Susceptibility of dermatophytes to *Aloe vera* juices using agar diffusion and broth dilution techniques

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ABSTRACT

Dermatophytes are kerationophylic fungi that cause various skin diseases known as dermatophytoses (ringworms) in humans. In other to predict the ability of a given antimycotic agent to eradicate the fungi isolate, in vitro susceptibility testing becomes helpful. This study, therefore, determined the susceptibility of dermatophytes clinical isolates to *Aloe vera* juices using agar diffusion and broth dilution techniques. Comparative mean zones of inhibition of *Aloe vera* juices against the dermatophytes clinical isolates using agar disc and well diffusion methods showed no significant difference in the two cultural methods for determining antifungal activities of *Aloe vera* juices against dermatophytes ($P>0.05$). No discrepancy was obtained between the results of Minimum Inhibitory Dilutions (MID) and Minimum Fungicidal Dilutions (MFD) of *Aloe vera* juices obtained from two Nigerian cities against *Epidermophyton floccosum* using macro broth dilution methods. SH-1 and SH-3 *Aloe vera* juices had the highest and lowest MID and MFD respectively in Shagamu against *E .floccosum* while in Ilorin, IL-5 , IL-6 recorded the highest and lowest MID and MFD respectively against *E. floccosum*. No activity against *E. floccosum* was obtained in SH-4, IL-7 and IL-8 against *E. floccosum*. The results of this study show that both agar diffusion and broth dilution invitro techniques can be used to determine susceptibility of dermatophytes to antifungal juices of *Aloe vera*.

Keywords: *Aloe vera* juices, dermatophytes, susceptibility test

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INTRODUCTION

Dermatophytoses rank among the most common and wide spread infectious diseases worldwide.[1] These groups of cutaneous diseases are caused by filamentous fungi called moulds which belong to a general group known as dermatophyte.

The increased use of antifungals, often for prolonged periods, has led to the recognition of the phenomenon of acquired antifungal resistance.[2] Furthermore, the rapid increase in fungal infections and the growing number of new antifungal agents [3] indicate an increase need for rapid and accurate methods for antifungal susceptibility testing. [4] Recent years have witnessed emergence of resistance of dermatophytic agents to synthetic and routinely prescribed antifungal agents including azoles and other antifungal compounds. [5]Hence, the search for alternative, cheaper and readily available newer compounds with potential antifungal activities is increasing.[5,6,7,8,9]
Plants seem to be the readily available natural alternative sources. Plants belonging to genus *Aloe* have been known for their medicinal values.[10] Researchers also reported that the juice of *Aloe vera* is useful in treating wounds from thermal burns and radiation injury, dry and moist epidermis and preventing dermatitis, eczema and other cutaneous infections.[11]

Successful treatment of dermatophytoses depends on the ability of a given antimycotic agent to eradicate the fungi isolate.[11] In other to predict this ability, in vitro susceptibility testing becomes helpful because it can help clinicians to choose the correct treatment for their patients .The Clinical and Laboratory Standards Institutes (CLSI, formerly the NCCLS)[12] approved standard protocol M38-A (CLSI, 2002) does not provide a methodology for testing susceptibility of dermatophytes to antifungal drugs. The protocol has generated a great number of methodologies proposed by many researchers, which makes comparison of results difficult. However, some conditions for performing the tests have been evaluated and have demonstated reproducibility and reliability.[11,13]
Hence the present study was carried out to access the antidermatophytic activities of *Aloe vera* juices obtained from Ilorin and Shagamu, Nigeria against six dermatophytes isolated from the skin lesions of Nigerian primary school pupils using agar disc diffusion and agar well diffusion methods, which have been validated for antibiotic susceptibility test of bacteria. Attempt was also made to determine the minimum inhibitory dilution (MID) and minimum fungicidal dilution (MFD) of *Aloe vera* juices against the isolates of dermatophytes.

MATERIALS AND METHODS

Sources and Identification of Dermatophytes

Six species of dermatophyte isolates namely: *Microsporum audouinii*, *Microsporum ferrugineum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton*

schoeleinii and *Epidermophyton floccosum* were obtained from Raheem Ademola of the Department of Community Medicine and Primary Care of Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria. The dermatophytes were initially isolated from skin lesions of various body parts of primary school pupils in Shagamu Ogun State Nigeria during an epidemiological study. The identity of the isolates were further confirmed using microscopy, cultural characteristic, growth rate and biochemical test such as urease as described by Larone (2002)[14] and Jessup *et al*.(2000).[15]

Sources of *Aloe vera* juices

Four types of *Aloe vera* were collected from home garden of Prof. Oluwadun in Shagamu, Ogun State Nigeria and thirteen types were collected from, Olulande 1, Olulande 2, Olorunshogo, Airport, and Eleko in Ilorin, Kwara State, Nigeria. The body of *Aloe vera* plants were washed with running tap water and were cut horizontally with sterile scalpel blade and the yellow juices were collected into sterile universal glass bottles. The contents were autoclaved at 121^{0}C for 15minutes.

Antifungal susceptibility test

Preparation of Aloe vera Discs

Six mililitre diameter discs were prepared from Whatman No.1 filter paper and were used to absorb 30ul of *Aloe vera* juices according to method described by Ahmad *et al* .(1998).[16] The discs containing juices were sterilized by autoclaving at 121^{0}C for 15minutes and then stored in refrigerator at 4^{0}C for further use.

Preparation of inoculum

Stock inoculums of *T. rubrum, T. mentagrophytes, T. schoenleinii, M. ferrugineum, M. audouinii, and E. fluccosum* strains isolated from the primary school pupils were prepared from 7 to 14 day cultures grown on Sabouraud Dextrose Agar as described by (Espinel-Ingroff, 2000)[17] fortified with 50mg chloramphenicol and 400mg cycloheximide.[14] Then 2 to 3ml sterile normal saline (0.85% NaCl) was added and the suspensions were made by gently scraping the colony with the tip of a sterile Pasteur pipette. The resulting suspended mixture was withdrawn and transfered to a sterile tube. Heavy particles of the suspension present were allowed to settle for 15 minutes at room temperature and the upper homogenous suspensions were mixed using vortex mixer for 15 seconds and adjusted with sterile normal saline to match an opacity of 0.5 McFarland's standard.[18] The inoculums were adjusted to between 1.0 x 10^6 and 5.0x 10^6 spores per ml by microscopic enumeration with a cell counting haemocytometer (Neubauer counting chamber).

Agar Disc diffusion method

Sterile Sabouraud dextrose agar (SDA) plates were prepared and dried in the oven. The prepared dermatophytes inoculums were seeded over the surface of the SDA plates, and allowed to dry. The impregnated discs with *Aloe vera* juices were placed on the surface of the plates. Incubation was done at room temperature for 5-10days. The zones of inhibition were measured and recorded in millimeters. The procedure was repeated in triplicate in other to find the mean zones of inhibition.

Agar well diffusion method

The prepared concentration of standardized dermatophytes inoculums were seeded over the surface of sterile SDA plates, and allowed to dry. Then, 6mm corkborer was used to bore holes of 6mm in diameter equidistant to each other and 0.1ml of *Aloe vera* juices were dispensed into their respective wells. The plates were allowed to stand on the bench for five minutes so that the *Aloe vera* juices could diffuse into their respective wells. The plates were incubated at room temperature for seven days, after which the mean zones of inhibition were measured and recorded in millimetre.

Serial dilution of *Aloe vera* juices

Twelve sterile tubes were arranged in the rack and 1ml of sabouraud dextrose broth was added to tubes 2 to 12 (except tubes 1 and 11) and 2.0ml of *Aloe vera juice* were put into tube 1. Then, 1.0ml of *Aloe vera* juice was transferred from tube1 to tube 2. Serial doubling dilution was made from tubes 2 to 10 by transferring 1.0ml of the homogeneous tube 2 content to tube 3, and from 3 to 4 and so on to 10 and the remaining 1ml was discarded. Then, 1ml of the *Aloe vera* juice was added to tube 11 (negaitive control) and 1ml of sabouraud dextrose broth was added to tube 12 (positive control).

Determination of minimum inhibitory dilution (MID) of *Aloe vera* juice

1.0 ml of 0.5 Mcfarland turbidity of dermatophyte spores was added to the contents of all the tubes and incubated at room temperature for 7days. The highest dilutions showing no turbidity were observed as the MID.

Determination of minimum fungicidal dilution (MFD) of *Aloe vera* juice

Mininmum fungicidal dilutions of the *Aloe vera* juices were determined by plating each MID on the Sabouraud dextrose agar plate and incubated at room temperature for 7days. Lack of growth indicated fungicidal activity of the juices while growth indicated fungistatic. [19]

Statistical Analysis of data

All tests were performed in triplicates. The data were analysed using INSTAT statistical package. The descriptive statistical tools used include the students t-test and bar charts. The level of significance was taken as $P< 0.05$.

RESULTS AND DISCUSION

The present study was carried out to access the antidermatophytic activities of *Aloe vera* juices obtained from Ilorin and Shagamu, Nigeria against six dermatophytes isolated from the skin lesions of Nigerian primary school pupils using agar disc diffusion and agar well diffusion methods which have been validated for antibiotic susceptibility test of bacteria. Attempt was also made to determine the minimum inhibitory dilution (MID) and minimum fungicidal dilution (MFD) of *Aloe vera* juices against the isolates of dermatophytes.

Table 1 shows comparative mean zones of inhibition of *Aloe vera* juices against dermatophytes using agar disc diffusion and well diffusion methods.

There was no significant difference in the two cultural methods for determining antifungal activities of *Aloe vera* juices against dermatophytes ($p>0.05$). This is not surprising because the two methods have the same physical principle of *Aloe vera* molecules moving from the discs and wells to the dermatophytes cultured on the Sabouraud dextrose agar plates. Such movement is called diffusion, which is a physical process that involves the movement of molecules from the region of higher concentration to lower concentration. This, therefore, implies that any of these two in vitro antifungal susceptibility tests could be applied to give reproducible results in the determination of susceptibility of dermatophytes, which are filamentous moulds, to the juices of *Aloe vera* which have antidermatophytic activities.

Figure 1 shows the minimum inhibitory dilutions (MID) of *Aloe vera* juices obtained from Shagamu against *Epidermophyton floccosum* using macrodilution method. SH-1 had the highest MID of 1 in 16 and SH-3 exhibited the lowest MID with value 1 in 4. SH-2 showed inhibition up to 1 in 8 against *Epidermophyton floccosum* and the neat of SH-4 had no inhibitiory activity against *Epidermophyton floccosum.* The MID ranged from 1 in 4 to 1 in 16. Figure 2 shows the minimum fungicidal dilution (MFD) of *Aloe vera* obtained from Shagamu against *Epidermophyton floccosum* using macrodilution method. SH-1 had the highest MFD of 1 in 8, SH-3 had the least MFD of 1 in 2. SH-2 showed inhibition up to 1 in 4 and SH-4 showed no inhibition against *Epidermophyton floccosum* . The MFD ranged from 1 in 2 to 1 in 8.

Figure 3 shows the minimum inhibitory dilution (MID) of *Aloe vera obtained* from Ilorin *against Epidermophyton floccosum* using macrodilution method. IL-5 had the highest MID of 1 in 8 and IL-6 had the lowest MID of 1 in 4. The neats of IL-7 and IL-8 showed no inhibition *against Epidermophyton floccosum.* The MID ranged from 1 in 4 to 1 in 8. Figure 4 shows the minimum fungicidal dilution (MFD) of *Aloe vera* obtained from Ilorin against *Epidermophyton floccosum* using macrodilution method. IL-5 had the highest MFD and IL-6 had the lowest MFD. IL-7 and IL-8 had no inhibition activity against *Epidermophyton floccosum.* The MFD ranged from 1 in 2 to 1 in 4.

The results depicted in figures 1 to 4 show the degree of reproducibility of broth dilution technique which was used in determining minimum inhibitory dilutions and minimum fungicidal dilutions of juices from the *Aloe vera* plant. In drug development from medicinal plant, it is important to have validated in vitro laboratory methods that will not only be sensitive but will also give consistent and reliable results that will allow comparison. Otherwise, it will be difficult to compare intra and inter laboratory results during drug development when incompactible techniques are employed in the assay of the same parameter of a product. Minimum inhibitory dilution and fungicidal dilution values play significant roles in the determination of the efficacy of antimicrobial agents against aetiology of infectious diseases such as dermatophytes.

CONCLUSION

From the above results it maybe concluded that agar diffusion and broth dilution techniques will give reproducible and comparable results when used in determining the susceptibility of dermatophytes to *Aloe vera* juices.

However, for these techniques to be relevant, the hyphae of the dermatophytes must be detached from the spores, as done in this study, so that the later could behave as unicellular microbial entities like bacteria which have been used to validate the two techniques.

Table 1. Comparative mean zone of inhibition of *Aloe vera* against dermatophytes using agar disc diffusion and well diffusion methods

Dermatophytes	Agar disc diffusion, mean ZI(mm)	Agar well diffusion, mean ZI(mm)	t-value	p-value
MA	5.67	9.58	0.86	>0.05
TR	3.25	3.17	0.026	>0.05
TM	2.67	3.17	0.17	>0.05
TS	4.92	6.67	0.47	>0.05
MF	6.92	10.00	1.03	>0.05
EF	5.67	10.75	1.02	>0.05

KEY: EF – *Epidermophyton floccosum*, MA – *Microsporum audouinii*, TM – *Trichopyton mentagrophytes*, TR – *Trichophyton rubrum*, TS – *Trichophyton schoenleinii*, MF – *Microsporum ferrugineum*. ZI – Zone inhibition.

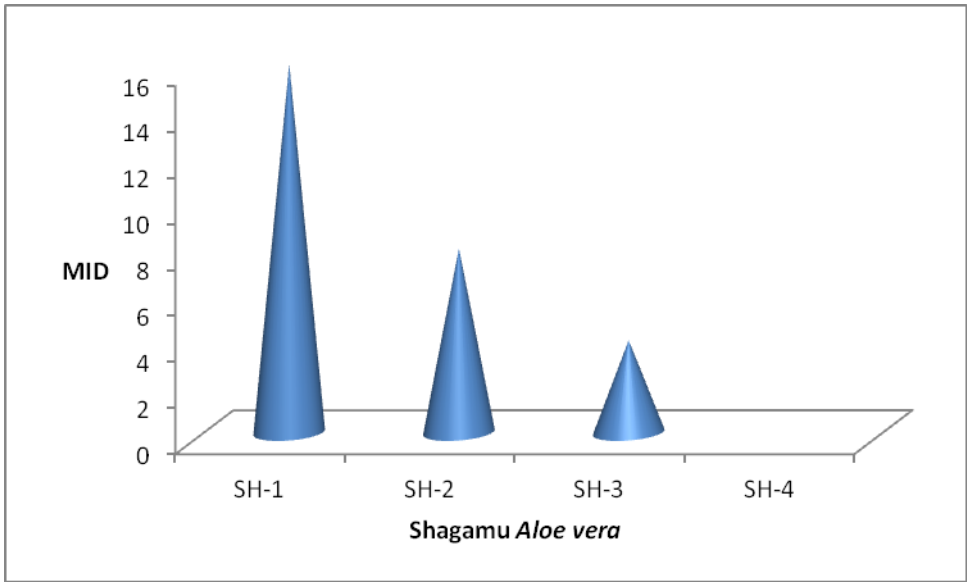

Figure 1 MID of *Aloe vera* from Shagamu against *Epidermophyton floccosum* using macrodilution method.

Sh= Shagamu, MID= Minimum inhibitory dilution.

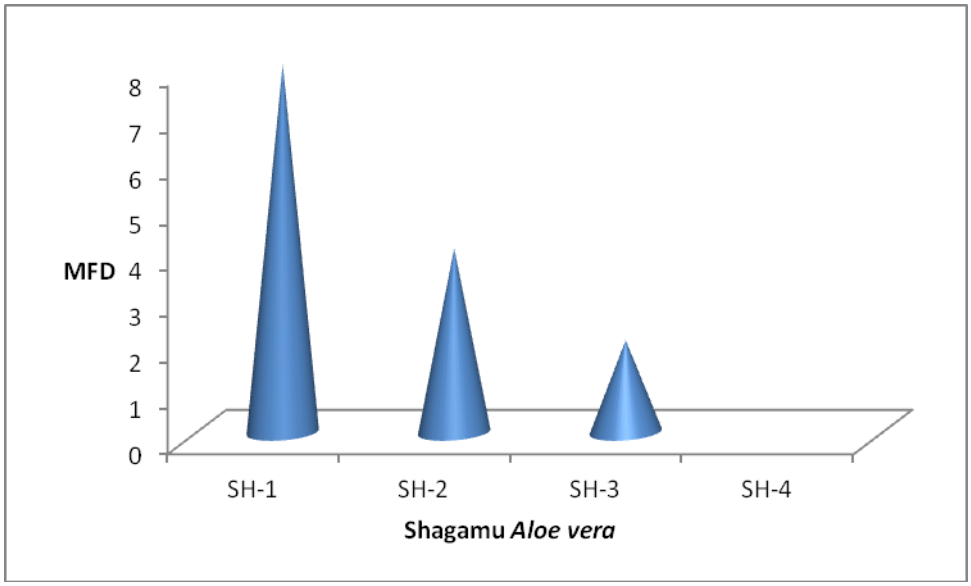

Figure 2: MFD of *Aloe vera* from Shagamu against *E. floccosum* using macrodilution method.

Sh= Shagamu, MFD= Minimum fungicidal dilution.

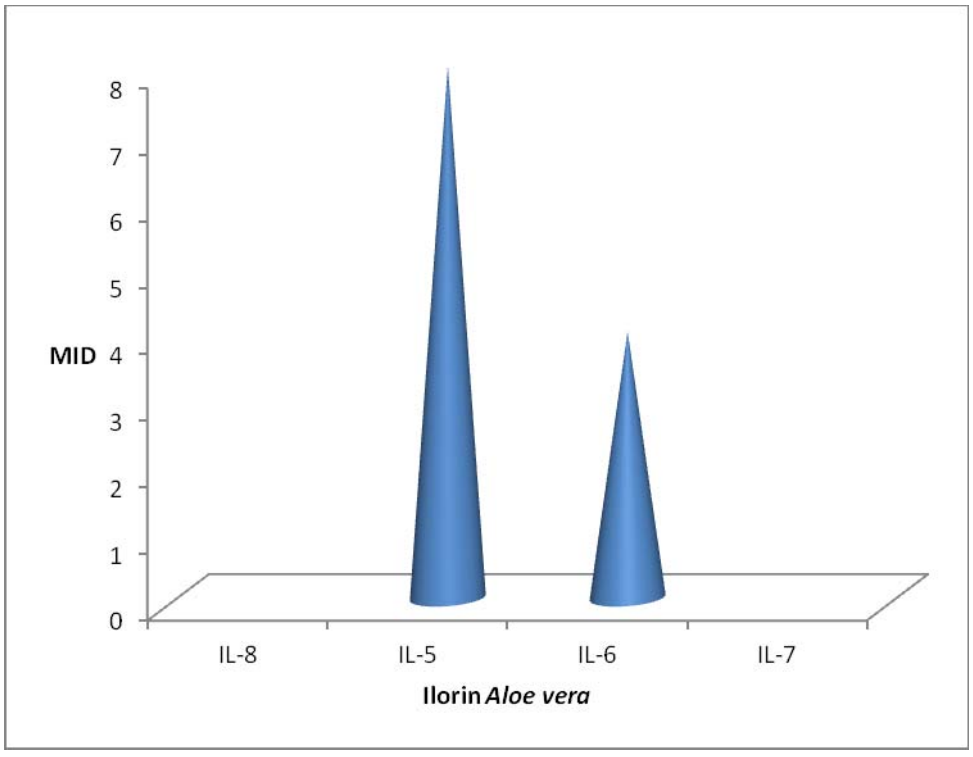

Figure 3: MID of *Aloe vera* from Ilorin against *E. floccosum* using macrodilution method.

Il= Ilorin, MID= Minimum inhibitory dilution.

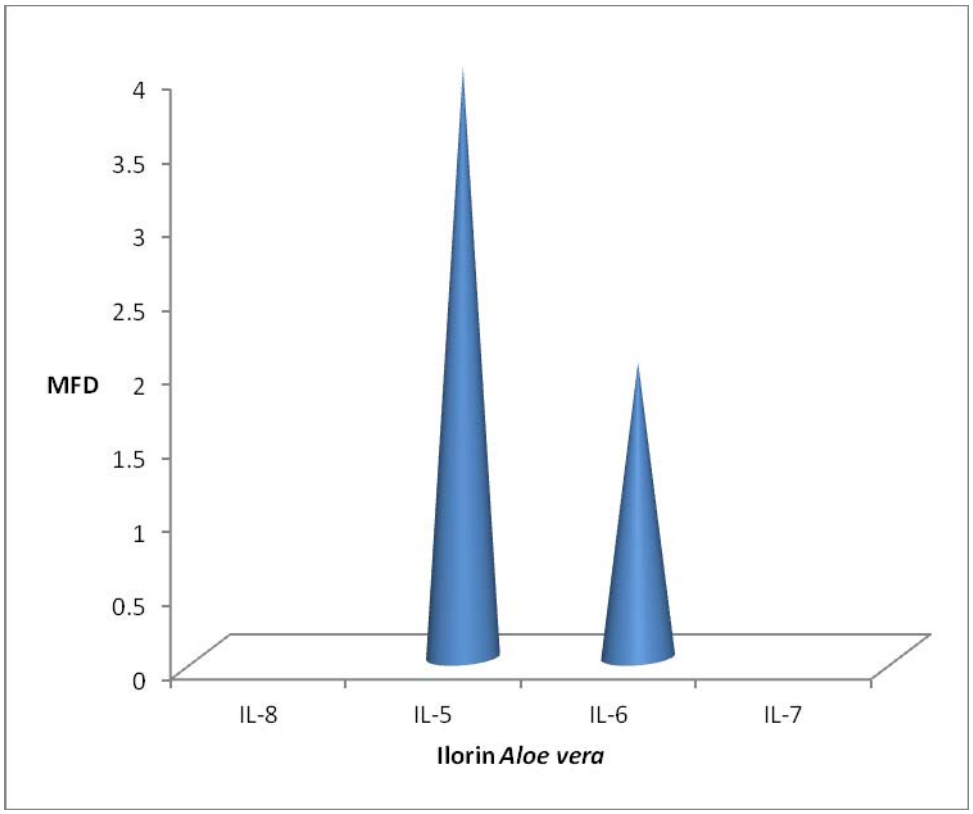

Figure 4.: MFD of *Aloe vera* juice from Ilorin against *E. floccosum* using macrodilution method.

Il= Ilorin, MFD= Minimum fungicidal dilution

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