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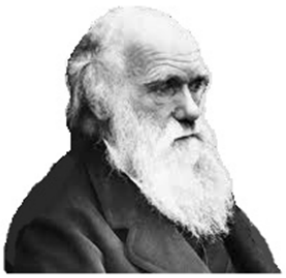

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Antifungal activities of moulds associated with selected stored grains and legumes

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ABSTRACT

An antifungal agent can either kill or inhibit the growth of fungi by interfering with the formation of fungal cell membrane, weakening it and hindering cell division. Antifungal agents of amphotericin B, ketoconazole, fluconazole and voriconazole of Thermo Fisher Scientific limited were used for this study. Cultural analysis of stored grains and legumes (rice, maize, wheat, groundnut and beans) from Imo State was done using streak plate method. Sabouraud dextrose agar was used for the culture while Mueller Hinton agar was used for Antifungal sensitivity test. Moulds were further identified using 18s rRNA gene sequencing method. The antifungal sensitivity profile of isolated and identified moulds was evaluated using the clinical laboratory and standard institute approved methods for testing of moulds, the disk diffusion and tetrazolium chloride test. The results showed that *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus* and *Penicillium chrysogenum* were the moulds isolated and identified using both cultural and 18S rRNA sequence. The fungal isolates were susceptible to ketoconazole and voriconazole. Amphotericin B was both resistant and susceptible to different moulds. The fungal isolates were resistant to fluconazole. Inhibition effects were more with the antifungal disc than with tetrazolium salts. All the isolates were resistant to tetrazolium chloride and gave no zone of inhibition. Combination of antifungal agent and tetrazolium chloride showed sensitivity only to ketoconazole. Antifungal disc alone gave a better zone of inhibition than the combination of antifungal agents with tetrazolium salts. This study showed that ketoconazole has greater inhibitory potential than other antifungal agents. Ketoconazole remains the best drug of choice among the studied antifungal agents for fungal infections. Therefore antifungal drugs can be used against moulds of economic importance in the country.

***Keywords*:** Antifungal agents, *Aspergillus* spp., Disk diffusion method, Stored grains and legumes, *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, *Penicillium chrysogenum*

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1. INTRODUCTION

In Africa, cereals like rice (*Oryza sativa*), maize *(Zea mays*), wheat (*Triticum aestivum*) and legumes like groundnut (*Arachis hypogaea*) and cowpea (*Vigna sinensis*) are important food crops. Cereals and legumes provide cheap sources of energy and protein [1]. A mould is a fungus that grows in the form of multicellular filaments called hyphae. Common genera of moulds include: *Acremonium, Alternaria, Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium, Rhizopus, Stachybotrys, Trichoderma* and *Trichophyton* [2]. Antifungal drugs either kill or inhibit the growth of fungi. The mode of action of antifungal drugs is to eliminate or destroy sensitive fungi by interfering with the formation of the fungal cell membrane, weakening it and hindering cell division [3]. According to [4] treatment of fungal infections are generally less successful than those of bacterial infections because as eukaryotes, fungal cells are more similar to human cells than bacterial cells. In 1985, the National committee for clinical laboratory standards (NCCLS), formed a subcommittee on antifungal susceptibility testing using the standard disk diffusion method to test antifungal drugs for non-dermatophyte filamentous fungal isolates (M51-A and supplement M51-S1). This document defined reference strains with ranges of Minimal Inhibitory Concentrations (MIC) and Break Points (BPs) for some antifungals and their action against yeasts and moulds [5].

Among antifungal agents, the azoles that are available for systemic use can be classified into two groups: the triazoles (fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole) and the imidazoles (ketoconazole) [5]. The reference CLSI documents include antifungal susceptibility testing of amphotericin B, flucytosine, fluconazole, ketoconazole, itraconazole, and the new triazoles (posaconazole, ravuconazole, and voriconazole). The method is based on visual reading of minimum inhibitory concentration (MIC, μg/ml) values [6].

Tetrazolium salts (TTC) serve as convenient indicators of dehydrogenase assay. In the TTC assay (also known as TTC test or tetrazolium test) is used to differentiate between metabolically active and inactive tissues. It forms colourless to yellowish (practically clear) solution when dissolved in ethanol or water. The white compound is enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living tissues due to the activities of various dehydrogenases (enzymes important in oxidation of organic compounds and thus cellular metabolism), while it remains in its unreacted state in areas of necrosis since these enzymes have either been denatured or degraded [7].

The objectives of the study were to determine moulds isolated from stored grains and legumes. To determine the antifungal profile of some selected antifungal agents against isolated moulds and to determine the effect of tetrazolium salt alone and in combination with antifungal agents.

2. MATERIALS AND METHODS.

Study area

Imo state is located in the south eastern part of Nigeria and is bounded to the North by Anambra State, to the East by Abia State, to the South by Rivers State and to the West by Delta State. Imo State is made up of 3 geo-political zones, Orlu, Owerri and Okigwe. The State lies between latitude 5° 30' and 6° 15' North. Longitude 6° 38' and 7° 18' East.

Sample collection

Whole/fine powder of rice, maize, wheat, groundnut and beans were stored in 4 different storage materials namely sack, polyethene (cellophane), plastic containers and metal containers for a period of 4 months. Two hundred samples were used, each weighing 30g. Stored grains and legume samples were labeled and transported immediately to laboratory and kept in cool place for mycological analysis.

Isolation and identification of moulds

Standard dilution and streaking technique methods were used. The samples were serially diluted up to dilution factor of 10^{-3} and 10^{-5}. An antibacterial agent chloramphenicol 50 mg/l was used to inhibit the growth of bacteria while lactic acid at a concentration of 0.1ml was added to prevent the growth of yeast [8] and 0.1ml of diluents were inoculated onto prepared plates containing 15ml sabouraud dextrose agar (SDA).The plates were incubated at 28 ±2 °C for 7 days in an incubator and examined daily for fungal growth. Fungal colonies grown on media were sub-cultured on various media [9]. The results were expressed in colony forming unit per gram (cfu/g). Mueller Hinton agar was used for sensitivity test.

Morphological and Microscopic identification.

The isolated moulds were identified according to colonial morphology and microscopic examination. The fungal isolates were transferred to sterilized plates for purification and identification. The grown fungi were mounted on a slide, stained with lactophenol cotton blue to detect fungal structures, covered with a cover slip, examined under microscope, identified on the bases of their colony morphology and spore characteristics [10].

Molecular identification

The deoxyribonucleic acid (DNA) was extracted using Zymo research fungal DNA mini prep extraction kit. The qualitative estimation of genomic DNA was done using agarose gel electrophoresis. The extracted DNA was amplified using Polymerase chain reaction amplification protocol. Sequencing protocol. PCR products were cleaned using ExoSAP Protocol. Fragments were sequenced using the nimagen brilliant dye terminator cycle sequencing kit according to manufacturer's instructions [11].

Determination of antifungal susceptibility test

The CLSI have standardized disk diffusion methodology for testing filamentous fungi (moulds) that cause invasive disease. The CLSI have approved methods for testing of moulds. They use the CLSI M38-A standard for moulds [12].

The reference method DISK DIFFUSION METHOD was performed according to (Clinical and laboratory standards institute) proposed guidelines and as described by [12]. The following four antifungal agents were used: amphotericin B, fluconazole, ketoconazole and voriconazole. Antifungal susceptibility using tetrazolium chloride and antifungal agents.

In the presence of moulds, 2,3,5,-triphenyltetrazolium chloride (TTC) is reduced to red formazan which indicated the activities and viability of the cells. 1g of TTC was diluted in 10ml of sterile distilled water. Then 0.1g/ml of the concentration was added to Sabouraud dextrose

agar (SDA) which was prepared by mixing TTC with molten SDA before the agar solidifies [13].(Shaaban *et al*., 2013).

Drug-impregnated disks containing the test agents were placed onto the surfaces of inoculated TTC plates with sterile pair of forceps. Fungal plates were incubated at 28 °C for 4 to 7 days to allow for fungal growth (Espinel-Ingroff *et al.,* 2010) [12].

3. RESULTS

Moulds were isolated from the different stored grains and legumes collected from different markets. The isolated species were first identified morphologically and microscopically. They were further identified genetically by sequencing of 18S rRNA gene using ITS1 and ITS4 primers. The resulting mould species were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum* as seen in Table 1.

Result from Table 2 showed that all the isolates were susceptible to ketoconazole and voriconazole though at different millimeters. Amphotericin B, *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus brunneoviolaceus*, were sensitive and *Aspergillus niger* and *Penicillium chrysogenum* were resistant. For fluconazole, *Aspergillus brunneoviolaceus* was sensitive while the other isolates were resistant.

All the isolates (*Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum*) were resistant to tetrazolium test.

According to Table 3, All the isolates were sensitive to ketoconazole and TTC, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus* were sensitive to voriconazole and TTC while *Aspergillus flavus* and *Penicillium chrysogenum* were intermediate. For Amphotericin B and TTC, *Aspergillus niger*, *Aspergillus brunneoviolaceus* were sensitive while *Aspergillus flavus*, *Aspergillus tamarii* were intermediate, *Penicillium chrysogenum* was resistant. *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, and *Penicillium chrysogenum* were resistant to fluconazole and TTC while *Aspergillus brunneoviolaceus* was intermediate. Antifungal agents alone gave a better zone of inhibition than the combination of antifungal agents with tetrazolium test and tertazolium test alone.

Statistical analysis done using the analysis of variance showed that there was no significant difference ($P<0.05$) between ketoconazole, voriconazole and amphotericin B but there was a significant difference ($P<0.05$) between, ketoconazole, voriconazole amphotericin B and fluconazole.

Table 1. Colonial and Microscopic Characteristic of Identified Isolates.

Colonial morphology	Microscopy	Probable fungus
Colonies are greenish or yellow brown, smooth texture, 4-8 cm	Hyphae are septate and hyaline.	*Aspergillus flavus*
Rusty brown or dark brown Colonies, smooth texture, size of 6-15 cm	long chain of conidia head	*Aspergillus tamarii*

Black colonies, rough texture, size of 4-5 cm	Septated hyphae, long smooth and colourless	*Aspergillus niger*
Brown to dark brown colonies, smooth texture, size of 6-10 cm	Hyaline or pigmented longer stipes	*Aspergillus brunneoviolaceus*
Blue green with a yellowish pigment, smooth texture, 4.5-5 cm	Septate hyphae branched	*Penicillium chrysogenum*

Table 2. Molecular identification of various moulds.

S/N	SEQQUENCE ID	PERCENTAGE (%)	NCBI MATCH	ISOLATES
1	NR111041.1	99	*Aspergillus flavus* NR135325	*Aspergillus flavus*
2	NR138279.1	97	*Aspergillus brunneoviolaceus* NR138279	*Aspergillus brunneoviolaceus*
3	AY373852.1	91	*Aspergillus niger* AY373852	*Aspergillus niger*
4	NR138306.1	99	*Penicillium chrysogenum* MH793845	*Penicillium chrysogenum*
5	AF004929.1	100	*Aspergillus tamarii* MN339986	*Aspergillus tamarii*

Table 3. Susceptibility of the isolated fungi to selected antifungal agents.

SAMPLES	Amphotericin B (20 µg/ml)	Ketoconazole (10 µg/ml)	Flucanozole (25 µg/ml)	Vorioconazole (1 µg/ml)
Aspergillus flavus	20.00 ±0.577(S)	35.00 ±2.886(S)	0.00 ±0.000(R)	32.00 ±1.154(S)
Aspergillus tamarii	23.00 ±0.577 (S)	38.00 ±2.081(S)	0.00 ±0.000(R)	36.00 ±1.732(S)
Aspergillus niger	11.00 ± 0.577 (R)	20.00 ±0.577(S)	0.00 ±0.000(R)	25.00 ±1.527(S)
Aspergillus brunneoviolaceus	30.00 ±2.000 (S)	39.00 ±1.000(S)	21.00 ±12.124(S)	36.00 ±2.000(S)
Penicillium chrysogenum	7.00 ±0.577(R)	20.00 ±1.527(S)	0.00 ±0.000(R)	17.00 ±1.527(S)

Key: Susceptible - (S), Resistant (R) measured in mm.
Minimum Inhibition Concentration (MIC) endpoint reading.

- Susceptible ≥ 17 mm (azole) and ≥15 mm (amphotericin B)
- Intermediate, 14 to 16 mm (azole) and 13 to 14 mm (amphotericin B)
- Resistant, ≤ 13 mm (azole) and ≤ 12 mm (amphotericin B).

Table 4. Susceptibility of the isolated fungi to selected antifungal agents and combination of tetrazolium chloride.

SAMPLES	Amphotericin B (20 µg/ml) + TTC (mm)	Ketoconazole (10 µg/ml) + TTC (mm)	Flucanozole (25 µg/ml) + TTC (mm)	Vorioconazole (1 µg/ml) + TTC (mm)
Aspergillus flavus	13.00 ± 0.577(I	25.00 ± 1.527(S)	0.00 ± 0.000(R)	15.00 ± 1.000(I)
Aspergillus tamarii	13.00 ± 1.154(I)	28.00 ± 0.577(S)	0.00 ± 0.000(R)	21.00 ± 1.000(S)
Aspergillus niger	21.00 ± 1.000(S)	25.00 ± 2.000(S)	8.00 ± 0.577(R)	30.00 ± 2.000(S)
Aspergillus brunneoviolaceus	27.00 ± 1.000(S)	36.00 ± 2.000(S)	16.00 ± 0.577(I)	32.00 ± 3.214(S)
Penicillium chrysogenum	9.00 ± 0.000(R)	19.00 ± 1.154(S)	0.00 ± 0.000(R)	14.00 ± 0.577(I)

Key: Susceptible - (S), Intermediate – (I), Resistant (R) measured in mm.
Minimum Inhibition Concentration (MIC) endpoint reading.

- Susceptible ≥ 17 mm (azole) and ≥15 mm (amphotericin B)
- Intermediate, 14 to 16 mm (azole) and 13 to 14 mm (amphotericin B)
- Resistant, ≤ 13 mm (azole) and ≤ 12 mm (amphotericin B).

4. DISCUSSION

The study of [14] showed that the standard disk diffusion method of analysis was a good method for testing the susceptibility of various moulds to different antifungal agents, which agreed with this work.

This study had similarity with the work of [15] where ketoconazole had the highest minimum inhibitory concentration among different antifungal agents.

Another study of [16] had it that all isolates in the study were sensitive to amphotericin-B and ketoconazole and a high frequency of fluconazole resistance was observed. The study of [17] observed that amphotericin B was known as the main antifungal agent against invasive fungal infections which did not agree with this work. [17-22] also observed that *Aspergillus* species were not susceptible to fluconazole but were susceptible to other azoles like ketoconazole, voriconazole and itraconazole which was similar to this research work.

5. CONCLUSION

Ketoconazole gave the highest zone of inhibition followed by voriconazole and amphotericin B while fluconazole gave the least inhibition. Ketoconazole had greater inhibitory potential than other antifungal agents. Tetrazolium chloride did not have much inhibitory effect when compared with antifungal agents. Therefore ketoconazole remain the best drug of choice for fungi infections.

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