ISOLATION AND SUSCEPTIBILITY OF DERMATOPHYTIC FUNGI SPORES TO ANTIFUNGAL AGENTS IN ZARIA, NIGERIA

BY

ADEDOYIN EBERE EKEOMA

(MSc/Pharm Micro/18984/07-08)

A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL AHMADU BELLO UNIVERSITY ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN PHARMCEUTICAL MICROBIOLOGY

DEPARTMENT OF PHARMACEUTICS AND PHARMACEUTICAL MICROBIOLOGY,

FACULTY OF PHARMACEUTICAL SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA

DECLARATION

I hereby declare that the work reported in this thesis " I**solation and susceptibility of dermatophytic fungi spores to selected antifungal agents in zaria, nigeria** was carried out by me under the supervision of Prof. J.O. Ehinmidu and Dr. Mrs G.O. Adeshina. both of the Department of Pharmaceutics and Pharmaceutical Microbiology. Faculty of Pharmaceutical Sciences. Ahmadu Bello University, Zaria. It has not been presented in any previous application for degree. The work of other investigators are acknowledged and referred to accordingly.

---------- ----------

ADEDOYIN EBERE EKEOMA DATE
Department of Pharmaceutics and Pharmaceutical Microbiology.
Faculty of Pharmaceutical Sciences.
Ahmadu Bello University, Zaria

CERTIFICATION

This thesis titled "**ISOLATION AND SUSCEPTIBILITY OF DERMATOPHYTIC FUNGI SPORES TO SELECTED ANTIFUNGAL AGENTS IN ZARIA, NIGERIA**", by Adedoyin Ebere Ekeoma, meets the regulation governing the award of the degree of Masters of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

---------- ----------

EXTERNAL EXAMINER DATE
PROF CHARLES ESIMONE,
B Pharm., M. Pharm, (U N N)
Ph.D. Pharm. Microbiol. (U.N.N)
Department of Pharmaceutics,
Nnamdi Azikiwe University Akwa.

---------- ----------

MAJOR SUPERVISOR DATE
PROF. J.O. EHINMIDU.
B Sc. Biol (UniBen)
MSc Pharm. Microbiol (UniBen), PhD. Pharm. Microbiol (A. B. U).
Department of Phsrmaceutics and Pharmaceutical Microbiology.
Ahmadu Bello University, Zaria, Nigeria.

---------- ----------

MEMBER OF SUPERVISORY TEAM DATE
DR. (MRS) G.O. ADESHINA.
B.Sc Microbiology (Unilorin),
M.Sc Pharm. Microbiol., Ph.D Pharm.Microbiolgy (A.B.U)
Department of Pharmaceutics and Pharmaceutical Microbiology.
Ahmadu Bello University, Zaria, Nigeria.

---------- ----------

HEAD OF DEPARTMENT DATE

DR B.A ISAH

B. Sc (Pharm); M.Sc (Pharm, Chealsea, London); Ph.D (A.B.U).
Department of Pharmaceutics and Pharmaceutical Micribiology
Ahmadu Bello University, Zaria.

---------- ----------

DEAN OF POST GRADUATE SCHOOL DATE

PROF ADEBAYO. A. JOSHUA

B.A, M. A, Ph.D (A.B.U).

DEDICATION

This work is dedicated to my Heavenly Father, the source of my strength..Thank you LORD

ACKNOWLEDGEMENT

I wish to express my profound gratitude to my supervisor, Prof. J O Ehinmidu for his immense efforts, and contributions to this work. May God bless you.

I also thank Dr (Mrs) G. O Adeshina , my co- supervisor for her contributions to this work.

My thanks also goes to Prof Olonitola, Dr Tytler, for their contributions as members of the supervisory committee.

I thank all my Lecturers in the name of Prof Y. K.E. Ibrahim and Prof J. O. Onolapo for imparting knowledge into me.

My appreciation goes to the entire staff of the Department of Pharmaceutics and Pharmaceutical Microbiology. Faculty of Pharmaceutical Sciences.

I thank my husband and my Miracle for their support and understanding during the entire period of my study. May God bless both of you

My sincere appreciation goes to my elder sisters, Dr Mrs Dandam, Mrs Oriwoh, Mrs Uba, Mrs Osamwonyi, and to my brother Mr Archy, and all other members of my family. May God bless you all.

I also acknowledge my friend Emmanuel, who helped me out in typing this work, even from the sick bed. I also appreciate the efforts you made to help me achieve this goal. May God bless and heal you in Jesus Name. Amen.

I thank my co students in the programme, and my students in Zamfara, May God bless you all.

Finally my greatest thanks go to my Father ‘’Him who is able to do exceedingly, abundantly more than I can ever ask from Him,’’To Him be all the glory now and for evermore. Amen.

ABSTRACT

Tinea capitis is a highly contagious infection of the scalp and hair caused by dermatophytes. In Nigera, as much as 30.6% of children have been reported to have these dermatophytic fungal infection. Dermatophytic fungal isolates from pupils in three primary schools in Zaria Nigeria were cultured, characterized and identified using standard microbiological methods. Antifungal activities of five selected commonly used chemical agents against five most resistant dermatophytic fungal isolates viz *Trichophyton rubrum* (Tr10), *Trichophyton mentagrophytes* (Tm07), *Trichophyton* tonsurans (Tt06), *Trichophyton verucosum* (Tv03), and *Trichophyton* soudanese (Ts01), were investigated using parameters such as Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC), Fractional Inhibitory Concentration (FIC), for Antifungal Agents Combinations and the effect of time on sporicidal activities of test chemical agents on the most resistant dermatophytic fungal spores. A total of 81 dermatophytic fungi were isolated from 150 swabs and samples collected from the volunteered primary school pupils. The dermatophytes were *Trichophyton rubrum* (12), *Trichophyton mentagrophytes* (18), *Trichophyton verucosum* (14) *Trichophyton soudanese* (16), *Trichophyton tonsurans* (13), *Trichophyton violaceum* (4) and *Microsporum canis* (4). Test fungal isolates with the highest incidences were *Trichophyton mentagrophytes* 18(22.2%), followed by *Trichophyton soudanese* 16(19.8%). *Microsporum canis* and *Trichophyton violaceum* were the least isolated, 4(4.9%) each. The M.I.C values of Flucytosine, Terbinafine HCL, and Tioconazole range from 0.50 to 100.00µg/ml against the five selected test fungi spores. The antifungal activity of Sodium propionate ranges from 50.0 to 250.0 µg/ml against the five dermatophytic spores. The order of inhibitory antifungal activity was Fluconazole > flucytosine >Terbinafine > Tioconazole > Sodium propionate. Similarly the Minimum Fungicidal Concentration of the test antifungal agents against the five selected test fungi spores showed the order of the antifungal activities as Fluconazole, > Terbinafine > Flucytosine > Tioconazole > Sodium Propionate. The most resistant dermatophyte was *Trichophyton rubrum*. Biocidal activities of the test compounds at 0.156µM concentration, displayed marked fungicidal activities in the order of Terbinafine HCl >Tioconazole > sodium propionate > Fluconazole, with one log cycle reduction time of 5.0, 7.5, 15.0, and 20.0 minutes respectively. After the first 30 minutes contact time in the admixtures, the log cycles reduction of resistant *T.rubrum* spores were in the following order; Terbinafine HCL (3.90) > Tioconazole (2.40) > Sodium Propionate (1.50) > Fluconazole (1.20) log cycles reductions. Generally, there was rapid rate of fungicidal activity among the test antifungal agents within the first 50 minutes. Based on the Minimum Fungicidal Concentration (MFC) values of the test antifungal agents, six new potential combined antifungal agents were formulated with tenfold their M.F.C. Results of the combinations of test antifungal agents against the most resistant *T. rubrum* showed synergism for Terbinafine /Sodium propionate, Fluconazole/ Sodium propionate, and Tioconazole /sodium propionate. The observed results in this study proved that the test antifungal agents singly and in combination possessed appreciable activities against the isolated dermatophytic fungi spores in Zaria Nigeria

TABLE OF CONTENT

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

LIST OF FIGURES

LIST OF TABLES

PAGE

LIST OF PLATES **PAGES**

LIST OF APPENDICES

PAGES

CHAPTER ONE

1.0 INTRODUCTION

Dermatophytic infections incidence has markedly increased as a significant clinical problem in recent years (Birgit *et al.,* 1997). Several factors have contributed to this Dermatophytic infections such as the use of immunosuppressive drugs, prolonged use of broad-spectrum antibiotics, widespread use of indwelling catheter and Immunodeficiency associated with HIV patients (Fuller *et al., 2003)*

Among the different classes of dermatophytic infections, *Tinea capitis* appears to be predominant among school children in Nigeria (Enweani *et al.,* 2009). The dermatophytic fungi spores are transmitted by either direct contact with infected host (human or animal) or indirect contact with fungi spores infecting exfoliated skin or hair in combs, hair brushes, clothing, furniture, theatre seats, caps, bed linens, towels, hotel rugs and locker room floors (Fuller *et al., 2001).*

Depending on the species, the infective fungi spores may be viable in the environment for up to 15 months. There is an increased susceptibility to infection when there is a pre existing injury to the skin such as scars, burns, excessive temperature and humidity (Fuller *et al., 2003)*

Dermatophytes cause a variety of clinical conditions such as dermatophytosis. From the site of infection the fungal hyphae grow centrifugally into the stratum corneum. The fungus continues downward growth into the hair invading keratin as it is formed. The zone of involvement extends upward at the rate at which the hair grows and it has been reported to be visible above the skin surface by 12-14 days of infection, and Infected hair, by the third week, it breaks off. (Fuller *et al*., 2001)

Dermatophytes are classified as anthropophilic, zoophilic or geophilic according to their normal habitat.

- Anthrophilic dermatophytes are restricted to human hosts and produce

  a mild, or chronic inflammation.

- Zoophilic organisms are found in animals and cause marked inflammatory reactions in humans who have contact with infected cats, dogs, cattle, horses, birds, or other animals.
- Geophilic organisms are usually contracted from the soil. They cause a marked inflammatory reaction, which spreads in a limited manner, and may disappear spontaneously, leaving scars.

Tinea capitis (scalp ringworm) is a highly contagious infection of the scalp and hair caused by dermatophytic fungi such as *Microsporum canis*, *Microsporum audounii* and *Trichophyton* species. It occurs in all age group, but predominantly children. It is endemic in some of the poorest and developing countries (Gonzalez *et al.,* 2007.)

Tinea capitis is the most common pediatric dermatophyte infection worldwide. It affects mostly children of primary school age. The increased incidence of *Tinea* among pre pubertal children has been attributed to reduced fungistatic properties of the child's sebum. (Gonzalez *et al.,* 2007).

A physiologic basis for the common occurrence of *Tinea capitis*, primarily in children has been debated for many years. It has been suggested that these fungi grow in the hair of children, because of the predominance of even-numbered specific fatty acids in the sebum surrounding the hair of children, as opposed to the odd- numbered fatty acids surrounding the hair of adults. Gonzalez *et al*., (2007) stated that the odd-numbered fatty acids are more effective in preventing the growth of

fungi. The increase in the production of saturated fatty acids with fungistatic activity in the sebum by the action of androgen hormones could be responsible for the spontaneous cure of *Tinea capitis* which occur right after puberty (Gonzalez *et al.,* 2007).

Tinea capitis, therefore, primarily is a disease of children (Williams 2005; Gupta and Summerbell 2000) It is a public health problem in some countries, because of increased incidence and epidemic transmission. Incidence of *Tinea capitis* may vary by sex, depending on the causative fungal organism. In *Microsporum audouinii*–related tinea capitis, boys are affected much more commonly (Gonzalez *et al*., 2007). The infection rate has been reported to be up to 5 times higher in boys than in girls probably because short hairs help implantation of spores .(Gonzales *et al.,* 2007). However, the reverse is true after puberty, possibly as a result of increased exposure to infected children by women and to hormonal factors. In infection by *M canis*, the ratio varies, and the infection rate usually is higher in male children. Girls and boys are affected equally by *Trichophyton* infections of the scalp, but in adults, women are infected more frequently than are men. *Tinea capitis* occurs primarily in children and occasionally in other age groups. It is seen most commonly in children younger than 10 years. Peak age range is in patients aged 3-7 years.

In adults, it affects mostly women (Bronson *et al.,* 1993) and the site of infection is usually the occipit. There is usually a trigger factor such as diabetes mellitus, pulmonary tuberculosis, immunodefficiency, malnutrition, drugs or other factors that causes immunossupression. It is not infrequent in transplanted patients or in those with systemic lupus erythematosus (Barlow and Saxe, 1998).

The epidemiology of *Tinea capitis* in the United Kingdom has recently changed dramatically (Bronson *et al.,* 1993). In the United Kingdom it is becoming a major public health problem, and

particularly among afro-caribbean children who are particularly affected by *Microsporum canis.* (Fuller *et al.,* 2003). *Trichophyton tonsurans* causes 90% of cases in the United Kingdom and in United States (Higgins *et al.,* 2000). *Trichophyton tonsurans* is an Anthropophilic fungus, which spreads from person to person. The reason for this is unclear, but hairdressing practices such as shaving the scalp, plating and using hair oils may increase the spread (Higgins *et al.,* 2000).

This variation in the epidemiology of *Tinea captis* reflects people's habits, standard of hygiene, climatic conditions and levels of education. Interestingly, increased education may increase the number of patients seeking medical attention for their scalp lesions, which in turn increase the diagnosed level of *Tinea capitis* in a given area.

The frequency of *tinea capitis* compared to other types of dermatophytosis varies from one location to another. *Tinea capitis* is considered the most frequent cause of dermatophytosis in the Islamic Republic of Iran and Jordan (Khosravi *et al*., 1994) and the second most frequent form of dermatophytosis in Mosul (Iraq) after *Tinea corporsis*. In contrast, there has been a marked decline in the incidence of *Tinea capitis* in Mexico City, down from 31.0% of all cases of dermatophytosis between 1940 and 1950 to 1.6% between 1986 and 1992 (Gayosso and Mendez, 1994).

Tinea capitis is widespread in some urban areas in North America, Central America, and South America. It is common in some parts of Africa and India. In Southeast Asia, the rate of dermatophytic infection has been reported to decrease dramatically from 14% (average of male and female children) to 1.2% in the last 50 years because of improved general sanitary conditions and personal hygiene while in Northern Europe, the disease is sporadic (Gupta *et al.*, 1999).

Kao (2008) found out that in the State of Amazon Country, among 115 cases of *Tinea capitis*, 91.7% was caused by *T. tonsurans*, out of these 13.9% were adults and of which 52.2% of these

were women. In Rio de Janeiro, Brazil, some cases of *Tinea capitis* in adults due to *M. canis* and *T. tonsurans* have been reported. Gupta *et al*., (1999), reported that *Microsporum audouinii* was the classic causative agent in Europe and America while *Microsporum ferrugineum* was most common in Asia. *Microsporum audouinii* and *M canis* remain prevalent in most parts of Europe, although *T. violaceum* is common in Romania, Italy, Portugal, Spain, and the former USSR, as well as in Yugoslavia. (Gupta *et al*., 1999). In Africa, *T. violaceum*, *T. schoenleinii*, and *M canis* commonly are isolated. (Gupta *et al*., 1999). *T. violaceum* and *M canis* are prevalent agents in Asia. *Trichophyton schoenleinii* is common in Iran and Turkey, while *M canis* is common in Israel. *Epidermophyton floccosum* and *T. concentricum* do not invade scalp hair. *Trichophyton rubrum*, which is the most common dermatophyte isolated world wide, is a common cause of *Tinea capitis*.

The geographical distribution and prevalence of dermatophytes are not static, but change under the influence of various forces such as climate, migration of people and developments in prophylaxis and therapy (Gupta *et al*., 1999).

Trichophyton rubrum is now the major cause of *Tinea capitis* in the USA and in United Kingdom. (Kao 2008). But until some years ago, it was *Microsporum canis* and *Microsporum audouinii*. These fungi have been reported to be the major causes of *Tinea capitis* infection in Chicago over the past 20 years (Bronson *et al.*, 1993).

In South Africa, *T. violaceum* was found to be the causative agents of Tinea capitis (Barlow and Saxe, 1998). *Tinea capitis* continues to be a common problem of infancy and childhood, particularly among those living in unhygienic and over crowded conditions

In Nigeria, the incidence of the dermatophytes causing *Tinea capitis* varies greatly in Ile-Ife. *M. audouinii* was found to be the major causative organism (Ajao and Akintunde, 1985). Ayanbimpe *et*

al. (2008) in a related study in Jos Plateau state and in Gboko Benue state discovered that the aetiological organism of *Tinea capitis* is *Trichophyton soudanese* 76 (30.6%), followed by M*icrosporum ferrugineum* 19 (7.7%) and M*icrosporum audouinii* 19(7.7%). .

The prevalence and aetiology of *Tinea capitis* in Anambra state of Nigeria showed that out of 47,723 primary school children residing in different regions of the State screened for *Tinea capitis* between 2002 and 2005. 4498 (9.4%) had *Tinea capitis*. The highest prevalence of the disease occurred in the Southern region of Anambara state (12.6%). Schools in urban areas recorded lower prevalence of the disease. Male: female prevalence ratio of 1:1: 6 was recorded. *Tinea capitis* occurred significantly more in children below 10 years of age). Of 502 isolates recovered in culture *Microsporum audouinii* was the most prevalent (42%), followed by *M. ferrugineum* (17%) and *Trichophyton mentagrophytes* (16%). The reported results showed that *Tinea capitis* remains an important public health problem in Anambra State of Nigeria (Barlow and Saxe, 1998). In a related study, Mbakwem Aniebo and Ezekwem (2010) found the etiology agent for Tinea capitis in Mgbuoba were *Microsporum canis* (20%), *Microsporum gypseum* (20%), *Trichophyton soudanese* (13.3%), *Trichophyton mentagrophytes* (6.67%), and *Trichophyton tonsurans* (6.67%). Where as those identified in Akpulu were *Microsporum audounii* (35%), *Trichophyton tonsurans* (30%), *Trichophyton mentagrophytes* (10%), *Trichophyton schoenleinii* (10%), *Microsporum canis* (10%), and *Microsporum gypseum* (5%).

Also out of 1400 pupils from two public primary schools in Ekpoma, Edo State, Nigeria, who were screened for dermatophyte infection, 188 (13.4%) were infected. The causative agents isolated included *Microsporum audouinii* in 88 (46.8%), *Trichophyton mentagrophytes* in 48 (25.5%), *T. rubrum* in 40 (21.3%), *T. tonsurans* in four (2.1%) and *Epidermophyton floccosum* in 8 (4.3%).

There were significant differences in the rate of infection between male and female school children as well as between children from different socio-economic backgrounds. (Enweani *et al*., 2009).

A total of 2184 primary school children were screened for superficial mycoses in Anyigba, town in Kogi State, Nigeria. Of the 2184 pupils sampled randomly from four schools, 144 (6.6%) had lesions suggestive of superficial mycoses. In a total of 155 samples collected, 108 (69.67%) yielded significant growth by culture. The distribution of superficial mycoses is dependent on age and sex. Eight species of fungi belonging to two genera were isolated, including: *Microsporum gypseum* (13.5%). *M canis* (12.4%), *M. ferrugineum* (3.4%), *Trichophyton rubrum* (30.3%), *T. tonsurans* (12.4%), *T. soudanense* (5.6%), *T. verrucosum* (11.2%) and *T. schoenleinii* (11.2%). Poor infrastructure (residential house and classrooms), contact with soil during outdoor activities (especially in children), intimate association with pet animals and poor personal hygiene may contribute to the spread of these infections among children. (Enemuor, and Amedu. 2009). Most dermatophytes are world wide in distribution, but some species show a higher incidence in certain regions than in others (e.g *Trichophyton schoenleinii* in the meditereanian, *Trichophyton rubrum* in tropical climates).

1.1 STATEMENT OF PROBLEM

Several antifungal agents such as griseofulvin, terbinafine and azole compounds were reported well effective when used in the treatment of *Tinea capitis* (Micol, 2007). However there has been emerging reports of toxicity associated with the use of these drugs. (Micol, 2007). Also the increase in dermatophytosis and the high level of therapeutic failure warrants the search for new therapeutic strategies (Tasunmi *et al*., 2002)

The high cost makes the antifungal drug unaffordable. There have also been some reports of fungi resistance due to inability of the patient to procure the appropriate drug due to high cost. (Micol, 2007). In many developing nations, a lack of drug access means patients are forced to use substandard or truncated therapies that invariably lead to a more rapid development of resistant microorganisms. In such conditions, patients tend to exhibit prolonged illness which results in the increased likelihood of developing and spreading resistant strains. In addition to this is the issue of counterfeit drugs. Many of these drugs provide minimal quantities of antifungal agents, so instead of wiping out the infection altogether, the treatment kills only the weaker, non resistant organisms, leaving the resistant ones to develop, replicate, and spread resistance genes. There is also the antifungal resistance that is clearly linked to over-the-counter (OTC) use of antifungal drugs. (Hudson, 2001).

1.2 JUSTIFICATION OF THE STUDY

Tinea capitis is thought to be the world's most common Dermatophytosis (Cohen *et al.,* 2005). *Tinea capitis* usually responds to antifungal agents, such as Terbinafine, Econazole, Ketoconazole, cicloprox, Griseofulvin, Itraconazole, and Fluconazole. However fungi resistance to the Azole compounds has been reported with *Trichophyton* species (Micol, 2007). Some of the resistant cases were attributed to patients with immune suppressed conditions, such as diabetics (Kao, 2008). Hence the need to study the prevalence, susceptibility and resistance patterns of the test pathogenic dermatophytic fungal spores isolated from primary school pupils in Zaria, Nigeria.

The anti- dermatophytic susceptibility testing will evaluate the sensitivity profile of the test fungal spores isolated from volunteers among primary schools in Zaria, Kaduna State, Nigeria.

1.3 GENERAL AIMS AND OBJECTIVES.

To determine the prevalence and antifungal susceptibility profile of Dermatophytic fungal spores associated with *Tinea capitis* among the primary school pupils in Zaria Kaduna state Nigeria.

1.3.1 SPECIFIC RESEARCH OBJECTIVES

1 To isolate dermatophytic fungi spores associated with *Tinea capitis* among the target volunteers in Zaria Nigeria.

2 To determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of test antifungal agents against different species of the isolated fungal spores.

3 To determine the combined MICs and calculate the Fractional Inhibitory Concentration (FIC) of the combined test antifungal agents.

4 To determine the survival of the most resistant fungi spores using biocidal concentrations of test antifungal agents.

5 To determine the effect of Temperature and pH on the antifungal activities of potential test antifungal agents

6 To determine the antifungal activities of the potential combined test antifungal agents.

CHAPTER TWO

2.0 **LITERATURE REVIEW**

2.1 DERMATOPHYTES

Dermatophytes are fungi that can cause infections of the skin, hair and nails due to their ability to utilize keratin. The organisms colonize the keratin tissues and inflammation is caused by host response to metabolic bye-products. These infections are known as Tinea, in association with the infected body part. Occasionally, the organisms do invade the subcutaneous tissues, resulting in kerion development.

2.1.1 MORPHOLOGY AND IDENTIFICATION OF DERMATOPHYTES

Dermatophytes can be classified into three groups; namely *Epidermophyton, Microsporum* and *Trichophyton*. In keratinized tissue, dermatophytes form only hyphae and arthrospores. Dermatophytes are worldwide in distribution, with some species displaying a higher incidence in certain regions than in others, for example, *Trichophyton schoenleinii* in the mediterranian, and *Trichophyton rubrum* in tropical climates.

Representative colonies form on Sabouraud dextrose agar at room temperature. Conidia formation may be observed by means of slide cultures. Sabouraud dextrose medium is the most suitable medium for the isolation of dermatophytes with the addition of cycloheximide, which inhibits non-pathogenic environmental fungi contaminants. .

2.1.2 TRICHOPHYTON

Trichophyton is a dermatophyte which thrives in the soil, humans or animals. Based on its natural habitats, the genus includes anthropophilic, zoophilic and geophilic species. Some species are

cosmopolitan, others have a restricted geographical distribution. *Trichophyton concentricum*, for example, is endemic to the Pacific Islands, Southern Asia, and Central America. Trichophyton is one of the leading causes of hair, skin and nail infections in humans.

The genus Trichophyton is divided into several species. Most common being *Trichohyton mentagrophytes, Trichophyton rubrum, Trichophyton schoenlenii*.

T. rubrum is the commonest causative agent of dermatophytoses world wide. *Trichophyton* species may cause invasive infections in immunocompromised hosts. The growth rate of T*richophyton* colonies is slow to moderately rapid. The texture is waxy, glabrous to cottony. From the above, the colour is white to bright yellowish or red violet. The reverse or below is pale, yellowish brown or reddish brown (Dehoog *et al.,* 2000). *Trichophyton* has septate hyaline hyphae, conidiophores, microconidia, macroconidia and athroconidia. Chlamydospores may also be produced. Conidiophores are poorly differentiated from the hyphae. Microconidia (also known as the microaleuriconidia) are one –celled and round or pyriform in shape. They are numerous and can be solitary or arranged in clusters. Microconidia are often the predominant type of conidia produced by *Trichophyton*. Macroconidia (also known as the macroaleuriconidia) are multicellular (2 or more celled), smooth, thin or thick-walled and cylindrical, clavate or cigar shaped. They are usually not formed or produced in very few numbers. Some species may be sterile and the use of specific media is required to induce sporulation (Dehoog *et al., 2000).* Trichophyton differs from microsporum and epidermophyton by having cylindrical, clavate to cigar shaped, thin walled or thick walled smooth macroconidia.

Plate 2.1 Front view of the cultural Morphology of T*richophyton mentagrophytes.*

(Ellis and Hermanis, 2003)

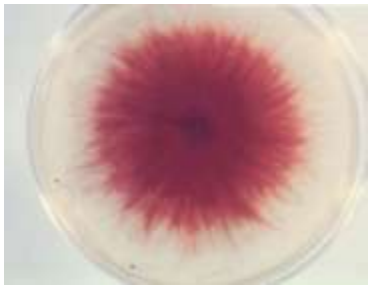


Plate 2.2. Reverse view of cultural morphology of T*richophyton mentagrophytes*

(Ellis and Hermanis, 2003).

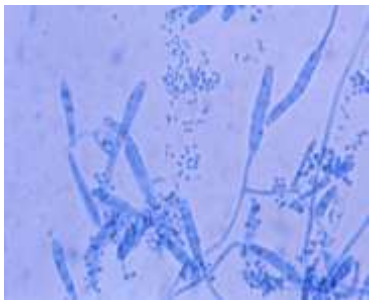


Plate 2.3 Microscopic morphology of T*richophyton mentagrophytes.*

(Ellis and Hermanis, 2003).

2.1.3 MICROSPORUM

Microsporum is a filamentous keratiophilic fungus included in the group of dermatophytes. The natural habitat of some of the *Microsporum* species is the soil (the geophilic species), others primarily affect various animals (the zoophilic species) or human (the anthropophilic species). Some species are isolated from both soil and animals (geophilic and zoophilic). Most of the *Microsporum* spp. are widely distributed in the world while some have restricted geographic distribution. *Microsporum* has the asexual state, that is the telemorph phase which is usually referred to as arthroderma (De hoog *et al*., 2000)

The genus *Microsporum* includes 17 conventional species. Among these, the most significant are: *M. canis, M. audouini, M. nanum, M. gypseum, M. distortum, M. ferrugineum, M. gallinae.Microsporum* colonies are glabrous, downy, wooly or powdery. The growth on sabourand dextrose agar at 25^0 C may be slow or rapid and the diameter of the colony varies between 1 to 9 cm after 7 days of incubation. The colour of the colony varies depending on the species. It may be white or yellow to cinnamon. From the reverse, it may be yellow to red-brown (Germain and Summerbell 1999). *Microsporum* macroaleurioconidia are hypae-like. Microaleurioconidia are unicellular, solitary, oval to clavate in shape, smooth, hyaline and thin-walled. Macroaleuriconidia are hyaline, echinulate to roughened, thin- to thick-walled, typically fusiform (spindle in shape) and multicellular (2-15 cells). They often have an annular trill. Inoculation on specific media, such as Potato dextrose agar or Sabouraud dextrose agar supplemented with 3 to 5% sodium chloride may be required to stimulate macroconidia production of some strains. *Microsporum* differs from *Trichophyton* and *Epidermopyhyton* by having spindle-shaped macroconidia with echinulate to rough walls (Germain and Summerbell, 1999).

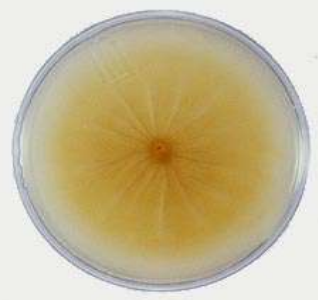


Plate 2.4 Front view of the Cultural morphology of *Microsporum canis.* (Ellis and Hermanis, 2003).

Plate 2.5. Reverse view of cultural morphology of *Microsporum canis* (Ellis and Hermanis, 2003)

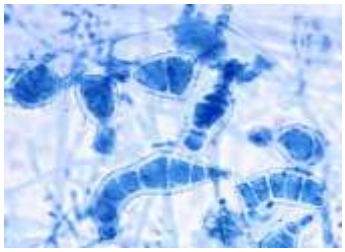


Plate 2.6 Microscopic morphology of *M. canis.* (Ellis and Hermanis, 2003)

2.1.4 EPIDERMOPHYTON

Epidermophyton is a filamentous fungus. It is distributed worldwide. Man is the primary host of *Epidermophyton floccosum*, the only species which is pathogenic. The natural habitat of the related but non pathogenic species *Epidermophyton stockdales* is the soil (Dehoog *et al*., 2000).

The genus *Epidermophyton* contains two species; *Epidermophyton floccosum* and *Epidermophyto n stockdales,* and it is known to be non pathogenic, leaving *E. floccosum* as the only species causing

infections in humans. The colonies of *E. floccosum* grow moderately rapidly and mature within 10 days. Following incubation at 25^0 C on potato dextrose agar, the colonies are brownish yellow to olive gray or khaki from the front. From the reverse, they are orange to brown with an occasional yellow border. The texture is flat and grainy initially and become radially grooved and velvety by aging. The colonies quickly become downy and sterile (Dehoog *et al*, 2000; Germain and Summerbell, 1998). Septate hyaline, macroconidia and occasionally, chlamydoconidium-like cells are seen. Microconidia are typically absent. Macroconidia (10-40 X 6-12µm) are thin-walled, 3 to 5-celled, smooth, and clavate-shaped with rounded end. They are found singly or in clusters. Chlamydoconidium-like cells, as well as arthroconidia, are common in older cultures (Dehoog *et al*., 2000; Germain and Summerbell 1998). *Epidermophyton floccosum* is differentiated from *Microsporumm* and *Trichophyton* by the absence of microconidia.

Plate 2. 7. Front view of the cultural morphology of *Epidermophyton flocossum* (Ellis and Hermanis, 2003).

Plate 2.8 Reverse view of cultural morphology of *Epidermophyton flocossum* (Ellis and Hermanis, 2003)

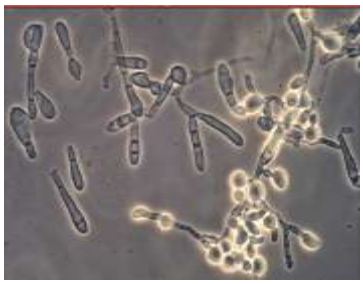


Plate 2.9 Microscopy of *E. floccosum* (Ellis and Hermanis, 2003)

Table 2.1.The characteristics of some commonly isolated Dermatophytes

DERMATOPHYTES	COLONIAL MORPHOLOGY	INCUBATION DURATION.	MICROSCOPIC IDENTIFICATION
M. canis	Colony is usually membranous with feathery periphery; centre of colony is white to butt over orange-yellow or lemon yellow.The reverse is tanned.	1 week	Thick walled, spindle shaped, multiseptate, rough walled, macroconidia some with macroconidia rarely seen.
Microsporum gypseum	Cinnamon coloured, powdery, colony.The reverse is light tan.	1 week	Thick-walled, rough, elliptical, multisaptate, macroconidia, microconidia few or absent.
Epidermophyton floccosum	Centre of colony tends to be folded and is khaki green, periphery is yellow; reverse is yellowish brown with folds.	1 week	Macroconidia large, smooth-walled multiseptate, clavate and borne singly or in cluster of two or three microconidia not formed
Trichophyton mentagrophytes	Different colonial types; white or pinkish, granular and fluffy varieties; occasional light yellow periphery in	7-10 days	Many round to globose microconidia most commonly borne in grapelike cluster or laterally along the hyphae; spiral hyphae in 30% of isolates, macroconidia are

	younger cultures, reverse buff to reddish brown.		thin-walled, smooth, club-shaped, and multiseptate, numerous or are depending upon strain.
Trichophyton rubrum	Colonial types vary from white dowry to pink granular, rugal folds are common, reverse yellow when colony is young however, wine red colour commonly develop with age.	2 weeks	Microconidia usually teardrop, most commonly borne along sides of the hyphae, macroconidia usually absent, but when present are smooth thin-walled and pencil-shaped.
Trichophyton tonsurans	White, tan to yellow or rust, suedelike to powdery; wrinkled with heaped or sunken centre; reverse yellow to tan to rust red.	7-14 days.	Micrococonidia are teardrop or club shaped with flat bottoms; vary in size but usually larger than other dermatophyte; microcodondia rare and ballon forms found when present.
Trichophyton schoonleinii	Irregularly heaped, smooth creamy white colony with radiating grooves, reverse white.	2-3 weeks	Hyphae usually sterile, many anther-type hyphae seen (favic chandeliers).
Trichophyton violaceum	Pot wine in deep violet colony, may be heaped or flat with waxy-glabrous-surface; pigment may be lost on subculture.	2-3 weeks	Branched, tortuous hyphae that are sterile; chlmydospores commonly alighned in chains.
Trichophyton verrucosum	Glabrous to velvety white colonies; rare stains produce yellow-brown colour; rugal folds with tendency to sink into agar surface.	2-3 weeks	Microconidia rare; large and tear-drop when seen; macroconidia extremely rare, but forms characteristic retail types when seen; many chlamydospores seen in chains, particularly when colony is incubated at 37^0 C.

(Summerbell, *and* Germain, 1999*)*

2.2 TYPES OF *TINEA CAPITIS*

Tinea capitis is classified according to how the fungus invades the hair shaft.

2.2.1 ectothrix infection. This is a fungus that grows inside the hair shaft, but produces a conspicuous external sheath of spores. Ectothrix hair infection reported causative agents are *M. canis, M. audouinii, M. distortum, M. ferrugineum, M. gypseum, M. nanum*, and *T. verrucosum*. This infections can be identified by Woods light (long wavelength ultraviolet light) examination of the affected area. The hair will flourescence green if infected with *M. canis* (Kao, 2008).

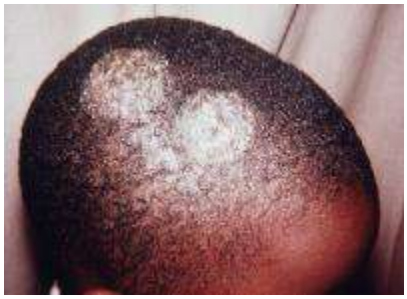


plate 2.10. Ectothrix hair infection.

(Kao, 2008).

2.2.2 endothrix infection. The growth and spore production are confined chiefly within the hair shaft. Endothrix hair invasion usually results from infection caused by *T. tonsurans, T. violaceum and T. soudanense*. The hair shaft is filled with fungal hyphae and spores

2.2.3 favus. This is a disease usually affecting the scalp but occurring occasionally on any part of the skin, and even at times on mucous membrane. Favus is caused by *T. schoenleinii* infection, which results in destruction of the hair shaft (Kao, 2008).

2.3 ANTIFUNGAL AGENT

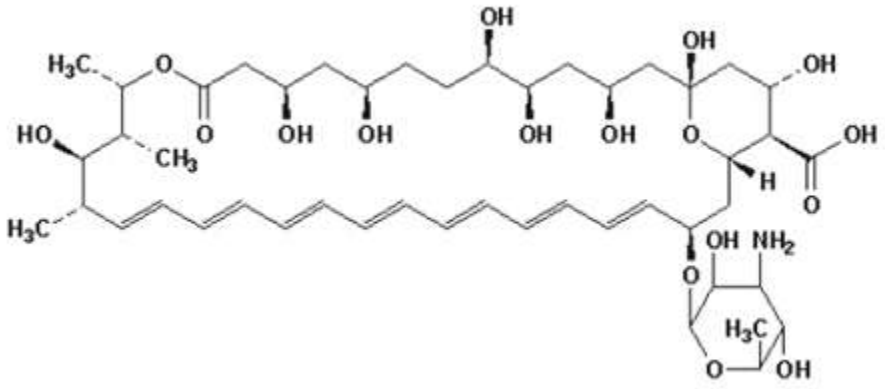
An antifungal agent is a compound that inhibit or kill fungal pathogens from a host with minimal or without toxicity to the host. The development of antifungal agents has lagged behind that of antibacterial agents. This is a predictable consequence of the cellular structure of the organisms involved. Bacteria are prokaryotic and hence offer numerous structural and metabolic targets that differ from those of the human host. Fungi, in contrast, are eukaryotes, and consequently most agents toxic to fungi are also toxic to the host. Fungi generally grow slowly and often in multicellular forms so they are more difficult to quantify than bacteria. This difficulty complicates experiments designed to evaluate the in vitro or in vivo properties of a potential antifungal agent. There are different classes of antifungal agents and they include the following:

2.3.1 Polyene Antifungal Drugs

The polyene compounds are so named because of the alternating conjugated double bonds that constitute a part of their macrolide ring structure. The polyene antifungal agents are all products of streptomyces species. These antifungal agents interact with sterols in cell membranes (ergosterol in fungal cells; cholesterol in human cells) to form channels through the membrane, causing the cells to become leaky. The polyene antifungal agents include nystatin, amphotericin B, and pimaricin.

Amphotericin B is a polyene antifungal agent, first isolated from *streptomyces nodosus* in 1955. It is an amphoteric compound composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic hydrocarbon chain on the other. Amphotericin B is poorly soluble in water (Baginski and Czub, 2009).

2.3.1.1 amphotericin B



Amphotericin B

a. Mechanism of action.

From the 1950s until the discovery of the azoles, polyene antifungal agents such as amphotericin B represented the standard of therapy for systemic fungal infections. There is an association between polyene susceptibility and the presence of sterols in the plasma membrane of the cells. All organisms susceptible to polyenes, e.g., yeasts, algae, and protozoa, contain sterols in their outer membrane, while resistant organisms do not.The importance of membrane sterols for polyene activity is also supported by earlier studies, where it was shown that fungi can be protected from the inhibitory action of certain polyenes by the addition of sterol to the growth medium. It was suggested that this effect is due to a physicochemical interaction between added sterols and the polyenes, which prevents the drug from binding with the cellular sterols. The interaction between the sterols and polyenes is further supported by direct spectrophotometric evidence that adding sterols to aqueous solutions of the polyene filipin or nystatin decreases the UV absorbance significantly, suggesting a direct interaction between the added sterol and the antifungal agent.

For larger polyenes, such as amphotericin B, it has been proposed that the interaction of the antifungal with membrane sterol resulting in the production of aqueous pores consisting of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane sterols. This configuration gave rise to a pore in which the polyene hydroxyl residues face inward, leading to altered permeability, leakage of vital cytoplasmic components, and death of the organism. The fatty acyl composition of the phospholipids has also been implicated in polyene susceptibility of yeast. In addition, killing of *C. albicans* has been attributed to oxidative damage caused by polyenes.

Amphotericin B binds to sterols, preferentially to the primary fungal cell membrane sterol, ergosterol. This binding disrupts osmotic integrity of the fungal membrane, resulting in leakage of intracellular potassium, magnesium, sugars, and metabolites leading to cellular death (Jill and Richard 2002).

b Spectrum of antifungal activities

Amphotericin B has a very broad range of activity and is active against most pathogenic fungi e.g *Coccidiodes immitis, Histoplasm capsulatum, Blastomyces dermatitidis and Paracoccidioides brasiliensis*. Notable exceptions include *Trichosporon beigelii*, *Aspergillus tereus* (Sutton *et al.*, 1990), *Pseudallescheria boydii Malassezia furfur*, and *Fusarium* spp. Among the *Candida spp*. Isolates of *C. albicans, C. guilliermondii, C. lipilitica, C. lusitaniae, C. norvegensis, C. tropicalis, C. glabrata, and C. krusei* have been reported to be relatively resistant to amphotericin B. Reduced susceptibility has been observed specifically at fungicidal levels for *C. parapsilosis* (Michallet *et al.*, 2009).

c Adverse side effects

The most commonly observed infusion-related side effects of amphotericin B are fever, chills, and myalgia. These can be partially overcome by premedication with diphenhydramine and/or acetaminophen .

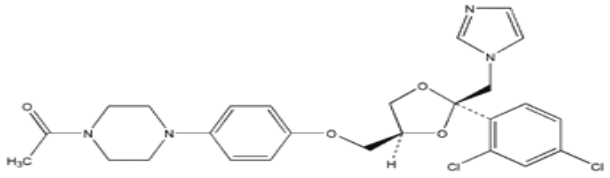
Nephrotoxicity is the major adverse effect limiting the use of amphotericin B. the manifestations of nephrotoxicity are azotemia, decreased glomerular filtration, loss of urinary concentrating ability, renal loss of sodium and potassium, and renal tubular acidosis . The renal injury reduces erythropoietin production and leads to a normochromic and normocytic anemia.Thrombophlebitis may occur at the site of infusion.Thrombocylopenia may rarely be observed (Laniado, 2009).

2.4. Azole Antifungal Drugs

The azole antifungal agents have five-membered organic rings that contain either two or three nitrogen molecules (the imidazoles and the triazoles respectively. The clinically useful imidazoles are clotrimazole, miconazole, and ketoconazole. Two important triazoles are itraconazole and fluconazole. The azoles inhibit fungal cytochrome P450 14α -demethylase which is responsible for the conversion of lanosterol to ergosterol. This leads to the depletion of ergosterol in the fungal cell membrane. The in-vitro antifungal activity of the azoles varies with each compound, and the clinical efficacy of each compound may not coincide exactly with in-vitro activity. The azoles are primarily active against *C. albicans, C. neoformans, C. immitis, H. capsulatum, B. dermatitidis, P. brasiliensis, C. glabrata, Aspergillus spp*. and *Fusarium spp*. but zygomycetes are resistant to currently available azoles. (Benetth *et al*., 2008).

2.4.1 ketoconazole

Ketoconazole is an imidazole antifungal agent. It has five-membered ring structures containing two nitrogen atoms. Ketoconazole member of the imidazole class, has been reportedly used for the treatment of systemic infections. Ketoconazole is a highly lipophilc compound. This property leads to high accumulation of ketoconazole in fatty tissues and purulent exudates. The distribution of ketoconazole into cerebrospinal fluid is poor in the presence of inflammation. Its oral absorption and solubility is optimal at acidic gastric pH. (Lewis *et al*., 2007).



ketoconazole

a. Mechanism of action

As with all azole antifungal agents, ketoconazole works principally by inhibition of cytochrome P450 14α-demethylase (P45014DM). This enzyme is in the sterol biosynthesis pathway that catalyses the conversion of lanosterol to ergosterol ((Lewis *et al.*, 2007). The affinity of ketoconazole for fungal cell membranes is lower compared to that of fluconazole and itraconazole. Ketoconazole has been reported to affect mammalian cell membranes (Ghannoum and Rice, 1999).

b. Spectrum of activities

Ketoconazole is active against *Candida spp* and *Cryptococcus neoformans*. However, its activity is limited compared to that of fluconazole and furthermore, due to its limited penetration ability to cerebrospinal fluid, it is clinically ineffective in meningeal cryptococcosis. Its activity against the dimorphic moulds, *Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides immitis,*

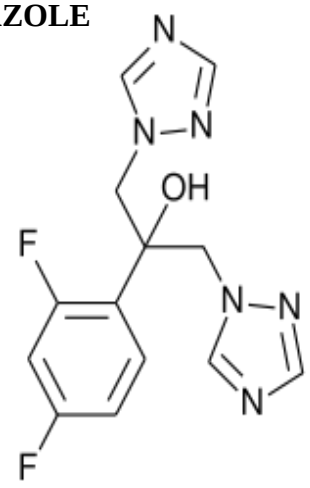
Sporothrix schenckii, Paracoccidioides brasilliensis, and Penicillium marneffei is favourable. However, fluconazole and itraconazole are at least as effective as ketoconazole against these fungi and are safer. Thus, ketoconazole remains as an alternative second-line drug for treatment of infections due to dimorphic fungi. Ketoconazole is not recommended for treatment of meningeal infections due to its limited penetration to cerebrospinal fluid (Kenneth *et al*., 2007).

Ketoconazole is also active against *Pseudallescheria boydii* and is a good alternative for treatment of pseudallescheriasis. It is also effective in *Pityriasis versicolor*.Ketoconazole has practically no activity against *Aspergillus spp. Fusarium spp*. and zygomycetes order of fungi. (Kenneth *et al*., 2007).

c Adverse side effects

The major drawbacks of ketoconazole therapy are from the occasionally seen adverse reactions. It may induce anorexia, nausea and vomiting. Increase in transaminase levels and hepatoxicity may occur. Ketoconazole may decrease testosterone and cortisol levels, resulting in gynecomastia and oligospermia in men and menstrual irregularities in women (Lewis *et al*., 2007).

2.4.2 FLUCONAZOLE



Fluconazole (2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-olis 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. (It is commonly marketed under the trade name Diflucan or Trican Pfizer). The molecular formula is $C_{13}H_{12}F_2N_6O$. The molecular mass is 306.271g/mol. (Ghannoum and Rice 1999).

a Chemical pathway of interaction.

Consistent with expectations based on human in vitro microsomal experiments, administration of fluconazole (400 mg/day) for 6 days to six human volunteers significantly reduced the cytochrome P450 (P450)-dependent metabolic clearance of the warfarin enantiomers. In particular, P4502C9 catalyzed 6- and 7-hydroxylation of (S)-warfarin, the pathway primarily responsible for termination of warfarin's anticoagulant effect, was inhibited by approximately 70%. The change in (S)-warfarin pharmacokinetics caused by fluconazole dramatically increased the magnitude and duration of warfarin's hypoprothrombinemic effect. These observations indicate that co-administration of fluconazole and warfarin will result in a clinically significant metabolically based interaction. The major P450-dependent, in vivo pathways of (R)-warfarin clearance were also strongly inhibited by fluconazole. Ten -Hydroxylation, a metabolic pathway catalyzed exclusively by P4503A4, was inhibited by 45% whereas 6-, 7-, and 8-hydroxylations were inhibited by 61, 73, and 88%, respectively. The potent inhibition of the phenolic metabolites suggests that enzymes other than P4501A2 (weakly inhibited by fluconazole in vitro) are primarily responsible for the formation of these metabolites in vivo as predicted from in vitro kinetic studies. These data suggest that fluconazole can be expected to interact with any drug whose clearance is dominated by P450s 2C9, 3A4, and other as yet undefined iso forms. Overall, the results strongly support the hypothesis that

metabolically based in vivo drug interactions may be predicted from human in vitro microsomal data (Bavisotto *et al., 2011*).

b. Mechanism of action of fluconazole

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. (Bavisotto *et al., 2011*).

Fluconazole interacts with 14-α demethylase, a cytochrome P-450 enzyme necessary to convert lanosterol to ergosterol. As ergosterol is an essential component of the fungal cell membrane, inhibition of its synthesis results in increased cellular permeability causing leakage of vital cellular contents. Fluconazole may also inhibit endogenous respiration, interact with membrane phospholipids, inhibit the transformation of yeasts to mycelial forms, inhibit purine uptake, and impair triglyceride and/or phospholipid biosynthesis (Pfaller *et al*., 2004.)

c. Spectrum of activity.

Fluconazole is generally considered a fungistatic agent. It is principally active against *Candida spp* and *Cryptococcus spp*. however, *Candida krusei* is intrinsically resistant to fluconazole. In addition, isolates of *Candida glabrata* has been reported to display high MICs. Acquired resistance to fluconazole among *Candida albicans* strains has been reported particularly in HIV-infected patients (Pfaller *et al*., 1999 and Pfaller *et al*., 2004)

Fluconazole has useful activity against *Coccidioides immitis* and is often used to suppress the meningitis produced by the fungus. It has limited activity against *Histoplasma capsulatum*.

Blastomyces spp, and *Sporothrix schenckii*, and is sometimes used a second-line agent in these diseases. Fluconazole has no activity against *Aspergillus* spp. or most other mould fungi. Fluconazole is generally quite well tolerated. In common with all azole antifungal agents, fluconazole may cause hepatotoxicity (Pfaller *et al*., 1999 and Pfaller *et al*., 2004).

Fluconazole is of the first-line antifungal agents, particularly in treatment of infections due to *Candida spp*. Other than *Candida krusei* and some *Candida glabrata* isolates. Fluconazole is commonly used also for prophylaxis in patient undergoing transplant (Pfaller *et al*., 2004 and Pfaller *et al*., 1999).

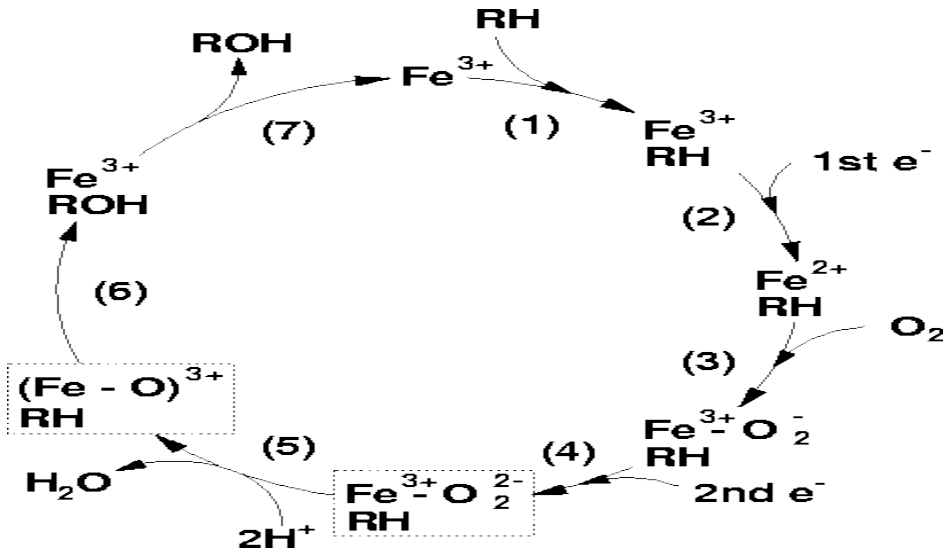
d The p450 catalytic cycle.

The principle physiological rôle of the P450 superfamily of enzymes is that of a monoxygenase. The catalytic reaction can be summarised

$$RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O.$$

Where RH can be one of a large number of possible substrates.

The catalytic cycle, is summarised below.



(Segall, 1997).

Figure 2.4.The catalytic cycle of cytochrome P450. The intermediate states enclosed in a dashed box have not been directly observed and are hypothetical.

1.Substrate binding

The binding of a substrate to a P450 causes a lowering of the redox potential by approximately 100mV which makes the transfer of an electron favourable from its redox partner, NADH or NADPH. This is accompanied by a change in the spin state of the haem iron at the active site. It has also been suggested that the binding of the substrate brings about a conformational change in the enzyme which triggers an interaction with the redox component

1. The first reduction

The next stage in the cycle is the reduction of the Fe^{3+}ion by an electron transfered from NAD (P) H via an electron transfer chain.

2. Oxygen binding

An O_2 molecule binds rapidly to the Fe^{2+} ion forming Fe^{2+} - O^2.There is evidence to suggest that this complex then undergoes a slow conversion to a more stable complex

3 Second reduction

$Fe^{3+} - O_2^{2-}$

A second reduction is required by the stoichiometry of the reaction. This has been determined to be the rate-determining step of the reaction. A comparison between the bond energies of O_2, O_2^-, and suggest that the complex is the most favourable starting point for the next stage of the reaction to occur. However, evidence from resonance Raman spectroscopy indicates the presence of a superoxide (O_2^-) complex .

4 O^2 cleavage

The O^2 reacts with two protons from the surrounding solvent, breaking the O-O bond, forming water and leaving an $(Fe - O)^{3+}$ complex.

5 Product formation

The Fe-ligated O atom is transferred to the substrate forming a hydroxylated form of the substrate.

6. .Product release

The product is released from the active site of the enzyme which returns to its initial state. (Segall, 1997). The structures of the transitional states following processes (4) and (5) have

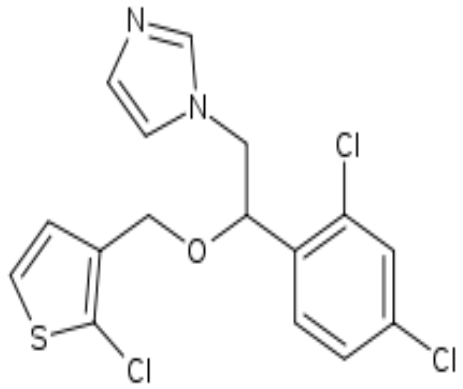
never been directly observed and are hypotheses based on analogy with other hemoproteins (Segall, 1997)

2.4.3 Tioconazole.

Tioconazole is classified into the group of antifungal agents called imidazole. Its chemical name is 1-2-(2-chloro-3-thienyl) methoxy)-2-(2, 4-Dichlorophenyl) ethyl) -1H-imidazole. The molecular formula is $\mathbf{C_{16}H_{13}Cl_3N_2OS}$, and the molar mass is 387.711 g/mol. It prevents fungal growth by interfering with the production of substances needed to preserve the cell membrane.

Several studies have shown that the imidazoles inhibit ergosterol synthesis in fungi by blocking C 14α demethylation.as a result, there is an accumulation of C14 intermediates like lanosterol and a decrese in ergosterol production in the fungal cell membrane formation.

Lanosterol cannot support yeast growth in the absence of ergosterol, and the inhibition of ergosterol synthesis or the accumulation of lanosterol may be the primary antifungal mechanism. (Bavisotto *et al., 2011*).



Lanosterol

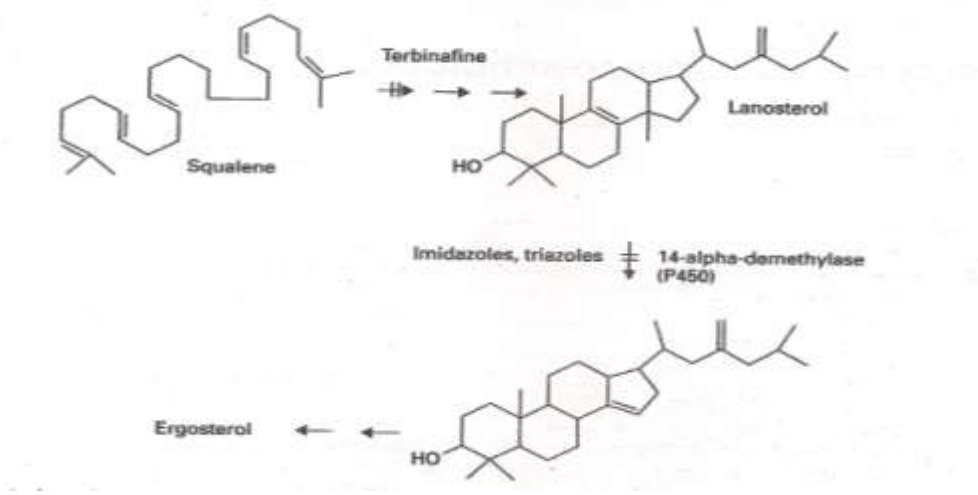


Figure 2.5 Pathway for synthesis of the essential fungal sterol ergosterol and the sites of inhibition by the antifungal agents Terbinafine, imidazoles and triazoles (Hugo and Russell's, 2004).

Ergosterol serves as a bioregulator of membrane fluidity and asymmetry and consequently of membrane integrity in fungal cells. Integrity of the cell membrane requires that. Inserted sterols lack C-4 methyl groups. Several lines of evidence suggest that the primary target of azoles is the heme protein, which co catalyzes cytochrome P-450-dependent 14α-demethylation of lanosterol). Inhibition of 14α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including 14α-methylated sterols (lanosterol, 4, 14-dimethyl zymosterol, and 24-methylene dihydrolanosterol), resulting in the formation of a plasma membrane with altered structure and function. The more recent triazole derivatives, such as fluconazole, itraconazole, and voriconazole (a triazole in development), owe their antifungal activity at least in part to inhibition of cytochrome P-450-dependent 14α-sterol demethylase. Compelling data in support of this mechanism of action comes from studies in which the cloned structural genes encoding the 14α-methyl sterol demethylase (*ERG11*) and the sterol desaturase (*ERG3*) from *C. glabrata* and used these cloned genes to create knockout mutants of each gene individually and both genes together. Phenotypic analysis revealed that the *ERG3* deletion mutant remained susceptible to fluconazole and

itraconazole. In contrast, the *ERG11* deletion mutant and a double mutant inwhich both genes were deleted were resistant to 100, 16, and 2 µg of fluconazole, itraconazole, and amphotericin B per ml, respectively.These data suggest an inhibitory interaction between azoles and 14α-demethylase.

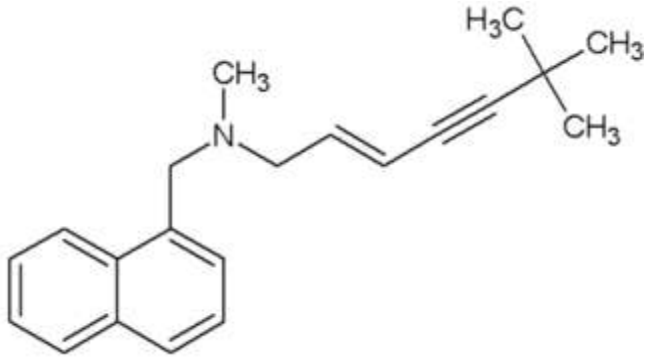
Although more recent azole antifungals are 14α-demethylase inhibitors, there exists a heterogenity of action among these antifungals. The earlier imidazole derivatives (such as miconazole, econazole, and ketoconazole) have a complex mode of action, inhibiting several membrane-bound enzymes as well as membrane lipid biosynthesis. An accumulation of zymosterol and squalene synthesis was observed when *C. albicans* cells were treated with voriconazole. It is unclear whether the accumulation of these intermediates results from voriconazole interaction with various (non-14α-demethylase) enzymes involved in ergosterol synthesis or from secondary effects of 14α-demethylase inhibition. Azole activity may also vary with the genus tested. In addition to inhibiting the 14α-demethylase in *Cryptococcus neoformans*, fluconazole and itraconazole affect the reduction of obtusifolione to obtusifoliol, which results in the accumulation of methylated sterol precursors. Mammalian cholesterol synthesis is also blocked by azoles at the stage of 14α-demethylation; however, the dose required to effect the same degree of inhibition is much higher than that required for fungi.It has been showed that voriconazole had a 50% inhibitory concentration of 7.4 µM against P-450-dependent 14α-sterol demethylase ($P\text{-}450_{DM}$) of rat liver cholesterol. In contrast, the 50% inhibitory concentration of this antifungal agent against fungal $P\text{-}450_{DM}$ was as low as 0.03 µM (about 250- fold more active against the fungal enzyme than against the mammalian enzyme). The clinical effects of inhibition of human sterol biosynthesis are most prominently seen with ketoconazole. (Ghanoum and Rice, 1999).

2.5 Allylamine and Morpholine Antifungal Drugs

Allylamine (naftifine, terbinafine) inhibit ergosterol biosynthesis at the level of squalene epoxidase. The morpholine drug, amorolfine, inhibits the same pathway at a later step.

2.5.1 Terbinafine

Terbinafine hydrochloride is an allylamine structurally related to naftifine. It is a synthetic antifungal agent. It is highly lipophilic in nature and tends to accumulate in skins, nails and fatty tissues. Terbinafine is a white to off white fine crystalline powder. It is freely soluble in methanol, ethanol, and slightly soluble in water. The chemical formula is (6, 6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthalene methanamine hydrochloride. The empirical formula is $C_{21}H_{26}CIN$ with a molecular weight of 327.90g/mol. (Marcin *et al*., 2010).



Terbinafine

A Mechanism of action

Terbinafine inhibits ergosterol biosynthesis via inhibition of squalene epoxidase. This enzyme plays a vital role in the fungal sterol synthesis pathway that enhances the production of sterols needed for functional fungal cell membrane. Squalene epoxidase (SE) is a key flavin adenine dinucleotide (FAD)-dependent enzyme of ergosterol and cholesterol biosynthetic pathways and an attractive potential target for drugs used to inhibit the growth of pathogenic fungi or to lower cholesterol level. Many studies on allylamine antifungal drugs activity have been published during the last 30 year.

Docking studies followed by molecular dynamics simulations and quantum interaction energy calculations [MP2/6-31 G (d)] showed the identification of the terbinafine−squalene epoxidase mode of interaction. (Marcin *et al*., 2010). In the energetically most likely orientation of terbinafine its interaction energy with the protein is 120 kJ/mol. In the favorable position the terbinafine lipophilic moiety is located vertically inside the squalene epoxidase binding pocket with the *tert*-butyl group oriented toward its center. Such a position results in the SE conformational changes and prevents the natural substrate from being able to bind to the enzyme's active site. That would explain the non competitive manner of SE inhibition. It was reported that there was strong interaction between terbinafine and SE arising from hydrogen bonding between hydrogen-bond donors, hydroxyl group of Tyr90 and amine nitrogen atom of terbinafine. Moreover, strong attractive interactions were recorded for amino acids whose mutations resulted in terbinafine resistance. (Marcin *et al*., 2010).

B **Spectrum of activities**

Spectrum of activity In vitro, terbinafine demonstrates excellent fungicidal activity against many dermatophytes including *Trichophyton rubrum, T. mentagrophytes, T. tonsurans, Microsporum canis* and *Epidermophyton floccosum*. However, terbinafine demonstrates variable and somewhat poor in vitro activity against many types of yeast. It generally demonstrates fungicidal activity against *C. parapsilosis* but it is fungistatic against *C. albicans* and other *Candida* spp. The in vitro spectrum of activity also includes *Aspergillus* spp., some dimorphic fungi, *S. schenckii*, and others. Initial animal studies in mice showed no activity in vivo against systemic pathogens, and the drug was abandoned for these indications. Terbinafine has been reported to be mainly effective on a specific group of fungi such as dermatophytes. Terbinafine yields lower MICs compared to fluconazole, itraconazole and griseofulvin, an indication of likely better performance. Terbinafine has also been reported to have better in vitro activity against most *Candida spp. Aspergillus spp*.

Sporothrix schenckii, *Penicillium mameffei*, *Trichophyton spp*. and Blastoschizomyces. (Pfaller 2009; *Elias et al.,* 2009)

C Adverse side effects

Common terbinafine side effects include headache, diarrhea, or unexplained rash. Side effects that are less common include fatigue, serious skin reaction, or vomiting. Some side effects of the drug are potentially serious such as difficulty breathing or swallowing and hives etc. Other serious Terbinafine Side Effects are

- Signs of liver proble problems, including yellow eyes or skin, itching, dark urine, or pain in the upper-right abdomen
- Vision changes, including blurred or double vision
- Unexplained skin rash
- Unexplained blisters or peeling of the skin
- Loss of smell, or other changes in smell
- Itching
- Hives
- Unexplained swelling
- Wheezing
- Difficulty breathing or swallowing.

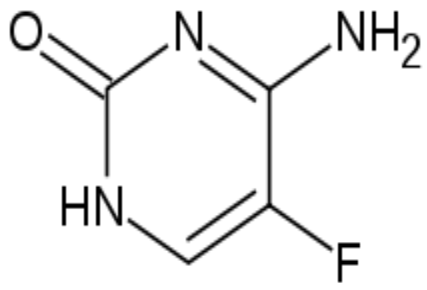
2.6. Antimetabolite Antifungal Agents.

5-Fluorocytosine acts as an inhibitor of both DNA and RNA synthesis via the intracytoplasmic conversion of 5-fluorocytosine to 5-fluorouracil

2.6.1 FLUCYTOSINE

Flucytosine (5-fluorocytosine;5-FC,4-amino-5-fluoro-2-pyrimidone) is an antimetabolite type of antifungal drug. It is chemically a pyrimidine. It is activated by deamination within the fungal cells to 5-fluorouracil. It is One of the oldest antifungal agents. 5-fluorocytosine (flucytosine; 5-FC), is a fluorinated analogue of cytosine. 5-FC was synthesized in 1957, as a potential anti-tumour agent but it was not sufficiently effective against tumours. Four years later, 5-FC proved to be active in experimental candidosis and cryptococcosis in mice and, in 1968, it was used to treat human candidosis and cryptococcosis. In addition to its activity against *Candida* spp. and *Cryptococcus neoformans*, 5-FC is active against fungi causing chromoblastomycosis (Vermes *et al.*, 2000).

Interest in 5-FC has been renewed as a result of two recent developments: (i) it is now used increasingly in combination with a number of azole antifungal agents, such as ketoconazole, fluconazole and itraconazole; (ii) it plays an important role in a new therapeutic approach in the treatment of certain tumours, especially colorectal carcinoma (Vermes *et al.*, 2000).



Flucytosine (5-FC).

A Mechanism of action

5-FC itself has no antifungal activity; its antimycotic activity results from the rapid conversion of 5-FC into 5-FU within susceptible fungal cells. 5-FC is taken up by these cells by the enzyme cytosine permease, which is also the transport system for adenine, hypoxanthine and cytosine. The latter

compounds competitively antagonize the uptake of 5-FC. This carrier system is energy-dependent and coupled to a proton gradient. Once inside the fungal cell, 5-FC is rapidly deaminated to 5-FU by means of the enzyme cytosine deaminase. The specificity of this step is crucial for the narrow antifungal spectrum of 5-FC. Fungi lacking cytosine deaminase are not sensitive to 5-FC, since no conversion to the active metabolite takes place. 5-FU, on the other hand, cannot be used as an antimycotic drug, since it is highly toxic to mammalian cells and also because it is only poorly taken up by fungal cells (Vermes *et al.*, 2000).

After uptake of 5-FC into the fungal cell and conversion into 5-FU, two mechanisms can be distinguished by which 5-FU exerts its antifungal activity. The first mechanism involves the subsequent conversion of 5-FU through 5-fluorouridine monophosphate (FUMP) and 5-fluorouridine diphosphate (FUDP) into 5-fluorouridine triphosphate (FUTP). FUTP is incorporated into fungal RNA in place of uridylic acid; this alters the amino-acylation of tRNA, disturbs the amino acid pool and inhibits protein synthesis. The second mechanism is the metabolism of 5-FU into 5-fluorodeoxyuridine monophosphate (FdUMP) by uridine monophosphate pyrophosphorylase. FdUMP is a potent inhibitor of thymidylate synthetase, which is a key enzyme in the biosynthesis of DNA, since thymidylate synthetase is a crucial source of thymidine. Consequently, fungal DNA synthesis is inhibited (Vermes *et al*., 2000).

It is not clear whether these two different pathways of 5-FC activity are equally important for the total antifungal effect of 5-FC. Using 5-FC-susceptible *Candida albicans* strains, it has been shown that there is a positive correlation between the degree of 5-FC susceptibility and the inhibition of both RNA and DNA synthesis, incorporation of 5-FU into RNA, inhibition of ribosomal protein synthesis and levels of FdUMP. However, some *C. albicans* strains have a reduced incorporation of 5-FU or reduced FdUMP concentrations, suggesting that these two pathways are not necessarily

linked to each other and that both may be responsible for 5-FC activity. Despite these findings, there is controversy about the importance of the inhibition of fungal DNA synthesis by 5-FC (Vermes *et al*., 2000).

B Spectrum of activity

5-FC is most active against yeasts, including *Candida*, *Torulopsis* and *Cryptococcus* spp., and against the dematiaceous fungi causing chromomycosis (*Phialophora* and *Cladosporium* spp.) and *Aspergillus* spp. The MICs of 5-FC vary from 0.1 to 25 mg/L for these fungal species. (Vermes *et al*., 2000).

In *Emmonsia crescens, Emmonsia parva, Madurella mycetomatis, Madurella grisea, Pyrenochaeta romeroi*, *Cephalosporium* spp., *Sporothrix schenckii* and *Blastomyces dermatitidis*, MICs vary from 100 to 1000 mg/L. 5-FC is also active against some protozoa, including *Acanthamoeba culbertsoni* both *in vitro* and *in vivo* and *Leishmania* spp. in patients. (Vermes *et al*., 2000).

The mode of action of 5-FC and the essential role of cytosine deaminase has been proven in *Saccharomyces cerevisiae* and *C. albicans* and are probably also valid for other sensitive fungi. However, specific research in this field is lacking. (Vermes *et al*., 2000).

c. **Chemical pathway of interaction.**

Flucytosine (5-FC) is a synthetic antimycotic compound, first synthesized in 1957. It has no intrinsic antifungal capacity, but after it has been taken up by susceptible fungal cells, it is converted into 5-fluorouracil (5-FU), which is further converted to metabolites that inhibit fungal RNA and DNA synthesis. Monotherapy with 5-FC is limited because of the frequent development of resistance. In combination with amphotericin B, 5-FC can be used to treat severe systemic mycoses, such as

cryptococcosis, candidosis, chromoblastomycosis and aspergillosis. Recently, 5-FC has been combined with newer azole antifungal agents; it also plays an important role in a new approach to the treatment of cancer. (Vermes *et al*., 2000).

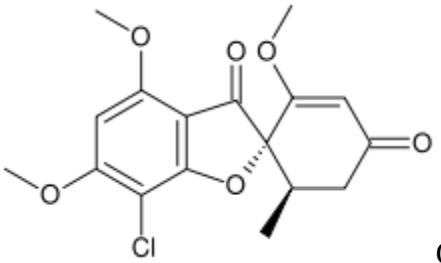
D Adverse side effects.

The adverse side effect of flucytosine has been reported to include gastrointestinal intolerance and bone marrow depression. Rash, hepatotoxicity, headache, confusion, hallucinations, sedation and euphoria have also been observed (Vermes *et al*., 2000)

Since flucytosine is commonly combined with amphotericin B, the renal impairment caused by amphotericin B has been speculated to increase the flucytosine levels in the body and thus potentiate its toxicity. The increase in toxicity of flucocytosine is presumably ascribed to 5-fluorouracil produced from flucytosine released by bacteria in gut lumen (Vermes *et al*., 2000).

2.7 GRISEOFULVIN

For the treatment of ringworm infections of the skin, hair, and nails, namely: *Tinea corporis, Tinea pedis, Tinea cruris, Tinea barbae*, cradle cap or other conditions caused by *Trichophyton* or *Microsporum* fungi.



Griseofulvin

Griseofulvin is used orally to provide systemic treatment for dermal fungal infections. It is moderately well absorbed. Taking the drug with a fatty meal will greatly increase its absorption.

Additionally, it is available in micro size and ultramicrosize formulations, which also increases the absorption of the drug.

Griseofulvin is effective on a specific group of fungi such as dermatophytes. Griseofulvin is a mycotoxic metabolic product of *Penicillium spp.* It was the first available oral agent for the treatment of dermatophytoses and has now been used for more than forty years. Griseofulvin is fungistatic with in vitro activity against various species of *Microsporum Epidermophyton,* and *Trichophyton*. It has no effect on bacteria or on other genera of fungi. ((Bossche *et al*., 2003)

a. Mechanism of action.

Following oral administration, griseofulvin is deposited in the keratin precursor cells and has a greater affinity for diseased tissue. The drug is tightly bound to the new keratin which becomes highly resistant to fungal invasions. Once the keratin-Griseofulvin complex reaches the skin site of action, it binds to fungal microtubules (tubulin) thus altering fungal mitosis. Griseofulvin is fungistatic. It inhibits fungal mitosis by inhibiting mitotic spindle formation (similar to colchicine). It is highly distributed to dermal tissues (keratophilic), which is beneficial in the treatment of dermal fungal infections. NOTE that therapy is long term (up to one year) and that the fungus may return when the drug is discontinued. This is due at least in part to the fungistatic nature of griseofulvin. (Bossche *et al*., 2003).

b Spectrum of activity.

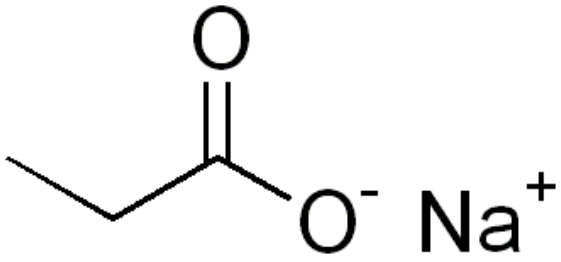
The spectrum of activity of griseofulvin is limited to *Trichophyton, Epidermophyton* and *Microsporum* species. This is due to the fact that dermatophytes possess a prolonged energy-dependent transport system for this antibiotic, where as in insensitive cells, such as *Candida albicans*, this is replaced by a short energy-independent transport system. Differences, depending on

the test method used, have to be taken into account when comparing minimum inhibitory concentration (MIC) data in the literature. The susceptibility of dermatophyte strains to griseofulvin, itraconazole, sertaconazole, terbinafine and cyclopiroxolamine was compared by using an agar macrodilution and broth microdilution test. For all five antimycotics, MIC data were three to seven folds lower in the microdilution test system used to compare the susceptibility of *T. rubrum* and *T. mentagrophytes*. The spectrum of activity of griseofulvin is limited to *Trichophyton, Epidermophyton* and *Microsporum* species. This is due to the fact that dermatophytes possess a prolonged energy-dependent transport system for this antibiotic, whereas in insensitive cells, such as *Candida albicans*, this is replaced by a short energy-independent transport system (Bossche *et al*., 2003).

c. Adverse side effects

Adverse reactions of griseofulvin are uncommon. Nausea, diarrhea, headache, skin eruptions and photosensitivity are occasionally observed. Hepatotoxicity and neurological side effects are rarely observed. Griseofulvin has been the first antifungal drug for the treatment of dermatophytosis in many years. However, following the emergence of alternatives such as itraconazole and terbinafine, its use has been limited. The major advantages of these newer agents over griseofulvin are their reduced toxicity, enhanced efficacy, and shorter duration of therapy (Bossche *et al*., 2003).

2.8 SODIUM PROPIONATE.



This chemical antifungal agent is also known as propionic acid or sodium salt. It has a chemical formula as follows CH_3 CH_2 COONA. It has a molecular Weight: of 96.06g/mol. It is transparent, crystal, granular, deliquescent in moist air, neutral to slightly alkaline in reaction to litmus. One gram dissolves in approximately one ml of water, in approximately 24ml of alcohol. It is most active at an acid pH (Budavari, 1996). Propionic acid occurs naturally as the result of metabolic processes. It dissolves very easily in water, in alcohol and slightly dissolvable in acetone. Sodium propionate is used as a fungicide because of its ability to retard growth of fungi in some food items. It is commonly used as a food additive, particularly in baked foods, confectionaries, and gelatine. It is also used as preservatives in cosmetics. It is used as a topical antifungal agent in livestock, and also as a preservative for hay and silage ((Bossche *et al*., 2003).).

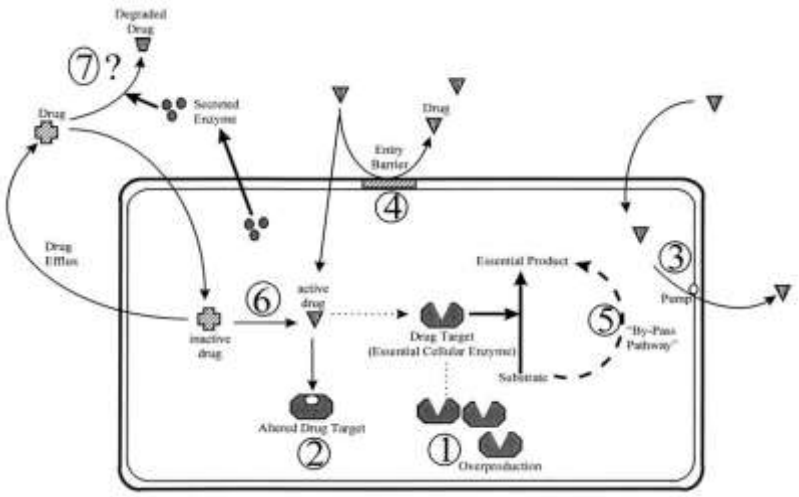
2.9 EMERGENCE OF ANTIFUNGAL RESISTANCE IN FUNGI

In spite of the availability of effective antifungal agents, the battle against fungi infectious diseases is far from being over. Not only do they continue to cause a large number of infections and deaths, particularly in developing countries, but the emergence and spread of antifungal resistance is now threatening to undermine the ability to treat fungi infections and save lives.

In general, antifungal agents act by interfering with specific processes that are essential for the growth and/or replication of fungus cell. Agents are commonly separated into groups based on their specific antifungal mechanisms of action. Inhibitors of cell membrane synthesis, such as terbinafine Hcl, polyenes, and imidazoles , direct and indirect inhibitors of nucleic acid synthesis flucytosine, in addition, antifungal agents are classified as either fungicidal (those that kill the target fungus or fungistatic (those that inhibit the microorganism’s growth).

2.9.1 Mechanisms of resistance to azoles.

As noted above, there are as yet no reports of modification of azole antimicrobials as a mechanism of resistance. Resistant strains therefore either exhibit a modification in the quality or quantity of target enzyme, reduced access to the target, or some combination of these mechanisms. These mechanisms are discussed in detail below and are summarized in the Figure and Table below .



(Ghannoum and Rice, 1999).

Figure 2.9. Model of different routes of antifungal agent resistance

1, The target enzyme is overproduced, so that the antifungal agent does not inhibit the biochemical reaction completely. 2, The antifungal agent target is altered so that the antifungal agent cannot bind to the target. 3, The antifungal agent is pumped out by an efflux pump. 4, The entry of the antifungal agent is prevented at the cell membrane/cell wall level. 5, The cell has a bypass pathway that compensates for the loss-of-function inhibition due to the antifungal activity. 6, Some fungal "enzymes" that convert an inactive antifungal agent to its active medium, which degrade the antifungal agent.(Ghannoun and Rice,1999). Many fungi can posses any number of these mechanisms simultaneously.

Table 2.9.2Biochemical basis of azole resistance.

Mecanisms	**Caused by**	**Comments**
Alteration in drug target(14α-demethylase).	Mutation which alter drug binding but not binding of the endogenous substrate.	Target is active i e, can catalyse demethylation) but has reduced affinity towards azoles.
Alteration in sterol biosynthesis	Lesions in the desaturase.	Results in accumulation of 14α- methyl fecosterol instead of ergosterol.
Reduction in the intracellular concentration of target enzyme.	Change in membrane lipid and starols overexpression of specific drug efflux pumps.(CDR1, PDRS, BEN1).	Poor penetration across the fungal membrane; active drug efflux.

(Ghannoum and Rice, 1999).

There are three basic mechanisms by which fungi resist antifungal agents

i. Alteration of the antifungal receptor.
ii. Alteration of antifungal entry or removal (efflux)
iii. Development of resistant metabolic pathways.

a).**modification of target**: Several lines of evidence implicate a modification in the quantity or quality of 14α-demethylase in the expression of resistance to azole antifungal agents. A recent study examined the biochemical mechanisms for resistance to fluconazole by comparing sterol composition, fluconazole accumulation, and inhibition of 14α-demethylase by fluconazole in two

clinical *C. krusei* strains (expressing intrinsic resistance to fluconazole) and a susceptible *C. albicans* isolate. No significant differences in the sterol content of *C. krusei* and *C. albicans* were detected (eregosterol was the major sterol in both species). Studies performed on cell extracts indicated that the concentration of fluconazole required to inhibit the synthesis of ergosterol by 50% was approximately 24-to-46-fold higher in *C. krusei* than in *C. albicans*, suggesting that affinity of the target enzyme is different in the two species. .(Ghannoum and Rice, 1999).

Other studies have implicated altered 14α-demethylase in resistance to azoles. Reduced susceptibility of *C. albicans* B41628 (isolated from a patient with chronic mucocutaneous candidiasis who relapsed following an extended period of treatment with ketoconazole) to miconazole, ketoconazole, itraconazole, and fluconazole was attributed to differences in the microsomal cytochrome P-450 enzyme. Analysis of carbon monoxide (CO) difference spectra of microsomes from this strain revealed thatit contained cytochrome p-450 with a soret peak different from that characteristic of the cytochrome in azole-susceptible cells. Actually, the enzyme had a low binding affinity for azole antifungals. Whether the altered 14α-demethylase is solely responsible for the level of resistance observed in this strain is unclear, since *C. albicans* b41628 is a clinical isolate and the contribution of other resistance mechanisms to the reduced susceptibility of this isoslate cannot be excluded (Ghannoum and Rice, 1999).

(b) **Over expression** of 14α-demethylase has also been implicated as a mechanism of resistance to azole antifungals. An azole-resistant *C. glabrata* strain showed that its ergosterol content was increased compared with that of pretreatment isolate. This increase in ergosterol synthesis was accompanied by a decrease in susceptibility to both azoles and amphotericin B. The increase in ergosterol synthesis was attributed to an elevated microsomal P-450 content in the resistant strain, suggesting an overexpression of the enzyme. Although the intracellular content of fluconazole in the

resistant strain was 1.5-3-fold lower than that in the pretreatment isolate, suggesting active efflux of this antifungal, the amount of itraconazole retained by the resistant strain did not differ from that found in the pretreatment isolate.This finding suggests that the increased P-450 levels were responsible for the cross resistance to these two triazoles. The scarcity of clinical isolates in which over-production of 14α-demethylase has in *C. glabrata* appeared to show that other resistance mechanisms may be operative in the same strain. Over expression of target enzyme may appear to plays only a limited role in clinical resistance to the azoles. (Ghannoum and Rice,1999).

It has been reported that a single point mutation of single amino acid substitution between two residues known to be involved in interactions with the heme moiety in the active site of the enzyme. A similar point mutation (T315A) that alters the susceptibility of the target enzyme has been observed in close proximity to the active site of this enzyme in *C. albicans.* A second significant change observed in ERG11 gene of the resistant *C. albicans* has been reported with loss of allelic variation in the ERG11 promoter and in the downstream THR1 gene (which encodes homoserine kinase, which is involved in threonine synthesis). (Ghannoum and Rice, 1999).

C) **Active efflux**: Considerable evidence has now been accumulated to suggest that active efflux is an important mechanism of resistance to azole antifungals. Recent studies indicate that fungi posses at least two efflux systems: (i) proteins belonging to the major facilitator superfamily (MFS) and (ii) ATP-binding cassette (ABC) super family of proteins. The MFS drug efflux proteins are associated with the transport of structurally diverse compounds and account for a range of resistance to antifungal compounds in fungal cells. An example of MFS protein associated with antifungal resistance in Candida is BEN (CaMDR1), which is implicated in resistance to several antifungal, including benomyl, methotroxate, and fluconazole. The ABC superfamily of proteins bind ATP, which is essential for substrate transport, through a highly conserved amino acid sequence known as

the ATP binding cassette . Four familes of ABC transporters have been identified in *sacharomyces cerevisiae* (MDR, CFTR, YEF, and PDR). These transporters have a common four-core domain structure consisting of two integral membrane domains that span the membrane multiple times and two ATP-binding cytoplasmic domains that couple ATP hydrolysis to substrate transport. To date, eight genes for ABC transporters have been identified in *Candida spp*. An example of an ABC transporter found in both *Candida spp* and, more recently, in *Cryptococcus neoformans* is CDR1, which is involved in resistance to fluconazole and other azoles. The gene encoding this transporter was cloned, and appears to be similar in structure to human P-glycoprotein, which functions as a multidrug pump and is associated with resistance to a number of chemotherapeutic agents in neoplasms. Recent evidence have shown that *C. albicans* may possess one or more additional genes encoding ATP-binding cassettes MDR-like proteins that are distinct from CDR1, which could participate in the development of azole resistance. In this regard, five cdr genes (Cdr1 to Cdr5) which belong to the PDR family have been identified in *C. albicans*. Additionally, one member each of the MDR, CFTR, and YEF families were identified (HST6, YCF1, and ELF1, respectively). (Ghannoum and Rice, 1999).

Evidence implicating drug efflux as a mechanism of resistance in *Candida spp* has been forthcoming recently. Comparism between pretreatment (susceptible) and psottreatment (resistant) isolates of *C. glabrata* showed that while no change in sterol biosynthesis between these two isolates was observed; the resistant isolate accumulated less fluconazole than susceptible one did. The reduced ability of the resistant strain to accumulate fluconazole was a consequence of energy-dependent drug efflux. In an extension of these studies. Other workers examined the mechanism of resistance to azoles in *C. albicans, C. glabrata,* and *C. krusei* by using the fluorescent dye rhodamine 123 (Rh123), which is known to be transported by a number of MDR (multidrug-resistant) organisms

(Clark, et al., 1996). Their results showed that resistant isolates accumulates less Rh123 than susceptible cells did. Furthermore, active efflux of Rh123 was observed in azole-resistant isolates of *C. albicans* and *C. glabrata*, consistent with the activity of an MDR transporter. The efflux mechanism associated movement of Rh123 appears to play a role in azole resistance in *C. glabrata* but not in *C. albicans*, suggesting that azole resistance in *C. albicans* may be mediated by an alternative efflux pump (Ghannoum and Rice,1999).

C. albicans isolates obtained from five AIDS patients showed increasing fluconazole resistance following prolonged treatment. In some of these resistant strains, decreased accumulation of fluconazole was association of fluconazole was associated with up to a 10-fold increase in the mRNA levels of the CDr1 gene. Other resistant isolates overexpressed mRNA from the gene encoding BEN1 (CaMDR1) and had normal levels of CDR1 Mrna. Data from this study suggests that CDR1 is involved in the export of several azole derivatives (including fluconazole, itraconazole, and ketoconazole), while BEN1 confers resistance especially to fluconazole. *C. albicans* isolates were cultured from a patient with recurrent episodes of oropharyngeal candidiasis and treated with progressively higher doses of fluconazole to control the infection. Over a 2-year period, the patient experienced 15 relapses, each of which was treated with fluconazole. Isolates from the early relapses had fluconazole MICs of >8 µg/ml, and the infection responded to fluconazole (100mg/day). Fluconazole MICs for subsequent isolates rose steadily to 64 µg/ml, requiring progressively greater doses of fluconazole to produce a clinical response. Fluconazole was ineffective after the 14^{th} relapse in which the minimum effective dose of fluconazole at each relapse is plotted against the MIC .for the isolate from that episode. The approximate breakpoint suggested by these data correlate roughly with achievable levels of fluconazole in blood, 100mg/day produces peak concentrations of approximately 6µg/ml and the linear pharmacokinetics of fluconazole would predict concentrations

of 40 to 60 µg/ml in serum at 800mg/day. Analysis of all isolates by contour –clamped homogenous electric field electrophoresis confirmed the persistence of the same *C albicans* strain throughout all infectious episodes (Ghannoum and Rice, 1999).

The sterol content did not differ between susceptible and resistant isolates in this collection, suggesting that the mechanisms of resistance does not involve alteration in sterol composition. A number of intresting findings were reported (!) MDR1 expression was increased early in the series while the CDR1 Mrna level was increased only in the final isolates.(ii) ERG16 signal increased towards the end of the series. (iii) increases in mRNA levels of ERG16 and CDR1 correlated with increase resistance to ketoconazole and itraconazole but not to amphotericin B and (iv) no changes in the mRNA signals for genes encoding members of the YEF and CFTR gene families (members of the ABC transporter family) were detected in the series, and no expression of ERG1 and ERG7 genes involved in the ergosterol biosynthetic pathway) was detected. These data suggest that high level azole resistance, at least in this series of isolates, results from the contributions of several mechanisms. They also suggest that prolongrd exposure of a strain to one azole may lead to over expression of genes, such as ERG 16 and CDR1, that result in cross resistance to other azoles. (Ghannoum and Rice, 1999).

(d) Alteration in membrane composition. Interactions between sterols and phospholipids in the cytoplasmic membrane affect membrane fluidity and asymmetry and consequently influence the transport of materials across the membranes. A decrease in the amount of drug taken up by the fungal cell may result from changes in the sterol and/or the phospholipid composition of the fungal cell membrane using cerulenin as a lipid modulator. It has been demonstrated that altered pospholipid and fatty acid profiles affected *C albicans* cell permeability and rendered the cell more resistant to miconazole. It has been showed that an azole and polyene resistant *C. albicans* mutant

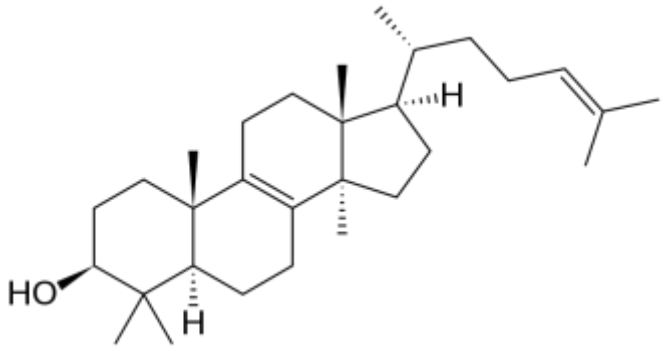
had a larger lipid content and lower polar lipid to neutral lipid ratio than did strains susceptible to azoles. However, the most significant change in the lipid of the resistant strain was in the membrane sterol pattern, where ergosterol was replaced by methylated sterols, such as methyl fecosterol. The authors hypothesized that an altered membrane sterol pattern is responsible for the doubly resistant phenotype observed in this strain (Ghannoum, and Rice,1999).

2.9.3 Mechanisms of resistance to polyenes.

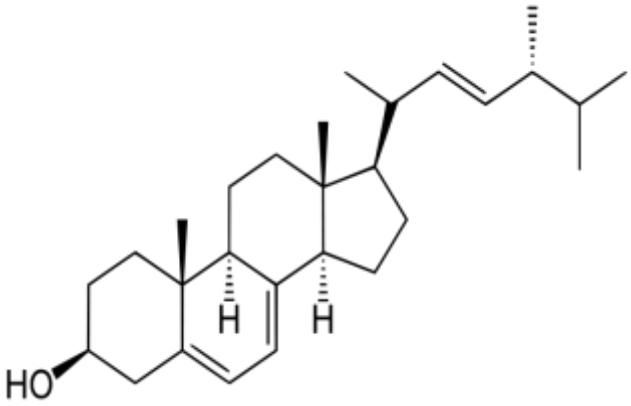
Despite more than 30 years of clinical use, resistance to antifugals such as amphotericin B and nystatin, is rare, withnresistant isolate being confirmed mostly to the less common species of *Candida*, such as *C. lusitaniae*, *C. glabrata* and *C. guilliermondii*. It has been suggested that development of resistance occurs by selection of naturally occurring resistant cells produce modified sterols that bind nystatin with lower affinity. The growth rate in the presence of nystatin is therefore dependent upon the normal growth rate (in the absence of nystatin) and the rate at which nystatin causes cell membrane damage. This later rate is presumed to be a function of the affinity of nystatin for the membrane sterols, the greater the nystatin sterol affinity, the greater the rate of membrane damage. Most of our knowledge of the mechanisms of resistance to polyenes in fungal species has come from studies using mutants generated by (i) growing cells in the presence of increasing concentrations of antifungal agents (multistep mutant), (ii) exposing the cell to a gradient concentration, or (iii) creating mutants by one step mutation with mutagenic agents. It has been demonstrated that development of inducible resistance (induced by adaptation mechanism) in a strain of *C albicans* was accomplished by a decrease in the ergosterol content was due not to enzymatic degradation of preformed ergosterol but to inhibition of its synthesis. Similarly, 27 polyene-resistant *C. albicans* isolates obtained from neutropenic patients showed that these strains

had a 74 to 85% decrease in their ergosterol content. Thus, decreased ergosterol content may lead to decrease susceptibility to polyenes (Ghannoum and Rice, 1999).

2.10 CHEMICAL STRUCTURE OF LANOSTEROL



2.11 CHEMICAL STRUCTURE OF ERGOSTEROL.



(Hugo and Russels, 2004)

2.12 PREVENTION AND CONTROL OF ANTIFUNGAL RESISTANCE

Strategies to avoid and suppress the emergence of antifungal resistance have not been widely practised. However, approaches analogous to those recommended for antifungals could be suggested. These measures include (i) prudent use of antifungals (ii) appropriate dosing with special emphasis on avoiding treatment with low antifungal dosage, (iii) therapy with combinations of

existing agents, (iv) treatment with the appropriate antifungal (in cases where the etiological agent is known), and (v) use of surveillance studies to determine the true frequency of antifungal resistance. It should be emphasized that data supporting the use of the suggested measures is largely lacking, this report studies may provide some specific guidelines in the near future. Additionally, advances in rapid diagnosis of fungi may be helpful in reducing the use of appropriate antifungals to treat organisms that are resistant to a particular agent. Unfortunately, progress in developing diagnostic methods specific to fungi has been slow. The recent approval of a reference method for the antifungal susceptibility testing of yeast is encouraging and provides a means for performing surveillance studies.(Ghannoum and Rice,1999).

2.13 THE 'IDEAL' CHEMICAL ANTIFUNGAL AGENT

The ideal antifungal agent remains an elusive goal for treatment of life-threatening systemic fungal infections. Such an agent would have broad antifungal activity, low rates of resistance, flexible routes of administration, few associated adverse events, and limited drug-drug interactions. Only three of the seven classes of antifungal agents currently available are suitable for treatment of systemic infection: the polyenes, the azoles, and the echinocandins. The academia and industry need to collaborate in the search for new lead antifungal compounds using traditional screening methods as well as the new pharmacogenomics methods. Enhancing efficacy and reducing toxicity of the currently available therapeutic agents is also another important avenue of study. As an example, the Mycosis Research Center at the University of Mississippi Medical Center has identified pyogenic polyenes in commercial preparations of amphotericin B deoxycholate which correlate with infusion related toxicities. A highly purified formulation of amphotericin B appears promising, with a better therapeutic index compared to its parent compound as evidenced by results of *in vitro* and *in vivo* studies reviewed in this presentation .(Chapman, 2008).

2.14 CHARACTERISTICS OF COMBINATIONS OF ANTIFUNGAL AGENTS.

a. The combination should have a faster sterilizing time against the test-organisms than the equivalent concentration of the sum of both compounds used individually e.g for ophthalmic solutions, this sterilizing time should not be more than one hour for 10^6 cells.

b. The combination should still be effective when the test-organism has acquired resistance to either of the antifungal in the combination. (Hugo and Russels, 2004)

c. The spectrum of activity of the combination should include pathogenic fungi. In addition, the following properties were suggested as being desirable for individual members of the combination

d. One member of the combination should be chosen for its rapid action against a wide spectrum of fungi.

e. The other member of the combination should have properties that enable it to potentiate the action of the first antifungal agent, particularly against organisms that have developed resistance to the first antifungal agent. (Hugo and Russels, 2004)

2.15 DEFINITION AND CLASSIFICATION OF COMBINED ANTIFUNGAL ACTION

In comparison with the action of single antifungal drugs, a combination of two drugs may exhibit;

(i) Increased activity either additive or synergistic.

(ii) Decreased activity, antagonistic or

(iii) No increase or decrease in activity i.e indifference (Hugo and Russels, 2004)

A simple additive effect in (i), is useful only as a means for achieving higher overall concentration for drugs for low solubilities or broader spectrum of activity. The most desirable effect resulting

from the simultaneous application of two drugs is synergistic action, that is, an effect greater than to be expected from simple summation of the action of drugs considered.

The reverse of this effect, that is, production of a lesser effect than the sum of the separate drug action constitutes antagonism. Evaluation of a combined activity defined synergism and antagonism as a combined response of more or less than expectation according to the criteria of drug equivalence, that is, observed combined response surpasses additivity (synergism) or less than additivity (antagonism), in term of drug equivalence.A comprehensive Classification of combined responses, that have been widely recognized as less-than-additive, additive and more-than-additive was evolved; the three special cases being antagonism, additive and synergism respectively.

It has also been reported that where the combined response exceeds that of an active drug in admixture with an inactive member or where a response is produced by a combination of two inactive drugs, then the term synergism could be applied. Conversely, where the combined response is less than that of either drug alone, or where one drug in the combination becomes inactive due to adsorption onto sites of secondary importance to the total action, the term antagonism is warranted. (Hugo and Russels, 2004)

2.16 RATIONALES FOR THE USE OF ANTIFUNGAL COMBINATIONS

The availability of new antifungal agents with novel mechanisms of action has stimulated renewed interest in combination antifungal therapies in particular, and despite the limited clinical data. The high mortality of mould infections and the relatively limited efficacy of current agents have produced significant interestin polyene-, extended-spectrum azole-, and echinocandin-based combinations for these difficult-to-treat infections. With the recent publication of the first large randomized trial of antifungal combination therapy to be conducted in two decades and the rapid

proliferation of new in vitro and in vivo data on antifungal combinations, we have sought to review the recent work and future challenges in this area.

The focus of this review is on the efficacy of antifungal drugs in combination with respect to the extent or rate of killing of the fungal pathogen, although other potential interactions (such as pharmacokinetic drug interactions) can impact efficacy when these agents are used together. The value of giving two drugs because each is separately effective against a group of organisms exhibiting a variety of types of resistant is not specifically discussed, but this also is an obvious and straight forward reason to use a combination of agents. (Johnson *et al*., 2004; Cuenca 2004, and Pearson *et al.*, 2004).

It cannot be simply assumed that the use of two or more effective drugs with different mechanisms of action will produce an improved outcome compared to the results seen with a single agent. Combination antifungal therapy could reduce antifungal killing and clinical efficacy, increase potential for drug interactions and drug toxicities, delay emergence of resistant strains, and carry a much higher cost for antifungal drug expenditures without proven clinical benefit. Thus, it is important to critically evaluate the role of combination therapy as new data become available. (Johnson *et al* ., 2004; Cuenca, 2004, and Pearson *et al.*, 2004)

2.17 CONCEPTUAL MODELS AND TERMINOLOGY

Methods for studying antifungal combinations in vitro and in vivo have differed considerably over time and among investigators. These tools do not differ with respect to their application to combination antibacterial or antiviral therapies and have been discussed extensively. In brief, all approaches to evaluating combinations can be reduced to two elements: (i) a conceptual model for predicting the expected result for a combination and (ii) a set of phrases used to categorize results

that are better than expected, worse than expected, or as expected. Although many subtle variations are possible, the underlying mathematical model is based on either the assumption of additive interactions or the assumption of probabilistic (multiplicative) interactions. On the basis of the terminology employed by the author who first carefully described each of these models, the two models can be usefully referred to as the Loewe additivity model and the Bliss independence model. (Johnson *et al*., 2004; Cuenca, 2004, and Pearson *et al.*, 2004)

The terminology used to place results into interpretive categories is often the subject of debate and confusion. It has been proposed that a set of consensus phrases are instructive. In this proposal, synergism and antagonism have clear and intuitive meanings. The phrases used to describe results that are neither synergistic nor antagonistic are, however some what tricky. Mathematically, the term "additive" is indeed logical for the additive model just as the term "independent"is logical for the probabilistic Bliss model. Unfortunately,the term "additive" often conveys an imprecise message and may be misinterpreted as referring to a positive interaction. Coined terms such as "subadditive" only reinforce this erroneous conception (Johnson *et al*., 2004; Cuenca, 2004, Pearson *et al.*, 2004)

This situation has no perfect resolution. Possible alternativesto the term "additive" include the terms "summation", "nointeraction", and "indifferent." The term "summation"unfortunately still carries a hidden positive message. Although some what imprecise, the terms "indifferent" and "no interaction"have an inherently conservative emotive nature and can be used to describe Loewe additivity and Bliss independence and also to describe results in cases in which the underlying model is not clearly specified. The use of these terms provides the reader with a constant reminder of the neutral nature of the result although there can be value in an indifferent (additive) interaction, the biological relevance of such an interaction is not always obvious. For reasons related mostly to ease of expression (it is simpler to speak of indifference and indifferent interactions than to speak of non

interaction and non interactive interactions, this review uses the phrases synergistic, indifferent, and antagonistic when interpretive categories are required.

With respect to the underlying mathematical model, Loewe additivity most often seems appropriate for combinations of antifungal agents. This result follows from the detailed comparison of the strengths and weaknesses of the additive and multiplicative models found in the review. The key argument for us is that Loewe additivity supports the experiment in which an agent combined with itself is neither synergistic nor antagonistic. (Johnson *et al*., 2004; Cuenca, 2004, Pearson *et al.*, 2004)

2.18 QUANTITATIVE ANALYSES: THE FRACTIONAL INHIBITORY CONCENTRATION INDEX.

Calculation of the fractional inhibitory concentration (FIC) index (FICI) by the use of the checkerboard method has long been the most commonly used way to characterize the activityof antifungal combinations in the laboratory. The FICI represents the sum of the FICs of each drug tested, where theFIC is determined for each drug by dividing the MIC of each drug when used in combination by the MIC of each drug when used alone. Stated in terms of the Loewe additivity model, the FICI model assumes that indifference is seen when this equation istrue: $1 = (MIC_{\text{drug A in combination}}/MIC_{\text{drug A alone}}) + (MIC_{\text{drugB in combination}}/MIC_{\text{drug B alone}})$. To make this concrete, imagine an organism for which the fluconazole MIC is 2 µg/ml.If we perform a checkerboard study of fluconazole versus fluconazole,we should find that the well which receives the combination of 1 µg/ml + 1 µg/ml produces an effect identical to that of the wells containing 2 µg/ml [or FICI = (1/2)+ (1/2) = 0.5 + 0.5 = 1, which indicates indifference or Loewe additivity].(Johnson *et al*., 2004; Cuenca, 2004, Pearson *et al.*, 2004).

Reproducible variations from an FICI of 1 represent non indifference of at least some magnitude. However, the experimentalist must consider both the inherent inaccuracy of MIC methodologies and the question of biological relevance. Thus, it has been proposed that synergy be declared when the FICI is less than or equal to 0.5 and that antagonism be declared when the FICI is greater than 4. (Johnson *et al*., 2004; Cuenca, 2004, Pearson *et al*., 2004).

The logic behind these interpretive categories is worth discussing.They are based on the related assumptions that (i) testing employs concentrations separated by a factor of 2 (e.g., a sequenc esimilar to 0.25, 0.5, 1, 2, and 4 µg/ml) and (ii) 1-dilution-step MIC changes are within experimental error ranges. For a result to be synergistic, these rules require that both drugs show a minimum drop in MIC of at least two dilution steps and thus, a fourfold drug concentration drop. As an example, consider drugs A and B, each of which has a MIC of 2 µg/ml fora given isolate. Synergy would only be declared when both drugs in combination showed a MIC of 0.5 µg/ml or less. Mathematically, this would be FICI = (0.5/2) + (0.5/2) = 0.25 + 0.25 = 0.5.Importantly, a FICI of 0.500 does not meet the definition of synergy. For instance, if the MIC of one drug in our example were to drop to only 1 when used in combination, synergy could not occur under these rules no matter low the other drug's MIC in combination were to become: FICI = (1/2) + (??/2) = 0.5+ some value greater than 0. This would result in a FICI value slightly greater than 0.5 and would thus be defined as indifference. (Johnson *et al*., 2004; Cuenca, 2004, Pearson *et al.*, 2004).

Conversely, antagonism is declared when at least one drug has a four fold increase in MIC. To understand this rule, consider the boundary condition under which both drugs shows a precisely two fold increase in MIC. Continuing with the same example, this would be the situation when the MICs of both agents increase to 4 and thus, FICI = (4/2) + (4/2) = 2 + 2 = 4. This result remains within experimental error limits; a FICI of precisely4.00 is defined as indifferent (or additive), where as any

value greater than 4 is defined as antagonistic. The rule that FICI of >4 defines antagonism also handles the situation where in a small amount of one drug dramatically increases the MIC for the other drug. `

2.19 MECHANISM OF INTERACTION OF THE ANTIFUNGAL AGENTS.

2.19.1 Mechanisms of synergy

There are several mechanisms proposed for antifungal synergy. (i) Inhibition of different stages of the same biochemical pathway represents one type of interaction. An example is the combination of terbinafine and azoles, in which both compounds inhibit ergosterol biosynthesis and, thus, impair the function of fungal cell membranes. (ii) Increased penetration of an antifungal agent as a result of cell wall or cell membrane antifungal activity from another agent is possible. This interaction has been proposed for combinations of amphotericin B or fluconazole. These agents easily penetrate the fungal cell membrane to reach their target of fungal DNA synthesis. Such a mechanism may also explain potential synergism between azoles or amphotericin B and flucytosine, in which case the azole or polyene damages the fungal cell membrane, enabling increased uptake of flucytosine. (iii) A transport interaction is proposed for amphotericin B-flucytosine, whereby amphotericin B acts on the fungal cell membrane and inhibits flucytosine transport across the cell membrane and out of the yeast cell. In this scheme, flucytosine exerts its lethal effects on any surviving fungus when amphotericinB degrades the cell membrane via an oxidative decay, allowing flucytosine to remain at the site of its action within the cell. (iv) Simultaneous inhibition of different fungal cell targets, such as cell wall and membrane targets, is also possible. This mechanism has been suggested for both the apparently synergistic interactions between echinocandins (cell wall active) and amphotericin B. (v) Potent initial activity of a rapidly fungicidal agent, such as amphotericin B, to reduce fungal burden, which then allows another agent to subsequently work well as consolidation or clearance therapy on

a reduced number of fungal cells, is another possible mechanism. Some of these effects have been observed with certain fungal pathogens but not with others, so these interactions may depend on and differ according to certain target cell factors and even between different fungal species .(Johnson *et al*., 2004; Cuenca, 2004, Pearson *et al.*, 2004).

2.19.2 Mechanism of antagonism Antagonism among antifungal agents might occur in one of several ways. (i) Direct antifungal action at the same site results in decreased ability of other agents to exert their competitive effects on that site or at an altered target, as proposed for the azoles and amphotericin B. Azoles block the synthesis of ergosterol in the fungal cell membrane and may thus render amphotericin B inactive, since this agent exerts its activity by binding to ergosterol in the cell membrane. (ii) Adsorption to the cell surface by one agent inhibits binding of another antifungal agent to its target site of activity. This mechanism is proposed for lipophilic azoles (such as itraconazole and ketoconazole) which may adsorb to the fungal cell surface and inhibit binding of amphotericinB to fungal cell membrane sterols. (iii) Modification of a target upon exposure to an antifungal agent occurs that renders the pathogen less susceptible to the effects of other antifungals. This mechanism has been proposed for sequential antagonism observed with azoles and amphotericin B, where by pre exposure to an azole compound causes replacement of membrane ergosterol with a methylated sterol derivative to which amphotericin B binds less well. (iv) Other unknown antagonistic mechanisms may exist such as that observed for polyenes and flucytosine. Some have suggested that antagonistic interactions between these agents might be related to changes in fungal cell membrane function due to the effects of amphotericin B however, additional data are needed to explain this phenomenon, because these two drugs generally display an interaction which tends towards synergy. (Johnson *et al*., 2004; Cuenca 2004, Pearson *et al.*, 2004.)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of sample collection centre.

Three different primary schools in Zaria Nigeria, namely Fahimta primary school,Vintage primary school and Aminu Primary school. Fahimta primary school and Aminu primary School are located in Sabon Gari, while Vintage Shools is located in Hanwa area.

3.2 Recruitment of volunteers for this study

A total of 150 samples will be collectd from the target volunteers. These are pupils in these schools who present clinical symtoms of *tinea capitis* on thetr scalp.

3.3 MATERIALS

3.3.1 Culture media and suspending media

a). Sabouraud Dextrose Agar (Fluka Germany)

b). Sabouraud Dextrose Broth (Fluka Germany)

(c). Constituents of harvesting

3.3.2. Antifungal agents used

a) Fluconazole pure sample was a kind gift from Pfizer Nigeria Plc.

b) A brand of Terbinafine Hydrochloride (Evans Nigeria plc)

c) Sodium Propionate (BDH UK)

d) Flucytosine pure sample was a kind gift from (Fluka Germany.

e) Tioconazole pure sample was a kind gift from Neimeth Nigeria Plc.

3.1.3 Other materials used

Methanol (BDH UK).

Tween 80 (Riedel-de Haen, UK).

Lactophenol (BDH, UK).

Streptomycin. (Neimeth Nigeria Plc)

Cycloheximide (Oxoid, UK).

3.2 METHODS

3.2.1 collection of samples.

The consent of the school authorities was obtained in order to collect samples from the school pupils. Samples were collected from volunteered male and female pupils (5-14 years age range.) using sterile cotton swab sticks, after disinfecting the affected scalp area. This sterile cotton wool swab stick was first dipped in sterile normal saline with 0.05% tween 80 and was used to swab the surface of the affected scalp area and cultured appropriately.

3.3 CULTIVATION OF ORGANISMS

The swab sticks from 150 volunteer pupils were inoculated on Sabouraud Dextrose Agar (SDA) plates supplemented with 0.5% w/v cycloheximide and 1% streptomycin and incubated at 30^0C for 7days. The identified dermatophytic fungal isolates were then sub-cultured onto slanted Sabouraud Dextrose Agar in five bottles and Incubated at a slant angle at 30^0C for 7 days. The five slants of

Sabouraud Dextrose Agar SDA of the test organisms were kept in the refrigerator at 4^0C until when required. Subsequently subculturing was carried out at six months regular time intervals and stored in the refrigerator at 4^0C.

3.4 TESTS FOR IDENTIFYING DERMATOPHYTES.

3.4.1 Microscopy and Cultural characteristics.

Slides were prepared from the appropriate fungi colonies on the plates showing fungi spores. A drop of cotton blue lactophenol was put on the slide. A pinch of the fungi culture was cut and spread on the slide with the aid of an inoculating pin. This was mounted on the microscope using Lactophenol cotton blue, and the morphological characteristics examined and identified using standards. (Summerbell and Germain, 1998)

TABLE 3.0 CHARACTERIZATION OF FUNGI ISOLATES

organisms	**Characteristics in Culture**	**Microscopy**
Trichophyton verrucosum	Slow growing on SDA. Colony is Moist, waxy, flattened, usually white, but may become yellow to buff.	Macroconidia and microconidia usually absent. There is frequent chlamydospores and hyphal swellings.
Trichophyton soudanese	Slow growing flat and folded, suede-lke texture, yellow dried apricot in colour.	Macroconidia absent. Microconidia club shaped characterised by segmentation into arthrospore and reflexive branching.
Trichophyton violaceum	On SDA, Slow growing, waxy wrinkled colony which becomes violet.	Macroconidia and microconidia rarely seen. Numerous hyphal swellings and chlamydospores.
Trichophyton mentagrophytes	Rapid growth, granular, cream white. Reverse side is reddish brown	Macroconidia few but may be numerous, cylindrical and thin walled. Microconidia is usually few, or numerous, round to pear shaped like grape like clusters.

organisms	Characteristics in Culture	Microscopy
Trichophyton rubrum	White velvet cotton growth after two weeks of incubation. Wine red diffusible pigment.	Macroconidia rare in cottony colonies but usually numerous in powdery colonies. Microconidia are numerous, elongated and spiral.
Microsporum canis	On SDA, buff-tan in colour with a characteristic yellow orange pigment	Macroconidia numerous, spindle shaped. Microconidia long and baton shaped along hyphae
Trichophyton tonsurans	Slow growing on SDA. Flat initially, later becomes like crater, powdery white colony, with brown to buff colour.	Macroconidia and hyphal swellings and chlamydospores are numerous. Macroconidia may be distorted if present.

(Elmer, et al., 1997)

3.5 PREPARATION OF FUNGI SPORES SUSPENSION

Spores suspension was prepared from dermatophyte slant culture. The spores were harvested with sterile normal *Saline* containing 0.05% *Tween* 80 to obtain homogenous spores suspension, with the aid of sterile glass beads and standardized to give approximately 10^6 spores per ml, as measured by using a spectrophotometer (Spectronic 20, Bausch and Lamb at 360nm wavelength) Olurinola *et al*.,(1992). The spore suspension was kept in the refrigerator at 4^0 C for subsequent use.

3.6 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) USING AGAR DILUTION METHOD.

Ten milliliters (10mls) volume of varying concentrations of the anti fungal agents such as Fluconazole (FLU) in µg/ml viz 2.00, 5.00, 10.00, 20.00, 40.00, 60.00, 100.00, 200.00, 500.00, 1000.00, 2000.00 and 4000.00 (µg/ml) were prepared. Terbinafine (TB), Flucytosine (FLUCY), Sodium propionate (SP), and Tioconazole (TIO) were prepared similarly. The slant SDA fungi culture stored in the fridge was harvested and standardized (Ehinmidu, 2003). The melted 10ml double strength SDA (45^0C) was aseptically mixed with varying concentration of the antifungal agents. Other antifungal agent SDA admixtures such as Terbinafine (TB), Flucytosine (FLUCY), Sodium propionate (SP) were similarly prepared. Each freshly harvested fungal spores was standardized aseptically and inoculated in duplicates on sterile filter discs placed at equidistance on SDA seeded with varying concentration of antifungal agents suspension.

The inoculated fungus was allowed to diffuse for a period of 30mins. The inoculated plates were then incubated at 30^0 C for 48hrs. Controls were also set up. The susceptibility of each of the test

fungi spores to each drug was recorded using lowest concentration of each compound that inhibits the fungal spores as MIC. (Olurinola *et al*., 1992; Shettima *et al*., 2000,; Ehinmidu 2003).

3.7 DETERMINATION OF THE MINIMUM FUNGICIDAL CONCENTRATION (MFC)

In determining the MFC of the different anti-fungal agents, the filter paper disc that showed no growth during MIC determination was aseptically subcultured into 5ml Sabouraud Dextrose broth, supplemented with 5%v/v polysorbate 80 as inactivator. This medium plus filter paper discs was incubated at 30^0C for 5 days (Olurinola et *al*., 1992,; Shettima *et al*., 2000,; Shettima and Baba., 2005). Visual observations for any visible growth were made. The lowest concentration of the anti-fungal agents that showed no visible growth was taken as the MFC of the test antifungal agents. Control were also set up

3.8 DETERMINATION OF FRACTIONAL INHIBITORY CONCENTRATION (FIC) OF COMBINED ANTIFUNGAL AGENTS IN ADMIXTURES USING AGAR DILUTION METHOD.

Each varying sub inhibitory concentrations of the test anti fungal (e.g. Sodium Propionate 50, 100, 200, 300, 500µg/ml) in 5mls volume each was mixed with fixed sub-inhibitory concentration of another test anti fungal agents (e.g. Fluconazole) in same 5mls. Each of these admixtures in 10ml volume was mixed with melted 10ml volume of sterile double strength SDA aseptically in a Petri-dish. This was allowed to set. Ten microlitres(µl) of standardized harvested most resistant test fungal spores of the suspension (10^6cfu/ml) was inoculated on sterile duplicate filter paper disc aseptically placed at equidistance on the test anti- fungal agents contained in the SDA.

The fungi spore inoculum was allowed to diffuse into the SDA for 30mins. This is incubated at 30^{o} C for 48hrs and the lowest mixed concentrations that showed no growth was taken as the combined MIC of the antifungal agents.

The same procedure was carried out for other test anti fungal agents. (Combination such as Terinafine/Sodium propionate, Tioconazole/ Sodium propionate, fluconazole/ Sodium propionate, and flucytosine/ Sodium propionate, at different fixed concentrations (e.g. 0.5, 1.0, 2.0, 10, 20, 50, 100, 200µg/ml) in 5mls volume).The fractional inhibitory concentration was calculated as reported by Ehinmidu, (2004). i e Fractional Inhibitory Concentration = A + B

Where:

• A = (MIC of combination X + Y) / (MIC of drug X alone)

• B = (MIC of combination X + Y) / (MIC of drug Y alone)

Interpretation:

• Synergistic: FIC </= 0.5

•Additive: FIC = 1

•Antagonistic: FIC >/= 2

Indifference FIC >/= 0.50

3.9 DETERMINATION OF FRACTIONAL FUNGICIDAL CONCENTRATION (FFC) OF COMBINED ANTIFUNGAL AGENTS.

In determining the combined MFC of admixture of test anti-fungal agents, the filter paper disc that showed no visible growths were aseptically transferred into 5ml volume of the sterilized Sabouraud's Dextrose broth, supplemented with 5% tween 80 as inactivator.These was incubated at 30^{0}C for 5 days, the lowest concentration of combined anti-fungal agents that showed no growth was

taken as the combined MFC. The fractional fungicidal concentration (FFC) was calculated as reported by Ehinmidu, (2004).

Fractional fungicidal Concentration Index (FFCI) is the sum of the FFC for each drug. The FFC is defined as the MFC of the drug when used in combination divided by the MFC of the drug when used alone. The Interaction is defined as synergistic if the FFC index is ≤ 0.50, additive . If the FFC is > 0.50 – 1.0, indifferent, If the FFC index is > 1.0 – 2.0 as antagonistic.

3.10 RATE OF KILL OF TEST ANTIFUNGAL AGENTS AGAINST THE MOST RESISTANT TEST DERMATOPHYTIC PATHOGENIC FUNGAL SPORES.

The effect of the fungicidal concentrations of test antifungal agents alone and admixtures on the viability of the spores of test fungi was studied. 10ml volume solutions of the desired single and combined concentrations of antifungal agents were prepared aseptically. These were inoculated with 1.0ml of 10^8 spores of the resistant test fungi.

At different specified time interval, 1.0ml was taken from the reaction mixtures and ten-fold dilutions were made in an inactivating tween 80 diluents (9ml sterilized normal saline with 0.5%w/v tween 80). One millimeter of each dilution was mixed with 19ml of melted SDA at 45^0C and plated in triplicates. The viability of the test fungal spores and the sterility of the SDA medium used were also determined as control. The fungal spores that survived and developed into colonies were counted and documented. These data were used to plot the graph. From the graph plotted the K values and D values will be determined for each of the antifungal agents used, using the equation $K = 2.303/t\{\log_{10} N/No\}$. $D = 1/K$

3.11 DETERMINATION OF EFFECTS OF VARYING pH VALUES ON THE ANTIFUNGAL ACTIVITIES OF POTENTIAL ANTIFUNGAL AGENT COMBINATIONS.

Three different pH (pH 4, 7 and 9) were employed. The various buffers were prepared according to the manufacturers instructions with the combined antifungal agents products and the volume made up to 10mls with sterile distilled water. This was allowed to stand for 30mins and the pH determined. The standardized culture of the test fungi spores (2ml) was used to flood the Sabouraud dextrose agar. The plates were allowed to dry at 37^0C in a sterile incubator. Adopting the cup plate method, a sterile cork borer no 4 (6mm diameter) was used to bore hole in the agar plates. The bottom of the well was sealed with the appropriate molten sterile SDA. Using a micropipette, 0.1ml, appropriate suspensions of the fixed pH buffer with test antifungal agents were inoculated (4%v/v ethanol in sterile distilled water) into the holes. Sterile pH diluents without antifungal agents served as control. These were allowed to diffuse into the agar at room temperature for one hour before incubation at 30^0C for 48hrs.The zones of inhibition of test organisms was measured to the nearest millimeter. This experiment was carried out in duplicates and was repeated for the other pH values used.

3.12 DETERMINATION OF THE EFFECT OF VARYING TEMPERATURE ON THE ANTIFUNGAL ACTIVITY OF POTENTIAL COMBINED ANTIFUNGAL AGENTS.

The procedure in the case of pH study was used for this investigation. The admixture was dissolved in 10mls of sterile diluent, giving a known concentration of the test antifungal agent. The set up was maintained at different temperatures (27^0C, 37^0C, 45^0C, 70^0C, and 100^0C) in the water bath for 30 mins. A 0.1ml of the antifungal admixtures was aseptically dispensed into the hole on the sabouraud dextrose agar plate with test fungal spores flooded and drained. This was allowed to stand for 30

minutes to diffuse before incubation at 30^0C for 48 hours. The zone of inhibition of test fungi growth was measured and documented. Controls were also set up and measured after incubation.

3.13 DETERMINATION OF THE EFFECT OF FORMULATION ON THE ANTIFUNGAL ACTIVITY OF POTENTIAL COMBINED ANTIFUNGAL AGENTS.

The solutions of the combined antifungal agents were prepared in 10ml volume and stored aseptically in a clean dry place. Samples (0.1ml) were taken and assessed for the antifungal activity of the formulation using the agar well diffusion method. 0.1 ml of the combined antifungal agents was aseptically dispensed into the hole bored on the agar plate containing the test fungi spores. The plates were incubated appropriately and the zones of inhibition of test organisms were then measured using calibrated metre ruler. Sterile diluents (5% v/w ethanol in water) were used as the negative control. (Shettima *et al*., 2000)

3.14 FORMULATION OF POTENTIAL FUNGICIDE COMBINATIONS.

Based on the Minimum Fungicidal Concentration (MFC) values of the test antifungal agents, six new products were formulated with ten fold their MFC per gram.

Product A.

Terbinafine ……..5mg

Sodium propionate……750mg

Menthol…….....1gm

Camphor…….3gm

Methanol…….6mls

White soft paraffin…….45gms

Product B

Terbinafine ……5mg

Menthol……..1gm

Camphor……3gm

Methanol……6mls

White soft paraffin……45.5gm

Product C

Sodium propionate…….……750mg

Menthol……..1gm

Camphor…….3gm

Methanol…….……6mls

White soft paraffin…….…….45gms

Product D

Fluconazole……..25mg

Sodium propionate……..…….750mg

Menthol…….…….1g

Camphor……3gm

Methanol…….……..6mls

White soft paraffin……45gms

Product E

Fluconazole……25mg

Menthol…….....1g

Camphor…….3gm

Methanol…….…….....6mls

White soft paraffin……46.0gms

Product F

Tioconazole……250mg

Sodium propionate……750mg

Menthol……1g

White soft paraffin…….…….45gms

All solids were first dissolved in 6mls of methanol. The resultant mixture was gradually mixed with a small quantity of the base by titrating on a tile until smooth. The rest of the base was then added and the resultant ointment packed aseptically into an – tight open mouth jar. (Carter S. J, 1982).

CHAPTER FOUR

4.0 RESULTS.

The organisms isolated are *Trichophyton mentagrophytes, Trichophyton soudanese, Trichophyton verrucosum, Trichophyton violaceum, Trichophyton rubrum, Trichophyton tonsurans* and *Microsporum canis*. Eighty – one dermatophytic fungi were characterized. Susceptibility of the 81 isolates was carried out before the most resistant ones were isolated for the study using agar diffusion method. Five most resistant dermatophytic fungi isolates namely *Trichophyton rubrum* (Tr10), *Trichophyton mentagrophytes* (Tm 07), *Trichophyton tonsurans* (Tt 06), *Trichophyton verrucosum* (Tv 03), *Trichophyton soudanese* (Ts 01), were selected after preliminary susceptibility investigation of eighty – one fungal isolates. An analysis of the result showed that 52 samples was collected from Fahimta Primary school, 68 samples from Vintage primary school, Zaria Nigeria and 30 samples from Aminu primary school. The incidence of dermatophytic fungi isolates from male and female volunteers in the three schools showed that males had higher number of isolates 59, and *Trichophyton* species were much in number 77 when compared to *Microsporum canis* 4. (Table 1).

Table 4.1. INCIDENCE OF DERMATOPHYTIC FUNGI ISOLATES FROM MALE AND FEMALE VOLUNTEERS IN THREE PRIMARY SCHOOLS IN ZARIA.

Schools	**No of volunteers.**	**No of fungi isolates from each sex**		**No of isolates according to different genera.**	
		Male	**Female**	***Trichophyton* species.**	***Microsporum canis.***
Vintage	**68**	**17**	**8**	**25**	**-**
Aminu	**30**	**26**	**4**	**28**	**2**
Fahimta	**52**	**16**	**10**	**24**	**2**
Total	**150**	**59**	**22**	**77**	**4**

The results showed that T*richophyton mentagrophytes* had the highest 18, followed by *Trichophyton soudanese* 16. *Microsporum canis* and *Trichophyton violaceum* had the least 4 isolates each. The total isolates were 81 as shown in figure 4.2.

TABLE 4.2 DISTRIBUTION AND PERCENTAGE FREQUENCIES OF DERMATOPHYTIC FUNGI ISOLATED FROM SCHOOL CHILDREN WITH CLINICAL SYMPTOMS OF *TINEA CAPITIS* IN ZARIA NIGERIA.

Organisms	**No of isolates**	**% Frequency**
T. mentagrophytes	18	22.2
T. soudanese	16	19.8
T. verrucosum	14	17.3
T.tonsurans	13	16
T.rubrum	12	14.8
T. violaceum	4	4.9
Microsporum canis	4	4.9
Total	81	100%

4.2 CLINICAL MICRO ORGANISMS ISOLATED AND USED IN THIS STUDY ARE AS FOLLOWS.

a). *Trichophyton tonsurans*

b). *Trichophyton verrucosum*

c). *Trichophyton mentagrophytes*

d). *Trichophyton soudanese*

e). *Trichophyton rubrum*

4.3 MINIMUM INHIBITORY CONCENTRATION (MIC) OF THE TEST ANTIFUNGAL AGENTS AGAINST DERMATOPHYTE FUNGI SPORES FROM THE INFECTED SCHOOL CHILDREN.

The Minimum Inhibitory Concentration of the test antifungal agents on the test organisms are as shown in table 4.2. The M.I.C values of Flucytosine, Terbinafine HCL, and Tioconazole range from 0.50 to 100.00µg/ml against the five selected test fungi spores. The antifungal activity of Sodium Propionate ranges from 50.0 to 250.0 µg/ml against the five dermatophytic spores. It appears that the order of inhibitory antifungal activity is Fluconazole>flucytosine>Terbinafine> Tioconazole > Sodium propionate as displayed.

TABLE 4.3 MINIMUM INHIBITORY CONCENTRATION VALUES OF TEST ANTIFUNGAL AGENTS AGAINST THE FUNGAL ISOLATES (µg/ml)

ANTIFUNGAL AGENTS	Tm	Ts	Tt	Tr	Tv
Flucytosine	0.50	0.50	0.50	2.50	0.50
Tioconazole	1.00	1.00	25.00	100.00	1.00
Terbinafine	0.50	0.50	10.00	1.00	0.50
Fluconazole	0.50	0.50	0.50	5.00	0.50
Sodium Propionate	50.00	50.00	50.00	250.00	50.00

KEY. Tm = *Trichophyton mentagrophytes*

Ts = *Trichophyton soudanese*

Tt = *Trichophyton tonsurans*

Tr =*Trichophyton rubrum*

Tv = *Trichophyton verucosum*

4.4 MINIMUM FUNGICIDAL CONCENTRATION (MFC) OF THE TEST ANTIFUNGAL AGENTS AGAINST DERMATOPHYTIC FUNGI SPORES FROM THE INFECTED SCHOOL CHILDREN.

Similarly the Minimum Fungicidal Concentrations of the test antifungal agents against the five selected test fungi spores are shown in table 4.3. A perusal of this results in the tables, showed that the order of the antifungal activities of the test chemical compounds is Fluconazole, > Terbinafine > Flucytosine > Tioconazole > Sodium Propionate.

TABLE4.4. MINIMUM FUNGICIDAL CONCENTRATION VALUES OF FLUCYTOSINE, TERBINAFINE, FLUCONAZOLE, TIOCONAZOLE, AND SODIUM PROPIONATE AGAINST TEST FUNGAL SPORES ISOLATES (µg/ml).

ANTIFUNGAL AGENTS	**Tm**	**Ts**	**Tt**	**Tv**	**Tr**
Flucytosine	2.50	250.00	1.00	1.00	25.00
Tioconazole	2.50	100.00	100.00	2.50	500.00
Terbinafine	1.00	1.00	25.00	1.00	10.00
Fluconazole	5.00	1.00	1.00	1.00	25.00
Sodium Propionate	100.00	250.00	250.00	100.00	1500.00

KEY. Tm =*Trichophyton mentagrophytes*

Ts =*Trichophyton soudanese*

Tt =*Trichophyton tonsurans*

Tr =*Trichophyton rubrum*

Tv =*Trichophyton verucosum*

4.5 DETERMINATION OF MINIMUM FUNGICIDAL CONCENTRATIONS OF THE TEST ANTIFUNGAL AGENTS IN ADMIXTURES.

The results of the combination of test antifungal agents against the most resistant *T rubrum* generally showed that the result were synergistic for Terbinafine/Sodium Propionate combinations , Fluconazole/Sodium Propionate combinations, against the test organism. (Table 4.3 and 4.4)

TABLE4.5 FRACTIONAL INHIBITORY CONCENTRATION (FIC) OF TERBINAFINE HYDROCHLORIDE AND SODIUM PROPIONATE AGAINST *TRICHOPHYTON TONSURANS* (10^6 spores/ml) AFTER 7 DAYS INCUBATION.

TB MIC 10.00	**TB COMB**	**FIC**	**SP MIC 50.00**	**SP COMB**	**FIC**	**∑FIC**
	0.10	0.01		5.00	0.10	0.11
	0.20	0.02		5.00	0.10	0.12
	0.30	0.03		5.00	0.10	0.13
	0.40	0.04		5.00	0.10	0.14
	0.80	0.08		5.00	0.10	0.18
						0.68

∑FICs/n = 0.68/5 =0.14. Markedly synergistic.

4.6 SUMMARY OF THE FICs OF THE TEST ANTIFUNGAL AGENTS IN COMBINATION

The sum of FICs were found to be markedly synergistic as showed in Table 4.4

TABLE 4.6. SUMMARY OF THE FICs OF THE TEST ANTIFUNGAL AGENTS IN COMBINATION.

combination of antifungal agents	***T. mentagrophytes***	***T. soudanese***	***T. verrucosum***	***T. tonsurans***	***T. rubrum.***
TerbinafineHCl/ Sodium Propionate	0.60	0.60	0.60	0.14	0.38
Fluconazole/Sodium Propionate	0.78	0.70	0.88	0.83	0.53
Tioconazole/Sodium propionate.	1.40	1.40	1.40	1.10	0.26
Flucytosine/Sodium Propionate.	2.80	2.80	2.80	2.80	0.55

KEY. FICs < 1= Synergistic

FICs ≥ 1 ≤ 4 = Additive

FICs > 4 =Antagonistics.

4.7 BIOCIDAL EFFECT OF 0.156µM CONCENTRATIONS OF TEST ANTIFUNGAL AGENTS AGAINST RESISTANT *T. RUBRUM* SPORES SUSPENSIONS.

Observation from the biocidal activities of the test compounds showed that at 0.156µM concentration, all the test antifungal agents displayed marked fungicidal activities. The order of

observed fungicidal activity at 0.156µM concentration was Terbinafine HCl >Tioconazole >Sodium propionate > Fluconazole, with one log cycle reduction time of 5.0, 7.5, 15.0, and 20.0 minutes respectively (Table 4.6.)

TABLE 4.7. BIOCIDAL ACTIVITIES OF 0.156µM OF TEST ANTIFUNGAL AGENTS AGAINST RESISTANT *T. RUBRUM* SPORES.

Test Antifungal Agents	D-10 values (minutes)	K-Values (minutes)
Terbinafine	5.0	0.46
Tioconazole	7.5	0.31
Sodium Propionate	15.0	0.15
Fluconazole	20.0	0.12

After the first 30 minutes contact time in the admixtures, the log cycles reduction of resistant *T.rubrum* spores were in the following order;

Terbinafine HCL (3.90) > Tioconazole (2.40) > Fluconazole (1.20) > Sodium Propionate (1.50) log cycles reductions. (Fig 4.1). This observation confirmed the results of the D_{10} –values of the test antifungal agents profiles mentioned above. Generally, there was rapid rate of fungicidal activity among the test antifungal agents within the first 50 minutes. Thereafter there was a slow rate of fungicidal activity as shown in figures 4.1 below.

The K values of the test antifungal agents at 0.156µM against T.*rubrum* showed that there was a general reduction in the test fungal spores viability with increase in contact time. Furthermore there was initial rapid rate of test fungal spores kill of the most susceptible ones. As time progresses, the rate of spores death per unit time gradually reduces until it remained fairly constant, figures 4.2.

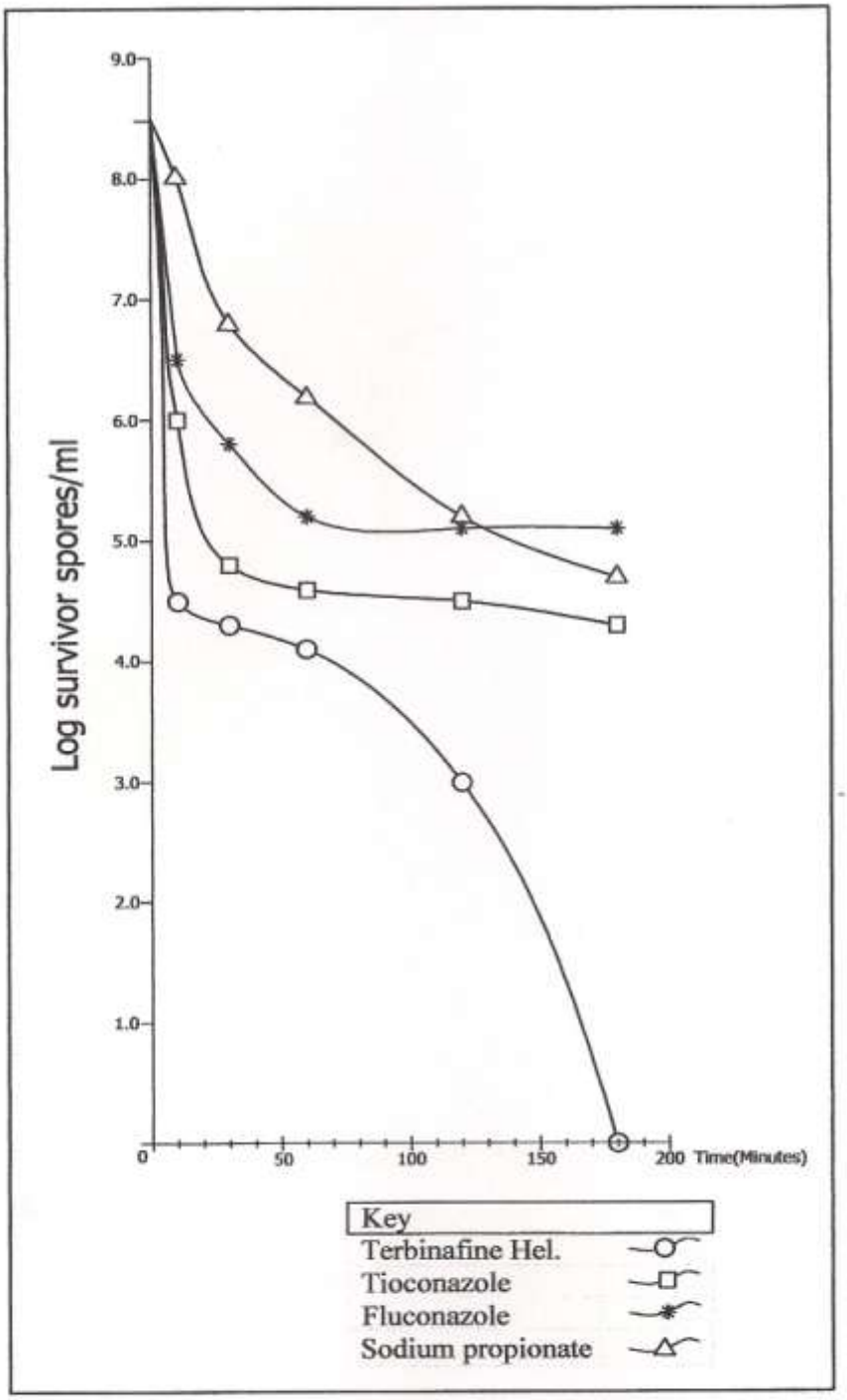


Fig 4.1 The biocidal effect of 0.156µM Terbinafine Hcl, Tioconazole, Sodium Propionate and Fluconazole on the survival resistant *T. rubrum* spores at 25 ± 2°c.

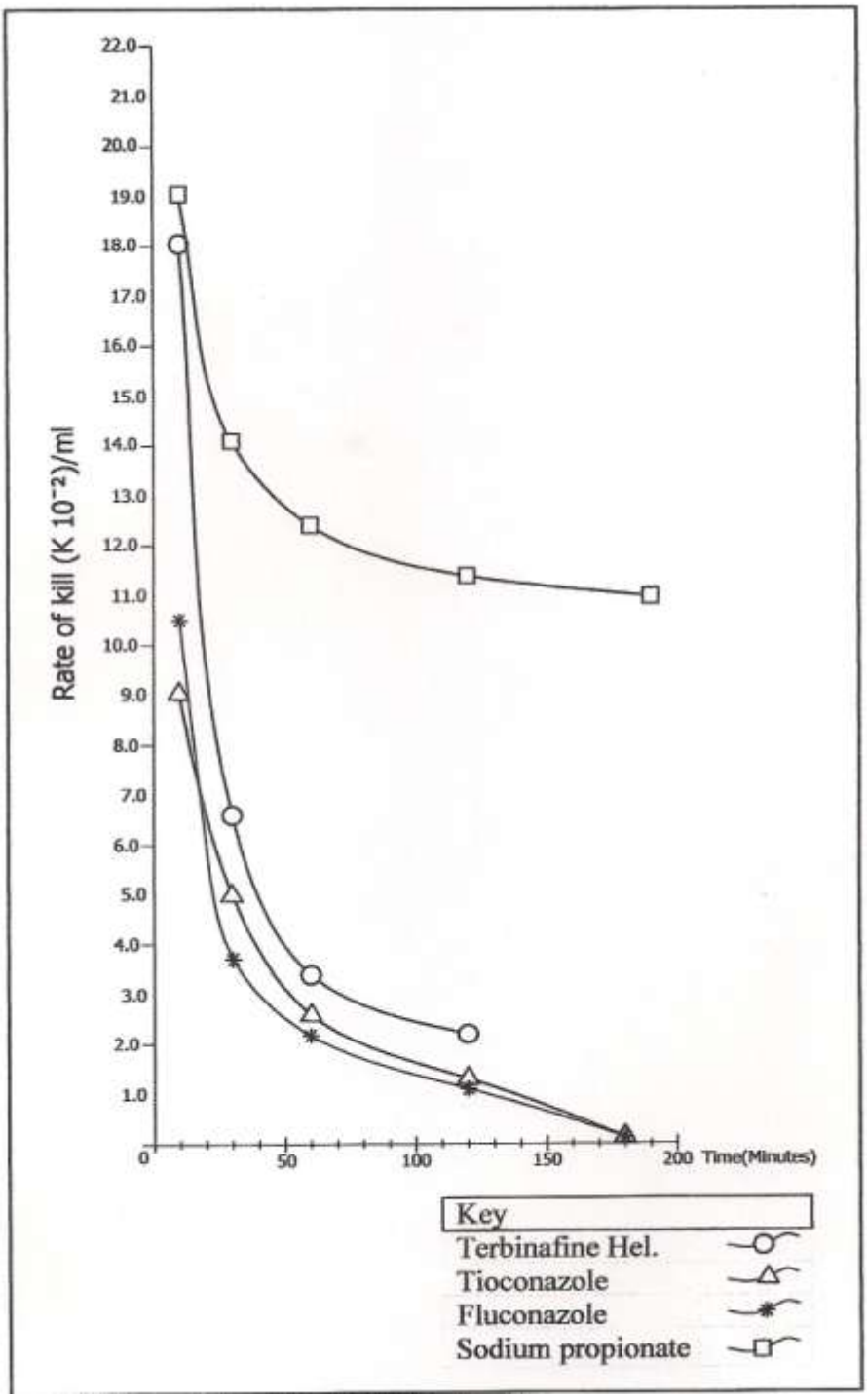


Fig 4.2. influence of contact time on Terbinafine HCl, Sodium propionate, Tioconazole, and Fluconazole at 0.156µM fungicidal concentration on the rate of kill of *T. rubrum*

4.8 EFFECT OF VARYING BIOCIDAL CONCENTRATION OF TEST ANTIFUNGAL AGENT AT 30 MINUTES FIXED CONTACT TIME.

Observation of biocidal activities of test antifungal agents at varying concentrations indicated a rapid initial lethal phase generally.The fungicidal activities decrease with increase in concentration, e.g as from 250µg/ml and above, for fluconazole, there was generally no significant increase in the biocidal activities against the test fungal spores. Hence, 250µg/ml appears to be effective threshold concentration for fluconazole. Similarly Sodium propionate displayed 15,000µg/ml as it's threshold fungicidal concentrations as observed in this study. For tioconazole and fluconazole, there was no threshold values observed (Fig 4.3 – 4.6).

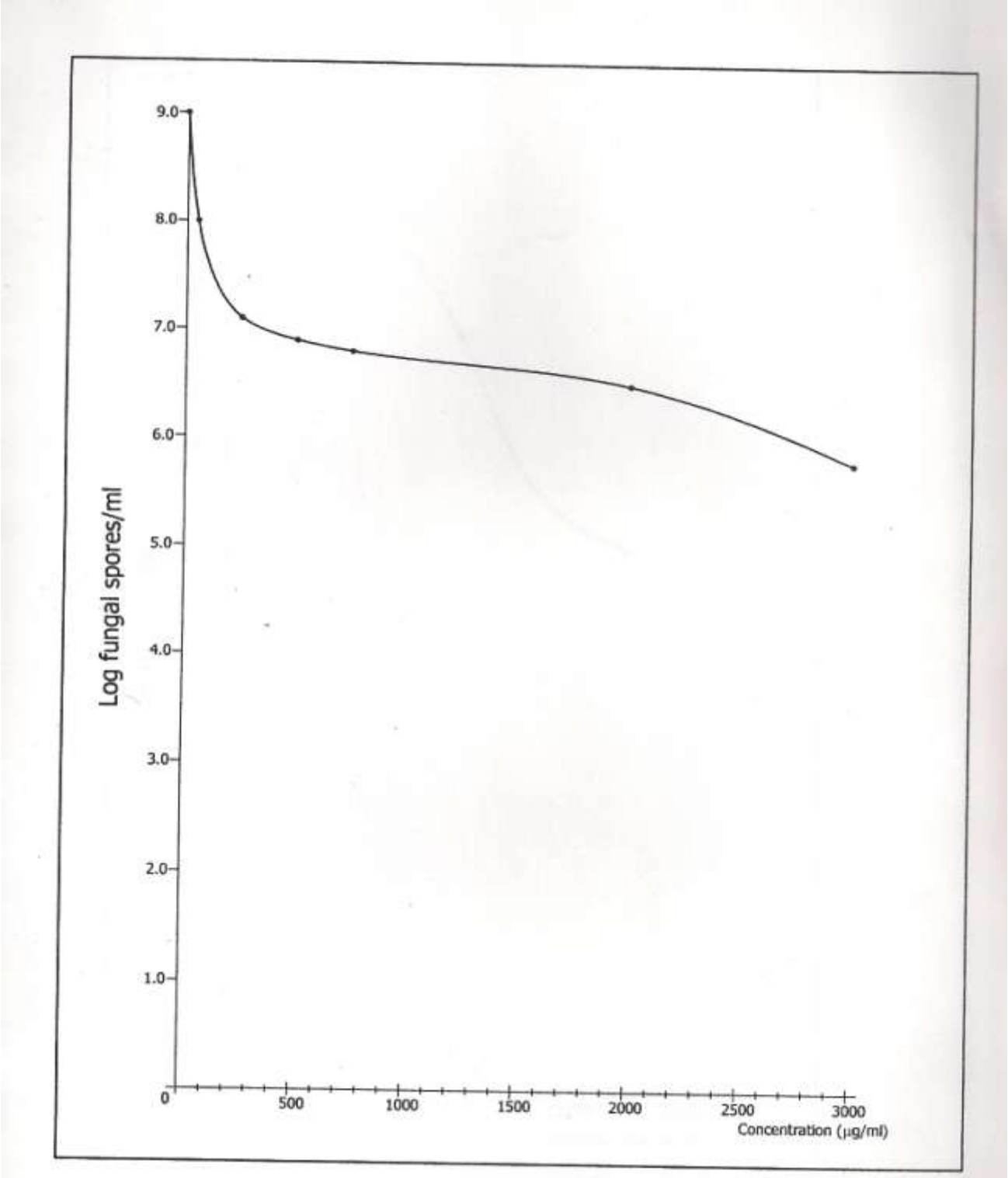


Fig 4.3 Biocidal effect of different concentration of Fluconazole on resistant spores of *T.rubrum* at 30 mins contact time

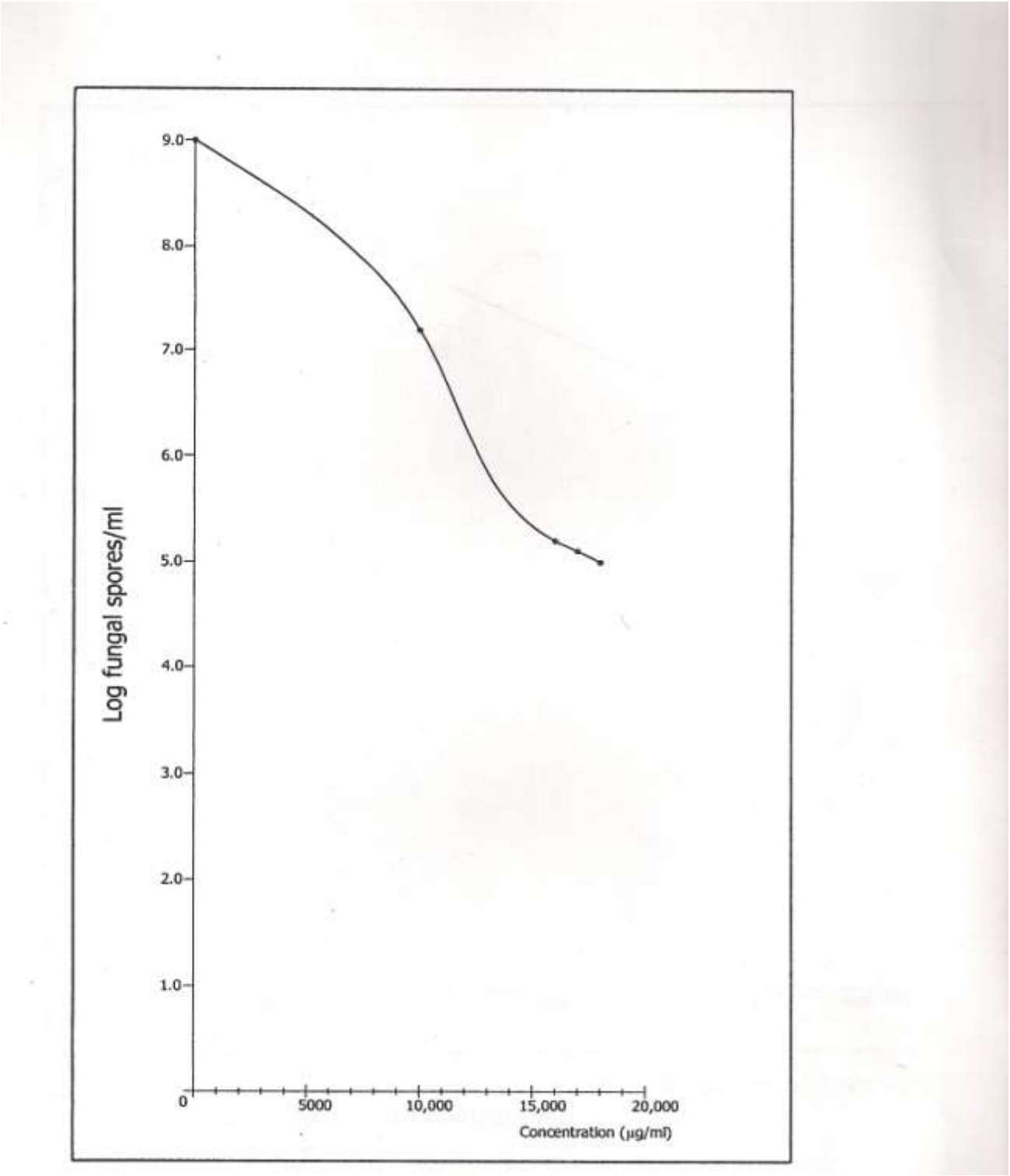


Fig 4.4 Biocidal effect of different concentration of Sodium propionate on resistant spores of *T.rubrum* at 30 mins contact time

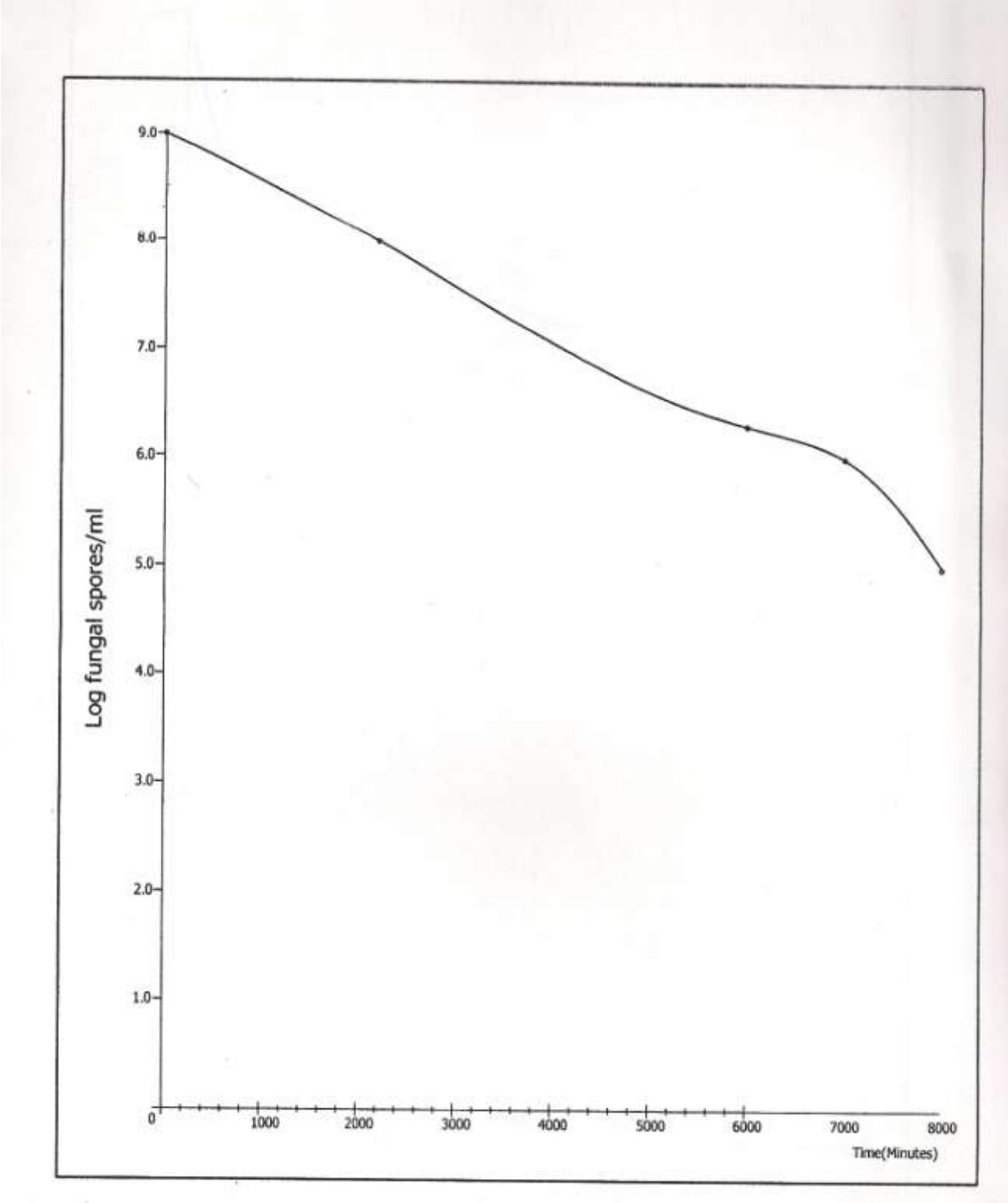


Fig 4.5 Biocidal effect of different concentration of Tioconazole on resistant spores of *T. rubrum* at 30 mins contact time

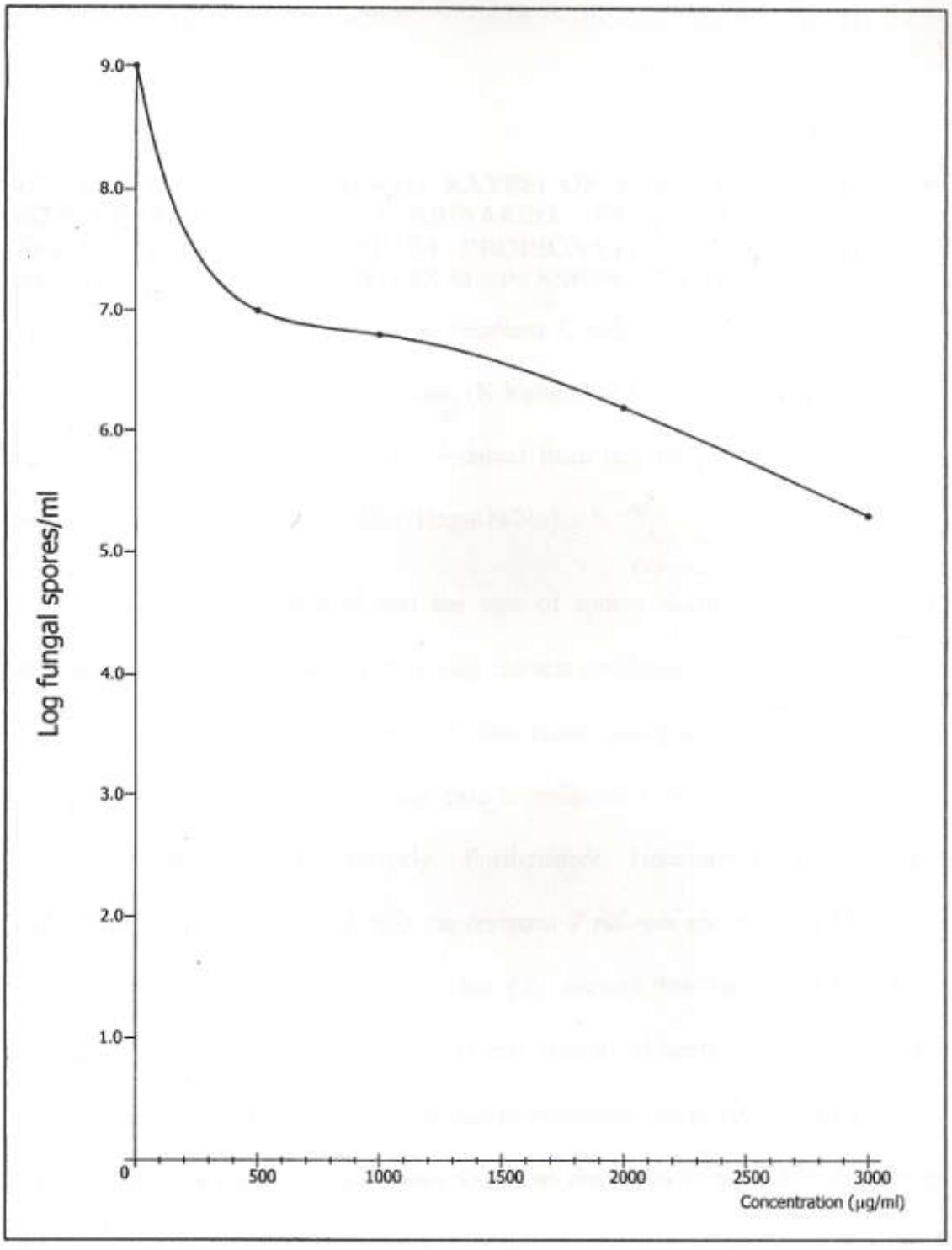


Fig 4.6 Biocidal effect of different concentration of Terbinafine HCL on resistant spores of *T.rubrum* at 30 mins contact time

4.9 THE K- VALUES (DEATH RATES) OF VARYING FUNGICIDAL CONCENTRATIONS OF TERBINAFINE HCL, FLUCONAZOLE, TIOCONAZOLE AND SODIUM PROPIONATE ON 10^8CFU/ML OF RESISTANT *T. RUBRUM* SPORES SUSPENSION AT 27 ± 2^0C.

Table 4.8 shows the rate of kill of the resistant *T. rubrum spores* suspension after 30 minutes contact time. The death rate, (K Values) of 10^8CFU/ml of spores of test fungi was calculated from the data obtained from test spores survivors at fixed time.

The observed results showed that the rate of spore death was concentration dependent. These K-values varies among the test antifungal agents. {For example Terbinafine HCl and Fluconazole at the same concentration of 3000µg/ml displayed 4.50 and 4.40 x 10^{-2} death rate of resistant *T. rubrum* spores after 30 minutes contact time respectively. Furthermore Tioconazole at the same concentration of 3000 µg/ml killed the resistant *T rubrum* spores (10^8CFU/ml) at 4.20 x 10^{-2} K-values. These results (Table 4.8) showed that the rate of kill of the resistant *T rubrum* spores suspension is test antifungal agent specific. Although Fluconazole and Tioconazole both belong to imidazole antifungal agents yet their K values against the test fungi spores varies as displayed in fig 4.7.

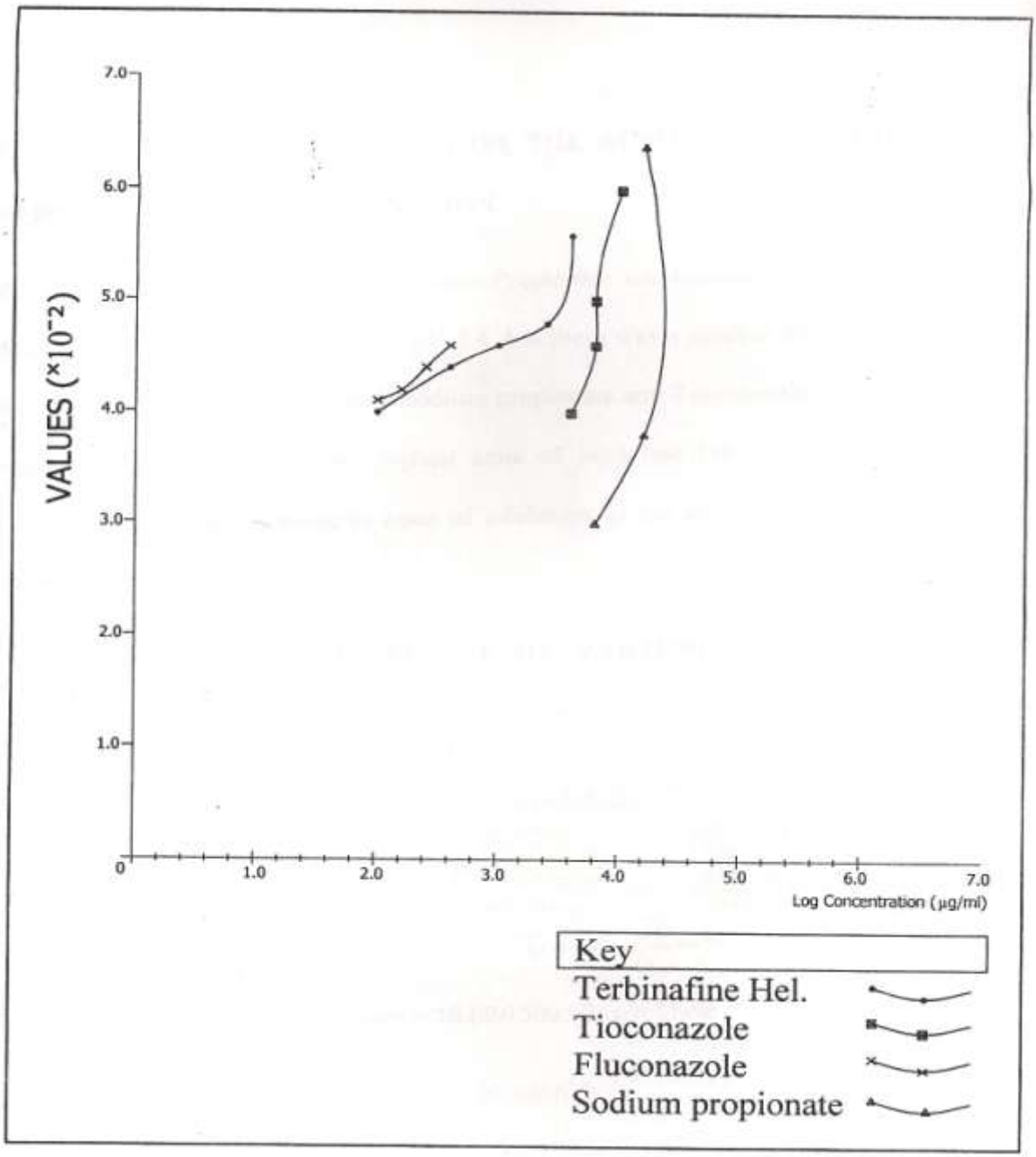


Fig. 4.7. The effect of varying fungicidal concentration after 30 mins contact time on the rate of kill (k value) of test *T.rubrum* spores suspension at 27 ± 2^0C.

4.10 THE EFFECT OF VARYING pH ON THE ACTIVITIES OF TEST ANTIFUNGAL AGENTS COMBINATION.

Table 4.10 showed that Terbinafine / Sodium propionate combination displayed a sharp increase in zone of inhibition at pH 7.4, but there was a gradual decrease in zone at pH 9.0. However fluconazole/sodium propionate and Tioconazole /sodium propionate combinations showed highest zone of inhibition (40mm) at pH 4 (acidic), with gradual decrease in zone of inhibition as the pH moved towards alkaline.

TABLE 4.10. THE EFFECT OF VARYING pH ON THE ZONE OF INHIBITION (MM) OF DIFFERENT ANTIFUNGAL AGENTS AFTER 24HRS AT 30 ^{O}C.

pH	**Antifungal Agents combinations**		
	Terbinafine/Sodium propionate	**Fluconazole/Sodium Propionate**	**Tioconazole/Sodium Propionate**
4.0	13±1.00	23.25±0.80	40±0.00
7.4	35±5.00	21.20±1.0	35±0.00
9.0	27.25±2.5	20±0.00	30±0.00

Key

Terbinafine HCL/Sodium propionate = 10.00/1500.00µg/ml/hole

Fluconazole/sodium propionate = 25.00/1500 µg/ml/hole

Tioconazole/sodium propionate =5.00/1500 µg/ml/hole

4.11 THE EFFECT OF VARYING TEMPERATURE ON THE ACTIVITIES OF TEST ANTIFUNGAL AGENTS COMBINATIONS.

Table 4.11 shows that the three different combinations displayed a pattern of gradual increase in zone of inhibition as the temperature increase from 27^0C to37^0C. However, there was a sharp reduction in the zone of inhibition as the temperature increased to 100^oC.

TABLE 4.11 THE EFFECT OF VARYING TEMPERATURE ON ZONE OF INHIBITION OF DIFFERENT ANTIFUNGAL AGENTS AFTER 24HRS AT 30 ^{O}C.

Temperature ^{O}C.	**Antifungal Agents Combinations.**		
	Tioconazole/Sodium propionate	**Terbinafine/Sodium propionate**	**Fluconazole/Sodium propionate**.
27	26.00±0.70	27.50±1.10	27.50±2.50
37	35.50±0.50	38.00±0.12	35.50±2.50
45	26.25±0.80	25.05±0.70	30.00±0.05
70	23.00±2.10	19.50±1.80	30.0±0.02
100	22.00±1.30	20.00±0.00	28.50±0.01

Key

Terbinafine HCL/Sodium propionate = 10.00/1500.00µg/ml/hole

Fluconazole/sodium propionate = 25.00/1500 µg/ml/hole

Tioconazole/sodium propionate =5.00/1500 µg/ml/hole

27 ^{0}C (room temperature) was used as the control.

TABLE 4.12 ZONE OF INHIBITION OF COMBINED ANTIFUNGAL AGENTS AT 30^0C FOR 24HRS ON SABOURAUD DEXTROSE AGAR (MM).

(A) Terbinafine/Sodium Propionate	**(B) Fluconazole/Sodium propionate**	**(C) Tioconazole/sodium propionate**	**(D) .**
39.00±1.00	38.00±0.05	38.00±0.20	36.00±0.25

Key

(A)Terbinafine HCL/Sodium propionate = 1.0/150.0µg/hole

(B)Fluconazole/sodium propionate = 2.50/150.0 µg/hole

(C) Tioconazole/sodium propionate =50.0/150.0 µg/hole

(D) Trosyds (Tioconazole) = 1000.00 µg/hole.

The results obtained showed that the chemical agents in the combinations displayed potential antifungal activities against the resistant *Trichophyton rubrum* spores. The order of the observed zone of inhibition was TerbinafineHCl/Sodium propionate (39.00mm) > Fluconazole/Sodium propionate (38.00mm), and Tioconazole/Sodium Propionate (38.00mm) > trosyds (36.00mm).

CHAPTER FIVE

5.0 DISCUSSION CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

Tinea capitis is a highly contagious infection of the scalp and hair caused by dermatophytes such as *Microsporum canis, Trichophyton mentagrophytes* etc.It occurs in all age groups but predominantly in children (*Fuller et al., 2003).* It is endemic in some of the poorest countries (*Gonzalez et al., 2004,)* . One of the greatest problem hindering the eradication and prevention of *Tinea capitis* is the presence of healthy asymptomatic carriers. It has been reported that asymptomatic carriers might be equal to symptomatic sufferers *(Gonzalez, et al., 2004).* As many as 14% of asymptomatic children have been found to be carriers of causative dermatophytes for *Tinea capitis* in a primary school in Philadelphia (William *et al.,* 1995). In Nigeria, as much as 30.6% of children have been reported to have *Tinea capitis* in Jos, Plateau State and in Gboko, Benue state (Ayanbimpe *et al*., 2008). In the South East Anambara State 42% had *Tinea capitis*.*Tinea capitis* has been declared a major public health problem. (Barlow and Saxe, 1998). In Ile Ife, the incidence of *Tinea capitis* varied greatly as reported by Ajao and Akintunde (1985).

5.2 THE PREVALENCE OF DERMATOPHYTIC INFECTION IN PRIMARY SCHOOL IN SABON GARI, LOCAL GOVERNMENT ZARIA, NIGERIA.

Tinea capitis has been reported to be caused by *Trichophyton* species which infects mostly children of primary school age. (*Gonzalez et a.*, 2004,*). T*he result of the isolation from volunteered school children in this study showed that *Trichophyton* species (96-0%) table 4.0 were the most prevalent isolated organisms. This agreed with the works of Higgins *et al*., (2000), that *Trichophyton tonsurans* was found to be the major cause of *Tinea capiti*s in USA, and in United Kingdom.

Ayanbimpe *et al*., (2008) in a related study found out that *Trichophyton soudanese* had the highest percentage (30 .6%) as the causative organism for *Tinea capitis* in Benue and Jos regions, while in Anambara State it was *Trichophyton rubrum* (Barlow and Saxe, 1998).

The high prevalence of *Tinea capitis* among school children has been attributed to the presence of even numbered fatty acids in the sebum surrounding the hair of children, as opposed to the odd numbered fatty acids surrounding the hair in adults. (Gonzalez 2007). It was also stated that the odd number fatty acids are more effective in preventing the growth of fungi spores. The increase in production of saturated fatty acids with fungistatic activity in the sebum by the action of androgen hormones could be responsible for the spontaneous reduction in the prevalence of *Tinea capitis* after puberty.

5.3 THE MICs AND MFCs OF THE TEST ANTIFUNGAL AGENTS.

The increase in dermatophytes infection rates and the high level of therapeutic failures warrant the search for new therapeutic strategies. (Goldstein, A. O and Goldstein B. G. 2011.., Tasumi *et al*., 2002). Griseofulvin has been the drug of choice for the treatment of *Tinea capitis*. (Fuller *et al.*, 2003 and Gupta *et al*., 2001). However in a recent survey of Griseofulvin treatment of *Tinea capitis*, approximately 40% of patients did not respond to the drug and required additional treatment (William and Wyandt, 2005).Five antifungal agents namely, Fluconazole, Flucytosine, Terbinafine, Sodium Propionate and Tioconazole were used in this study. These antifungal agents were effective against dermatophytic fungal spores, (*Trichophyton mentagrophytes, Trichophyton verucosum, Tichophyton tonsurans, Trichophyton soudanese* and the most resistant *Trichophyton rubrum*) isolated from school children.

The Minimum Inhibitory Concentrations values obtained in this study showed that flucytosine was the most effective antifungal agent with MIC values range from 0.5 to 2.50 µg/ml.The order of inhibitory antifungal activity showed flucytosine (0.50-2.50 µg/ml)> Fluconazole (0.50-5.0 µg/ml)>Terbinafine (0.50-10.00µg/ml)>Tioconazole (1.00-100.00 µg/ml)> Sodium propionate (50.00-250.00 µg/ml) against the test dermatophytic fungi spore (10^6CFU/ml). Fluconazole (0.00-0.50 µg/ml) and Flucytosine (0.50-2.50 µg/ml) displayed highly effective antifungal activities at low concentrations (MIC) compared to terbinafine HCL (0.50-10.00 µg/ml) Tioconazole (1.00-100 µg/ml) and Sodium propionate (50.0-250.0 µg/ml). Fluconazole belongs to the new generation antifungal agents introduced into Nigerian market approximately 20 years ago, with high cost. Flucytosine is also an expensive drug. Therefore it may be that their high cost reduces their abuse, hence the reason for the high level of antifungal activities observed.

Trichophyton mentagrophytes and *Trichophyton verucosum* were the most susceptible to the antifungal agents, while *Trichophyton rubrum* was the most resistant test dermatophytic spores under this investigation. Observation from this study showed that the order of fungicidal activities of the test antifungal agents was Terbinafine HCl (1.0-25.0µg/ml) = Fluconazole (1.0-25.0 µg/ml) > Flucytosine (1.0-250 µg/ml) >Tioconazole (2.5-500.0 µg/ml) > Sodium propionate. The high cost of of Flucytosine and its mode of administration probably limit the abuse of this drug, hence its high antifungal activity compared to Tioconazole and Sodium propionate.

5.4 Activities of Combined Antifungal Agents

The FIC values of the combined antifungal agents in this study, showed synergistic antifungal activities For example, when Terbinafine HCl was added to sodium propionate, the FIC values was 0.14 with *T. tonsurans* spores. This value indicated a strong synergistic action. All the antifungal

agents combinations studied, strongly showed synergistic actions against the most resistant *T. rubrum spore* (Table 4.5). Imidazole and Terbinafine HCl have been reported to inhibit fungal cytochrome p450 enzyme 14α-demethylase, and squalene epoxidase enzymes respectively. These antifungal agents thereby render the fungal membrane more porous to cytoplasmic poisoning activity of sodium propionate. Thus little quantity of Sodium propionate will be required in combination than when acting alone. Hence the synergistic effects observed in this study.

This observation suggests the probable potential of the studied antifungal agents combinations for a useful formulation to combat the problem of *Tinea capitis*. The reduction in antifungal agents concentrations offer a cost effective formulation with less toxicity challenges.

5.5 Rate of kill of Resistant *T. rubrum*.

The results of the rate of kill of spores of resistant *T. rubrum* at 0, 10, 30, 50, 60, 180 minutes by the same molecular weight test antifungal agents showed rapid fungicidal activities as displayed by figure 4.1 It was observed that the fungicidal activities of each test antifungal agents were different. For example, Tioconazole and Fluconazole displayed an initial rapid rate of kill and there after, the rate of kill was drastically reduced until it became constant at the same concentrations of 0.156µM. After 30 minutes contact time the log cycles reductions of resistant *T. rubrum* showed that the order of fungicidal activity was Terbinafine HCl (3.90)>Tioconazole (2.40) >Sodium propionate(1.50)> Fluconazole (1.20) Figure (4.1).

K values (death rate) of the test antifungal agents at 0.156µM concentrations against resistant *Trichophyton rubrum*s spores *showed* that there was an initial rapid rate of kill of the most susceptible fungal spores. This was followed by the ‘shoulder’ and ‘tailing’ effect, as was described by Denyer and Hugo (2004). The killing dynamics was of a first order reaction whereby one log

reduction (D value) was achieved within the first 10 minutes (fig 4.2). Calculation of D values assumes a linear type E survival curve (Denyer and Hugo, 2004).The biocidal effect of different antifungal agents at varying concentrations at 30minutes contact time displayed a rapid lethal phase. The fungicidal activity decreases with increase in concentration of the antifungal agent. Fluconazole at threshold concentration of 250µg/ml did not display any further significant increase in biocidal activity against the test fungal spores. Also, sodium propionate at threshold concentration of 15000µg/ml followed the same pattern as fluconazole above (fig 4.3 -4.6).The death rate showed that the rate of spore death was concentration dependent (Table 4.8).Terbinafine HCl and fluconazole at the same concentration of 300µg/ml displayed 5.70 and $5.20x10^{-2}$ death rates of resistant *T. rubrum* spores after 30 minutes. Tioconazole at the same concentration of 3000µg/ml killed resistant *T. rubrum* (10^{8}CFU/ml at $4.20x10^{-2}$). This observation agrees with Woakes et al., (1991), that the killing of cells is chiefly a function of time and concentration range. This result showed that the rate of kill of the resistant *T. rubrum* spores suspension was test antifungal specific. Although tioconazole and fluconazole belong to the azole antifungal agent, yet their K values against the test fungi spores vary (Table 4.8 and fig 4.7).

The activity of different antifungal agents appears to be affected by changes in pH. At acidic pH there is a general increase in antifungal activity displayed by terbinafine /sodium propionate combinations (pH 7.4), but there was a gradual decrease in zone as pH moved towards alkaline. This is supported by the findings of Ozcan *et al*., (2009), that adding salts to terbinafine increases the antifungal capability of terbinafine at pH 2.0 to pH 5.8.

Tioconazole/sodium propionate and fluconazole/sodium propionate displayed the highest zone of inhibition (40mm) at pH 4(acidic) with a gradual decrease in zone of inhibition as the pH moved towards alkaline (Table4.9). The imidazoles have a degree of dissociation (referred to as alpha) of

0.55 in the intracellular compartment and these remain constant despite changes in temperature (ie the pK is changing with change in temperature). This theory about the constancy of the imidazole alpha value as proposed by Kerry (2006) has been termed the imidazole alphastat hypothesis and is also stated by Woakes et al., (1991).

Also Imidazole (1,3-diaza-2,4-cyclopentadiene) is a planar five-member ring system with 3C and 2N atom in 1 and 3 positions The simplest member of the imidazole family is imidazole itself, a compound with molecular formula $C_3H_4N_2$. The systemic name for the compound is 1, 3 diazole, one of the annular N bear a H atom and can be regarded as a pyrole type N. It is soluble in water and other polar solvents. It exists in two equivalent tautomeric forms because the hydrogen atom can be located on either of the two nitrogen atoms. Imidazole is a highly polar compound, as evidenced by a calculated dipole of 3.61D, and is entirely soluble in water. The compound is classified as aromatic due to the presence of a sextet of π-electrons, consisting of a pair of electron from the protonated nitrogen atom and one from each of the remaining four atoms of the ring.Imidazole is amphoteric, *i.e.* it can function as both an acid and as a base. As an acid, the p*K*a of imidazole is 14.5, making it less acidic than carboxylic acids, phenols, and amides, but slightly more acidic than alcohols. The acidic proton is located on N-1. As a base, the p*K*a of the conjugate acid (cited above as p*K*BH + to avoid confusion between the two) is approximately 7, making imidazole approximately sixty times more basic than pyridine. The basic site is N-3 (Hatnager *et al*., 2011).

The antifungal activity of the different combinations of antifungal agents at different temperature displayed an increase in activity as the temperature moved from 27^{o}C to 45^{o}C. However there was a gradual decrease in activity as the temperature moved from 45^{o}C to 100^{o}C.This could be attributed to the thermal denaturing of the chemical structure of the compounds as temperature moved from 45^{o}C to 100^{o}C. Langner and Maibach (2010), have observed that an increase in heat or temperature

is not always accompanied by an increase in kinetic energy or potential energy. They further noted that in some instance the heat could be used to change the bonding between the molecules rather than be used to speed up the molecules. The result of the potential combined antifungal agents against resistant *Trichophyton rubrum* displayed significant antifungal activities as showed in table 4.11. For example Terbinafine HCl/sodium propionate (1.0/150.0µg/hole) combinations displayed the highest zone of inhibition (39mm), while trosyds (1000.0 µg/hole) showed the least zone of inhibition. The study further showed that the adjuvants in these combined fungicides have no significant negative effects observed on the antifungal activity of antifungal agents combinations in the formulation. It is interesting to note that Tioconazole concentration in formulation D was twenty folds greater than Tioconazole concentration in combination C, however the antifungal activities displayed by combination C (38.00mm zone of inhibition) was superior to formulation D (36.00mm zone of inhibition). This is an evidence of synergistic activities of the Sodium propionate added to the lesser Tioconazole in the combination.

5.6 CONCLUSION

The following conclusions were drawn from the result of the work carried out on the schools

(a) *Trichophyton rubrum*, *Trichophyton verucosum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans, and Trichophyton soudanese* were isolated from pupils with clinical symptoms of *Tinea capitis* from some selected primary schools in Zaria, Nigeria.

(b) Fluconazole, TerbinafineHCL, Tioconazole, and Sodium propionate were found to posses appreciable antifungal activity against isolated dermatophytic fungi spores in Zaria, Nigeria

(c) Fluconazole and Terbinafine HCl were found to be the most effective antifungal agent against resistant *Trichophyton rubrum* spores in Zaria Nigeria.

(d) The combination of Terbinafine HCl/Sodium propionate, Fluconazole/Sodium propionate, Tioconazole/Sodium propionate were found to display marked synergistic activity, both at sub inhibitory concentrations and at fungicidal level against the resistant *T. rubrum* spores in Zaria, Nigeria.

(e) The threshold value of Fluconazole was 250µg/ml while Sodium propionate displayed a threshold value of 15000µg/ml. An increase in concentration did not produce a significant inhibition of the resistant *T. rubrum* spores under study.

(f) The antifungal agents were found to be most effective at temperature range of 37^{o}C -45^{o}C,.

(g) The combined antifungal agents displayed better antifungal activity at acidic pH and there was a gradual decrease in antifungal activity at alkaline pH.

5.7 RECOMMENDATIONS.

From the results obtained in this study it is recommended that further studies should be carried out to determine the relationship between in vitro and in vivo effects of these combinations. This will help to study the antifungal activities of these combinations at the target sites, before these combinations are produced in commercial formulations.

5.8 LIMITATIONS

The limitation encountered in this research includes the issue of patenting the formulation. In addition is the issue of carrying out toxicologic studies on the formulation. Other limitations includes lack of access to swab the scalp of some school pupils. This was due to the fact that the school authorities refused authorization.

REFERENCES

Ajao A O, Akintunde C (1985). *Studies on the prevalence of Tinea capitis* infection in Ile-Ife, Nigeria. *Mycopathologia.* 89(1):43-48.

Ayanbimpe G. M, Taghir H, Diya A, and Wapwera, S. (*2008*). *Tinea capitis* Among Primary School Children in Some Parts of Central Nigeria. *Mycoses.* 51(4): 336-40.

Baginski. M, Czub. J. (2009) Amphotericin B and its new derivatives - Mode of Action . 10(5):459-69.

Barlow, D. and Saxe, N. (1998). *Tinea Capitis* in Adults. *International Journal of Dermatology*, 27(6): 388–390.

Bennett, D. I. and Zonios, J. E. (2008). Update on Azole Antifungals. *Journal of antimicrobial chemotherapy* . 48 (3): 345-350.

Birgit W. Gerd K, Sabine E, Elisabeth P, Gerhard K, Ferdinand H, Petra A (1997). Disseminated Infection with *Nattrassia mangiferae* Onchomycosis. *Journal of Clinical Microbiology*/42/1/478

Bossche V. N, Engelen, M, Rochette, F (2003).Antifungal Agents of Use in Animal Health, Chemical, Biochemical and Pharmacological Aspects. Belgium. *Journal Vetinary Pharmacol. therapy.26, 5-29.*

Bronson D, M, Desai D R, Barsky S, Foley J (1999) Epidemiological Study of *Tinea* C*apitis* in Puerto Rico. *Puerto Rico Health Sci* 12: 287–289.

Budavari, S. and Hernando, F (1996). *Mycosis vaccines* - US Patent 6290950.

Carter S .J. (1982). Cooper and Gunns Dispensing for pharmaceutical students 12th Edition. Pitman publishing Inc. Massachusetts U.S.A. Pp 192-231.

Chapman S. W. (2008). In search of The Holy Grail of Antifungal Therapy *Trans Am Clin Climatol Assoc* V.119; 197-215.

Cohen B. A, Ghannoum, M., Isham, N., McBride A. (2005). Prevalence of *Tinea Capitis* and *Tinea Pedis* in Barcelona Schools**.** *Pediatric Infectious Disease Journal*: 24 (2) Pp 137-141.

Cuenca, E . M. (2004). Combinations of Antifungal Agents in Therapy *Journal of Antimicrobial Chemotherapy*. 54, 854–869.

De Hoog, G.S., Guarro, J., Gene, J., Figueras, M.J. *(*2000). Canine Dermatophytosis cased by *Trichophyton tonsurans***.** Journal of Medical Microbiology, 55, 1583–1586

Denyer and Hugo (2004). Pharmaceutical Microbiology. Pp 356, 435. Blackwell publishers USA

Ellis and Hermanis (2003). *Dermatophytes.* (Imagebank) (n.d) Geraldine Kaminski Medical Mycology Laboratory. Retrieved April 8, 2010 from www.drfungus.com

Elias J. A, Michael R. M, Gubbins O. P. (2009). *Antifungal Therapy* Spectrum of Activity *of T*erbinafine and *Mechanism of Actions*. Springer London. Retrieved from June 6, 2010, From Springeramazon.com Pg 317 - 322

Enemour, S.C and Amedu. S.C. (2009). Prevalence of Superficial Mycoses in Primary School Children in Anyigba, Kogi State, Nigeria. *African Journal of Microbiology Research* 3 (2) . 062-065.

Enweani I B, Agbonlahor, R. N, Ozan, D. E. *C.* (2009), Dermatophytosis in School Children in Ekpoma, Nigeria. *Dermatology Journal Home* (39) 7-8.

Ehinmidu, J .O (*2003*)**.** Antibiotic Susceptibility Patterns of Urine Bacterial Isolates in Zaria, Nigeria. *Tropical Journal of pharmaceutical Research*. 2.(2) 223-228.

Ehinmidu, J.O (*2004*). Isolation and Antibiotic Susceptibility Profile of *Neisseria gonorrhea* Isolated from Urine Samples in Zaria Northern Nigeria. *Journal of Phytomedcine and Therapeutics*. Vol 9**. (**Jan- Dec, 2004). 20-24.

Elmer, N.K, Stephen, D.A, Dowell, V.R, Herbert, M.(1997). Colour Atlas and Textbook of Diagnostic Microbiology. Lippincott Company U.S.A. 5th ed. Pp 86 - 91

Fuller L C, Child F J, Midgley G, Higgins E M. (2001). Diagnosis and Management of Scalp Ringworm. *British Medical journal*. London. 326: 539-41.
.

Fuller, L. C Child, F..J, Midgley, G. Higgins, E.M (2003).*Guidelines for the management of Dermatophyte infections of the skin.. British Medical Journal* 326 (7388): 539–541.

Gayosso, P Manzano, L J Méndez-Tovar (1994). *Dermatophytosis in Mexico*. 36(1) 17-20. National Center for Biotechnology U.S. National Library of Medicine Rockville Pike**,** Bethesda MD**, USA**

Germain. G**,** and Summerbell**,** R.(1999). Identifying Filamentous Fungi: **A Clinical Laboratory Handbook.** Star Publishing, Belmont. 147 (1, 61, 314).

Ghannoum, M. A., Rice, L. B. *(*1999*).* In Vitro and In Vivo Effects of Itraconazole on Cytochrome P-450 14alpha -Demethylase (ERG11) Depletion. USA. *Journal of American Society for Microbiology*. 12(4): 501–517

Goldstein, A. O and Goldstein B. G. (2011). Dermatophyte infections are common disorder worldwide, and dermatophytes . *Oral terbinafine, itraconazole, and fluconazole are additional therapeutic options*. Proceedings of Pharmacy and Therapeutics Committee Meeting at Alabama Medicaid Agency. November 9, 2011. Series No 27. Alabama

González, U., Seaton, T. Bergus, G. Jacobson J, Martínez-Monzón C (2007) Systemic antifungal therapy for *Tinea Capitis* in Children. .*Cochrane Database Sys Rev.* Retrived on May 29, 2009 from www.thecochranelibrary.com.

Gupta, A. K Hofstader, S.L. R , Adam P , Summerbell .R.C sophie L R,(1999).*Tinea Capitis*: An Overview with Emphasis on Management. *Pediatric Dermatology* , 16,(3), pp 171–189, .

Gupta A. K and Summerbells R C (2000).Tinea *capitis* is a common dermatophyte Infection of the Scalp in Children. *Med Mycol*.38(4):255-87.

Hatnager, A, Sharma P. K, Kurma N, (2011). A Review on Imidazoles, Their Chemistry and Pharmacological Potentials. India. *International journal of PharmTech Research*. 3.(1), 268-282.

Higgins E. M, .Fuller L C, Child F. J, Midgley G, (2000). The epidemiology of *Tinea capitis*. *International journal of .Epidemiology* ,22 325: 461:

Hudson M. M.(2001).Antifungal Resistance. *Journal of Antimicrobial Chemotherapy*. 48 (3) : 345-350.

Hugo and Russell (2004). Pharmaceutical Microbiology. 7th edition.12 219. Blackwell. USA.

Jill A. M and Richard T. P**.** (2002). Amphotericin B (AmBisome): Liposomal Formulation, Structure, Mechanism of Action and Pre-Clinical Experience. *The British journal for Antimicrobial Chemotherapy*. 49, 21–30.

Johnson, M. D. MacDougall, C., and Ostrosky Z. L (*2004*). Combination Antifungal Therapy**.** *Antimicrobial Agents Chemotherapy* **2004**;48:693-715.

Kao, G F (2008).*Tinea capitis* is a disease caused by superficial fungal**.** *E medicine*. 8 October 2009. www.medscape.com/article/1091351-overview:

Kenneth A. A, Jeffrey T. S. Hsu H. S (2007). *Manual of dermatologic therapeutics (9780781760584).* Lippincot Williams & Wilkins London. Retrieved 10 May 2009 from www. books.google.com.ng/books.

Kerry, B. (2006). *1.6 Alphastat hypothesis of imiazoles. Acid-base physiology*. Retrieved May 14, 2010, from Http;//www.anaesthesiamcq/acidbasebook/.com.

Khosravi, A.R. Aghamirian M. R, and. Mahmoudi, M (2009). Dermatophytoses in Iran. *Mycoses*, 37: 43-48.

Kunze K. L, Wienkers L C, Thummel K. E, Trager W. F (2005). Warfarin-fluconazole Inhibition of the human cytochrome. P450 dependent metabolism of wafarian by fluconazole. *Drug Metab Dispos* 24(4): 414-21.

Langner, M. D and Maibach, H .I (2010). Many Common Drugs in Dermatology are Light,Temperature or Moisture Sensitive.Skin Care Guide USA. Vol 16. 22-28.

Laniado L. A, and Maria N. V. (2009) Amphotericin B. Adverse Effects. Toxicity.and Side Effects. *Mexico.Ther Clin Risk* 4(6) 1261 -80

Lewis, J. H., H. J. Zimmerman, G. D. Benton, and K. G. Ishak,K. J. McLean, P. Carroll, D. G., A. J. Dunford, H. E. Seward, R. Neeli,.(2007). In Vivo and In Vitro Characterization of a First in Class Novel Azole. *Mol Pharmacol* 80: 124 – 135.

Marcin N, Marcin H, Lucjan S Wyrwicz,, Piotr S, Dariusz M., Plewczynski, Michal L, Krzysztof G., and Leszek
R.(2010). Detailed Mechanism of Squalene Epoxidase Inhibition by Terbinafine. *J. of Chem. Inf. and Modelling.* 51 (2), 455-462.

Mbakwem-Aniebo, C. and *Ezekwem,* L.C .(2010)**. C**omparable Etiology of T*inea capitis* in Mgbuoba, Rivers State. *Nigerian journal of microbiology* (NJM) ISSN 07941293.

Micol, I. (2007). New generation azole antifungals in clinical investigation. *Curr clin pharmacol* 2, 37 – 55.5.

Michallet M. Sutton D A, Sanche S E, Revankar S G, (2009). Approaches to the Management of Invasive Fungal Infections in Amphotericin *B. S*pectrum of activity. *Journal of clinical onchology*. vol. 27. (20) 3398-3409

Olurinola *P F,* Onaolapo J A, and Olayinka A. T.(1992). A Survey of Dermatophytes Associated with Athlete's Foot. *Sokoto Journal of Veterinary Sciences Vol.1 No*. 1. 6.

Ozcan, I, Abaci O, Uztan A. H, Aksu B, Ozer, O (2009).
Enhanced topical delivery of terbinafine hydrochloride. *NCBI NIH*. AAPSPharmSciTech vol 10(3)

Pearson M M, Rogers P. D, Cleary J. D, Chapman S. W.Wang E. J, Lew K, Casciano C. (2004).
Interactions with Azole Antifungal Agents in Transplant Recipients. *Exp. Rev. Anti Infect Ther* : 2: 485-97.

Pfaller M.A, Boyken L, Messer S.A, Hollis R.J, Diekema D.J. (2004).Stability of Mueller-Hinton agar supplemented with glucose and methylene blue for disk diffusion testing of fluconazole and voriconazole. *J Clin Microbiol* 2004;42:1288-9.

Pfaller, M.A., Messer, S.A., Hollis, R.J., Jones, R.N., Doern, G.V., Brandt, M.E., Hajjeh, R.A. (1999).Trends in species distribution and susceptibility to fluconazole among blood stream isolates of Candida species in the United States. Diagn Microbiol Infect Dis., 33, 217-222.

Segall M (1997**).** An ab initio study of biological systems. The catalytic cycle of cytochrome P450**.** Unpublished MSc Thesis. University of Cambridge. London.

Shettima ,Y.Y, Karumi E. O, Addy G. I, Anyanwu , U, Omoregie, H. C, Opara, V. V. Yarling, J. S, Yanaga**,** E. T, Fadipe, A.I Ehinmidu, J. O, Haruna A. K and Ilyas, M (2000). Patterns of Dermatophytosis in Jos North, Plateau State of Nigeria. *Nigerian Journal of Experimental and Applied Biology*. Vol 1 No 1. Pp 30

Shettima K. and Baba, A. T. (*2005*,).
Response of tomato (Lycopersicon esculentum Mill) to foliar application as an Alternative to Griseofulvin in the Treatment of *Tinea* Cruris. *Sokoto Journal of Veterinary Sciences* Vol.1 No. 1. Pp 35 -65

Sutton DA, Sanche S E, Revankar S. G (1990).
Amphotericin B in the treatment of invasive fungal Disease. *Journal of Clinical Infectious Disease*. 2005; 40: 392- 400

Tatsumi, Y, Mamoru Y, Hisato S, and Kazuaki K (2002).
Therapeutic Efficacy of Topically Applied KP-103 against Experimental *Tinea Unguium* in Guinea Pigs. *Antimicrob Agents Chemother*. 46(12) 3797–3801.

Vermes A, Guchelaar, and Dankert, J. (*2000*). Flucytosine: a review of its pharmacology, clinical Indications, pharmacokinatics, toxicity and drug interactions. *Journal of Antimicrobial Chemotherapy*. (46).171- 179.

Williams J. V, Honig P. J, McGinley K. J, Leyden J. J. (2005) Semiquantitative study of*Tinea capitis* and the asymptomatic carrier state in inner-city school children Boulevard. *American Academy of Pediatrics*.(96) :265– 267.

Woakes, A.J, Grieshaber M.K, Bridges, C .R (1991).
Physiological Strategies for Gas Exchange and Metabolism. Booktopia pty Ltd. Australia. www.booktopia.com.au. Pp 106

APPENDIX

APP 1. PREPARATION OF MEDIA

i) Preparation of Sabourad's Dextrose Agar. (SDA)

A Single strength; sixty two grammes (62.0g) of SDA was weighed and dissolved in 1000mls of distilled water. The mixture was brought to boiling with continuous stirring to dissolve. 10mls was dispensed into Mac Cartneys bottles and sterilized by autoclaving at 121^0C for 15mins. The bottles were then slanted and the prepared SDA were allowed to solidify.

B Double strength; one hundred and twenty four grammes of SDA (124g) was weighed and dissolved in 1000mls of distilled water. The mixture was brought to boiling with continuous stirring to dissolve. 10mls was dispensed into MacCartney bottles and sterilized by autoclaving at 121^0C for 15mins. The bottles were then cooled and the prepared SDA were kept in a clean environment until required.

ii) Preparation of Sabourad's dextrose liquid media.

Thirty grammes (30.0g) of the powder will be weighed and dissolved in 1000mls of distilled water. 5mls were then dispensed into MacCartney bottles and sterilized by autoclaving at 121^0C for 15mins. The bottles were then kept in aseptic environment until required,

iii) Preparation of inactivating media

The medium will be prepared by dissolving 0.9g of NaCl and a 5%v/vTween 80 respectively. The mixture was then heated to enhance solubility.9mls was dispensed into MacCartney bottles and sterilized by autoclaving at 121^0C for 15mins.

iv) Preparation of dispersing media

Normal saline (0.9g of NACL) was weighed and dissolved in 100mls of distilled water. 0.05mls of Tween 80 was added and heated to enhance solubility.9mls was dispensed into MacCartney bottles and sterilize by autoclaving at 121^0C for 15mins and kept until required.

APP 2. PREPARATION OF STOCK SUSPENSION OF THE ANTI-FUNGAL AGENTS

i) **FLUCONAZOLE**

Fluconazole powder (100g) was weighed and dissolved in 10mls of 50%v/v ethanol and was made up to 20mls with sterile distilled water aseptically to give a stock concentration of 5,000µg/ml. This solution was kept at 4oc until required for use.

ii) **TERBINAFINE.**

Terbinafine (250mg) powder was dissolved in 10mls of 50%v/v ethanol and was made up to 20mls with sterile distilled water to give a stock concentration 12,500µg/ml

ii) SODIUM PROPIONATE

The sodium propionate solution was prepared by weighing 60grams of the compound and dissolved in 250ml of sterile distilled water.This was used in the investigation.

TABLE APP 3. K VALUES OF VARYING FUNGICIDAL CONCENTRATION OF TEST ANTIFUNGAL AGENTS AFTER 30 MINUTES CONTACT TIME WITH 10^8CFU/ML OF RESISTANT *T. RUBRUM* SPORES SUSPENSION AT 27±2^{O}C.

Antifungal Agents	K-Values ($x10^{-2}$) (time)	Log concentrations.
Terbinafine	4.00	2.00
	4.40	2.70
	4.50	3.00
	4.80	3.30
	5.70	3.50
Tioconazole	4.20	3.00
	4.80	3.80
	5.00	3.90
	6.00	4.00
Fluconazole	4.20	2.40
	4.30	2.70
	4.40	3.00
	4.60	3.30
	5.20	3.50
Sodium Propionate	4.60	4.10
	5.80	4.20
	5.90	4.30
	6.00	4.40

TABLE APP 4.SYNERISTIC EFFECT OF SUB –INHIBITORY CONCENTRATION OF FLUCONAZOLE AND SODIUM PROPIONATE AGAINST TRCHOPHYTON SOUDANESE(10^6SPORES/ML) AT 30^0C FOR 7 DAYS.).

FLUC MIC 0.5	FLU COMB	FIC	SP MIC 50	SP COMB	FIC	∑FIC
	0.1	0.2		15	0.3	0.5
	0.2	0.4		15	0.3	0.7
	0.3	0.6		5	0.1	0.7
	0.4	0.8		5	0.1	0.9

MEAN OF ∑FIC/N=2.8/4=0.70
COMB OF FIC =0.70 = SYNERGISTICS.

TABLE APP 5. SYNERISTIC EFFECT OF SUB –INHIBITORY CONCENTRATION OF FLUCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON VERRUCOSUM (10^6SPORES/ML) AT 30^0C FOR 7 DAYS.).

FLUC MIC 0.5	FLU COMB	FIC	SP MIC 50	SP COMB	FIC	∑FIC
	0.1	0.2		30	0.6	0.8
	0.2	0.4		15	0.3	0.7
	0.3	0.6		15	0.3	0.9
	0.4	0.8		15	0.3	1.1

MEAN OF ∑FIC/N=3.5/4=0.88
COMB OF FIC =0.88 = SYNERGISTICS.

TABLE APP 6. SYNERISTIC EFFECT OF SUB –INHIBITORY CONCENTRATION OF FLUCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON RUBRUM (10^6SPORES/ML) AT 30^0C FOR 7 DAYS.).

FLUC MIC 5.00	FLU COMB	FIC	SP MIC 250	SP COMB	FIC	∑FIC
	0.1	0.2		15	0.06	0.26
	0.2	0.4		5	0.02	0.42
	0.3	0.6		5	0.03	0.62
	0.4	0.8		5	0.02	0.82

MEAN OF ∑FIC/N=2.12/4=0.53
COMB OF FIC =0.53 = SYNERGISTICS.

TABLE APP 7. SYNERISTIC EFFECT OF SUB –INHIBITORY CONCENTRATION OF FLUCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON TONSURANS (10^6SPORES/ML) AT 30^0C FOR 7 DAYS.).

FLUC MIC 0.5	FLU COMB	FIC	SP MIC 50	SP COMB	FIC	∑FIC
	0.1	0.2		30	0.6	0.8
	0.2	0.4		15	0.3	0.7
	0.3	0.6		15	0.3	0.9
	0.4	0.8		5	0.1	0.9

MEAN OF ∑FIC/N=33/4=0.83
COMB OF FIC =0.83 = SYNERGISTICS.

TABLE APP 8. SYNERISTIC EFFECT OF SUB –INHIBITORY CONCENTRATION OF FLUCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON MENTAGROPHYTES (10^6SPORES/ML) AT 30^0C FOR 7 DAYS.).

FLUC MIC 0.50	FLU COMB	FIC	SP MIC 50	SP COMB	FIC	∑FIC
	0.1	0.2		30	0.6	0.8
	0.2	0.4		15	0.3	0.7
	0.3	0.6		5	0.1	0.7
	0.4	0.8		5	0.1	0.9

MEAN OF ∑FIC/N=3.1/4=0.78
COMB OF FIC =0.78 = SYNERGISTICS.

TABLE APP 9. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TERBINAFINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON RUBRUM (10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TERB MIC 1.00	TERB COMB	FIC	SP 250	MIC	SP COMB	FIC	∑FIC
	0.1	0.1			5	0.02	0.12
	0.2	0.2			5	0.02	0.22
	0.3	0.3			5	0.02	0.32
	0.4	0.4			5	0.02	0.42
	0.8	0.8			5	0.02	0.82

MEAN OF ∑FIC = 1.9/5 = 0.38 SYNERGISTICS.

TABLE APP 10. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TERBINAFINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON TONSURANS (10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TERB MIC	TERB COMB	FIC	SP MIC	SP COMB	FIC	∑FIC
10.00			250			
	0.1	0.01		5	0.10	0.11
	0.2	0.02		5	0.10	0.12
	0.3	0.03		5	0.10	0.13
	0.4	0.04		5	0.10	0.14
	0.8	0.08		5	0.10	0.18

MEAN OF ∑FIC = 0.68/5 = 0.14 SYNERGISTICS.

TABLE APP 11. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TERBINAFINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON VERRUCOSUM(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TERB MIC	TERB COMB	FIC	SP MIC	SP COMB	FIC	∑FIC
0.50			50.00			
	0.1	0.2		5	0.1	0.30
	0.2	0.4		5	0.1	0.50
	0.3	0.6		5	0.1	0.70
	0.4	0.8		5	0.1	0.90

MEAN OF ∑FIC = 2.4/4 = 0.6 SYNERGISTICS.

TABLE APP 12. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TERBINAFINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON SOUDANESE(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TERB MIC	TERB COMB	FIC	SP MIC	SP COMB	FIC	∑FIC
0.50			50.00			
	0.1	0.2		5	0.1	0.30
	0.2	0.4		5	0.1	0.50
	0.3	0.6		5	0.1	0.70
	0.4	0.8		5	0.1	0.90

MEAN OF ∑FIC = 2.4/4 = 0.6 SYNERGISTICS.

TABLE APP 13. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TERBINAFINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON MENTAGROPHYTES(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TERB MIC	TERB COMB	FIC	SP MIC	SP COMB	FIC	∑FIC
0.50			50.00			
	0.1	0.2		5	0.1	0.30
	0.2	0.4		5	0.1	0.50
	0.3	0.6		5	0.1	0.70
	0.4	0.8		5	0.1	0.90

MEAN OF ∑FIC = 2.4/4 = 0.6 SYNERGISTICS.

TABLE APP 14. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TIOCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON MENTAGROPHYTES(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TIO	MIC	TIO COMB	FIC	SP	MIC	SP COMB	FIC	∑FIC
1.O				50.00				
		0.2	0.2			45	0.9	1.10
		0.4	0.4			45	0.9	1.30
		0.6	0.6			45	0.9	1.50
		0.8	0.8			45	0.9	1.70

MEAN OF ∑FIC = 5.6/4 = 1.4

TABLE APP 15. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TIOCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON SOUDANESE(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TIO	MIC	TIO COMB	FIC	SP	MIC	SP COMB	FIC	∑FIC
1.0				50.00				
		0.2	0.2			45	0.9	1.10
		0.4	0.4			45	0.9	1.30
		0.6	0.6			45	0.9	1.50
		0.8	0.8			45	0.9	1.70

MEAN OF ∑FIC = 5.6/4 = 1.4

TABLE APP 16. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TIOCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON VERRUCOSUM(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TIO	MIC	TIO COMB	FIC	SP	MIC	SP COMB	FIC	∑FIC
1.0				50.00				
		0.2	0.2			45	0.9	1.10
		0.4	0.4			45	0.9	1.30
		0.6	0.6			45	0.9	1.50
		0.8	0.8			45	0.9	1.70

MEAN OF ∑FIC = 5.6/4 = 1.4

TABLE APP 17. SYNERGISTIC EFFECT OF SUB INHIBITORY CONCENTRATION OF TIOCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON TONSURANS(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.)

TIO	MIC	TIO COMB	FIC	SP	MIC	SP COMB	FIC	∑FIC
25.0				50.00				
		0.8	0.032			45	0.9	0.932
		2.0	0.030			45	0.9	0.980
		5.0	0.200			45	0.9	1.100
		10.0	0.400			45	0.9	1.300

MEAN OF ∑FIC = 4.312/4 = 1.078

TABLE APP 18. SYNERGISTICS EFFECT OF SUB INHIBITORY CONCENTRATION OF TIOCONAZOLE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON RUBRUM(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.).

TIO MIC 100	TIO COMB	FIC	SP MIC 250	SP COMB	FIC	∑FIC
	0.2	0.002		45	0.18	0.182
	0.4	0.004		45	0.18	0.184
	0.6	0.006		45	0.18	0.186
	0.8	0.008		45	0.18	0.188
	2.0	0.020		45	0.18	0.200
	5.0	0.050		45	0.18	0.230
	10.0	0.100		45	0.18	0.280
	25.0	0.250		45	0.06	0.310
	50.0	0.500		45	0.06	0.560

MEAN OF ∑FIC = 2.32/9 = 0.26

TABLE APP 19. SYNERGISTICS EFFECT OF SUB INHIBITORY CONCENTRATION OF FLUCYTOSINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON RUBRUM(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.).

FLUCY MIC 2.50	FLUCY COMB	FIC	SP MIC 250	SP IN COMB	FIC	∑FIC
	0.4	0.20		100	0.40	0.60
	0.8	0.32		45	0.18	0.50

MEAN OF ∑FIC/N = 1.10/2=0.55

TABLE APP 20.SYNERGISTICS EFFECT OF SUB INHIBITORY CONCENTRATION OF FLUCYTOSINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON MENTAGROPHYTES(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.).

FLUCY MIC 0.50	FLUCY COMB	FIC	SP MIC 50.0	SP IN COMB	FIC	∑FIC
	0.4	0.8		100 45	2.00	2.80

MEAN OF ∑FIC/N = 2.80/1=2.8O

TABLE APP 21. SYNERGISTICS EFFECT OF SUB INHIBITORY CONCENTRATION OF FLUCYTOSINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON SOUDANESE(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.).

FLUCY MIC 0.50	FLUCY COMB	FIC	SP MIC 50.0	SP IN COMB	FIC	∑FIC
	0.4	0.8		100 45	2.00	2.80

MEAN OF ∑FIC/N = 2.80/1=2.8O

TABLE APP 22.SYNERGISTICS EFFECT OF SUB INHIBITORY CONCENTRATION OF FLUCYTOSINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON TONSURANS(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.).

FLUCY MIC 0.50	FLUCY COMB	FIC	SP	MIC	SP COMB	IN	FIC	∑FIC
	0.4	0.8			100 45		2.00	2.80

MEAN OF ∑FIC/N = 2.80/1=2.8O

TABLE APP 23. SYNERGISTICS EFFECT OF SUB INHIBITORY CONCENTRATION OF FLUCYTOSINE AND SODIUM PROPIONATE AGAINST TRICHOPHYTON VERRUCOSUM(10 ^{6}SPORES/ML AT 30 ^{0}C FOR 7 DAYS.).

FLUCY MIC 0.50	FLUCY COMB	FIC	SP	MIC	SP COMB	IN	FIC	∑FIC
	0.4	0.8			100 45		2.00	2.80

MEAN OF ∑FIC/N = 2.80/1=2.8O

TABLE APP 24. K values of Tb, Flu, SP, and Tio at 0.156µM fungcidal concentration on T. rubrum at varying time interval. K values (x10^{-2}).

Time	TB	FLU	TIO	SP
10	18.7	10.8	11.8	11.5
30	6.5	3.7	5.0	4.1
60	3.4	2.2	2.6	2.4
120	2.3	1.1	1.3	1.4
180	0.0	0.8	0.9	1.0

Performing the t test on the table above

HO. The rate of kill of *T. rubrum* by Tb, and Flu at 0.156 µM cidal concentrations is the same.

HA. The rate of kill of *T. rubrum* by Tb, and Flu at 0.156 µM bio cidal concentrations is not the same

TABLE APP 25. Terbinafine (Tb) and Floconazole (Flu)

.

X_1	X_2	$(X_1 - X_2) = D$	$(D - \check{D}) = d$	D^2
18.7	10.8	7.9	5.44	29.60
6.5	3.7	2.8	1.24	1.54
3.4	2.2	1.2	-1.26	1.59
2.3	1.1	1.2	-1.26	1.59
0.0	0.8	-0.8	-3.26	10.63
$\sum$ 30.9/5=6.18 = $\overline{X}$	$\sum$ 18.6/5=3.72			$\sum$34.95

$\check{D} = \dot{X}_1 - \dot{X}_2$

= 6.18 – 3.72 = 2.46

t = $\frac{\check{D}}{SD}$ Where $\check{D}$ = 2.46

S$\check{D}$= Standard error of mean

But we calculate variance first

$SD^2 = \frac{\sum d^2}{n-1} = \frac{34.95}{4} = 8.7375$

$S\check{D} = \frac{\sqrt{SD^2}}{n} = \frac{\sqrt{8.7375}}{5} = \sqrt{1.7475} = 1.321$

$t = \frac{\check{D}}{S\check{D}} = \frac{2.46}{1.321} = 1.862$

Cal t = 1.862. Tab t = 2.775

The rate of biocidal activities of Terbinafine and Fluconazole at 0.156µM concentration at the different contact time are the same at 95% confident limit (cal t = 1.862 < Tab t 2.775)

TABLE APP 26. FLUCONAZOLE (Flu) and TIOCONAZOLE (TIO)

X_1	X_2	$(X_1-X_2)=D$	$(D-\check{D})=d$	d^2
11.8	10.8	1	0.4	0.16
5.0	3.7	1.3	0.7	0.49
2.6	2.2	0.4	-0.2	0.04
1.3	1.1	0.2	-0.4	0.16
0.9	0.8	0.1	-0.5	0.25
21.6%	18.6%			$\sum d^2=1.1$

S=4.32 ($\dot{X}_1$) S=3.72 ($\dot{X}_2$)

$\check{D} = \dot{X}_1 - \dot{X}_2 = 4.32 - 3.72 = 0.6$

$SD^2 = \frac{\sum d^2}{n-1} = \frac{1.1}{4} = 0.275$

$S\check{D} = \frac{\sqrt{SD^2}}{n} = \frac{\sqrt{0.275}}{5} = \sqrt{0.055} = 2.345$

$t = \frac{\check{D}}{S\check{D}} = \frac{0.6}{2.35} = 2.553$

At 95% confident limit, Cal t is less than Tab t. we accept Ho. Hence the rate of kill of fluconazole and tioconazole, though in the same imidazole group were the same at 95% confident limit. (cal t = 2.553 < Tab t 2.775)

TABLE APP 27.FLUCONAZOLE (Flu) and SODIUM PROPIONATE (SP)

X_1	X_2	$(X_1-X_2)=D$	$(D-\check{D})=d$	d^2
11.5	10.8	1	0.4	0.16
4.1	3.7	1.3	0.7	0.49
2.4	2.2	0.4	-0.2	0.04
1.4	1.1	0.2	-0.4	0.16
1.0	0.8	0.1	-0.5	0.25
$(\dot{X}_{1)} =$ 20.4/5 =4.08	$(\dot{X}_{2)} =$ 18.6 S=3.72			$\sum d^2$=0.2604

$\check{D} = \dot{X}_1 - \dot{X}_2$ = 4.08 – 3.72 = 0.36

t = $\frac{\check{D}}{S\check{D}}$ Where $\check{D}$ = 2.46

$S\check{D} = \sum \frac{d^2}{n-1} = \frac{0.2604}{4} = 0.0651$

$S0^- = \frac{\sqrt{S0}}{n} - \frac{\sqrt{0.0651}}{5} - \sqrt{0.01302} = 0.114$

t = $\frac{\check{D}}{S\check{D}} = \frac{0.36}{0.114} = 0.497$

At 95% confident limit, Tab t = 3.157 is greater than cal t = 0.497, so we reject Ho.

The rate of kill of 0.156µM of fluconazole and sodium propionate are not the same.