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Phytochemical, Toxicological and In vitro Antifungal Studies of *Mitracarpus hirtus* L.

Hassan Abubakar[1] ✉, Alam, O.[2], Tukur Mokhtar[1], Muazu A. S.[1], Yusuf A. J.[3]

[1]Department of Chemistry, Sokoto State University, Sokoto, Nigeria. E-mail: hassan.abubakar@ssu.edu.ng
[2]Department of Pharmaceutical Chemistry, Jamia Hamdard, New Delhi, India. E-mail: oalam@jamiahamdard.ac.in
[3]Department of Chemistry, Sokoto State University, Sokoto, Nigeria. E-mail: tukur.mukhtar@ssu.edu.ng
[4]Department of Chemistry, Sokoto State University, Sokoto, Nigeria. E-mail: safgummi@yahoo.com
[5]Department of Pharmaceutical and Medicinal Chemistry, Usmanu Danfodiyo University, Sokoto, Nigeria. E-mail:yusufaj@ymail.com
Corresponding Author, Department of Chemistry, Sokoto State University, Sokoto, Nigeria. E-mail: hassan.abubakar@ssu.edu.ng

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Abstract

Objective: In this work, phytochemical screening, acute toxicity and antifungal evaluation on the methanol extracts of different parts (leaves, seed and aerial part) of *Mitracarpus hirtus* against *Candida albicans*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus niger*, *Histoplasm capsulatum*, *Mucor* sp, *Microsporum canis*, *Microsporum gallinae*, *Trichophyton mentagrophyte*, and *Trichophyton rubrum* using agar well diffusion and broth dilution techniques is investigated.

Material and Methods: The phytochemical screening of the methanol extracts of different parts of the plant revealed the presence of tannins saponins, flavonoids, alkaloids, steroids, and terpenoids and the median lethal dose (LD50) was estimated to be greater than 5000 mg/kg which indicates that the plant is non-toxic.

Results: Susceptibility test for the methanol extracts showed mean zone of inhibition ranging from 19.33±0.58-23.33±0.56 mm against the test organisms while the standard drug, Fulcin had 24.33±0.58 – 31.67±0.58 mm. The minimum inhibitory concentration (MIC) of the extracts ranges between 0.25-0.50 mg/mL while the minimum fungicidal concentration (MFC) ranges between 0.25-1.00 mg/mL against some of the fungal pathogens.

Conclusion: The findings of this study have revealed the antifungal efficacy of methanol extracts of the different part of *M. hirtus* which supports the ethno-medicinal claim of the use of the plant in the treatment of fungal infections.

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Introduction

Fungal illnesses were only recognized as a major public health issue in the second part of the twentieth century, when breakthroughs in medicine collided with the start of the HIV epidemic, resulting in vast numbers of human hosts with compromised immunity [1]. The most common fungal infections affecting humans are Candidiasis and Mucomycosis [2]. Dermatomycoses have become more common in recent years, and modern anti-dermatophytosis drugs have a variety of drawbacks, including adverse effects, limited efficacy, and an increase in drug resistance in human pathogens [3]. Fungal infections especially those caused by Candida albicans which is usually resistant to fluconazole, has long been the most common cause of invasive and mucocutaneous infections. However, as antifungal medications and diagnostic procedures become more widely utilized, the epidemiology of Candida infections is changing, resulting in an increase in infections caused by species with less expected antifungal susceptibility [4].

Modern and traditional medicine both rely on medicinal plants that contain bioactive chemicals. Indigenous plants have historically offered superior raw materials for the pharmaceutical, food, cosmetic, and fragrance industries [5]. Scientists have been investigating different plants to discover their potential biological impacts due to the existence of various phytochemical constituents in plants [6]. M. hirtus L. is a plant in the Rubiaceae family that is generally known as Button grass in English and Harwastii in Hausa. It is found in tropical and subtropical regions such as India, the United States of America, Malaysia, Thailand, and East and West African countries [7]. Ethnobotanical surveys of M. hirtus have revealed that the plant is used to cure fungal infections, skin illnesses like eczema, ringworm, rashes, itching, toothache, and venereal diseases by applying the leaf sap, rubbing the leaves on the skin, or taking the leaves orally [7].

Mitracarpus species including M. hirtus have been shown to demonstrate different pharmacological activities such as antioxidant [8], antibacterial [9], antifungal [10, 11], anti-imflammatory [12], anticancer [13], anti-diabetic [14], anti-direreal [15] and hepatoprotective activities [16]. Phytochemical and spectroscopic investigation on the extract of M. hirtus and other Mitracarpus species led to the identification and isolation of various phytochemical constituents including alkaloids, tannins, cardiac glycosides, saponnins, flavonoids, steroids/terpenoids [17], kaempferol [18], quercetin [19], rutin and kaempferol-3-O-rutinoside [20].

Thus, in this work, phytochemical screening, acute toxicity and antifungal evaluation on the methanol extracts of different parts (leaves, seed and aerial part) of *Mitracarpus hirtus* against *Candida albicans*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus niger*, *Histoplasm capsulatum*, *Mucor* sp, *Microsporum canis*, *Microsporum gallinae*, *Trichophyton mentagrophyte*, and *Trichophyton rubrum* using agar well diffusion and broth dilution techniques.

Materials and Method

Material

Ohaus digital weighing balance (Champ 11 CH15R, Ohaus Corporation, Pinebrook NJ, USA) Dimethyl Sulfoxide (LOBA Chemie Pvt Ltd, India), Methanol (LOBA Chemie Pvt Ltd, India), Biological safety cabinet (NZS 2208 ESCOBIOTEH), Portable pressure steam sterilizer YX-280A, Ocean med. England), UV- Spectrophotometer (41200) Abrera (BARCELONA) Spain, Vertical automatic electro thermal pressure steam sterilizer (LX-C35L) HEFEI HUATAI Medical equipment Co. LTD), Cork borer 6mm, Disposable Petri Dishes, Sabouroud dextrose Agar (Himedia Laboratoies Pvt Ltd, india), Sabouroud dextrose broth (Himedia Laboratoies Pvt Ltd, india).

Methodology

Collection and identification of plant material

The M. hirtus samples were collected at the premises of Usmanu Danfodiyo University Teaching Hospital in Sokoto State. It was assessed and identified by Musa Abubakar at the herbarium unit of the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. A voucher number (PCG/UDUS/RUBI/002) was issued after identification.

Preparation and extraction of plant material

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Different samples (leaf, seed and aerial) of the plant *M. hirtus* were shed dried, pulverized into powder using pestle and motor and stored at room temperature for future use. About 300 g each of the powdered plant materials were extracted with methanol using maceration method for 3 days each with occasional agitation. The extracts were filtered and evaporated *in-vacuo* using rotary vacuum evaporator at 40 °C to afford a concentrated residue known as crude methanol leaf (MLE), crude methanol aerial (MAE) and crude methanol seed (MSE) extracts, respectively.

Preliminary phytochemical investigation

Different chemical tests were carried out on the methanol extracts to identify the presence of various phytochemical constituents such as alkaloids, flavonoids, and tannins etc. using standard procedures [21].

Toxicological Studies

Experimental animals

Animal House, Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria, provided locally bred adult Swiss albino mice of either sex (22–30 g body weight). The animals were fed a laboratory diet and given free access to water, and they were kept in clean cages under regular 12 hr light/12 h dark cycles. All experimental methods were followed, as well as the ethical requirements for the care and use of laboratory animals.

Acute Toxicity Study (ATS)

ATS was performed on MLE, MAE and MSE according to the method described by Lorke's [22] via oral route. Nine mice were randomly divided into three groups of three mice each in the first phase; Groups 1, 2 and 3 were administered with the different doses MLE at 10, 100, and 1000 $mgkg^{-1}$ and they were monitored for 24 h for any signs of toxicity or mortality. Based on the results of the first phase, three more precise dosages of MLE at 1600, 2900, and 5000 mg/kg body weight were given to three new mice in the second phase, and they were also examined for 24 h. The median lethal dose (LD_{50}) was calculated as the geometric mean of the lowest lethal dose and the maximum dose survived by the animal. The above procedure was repeated for MSE and MAE

Antifungal Studies

Test Organisms

Clinical isolates *Candida albicans*, *Candida tropicalis*, *Aspergillus fumigatus*, *Aspergillus niger*, *Histoplasm capsulatum*, *Mucor* sp, *Microsporum canis*, *Microsporum gallinae*, *Trichophyton mentagrophyte*, and *Trichophyton rubrum* were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. All the isolates were checked for purity and maintained in slants of sabouraud dextrose agar.

Standardization of inoculum

A 0.5 MC-Farlands turbidity standard scale was developed. Continuous dilutions of the organism suspension were made with sterile normal saline until the turbidity matched Mc-Farland's scale visually [23].

Preparation of the plant materials.

About 0.01 g of each of the extracts was weighed and dissolved in 10 mL of DMSO to obtain a stock concentration of 1 mg/mL. This was the initial concentration of the extract used to determine its antifungal activities.

Antifungal Screening

The antifungal screening of the extracts was determined using Agar well diffusion method [24]. Sabouraud dextrose agar was the medium used as the growth medium for the microbes. The medium was prepared according to the manufacturer instructions, sterilized at 121°C for 15 mins, poured into the sterile petri dishes and was allowed to cool and solidify. The sterilized medium was seeded with 0.1 mL of the standard inoculum of the test microbes, the inoculums were spread evenly over the surface of the medium with the aid of a sterile swab. Using a standard cork borer of 6mm in diameter a well was bored at the center of each inoculated medium. 0.1 mL solution of each of the extract of 1 mg/mL concentration was then introduced into the well on the inoculated medium, separately. Incubation was made at 30 °C

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for 1–7 days, after which the plates of the media were observed for the zone of inhibition of growth, the zone was measured with a transparent ruler and the result recorded in millimeter. The experiment was carried out in triplicate for each extract.

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of the extracts was determined using the broth dilution method [25]. Sabouraud dextrose broth was prepared, 10 mL was dispensed into test tubes and was sterilized at 121 ^{o}C for 15 mins and the broth was allowed to cool. MC- Farland's turbidity standard scale number 0.5 was prepared. The test microorganism was injected and incubated at 30 ^{o}C for 6 h after 10 mL of normal saline was poured into a sterile test tube. By visual inspection, the microorganism was diluted in normal saline until the turbidity matched that of the MC- Farland turbidity scale; the test microorganism has a concentration of around $1.5X10^{8}$ cfu/mL at this moment. The extracts were serially diluted twice in sterile broth to generate concentrations of 1, 0.5, 0.25, 0.125 and 0.063 mg/mL. The initial concentration was obtained by dissolving 0.01 g of each of the extract in 10 mL of the sterile broth. Having obtained the different concentrations of the extract in the sterile broth, 0.1 mL of the test microbe in the normal saline was then inoculated into the different concentrations, incubation was made at 30^{0}C for 1-7 days, after which the test tubes of the broth were observed for turbidity (growth) the lowest concentration of the extract in the sterile broth which shows no turbidity was recorded as the MIC. .

Determination of the Minimum Fungicidal Concentration (MFC)

The MFC was determined in accordance with method [26]. It was used to check if the extracts were able to kill or inhibit the growth of the test microorganisms. Sabouraud dextrose agar was sterilized at 121°C for 15 minutes, then placed into sterile petri dishes to cool and solidify. The contents of the MIC in serial dilutions were then sub-cultured onto the prepared medium, incubated at 30 °C for 1–7 days, and the plates of the medium were observed for colony growth. The plates with the lowest concentration of the extracts without colony growth were recorded as the MFC.

Statistical Analysis

The data were expressed as mean±S.E.M, and they were assessed for significance using analysis of variance (ANOVA) and values were considered significant at $p<0.05$.

Results

Percentage Yield

Percentage yield of MLE, MSE and MAE of *M. hirtus* obtained from the powdered samples (300 g) via maceration method were 9.79, 7.04 and 4.87 %, respectively (Table 1).

Phytochemical screening

Preliminary Phytochemical screening of MLE, MSE and MAE of *M. hirtus* revealed the presence of carbohydrates, cardiac glycosides, tannins, saponins, alkaloid, flavonoids, steroids and triterpenes in all the extracts (Table 2).

Acute toxicity test

The median lethal dose (LD_{50}) of MLE, MSE and MAE of *M. hirtus* was estimated to be ≥ 5000 mg/kg which indicates that the plant is non-toxic (Table 3). No mortality was recorded for all the extracts.

Susceptibility test

Antifungal susceptibility test results indicated significant effect with mean zone of inhibition ranging between 19.33 – 23.33 mm against the test organisms. No activity was recorded against *A. niger*, *M. specie*, *M. gallinae* and *C. tropicalis* but MSE was effective against *M. canis* (Table 3). The MAE and MES were the most active extracts (20.33 – 23.33 mm) while MLE was the least active (19.33 – 22.33 mm). The MIC and MFC of the extracts ranges between 0.25 – 0.5 mg/mL and 0.25 – 1.0 mg/mL against the susceptible organisms respectively (Tables 5 & 6).

The results of the minimum inhibitory concentration (MIC) showed that crude methanol extract of both seed and aerial part exhibited MIC at 0.25 mg/mL against *A. fumigatus, H. capsulatum, T. mentagrophyte* and *T. rubrum*, and *Candida albican* while leave extract recorded MIC range

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between 0.25 mg/mL - 0.5 mg/mL against the same fungal pathogens of *A. fumigatus, H. capsulatum, T. mentagrophyte* and *T. rubrum. H. capsulatum* and *Candida albican.*

The results of minimum fungicidal concentration (MFC) of methanol extracts of both the leave and the aerial parts exhibited MFC at 1 mg/mL, while that of seed extract range between 0.5 – 1 mg/mL against the susceptible organisms.

Table 1: *Percentage Yield of MLE, MSE and MAE of M. hirtus*

Extract	Weight (g)	Yield (%)	Colour
MAE	14.63	4.87	Light green
MLE	29.39	9.79	Dark green
MSE	21.11	7.04	Dark green

Key: MLE=methanol leaf extract, MSE= methanol seed extract, MAE= methanol aerial extract

Table 2: *Phytochemical Screening of MAE, MLE and MSE of M. hirtus*

Phytoconstituent	Test	MAE	MLE	MSE
Saponins	Frothing	++	++	+
Carbohydrates	Molisch's	++	++	++
	Fehling's	++	++	++
Tannins	Ferric chloride	++	++	++
	Lead acetate	++	++	++
Steroids/Terpenes	Liebermann-Burchard's	++	++	++
	Salkowski's	++	++	++
flavonoids	Shinoda	++	++	++
	Alkaline	++	++	++
	Ferric chloride	++	++	++
Cardiac glycosides	Killer-Killiani's	++	++	+
Anthraquinones	Bontrager's	-	-	-
Alkaloids	Mayer's	N.D	N.D	N.D
	Wagner's	N.D	N.D	N.D
	Dragendorff's	++	++	++

Key: - = absent; + = present; ++ = moderate; MLE=methanol leaf extract, MSE= methanol seed extract, MAE= methanol aerial extract, N.D = not detected

Table 3: *Median lethal Dose (LD_{50}) of MLE, MSE and MAE of M. hirtus*

Experiment	Dose (mg/kg)	No. of mortality within 24 h		
		MAE	MLE	MSE
Phase 1	10	0/3	0/3	0/3
	100	0/3	0/3	0/3
	1,000	0/3	0/3	0/3
Phase 2	1,600	0/1	0/1	0/1
	2,900	0/1	0/1	0/1
	5,000	0/1	0/1	0/1

Key: MSE= MAE= methanol aerial extract, MLE=methanol leaf extract, methanol seed extract

Table 4. *Antifungal susceptibility test on methanol extract of three different part of M. hirtus*

	Mean zone of inhibition (mm)			
Organisms	MAE	MLE	MSE	Fulcin
A. fumigatus	20.33±0.58	19.33±0.58	20.67±0.58	27.00±1.00
A. niger	-	-	-	31.67±0.58
H. capsulatum	22.80±0.83	22.00±0.58	23.33±0.56	-
M. specie	-	-	-	28.67±0.58
M. canis	-	-	20.33±0.58	24.33±0.58

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M. gallinae	-	-	-	-
T.mentagrophyte	21.33±0.58	19.33±0.58	22.33±0.58	-
T. rubrum	20.67±0.58	20.33±0.58	20.33+0.58	31.33±0.58
C. albican	23.33±0.58	20.33±0.58	22.33±0.58	-
C. tropicalis	-	-	-	-

Values are presented as mean inhibition zone (mm) ± S.E.M of three replicates;

Key; MAE=Methanol Aerial part extract, MLE= Methanol leave Extract, MSE= Methanol Seed Extract

Table 5: *MIC of MLE, MSE and MAE of M. hirtus against the test isolates*

Extract	Conc. (mg/mL)	Test organisms					
		A. fumigatus	H. capsulatum	M. canis	T. mentagrophyte	T. rubrum	C. albican
MAE	1	-	-		-	-	-
	0.5	-	-		-	-	-
	0.25	O*	O*		O*	O*	O*
	0.125	+	+		+	+	+
	0.0635	++	++		++	++	++
MLE	1	-	-		-	-	-
	0.5	O*	-		O*	-	-
	0.25	+	O*		+	O*	O*
	0.125	++	+		++	+	+
	0.0635	++	++		++	++	++
MSE	1	-	-	O*	-	-	-
	0.5	-	-	+	-	-	-
	0.25	O*	O*	++	O*	O*	O*
	0.125	+	+	++	+	+	+
	0.0635	++	++	++	++	++	++

Key -=> No turbidity (no growth), O*=>MIC, +=>turbid (light growth), ++=>Moderate turbidity, +++=>High turbidity, conc. = concentration

Table 6: *MFC of MLE, MSE and MAE of M. hirtus against the test isolates*

Extract	**Conc. (mg/mL)**	**Test organisms**					
		A. fumigatus	***H. capsulatum***	***M. canis***	***T. mentagrophyte***	***T. rubrum***	***C. albican***
MAE	**1**	O*	O*	-	O*	O*	-
	0.5	+	+	-	+	+	O*
	0.25	++	++	-	++	++	+
	0.125	++	++	-	++	++	++
	0.0635	++	++	-	++	++	++
MLE	**1**	O*	O*	-	O*	O*	O*
	0.5	+	+	-	+	+	+
	0.25	++	++	-	++	++	++
	0.125	++	++	-	++	++	++
	0.0635	++	++	-	++	++	++
	1	O*	-	O*	O*	O*	O*

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	0.5	+	O*	+	+	+	+
MSE	**0.25**	++	+	++	++	++	++
	0.125	++	++	++	++	++	++
	0.0635	++	++	++	++	++	++

Key=- No colony, o*=>/MFC, +=>Scanty colony growth, ++=>Moderate colony growth, +++=>Heavy colony growth, conc. = concentration

Discussion

The percentage yield of MLE was higher compared to that of MAE and MSE suggesting that the leaves of *M. hirtus* contain polar constituents compared to the seeds and the aerial parts. Phytochemical screening of the crude methanol extracts (MLE, MSE, and MAE) of the plant revealed the presence of carbohydrates, cardiac glycosides, tannins, saponins, alkaloids, flavonoids, steroids and triterpenes, these constituents are in close agreement to the findings reported for the ethanol leaves, roots and stem bark extracts of *M. hirtus* [11, 17]. More so, these phytochemicals have been reported to be responsible for most of the pharmacological activities of the plant extracts including antimicrobial effects [27-29].

The acute toxicity studies of the methanol extracts of *M. hirtus* in mice showed that no mortality was observed after administration of the different doses of the extracts within 24 hours; but the most common sign of toxicity that was observed between 0 – 10 minutes after oral administration for all the animals include; fur erection, difficulty in breathing, itching of mouth and gasping. These signs of toxicity were not observed with the groups that received lower doses of the extracts but as the doses were increased, the toxicity signs became more apparent. The LD_{50} was found to be greater than 5000 mg/kg B.W indicating that all the three parts of *M. hirtus* used in this study are non-toxic [22]. Hence, the findings have corroborated what was reported for the aerial part of *M. hirtus* [11].

Studies on the ethnomedicinal uses and the pharmacological actions of the plant revealed that the M. hirtus contain some bioactive secondary metabolites with promising antifungal activities [11, 30]. The present study, tested the antifungal activity of the different parts of M. hirtus against some fungal isolates including C. albicans, C. tropicalis, A. fumigatus, A. niger, H. capsulatum, Mucor sp, M. canis, M. gallinae, T. mentagrophyte, and T. rubrum. The findings revealed that MLE, MAE and MSE exhibited good antifungal effect against the test organisms with the exception of A. niger M. specie M. gallinae C. tropicalis; though the standard antifungal agent, fluconazole exhibited higher effect compared to the extracts.

The minimum inhibitory concentration results showed that MAE and MSE inhibited the growth of the susceptible organisms at a lower MIC value of 0.25 mg/mL suggesting that this extracts could be used in the management of skin infections caused by dermatophytes, candida and histoplasmosis species. Extracts of *M. hirtus* and some *Mitracarpus* species have in various studies exhibited lower MIC values when tested against different fungal pathogens signifying the efficacy of the *M. hirtus* used in this study [21]. Minimum fungicidal concentration was observed to be higher than the MIC against some test isolates suggesting that at a higher concentration, the extracts have fungicidal effects but at lower MIC it tends to exhibit fungistatic effects. Antifungal drugs in clinical use primarily target the fungal cell wall, cell membrane, DNA and protein synthesis, and signal transduction pathways [31-33]. In addition, extracts and essential oils from plants have been shown to limit fungal growth by a variety of processes, including membrane disruption and the production of reactive oxygen species [34, 35]. Thus, the extracts of *M. hirtus* might elicit their antifungal effects via the same mechanisms.

As part of our recommendations, further studies to established the *in vivo* antifungal activity of *M. hirtus* and mechanism of action involved as well as the isolation and characterization of the bioactive constituents responsible for the observed effect and *In silico* modelling studies of the compounds from the plant should be conducted because the scope of this study was restricted to phytochemical, toxicological and *in vitro* antifungal activity.

Conclusion

The results obtained from this study indicated that methanol extracts of the different parts of *M. hirtus* exhibited significant antifungal activity against the susceptible

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organisms with inhibitory and fungicidal effects at a lower concentration. The antifungal efficacy of *M. hirtus* in this investigation supports its ethno-medicinal use in the treatment of fungal infections.

Conflict of interest

There is no conflict of interest among the authors

Consent for publications

The authors approved the manuscript for publication

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