EVALUATION OF THE ANTI-FUNGAL ACTIVITIES OF CRUDE EXTRACTS OF THE AERIAL PARTS OF *MITRACARPUS VILLOSUS* (Sw.) DC.

By

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A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES AHMADU BELLO UNIVERSITY, ZARIA.

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APRIL, 2014

Declaration

I hereby declare that the work in this thesis entitled "Evaluation of the Anti-Fungal Activities of Crude Extracts of the Aerial Parts of *Mitracarpus villosus* (Sw.) DC" was performed by me in the Department of Pharmaceutics and Pharmaceutical Microbiology under the supervision of Dr B.O. Olayinka and Dr. (Mrs) G. O. Adeshina. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work was previously presented for another degree or diploma at this or any institution.

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Mercy Itohan ABOH Signature Date

Certification

This thesis titled "Evaluation of the Anti-Fungal Activities of Crude Extracts of the Aerial Parts of *Mitracarpus Villosus* (Sw.) Dc Obtained in Abuja, Nigeria" by Mercy Itohan ABOH meets the regulations governing the award of the degree of Master of Science (Pharmaceutical Microbiology) of Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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Dedication

This work is dedicated to the Lord Jesus Christ for giving me wisdom, sound health, favour and life. To Him alone belong all the glory, adoration, worship and praise and my loving husband Anthony who stood by me at all times.

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ABSTRACT

*Plants have provided a source for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. The aerial parts of Mitracarpus villosus were powdered, screened for phyto-compounds and the phyto-compounds detected were isolated. This was followed by successive extraction of the plant (hexane, ethyl acetate, ethanol and water) and fractionation of the ethyl acetate extract. Antifungal activities of the crude extracts, fractions and extracted secondary metabolites against clinical isolates of Candida albicans, Candida krusei, Trichophyton verrucosum, Trichophyton mentagrophytes, A*spergillus fumigatus *and* Aspergillus niger *were investigated using agar diffusion, broth dilution and micro broth dilution methods. The antibiotic susceptibility profiles of the fungal isolates to standard antifungals such as fluconazole and ketoconazole were determined using agar diffusion and broth dilution methods. The effects of temperature and different storage conditions on the antifungal activity of the crude plant extracts, as well as the rate of death / survival of the fungal isolates on exposure to crude extract and standard drugs were also investigated. Phytochemical screening powdered plant revealed the presence of tannins, saponins, flavonoids, terpenes, phenols and resins. The ethyl acetate extract of the plant aerial parts produced the highest antifungal activity when compared with the other solvent extracts (hexane, ethanol and water) with inhibition zone diameter ranging from 18.67 – 21.67 mm at an exposure concentration of 12.5 mg/mL. The crude tannin, saponin and phenol extracts produced zones of inhibition ranging from 19.67- 24.0 mm, 19.0 –24.67 mm and 18.33- 22.0 mm respectively. The minimum inhibitory concentration and minimum fungicidal concentration values of the ethyl acetate extract against all the fungal isolates tested were 0.50 – 2.00 mg/ml and 2.00 – 8.00 mg/ml respectively. The minimum inhibitory concentrations of the crude tannins, crude saponin and phenol extracts were 0.50 – 2.00 mg/mL, 0.50 – 4.00 mg/ml and*

0.50 – 8.00 mg/ml respectively, while their minimum fungicidal concentrations were 1.00 – 8.00 mg/ml, 1.00 – 8.00 mg/ml and 1.00 – 16.00 mg/ml respectively. The ethyl acetate fractions (M1- M6) had stronger antifungal effects on the test fungi than the crude extracts and isolated phyto-compounds. M2 and M3 had minimum inhibitory concentration values of 250.00 – 2000.00 µg/ml, M1 and M5 had minimum inhibitory concentration values ranging from 250.00 – 4000.00 µg/ml while that of M5 and M6 was 500.00 – 4000.00 µg/ml. The minimum fungicidal concentration values for M2 and M3 was 500.00 – 4000.00 µg/ml while the minimum fungicidal concentrations of fractions M1, M4, M5 and M6 ranged from 500.00 – 16000.00 µg/ml against the fungal isolates tested. Crude extracts maintained their antifungal activity within the temperature range tested and over a storage period of nine months. The pattern of kill of the fungal isolates by the ethyl acetate extract and fraction M2 of were comparable with that of the standard drugs. In conclusion, the crude extracts of Mitracarpus villosus aerial parts possess strong antifungal activities and stable over nine months storage over a temperature range of 25 – 45 ^{o}C. Further investigations should be carried out to isolate pure compounds and determine the mechanisms of action of the plant.

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CHAPTER 1

1.0 INTRODUCTION

Plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being (Avijgan *et al.*, 2010). Early humans recognized their dependence in nature in both health and illness. Led by instinct test and experience, primitive people treated illness by using plant, animal parts and minerals that were not part of their diet (Anwanni *et al.*, 2005).

In recent years, there has been a gradual revival of interest in the use of medicinal plants in developing countries because herbal medicines have been reported to be well tolerated when compared with synthetic drugs (Iniaghe *et al*., 2009). In addition, there has been little or no report of any form of microbial resistance during the administration and use of herbal medicines. More importantly in Africa, particularly West Africa, new drugs are often beyond the reach of the poor such that up to 80% of the population uses medicinal plants as remedy against infections and diseases (Ezekiel *et al.*, 2009). There has been renewed interest in herbal product partly due to high cost involved in the development of patentable chemicals/ drugs. There is also evidence that medicinal plants contain synergistic and side effect neutralizing combination (Gilani *et al*., 2005).

The search for antimicrobial agents from plants has been a growing interest in the last few decades. However, results generated from many of these studies cannot be directly compared due to the lack of standardization in particular antimicrobial methods employed; *standard* criteria for evaluation of *plant antimicrobial* activity are *lacking* and results greatly differ between authors (Ncube *et al*., 2008). The need to establish methods with consistent results for the evaluation of antimicrobial activities from plant extracts has been proposed by many researchers (Othman *et al*., 2011). Research have shown that plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Cowan, 1999).

1.1 STATEMENT OF PROBLEM

In the past few decades, a worldwide increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some species of fungus to different fungicidal agents used in medicinal practice (Abad *et al*., 2007). Fungi are one of the most neglected pathogens, as demonstrated by the fact that the amphotericin B, a polyene antibiotic discovered as long ago as 1956, is still used as a "gold standard" for antifungal therapy. The last two decades have witnessed a dramatic rise in the incidence of life threatening systemic fungal infections (Richardson, 2005). The challenge has been to develop effective strategies for the treatment of candidiasis and other fungal diseases, considering the increase in opportunistic fungal infections in human immunodeficiency virus-positive patients and in others who are immunocompromised due to cancer chemotherapy and the indiscriminate use of antibiotics. The majority of clinically used antifungals have various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains (Abad *et al*., 2007).

Mycosis constitutes a common health problem, especially in tropical and subtropical developing countries including Nigeria; dermatophytes, *Malassezia* spp. and *Candida* spp. being the most frequent pathogens in humans and animals (Fontenelle *et al*., 2007). Dermatophytes are the major cause of superficial mycoses and remain a public health problem. They have the ability to invade keratinized tissues and cause dermatophytosis, the most common human contagious fungal disease (Nasrin *et al.*, 2007). Microorganisms have developed resistance to many antifungals and as a result, immense clinical problems have been created. The resistance of the organisms increased due to indiscriminate use of antifungal drugs for the treatment of infectious disease. *Candida* is a common opportunistic pathogen of immunocompromised

humans, and the incidence of candidiasis has risen over the years due to the AIDS epidemic and the use of therapeutics during immunosuppressive drug therapy (Kullberg and Filler, 2002). Infections with mould pathogens have emerged as an increasing risk faced by patients under sustained immunosuppression Species of the *Aspergillus* family account for most of these infections and in particular *Aspergillus fumigatus. Trichophyton verrucosum* and *Trichophyton mentagrophytes* are the major causes of dermatomycosis (commonly known as ringworm) in cattle in many parts of the world (Adejumo and Bamidele, 2009). Transmission of these ringworm-causing organisms from infected animals to people has been reported (Shams-Ghahfarokhi *et al*., 2009). The zoonotic nature of the infection demonstrates the potential for animal-derived ringworm in humans, and indicates the need for an integrated approach to control of ringworm infections (Wabacha *et al.*, 1998). The relatively sudden increase in the numbers of patients with invasive fungal infection coupled with the toxicity, limited spectrum of activity, and the propensity to induce or select for resistant strains of current antifungal drugs create a need for novel antifungals belonging to a wide range of structural classes, selectively acting on new targets with fewer side effects. (De Pauw, 2002). These situations have forced researchers to search for new antimicrobial substance from various sources including medicinal plants.

1.2 JUSTIFICATION

Dermatophytosis (syn. ringworm, *Tinea*), a zoonotic skin infection of keratinized tissues caused by a specialized group of fungi named dermatophytes, has worldwide distribution and it is to be public health problem all over the world. *Trichophyton verrucosum* is the usual zoophilic dermatophyte involved in cattle ringworm throughout the temperate regions of the world (Patel *et al*., 2010). Aside from animal

involvement, several human outbreaks of *T. verrucosum* infection have been reported so far by direct contact with infected animals or indirect contact with infectious propagules in the environment (Shams-Ghahfarokhi *et al*., 2009). The search for antimicrobial agents from plants has been a growing interest in the last few decades. However, results generated from many of these studies cannot be directly compared due to the lack of standardization in particular antimicrobial methods employed *standard* criteria for evaluation of *plant antimicrobial* activity are *lacking* and results greatly differ between authors (Ncube *et al*., 2008).

Mitracarpus villosus plant is found in different parts of Nigeria. However, there is limited information on the evaluation of the antifungal activities of the ethyl acetate extracts and its isolated secondary metabolites and fractions on *Candida albicans, Candida krusei, Trichophyton verrucosum, Trichophyton mentagrophytes, Trichophyton verrucosum, Aspergillus fumigatus* and *Aspergillus niger.*

The high prevalence of fungal infections worldwide which is further complicated in immunocompromised states as well as the increasing rate of resistance of most pathogenic fungi to existing orthodox antifungals, justifies the need for this study.

1.3 MAIN OBJECTIVE

The main objective of this study is:

To evaluate the anti-fungal activities of the crude extracts of the aerial parts of *Mitracarpus villosus* (Sw.) DC.

1.4 SPECIFIC OBJECTIVES

The specific objectives of this study are as follows:

i. To determine the antifungal activities of the crude extracts of *Mitracarpus villosus* using zone of inhibition, fungistatic and fungicidal criteria.

ii. To identify and quantify the active phyto-compounds in the plant responsible for its antifungal activity.

iii. To determine the rate of kill of the test fungal cells by the most active extract of *Mitracarpus villosus*.

iv. To determine the influence of temperature and storage duration on the antifungal properties of the most active extract of *Mitracarpus villosus*.

1.5 RESEARCH HYPOTHESIS

NULL HYPOTHESIS (H_0):

- *Mitracarpus villosus* extracts donot possess antifungal activities against *Candida albicans, Candida krusei, Trichophyton verrucosum, Trichophyton mentagrophytes, Trichophyton verrucosum, Aspergillus fumigatus* and *Aspergillus niger.*

ALTERNATE HYPOTHESIS (H_1):

- *Mitracarpus villosus* extracts possess antifungal activities against *Candida albicans, Candida krusei, Trichophyton verrucosum, Trichophyton mentagrophytes, Trichophyton verrucosum, Aspergillus fumigatus* and *Aspergillus niger.*

1.6 RESEARCH LIMITATIONS

- Only *Candida albicans, Candida krusei, Trichophyton mentagrophytes, Trichophyton verrucosum, Aspergillus fumigatus* and *Aspergillus niger.* is used in the study.
- Only the extract with highest zone of inhibition was used subsequently for the study.
- No typed culture of test organisms was used in the study.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 MITRACARPUS VILLOSUS (Sw.) DC

The plant *Mitracarpus villosus* (S.W) D.C belongs to the family Rubiaceae. It was formerly known as *Mitracarpus scaber* Zucc. In Nigeria, it is known as 'Irawo Ile' by Yorubas (Gbile, 1984), 'Obuobwa' by Ibos and 'Gududal' by Sokoto Fulanis (Hutchinson and Dalziel, 1963).

2.2 DESCRIPTION OF PLANT

Mitracarpus villosus is an erect perennial annual herb up to 60cm high. The stem is angled, hairy and sparsely branched. It is woody at the base, segmented into nodes with each internode bearing a pair of leaves. The fresh leaves are green in colour with the characteristic mild odour, bitter and peppery taste. The leaf is simple in composition, opposite/decussate in arrangement, lanceolate in shape, entire in margin with a cuneate base and an acute apex. Venation is parallel, without petiole i.e. sessile and with an internode length of 5.2 – 7.2 cm. Veins are more prominent on lower surface with nearly glabrous upper and lower surfaces with hairs on mid-rib region. The matured leaf size is 3.5 cm (length) and 0.8- 1.3 cm (breath). Fruits are small capsules while seeds are more or less rectangular shaped and yellowish- brown in colour (Onawunmi *et al.*, 2012).

Qualitative and quantitative studies indicated presence of amphicribal vascular bundle arrangement, characteristic asperites, cone-shaped clothing trichomes, simple leaf arrangement lanceolate shape, entire margin, cuneate base, parallel venation and opposite/decussate arrangement. Other features include presence of calcium oxalate crystals, lignin and oil globules with palisade ratio of 4 – 7 and stomatal number of 13.5 (Jegede *et al.*, 2005). Microscopical studies indicated presence of simple leaf whorled arrangement, an entire margin with lanceolate shape, acute apex and base, parallel venation, thin and wavy anticlinal walls with numerous calcium oxalate crystals. Stomata arrangement was reported to be anomocytic with numerous covering trichomes on both surfaces. Chemomicroscopic characters present include lignin, starch, cellulose, mucilage and calcium oxalate crystals (Abere *et al.*, 2007).

Figure 1.0 *Mitracarpus villosus* (S.W) D.C

2.3 ORIGIN AND GEOGRAPHICAL LOCATIONS

It is found along the West African region ranging from Senegal to Nigeria (Jegede *et al*., 2005). It is often confused with *Borreria ocymoides*, with which it was formerly grouped. It grows as a weed on old and abandoned farmlands. It is distributed widely from forest to savanna zones of the tropics.

2.4 PHYTOCHEMICAL PROPERTIES OF *MITRACARPUS VILLOSUS* (Sw.) DC

Phytochemical evaluation of the leaves of this plant found in other places has been reported to show the presence of alkaloids, tannins, cardiac glycosides and saponins. (Ubani *et al.*, (2012), showed that *Mitracarpus villosus* found in South East and South Southern Nigeria, contain tannins, saponins, alkaloid, soluble carbohydrate, flavonoids, reducing sugar, cyanide, glycoside, steroid and terpenoid in varying concentration depending on the environment where the leaves were harvested. In Republic of Congo Makambila-Koubemba *et al.*, (2011) also reported the presence of terpenoids, saponins, tannins and flavonoids in the whole plant.

2.5 MEDICINAL PROPERTIES

In various parts of tropical Africa, it is traditionally used for treatment of sore throat. It has been used in traditional medicine practices in West Africa for the treatment of headaches, toothaches, amenorrhoea, dyspepsia, hepatic diseases, veneral diseases as well as leprosy (Dalziel, 1937). In Nigeria, it has been used to treat ringworm and eczema, fresh cuts, wounds and ulcer. The aerial portion of this plant has also been used to make lotion and skin ointment used for skin diseases and infections (Bisignano *et al*., 2000). In Nigeria, the extracted juice from aerial parts is topically applied against skin diseases and on wounds (Fluck, 1976). Internally it is used as an antidote to arrow poison, anti-diarrhea, and anti-dysentery (Abere *et al*., 2007).

The extract from the native plant of *Mitracarpus villosus* is used treat the infection known as *Dermatophilus congolensis* of Cattle (Gbaguidi *et al*., 2005). A decoction of its aerial part have been reported to have significant hepatoprotective

effect against induced liver injury, both *in vivo* or *in vitro* (Germano *et al*., 1999). Hexane extract of the leaves was found to have a dose dependent anti-inflammatory activity (Ekpendu *et al*., 1994). The methanol extract is active against a wide range of test microorganisms, most remarkably *Pseudomonas aeruginosa* (Jegede *et al.*, 2005).

2.6 SECONDARY METABOLITES

Plants can produce far more compounds than are necessary for their propagation. These secondary metabolites specific with diverse structures and bioactivities (like flavours, colours, dyes, insecticides and drugs), are synthesized mainly for defense against predators. Secondary compounds are often involved in key interactions between plants and their abiotic and biotic environments. Relationship between intermediate metabolites and secondary compounds in plants throughout history has been utilized by humanity. The plant metabolites can be broadly grouped into phenolics (anthro cyanins, coumarins, flavonoids, quinons and tannins), terpenoids (essential oil, saponins, sterols and cucurbitacins), alkaloids, proteins and peptides. (Edwards *et al.*, 1999).

2.6.1 Flavonoids

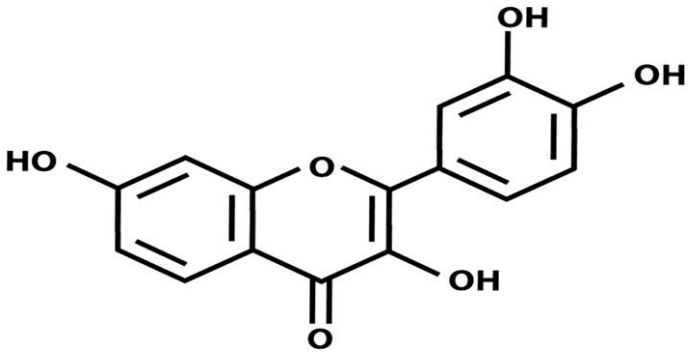

Fig. 2.1: Structure of flavonoids

The flavonoids are a large group of natural products widespread in higher plants, and are also found in some lower plants including algae. The flavonoids are phenolic compounds possessing 15 carbon atoms and comprise two benzene rings joined by a linear three carbon chain (Leon *et al*., 2012). Flavonoids constitute one of the most characteristic classes of compounds in higher plants. Many flavonoids are easily recognized as flower pigments in most angiosperm families (flowering plants). However, their occurrence is not restricted to flowers but includes all parts of the plant. They are low molecular weight substances found in all vascular plants. In the broad sense, they are virtually universal plant pigments. The anthocyanidins are responsible for flower colour in the majority of angiosperms, but colourless flavonoids are also widespread and abundant. They are phenyl benzopyrones with an assortment of basic structures usually found conjugated to sugars, which have been identified in nature. Flavonoids have been reported to occur in several structurally and biosynthetically related classes and are important constituents of the human diet, being derived largely from fruits, vegetables, nuts, seeds, stems and flowers (Harborne, 1977).

Several members of the flavonoids family have been reported to possess antiviral and anti-inflammatory properties, vasculo-protector and anti-thrombotic action, spasmolytic activity, estrogenic actions, antioxidant and liver protecting effects (Middleton and Kandaswami, 1994). Some flavonoids, like quercetin and gossypin, have recently been shown to possess sedative and analgesic effects (Picq *et al*., 1991). Another flavonoid, and biflavonoid derivatives, isolated from *Gingko biloba* have been shown to increase blood flow and reduce neuronal oxidative metabolism (Picq *et al.,* 1991).

Report from research indicates that flavonoids may modify allergens, viruses, and carcinogens, and so may be biological "response modifiers". *In vitro* studies have also shown that flavonoids also have anti-allergic, anti-inflammatory (Spencer and Jeremy 2008), anti-microbial (Cushnie and Lamd, 2005), anti-cancer (Sousa *et al.*, 2007) and anti-diarrheal activities (Schuier *et al.*, 2005). Flavonoids (both flavonols and flavanols) are most commonly known for their antioxidant action (Aqil and Mejmood, 2006)

2.6.2 Alkaloids

Alkaloids are a chemically heterogenous group of basic nitrogen containing substances found predominantly in higher plants and also occur in lower plants, animals, microorganisms and marine organisms (Garba and Okeniyi, 2012). Alkaloids usually contain one or two nitrogen atoms although some like ergotamine may contain up to five nitrogen atoms (Jin-Jian *et al*., 2012)

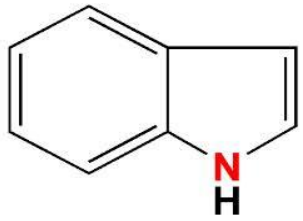

Fig. 2.2: Molecular structures of indole alkaloids.

Alkaloids were so named because of their alkali-like nature. 'True Alkaloids' were defined as compounds meeting the additional four qualifications namely:

i) Nitrogen is a part of heterocyclic ring

ii) The occurrence of compound is restricted to plant kingdom

iii) The compound has complex molecular structure

iv) The compound manifests significant physiological activity

Their manifold pharmacological activities have always excited man's interest, and since early times selected plant products have been used as poisons, euphoriants, psychedelics, stimulants or as medicines (Campbell *et al*., 2012). Many of our modern drugs now contain the same compounds or synthetic analogues, and the pharmacological and toxicological properties of these compounds are thus of immense interest and importance. Alkaloids often have pronounced bioactivities and are therefore thought to play an important role in the interaction of plants with their environment. Alkaloids and extracts of alkaloid-containing plants have been used throughout human history as remedies, poisons and psychoactive drugs (Fester, 2010). The biosynthesis of alkaloids often follows complex pathways and includes stereo specific steps. Alkaloids often have pronounced bioactivities and are therefore thought to play an important role in the interaction of plants with their environment.

Garba and Okeniyi, (2012) evaluted the antimicrobial activity of the total alkaloids from five medicinal plants namely *Jatropha curcas, Calotropis procera, Magifera indica, Carica papaya* and *Psidium guajava,* commonly used in northern Nigeria for the treatment of various ailments. It was reported that The results obtained showed that all the plants contained various level of alkaloids and an appreciable level of antimicrobial activities were observed at a concentration level of $6x10^2$ µg/ml indicating that the plants could be a potential source of alkaloids that may be used for the treatment of various microbial diseases.

2.6.3 Phenylpropanoids

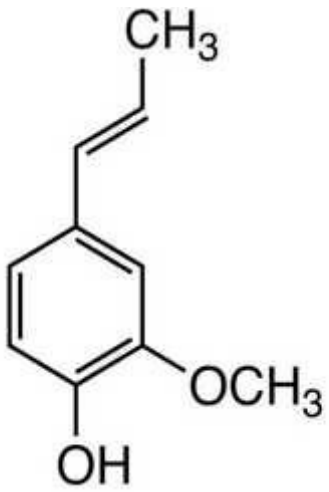

Fig. 2.3: Molecular structure of phenylpropanoids

These are a diverse family of organic compounds that are synthesized by plants from the amino acid phenylalanine. Their name is derived from the six-carbon, aromatic phenyl group and the three-carbon propene tail of cinnamic acid, which is synthesized from phenylalanine in the first step of phenylpropanoid biosynthesis. Phenylpropanoids are found throughout the plant kingdom, where they serve as essential components of a number of structural polymers, provide protection from ultraviolet light, defend against herbivores and pathogens, and mediate plant-pollinator interactions as floral pigments and scent compounds (Korkina, 2007). Plant-derived phenylpropanoids (PPPs) compose the largest group of secondary metabolites produced by higher plants, mainly, for the protection against biotic or abiotic stresses such as infections, wounding, ultra violet (UV) irradiation, exposure to ozone, pollutants, and herbivores. PPPs are parent molecules for biosynthesis of numerous structurally and functionally diverse plant polyphenols (simple phenolic acids and esters, glycosylated derivatives of primary PPPs, flavonoids, isoflavonoids, stilbenes, coumarins, curcuminoids, lignans, etc.), which play multiple essential roles in plant physiology (Ivanauskas *et al*., 2008). During the last few decades, extensive research has been dedicated to natural and

biotechnologically produced PPPs for medicinal use as antioxidants, UV screens, anticancer, antiviral, anti-inflammatory, wound healing, and antibacterial agents (Korkina *et al*., 2011).

2.6.4 Triterpenoids

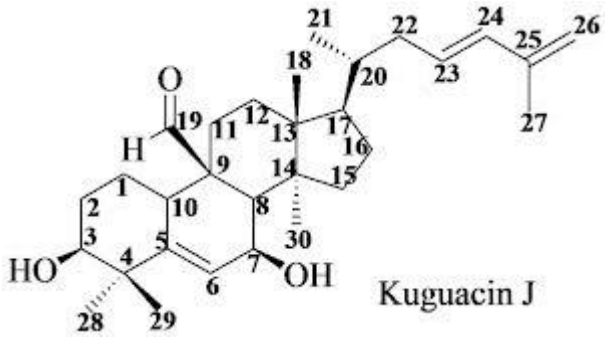

Fig. 2.4: Structure of Kuguacin J (a triterpenoid)
Adapted from www.intechopen.com., accessed 10th March, 2012.

Triterpenoids are non-steroidal secondary metabolites which have been isolated from plants. The physiological function of triterpenoids is generally believed to be a chemical defense against pathogens and herbivores. Throughout the plant and animal kingdoms, terpenoids are known to have a wide range of functions. They can act as defensive substances in plants (allomones) and animals, they can be used by plants to deter herbivores or attract natural enemies of herbivores (synomones). Plant hormones are often derivatives of terpenoids, such as cytokinins, gibberellins and abscisic acid. It is therefore expected that terpenoids should act against certain pathogens causing human and animal diseases (Mahato and Sen, 1997), although medicinal use of this class of compounds is rather limited, possibly due to their hydrophobic nature, recent work in this regard indicated their great potential as drugs. Moreover, despite the

remarkable diversity already known to exist, new variants continue to emerge (Mahato and Sen, 1997).

Terpenoids have been shown to be active against bacteria, fungi and viruses (Taylor *et al.,* 1996; Suresh *et al.,* 1997; Xu *et al.,* 1996), though their mechanism of action is not fully understood. Capsaicin, a constituent of chili peppers, is bactericidal to *Helicobacter pylori*, although possibly detrimental to the human gastric mucosa (Jones *et al.,* 1997). Another terpenoids called petalostemumol, isolated from the prairie clover (*Dalea* sp) showed excellent activity against *Bacillus subtilis* and *Staphylococcus aureus* as well as *Candida albicans* (Cowan, 1999).

Gbaguidi *et al*., (2005), isolated two triterpenoid compounds (oleanolic acid and ursolic acids) from the alcoholic extract of M. villosus which was shown to inhibit the growth of *Dermatophilus congolensis* the causative organism of dermtophilosis in African animals with minimum inhibitory concentration of 15 µg/ml.

2.6.5 Glycoside

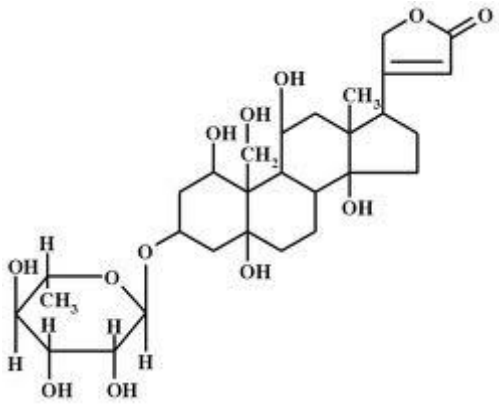

Fig. 2.5: Structure of Ouabain a cardiac glycoside

Glycosides are compounds containing a carbohydrate and a non-carbohydrate residue in the same molecule. The carbohydrate residue is attached by an acetyl linkage at carbon atom 1 to a non-carbohydrate residue or aglycone. The sugar component is called the glycone. If the carbohydrate portion is glucose, the resulting compound is a glucoside (Edwards *et al.*, 1999). Aglycones belong to terpenoid, steroid, flavonoid, quinonoid, lignan, other simple phenolics, and isothiocyanate. However, biological activities of glycosides are, in many cases, susceptible to the nature of sugar moieties, even though their aglycone is the same. Saponin glycosides are divided into two based on the chemical structure of their aglycone (sapogenins). Saponins on hydrolysis yield an aglycone known as 'sapogenin'.

Saponin = Sugar (glycone) + Sapogenin (aglycone).

The so-called neutral saponins are derivatives of steroids with spiroketal side chains. The acid saponins possess triterpenoid structures.

The roles of glycosides in plant on microorganism are as follows:

- Conversion of toxic materials to none or less toxic.
- Transfer water insoluble substances by using monosaccharide.
- Source of energy (sugar reservoir).
- Storage of harmful products such as phenol.
- Regulation for certain functions (growth). Some have beautiful colours (pollination process).

2.7 ANTIFUNGAL CHEMOTHERAPEUTIC AGENTS

Chemotherapy refers to the control of any disease using drugs. However, the term chemotherapy is usually associated with the treatment of cancer by anti-tumor drugs. In the case of infectious disease, this involves the use of drugs that will kill or limit the growth of microorganisms. Until the early part of the twentieth century, no effective antifungal chemotherapeutic drugs existed (Lewis, 2011). For such a drug to be effective, it must be able to kill or limit the growth of microorganisms without severely affecting cells of the host, referred to as selective toxicity.

Fungal infections are caused by microscopic organisms that can invade the epithelial tissue. The fungal kingdom includes yeasts, molds, rusts and mushrooms. Fungi, like animals, are heterotrophic, that is, they obtain nutrients from the environment, not from endogenous sources (like plants with photosynthesis). Most fungi are beneficial and are involved in biodegradation; however, a few can cause opportunistic infections if they are introduced into the skin through wounds, or into the lungs and nasal passages if inhaled. Diseases caused by fungi include superficial infections of the skin by dermatophytes in the *Microsporum*, *Trichophyton* or *Epidermophyton* genera. These dermophytic infections are named for the site of infection rather than the causative organism. The humid weather, overpopulation and poor hygienic conditions are conductive factors to the growth of dermatophytes (Nasrin *et al.*, 2007).

2.7.1. Mechanisms of Action of Antifungal Drugs

Fungal cells are complex organisms that share many biochemical targets with other eukaryotic cells. Therefore, agents that interact with fungal targets not found in eukaryotic cells are needed. The fungal cell wall is a unique organelle that fulfills the criteria for selective toxicity. The fungal cell wall differs greatly from the bacterial cell

wall and is not affected by antibacterial cell wall inhibitors such as the β-lactams or vancomycin.

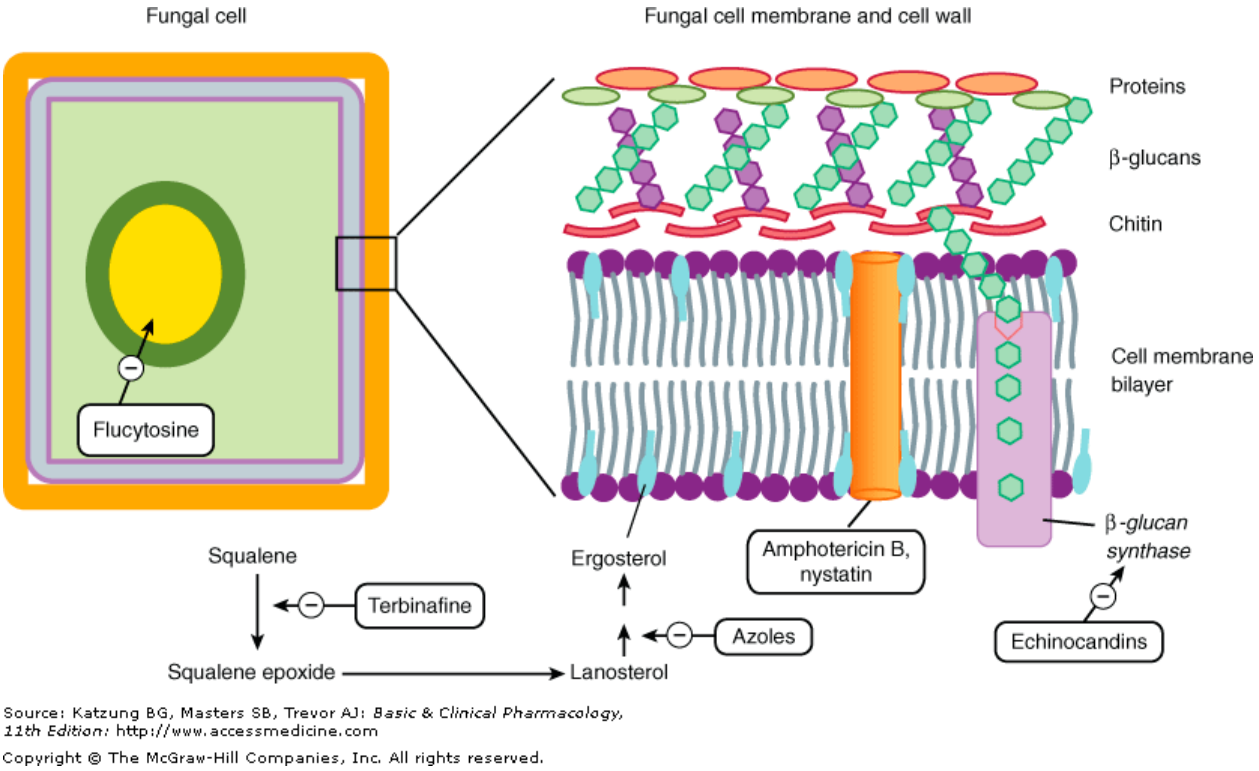

Figure 2.6: Mechanism of action of anti-fungal drugs

Arrangement of the biomolecular components of the cell wall accounts for the individual identity of the organism. Although, each organism has a different biochemical composition, their gross cell wall structure is similar. There are three general mechanisms of action for the antifungal agents: cell membrane disruption, inhibition of cell division and inhibition of cell wall formation (Bennett, 2011).

2.7.1.1 Inhibition of Cell Wall Formation

Interference with fungal cell wall biosynthesis has not been as successful and effective as penicillins and cephalosporins against bacteria. Drugs have been developed that interfere with various steps in fungal cell wall synthesis with excellent antifungal activity *in vitro* (Urbina *et al*., 2000). Unfortunately, development of these agents into

useful drugs has proven very difficult. Many of these agents are developed to target β-glucan synthesis.

2.7.1.2 Cell Membrane Disruption

Antifungal agents that disrupt the cell membrane do so by targeting ergosterol, either by binding to the sterol, forming pores and causing the membrane to become leaky (as with polyene antifungals), or inhibiting ergosterol biosynthesis (as seen with azole antifungal agents: White *et al*., 1998). Ergosterol is similar to mammalian cholesterol, thus agents binding ergosterol may have acytotoxic effect in the host tissue. Ergosterol has two conjugated double bonds that are lacking in mammalian sterols (Heeres *et al*., 1979).

2.7.1.3 Inhibition of Cell Division

Nucleoside antifungal agents affect cell division by targeting the microtubule effects in forming the mitotic spindle. They can also act by inhibiting DNA transcription.

2.7.2 Classification of Antifungal Agents

Most antifungal drugs interfere with biosynthesis or integrity of ergosterol, the major sterol in the fungal cell membrane (Georgopadakou *et al*., 1996). Others cause disruption of the fungal cell wall (Kauffman, 2004). Based on their mechanism of actionthe major agents can be grouped into five classes: polyenes; azoles; allylamines; echinocandins; and other agents, including griseofulvin and flucytosine.

2.7.2.1 Polyenes

The polyenes are a class of antifungal drugs that target membranes containing ergosterol (Georgopadakou *et al*., 1996). These drugs, which include amphotericin B and nystatin, are amphipathic, having both hydrophobic and hydrophilic sides. The drugs are thought to intercalate into membranes, forming a channel through which cellular components, especially potassium ions, leak and thereby destroying the proton gradient within the membrane. The drugs are less likely to interact with membranes containing cholesterol (Vanden Bossche et al., 1994). It has also been suggested that amphotericin B causes oxidative damage to the fungal plasma membrane (Vanden Bossche *et al*., 1994).

2.7.2.2 Azole Antifungal Agents

These are the most widely used antifungal drugs, and act primarily by inhibiting the fungal cytochrome P450 enzyme, 14α-demethylase (Georgopadakou *et al*., 1996). There are two groups in clinical use: the imidazoles (ketoconazole, miconazole, clotrimazole, and econazole) and the triazoles (fluconazole, itraconazole, voriconazole, and posaconazole). As the triazoles have greater affinity for fungal compared with mammalian P450 enzymes, their safety profile is significantly improved over the imidazoles. The use of imidazoles is limited to treating superficial mycoses. The triazoles have broad application in therapy of both superficial and systemic fungal infections. In general, the triazoles are relatively safe, even when used for prolonged periods. All triazoles can cause hepatotoxicity, but only 5%–7% of patients require treatment cessation (Boucher *et al*., 2004). The allylamines (i.e., naftifine and terbinafine) and thiocarbamates (i.e., tolnaftate and tolciclate) inhibit the conversion of squalene to 2,3-oxidosqualene by the enzyme squalene epoxidase interact with enzymes involved in the synthesis of ergosterol from squalene, which is produced from

acetate through acetyl coenzyme A, hydroxymethylglutaryl coenzyme A, and mevalonate (Favre et al., 1996). Ergosterol is an important sterol for fungi, since it is the predominant or "bulk" sterol in fungal plasma membranes. In addition, it has an essential "sparking" function in which trace amounts of ergosterol are necessary for the cells to progress through the cell cycle. This sparking function is independent of the bulk sterol in the fungal membranes, since certain sterols can replace the bulk sterol of the membrane without supplying the sparking function for the cell cycle (Hitchcock, 1993).

2.7.2.3 Echinocandins

There are three clinically important echinocandins (Denning, 2003). Caspofungin is the first to be licensed in Australia. Micafungin is only approved in Japan and the United States. Anidulafungin is currently undergoing review for marketing in Australia. All three show good in vitro activity against *Candida* and *Aspergillus* species, but are not active against *C. neoformans* or non-*Aspergillus* moulds (Boucher *et al*., 2004).

2.7.2.4 5-Flucytosine

5-Flucytosine (5-FC) has an entirely distinct mode of action from the azoles. 5-FC is taken up into the cell by a cytosine permease and deaminated into 5-fluorouracil (FU) by cytosine deaminase. 5-FC is fungus specific since mammalian cells have little or no cytosine deaminase (Vanden Bossche *et al*., 1994). FU is eventually converted by cellular pyrimidine-processing enzymes into 5-fluoro-dUMP (FdUMP), which is a specific inhibitor of thymidylate synthetase, an essential enzyme for DNA synthesis, and 5-fluoro-UTP (FUTP), which is incorporated into RNA, thus disrupting protein synthesis (Vanden Bossche *et al*., 1987).

2.7.3 Antifungals Used in the Study.

2.7.3.1 Fluconazole

Fluconazole is a triazole antifungal drug used in the treatment and prevention of superficial and systemic fungal infections. In a bulk powder form, it appears as a white crystalline powder, and it is very slightly soluble in water and soluble in alcohol.

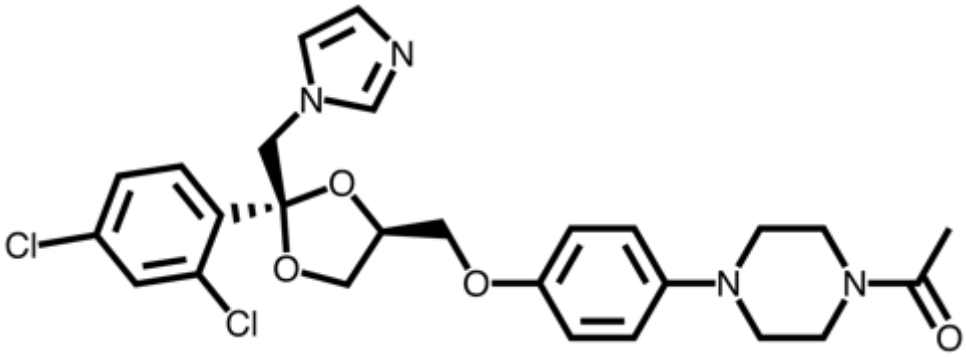

Figure 2.7: Chemical Structure Fluconazole

Mechanism of antifungal action:

Like other imidazole- and triazole-class antifungals, fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase. Mammalian demethylase activity is much less sensitive to fluconazole than fungal demethylase. This inhibition prevents the conversion of lanosterol to ergosterol, an essential component of the fungal cytoplasmic membrane, and subsequent accumulation of 14α-methyl sterols. Fluconazole is primarily fungistatic; however, it may be fungicidal against certain organisms in a dose-dependent manner, specifically *Cryptococcus* (Longley *et al*., 2008).

Therapeutic uses:

Fluconazole is indicated for the treatment and prophylaxis of fungal infections where other antifungals have failed or are not tolerated (e.g., due to adverse effects),

including: Candidiasis (caused by susceptible strains of *Candida*), *Tinea corporis*, *Tinea cruris* or *Tinea pedis* (Longley *et al*., 2008). It is also used in the treatment of onychomycosis and cryptococcal meningitis.

2.7.3.2 Ketoconazole

Ketoconazole is a synthetic antifungal drug used to prevent and treat fungal skin infections, especially in immunocompromised patients such as those with AIDS or those on chemotherapy. Ketoconazole is very lipophilic, which leads to accumulation in fatty tissues. The less toxic and more effective triazole compounds fluconazole and itraconazole are sometimes preferred for internal use (Evans *et al.*, 2004).

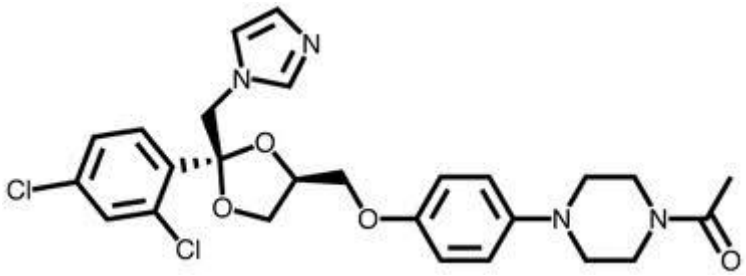

Figure 2.8 Chemical Structure Ketoconazole

Mechanism of anti fungal action

Ketoconazole is structurally similar to imidazole, and interferes with the fungal synthesis of ergosterol, a constituent of fungal cell membranes, as well as certain enzymes. As with all azole antifungal agents, ketoconazole works principally by inhibiting the enzyme cytochrome P450 14-alpha-demethylase (P45014DM). This enzyme participates in the sterol biosynthesis pathway that leads from lanosterol to ergosterol (Loose *et al.*, 1983).

Spectrum of activity:

Ketoconazole inhibits growth of dermatophytes and yeast species such as *Candida albicans*.

Therapeutic uses:

Ketoconazole is usually prescribed for topical infections such as athlete's foot, ringworm, candidiasis (yeast infection or thrush), and jock itch (Evans *et al.*, 2004). The over-the-counter shampoo version can also be used as a body wash for the treatment of *Tinea versicolor*. Ketoconazole is used to treat eumycetoma, the fungal form of mycetoma (Loli *et al.*, 1986).

2.8 CHARACTERISTICS OF THE FUNGAL SPECIES USED IN THE STUDY

2.8.1. *Candida albicans*

Candida albicans is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans. On Sabouraud dextrose agar (SDA) it grows as a cream coloured, smooth surfaced and glabrous colonies (McClary, 1952) . It is Gram positive and germ tube positive. Systemic fungal infections (fungemias) including those by *C. albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g. AIDS, cancer chemotherapy, organ or bone marrow transplantation). *Candida albicans* biofilms may form on the surface of implantable medical devices. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns (Jabra-Rizk *et al*., 2004).

Candida albicans is commensal and a constituent of the normal gut flora comprising microorganisms that live in the human mouth and gastrointestinal tract (Ryan and Ray, 2004). *Candida albicans* can be found in 80% of the human population without causing harmful effects, although overgrowth of the fungus results in candidiasis (candidosis). Candidiasis is often observed in immunocompromised individuals such as HIV-infected patients. A common form of candidiasis restricted to the mucosal membranes in mouth or vagina is thrush, which is usually easily cured in people who are not immunocompromised.

Although often referred to as "dimorphic", *C. albicans* is in fact polyphenic. When cultured in standard yeast laboratory medium, *C. albicans* grows as ovoid "yeast" cells. However, mild environmental changes in temperature and pH can result in a morphological shift to pseudohyphal growth. Pseudohyphae share many similarities with yeast cells (Berman and Sudbery, 2002), but their role during candidiasis remains unknown. When *C. albicans* cells are grown in a medium that mimics the physiological environment of a human host, they grow as "true" hyphae. Its ability to form hyphae has been proposed as a virulence factor, as these structures are often observed invading tissue, and strains that are unable to form hyphae are defective in causing infection (McClary, 1952). *Candida albicans* is widely recognized as being the most pathogenic yeast species and in the majority of epidemiological studies has been found to be the most common cause of superficial and systemic infections (Moran *et al.*, 1997).

2.8.1.2 *Candida krusei*

Candida krusei is a budding yeast (a species of fungus) involved in chocolate production. On SDA colonies are white to cream coloured, smooth surfaced and glabrous. Colonies are Gram positive and germ tube negative (Yuthika *et al*., 1994). *Candida krusei* is an emerging fungal nosocomial pathogen primarily found in the immunocompromised and those with hematological malignancies. It has natural resistance to fluconazole, a standard antifungal agent. It is most often found in patients who have had prior fluconazole exposure, sparking debate and conflicting evidence as to whether fluconazole should be used prophylactically. Mortality due to *C. krusei* fungemia is much higher than the more common *C. albicans* **(**Yuthika *et al*., 1996). The most common clinical manifestation of *C. krusei* is disseminated fungaemia in compromised patients, especially leukaemia patients. The advent of HIV infection and the widespread use of the newer triazole fluconazole to suppress fungal infections in these individuals have contributed to a significant increase in *C. krusei* infection, particularly because of the high incidence of resistance of the yeast to this drug. Other focal infections due to *C. krusei* include endophthalmitis, arthritis and endocarditis, which are usually related to invasive procedures superimposed on a compromised host defence system. Thus the epithet "an emerging pathogen" could justifiably be given to this yeast, not least because of a putative increasing incidence of C. *krusei* infection, due partly to the HIV pandemic

2.8.1.3 *Aspergillus fumigatus*

It is a fungus of the genus *Aspergillus*, and is one of the most common *Aspergillus* species to cause disease in individuals with an immunodeficiency. On SDA plates they appear as velvety to powdery colonies turning smoky green. Conidia are produced in

biseptal succession (O'Gorman, 2008). *Aspergillus fumigatus*, is a saprotroph widespread in nature, being typically found in soil and decaying organic matter, such as compost heaps, where it plays an essential role in carbon and nitrogen recycling. Colonies of the fungus produce from conidiophores thousands of minute grey-green conidia (2–3 μm) that readily become airborne. For many years, *Aspergillus fumigatus* was thought to only reproduce asexually, as neither mating nor meiosis had ever been observed. In 2008, however, *Aspergillus fumigatus* was shown to possess a fully functional sexual reproductive cycle, 145 years after its original description by Fresenius (O'Gorman, 2008).

The fungus is capable of growth at 37 C (normal human body temperature), and can grow at temperatures up to 50 C, with conidia surviving at 70 C conditions it regularly encounters in self-heating compost heaps (Nieminen *et al*., 2002). Its spores are ubiquitous in the atmosphere, and it is estimated that everybody inhales several hundred spores each day; typically these are quickly eliminated by the immune system in healthy individuals. In immunocompromised individuals, such as organ transplant recipients and people with AIDS or leukemia, the fungus is more likely to become pathogenic, over-running the host's weakened defenses and causing a range of diseases generally termed aspergillosis (Ben-Ami *et al*., 2010).

Aspergillus fumigatus is the most frequent cause of invasive fungal infection in immunosuppressed individuals, which include patients receiving immunosuppressive therapy for autoimmune or neoplastic disease, organ transplant recipients, and AIDS patients (Brakhage, 2005). *Aspergillus fumigatus* primarily causes invasive infection in the lung and represents a major cause of morbidity and mortality in these individuals

(Ben-Ami *et al.*, 2010). Additionally, *Aspergillus fumigatus* can cause chronic pulmonary infections or allergic disease in immunocompetent hosts (Hohl and Feldmesser, 2007).

2.8.1.4 *Aspergillus niger*

It is a fungus and one of the most common species of the genus *Aspergillus*. On SDA plates they appear as deep brown to black colonies with densely stippled surface. Conidia heads are dark brown to black and large, radiate, biseriate with metulea twice as long as the phialides (Schuster *et al*., 2002). It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. According to Samson *et al.* (2001), it is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called "black mould").

Aspergillus niger is less likely to cause human disease than some other *Aspergillus* species, but, if large amounts of spores are inhaled, a serious lung disease, aspergillosis can occur. Aspergillosis is, in particular, frequent among horticultural workers that inhale peat dust, which can be rich in *Aspergillus* spores (Schuster *et al*., 2002). It has been found on the walls of ancient Egyptian tombs and can be inhaled when the area is disturbed. *Aspergillus niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane. *Aspergillus niger* is often used as a

challenge organism for cleaning validation studies performed within sterile manufacturing facilities (Abarca *et al*., 1994).

2.8.1.5 *Trichophyton mentagrophytes*

It is a zoophilic dermatophyte of wild and domestic rodents which is occasionally transmitted to man and other animals by direct contact with an infected animal or asymptomatic carrier or with contaminated material (hair and scales) from the environment (Ljiljana *et al*., 2004). On SDA plates they appear as flat, white to cream in colour, with powdery to granular surface. Conidia are numerous and single celled in dense clusters. Colonies are positive to hair perforation test (Brakhage, 2005). *Trichophyton mentagrophytes* and *Trichophyton verrucosum* are the major causes of dermatomycosis (commonly known as ringworm) in cattle in many parts of the world. The infection is mainly spread by contact between infected and susceptible animals or via a contaminated environment such as bedding and walls. *Trichophyton mentagrophytes* is also the second most commonly isolated fungus causing so-called tinea infections in humans, and the most common fungus that causes zoonotic skin disease (i.e. transmission of mycotic skin disease from humans to animals, and vice versa). The fungus has a major natural reservoir in rodents, but can also infect pet rabbits, dogs and horses (Zaias and Rebell, 2003).

2.8.1.6 *Trichophyton verrucosum*

Trichophyton verrucosum is the usual zoophilic dermatophyte involved in cattle ringworm throughout the temperate regions of the world (Nasrin *et al.*, 1984). On SDA colonies are slow growing, small, button or disc shaped, white to cream coloured, with suede like to velvety surface. Reverse pigment may vary from non-pigmented to

yellow. Macro conidia are rarely produced, tips of hyphae are broad, club shaped and occasionally divided (Shams-Ghahfarokhi *et al.*, 2009). It also affects, but with lower prevalence, sheep, goat and other ruminants. Aside from animal involvement, several human outbreaks of *Trichophyton verrucosum* infection have been reported so far by direct contact with infected animals or indirect contact with infectious propagules in the environment. Although various parts of human body may be involve by the fungus, the face and the body is mainly affected during the fungal infection. The fungus is able to survive in skin scales of infected animals for several months in moist and dark places where it can be easily transmitted to human and other animals (Shams-Ghahfarokhi *et al.*, 2009).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment

Soxhlet extractor (Quick- Fit, England)

Chromatographic column (Supelco, Italy)

Class 2 Biosafety cabinet (Esco, USA)

Light Microscope (Model XSZ-107BN, Olympus Optical Company, Ltd., Japan)

Refrigerator (Thermocool Engineering Company Ltd., Nigeria)

Incubator (Memmert Karl Kolb Scientific Technical Supplies, West Germany)

Autoclave (Adelphi MFG Co Ltd, Portland)

Electronic weighing balance (Ohaus, AR2140, U.S.A,).

Water bath (Gasellschaft fur Labortechnik, Germany)

Rotary evaporator (Buchi Rotavapor, Sigma- Aldrich, U.S.A.)

Oven (Gallenkamp, England).

Single-beam Spectrophotometer (SPECTRONIC 20D; Milton Roy Company, Madrid, Spain)

3.1.2 Glass wares

Separating funnel

Beakers

3.1.3 Reagents.

Absolute Ethanol (Sigma Aidrich- Missouri, U.S.A.)

Methanol (Sigma Aidrich- Missouri, U.S.A.)

Ethyl acetate (Sigma Aidrich- Missouri, U.S.A.)

N-Butanol (Sigma Aidrich- Missouri, U.S.A.)

Hexane (Sigma Aidrich- Missouri, U.S.A.)

Di ethyl ether (Sigma Aidrich- Missouri, U.S.A.)

Petroleum ether (Sigma Aidrich- Missouri, U.S.A.)

Chloroform (Sigma Aidrich- Missouri, U.S.A.)

Tween 80 (BDH, England.)

Silica gel G(Merck, Germany)

3.1.4 Media

Saboraud Dextrose Agar (Oxoid, Basingstoke, U.K.)

Saboraud Dextrose Broth (Oxoid, Basingstoke, U.K.)

3.1.5 Reference Antibiotic

Fluconazole powder (Cat No. F8929, Sigma Aldrich, U.S.A.)

Ketoconazole tablets (Nizoral^{R}, Janssen Pharmaceuticals Inc. U.S.A.)

3.1.6 Test Organisms

Clinical isolates, obtained from the Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria, include: *Candida albicans* (4), *Candida krusei* (1), *Aspergillus fumigatus, Aspergillus*

niger and *Trichophyton mentagrophytes*. *Trichophyton verrucosum* was collected from the Department of dermatophytosis, National *Veterinary Research* Institute (NVRI), *Vom*, Plateau State, Nigeria.

3.2 METHODOLOGY

3.2.1 Collection and Identification of Plant Materials

The fresh aerial parts of *Mitracarpus villosus* was collected from the National Institute for Pharmaceutical Research and Development (NIPRD) garden in October 2011. The plant was identified and authenticated in the herbarium of the Department of Medicinal Plant Research and Traditional Medicine, NIPRD, Abuja, Nigeria and given voucher specimen number NIPRD/ H/ 4208.

3.2.2 Preparation of Crude Plant Material.

Mitracarpus villosus aerial parts were air dried at room temperature for 10 days. The dried aerial parts were crushed to coarse powder by grinding with wooden mortar and pestle.

3.2.3 Preliminary Extraction of Plant Material

Eight hundred (800 g) powdered sample was extracted successively and exhaustively using hexane as solvent. This process was repeated using ethyl acetate and ethanol respectively. After successive extraction with these solvents, the residue (marc) was

collected, dried (to remove any residue of the other solvents) and then macerated with water to obtain the water extract. After each extraction, the extract was concentrated, dried and weighed.

3.2.4 Chromatographic Analysis of Ethyl Acetate Extract (EAE)

A portion (50 g) of crude ethyl acetate extract was subjected to column chromatography using silica gel G as an absorbent (Aiswarya *et al.,* 2010). The column was successively eluted with three hundred milliliters each of hexane (100%), hexane–ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90%), ethyl acetate (100%), ethyl acetate- ethanol (90:10, 80:20) and ethanol (100%). The fractions were collected and combined on the basis of their thin layer chromatography (TLC) profiles.

3.2.5 Preparation of Test Organisms

Test organisms were aseptically purified, sub cultured and grown on 10 ml Sabouraud dextrose agar slants and thereafter kept in the refrigerator at $2 – 8^0$C.

3.2.6 Preparation of Reference Antifungal Agents

Stock solutions of fluconazole was prepared by dissolving appropriate quantity of the antifungal agent in dimethyl sulfoxide (DMSO) while ketoconazole was dissolved in methanol and later diluted to their required concentrations with broth.

3.2.7 Preparation of Culture Media

The required quantities of the dehydrated fungal media were weighed and prepared with distilled water according to the manufacturer's specifications. Where necessary gentle heating was applied to aid dissolution and the media were dispensed into 10 or 20 ml sizes and sterilized by autoclaving at 121 ^{o}C for 15 minutes. These were kept aseptically until required.

3.2.8 Preparation of Sterile Solutions

a. Sterile distilled water: distilled water was dispensed in volumes of 100 ml and 200 ml in clean bottles and sterilized by autoclaving at 121 ^{o}C for 15 minutes.
b. Sterile normal saline with 3% Tween 80: 9 g of sodium chloride and 30 mL of Tween 80 were dissolved in 1 liter of distilled water. This solution was dispensed in volumes of 9 ml or 10 mL and sterilized by autoclaving at 121 ^{o}C for 15 minutes.

3.3 PHYTOCHEMICAL ANALYSIS

The powdered plant sample was evaluated for the presence of carbohydrate, tannins, flavonoids, phlobatannins, cardiac glycosides, saponins, alkaloids, terpenes, steroids and anthraquinone using simple qualitative and quantitative methods described by Trease and Evans (1989) and Sofowora (1993).

3.3.1 Carbohydrate Tests

(a) Molish's Test: A few drop of Molich's reagents was added to the extract dissolved in water, followed by addition of 1 ml of concentrated sulphuric acid down the side of the test tube. The mixture was allowed to stand for 2 minutes. 5 mls of water was used to dilute the mixture. A red coloration at the interphase indicated the presence of carbohydrate (Trease and Evans, 1989).

(b) Fehling's Test: A small quantity (0.1 g) of the extract was dissolved in 5 mls of water. This was filtered and the filtrate will be treated with 5 mls of Fehling's solution A and B. Formation of brick-red precipitate indicated the presence of reducing sugars (Trease and Evans, 1989).

3.3.2 Test for anthraquinones

Borntrager's Test: A small quantity (0.1 g) of extract was dissolved in 10 ml of benzene, filtered and 5 ml of 10% ammonia solution was added to the filtrate. The production of pinkish colour in the lower ammonical layer signified the presence of anthraquinones (Sofowora, 1993).

3.3.3 Test for Saponins

The extract (0.1 g) was added to 10 ml of distilled water in a test tube. The test tube was corked and shaken. Production of frothing which persists on heating indicated the presence of saponins (Sofowora, 1993).

3.3.4 Test for Flavonoids

(a) Shinoda test: The extract (0.1 g) was dissolved in ethanol, warmed and filtered, three pieces of magnesium chips was added, followed by a few drops of concentrated sulphuric acid. An orange colour indicated the presence of flavones, flavonols and flavonoids (Silva *et al.*, 1998).

(b) Ferric Chloride Test: The extract was boiled with water and filtered. To 2 ml of the filtrate, 2 drops of freshly prepared ferric chloride solution was added. A dark green coloration indicated the presence of phenolic hydroxyl group. (Trease and Evans, 1989).

(c) Sodium Hydroxide Test: The filtered extract (2 mL) was dissolved in 10% aqueous sodium hydroxide solution to give a yellow coloration. A change in colour from yellow to colourless on addition of dilute Hydrochloric acid indicated the presence of flavonoids (Trease and Evans, 1989).

3.3.5 Tannin Test

Ferric Chloride Test: A small portion of the extract was dissolved in 10ml of water and filtered. Two drops of freshly prepared 5% ferric Chloride solution was added to the filtrate. A bluish black colour indicated the presence of condensed tannins. (Trease and Evans, 1989).

3.3.6 Test for Alkaloids

The extract (0.5 g) was dissolved in 5 ml 0.1 % Hydrochloric acid on a steam bath. 1 m1 of the filtrate was treated with a few drops of Mayer's reagent. Another portion of the extract was treated with Dragendroffs reagent and the third portion was also treated with Wagner's reagent. Formation of precipitates with any of the reagents indicated the presence of alkaloids (Trease and Evans, 1989).

3.3.7 Test for Steroids and Triterpenes

(a) Salkowski Test: The extract (0.1 g) was dissolved in 2 ml of chloroform. Concentrated sulphuric acid was added to form a lower layer. A reddish brown colour at the interphase is positive for the presence of steroidal ring (Soforowa, 1993).

(b) Lieberman Burchard Test: A little portion of the extract was added to few drops of acetic acid anhydride and 2 ml of sulphuric acid was added down the wall of the test tube. A colour change from violet to blue on standing and then to bluish green indicated the presence of steroidal nucleus (an aglycone portion of cardiac glycosides) (Shopee, 1982).

3.3.8 Test for Resin

a) Fifteen milliliters of petroleum ether extract were prepared using 0.1 g of the powdered stem bark and root, filtered into a test-tube. An equal volume of copper acetate solution was added and shaken vigorously then allowed to separate. The colour change was noted (Trease and Evans, 1989).

b) 0.5 g each of the powdered stem bark and root were dissolved in acetic anhydride and one drop of concentrated sulphuric acid was added. The colour change was noted.

3.4 EXTRACTION OF SECONDARY METABOLITES

3.4.1 Extraction of Tannins

One hundred milliliters of distilled water was added to 20 g of powdered plant. This was allowed to boil for a minute and then filtered. The filtrate was mixed with 100.0ml of ethyl acetate in a separating funnel. The ethyl acetate layer was evaporated to dryness and the residue was weighed (Marcek, 1972).

3.4.2 Extraction of Saponins

The sample (50 g) was extracted with 1 liter of aqueous methanol (50%) by cold extraction for 72 h. The extract was filtered using Whatman filter paper No. 2, and concentrated on a rotary evaporator at 45ºC to give the crude methanol extract. The methanol crude was suspended in water saturated with n-butanol in a separating funnel. The n-butanol portion separated and collected from the aqueous portion. Diethyl ether (200 ml) was added to the n-butanol portion in a pre-weighed container, and crude saponin was precipitated (Aliyu *et al.*, 2011).

3.4.3 Extraction of Flavonoids

50 g of the powdered plant was completely denatured with acetone. The acetone was then completely evaporated on a water bath. The residue was dissolved in warm water and the mixture filtered while hot and allowed to cool (Marcek, 1972).

3.4.4 Extraction of Phenols

200 g of the dried powdered plant was heated with 1 L of 2M Hydrochloric acid for 40 minutes in a water bath. The mixture was allowed to cool and the cold solution filtered. The phenol was extracted with diethyl ether three times and the combined extract evaporated to dryness and weighed (Har and Ismail 2012).

3.5 DETERMINATION OF ANTIFUNGAL ACTIVITY OF *MITRACARPUS VILLOSUS*

3.5.1 Cultivation and Standardization of Test Fungi

Eighteen-hour solid culture of the test *Candida* spp. was suspended into sterile Sabouraud dextrose liquid medium. It was standardized according to Clinical Laboratory Standards Institute (CLSI, 2002) by gradually inoculating in normal saline to compare its turbidity to McFarland standard of 0.5 which is approximately 1.0×10^6 cfu/ml. However, for *Trichophyton* spp. and *Aspergillus* spp., fungal spores were harvested from 7 day old Sabouraud dextrose agar (SDA) slant cultures by washing with 10 ml sterile normal saline containing 3 % w/v Tween 80 with aid of sterile glass beads to help in dispersing the spores (Olowosulu *et al.,* 2005). Thereafter, the spore

suspension was standardized to 1.0 x 10^6 spores / ml by using a single-beam spectrophotometer at 530 nm (OD530) adjusted to 80 – 85 % transmittance (*Aspergillus* spp.) and 70 -72 % transmittance (*Trichophyton* spp.). All adjusted suspensions were quantified by spreading 100 µl on Sabouraud dextrose agar plate and incubated at 37°C for 18 hours for yeast and 30°C for 72 hours for dermatophytes and moulds (Aberkane *et al*., 2002). All cultures were checked for purity (by morphological growth on media, staining and biochemical tests) and maintained on Sabouraud dextrose agar (SDA) for the fungal test organisms respectively at 4º C (in the refrigerator) until required for use.

3.5.2 Antifungal Screening of the Solvent Extracts

Eighteen hours overnight cultures of *Candida spp.* and innoculum suspensions of the moulds and dermatophytes prepared from fresh, mature (3- to 5-day-old) cultures (in some cases an extended incubation was required for proper sporulation of the isolate) in Sabouraud dextrose liquid medium were standardized to produce inoculum size of 10^6 cfu/ml. 1 mL of the diluted culture of each test organism was used to flood Sabouraud dextrose agar media and excess aseptically drained.

The plates were allowed to dry at 37°C temperature in a sterilized incubator. Adopting the agar diffusion cup plate method (Olowosulu *et al.*, 2005), a sterile cork borer (6 mm) was used to bore holes in the agar plates. The bottoms of the wells (holes) were sealed with the appropriate molten Sabouraud dextrose agar. Using micropipette, 0.1 ml each of 200mg/ml concentration of the solvent extracts of the crude plants and extracted secondary metabolites of the plant were dispensed into the holes. Distilled

water and the solvents used in diluting the extracts were used as control. These were allowed to diffuse into the agar at room temperature for one hour before incubation at 37°C for 18 hours (yeast) and 30°C for 72 hours (dermatophytes and moulds). The zones of inhibition of the test organisms were measured to the nearest millimeter, using a well-calibrated meter ruler. The experiment was carried out in duplicates.

3.5.3 Antifungal Screening of the Ethyl Acetate Extracts and Secondary Metabolites

Eighteen hours cultures of *Candida spp.* and innoculum suspensions of the moulds and dermatophytes prepared from fresh, mature (3- to 5-day-old) cultures (in some cases an extended incubation was required for proper sporulation of the isolate) in Sabouraud dextrose liquid medium were standardized to produce inoculum size of 10^6 cfu/ml. 1 ml of the diluted culture of each test organism was used to flood Sabouraud dextrose agar media and excess aseptically drained.

The plates were allowed to dry at 37°C temperature in a sterilized incubator. Adopting the agar diffusion cup plate method (Olowosulu *et al.*, 2005), a sterile cork borer (6mm) was used to bore holes in the agar plates. The bottoms of the wells (holes) were sealed with the appropriate molten Sabouraud dextrose agar. Using micropipette, 0.1 ml each of the different graded concentrations of the ethyl acetate extract was dispensed into the holes marked 'A' (100 mg/ml), 'B' (50 mg/ml), 'C' (25 mg/ml), 'D' (12.50 mg/ml) and 'E' (6.25 mg/ml). Distilled water and the solvents used in diluting the extracts were used as control. These were allowed to diffuse into the agar at room

temperature for one hour before incubation at 37°C for 18 hours (yeast) and 30°C for 72 hours (dermatophytes and moulds). The zones of inhibition of the test organisms were measured to the nearest millimeter, using a well-calibrated meter ruler. The experiment was carried out in duplicates (Otimenyin *et al*., 2008).

3.5.4 Determination of Minimum Inhibitory Concentration (MIC) of the extracts, secondary metabolites and fractions

The MIC of the extracts, secondary metabolites, fractions and reference antibiotics to the test fungi determined by using serial broth microdilution method (Shanmugapriya *et al* 2012). This assay was performed using flat bottom 96-well clear microtitre plates. The wells in column A of each row were left blank and the last seven wells from column B to H were filled with 50µl of sterilized Saboraud dextrose broth. Working solution of plant extracts were added to the wells in column A and B of each row and an identical two-fold serial dilution were made from column B to the column G. The last wells in column H was served as drug-free controls. Water was included as negative control. Lastly, 50 µl of standardized fungal inoculum (10^6 cfu ml^{-1}) were added in all the wells from column A to H and mixed thoroughly to give final concentrations. Tests were done in triplicates. The cultured microplates were sealed with parafilm and incubated at 37°C for 24 hours (bacteria and yeast) and 30°C for 48 hours (dermatophytes and moulds). Minimum inhibitory concentration was defined as the first well with no visible growth after 24 hours.

3.5.5 Minimum Fungicidal Concentration (MFC) of the extracts and fractions.

50 µl of the wells that did not show any visible growth after MIC determination were inoculated in fresh wells containing Saboraud dextrose broth and incubated at 37°C for 18 hours (yeast) and 30°C for 48 hours (dermatophytes and moulds). Minimum fungicidal concentrations were determined as the lowest concentration resulting in no growth on subculture (Hafidh *et al.,* 2011).

3.5.6 Rate of Kill of Test Antifungal Agents against Test Organisms.

To 9 ml of fungicidal concentrations of *Mitracarpus villosus* ethyl acetate extract, 1ml of standardized culture (10^6 cfu ml^{-1}) of fungal test organism was added and mixed. At different time intervals (0, 30, 60, 120, 180, 240, 300 and 360 minutes), 1 ml of the admixture was taken and diluted ten-fold with inactivating 9ml sterile normal saline supplemented with 3% Tween 80. These dilutions were plated out on 20ml of melted (45°C) Sabouraud dextrose agar containing 5% Tween 80. The plates were allowed to set and incubated at 37°C for 18 hours (yeast) and 30°C for 48 hours (dermatophytes and moulds) and the colonies observed and counted. The same procedure was repeated for fluconazole (*Candida* spp.) and ketoconazole (dermatophytes and moulds). The log of CFU versus time was plotted to display the rate of kill of the test organisms by the extract, fraction and standard antifungal drugs **(**Intzar *et al*., 2010**).**

3.5.7 Determination of Effects of varying Temperature on the Antifungal Activity of *Mitracarpus villosus* ethyl acetate extract.

The extract (500 mg) was dissolved in 10 mls of sterile distilled water. This gave a concentration of 50 mg/ml. The set up was maintained at different temperatures (25 °C, 37°C and 45°C) in the water bath for three hours. The standardized cultures of the test organisms were used to flood Sabouraud dextrose agar. The plates were allowed to dry at 37 °C in a sterile incubator. Adopting the cup plate method, a sterile cup borer no. 4 (6 mm diameter) was used to bore holes in the agar plates. The bottoms of the well were sealed with the appropriate molten sterile agar.

Using a micropipette, 0.1 ml of the extract solution was aseptically dispensed into the holes on the agar plate and incubated 37°C for 18 hours (yeast) and 30 °C for 48 hours (dermatophytes). Sterile distilled water was used as negative control. The zones of inhibition of test fungi were measured using calibrated meter ruler (Durairaj *et al*., 2009).

3.5.8 Determination of the Effect of Storage Conditions and Duration on the Antifungal Activity of the Ethyl Acetate Extracts of *M. villosus*.

The ethyl acetate of *M. villosus* extract in 30% dimethyl sulfoxide was prepared (50 mg/ml) in 10ml volume and stored aseptically under different conditions (dark compartment, direct sunlight and in an amber coloured bottle) for a duration of nine months. At 3 months intervals samples were taken and assessed for antimicrobial activity using the agar diffusion method. 0.1 ml of the extracts was aseptically dispensed into the holes bored on the agar plate containing the test organism. The plates were incubated appropriately and the zones of inhibition of test organisms were

measured using calibrated meter ruler. Sterile distilled water was used as the negative control (Oyi *et al*., 2007).

3.5.9 Statistical analysis

Results obtained were expressed as mean± standard deviation and analysed for significance using one way ANOVA (Smith's Statistical Package version 2.80) at $p < 0.05$.

CHAPTER 4

RESULTS

4.1. SUCCESSIVE EXTRACTION OF THE AERIAL PARTS OF *MITRACARPUS VILLOSUS* (Sw.) DC

The percentage yield of the solvent extracts from the plant parts showed that the ethanol extract had the highest yield and the ethyl acetate extract had the lowest yield as shown in Table 4.1. The more polar solvents such as water and ethanol yielded greater quantities than the less polar solvents like hexane and ethyl acetate. The percentage yield of the ethanol (8.0) extract of *M. villosus* was greater than that of water extract (7.41). This was followed by the hexane extract (5.36) and the least ethyl acetate (5.17).

Table 4.1: Percentage yield of extracts using various solvents.

Solvents	Yield (g)	Percentage Yield
Hexane	42.85±0.33	5.36±0.33
Ethyl acetate	41.34±0.33	5.17±0.33
Ethanol	64.00±0.67	8.00±0.67
Water	59.30±0.00	7.41±0.00

4.2 PHYTOCHEMICAL SCREENING OF THE AERIAL PARTS OF *MITRACARPUS VILLOSUS* (Sw.) DC

The phytochemical screening of the powdered plant part revealed the presence of tannins, saponin, flavonoids, terpenes, phenols and resins; alkaloid, sugars, carbohydrates, balsams and anthraquinones were not detected (Table 4.2).

Table 4.2: Phytochemical analysis of *M. villosus.*

Secondary metabolites	Inference
Carbohydrates	-
Terpenes	+
Sterols	-
Saponins	+
Tannins	+
Anthraquinones	-
Balsams	-
Resins	+
Alkaloids	-
Flavonoids	+
Phenols	+
Volatile oil	-

Key + present; - absent

4.3 EXTRACTION OF SECONDARY METABOLITES FROM THE AERIAL PARTS OF*MITRACARPUS VILLOSUS* (Sw.) DC

From the dried powdered sample tannins, saponins, flavonoids and phenols were extracted and quantified as shown in Table 4.3.

Table 4.3: Extraction of secondary metabolites from the aerial parts of *Mitracarpus villosus*

Secondary metabolite	Weight of sample (g)	Yield (g)	Percentage yield
Tannins	80.00±0.00	1.12±0.33	1.40±0.33
Saponins	10.00±0.00	1.70±0.00	17.00±0.00
Phenols	20.00±0.00	1.80±0.33	9.00±0.33

The crude saponins from *M. villosus* gave the highest yield.

4.4 CHROMATHOGRAPHIC ANALYSIS OF ETHYL ACETATE EXTRACT OF *MITRACARPUS VILLOSUS* (Sw.) DC

A total of 30 fractions were collected from the elution of the ethyl acetate extract. Identical fractions were combined giving 6 fractions altogether as follows:

M1 -Combination of fractions 1-6

M2 - Combination of fractions 7- 9

M3 - Combination of fractions 10–12

M4-Combination of fractions 13-14

M5-Combination of fractions 15-25

M6-Combination of fractions 26-30

The percentage yield of the fractions is shown in table 4.4.

Table 4.4: Percentage yield of fractions from ethyl acetate extracts of *Mitracarpus villosus*

Fractions	Yield (g)	Percentage Yield (g)
M1	3.50±0.33	7.00±0.33
M2	1.00±0.00	2.00±0.00
M3	7.57±0.67	15.10±0.67
M4	5.32±0.00	10.60±0.00
M5	6.06±0.00	12.10±0.00
M6	2.00±0.00	4.00±0.00

Fraction M3 had the highest yield**.**

4.5 ANTIFUNGAL ACTIVITIES OF THE AERIAL PARTS OF *M. VILLOSUS* (Sw.) DC

4.5.1. Susceptibility of the Test Fungi to the Solvent Extracts, Ethyl acetate Fractions and Secondary Metabolites of *M. villosus*.

The result of susceptibility testing is shown in Tables 4.5 – 4.9. Generally, an increase in the concentration of the extracts/ fraction led to an increase in antifungal activity shown with the increase in the diameter of zone of inhibition. The ethyl acetate fraction of *M. villosus* had the highest antifungal activity on all the fungal isolates tested. This was followed by the hexane extracts which inhibited the growth of *Candida sp.* but not on the moulds and finally, ethanol extract of *M. villosus* which had antifungal activity on only *T. verrucosum*. The water extracts of *M. villosus* had no antifungal activity on all the isolates tested

4.5.2. The Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentration (MFC) of Fungal Isolates

The minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of the ethyl acetate extracts, fractions and secondary metabolite extracts on the fungal isolates of *Mitracarpus villosus* are represented in Table 4.10 and 4.11.

Table 4.5:Susceptibility of the test fungi to the solvent extracts of *M. villosus* at concentration of 100mg/ml

Organisms	Zone of Inhibition (mm)					
	HEX	ETA	ET	W	FCZ (50 µg/ml)	KTZ (50 µg/ml)
Ca1	11.00±0.57	25.00±0.00	IA	IA	25.00±0.57	NA
Ca2	10.67±0.33	25.00±0.57	IA	IA	25.67±0.33	NA
Ca3	10.67±0.33	25.00±0.00	IA	IA	26.67±0.33	NA
Ca4	11.33±0.57	25.00±0.00	IA	IA	26.33±0.33	NA
Ck	10.67±0.33	25.0±0.00	IA	IA	20.00±2.00	NA
AF	IA	20.67±0.33	IA	IA	NA	25.67±0.33
AN	IA	24.33±0.33	IA	IA	NA	26.33±0.33
TM	IA	25.67±0.33	IA	IA	NA	25.33±0.33
TV	IA	26.00±0.00	18.67±0.33	IA	NA	27.33±0.33

Values are mean inhibition zone (mm) ± S.D of three replicatIes

Key: HEX- hexane; ETA- ethylacetate; ET- ethanol ; W- water
FCZ- fluconazole; KTZ- ketoconazole; NA- not applicable ; IA inactive
Ca- *Candida albicans*; CK- *Candida krusei*; AN- *Aspergillus niger*; AF- *Aspergillus fumigatus*
TM- *Trichophyton mentagrophytes*; TV- *Trichophyton verucossum*

Table 4.6:Antifungal susceptibility testing of ethyl acetate extracts of *Mitracarpus villosus*

Organisms	Zone of Inhibition (mm)				
	100 mg/ml	50 mg/ml	25 mg/ml	12.50 mg/ml	6.25 mg/ml
Ca1	25.00±0.00	22.00±0.57	20.00±0.00	18.67±0.33	18.00±0.00
Ca2	25.00±0.57	22.00±0.00	20.00±0.00	19.33±0.33	18.67±0.33
Ca3	25.00±0.00	23.33±0.33	20.00±0.00	20.33±0.33	19.33±0.33
Ca4	25.00±0.00	23.00±0.00	20.33±0.33	18.67±0.33	17.67±0.33
Ck	25.00±0.00	20.00±0.00	18.67±0.33	18.33±0.33	18.67±0.33
AF	20.67±0.33	19.67±0.33	19.00±0.57	18.67±0.33	17.00±0.00
AN	24.33±0.33	23.67±0.887	20.00±0.00	18.33±0.88	17.67±0.67
TM	25.67±0.33	24.67±0.33	22.00±0.00	20.67±0.33	20.00±0.00
TV	26.00±0.00	25.67±0.33	23.67±0.33	21.67±0.33	21.00±0.00

Values are mean inhibition zone (mm) ± S.D of three replicates

Key Ca- *Candida albicans*; CK- *Candida krusei*; AN- *Aspergillus niger*

AF- *Aspergillus fumigatus*; TM- *Trichophyton mentagrophytes* TV-*Trichophyton verucossum*

Table 4.7:Antifungal susceptibility testing of crude tannin from *Mitracarpus villosus*

Organisms	Zone of Inhibition (mm)				
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.50 mg/ml
Ca1	27 .67±0.33	26.67±0.33	22.67±0.33	22.00±0.00	21.67±0.33
Ca2	28.67±0.33	28.00±0.00	25.0±0.0	24.00±0.00	23.67±0.33
Ca3	31.33±0.33	31.00±0.00	27.00±0.00	24.67±0.33	24.00±0.00
Ca4	31.00±0.00	30.67±0.33	27.00±1.00	24.67±0.33	23.67±0.33
CK	28.67±0.33	26.67±0.33	26.67±0.33	21.67±0.33	21.00±0.00
AF	24.33±0.33	22.00±0.00	20.67±0.33	19.67±0.33	19.67±0.33
AN	25.67±0.33	24.67±0.33	21.67±0.33	20.67±0.33	20.00±0.00
TM	27 .67±0.33	26 .67±0.33	24 .67±0.33	23.00±0.00	22.00±0.00
TV	28.00±0.00	26 .67±0.33	25.00±0.00	24.00±0.00	22 .67±0.33

Values are mean inhibition zone (mm) ± S.D of three replicates

Key Ca- *Candida albicans*; CK- *Candida krusei* ; AN- *Aspergillus niger*

AF- *Aspergillus fumigatus*; TM- *Trichophyton mentagrophytes*; TV-*Trichophyton verucossum*

Table 4.8:Antifungal susceptibility testing of crude phenol from *Mitracarpus villosus*

Organisms	Zone of Inhibition (mm)				
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.50 mg/ml
Ca1	24.00±0.00	23.67±0.33	21.00±0.58	20.67±0.33	20.33±0.33
Ca2	23.67±0.33	23.00±0.00	20.00±0.00	21.000±0.0	20.67±0.33
Ca3	25.67±0.33	24.67±0.67	22.67±0.33	22.00±0.00	21.67±0.33
Ca4	26.67±0.33	26.00±0.00	22.67±0.33	21.00±0.00	20.00±0.00
Ck	22.67±0.33	20.67±0.33	20.00±0.00	18.67±0.33	18.33±0.33
AF	23.00±0.00	22.00±0.00	20.67±0.33	20.00±0.00	19.67±0.33
AN	25.67±0.33	24.67±0.33	21.67±0.33	20.33±0.33	20.00±0.00
TM	25.67±0.33	24.67±0.33	23.00±0.00	22.00±0.00	22.00±0.00
TV	26.67±0.33	25.67±0.33	24.00±0.00	23.00±0.00	22.00±0.00

Values are mean inhibition zone (mm) ± S.D of three replicates

Key Ca - *Candida albicans*; CK- *Candida krusei*; AN- *Aspergillus niger*
AF- *Aspergillus fumigatus*; TM- *Trichophyton mentagrophytes*; TV- *Trichophyton verucossum*

Table 4.9:Antifungal susceptibility testing of crude saponin from *Mitracarpus villosus*

Organism	Zone of Inhibition (mm)			
	100 mg/ml	50 mg/ml	25 mg/ml	12.50 mg/ml
Ca1	23.67±0.33	22.57±0.33	21.67±0.33	20.67±0.33
Ca2	26.00±0.57	24.67±0.33	23.00±0.00	22.00±0.00
Ca3	25.00±0.00	24.00±0.00	23.00±0.00	21.0±0.00
Ca4	25.0±0.0	24.00±0.00	23.00±0.00	20.67±0.33
Ck	22.00±0.00	20.67±0.33	19.67±0.33	19.00±0.00
AF	32.67±0.33	28.67±0.33	24.67±0.33	21.33±0.67
AN	31.33±0.67	27.67±0.33	24.67±0.33	22.67±0.33
TM	29.33±0.67	27.00±0.00	25.67±0.33	23.67±0.33
TV	29.33±0.67	28.00±0.00	26.67±0.33	24.67±0.33

Values are mean inhibition zone (mm) ± S.D of three replicates

Key: Ca- *Candida albicans*; CK- *Candida krusei*; AN- *Aspergillus niger*

AF- *Aspergillus fumigatus*; TM- *Trichophyton mentagrophytes*; TV *Trichophyton verucossum*

Table 4.10: Minimum inhibitory concentration of ethyl acetate extract, secondary metabolites and fractions of *Mitracarpus villosus*

ORG	Minimum Inhibitory Concentration (µg mL^{-1})											
	ETA	Tannins	Saponins	Phenols	M1	M2	M3	M4	M5	M6	FCZ	KCZ
Ca1	1000.00 ±0.33	1000.00±0.33	1000.00± 0.00	8000.00± 0.00	2000.00± 0.57	1000.00± 0.00	1000.00± 0.00	500.00± 0.57	2000.00± 0.00	2000.00± 0.00	25.00± 0.00	NA
Ca2	1000.00± 0.57	1000.00± 0.0	1000.00± 0.57	8000.00± 0.00	2000.00± 0.00	1000.00± 0.00	1000.00± 0.00	2000.00± 0.33	500.00± 0.00	2000.00± 0.57	25.00±0.33	NA
Ca3	500.00± 0.00	500.00±0.33	1000.00± 0.33	4000.00±0.33	1000.00± 0.00	1000.00±0.33	2000.00± 0.00	2000.00± 0.00	250.00± 0.00	1000.00± 0.00	25.00± 0.00	NA
Ca4	500.00± 0.00	500.00± 0.00	1000.00± 0.57	4000.00± 0.00	2000.00± 0.00	500.00± 0.00	500.00± 0.33	1000.00± 0.33	250.00± 0.00	1000.00± 0.00	25.00±0.33	NA
Ck	2000.00± 0.33	2000.00± 0.00	4000.00± 0.33	8000.00± 0.00	4000.00±0.33	500.00± 0.00	500.00± 0.00	1000.00± 0.00	500.00± 0.00	1000.00± 0.33	200.00± 0.00	NA
AF	2000.00± 0.00	2000.00±0.33	1000.00± 0.00	1000.00± 0.00	2000.00± 0.00	2000.00±0.33	2000.00± 0.00	4000.00± 0.00	4000.00± 0.33	4000.00± 0.00	NA	25.00±0.33
AN	1000.00± 0.57	1000.00± 0.00	1000.00± 0.57	1000.00± 0.00	2000.00± 0.33	500.00± 0.00	1000.00± 0.00	2000.00± 0.57	4000.00± 0.00	4000.00± 0.33	NA	25.00±0.57
TM	2000.00± 0.00	1000.00± 0.00	500.00± 0.00	500.00± 0.58	1000.00± 0.00	1000.00± 0.00	1000.00± 0.00	1000.00± 0.00	500.00± 0.00	500.00± 0.00	NA	8.00±0.33
TV	500.00± 0.33	500.00± 0.00	500.00± 0.00	500.00± 0.00	250.00± 0.00	250.00± 0.00	250.00± 0.00	250.00± 0.00	500.00± 0.00	500.00± 0.57	NA	4.00± 0.00

Values are mean minimum inhibitory concentration (mg mL^{-1}) ± S.D of three replicates

Key: Ca - *Candida albicans*; CK- *Candida krusei*; AN- *Aspergillus niger*; AF- *Aspergillus fumigatus*

TM- *Trichophyton mentagrophytes*; TV- *Trichophyton verucossum* ; ETA -Ethyl acetate extract; ORG – Organisms

M -- *M. villosus* fraction; FCZ- Fluconazole; KCZ – Ketoconazole; NA- Not applicable

Table 4.11: Minimum fungicidal concentration of ethyl acetate extract, secondary metabolites and fractions of *Mitracarpus villosus*

ORG	Minimum Fungicidal Concentration (µg/mL)											
	ETA	Tannins	Saponins	Phenols	M1	M2	M3	M4	M5	M6	FCZ	KCZ
Ca1	4000.00± 0.33	4000.00± 0.33	8000.00± 0.00	8000.00± 0.00	8000.00±0. 00	2000.00± 0.33	4000.00± 0.33	2000.00± 0.00	4000.00± 0.57	4000.00± 0.00	25.00± 0.33	NA
Ca2	4000.00± 0.00	4000.00± 0.33	4000.00± 0.33	8000.00± 0.33	4000.00± 0.33	500.00± 0.00	4000.00± 0.00	4000.00± 0.57	4000.00± 0.33	8000.00± 0.33	25.00± 0.00	NA
Ca3	4000.00± 0.33	4000.00± 0.00	4000.00± 0.00	8000.00± 0.00	8000.00± 0.00	4000.00± 0.00	4000.00± 0.00	4000.00± 0.00	1000.00± 0.00	4000.00± 0.00	25.00± 0.57	NA
Ca4	4000.00± 0.00	4000.00± 0.00	8000.00± 0.00	8000.00± 0.57	8000.00± 0.00	2000.00± 0.33	4000.00± 0.57	8000.00± 0.33	8000.00± 0.57	16000.00± 0.00	25.00± 0.00	NA
Ck	8000.00± 0.57	8000.00± 0.57	8000.00± 0.33	8000.00± 0.00	8000.00± 0.33	1000.00± 0.00	4000.00± 0.00	8000.00± 0.00	8000.00± 0.00	4000.00± 0.57	2000.00± 0.00	NA
AF	4000.00± 0.33	8000.00± 0.00	4000.00± 0.00	16000.00±0. 00	4000.00± 0.00	4000.00± 0.33	4000.00± 0.00	8000.00± 0.33	8000.00± 0.57	8000.00± 0.00	NA	50.00±0.33
AN	4000.00± 0.00	8000.00± 0.00	4000.00± 0.00	16000.00±0. 00	4000.00± 0.33	1000.00± 0.00	2000.00± 0.57	4000.00± 0.00	8000.00± 0.00	8000.00± 0.57	NA	50.00±0.33
TM	4000.00± 0.33	2000.00± 0.57	2000.00± 0.33	2000.00± 0.33	2000.00± 0.00	2000.00± 0.57	2000.00± 0.00	2000.00± 0.33	1000.00± 0.33	1000.00± 0.00	NA	16.00±0.33
TV	2000.00± 0.00	1000.00± 0.00	1000.00± 0.00	1000.00± 0.57	500.00± 0.00	500.00± 0.00	500.00± 0.00	500.00± 0.00	1000.00± 0.00	1000.00± 0.33	NA	16.00± 0.00

Values are mean minimum fungicidal concentration (mg mL^{-1}) ± S.D of three replicates

Key Ca- *Candida albicans*; CK- *Candida krusei*; AN - *Aspergillus niger*; AF- *Aspergillus fumigatus*

TM- *Trichophyton mentagrophytes* ; TV - *Trichophyton verucossum*; ETA- ethyl acetate extract; NA- Not applicable

M- *villosus* fraction; FCZ - fluconazole; KCZ- ketoconazole; ORG- organism

4.5.3. Effect of Temperature on the Antifungal Activity of the Ethyl Acetate Extract of *M. villosus*.

The values on Table 4.12 showed that temperature had no much effect on the activity of the ethyl acetate extract, as there was no significant difference (p= 0.3931- 0.9203; p > 0.05) in the zones of inhibition with temperature increase. The ANOVA summary tables (Table 4.14- 4.22) are shown in the appendix.

Table 4.12: Effect of varying temperature on the antifungal activity of the ethyl acetate *M. villosus* at concentration of 50 mg/ml against the test fungal isolates.

Organism	Zone of Inhibition (mm)		
	25^{o}C	37 ^{o}C	45 ^{o}C
Ca1	20.33±0.33	20±0	19.67±0.33
Ca2	23.33±0.33	21.67±0.33	21.33±0.33
Ca3	22.67±0.33	22.33±0.33	22.67±0.33
Ca4	21.33±0.33	20.33±0.33	20.33±0.33
Ck	20.67±0.33	20.33±0.33	20.33±0.33
AF	22.00±0.00	21.67±0.33	21.33±0.33
AN	24.67±0.33	25.33±0.33	24.67±0.33
TM	24.67±0.33	25.0±0.33	24.67±0.33
TV	25.67±0.33	25.67±0.33	25.00±0.33

Values are mean inhibition zone (mm) ± S.D of three replicates

Key Ca- *Candida albicans*; CK- *Candida krusei* ;

AN- *Aspergillus niger*; AF- *Aspergillus fumigatus*;

TM- *Trichophyton mentagrophytes*; TV *Trichophyton verucossum*

4.5.4. Effect of Storage Duration on the Antifungal Activity of the Ethyl Acetate Extracts *M. villosus*.

The values on Table 4.13 showed that there was no marked difference in the antifungal activities of the ethyl acetate extract of on the fungal isolates tested when stored over a period of nine months, as there was no significant difference ($p= 0.0983- 0.7293$; $p > 0.05$) in the zones of inhibition of the fungal isolates over the time of storage. Also there was no significant difference ($p= 0.0983- 0.7293$; $p > 0.05$) in antifungal activities between the different storage conditions. The ANOVA summary tables (Table 4.23- 4.29) are shown in the appendix.

Table 4.13: Effect of storage on ethyl acetate extracts of *Mitracarpus villosus* at concentration of 50 mg/ml

Organism	Zone of Inhibition (mm)								
	3 Months			6 Months			9 Months		
	AB	DS	D	AB	DS	D	AB	DS	D
Ca1	21.67±0.33	21.67±0.33	21.67±0.33	22.00±0.00	21.67±0.33	21.67±0.33	22.0±0.0	22.00±0.00	22.00±0.00
Ca2	21.67±0.33	22.00±0.00	22.33±1.15	21.67±0.33	22.00±0.33	22.00±0.57	21.67±0.33	22.33±0.33	22.00±0.00
Ca3	22.67±0.33	23.00±0.00	22.67±0.33	23.00±0.00	23.00±0.00	22.67±0.33	23.00±0.00	23.00±0.00	23.33±0.33
Ca4	22.67±0.33	23.00±0.00	22.67±0.33	22.67±0.33	22.67±0.33	23.00±0.00	23.33±0.00	23.00±0.00	23.33±0.33
Ck	19.67±0.33	19.67±0.33	19.67±0.33	20.00±0.00	20.33±0.33	19.67±0.33	20.00±0.00	20.00±0.00	20.00±0.00
AF	19.67±0.33	19.67±0.33	19.67±0.33	19.67±0.33	19.67±0.33	19.67±0.33	19.00±0.00	20.00±0.00	19.00±0.00
AN	23.67±0.33	23.67±0.33	23.67±0.33	23.67±0.33	24.00±0.33	23.67±0.33	24.00±0.00	23.33±1.15	24.00±0.00

Values are mean inhibition zone (mm) ± S.D of three replicates

Key Ca- *Candida albicans*; CK- *Candida krusei*; AN- *Aspergillus niger*
AF- *Aspergillus fumigatus*; TM- *Trichophyton mentagrophytes*; TV-*Trichophyton verucossum*
AB- amber bottle; DS- Direct sunlight; D- Dark compartment

4.5.5. Rate of Kill Studies.

The result obtained from the rate of kill experiment using ethyl acetate extract, fluconazole (against yeast) and ketoconazole (against mould) as shown in Figures 4.1 - 4.9 indicate that these compounds possess significant biocidal activity against the test fungal isolates which was generally concentration- dependent. Killing of cells occur as a function of time within a concentration range of 0.025 – 8.0 mg ml^{-1}. Fig 4.1 shows the time kill curve of the most resistant *Candida albicans* (Ca4) isolate to fixed concentrations of ethyl acetate extract, M2 fraction and fluconazole. As depicted in the graph, the test fungal isolate was affected, with gradual decrease in cell population up to 300 mins, after which there was a total kill. The survival was similar to that of fluconazole (0.025 mg ml^{-1}); initial gradual decrease followed by complete kill at 240 minutes. M2 (2 mg/ml) showed a rate of kill pattern similar to fluconazole with total kill at 240 minutes. The pattern of kill of *Candida krusei* by the extract (8 mg/ml) was slower than that of *Candida albicans*, with *Candida krusei* surviving up to 420 minutes. The survival rate of the organism to M2 (1 mg/ml) was similar to fluconazole (0.025 mg/ml) with a total kill observed at 360 minutes. As shown in Fig. 4.3, *Aspergillus fumigatus* cells decreased slowly up to 420 min where there was a total kill. The survival rate of M2 (4 mg/ml) was similar to that of ketoconazole (0.05 mg/ml); initial gradual decrease followed by complete kill at 360 minutes. The killing rate of *Aspergillus niger* upon exposure to M2 (1 mg/ml) was similar to ketoconazole with total killing time of 360 minutes each. The extract (4 mg/ml) on the other hand killed all the cells at 420 minutes. The time kill curve of *Trichophyton mentagrophytes* and *Trichophyton verrucosum* are shown in Fig.4.5 and 4.6. respectively. *Trichophyton mentagrophytes* when exposed to the ethyl acetate extracts (4 mg/ml), M2 (2 mg/ml) and

ketoconazole (0.05mg/ml) produced the same total killing time of 360 min. *Trichophyton verrucosum*, on the other hand on exposure to the extract (2mg/ml) and M2 (0.5 mg/ml) showed gradual decrease in cell population up to 360 mins, after which there was a total kill. The survival rate of the organisms to the ethyl acetate extract and M2 were also similar to that of ketoconazole (0.05 mg/ml); with an initial gradual decrease in the cells followed by complete kill at 300 minutes.

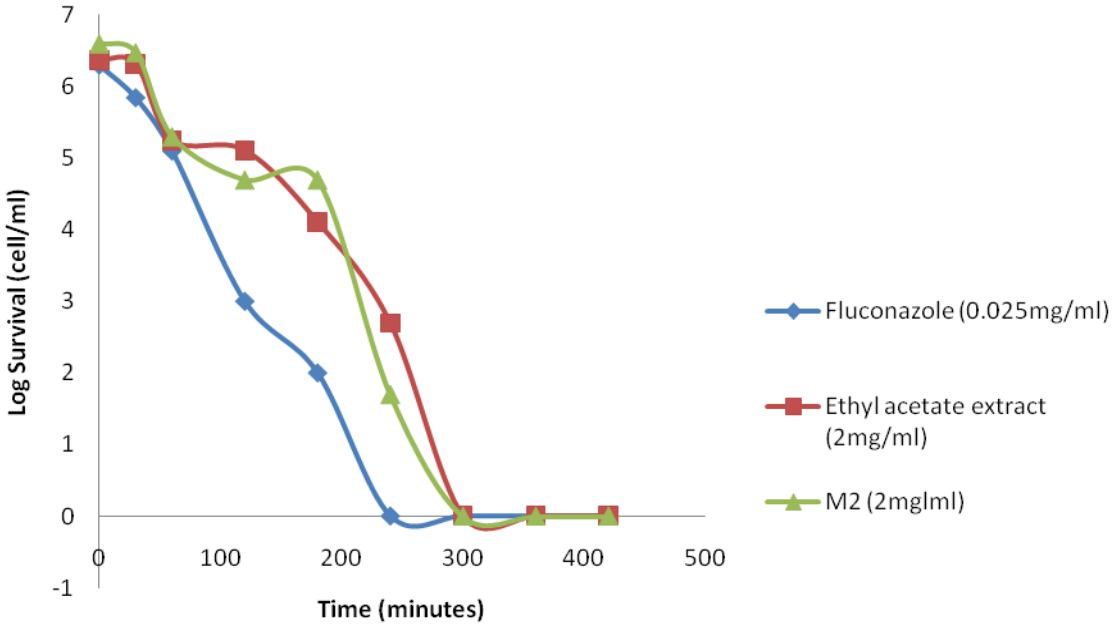

Fig 4.1: Biocidal activity of fixed concentration (0.025mg/ml) of fluconazole, ethyl acetate extract (2 mg/ml) and M2 (2mg/ml) against *C. albicans* at different time interval

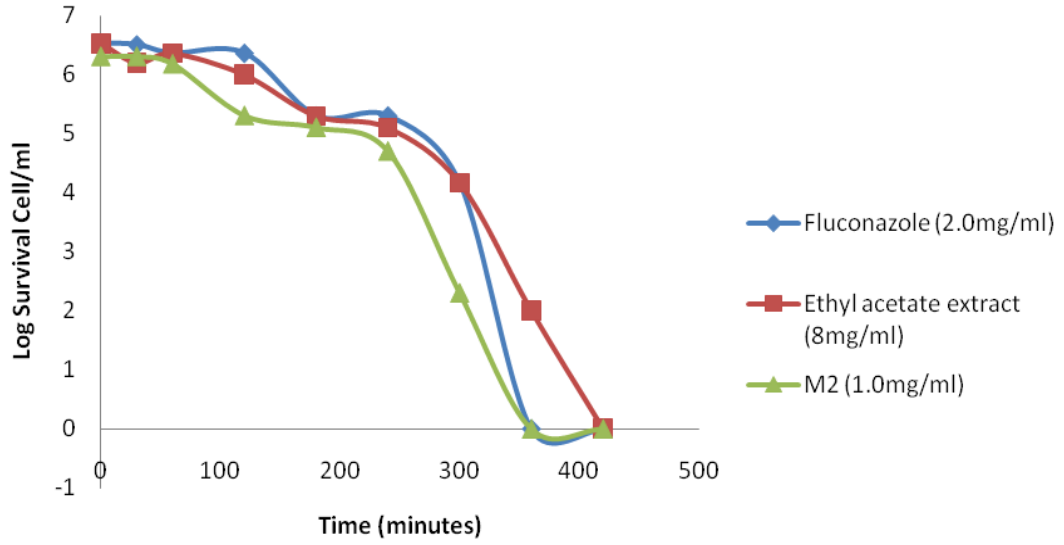

Fig. 4.2: Biocidal activity of fixed concentration (0.2mg/ml) of fluconazole, ethyl acetate extract (4 mg/ml) and M2 (1mg/ml) against *C. krusei* at different time interval

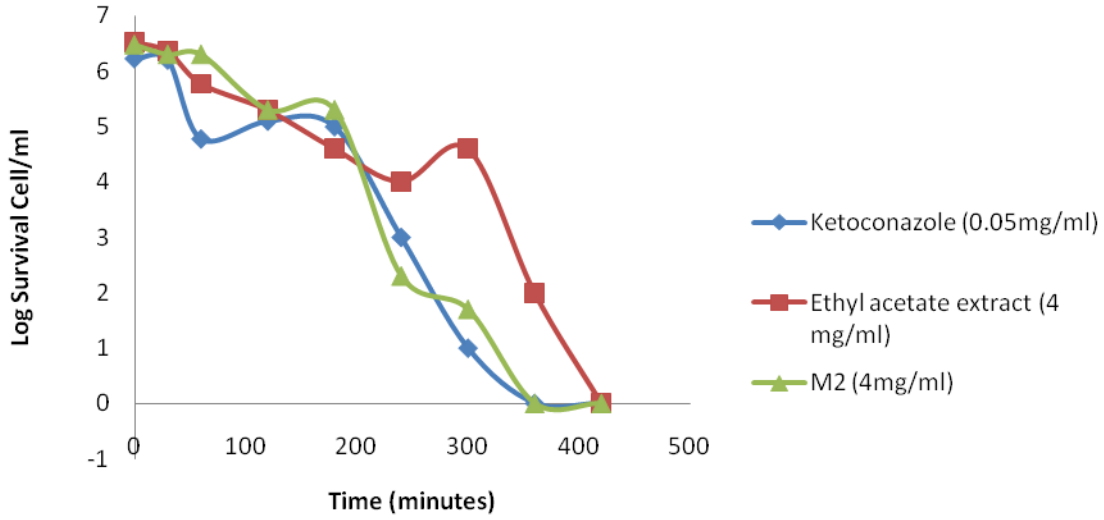

Fig 4.3: Biocidal activity of fixed concentration (0.05mg/ml) of Ketoconazole, ethyl acetate extract (4mg/ml) and M2 (4mg/ml) against *A. fumigatus* at different time interval

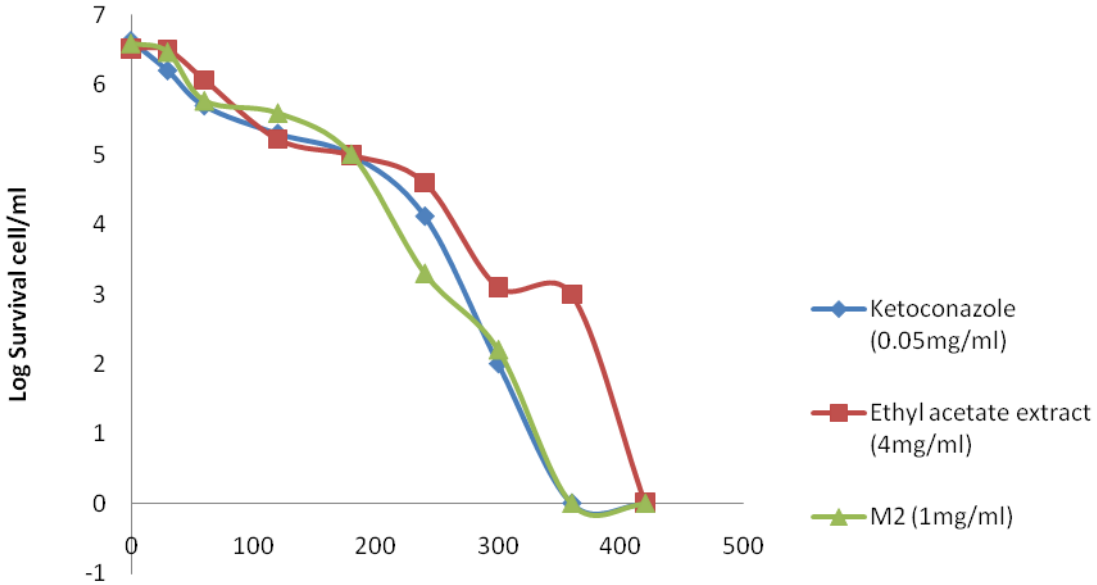

Fig 4.4: Biocidal activity of fixed concentration (0.05mg/ml) of Ketoconazole ethyl acetate extract (4 mg/ml) and M2 (1mg/ml) against *A. niger* at different time interval

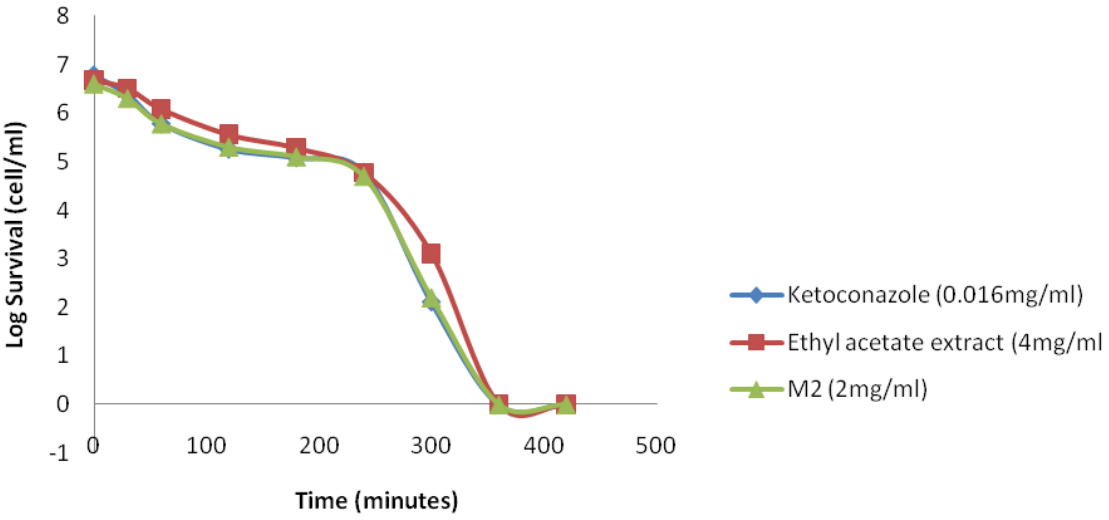

Fig 4.5: Biocidal activity of fixed concentration (0.016mg/ml) of ketoconazole, ethyl acetate extract (4 mg/ml) and M2 (2mg/ml) against *T. mentagrophytes* at different time interval

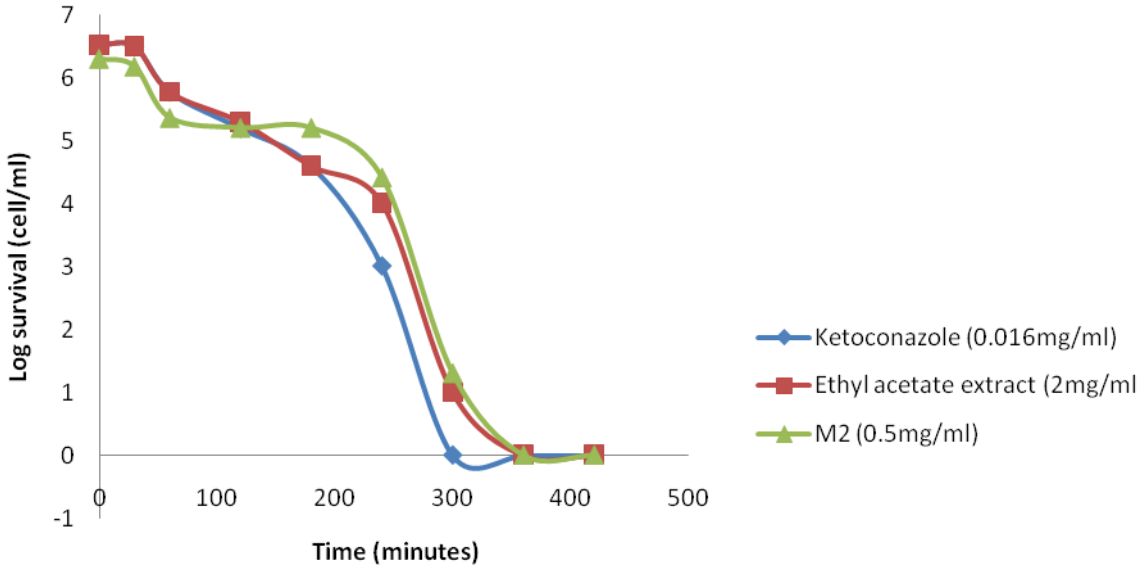

Fig 4.6: Biocidal activity of fixed concentration (0.016mg/ml) of Ketoconazole ethyl acetate extract (2 mg/ml) and M2 (0.5mg/ml) against *T. verrucosum* at different time interval

CHAPTER 5

5.0 DISCUSSIONS

5.1 EXTRACT YIELD

The different solvents used in the successive extraction of *M. villosus* yielded different proportion of extract. The higher yield of the ethanol extract over the water extract and other less polar solvents could be due to their difference in polarity. During extraction solvents diffuse into the solid plant material and solubilize compounds with similar polarity (Prashant *et al.,* 2011; Ncube *et al.,* 2008). Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Prashant *et al.*, 2011). The extraction yield and biological activity of extracts have been shown to be highly dependent on solvent polarity (Zohra and Fawzia, 2011). In a study by Cimanga *et al*., (2004), the percentage yield of the hexane extract of *M. villosus* was reported to be 14.80. This was higher than the percentage yield of hexane extract recorded in this study. This could be due to the difference in the method of extraction used. Successive extraction was employed in this study, while cold maceration was employed in the study by Cimanga *et al*., (2004).

5.2 PHYTOCHEMICAL SECONDARY METABOLITES

The phytochemical screening of the powdered plant part revealed the presence of tannins, saponin, flavonoids, terpenes, phenols and resins while alkaloid, sugars carbohydrates, balsams and anthraquinones were not detected. Plants have been known to posses bioactive constituents as protective substances against bacteria, fungi, viruses and pests (Marjorie, 1999). Phytochemical studies have shown that plants with antimicrobial activity contain

bioactive constituents such as tannins, flavonoids, alkaloids and saponins which are responsible for the biological properties of such plants (Thamaraiselvi *et al.,* 2012). Research in Nigeria have reported the presence of tannins, flavonoids, saponins and phenols from extracts of *M. villosus* (Abere *et al.,* 2007; Onawunmi *et al.,* 2012; Ubani *et al.,* 2012 ; Edeoga *et al*., 2005). In a study by Ameh *et al.* (2011), tannins, saponins and anthraquinone glycosides were detected in the aerial parts of *M. villosus*.

5.3 ANTIFUNGAL ACTIVITIES

5.3.1 Crude Extracts

The biological activities of extracts have been shown to be highly dependent on solvent polarity (Zohra *et al.,* 2011). From the results of this study, the degree of antifungal activities of the test plant varied from one test organism to another. It was observed that there was an increase in antifungal activity with increase in the concentration of extract used. In the susceptibility test of the fungi to the different extracts, it was observed that the ethyl acetate extract of *M. villosus* produced the highest antifungal activity against all the test fungi. This was indicated by the diameter of zone of inhibition which was shown to increase with the increase in concentration of the extracts.

The increased antifungal activity of the ethyl acetate extract over the other solvent extracts (hexane, ethanol and water) of *M. villosus* is an indication that ethyl acetate was able to extract out most of the active components of the plant. The zones of inhibition of the ethyl acetate extracts against all the fungi tested at 50 mg/ml was comparable to the standard drugs (fluconazole and ketoconazole) used. .The mode of action of ethyl acetate extract

could be related to their ability to alter membrane properties leading to cell death (Aquel *et al*., 2012). The hexane extract (100 mg/ml) on the other hand, was only active against *C. albicans* and *C. krusei*, with zones of inhibition lower than fluconazole (at 50 µg/ml). The antifungal effect of the hexane extract of *M. villosus* agrees with a study reported by Onawunmi *et al*., 2012 who reported its inhibitory action on *Candida albicans*. In another study by Shinkafi and Manga, (2011), the hexane and chloroform extracts of *M. villosus* (10 mg/ml) inhibited the growth of *Trichophyton rubrum, T. mentagrophytes* and *Microsporum gypseum*, while the petroleum ether extract (160 mg/ml) of *M. villosus* had no activity on any of the three organisms.

The absence of anti fungal activity of the aqueous extracts of *M. villosus* agrees with a work by Irobi and Daramola (1994), who reported that aqueous extracts of *M. villosus* had no inhibitory action against *Trichophyton rubrum, Candida albicans* and *Aspergillus niger*. In a study by Ameh *et al*. (2007), the thin layer chromatographic results of different solvent extracts of *M. villosus* revealed the presence of more spots in the dried ethyl acetate and hexane extracts of *M. villosus* than the water and ethanol extract. This could be responsible for their better antifungal activities of the ethyl acetate and hexane extracts of *M. villosus* over the ethanol and aqueous extracts.

5.3.2 Secondary Metabolites

All the secondary metabolites were active against the tested fungi. Phytochemical compounds isolated from plants have been reported to possess anti fungal properties (George *et. al*., 2002; Ubani *et al*., 2012). No work has been documented on the antifungal activities of extracted secondary metabolites from *M. villosus*. In this study, the crude tannin from *M. villosus* had strong antifungal activities against all the fungi tested with anti fungal activities comparable with the standard drugs used. Adekunle and Ikumapayi (2006), reported that tannins act as an antifungal agent at higher concentrations by coagulating the protoplasm of the micro-organism. Similarly, Harekrishna *et al*., (2010) stated that the possible mechanism of tannins may be to interfere with energy generation by uncoupling oxidative phosphorylation or they may interfere with glycoprotein of cell surface.

Saponins are naturally occurring surface-active glycosides. They have been reported to possess strong antifungal activities (George *et. al*., 2002). Many saponins are known to possess antimicrobial properties inhibiting the growth of moulds and dermatophytes. The major mechanism suggested for the antifungal activity of saponins is their interaction with membrane sterols (George *et al*., 2002). The antifungal activities of the crude saponin from *M. villosus* against the test fungi were comparable with the standard drugs. The crude saponin at a concentration of 12.50 mg/ml exhibited good antifungal activity as indicated by high zones of inhibition against the yeast, moulds and dermatophytes, which was comparable with fluconazole and ketoconazole at a concentration of 0.05 mg/ml. This strong antifungal activity has been linked to possess to the formation of complexes with

sterol in fungal plasma membrane leading to death by destruction of cellular semipermeablility (David Hoffman, 2003).

Natural phenolic compounds are widespread in the plant kingdom. They are found in leaves, fruits, barks and wood and can accumulate in large amounts in particular organs or tissues of the plant (Nitiema *et al*., 2012) Antimicrobial activity of plant phenolics has been intensively studied, and, in addition to controlling invasion and growth of plant pathogens, their activity against human pathogens has been investigated to characterize and develop new healthy food ingredients, medical compounds, and pharmaceuticals (Nohynek *et al*., 2006) The result of this study revealed that the phenol extracts of *M. villosus* produced the least antifungal activity against the test fungi than the tannin and saponin extracts of *M. villosus.* Its zones of inhibition at a concentration of 12.50 mg/ml, was lower than the standard drugs at 0.05 mg/ml concentration, however it had a better antifungal activity on *Trichophyton* species than *Aspergillus* species. The mode of antifungal action of phenolic extract might be related to their ability to inactive adhesions, enzymes, cell envelope transport proteins (Chabot *et al*., 1992) or related to the sites and number of OH group on the phenolic rings (Cowan, 1999).

5.3.3 Ethyl acetate Fractions

The ethyl acetate fractions (M1- M6) of *M. villosus* had varied effect on the fungal isolates (moulds, dermatophytes and yeast). This is an indication that different chemical substances are present in the extract depending on the type of eluent used. This agrees with a report by Prashant *et al.,* (2011), who stated that successful determination of biologically active

compounds from plant material is largely dependent on the type of solvent used in the extraction procedure Generally, the fractions produced higher antifungal activities than the crude ethyl acetate extracts. With respect to the results on the minimum inhibitory concentrations of the ethyl acetate fractions, M1 and M2 exhibited the highest anti fungal activities than the other fractions. These increased antifungal activities can be linked to the higher volume of ethyl acetate used in extracting these fractions.

5.3.4 Effect of Temperature on the Antifungal Activity of the Ethyl Acetate Extract of *M. villosus*

The result of the effects of temperature on antifungal activity of the ethyl acetate extract of *M. villosus* (Table 4.12) showed that varying the temperature did not significantly ($p > 0.05$) affect the antifungal activity of the extract over a temperature range of 25^oC -45^oC with respect to the zones of inhibition produced. The extract was stable within this temperature range as the diameter of zone of inhibition produced on all the fungal isolates did not vary markedly. This agrees with a work by Abere *et al*. (2007), who reported that subjection of *M. villosus* extracts to heat did not affect its antifungal activity.

5.3.5 Effect of Storage Conditions and Duration on the Antifungal Activity of the Ethyl Acetate Extract of *M. villosus*

The effect of varying storage conditions and duration on the antifungal activities of the ethyl acetate extract of *M. villosus* as shown in Table 4.15, indicated that the extract was stable over the nine month storage period. There was also no significant difference ($p > 0.05$) in the zones of inhibition of extract stored in the amber bottle, plain glass bottle or in

a dark enclosure. This agrees with the work reported by Ameh *et.al*. 2011, which stated that the ethyl acetate extract of *M. villosus* was stable over thirty nine months of storage.

5.3.6 Rate of Kill of the Ethyl Acetate Extract of *M. villosus*.

The biocidal activities of the ethyl acetate extract of *M. villosus,* fraction M2 and reference drugs (Fluconazole and Ketoconazole) are represented in Fig 4.1 to 4.6. The ethyl acetate extract and fraction M2 showed similar kill pattern as the reference drugs against the fungi tested with slight variations. Generally the extracts and fraction M2 killed the cells and spores gradually at the initial stage, followed by a sharp reduction of the cells. The effective killing of the cells by the ethyl acetate extract of *M. villosus* could be linked to the combined effect of the secondary metabolites present. Killing of cells occur chiefly as a function of time within a range of concentrations and this possibly explain the increased lethal activity after a longer time of incubation with the antifungal agents. The time-kill dynamic process is used for the evaluation of new antimicrobial agents. This makes it possible to determine if the agent has a fungistatic or fungicidal effect, and if the killing process is concentration or time-dependent (Pfaller *et al*., 2004).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

1. The aerial parts of *Mitracarpus villosus* contained tannins, saponin, flavonoids, terpenes, phenols and resins.

2. Ethyl acetate extract of *Mitracarpus villosus* ethyl acetate extracts obtained from Abuja, Federal Capital Territory, Nigeria was found to possess antifungal action against *Candida albicans, Candida krusei*, *Aspergillus fumigatus, Aspergillus niger*, *Trichophyton mentagrophytes* and *Trichophyton verrucosum* isolates.

3. The crude ethyl acetate extract and secondary metabolites of *M. villosus* have shown a consistent inhibition of fungal isolates, although higher concentrations were required to produce zones of inhibitions when compared to the reference antibiotics.

4. The fractions of the ethyl acetate extract showed the highest antifungal activity (M.I.C. and M.F.C.) than the secondary metabolites and crude ethyl acetate extracts.

5. The crude extracts had a stable antifungal activity over a temperature range of $25^{o}C$ – $45^{o}C$ and over a storage period of nine months.

6. The pattern of kill of the fungal isolates by the ethyl acetate extract and fraction M2 was comparable with the pattern of kill produced by the standard drugs.

6.2 RECOMMENDATIONS

1. The findings of this study have shown the need for further investigation to establish the economic viability of exploiting *M. villosus* plant to address fungal infections.
2. Toxicology test for safety is required with further purification for economical purposes.
3. Further investigations should be carried out on the plant with the aim of isolating pure compounds, with potential of solving the drug resistance dilemma.
4. Further studies to determine the specific activities as well as the mechanism of action of the ethyl acetate fractions of *M. villosus*.
5. Steps should be put in place to encourage cultivation and domestication of the plant.

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APPENDICES

Appendix I: Phytochemical analysis of *M. villosus.*

TEST	APPEARANCE	INFERENCE
1.STEROLS a. Lieberman-Buchard	Production of blue green, red or orange color that change with time	-
b. Salkowski test	Formation of red or yellow coloration was obtained	-
2.FLAVONOIDS a. Shinoda test	Appearance of an orange, pink or red to purple color was obtained	+
b. Sulphuric acid	Color change was observed	+
c. Ferric chloride	Green, blue or violet coloration indicate the presence of phenolic hydroxyl group	+
d. Sodium Hydroxide	A change in color from yellow to colorless on addition of dilute HCl	+
3. ALKALOID a. Dragendoff reagent	Formation of yellow to brown precipitate	-
b. Mayer's reagent	Formation of white to yellowish or green color precipitate	-
c. Wagner's reagent	Formation of brown or reddish brown precipitate No precipitate occurred in ethyl acetate extract.	-

4.TANNINS a. Lead sub-acetate	Formation of light brown precipitate.	+
b. Bromine water	A blue-black precipitate was observed	+
c. Ferric Chloride	Blue mixture was obtained in ethanol and ethyl acetate. Dirty green coloration was obtained in water extract.	+
d. Ferric Ammonium citrate test	A brown mixture was obtained	+
5.SAPONIN a. Frothing test	Frothing which persisted for 15 minutes	+
b. Emulsion test or Olive oil test	Formation of stable emulsion	+
c. Fehling's test	Bluish-green precipitate observed in ethanol and water extract. No color change was observed in ethyl acetate.	+
d. Haemolytic test	Formation of haemolysis was seen in ethanol and water but not visible change/signs of haemolysis in ethyl acetate portion	+
6.CARBOHYDRATE TEST a. Molisch test	Appearance of reddish color is observed	-
b. Fehling's test for reducing sugar	Appearance of brick red precipitate	-
7. RESIN	The presence of green precipitate is a positive test	+
8. ANTHRAQUINONES a. Free anthraquinones	Presence of pink, red or violet colour in the ammoniacal layer is observed	-

b.Combined Anthraquinones	A pink, red colouration in the ammonia phase (lower phase) indicate the presence of combined anthraquinones or anthraquinone derivative	-

Key: + = Present - = Absent

Appendix II: Biocidal activity of fixed concentration (0.025 mg/ml) of fluconazole, ethyl acetate extract (2 mg/ml) and M2 (2 mg/ml) against *C. albicans* at different time interval.

Time (minutes)	Fluconazole (Log count)	Ethyl acetate extract (Log count)	M2 (Log count)
0	6.301	6.38	6.602
30	5.845	6.322	6.477
60	5.10	5.255	5.301
120	3.0	5.114	4.699
180	2.0	4.114	4.699
2400	0	2.699	1.699
300	0	0	0
360	0	0	0
420	0	0	0

Appendix III: Biocidal activity of fixed concentration (0.2 mg/ml) of fluconazole, ethyl acetate extract (4 mg/ml) and M2 (1 mg/ml) against *C. krusei* at different time interval.

Time (minutes)	Fluconazole (Log count)	Ethyl acetate extract (Log count)	M2 (Log count)
0	6.531	6.531	6.301
30	6.505	6.203	6.3013
60	6.362	6.362	6.176
120	6.362	6.0	5.301
180	5.301	5.301	5.101
2400	5.301	5.101	4.699
300	4.17	4.17	2.301
360	0	2.0	0
420	0	0	0

Appendix IV: Biocidal activity of fixed concentration (0.05 mg/ml) of ketoconazole, ethyl acetate extract (4 mg/ml) and M2 (4 mg/ml) against *A. fumigatus* at different time interval.

Time (minutes)	Fluconazole (Log count)	Ethyl acetate extract (Log count)	M2 (Log count)
0	6.230	6.53	6.477
30	6.204	6.362	6.301
60	4.778	5.778	6.301
120	5.10	5.3011	5.301
180	5.0	4.602	5.301
2400	3.0	4.01	2.301
300	1.0	4.602	1.699
360	0	2.0	0
420	0	0	0

Appendix V: Biocidal activity of fixed concentration (0.05 mg/ml) of ketoconazole, ethyl acetate extract (4 mg/ml) and M2 (1 mg/ml) against *A. niger* at different time interval.

Time (minutes)	Fluconazole (Log count)	Ethyl acetate extract (Log count)	M2 (Log count)
0	6.63	6.331	6.602
30	6.204	6.505	6.474
60	5.70	6.079	5.778
120	5.301	5.225	5.602
180	5.0	5.0	5.01
2400	4.115	4.602	5.301
300	2.0	3.101	2.204
360	0	3.0	0
420	0	0	0

Appendix VI: Biocidal activity of fixed concentration (0.016 mg/ml) of ketoconazole, ethyl acetate extract (4 mg/ml) and M2 (2 mg/ml) against *T. mentagrophytes* at different time interval.

Time (minutes)	Fluconazole (Log count)	Ethyl acetate extract (Log count)	M2 (Log count)
0	6.778	6.678	6.602
30	6.38	6.508	6.301
60	5.778	6.08	5.778
120	5.255	5.555	5.101
180	5.079	5.299	4.699
2400	4.778	4.778	2.202
300	2.114	3.114	0
360	0	0	0
420	0	0	0

Appendix VII: Biocidal activity of fixed concentration (0.016 mg/ml) of ketoconazole, ethyl acetate extract (2 mg/ml) and M2 (0.5 mg/ml) against *T. verrucosum* at different time interval.

Time (minutes)	Fluconazole (Log count)	Ethyl acetate extract (Log count)	M2 (Log count)
0	6.531	6.531	6.301
30	6.505	6.505	6.176
60	5.778	5.718	5.361
120	5.201	6.30	5.204
180	4.602	4.602	5.204
2400	3.01	4.01	4.15
300	0	1.0	1.301
360	0	0	0
420	0	0	0

Appendix VIII: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. albicans* (Ca1).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	1.556	2	0.1111	3.500	0.0983	5.1433
Within groups	1.333	6	0.3333			
Total	2.889	8				

Appendix IX: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate Extract of *M. villosus* on *C. albicans* (Ca2).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	1.556	2	0.7778	3.000	0.1780	5.1433
Within groups	2.000	6	0.3333			
Total	3.556	8				

Appendix X: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. albicans* (Ca3).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	0.2222	2	0.1111	2.333	0.1780	5.1433
Within groups	2.000	6	0.3333			
Total	2.222	8				

Appendix XI: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. albicans* (Ca4).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	2.000	2	1.000	3.000	0.0978	5.1433
Within groups	2.000	6	0.3333			
Total	4.000	8				

Appendix XII: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. krusei* (Ck).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	0.2222	2	0.1111	0.3333	0.7290	5.1433
Within groups	2.000	6	0.3333			
Total	2.222	8				

Appendix XIII: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *Aspergillus fumigatus* (AF).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	0.6667	2	0.3333	1.500	0.2963	5.1433
Within groups	1.333	6	0.2222			
Total	2.000	8				

Appendix XIV: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *Aspergillus niger* (AN).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	1.556	2	0.7778	2.333	0.1780	5.1433
Within groups	2.000	6	0.3333			
Total	3.556	8				

Appendix XV: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *Trichophyton mentagrophytes* (TM).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	0.2222	2	0.1111	0.5000	0.6297	5.1433
Within groups	1.333	6	0.2222			
Total	1.556	8				

Appendix XVI: ANOVA summary on the effect of temperature on the antifungal activities of ethyl acetate extract of *M. villosus* on *Trichophyton verrucosum* (TV).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	0.8889	2	0.4444	2.000	0.2160	5.1433
Within groups	1.333	6	0.2222			
Total	2.222	8				

Appendix XVII: ANOVA summary on the effect of storage conditions and duration on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. albicans* (Ca1).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	0.6667	8	0.08333	0.3750	0.9203	2.51
Within groups	4.000	18	0.2222			
Total	4.667	26				

Appendix XVIII: ANOVA summary on the effect of storage conditions and duration on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. albicans* (Ca2).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	2.000	8	0.2500	1.125	0.3931	2.51
Within groups	4.000	18	0.2222			
Total	6.000	26				

Appendix XIX: ANOVA summary on the effect of storage conditions and duration on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. albicans* (Ca3).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	0.7407	8	0.09259	0.5000	0.8405	2.51
Within groups	3.333	18	0.1852			
Total	4.074	26				

Appendix XX: ANOVA summary on the effect of storage conditions and duration on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. albicans* (Ca4).

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	2.741	8	0.3426	1.321	0.2947	2.51
Within groups	4.667	18	0.2593			
Total	7.407	26				

Appendix XXI: ANOVA summary on the effect of storage conditions and duration on the antifungal activities of ethyl acetate extract of *M. villosus* on *C. krusei* (Ck)

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	1.407	8	0.1759	0.7917	0.6168	2.51
Within groups	4.000	18	0.2222			
Total	5.407	26				

Appendix XXII: ANOVA summary on the effect of storage conditions and duration on the antifungal activities of ethyl acetate extract of *M. villosus* on *Aspergillus fumigatus* (AF)

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	4.519	8	0.5648	0.4919	0.8462	2.51
Within groups	20.67	18	1.148			
Total	25.19	26				

Appendix XXIII: ANOVA summary on the effect of storage conditions and duration on the antifungal activities of ethyl acetate extract of *M. villosus* on *Aspergillus niger* (AN)

Source of variation	SS	DF	MS	F	P value	F critical
Between groups	1.185	8	0.1481	0.6667	0.7139	2.51
Within groups	4.000	18	0.2222			
Total	5.185	26				