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Antifungal Activities of Some Nigerian Medicinal Plants against Non-dermatophyte Molds Isolated from Cases of Onychomycosis among Rice Farmers in Anambra State, Nigeria

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Research Article

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ABSTRACT

Aims: To investigate *in vitro* antifungal activities of methanol, hexane and cold water extracts of *Cassia alata, Mitracarpus villosus* and *Lawsonia inermis* against non-dermatophyte molds isolated from rice farmers with onychomycosis in Anambra State, Nigeria.
Study Design: Examination of antifungal activity of medicinal plants among cross-section of farmers.
Place and Duration of Study: Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University Awka. Anambra State, Nigeria between November 2009 and June 2011.
Methodology: Clinical samples were collected from 135 rice farmers in Anambra State, Nigeria and identified. Dried leaves of *C. alata, M. villosus* and *L. inermis* were extracted by soxhlet using methanol and hexane as solvents. Cold water extraction was also carried out using fresh leaves. The extracts were tested against the isolated non-dermatophyte molds using disc diffusion method at varying concentrations (10mg, 20mg, 40mg, 80mg). Discs impregnated with 2% dimethylsulphoxide were used as negative control while those impregnated with 2mg/disc ketoconazole served as positive control. The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MIC) of the crude extracts were assayed for against the test isolates.
Results: The organisms recovered included *Aspergillus terrus, Aspergillus sclerotiorum, Aspergillus flavus, Fusarium* sp., *Chrysosporium* sp. and *Scopulariopsis* sp. The organisms were sensitive to all the methanol extracts of medicinal plants with minimum inhibitory concentration range of 10-40mg/disc except *A. flavus* which was inhibited only by *L. inermis* extract at concentration of 40mg/disc. The mean zone of inhibition produced ranged

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between 6.0mm and 14.2mm with *C. alata* extract showing the highest zone of inhibition (14.2mm) against *Fusarium* sp. The standard Ketoconazole range was 6.0-12.4mm diameter. Hexane extract of *L. inermis* inhibited all isolates at 20-40mg/disc concentration. *A. flavus* resisted hexane extracts of *C. alata* and *M. villosus*, while *A. terrus* resisted extract of *M. villosus* alone. All isolates except *A. flavus* were sensitive to cold water extract of *L. inermis* with diameter zone of inhibition range of 6.2-8.2mm. Methanol extracts of *C. alata* and *L. inermis* showed fungicidal actions against all test isolates at 10-40mg/disc range except for *A. flavus.*

Conclusion: The various antifungal extracts showed inhibitory/fungicidal effect against the isolated non-dermatophyte molds which compared favorably with that of standard antifungal drug, ketoconazole. The plant leaves could serve as sources for development of new antifungal drugs.

Keywords: Non-dermatophyte mold; onychomycosis; plant extracts; antifungal activity; rice farmers.

1. INTRODUCTION

Onychomycosis, defined as fungal infection of the nail, is a worldwide problem especially in tropical areas (Hanan et al., 2005). Causative agents of onychomycosis belong to three groups of fungi: dermatophytes, responsible for most of the infections, yeasts and non-dermatophyte filamentous fungi (Souza et al., 2010). Epidemiological studies have shown that A*spergillus* sp. is the emerging fungal agents of toe nail onychomycosis and is now ranked second to *Scopulariopsis* sp. in non-dermatophyte onychomycosis (Gianni and Romano, 2004). It had been reported that this infection, though expensive, difficult and requires a longer time to eradicate, could be treated with antifungal agents (Barry, 2003). Due to report of increasing developments of drug resistance in human pathogen as well as undesirable side effects of certain antimicrobial agents (Phongpaichit et al., 2004), it is necessary to search for new agents that are better, cheaper and without side effect.

Medicinal plants have been used for centuries as remedies for human diseases. They constitute an effective source of both traditional and modern medicine. The acceptance of traditional medicine as an alternative form of health care hassled researchers to further investigate antimicrobial activity of medicinal plants. Countries in Africa, Asia and Latin America use traditional medicine to help meet some of their primary health care needs (Sule et al., 2010). In developing countries notably in West Africa, new drugs are often not affordable, thus up to 80% of the population use medicinal plants as remedies (Okoro et al., 2009). In eastern Nigeria, a wide variety of plant/natural products are used in the treatment of infections. Among them are extracts of leaves of *Cassia alata* (Leguminosa), *Mitracarpus villosus* (Rubiacea) and *Lawsonia inermis* (Lythracea) which are topically applied as antimicrobial agents in the treatment of skin infections. These plants have organic chemicals and bioactive molecules that possess antimicrobial agents.

C. alata is a pan-tropical ornamental shrub that grows up to 3-4M tall. Its common names include guajava, bajagua and ringworm shrub. It is a native to Central America but has also been introduced into many tropical countries and Islands including Nigeria (Sule et al., 2010). It is now widely considered a weed. The leaves or sap of *C. alata* are greatly used to treat fungal infections (ringworm), bacterial, viral and parasitic diseases (Henebelle et al.,

2009). They are useful in treating convulsion, heart failure, abdominal pains and oedema (Ogunti and Elujoba, 1993). *M. villosus* having a vernacular name 'obunezi', is a shrub that measures up to 8 inches in height. It is a common weed in upland areas from the forest to the savanna zones (Abdalbasit and Bertrand, 2008). Extracts of *M. villosus* leaves had been shown to have antifungal and antibacterial activities. It is very good in treatment of boils (Irobi and Daramola, 1993). *L. inermis* locally called 'lalli' in eastern Nigeria is a native to tropical and sub-tropical regions in Africa, Southern Asia and Northern Australasia in semi-arid zone. They are small shrub 2-6M high. The leaves of this shrub are used to dye skin, hair, finger nails, leather, silk and wool (Auboyer, 2002), in treating fungal infections, dysentery, leprosy, anemia, hemorrhages, fever and cough. They are also used as diuretic, anti-inflammatory agents and as preservative for leather and cloth (Reddy, 1988; Bosoglu et al., 1998).

A lot of work had been carried out on antifungal susceptibility test of these medicinal plants against dermatophytes (Irobi and Daramola, 1993; Babu and Subharsree, 2009; Sule et al., 2010), but not much is known about the antifungal activity of these plant extracts against non-dermatophyte molds. In this study, the *in vitro* antifungal activity of methanol, hexane and cold water extracts of *C. alata*, *M. villosus* and *L. inermis* against non-dermatophyte molds involved in onychomycosis among rice farmers in Anambra State, Nigeria were investigated.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

The leaves of the different plants- *C. alata, M. villosus* and *L. inermis* used in this study were collected from their wild sources from Anambra State in eastern part of Nigeria. These plants were identified by Dr. E. I. Mbaekwe of Botany department, Nnamdi Azikiwe University, Awka, Nigeria and voucher sample deposited in herbarium of the department. The leaves were washed, dried overnight in an oven at 40^{o}C and then grinded into fine powder before storing in an airtight container for further use.

2.1.1 Extraction of crude extracts

Extraction was carried out by Soxhlet procedure as described by Horowitz (1984). Thirty grams of the dried plant leaf was extracted with 300ml of methanol (BDH). The process was repeated using 500ml hexane (BDH) as solvent. The extracts were recovered from the solvent using rotavapour apparatus and stored in a freezer (-20^{o}C) for subsequent antifungal activity. Fresh leaf (20g) was blended with 10ml of water in a moulinex blender for 5min. and the suspension filtered through muslin cloth. A clear filtrate obtained by further filtration of the suspension through Whatman No 1 filter paper and sterilized at 121ºC for 15min was used to impregnate discs (6mm) prepared from Whatman No 1 filter paper (Igbal et al., 2001).

2.2 Collection, Isolation and Characterization of Fungal Organisms

Clinical specimens were collected from 135 rice farmers with onychomycosis in Anambra State, Nigeria between November 2009 and March 2010. The nails were first cleaned with 70% alcohol and specimen collected using sterile nail clips. Samples were collected in sampling pockets, transferred to Microbiology laboratory, Nnamdi Azikiwe University, Anambra State, Nigeria and processed within 2h.

A portion of each specimen was examined in 20% KOH mount and the remaining portion was inoculated into duplicate plate of Sabouraud's Dextrose Agar (SDA) (Biotech) supplemented with 0.05mg/ml chloramphenicol and 0.5mg/ml cyclohexamide. Another duplicate plate supplemented with 0.05mg/ml chloramphenicol only was also inoculated with the specimen. The agar plates were incubated at 27ºC and observed for 3–4 weeks for fungal growth. The fungal isolates were purified, stored in SDA slant and sent to Nationales Konsiliarlabour fur Dermatophyten, Charite Institute fur Microbiologie und Hygiene, Universitatsmedizin, Berlin Germany for characterization.

2.2.1 Inoculum preparation/antifungal screening

A four day old fungal isolate grown on SDA plate was aseptically scraped and transferred into bottle containing 10ml of sterile water (Espinel Ingroff et al., 1998). The suspension was vigorously shaken, diluted Ten-fold and used for antifungal screening.

The antifungal activity of the plant extracts were investigated using disc diffusion method as described by Duraipandiyan and Ignacimuthu (2007). Sterilized discs (6mm) prepared from Watman no 1 filter paper, were impregnated with different concentrations (10mg, 20mg, 40mg, 80mg) of methanol and hexane extracts dissolved in 2% DimethylSulphoxide (DMSO) and placed on SDA plates spread inoculated with 0.1ml of 10^{-4} dilution of the inocula preparation. The plates were incubated at 27ºC and average diameter zone of inhibition recorded after 48h. The antifungal activities of the cold water extracts in the impregnated discs were similarly examined as described earlier. Discs prepared with 2% DMSO served as negative control while those with ketoconazole (2mg/disc) served as positive control. Plates were prepared in duplicates.

2.2.1.1 Minimum Inhibitory Concentration/ Minimum Fungicidal Concentration of crude extracts

The Minimum Inhibitory Concentration (MIC) of different extracts was determined by broth dilution method as described by Dash and Murthy, 2011. Extracts were first dissolved in 2% DMSO and diluted to highest concentration of 200mg/ml (80mg/disc). These were serially diluted two-fold in a concentration range from 25mg/ml to 200mg/ml in sterile water. For broth dilution, 0.1ml of standardized suspension of test non-dermatophyte molds (10^4 CFU/ml) separately was added to each tube containing various extracts at concentrations of 0 (control), 25, 50, 100 and 200mg/ml in the broth medium. These were done in duplicates. The tubes were incubated at 27ºC for 48h and observed for visible growth after vortexing the tube gently. The lowest concentration of test extract in a tube that failed to show any visible macroscopic growth was considered as its MIC.

Minimum Fungicidal Concentration (MFC) was used to determine if the crude extracts were fungistatic or fungicidal in their effect using Cheesbrough (2006) method. Tubes with MIC and the preceding ones were used in this test. A loopful from each of these tubes were sub-cultured into appropriate labeled sterilized SDA plates without drug supplement using sterile inoculating needle. These were incubated for 14 days at 27ºC after which they were observed for growth. MFC was the quadrant with the lowest concentration of crude extract without growth.

3. RESULTS AND DISCUSSION

Fungal isolates were identified as *Aspergillus terrus, Aspergillus sclerotiorum, Aspergillus flavus, Fusarium* sp., *Chrysosporium* sp. and *Scopulariopsis* sp.

The antifungal activity of the methanol and hexane extracts of medicinal plants showing the Minimum Inhibitory Concentration (MIC), average diameter Zone of Inhibition (ZOI) (measured in millimeter) and Minimum Fungicidal Concentration (MFC) are presented in Table 1 and 2. Table 3 shows the average zone of inhibition (mm) of cold water extracts of the medicinal plants.

As shown in Table 1, methanol extracts of the various medicinal plants were found exhibiting high antifungal activity against all test fungi at concentration ≤40mg/disc except on *A. flavus* which was inhibited only by extract of *L. inermis* at 40mg/disc. No fungicidal activity was observed with the methanol extract of *M. villosus* on *A. terrus*, *Chrysosporium* sp. and *Scopulariopsis* sp. (Table 1).

As presented in Table 2, hexane extract of *L. inermis* inhibited all test isolates at concentration of ≤40mg/disc. *A. flavus* was resistant to extracts of *C. alata* and *M. villosus* while *A. terrus* was not inhibited by *M. villosus*. Hexane extract of *C. alata* and *M. villosus* did not show any fungicidal activity on all the test organisms except on *Fusarium* sp. (Table 2).

Table 3 shows that, *A. flavus* was not inhibited by cold water extract of all the medicinal plants, while *A. sclerotiorum* and *Scopulariopsis* sp. were resistant to cold water extracts of *C. alata* and *M.villosus* respectively.

Aspergillus terrus, A. sclerotiorum, A. flavus, Fusarium sp., *Chrysosporium* sp. and *Scopulariopsis* sp. were isolated from cases of onychomycosis among rice farmers in Anambra State, Nigeria. That these non-dermatophyte molds are involved in onychomycosis is in line with the reports of Bonifaz et al. (2007) and Souza et al. (2010), even though their studies were among hospital patients.

Methanol and hexane extracts of *L. inermis* (Tables 1 and 2) showed definite antifungal activity against the isolates at MIC and MFC range of 10-40mg/disc and 10-80mg/disc respectively. These results are supported by the work of Singh and Pandey (1989), Babu and Subharsree (2009) and Sharma and Sharma (2011), who reported absolute toxicity of *L. inermis* against fungal pathogens. They attributed this to the presence of Lawsome, 2 hydroxy-1,4-naphthoquinones contained in the leaves. *A. flavus* was not inhibited by water extract of *L. inermis* which is contrary to the report of Abdulmonein (2007). It was noted that the water extract of *L. inermis* showed better antifungal activity than methanol and chloroform extracts. The effectiveness of *C. alata* against both dermatophyte, non dermatophyte mold (*Penicillium marneffei* and *A. flavus*) and yeast have been reported by many researchers (Phongpaichit et al., 2004; Makinde et al., 2007). However, this experimental study recorded resistance of *A. flavus* to *C. alata* leaf extract which is contrary to the findings of Makinde et al. (2007). The resistance of *A. flavus* to cold water extract of *C. alata* (Table 3) observed in this study is supported by the work of Somchit et al., (2003), even though the fungal organism used was *Aspergillus fumigatus.*

Table 1. Antifungal activity of methanol extracts of medicinal plants

Isolates	*C. alata*			*M. villosus*			*L. inermis*		Ketoconazole (2mg/disc)	
	Concentration (mg/disc), Diameter zone of inhibition (mm)									
	MIC	ZOI	MFC	MIC	ZOI	MFC	MIC	ZOI	MFC	ZOI
A. terrus	10	6.8	40	40	6.8	-	10	11.2	20	6.4
A. sclerotiorum	20	6.6	40	20	6.4	80	10	7.2	20	6.8
A. flavus	-	-	ND	-	-	ND	40	6.8	80	6.0
Fusarium sp.	10	14.8	10	10	9.4	40	10	9.2	10	-
Chrysosporium sp.	10	9.2	20	10	8.2	-	10	9.0	20	12.4
Scopulariopsis sp.	10	7.4	20	40	7.7	-	10	6.6	20	7.6

MIC= Minimun inhibitory concentration, ZOI= Zone of inhibition, MFC= Minimum fungicidal concentration, ND= Not done, - = No activity.

Table 2. Antifungal activity of hexane extracts of medicinal plants

Isolates	*C. alata*			*M. villosus*			*L. inermis*		Ketoconazole (2mg/disc)	
	Concentration (mg/disc), Diameter zone of inhibition (mm)									
	MIC	ZOI	MFC	MIC	ZOI	MFC	MIC	ZOI	MFC	ZOI
A. terrus	40	6.2	-	-	-	-	40	8.4	80	6.4
A. sclerotiorum	40	6.0	-	40	6.2	-	40	6.0	80	6.8
A. flavus	-	-	ND	-	-	ND	40	6.0	80	6.0
Fusarium sp.	10	8.4	40	20	8.6	80	20	7.6	40	-
Chrysosporium sp.	40	6.8	-	40	7.2	-	20	6.2	80	12.4
Scopulariopsis sp.	80	6.2	-	80	6.0	-	20	6.2	80	7.6

MIC= Minimum inhibitory concentration, ZOI= Zone of inhibition, MFC=Minimum fungicidal concentration, ND= Not done, - = No activity.

Table 3. Antifungal activity of cold water extracts of medicinal plants

Isolates	Diameter zone of inhibition in mm			
	C. alata	***M. villosus***	***L. inermis***	**Ketoconazole (2mg/disc)**
A. terrus	6.2	6.4	6.8	6.4
A. sclerotiorum	-	6.0	6.2	6.8
A. flavus	-	-	-	6.0
Fusarium sp.	6.8	7.2	8.2	-
Chrysosporium sp.	6.4	6.2	6.8	12.4
Scopulariopsis sp.	6.0	-	6.2	7.6

All the test fungi were found to be susceptible to methanol extracts of *M. villosus* except *A. flavus* (Table 1), which is in line with the work of Irobi and Daramola (1993), although the extraction of the leaf was with ethanol. However, their MIC range (10-20.5mm) was observed to be higher than the range (6.4-9.4mm) obtained in this study. This difference may have been influenced by the solvent used for the extraction.

Although this experimental study showed that Scopulariopsis sp. (which is ranked first as the cause of non-dermatophyte onychomycosis) and *Chrysosporium* sp. were inhibited by both methanol and hexane extracts of the medicinal plants (Tables 1 and 2), there had not been reports of antifungal activity of these medicinal plants against these fungi.

As observed in this study, methanol plant extracts showed better inhibitory and fungicidal activities against most tested fungi with greater zones of inhibition range (6.4-14.8mm) than hexane (6.0-8.6mm) and cold water (6.0-8.2mm) plant extracts (Tables 1, 2 and 3). Standard ketoconazole drug recorded diameter zone of inhibition range of 6.0-12.4mm. This shows methanol to be a better solvent in the extraction of bioactive substances present in the medicinal plant leaves, which are responsible for the inhibitory actions on fungal organisms. The antifungal activity of cold water extract of medicinal plants as shown by their average zone of inhibition (Table 3), supports the claim by traditional healers in the use of these medicinal plants in treatment of skin infections. The results of the antifungal activities of these plant extracts also compared favorably with that of the standard ketoconazole drug.

4. CONCLUSION

The tested medicinal plants had efficacy against the isolated non-dermatophyte molds (which are now the emerging cause of onychomycosis), showing their great potential as sources for development of new antifungal drugs for treatment and control of fungal nail infections which is usually expensive and difficult to treat.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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