Evaluation of the Antifungal Activities of Local Herbs on Dermatophyte species isolated from Rice Farmers in Ebonyi State, Nigeria

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Abstract: **The objective of the study was to evaluate the antifungal activities of locally used herbs on dermatophyte species isolated from rice farmers in Ebonyi State. Extracts of five medicinal plants; Azadirachta indica, Cymbopogon citratus, Emilia sonchifolia, Senna alata and Senna occidentalis used by the farmers to treat dermatophyte infection were used in the study. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the plant extracts were evaluated using broth microdilution method and spread plate methods respectively. Terbinafine (125 mg) oral granules were used in the study as quality control antifungal agent. The range of MIC for n-hexane, chloroform, petroleum ether and hot water extracts of the medicinal plants against the fungal isolates tested was 2 - 15 µg/ml, 8 - 32 µg/ml, 4 - 18 µg/ml and 10 - 32 µg/ml respectively. The dermatophyte species were susceptible to n-hexane, chloroform and petroleum ether extracts used in the study but less susceptible to hot water extract. Minimum fungicidal concentration (MFC) values ranged from 4 - 32 µg/ml. The most significant MFC (4 µg/ml) was observed in n-hexane extracts of C. citratus and S. alata. The comparative results of antifungal susceptibility patterns of the various isolates to examined plant extracts indicated that the sensitivity to terbinafine (2 µg/ml) by dermatophyte species was higher than the plant extracts (4 µg/ml) with significant difference ($P<0.05$). These plant materials should be harnessed as they hold promising potential source of new drugs for the management and treatment of dermatophytosis.**

Keywords: **medicinal plants, susceptibility, harnessed, treatment, dermatophyte infection**

I. INTRODUCTION

Dermatophytes are the major cause of dermatophytosis. They are classified into three genera; *Trichophyton*, *Microsporum* and *Epidermophyton*, based on the formation and morphology of their conidia. Dermatophytosis had been extensively reported to be a public health problem in Nigeria and in many countries with high humid environment. These mycoses cannot be overemphasized as the contagious nature, high cost of treatment, difficulty of control and the public health consequences explain their great importance.

Plant-derived drugs in clinical use have been discovered through follow-up investigation of the ethno medicinal uses of plants. Nigeria is endowed with a variety of plant species. The vast arrays of plants are traditionally used for the treatment of various ailments and diseases. These plants, mostly employed in a synergistic combination, have shown to be as effective and are often preferred to the commercially available drugs by a larger portion of the society (Ajose, 2007). The vast diversity of plants in Nigeria is a promising source of novel compounds that are still relatively unexplored. The control of fungal infections requires the use of fungicidal or fungistatic drugs that more specifically target the infecting agent to avoid damage to the host. With developing microbial resistance and need for safe and cost-effective antifungal drugs, screening of plant organs for potential bioactive secondary metabolites becomes indispensible. Thus the indigenous knowledge of traditional medicinal plants is a valuable tool for targeting potentially active species from the wealth of medicinal plants in Nigeria.

II. MATERIALS AND METHODS

2. 1. Collection and Preparation of Plant Materials

Fresh leaves of five medicinal plants; *Azadirachta indica*, *Cymbopogon citratus*, *Emilia sonchifolia*, *Senna alata* and *Senna occidentalis* were collected from herbalists in the study area. They were identified by Prof. F. N. Mbagwu of the Department of Plant Science and Biotechnology, Imo State University, Owerri, Imo State. The leaves were rinsed with distilled water and air-dried at room temperature for 7 days. The dried leaves were ground into fine powder using an electric blender. 50g of finely grounded leaves of each medicinal plant was extracted separately using 200ml of petroleum ether, n-hexane, chloroform and hot water in a soxhlet apparatus. The obtained liquid extracts were gently concentrated to dryness in a water bath at 40^{o}C for 12 hours and subsequently air-dried.

2. 2. Test Organisms

Pure cultures of dermatophyte species used in this research work; *Trichophyton tonsurans, T. mentagrophytes, T. rubrum, T. soudanense, Microsporum gypseum* and *M. canis* were isolated from rice farmers in some parts of Ebonyi State, Nigeria. The rice farmers were screened for lesions suggestive of dermatophytosis on their skin, hair and nails. The dermatophyte isolates were identified based on detailed conventional methods, compared with the standard description given by *Ellis et al.* (2007) and confirmed by sequencing the

internal transcribed spacer (ITS) region of the ribosomal DNA.

2. 3. Preparation of Inocula

Inoculum suspensions prepared from a 7 day old culture of dermatophyte species grown on SDA plates were made as described by Santos and Hamdan (2005). The fungal colonies were flooded with 5 ml sterile saline (0.9%) and suspensions were made by gently probing the surface of the culture plate with a sterile glass slide. The suspensions were filtered with sterile cotton gauze. A ten-fold serial dilution was carried out by diluting 0.1 ml inoculum suspension in 0.9 ml Sabouraud dextrose broth (SDB). A 0.1 ml aliquot of 10^{-2} dilution was inoculated on SDA plate and incubated at 25 ^{o}C for 7 days. The inoculum suspensions were quantified by counting microconidia in a haemocytometer to obtain a cell number of 2×10^5 CFU/ml.

2. 4. Determination of Minimum Inhibitory Concentration (MIC) of Plant Extracts

A 125 mg terbinafine oral granules (Novartis, UK) was used in the study as quality control antifungal agent. Stock solution (128 µg/ml) was prepared by dissolving 12.8 mg of the drug in 100 ml DMSO (100 %) and stored at 4 ^{o}C. Minimum inhibitory concentration (MIC) was determined using broth microdilution assay in accordance with protocol M38-A2 of the Clinical and Laboratory Standards Institute, CLSI (2008). Flat-bottomed microdilution plates (Nunc, Denmark) were set up. Stock solutions (128 µg/ml) of the plant extracts were prepared from n-hexane, petroleum ether, chloroform and aqueous extracts of *A. indica, C. citratus, E. sonchifolia, S. alata* and *S. occidentalis* respectively by dissolving 12.8 mg of each plant extract in 100 ml dimethyl sulfoxide (100%). A 100 µl SDB medium was added to all 96 wells of the microdilution plate. Serial twofold dilutions were prepared in microdilution plate with 100 µl of the extracts with the 128 µg/ml concentrations in the first column wells, followed by further dilutions in SDB media. The plant extracts and the standard antifungal control agent were prepared in ranges from 2-64 µg/ml in the microdilution plate. Each microdilution well containing 100 µl of the twofold extract/drug concentrations was inoculated with 100 µl of inoculum suspension. For each test plate, the twelve vertical column wells were used for duplicate assay of the five different plants materials and terbinafine (antifungal control agent) while the eight horizontal row wells were used for the six different concentrations (64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml and 2 µg/ml) of each extract and two extract-free controls: one with 100 µl of medium and 100 µl of inoculum suspension (growth control) and the other with medium alone (sterility control). Empty microdilution plates were placed on top of the test plates to prevent the test plates from dehydration during incubation. The microdilution plates were incubated at 25°C for 7 days.

Endpoint values were determined visually every 24 hour until the indication of turbidity in the growth control wells. The mean MIC was obtained for each species-extract-standard agent combination tested. MICs of the plant extracts and the standard agent were reported as the concentration at which 90% (MIC_{90}) and 100% (MIC_{100}) of the isolates were inhibited when compared to the growth control respectively.

2. 5. Determination of Minimum Fungicidal Concentration (MFC) of Plant Extracts

This was carried out as described by Cheesbrough (2010). Emphasis was mostly placed on the wells with MIC and the preceding wells. A loopful from each of these wells was subcultured onto appropriately labeled SDA plate. The plates were incubated at 25°C for 7 days. The MFC was recorded as the lowest concentration of the extract without growth, that is, the lowest concentration at which 100% of the isolates were inhibited.

2. 6. Statistical Analysis

The analysis of variance (ANOVA) statistical test was used at $p<0.05$ significant level for the comparative results of antifungal susceptibility of the various fungal species to examined plant extracts.

III. RESULTS

The results of the in vitro antifungal activity testing of n-hexane, chloroform, petroleum ether and hot water extracts of the plant materials against the fungal species isolated from the rice farmers are presented below. The minimum inhibitory concentrations (MICs) of the plant extracts against the isolates were determined after 7 days at 25^{o}C incubation temperature**.** *Table 1* shows the effects of n-hexane, chloroform, petroleum ether and hot water extracts of *A. indica, C. citratus, E. sonchifolia, S. alata* and *S. occidentalis* on dermatophyte species. The n-hexane extracts of *C. citratus* and *S. alata* had minimum inhibitory effect on the test dermatophytes while the petroleum ether extracts of *C. citratus* and *S. occidentalis* minimum inhibition on the test dermatophytes. At a concentration of 2.0 µg/ml, n-hexane extracts of *C. citratus* and *S. alata* had minimum inhibition on *M. gypseum* and *M. canis* respectively. The least activity was observed on n-hexane extract of *E. sonchifolia* against *T. tonsurans* and *M. gypseum* at a concentration of 15.0 µg/ml. Petroleum ether extracts of *C. citratus* and *S. occidentalis* were observed to be more active against *T. tonsurans* at a concentration of 4.0 µg/ml. Petroleum ether extracts of *E. sonchifolia* had slight effect (18.0 µg/ml) on *M. gypseum.* Chloroform and hot water extracts of the medicinal plants had the least effect on the test dermatophytes. Chloroform extract of *E. sonchifolia* had the least activity (28.0 µg/ml) on *M. gypseum* while hot water extracts of *C. citratus, E. sonchifolia* and *A. indica* had the least activity (32.0 µg/ml) on *T. mentagrophytes, T. rubrum* and *M. gypseum* respectively. The range of MIC for n-hexane, chloroform and petroleum ether extracts was 2 - 15 µg/ml, 8 - 28 µg/ml and 4 - 18 µg/ml respectively. The value for hot water extract was 12 - 32 µg/ml. All the isolates of dermatophytes tested were susceptible to the five plant extracts used in the study. The test dermatophytes were less

susceptible to hot water extract. Terbinafine was the most effective against the test dermatophytes with MIC ranging from 2 - 5 µg/ml.

The order of in vitro antifungal activity of the plant extracts was n-hexane extract > petroleum ether extract > chloroform extract > hot water extract. The comparative results of antifungal susceptibility of the various fungal species to examined plant extracts indicated that the sensitivity to terbinafine in the different species was higher than the plant extracts with significant difference ($P<0.05$).

The minimum fungicidal concentration (MFC) values of n-hexane and petroleum ether extracts of the plant materials against the test dermatophytes were 4 µg/ml and 6 µg/ml respectively while chloroform and hot water extracts showed 8 µg/ml and 15 µg/ml respectively (*Table 2*). MFC values against the test dermatophytes ranged from 4 - 32 µg/ml.

Table 1: In vitro antifungal activity testing of 5 medicinal plants against dermatophytes species by microbroth dilution assay

Species	Plant Extracts	MIC				
		HEX	CHL	PET	HWE	TER
		(Extract Concentration +2,4,8,16,32 and 64ug/ml)				
T. tonsurans	*A. indica*	5.3	12	6.6	12	3
	C. citratus	4.7	8	4	13	2.8
	E. sonchifolia	15	16	10	16	2.2
	S. alata	5.2	8.5	8.1	16	3.1
	S. occidentalis	4	8.2	4	30	4
T. mentagrophytes	*A. indica*	5.4	10	7.2	16	2.4
	C. citratus	4.6	8.6	5.8	32	2.6
	E. sonchifolia	7.2	8.3	15	16.2	4
	S. alata	5.6	17	6.1	15	4.4
	S. occidentalis	4.5	12	5	22.4	2
T. rubrum	*A. indica*	11	16	5.8	16	4
	C. citratus	5	8.5	4.8	14	3
	E. sonchifolia	6.2	26.1	7	32	4
	S. alata	4.1	16	5.3	12	2.2
	S. occidentalis	8	16.2	5.1	16	4
T. soudanense	*A. indica*	10.5	18	6.1	13.7	2
	C. citratus	3	8.1	6	18	2.5
	E. sonchifolia	8	11.1	7.5	16	2.7
	S. alata	3.6	8.2	4.4	23.5	5
	S. occidentalis	4.1	21.8	4.2	31	4.2
M. gypseum	*A. indica*	8	12	6.5	32	4.1
	C. citratus	2	8	4.3	15	3.3
	E. sonchifolia	15	28	18	22	2
	S. alata	4.7	16	4.6	18	2.2
	S. occidentalis	12.6	16.3	14.2	29.1	3
M. canis	*A. indica*	5	16	4.7	15	2
	C. citratus	3.2	12.4	8.5	16	4.2
	E. sonchifolia	8	16	16	20	3.4
	S. alata	2	8	8	14.3	2
	S. occidentalis	10	16.1	4.1	16.5	4.4

Key: HEX = n-hexane extract CHL = chloroform extract PET = petroleum ether extract

HWE = hot water extract TER = terbinafine

Table 2. MFCs (µg/ml) of plant extracts against dermatophyte species

Species	Plant Extracts	HEX	CHL	PET	HWE
T. tonsurans	A. indica	7	14	7	16
	C. citratus	5	8	6	1
	E. sonchifolia	16	18	10	20
	S. alata	6	12	10	17
	S. occidentalis	6	10	7	32
T. mentagrophytes	A. indica	6	15	8	16
	C. citratus	5	12	6	32
	E. sonchifolia	8	11	16	18
	S. alata	6	18	8	16
	S. occidentalis	5	14	8	24
T. rubrum	*A. indica*	14	20	8	20
	C. citratus	6	10	6	16
	E. sonchifolia	8	28	12	32
	S. alata	5	16	8	15
	S. occidentalis	10	18	8	16
T. soudanense	*A. indica*	12	18	10	15
	C. citratus	4	12	8	18
	E. sonchifolia	8	12	11	16
	S. alata	6	10	7	25
	S. occidentalis	6	24	6	32
M. gypseum	*A. indica*	10	15	8	32
	C. citratus	4	11	6	16
	E. sonchifolia	16	30	18	24
	S. alata	5	16	7	20
	S. occidentalis	14	18	15	30
M. canis	*A. indica*	8	16	8	17
	C. citratus	4	14	10	18
	E. sonchifolia	12	17	16	22
	S. alata	4	8	8	16
	S. occidentalis	11	18	6	18

Key: HEX = n-hexane extract CHL = chloroform extract

PET = petroleum ether extract HWE = hot water extract

IV. DISCUSSION

Five plants were frequently mentioned and highly recommended by both the traditional healers and rural dwellers. These include: *Azadirachta indica*, *Cymbopogon citratus*, *Emilia sonchifolia*, *Senna alata* and *Senna occidentalis*. The leaves were reported to be the most used part of the plants, followed by the roots and seeds. Corms, twigs and whole plant were rarely used for the preparation of the medicines. The most common method of herbal preparation was infusion. This is made by boiling pulverized or loose plant materials in water. Application of the herbal remedies was generally by drinking, usually on daily basis for very long period of time. Information from the literature revealed that these plants are used for the treatment of skin diseases like eczema, rashes and ringworm (Porter, 2006; Tanaka and Van Ke, 2007).

In the present study, antifungal activity of n-hexane, petroleum ether, chloroform and hot water extracts of these medicinal plants were evaluated against dermatophytes species. It was observed that the various extracts evaluated showed a marked inhibitory/fungicidal effect against the isolated fungi which compared favorably with the standard antifungal control, terbinafine. The potency of these plant extracts could be due to phytochemicals contained in the extracts which have similar mode of action to that of terbinafine (Onwuliri and Wonang, 2005; Ayodele *et al*. 2009). It was also observed that hot water extracts had the least effect on the fungal isolates while n-hexane and petroleum ether extracts were the most effective. This might be due to the failure of the active ingredient to dissolve sufficiently in water. The significant antifungal activity of n-hexane and petroleum ether extracts over hot water extracts on dermatophyte species justified the principle observed in herbal practitioners' preference for using local gin as extraction agent. It was also observed that *E. sonchifolia* extracts were less effective in the control of the fungal isolates as compared to other extracts at lower concentrations; this may be due to the fact that the phytochemicals contained in other plant extracts were stronger and more effective than those of *E. sonchifolia*. This finding corroborates that of Igbinosa *et al*. (2009) on antifungal activity of medicinal plants on fungal pathogens.

Overall, terbinafine was the most potent active drug, concurring with report by Fernandez-Torres *et al*. (2000). In Nigeria, terbinfine is relatively new and not readily available, affordable and widely used. This buttresses our initial observation that most farmers with dermatophytosis resort to use of some medicinal plants as a preferred treatment choice apparently due to inability to afford the orthodox drugs. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries (Alim *et al*., 2009). The results obtained may contribute to the alternative use of these plant materials for medicinal purposes.

V. CONCLUSION

Our findings demonstrated that extracts from leaves of *Azadirachta indica*, *Cymbopogon citratus*, *Emilia sonchifolia*, *Senna alata* and *Senna occidentalis* used by the rice farmers in the treatment of dermatophytosis have fungicidal activity against dermatophyte species. The results obtained may contribute to the alternative use of these plant materials in the treatment of dermatophytosis. However, further toxicity studies need to be performed as well as assays to clarify the mechanism of action and possible interactions with antifungals or other compounds to guarantee their safety.

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