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Kinetics of Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of Ethyl Acetate and Butanol Leaf Extract of *Moringa oleifera* against some Dermatophytes

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ABSTRACT

This study was designed to assess the antifungal activity of Moringa oleifera ethyl acetate and butanol leaf extracts against some dermatophytes viz: Epidermophyton floccossum, Microsporium audouinii and Trichophyton rubrum. Scalp scrappings and nail clippings were collected from 50 pupils in primary school and were processed by potassium hydroxide (KOH) for direct microscopy and culture. Fresh leaves of Moringa oleifera *were also collected and extracted with butanol and ethyl acetate to investigate their antifungal activity in vitro. Phytochemical analysis of the M. oleifera leaf extracts was carried out using a well known standard method. The antifungal effect of the leaf extracts against the dermatophyte was assessed by agar well diffusion method. The fungicidal activity was determined using Kill-Time Kinetics. Results showed that 20% of the samples analyzed were positive for dermatophytes while 80% did not yield any dermatophyte species. Dermatophytes isolated include: Epidermophyton floccossum (8%), Trichophyton rubrum (6%) and Microsporium audouinii (6%). Males showed higher prevalence to dermatophytosis (14%) than their female counterparts (6%). Phytochemical analysis revealed the presence of flavonoids and tannins in the leaf extracts of M. oleifera. The results revealed that Epidermophyton floccossum was more susceptible to the extracts followed by Microsporium audouinii while T. rubrum was least susceptible. The butanol fraction of Moringa oleifera showed the highest antifungal activity against the dermatophyte isolates; E. floccossum, M audouinii and T. rubrum, with MIC values of 31.23 mg/ml, 62.5 mg/ml and 500 mg/ml, respectively in relation to ethyl acetate fraction. The fungicidal activity revealed that both of the extracts were fungicidal at the 8th hour. It can be concluded that M. oleifera leaves possess inhibitory properties, thus can serve as an alternative therapy for certain fungal infections and also a good source of nutrients supplements.*

Keywords: Kinetics, antifungal activity, *Moringa oleifera,* Butanol fraction, Ethyl acetate fraction and dermatophytes.

INTRODUCTION

Dermatophytes are responsible for serious human pathogenic infections that have increased during the last decades Pfaller [1]. These infections are a major cause of morbidity associated superficial mycoses, with frequent relapses and often refractory to therapy [2].

A few antifungal agents are available and licensed for use in veterinary practice or human treatments. The use of systemic drugs is limited to treat man or animal due to their high toxicity and problems of residues in products intended for human consumption [3].

The "*Moringa*" tree is considered one of the world's most useful trees, as almost every part of the *Moringa* tree can be used for food or has other beneficial properties [4]. The *Moringa* plant provides a rich and a rare combination of zeatin, quercetin, kaemferom and many other phytochemicals. It is very important for its medicinal value. Various parts of the plants such as the leaves, roots, seeds bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants, possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer. Other important medicinal properties of the plant include antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities[5].

In this view, this research was set up to determine the antifungal activity of *Moringa* leaf extracts against some dermatophytes.

AIMS AND OBJECTIVES

- To determine the prevalence of dermatophytosis in primary school pupil.
- Isolation and identification of dermatophytes.
- To determine phytochemical analysis of ethyl acetate and butanol leaf extract of *Moringa oleifera.*
- To determine the kinetics of MIC and MFC of ethyl acetate and butanol leaf extract of *Moringa oleifera* leaf extract against some dermatophytes.

MATERIALS AND METHODS

COLLECTION OF SPECIMEN

Scalp scrappings and nail clippings were collected from 50 pupils in Community Primary School Obuofia, in Nkanu Local Government area, in Enugu State, Nigeria.

The affected area was swabbed with methylated spirit and specimen was collected by scrapping the scalp with blunt scalpel. Nail specimen was collected by taking clippings of infected part and scrappings beneath the nail. The specimen was collected in clean

white paper packs to reduce humidity and multiplication of bacteria and the specimen were transported to the laboratory.

MICROSCOPY AND CULTURE

DIRECT MICROSCOPY EXAMINATION

The scalp scrapings and nail clippings were placed in a drop of 10% KOH on a slide and a coverslip placed over it. The slide was gently passed over a burnsen burner.

CULTURE

The scalp scrappings and nail clippings were inoculated onto a selective media (dermatophyte test medium), they were incubated at 28°C for 7 days and observed for colour change.

Fungal isolates were identified based on colonial morphology and pigmentation. Pure fungal colonies were also subjected to lactophenol cotton blue staining for microscopic examination of their specialized hyphae and morphology of their macroconidia, microconidia and chlamydospores. Urease test was also used to confirm *T.rubrum*.

Each fungal colony was transferred to a new SDA plate to obtain a pure growth which was then stored in SDA agar slants for further studies.

COLLECTION AND PROCESSING OF PLANT MATERIALS

Fresh leaves of *Moringa oleifera* were collected from the Department of Agricultural Education, Enugu State College of Education Technical Enugu, Nigeria in the month of May 2013 and were identified by a taxonomist Prof. J. C. Okafor of the Department of Applied Biology, Enugu State University of Science and Technology, Enugu.
The leaves were air-dried in a well-ventilated room for four weeks, until they became friable. The dried leaves were ground into powder form using an electrical mill and stored in an air tight bottle until further use.

Soxhlet extraction method was employed to extract the constituents of *Moringa oleifera* leaf. Butanol and ethyl acetate were used in the extraction of the *M. oleifera* leaf constituents for 6 hour. The extract was then collected and concentrated almost to dryness under vacuum at 45°± 5°C using rotary evaporator. The extract obtained was stored at 4°C.

PROCEDURE FOR QUALITATIVE PHYTOCHEMICAL ANALYSIS OF *Mornga oleifera* LEAF EXTRACTS.

Phytochemical analysis was carried out as described by Harborne, (1973), [6].

STANDARDIZATION OF DERMATOPHYTE ISOLATES

All dermatophyte isolates were inoculated onto SDA plates and incubated at 25°C for 7–10 days to obtain a young, actively growing culture consisting of mycelia and conidia. A mycelial disc, 5 mm in diameter, was cut from the periphery of the 7–10 day old culture, and was aseptically inoculated into tubes containing Sabouraud's dextrose broth. The tubes were incubated at 25°C for 2-3 days. After incubation, the tubes were placed on a vortexing machine and vortexed for about 15-20 mins to properly disperse the cells in the broth. The concentration of organisms in the tubes was standardized by adjusting to a concentration of about 10^4 CFU/ml.

PRIMARY SCREENING OF CRUDE EXTRACTS/FRACTIONS OF *M. oleifera* FOR ANTI-DERMATOPHYTE ACTIVITY

The anti-dermatophyte activities of the plant extracts were determined by the agar well diffusion method as described by Perez et al., 1990. Dilutions of 250, 125, 62.5, 31.25, and 15.125 mg/mL were prepared from the 500 mg/mL stock solution of the extracts. 20 mL of molten SDA was poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations of culture of test isolates grown in Sabouraud's dextrose broth were swabbed aseptically on the agar plates and holes (6mm) were made in the agar plates using a sterile metal cork-borer. 20 µl of the various dilutions of each formulation and controls were put in each hole under aseptic condition, kept at room temperature for about 30 minutes to allow the drugs to diffuse into the agar medium and incubated accordingly. Ketoconazole (50 µg/mL) was used as the positive control, while DMSO or sterile distilled water (depending on the nature of the extracts and their solubility in either water or DMSO) was used as the negative control. The plates were then incubated at room temperature for fungal isolates for 24-28 hours and the inhibition zones diameters (IZDs) were measured and recorded. The size of the cork borer (6mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicate and the mean IZDs calculated and recorded.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF *M. oleifera* LEAF CRUDE EXTRACTS ON THE DERMATOPHYTES

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that inhibits the fungal growth. The MIC of the plant extracts on the dermatophyte isolates was determined by the agar dilution method as described by [7]. The stock solution (5000 mg/mL) was further diluted in a 2-fold serial dilution to obtain the following concentrations: 2500, 1250, 625, 312.5, and 156.25 mg/ml. Agar plates

were prepared by pouring 9mL of molten double strength SDA into sterile Petri plates containing 1mL of the various dilutions of the extract making the final plate concentrations to become 500, 250, 125, 62.5, 31.25, and 15.625 mg/ml.

The dermatophyte isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the surface of the agar plates containing dilutions of the extract. The SDA plates were incubated at room temperature (25-27^{o}C) for 5-7 days, after which all plates were observed for growth. Control plates, which contained no plant extracts, were also made with the test. The lowest concentration of the extracts completely inhibiting the growth of each organism was taken as the MIC[8].

DETERMINATION OF MINIMUM FUNGICIDAL CONCENTRATIONS (MFCS) OF *M. oleifera* CRUDE EXTRACTS ON DERMATOPHYTE ISOLATES

The minimum fungicidal concentration (MFC) is defined as the minimum concentration of the antimicrobial agent the kills off all the fungal cells. The MFC of the plant extracts was derived by sub culturing portions of the agar from plates that showed no growth in the tests for determination of MFCs. These agar portions were transferred into plates containing freshly prepared SDA. These plates were incubated at 25-27^{o}C for 5-7 days and were observed daily for mycelial growth. The absence of growth at the end of incubation period signifies total cell death. The minimum concentration of the plant extracts that produces total cell death is taken as the MFC[9].

DETERMINATION OF THE FUNGICIDAL ACTIVITIES OF *M.oleifera* CRUDE EXTRACT ON DERMATOPHYTE ISOLATES BY TIME-KILL ASSAY

Standardized concentrations (10^{4}CFU/mL) of logarithmic phase culture of test isolates were prepared. An appropriate quantity of the extract was added to a sterile test tube containing Sabouraud's dextrose broth, and 1mL of the standardized test culture was added to 9 mL of the extract-broth mixture to give a microbial concentration 10^{3} CFU/mL and a concentration equal to the MFC of the extract[10].

Sterile molten SDA was poured into sterile Petri plates and allowed to set. 0.1mL of the extract-broth-culture mixture was put onto the agar and spread with a sterile spreader. This is to give control time 0 minutes count. Samples were taken after 1hr, 4hr, 8hr, and 24hr intervals. The procedure was carried out in triplicate to ensure accuracy. Plates were incubated at 25-27^{o}C for 5-7hrs and observable colonies were counted. For controls, 1 strain each of *Epidermophyton floccosum, Microsporum audouinii,* and *Trichophyton rubrum* was grown in tubes containing broth with no added plant extract and samples taken at the indicated time intervals. Control plates were also incubated.

The number of colony forming unit (CFU) were counted after the period of incubation. A graph of percentage viable count against time in hour was plotted]11].

STATISTICAL ANALYSIS

Results were analyzed by SPSS Version 20 using One-way ANOVA and expressed as mean ± standard error of mean (SEM). Differences between means were considered significant at $P<0.05$.

RESULTS

Table 1: Gender Distribution of Dermatophytes

GENDER	NO SAMPLE D (%)	NO OF POSITIVE (%)	NO OF NEGATIVE (%)	DERMATOPHYTE ISOLATED
Male	50%	14%	36%	*E.floccosum, M.audouinii*
Female	50%	6%	44%	*T.rubrum, M.audouinii*
Total	100%	20%	80%	

Table 1 shows the distribution of dermatophytes in the study group. Out of 50 samples, the male were more affected with 7cases (14%) and female 3 cases (6%) while a total number of 40 (80%) did not yield any dermatophyte.

Table 2: Frequency of Dermatophytes Isolated According to Age Group of Pupils Sampled

AGE GROUP	NO SAMPLED (%)	NO OF POSITIVE (%)	NO OF NEGATIVE (%)	DERMATOPHYE ISOLATED
6-8	30%	6%	24%	*M.audouinii*
9-11	40%	8%	32%	*M audouinii, T. rubrum*
12-14	30%	6%	24%	*E.floccosum, T.rubrum*
Total	100%	20%	80%	

Table 2 shows the frequency of dermatophytes isolated according to age group of pupils sampled. Out of 50 samples analysis (20%) were positive for 3 different dermatophyte species including *Epidermophyton floccosum* (8%) *Trichophyton rubrum* (6%) and *Microsporum audouinii* (6%) while 80% of the sample analyzed did not yield any dermatophyte.

Table 3: Phytochemical Characteristics of *M. oleifera* Leaf Crude Extracts

Phytochemical constituents	Crude extracts	
	Ethyl acetate fraction	**Butanol fraction**
Alkaloids	-	-
Saponins	-	-
Steroids	-	-
Terpenoids	-	-
Flavonoids	+++	+++
Tannins	+++	++

+ means present, - means absent, +++ means highly present

Table 3 shows the result of phytochemical screening of the butanol and ethyl acetate crude extract of *Moringa oleifera* leaf. Flavonoids and tannins were observed in the Ethyl acetate extract and butanol extract.

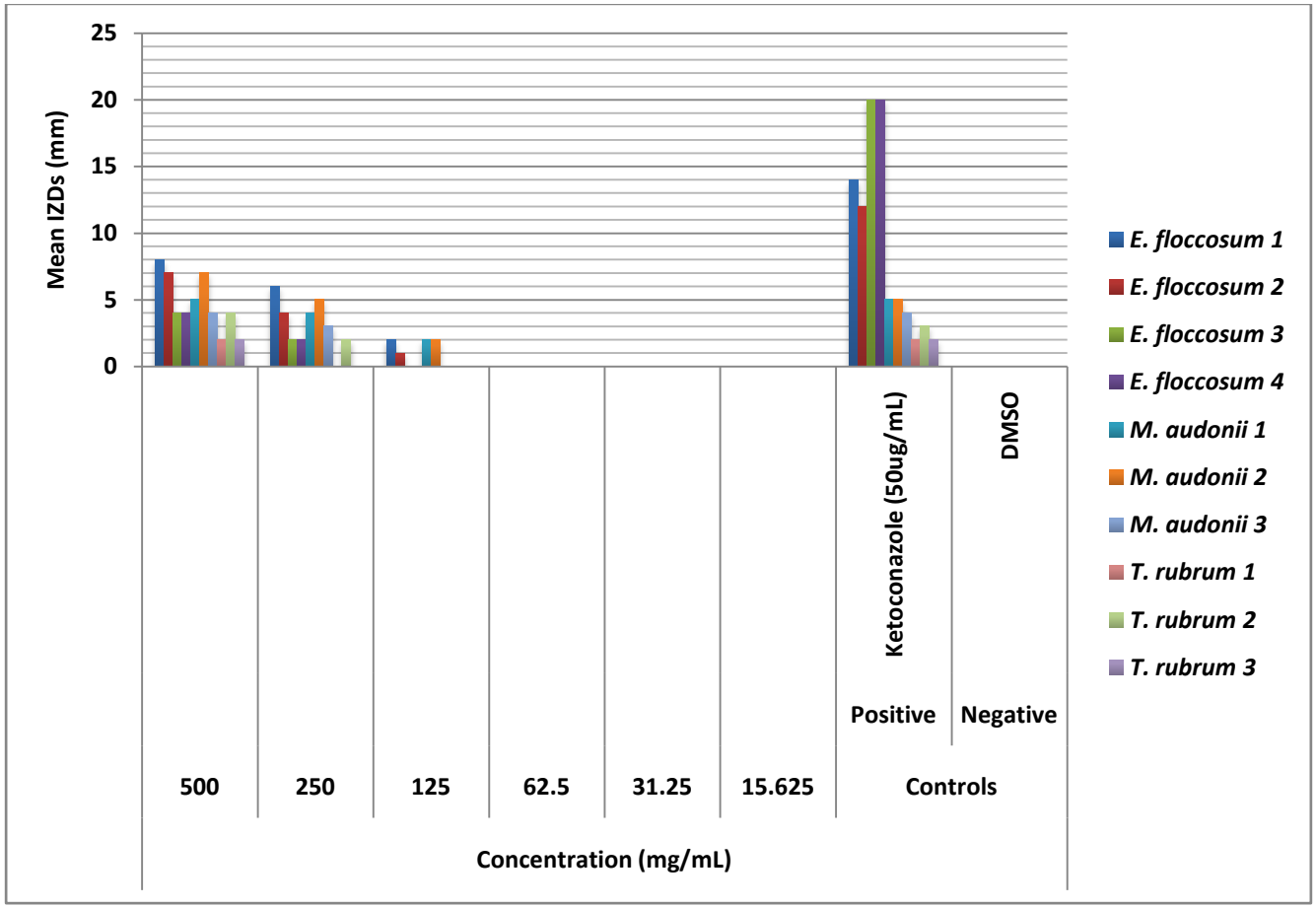

Fig 1: Inhibition Zone Diameters (IZDs) Produced by the Ethyl Acetate Extract of *Moringa oleifera* leaf on Dermatophyte Isolates

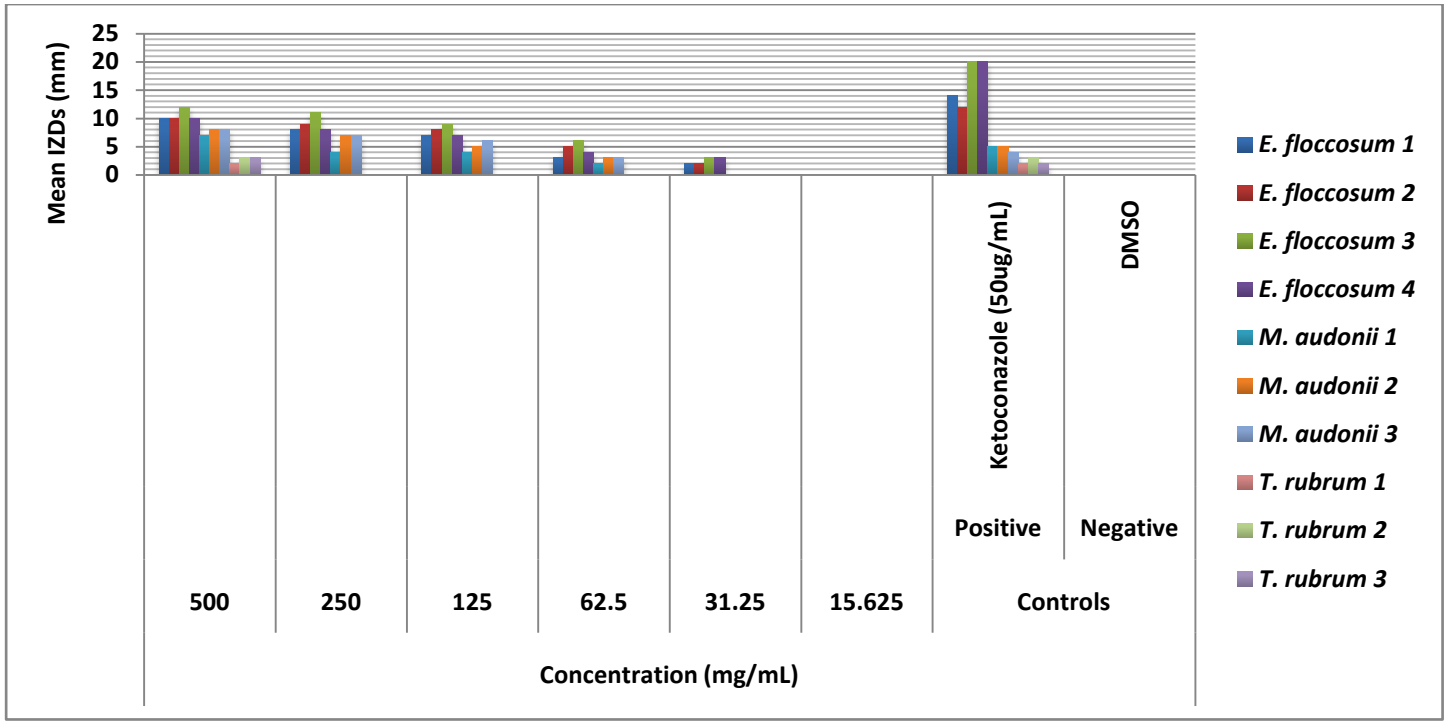

Fig 2: Inhibition Zone Diameters (IZDs) Produced by the Butanol Extract of *Moringa oleifera* leaf on Dermatophyte Isolates

Table 4: Minimum Inhibitory Concentrations (MICs) of Crude extracts of *Moringa oleifera* leaf

Dermatophyte Isolates	MICs of the Crude extracts (mg/mL)	
	ethyl Acetate Extract	**Butanol Extract**
E. floccosum 1	62.5	31.25
E. floccosum 2	125	31.25
E. floccosum 3	125	31.25
E. floccosum 4	125	31.25
M. audonii 1	125	62.5
M. audonii 2	125	62.5
M. audonii 3	125	62.5
T. rubrum 1	500	500
T. rubrum 2	250	500
T. rubrum 3	500	500

Table 5: Minimum Fungicidal Concentrations (MFCs) of the Crude extracts of *Moringa oleifera* leaf

Dermatophyte Isolates	MFCs of the various Extracts (mg/mL)	
	Ethyl Acetate Fraction	**Butanol Fraction**
E. floccosum 1	125	31.25
E. floccosum 2	125	31.25
E. floccosum 3	125	31.25
E. floccosum 4	125	31.25
M. audonii 1	125	62.5
M. audonii 2	125	62.5
M. audonii 3	250	62.5
T. rubrum 1	500	500
T. rubrum 2	250	500
T. rubrum 3	-	500

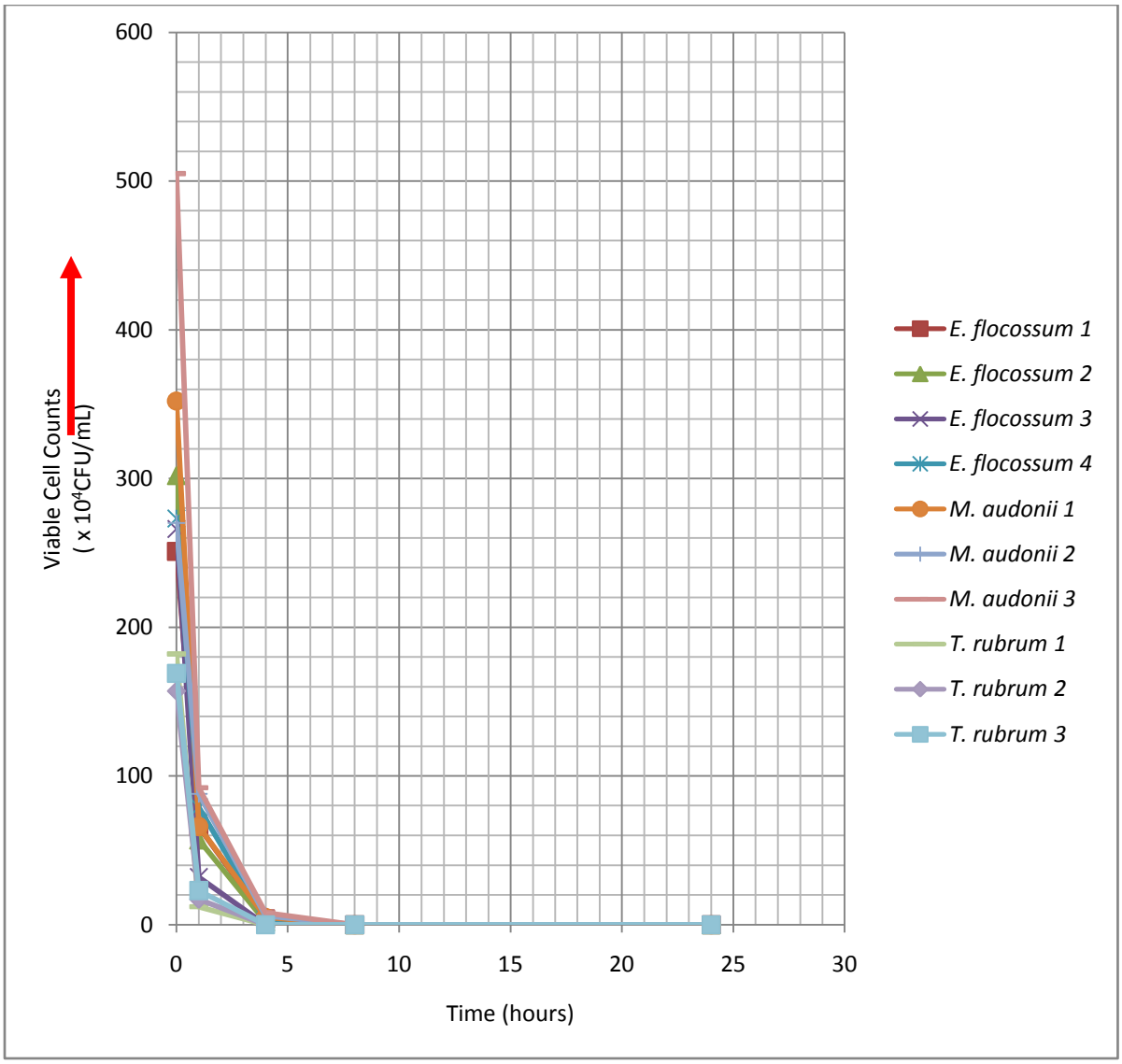

Fig 3: Fungicidal Activity of Ethyl Acetate Extract of *M. oleifera* on Dermatophytes showing the Viable Cell Counts with Time

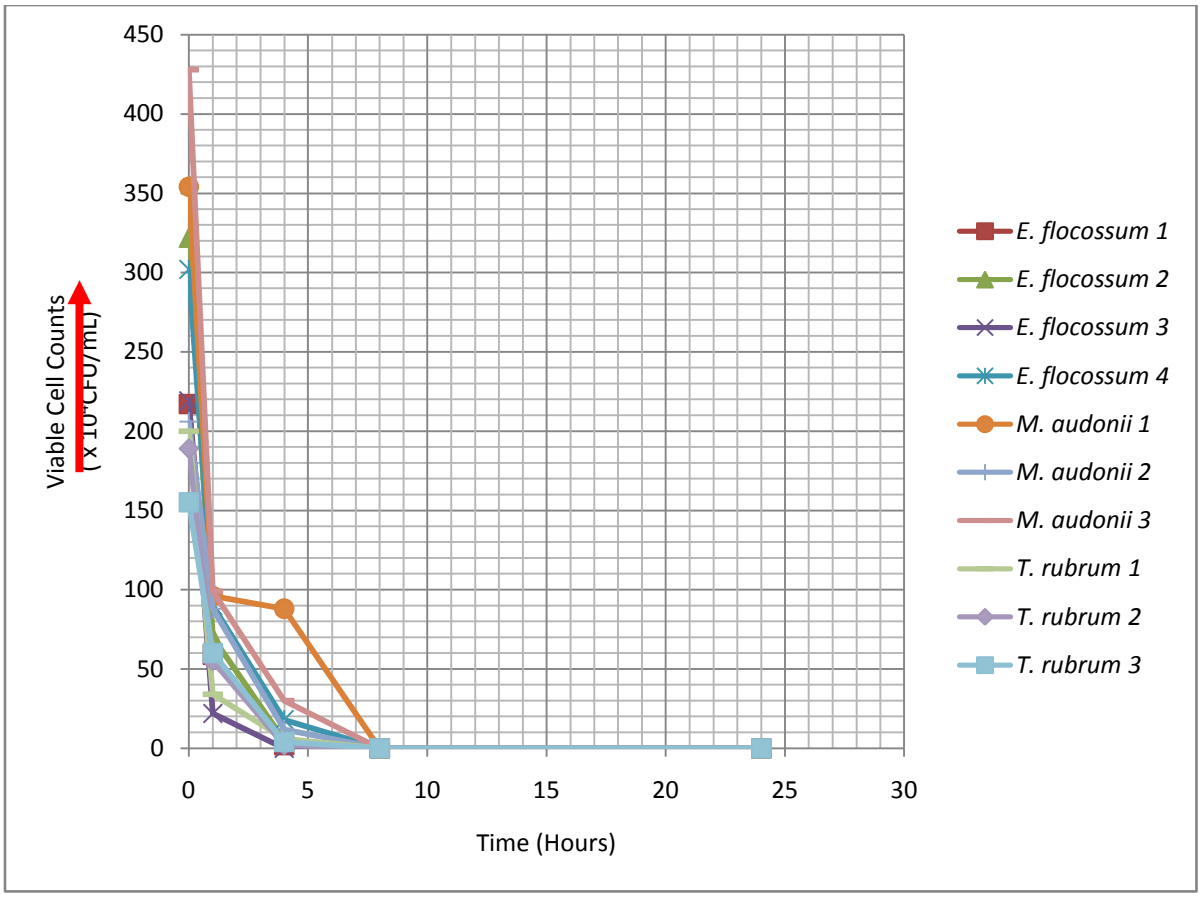

Fig 4: Fungicidal Activity of Butanol Extract of *M. oleifera* on Dermatophytes showing the Viable Cell Counts with Time

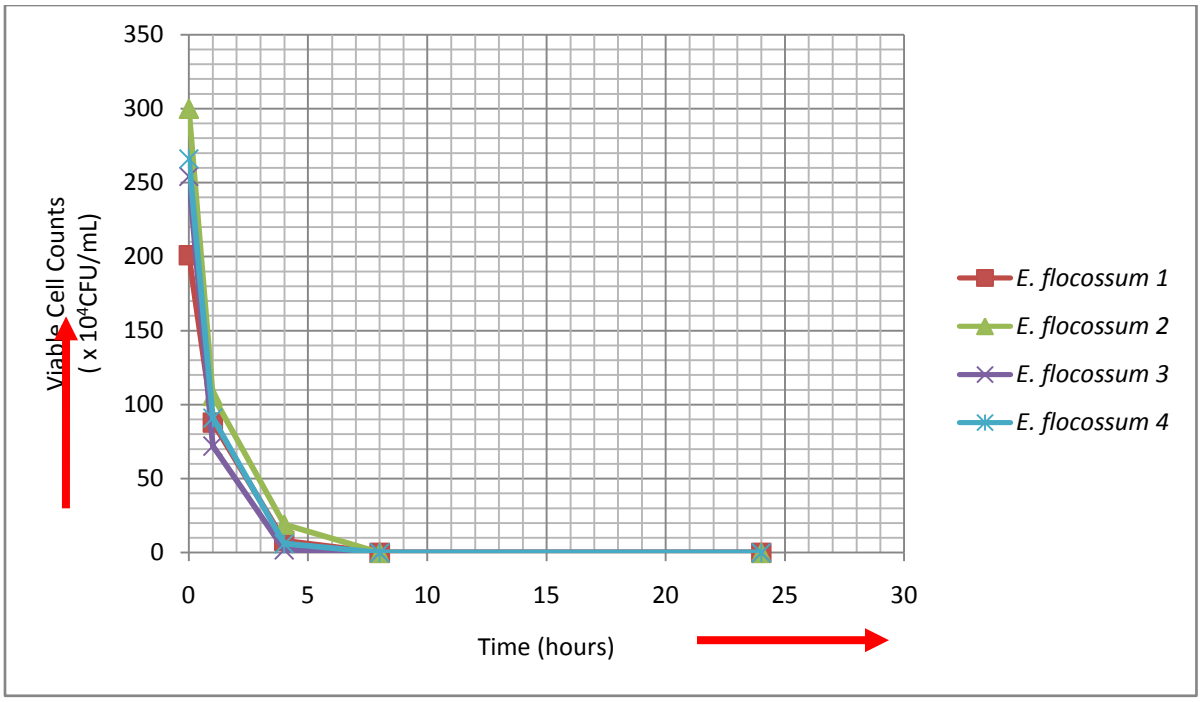

Fig 5: Viable Cell Counts of Control Dermatophytes with Time

DISCUSSION

In the study of prevalence of dermatophytosis, a higher prevalence in males was observed, similar to other reports from various countries of the world Abalaka, Daliyan, Oyeleke and Adeyemo (2012)[12]. This male preporiderance to dermatophytosis could be attributed to level of hygiene which encourage the growth and spread of dermatophytes and other fungi in the human body. It was reported by Acholonu and Nwobu (1983)[9], in Luth that rate of dermatophytic infection was higher in males (63.9%) than in females (36.1%). According to Enweani, *et al.,* (1996)[8], the higher infection rate in boys (61.7%) than in girls (38.3%) may be due to boys taking part in more outdoor activities than girls and therefore being more prone to come in contact with dermatophyte spores. The dermatophyte *Epidermophyton floccossum* showed the highest prevalence in this study contrary with reports from some researchers which have constantly reported this organism as the least etiological agent of dermatophytosis [13 and 14]. Enweani *et al.(*1996*)*[8], also reported high rate of *M. audouinii* infection (68.1%) in primary school pupils.

There were no differences in the dermatophyte infection depending on the age (6-14) of the school children examined, this is in line of the reports of Enweani *et al.(*1996*)*[8]. This could be because children in this age range have very similar life style. At this age, the self-

limiting effect of the sebaceous gland secretions that occur at the onset of puberty and persist until old age had not yet come into play [15].

In the plant study, *M. oleifera* was identified as belonging to the family *Moringaceae*, genus *Moringa* and species *oleifera*. The result of the phytochemical screening of butanol and ethyl acetate extracts of *Moringa oleifera* leaf revealed the presence of phytochemicals such as flavonoids and tannins which are in close agreement with the findings of Abalaka Daliyan, Oyeleke and Adeyemo (2012)[12]. Farooq *et al*, (2007), [13], reported that plants occur in varying habitats, a great magnitude of variation in the concentration and composition of phytochemicals ingredients in the different parts of such plants is expected. Moreover, chemicals are produced in response to perceived threats by the plants, therefore variation exist in the production of these phytochemicals depending on the type and amount of threat encountered by the plant[13].

The antifungal activity as shown in figure 1 and 2 showed that the plant extract had antifungal activity on some dermatophytes. The result showed that butanol extract of *Moringa oleifera* had the highest activity against the dermatophyte isolates; *E. floccosum*, *M.audouinii* and *T. rubrum*, with MIC values of 31.23 mg/ml, 62.5 mg/ml and 500 mg/ml respectively in relation to ethyl acetate extract. Meanwhile, ethyl acetate extract had appreciable inhibitory effect against the dermatophytes; *E. floccosum*, *M.audouinii* and *T. rubrum*, ethyl acetate had MIC values ranging from 62.5 -125 mg/ml, 125 mg/ml and 250-500 mg/ml respectively.

The result also revealed that *E. floccosum* was more susceptible to the *M. oleifera* leaf extracts followed by *M. audouinii,* while *T. rubrum* was resistant to *M. oleifera* extracts. The basis of varying degree of sensitivity of test organisms of fungi may be due to the intrinsic tolerance of microorganisms and the nature and combinations of phytocompounds present in the extracts [14].

The fungicidal activity fig (3-4) shows that at 0hr, all the dermatophyte isolates had the highest viable cell counts. As the time increased, the viable cell counts of the dermatophyte isolate decreased. This result also revealed that all the extracts were fungicidal at the 8th hour. The antifungal activities of the extracts were compared with a standard drug Ketoconazole, which was found effective at a much lower concentration than the butanol and ethyl acetate crude extracts.

The leaves of *M. oleifera* have also been known to contain a number of phytochemicals such as flavonoids, saponins, tannins and other phenolic compounds that have antimicrobial activities [15 and 16]. This would suggest that the antimicrobial activities observed in this study could be attributed to such compounds. The mechanisms of actions of these compounds have been proven to be via cell membranes perturbations [17]. This coupled with the action of β-lactams on the trans-peptidation of the cell wall could lead to an enhanced antimicrobial effect of the combinations [17].

Their mechanism of actions may include enzyme inhibition by the oxidized compounds and act as a source of stable free radical and often leading to inactivation of the protein and loss of function. They have the ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls and disrupt microbial membranes [3].

CONCLUSION

The extracts of *Moringa oleifera* leaf have high potential as antimicrobial agent. It showed varying degrees of activities against the tested dermatophytes with better antifungal activity against *E.floccossum* and *M. audouinii*.

However, this finding provides an insight into the usage of this plant in traditional treatment of foot infections, parasitic infections, venereal diseases and other diseases associated with bacterial and fungal infections. It also suggests that a great attention should be paid to medicinal plants which are found to have plenty of pharmacological properties that could be sufficiently better when considering a natural food and feed additives to improve human and animal health.

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