



Susceptibility Pattern of Different Parts of *Moringa oleifera* against Some Pathogenic Fungi, Isolated from Sputum Samples of HIV Positive Individuals Co-Infected with Pulmonary Tuberculosis

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Authors' contributions

This work was carried out in collaboration among all authors. Authors IBE, VCO and OEO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VCO and IBE managed the analyses of the study. Author OEO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Opportunistic fungal infection in HIV positive individuals has a high risk of morbidity and mortality as it could be a pointer to systemic infection. *Moringa oleifera* is one of the 14 species of the family Moringaceae with traditional effectiveness in anti-helminthic, analgesic, anti-ulcerative, anti-microbial, etc.

Aim: This study was designed to determine the susceptibility pattern of different parts of *Moringa oleifera* against some pathogenic fungi using dried *Moringa oleifera* seed, bark, pod, and leaf extracts/fractions as agents.

Materials and Methods: A total of 132 subjects (62 males and 70 females) attending outpatient

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clinics (OPD) at Nnamdi Azikiwe University Teaching Hospital Newwi, and Holy Rosary Hospital Emekuku Owerri, Nigeria were recruited for this study. The HIV status of the test subjects was confirmed using immuno-chromatographic technique and their sputum screened for Tuberculosis (TB) using Ziehl Nelsen staining technique. The sputum samples were also cultured on Sabouard dextrose agar with and without chloramphenicol incubated at 25°C with examination of growth every 3 days for two weeks. Identification of isolates was done using Gram staining methods, chromogenic media and slide culture. 700g, 220g, 500g, and 250g each of the different parts of the pulverized *Moringa oleifera* (seeds, barks, pods, and leaves) were weighed and extracted using Soxhlet extractor and methanol as solvent.

Results: The prevalence of fungi isolates from test subjects shows that *Candida albicans* has the highest frequency of 25(26.6%), with the least prevalent being *Aspergillus flavus* and *Phialemonium curvatum* at 2(2.1%) respectively. There was also a positive significant correlation between TB and fungi infection in HIV-sero positive subjects ($P < 0.05$). The *invitro* antifungal activity of these *Moringa oleifera* extracts/fractions against the test isolates showed no antifungal activity except for ethyl acetate fraction which demonstrated the most prominent activity against *A. fumigatus* with zones of inhibition of 3-10mm diameter with the least activity against *C. krusei* with zones of inhibition of 3-6mm diameter. However, the antifungal activities of fluconazole which served as a control in the antifungal susceptibility against the test isolates were more effective than the extract/fractions against the test isolates with a minimum inhibition concentration (MIC) of 50-200mg/ml against 25-100mg/ml for ethylacetate fraction.

Conclusion: The comparable antifungi effect of ethylacetate fraction of *M. oleifera* leaf with that of fluconazole, suggests that ethylacetate fraction of *M. oleifera* leaf could aid the treatment of pathogenic fungi due to its potential applications in Pharmaceutical industry, thereby ameliorating the morbidity and mortality rate of HIV positive co-infected TB subjects.

Keywords: *Moringa oleifera*; HIV; Tuberculosis (TB); fungi infections; CD4 count; co-infection.

1. INTRODUCTION

The Human Immune deficiency Virus (HIV) is a lentivirus (slowly replicating retrovirus) that causes Acquired Immuno Deficiency Syndrome (AIDS), a condition in humans in which progressive failure of the immune system allows life threatening opportunistic infections and cancers to thrive [1]. Its modes of transmission ranges from sexual intercourse with an infected partner (including anal, oral sex), sharing of an infected objects eg needles, razor, and syringes with an infected person, person to person through contaminated blood and other body fluids, and from mother to unborn child during pregnancy, delivery or breastfeeding, and through transfusion with an infected blood and blood products. HIV has been found in saliva and tears in low concentrations in some AIDS patients. However, contact with saliva, tears or sweat has shown to result in transmission [2]. HIV infects vital cells in the human immune system such as helper T cells specifically CD4+ T cells, macrophages, and dendritic cells [3]. HIV infection leads to low levels of CD4+ T cells through a number of mechanisms including apoptosis of uninfected bystander's cells via direct viral killing of infected CD4+ T cells by the CD8 cytotoxic lymphocytes that recognize infected cells [4]. When CD4+ T cells number

declines below a critical level cell mediated immunity is lost, the body becomes progressively more susceptible to opportunistic infections.

UNAIDS and WHO estimates that AIDS has killed more than 25million people since it was first recognized in 1981, making it one of the most destructive pandemics in recorded history [5]. Despite recent improved access to antiretroviral treatment and care in many regions of the world, the AIDS pandemic claimed an estimated 2.8million (between 2.4 and 3.3 million) lives in 2005 of which more than half a million (570,000) were children. Sub-Saharan Africa remains by far the worst affected region with an estimated 21.6 to 27.4 million people currently living with HIV. About 2 million of them are children younger than 15 years of age [6]. More than 64% of all people living with HIV are in Sub-Sahara Africa, as more than 3 quarters of all women living with HIV. In Nigeria, a prevalence rate of 3.1% has been reported [6].

Fungi are widely distributed throughout the environment. The lungs are the common portal of entry for most fungi into the human host. Fungal infection is a predominant source of morbidity and mortality among HIV positive individuals in late stages of HIV infection and low CD4 counts below 500cells/ μ m, is opportunistic infection

caused by agents that rarely infect immune competent individual [7]. The occurrence of opportunistic fungal infection has risen progressively in recent years. Invasive fungal infections have been reported in recent years in 26% of chronically and intensively immune suppressed patients [8] as cited by [9]. Infection with *Candida albicans* appears when CD4 counts is between 500-200 cells/ μm and may be the first indication of immunodeficiency. Cryptococcal infection occurs when CD4 count has fallen below 150 cells/ μm . Penicilliosis is observed in patients with CD4 count less than 100 cells/ μm [10]. HIV positive individuals are prone to get recurrent fungal infection due to damages on the cells that gives protection on humans, and therefore more vulnerable to develop pathogenic yeasts and mould infections. The pathogenic yeasts and moulds infections depend on the exposure to sufficient inoculum size of the organism and general resistance of the host.

Tuberculosis and HIV constitute the main burden of infectious disease in resource-limited countries. Estimates by the World Health Organization (WHO) indicate that there are more than 9 million new activate case of TB and close to 2 million death per year [11], and that 2.6 million new cases of HIV infection and 1.8 million AIDS related deaths occur per year [5]. *Mycobacterium tuberculosis*- HIV co-infections pose particular diagnostic and therapeutic challenges and exert immense pressure on health care systems in Africa and Asian countries with large population of co-infected individuals.

In the individual host with the two pathogens, *M. tuberculosis* and HIV potentiate one another, accelerating the deterioration of immunological functions and resulting in premature death if untreated. Some 14 million individuals worldwide are estimated to be dually infected [12]. TB is the largest single cause of death in the setting of AIDS, accounting for about 26% of AIDS related deaths [12] and 99% in developing countries [13]. Both TB and HIV have profound effects on the immune system, as they are capable of disarming the hosts immune responses through mechanisms that are not fully understood. HIV co-infection is the most powerful known risk factor for progression of *Mycobacterium tuberculosis* infection to active disease increasing the risk of latent TB reactivation 20-fold [12].

Moringa oleifera is one of the 14 species of family *Moringaceae*, native to India, Africa, Arabia, South Asia, South America, Pacific and

Caribbean Islands. *Moringa oleifera* has been naturalized in many tropic and subtropics regions worldwide. The plant is referred to with a number of names such as horse radish tree, drumstick tree, ben oil tree, miracle tree and mother's best friend [14]. The plants is commonly called horse-radish tree or the miracle tree and locally known as "Zogale- gandi" in Hausa, "Eweigbale" in Yoruba and "Okweoyibo" in Igbo [15]. *Moringa oleifera* is well known for its nutritional and medicinal values by many communities in northern Nigeria.

According to World Health Organization, more than 80% of the world's population relies on traditional medicines for their primary health care needs [16]. The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on human body. The most important bioactive compounds of plants are alkaloids, flavonoids, tannis and phenolic compounds. The phytochemical researches based on ethnopharmacological information are generally considered an effective approach in the discovery of new anti-infective agents from higher plants [17].

The targeted plant *Moringa oleifera* has many uses in traditional medicines eg root and barks are used as astringent, anti-helminthic analgesic (useful in heart complains), eye disease, dyspepsia, enlargement of the spleen, tuberculous glands in the neck, tumors, earache, stuttering and in ulcers. The plant is also considered to be useful by tribals (sandals) in burns, sores, epilepsy, scabies, cholera, dysentery and retention of urine [18]. Traditionally its roots are also applied as plaster to reduce the swelling and rheumatism. The root, flower, fruit and leaf have analgesic and inflammatory activity.

Important medicinal properties of the plant include antipyretic, antiepileptic anti-inflammatory, anti-ulcerative [19] antihypertensive cholesterol lowering [20], antioxidant anti-diabetic, hepato-protective [21], antibacterial and antifungal activities. In addition *Moringa oleifera* seeds possess water purifying powers. [22]. They are known to be anti-helminthic, antibiotic, detoxifiers, immune builders and have been used to treat malaria [23]. It can also be used as a less expensive bio-absorbent for the removal of heavy metals [24]. *Moringa* leaves contains phytochemical having potent anticancer such as benzyl isothiocyanate, etc. and hypotensive activity and are considered

full of medicinal properties and used in Siddha medicine [25]. The leaves of this plants contains a profile of important trace elements such as zinc, iron, copper etc. [26] and are a good source of proteins, vitamins, beta-caroteine, amino acids and various phenolics [27]. With all these attributes of *Moringa oleifera* leaves one wonders why very few people and media are promoting the use of *Moringa oleifera* leaves in a country where malnutrition among children below 5 years stands at 15% while 45% children below 5 years are stunted [28]. The whole *Moringa oleifera* plant is used in the treatment of psychosis, eye diseases, fever and as in aphrodisiac, the aqueous extracts of roots and barks were found to be effective in preventing implantation, aqueous extracts of fruits have shown significant anti-inflammatory activity, methanolic extracts of leaves have shown anti-ulcer activity and ethanolic extracts of seeds exhibited anti-tumour activity *Moringa oleifera* is used as drug by many ayurvedic practitioners for the treatment of asthma [29].

1.1 Statement of Research Problem

There had been an increase in the resistance of anti fungals used in the treatment of HIV patients co-infected with pulmonary tuberculosis. Fungal infection as an opportunistic infection impacts negatively to the health status of HIV positive individuals resulting to increasing morbidity and mortality among HIV affected individuals in late stages of HIV infection. This has been challenging in the management of these group of patients. This study will help to ascertain the antifungal activity of the different parts of *Moringa oleifera* against the isolated fungi.

1.2 Justification of Research Problem

The *Moringa oleifera* plant provides a rich and rare combination of zeatin, quercetin, kaempferom and many other phytochemicals. It is very important for its medicinal value. Various parts of the plants such as the leaves, roots seeds, bark, fruits, flowers and immature pods act as cardiac and circulatory stimulants, possess anti-tumor [30] antipyretic, antispasmodic antihypertensive, antidiuretic, antiepileptic anti-inflammatory, antiulcer [19]. Hence, this was a cross-sectional study, designed to determine the susceptibility pattern of different parts of *moringa oleifera* against some pathogenic fungi using dried *Moringa oleifera* seed, bark, pod, and leaf extracts/fractions as agents.

2. MATERIALS AND METHODS

2.1 Study Location

This study was carried out both in Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Anambra State and Holy Rosary Hospital Emekuku, Owerri (HRH), Imo State all in Nigeria. It is the only mission hospital in Owerri that serves as a treatment centre for patients with both *Mycobacterium tuberculosis* and HIV/AIDS from Owerri and beyond. Nnewi is coordinated at 6°11'N, 6°55'E/6.017°N,6.917°E of Nigeria comprising of both Nnewi North and North South with an estimated population of 391,227 according to the Nigeria census. The city spans over 1,076.9 square miles (2,789 km²) in Anambra State. NAUTH is a tertiary health institution serving patients of high, middle and lower socio economic status and one of the Federal Teaching Hospital in the South Eastern Nigeria and the only one in the state. It houses major HIV/AIDS centre (IHVN Clinic) and *Mycobacterium tuberculosis* serving patients from all parts of the state and beyond.

2.2 Study Population

A total of 153 subjects comprising of 51 HIV-sero positive patients on ART, 51 HIV-sero positive subjects not on ART attending Out Patient Clinic at NAUTH, Nnewi and HRH Emekukwu, Owerri and 51 HIV-sero negative individuals.

2.2.1 Inclusion criteria

This study includes HIV-sero positive patients on HAART, HIV-sero positive individuals not on HAART; HIV-sero positive patients co-infected with pulmonary tuberculosis as test subjects and HIV-sero negative patients serving as controls.

2.2.2 Exclusion criteria

This study excluded patients that refused to give their consent.

2.3 Collection and Processing of Plant Materials

The different parts of *Moringa oleifera* plant (seeds, barks, pods, and leaves) were collected from a *Moringa oleifera* tree in Owerri, Imo State and was identified by a herbarium collector Mr. Paulius O. Ugwuozor of the department of botany Nnamdi Azikiwe University, Awka. The different parts were rinsed with water and

allowed to air dry under room temperature for one month and ground into powder.

2.3.1 Extraction

A total of 1670 g (700 g, 220 g, 500 g, and 250 g each) of the different parts of the pulverized *Moringa oleifera* (seeds, barks, pods, and leaves) were weighed out with an automatic weighing balance. The Soxhlet extractor was used for extraction using methanol as solvent.

2.3.2 Principle

It is used where the desired compound has a significant solubility in a solvent and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance.

2.3.3 Method

The weighed pulverized seeds, barks, pods, and leaves of *Moringa oleifera* are placed inside a thimble made from thick filter paper. The solvent (methanol) is poured into the thimble. This is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent (methanol). It is, then, equipped with a condenser and the methanol heated to reflux. The solvent (methanol) vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent (methanol). When the Soxhlet chamber is almost full the chamber is automatically emptied by a siphon side arm with the solvent running back down to distillation flask. The thimble ensures that rapid motion of solvent does not transport any solid material to the still pot. This cycle is allowed to repeat many times, over hours and days. During each cycle a portion of the non-volatile compound dissolves in the solvent (methanol) and the desired compound is concentrated in the distillation flask. After the extraction the solvent (methanol) is removed by means of a rotary evaporator at a reduced pressure of $45^{\circ}\text{C} \pm 5^{\circ}\text{C}$ yielding the extracted compound. The non-insoluble portion of the extracted solid remains in the thimble and is usually discarded. The different fractions obtained (seeds, barks, pods, and

leaves) were screened for phytochemical constituents and stored at 4°C .

The crude methanol extract of *Moringa oleifera* leaves was fractionated using liquid-liquid extraction method with n-hexane, ethylacetate, butanol, and water in the order of increasing polarity.



Fig. 1. *Moringa oleifera* extraction process using Soxhlet extractor at Faculty of Pharmaceutical Sciences of Nnamdi Azikiwe University, Awka



Fig. 2. Fractionation of *Moringa oleifera* leaf at Faculty of Pharmaceutical Sciences of Nnamdi Azikiwe University, Awka

2.3.4 Procedure for phytochemical analysis of *Moringa oleifera* seeds, barks, pods, and leaves

The extracts of the different parts of *Moringa oleifera* were analyzed by the following procedure according to Aggarawal et al. [31]. This test for the presence of alkaloids, tannis, saponins, terpenoids, flavonoids, glycosides, volatile oils, and reducing sugars.

2.3.5 Tannis

To a portion of the extract diluted with water, 3-4 drops of 10% ferric chloride was added. A blue colour is observed for gallic tannis and green colour indicates for catecholic tannis.

2.3.6 Reducing sugar

To 0.5 ml of the extract, 1 ml of water and 5-8 drops of Fehling's solution was added and heated over water bath. Brick red precipitate indicates the presence of reducing sugars.

2.3.7 Glycosides

Twenty five (25) ml of diluted sulphuric acid was added to 5ml of the extract in a test tube and boiled for 15 minutes, cooled and neutralized with 10% sodium hydroxide, and then 5ml of Fehling solution added. Glycosides are indicated by brick red precipitate.

2.3.8 Alkaloids

Two (2) ml of the extract was added into a test tube with 2ml of picric acid solution. An orange colouration indicates presence of alkaloids while no colour indicates absence of alkaloids.

2.3.9 Favonoids

Four (4) ml of the extract was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution 5-6 drops of concentrated hydrochloric acid was added red colour indicates flavonoids and orange colour indicates flavones present.

2.3.10 Volatile oils

Two (2) ml of the extract was shaken with 0.1ml of dilute NaOH and a small quantity of dilute HCL was added. A white precipitate indicates volatile oils present while no precipitate indicates absences of volatile oils.

2.3.11 Terpenoids

Four (4) ml of extract was treated with 0.5ml of acetic anhydride and chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet colour indicates terpenoids present

2.3.12 Saponins

Saponins were detected using froth test; 1 g of the extract was weighed into a conical flask containing 10ml distilled water, boiled for 5 minutes. The mixture was filtered and 2.5 ml of filtrate was added to 10ml of distilled water in a test tube. The tube was stoppered and shaken vigorously for 1minute; it was then allowed to stand for 30minutes. Presence of honeycomb froth indicates presence of saponins.

2.4 Data Collection

Questionaries were distributed to the HIV positive and HIV negative individuals. Socio-demographic data and clinical records were collected for the mycological study.

2.5 Sample Collection

2.5.1 Sputum for Acid Fast Bacilli (AFB)

Three (3) early morning sputum samples were collected in 3 consecutive days from each of the HIV patients. Patients were asked to collect the sample in a sterile wide mouth container. According to the methods described by [32].

2.5.2 Sputum for culture

A wide mouthed sterile labeled container was given to the HIV positive and HIV negative patients. Patients were asked to produce the sputum into the container.

Blood: a total of 4ml of blood was collected from each participant according to the methods described by Chessbrough, [32] with EDTA container for HIV screening and CD4 cell count. All aseptic conditions were observed accordingly.

2.6 Analysis of Samples

2.6.1 Sputum sample

2.6.1.1 Cultural technique

Each sputum sample were inoculated on two sets of Sabourand's dextrose agar (SDA)

impregnated with antibiotic, chloramphenicol and SDA without antibiotic). Media plates were incubated at 25°C with examinations for growth on every three days for two weeks. SDA plates with growth were identified macroscopically and microscopically. Criteria for positive growth were based on two plates from the same patients yielding the same growth.

2.6.2 Identification of fungal isolates

The different fungal isolates were identified macroscopically and microscopically by the methods described in Mackie and McCantey, [33].

2.6.3 Macroscopic identification

This was based on their colonial morphology of the isolates appearing on the laboratory media. Visual examination is based on the physical appearance such as colour, texture, colonial topography and diffusible pigments. Mucoid yeast-like growth was processed by Gram staining, capsular staining, urease test and inoculation on CHROMagar Candida.

2.6.4 Microscopic identification

The definitive identification was based on the morphology of the spores and hyphae on the preparation with lactophenol cotton blue (LCB) on wet mount technique. The morphological features were observed and identified [32].

2.6.5 Gram's staining

Gram's staining was done to all the isolates with mucoid yeast-like growth and observed for gram positive budding yeast cells [32].

Reagents: Crystal violet, acetone, Lugol's iodine and neutral red.

Methods:

- A loopful of the culture was smeared on a grease free slide.
- The dried smear was heat-fixed.
- It was covered with crystal violet stain for 30-60 seconds.
- The stain was washed off with water.
- The smear was covered with Lugol's iodine for 30-60 seconds
- It was washed with water and then decolorized with acetone and washed immediately with water.

- The smear was covered with neutral red for 2 minutes and then washed off with water.
- It was dried and examined microscopically using 100X objectives for budded yeast cells.

2.6.6 Capsular staining for confirmation of *Cryptococcus neoformans*

All the isolates with mucoid and yeast like growth were tested for capsulated budding yeast cells for identification of *Cryptococcus neoformans* using Indian ink preparation as described in [32].

2.6.7 Indian ink preparation for confirmation of *C. neoformans*

- A drop of India ink was placed on a cleaned grease free slide.
- A loopful of the culture was emulsified on the stain.
- It was covered with coverslip and then viewed with 100x immersion objective for capsulated organism.

2.6.8 Urease test

Christensen's urease test was used for confirmation of *Cryptococcus neoformans* as described in [34].

Urease test using Christensen's (modified) urea broth.

- The test organisms were inoculated in a bifo bottle containing 3mls of sterile Christensen's modified broth.
- It was incubated at 37°C for 12 hours.
- It was observed for presence or absence of pink colour.

Results:

Pink colour indicates positive urease test. No pink colour indicates Negative urease test.

2.6.9 Identification of *Candida* species with CHRO Magar candida

Different *Candida* species were identified on a selective and differential chromogenic media (CHROMagar Candida). The *Candida* CHROMagar was prepared according to manufacturer's instructions. The *Candida* species were identified based on their chromogenic reaction as described in the instruction by the manufacturer's instruction.

Candida albicans (green), *Candida tropicalis* (blue), *Candida glabrata* (pink), and *Candida krusei* (dry pink). See appendix Candida species were identified on a selective and differential medium known as CHROMagar Candida [34].

Procedure:

- A litre (1000 ml) of water was autoclaved.
- 33 g of the CHROMagar Candida powder was dissolved in it.
- It was heated on a bunsen flame until complete dissolution of the agar.
- It was poured into the petri-dishes and allowed to solidify.
- The Candida isolated were cultured on it and then incubated for upto 48hrs
- The Candida species were identified based on their chromogenic reaction.

2.6.10 Identification of the growth appearance with slide culture

The fungal isolates were cultured onto potato dextrose agar for slide culture. This helps in the microscopic examination of the features of fungi on which classification depends on the spores and the spore-bearing apparatus. Riddels initiates slide culture in order to accurately identify many fungi by the arrangement of their conidiophores and the way in which spores are produced (conidial ontogeny) [35].

2.6.11 Procedure for slide culture

- Complete identification of molds is often dependent upon observation of the undisturbed relationship of the sporulation structures.
- Using aseptic technique a prepared plate of Potato Dextrose Agar was cut into sections approximately 1 cm square and 2-3 mm deep.
- A bent glass rod was placed in a petri dish and balanced a sterile glass slide on top.
- One block of agar was placed on the center of the slide.
- Each of the four sides of the block were inoculated with the fungi for study and covered with a sterile coverslip.
- 8 ml of sterile water was added to the bottom of the dish to maintain a moist environment followed by incubation at room temperature 25°C until sporulation occurs.
- A drop of Lacto-phenol cotton-blue was placed on the end of a second glass slide.

- After the agar block was discarded, a drop of Lacto-phenol cotton-blue was placed in the center of the growth area and covered with a second coverslip.
- Excess fluid was then blotted and the edges sealed with fingernail polish or appropriate varnish followed by microscopically examination with the atlas used to confirm each arrangement.

2.7 HIV Screening

The patients were rescreened for HIV using immunochromatographic technique.

HIV screening was done using Determine HIV-1/2 test kit, Uni-Gold kit (Trinity Biotech) and STAT-PAK (Chembio Diagnostic System, Inc.) was used for the HIV screening [35].

2.7.1 Principle

Is based on immune-chromatographic sandwich principle. Algorithm is defined as combination and sequence of specific tests in a given testing strategy. A HIV positive status should be based upon the outcome of two or more tests. Serial testing means sample tested by first test kit, the results of the first determine whether additional testing is required. If the first kit showed non reactive; the sample will be reported as HIV negative. If the first kit showed a reactive result, sample will be further tested by the second test kit; if the second test kit showed a reactive result, the tested sample will be reported as HIV positive. When the two test results disagree (the first is reactive and the second is non-reactive), a third test will be performed; the result of third test is the final test result.

2.8 CD₄ T- Cell Count

2.8.1 Principle

The calibration is based on the use of count check beads to verify counting cycle, signal position on the scale and peak or signal characteristics. The stained CD4+ Tcell is energized by the application of the laser beam, which invariably interprets, and present CD4+ Tcells on liquid crystal display (LCD) screen as peaks [35].

2.8.2 Procedure

Flow cytometry method was used for CD₄ count. 20µl of whole blood was taken from the

potassium EDTA tube and transferred into the Partec test tube, monoclonal antibody was added with the buffer given in the kit. The components were mixed thoroughly and the tubes incubated in the dark for 15 minutes. The samples were run using cyflow count machine (Partec Germany). Results were expressed as number of cells/ μ L. Reference range for CD₄ was taken as 600-1200 cells/ μ L.

2.8.3 Preparation of stock solutions

For the primary screening of the extracts/fractions of *M. oleifera* seed, bark, pod, and leaf, for antifungal activity, stock solution of the plant extracts were prepared by dissolving 1000 mg of the extract in 2 mL of DMSO to obtain a final concentration of 500 mg/mL. For determining the MICs, stock solution of the plant extract was prepared by dissolving 4000 mg of the extract in 2 mL of DMSO to attain a final concentration of 2000 mg/mL. These were transferred to a screw capped bottle and stored at 4°C.

2.8.4 Standardization of test organisms

All test isolates were inoculated onto SDA plates and incubated at 25°C for 2-5 days to obtain young, actively growing cultures which was then aseptically inoculated into sterile tubes containing Sabouraud dextrose broth. The tubes were then incubated at 25°C for 2 days. After incubation, the concentration of organisms in the tubes was standardized by adjusting to McFarland 0.5 standards (i.e. a concentration of about 10⁸ CFU/mL).

2.8.5 Primary screening of plant extracts/fractions for antifungal activity

The antifungal activities of the plant extracts/fractions were determined by the agar well diffusion method as described by Perez *et al.*, [36]. Dilutions of 500, 250, 125, 62.5, 31.25, and 15.125 mg/mL were prepared from the 500 mg/mL stock solution of the extracts/fractions. Twenty (20) mL of molten SDA was poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations of cultures of test isolates grown in Sabouraud dextrose broth were swabbed aseptically on the agar plates and holes (6 mm) were made. Standardized concentrations (10⁴ 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 Dextrose Broth, and 1 mL of the standardized test culture was added to 9 mL of the extract-broth mixture to give a

microbial concentration 10³ CFU/mL and a concentration equal to the MBC of the extract.

Sterile molten SDA was poured into sterile Petri plates and allowed to set. A volume of 0.1 mL 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 1 hrs, 2 hrs, 4 hrs, 8 hrs, and 24 hrs intervals. The procedure was carried out in triplicate to ensure accuracy. Plates were incubated at 25-27°C for 2-3 days and observable colonies were counted. For controls, 1 strain each of *Candida albicans*, *C. tropicalis*, *C. krusei*, *Aspergillus niger*, *A. fumigatus*, *A. flavus*, *Penicillium marneffeii* and *Phialemonium curvatum* 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 in the agar plates using a sterile metal cork-borer. Twenty (20) μ L of the various dilutions of each extract/fraction and controls were put in each hole under aseptic condition, kept at room temperature for about 30 minutes to allow the agents to diffuse into the agar medium and were then incubated accordingly. Fluconazole (50 μ g/mL) was used as the positive control, while DMSO or sterile distilled water (depending on the nature of the extracts/fractions and their solubility in either water or DMSO) was used as the negative control. The plates were then incubated at room temperature for 2-3 days hours and the inhibition zones diameter (IZD) were measured and recorded in millimeters. The size of the cork borer (6 mm) was deducted from the values recorded for the IZD to get the actual diameter. This procedure was conducted in triplicate and the mean IZD calculated and recorded.

2.9 Determination of the Fungicidal activities of Extracts/Fractions of the *M. oleifera* on Test Isolates by Time-Kill Assay (Kill-Time Kinetics)

Standardized concentrations (10⁴ 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 Dextrose Broth, and 1 mL 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.

Sterile molten SDA was poured into sterile Petri plates and allowed to set. A volume of 0.1 mL 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 1hrs, 2hrs, 4hrs, 8hrs, and 24hrs intervals. The procedure was carried out in triplicate to ensure accuracy. Plates were incubated at 25-27°C for 2-3 days and observable colonies were counted. For controls, 1 strain each of *C. albicans*, *C. tropicalis*, *C. krusei*, *A. niger*, *A. fumigatus*, *A. flavus*, *P. marneffeii*, and *P. curvatum* 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.

2.10 Determination of Minimum Inhibitory Concentration (MIC) of the Extracts/Fraction of the *M. oleifera* Leaf on Test Isolates

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that inhibits the fungal growth as described by Russell and Furr [37]. The MIC of the plant extract on the test isolates was determined by the agar dilution method. The stock solution (2000 mg/mL) was further diluted in a 2-fold serial dilution to obtain the following concentrations: 1000, 500, 250, 125, and 62.5 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 200, 100, 50, 25, 12.5, and 6.25 mg/mL.

The test isolates which were grown overnight in broth were adjusted to 0.5 McFarland 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°C) for 2-3 days, after which all plates were observed for growth. Control plates, which contained no plant extracts, were also made with the test. The minimum dilution (concentration) of the extracts completely inhibiting the growth of each organism was taken as the MIC.

2.11 Determination of Minimum Fungicidal Concentrations (MFCs) of Extracts/Fractions of the *M. oleifera* on Test Isolates

The minimum fungicidal concentration (MFC) is defined as the minimum concentration of the antifungal agent that kills off all the fungal cells. The MFC of the plant extracts/fractions was derived by subculturing portions of the agar from plates that showed no growth in the tests for determination of MICs. These agar portions were transferred into plates containing freshly prepared SDA. These plates were incubated at 25-27°C for 2-3 days and were observed daily for growth. The absence of growth at the end of incubation period signifies total cell death. The minimum concentration of the plant extracts/fractions that produces total cell death is taken as the MFC.

2.11.1 Quality control

The following controls were used to ensure accuracy and precision throughout the culturing process.

Control 1 contained the broth, extract dilutions without the microorganism (Negative control).

Control 2 contained the broth and the fungal organism without the extract (Positive control) which shows that the organism is capable of growing in the medium used for the study and was only one tube in each batch.

Control 3 contained the dilutions of the fluconazole and the microorganism without the extract and served also as a positive control showing that certain chemical agents are capable of inhibiting the growth of the organism.

Different sterile pipette tips were used for each extract as well as fluconazole in each batch during the experiment. This is to avoid mixing one extract with another and ensure accuracy.

2.12 Statistical Analysis

Statistical analysis was done using chi-square and correlation set as 95% confidence limit and 0.05 level of significance respectively. Statistical package for socio science version 17 was used for the evaluation.

3. RESULTS

Out of the 132 HIV-sero positive subjects sampled, 60 patients were on antiretroviral drugs while 72 were not on ART. 16 out of the 60

subjects on ART were TB positive while 30 out of the 72 subjects not on ART were TB positive. More so, 36(60.0%) individuals on ART were positive for fungi infection while 58(80.0%) of subjects not on ART were positive for fungi infection (P<0.05) respectively.

respectively. However, among the HIV sero-negative individuals, *Candida albicans* was of the highest prevalence with 7(53.9%), followed by *Candida tropicalis* with 3(23.1%) and *Aspergillus fumigatus* and *A. flavus* with the least prevalence of 1(7.7%) respectively.

Table 2 shows the frequency of the different single fungal isolates from the sputum samples of HIV-sero positive and negative individuals. A total of 8 different fungi were isolated comprising of *Candida albicans* with the highest frequency of fungi isolates amongst test subjects 25(26.6%), followed by *Penicillium marneffeii* with 18(21.6%), followed by *Candida tropicalis* with 13(13.8%), followed by *Aspergillus fumigatus* with 5(5.3%), followed by *Aspergillus niger* with 3(3.2%), followed by *Aspergillus flavus* and *Phialemonium curvatum* with same prevalence of 2(2.1%)

Table 3 shows the mixed fungi infection isolated from both HIV-sero positive and negative subjects with *candida albicans* and *P. marneffeii* occurring highest with 11(11.7%), followed *P. marneffeii* and *A. niger* with a prevalence of 3(3.2%) and *C. albicans A. flavus* and *P. marneffeii* occurring least with a prevalence of 1(1.1%) in test individuals respectively. In HIV-sero negative subjects, *C. albicans* and *A. fumigatus* was only the mixed fungi isolate recorded with a prevalence of 1 (7.7%).

Table 1. TB and fungi infection status of HIV-sero positive subjects on ART and those not on ART

Status of HIV Positive Subjects	ART status of test subjects	TB status of test subjects	Fungi status of test subjects	X ² -value	p-value
On ART	60	16	36(60.0)		
Not on ART	72	30	58(80.6)		
Total	132	46	94(71.2)	25.30	0.000*

Keys %:=Percentage, No: =Number, X²: =Chi-square, *: =significant, TB: =Tuberculosis, Pts: =Patients

Table 2. Prevalence of single fungal isolates from sputum samples of HIV-sero positive and sero negative individuals

Fungi Isolates (Single)	No (%) of isolates from HIV positive subjects	No (%) of isolates from HIV negative subjects
<i>Candida albicans</i>	25(26.6)	7(53.9)
<i>Penicillium marneffeii</i>	16(17.0)	0(0.0)
<i>Candida tropicalis</i>	13(13.8)	3(23.1)
<i>Candida krusei</i>	8(8.5)	0(0.0)
<i>Aspergillus fumigates</i>	5(5.3)	1(7.7)
<i>Aspergillus niger</i>	3(3.2)	0(0.0)
<i>Aspergillus flavus</i>	2(2.1)	1(7.7)
<i>Phialemonium curvatum</i>	2(2.1)	0(0.0)
Total	74(78.6)	12(62.4)

X² = 0.06, P=1.00 X²=0.23 P=0.672

Table 3. Prevalence of mixed fungal isolates from sputum samples of HIV-sero positive and sero negative individuals

Fungi Isolates (Mixed)	No / % of isolates from HIV positive subjects	No / % of isolates from HIV negative subjects
<i>C. albicans</i> and <i>P. marneffeii</i>	11(11.7)	0(0.0)
<i>P. marneffeii</i> and <i>A. niger</i>	3(3.2)	0(0.0)
<i>C. tropicalis</i> and <i>A. fumigates</i>	2(2.1)	0(0.0)
<i>C. albicans</i> and <i>A. niger</i>	2(2.1)	0(0.0)
<i>C. albicans</i> and <i>A. fumigates</i>	1(1.1)	1(7.7)
<i>C. albicans A. flavus</i> and <i>P. marneffeii</i>	1(1.1)	0(0.0)
Total	20(21.3)	1(7.7)

X²=3.85, (P=0.277) Keys: %= Percentage, No=Number, X²=Chi square

Table 4. Prevalence of fungi infection amongst HIV-sero positive and negative individuals in relation to their CD4 count

CD4 Count Cells/ μ l	HIV-Seo Positive Subjects		HIV-Seo Negative Subjects	
	No tested	No (%) of fungi infection	No tested	No (%) of fungi infection
<350	112	88(78.6)	0	0(0.0)
350-500	19	6(31.6)	0	0(0.0)
501-800	0	0(0.0)	43	12(27.9)
801-1000	0	0(0.0)	20	1(5.0)
>1000	0	0(0.0)	5	0(0.0)
Total	132	94(71.2)	68	13(19.1)

$\chi^2=0.00$, ($P= 1.000$) Key %= Percentage, μ l= Micro litre, χ^2 = Chi-square, $\chi^2 = 0.667$ ($P= 0.955$)

Table 5. Correlation coefficient of TB, CD4 count and fungi infection among HIV-sero positive and negative subjects

Parameters	Correlation coefficient (r)	P-value
HIV Positive Subjects		
TB Vs Fungal infection	0.813	0.002
CD4 count Vs Fungal infection	-0.210	0.720
Control Subjects		
TB Vs Fungal infection	0.317	0.022
CD4 count Vs Fungal infection	-0.310	0.620

Table 6. Extraction yield of the different parts of *Moringa oleifera*

Extract	Yield	% Yield
<i>Moringa oleifera</i> seed	16.8	2.4
<i>Moringa oleifera</i> bark	3.4	0.68
<i>Moringa oleifera</i> pods	2.8	1.3
<i>Moringa oleifera</i> leaf	39.6	15.8

Key: %=Percentage

Table 7. Fractionation yield of *Moringa oleifera* leaf

Fraction	Yield	% Yield
N-hexane	7.4	2.96
Ethyl acetate	4.5	1.80
Butanol	15.8	6.32
Water	11.9	4.76

Key: %=Percentage

Table 4 shows the prevalence of fungi infection amongst HIV-sero positive and negative subjects in relation to their CD4 counts. Patients with CD4 count <350cells/ μ l has the highest frequency of fungi infection with 34(97.1%), followed by those with CD4 count value between 351-500cells/ μ l with a prevalence rate of 6(31.6%) whereas those with CD4 count >500cells/ μ l had no fungi infection isolated in HIV-sero positive subjects. However in control subjects (HIV-sero negative subjects), the highest prevalence of fungi infection was recorded in subjects with CD4 count between 501-800cells/ μ l with 12(27.9%), followed by those with CD4 count 801-1000cells/ μ l with 1(5.0%) respectively.

Table 5 shows the correlation coefficient of the different parameters studied in relation to the fungal infection. There was a positive significant correlation between TB and fungi infection in HIV-sero positive subjects ($P<0.05$) while CD4 count and fungal infection showed a non-significant negative correlation ($P>0.05$).

Table 6 shows the various yields and their percentage yield after crude methanol extraction from the dried 700g, 500g, 220g and 250g seed, bark, pods, and leaf of *Moringa oleifera*. Their yields are as follows seed 16.8(2.4%), bark 3.4(0.68%), pods 2.8(1.3%) and leaf 39.6(15.8%).

Table 7 shows the different yields from the fractionated *Moringa oleifera* leaf using 4 different solvents based on their solubility. Their yields are as follows n-hexane 7.4(2.96%), ethyl acetate 4.5(1.80%), butanol 15.8(6.32%) and water fraction 11.9(4.76%).

Table 8 shows the phytochemical constituent of the different parts of *Moringaoleifera* including seed bark and leaf using methanol as solvent. Alkaloids and Tannis were found in the seed, bark, pods, and leaf respectively while Steroids were absent in the 3 plant parts tested. Volatile oil was only present in the *Moringa* seed tested. Saponins were only present in the *Moringa oleifera* leaf while Terpenoids was only found in the leaf. Flavonoid was found in the *Moringa oleifera* barks, pods, and leaf.

Table 9 shows the phytochemical constituent of the different fraction of *Moringa oleifera* leaf showed alkaloids to be present in the n-hexane fraction of the leaf. Flavonoids and Tannis were present in the ethyl acetate, butanol, and water fractions and absent in the n- hexane fraction. Treprenoids volatile oil and steroids were all absent in the solvents used while Saponins was

only present in water fraction and absent in the other fraction.

Table 10 shows the antifungal activity of the crude methanol extract of the seed shows that there was no activity recorded at the different concentrations.

Table 11 shows the antifungal activity of the crude methanol extract of the bark shows that there was no activity recorded at the different concentrations.

Table 12 shows the antifungal activity of the crude methanol extract of the pods shows that there was no activity recorded at the different concentrations.

Table 13 shows the antifungal activity of the crude methanol extract of the leaf shows that there was no activity recorded at the different concentrations as seen in Table 4.

Table 14 shows the antifungal activity of the n-hexane fraction of the leaf extract shows that there was no activity recorded at the different concentrations.

Table 8. Phytochemical constituents of different parts of crude Methanol extracts of *Moringa oleifera*

Phytochemical constituents	Inference			
	Seed	Bark	Pods	Leaf
Alkaloids	+++	+++	++	++
Saponins	-	-	-	+++
Steroids	-	-	-	-
Terpenoids	-	-	-	-
Flavonoids	-	+++	++	+++
Tannis	++++	++++	++	++++
Volatile oil	+++	-	+	-

Keys: - = Absent, + = Fairly Present, ++ = Moderately Present, +++ = Highly present

Table 9. Phytochemical constituents of the different fractions of *Morrnga oleifera* leaf extract using four different solvent

Phytochemical constituents	Fractions			
	N-hexane	Ethyl acetate	Butanol	Water
Alkaloids	+	-	-	-
Saponins	-	-	-	+++
Steroids	-	-	-	-
Terpenoids	-	-	-	-
Flavonoids	-	+++	+++	+++
Tannis	-	+++	++	+
Volatile oil	-	-	-	-

Keys: - = Absent, + = Present, +++ = Highly present

Table 10. Mean inhibition zone diameter (IZD) in millimeters (mm) produced by the crude extract of *Moringa oleifera* seed on test isolates

Test Isolates	Concentration (mg/mL) of crude methanol extract							Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [DMSO]	
<i>C. albicans</i>	0	0	0	0	0	0	6	0	
<i>C. krusei</i>	0	0	0	0	0	0	5	0	
<i>C. tropicalis</i>	0	0	0	0	0	0	5	0	
<i>A. niger</i>	0	0	0	0	0	0	14	0	
<i>A. fumigatus</i>	0	0	0	0	0	0	14	0	
<i>A. flavus</i>	0	0	0	0	0	0	6	0	
<i>Penicillium marneffeii</i>	0	0	0	0	0	0	7	0	
<i>Phialemonium curvatum</i>	0	0	0	0	0	0	6	0	

Table 11. Mean inhibition zone diameter (IZD) in mm produced by the crude extract of *Moringa oleifera* bark on test isolates

Test Isolates	Concentration (mg/mL) of crude methanol extract							Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [DMSO]	
<i>C. albicans</i>	0	0	0	0	0	0	6	0	
<i>C. krusei</i>	0	0	0	0	0	0	5	0	
<i>C. tropicalis</i>	0	0	0	0	0	0	5	0	
<i>A. niger</i>	0	0	0	0	0	0	14	0	
<i>A. fumigatus</i>	0	0	0	0	0	0	14	0	
<i>A. flavus</i>	0	0	0	0	0	0	6	0	
<i>Penicillium marneffeii</i>	0	0	0	0	0	0	7	0	
<i>Phialemonium curvatum</i>	0	0	0	0	0	0	5	0	

Table 12. Mean inhibition zone diameter (IZD) in mm produced by the crude methanol extract of *Moringa oleifera* podson test isolates

Test Isolates	Concentration (mg/mL) of crude methanol extract						Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [DMSO]
	<i>C. albicans</i>	0	0	0	0	0		
<i>C. krusei</i>	0	0	0	0	0	0	5	0
<i>C. tropicalis</i>	0	0	0	0	0	0	5	0
<i>A. niger</i>	0	0	0	0	0	0	14	0
<i>A. fumigatus</i>	0	0	0	0	0	0	14	0
<i>A. flavus</i>	0	0	0	0	0	0	6	0
<i>Penicillium marneffeii</i>	0	0	0	0	0	0	7	0
<i>Philamonium curvatum</i>	0	0	0	0	0	0	6	0

Table 13. Mean inhibition zone diameter (IZD) in mm produced by the crude methanol extract of *Moringa oleifera* leaf on test isolates

Test Isolates	Concentration (mg/mL) of crude methanol extract						Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [DMSO]
	<i>C. albicans</i>	0	0	0	0	0		
<i>C. krusei</i>	0	0	0	0	0	0	5	0
<i>C. tropicalis</i>	0	0	0	0	0	0	5	0
<i>A. niger</i>	0	0	0	0	0	0	14	0
<i>A. fumigatus</i>	0	0	0	0	0	0	14	0
<i>A. flavus</i>	0	0	0	0	0	0	6	0
<i>Penicillium marneffeii</i>	0	0	0	0	0	0	7	0
<i>Philamonium curvatum</i>	0	0	0	0	0	0	6	0

Table 14. Mean inhibition zone diameter (IZD) in mm produced by the n-hexane fraction of *Moringa oleifera* leaf on test isolates

Test Isolates	Concentration (mg/mL) of n-hexane fraction of the leaf						Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [DMSO]
	<i>C. albicans</i>	0	0	0	0	0		
<i>C. krusei</i>	0	0	0	0	0	0	5	0
<i>C. tropicalis</i>	0	0	0	0	0	0	5	0
<i>A. niger</i>	0	0	0	0	0	0	14	0
<i>A. fumigatus</i>	0	0	0	0	0	0	14	0
<i>A. flavus</i>	0	0	0	0	0	0	6	0
<i>Penicillium marneffeii</i>	0	0	0	0	0	0	7	0
<i>Phialemonium curvatum</i>	0	0	0	0	0	0	6	0

Table 15. Mean inhibition zone diameters (IZD) in mm produced by the ethyl acetate fraction of *Moringaoleifera* leaf on test isolates

Test Isolates	Concentration (mg/mL) of the ethyl acetate fraction of the leaf						Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [DMSO]
	<i>C. albicans</i>	6	5	4	2	0		
<i>C. krusei</i>	6	5	3	0	0	0	5	0
<i>C. tropicalis</i>	8	5	3	2	0	0	5	0
<i>A. niger</i>	10	6	4	0	0	0	14	0
<i>A. fumigatus</i>	10	8	7	5	3	0	14	0
<i>A. flavus</i>	9	8	5	2	0	0	6	0
<i>Penicilliumsp</i>	6	4	2	0	0	0	7	0
<i>Phialemonium curvatum</i>	7	5	3	0	0	0	6	0

Table 16. Mean inhibition zone diameters (IZD) in mm produced by the butanol fraction of *Moringaoleifera* leaf on test isolates

Test Isolates	Concentration (mg/mL) of the butanol fraction of the leaf							Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [DMSO]	
<i>C. albicans</i>	0	0	0	0	0	0	6	0	
<i>C. krusei</i>	0	0	0	0	0	0	5	0	
<i>C. tropicalis</i>	0	0	0	0	0	0	5	0	
<i>A. niger</i>	0	0	0	0	0	0	14	0	
<i>A. fumigatus</i>	0	0	0	0	0	0	14	0	
<i>A. flavus</i>	0	0	0	0	0	0	6	0	
<i>Penicillium</i> sp	0	0	0	0	0	0	7	0	
<i>Phialemonium curvatum</i>	0	0	0	0	0	0	6	0	

Table 17. Mean inhibition zone diameters (IZDs) in mm produced by the water fraction of *Moringa oleifera* leaf on test isolates

Test Isolates	Concentration (mg/mL)						Controls	
	500	250	125	62.5	31.25	15.625	Positive [Fluconazole(50ug)]	Negative [Water]
<i>C. albicans</i>	0	0	0	0	0	0	6	0
<i>C. krusei</i>	0	0	0	0	0	0	5	0
<i>C. tropicalis</i>	0	0	0	0	0	0	5	0
<i>A. niger</i>	0	0	0	0	0	0	14	0
<i>A. fumigatus</i>	0	0	0	0	0	0	14	0
<i>A. flavus</i>	0	0	0	0	0	0	6	0
<i>Penicillium marneffe</i>	0	0	0	0	0	0	7	0
<i>Phialemonium curvatum</i>	0	0	0	0	0	0	6	0

Table 18. Minimum inhibitory concentration (MIC) of the extracts/fractions of *Moringa oleifera* leaf, seed, pod, and bark on test isolates

Test Isolates	MICs of the various Extracts/Fractions (mg/mL)							
	Meth. extract of Seed	Meth.extr act of Bark	Meth. extract of Pod	Meth. extract of (Leaf)	N-hexane fractions of leaf	Ethyl Acetate fraction of Leaf	Butanol fraction of Leaf	Water fraction of leaf
<i>C. albicans</i>	-	-	-	-	-	50	-	-
<i>C. krusei</i>	-	-	-	-	-	100	-	-
<i>C. tropicalis</i>	-	-	-	-	-	50	-	-
<i>A. niger</i>	-	-	-	-	-	50	-	-
<i>A. fumigates</i>	-	-	-	-	-	25	-	-
<i>A. flavus</i>	-	-	-	-	-	50	-	-
<i>Penicillium marneffeii</i>	-	-	-	-	-	100	-	-
<i>Phialemonium curvatum</i>	-	-	-	-	-	100	-	-

Table 19. Minimum fungicidal concentrations (MFC) of the extracts/fractions of *Moringa oleifera* leaf, seed, pod, and bark on test isolates

Test Isolates	MFCs of the various Extracts/Fractions (mg/mL)							
	Methanol extract of Seed	Methanol extract of bark	Methanol extract of Pod	Methanol extract of Leaf	N-Hexane fraction of Leaf	Ethyl Acetate fraction of Leaf	Butanol fraction of Leaf	Water fraction of Leaf
<i>C. albicans</i>	-	-	-	-	-	100	-	-
<i>C. krusei</i>	-	-	-	-	-	200	-	-
<i>C. tropicalis</i>	-	-	-	-	-	100	-	-
<i>A. niger</i>	-	-	-	-	-	100	-	-
<i>A. fumigates</i>	-	-	-	-	-	50	-	-
<i>A. flavus</i>	-	-	-	-	-	100	-	-
<i>P.marneffeii</i>	-	-	-	-	-	100	-	-
<i>Phialemonium curvatum</i>	-	-	-	-	-	200	-	-

Table 15: The antifungal activity of the ethyl acetate fraction of the *Moringa oleifera* leaf as seen in Table 15 shows that *Aspergillus fumigatus* had the highest activity (sensitivity) at a concentration of 500 mg/ml with an inhibition zone of 10 mm diameter. It also had activity at concentration of 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml with inhibition zones of 8 mm, 7 mm, 5 mm and 3 mm diameter respectively. *Aspergillus niger* had antifungal activity at concentration of 500 mg/ml, 250 mg/ml, and 125 mg/ml with inhibition zones of 10 mm, 6 mm, and 4 mm respectively.. *Phialemonium curvatum* had antifungal activity at concentration of 500 mg/ml, 250 mg/ml, and 125 mg/ml with inhibition zones of 7 mm, 5 mm, and 3mm diameter respectively. *Penicillium marneffeii* had antifungal activity at concentration of 500 mg/ml, 250 mg/ml, and 125mg/ml with inhibition zones of 6mm, 4mm, and 2mm respectively. *Candida albicans* had antifungal activity at concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml with inhibition zones of 6 mm, 5 mm, 4 mm, and 2 mm respectively. *Candida tropicalis* had antifungal activity at concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5mg/ml with inhibition zones of 8 mm, 5 mm, 3 mm, and 2 mm respectively. *Aspergillus flavus* had antifungal activity at concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml with inhibition zones of 9 mm, 8 mm, 5 mm and 2 mm respectively. *Candida krusei* had the least antifungal activity at concentration of 500 mg/ml with inhibition zone

of 6mm diameter. It also had activity at concentration of 250 mg/ml and 125 mg/ml with inhibition zones of 5mm and 3mm diameter respectively.

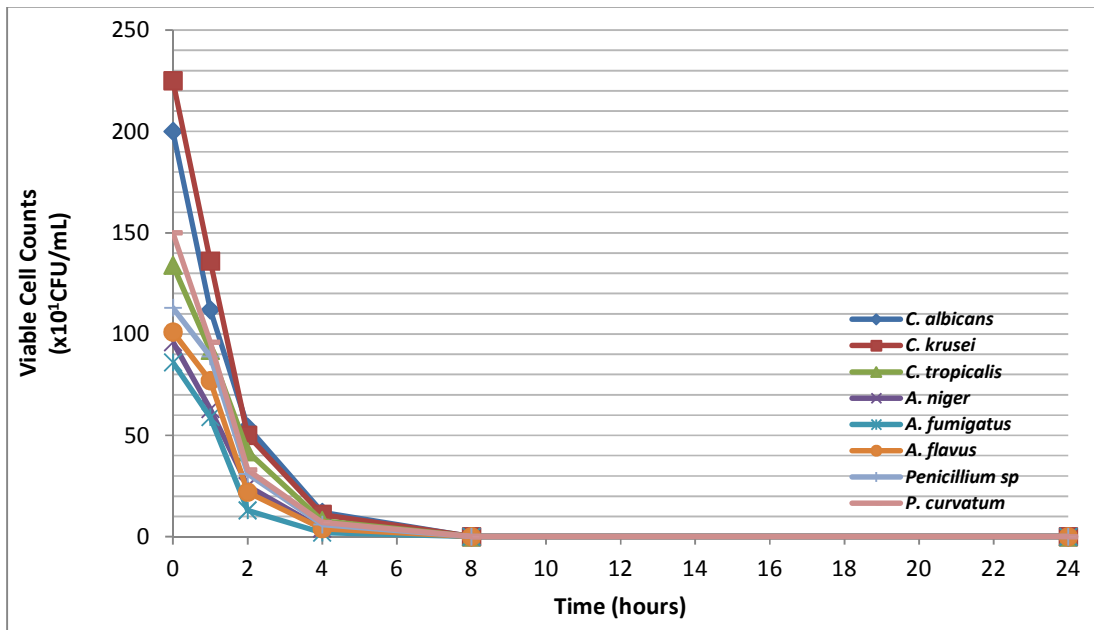
Table 16 shows the antifungal activity of the butanol fraction of the leaf extract shows that there was no activity recorded at the different concentrations.

The antifungal activity of the water fraction of the leaf extract shows that there was no activity recorded at the different concentrations as seen in Table 17.

Table 18 shows the Minimum inhibitory concentration (MIC) as the lowest concentration of the antifungal agent that inhibits the fungal growth. The MIC of of *A.fumigatus* at 25mg/ml, *C.albicans*, *A.niger*, *C.tropicalis*, and *A.flavus* at 50mg/ml while *P. curvatum*, *C.krusei*, and *P.marneffeii* at 100 mg/ml.

The minimum fungicidal concentration (MFC) defined as the minimum concentration of the antifungal agent that kills off all the fungal cells is shown in Table 19. *A. fumigatus* at 50mg/ml, *C.tropicalis*, *C.albicans*, *A.niger*, *A. flavus* and *P. marneffeii* at 100 mg/ml while *P.curvatum* at and *C.krusei* at 200 mg/ml.

Fig. 3. Shows the viable cell count shows and the rate of killing of the extract with time in a graphical representation



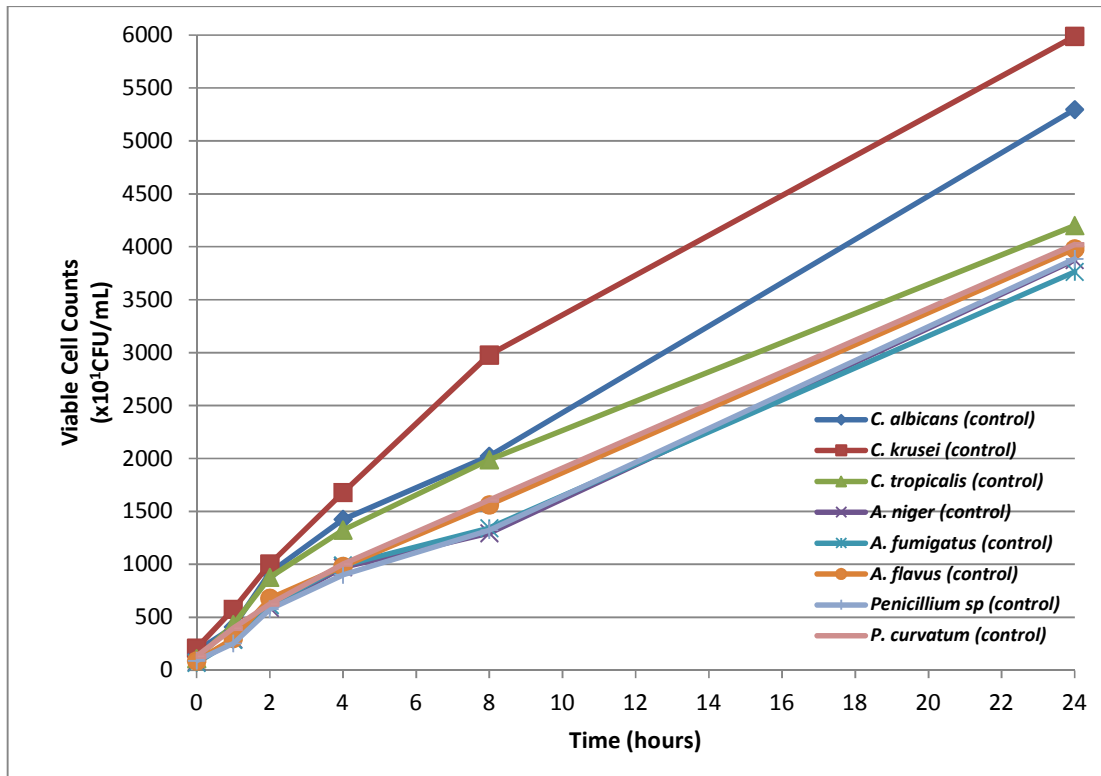


Fig. 3. Fungicidal activity of ethyl acetate fraction of *M. oleifera* on test fungi showing the viable cell counts with time

4. DISCUSSION

Tuberculosis is a disease associated with immune compromised state while HIV has also been known to be associated with a depressed immunity. Tuberculosis and HIV co-infection pose particular diagnostic and therapeutic challenges. Infections with HIV are the most powerful known risk factor predisposing for *Mycobacterium tuberculosis*. Results obtained showed that out of a total of 132 HIV positive individuals between age range 11-80 years screened for mycobacterium tuberculosis, 16(12.1%) had *Mycobacterium tuberculosis* consisting of 5 males and 11 females with ($P < 0.05$) showing that it is statistically significant in the study group. Out of the 68 HIV negative individuals with age range of 1-80 years screened for *Mycobacterium tuberculosis* 5(7.4%) had *Mycobacterium tuberculosis* consisting of 2 males and 3 females with ($P > 0.05$) showing that there is no statistical significant relationship between tuberculosis and HIV negative individuals in the study group. The correlation analysis between the HIV positive co-infected with tuberculosis and HIV negative

infected with tuberculosis and the prevalence of fungal infection is statistically significant at ($P < 0.05$) meaning there is a positive association. Previous studies have shown that tuberculosis is the most common opportunistic infection in HIV positive patients particularly those with CD4 counts less than 200 cells / μ l. Karim *et al.* [38] in his study on *Mycobacterium tuberculosis* spoligotypes from tuberculosis patients in South Western Uganda reported a high prevalence of TB-HIV co-infection reaching up to 65% of the study population which is not in accordance with the present study.

There is a high prevalence of fungal infection among HIV positive patients (71.2%) unlike the HIV negative subjects (19.1%) in the study. Esebelahie *et al.* [39] reported a prevalence rate of fungal infection as (52.5%) in HIV patients and (30.0%) in HIV negative individuals which is contrary to the present study. Out of the 132 sputum samples of HIV positive patients cultured in the study, 74(56.1%) samples yielded single fungal isolates, mixed fungal isolates were observed in 20(15.2%) and the rest of the 38(28.8%) yielded no fungal growth. Out of the

74 single isolates, 8 different fungal species were obtained which includes *Candida albicans* 25(26.6%), *Penicillium marneffeii* 16(17.0%), *Candida tropicalis* 13(13.8%), *Candida krusei* 8(8.5%), *Aspergillus fumigatus* 5(5.3%), *Aspergillus niger* 3(3.2%), *Aspergillus flavus* and *Phialemonium curvatum* 2(2.1%) each. Mixed fungal isolates obtained includes *Candida albicans* and *Penicillium marneffeii* 11(11.7%), *Candida albicans* and *Aspergillus fumigatus* 1(1.1%), *Aspergillus fumigatus* and *Candida tropicalis* 1(1.1%), *Candida albicans* and *Candida tropicalis* 1(1.1%), *Penicillium marneffeii*, *Aspergillus flavus* and *Candida albicans* 1(1.1%). This study is contrary to the work of Bharathi and Usha- Rani, [40] who sampled 100 sputum samples, 54 samples yielded single fungal isolates, mixed fungal isolates were observed in 20 samples and the rest 26 samples were no fungal growth. He also had *Candida albicans* in 42, *Cryptococcus neoformans* 4, *Penicillium sp.*

Aspergillus fumigatus and *Aspergillus niger* 2 each, with *Scedosporium apiospermum*, *Cunninghamella bertholletiae*, *sporothrix schenckii* etc. This is in support of the present study which had *Candida albicans* as the predominant specie. Out of the 68 sputum samples of HIV negative individuals cultured in the study, 12(17.7%) samples yielded single fungal isolates, mixed fungal isolate 1(1.5%) was observed and no fungal growth was observed in 55(80.1%) sputum samples. Of the 12 single isolates, 4 different fungal species were obtained which included *Candida albicans* 7(53.9%), *Candida tropicalis* 3(23.1%), *Aspergillus fumigatus* 1(7.7%), *Aspergillus flavus* 1(7.7%) while the mixed fungal isolates include *Candida albicans* and *Aspergillus fumigatus* 1(7.7%). This is contrary to the findings of Esebelahie et al. [41] whom isolated only 4 *Candida albicans* from 100 urine samples of HIV negative individuals. Also this study varies from the findings of Esebelahie et al., [40] who reported 3 *Candida* species as *Candida albicans*, *Candida krusei* and *Candida glabrata* from non HIV individuals. The number of opportunistic fungal infections has increased in general among HIV positive patients (71.2%) than HIV negative individuals (19.1%) from this study. Candidiasis from this study was found to be the most common fungal infection in HIV/AIDS patients 25(26.6%) and 7(53.9%) in HIV negative individual and is of prognostic value only as its presence indicates progression of immunodeficiency [42]. This is contrary to the findings of this study is contrary to the findings of Bharanti et al. [39], in their study on the

incidence of bacterial and fungal co-infection in some HIV infected Indian population reported a total of 6 different fungal isolates from sputum sample of which *Candida albicans* predominated. They also reported non- *Candida* species of which *Candida tropicalis* and *Candida krusei* was among. Esebelahie et al., [40] in her study isolated 5 different *Candida* species; *C.albicans* was the most prevalent followed by *C. krusei* and *C. parapsilosis* while *C.tropicalis* and *C.glabrata* were the least prevalent recovered only from stool specimen. They also reported that *Candida albicans* was the most predominant specie recovered irrespective of their HIV status and the type of specimen processed. From their study also *C. albicans* were the most prevalent specie isolated irrespective of their HIV status. This varies from the present study in that 3 species of *Candida* were isolated from sputum specimen. Esebelahie et al., [41] reported the prevalent of *C. albicans* 88.5%, *C. krusei* 8.57% and *C. parapsilosis* 2.86%. This varies with the species isolated in this present study as *C. albicans* predominates at 26.6%, *C.krusei* 8.5% and *C. tropicalis* 13.8%.

Penicillium marneffeii was the second most common fungi isolated in the present study. It had a total of 18 samples (19.1%) single isolates and 1 mixed isolates with *Candida albicans* and *Aspergillus flavus* (1.1%). Penicillosis after tuberculosis and cryptococcosis is the third common opportunistic infection in patients with AIDS (6). It is always associated with low CD4 count typically less than 100cells/ μ l [42]. It is very pathogenic and can mimic tuberculosis with 50% cases resulting to cough, dyspnea and hemoptysis. Bharanthi and Usha- Rani, (9) in their study of pathogenic fungal isolates in sputum of HIV positive patients in India had 2 isolates out of the 100 sputum samples. Ranjana et al. [43] reported 50 cases of disseminated *Penicillium marneffeii* infection in HIV infected patients in Manipur State, India. This is contrary to the present study. Bharathi et al. [40] from New Delhi isolated *Penicillium marneffeii* from HIV positive patients whose CD4 count were less than 100cell/ μ l. The finding from this study was in line with other study studies where *P.marneffeii* occurs at CD4 count less than 100cell/ μ l. *Penicillium marneffeii* has been noted as a very important emerging systemic fungal infection in immune suppressed individuals especially in HIV patients [40].

The next common isolate was *Aspergillus* species. Three different *Aspergillus* species were

isolated which include *Aspergillus fumigatus* 5(5.3%), *Aspergillus niger* 3(3.2%), *Aspergillus flavus* 2(2.1%) from HIV positive patient while 2 species were found from HIV negative individuals namely *Aspergillus fumigatus* 1(7.7%) and *Aspergillus flavus* 1(7.7%). Aspergillosis exposure is universal though invasive aspergillosis is uncommon except in compromised cases like HIV infection [6]. Bharathi et al. [40] in their study on the incidence of bacterial and fungal co-infection in some HIV infected Indian population reported 7 cases of *Aspergillus niger* in 2011. Bharathi and Usha-Rani, [9] reported 13 samples (13.5%) of *Aspergillus* species with *Aspergillus fumigatus* and *Aspergillus niger* each 6 samples and *Aspergillus flavus* from 1 sample. These are synonymous to the findings of the present study.

The study also revealed that there is a statistical significant difference between the prevalence of opportunistic fungal infection among those on antiretroviral therapy (ART) ($P < 0.05$). This shows that those not on ART are more infected with opportunistic fungal infection than those that are on ART. This could be attributed to the fact that ART reduces the chances of opportunistic infection by reducing the viral load and boosting the immune system of the HIV positive subjects. Also this is synonymous with the findings of Esebelahie et al. [41] that Candiduria was associated with HAART naïve HIV patients with CD4 T cell counts < 200 cells/ μ l. He also reported that a fungal agent such as *Candida* takes advantage of immune suppression seen in HIV patients as a result of CD4 T cell depletion. It also reported that HAART causes a decline in the incidence of some opportunistic infection in AIDS due to the restoration of immunity. This is in line with the present study which observed that fungal infection was higher among patients not on HAART than on HAART.

The correlation analysis between CD4 counts and prevalence of fungal infection was not statistically significant ($P > 0.05$) meaning there is negative association between CD4 count and the rate of fungal infection indicating that as CD4 count increases the rate of fungal infection decreases. No wonder that there was no *Penicillium marneffe* isolated among the HIV negative individuals in the study. These findings also concur with the study of Bharathi et al. [40] in India who reported that HIV positive with low CD4 counts had a higher prevalence of fungal infection. WHO (2011) in the manual for diagnosis of fungal opportunistic infection in

HIV/AIDS patients recorded that people with low CD4 counts usually develop more fungal opportunistic infection.

From the 700g, 500g, 220g and 250g of the dried *Moringa oleifera* seed, bark, pod and leaf extracted with methanol, their percentage yield are as follows 2.4%, 0.64%, 1.3% and 15.8% respectively while the fractionated n-hexane, ethyl acetate, butanol and water fractions of the leaf are as follows 2.96, 1.80, 6.32, and 4.76% respectively. This study is contrary to the work of Olufunmilayo et al. [44] who reported 500g of aqueous fraction of seed (6.3%), leaf (17.9%), and flower (42.4%) and the extractive yield in methanol, ethyl acetate, and butanol of leaf as 20.1%, 1.3%, and 0.6% and seed 9.7%, 2.2%, and 3.2% respectively.

The phytochemical screening of the methanol extracts of the seed, bark and leaf shows that alkaloids and tannins were present in all the plant parts. Steroids and terpenoids were absent in the plant parts. Flavonoids were only present in the bark and leaf extracts. Saponins were only present in the leaf. This correlates with the work of Oluduro, [45] who reported the presence of alkaloids, tannins, flavonoids, phenol and saponins and absence of steroids terpenoids and cardiac glycosides in *Moringa oleifera* leaf. Another work by Bukar et al. [46] reported the presence of flavonoids and saponins in the ethanol extracts of *Moringa oleifera* leaf and absence of alkaloids and tannins while alkaloids, tannins and saponins were present in the chloroform extract of the *Moringa oleifera* leaf and in the ethanol extract of the seeds contains only alkaloids, flavonoids, and tannins which are contrary to the findings of the present study.

Also anti-fungal activity was only recorded in the ethyl acetate fraction of *Moringa oleifera* from this study. *Aspergillus fumigatus* had the highest frequency of activity 3-10mm in diameter, *A. niger* at 4-10mm, *A. flavus* at 2-9mm, *C. tropicalis* at 2-8mm, *P. curvatum* at 3-7mm, *C. krusei* at 3-6mm, *C. albicans* at 2-6mm, and *P. marneffe* at 2-6mm. The standard fluconazole had activity at 5-14mm in diameter. This study varies with the work of Farjana et al. [46] who reported an antifungal activity of Compound-1 $100\mu\text{g disc}^{-1}$ against *C. albicans*, *A. niger*, *A. flavus*, *A. fumigatus*, *Trichoderma* spp., *Fusarium* spp. At 12, 10, 13, 11, 09, and 10 mm respectively while the crude extract $100\mu\text{g disc}^{-1}$ at 00, 00, 14, 00, 15, and 00 mm and the control nystatin $50\mu\text{g disc}^{-1}$ at 20, 22, 19, 22, 25 and 25

mm respectively. Also Bukar *et al.* [46] reported that the antifungal activities of chloform extracts *M. oleifera* seed inhibited the growth of *Mucor spp* and *Rhizopus spp.* By 100% at 1000µg/ml while the ethanol extract of *M.oleifera* seed at 75% for *Mucor spp.* and 50% for *Rhizopus spp.* at 1000µg/ml concentration each. Chloform extract of *Moringa oleifera* leaf inhibited the growth of *Mucor spp.* and *Rhizopus spp.* by 25% at 1000µg/ml while ethanol extract of *M. oleifera* inhibited growth of *Mucor spp.* by 50% and *Rhizopus spp.* by 100% each at 1000µg/ml concentration each while the standard control ketoconazole inhibited the growth of both fungi at 500µg/ml which is contrary to the work of the present study.

The Minimum Inhibitory Concentration (MIC) of the ethyl acetate fraction of *M.oleifera* leaf ranges from 25-100mg/ml while minimum fungicidal concentration ranges from 10-20mg/ml. This work varies from Abalaka *et al.* [47] reported an MIC from range of 10-20mg/ml and MBC from range of 20-40mg/ml. Also, Farjana *et al.* [48] recorded an MIC of 32-128µml⁻¹ which varied with the present study.

The ethyl acetate fraction of *Moringa oleifera* leaf demonstrated appreciable antifungal activity on the pathogenic fungal infection, indicating the antifungal potential of the plant and its effectiveness in the treatment of pathogenic fungal infections especially those caused by the test organism. The fraction contains phytochemicals that contributed to their activity against the test organism. The fraction also showed appreciable MIC and MFC that make them potential drugs for fungal infection caused by the test organisms. Fluconazole showed a more appreciable result in its antifungal activity.

5. CONCLUSION

The comparable antifungi effect of ethylacetate fraction of *M. oleifera* leaf with that of fluconazole, suggests that ethylacetate fraction of *M. oleifera* leaf could aid the treatment of pathogenic fungi due to its potential applications in Pharmaceutical industry, thereby ameliorating the morbidity and mortality rate of HIV positive co-infected TB subjects. The potential of *Moringa oleifera* as an antifungal agent requires that more studies be undertaken to know the best solvent for extraction and the best extraction method that can extract all the phytochemicals present in the plant and then increase the inhibitory activity of the plant extract against pathogens which can

complement or replace the conventional antimycotic.

CONSENT AND ETHICAL APPROVAL

Ethical clearance was sought for and obtained from the Ethics Committte of Nnamdi Azikiwe University Teaching Hospital, (NAUTH/CS/66/VOL.5/100) in accordance with the code of ethics for biological research involving human subjects in order for this research to be carried out. Individual consent was also obtained from the patients after proper explanation to them concerning the work using a well structured questionnaire.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDICES

Appendix 1. Identification of candida using CHROMagar



Plates 1 to 3. from left to right: *Candida albicans*; *Candida tropicalis* and *Candida krusei*

Appendix 2. identification of fungi isolates using slide culture

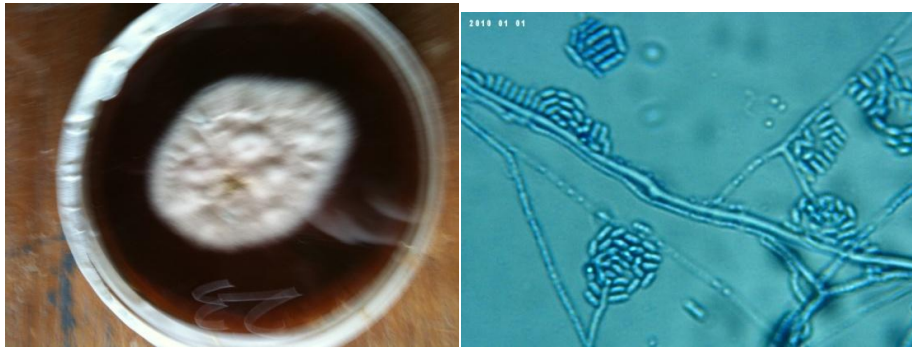


Plate 1. *Phialemonium curvatum* growth on SDA and Slide culture

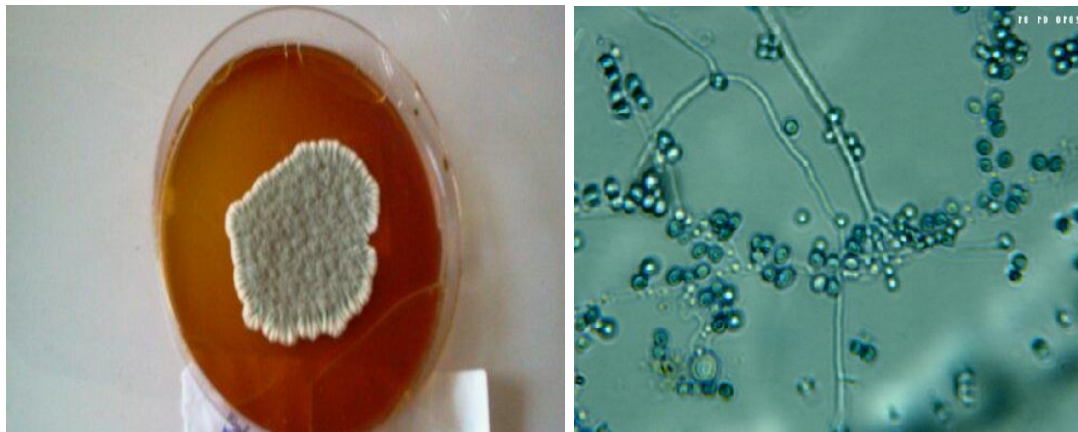


Plate 2. *Penicillium marneffei* on growth SDA and slide culture

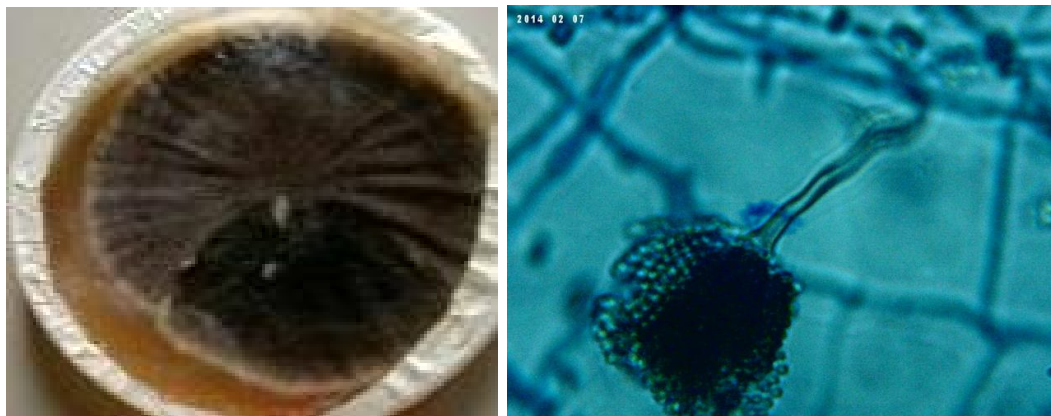
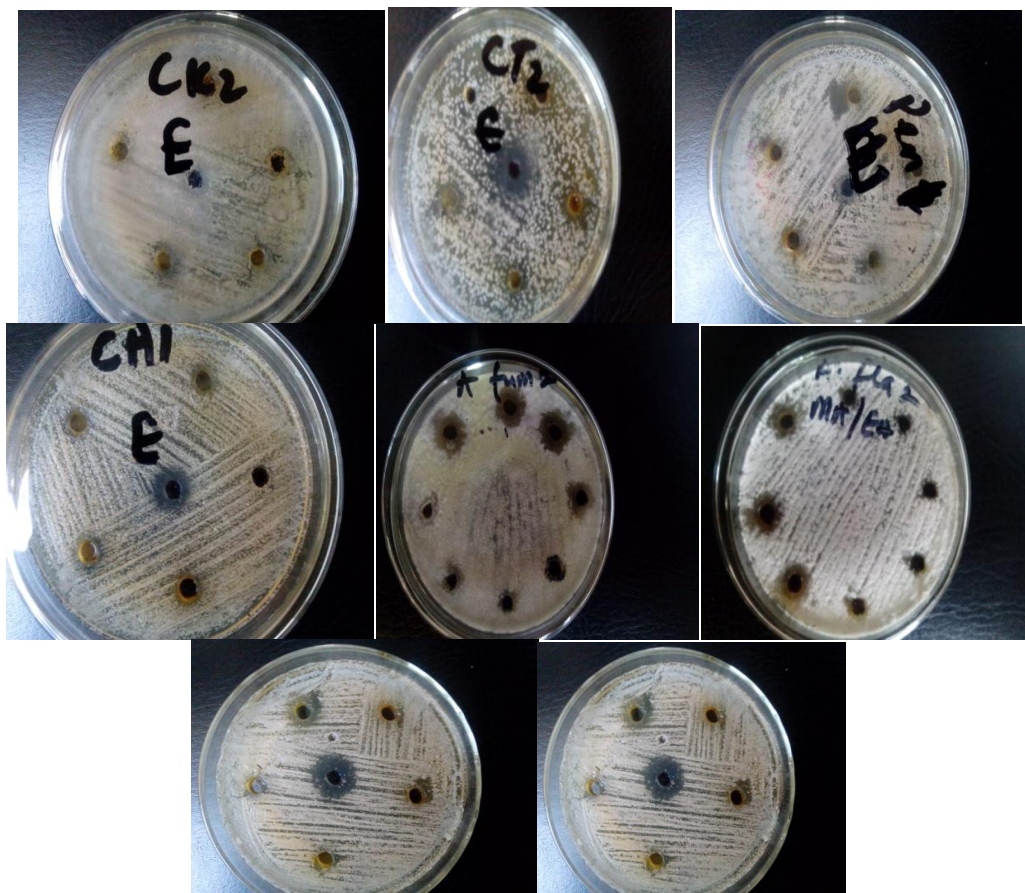


Plate 3. *Aspergillus fumigatus* on growth SDA and slide culture

Appendix 3. Antifungal activity of ethyl acetate fraction of *Moringa oleifera* leaf



Plates 1-3. *Aspergillus fumigatus* growth on SDA and slide culture



P. curvatum E

A.niger E

Plate 4-8. Antifungal activity of ethyl acetate fraction of *Moringa oleifera* leaf

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