2014) 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 (Abere et al., 2007). Several bioactive compounds have been isolated from the plant. These compounds included stigmasterols, urlolic acids, pentalongin, azanthroquinones, psoralen isolated from the aerial parts of *Mitracarpus villosus* (Adamu *et al*., 2016). Furthermore, reasonable quantities of gallic acid (1%), 3,4,5-trimethoxybenzoic acid (0.1%), 4-methoxyacetophenone (0.046%), 3,4,5-trimethoxyacetophenone (0.09%), kaempferol-3-O-rutinoside (0.07%), rutin (0.2%) and psoralen (0.09%) were reportedly isolated from methanol extract of the aerial part of the plant (Adamu *et al*., 2016). Oleanolic and ursolic acids were also identified from the alcohol extract of the plant and it was also observed that the presence of coumarin-like compounds were presumably responsible for the antifungal activity of the plant (Adamu *et al*., 2016).

The antifungal activities of different extracts of *Mitracarpus villosus* have been reported by several researchers. The methanol extract of *Mitracarpus villosus* had been established to possess antimycotic activities against *Candida albicans* strains and 4-methoxyacetophenone and 3, 4, 5-trimethoxyacetophenone isolated from *Mitracarpus villosus*, were also reported to effectively inhibit *Candida albicans* at MIC of 1.95mg/ml (Bisignano *et al*., 2000). Also, azaanthroquinones isolated from the plant demonstrated significant activity against *Candida Albicans* with MIC of 6.2ug/ml (Okunade et al., 1999).

The antifungal activities of ethyl acetate extract and fractions of *Mitracarpus villosus* against clinical isolates of *Candida albicans, Candida krusei* had been reported by Aboh *et al*. (2014). It has been reported that the ethyl acetate extract of *Mitracarpus villosus* aerial parts produced the highest antifungal activity with the minimum inhibitory concentration and minimum fungicidal concentration values of 0.50-2.00 mg/ml and 2.00-8.00 mg/ml respectively when compared with the other solvent extracts (hexane, ethanol and water) (Aboh *et al*., 2014). However, no report is available on the semisynthetic modifications of ethyl acetate extract of *Mitracarpus villosus* aerial part and the effects on the antimicrobial activities.

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 immunocom promised people especially, those receiving immunosuppressive therapy for autoimmune or neoplastic disease, organ transplant recipients, and AIDS patients (Ben-Ami et al., 2010). 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 (Irobi and Daramola, 1994).

Conclusion

From the above studies, it is concluded that the traditional plants may represent new sources of anti- microbials with stable, biologically active components that can establish a scientific base for the use of plants in modern medicine. These local ethnomedical preparations and prescriptions of plant sources should be scientifically evaluated and then disseminated properly and the knowledge about the botanical preparation of traditional sources of medicinal plants can be extended for future investigation into the field of pharmacology, phytochemistry, ethnobotany and other biological actions for drug discovery. The ethyl acetate extract and fractions of *M. villosus* possess antifungal activity against a broad spectrum of fungi ranging from *Pseudomonas aeruginosa* to *Malassezier furfur*. This medicinal plant holds great promise for the development of novel antifungal drug. Given the pharmacological activities of these plants, toxicity and clinical studies are required to promote their therapeutic uses.

Conflict of Interest
The authors declare no conflict of interest.

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