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Sensitivity Pattern of Some Antifungal Drugs on Fungi Isolated from Soil

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Abstract

Presently, no agar-based susceptibility testing method has been perpetuated for testing moulds. We describe a newly developed agar-based method using agar well diffusion method to examine the susceptibility of 6 moulds against 2 antifungals. Our findings show that the method is reproduceable. Fungi isolated from different soil samples for a period of eight weeks within the University of Ilorin, were analysed for their sensitivity to different concentrations of two antifungal tablets (Ketoconazole and Griseofulvin). The antifungal activities were evaluated using agar well diffusion method. The assay was carried out on the isolated and identified fungi: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Mucor racemosus*, *Rhizopus stolonifer*, and *Fusarium oxysporium*. The results showed that the fungal isolates were susceptible to griseofulvin and ketoconazole. This study showed that ketoconazole has greater inhibitory potential and broad spectrum of activity than griseofulvin.

Keywords: Antifungal, Agar-based, Drugs, Sensitivity, Soil.

1. Introduction

An antifungal drug is a term that is used to define a fungal agent that either kills or inhibits 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 hinder cell division (Bossche *et al*., 1988). According to Willey *et al.* (2011), treatment of fungal infections generally has been less successful than that of bacterial infections because as eukaryotes, fungal cells are much more similar to human cells than bacterial cells.

There are several classes of antifungal agents based on the cell membrane disruption (Amphotericin B, Nystatin, Natamycin, Ketoconazole, Clotrimazole), inhibition of cell division (Fluctytosine and Griseofulvin) and cell wall synthesis (Capsofungin, Anidulafungin) (Meyers, 2010). There are a lot of fungi in the soil which are of economic importance to man. Apart from fungi, soil also contains other living things such as plants,

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insects and bacteria. The presence of microorganisms in the soil has resulted into the development of chemotherapeutic agents in which fungi represent one of them. Soil contains many organisms, these organisms are called soil organisms and they are collectively called soil biota. Some of the soil organisms are microscopic. These organisms are called soil microorganisms. Soils are dynamic environments. A mineral soil contains less than 20% organic carbon, whereas an organic soil possesses at least this amount (Willey *et al.,* 2011).

Soil fungi are eukaryotic and microscopic cells that usually grow into thread like structures or hyphae that forms a mass called mycelium (Dix and Webster, 1995). Hyphae are usually only several thousandth of an inch. Soil represents an oligotrophic medium for the growth of fungi. Nutrients for fungal growth are extremely limited. Hence, most of the time, fungi are either dormant or they metabolize and grow very slowly utilizing a range of organic molecules (Nester *et al*., 2004). Gupte *et al.* (2002) reported that search for a new drug against fungal infections is a major challenge to current search in mycotic diseases.

This paper reports the physico-chemical properties of the soil, fungi present in uncultivated bare soil and antibiotic sensitivity of some antifungal tablets on the isolates.

Justification for the study

Antifungal vulnerability testing has been in normal use and has become a useful tool for clinicians who are faced with difficult treatment decisions. Although, antifungal susceptibility testing using commercial tablet is uprising, much misunderstanding still exists regarding the use of the findings. Sufficient data have to be generated to determine susceptibility trends of specific fungi against specific agents. Susceptibility testing of fungal moulds from different sources in which soil is one of them will help to provide information to assist the clinician on how to care for their patients.

2. Materials and Methods

Soil samples were collected from the bare ground around microbiology department, microbiology laboratory, biochemistry department, Computer Based Test (CBT) centre and plant biology department, all within the University of Ilorin.

Collection of Soil Sample

The overlying debris was removed from the soil surface. Sterile hand trowel was used to dig deep into the soil to about 20cm. Soil sample was collected into labelled sterile polythene bag. Collected samples were analysed mycologically in the laboratory.

Isolation and Identification of Fungi

Fungal isolation was done using potato dextrose agar (PDA) with the addition of streptomycin to prevent growth of bacteria. Petri dishes were inoculated with 1ml of 10^{-4} dilution (Pour plate method) and incubated at 25^{O}C for 3-5 days. The colonies obtained on each plate were observed both macroscopically and microscopically using the protocol of Fawole and Oso (2007). Characterised fungi were identified with reference to Alexopolous (1979) and Campbell and Stewart (1980).

Preparation of Antifungal Drugs

Two antifungal drugs were used in this work. They are ketoconazole and griseofulvin. The antifungal tablets were ground into fine powdery form using mortar and pestle. The grinded tablets were measured in different concentrations: 0.02g/ml, 0.04g/ml and 0.06g/ml and were dissolved in 95% ethanol and diluted with distilled water to make the different concentrations. Rotary shaker was used to homogenize the mixture.

Determination of Antibiotic Sensitivity Pattern

The antibiotic sensitivity patterns of the antifungal drugs against the isolated fungi were determined using agar well diffusion method. Currently, no agar-based susceptibility testing method has been standardized for testing moulds. We describe a newly developed agar-based method employing agar well method to test the susceptibility of mould isolates against 2 antifungals A standardized concentration of inoculums (10^{-4}) serial with a fixed volume (1ml) was spread evenly over the surface of the gelled agar medium of potato-dextrose agar medium. A fixed volume of different concentrations of the antifungal drugs were then introduced into the bored agar well. The plates were incubated at 25ºC for 72 hours and the zones of inhibitions were determined in mm using a measuring ruler.

Determination of Soil Moisture Content

Five grams of the soil sample was weighed in to pre weighed crucible. The weighed sample

was dried in the oven at 80 ^{o}C for 24hours and allowed to cool. The loss in weight of the sample was expressed as percentage weight of the sample (Fawole and Oso, 2007). The moisture content was calculated using the formula below;

$$\% \text{ moisture content} = \frac{\text{Loss in weight of the sample}}{\text{Initial weight of sample}} \times 100\%$$

Determination of Soil pH

Twenty grams of air-dried soil was weighed and introduced into a 100ml beaker. 20ml of distilled water was measured into the beaker and the suspension was left for twenty minutes, with occasional stirring with a glass rod to enable it reach equilibrium. At the end of twenty minutes, a pH with glass electrodes was used to determine the pH of the suspension. (Fawole and Oso, 2007).

3. Results and Discussion

The fungi isolated from the soil samples include *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Mucor racemosus*, *Rhizopus stolonifer* and *Fusarium oxysporium* (Table 2). Antifungal drugs such as ketoconazole and griseofulvin are a good source of antimicrobial agents against fungi isolated from soil. Soil is confirmed to be a good reservoir of microorganisms particularly fungi (Nester *et al*., 2004). The formation of organic matter and the growth of plants depend on the microbial community within the soil. The level of microbial diversity in soil exceeds that of any other habitat on Earth. These varieties are supported by the complexity of the physical and chemical habitats, which provide a vast array of microhabitats (Willey *et al*., 2011). Fungi can be influenced by physiochemical properties of soil, temperature, humidity, season and human activities.

Most of the different concentrations of antifungal tablets have effects on the isolates. However, as the concentration of the antifungal drugs increased, the diameter of zone of inhibition also increased (Table 3). Ketoconazole exhibited greater inhibitory potential than griseofulvin. This finding is in consonance with the work of Adam *et al*. (2008). The results of ketoconazole and griseofulvin have also been reported in the work by Gupte *et al*. (2002) where not all the isolates of a particular strain were sensitive to these drugs.

Willey *et al.* (2011) reported that antibiotics are fungistatic only as long as repeated application maintains high levels of unchanged antibiotic. However, a few drugs are useful in treating many major fungal diseases. In their work on agar-based disk diffusion assay for moulds, Nweze *et al*., (2010) showed that agar based disc diffusion tests can be developed for moulds and not used for yeasts only.

Table 1: pH and Moisture Content of the Soil

Sampling Location	pH	Moisture Content (%)
Microbiology Department	6.78 ± 0.01^{ab}	1.13 ± 0.03^{a}
Microbiology Laboratory	8.34 ± 0.02^{c}	1.97 ± 0.01^{b}
Biochemistry Department	6.80 ± 0.03^{b}	2.00 ± 0.03^{b}
CBT Centre	6.75 ± 0.01^{a}	1.10 ± 0.01^{a}
Plant Biology Department	6.81 ± 0.01^{b}	3.68 ± 0.05^{c}

Legend: Each value is a mean of three determinations ± standard deviation. Different superscripts on the columns are significantly different ($p<0.05$).

Table 2: Occurrences and Distribution of the Fungal Isolates in the Soil Sample

Fungal isolates	Sampling period (week)							
	1	2	3	4	5	6	7	8
Aspergillus niger	+	-	+	+	-	+	-	+
Aspergillus flavus	+	+	+	+	+	+	+	-
Aspergillus fumigates	+	-	-	-	+	-	-	+
Mucor racemosus	-	+	+	+	+	+	+	-
Rhizopus stolonifera	+	+	-	+	-	-	-	+
Fusarium oxysporium	-	+	+	+	-	+	+	-

Legend: + = Present; - = Absent

Table 3: Antibiotic sensitivity pattern of ketoconazole and griseofulvin on the Fungal isolates

Fungal isolates	Concentrations (g/ml)	Diameter of zone of inhibition (mm)		
		Ketoconazole	**Griseofulvin**	**Control (water)**
Aspergillus niger	0.02	20.00 ± 0.58^{e}	12.00 ± 0.58^{c}	0.00 ± 0.00^{a}
	0.04	22.00 ± 0.58^{f}	15.00 ± 0.58^{e}	0.00 ± 0.00^{a}
	0.06	26.00 ± 0.58^{i}	18.00 ± 0.58^{g}	0.00 ± 0.00^{a}
Aspergillus flavus	0.02	22.00 ± 0.58^{f}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	0.04	24.20 ± 0.00^{h}	20.00 ± 0.58^{h}	0.00 ± 0.00^{a}
	0.06	26.00 ± 0.58^{i}	20.20 ± 0.06^{h}	0.00 ± 0.00^{a}
Aspergillus fumigatus	0.02	21.30 ± 0.06^{f}	13.00 ± 0.06^{d}	0.00 ± 0.00^{a}
	0.04	22.00 ± 0.06^{f}	17.00 ± 0.12^{f}	0.00 ± 0.00^{a}
	0.06	23.00 ± 0.12^{g}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Mucor racemosus	0.02	0.00 ± 0.00^{a}	15.00 ± 0.03^{e}	0.00 ± 0.00^{a}
	0.04	21.00 ± 0.03^{f}	15.50 ± 0.06^{e}	0.00 ± 0.00^{a}
	0.06	23.00 ± 0.06^{g}	16.70 ± 0.06^{f}	0.00 ± 0.00^{a}
Rhizopus stolonifer	0.02	12.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	0.04	15.00 ± 0.06^{c}	9.00 ± 0.17^{b}	0.00 ± 0.00^{a}
	0.06	18.00 ± 0.29^{d}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Fusarium oxysporium	0.02	0.00 ± 0.00^{a}	20.00 ± 0.06^{h}	0.00 ± 0.00^{a}
	0.04	34.00 ± 0.029^{j}	23.00 ± 0.03^{i}	0.00 ± 0.00^{a}
	0.06	35.10 ± 0.06^{k}	26.00 ± 0.12^{j}	0.00 ± 0.00^{a}

Legend: Each value is a mean of three determinations ± standard error. Different superscripts on the columns are significantly different ($p<0.05$).

4. Conclusion

This report shows the possibility of using moulds isolated from the soil for antifungal studies. This work also shows that this method is reproducible, is not complex, and could be used to determine the antifungal properties of moulds.

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