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In Vitro Susceptibility of Dermatophytes to Anti-Fungal Drugs and Aqueous *Acacia nilotica* Leaf Extract in Lagos, Nigeria

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ABSTRACT

Dermatophytes were earlier reported to respond well to anti-fungal agents; however, an upsurge in resistance with the high cost of these agents increased the use of medicinal plants for treatment. This study investigated the sensitivity pattern of dermatophytes to oral anti-fungal drugs and aqueous leaf extract of the plant, *Acacia nilotica*. The extract was tested against seven strains of dermatophytes *Arthroderma otae*, *Trichophyton interdigitale*, *Trichophyton mentagrophyte*, *Microsporum ferrugineum*, *Arthroderma vespertilii*, *Arthroderma quadrifidum*, and *Arthroderma multifidum*, previously isolated from diabetic patients. The minimum inhibitory and fungicidal concentrations of the plant extracts and the standard antifungal agents were evaluated using modifications of the broth macro dilution method of the National Committee for Clinical Laboratory Standards M38-A2 protocol. There was a significant difference in the Minimum Inhibitory concentrations (MIC) of the dermatophytes to the three antifungal drugs tested ($p < 0.001$). The dermatophytes were mostly susceptible to itraconazole followed by Nystatin. All the dermatophytes tested were resistant to griseofulvin. *Acacia nilotica* had an inhibitory effect on all the dermatophytes tested, and showed anti-fungal activity in a dose-dependent relationship between 0.625 - 1.25 mg/ml. Though the inhibitions of the dermatophytes were significantly higher with the standard anti-fungal drugs as compared to the plant extract ($p < 0.001$); however, the dermatophyte, *Arthroderma*

***quadrifidum*, which was resistant to all the anti-fungal drugs, had the highest inhibition with *A. nilotica*. Some circulating dermatophyte strains in Nigeria are griseofulvin and/or itraconazole resistant which may influence the spread of infection and *A. nilotica* aqueous leaf extract showed a strong anti-dermatophytic activity.**

1. INTRODUCTION

Dermatophytosis is a fungi infection that affects the skin, hair, and nails. Dermatophytosis is called tinea based on the infection site. Dermatophytosis of the arms, trunk and legs are generally referred to as tinea corporis, those involving the scalp are called tinea capitis and that of the foot, tinea pedis [1]. Dermatophytosis is typically caused by dermatophytes, which are one of the most common fungal agents, belonging to the *Microsporum*, *Trichophyton*, or *Epidermophyton genera*, causing superficial skin infection globally [1-4]. The transmission of dermatophyte infection is mainly through contact with contaminated hair coats of animals and insects such as fleas or houseflies [3]. Dermatophytes can survive over a year in optimal humid and high temperature environments [tropical and sub-tropical regions], and socioeconomic factors and migrations also influence the transmission of infection, making dermatophytosis the most prevalently diagnosed skin disease in Africa [5].

In Africa, the disease is reported to affect over 20% of school-aged children in Western Africa, with a range of 10% and 70% in other African regions [6]. Some African studies have shown that the most susceptible age brackets for dermatophyte infection is in children 1 to 10 years, followed by adults 25 to 44 years [7, 8]. A study from India, however, identified 21 - 50 years as the most effected age group followed by the 1 - 20 years age bracket [9]. The increase in the prevalence of dermatophytosis in adult patients have been attributed to the increase in the number of immunocompromised patients with ailments such as HIV infection and other immune-deficiencies, diabetes mellitus, organ transplantation, and cancer [6, 10].

Dermatophytosis usually deface patients carrying the infection, though its treatment have been successful with oral or topical antifungal agent such as terbinafine, griseofulvin, fluconazole, itraconazole, and other oral azole antimycotics [4, 10-12]. The current expert consensus on the management of dermatophytosis in India suggests the use of topical azoles, and systemic use of Terbinafine and itraconazole for the treatment of dermatophytosis, with a minimum treatment duration of 2 - 4 weeks in new cases and >4 weeks in recurrent cases [13]. Problem of recurrence of infection, which is typical in dermatophytic infection, occur which results in long-term use of antifungal agents as seen in the consensus report in India [13]. Notwithstanding, there are increasing reports of resistance to common antifungal drugs used for the treatment of dermatophytic infections [14-17]. Treatment failure has been attributed to suboptimal dose, long duration of therapy, and even antifungal misuse by the patients due to incomplete adherence to the course of treatment [14]. Based on the problem of long duration of treatment, which can also lead to adverse effects such as liver and kidney failure, and emerging antifungal resistance, there is a need to identify natural products with antifungal activity that can be explored for the treatment of dermatophytosis.

Medicinal plants have been shown to contain countless phytochemicals which have been continuously identified for the prevention, mitigation, and/or treatment of various disease conditions. *Acacia nilotica* [*A. nilotica*] is one of such medicinal plants, referred to as a multipurpose medicinal plant [18, 19]. *A. nilotica* [family Fabaceae, subfamily Mimosoideae] is a leguminous plant which grows in the Northern part of Nigeria, where it is referred to as Gabaruwa. Interestingly, as dermatophytosis is prevalent in tropical and subtropical regions, *A. nilotica* occurs naturally in tropical and sub-tropical regions of the world. *A. nilotica* have been reported to have antifungal activity against fungal agents such as *Candida albicans*, *Aspergillus niger*, *Aspergillus fumigates*, *Aspergillus flavus*, *Dreschlera turcica*, and *Fusarium verticillioides* [20-22]. Though traditional ethnobotanical uses of *A. nilotica* for the treatment of skin infection have been reported [23-25]; however, there is a paucity of data on the biological activity of *Acacia nilotica* on dermatophytes which can further substantiate its use for the treatment of skin infections. Hence, the aim of this study was to evaluate the sensitivity pattern of dermatophytes to oral anti-fungal drugs and to aqueous leaf

extract of *Acacia nilotica*. The objectives of this study is to assess the efficacy of antifungal drugs against the circulating strains of dermatophytes in Lagos, Nigeria, and also validate local claims of the use of *A. nilotica* in treating fungal infection.

2. METHODS

2.1. Test Organisms

The dermatophytes *Arthroderma otae*, *Trichophyton interdigitale*, *Trichophyton mentagrophyte*, *Microsporum ferrugineum*, *Arthroderma vespertilii*, *Arthroderma quadrifidum*, and *Arthroderma multifidum*, were previously isolated from skin snips and nail clippings of diabetic patients. The patients were being managed for diabetes, presented with skin infection, and have been clinically diagnosed of dermatophyte infection at the skin clinic of the Lagos State University Teaching Hospital (LASUTH). Five samples, of each species, isolated from different patients were used in the analysis. Ethical approval for the isolation of the dermatophytes from diabetic patients and to carry out the anti-dermatophytes activity of different plant extracts was gotten from the Health Research and Ethics Committee of the Lagos State University Teaching Hospital with approval number LREC/10/06/554.

2.2. Collection and Preparation of *A. nilotica* Leaves

Fresh leaves of *A. nilotica* were collected form Birnin Kebbi, Kebbi State, Northern Nigeria. *A. nilotica* occurs naturally in the State and is unthreatened. However, the aerial leaves were collected from the plant which would not affect subsequent growth of the plant. The leaves were authenticated at the herbarium in the Department of Botany, University of Lagos with voucher number LUH 7553. The leaves were washed twice with tap water and once with distilled water, air dried at room temperature, blended into a fine powder, and extracted using standard methods [26-28].

2.3. Standard Antifungal Agents

The antifungal agents tested in this study are Nystatin (Sigma Chemical Co., St. Louis, Mo.), itraconazole (Sigma Chemical Co., St. Louis, Mo.), and griseofulvin (Sigma Chemical Co., St. Louis, Mo.).

2.4. Antifungal Susceptibility Testing

2.4.1. MIC Determination Using Broth Macrodilution Method

Before evaluating the minimum inhibitory concentration (MIC) of the plant extract. A preliminary test was done to check if the dermatophytes were susceptible to the plant extracts using the agar diffusion method. The susceptibilities of the dermatophytes to the plant extracts and to the anti-fungal drugs were then assayed using the broth macrodilution method using modifications of the National Committee for Clinical Laboratory Standards M38-A2 protocol as described by Norris *et al.* [29]. Inocula were prepared in RPMI 1640 with L-glutamine, without sodium bicarbonate, pH 7.0 (Carl Roth GMBH) with an inoculum density of 3×10^3 CFU/ml. The plant extracts were added into the medium to a final concentration ranging from 40 mg/ml to 0.3125 mg/ml. The three antifungal agents' griseofulvin, nystatin, and itraconazole were also tested in a final concentration ranging from 64 µg/ml to 0.0625 µg/ml. The tubes were incubated at room temperature (about 30˚C) for up to 7 days. The MIC was defined as the lowest drug concentration that caused 80% inhibition of visible fungi growth. Visual reading of the MIC was employed as visual evaluation has been reported to agree with spectrophotometric readings by several investigators [30-32]. The broth macrodilution assay was done in three replicates.

2.4.2. Determination of MFC

The minimum fungicidal concentration (MFC) was evaluated by streaking 100µl of the aliquots after incubation from the broth macro dilution method onto Saboraud dextrose agar. The plates were again incubated at 30˚C for up to 7 days, and observed for the presence of fungal colonies. The MFC was defined

as the lowest drug concentration at which no visible fungal colonies was seen.

2.5. Statistical Analysis

Statistical analysis was carried out on SPSS version 20. Analysis of variance (ANOVA) was used to check if there was a significant difference in the MIC of the different antifungal agents used.

3. RESULTS

3.1. *In Vitro* Antifungal Activity of Extracts on Dermatophytes

Aqueous extracts of *A. nilotica* leaves were able to inhibit the growth of dermatophytes in a dose dependent relationship (Table 1, Figure 1). Interestingly, all the dermatophytes tested in this study were resistant to griseofulvin, and even dermatophytes resistant to itraconazole, nystatin and griseofulvin were susceptible to the *A. nilotica* leaf extract (Table 1).

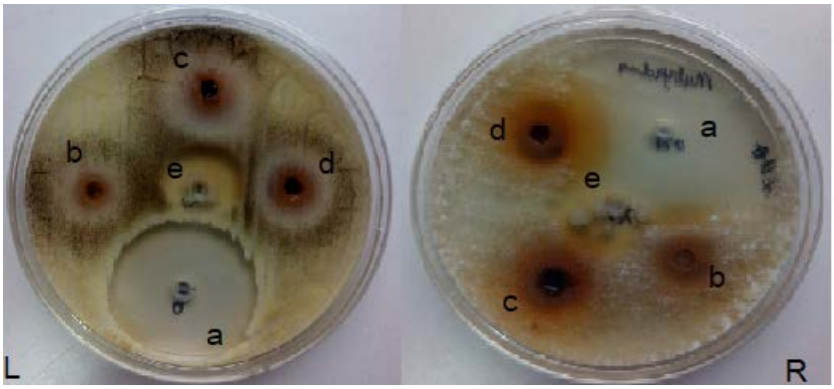

Figure 1. Plates showing the susceptibility of the dermatophytes to the antifungal agents and *A. nilotica*. (L = *T. mentagrophyte*, with a. 10 mg/ml Itraconazole; b. 10 mg/ml *A. nilotica*; c. 20 mg/ml *A. nilotica*; d. 40 mg/ml *A. nilotica*; e. 10 mg/ml Nystatin. R = *A. multifidum,* with a. 10 mg/ml Itraconazole; b. 10 mg/ml *A. nilotica*; c. 20 mg/ml *A. nilotica*; d. 40 mg/ml *A. nilotica*; e. 10 mg/ml Nystatin.)

Table 1. Mean zone of inhibition (mm ± SD) showing the antifungal activity of extracts on dermatophytes.

Organisms	Plant and Antibiotics Concentrations (mg/ml)					
	A (40)	A (20)	A (10)	I (10)	N(10)	G (10)
A. otae	17 ± 0.9	10 ± 1	8 ± 1.3	38 ± 1.5	18 ± 1.0	NI
T. interdigitale	15 ± 0.9	12 ± 0.4	10 ± 1.3	46 ± 1.5	26 ± 0.5	NI
T. mentagrophyte	12 ± 0.5	11 ± 0.8	8 ± 0.5	36 ± 0.5	18 ± 0.5	NI
M. ferrugineum	11 ± 0.9	10 ± 0.4	6 ± 1.0	36 ± 1.0	20 ± 1.0	NI
A. vespertilii	11 ± 0.8	11 ± 1.0	8 ± 1.0	42 ± 0	20 ± 1.4	NI
A. quadrifidum	27 ± 1.5	20 ± 1.5	18 ± 0.8	NI	NI	NI
A. multifidum	18 ± 1.5	16 ± 0.8	12 ± 1.3	50 ± 2.9	26 ± 1.5	NI

Key: A = Acacia nilotica leaf extract, I = Itraconazole, N = Nystatin, G = Griseofulvin, NI = No inhibition.

3.2. MIC of the Extract and Standard Antifungal Agents

The MIC and MFC of the three antifungal agents and the plant extract is presented in Table 2. Both the agar diffusion method and the broth macrodilution method showed that the dermatophytes used in this study were resistant to griseofulvin. All the strains tested in this study were susceptible to itraconazole and nystatin with the exception of *Arthroderma quadrifidum* which was resistant to all the antifungals tested. As seen in Table 2, the susceptibility of each species of the dermatophytes was similar in all the anti-fungals tested. The organisms were more susceptible to itraconazole when compared to the other plant extracts. The difference in susceptibility to itraconazole and nystatin was statistically significant ($p < 0.001$). With the exception of *A. quadrifidum*, the susceptibility of each specie of dermatophytes was similar in all the anti-fungal agents tested with no statistically significant difference ($p = 1.00$). The inhibitions of the dermatophytes were significantly higher with the standard anti-fungal drugs as compared to the plant extracts [$p < 0.001$]; however, *Arthroderma quadrifidum*, which was resistant to all the anti-fungal drugs, was the most susceptible to *A. nilotica*.

4. DISCUSSION

Therapies commonly used in the systematic treatment of dermatophytosis include the oral use of antifungal drugs like itraconazole, terbinafine, griseolfulvin and fluconazole [33]. A randomized controlled trial that compared the efficacy of itraconazole with griseofulvin in the treatment of tinea corporis and tinea cruris showed that patients had better outcome after 2 weeks of treatment with itraconazole when compared to griseofulvin [34]. There are very limited studies that have evaluated the susceptibility of dermatophytes in Nigeria. Nweze *et al.* [2007] tested the susceptibility of dermatophytes isolated from children against five antifungal agents [35]. The authors did not record any resistance to itraconazole, ketoconazole, fluconazole, terbinafine and griseofulvin [35]. In corroboration with the randomized controlled trial [34], the result of this study showed that itraconazole had the best MIC and MFC on the tested dermatophytes when compared to nystatin and griseofulvin, and was therefore a better therapy for the treatment of dermatophyte infection in Nigeria. However, caution should be employed in prescribing oral itraconazole to adults' patients with dermatophytosis considering that itraconazole is a CYP3A4 inhibitor that is capable of multiple drug interactions. Considering that the dermatophytes used in this study were isolated from adult patients with diabetes, care should be taken in prescribing this drug for adult patients

Table 2. MIC of the plant extracts and antifungal agents.

	MIC (mg/ml)	MFC (mg/ml)	MIC (µg/ml)			MFC (µg/ml)		
Organisms	*A. nilotica*	*A. nilotica*	I	N	G	I	N	G
A. otae	1.25	10	<0.0625	0.5	>64	1	4	>64
T. interdigitale	1.25	10	<0.0625	0.25	>64	0.5	2	>64
T. mentagrophyte	1.25	10	<0.0625	0.5	>64	0.25	2	>64
M. ferrugineum	1.25	10	<0.0625	0.5	>64	0.5	2	>64
A. vespertilii	1.25	10	<0.0625	0.5	>64	0.5	2	>64
A. quadrifidum	0.625	5	>64	>64	>64	>64	>64	>64
A. multifidum	1.25	10	<0.0625	0.25	>64	2	4	>64

Key: I = Itraconazole, N = Nystatin, G = Griseofulvin.

with other co-morbidities as it might have contraindications with other drugs. Alternatively, topical therapy alone should be prescribed as topical azoles are not or minimally absorbed through the skin [36].

In contrast to the study by Nweze *et al.* [35], all the dermatophytes tested in this study were resistant to griseofulvin. The hundred percent resistance to griseofulvin seen in this study could mean the emergence of griseofulvin resistance in circulating strains of dermatophytes in Nigeria. The emergence of resistance to griseofulvin means that further prescription of this antifungal agent for the treatment of dermatophytic infections may not be efficacious and may influence the spread of the infection.

A. nilotica is found mainly in the Northern part of the country were it grows naturally. The leaves and barks of the plant are used by the local folks for medicinal purposes but notably for the treatment of diarrhea diseases and skin infections. With the increase in multi drug resistance to different microbial infections and with *A. nilotica* termed "a plant of multipurpose medicinal use" there is a need to scientifically validate the efficacy of this plant in treating the different ailments it has been implicated to treat.

Aqueous extracts of *A. nilotica* leaves showed a good anti-dermatophytic activity against all the dermatophytes tested (**Table 1**). The zone of inhibition from the agar dilution method and the MIC and MFC from the broth macro-dilution method showed that the susceptibility to *A. nilotica* was comparable among all the dermatophytes tested with the exception of *A. quadrifidum* which showed a better susceptibility to the plant extracts. Interestingly, *A. quadrifidum* which was more susceptible to *A. nilotica* was resistant to all the reference antifungal drugs used in this study. Some studies have reported the antifungal activity of *A. nilotica* against fungi such as *Candida albicans*, *Aspergillus niger*, *Aspergillus fumigates*, *Aspergillus flavus*, *Dreschlera turcica*, and *Fusarium verticillioides* [20-22]. Though the absence of anti-fungal activity has also been reported for *A. nilotica* [33], however, this report represents the first data showing the anti-dermatophytic activity of *A. nilotica*.

Limitations of the Study

Some of the limitations of this study was that the dermatophytes used in this study were archived isolates cultured from adult diabetic patients in Lagos, Nigeria and only a total of thirty five isolates [5 of each species] were tested in this study. This may not reflect the overall circulating dermatophytes in Nigeria. Additionally, the anti-dermatophytic activity of the crude plant extract was used in this study. There is a need for further research to identify the main bioactive components in *A. nilotica* that is responsible for its anti-dermatophytic activity through activity based fractionation, isolation and characterization study of the most active fraction.

5. CONCLUSION

The findings from this study show that griseofulvin-resistant dermatophyte strains exists and are now circulating in Nigeria, and also itraconazole resistant strains of dermatophytes can be emerging and may also begin to circulate in Nigeria. This can influence lack of clinical response to these drugs. The aqueous leaf extract of *A. nilotica* showed a strong anti-dermatophytic activity. There is a need to study the effect of the plant extracts *in vivo* in an animal model [topical and oral use] and also further isolate, purify and identify the active components in the crude leaf extracts responsible for the activity seen. This is necessary to develop a novel anti-fungal agent that can be used to combat dermatophyte infection.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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