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Resistance to Azole Drugs by Fungal Species Isolated from Date Palm Samples within Lafia Metropolis, Nigeria

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Abstract

Fungal species infecting fruits are developing resistance to some antifungals. The study determined prevalent fungi in date palm fruits and their resistance to azole antifungals. Dried date palm fruit samples were collected from Modern Market, Old Market, Alhamis Market, Koro Market and Tomato Market in Lafia Metropolis, Nigeria and fungi isolated from them. Antifungal susceptibility test as a zone of inhibition of fungal mycelial growth were recorded for terbinafine, clotrimazole, nystatin and fluconazole. Dates from the old market had the highest contamination with $6.0\times10^3 \pm 4.2$ cfu/g, while samples from Koro market with $2.5\times10^3 \pm 1.7$ cfu/g were the least contaminated. A total of 84 fungi were isolated and *Aspergillus niger* with incidence of 58 (69.1%) was the most predominant species while *Aspergillus versicolor* with a value of 1 (1.2%) was the least predominant. Terbinafine was the most effective azole against the fungal species isolated with a zone of inhibition of 44.7 ± 6.8 mm against *Penicillium chrysogenum*, while fluconazole was the least effective with no zone of inhibition against a host of the isolates. The most resistant isolataes were *Fusarium graminaerum, A. fumigatus, A. versicolor* and *P. chrysogenum* at 0.0 mm inhibition. Results of this study indicated that, date palm fruits around the Lafia markets were more susceptible to *Aspergillus niger* contamination than other fungal species, and Terbinafine was the best azole antifungal drug. The study recommends that further research should be carried out with respect to the resistance developed against azoles by the fungal species.

Keywords: Fruits, azoles, *Aspergillus*, infection, contamination, resistance

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Introduction

Post-harvest pathogens especially fungi are a menace bringing huge losses to farmers in the form of reduced yields and income, foreign exchange losses, export rejection and labour loss [1]. The pathogens invade agricultural crops after harvesting due to poor hygienic attitudes and lack of adherence to the principle of Hazard Analysis Critical Control Point (HACCP) by stakeholders along the food chain [2]. Pathogens (fungi, bacteria, pests) are introduced to crops and other products derived from them along the food chain (farm-to-table). Fungi invade and colonize crops due to the conducive tropical climate (hot and humid) which encourages growth and multiplication. Fungi not only spoil food materials, they also decimate the quality of such crops and produce secondary metabolites known as mycotoxins which are lethal when consumed. Mycotoxin contamination of agricultural produce is reported to reduce safe crops for consumption thus creating a food security challenge worldwide [3]. Some of the common mycotoxins apart from causing mycotoxicosis have been designated carcinogenic, teratogenic, estrogenic, nephrotoxic immunosuppressive and neurotoxic [4].

Filamentous fungi like *Aspergillus*, *Penicillium* and *Talaromyces* are the most common post-harvest fungal genera. The fungi grow easily at low water levels peculiar to dried and stored crops, and the crops contain rich growth substrates making them ideal growth mediums. In Nigeria, most storage facilities are poorly aerated and humid, a condition that favours infection and proliferation. The date palm tree (*Phoenix dactylifera*) belongs to the *Arecaceae* family and possesses antimicrobial properties, primarily attributed to the presence of antioxidants in the fruits [5]. The antioxidants are phenolics, flavonoids, vitamin C, vitamin E, and glutathione which aid in reducing free radicals within cells thus preventing lipid peroxidation, DNA damage, and protein modification [6]. The fruit is commonly eaten in the Northern part of Nigeria due to the beneficial attributes it contains.

Azoles are a class of antifungal agents that target fungal cell membranes. They act by blocking the activity of 14-alpha-sterol demethylase, a member of the microsomal cytochrome P450 (CYP) family. This enzyme is essential for the production of ergosterol, an important component of the fungal cell plasma membrane [7, 8]. By inhibiting this enzyme, azoles cause the accumulation of 14-alpha-methylsterol, which disrupts the function of certain membrane-bound enzymes and affects the arrangement of acyl chains in phospholipids. This inhibits fungal growth.

Some azoles directly increase the permeability of fungal cell membranes and others hinder the conversion of lanosterol to ergosterol by inhibiting the activity of lanosterol 14α-demethylase [9, 10]. These compounds usually have a five-membered ring structure containing two or three nitrogen atoms. Azole antifungals are both substrates and inhibitors of CYP3A4 of the cytochrome P450 family and are compatible with calcium channel blockers, immunosuppressants, chemotherapy drugs, benzodiazepines, and tricyclic drugs. The aim of the study was to determine the azole resistance of fungi isolated from date palm fruits consumed in Lafia Metropolis, Nigeria.

Materials and Methods

Isolation of fungi

A total of 40 samples of dried date palm fruits were sourced from five markets in Lafia Metropolis, Nasarawa State. The markets were the Modern Market, Old Market, Alhamis Market, Koro Market and Tomatoes Market. Fruits of date palm were collected from different vendors in each market. The fruits were blended separately, weighed and homogenized in distilled water and inoculated on prepared Potato Dextrose Agar (PDA) and incubated at 37^{o}C for 6 days.

Identification of fungal isolates

Identification of isolated fungi was done by observing the colony morphology and the formation of spores by the isolates. After incubation and further purification, taxonomic identification of isolates was done by viewing colours and shapes of colonies macroscopically, and observing the fruiting structures and spores microscopically according to Dayan [11]. Identification was based on the protocol described by Cheesbrough [12].

Antibiogram with azoles and other anti-fungal antibiotics

The antifungal *in vitro* assay was carried out on fungal strains isolated in the study. The azole drugs tested were terbinafine, metronidazole, clotrimazole, nystatin and fluconazole. Discs of filter papers were prepared and soaked with a known concentration of the azole drugs. A concentration of 50 mg/mL per drug solution was prepared and the disks were inserted and then air-dried on petri dishes. The organisms were then introduced from the 7-day-old cultured sample of the isolates separately into prepared Potato Dextrose Agar (PDA). The drug-embedded disks were then placed without pressure but made to stick to the medium using forceps. The plates were kept at room temperature and observed for growth every 24 h, and the zones of inhibition were measured using a meter rule.

Results and Discussion

Total fungal counts in date palm fruit

Table 1 showed generally high fungal counts for all samples. Of the date fruits collected, those from the Old market were found to have the highest rate of contamination, measuring 6.00 ± 4.16 × 10^3 cfu/g. Comparatively, the Koro market samples within the Metropolis had the least contamination, measuring 2.50 ± 1.73 × 10^3 cfu/g though the obtained values were not statistically significant (p=0.081).

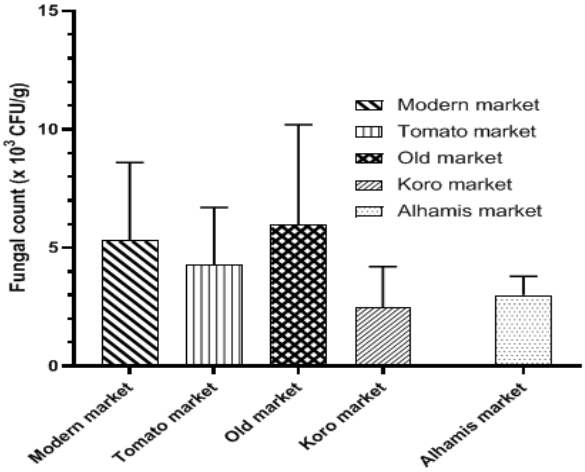

Figure 1: Total fungal counts in date palm fruits from markets (Values are expressed as Mean ± Standard Deviation)

Fungal species and frequency of occurrence from date palm fruit

Eight-four fungal species suspected according to their macroscopic and microscopic characteristics were isolated in the study (Table 1). These include *Candida* species which are smooth and creamy in colour and have short and inflated conidiophores, *Aspergillus niger* which is initially white and turn black with long conidiophores arising from septate hyphae, *Penicillium chrysogenum* which are olivaceous green with sterile white margin and branched conidiophores, *Aspergillus versicolor* which are initially white then turns orange cream with green spore at the centre and a smooth walled and brittle conidiophore, *Aspergillus fumigatus* which are smoky grey-green with short conidiophores, *Fusarium graminaerum* which are initially white then turns pale yellow with branched conidiophores, *Aspergillus carbonaruis* which are brownish-black in colour with biseriate conidiophores and finally *Rhizopus* species which are initially white then turns to grey or yellowish brown with a globose to ovoid sporangiospores.

Frequency of fungal occurrence in the different sampling markets

Eight fungal species were isolated and identified from date fruits collected in markets within Lafia as shown in Table 1. *Aspergillus niger* occurred most with a 69.1% occurrence rate followed by *Aspergillus carbonarium, Aspergillus fumigatus, Penicillium chrysogenum* and *Candida* species with 6.0% occurrence each, followed by *Rhizopus* species with 3.6%, then *Fusarium graminaerum* with 2.4% while *Aspergillus versicolor* had the least occurrence with 1.2% from the samples collected.

Table 1: Incidence of fungal species from the different sampling locations

Fungal isolates	Mm	Tm	Km	Om	Am	Freq. (%)
Candida species	0	0	0	1	4	5 (6.0)
A. niger	9	20	10	17	2	58 (69.1)
P. chrysogenum	2	1	1	0	1	5 (6.0)
A. versicolor	0	0	0	0	1	1 (1.2)
A. fumigatus	0	0	1	2	2	5 (6.0)
F. graminaerum	1	0	1	0	0	2 (2.4)
A. carbonarium	0	0	1	4	0	5 (6.0)
Rhizopus species	0	0	3	0	0	3 (3.6)
Total	**12**	**21**	**17**	**24**	**10**	

Mm = Modern market; Tm = Tomato market; Km = Koro market; Om = Koro market; Am = Koro market; Freq. = Frequency

Antibiogram with azoles and other anti-fungal drugs

The Antibiogram carried out using four different antifungal drugs (Table 2); terbinafine, clotrimazole, nystatin and fluconazole reveals that terbinafine was the most effective antifungal on the fungal species isolated than the other antifungal used with the highest zone of inhibition of 43 mm in diameter. The anti-fungal with the least effectiveness is Fluconazole with the least zone of inhibition of 0 mm. The organism that is most susceptible to the anti-fungal drugs during the research was *Aspergillus niger* and the organism that is most resistant to the anti-fungal drugs used was *Penicillium chrysogenum*. The market that has the most resistant organism is the modern market while the market with the most susceptible market is the Koro market.

Table 2: Radial growth (mm) of isolated fungi against test drugs

Isolated fungi	Markets	Anti-fungal drugs used			
		Terbinafine	Nystatin	Clotrimazole	Fluconazole
Aspergillus niger	Alhamis Market	41.0 ± 2.3 ^{a}	23.0 ± 2.0 ^{c}	20.7 ± 1.2 ^{c}	11.3 ± 1.5 ^{d}
Aspergillus niger	Old Market	32.7± 2.9 ^{b}	24.3 ± 2.1 ^{c}	25.3 ± 7.6 ^{c}	16 ± 1.0 ^{d}
Aspergillus niger	Tomato market	35.7 ± 5.0 ^{ab}	21.0 ± 3.5 ^{c}	20.7 ± 1.5 ^{c}	13.67 ± 0.6 ^{d}
Aspergillus niger	Modern market	36.0 ± 4.4 ^{a}	26.7 ± 2.1 ^{c}	27.3 ± 4.0 ^{c}	16.7 ± 2.9 ^{d}
Aspergillus niger	Koro market	31.7 ± 3.2 ^{b}	21.7± 1.2 ^{c}	19.3 ± 3.1 ^{cd}	11.7± 1.2 ^{d}
Aspergillus carbonarius	Koro market	38.0 ± 2.7 ^{a}	18.3 ± 1.2 ^{cd}	23.3 ± 1.2 ^{c}	16.7± 1.5 ^{d}
Aspergillus carbonarius	Old market	34.0 ± 4.0 ^{b}	22.7 ± 0.6 ^{c}	26.0 ± 2.0 ^{c}	12.3 ± 1.5 ^{d}
Aspergillus fumigatus	Old market	35.7± 5.0 ^{ab}	33.0 ± 0.6 ^{b}	19.3 ± 2.3 ^{cd}	0
Aspergillus fumigatus	Koro market	38.3 ± 1.5 ^{a}	15 ± 1.0 ^{cd}	21.7 ± 2.9 ^{c}	0
Aspergillus versicolor	Alhamis market	21.0 ^{c}	40.0 ^{a}	18.0 ± 2.0 ^{cd}	0
Penicillium chrysogenum	Alhamis market	40.7± 9.1 ^{a}	26.0 ± 2.7 ^{c}	29.0 ± 2.7 ^{b}	24.7± 2.1 ^{c}
Penicillium chrysogenum	Koro market	44.7± 6.8 ^{a}	25.3 ± 1.2 ^{c}	27.7 ± 0.6 ^{c}	0
Penicillium chrysogenum	Tomato market	38.0 ± 7.6 ^{a}	20.7 ± 0.6 ^{cd}	23.3 ± 4.9 ^{c}	0
Penicillium chrysogenum	Modern market	43.0 ± 4.4 ^{a}	0	24.0 ± 7.21 ^{c}	0
Candida species	Old Market	9.3 ± 0.6 ^{d}	0	15.7 ± 1.5 ^{cd}	0
Rhizopus species	Koro market	33.7± 3.1 ^{b}	20.0 ± 1.0 ^{cd}	21.0 ± 5.2 ^{c}	0
Fusarium graminaerum	Modern market	44.0 ^{a}	8.0 ± 13.9 ^{d}	27.0 ± 2.0 ^{c}	0
Fusarium graminaerum	Koro market	39.7± 4.2 ^{a}	24.3 ± 4.2 ^{c}	40.0 ± 1.7 ^{a}	21.0 ± 4.6 $^{c/}$

Values are Means of three triplicates and are expressed as Mean ± Standard Deviation. a[1]= Means with different letters are significantly different at P= 0.05 (Tukey HSD all-pairwise comparisons test)

The antibiogram reveals that terbinafine was the most effective antifungal against the fungal species isolated. This could be due to the rare use of the drugs as it is expensive and not commonly known. Terbinafine blocks the biosynthesis of fungal sterol by inhibiting squalene epoxidase [16]. Fluconazole with the least activity might be due to its frequent administration. It decreases ergosterol synthesis and inhibits cell membrane formation. The most resistant fungi isolated were *Penicillium chrysogenum* which could be due to the over-expression of 14α- sterol demethylase it habours while the most susceptible organism was *Aspergillus niger* which has limited 14α- sterol demethylase expression.

Different azoles have varying affinities for their target, which can explain the variations in their range of effectiveness. This is evident in the resistance profiles of different fungi [17]. Other factors that affected the effectiveness of the test drugs and resistance by fungal isolates were the variations in the structure of azoles which determines the patterns of cross-resistance among species of the same genera [18, 19], extensive use of an azole drug, and genetic mutation as noticed in the ERG11 gene. The gene encodes lanosterol C14a-demethylase in fungi. It acts by interfering with the disruption of the synthesis of ergosterol in the cell membrane of the fungus. It is important to note that multiple mechanisms can operate simultaneously in a particular fungal strain, resulting in additive effects.

Multiple mutations can exist within one gene, and their cumulative effects can be significant. In a study conducted by Lopez-Ribot et al. [20], it was discovered that certain strains of *Candida*, with decreased susceptibility to azoles, have higher levels of intracellular ERG11p compared to those that are more susceptible to azoles [21]. As a result, the antifungal agents become overwhelmed, and the standard therapeutic concentrations are no longer sufficient to effectively inhibit ergosterol synthesis. Target enzyme up-regulation can be achieved through gene amplification, increased transcription rate, or decreased degradation of the gene product [22]. However, this mechanism is unlikely to have a significant contribution to the overall resistance burden in *Candida* species, as only minor increases in enzyme levels are recorded.

When azole compounds come into contact with fungi, the resulting effect is the exhaustion of ergosterol in the fungal membrane. This exhaustion is accompanied by the accumulation of the hazardous byproduct 14a-methyl-3,6-diol, which brings about growth cessation. The prevention of the formation of 14a-methyl-3,6-diol from 14a-methylfecosterol is attributed to the mutation of the ERG3 gene [23]. If ergosterol is replaced with the latter product, it will result in functional membranes that will render azoles ineffective on the ergosterol biosynthetic pathway. The absence of ergosterol in the cell membrane causes *Candida* strains with ERG3 gene mutation to be resistant to polyenes. While uncommon, resistance to azole compounds among *Aspergillus* species is widely acknowledged, with the first resistance mechanism being the expression of efflux pumps that reduce the intracellular concentration of itraconazole [24, 25]. The second and more common mechanism of resistance involves the modification of the 14asterol demethylase enzyme, which is encoded by the cyp51A and cyp51B genes [26, 27]. A resistance phenotype with elevated MICs to all azoles is associated with amino acid substitutions at the M220 position, though that occur at specific sites within a genetic sequence are correlated with a phenotype that resists treatment, as evidenced by increased minimum inhibitory concentrations for all azole drugs. In contrast, mutations at the G54 site demonstrate cross-resistance to itraconazole and posaconazole.

Aspergillus fumigatus has recently been found to possess a new mechanism of azole resistance, in which mutations located in the promoter region of cyp51A result in the protein product being overexpressed [28]. With the rise in the use of new-generation azoles for the treatment and prevention of invasive aspergillosis, it is imperative to maintain surveillance for azole resistance and the extent of cross-resistance amongst *Aspergillus* species. It is worth noting that the efficacy of azoles against other emerging fungal pathogens, including Zygomycetes, *Fusarium*, and *Scedosporium* species, is inconsistent. While fluconazole has been consistently found to be ineffective against these organisms, new-generation azoles have varying degrees of effectiveness. Thus, susceptibility testing should be used to direct antifungal therapy.

Conclusions

The findings of the study indicate that *Aspergillus niger* is the most prevalent fungal species contaminating date palm fruits in the markets of Lafia. The study also revealed the presence of several other fungal species, which suggests that the fruits are susceptible to contamination, making them unsuitable for consumption by both humans and animals. Terbinafine from the study showed the best activity against the fungal isolates in the study. The study also confirmed that among the azoles antifungal used, the fungal species isolated were least inhibited by fluconazole.

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