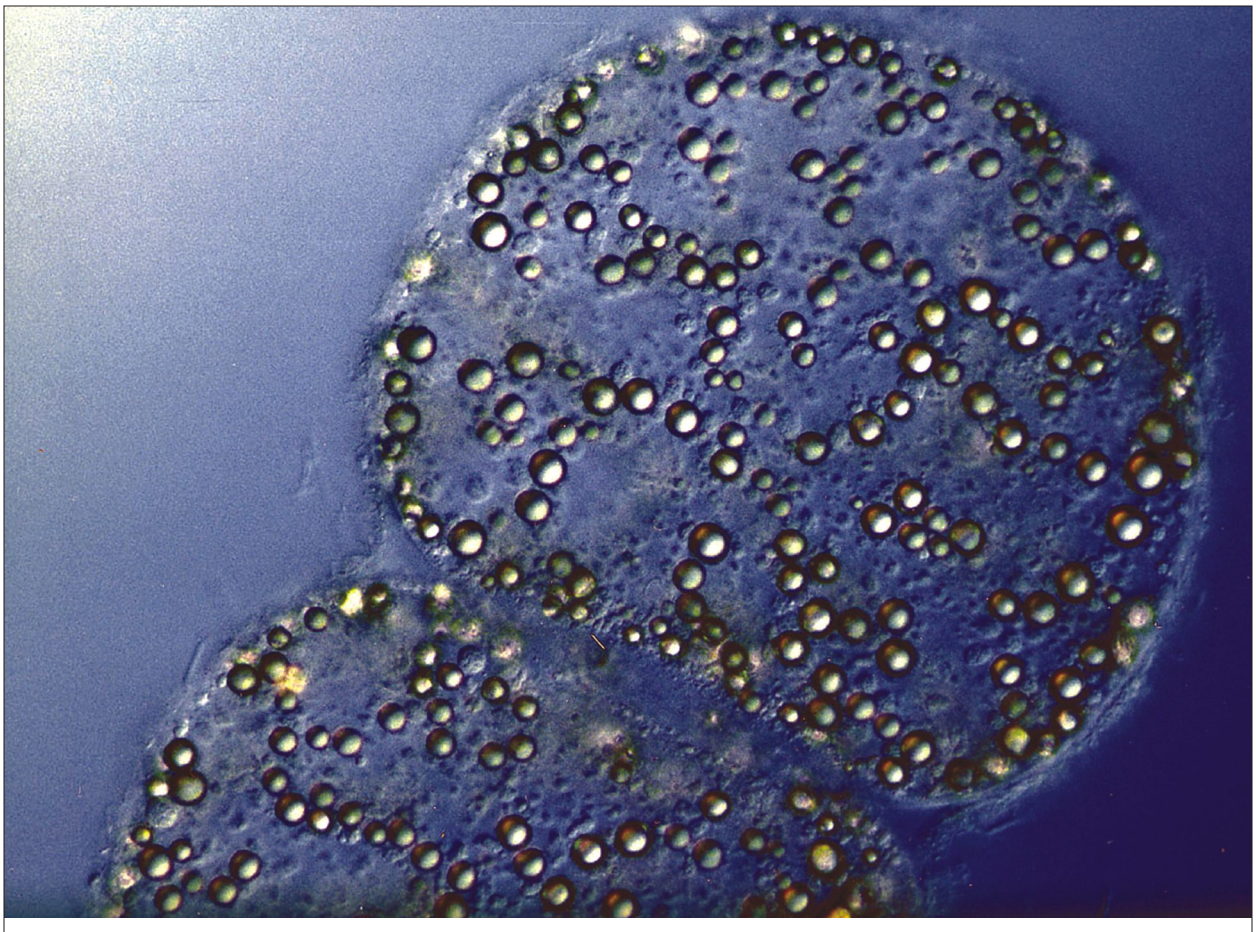

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In vitro Susceptibility Test of Some Antifungal Drugs on Selected Dermatophytes and Yeasts Isolated from Patients Attending Hospitals in Makurdi Environ

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ABSTRACT

The susceptibility testing of Ketoconazole, Itraconazole, Nystatin, Griseofulvin and Flucamed against *Trichophyton rubrum*, *Trichophyton mentagrophyte*, *Microsporum audouinii*, *Microsporum canis*, *Trichophyton schoenleienii* and *Candida albicans* was carried out using the Agar dilution method. The dermatophytes were incubated at room temperature (22°C) and the yeast at 37°C. Incubation of 2-7 days was found to be sufficient for prominent growth to be observed in the two controls. The study reveals Minimum Inhibitory Concentration (MIC) of 6.25-25 μg mL^{-1} for Itraconazole on all the dermatophytes and yeast isolates tested while Griseofulvin 50-100 μg mL^{-1}, Nystatin 6.25-100 μg mL^{-1}, Ketokonazole 25-100 μg mL^{-1} and Flucamed 6.25-50 μg mL^{-1} on the yeast and all the dermatophytes isolates tested. The MIC value for the entire antifungal agents analyzed against the dermatophytes and yeast isolates tested range between 3.13-6.25 μg mL^{-1} after seven days of incubation for dermatophytes and two days for yeast, while the range of greater than 6.25 μg mL^{-1} was obtained after fourteen days of incubation for dermatophytes and four days for yeast.

Key words: Antifungal drugs, susceptibility testing, dermatophytes, yeast

INTRODUCTION

The term fungus (plural; fungi) describes eukaryotic organisms that are spore-bearing, have absorptive nutrition, lack chlorophyll and reproduce sexually and asexually (Ochei and Kolhatkar, 2000).

They are microscopic plant organisms that consist mainly of cells such as mould and yeast. They cannot produce their own food thus, they behave as either parasites or saprophytes, absorbing nutrients from organic matter, such as human and animals (Willey *et al*., 2011).

The body or the vegetative structure of a fungus is called a thalus (plural; thalli). It varies in complexity and size, ranging from single cell microscopic yeast to multicellular moulds and macroscopic mushrooms. The fungi cell is usually encased in a cell wall of chitin. Chitin is a strong but flexible nitrogen containing polysaccharide consisting of N-acetylglucoseamine residues (Jawetz *et al*., 2007).

Yeast is a unicellular fungus that has a single nucleus and reproduces either asexually by budding and transverse division or sexually through spore formation (Ochei and Kolhatkar, 2000). Each bud that separates can grow in to a new cell and some group together to form colonies

(Leventin and Burge, 2007). Generally, yeast is larger than bacteria, varies considerably in size and is commonly spherical to egg shaped. They lack flagella and cilia but possess most other eukaryotic organelles (Ochei and Kolhatkar, 2000).

The thallus of a mould consists of long, branched, threadlike filaments of cells called hyphae (singular; hypha, Greek; hyphe) that form a mycelium (plural, mycelia), a tangled mass or tissue like aggregation of hyphae. In some fungi, protoplasm stream through hyphae, uninterrupted by cross walls. These hyphae are called coenoctic or aseptate hyphae. The hyphae of other fungi have cross walls called septa (singular, septum) that enable cytoplasmic streaming. These hyphae are termed septate hyphae (Ochei and Kolhatkar, 2000).

The key characteristics of fungi which set them apart from other organisms are their chitinous cell wall. This durable material, chitin, also makes up the cell of many insects (Anissimor, 2008). Fungi tend to grow in filamentous structures known as mycelium and reproduce either sexually or asexually via spores. In mushroom, the spores are visible as black dust underneath the cap (Anissimor, 2008).

Frequent inhalation of fungal agents has made some individuals to develop a level of immunity and during subsequent exposure, such individual may show little or no clinical sign or symptom (Ochei and Kolhatkar, 2000). Individuals that lack immunity to such agent when exposed will be infected. Indiscriminate use of antifungal drugs for such individual without susceptibility testing brings about resistance (Anissimor, 2008). The number of cell that is present in an infected tissue will have an effect on the anti fungal drug resistance. Since, the increase number of cell will increase the probability that a mutation can occur which confer resistance. Another study was conducted in which some fungal pathogen and antifungal agents found out that most of the antifungal drugs in the open markets are more or less fungistatic rather than being fungicidal.

Ezeronye (2002) worked on skin lesions suspected to be dermatophytic infection and found out that *Tinea capitis*, *Tinea corporis* and *Tinea verriscolor* occur in male than female.

Since, fungal infections are difficult to treat and *in vitro* susceptibility to antifungal agents is not a common practice in West African sub region as in the case of bacteria, this study is aimed at checking some first choice antifungal drugs if they can still be relied on as drug of choice without susceptibility test.

MATERIALS AND METHODS

Collection of specimen (drugs): Only antifungal drugs properly sealed in its sachets, to ensure sterility, were collected. Expiration date ranging from 2014-2015 and the National Agency for Food, Drug Administration and Control (NAFDAC) registration number of each drug were considered.

Source of fungal isolates: Clinical isolates were obtained from Bacteriological Laboratory of Tosema, Specialist Diagnostic Laboratory and Federal Medical Center Makurdi, Benue State Nigeria.

Source of antifungal drugs: A total of five different antifungal drugs were obtained from pharmacies in and around Makurdi Benue State Nigeria. They are as follows:

- Nystatin of polyene antifungal group manufactured by BDH industries LTD, Akurli Road Mumbai India and Manufactured Date: May 2012, Expiration Date: April 2014, National Agency for Food, Drug Administration and Control (NAFDAC) NO: A4-1510, Batch No: 020512

- Ketoconazole of Azole antifungal group manufactured by X Laboratories PVT LTD.E.1223, Phase I EXTN.Rajasthan India and Manufactured Date: January 2011, Expiration Date: April 2015, NAFDAC NO: A4-6022, Batch No: T791
- Itraconazole (sporanox) of azole antifungal drugs manufactured by BDH industries LTD, Akurli Road Munbai India and Manufactured Date: September, 2012, Expiration Date: October 2014, NAFDAC NO: 04-2404, Batch No: 020512
- Griseofulvin (fulsin) from others manufactured by Natong Jinghua Pharmaceutical Co, LTD, India, Manufactured Date: September 2011, Expiration Date: August 2014, NAFDAC NO: A4-6246, Batch No: 110901.
- Flucamed from others manufactured by Drug Field Pharmaceutical LTD, Lynson Chemical Avenue Manufactured Date: August 2012, Expiration Date: July 2014, NAFDAC NO: 04-4514, Batch No: 350801

Sterilization of materials: All glass wears were sterilized using hot air oven and media using autoclave. The media was prepared according to manufactures instruction.

Determination of Minimum Inhibitory Concentration (MIC) (Ochei and Kolhatkar, 2000): Fourteen day old culture of *T. rubrum*, *T. schoenleienii*, *T. mentagrophyte*, *M. canis* and *M. audouinii* isolated from skin and three day old *Candida albicans* isolated from High Vaginal Swab (HVS) were obtained from Federal Medical Center Makurdi and Tosema Specialist Diagnostic Laboratory Makurdi. Identification was done using morphological and biochemical characteristics. They were purified on Sabouraud Dextrose Agar (SDA). The dermatophytes were incubated at 22°C for fourteen days and the yeast incubated at 37°C for three days.

Preparation of the inoculums: Pure culture of *T. rubrum*, *T. schoenleienii*, *T. mentagrophyte*, *M. canis* and *M. audouinii* were harvested from SDA purity plate for spores by adding 5 mL of nutrient broth on the growth medium and carefully ten sterile glass beads were introduced, gently shake from cover to base to get the homogenous mixture while, pure culture of *Candida albicans* was harvested by using disposable sterilized wire loop. Loop full of *Candida albicans* was transferred to nutrient broth. The obtained spores' suspension, dermatophyte were decanted and dilute with normal saline to about No. 1 Mac farland turbidity likewise, the yeast isolates and allowed to stand on worktop.

Preparation of antifungal agents: The antifungal agent used includes, Nystatin (500 IU) Concentration, Ketoconazole (200 mg), Itraconazole (200 mg), Griseofulvin (Fulsin) (500 mg) and Flucamed (50 mg).

A solution of antifungal standard was prepared by dissolving the antifungal drug in distilled water to a concentration 1000 µg mL^{-1}. Working standard was prepared from this stock solution by diluting with distilled water to the following concentrations 500, 250, 125, 62.5 and 31.3 µg mL^{-1}.

Methods: The stock solution and the standards of the antibiotic described were each diluted 1:10 (1 volume of standard+9 volumes medium) with sterile agar culture medium cooled to 40-45°C after mixing thoroughly by shaking. The solutions were poured into a test tube and allowed to solidify to form a slant. The concentration now becomes 100, 50, 25, 12.5, 6.25 and 3.13 µg mL^{-1},

respectively. Control tubes were also prepared containing only distilled water in the nutrient medium without antifungal agent and no inoculation done on them and a "blank" was also prepared as another control containing agar medium only but inoculated with the test organism.

Inoculation: Loops full of the homogenized broth culture of the test organism prepared were then spread evenly over the surface of each agar slant. Inoculated tubes were incubated at the temperature which is considered optimal for the test organism, 25°C for dermatophyte and 37°C for yeast.

Determination of MIC (Minimum Inhibitory Concentration): The tubes were examined daily for growth. As soon as adequate growth was observed in the blank (control tube), growth on the agar slants were evaluated by visual inspection, the MIC was indicated by that tube of the dilution series containing the lowest concentration of antibiotic which does not permit growth of the test organism.

RESULTS

Susceptibity testing carried out on five dermatophytes and one yeast (*T. rubrum*, *T. mentagrophyte, T. schoenleienii, M. canis, M. audouin*ii and *Candida albicans*) using *Nystatin, Ketoconazole, Flucamed itraconazole* (sporanox) and *Griseofulvin* (Fulsin).

Flucamed had the highest MIC of 25 µg mLG^{1} and Nystatin had the lowest MIC of 3.13 µg mLG^{1} after seven days. Griseofulvin had the highest MIC of 100 µg mLG^{1} and Itraconazole had the lowest MIC of 12.5 µg mLG^{1} after fourteen days against *Trichophyton rubrum* (Table 1).

Table 2 shows that Flucamed and Griseofulvin had the highest MIC of 25 µg mLG^{1} and Nystatin had lowest MIC with 3.13 µg mLG^{1} after seven days. Ketoconazole had the highest MIC of 100 µg mLG^{1} and Itraconazole had lowest with 25 µg mLG after fourteen days against *Trichophyton mentagrophytes*.

Table 1: Minimum inhibitory concentration of *Trichophyton rubrum* at seven and fourteen days incubation

	MIC (µg mLG^{1}) after days	
Drugs	7	14
Itraconazole	6.25	12.5
Nystatin	3.13	50.0
Flucamed	25.00	50.0
Griseofulvin	6.25	100.0
Ketoconazole	12.50	25.0

Table 2: Minimum inhibitory concentration of *Trichophyton mentagrophyte* at seven and fourteen days incubation

	MIC (µg mLG^{1}) after days	
Drugs	7	14
Itraconazole	12.50	25
Nystatin	3.13	50
Flucamed	25.00	50
Griseofulvin	25.00	50
Ketoconazole	12.50	100

Table 3: Minimum inhibitory concentration of *Microsporum audouinii* at seven and fourteen days incubation

	MIC (µg mLG^{1}) after days	
Drugs	7	14
Itraconazole	6.25	12.5
Nystatin	3.13	25
Flucamed	3.13	25
Griseofulvin	12.50	50
Ketoconazole	12.50	25

Table 4: Minimum inhibitory concentration of *Microsporum canis at* seven and fourteen days incubation

	MIC (µg mLG^{1}) after days	
Drugs	7	14
Itraconazole	3.13	6.25
Nystatin	25	100
Flucamed	25	50
Griseofulvin	50	100
Ketoconazole	12.5	50

Table 5: Minimum inhibitory concentration of *Trichophyton schoenleienii* at seven and fourteen days incubation

	MIC (µg mLG^{1}) after days	
Drugs	7	14
Itraconazole	6.25	25
Nystatin	6.25	25
Flucamed	3.13	6.25
Griseofulvin	3.13	100
Ketoconazole	6.25	25

In Table 3, Griseofulvin and Ketoconazole gave the highest MIC 12.5 µg mLG^{1} and Nystatin and Flucamed had the lowest MIC of 3.13 µg mLG^{1} after seven days of incubation while, Griseofulvin had the highest MIC of 50 µg mLG^{1} at fourteenth days after incubation against *Microsporum audouinii*.

The study also showed that Griseofulvin gave the highest MIC value of 50 µg mLG^{1} while, Itraconazole, being very effective, gave the lowest MIC value of 3.13 µg mLG^{1} after seven days of incubation. Nystatin and Griseofulvin had MIC 100 µg mLG^{1} while, Itraconazole had 6.25 µg mLG^{1} after fourteen days of incubation against *Microsporum canis* (Table 4).

In Table 5, Itraconazole, Nystatin and Ketoconazole produced the highest MIC of 6.25 µg mLG^{1} while, Griseofulvin and Flucamed gave MIC of 3.13 µg mLG^{1} after seven days of incubation. Griseofulvin had the highest MIC of 100 µg mLG^{1} while, Flucamed had the lowest with 6.25 µg mLG^{1} after fourteen days of incubation against *Trichophyton schoenleienii*.

In Table 6, Itraconazole and Ketoconazole had the highest MIC of 6.25 µg mLG^{1} while Nystatin, Flucamed and Griseofulvin had lowest MIC of 3.13 µg mLG^{1} after 2 days of incubation. At the fourth day, Griseofulvin had the highest MIC of 50 µg mLG^{1} while, Nystatin and Flucamed had the lowest MIC of 6.25 µg mLG^{1} after four days against *Candida albicans*.

Table 6: Minimum inhibitory concentration of *Candida albicans* at two and four days incubation

	MIC (µg mLG^{1}) after days	
Drugs	2	4
Itraconazole	6.25	25
Nystatin	3.13	6.25
Flucamed	3.13	6.25
Griseofulvin	3.13	50
Ketoconazole	6.25	25

In this study, the MIC value for the entire antifungal agents analyzed against the dermatophytes and yeast tested range from 3.13-6.25 µg mLG^{1} after seven days of incubation for dermatophytes and two days for yeast, while the range of greater than 6.25 µg mLG^{1} was obtained after fourteen days of incubation for dermatopytes and four days for yeast.

DISCUSSION

In this study, the ideal incubation temperature used for the susceptibility testing of dermatophytes was 25°C (room temperature) and incubation time of 4-7 days was found to be sufficient to observe prominent growth in the control cultures as reported by Ochei and Kolhatkar (2000).

From the result, Itraconazole (Sporanox) produced a low MIC of 3.13-6.25 µg mLG^{1} after seven days of incubation against *T. rubrum*, *M. audouinii*, *M. canis*, *T. schoenleienii* and after two days against *C. albicans* which falls within the cumulative MIC parameter data from all sites by Craig and Andes (2004). The MIC after fourteen days incubation of all the dermatophytes with Itraconazone varies within 6.25-25 µg mLG^{1}. Gupta *et al.* (2001) reported that Itraconazole is best used in the treatment of dermatophytes.

Nystatin, Ketoconazole, Flucamed showed a considerable variation of MIC of 3.13-50 µg mLG^{1} against *T. schoenleienii*, *M. audouinii*, *T. rubrum*, *T. mentagrophytes* and *C. albicans* after seven days and fourteen days incubation for dermatophytes and two and four days for yeast which is in agreement with the value 0.02 to $64 µg mLG^{1} reported by NCCLS (2002).

Ketoconazole produced a high MIC of 100 µg mLG^{1} against *Trichophyton mentagrophyte* while, Nystatin and Griseofulvin also produced an MIC of 100 µg mLG^{1} against *Microsporum canis*. Flucamed and Nystatin showed MIC of 3.13-6.25 µg mLG^{1} against *Candida albicans* which agrees with Ochei and Kolhatkar (2000) who reported that Flucamed and Nystatin is better in the treatment of *C. albicans*. From the result obtained, Itraconazole is effective against *T. rubrum* and *M. audouinii*, Flucamed is effective against *T. schoenleienii* and *Candida albicans*. This agreed with Gupta *et al.* (1999) who reported that Griseofulvin and Itraconazole are effective against most fungal infection. The result is not different from Jawetz *et al.* (2007), who reported that oral Itraconazole or another of the azoles is the treatment of choice against *T. rubrum* and *M. audouinii*.

The increase variation with the other studies lie in the agreement with the report by Fernandez-Torres *et al.* (2000), that since 1980's, several studies on the *in vitro* susceptibility of dermatophytes to different antifungal drugs have been done and the result have shown considerable result variation. This variability is probably due to important methodological differences among the laboratories (Jessup *et al.*, 2000). Ochei and Kolhatkar (2000) reported that

fungal susceptibility testing suffers comparison of MIC data since investigation is tedious and time consuming and few have attempted to correlate *in vitro* MIC with the outcome of fungal therapy in West Africa.

Based on the result obtained, the *in vitro* activity of Griseofulvin is high (100 µg mL^{-1}) which can be said to be ineffective against dermatophytes, though, Artis *et al*. (1981) reported the resistance of dermatophytes against Griseofulvin using the micro-culture method for determining MIC.

Nystatin, Flucamed and Griseofulvin showed lower MIC of 3.13 µg mL^{-1} against *Candida albicans* after two days of incubation. This is in agreement with Anissimor (2008),who reported that Nystatin and Flucamed are effective antifungal agents in the treatment of systemic fungal infection.

From the result, Nystatin shows MIC of 3.13 µg mL^{-1} to *T. rubrum*, *T. mentagrophyte*, *M. audouinii* and *Candida albicans*. This agrees with Hay (2001), who reported that Nystatin can be used as a broad Spectrum antifungal drug in the treatment of both dermatophytes and yeast.

CONCLUSION

All the Minimum Inhibitory Concentration (MIC) obtained in the study is considerably far below the daily body concentration of drugs, which are 500-1000 mg and all the MIC results obtained fall within the range of commercially prepared antifungal kit for susceptibility test i.e., 0.8-100 µg mL^{-1}. The question of drug resistance is complicated by the limitations in the available susceptibility testing methodology and the ability to distinguish between microbiological and clinical drug resistance, the later typically occur when an inhibitory antifungal agents reaches the limit of its activity in a host with decreasing efficient immune system.

With the circulation of new antifungal agents like Itraconazole (Sporanox) and other groups, fatal infections can now be treated. However, as modern medicine continues to extend life through aggressive therapy of other life-threatening diseases such as cancer and risk for opportunistic fungal infections are increasing. Therefore, chemotherapeutic agents should be fungicidal and not just fungistatic. The search continues for fungicidal agents that are not toxic to the host.

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