PREVALENCE OF CUTANEOUS MYCOSES AMONG RICE FARMERS IN PARTS OF EBONYI STATE AND ANTIFUNGAL ACTIVITY PATTERNS OF LOCALLY USED HERBS

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DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING

FACULTY OF BIOSCIENCES

NNAMDI AZIKIWE UNIVERSITY, AWKA

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING, FACULTY OF BIOSCIENCES, NNAMDI AZIKIWE UNIVERSITY, AWKA, ANAMBRA STATE, NIGERIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D) IN MEDICAL MICROBIOLOGY

SUPERVISOR: PROF. C. O. ANYAMENE

CERTIFICATION

The research work embodied in the dissertation titled "Prevalence of Cutaneous Mycoses among Rice Farmers in Parts of Ebonyi State and Antifungal Activity Patterns of Locally Used Herbs", carried out by Ahuocha Pauline Amaka with registration number NAU/PG/PhD/2013487015F, under the supervision of Prof. C. O. Anyamene is original and has not been submitted in part or full for the award of any degree or diploma in this university or any other university.

……. ……..

Ahuocha, Pauline Amaka Date

(Student)

APPROVAL

This dissertation titled "Prevalence of Cutaneous Mycoses among Rice Farmers in Parts of Ebonyi State and Antifungal Activity Patterns of Locally Used Herbs", carried out by Ahuocha Pauline Amaka with registration number NAU/PG/PhD/2013487015F, has been approved after meeting the requirements for the award of the degree of Doctor of Philosophy (Ph.D) in Medical Microbiology, in the Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

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DEDICATION

This research work is dedicated to my wonderful family for always being there for me.

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ABSTRACT

Cutaneous mycoses caused by dermatophytic and non-dermatophytic fungi are highly contagious and represent significant public health problem in Nigeria and the world at large. The infection among rice farmers had not received much attention, hence, the prevalence of cutaneous mycoses among rice farmers in Parts of Ebonyi State and antifungal activity patterns of locally used herbs were studied. A total of 2130 rice farmers were randomly selected and screened for cutaneous mycoses. Hair fragments, skin and nail scrapings were collected from 182 rice farmers that presented with lesions suggestive of the infection using sterile scissors and scalpels. The samples were examined by direct microscopy using 5% KOH and cultured on Sabouraud dextrose agar supplemented with 0.05mg/ml chloramphenicol and 0.5mg/ml cycloheximide and incubated at 27^{o}C for 4 weeks. The fungal isolates were identified by macroscopic and microscopic methods, and confirmed by molecular analysis. The ability of the isolates to secrete extracellular virulence enzymes was screened on solid media. Phytochemical screening of five medicinal plants: *Azadirachta indica, Cymbopogon citratus, Emilia sonchifolia, Senna alata* and *Senna occidentalis*; used by the farmers to treat the infection was carried out. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the plant extracts were evaluated using broth microdilution method and spread plate methods respectively. Terbinafine (125 mg) oral granules were used in the study as quality control antifungal agent. Demographic data and factors influencing the infection were obtained through questionnaires and analyzed using analysis of variance (ANOVA) at $p<0.05$ significant level with SPSS version 22. A total of 197 fungal isolates were recovered and comprised of 54 (27.41%) dermatophytes and 143 (72.59%) non-dermatophytes. Dermatophytes isolated included *Trichophyton tonsurans* 23 (11.68%), *T. mentagrophytes* 14 (7.11%), *T. rubrum* 7 (3.55%), *T. soudanense* 5 (2.54%), *Microsporum gypseum* 3 (1.52%) and *M. canis* 2 (1.01%) while non-dermatophyte species isolated included *Aspergillus flavus* 64 (32.49%), *A. tamarii* 41 (20.81%), *A. nomius* 30 (15.23%) and *Candida albicans* 8 (4.06%). The overall prevalence rate of the infection among the rice farmers was 182 (8.54%). Age, gender, educational status and family size of the farmers and their interactions with domestic animals/pets influenced the distribution of the fungal species. Out of 182 rice farmers screened, the age-group 16-26 years were most infected with the infection 71 (39.01%), followed by 39 (21.43%) between the age-group 27-37 years and a decline between the age-group 49-59 years 15 (8.24%). The females were more infected 144 (79.12%) than the males 38 (20.88%). The infection among the females was found to be significant ($P<0.05$). Among the skin infections observed in the study areas, tinea capitis 76 (3.57%) was the most predominant type of infection followed by tinea ungium (onychomycosis) 59 (2.77%) and tinea corporis 47 (2.20%). Highest keratinase (23 mm), protease (24 mm), lipase (22 mm) and collagenase (17 mm) activities were observed in *T. rubrum* while the highest xylanase (17 mm) and cellulase (22 mm) activities were observed in *T. tonsurans* and *T. mentagrophytes* respectively. Phytochemical screening of the medicinal plants used in this study showed that they contained some metabolites like flavonoids, alkaloids, saponins, tannins, phenols, glycosides, terpenoids and steroids. The range of MIC for n-hexane, chloroform, petroleum ether and hot water extracts of the medicinal plants against the fungal isolates tested was 2 - 15 µg/ml, 8 - 32 µg/ml, 4 - 18 µg/ml and 10 - 32 µg/ml respectively. Both dermatophytes and non-dermatophytes species isolated were susceptible to the plant extracts used in the study. The dermatophyte species were less susceptible to hot water extract. Minimum fungicidal concentration (MFC) values ranged from 4 - 32 µg/ml. The most significant MFC (4 µg/ml) was observed in n-hexane extracts of *C. citratus* and *S. alata*. The comparative results of antifungal susceptibility patterns of the various isolates to examined plant extracts indicated that the sensitivity to terbinafine (2 µg/ml) by the different species was

higher than the plant extracts (4 µg/ml) with significant difference ($P<0.05$). These plant materials should be harnessed as they hold promising potential source of new drugs for the management and treatment of cutaneous mycoses.

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CHAPTER ONE

INTRODUCTION

1.1. Background of the Study

Superficial fungal skin infections are called cutaneous mycoses. They are caused by both dermatophytes and nondermatophytes, the most common being *Candida* species. Dermatophytes are a group of closely related filamentous fungi that invade keratinized tissues such as skin, hair and nails of humans and animals (Popoola *et al*., 2006; Ameen, 2010). They are classified into three genera; *Trichophyton*, *Microsporum* and *Epidermophyton*, based on the formation and morphology of their conidia. In addition, species of dermatophytes are divided into zoophilic, geophilic or anthropophilic depending on their primary habitat (animals, soil or humans respectively). Zoophilic species are responsible for about 30% of human infection and they often provoke acute inflammation; anthropophilic species represent about 70% of infection on these hosts, causing a chronic infection of slow progression, suggesting that the fungus has adapted to the human host (White *et al*., 2008).

The transmission of cutaneous mycoses occurs by direct contact with infected animals and humans or by indirect contact with contaminated fomites. The causative fungi produce keratinases which degrade the keratin and thus, invade the cutaneous skin tissue. The infections due to these pathogens are generally cutaneous and restricted to the non-living, cornified layers of the skin. However, in chronic conditions, the fungi may invade deeper tissues, particularly in concurrent infections with other organisms. The clinical forms vary according to the etiologic agent and the anatomical site involved. Symptoms may be mild or severe based on the host's immunologic condition and invasion of subcutaneous tissues or internal organs normally does not occur. Typical lesions of skin infections are circular, erythematous, pruritic and are the results of direct action of the fungus or hypersensitivity reactions to the microorganisms and/or their metabolic

products. In nail infections (onychomycosis), the nail may separate from its bed, may become thick and have white spots or even become dystrophic (Degreef, 2008). Although the infection is usually restricted to the superficial epidermis, the causative fungi may be invasive and cause a severe and disseminated infection in immunocompromised patients (Rodwell *et al.*, 2008). In general, the dermatophytes lack the ability to invade deeper tissues or organs of the host.

Cutaneous mycoses are highly contagious and represent a significant public health problem in Nigeria and the world at large (Anosike *et al*., 2005). Although not usually life-threatening, the infection can be persistent and symptomatic, with millions of Naira spent annually in their treatment (Brooks *et al*., 2007). According to the World Health Organization, cutaneous mycoses affect about 25% of the world population. It is estimated that about 30 to 70% of adults are asymptomatic hosts of these infection and that the incidence increases with age. Generally, the infections have been reported worldwide, though with variation in distribution, incidence, epidemiology, etiology and hosts from one location to another with time. Host susceptibility may be enhanced by moisture, warmth, specific skin chemistry, composition of sebum and perspiration, age, exposure and genetic predisposition (Brooks *et al*., 2007). Other factors such as personal hygiene, geographical location, climatic factors, social practices, health care, crowded living and poor sanitary conditions, socioeconomic status and immigration may influence the prevalence of cutaneous mycoses (Shenoi *et al*., 2005; Oyeka and Eze, 2008). The incidence is higher in hot humid climates and crowded living conditions.

Rice farming is one of the major occupations of the people of Ebonyi State, Nigeria. This grain, which is one of the worlds' most important food crops, is cultivated either in marshy low land areas with plenty of water or in plateau or hilly regions where natural rainfall provides adequate amount of water. The preparation of the land, ploughing the field, using manure, fertilizers, pesticides and herbicides, sowing of rice seeds, transplanting of rice seedlings and harvesting are

done manually by the farmers. The farmers are believed to be at high risk of cutaneous mycoses because of their occupational contact with soil. Farm workers that are exposed to various irritant agents namely; mud, cow dung or other types of manure, fertilizers, dust and soil are also predisposed to the infection (Oyeka and Okoli, 2003; Efuntoye *et al.,* 2011). In addition, during the ploughing and planting season and sometimes in the harvesting season, their feet are constantly immersed in water. These factors can predispose workers to cutaneous infection of the skin, hair and nail (Shenoi *et al*., 2005).

Nigeria is endowed with a variety of plant species. The vast arrays of plants are traditionally used for the treatment of various ailments and diseases. These plants, mostly employed in a synergistic combination, have shown to be as effective and are often preferred to the commercially available drugs by a larger portion of the society (Ajose, 2007). The vast diversity of plants in Nigeria is a promising source of novel compounds that are still relatively unexplored. The control of fungal infections requires the use of fungicidal or fungistatic drugs that more specifically target the infecting agent to avoid damage to the host. With developing microbial resistance and need for safe and cost-effective antifungal drugs, screening of plant organs for potential bioactive compounds cannot be overemphasized. Thus the indigenous knowledge of traditional medicinal plants is a valuable tool for targeting potentially active species from the wealth of medicinal plants in Nigeria.

1.2. Statement of Problem

Rice farming dominates the agricultural sector of Nigeria. It is the single most important industry in Ebonyi State, contributing to the gross domestic product of the country. These rice farmers are exposed to various agents such as mud, cow dung, fertilizers, dust and soil that predispose them to cutaneous mycoses. The water-rice field is a suitable place for the growth of bacteria, fungi,

viruses and parasites (Sunil, 2002). Most of the rice farmers work on bare feet and skin diseases are very common.

Cutaneous mycoses had been extensively reported to be a public health problem in Nigeria and in many countries with high humid environment. These mycoses cannot be overemphasized as the contagious nature, high cost of treatment, difficulty of control and the public health consequences explain their great importance. A number of studies on the prevalence and etiological aspects of cutaneous mycoses have been carried out in different parts of Nigeria which had school children as their target population (Ogbonna *et al*., 1985; Anosike *et al*., 2005; Ive, 1996; Obasi and Clayton, 1989; Egere and Gugnani, 1980). There is a paucity of epidemiological data in the literature to ascertain the prevalence of cutaneous mycoses among rice farmers in parts of Ebonyi State. Thus, studies in different geographic areas assessing the specific fungal etiology involved are of public health importance serving as baseline information for the management of the infection at the local level.

Due to the absence of health amenities, people living in remote rural areas are using indigenous plants as medicines against these mycoses. Most of the rural population of Ebonyi State lives below poverty level and depend on traditional medicine for all or most of their medicinal needs. With developing fungal resistance and need for safe and cost-effective antifungal drugs, screening of plant organs for potential bioactive secondary metabolites becomes indispensible.

1.3. Aim of the Study

The aim of this study is to determine the prevalence of cutaneous mycoses among rice farmers in parts of Ebonyi State and evaluate the antifungal activities of locally used herbs in the study area.

1.4. Objectives of the Study

The following were the objectives targeted in this study. Which include:

i. To determine the prevalence of cutaneous mycoses among the rice farmers by age, gender, educational status, family size and their interaction with domestic animals in Ikwo and Izzi Local Government Areas.

ii. To determine the dermatophytes and non-dermatophytes causing mycoses among rice farmers in Ikwo and Izzi Local Government Areas of Ebonyi State.

iii. To screen the fungal isolates for the production of extracellular enzymes.

iv. To determine the phytochemicals in five medicinal plants used in the study area as medicine against the infections.

v. To determine the minimum inhibitory concentration (MIC) of extracts of the five medicinal plants against the fungal isolates.

vi. To determine the minimum fungicidal concentration (MFC) of extracts of the five medicinal plants against the fungal isolates.

1.5. Significance of the Study

Cutaneous mycoses associated with agricultural practices in Nigeria have a major health impact on rice farmers and other associated people. This leads to reduced productivity through decreased human resources, famine and places an additional burden on health care requirements. In-depth knowledge of the etiologic agents of the infections and potential risk factors associated with rice farming in Ebonyi State could provide insights on control measures. In addition, effective reporting of the infections, risk awareness and appropriate intervention measures will assist in tackling the infections amongst rice farmers in Ebonyi State and the country at large.

Plant-derived drugs in clinical use have been discovered through follow-up investigation of the ethno medicinal uses of plants. Therefore, it is essential for drug discovery to record and preserve the traditional knowledge of medicinal plants that mostly depends on local practitioners and field surveys. This study evaluated the phytochemical constituents and antifungal potency of five medicinal plants on some fungi causing cutaneous mycoses with a view of providing potential source of new drugs for the management and treatment of cutaneous mycoses.

1.6. Scope of the Study

The study covers rice farmers in two Local Government Areas (Ikwo and Izzi Local Government Areas) of Ebonyi State to determine the prevalence of cutaneous mycoses among rice farmers during the period of June, 2015 to November, 2016 and evaluates the antifungal activities of locally used herbs.

CHAPTER TWO

LITERATURE REVIEW

2.1. Overview of Cutaneous Mycoses

Cutaneous mycoses are fungal infections due to dermatophytes and some opportunistic fungi such as *Candida*, *Aspergillus*, *Penicillium*, *Cryptococcus*, *Alternaria, Fusarium* or *Mucor* species. Dermatophytes are a group of closely related filamentous fungi that cause dermatophytosis (Peerapur *et al*., 2004). Dermatophytosis (plural, dermatophytoses) is a cutaneous mycotic condition characterized by the infection of keratinized tissues such as the skin, hair and nails. *Trichophyton***,** *Microsporum* and *Epidermophyton* are the genera of dermatophytes implicated in dermatophytosis and are related to organisms in the soil which are capable of digesting keratinous material. This group of fungi invades and grows in dead keratin. They have the ability to utilise keratin as a nutrient source, i.e. they have a unique enzymatic capacity (keratinase). They tend to grow outwards on skin; producing a circular rash (shaped like a ring), hence the term 'ringworm'. They are very common and affect different parts of the body. They can usually be successfully treated but success depends on the site of infection and on compliance with treatment.

Dermatophytes are the major cause of cutaneous mycoses. Less frequently, the infection is caused by nondermatophyte fungi such as *Aspergillus* and *Candida* species. These organisms are assuming greater significance due to the excessive use of immunosuppressive drugs for controlling serious infectious as well as non-infectious conditions. The infection due to these pathogens is generally cutaneous and restricted to the non-living, cornified layers of the skin. However, in chronic conditions, the fungi may invade deeper tissues, particularly in concurrent infections with other organisms. In general, the dermatophytes lack the ability to invade deeper tissues or organs of the host.

In recent years, fungal infections related to *Aspergillus* species have become a major focus of clinical microbiology and infectious disease studies as the number of patients infected with *Aspergillus* species has risen dramatically. Among the known *Aspergillus* species, *Aspergillus fumigatus* is the most common one causing infections in humans, whereas *Aspergillus flavus* is as important as *A. fumigatus* and is the second most common *Aspergillus* species associated with human infections. The other *Aspergillus* species commonly associated with human infections include *Aspergillus niger* and *Aspergillus terreus* (Chong *et al*., 2004). In immunocompetent hosts, *Aspergillus* species rarely cause serious illnesses.

2.2. Etiologic Agents of Cutaneous Mycoses

Some etiologic agents of cutaneous mycoses are briefly described below.

2.2.1. Dermatophyte species

- ***Trichophyton rubrum***

Trichophyton rubrum is a dermatophytic mould fungus in the phylum Ascomycota, class Euascomycetes. It is an exclusively clonal, anthropophilic saprotroph that colonizes the upper layers of dead skin. *T. rubrum* was first described by Malmsten in 1845 and is currently considered to be a complex species that comprises multiple, geographically patterned morphotypes, several of which have been formally described as distinct taxa, including *T. raubitschekii*, *T. megninii* and *T. soudanense* (Graser *et al*., 2008).

T. rubrum is one of the most commonly encountered dermatophytes that infect human keratinized tissue such as skin, nails and possibly hair. This pathogen causes well-characterized superficial infections and also produces skin infections in unusual parts of the body in immunodepressed patients (Cervelatti *et al*., 2004). Though it is usually not life-threatening, infections are long-lasting, recurring and incredibly difficult to cure. Nearly 80% of onychomycosis due to *T. rubrum*

and 90% of the chronic dermatophyte infections are caused mostly by *T. rubrum*, this pathogen developed mechanisms to avoid or suppress cell-mediated immunity (Baeza *et al*., 2006; Baeza *et al*., 2007).

Due to pleomorphism, many strains and varieties of *T. rubrum* have been described. For practical purposes, two types are distinguished: *T. rubrum* downy type and *T. rubrum* granular type.

Morphology of *T. rubrum* typical downy strain

Sabouraud's Dextrose Agar: Colonies are flat to slightly raised, white to cream, suede-like to downy, with a yellow-brown to wine-red reverse. Most cultures show scanty to moderate numbers of slender clavate to pyriform microconidia. Macroconidia are usually absent, however, closterospore-like projections may be present in some mounts. On primary isolation, some cultures may lack reverse pigmentation and fail to produce microconidia. These will need to be subcultured onto media like lactritmel agar or potato dextrose agar which stimulate pigmentation and sporulation. If sporulation still fails, subculture the fungus onto Trichophyton Agar No.1.

Lactritmel Agar: Flat, white, downy colonies with a deep wine-red reverse pigment. Microscopically, cultures show the typical downy type morphology of pyriform to slender clavate microconidia.

Trichophyton Agar No.1: Colonies are flat, white to cream, suede-like to downy with a deep wine-red reverse pigment.

Hydrolysis of Urea: Negative at 7 days.

Hair Perforation Test: Negative at 28 days.

Morphology of *T. rubrum* granular type

Sabourand's Dextrose Agar: Colonies are flat to slightly raised, white to cream, suede-like with a pinkish-red reverse. Microscopically, most cultures have numerous clavate to pyriform

microconidia and moderate numbers of smooth, thin-walled multiseptate, slender cylindrical macroconidia. Older cultures may show numerous chlamydospores with few clavate to pyriform microconidia.

Lactritmel Agar: Flat, white to rose pin, suede-like to granular colonies with a pinkish to wine-red reverse. Numerous broad clavate to pyriform microconidia and moderate numbers of smooth, thin-walled, slender cylindrical macroconidia are present. A few chlamydospores may be present in older cultures.

Trichophyton Agar No. 1: Colonies are white to cream, suede-like with a pinkish-red to wine-red reverse.

Hydrolysis of Urea: Positive at 7 days

Hair Perforation Test: Positive

- ***Trichophyton tonsurans***

Trichophyton tonsurans is a mating-incompetent, anthropophilic fungus with a worldwide distribution which causes inflammatory or chronic non-inflammatory finely scaling lesions of skin, nails and scalp. It is a common cause of tinea capitis in the Australian Aborigine and African Americans. Invaded hairs show an endothrix infection and do not fluoresce under Wood's ultra-violet light.

Morphology

Sabouraud's Dextrose Agar: On SDA, colonies show considerable variation in texture and colour. They may be suede-like to powdery, flat with a raised centre or folded, often with radial grooves. The colour may vary from pale-buff to yellow, (the sulfureum form which resembles *Epidermophyton floccosum*), to dark-brown. The reverse colour varies from yellow-brown to reddish-brown to deep mahogany. Hyphae are relatively broad, irregular, much branched with

numerous septa. Numerous characteristic microconidia varying in size and shape from long clavate to broad pyriform, are borne at right angles to the hyphae, which often remain unstained by lactophenol cotton blue. Very occasional smooth, thin-walled, irregular, clavate macroconidia may be present on some cultures. Numerous swollen giant forms of microconidia and chlamydospores are produced in older cultures.

Hair Perforation Test: Positive within 14 days.

- ***Trichophyton soudanense***

Trichophyton soudanense is an anthropophilic fungus which is a frequent cause of tinea capitis in Africa. Invaded hairs show an endothrix infection but do not fluoresce under Wood's ultra-violet light. Distribution is mainly in Africa with imported cases now reported from Europe, Brazil, Australia and USA. *T. soudanense* appears to be genetically related to *T. rubrum* and *T. violaceum* (Graser *et al*., 2007).

Morphology

Colonies (SDA) are slow-growing with a flat to folded, suede-like surface. Often there is a broad fringe of submerged growth. The surface mycelium and reverse pigment is characteristically a deep apricot-orange in colour. Microscopically, the hyphae often show reflexive or right-angle branching. Pyriform microconidia may occasionally be present and numerous chlamydospores are often found in older cultures.

- ***Trichophyton mentagrophytes***

T. mentagrophytes is a zoophilic fungus with a worldwide distribution and a wide range of animal hosts including mice, guinea-pigs, kangaroos, cats, horses, sheep and rabbits. It produces inflammatory skin or scalp lesions in humans, particularly in rural workers. Kerion of the scalp

and beard may occur. Invaded hairs show an ectothrix infection but do not fluoresce under Wood's ultra-violet light.

Morphology

Sabouraud's Dextrose Agar: Colonies are generally flat, white to cream in colour, with a powdery to granular surface. Some cultures show central folding or develop raised central tufts or pleomorphic suede-like to downy areas. Reverse pigmentation is usually a yellow-brown to reddish-brown colour. Numerous single-celled microconidia are formed, often in dense clusters. Microconidia are hyaline, smooth-walled and are predominantly spherical to subspherical in shape; however, occasional clavate to pyriform forms may occur. Varying numbers of spherical chlamydospores, spiral hyphae and smooth, thin-walled, clavate-shaped, multi-celled macroconidia may also be present.

Hydrolysis of Urea: Positive within 7 days (usually 3 to 5 days).

Hair Perforation Test: Positive within 14 days.

- ***Microsporum canis***

Microsporum canis is a pathogenic, asexual fungus in the phylum Ascomycota that infects the upper, dead layers of skin on domesticated cats and occasionally dogs and humans (Shafiee *et al*., 2014). Despite its species name ("canis" implies dogs); the natural host of *M. canis* is the domestic cat. However this species can colonize dogs and horses as well. In all cases, it resides on the skin and fur (Sharma *et al*., 2007). *M. canis* may also persist as dormant spores in environment for prolonged periods. *M. canis* species have a worldwide distribution (Shafiee *et al*., 2014). *M. canis* reproduces by means of two conidial forms, large, spindle-shaped, multicelled macroconidia and small, single-celled microconidia.

Morphology

Sabouraud's Dextrose Agar: Colonies are flat, spreading, white to cream-coloured, with a dense cottony surface which may show some radial grooves. Colonies usually have a characteristic bright golden yellow to brownish yellow reverse pigment due to the metabolites secreted by the fungus. The intensity of the yellow pigmentation peaks on the 6th day of colony growth and fades gradually making the identification of older colonies difficult. Some strains of *M. canis* fail to produce yellow pigment altogether, exhibit abnormally slow colony growth and form undeveloped macroconidia. Cultivation on polished rice tends to reestablish the typical growth morphology and is helpful for identification. Macroconidia are typically spindle-shaped with 5-15 cells, verrucose, thick-walled and often have a terminal knob, 35-110 x 12-25 µm. A few pyriform to clavate microconidia are also present. Macroconidia and/or microconidia are often not produced on primary isolation media and it is recommended that sub-cultures be made onto lactritmel agar and/or boiled polished rice grains to stimulate sporulation.

Growth on autoclaved Rice Grains: Good growth of white aerial mycelium with production of yellow pigment. Microscopy reveals numerous macroconidia and microconidia similar to those described above.

Lactritmel Agar: Flat, white suede-like to cottony colony with a bright yellow reverse. Microscopy reveals moderate numbers of thick-walled, multiseptate, long, spindle- shaped macroconidia, some of which show a terminal knob. Walls of macroconidia are slightly rough or echinulate especially at terminal knobs. Numerous clavate to pyriform microconidia are also present in this strain. Lactritmel agar with sterile soil added is also an excellent medium for the stimulation of macroconidial development in *M. canis*.

Reverse Pigment on Potato Dextrose Agar: Produces a lemon-yellow pigment that is easily visualized, due to the presence of aerial hyphae (both *M. audouinii* and *M. canis* var. *equinum* are salmon to pinkish-brown).

Hair Perforation Test: Positive at 14 days.

- ***Microsporum audouinii***

Microsporum audouinii is an anthropophilic fungus in the genus *Microsporum* (Roque *et al*., 2006). It is a type of dermatophyte that colonizes keratinized tissues (primarily hair) causing non-inflammatory infections of scalp and skin. Invaded hairs show an ectothrix infection and usually fluoresce a bright greenish-yellow under Wood's ultra-violet light (Griffin *et al.*, 2001).

Morphology

Sabouraud's Dextrose Agar: Colonies are flat, spreading, greyish-white to light tan-white in colour and have a dense suede-like to downy surface, suggestive of mouse fur in texture. Reverse can be yellow-brown to reddish-brown in colour. Some strains may show no reverse pigment. Macroconidia and microconidia are only rarely produced; most cultures are sterile or produce only occasional thick-walled terminal or intercalary chlamydospores. When present macroconidia may resemble those of *M. canis* but are usually longer, smoother and more irregularly fusiform in shape; microconidia when present are pyriform to clavate in shape and are similar to those seen in other species of *Microsporum*. So called pectinate (comb-like) hyphae and racquet hyphae (a series of hyphal segments swollen at one end) may also be present.

Growth on Autoclaved Rice Grains: Very poor or no visible growth and abundant brown pigment on the rice grains (Ellis, 2013). This is one of the features which distinguish *M. audouinii* from *M. canis*.

Reverse Pigment on Potato Dextrose Agar: Salmon to pinkish-brown or peach-coloured (*M. canis* is bright yellow).

Lactritmel Agar: Colonies are usually flat, spreading, with a fine, whitish suede-like surface and a very pale yellow-brown reverse. Microscopic morphology as described above.

Hair Perforation Test: Negative after 28 days.

- ***Microsporum gypseum***

Microsporum gypseum is a mating-competent geophilic fungus with a worldwide distribution which may cause infections in animals and humans, particularly children and rural workers during warm humid weather. It usually produces a single inflammatory skin or scalp lesion. Invaded hairs show an ectothrix infection but do not fluoresce under Wood's ultra-violet light.

Morphology

Colonies (SDA) are usually flat, spreading, suede-like to granular, with a deep cream to tawny-buff to pale cinnamon-coloured surface. Many cultures develop a central white downy umbo (dome) or a fluffy white tuft of mycelium and some also have a narrow white peripheral border. A yellow-brown pigment, often with a central darker brown spot, is usually produced on the reverse; however, a reddish-brown reverse pigment may be present in some strains. Cultures produce abundant, symmetrical, ellipsoidal, thin-walled, verrucose, four-to-six-celled macroconidia. The terminal or distal ends of most macroconidia are slightly rounded, while the proximal ends (point of attachment to hyphae) are truncate. Numerous clavate-shaped microconidia are also present, but these are not diagnostic.

- ***Epidermophyton***

Fungi of the genus *Epidermophyton* are club-shaped sac fungi and belong to the Arthrodermataceae family. The genus comprises the *Epidermophyton floccosum* and *Epidermophyton stockdaleae* fungi. *Epidermophyton* species may elicit skin diseases such as athlete's foot (tinea pedis), jock itch (tinea cruris) or body ringworm (tinea corporis). Of the two species that fall within the *Epidermophyton* species, *E. floccosum* is the only pathogenic species which is found world wide and its primary reservoir is humans. Like all dermatophytes, *E. floccosum* contains keratinase giving it the ability to breakdown keratin a protein commonly found within the skin, nails and hair. *E. floccosum* is spread by direct contact with the fungus where people aggregate and share inanimate objects such as towels in a gym setting (Mycology Online, 2010).

The major distinguishing factor that differentiates *E. floccosum* from the other dermatophytes, *Microsporum* and *Trichophyton* is the absence of microconidia, and the shorter, wide, smooth macroconidia. To distinguish between *E. floccosum* and *E. stockdaleae*, a hair perforation test can be done to see if the hyphae infiltrate the hair shaft. In this case *E. floccosum* will show a negative test while *E. stockdaleae* will show a postive test. *E. stockdaleae* will also grow in medium with NaCl, while *E. floccosum* will not grow (Mycology Online, 2010).

E. floccosum is mainly found infecting humans worldwide, but can also survive for short periods of time, with moisture, on inanimate objects. The other species, *E. stockdaleae* is also found worldwide, but is nonpathogenic and geophilic meaning found almost always in soil habitats.

2.2.2. Non-dermatophyte species

- ***Aspergillus flavus***

Aspergillus flavus is a pathogenic fungus in the phylum Ascomycota. It has a world-wide distribution and normally occurs as a saprophyte in soil and on many kinds of decaying organic matter, however, it is also a recognized opportunistic pathogen of humans and animals, causing cutaneous aspergillosis in immunocompromised individuals (Ellis *et al*., 2007*)*. It may be found in any type of climate, but it is most common in warm temperate zones and environments with low water levels and higher temperatures. After *Aspergillus fumigatus, A. flavus* is the second-leading cause of cutaneous infections. The deposition of certain spore sizes could be a leading factor for why *A. flavus* is a common etiological cause of fungal cutaneous infections and noninvasive fungal pneumonia.

Morphology

The hyphae of *A. flavus* are partitioned by a septum and are hyaline, giving them a glossy appearance. The organism is a circular vesicle, with protruding filamentous extensions. *A. flavus* has a very rapid rate of growth on SDA, maturing in about three days. Colonies are yellow-green to olive and may have a white border with cream to tan to yellowish reverse. Texture is often floccose, especially near the center and overall can be velvety to woolly. On Czapek Dox agar, colonies are granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age, reverse uncolored. On malt extract agar, colonies are dark green, reverse hyaline. Conidial heads are typically radiate, later splitting to form loose columns (mostly 300-400 µm in diameter), biseriate but having some heads with phialides borne directly on the vesicle (uniseriate). Conidiophore stipes are uncolored and coarsely roughened, often more noticeable near the vesicle. Conidia are globose to subglobose (3-6 µm in diameter), pale green and conspicuously echinulate. Some strains produce brownish sclerotia (Ellis *et al*., 2007*)*.

- ***Aspergillus tamarii***

Aspergillus tamarii is a species of *Aspergillus* that phenotypically resemble *A. flavus*. It is a typical soil fungus with a world-wide distribution. It has also been reported as causing disease in human and animals.

Morphology

Colonies on SDA produce olive green colour, floccose with brown reverse. The conidia are sparse, globose to subglobose and dark olive green. The conidiophores are light brown and slightly roughened with globose to subglobose vesicles and biseriate phialides.

- ***Aspergillus nomius***

Aspergillus nomius is morphologically a highly variable species which is difficult to classify by conventional criteria (Kumeda and Asao. 2001). *A. nomius* can be distinguished from other members of *Aspergillus* species by a number of molecular techniques including sequence analysis of the internal transcribed spacer (ITS) regions (Rigo *et al*., 2002). It was hypothesized that *A. nomius* diverged from a common ancestor prior to the divergence of *A. flavus* (Ehrlich *et al*., 2003). *A. nomius* occurs commonly in soil and is occasionally reported as a pathogen of humans and animals.

Morphology

Colony surface on SDA is velvety with floccose tufts and yellowish green and a dull yellow or orange-brown reverse. The conidiophores are hyaline with globose to subglobose vesicles and biseriate phialides. The conidia are sparse to moderately abundant and globose to subglobose.

- ***Candida albicans***

Candida albicans is an opportunistic pathogenic yeast in the phylum Ascomycota and family Saccharomycetaceae. It is usually a commensal organism, but can become pathogenic in immunocompromised individuals under a variety of conditions (Calderon and Clancy, 2012). It is one of the few species of the *Candida* genus that causes the human cutaneous candidiasis. *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata*, are together responsible for 50–90% of all cases of candidiasis in humans (Schlecht *et al*., 2015; Singh and Chakrabarti, 2017). It is generally referred to as a dimorphic fungus since it grows both as yeast and filamentous cells.

Morphology

C. albicans is easily identified using phenotypic method. The identification of the species relies on morphological and physiological features. Pseudohyphae and oval budding yeast cells can be detected in unstained potassium hydroxide (KOH) preparations. Colonies on SDA or potato dextrose agar after 24-48 hours incubation at 25-37°C are cream-colored or off white to grey, dull, smooth, soft or wrinkled or rough. Colonies cannot be dependably distinguished from other *Candida* species based on macroscopic morphology but the absence of terminal chlamydospores is characteristic. A positive germ tube test is the confirmatory test for *C. albicans*.

- ***Candida tropicalis***

Candida tropicalis is a species of yeast in the genus *Candida*. The organism can survive for up to 24 hours therefore be cross-transmitted to a second hand with a probability of 69% and to a third hand with 38% probability. It is responsible for approximately half of the beyond-surface *Candida* infections (Chai *et al*., 2010). *C. tropicalis* is the second most virulent *Candida* species (Zuza-Alves *et al*., 2017) that can significantly affect by spreading through the weakened immune system host and can occupy the gastrointestinal tract within 30 minutes of inoculation, all this

resulting in increased mortality (Chai *et al*., 2010). Impact of candidiasis caused by *C. tropicalis* has increased globally. *C. tropicalis* is virulent due to its ability to produce biofilm, secrete lytic enzymes, adhere to epithelial and endothelial cells, and undergo transition of bud to hyphae (Kothavade *et al*., 2010).

Morphology

C. tropicalis is a vegetative cell with the shape from round to oval ranging from approximately 2-10 micrometers (Murray *et al*., 2003). It exhibits dimorphism forming a single-celled yeast or blastoconidia which reproduces by simple budding. There are different media on which *C. tropicalis* can grow effectively. A common medium used is the Sabouraud's agar which contains peptone and sugar. This is enough for identifying the species but with a disadvantage of promoting mycelial growth and suppressing conidia formation. Another commonly used medium is the cornmeal agar which is useful in inducing formation of conidia. Potato-glucose, potato-carrot, tomato juice, lima bean and others are also types of media used for growth. The optimal temperature for growth is between 25-35 °C and growth is enhanced if sugar or fat is added in the medium. Colonies are white, smooth and butyrous with a fringed border. *C. tropicalis* is found to be urease negative (Wilson *et al*., 2015).

2.3. Transmission and Pathogenesis of Cutaneous Mycoses

The possible route of entry for the causative organism into the host body is injured skin, scars and burns. Infection is caused by arthrospores or conidia. Resting hairs lack the essential nutrient required for the growth of the organism. Hence these hairs are not invaded during the process of infection. The first step of cutaneous mycoses involves contact and adherence of the infectious elements from the organism. The transmission of the infection may occur by direct contact with infected animals and humans or by indirect contact with contaminated fomites (Peres *et al*.,

2010). The fungi adhere to the surface of the keratinized tissue to reach the epidermis by germination of arthroconidium and then the hypha enters the stratum corneum. In the pathogenesis of the infection, the initial interaction between the arthroconidia and the stratum corneum occurs 3 to 4 hours after contact. During the adhesion of arthroconidia to the stratum corneum surface, the formation of elongated fibrillary structures that appear to anchor and connect the arthroconidium to the tissue surface occurs. This may also prevent its removal from the host tissue. This would increase the contact surface with the tissue making greater adhesion and acquisition of nutrients possible. At present, the knowledge of the factors that mediate adherence of the fungi is little known. However, it has been suggested that the mannose and galactose that are present on the skin surface are carbohydrate-specific adhesins recognized by the fungi (Esquenazi *et al*., 2003; Esquenazi *et al*., 2004). These adhesins are probably involved in the first step of infection. Moreover, it has been suggested that fungi-secreted proteases are necessary for the adherence process (Baldo *et al*., 2008). Hence, the pathogen must produce and secrete proteases in response to the presence of the components of the epidermal extracellular matrix during tissue invasion.

In the early stages of the infection and in response to the acidic pH of human skin, the pathogen unsuppresses the synthesis of nonspecific keratinases and proteases that have optimal activity in acidic pH. They act in substrates, keratinous or not, producing peptides that are hydrolyzed to aminoacids, which are used by the fungus as a source of carbon, nitrogen and sulphur. The metabolization of some amino acids promotes the alkalinization of the host's microenvironment, making it suitable to the action of keratinases with optimal activity in alkaline pH, which allows the maintenance of the infection. Other important components found in the host tissue are lipids, collagen and esters which are also the target of fungal extracellular enzymes in the pathogenesis of the infection.

2.4. Clinical Forms of Cutaneous Mycoses

Clinical presentations of cutaneous mycoses vary largely. It depends on the causative agent, the affected area of the body and the condition of the patient's immune system. Although the causative agents can be found in more than one clinical form, most of them have preferred sites for infections based on their pathogenicity factors. The customary signs of inflammatory reactions such as redness, swelling, heat and alopecia are seen at the infection site. Inflammation causes the pathogen to move away from the site of infection and take residence at a new site. This movement of the organism away from the infection site produces the classical ringed lesion.

A. flavus is most commonly associated with primary cutaneous aspergillosis. Primary cutaneous aspergillosis may present as macules, papules, plaques or hemorrhagic bullae, which may progress into necrotic ulcers that are covered by a heavy black eschar (Longley, 1997). Signs and symptoms of candidiasis vary depending on the area affected (Gerald *et al*., 2010). Most candidal infections result in minimal complications such as redness, itching, and discomfort, though complications may be severe or even fatal if left untreated in certain populations. In healthy (immunocompetent) persons, candidiasis is usually a localized infection of the skin, fingernails or toenails (onychomycosis), or mucosal membranes, including the oral cavity and pharynx (thrush), esophagus, and the genitalia (vagina, penis, etc).

2.5. Laboratory Diagnosis of Cutaneous Mycoses

- **Collection and Transport of Specimens**

Filamentous fungi undergo radial growth. The centers of infected skin patches may consist of the older and poorly viable material, as may portions of older nail plate in onychomycosis. In skin infections where the ‘rings’ of ringworm are well defined, collection is best made by collecting epidermal scales from near the advancing edges of the rings. The lesion is lightly disinfected with

alcohol in gauze and then scraped from center to edge, crossing the lesion margin, using a sterile scalpel blade or equivalent. If the lesions have vesicles or bullae, the tops of the vesicles or bullae should be clipped and included in the sample. Suppurating lesions may be sampled with a swab when it is impractical to obtain scrapings. The skin scales are scraped in such a way that the whole infected area is represented, since an advancing margin is often not evident.

In scalp infection, the basal root portion of the hair is best for direct microscopy and culture. Wood's light may be used to allow detection of the most heavily infected hairs. Hairs are best sampled by plucking (not cutting) so that the root is included. If this is not possible due to hair fragility, a scalpel may be used to scrape scales and excavate small portions of the hair root. Brushes with stiff bristles, run firmly across the lesion, have also been used successfully.

The common distal-subungual type of nail infection is traditionally sampled, after light alcohol disinfection, by scraping the debris from beneath the distal end of the nail with a scalpel and collecting scrapings from near the nail bed, where viable inoculum is most likely to be encountered. Close clipping of the whole nail end is an alternative to this procedure, as is nail drilling. In difficult to sample, degraded nails, specialists may use a Skele curette, a surgical instrument with a small, spoon-like end with a sharpened edge. Superficial white onychomycosis is sampled by scraping material from the white spots on the surface of the nail. Discarding the uppermost layer of material is recommended in order to reduce the presence of contaminant inoculum.

Sample materials are best transported in dry, strong black paper folded in the manner of a herbarium packet. Bacteriological transport media should not be used as they may allow growth of contaminants and their viscosity may result in substantial loss of the available specimen. Moisture of any kind is to be avoided. Black paper allows easy visualization of small skin squames; it should be thin enough to fold tightly at the corners and not ‘‘leak’’ specimen.

- **Microscopic Examination and Culture**

Although false negative in 5 to 15% of cases in ordinary practice, direct microscopy is a highly efficient screening technique for dermatophyte species. Scrapings and hairs may be mounted for direct examination in 20% KOH or NaOH mixed with 5% glycerol, heated (e.g., for 1 h at 51 to 54^{o}C) to emulsify lipids, and examined under 3400 magnification for fungal structures. Another formulation is 20% KOH-36% dimethyl sulfoxide and two techniques for fluorescence microscopy; the calcofluor white technique and the Congo red technique may be used. Samples are usually cleared with KOH or other agents to help visualize dermatophyte structures. Various stains such as chlorazol black E, Parker blue-black ink, Swartz-Lamkin stain or Congo red stain may be added. A KOH preparation of hairs or skin scrapings from an affected area will show the segmented hyphae characteristic of dermatophyte infections.

Culture is a valuable adjunct to direct microscopy and is essential at least in all nail infections and in any infection to be treated by systemic medication. One of the most common media used to culture fungi in the laboratory is Sabouraud's dextrose agar (SDA). It consists of peptone, dextrose and agar. High concentration of sugar and a low pH (4.5-5.5) prevents growth of most bacteria and makes it selective for fungi. Other basal media used to grow fungi include potato dextrose agar (PDA) and malt extract agar. Most fungi are able to grow at room temperature while few pathogenic fungi (e.g., *Cryptococcus*, dimorphic fungi) can grow at 37^{o}C. Saprophytic fungi grow much quickly than pathogenic fungi (e.g. dermatophytes). In such situations the saprophytic fungi can be inhibited by the addition of cycloheximide to the SDA. Addition of antibiotics such as chloramphenicol, gentamicin or streptomycin to SDA serves to inhibit bacterial multiplication. An example of SDA amended with cycloheximide and chloramphenicol is Mycosel agar. Since some dermatophytes grow slowly, cultures should not be discarded for 4-6 weeks.

Dermatophyte test medium is an alternative; it normally shows alkalinity generated by dermatophyte growth as a color change to red in its constituent phenol red indicator. Some nonpathogenic dermatophyte (e.g., *Trichophyton terrestre*), however, induce the red color change, while some *Microsporum* isolates and bacterially contaminated isolates may give a false-negative reaction. Therefore, this medium is good but is not an absolute indicator of the growth of a dermatophyte. It has the disadvantage of not allowing visualization of colony reverse pigmentation, a character often important in identification.

Some specialized isolation media are used in specific circumstances. Casamino Acids-erythritol-albumin medium, a highly selective medium are used for isolating dermatophytes from lesions heavily contaminated by bacteria or by the cycloheximide-tolerant *C. albicans*. This medium contains cycloheximide, antibacterial agents, and suspended egg albumin. The albumin inhibits yeasts such as *C. albicans* which have an absolute requirement for exogenous biotin. This medium is most advantageous for showing the presence of etiologic dermatophytes in diabetics and other immunocompromised patients whose skin lesions may be profusely overgrown by *Candida* species. Another isolation medium is bromocresol purple (BCP)-casein-yeast extract agar, which grows all dermatophytes but is designed for the rapid recognition of microcolonies of *T. verrucosum*. This species elaborates a distinctive diffusing protease which produces a broad, distinct zone of clearing in the opaque casein solids surrounding the small colonies.

A presumptive diagnosis of primary cutaneous aspergillosis can be made by examining a potassium hydroxide preparation of a clinical specimen. Generally, however, the diagnosis of most primary and secondary *Aspergillus* infections requires sample of a skin lesion taken for culture. A skin specimen for a suspected fungal lesion should be taken from the center of the lesion and should reach the subcutaneous fat because *Aspergillus* tends to invade blood vessels of the dermis and subcutis, resulting in an ischemic cone above it. In the microbiology laboratory,

fungal hyphal structures can be stained directly from tissue specimens with the whitening agent calcofluor, which will fluoresce when exposed to UV light. The specimen should be minced and plated on medium specific for the recovery of mold (e.g., potato dextrose agar). The isolates from culture media are identified on the basis of colony morphology and sporulation.

Diagnosis of a yeast infection is done either via microscopic examination or culturing. For identification by light microscopy, a scraping or swab of the affected area is placed on a microscope slide. A single drop of 10% potassium hydroxide (KOH) solution is then added to the specimen. The KOH dissolves the skin cells, but leaves the *Candida* cells intact, permitting visualization of pseudohyphae and budding yeast cells typical of many *Candida* species. For the culturing method, a sterile swab is rubbed on the infected skin surface. The swab is then streaked on a fungal culture medium. The culture is incubated at 37 °C for several days, to allow development of oval yeast colonies. The characteristics (such as morphology and colour) of the colonies may allow initial diagnosis of the organism causing disease symptoms (Ferris *et al*., 2002).

- **Identification Characters and Diagnostic Media**

Many typical fungal isolates can be identified directly from primary isolation media, particularly, Sabouraud dextrose agar and potato dextrose agar. Colonies appear in 1 to 4 weeks, depending on the organism. Colony morphology can differ with the medium. Identification characters include colony pigmentation of the surface and reverse sides, texture, growth rate and distinctive morphological structures, such as microconidia, macroconidia, spirals, pectinate branches, pedicels, and nodular organs; biochemical characteristics such as urease production and nutritional requirements. Specialized tests such as the ability to penetrate hairs in vitro, or mating tests (which are usually available only at reference laboratories) may be used occasionally.

Differential media (e.g., bromocresol purple-milk solids glucose) can be helpful during differentiation.

The development of microscopic structures may be enhanced by use of sporulation media such as lactrimel, pablum cereal or oatmeal agars. Conditions for inducing macroconidial formation vary from species to species: e.g., for *M. canis*, somewhat depauperate media such as rice grains; for *T. mentagrophytes* and *M. persicolor*, Sabouraud agar with 3 to 5% added sodium chloride; and for *M. equinum*, niger seed medium (Purim *et al.*, 2009). A series of vitamin and amino acid test agars are available as the Trichophyton agars (Difco) and is used to confirm the identity of several species with distinctive responses to growth substance. An unknown but characteristic nutrient requirement of *M. audouinii* is elucidated on autoclaved polished rice grains. The organism grows poorly on the grains and secretes a brownish pigment; *M. canis*, the main dermatophyte of differential diagnosis, grows well and usually secretes a yellow pigment.

Urea agar or broth is used to facilitate recognition of the small number of urease-negative species. BCP-milk solids-glucose agar may be used to differentiate a number of dermatophytes, particularly, *T. rubrum, T. mentagrophytes, M. persicolor, M. equinum, T. soudanense* and *T. megninii*, on the basis of their differences in the release of ammonium ion from casein and the catabolite repression of this process by glucose. The most common use of this medium is to differentiate the constitutively ammonifying *T. mentagrophytes* from *T. rubrum*; in which ammonification is suppressed and radial growth is restricted by glucose for approximately the first 10 days of growth at 25^{o}C. With the former fungus, the BCP indicator in the medium turns from its original sky blue color to violet within 4 to 7 days, indicating a pH change to alkaline, whereas with the latter fungus, the sky blue color indicating neutral pH is maintained until after 10 to 14 days.

A confirmatory test for atypical isolates of dermatophyte is the in vitro hair perforation test of Ellis *et al*., (2007). This test relies on the development by certain dermatophytes of specialized perforating organs invading detached hairs and engendering conspicuous conical pits at right angles to the long axis of the hair. The most common use of this test is to differentiate atypical isolates of *T. mentagrophytes* (perforation positive) from atypical *T. rubrum* (negative), but it is also useful for many other determinations, including differentiation of atypical *M. canis* (positive) from *M. audouinii* and *M. equinum* (negative).

From a clinical point of view, for definition of species or for performance of an epidemiological study, it is important to have a reliable method for the identification of fungal species. Recently, several molecular studies have focused on sequence analysis of the ribosomal DNA (rDNA) and in particular on the internal transcribed spacer (ITS) regions that represent organism diversity. Sequence analysis of the ITS regions has proven to be a useful tool for phylogenetic delineation and for the identification of some fungal pathogens (Hasegawa and Yamaguchi, 2001; Iwen *et al*., 2002; Yoshida *et al*., 2006).

- **Internal Transcribed Spacer**

Internal transcribed spacer (ITS) refers to the spacer DNA (non-coding DNA) situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome or the corresponding transcribed region in the polycistronic rRNA precursor transcript. In bacteria and archaea, ITS is located between the 16S and 23S rRNA genes. On the other hand, there are two ITS's in eukaryotes; ITS1 is located between 18S and 5.8S rRNA genes, while ITS2 is between 5.8S and 25S (in plants, or 28S in animals) rRNA genes. ITS1 corresponds to the ITS in bacteria and archaea, while ITS2 originated as an insertion that interrupted the ancestral 23S rRNA gene (Lafontaine and Tollervey, 2001; Scott, 2011).

Genes encoding ribosomal RNA and spacers occur in tandem repeats that are thousands of copies long, each separated by regions of non-transcribed DNA termed intergenic spacer (IGS) or non-transcribed spacer (NTS). Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it;

i.) is easy to amplify even from small quantities of DNA (due to the high copy number of rRNA genes), and

ii.) has a high degree of variation even between closely related species. This can be explained by the relatively low evolutionary pressure acting on such non-functional sequences.

The ITS region is the most widely sequenced DNA region in molecular ecology of fungi (Peay *et al*., 2008) and has been recommended as the universal fungal barcode sequence (Schoch *et al*., 2012). It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small-and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1+ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences.

2.6. Treatment of Cutaneous Mycoses

This section contains a survey of established and recent trends in the treatment of cutaneous mycoses and is not intended to be prescriptive of therapy in individual cases. The most noteworthy recent trend in cutaneous mycoses therapy is the proliferation of new drugs and even new classes of drugs, such as the allylamines, the orally active triazoles and hydroxypyridones. The new agents are rendering some previously difficult to treat conditions susceptible to rapid resolution. The strong biological variability of the infection, however, has so far prevented the

emergence of a single agent or regimen effective against all manifestations of these diseases. The relative cost of different therapies has also been an important factor in bringing about therapeutic diversity.

2.7. Prevention and Control of Cutneous Mycoses

Prevention and control of cutaneous infections must take into consideration the area invaded and the source of infection.

In scalp infection, routine inspection of scalps of young children should be performed at the beginning of the school term. All outbreaks in schools or institutions should be reported to the proper authorities. Good hygiene should be impressed upon those infected, and they must be instructed not to share headgear, combs, and brushes. Barbershop instruments (combs, brushes, and scissors) must be disinfected after use. All those infected must be treated promptly to prevent further spread of the infection.

Since skin infection caused by anthropophilic fungi can be transmitted by infected clothing, towels, and bedding, these items should be disinfected after use and infected individuals should not permit others to share them. Good hygiene and sanitation and fungicidal sprays and washes have been effective in controlling these infections. Avoid trainers which can retain sweat and promote a warm, moist environment and wear clean, loose-fitting underwear (Nice, 2014). Cutaneous infections are common in personnel handling animals (dogs, cats, and rodents). Avoid touching pets with bald spots, as they are often carriers of pathogens. Many of these infections are subclinical; therefore, routine wearing of protective clothing, especially gloves, is recommended.

Prevention of toe infection may be enhanced by using good foot hygiene (includes regular washing of the feet, thorough drying, and application of foot powder); avoiding excessive moisture and occlusion by wearing sandals or other well-ventilated shoes; avoiding prolonged

wetting or dampness of the feet; avoiding trauma to the feet, especially blistering by ill-fitting footgear; and not sharing towels, socks, or shoes. Since the infection is considered contagious, i.e., transferred by infected shed skin scales, control may be accomplished by educating infected individuals not to expose others by walking barefoot near swimming pools, locker rooms, swamp and public showers and by not sharing footgear. Frequent hosing of floors of public baths, swimming pools, etc., and discouraging antifungal foot dips (which may harbor the pathogens) near swimming pools may be helpful as preventive measures.

2.8. Epidemiology and Risk Factors of Cutaneous Mycoses

Cutaneous mycoses are worldwide in geographical distribution. The distribution, epidemiology, frequency and the causative agents involved vary from place to place depending upon the geographic region studied, climatic variations, population density, health care, immigration, culture, socioeconomic level of the population, time of study, presence of domestic animals and age of the individual (Das *et al*., 2009; Venkatesan *et al*., 2007). According to the World Health Organization survey on the incidence of cutaneous mycoses, about 20% of the people worldwide present with the infection (Marques *et al.*, 2000). In the United States alone, this translates into an economic impact on the health care system estimated to exceed $400 million a year for treatment alone. The disease does not spare people of any age (Vander Straten *et al.*, 2003).

Variation in the distribution pattern of cutaneous infections among different countries of the world is evident in the studies of Ellabib and Khalifa (2001) and Anosike *et al*. (2005). This distribution pattern of cutaneous infections in different part of the world has been attributed to factors of climate, life-style, and prevalence of immunodeficiency diseases in the community and also the reluctance of patients to seek treatment because of embarrassment or minor nature of

disease unless the condition becomes sufficiently serious to affect the quality of life (Hashem al sheikh, 2009).

Scarcity of adequate comparative data has undermined information on hot spot zones and control management strategies of cutaneous mycoses especially among rice farmers in Nigeria. Ibrahim and Mohammed (2004) and Nweze (2001) observed that a constant interaction by human with domestic animals promotes the prevalence of cutaneous mycotic infections. In a study carried out in Kano State Nigeria, 2150 itinerant quranic scholars were screened. Only 9.5% were found to be infected and the age group 10-14 years was most affected. *T. rubum* (50.2%) was the most prevalent followed by *M. audouinii* (26.5%). *T. rubrum* was the only dermatophyte that was recovered from all sites apart from the buttocks (Adeleke *et al*., 2008).

In a recent and more expanded study involving several states in Central Nigeria, a total of 28,505 primary school children aged between 3 and 16 years were sampled from 12 primary schools. Tinea capitis was found to be the most prevalent superficial mycoses. According to Ayanbimpe *et al*. (2008), the most common aetiological agent was *T. soudanense* (30.6%), followed by *M. ferrugineum* (7.7%) and *M. audouinii* (7.7%).

In a study in Lagos Southwest Nigeria carried out by Nwobu and Odugbemi (1990), involving patients attending the Lagos State University Teaching Hospital, Lagos found that 162 (41%) of the patients were infected by cutaneous mycoses. *Microsporum* species were the most common species (74.1%), followed by *E. floccosum* (4.3%). Cutaneous mycosis of the skin was the most prevalent (59.3%), followed by that of the hairs (27.2%) while infection of the nails (13.6%) was the least. Those aged five years and below had the lowest isolation rate of 3.7%.

In a study carried out in Anambra State, 1624 children with clinically suggestive lesions were screened. These children aged between 4 and 16 years were sampled in selected urban and rural areas of the State. Data showed that tinea capitis was the predominant clinical type. *T. tonsurans*

was the most prevalent etiological agent while *M. audouinii* was the least in occurrence (Nweze and Okafor, 2005). Emele and Oyeka (2008) in another larger study which involved a total of 47723 primary school children residing in different regions of Anambra State, found that 4498 (9.4%) had tinea capitis. The highest prevalence of the disease occurred in the Southern region of the state (12.6%). There was a significant difference in the prevalence of cutaneous mycoses among children in the urban and rural areas. Schools in the urban areas recorded lower prevalence of the disease, thereby emphasizing the role of locality in cutaneous mycoses. Moreso, tinea capitis occurred significantly more in children below 10 years of age than in those above this age.

Although the ecological and host factors involved in developing symptomatic infection are poorly known, known risk factors include foot dampness and abrasion combined with likely exposure to high fungal inoculum in communal aquatic facilities such as swimming pools, showers and swamps. Exchange of clothing, towels, and linen, either directly or through substandard communal laundering, is another recognized risk which may lead to outbreaks. Asymptomatic infection is common, especially in tinea pedis. Damp foot conditions may lead to aggravated symptoms due to mixed infection by fungi and bacteria.

2.9. Enzymes Secreted by Cutaneous Mycotic Fungi

Cutaneous mycosis is one of the most common fungal infections and is caused by filamentous keratinophilic fungi called dermatophytes and some fungi that use keratin as nutrient source during skin, hair and nail infection. Keratin is a fibrous protein molecule of high molecular weight, rich in cysteine, whose disulfide bridges and acetamide bonds guarantee its stability. This protein is produced by humans and other animals and is the main component of skin, nails and shells, having the function to protect and cover (Fraser and Parry, 2005).

Dermatophytes and associated fungi secrete varieties of enzymes that have different substrate specificities such as keratinases, protease, lipase and cellulase. These fungal pathogens secrete these enzymes to obtain the nutrients to develop and survive. The macromolecules that are present in the host tissue are used as a source of carbon, nitrogen, phosphorus and sulfur for dermatophytes and associated fungi (Peres *et al*., 2010). The keratinases secreted by these fungal pathogens catalyse the degration of keratin present in the host tissue into oligopeptides or amino acids that may be then assimilated by the fungi. Moreover, it had been suggested that released enzymes from these fungal pathogens also act as antigens and induce various degrees of inflammation (Jensen *et al*., 2007).

Among the wide variety of enzymes secreted by dermatophytes and associated fungi, protease enzymes are the most studied and are the major enzymes from dermatophytes and associated fungi involved in invasion and utilization of the stratum corneum of the host (Liu *et al*., 2014). It has been suggested that these pathogens secrete proteases in response to the presence of the components of the skin during tissue invasion. Some authors suggest that these pathogens secrete proteases to facilitate and are even necessary for an efficient adhesion of these pathogens to the host tissue. Furthermore, secreted proteases from these pathogens also trigger immune response (Peres *et al*., 2010). Although protease enzymes from these pathogens were initially studied, other enzymes apart from proteases were also studied.

2.10. Rice Field Work and its Occupational Hazards

Rice is one of the world's most important food crops. More than half of the people in the world eat this grain as the main part of their meals. Rice is a cereal grain and belongs to the grass family. But unlike other grains rice grow in shallow water. Farmers usually flood rice fields to supply the growing plants with moisture and kill weeds and other pests. Rice farming dominates

the agricultural sector of Nigeria, which itself dominates the economy. It is the single most important industry in Ebonyi State, Nigeria; contributing to the gross domestic product (GDP) of the country.

The initial preparation of the land (flooding, plowing the field, using manure, sowing the rice seeds in the soil) is done exclusively by men in the months of April and May. Seedlings (rice sprouts) are transplanted in the same field or a different one. This work is done exclusively by women. For the next 3 months, the rice plants are left to grow into paddy. Male laborers use pesticides during this time. Once the paddy turns light brown, water is drained and the field is left to dry. After the drained field is completely dry and the plants turn hay-color, paddy harvesting is done by females, usually in October and November (Shenoi *et al.*, 2005). Rice field workers are exposed to various agents including irritants like mud, cow dung or other manure, fertilizers, pesticides and dust from the dried plant and grains during thrashing. The outdoor work also contributes to the effect of sunlight and wind. In addition, during the plowing and planting season, and sometimes in the harvesting season, the feet are constantly immersed in water. These factors can predispose workers to dermatoses of the face, hand and feet (Shenoi *et al*., 2005).

The water-rice field is a suitable place for the growth of several types of bacteria, virus, fungi and different parasites. In the developing countries, diseases like malaria, tetanus, hookworm, leptospirosis, schistosomiasis, hay fever, farmer's lung, dermatitis, conjunctivitis and cold are very common. The very common occupational diseases are skin diseases (Sunil, 2002). The facts enabling the transmission of the different biological agents are poor housing, low sanitary standards, and inadequate nourishment and need to drink large quantity of water, which is not always pure. It may lead to general weakness and fatigue, possible sunstroke, intestinal troubles and diarrhea. In the developing countries, most of the workers in the rice field work on bare feet and as it is a family business all children are also involved in the work. Respiratory diseases due

to organic dust from field preparation and harvesting, dust mites, molds and other organic antigens, as well as smoke from burning rice stubble are also common in the developing countries.

Chemical agents, such as fertilizers, herbicides, pesticides and other extensively used substances increase the hazards for the workers. Human occupational exposure to pesticides is a significant cause of deaths worldwide and is suspected to contribute to serious long-term and chronic health hazards. The World Health Organization estimates that there is a minimum of three million acute, severe cases of pesticide poisonings and 20,000 unintentional deaths each year related to pesticide use in agriculture worldwide. Epidemiological research has revealed that farmers are at a higher risk for certain types of cancer than the general population. The greater incidence of cancers such as soft tissue sarcoma, non-Hodgkin's lymphoma and stomach cancer has been linked with exposure to pesticides and nitrates. Psychological stress factors such as economic problems, sense of insecurity, lack of social standing, lack of educational opportunities, lack of prospects and risk of unexpected calamities like heavy rain, storms and lightning are common in the developing countries (Sunil, 2002).

2.11. Overview of Medicinal Plants Used in the Study

- ***Azadirachta indica***

Azadirachta indica, also known as Neem or Nimtree (English) and Dogonyaro (Hausa) is a tree in the mahogany family Meliaceae. It is a species of the genus *Azadirachta*, and is native to India and the Indian subcontinent including Nepal, Pakistan, Bangladesh and Sri Lanka. It is typically grown in tropical and semi-tropical regions. Neem trees now also grow in islands located in the southern part of Iran. Its fruits and seeds are the source of neem oil.

Neem is a fast-growing tree that can reach a height of 15-20 metres (49-66 ft), and rarely 35-40 metres (115-131 ft). It is evergreen, but in severe drought it may shed most or nearly all of its leaves. The branches are wide and spreading. The fairly dense crown is roundish and may reach a diameter of 15-20 metres (49-66 ft) in old, free-standing specimens. The neem tree is very similar in appearance to its relative, the Chinaberry (*Melia azedarach*). The opposite, pinnate leaves are 20-40 centimetres (7.9-15.7 in) long, with 20 to 31 medium to dark green leaflets about 3-8 centimetres (1.2-3.1 in) long. The terminal leaflet is often missing. The petioles are short. The (white and fragrant) flowers are arranged in more-or-less drooping axillary panicles which are up to 25 centimetres (9.8 in) long. The inflorescences, which branch up to the third degree, bear from 150 to 250 flowers. An individual flower is 5-6 millimetres (0.20-0.24 in) long and 8-11 millimetres (0.31-0.43 in) wide. Protandrous, bisexual flowers and male flowers exist on the same individual tree. The fruit is a smooth (glabrous) olive-like drupe which varies in shape from elongate oval to nearly roundish, and when ripe is 1.4-2.8 centimetres (0.55-1.10 in) by 1.0-1.5 centimetres (0.39-0.59 in). The fruit skin (exocarp) is thin and the bitter-sweet pulp (mesocarp) is yellowish-white and very fibrous. The mesocarp is 0.3-0.5 centimetres (0.12-0.20 in) thick. The white, hard inner shell (endocarp) of the fruit encloses one, rarely two or three, elongated seeds (kernels) having a brown seed coat.

Medicinal Uses

Products made from neem trees have been used in India for over two millennia for their medicinal properties. Neem products are believed by Siddha and Ayurvedic practitioners to be antihelmenthic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative. It is considered a major component in siddha medicine and Ayurvedic and Unani medicine and is particularly prescribed for skin diseases. Neem oil is also used for healthy hair, to improve liver function, detoxify the blood, and balance blood sugar levels. Neem leaves have also been used to

treat skin diseases like eczema, psoriasis, etc (Porter, 2006). However, insufficient research has been done to assess the purported benefits of neem.

- ***Cymbopogon citratus***

Cymbopogon citratus commonly known as lemongrass belongs to the family Poaceae (grasses) (Shadab *et al.*, 1992). This plant is commonly cultivated as culinary and medicinal herb because of its scent, resembling that of lemon (*Citrus limon*). *Cymbopogon citratus* is a tall, perennial grass which is native to India and tropical regions of Asia. It is a coarse and tufted plant with linear leaves that grows in thick bunches, emerging from a strong base. It grows to as high as 3 meters with leaves of 1 to 1.5 centimeters in width that grows from a stalk of about 30 to 80 cm long with tough bulbous base. The leaves are used medicinally and are the source of essential oil. Its bright green leaves with sharp edges feature in appearance similar to that of grass. It flourishes in fertile, well-draining sandy soils under tropical climates receiving heavy rain. The plant usually grows wild and is easily promulgated. Propagation is by dividing the root clumps. Lemongrass does not usually produce seeds.

Medicinal Uses

The main chemical component found in lemongrass is citral, an aromatic compound, also known as lemonal. It is the presence of citral which account for lemongrass' lemon scent. It is an antimicrobial and therefore effective in destroying or inhibiting microorganisms. Fresh *C. citratus* grass contains approximately 0.4% volatile oil. Lemongrass is widely used as a culinary herb in Asian cuisine and also as medicinal herb in India. It has a subtle citrus flavor and can be dried and powdered, or used fresh. It is often used as a tea and in preparations such as *kadha*, which is a traditional herbal 'soup' used against coughs, colds, etc in African countries such as Togo and the Democratic Republic of the Congo and Latin American countries such as Mexico. Research

shows that lemongrass oil has antifungal properties (Shadab *et al.*, 1992). The effect is attributed in part to the geraniol (alpha-citral) and neral (beta-citral) constituents. In a 13-oil study, lemongrass oil was found to be among the most active against human fungal strains, inhibiting 80% of strain with inhibition zones more than 10 mm in diameter.

- ***Senna alata***

Senna alata Linn is an important medicinal plant, as well as an ornamental shrub in the family Fabaceae. It is known as emperor's candlesticks (Natural Resources Conservation Service, 2008), candle bush, ringworm shrub, or candletree. It is also commonly known as 'Rai dore' in Hausa, 'Asuwon oyinbo' in Yoruba, and 'Omirima' in Igbo (Arbonnier, 2004). *S. alata* can be found in Nigeria, Malaysia, Australia, Thailand, tropical America and many other parts of the world. In the tropics, it grows up to an altitude of 1,200 m. The shrub stands 3-4 m tall, with leaves 50-80 cm long. The leaves close in the dark. The inflorescence looks like a yellow candle. The fruit, shaped like a straight pod, is up to 25 cm long. Its seeds are distributed by water or animals. The seed pods are nearly straight, dark brown or nearly black, about 15 cm long and 15 mm wide. On both sides of the pods is a wing that runs the length of the pod. Pods contain 50 to 60 flattened, triangular seeds. This species is easy to grow from the seed. They may either be sown directly or started in a nursery.

Medicinal Uses

Senna alata is recommended for primary health care in Thailand to treat ringworm and skin diseases (Farnsworth and Bunyaprapatsara, 1992). It is locally used in Nigeria in the treatment of several infections which include ringworm and parasitic skin diseases. The leaf of this plant was reported to be useful in treating convulsion, abnormal pain, oedema as and as purgative but it was especially useful in treating cutaneous mycoses (Ogunti and Elujobi, 1993).

- ***Senna occidentalis***

Senna occidentalis (Linn.), also known as coffee senna, septicweed or antbush in English is an herbaceous species that is native to the tropical and subtropical regions of the America. It belongs to the family Fabaceae (Leguminosae) and subfamily Caesalpinioideae (Silva *et al*., 2011). It is a low growing, sparsely branching annual or short-lived perennial plant up to 0.5-2 m high and having a characteristic foetid odour. The stems are reddish purple, erect, 4-angled when young, becoming rounded with age. The plant has a robust primary root with several laterals. The leaves are pale green on reddish stalks; alternate, pinnate, with 3-5 (sometimes 6) pairs of opposite ovate to lanceolate-elliptic leaflets, 25-100 mm long, 20-30 mm wide, rounded at the base. A conspicuous, dark-coloured gland occurs at the base of the petiole (leaf stalk) but not on the stalks of the leaflets. The flowers are pale to bright yellow, 20-30 mm in diameter, in 2-6 flowered axils of the upper leaves; sepals are red veined; 5 petals per flower, the 2 anterior ones are smaller than the others; fertile stamens 6, the two basal ones longer than the rest, 4 infertile stamens are reduced to tiny petal-like staminodes. The fruit is a dark brown, flattened, sickle-shaped pod with paler stripes along the edges when mature. Pods are 75-130 mm long, 8-10 mm wide, containing a single row of 25-35 seeds. The seeds are dark brown, flattened, hard, 5 mm long and 3 mm wide. The plant reproduces entirely by seed.

Medicinal Uses

Senna occidentalis is used in various traditional medicines for curing various diseases, with antibacterial, antifungal, anti-diabetic, anti-inflammatory, anticancer, antimutagenic and hepatoprotective activities (Yadav *et al.*, 2010). Its extract is reported to be beneficial in treating many skin diseases like eczema, rashes, ringworm, the seeds are roasted and boiled in water to produce tea as folk medicine. The paste of its leaves is externally applied on healing wounds, sores, itch, bone fracture, fever, ringworm and other skin diseases.

- ***Emilia sonchifolia***

Emilia sonchifolia (L.), also known as lilac tasselflower or cupid's shaving brush is a tropical flowering plant in the Asteraceae family (Natural Resources Conservation Service, 2008). It is a branching, perennial herb up to 40 cm (15.5 in) tall. Leaves are lyrate-pinnate with large terminal lobe, up to 10 cm (4 in) long, sometimes becoming purplish as they get old. One plant can produce several lilac or purplish flower heads. Fruits are oblong, reddish brown or off-white and containing many seeds. Seeds are long, compressed, having terminal tuft of soft hairs for wind dispersal. It is widespread in tropical regions around the world, apparently native to Asia and naturalized in Africa, Australia, the Americas and various oceanic islands.

It is an easily grown plant, propagated by seed and succeeding in most well-drained soils in a sunny position. Plants flower better when growing on nutritionally poor soils, producing much lusher growth on rich soils. Plants are drought tolerant once established.

Medicinal Uses

Emilia sonchifolia is a medicinal herb. It is used in traditional medicine for the treatment of fever, sore throat, diarrhoea, eczema, cuts and wounds and as an antidote for snake bites (Tanaka and Van Ke, 2007). The flower heads are chewed and kept in the mouth for about 10 minutes to protect teeth from decay (Manandhar, 2002).

CHAPTER THREE

MATERIALS AND METHODS

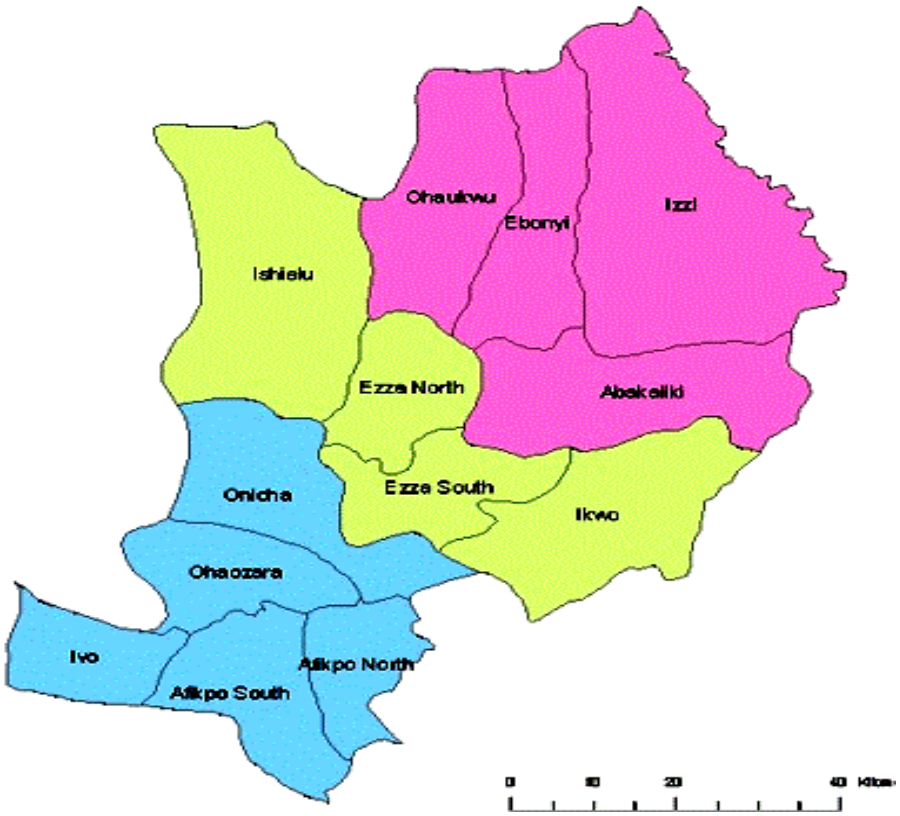
3.1. Study Area

Ebonyi State is located in the South Eastern part of Nigeria and is bounded to the North by Benue State, to the East by Cross River, to the South by Abia State and to the West by Enugu State. It is a tropical climatic zone. It has an average temperature of 28°C and humidity of 80–90% in dry season. The vegetation is guinea savanna characterized by a mixture of deciduous trees, shrubs, and grasses. Majority of the people in the study area are farmers who cultivate large quantities of rice, yam and cassava for sale. There are significant patches of swamp which form the basis of their agricultural activities. There are ponds, streams, springs and other stagnant water bodies scattered all over the area (Nworie *et al.*, 2014). Six communities including Okpera, Omega achara, Nkalafor, Agubia, Ndechi and Igbudu in Ikwo Local Government Area and Iboko, Ikeanyi, Amaguanyim, Sharon, Mgbo and Ezza Ohu in Izzi Local Government Area of Ebonyi State (Figure 1) were randomly chosen for this study.

3.2. Collection of Specimens

The study was approved by the Ethical Committee of the Federal Teaching Hospital, Abakiliki (Appendix 1) and an informed consent (Appendix 2) was taken prior to the collection of specimens. Specimens were collected during field visits undertaken during the transplanting and harvesting seasons (June, 2015-November, 2016). Specimens were also collected during house-to-house visit with the help of a guide who knew the local residents involved in rice field work. The farmers were randomly chosen and interviewed.

A.



B.

Figure 1: (A) Map of Nigeria showing Ebonyi State
(B) Map of Ebonyi State showing Ikwo and Izzi LGAs (www.joshuaproject.net)

A total of 2130 rice farmers including adults and children were screened for lesions suggestive of cutaneous mycoses on their skin, hair and nails. The method of Singh and Masuku (2014) was used to determine the sample size (Appendix 3). The lesions were evaluated for scaling, fissuring, redness of the skin and dystrophy of the nails. For obtaining the samples aseptically, the infected areas or lesions were cleaned with cotton wool soaked in 70% ethanol before collecting scrapings from the affected areas using sterile scapels. The scrapings were put into drug dispensary polythene bags and transported to Imo State University Owerri, Microbiology Laboratory for examination. The examination was carried out within 1 to 3 days. The information about the age, gender, location of lesion, educational level, size of family, nature of families' living accommodation, interaction with pets/domestic animals, use of manure, fertilizer, herbicides or pesticides and applications of antifungal therapy was obtained through questionnaire (Appendix 4).

3.3. Processing of Specimens

3.3.1. Examination of Direct KOH Mount

Direct microscopic examination was carried out as described by Cheesbrough (2010). A part of the hair follicles, scrapings of skin and nails was treated with two drops of a 5% potassium hydroxide (KOH) using a sterile forceps for 10 minutes to allow clearing of epithelial tissue surrounding the fungi so that the fungal structures can be exposed. The preparation was mounted on a clean glass slide and examined by direct microscopy for the presence of fungal hyphae and arthrospores under low power (X10) and high power (X40) magnification. The positive samples were processed for the isolation of fungal species on Sabouraud Dextrose Agar (SDA; Lab M).

3.3.2. Isolation of Fungi

Culture of specimen was carried out as described by Cheesbrough (2010). A portion of the specimen was inoculated on the prepared culture media - SDA supplemented with 0.5mg/ml cycloheximide (Sigma, USA) and 0.05mg/ml chloramphenicol (Yangzhou, China) in duplicates using spot inoculation technique. The inoculated plates were incubated at room temperature (28°C ± 2°C) for 7 days. Where fungal growth was observed, the organism was subcultured onto fresh media to obtain pure isolates used for identification.

3.4. Conventional Methods of Identification of Fungal Isolates

The fungal isolates were identified based on detailed conventional methods: colony morphology, pigmentation on reverse side of colony, growth rate, slide culture, hair perforation, urease test, rice grain test and germination tube test. The morphologies were compared with the standard description given by Ellis *et al.* (2007).

3.4.1. Lactophenol Cotton Blue (LCB) Mount Microscopy

Two drops of LCB were placed on a clean slide and using an inoculating needle, a small piece of the fungal mycelium free of the medium was removed. The mycelium was then transferred to the LCB, spread out and the slide was covered with a cover slip. The slide was then observed under low (x10) as well as high power (x40) of light microscope (Ellis *et al.*, 2007). The identification was based on the nature of hyphae, fruiting bodies, arrangement of stengmata, phialides and conidia.

3.4.2. Slide Culture Technique

The modified slide culture technique as described by Wijedasa and Liyanapathirana (2012) was used to observe the fungal isolate in its natural conditions. Petri dishes each containing a slide, cover slip and a filter paper were sterilized. SDA was prepared, aliquoted into small containers and sterilized. An aliquote of the media was pipetted aseptically onto the centre of the slide with a sterile Pasture pipette and allowed to set for 5 minutes. Each fungal isolate to be identified was inoculated onto the medium using a sterile straight wire and a sterile cover slip was placed on top of it. The filter paper was moistened with sterile distilled water to humidify the culture environment and the Petri dishes were incubated at room temperature. Fungal growth was observed using x10 and x40 light microscope on a daily basis. When sufficient growth had occurred, the cover slip was slightly raised and a drop of LCB was applied using a Pasture pipette. The slide was examined under x10 and x40 light microscope.

3.4.3. Hair Perforation Test

Hair perforation test was carried out as described by Ellis *et al*. (2007). The test was carried out to distinguish between isolates of dermatophytes, particularly *T. mentagrophytes* and *T. rubrum*. Sterilized human hair cut into pieces (1cm) was placed in a Petri dish to which 5ml of sterile distilled water and 3 drops of yeast extract were added. The plate was inoculated with small fragments of the test fungus from a 7-day-old SDA culture and incubated at room temperature ($28^oC \pm 2^oC$). Individual hair is removed at intervals up to 7 days and examined microscopically in 2 drops of lactophenol cotton blue stain for the presence or absence of perforations.

3.4.4. Urease Test

Urease test was carried out as described by Cheesbrough (2010). The test was done to differentiate between *T. mentagrophytes* and *T. rubrum*. Urea agar (Oxoid, UK) was prepared in test tubes according to manufacturer's instruction. The tubes were allowed to cool in a slant position. The test fungus was inoculated on the slant (one tube was left uninoculated to serve as control) and incubated at room temperature for up to 7 days. The appearance of a pink color indicated a positive test and a yellow-orange color indicated a negative test.

3.4.5. Rice Grain Test

Rice grain test was carried out as described by Ellis *et al*. (2007). The test was carried out for differentiation of *M. audouinii* and *M. canis.* Eight grams rice grains in 25 ml distilled water were autoclaved at 121°C and 15 psi of pressure for 15 minutes. The media was dispensed in Petri dishes and then the test fungi were inoculated on the rice grains and incubated at room temperature for 7 days.

3.4.6. Germination Tube Test

This test was carried out as described by Alexopolous (2004) to distinguish between isolates of *Candida*. A suspension of the *Candida* colonies was made on 0.5 ml serum in a test tube by picking 3 smaller colonies with a sterile inoculating loop. The tube was incubated for 30 minutes at 37°C. A drop of the suspension was placed on a slide using a Pasteur pipette and covered with a coverslip. The slide was examined microscopically using x40 for the production of long tube-like projections with no constrictions extending out from the yeast cells.

3.5. Molecular Method

Ten fungal isolates were sent to Macrogen Inc, Amsterdam, Netherlands for molecular identification using sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA. All isolates were maintained on SDA medium and stored at 4°C.

3.5.1. DNA Isolation

DNA was extracted from fresh fungal cultures on Sabouraud's agar medium. Colonies were picked up with sterilized toothpick, suspended in 100µl of sterilized saline in a PCR tube and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 50µl of InstaGene Matrix (Bio-Rad, USA). Incubated at 56 ^{o}C for 30 min and then heated at 100 ^{o}C for 10 min.

3.5.2. PCR Amplification, Purification and DNA Sequencing

Internal transcribed spacer 1 and 4 (ITS1 and ITS4) used for the PCR was designed as ITS1 forward primer 5' (TCC GTA GGT GAA CCT GCG G) 3' and ITS4 reverse primer 5’ (TCCTCC GCT TAT TGA TAT GC) 3’. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30µl reaction mixture by using a *EF-Taq* (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2 minutes, 35 cycles of 95°C for 1 minute, 55°C, and 72°C for 1minute each were performed, finishing with a 10 minute step at 72 °C.

The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM Big Dye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min,

followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

3.6. Screening for Extracellular Enzymes

3.6.1. Keratinase Production

The modified method of Wawrzkiewicz *et al*. (1991) was used to screen the test fungi for keratinase production on mineral salt media. Keratin azure (Sigma, USA) was used in place of chicken feathers as a source of keratin. Four (4) mg finely chopped keratin azure was suspended in 1 ml dimethylsulphoxide (DMSO) and the resulting suspension was added to 200 ml mineral salt medium. 15ml of this medium was dispensed aseptically into Petri dishes. The plates were inoculated with spore suspension of the test fungi and incubated at 25^{o}C for 7 days. All test fungi were assayed in duplicate. Keratinase activities of the test fungi were detected as clear zones around the colonies. The diameter of clear zone was measured to quantify activity.

3.6.2. Protease Production

The test was performed using the technique described by Vijayaraghavan and Vincent (2013). A 200 ml PDA incorporated with 10 g casein (Sigma, USA) as protein substrate was sterilized and dispensed into sterile Petri dishes. The plates were inoculated with spore suspension of the test fungi and incubated at 25^{o}C for 7 days. Colonies which showed clear zones around them indicated protease production. All test fungi were assayed in duplicate.

3.6.3. Lipase Production

Screening for lipase production was carried out using the gel diffusion assay described by Rua *et al.* (1993). Ten (10) µl of spore suspension of the test fungi was inoculated at the center of a 20ml

PDA media incorporated with 4ml of olive oil emulsion as lipid substrate. The olive oil emulsion consisted of 25% of olive oil and 75% of a 10% (w/v) gum arabic (Plus Chem., India) solution in water, mixed in a homogeneizer on an ice-bath for 15min. The plates were incubated at 25°C for 7 days. A clear zone of hydrolysis beneath and around the colony indicated a positive lipase activity.

3.6.4. Xylanase Production

The screening of xylanase enzyme was carried out as described by Dhulappa and Lingappa (2013). Ten plates containing PDA were added oat meal xylan 1% (v/v) as carbon source and inoculated with mycelia and spores of the test fungi. Crude xylan was recovered from breakfast oat meal purchased from a grocery store. The plates were incubated at 25^{o}C for 7 days. Colonies which showed clear zones around them were considered positive, regarding to xylanase enzyme production.

3.6.5. Cellulase Production

The fungal isolates were subjected to screening for their cellulolytic properties using the plate assay method of Whitaker *et al.* (2002). Ten (10) µl of spore suspension of each isolate was inoculated onto the center of PDA incorporated with 1% carboxylmethyl cellulose (CMC; Sigma) as carbon source. The plates were incubated at 25°C for 7 days before flooding with 0.4% Congo red and observed for lysis. The hydrolysis zones on the plate media were measured and recorded.

3.6.6. Collagenase Production

The ability of the test fungi to synthesize collagenase was screened on agar plates containing 20 g/L of peptone agar and 10 g/L of chicken sternal cartilage collagen as substrate. This method is a

modification of the method described by Cooper and Davidson (1965) and it differs in the use of chicken sternal cartilage collagen as substrate instead of calf skin collagen. Chicken sternal cartilage collagen was prepared and kept in 0.47 M acetic acid at 4ºC. Prior to use, the collagen was clarified by centrifugation and dialysed against 0.1 M sodium phosphate buffer, pH 7.6. The collagen was poured over the surface of peptone agar plates and allowed to gel. The plates were inoculated with the fungal strain and incubated at 25°C for 7 days. Collagenase-positive colonies were identified by a zone of hydrolysis around the colonies.

3.7. Collection and Preparation of Plant Materials

Fresh leaves of five medicinal plants; *Azadirachta indica*, *Cymbopogon citratus*, *Emilia sonchifolia*, *Senna alata* and *Senna occidentalis* were collected from herbalists in the study area. They were identified by Prof. F. N. Mbagwu of the Department of Plant Science and Biotechnology, Imo State University, Owerri, Imo State. The leaves were rinsed with distilled water and air-dried at room temperature for 7 days. The dried leaves were ground into fine powder using an electric blender. 50g of finely grounded leaves of each medicinal plant was extracted separately using 200ml of petroleum ether, n-hexane, chloroform and hot water in a soxhlet apparatus. The obtained liquid extracts were gently concentrated to dryness in a water bath at 40ºC for 12 hours and subsequently air-dried. The percentage yield of the solid extracts were evaluated and the extracts were stored in a refrigerator at 4°C until when required (Ibrahim *et al.*, 1997).

3.8. Qualitative and Quantitative Phytochemical Analysis of Plant Extracts

3.8.1 Qualitative Determination of Plant Secondary Metabolites

Hexane, chloroform, petroleum ether and hot water extracts were used for qualitative phytochemical analyses using standard procedures as described by Harborne (1998) and Kokate *et al*. (2004). The following qualitative tests were done:

- **Test for Flavonoid**

Lead acetate test: 2 mg of each plant extract was taken and 2 drops of 10% lead acetate solution was added. Appearance of yellow colour precipitate indicates the presence of flavonoid.

- **Test for Alkaloid**

Wagner's test: 2 mg of each plant extract was taken and 2 drops of Wagner's reagent were added and the formation of a reddish brown precipitate indicates the presence of alkaloid.

- **Test for Saponin**

Foam test: 5 mg of each plant extract was diluted with 20 ml distilled water and shaken well in a graduated cylinder for 15 min. The formation of foam to a length of 1cm indicated the presence of saponin.

- **Test for Tannin**

Ferric chloride test: 2 mg of each plant extract was taken and 1 ml of 5% ferric chloride was added. The development of dark bluish black color indicates the presence of tannin.

- **Test for Glycoside**

Glycoside test: 2 mg of each plant extract was dissolved in 1 ml of water and then aqueous NaOH solution was added. Formation of yellow color indicates the presence of glycoside.

- **Test for Terpenoid and Steroid**

Salkowski's test: 5 mg of each plant extract was dissolved in 2 ml chloroform and 2 ml concentrated sulphuric acid was added along the sides of the test tube. The upper layer turns red and lower layer turns yellow with green fluorescence, indicating the presence of the terpenoid and steroid compound in the extract.

3.8.2 Quantitative Determination of Plant Secondary Metabolites

The quantity of flavonoid, alkaloid, saponin, phenol and tannin in each plant material was determined using standard procedures as described by Harborne (1998) and Krishnaiah *et al.* (2009).

- **Determination of Total Flavonoid Content of Plant Material**

One gram of each plant sample was repeatedly extracted with 100ml of methanol at room temperature. The mixture was filtered through a Whatman filter paper into a pre-weighed 250ml beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed.

- **Determination of Total Alkaloid Content of Plant Material**

One gram of each plant sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and allowed to stand for 4 h. The mixture was filtered and the extract was

concentrated on a water bath to one quarter of the original volume. Concentrated NH_4OH was added by drop wise to the extract until the precipitation was complete. The solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH and then filtered. The alkaloid residue was dried and weighed.

- **Determination of Total Saponin Content of Plant Material**

One gram of each plant sample was put into a beaker and 100 ml of 20% C_2H_5OH was added. The sample was heated over a hot water bath for 4 hours with continuous stirring at 55^oC. The mixture was then filtered and the residue re-extracted with another 200 ml of 20% ethyl alcohol. The extract was reduced to 40 ml over a water bath at 90^oC. The concentrated extract was then transferred into a 250 ml separating funnel and 20 ml of $(CH_3CH_2)_2O$ was added to the extract and vigorously shaken. The aqueous layer was recovered while the $(CH_3CH_2)_2O$ layer was discarded and the purification process repeated. 60 ml of n-C_4H_9OH was added and the combined n-C_4H_9OH-extract was washed twice with 10 ml of 5% NaCl. The remaining solution was then heated in a water bath and allowed to evaporate to dryness and weighed.

- **Determination of Total Phenol Content of Plant Material**

The quantity of phenol was determined using spectrophotometric method. One gram of each plant sample was boiled with 50 ml of ether for 15 min. Five (5) ml of the boiled sample was pipetted into 50 ml flask and then 10 ml of distilled water was added. Two (2) ml of NH_4OH solution and 5 ml of concentrated amyl alcohol were added to the mixture. The sample was made up to the mark, left to react for 30 min for colour development and measured at 505 nm wavelength using spectrophotometer.

- **Determination of Total Tannin Content of Plant Material**

The quantity of tannin was also determined using spectrophotometric method. One gram of each plant sample was weighed into a 50 ml plastic bottle. 50 ml of distilled was added and stirred for 1 hour. The sample was filtered into a 50 ml volumetric flask and made up to the mark. 5 ml of the filtered sample was then pipetted into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M $K_4Fe(CN)_6.3H_2O$. The absorbance of the sample was measured with a spectrophotometer at 395 nm wavelength within 10 minutes.

3.9. In vitro Antifungal Activity Testing of Plant Extracts

3.9.1. Standard Antifungal Control Agent

A 125 mg terbinafine oral granules (Novartis, UK) was used in the study as quality control antifungal agent. Stock solution (128 μg/ml) was prepared by dissolving 12.8 mg of the drug in 100 ml DMSO (100 %) and stored at 4 ^oC.

3.9.2. Preparation of Inocula

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

3.9.3. Determination of Minimum Inhibitory Concentration (MIC) of Plant Extracts

Minimum inhibitory concentration (MIC) was determined using broth microdilution assay in accordance with protocol M38-A2 of the Clinical and Laboratory Standards Institute, CLSI (2008). Flat-bottomed 96 wells (8 columns and 12 rows) microdilution plates (Nunc, Denmark) were set up. Stock solutions (128 µg/ml) of the plant extracts were prepared from n-hexane (3.1, 2.4, 2.6, 3.4 and 3.8 grams); petroleum ether (2.2, 2.3, 2.6, 2.8 and 3.3 grams); chloroform (1.3, 1.1, 1.4, 2.1 and 2.1 grams) and aqueous (1.8, 1.6, 1.1, 1.5 and 2.1 grams) extracts of *A. indica, C. citratus, E. sonchifolia, S. alata* and *S. occidentalis* respectively by dissolving 12.8 mg of each plant extract in 100 ml dimethyl sulfoxide (100%). A 100 µl SDB medium was added to all 96 wells of the microdilution plate. Serial twofold dilutions were prepared in microdilution plate with 100 µl of the extracts with the 128 µg/ml concentrations in the first column wells, followed by further dilutions in SDB media. The plant extracts and the standard antifungal control agent were prepared in ranges from 2-64 µg/ml in the microdilution plate. Each microdilution well containing 100 µl of the twofold extract/drug concentrations was inoculated with 100 µl of the diluted inoculum suspension. For each test plate, the twelve vertical column wells were used for duplicate assay of the five different plants materials and terbinafine (antifungal control agent) while the eight horizontal row wells were used for the six different concentrations (64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml and 2 µg/ml) of each extract and two extract-free controls: one with 100 µl of medium and 100 µl of inoculum suspension (growth control) and the other with medium alone (sterility control). Empty microdilution plates were placed on top of the test plates to prevent the test plates from dehydration during incubation. The microdilution plates were incubated at 25°C for 7 days.

Endpoint values were determined visually every 24 hour until the indication of turbidity in the growth control wells. The mean MIC was obtained for each species-extract-standard agent

combination tested. MICs of the plant extracts and the standard agent were reported as the concentration at which 90% (MIC_{90}) and 100% (MIC_{100}) of the isolates were inhibited when compared to the growth control respectively.

3.9.4. Determination of Minimum Fungicidal Concentration (MFC) of Plant Extracts

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

3.10. Statistical Analysis

The prevalence of cutaneous mycoses among rice farmers in Ebonyi State was expressed in percentage. In order to establish association of age, gender, location of lesion, educational level, size of family, nature of families' living accommodation and interaction with pets/domestic animals with prevalence of cutaneous mycoses among the farmers, the analysis of variance (ANOVA) statistical test was used at $p<0.05$ significant level. Statistical computation was done using Statistical Package for Social Science (SPSS) version 22 for windows.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of Rice Farmers with Cutaneous Lesions

A total of 2130 rice farmers were sampled from six communities each of Ikwo and Izzi Local Government Areas of Ebonyi State (Tables 1 and 2). Of the 2130 rice farmers screened, only 182 (8.54%) persons presented with lesions suggestive of cutaneous mycoses and comprised of 22 (1.03%) males and 90 (4.23%) females in Ikwo LGA (Table 3) and 16 (0.75%) males and 54 (2.53%) females in Izzi LGA (Table 4). The tables showed that the females were more infected with cutaneous mycoses than their male counterparts. The 16-26 years age group was the most infected with the infection in both Ikwo LGA 48 (2.25%) and Izzi LGA 23 (1.08%).

4.2 Isolation and Identification of Fungi

Of the 182 specimens collected, 106 were positive by KOH mount whereas all the specimens collected were positive by culture. The positive KOH mount showed fragments of septate and nonseptate hyphae, and budding yeast cells. A total of 197 fungal isolates differentiated into 54 (27.41%) dermatophytes (48 rice farmers were infected each with one species while 6 rice farmers were infected with two different dermatophytes) and 143 (72.59%) non-dermatophytes (134 farmers were infected each with one species while 9 farmers were infected with two different species) were recovered from the specimens (Figure 2). The macroscopically and microscopically (Figure 3) identified fungal species were further confirmed by molecular identification using internal transcribed spacer (ITS) region. The ITS regions were successfully amplified and sequenced from all the fungal isolates by the fungus-specific universal primers ITS1 and ITS4 at Macrogen Inc, Amsterdam, Netherlands. The phylogenetic trees of the identified fungal species are shown in Appendix 5. Most *Trichophyton* species had identical sizes.

Each identified fungal species was shown to have ITS1 base pair sequences identical to that of the respective standard strain. The results showed that the similarities among the fungal genera recovered are higher, which is more than 90% identities.

4.3 Prevalence and Distribution of Cutaneous Mycoses

The prevalence rates of cutaneous mycoses in Ikwo and Izzi Local Government Areas of Ebonyi State were 5.26% (112/2130) and 3.28% (70/2130) respectively (Figure 4) whereas the overall prevalence rate of the infection among rice farmers in both LGAs was 8.54% (182/2130). *Trichophyton tonsurans* was the predominant dermatophyte species found in 8.12% (16/197) cases of fungi species isolated in Ikwo LGA, followed by *T. mentagrophytes* 4.57% (9/197), *T. rubrum* 1.52% (3/197), *T. soudanense* 1.52% (3/197), *Microsporum gypseum* 0.51% (1/197) and 0.51% (1/197) cases of *M. canis* (Figure 5). The distribution of dermatophyte species isolated in Izzi LGA also showed that *T. tonsurans* was the predominant dermatophyte species 3.55% (7/197), followed by *T. mentagrophytes* 2.54% (5/197), *T. rubrum* 2.03% (4/197), 1.02% (2/197) cases of *T. soudanense* and *M. gypseum*, and *M. canis* 0.51% (1/197) (Figure 6). *Aspergillus flavus* was the predominant non-dermatophyte species found in 34 (17.26%) cases of fungi isolated, followed by *A. tamarii* 15 (7.61%), *A. nomius* 15 (7.61%) and 5 (2.54%) cases of *Candida albicans* in Ikwo LGA (Figure 7). Figure 8 showed that *A. flavus* 30 (15.23%) was the predominant non-dermatophyte species isolated, followed by *A. tamarii* 26 (13.20%), *A. nomius* 15 (7.61%) and *C. albicans* 3 (1.52%) in Izzi LGA.

Table 1: Distribution of rice farmers screened for lesions suggestive of cutaneous mycoses in Ikwo LGA of Ebonyi State

Community	Number sampled	Number infected
Okpera	388	41
Omega achara	201	10
Nkalafor	274	18
Agubia	90	10
Ndechi	41	7
Igbudu	150	26
Total	1144	112

Table 2: Distribution of rice farmers screened for lesions suggestive of cutaneous mycoses in Izzi LGA of Ebonyi State

Community	Number sampled	Number infected
Iboko	142	12
Ikeanyi	297	28
Amaguanyim	180	8
Sharon	118	5
Mgbo	122	7
Ezza ohu	127	10
Total	986	70

Table 3: Demographic characteristics of rice farmers that presented with lesions suggestive of cutaneous mycoses in Ikwo LGA of Ebonyi State

Age Groups (Years)	Gender		Number of specimens collected
	Male	Female	
5-15	1	9	10
16-26	5	43	48
27-37	10	11	21
38-48	3	20	23
49-59	3	7	10
Total	22	90	112

Table 4: Demographic characteristics of rice farmers that presented with lesions suggestive of cutaneous mycoses in Izzi LGA of Ebonyi State

Age Groups (Years)	Gender		Number of samples collected (%)
	Male (%)	Female (%)	
5-15	1	8	9 (0.42)
16-26	2	21	23 (1.08)
27-37	7	11	18 (0.85)
38-48	5	10	15 (0.70)
49-59	1	4	5 (0.23)
Total	16 (0.75)	54 (2.53)	70 (3.28)

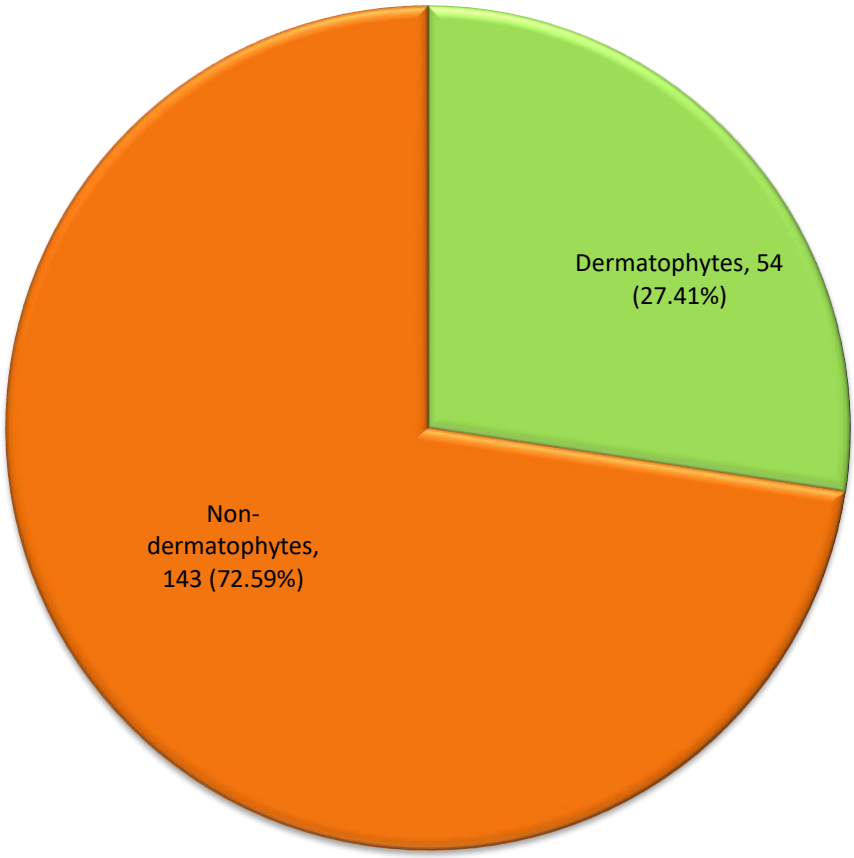
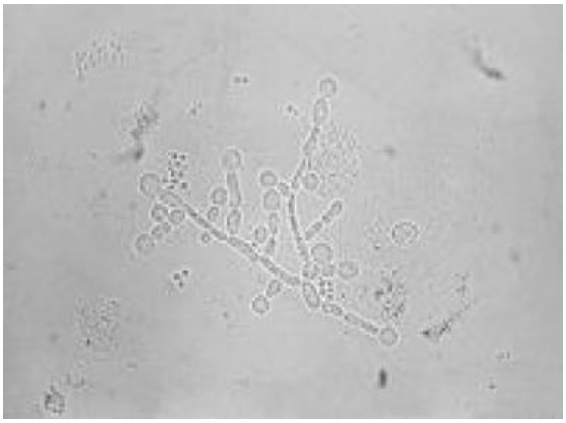
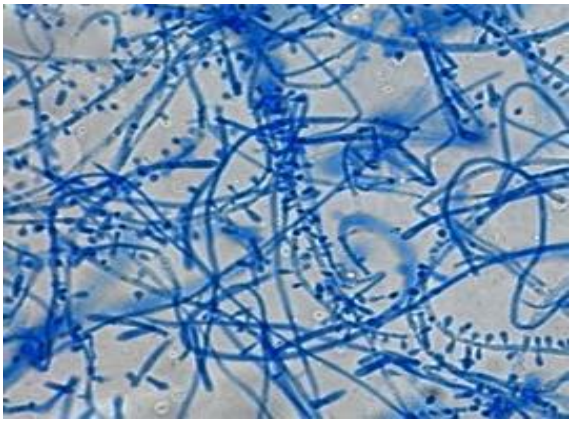


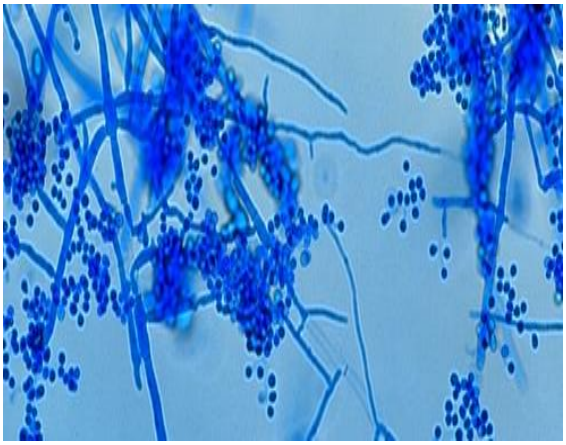
Figure 2: Percentage occurrence of dermatophytes and nondermatophytes isolated from rice farmers in Ikwo and Izzi LGAs of Ebonyi State



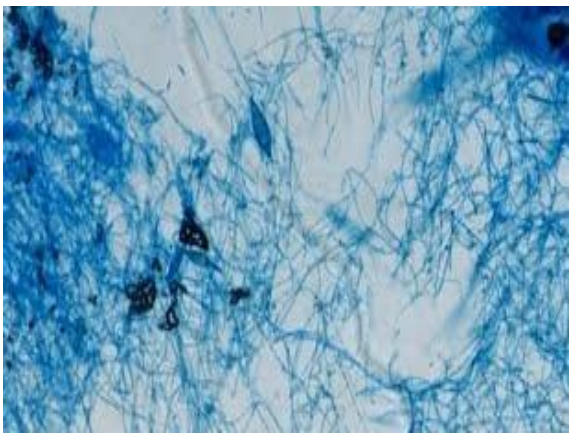
KOH mount showing fungal hyphae



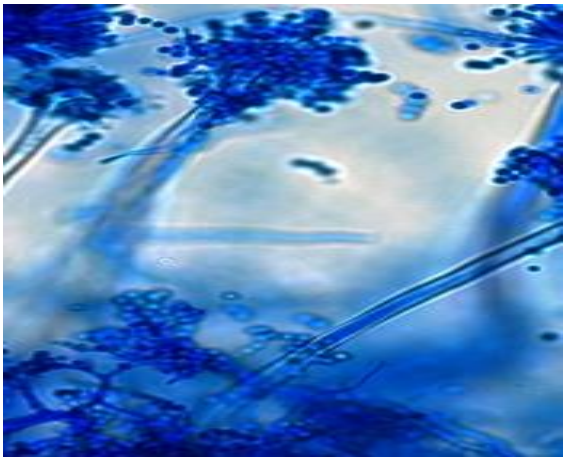
T. mentagrophytes showing numerous tear-drop microconidia and club-shape macroconidia



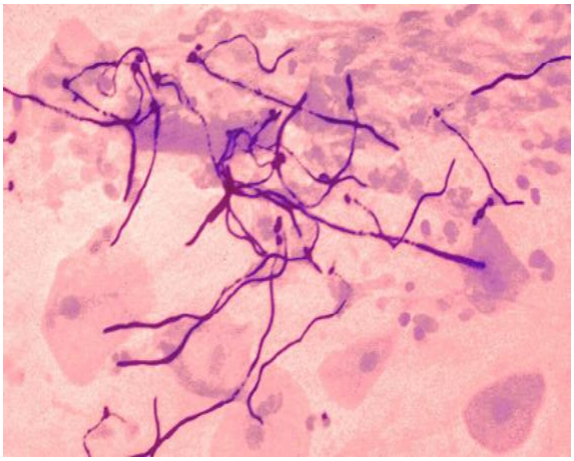
T.rubrum showing showing clavate micro-conidia and pencil-shaped macroconidia



Microsporum canis showing clavate micro-conidia and spindle-shaped macroconidia



Aspergillus flavus showing rough, radial, biseriate conidial heads and uncoloured conidiophore



Candida albicans showing pseudohyphae

Figure 3: Microscopic characteristics of fungal isolates

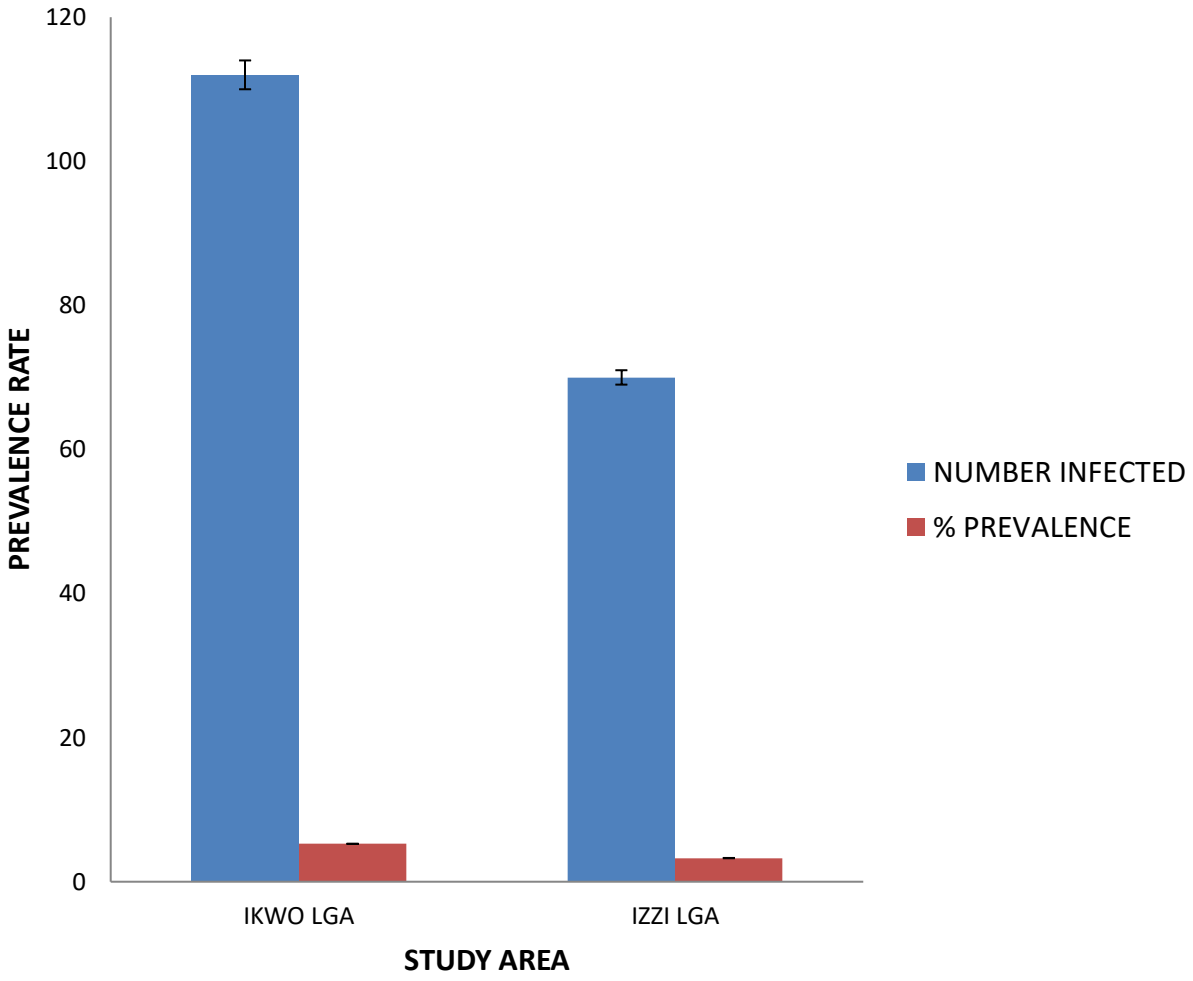


Figure 4: Prevalence rates of cutaneous mycoses among rice farmers in Ikwo and Izzi LGAs of Ebonyi State

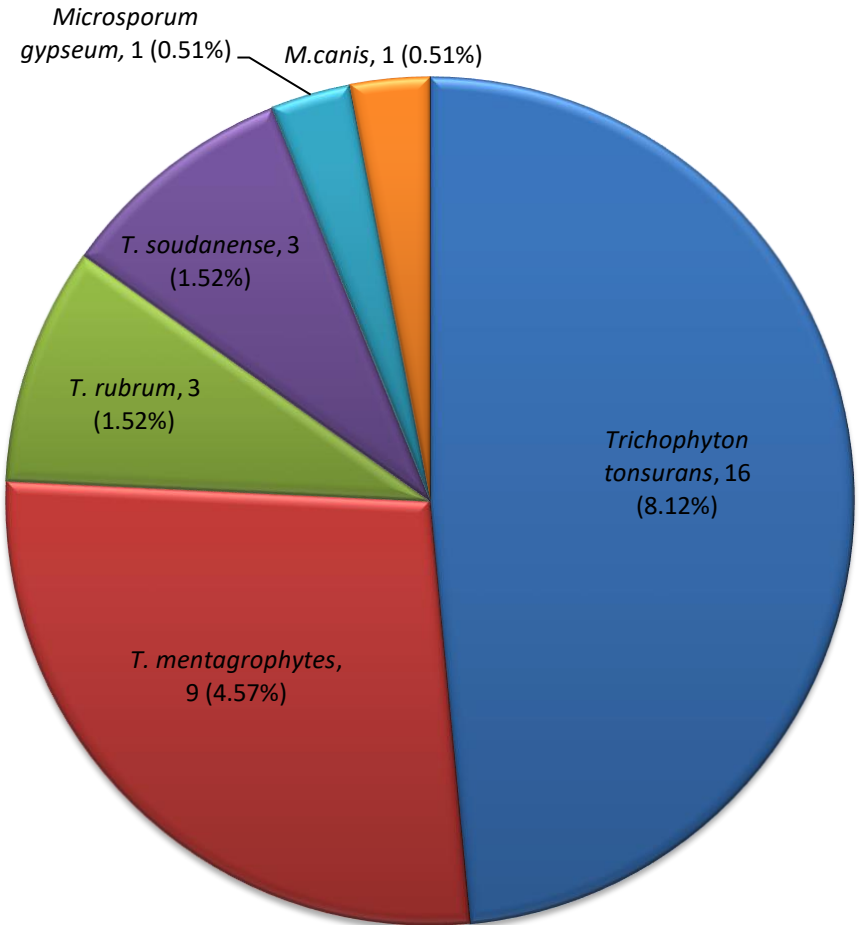


Figure 5: Percentage distribution of dermatophyte species isolated from rice farmers in Ikwo LGA of Ebonyi State

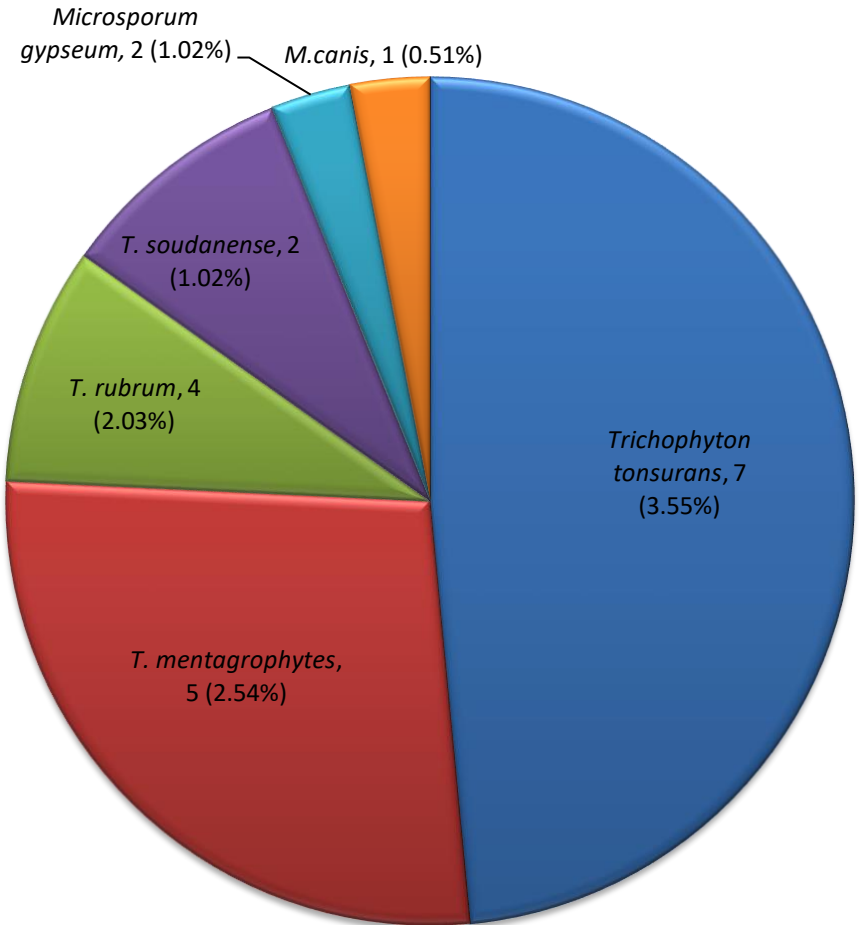


Figure 6: Percentage distribution of dermatophyte species isolated from rice farmers in Izzi LGA of Ebonyi State

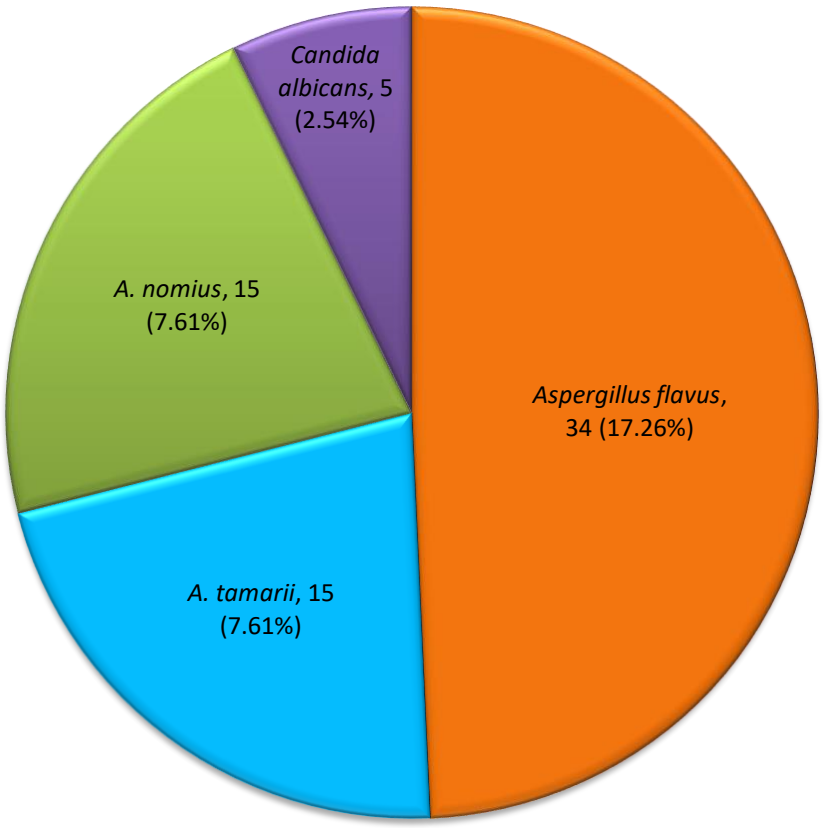


Figure 7: Percentage distribution of non-dermatophyte species isolated from rice farmers in Ikwo LGA of Ebonyi State

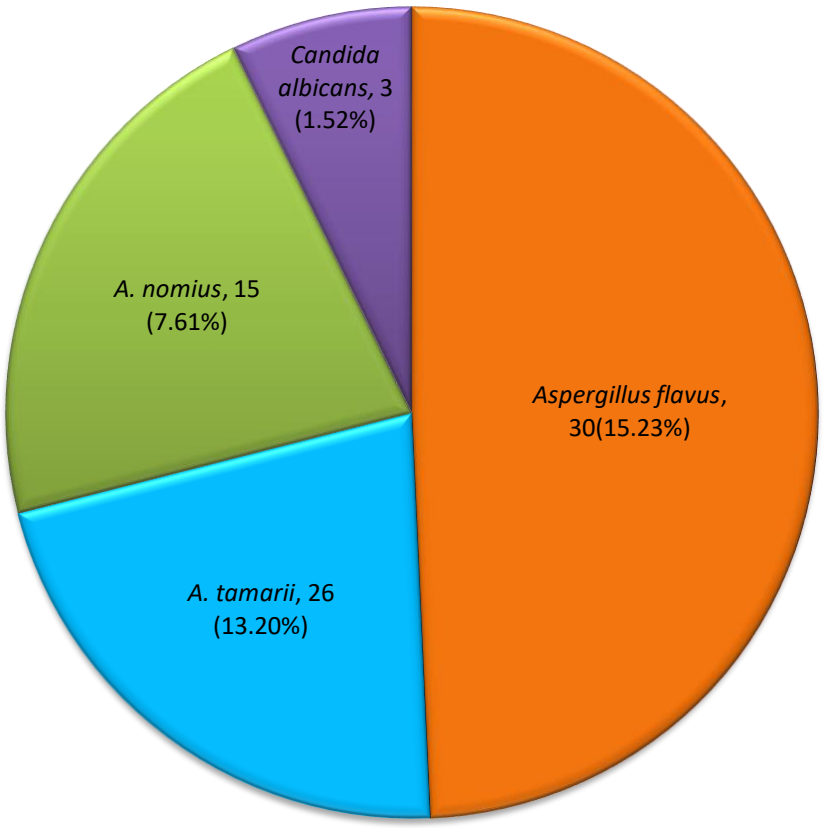


Figure 8: Percentage distribution of non-dermatophyte species isolated from rice farmers in Izzi LGA of Ebonyi State

A total of 54 dermatophyte species were identified, out of which 33 (16.75%) and 21 (10.66%) were isolated from Ikwo and Izzi LGAs respectively. Highest percentage frequency of 10 (5.08%) dermatophytic fungi was observed among rice farmers in Okpera community, followed by 8 (4.06%) in Igbudu, Nkalafor 6 (3.04%), Agubia 4 (2.03%), Ndechi 3 (1.52%) and Ndechi 3 (1.52%) in Ikwo LGA. *T. tonsurans* 5 (2.54%) was a major cause of the infection in Okpera and Igbudu communities whereas *T. mentagrophytes* 3 (1.52%) caused most of the infection in Agubia (Figure 10). Ikeanyi community in Izzi LGA recorded the highest percentage frequency of dermatophytic fungi 7 (3.55%), followed by Amaguanyim 4 (2.03%). Iboko and Ezza ohu recorded 3 (1.52%) each while Sharon and Mgbo recorded 2 (1.02%) each. *T. tonsurans* 3 (1.52%) was the most frequently isolated species in Ikeanyi community (Figure 11). Of the 143 non-dermatophyte species identified in the study, 69 (35.03%) and 74 (37.56%) were isolated from Ikwo and Izzi LGAs respectively. Highest percentage frequency 17 (8.63%) of non-dermatophytic fungi was observed among rice farmers in Nkalafor community followed by Agubia 15 (7.61%), Okpera and Ndechi had 10 (5.07%) each while Igbudu and Omega achara had 9 (4.57%) and 8 (4.08%) respectively in Ikwo LGA. *A. flavus* 13 (6.60%) and *A. nomius* 6 (3.04%) were the most frequently isolated species in Nkalafor and Agubia respectively whereas *A. tamarii* 4 (2.03%) and *C. albicans* 2 (1.02%) were the most frequently isolated species in Omega achara and Nkalafor respectively (Figure 12). In Izzi LGA, highest percentage frequency 14 (8.63%) of non-dermatophytic fungi was observed among rice farmers in both Ikeanyi and Ezza ohu communities, followed by 12 (6.09%) in both Amaguanyim and Sharon, and 11 (5.58%) in both Iboko and Mgbo. *A. flavus* 8 (4.06%), *A. tamarii* 6 (3.04%), *A. nomius* 5 (2.54%) and *C. albicans* 2 (1.02%) occurred mostly in Agbanyim, Sharon, Ikeanyi and Mgbo respectively (Figure 13).

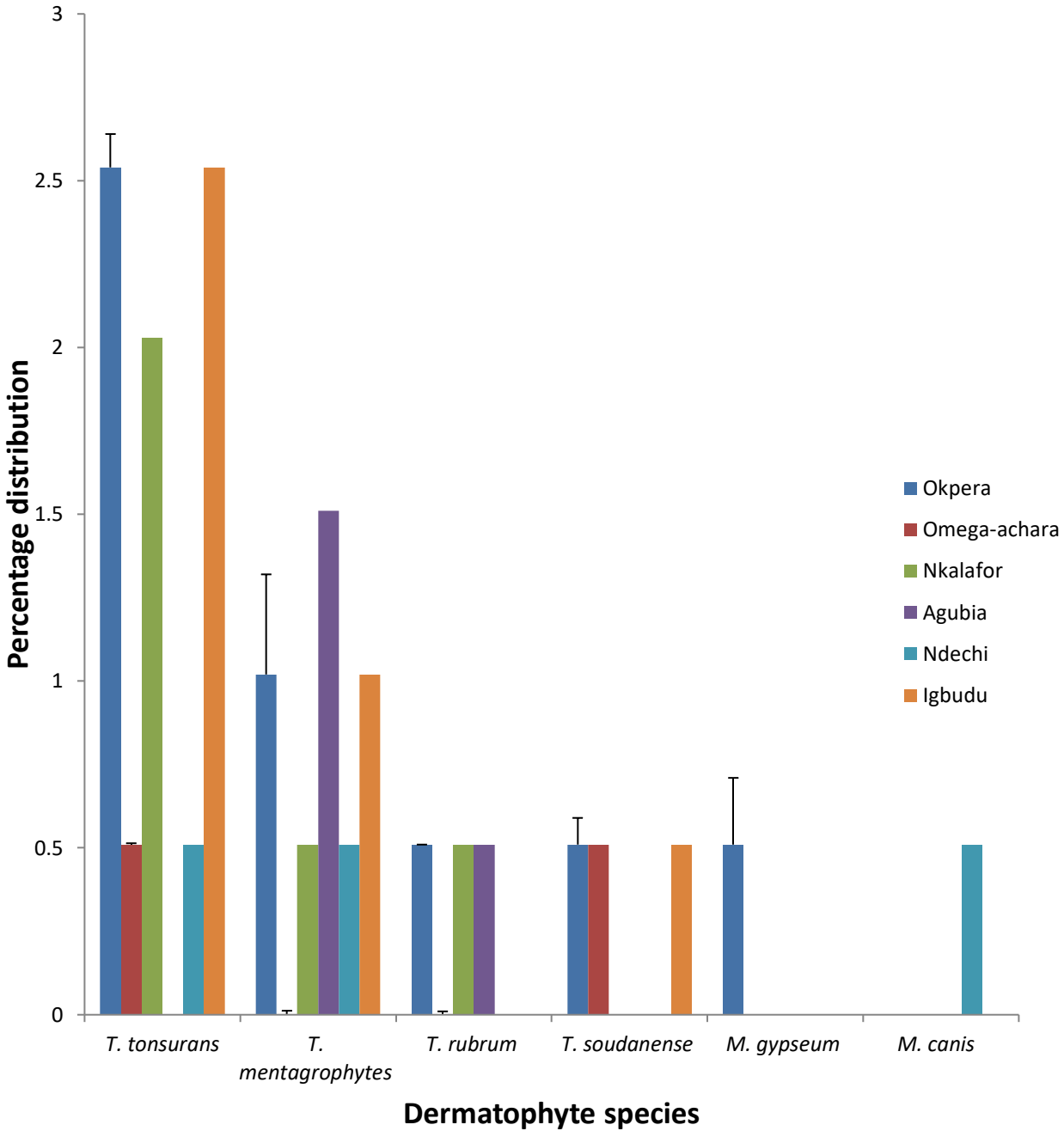


Figure 9: Percentage distribution of dermatophyte species by communities in Ikwo LGA of Ebonyi State

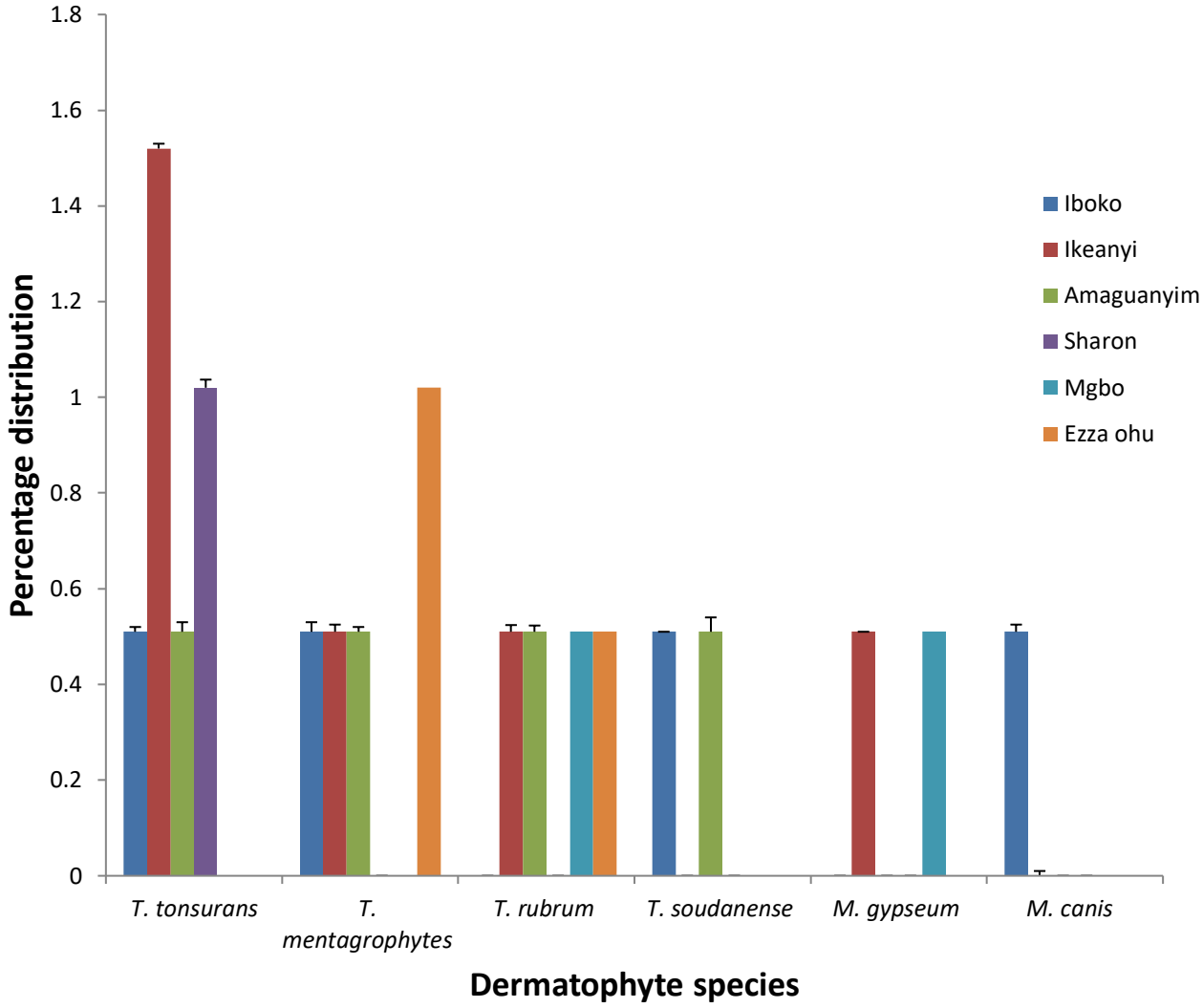


Figure 10: Percentage distribution of dermatophyte species by communities in Izzi LGA of Ebonyi State

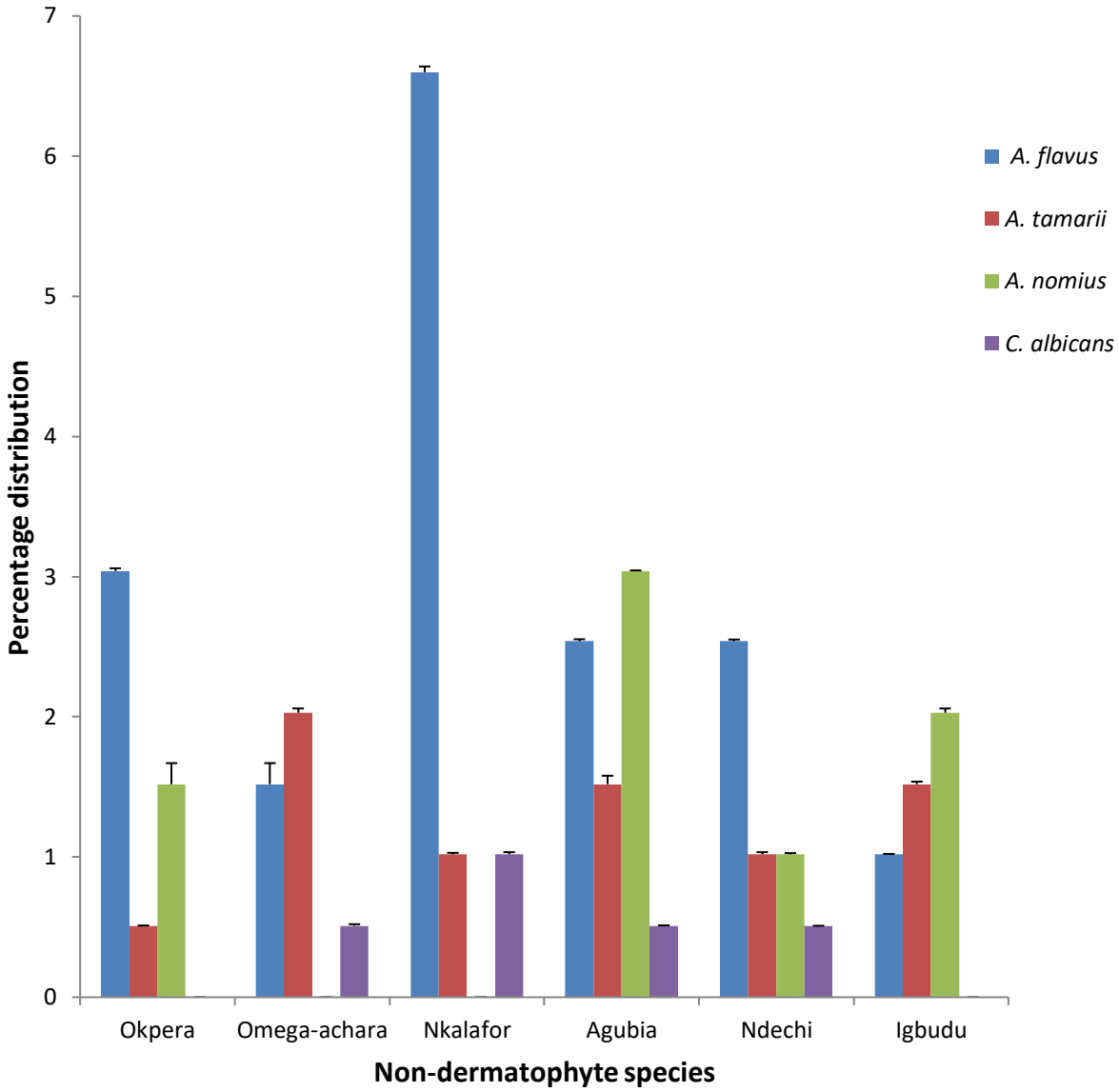


Figure 11: Percentage distribution of non-dermatophyte species by communities in Ikwo LGA of Ebonyi State

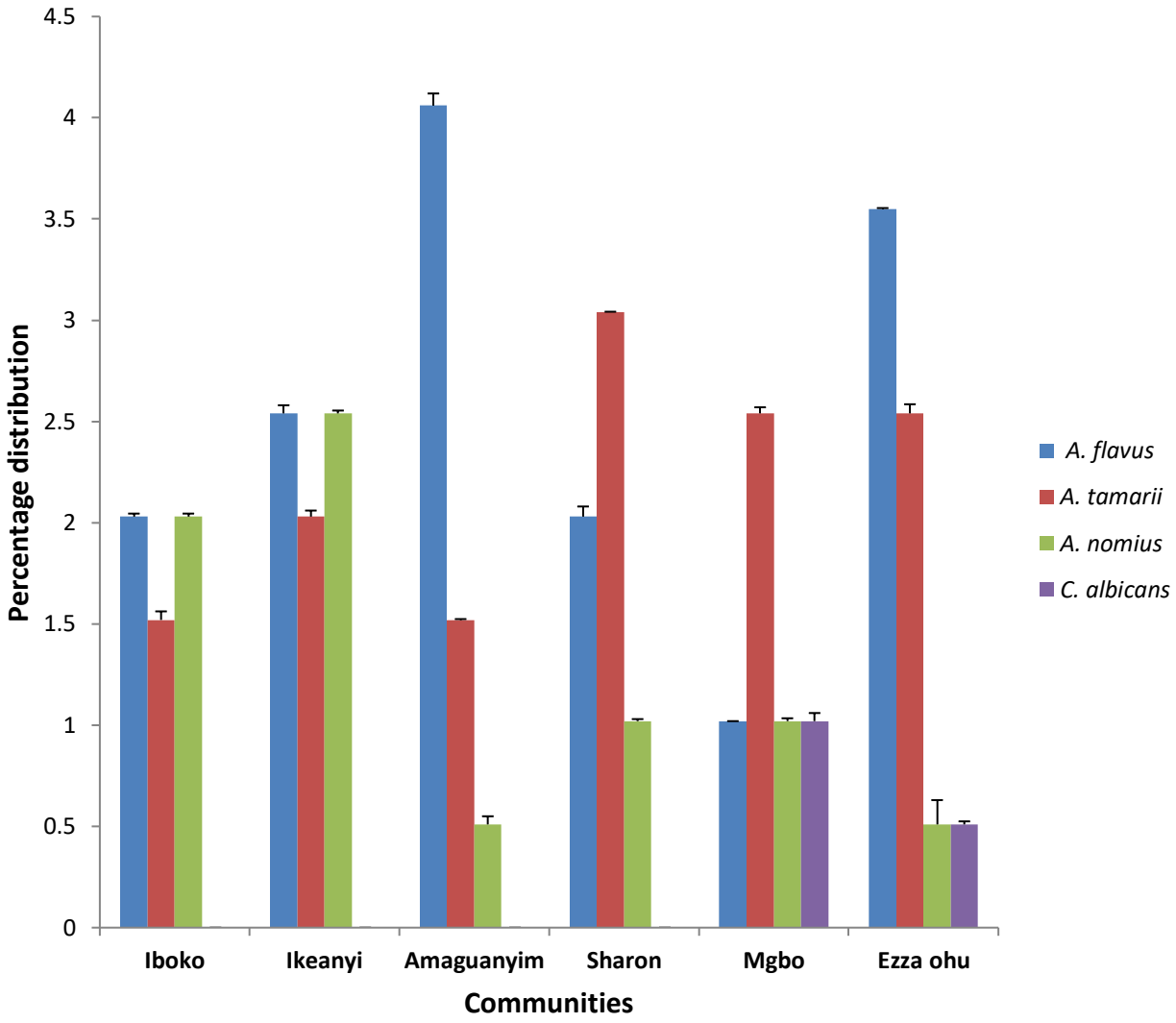


Figure 12: Percentage distribution of non-dermatophyte species by communities in Izzi LGA of Ebonyi State

The distribution of dermatophytic fungi based on age groups in Ikwo and Izzi LGAs showed an increase in the percentage frequency of dermatophyte species between ages 16-26 years 13 (6.60%), followed by 27-37 years 8 (4.07%), 5-15 years 6 (3.04%), 38-48 years 5 (2.54%) and a decline between ages 49-59 years 1 (0.51%) in Ikwo LGA than in Izzi LGA with age-group 16-26 years 7 (3.55%), 27-37 years 6 (3.04%), 38-48 years 4 (2.03%) and a decline between ages 5-15 years 1 (0.51%) and 49-59 years 1 (0.51%). *T. tonsurans* caused infection in all age groups examined in the study. The age-group 16-26 years was most infected with *T. tonsurans* 7 (3.55%) and *T. mentagrophytes* 3 (1.52%) in Ikwo LGA and Izzi LGA respectively (Figure 13). The distribution of non-dermatophytic fungi within age groups in Ikwo and Izzi LGAs showed that the age-group 16-26 years 30 (15.24%) was the most infected with non-dermatophyte species, followed by 27-37 years 15 (7.61%), 38-48 years 12 (6.09%), 49-59 years 7 (3.5%) and 5-15 years 5 (2.54%) in Ikwo LGA than in Izzi LGA with 16-26 years 27 (13.70%), 27-37 years 19 (9.65%), 38-48 years 17 (8.63%), 5-15 years 6 (3.04%), 49-59 years 5 (2.54%). *A. flavus* caused infection in all age groups examined in the study. The age-group 16-26 years was most infected with *A. flavus* 17 (8.62%) and *A. tamarii* 10 (5.08%) in Ikwo LGA and Izzi LGA respectively (Figure 14). The age-group 5-15 years was most infected with *C. albicans* 3 (1.52%) in Ikwo LGA followed by 2 (1.02%) in Izzi LGA. Statistical analyses revealed significant difference ($P<0.05$) in the prevalence rates of cutaneous mycoses among rice farmers in all age groups.

The gender-specific distribution of dermatophytic fungi in Ikwo LGA was 10 (5.07%) and 23 (11.68%) while the distribution of dermatophytic fungi in Izzi LGA was 5 (2.54%) and 16 (8.12%) for male and female farmers respectively. The females were more infected with dermatophytic fungi in both LGAs (Figure 15) and *T. tonsurans* was the most frequently isolated species in Ikwo LGA 12 (6.09%) and Izzi LGA 6 (3.04%). The distribution of non-dermatophytic fungi based on gender in Ikwo LGA was 12 (6.09%) and 57 (28.94%) while in Izzi LGA was 16

(8.12%) and 58 (29.44%) for male and female farmers respectively. The females were also more infected with non-dermatophytic fungi in both LGAs. *A. flavus* 30 (15.23%) and *A. tamarii* 24 (12.18%) were the most frequently isolated species in Ikwo and Izzi LGAs respectively (Figure 16). Comparison of the prevalence rates of cutaneous mycoses of males and females showed that female rice farmers had a significantly higher ($P<0.05$) prevalence rate.

Figure 17 shows the distribution of dermatophytic fungi according to educational status of rice farmers examined. Lower percentage frequency 1 (0.51%) of dermatophytic fungi was observed among rice farmers with tertiary education in both Ikwo and Izzi LGAs. Both Ikwo and Izzi LGAs recorded 4 (2.03%) among rice farmers with secondary education whereas rice farmers with primary education recorded 12 (6.09%) and 5 (2.54%) in Ikwo and Izzi LGAs respectively. Rice farmers with no formal education recorded the highest frequencies 16 (8.13%) and 11 (5.58%) of dermatophytic fungi in Ikwo and Izzi LGAs respectively. Higher frequency of dermatophytic fungi was observed among rice farmers in Ikwo LGA than Izzi LGA. *T. tonsurans and T. rubrum* caused infection in all the educational groups examined in both Ikwo and Izzi LGAs. *T. tonsurans* occurred mostly among rice farmers with no formal education in Ikwo LGA 9 (4.57%) than Izzi LGA 5 (2.54%) while *T. mentagrophytes* occurred mostly among rice farmers with primary education in Ikwo LGA 5 (2.54%) than Izzi LGA 1 (0.51%). Highest frequencies of non-dermatophytic fungi 35 (15.23%) and 44 (15.23%) was observed among rice farmers with no formal education in Ikwo and Izzi LGAs respectively. The prevalence was more in Izzi LGA. The least frequencies 4 (2.03%) and 2 (1.02%) of non-dermatophytic fungi was observed among those with tertiary education in Ikwo and Izzi LGAs respectively, followed by secondary education 15 (7.61%) and 12 (6.09%), and primary education 15 (7.61%) and 16 (8.13%) in Ikwo and Izzi LGAs respectively. *A. flavus* and *A. nomius* caused infection in all the educational groups

examined in both Ikwo and Izzi LGAs (Figure 18). *A. flavus* was most frequently isolated in Izzi LGA 23 (11.68%) than Ikwo LGA 13 (6.60%).

The distribution of dermatophyte species based on family size of the rice farmers showed highest percentage frequency 13 (6.60%) of dermatophytic fungi in Ikwo LGA from those with families of 12-14 persons, followed by 10 (5.08%), 7 (3.55%) and 3 (1.52%) from those with families of 9-11, 6-8 and 3-5 persons respectively compared to 9 (4.57%), 7 (3.55%), 4 (2.03%) and 1 (0.51%) among those with families of 12-14, 9-11, 6-8 and 3-5 persons respectively in Izzi LGA. The infection was reported more among families living in crowded accommodation. *T. mentagrophytes* and *T. tonsurans* were isolated from all family sizes of rice farmers examined in Ikwo and Izzi LGAs (Figure 19). *T. tonsurans* was the most frequently isolated species from rice farmers with family size of 12-14 persons in Ikwo LGA 8 (4.06%) than Izzi LGA 3 (1.52%). *M. canis* only caused infection on rice farmers with families of 9-11 persons 1 (0.51%) in both Ikwo and Izzi LGAs. Highest percentage frequency 32 (16.24%) of non-dermatophytic fungi was observed among rice farmers with families of 12-14 persons in Izzi LGA compared to 24 (12.18%) in Ikwo LGA, followed by 9-11 persons 26 (13.20%) and 18 (9.14%), 6-8 persons 14 (7.11%) and 14 (7.11%), 3-5 persons 5 (2.54%) and 10 (5.07%) in Ikwo and Izzi LGAs respectively. *A. flavus and A. nomius* were the most frequently isolated species found on all family size of rice farmers examined in both Ikwo and Izzi LGAs. *A. flavus* 13 (6.60%) occurred most among rice farmers with both family sizes of 9-11 and 12-14 persons in Ikwo and Izzi LGAs respectively (Figure 20). Infection due to *C. albicans* was not observed among rice farmers with families of 3-5 persons in both Ikwo and Izzi LGAs.

The infection was also reported among those that interact with pets/ domestic animals such as dogs, cats, goats, rabbits and poultry birds. Highest percentage frequencies 21 (10.66%) and 12 (6.09%) of dermatophytic fungi were observed among rice farmers with no interaction with pets/

domestic animals in Ikwo and Izzi LGAs respectively compared to rice farmers with interaction with pets/ domestic animals (Tables 5 and 6). Highest percentage frequencies 59 (29.95%) and 68 (34.52%) of non-dermatophytic fungi were also observed among rice farmers with no interaction with pets/ domestic animals in Ikwo and Izzi LGAs respectively (Tables 7 and 8). The locations of cutaneous mycotic lesions and the organisms recovered are presented in Table 9. The table showed that the scalp (tinea capitis) 76 (3.57%) was the commonest affected site of the body among the suspected cases. This was followed by the nail (tinea ungium) 59 (2.77%) and the skin (tinea corporis) 47 (2.20%). *T. tonsurans* and *T. rubrum* were recovered from all body sites examined in the study. Figure 21 shows the cutaneous lesions observed among rice farmers in Ikwo and Izzi LGA. The majority of the farmers examined in Ikwo LGA had scales 36 (1.69%); other work-related symptoms were nail dystrophy 27 (1.27%), erythema 16 (0.75%) and fissures 11 (0.52%). Izzi LGA recorded the following: scales 20 (0.94%), nail dystrophy 16 (0.75%), erythema 13 (0.61%) and fissures 4 (0.19%). Twenty-two farmers (1.03%) complained of itching during and after work, especially during harvesting and thrashing in Ikwo LGA whereas Izzi recorded 17 (0.80%). These dermatoses were less common among farmers in Izzi LGA (Figure 22).

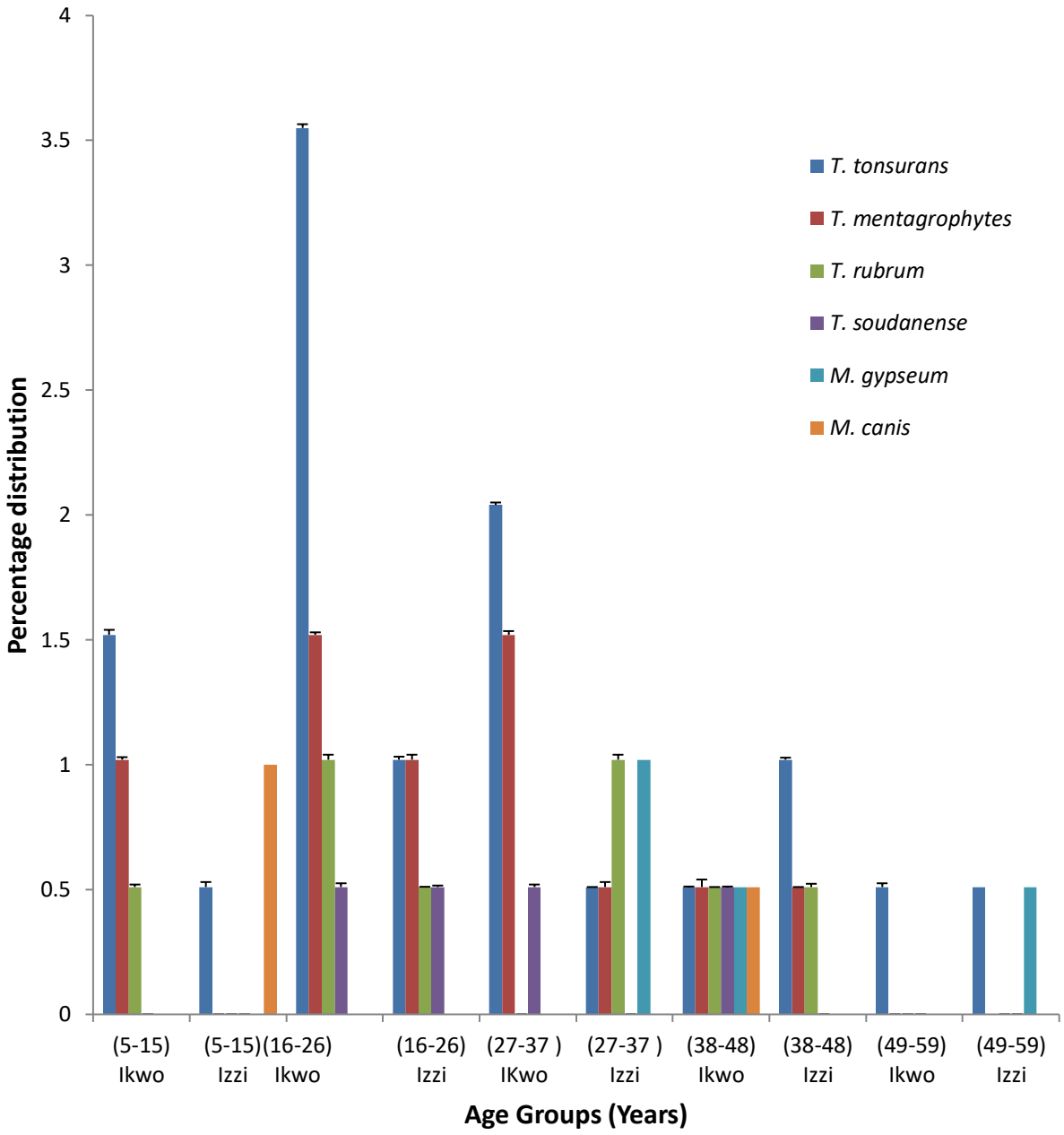


Figure 13: Percentage age distribution of dermatophyte species among rice farmers in Ikwo and Izzi LGAs

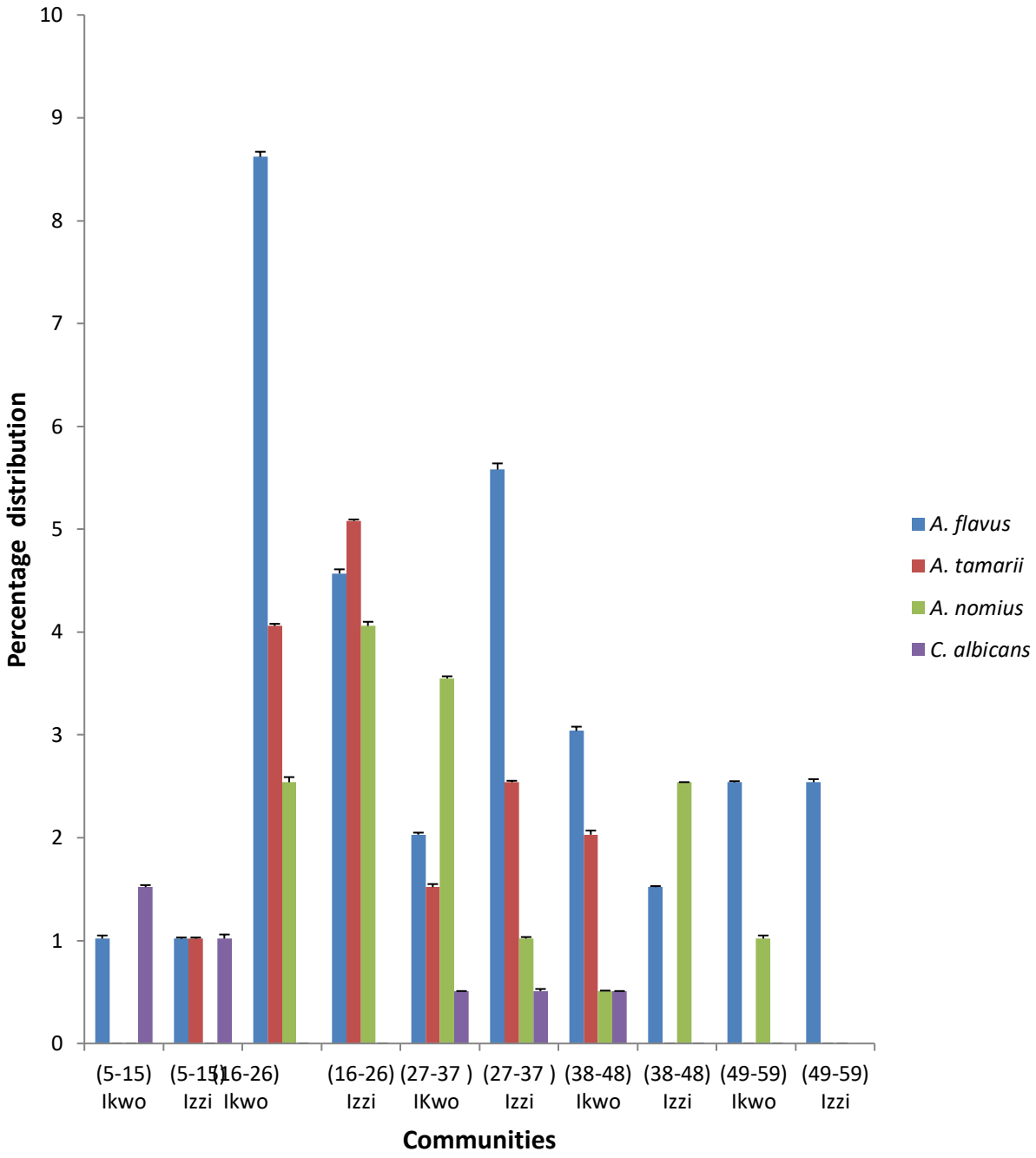


Figure 14: Percentage age distribution of non-dermatophyte species among rice farmers in Ikwo and Izzi LGAs

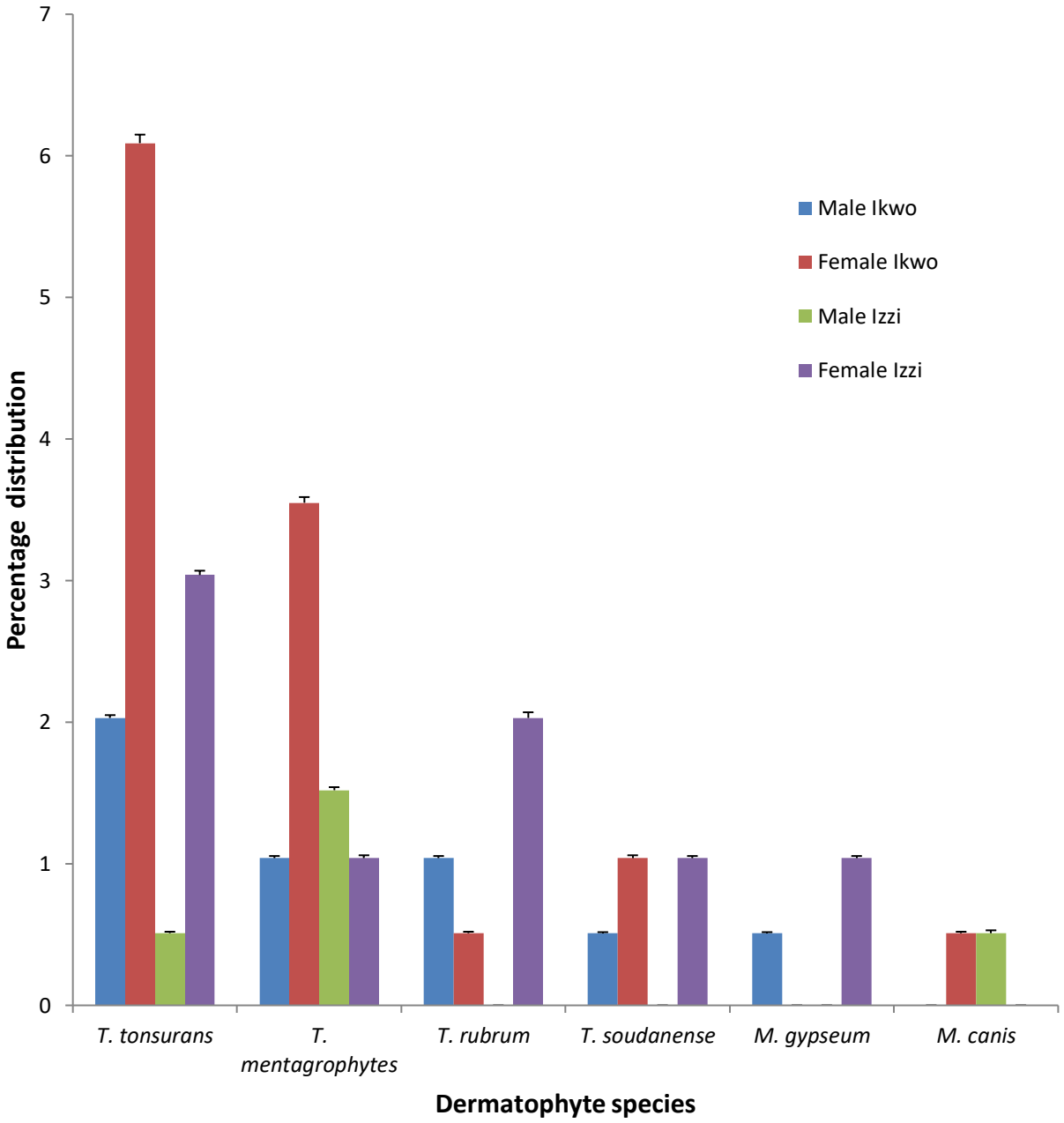


Figure 15: Percentage gender distribution of dermatophyte species among rice farmers in Ikwo and Izzi LGAs of Ebonyi State

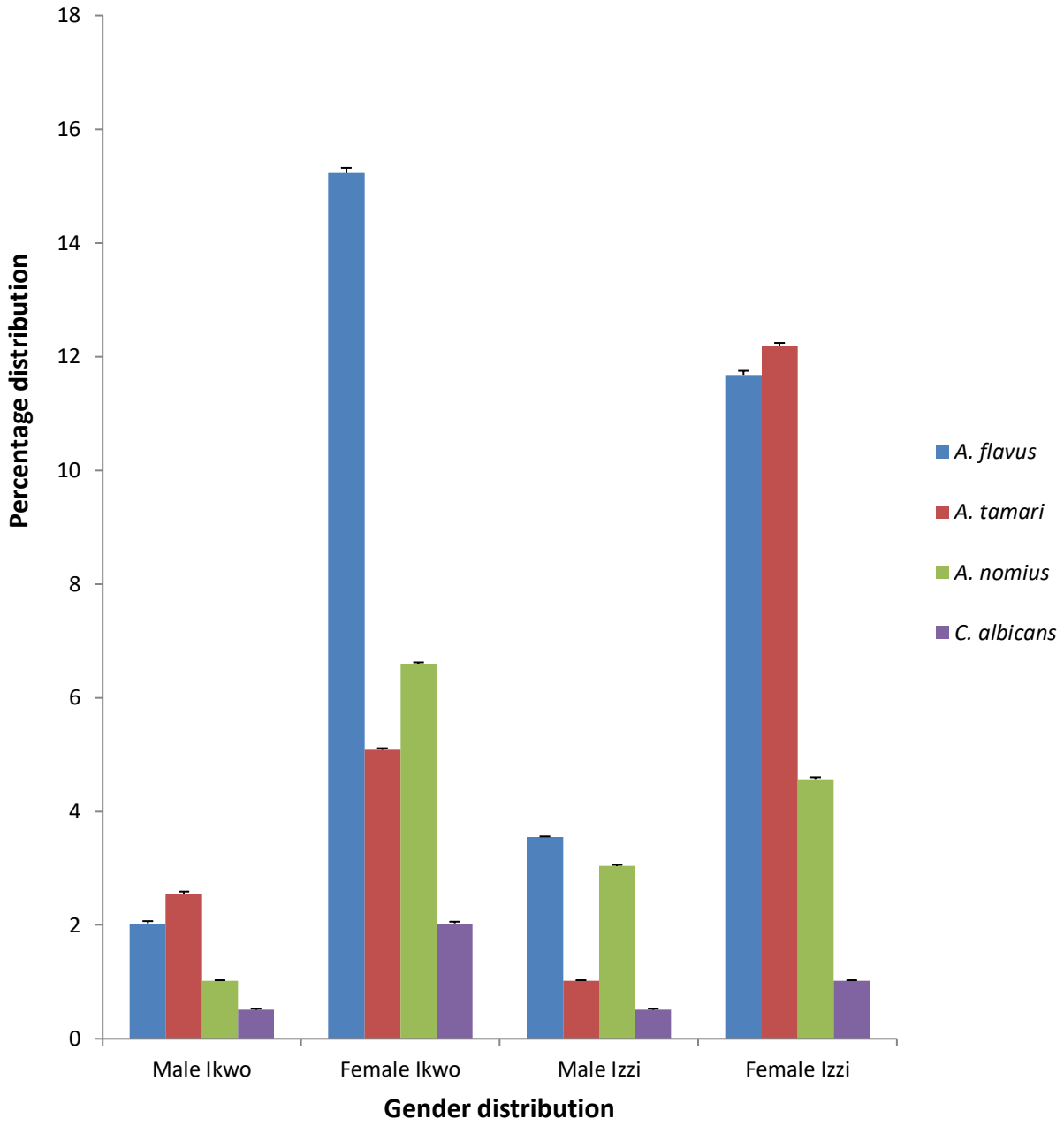


Figure 16: Percentage gender distribution of non-dermatophyte species among rice farmers in Ikwo and Izzi LGAs of Ebonyi State

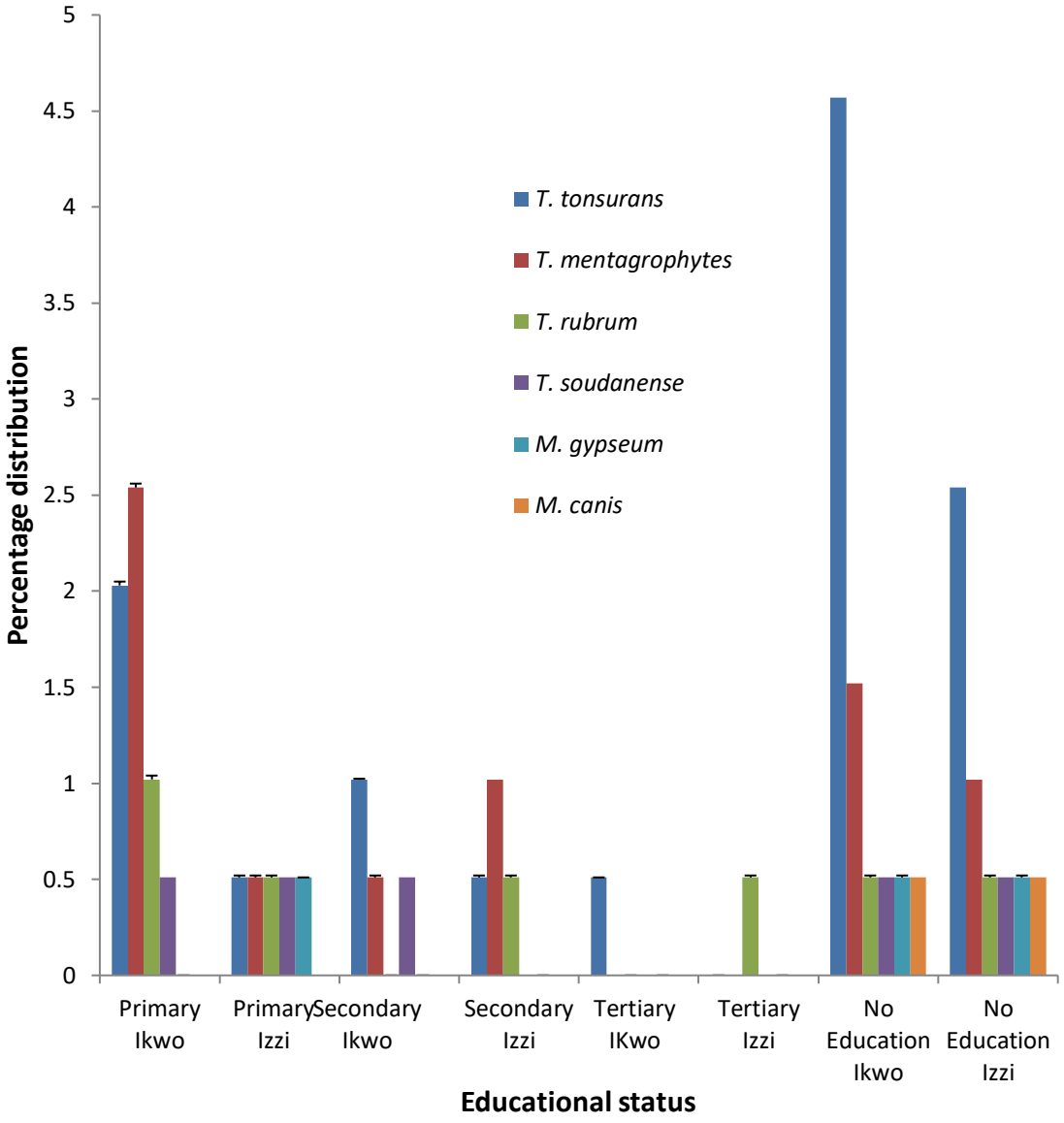


Figure 17: Percentage distribution of dermatophytic fungi based on educational status of rice farmers in Ikwo and Izzi LGAs of Ebonyi State

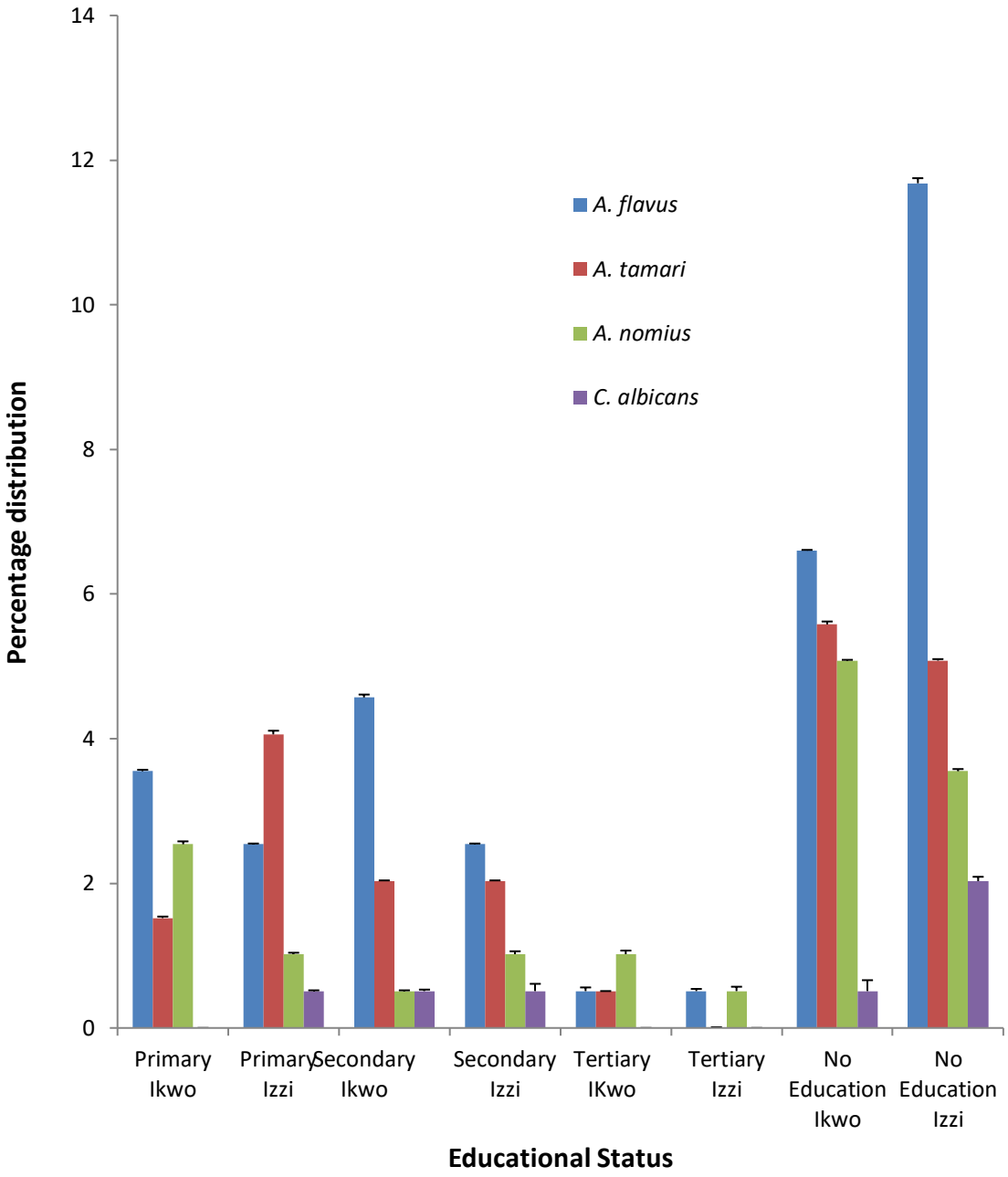


Figure 18: Percentage distribution of non-dermatophytic fungi based on educational status of rice farmers in Ikwo and Izzi LGAs of Ebonyi State

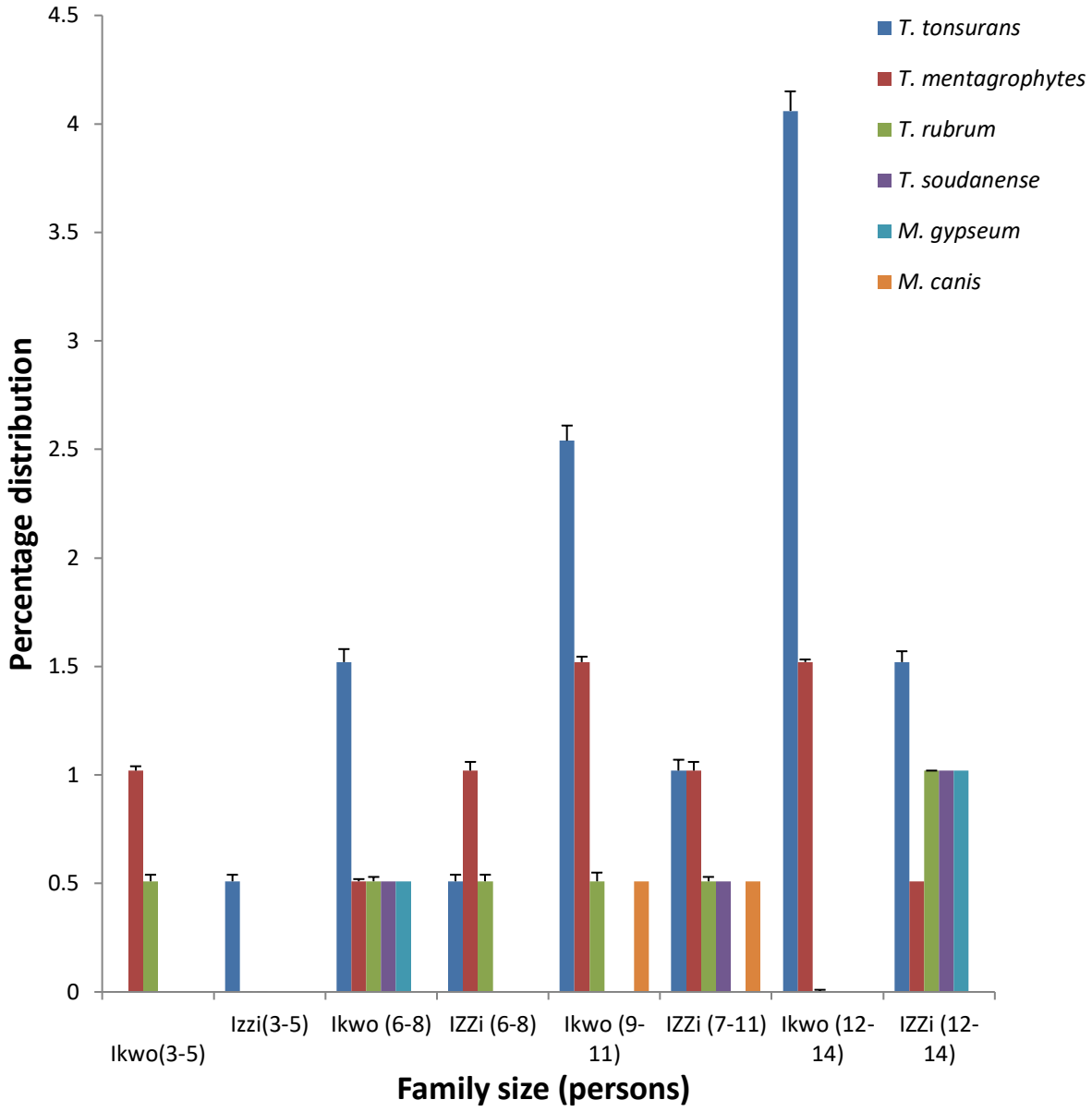


Figure 19: Percentage distribution of dermatophyte species based on family size of rice farmers in Ikwo and Izzi LGAs of Ebonyi State

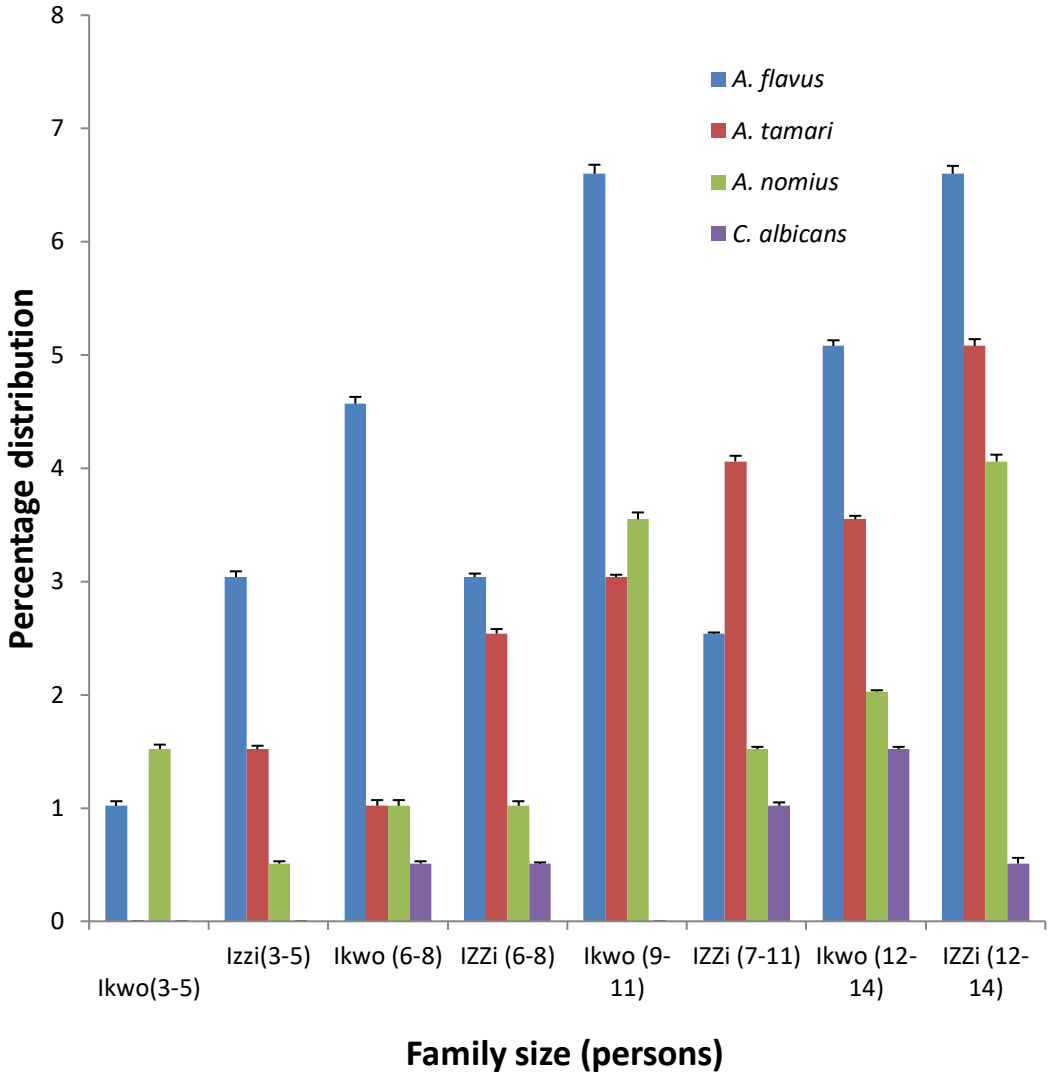


Figure 20: Percentage distribution of non-dermatophyte species based on family size of rice farmers in Ikwo and Izzi LGAs of Ebonyi State

Table 5: Percentage distribution of dermatophytic fungi among rice farmers in Ikwo LGA based on their interactions with domestic animals

Domestic Animals	Dermatophyte species	No. of Rice Farmers	No. of Isolates (%)
Goats	*T. tonsurans* (1)	3	3 (1.52)
	T. mentagrophytes (2)		
Poultry	*T. tonsurans* (2)	6	6 (3.04)
	T. mentagrophytes (2)		
	T. soudanense (1)		
	T. rubrum (1)		
Dogs	*M. canis* (1)	1	1 (0.51)
Cats	*T. mentagrophytes* (1)	1	1 (0.51)
Rabbits	*T. rubrum* (1)	1	1 (0.51)
No animal	*T. tonsurans* (11)	21	21 (10.66)
interaction	*T. rubrum* (2)		
(soil and wet works)	*T. mentagrophytes* (2)		
	T. soudanense (3)		
	M. gypseum (1)		
Total			33 (16.75)

Table 6: Percentage distribution of dermatophytic fungi among rice farmers in Izzi LGA based on their interactions with domestic animals

Domestic Animals	Dermatophyte species	No. of Rice Farmers	No. of Isolates (%)
Goats	*T. tonsurans* (1)	1	1 (0.51)
Poultry	*T. tonsurans* (2)	3	3 (1.52)
	T. mentagrophytes (1)		
Dogs	*T. rubrum* (1)	1	1 (0.51)
Cats	*M. canis* (1)	1	1 (0.51)
	M. canis (1)		
Rabbits	*T. mentagrophytes* (1)	2	3 (1.52)
	T. rubrum (1)		
	M. gypseum (1)		
No animal	*T. tonsurans* (5)	10	12 (6.09)
interaction	*T. rubrum* (1)		
(soil and wet works)	*T. mentagrophytes* (4)		
	T. soudanense (1)		
	M. gypseum (1)		
Total			21 (10.66)

Table 7: Percentage distribution of non-dermatophytic fungi among rice farmers in Ikwo LGA based on their interactions with domestic animals

Domestic Animals	Non-dermatophyte species	No. of Rice Farmers	No. of Isolates (%)
Goats	*A. flavus* (2)	2	2 (1.02)
Poultry	*A. flavus* (3)	5	5 (2.54)
	A. nomius (2)		
Cats	*A. flavus* (1)	3	3 (1.52)
	A. nomius (2)		
No animal	*A. flavus* (21)	59	59 (29.95)
interaction	*A. tamarii* (17)		
(soil and wet works)	*A. nomius* (18)		
	C. albicans (3)		
Total			69 (35.03)

Table 8: Percentage distribution of non-dermatophytic fungi among rice farmers in Izzi LGA based on their interactions with domestic animals

Domestic Animals	Non-dermatophyte species	No. of Rice Farmers	No. of Isolates (%)
Goats	*A. flavus* (1)	1	1 (0.51)
Poultry	*A. flavus* (1)	3	3 (1.52)
	A. tamarii (2)		
Dogs	*A. nomius* (1)	1	1 (0.51)
Cats	*A. flavus* (1)	1	1 (0.51)
No animal	*A. flavus* (34)	46	68 (34.51)
interaction	*A. tamarii* (22)		
(soil and wet works)	*A. nomius* (7)		
	C. albicans (5)		
Total			74 (37.56)

Table 9: Distribution of cutaneous mycoses according to body sites

Body site affected	No. sampled (%)	Organisms recovered
Hair	76 (3.57)	*T. tonsurans, T. mentagrophytes, T. rubrum, T. soudanense, M. gypseum, M. canis, A. flavus, A. tamarii, A. nomius*
Nail	59 (2.77)	*T. tonsurans, T. rubrum, A. flavus, A. nomius, C. albicans*
Skin	47 (2.20)	*T. tonsurans, T. rubrum, M. gypseum, M. canis, A. tamarii, C. albicans*
Total	182 (8.54)	

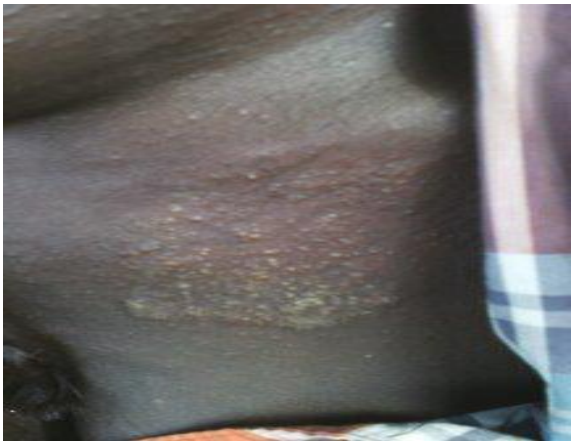


Fig. 21a: Skin lesion caused by *C. albicans*

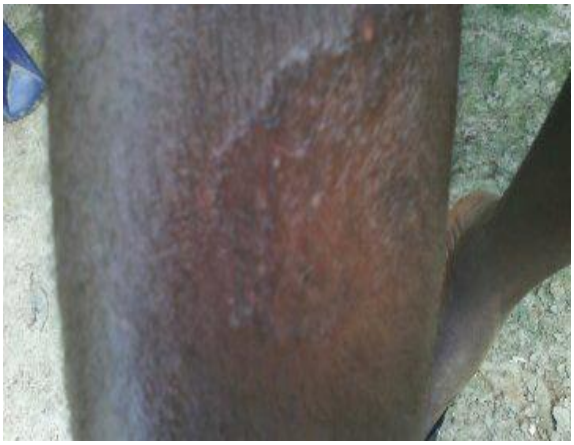


Fig. 21b: Skin lesion caused by *T. rubrum*

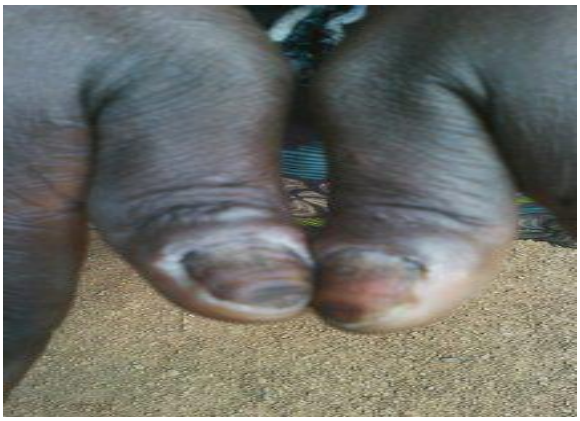


Fig. 21c: Nail lesion caused by *T. tonsurans*

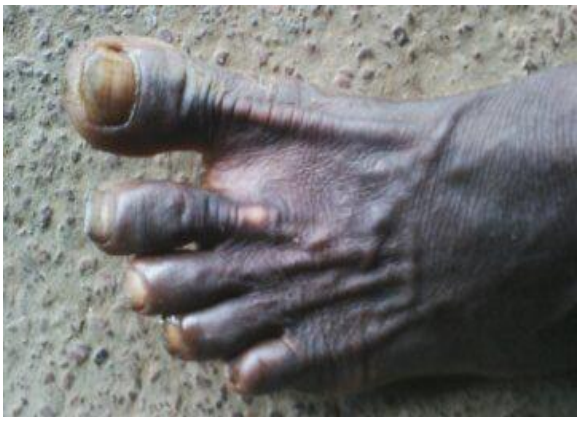


Fig. 21d: Nail lesion caused by *A. flavus*

Fig. 21e: Scalp lesion caused by *M. canis*

Fig. 21f: Scalp lesion caused by *M. gypseum*

Figure 21: Cutaneous lesions observed

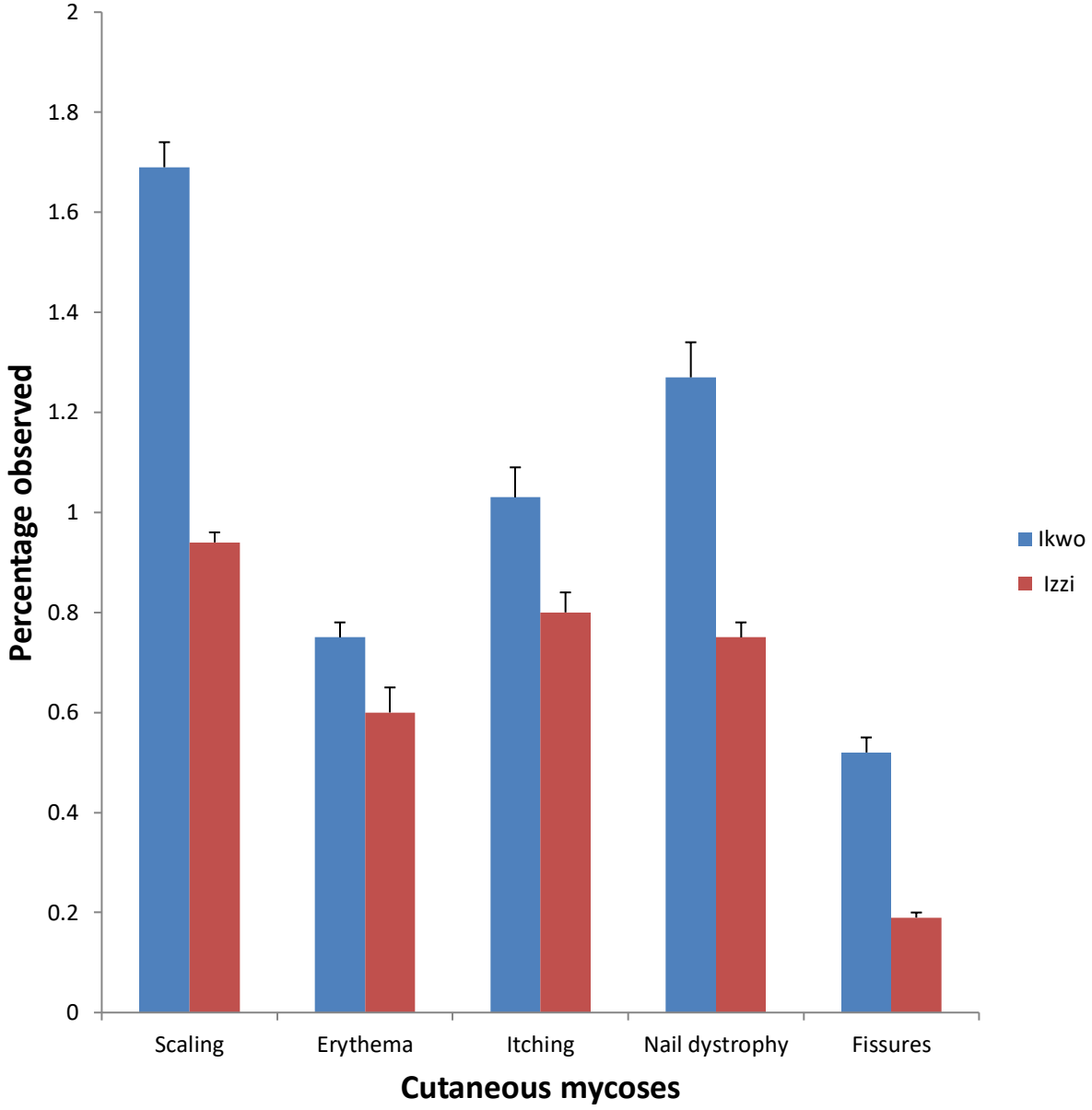
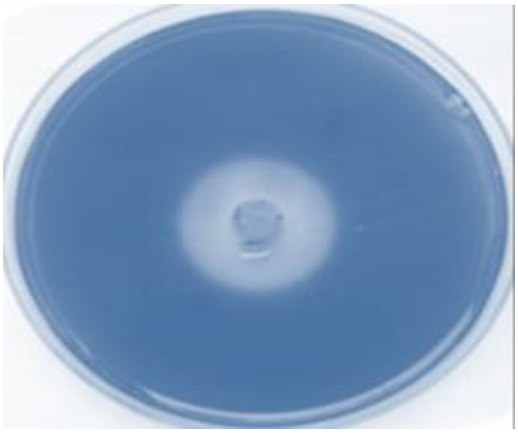


Figure 22: Percentage distribution of cutaneous mycoses observed among rice farmers in Ikwo and Izzi LGAs of Ebonyi State

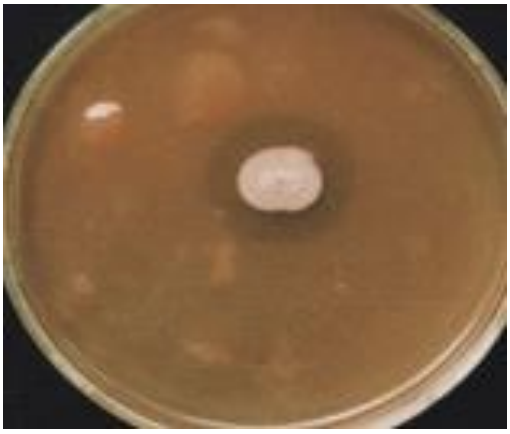
4.4 Screening for Extracellular Enzymes

The ability of the fungal isolates to secrete extracellular enzymes was screened on solid media (Figure 23). Highest keratinase activity (23 mm) was observed in *T. rubrum,* followed by *T. tonsurans, T. mentagrophytes* and *M. gypeum* (22 mm). *T. soudanense* recorded the least keratinase activity (16 mm) as enumerated in Figure 24. Highest protease activity (24 mm) was observed in *T. rubrum,* followed by *M. canis* (23 mm). Protease activity recorded for *T. mentagrophytes* was the least (16 mm). The highest lipase activity of 22 mm was recorded for *T. rubrum,* followed by *T. tonsurans* (21 mm). The least activity was observed in *M. canis* (15 mm). In screening dermatophyte species for their xylanase activity, the figure shows that *T. tonsurans* had the highest xylanase activity of 20 mm, followed by *T. soudanense* (18 mm). *T. mentagrophytes* and *T. rubrum* had the least xylanase activity (16 mm). *T. mentagrophytes* and *T. rubrum* had the highest (22 mm) and least (10 mm) cellulase activity respectively. The collagenase activity of 17 mm recorded for *T. rubrum* was the highest and the least was 14 mm for *M. canis*. Highest keratinase, protease, lipase and collagenase activities were observed in *T. rubrum* while the highest xylanase and cellulase activities were observed in *T. tonsurans* and *T. mentagrophytes* respectively. In screening non-dermatophyte species for their ability to secrete extracellular enzymes, Figure 25 shows that *A. flavus* produced the highest keratinase enzyme (15 mm) followed by *A. nomius* (10 mm). *C. albicans* produced the least keratinase (5 mm). *A. flavus* and *A. nomius* had the highest (16 mm) and least (10 mm) protease activity respectively. The lipase activity of 18 mm recorded for *A. flavus* was the highest, followed by 10 mm for *A. tamarii* and the least was 5.5 mm for *A. nomius*. Highest xylanase activity (11.5 mm) was observed in *A. flavus*, followed by *A. tamarii* and *C. albicans* (10 mm). Xylanase activity recorded for *A. nomius* was the least (8.5 mm). The highest cellulase activity of 14 mm was obtained for *A. flavus*, followed by *C. albicans* (10 mm). The least activity was observed in *A. nomius* (5 mm). *A.*

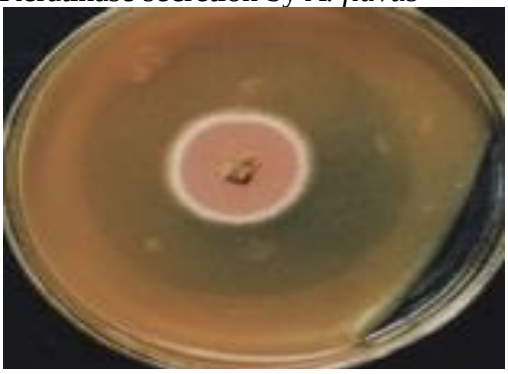
nomius produced the highest collagenase (10.5 mm) while *A. tamarii* produced the least collagenase (6 mm). Highest keratinase, protease, lipase, xylanase and cellulase activities were observed in *A. flavus* while the highest collagenase activitiy was observed in *A. nomius*. The results of this study showed that the isolated cutaneous mycotic fungi produced keratinase, protease, lipase, xylanase, cellulase and collagenase virulence enzymes.



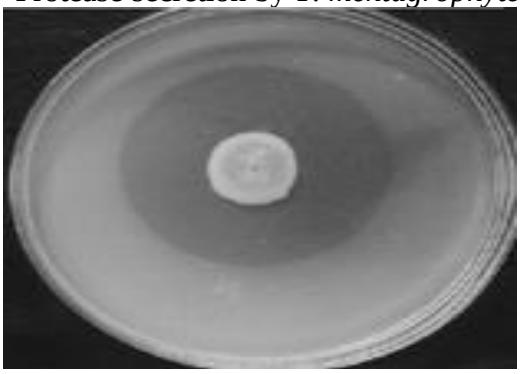
Keratinase secretion by *A. flavus*



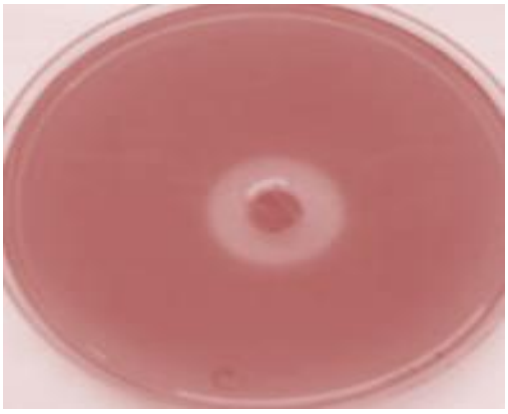
Protease secretion by *T. mentagrophytes*



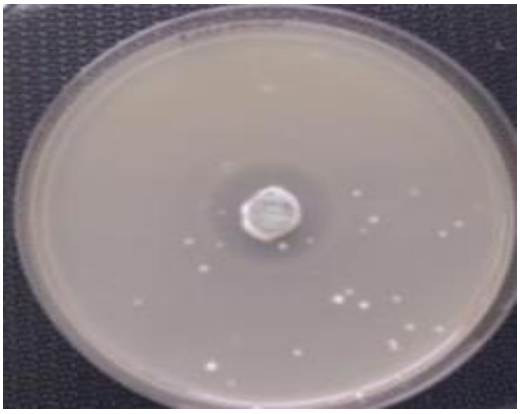
Lipase secretion by *T. rubrum*



Xylanase secretion by *T. tonsurans*



Cellulase secretion by *Candida albicans*



Collagenase secretion by *M. gypseum*

Figure 23: Extracellular enzyme activities of fungal isolates on solid media showing zones of clearance

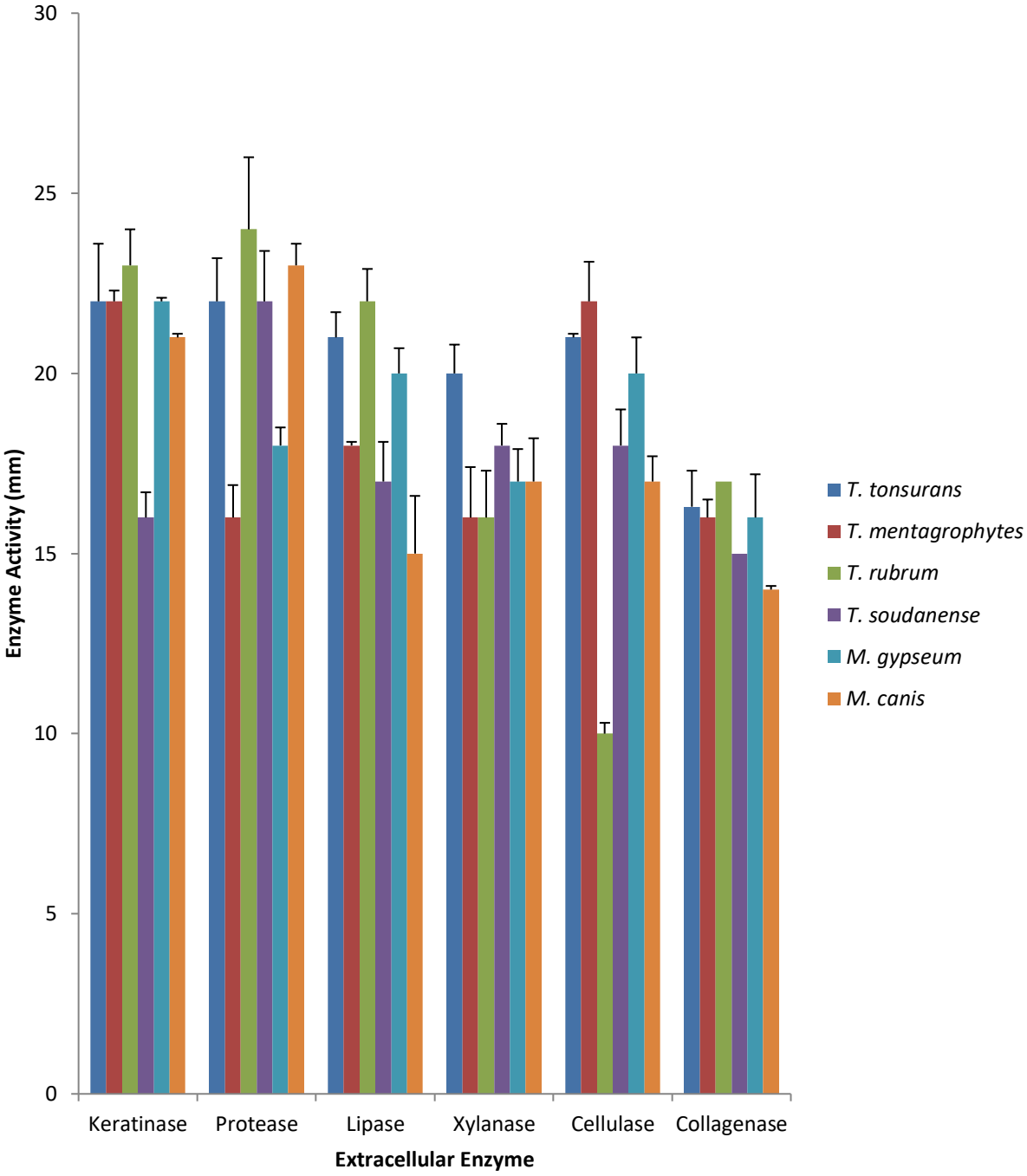


Figure 24: Relative enzyme activities of dermatophyte species on solid media

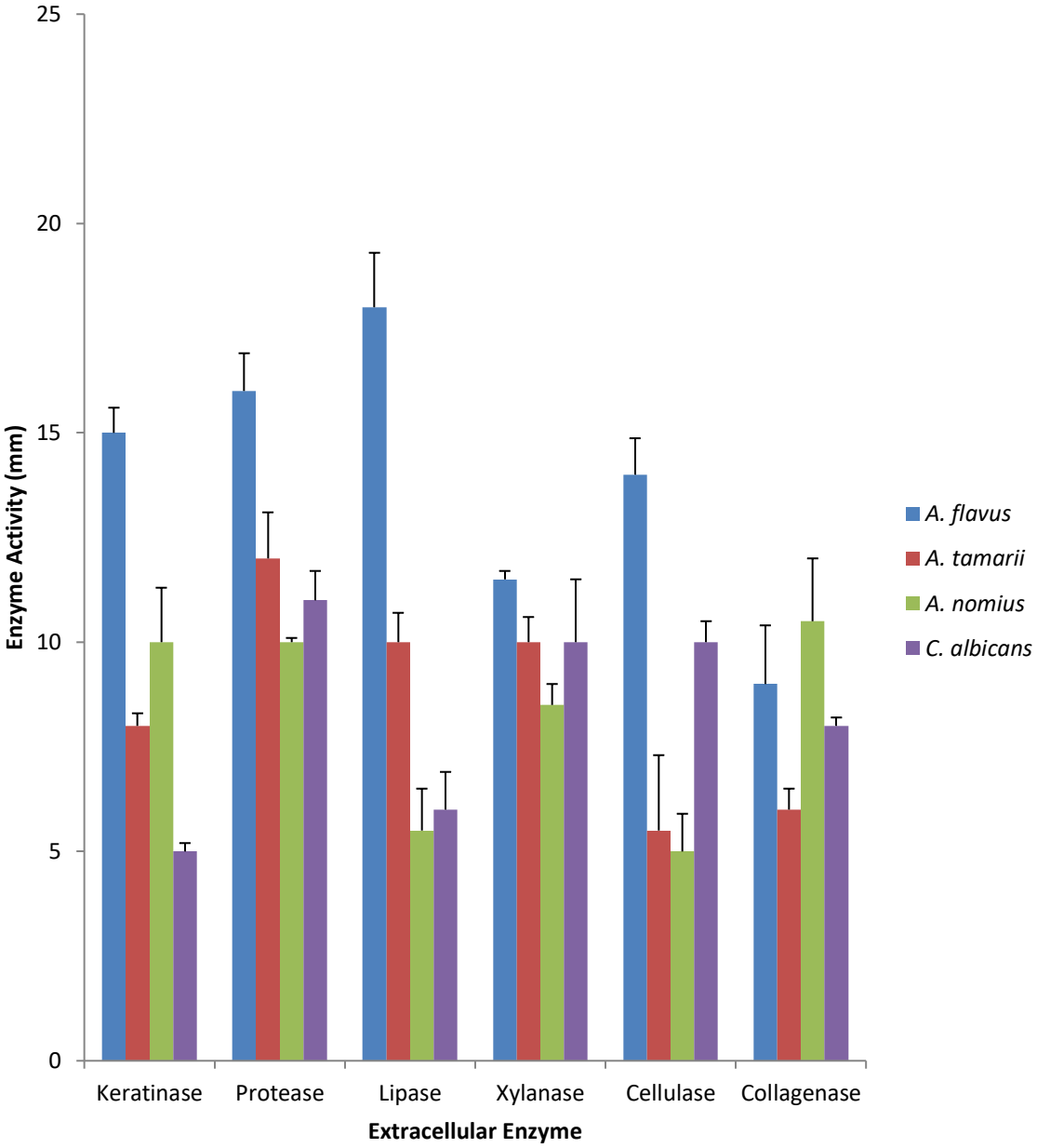


Figure 25: Relative enzyme activities of non-dermatophyte species on solid media

4.5 Extraction Yield of Plant Materials

Figure 26 shows the extractive yield of the various plant extracts used. The n-hexane extracts were found to have the highest extractive yield 3.1, 2.4, 2.6, 3.4 and 3.8 grams; followed by the petroleum ether extracts 2.2, 2.3, 2.6, 2.8 and 3.3 grams for *Azadirachta indica, Cymbopogon citratus, Emilia sonchifolia, Senna alata* and *Senna occidentalis* respectively. The chloroform (1.3, 1.1, 1.4, 2.1 and 2.1 grams) and aqueous (1.8, 1.6, 1.1, 1.5 and 2.1 grams) extracts were found to have lower extractive yield. The extraction process gave highest yield from *Senna occidentalis* plant followed by *Senna alata* plant.

4.6 Phytochemical Screening

Qualitative and quantitative determination of phytochemical constituents of the medicinal plants used in this study showed that they contain secondary metabolites like flavonoids, alkaloids, saponins, tannins, phenols, glycosides, terpenoids and steroids as presented in Table 10. The phytochemical analysis of *A. indica, C. citratus* and *S. alata* extracts revealed the presence of all the secondary metabolites. *E. sonchifolia* extracts gave positive reactions for all the secondary metabolites except glycosides and steroids. *S. occidentalis* extracts tested positive for all the secondary metabolites except terpenoids. Flavonoids, alkaloids, saponins, tannins and phenols were detected as the major phytochemicals while glycosides, terpenoids and steroids were detected in minute concentrations (Figure 27). The figure shows that *C. citratus* gave the highest concentration of flavonoid (25.72±0.14 mg/g), phenol (75.30±0.05 mg/g) and tannin (14.58±0.02 mg/g) while *S. alata* gave the highest concentration of alkaloid (42.14±0.10 mg/g) and saponin (22.16±0.13 mg/g).

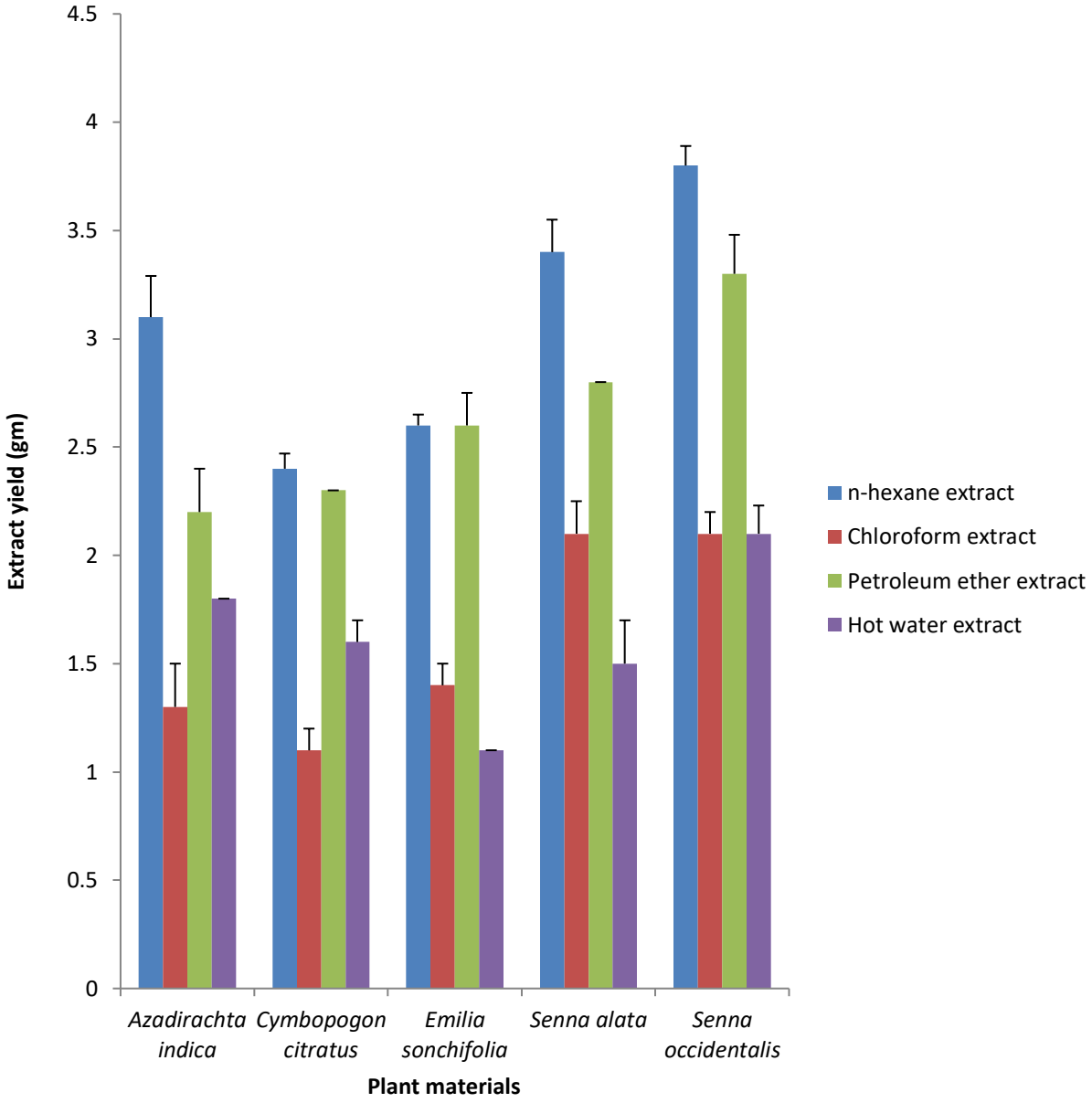


Figure 26: Extract yield of plant materials

Table 10: Qualitative analysis of phytochemical constituents

Extract	Phytochemicals						
	FLA	ALK	SAP	TAN	GLY	TER	STE
Azadirachta indica							
HEX	-	-	+	-	+	+	+
CHL	-	-	+	+	-	+	+
PET	+	+	+	+	+	-	-
HWE	+	-	+	-	+	-	-
Cymbopogon citratus							
HEX	+	-	+	+	-	-	+
CHL	+	-	-	-	-	+	+
PET	+	+	+	+	+	-	-
HWE	-	-	+	+	-	+	-
Emilia sonchifolia							
HEX	-	+	+	+	-	+	-
CHL	-	-	+	-	-	-	-
PET	+	+	-	+	-	-	-
HWE	+	+	-	+	-	-	-
Senna alata							
HEX	+	+	+	+	+	-	-
CHL	+	+	+	+	+	-	+
PET	+	+	+	-	+	-	-
HWE	-	+	+	+	+	+	-
Senna occidentalis							
HEX	+	-	-	-	+	-	-
CHL	-	+	+	+	-	-	-
PET	-	+	-	+	+	-	+
HWE	+	-	+	+	-	-	+

Key

FLA = flavonoids ALK = alkaloids SAP = saponins TAN = tannins

GLY = glycosides STE = steroids TER = terpenoids HEX = n-hexane extract

CHL = chloroform extract PET = petroleum ether extract HWE = hot water extract

+ = present - = absent

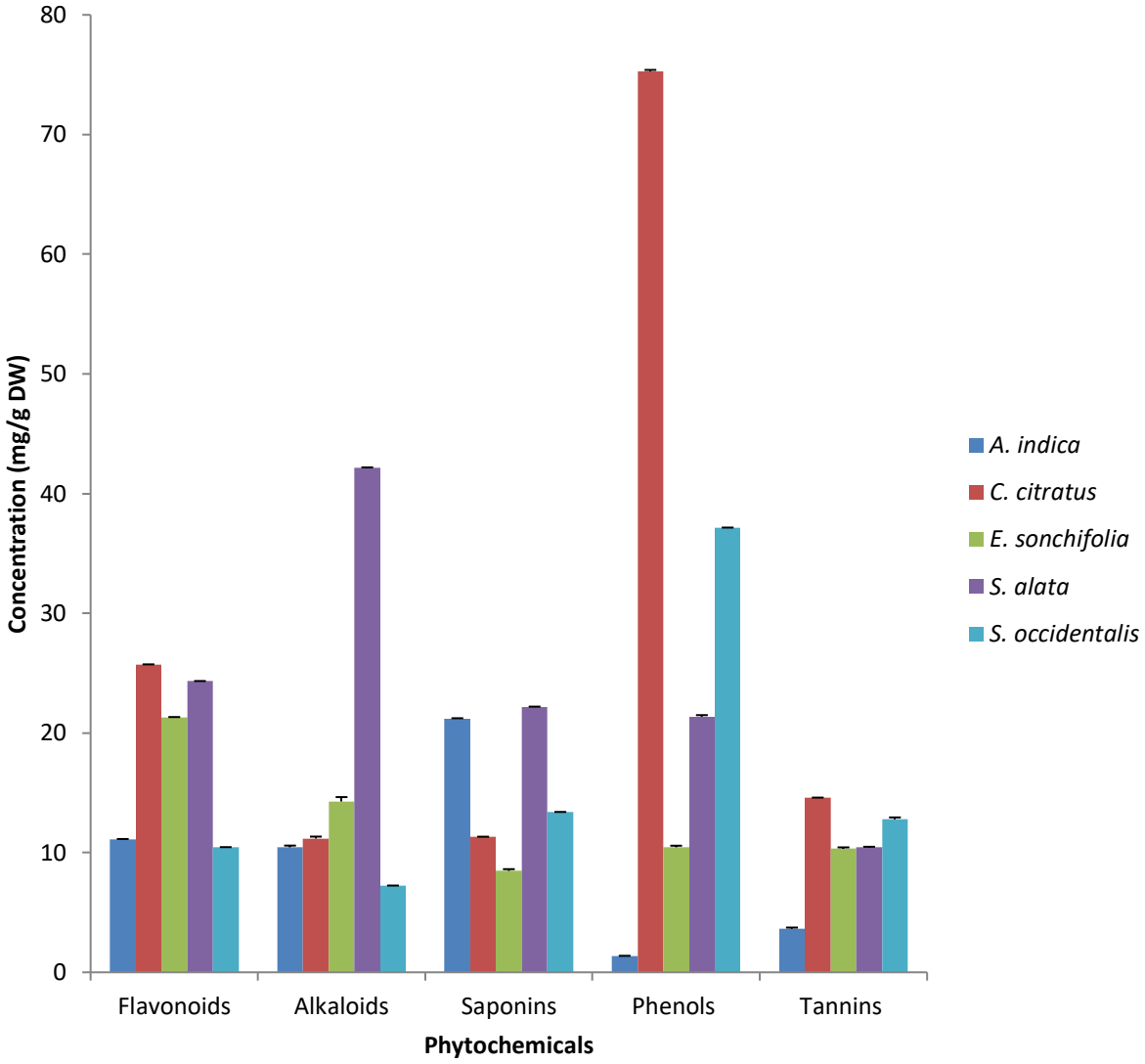


Figure 27: Quantitative analysis of phytochemical constituents

4.7 In vitro Antifungal Activity Testing of Plant Extracts

The results of the in vitro antifungal activity testing of n-hexane, chloroform, petroleum ether and hot water extracts of the plant materials against the fungal species isolated from the rice farmers are presented below. The minimum inhibitory concentrations (MICs) of the plant extracts against the isolates were determined after 7 days at 25ºC incubation temperature. Table 11 shows the effects of n-hexane, chloroform, petroleum ether and hot water extracts of *A. indica, C. citratus, E. sonchifolia, S. alata* and *S. occidentalis* on dermatophyte species. The n-hexane extracts of *C. citratus* and *S. alata* had minimum inhibitory effect on the test dermatophytes while the petroleum ether extracts of *C. citratus* and *S. occidentalis* minimum inhibition on the test dermatophytes. At a concentration of 2.0 µg/ml, n-hexane extracts of *C. citratus* and *S. alata* had minimum inhibition on *M. gypseum* and *M. canis* respectuvely. The least activity was observed on n-hexane extract of *E. sonchifolia* against *T. tonsurans* and *M. gypseum* at a concentration of 15.0 µg/ml. Petroleum ether extracts of *C. citratus* and *S. occidentalis* were observed to be more active against *T. tonsurans* at a concentration of 4.0 µg/ml. Petroleum ether extracts of *E. sonchifolia* had slight effect (18.0 µg/ml) on *M. gypseum.* Chloroform and hot water extracts of the medicinal plants had the least effect on the test dermatophytes. Chloroform extract of *E. sonchifolia* had the least activity (28.0 µg/ml) on *M. gypseum* while hot water extracts of *C. citratus, E. sonchifolia* and *A. indica* had the least activity (32.0 µg/ml) on *T. mentagrophytes*, *T. rubrum* and *M. gypseum* respectively. The range of MIC for n-hexane, chloroform and petroleum ether extracts was 2 - 15 µg/ml, 8 - 28 µg/ml and 4 - 18 µg/ml respectively. The value for hot water extract was 12 - 32 µg/ml. All the isolates of dermatophytes tested were susceptible to the five plant extracts used in the study. The test dermatophytes were significantly less susceptible to hot water extract. Terbinafine (standard antifungal control) was the most effective against the test dermatophytes with MIC ranging from 2 - 5 µg/ml.

Table 12 shows the effect of n-hexane, chloroform, petroleum ether and hot water extracts of *A. indica, C. citratus, E. sonchifolia, S. alata* and *S. occidentalis* on non-dermatophyte species. The n-hexane extract of *C. citratus* had minimum inhibitory effect on *C. albicans* at the lowest concentration (2.0 µg/ml) while the least activity (15.0 µg/ml) was observed in *E. sonchifolia* extracts against *A. flavus*. The petroleum ether extract of *C. citratus* had minimum inhibition on *A. nomius* at a concentration of 4.0 µg/ml. Chloroform and hot water extracts had slight effect on the test non-dermatophytes. Chloroform and hot water extracts of *A. indica* had the least activity (32.0 µg/ml) against *A. nomius* and *A. flavus* respectively. The range of MIC for n-hexane, chloroform, petroleum ether and hot water extracts was 2 - 15 µg/ml, 8 - 32 µg/ml, 4 - 16 µg/ml and 10 - 32 µg/ml respectively. All the isolates of non-dermatophytes tested were susceptible to the five plant extracts used in the study. The test non-dermatophytes were significantly less susceptible to hot water extract. Terbinafine was also most effective against the test non-dermatophytes with MIC ranging from 2 - 6 µg/ml. The order of in vitro antifungal activity of the plant extracts was n-hexane extract > petroleum ether extract > chloroform extract > hot water extract. The comparative results of antifungal susceptibility of the various fungal species to examined plant extracts indicated that the sensitivity to terbinafine in the different species was higher than the plant extracts with significant difference ($P<0.05$).

The minimum fungicidal concentration (MFC) values of n-hexane and petroleum ether extracts of the plant materials against the test dermatophytes were 4 µg/ml and 6 µg/ml respectively while chloroform and hot water extracts showed 8 µg/ml and 15 µg/ml respectively (Table 13). MFC values against the test dermatophytes ranged from 4 - 32 µg/ml. The minimum fungicidal concentration (MFC) values of n-hexane, chloroform, petroleum ether and hot water extracts of the plant materials against non-dermatophytes were 5 µg/ml, 12 µg/ml, 8 µg/ml and 12 µg/ml respectively (Table 14). MFC values ranged from 5 - 32 µg/ml. The most significant MFC was

observed on n-hexane extract of *C. citratus* and *S. alata* against against dermatophytes, and n-hexane extract of *C. citratus* against non-dermatophytes.

Table 11: In vitro antifungal activity testing of 5 medicinal plants against dermatophytes species by microbroth dilution assay

Species	Plant Extracts	MIC				
		HEX	CHL	PET	HWE	TER
		(Extract Concentrations = 2, 4, 8, 16, 32 and 64 µg/ml)				
T. tonsurans	*A. indica*	5.3	12.0	6.6	12.0	3.0
	C. citratus	4.7	8.0	4.0	13.0	2.8
	E. sonchifolia	15.0	16.0	10.0	16.0	2.2
	S. alata	5.2	8.5	8.1	16.0	3.1
	S. occidentalis	4.0	8.2	4.0	30.0	4.0
T. mentagrophytes	*A. indica*	5.4	10.0	7.2	16.0	2.4
	C. citratus	4.6	8.6	5.8	32.0	2.6
	E. sonchifolia	7.2	8.3	15.0	16.2	4.0
	S. alata	5.6	17.0	6.1	15.0	4.4
	S. occidentalis	4.5	12.0	5.0	22.4	2.0
T. rubrum	*A. indica*	11.0	16.0	5.8	16.0	4.0
	C. citratus	5.0	8.5	4.8	14.0	3.0
	E. sonchifolia	6.2	26.1	7.0	32.0	4.0
	S. alata	4.1	16.0	5.3	12.0	2.2
	S. occidentalis	8.0	16.2	5.1	16.0	4.0
T. soudanense	*A. indica*	10.5	18.0	6.1	13.7	2.0
	C. citratus	3.0	8.1	6.0	18.0	2.5
	E. sonchifolia	8.0	11.1	7.5	16.0	2.7
	S. alata	3.6	8.2	4.4	23.5	5.0
	S. occidentalis	4.1	21.8	4.2	31.0	4.2
M. gypseum	*A. indica*	8.0	12.0	6.5	32.0	4.1
	C. citratus	2.0	8.0	4.3	15.0	3.3
	E. sonchifolia	15.0	28.0	18.0	22.0	2.0
	S. alata	4.7	16.0	4.6	18.0	2.2
	S. occidentalis	12.6	16.3	14.2	29.1	3.0
M. canis	*A. indica*	5.0	16.0	4.7	15.0	2.0
	C. citratus	3.2	12.4	8.5	16.0	4.2
	E. sonchifolia	8.0	16.0	16.0	20.0	3.4
	S. alata	2.0	8.0	8.0	14.3	2.0
	S. occidentalis	10.0	16.1	4.1	16.5	4.4

Key: HEX = n-hexane extract CHL = chloroform extract PET = petroleum ether extract HWE = hot water extract TER = terbinafine (antifungal control agent)

Table 12: In vitro antifungal activity testing of 5 medicinal plants against non-dermatophytes species by microbroth dilution assay

Species	Plant Extracts	MIC				
		HEX	CHL	PET	HWE	TER
		(Extract Concentrations = 2, 4, 8, 16, 32 and 64 µg/ml)				
A. flavus	*A. indica*	8.0	18.0	7.5	32.0	5.4
	C. citratus	6.0	10.0	8.0	13.0	6.0
	E. sonchifolia	15.0	22.0	6.4	16.0	5.8
	S. alata	10.0	11.0	14.8	12.0	5.0
	S. occidentalis	7.0	14.0	8.0	15.0	4.4
A. tamarii	*A. indica*	10.0	16.0	8.2	17.0	4.0
	C. citratus	5.0	8.0	5.0	14.2	2.5
	E. sonchifolia	12.0	26.0	12.4	14.0	5.2
	S. alata	6.0	11.6	8.0	20.0	4.0
	S. occidentalis	5.5	16.0	6.4	26.0	4.5
A. nomius	*A. indica*	13.0	32.0	16.0	15.8	6.0
	C. citratus	4.0	16.0	4.0	22.0	2.0
	E. sonchifolia	10.0	22.0	17.0	18.1	5.0
	S. alata	4.5	10.0	5.2	10.4	3.0
	S. occidentalis	4.0	13.0	5.2	12.0	4.6
C. albicans	*A. indica*	5.0	18.0	8.6	16.5	2.0
	C. citratus	2.0	11.0	5.5	12.0	4.0
	E. sonchifolia	8.0	13.4	10.0	12.1	5.0
	S. alata	5.2	12.0	7.0	20.0	3.2
	S. occidentalis	10.0	12.2	6.6	15.2	3.0

Key: HEX = n-hexane extract CHL = chloroform extract PET = petroleum ether extract HWE = hot water extract TER = terbinafine (antifungal control agent)

Table 13: MFCs (µg/ml) of plant extracts against dermatophyte species

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

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

PET = petroleum ether extract HWE = hot water extract

Table 14: MFCs (µg/ml) of plant extracts against non-dermatophyte species

Species	Plant Extracts	HEX	CHL	PET	HWE
			(MFC range = 5 - 32 µg/ml)		
A. flavus	*A. indica*	8	24	12	32
	C. citratus	7	12	8	14
	E. sonchifolia	18	26	10	16
	S. alata	12	15	16	12
	S. occidentalis	8	16	10	16
A. tamarii	*A. indica*	12	16	12	18
	C. citratus	6	12	8	15
	E. sonchifolia	15	30	16	16
	S. alata	8	13	11	22
	S. occidentalis	6	18	8	30
A. nomius	*A. indica*	16	32	18	18
	C. citratus	6	16	8	22
	E. sonchifolia	13	24	18	22
	S. alata	7	14	8	12
	S. occidentalis	6	15	8	14
C. albicans	*A. indica*	8	21	12	20
	C. citratus	5	13	10	12
	E. sonchifolia	8	16	13	15
	S. alata	8	16	11	21
	S. occidentalis	12	14	8	17

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

PET = petroleum ether extract HWE = hot water extract

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. DISCUSSION

Cutaneous mycoses constitute an important public health problem among rice farmers worldwide, including Nigeria. This disease remains endemic in Nigeria, largely because of lack of information on its prevalence and absence of control measures (Anosike *et al*., 2005). The present study highlights the prevalence of cutaneous mycoses and the antifungal susceptibility pattern of locally used herbs among rice farmers in Ebonyi State, Nigeria. Ikwo and Izzi, the two Local Government Area chosen for the study have relatively higher population density consisting primarily of rice farmers. In this study, there were more females than males due to the fact that rice farming in the study areas was done exclusively by females.

The study revealed that 182 (8.54%) of rice farmers in a random population of 2130 farmers were infected with various species of cutaneous mycotic fungi. The prevalence of cutaneous mycoses observed in the study was insignificant and was unexpected because almost all parameters known to favour person to person transmission, such as the presence of skin lesions, practice of sharing personal belonging, overcrowding in the home, practice of keeping domestic animals/pets and factors that suggest unhygienic life style of the community with low socio-economic background are present in the rice farmers surveyed. Moreover, rice farming is generally associated with higher risk of developing cutaneous mycotic infection, both due to contact with many potential sources of infection such as animals, soil and wet work which promote infection (Sunil, 2002; Oyeka and Okoli, 2003; Shenoi *et al*., 2005; Efuntoye *et al.,* 2011). Similar observations were reported from a study among rice farmers in Anambra State (Ekwealor and Oyeka, 2013). The low prevalence may be due to variation in environmental and climatic conditions favourable for the growth of the pathogens in the area studied. Another possible explanation for the low

prevalence of cutaneous mycoses observed in this study is that the study only looked at those with detectable signs of fungal infection. This has the potential of missing asymptomatic carriers.

In this study, non-dermatophyte molds were found to be more frequently isolated than dermatophytes with *Aspergillus* species as predominant fungal agents. Several researchers have reported the association of dermatophytes and other fungi with cutaneous mycoses world over (Havlickova *et al*., 2008; Enemuor and Amedu, 2009; Prasad *et al*., 2013). The finding of non-dermatophytes as increasing cause of cutaneous mycotic infection as in this study has also been reported by El-Batawi *et al*. (2006), and Ekwealor and Oyeka (2013). The prevalence of non-dermatophytic fungi among the sampled population of rice farmers was significant compared to dermatophytic fungi ($P<0.05$). The high frequency in the isolation of non-dermatophytic molds as observed in this study would suggest that those unhygienic practices that establish non-dermatophytic infection specifically and cutaneous mycoses generally, are still much present within the area surveyed. It is also not known whether non-dermatophytic molds overgrown dermatophytes in coinfection due to resistance to cycloheximide. A high frequency in isolation of non-dermatophyte molds was also recorded by Oyeka and Okoli (2003) and Cribier and Bakshi (2004) and was attributed to their regular isolation from water, air, soil and vegetation. This would have to be dealt with in order to achieve reduction in the load of infection with fungal organisms generally as rice farming poses high chances of their getting in contact with human skin, giving rise to opportunistic infection.

T. tonsurans was the predominant dermatophyte species followed by *T. mentagrophytes* and *T. rubrum* while *A. flavus* was the predominant non-dermatophyte species followed by *A. tamarii* and *A. nomius*. It can be deduced that *T. tonsurans* was the major aetiologic agent of dermatophytoses while *A. flavus* was the major aetiologic agent of non-dermatophytic infection and cutaneous mycoses in general, among rice farmers in the study area. The finding of *T.*

tonsurans as the predominant dermatophyte species was at variance with similar research by Ekwealor and Oyeka (2013) who reported *T. rubrum* as the commonest etiologic agent of cutaneous mycoses in Anambra State. However, because no such study had been previously carried out in the study area among the studied population, the assertion of a variation in etiology is possible.

Species recognition of pathogenic fungi has important epidemiological implications in relation to the acquisition and spread of human infection (Richardson and Warnock, 2003). For example, currently, the predominant species causing tinea capitis in the United States is *T. tonsurans* (Foster *et al*., 2004). By contrast, *T. violaceum* and *T. soudanense*, which are common causes of tinea capitis in parts of Africa and West Asia (Ellabib *et al*., 2002), are rarely isolated from patients in the United States. Recently, Magill *et al*. (2007) found that the isolation rates of *T. violaceum* and *T. soudanense* significantly increased in the Baltimore, MD, metropolitan area. They concluded that the changing epidemiology was due to the introduction of a "new" pathogen by increased immigration to the Baltimore area, where the two species were not endemic.

Although rice farmers of all ages were susceptible to cutaneous mycoses, most of them belonged to the age group 16-26 years. Similar observation was made by Ekwealor and Oyeka (2013). The age group 16-30 years is more actively involved in rice farming and exposed to the hot and humid climate than the younger and older age groups and tends to sweat more. Gender-related studies on the prevalence of cutaneous mycoses consist of fragments, with some studies claiming that males predominate (Nweze, 2001; Ali-Shtayeh *et al.* 2002) while in some, it is the females (Omar, 2000; Anosike *et al*., 2005; East-Innis *et al*., 2006). This study also found more females than males infected with cutaneous mycoses. The probable reason for the higher prevalence of cutaneous mycoses amongst females in the study could be because there were more females in the areas

surveyed than males as well as more females that presented with cutaneous mycoses, thus this may have made the gender prevalence of the males to be lower than that of the females.

The present study showed a relationship between the level of education and the prevalence of cutaneous mycoses in the population studied. Lower frequencies of cutaneous mycotic fungi were observed among rice farmers who had formal education compared to those without formal education. A study in Iraq revealed a higher prevalence of fungal infections in families which had parents with no formal education or low level of education (Fathi and Al-Samarai, 2000), though the study was not amongst rice farmers. Poor personal hygiene and illiteracy are other major factors that influence cutaneous mycoses in this part of the country. Enlightenment programmes and health education are important in the control of the infection.

The infection was reported more among families living in crowded accommodation and those that interacted with pets/ domestic animals such as dogs, cats, goats, rabbits and poultry birds. The reports of Gugnani and Oyeka (1989) support the findings of this study. It was observed that families share their residential houses with domestic animals such as cats, dogs, goats and poultry birds. Although, rice farmers with no interaction with domestic animals were more infected, the isolation of *M. canis* from the farmers is an indication of animal to human transmission. Unhygienic conditions among these farmers may be one of the contributing epidemiological factors influencing transmission of infection (Oyeka and Gugnani, 1992). Domestic animals which are usually carriers or infected should be bathed with disinfectant at least once a week to reduce the spread of spores. Inadequate and poor infrastructures were also observed in the study areas and could be deduced as one of the factors influencing the prevalence of cutaneous mycoses among the farmers.

T. tonsurans and *T. rubrum* were isolated from all three affected body sites examined in the study. The finding of *T. rubrum* as the predominant agent of cutaneous mycoses of different parts of the

body in the study areas was in agreement with similar research by Ekwealor and Oyeka (2013) who reported *T. rubrum* as the most common agent of dermatophytoses of different parts of the body. The results of the study showed that scalp infections were more frequent than nail infections. It was observed that some of the farmers used footwears in the fields whereas most of the farmers did not use hand gloves during their farm work. It can therefore be deduced that the prevalence of scalp lesions (tinea capitis) was much higher in the study population. This was followed by tinea ungium and tinea corporis. This disagrees with the findings of Ekwealor and Oyeka (2013) who reported a high rate of infection of the finger and toe nail among rice farmers in Anambra State, Nigeria. This disagrees also with the findings of Shenoi *et al*. (2005) who reported a high rate of nail infection among paddy field workers in India. Scalp infection was mostly seen in children. This can be attributed to the fact that children are also involved in rice farming.

Dermatophytes and other fungi are the major cause of cutaneous mycoses (Weitzman and Summerbell, 1995). These fungi have the capacity to invade keratinized tissue of humans or animals to produce infections that are generally restricted to the corneocytes of the skin, hair and nails. The keratinolytic activity of keratinophilic fungi is important for ecology and has attracted many researchers' attention around the world. Keratinophilic fungi play an important role in the natural degradation of keratinized residues in the soil (Fillipello, 2000). Some of these fungi live in soil and could be transmitted to humans as well as animals and cause cutaneous mycotic infection (Spiewak and Szostak, 2000). The results of this study showed that the isolated dermatophytes and associated fungi produced keratinase, protease, lipase, xylanase, cellulase, and collagenase enzymes. Highest keratinase activity was observed in *T. rubrum* and *A. flavus*. In a similar study by Sharma *et al*. (2012), *T. rubrum* produced the highest activity of keratinase that concurs with our findings. Contrarily, in another study by Muhsin and Salih (2001), high

keratinase activity was expressed by *T. mentagrophytes* and *M. gypseum*. They also showed high protease activity that was contrary to our result. In overall, the difference in extracellular enzyme activities in the different fungal species was shown to be statistically significant. The ability of these organisms to produce the above mentioned enzymes could explain their ability to degrade the keratinized areas of the skin, indicating the importance of these enzymes in the pathogenesis of cutaneous mycoses.

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

Phytochemical analysis revealed the presence of important secondary metabolites such as flavonoids, alkaloids, saponins, tannins, phenols, glycosides, terpenoids and steroids in the plants thus indicating the therapeutic potentials of the plants. Different phytochemicals have been found to possess a wide range of medicinal properties, which may help in protection against various diseases. For example, flavonoids are a group of polyphenolic compounds that have potent antioxidant, antimicrobial and anti-inflammatory (Okwu, 2001a; Okwu, 2001b) actions. Alkaloids are bitter to taste and are toxic to microorganisms (Gupta *et al*., 2010) and hence act in inhibiting microbial growth. Saponin-containing plants are important for their haemolytic, expectorative, anti-inflammatory and immune-stimulating activities (Zwane *et al*., 2011). Beyond these, saponin

demonstrates antimicrobial properties particularly against fungi, bacteria and protozoa (Ray, 2016). Tannins have antioxidant, antimicrobial and anti-inflammatory properties (Killedar and More, 2010). Phenols possess anti-inflammatory and anti-apoptotic properties. Steroids and terpenoids show analgesic properties. These results support the work of Owoyale *et al.* (2005) which reports the antifungal activity these secondary metabolites on fungal genus. The differences in the above results in the respective solvents may be due to the interaction of the phytoconstituents with the solvent system or the process employed for extraction.

Extract of *C. citratus* showed high concentration of phenols (75.30±0.05 mg/g DW) than the other plant extracts. This is followed by *S. occidentalis* extract. Extract of *C. citratus* usually contain high amount of phenolic content as reported by Mirghani *et al*. (2012). Flavonoids, alkaloids, saponins and tannins were moderately concentrated. The present investigation showed significant variation in the contents like phenol, flavonoids and tannin when compared to previous reports. These variations may be due to species, variety and part of the plant, conditions of growth (soil, water and temperature), season of harvest, age of the plant and a number of environmental factors such as climate, altitude and rainfall.

In the present study, antifungal activity of n-hexane, petroleum ether, chloroform and hot water extracts of these medicinal plants were evaluated against dermatophytes and associated fungi isolated. It was observed that the various extracts evaluated showed a marked inhibitory/fungicidal effect against the isolated cutaneous mycotic fungi which compared favorably with the standard antifungal control, terbinafine. The potency of these plant extracts could be due to phytochemicals contained in the extracts which have similar mode of action to that of terbinafine (Onwuliri and Wonang, 2005; Ayodele *et al*. 2009). It was also observed that hot water extracts had the least effect on the fungal isolates while n-hexane and petroleum ether extracts were the most effective. This might be due to the failure of the active ingredient to

dissolve sufficiently in water. The significant antifungal activity of the n-hexane and petroleum ether extracts over the hot water extracts on the test dermatophyte and non-dermatophyte species justified the principle observed in herbal practitioners' preference for using local gin as extraction agent. It was also observed that *E. sonchifolia* extracts were less effective in the control of the fungal isolates as compared to other extracts at lower concentrations; this may be due to the fact that the phytochemicals contained in other plant extracts were stronger and more effective than those of *E. sonchifolia*. This finding corroborates that of Igbinosa *et al*. (2009) on antifungal activity of medicinal plants on fungal pathogens.

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

5.2. CONCLUSION AND RECOMMENDATIONS

In this study, cutaneous mycoses caused by dermatophyte species were observed to be less prevalent among rice farmers than we had expected. Cutaneous mycoses caused by non-dermatophytes were observed to be more prevalent and leading to these rice farmers' worse quality of life and economic burden. The confirmation of both dermatophytes and non-dermatophytes from the sampled population suggests a relationship that requires further investigation and whose complete understanding has a strong implication for the efficient screening, management, reduction and treatment of the infection among rice farmers, especially in

countries like Nigeria where the infection have become a public health problem and have remained endemic. *T. tonsurans* and *A. flavus* were the most predominant dermatophyte and non-dermatophyte species respectively, which validates etiologic agent variation with respect to a particular geographical location, socioeconomic status and life style of the studied population. Age, gender, educational status and family size of the farmers and their interactions with domestic animals/pets influenced transmission of the infection. Among the tinea infections observed in the community, tinea capitis was the most predominant type of infection followed by tinea ungium (onychomycosis) and tinea corporis.

Our results further indicated the importance of keratinase, protease, lipase, xylanase, cellulase, and collagenase enzymes in the pathogenesis of cutaneous mycoses. An in vivo study of adherence and invasion of cutaneous mycotic fungi to the stratum corneum would be further studied. The study will contribute to a better understanding of the nature of the interaction between dermatophytes and associated fungi and skin cells in cutaneous mycoses process. In addition, future studies to characterize these enzymes and the sequences of their DNA where they are produced is necessary for the proper understanding of how these fungi behave during infection of their hosts as well as its function as saprophytes in the natural environment. Our findings demonstrated that extracts from leaves of medicinal plants used by the rice farmers in the treatment of cutaneous mycoses have fungicidal activity against dermatophyte and associated fungal species. The results obtained may contribute to the alternative use of these plant materials in the treatment of cutaneous mycoses. However, further toxicity studies need to be performed as well as assays to clarify the mechanism of action and possible interactions with antifungals or other compounds to guarantee their safety.

Although, the present study is a random study that focuses primarily on the prevalence of cutaneous mycoses among rice farmers in Ebonyi State, more systematic study covering larger

population and over a longer period of time would give a better insight into the epidemiology of the infection among rice farmers in the state. It is expected that the study will be an eye-opener to the government, non-governmental organizations as well as community-based organizations to execute various intervention programmes like public health education, improved working conditions, housing and sanitary standards, accessible potable water, protective equipment such as long rubber boots, masks and gloves, periodic surveillance and economic stability to help reduce the burden of the infection in the area. As most of the chemicals applied for the eradication of pests and different diseases can cause serious health hazards, it is recommended to plant different variety of crops that can resist diseases and pests.

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APPENDIX ONE

ETHICAL CLEARANCE

FEDERAL TEACHING HOSPITAL, ABAKALIKI

P.M.B. 102 ABAKALIKI, EBONYI STATE, NIGERIA *Website:www.fetha.ng Email:info@fetha.ng*

DR. ONWE EMEKA OGAH
MB. BS, FWACP
Chief Medical Director

CHIEF C. C OGBU JP, KSM
B.Sc. (Hons), MBA, M.Sc
FCAL, FNIMN, FHAN, MNIM
Chief Medical Director

Our Ref: FETHA/AD/ECMC/VOL.1/17

Date: 18th May, 2015

PAULINE A. AHUOCHA
Department of Applied Microbiology & Brewing
Nnamdi Azikiwe Univeristy, Awka

ETHICAL CLEARANCE

Following your application and subsequent interview, you are hereby informed that Ethical Clearance Management Committee of Federal Teaching Hospital Abakaliki has approved PhD Project Research Thesis 'Prevalence and Epidemiology of Dermatophytoses among rice farmers in Ebonyi State, Nigeria' by Pauline Amaka Ahuocha with registration number: 2013487015F, Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, to be carried out in the State.

The management believed that you are going to carry out this research according to rules and regulation guiding professional ethic.

Yours faithfully

Chief C. C. Ogbu
Director, Administration

APPENDIX TWO

CONSENT LETTER

Department of Applied Microbiology and Brewing,
Faculty of Biosciences,
Nnamdi Azikiwe University,
P.M.B. 5021, Awka,
Anambra State,
4th May, 2015.

Dear Respondent,

RE: Prevalence and Epidemiology of Dermatophytoses among Rice Farmers in Ebonyi State

The researcher, Ahuocha Pauline Amaka is a post graduate student of Nnamdi Azikiwe University, Awka. She is interceded in determining the prevalence and epidemiology of dermatophytoses among rice farmers in Ebonyi State.

She is therefore soliciting your assistance to enable her complete the research. You are requested to assist by completing the attached questionnaire. There is no right to wrong answer; it is basically on your personal experience. Be assured that all your responses shall be treated with utmost confidentiality.

Thanks in anticipation of your positive responses.

Yours faithfully,

Prof. C. O. Anyamene
(Project Supervisor)

APPENDIX THREE

DETERMINATION OF SAMPLE SIZE

Sample size, $n = 4 \times p (1-p)/ L^2$

Where,

p = approximate prevalence rate

L = permissible error

A 95% confidence level, 5% permissible error and p= 0.5 is assumed.

$n = 4 \times 0.5 (1-0.5)/ (0.05)^2$

$n = 4 \times 0.25/ 0.0025$

$n= 1/ 0.0025$

$n = 400$

This implies that the minimum sample size for this study is 400 samples

APPENDIX FOUR

QUESTIONNAIRE

A. For each statement, please tick/write the appropriate answer

1. Gender: Female ( ); Male ( )
2. Age? (In years)_____
3. Occupation _____
4. What is your religious affiliation? Christian ( ); Islam ( ); Other ( ); None ( )
5. What was the last level of schooling that you completed?
No formal education ( )
Primary ( )
Secondary/vocational ( )
Post-secondary ( )
Other, please specify: _____
6. Marital Status: Married ( ) Single ( ); Separated ( ); Divorced ( );
Widowed ( ); Unknown ( )
7. No of children (if married) _____
8. What is the nature of your family's living accommodation? _____
9. Have you had any information on dermatophytoses? Yes ( ); No ( )
10. Have you ever seen anybody with dermatophytoses? Yes ( ), No ( ); which year? _____
11. Are you living with dermatophytoses? Yes ( ) No ( )
12. Do you have anybody living dermatophytoses? Yes ( ) No ( )
13. Where is/are the location of the infection on your body? _____
14. How long have you noticed the infection? _____
15. Does there seem to be a seasonal influence? Yes ( ) No ( ); if yes, which season? _____
16. Presence of pets/domestic animals in the house? Yes ( ) No ( )
17. When did you adopt the pet/domestic animal? _____
18. Where did you adopt the pet/domestic animal? _____
19. Does your pet/domestic animal experience any of the following?
Itching ( ), Flaky skin ( ), Loss of nails ( ), Curving/cracking/breaking nails ( ), Hair loss ( ), Red skin ( ), Odour ( ), others _____
20. How long have you noticed the signs/symptoms on your pet/domestic animal?
21. Use of manure ( ), herbicides ( ) or pesticides ( )
22. Are you on any antifungal drug? Yes ( ) No ( )
23. Does the drug seem helpful? Yes ( ) No ( )
24. Travel history/ Recent move? _____

B. For each statement; please tick "True", "False", or "I don't know". If you do not know, please do not guess; instead, please tick "I don't know".

SN	Question	True	False	I don't know
1.	Dermatophytosis is common among rice farmers in Ebonyi State			
2.	The infection can give rise to adverse effects			
3.	The infection is caused through sharing of towels			
4.	The infection is caused through sharing of foot wears			
5.	A person can get the infection by sharing a glass of water.			
6.	The infection is caused by frequent use of water.			
7.	The infection is easily cured by cleanliness			
8.	We can use drugs to cure the infection			
9.	I can get the infection by not taking by bath daily			
10	The infection is caused due to my occupation.			
11.	The infection is caused due to poor skin hygiene			
12.	I believe that the infection heal itself			
13.	The infection is unavoidable and it is a normal condition			
14.	The infection can be dangerous to the society			
15.	I can get the infection during my working time.			
16.	The chemicals I am using can expose me to the infection.			
17.	Sharing of cloths between neighbours can predispose me to the infection.			
18.	The infection is contagious			
19.	Anybody can get the infection.			
20.	Children can get the infection through their parents			
21.	The infection could be spread through shaking of hands.			
22.	I can get the infection from my pets/domestic animals			
23.	I have fungal infections on my toe/nail/hair/groin/neck			

APPENDIX FIVE

MEDIA AND REAGENT PREPARATION

20% Potassium Hydroxide (KOH) Solution

Composition

Potassium Hydroxide	20 g
Distilled Water	100 ml

Directions

1. Weigh 20g KOH pellets.
2. Dissolve in100 ml distilled water.
3. Store the solution at 25-30°C.

Sabouraud Dextrose Agar (SDA)

Composition

Dextrose (Glucose)	40 gm
Peptone	10 gm
Agar	15 gm
Distilled Water	1000 ml

pH 5.6 at 25^{o} C

Directions

1. Suspend 65 g of the medium in 1000 ml distilled water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121° C for 15 minutes.
4. Cool to 45 - 50°C and pour into Petri dishes or tubes for slants.

Sabouraud Dextrose Broth

Composition

Dextrose (Glucose)	20 gm
Peptone	10 gm
Distilled Water	1000 ml

pH 5.6 at 25^{o} C

Directions

1. Suspend 30 g in 1000 ml distilled water.
2. Heat if necessary to dissolve the medium completely.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Mix well and dispense as desired.

Cycloheximide Reagents (0.5 mg/mL stock solution)

Composition	Stock solution volume (200 ml)
Cycloheximide	100 mg
Acetone	200 mL

Directions

1. Weigh the cycloheximide in the fume hood.
2. Add the acetone and mix to dissolve.
3. Store in a foil covered, sealed bottle in cold storage (4°C).

Volume of cycloheximide stock solution (0.5 mg/mL) required for mL agar

250 mL agar	500 mL agar	1000 mL agar
5 mL	10 mL	20 mL

Chloramphenicol (0.05 mg/ml stock solution)

Composition

10 mg chloramphenicol

200 ml 95% ethanol

Directions

1) Dissolve 10 mg of chloramphenicol into 200 ml 95% ethanol.
2) Mix/vortex vigorously so all the chloramphenicol goes into solution.
3) Aliquot and store at -20°C.
4) Use at 1:1000 dilution

Yeast Extract Agar

Composition

Yeast Extract	3.0 g/L
Peptone	5.0 g/L
Agar	15.0 g/L

pH 7.2 ± 0.2 @ 25 °C

Directions

1. Suspend 23 g in 1000 mL of distilled water.
2. Bring to boil to dissolve completely.
3. Sterilize by autoclaving at 121 °C for 15 minutes.
4. Dispense and store at 2 - 8 °C.

Urea Agar Base

Composition

Peptone	1.0 g/L
Glucose	1.0 g/ L
Sodium chloride	5.0 g/L
Disodium phosphate	1.2 g/L
Potassium dihydrogen phosphate	0.8 g/L
Phenol red	0.012 g/L
Agar	15.0 g/L

pH 6.8 ± 0.1

Directions

1. Suspend 2.1g in 95ml of distilled water.
2. Bring to boil to dissolve completely.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Cool to 50 °C and aseptically introduce 5ml of sterile 40% Urea Solution (Urea No 8487).
5. Mix well, dispense 10 ml amounts into sterile tubes and allow solidifying in slant position.
6. Store at 2 - 8°C.

Rice Grain Media

Composition

Unfortified white rice grains	8.0 g
Distilled water	25.0 ml

Directions

1. Mix ingredients in 125 ml flask.
2. Autoclave for 15 minutes at 121 ºC.
3. Store at -4 ºC

Potato Dextrose Agar

Composition

Potatoes extract	4.0 g/L
Glucose	4.0 g/L
Agar	15.0 g/L
Distilled water	1000 mL

pH 5.6 ± 0.2 @ 25°C

Directions

1. Suspend 39g in 1000 ml distilled water.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Sterilize by autoclaving at 121°C for 15 minutes.
4. Mix well and dispense in Petri dishes and tubes.

Olive Oil Emulsion

Composition

Virgin olive oil	25 ml
Gum Arabic	10 g
Distilled water	100 ml

Directions

1. Disperse 10 g gum arabic powder in 100 ml distilled water to form solution.

2. Slowly add 25 mL olive oil into 75 mL gum arabic solution.
3. Emulsify in a homogeneizer on an ice-bath for 15min.
4. The emulsion is stable at 4^{o}C.

Oat Meal Xylan

Composition

Oat meal	25 g
Distilled water	300 ml

Directions

1. Suspend 25 g oat meal in 200 ml distilled water.
2. Mix very well by stirring for 15 min using magnetic stirrer.
3. Strain the content using strainer.
4. Store the filtrate on ice for 1 hour.
5. Add 100 ml water to the sediment and stir again for 15 min.
6. Repeat 3.
7. Pool both filtrate and transfer the content in a container.
8. Store the filtrate in a refrigerator overnight.
9. Gently transfer the content into a centrifuge tube without disturbing the sediment at the bottom.
10. Spin 10,000 rpm for 30 min.
11. Transfer the supernatant into a flask and autoclave for 15 min at 121 ^{o}C.
12. Add nutrients and make your medium which will have xylan as the major carbohydrate.

Carboxymethyl cellulose (CMC) Agar

Composition

$NH_4H_2PO_4$	1.0 g/L
KCl	0.2 g/L
$MgSO_4 . 7H_2O$	1.0 g/L
Yeast extract	1.0 g/L
Distilled water	1000 mL

Directions

1. Add 15g agar and 1.5g carboxymethyl cellulose (CMC) substrate to 1L distilled water and autoclave to sterilize.
2. Allow medium to cool.
3. Gently mix and dispense into Petri dishes.

Chicken Sternal Cartilage Collagen

Directions

1. Digest 1 g of sternal cartilage pieces in 100 ml of pepsin solution for 32 h at 20°C.
2. Centrifuge the resulting viscous solution at 8.000 rpm at 4°C for 30 min.
3. Allow to precipitate for 16 h in 0.9 M NaCl solution.
4. Dissolve the precipitated collagen in 0.47 M acetic acid (pH 2.5).
5. Store at 4 ^{o}C.