



Phenotypic and Genotypic Identification and Antifungal Susceptibility of Some Fungi Isolated from *Tympanotonus fuscatus* var. *radula*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AUK and OSO designed the study. Authors AUK, OSO and MMD wrote the protocol. Authors AUK and JEO wrote the first draft of the manuscript. Authors OSO, MMD and JEO performed the statistical analysis and helped with the analyses of the work. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study aimed to identify fungi isolated from *Tympanotonus fuscatus* var. *radula* and evaluate its level of susceptibility to known antifungal compounds.

Place and Duration of Study: Biotechnology Advanced Research Centre, Sheda Science and Technology Complex, Abuja between September and December 2019.

Methods: *Tympanotonus fuscatus* var. *radula* samples were purchased from the Keffi, Masaka, and Orange markets in Nasarawa State, Nigeria. Fungal isolation was achieved using Sabouraud dextrose agar supplemented with chloramphenicol and incubated at 28°C for 5 days. ITS-1 and ITS-4 primers were used at 94°C for 2 min, 52°C for 1 min, and 72°C for 2 min for the polymerase chain reaction before sequencing at Inqaba Biotech South Africa. Disk diffusion technique was employed for antifungal susceptibility testing.

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Results: Data obtained revealed that the suspected fungal species exhibited a generally higher level of resistance (19-40 mm) to 1 µg voriconazole in addition to a 20-35.5 mm zone of inhibition against 10 µg ketoconazole. Blast sequence analysis of the isolated samples revealed a 99.65% sequence homology to *Meyerozyma guilliermondii*, 99.38% to *Fusarium oxysporium isolate E-225 1* and 96.23% to *Aspergillus terreus isolate A2S4*.

Conclusion: Food safety involves isolating and accurately identifying disease causing pathogens such as fungi in food. Based on the fungal load obtained from this study, proper cooking and handling of sea-food which would otherwise cause disease, is highly recommended.

Keywords: Molecular; fungi; *Tympanotonus fuscatus var. radula*; antifungal; Nigeria.

1. INTRODUCTION

Tympanotonus fuscatus var. radula are invertebrates with shells that have blackish-brown stripes and tapering ends. It is distinguished from other varieties by the absence of spiny tubercles on the shell [1]. *Tympanotonus fuscatus var. radula* is easily obtainable in aquatic environments such as but not limited to water banks, mangrove estuaries, swamps, etc, thus classified as prosobranch gastropod common in many [2]. It is the most abundant molluscs obtained within such environments particularly in West Africa [3]. It is a relatively cheap source of high-quality protein, minerals, and amino acids [4,5]. *Tympanotonus fuscatus var. radula* is used in the preparation of many Nigerian and West African cuisines with or without the shells. These delicacies include ekpang nkukwo and edikang ikong soup in Southern Nigerian states; Akwa Ibom, Bayelsa and Cross River, where they are known locally as mfi. In Bayelsa State, they are used to prepare isemi fulo and foi isemi.

However, studies on the microbiological evaluation of *Tympanotonus fuscatus var. radula* shows that they harbor fungi and other pathogenic microorganisms which have been implicated in the outbreak of foodborne diseases in many parts of the world [6]. In times past, fungi were only identified by examining their microscopic and macroscopic features.

The disadvantage of this technique is that some fungi do not produce features such as fruiting bodies that are easily noticed while some do not produce at all and this could result in the wrong presentation of the fungal load. Fungal identification based on microscopic features such as spore producing structures after media culturing is also not reliable due to the inability of some fungi to grow in culture and the difficulty of identifying fungi to species level based on microscopic features. Recombinant DNA

technologies geared towards species diagnostics proffers a measure by which closely related microorganisms can be individually identified based on their highly distinguishable genetic make-up even when different species share common morphological and biochemical features [7]. Molecular identification is an indispensable technique which enables scientists to delve further in the study of fungal pathology, physiology, taxonomy, fungal evolution, community genomics as well as in fungus-plant interactions [8].

At the turn of the millennium, genetic techniques have aided in the specie-specific identification of filamentous fungi. This has in-turn triggered the re-classification of several organisms previously thought to belong to one or more different families without consideration for their close relationships with other families of fungi [9].

Preceding an accurate identification of pathogenic fungi, it is expedient to do an antifungal susceptibility test to determine antifungal efficacy, eliminate the chances of drug resistance and chemotherapeutic failure. This work is aimed at molecular diagnostics as well as the elucidation of antifungal drug resistance of fungi isolated from periwinkle (*Tympanotonus fuscatus var. radula*) sold in markets in Nasarawa State, Nigeria.

2. MATERIALS AND METHODS

2.1 Description of Study Area

The study area is Nasarawa State, Nigeria. Nasarawa State is known as the state of solid minerals and it is bounded in the north by Kaduna State, in the west by Abuja, Federal Capital Territory, in the south by Kogi and Benue States, and in the east by Taraba and Plateau States. Nasarawa State is located in the middle belt of Nigeria with a total area of about 27, 117 km² and a total population of about 1, 869,377

people. The Coordinates of Nasarawa State is 8°32'N 8°18'E / 8.533°N. Lafia is the capital of Nasarawa State. Three markets in Nasarawa State were randomly selected. Keffi market in Keffi Local Government Area; Masaka and Orange markets both in Karu Local Government Area of Nasarawa State.

2.2 Macroscopic and Microscopic Examination of Isolation of Fungal Isolates

Sabouraud dextrose agar (SDA) + chloramphenicol (Laboratorios CONDA, South Africa) was used for total fungal counts and incubated at room temperature (28°C) for 5 days [10]. The fungal morphology was examined macroscopically by observing the colony features such as pigmentation, hyphae, shape, size, and kind of asexual spore, presence of special structure such as foot cell, sporangiophore or conidiophores, and the characteristics of the spores. Reproductive spores were microscopically examined by picking a small portion of the mycelia growth carefully with the aid of sterile dissecting needles and placed on a drop of lactophenol cotton blue stain on a clean glass slide covered with a coverslip. The wet mount preparation was examined under the electron microscope (Zeiss, Germany) for branched and unbranched hyphae [11].

Fungal isolates were picked with a sterile wire loop and sub-cultured onto freshly prepared sabouraud dextrose agar + chloramphenicol. They were further incubated at room temperature (28°C) for 5 days to obtain pure cultures which were sub-cultured into McCartney bottles and stored in the refrigerator at -4°C. All aseptic techniques were carried out under the Purifier Biosafety Cabinet (Model Delta series, LABCONCO, USA).

2.3 Molecular Identification

Species diagnostics of each isolate was achieved via the amplification and sequence analysis of the ribosomal DNA internal transcribed spacer region (ITS) using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers [12]. This polymerase chain reaction (PCR) was carried out in a final volume of 50µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, each dNTPs at a concentration of 0.2 mM, 1.25 IU of Taq polymerase, each primer at a concentration of 0.2 mM and 10 µl of DNA (50ng) template.

The PCR cycle ran at 94°C for 2 min, 52°C for 1 min, and 72°C for 2 min. The PCR product was purified using Zymo DNA clean and concentrator (DCC) kit (Zymo Research) and sequenced with a 3500 ABI genetic analyser at Inqaba Biotech Ltd, South Africa using the same forward and reverse primers employed during PCR amplification.

The results obtained were further imported into MEGA software for the construction of a phylogenetic tree using Bootstrap analysis and the statistical method used was Neighbor-joining. Taxonomic identification was achieved by comparing the obtained nucleotide sequences for similarity with reference strains by a BLAST search within the NCBI gene bank [13].

2.4 Antifungal Susceptibility Testing of the Isolates

Antifungal susceptibility testing was carried out using disc diffusion method. All glassware used were sterilized using a hot air oven (Mettler, Germany) at 160°C for one hour and media with autoclave (MSE, United Kingdom) at 121°C for 15 minutes. Media were also prepared according to manufacturers' instructions. 1mm diameter of 24-hour old pure colonies of the fungal isolates from Sabouraud dextrose agar + Chloramphenicol were inoculated into 5ml sterile 0.85% (w/v) NaCl (normal saline) and the turbidity was adjusted to the turbidity equivalent to 1.5 McFarland standard. The McFarland's standard was prepared by adding 0.5ml of 1.172% (w/v) BaCl₂·2H₂O into 99.5 ml of 1% (w/v) H₂SO₄. A sterile cotton bud was moistened in the adjusted inoculum suspension, excess fluid was removed by rolling the swab on the surface of the tube above the fluid surface. The dried surface of the plates of Mueller-Hinton agar (Titan Biotech, Rajasthan, India) were streaked with the isolate. Disc diffusion technique was employed in the antifungal susceptibility test. The antifungal disc was aseptically placed on the surface of the Mueller Hinton agar supplemented with 2% glucose and covered with 0.5mg/ml methylene blue agar as proposed by Clinical Laboratory Standard Institute (CLSI) M44A document. The antifungal disk was: itraconazole (10µg), fluconazole (10µg), Ketoconazole (25 µg), voriconazole (1 µg). The plates were then incubated at 35°C for 48 hours. Zones of inhibition were measured in millimeters using a transparent ruler. The measurement was from the point of marked decrease in fungal density to the point of 100% inhibition of growth. Interpretation was done using CSLI standards.

2.5 Statistical Analysis

Data were presented as means standard deviation of triplicate determinations. All statistical analyses were carried out using SPSS for windows version 21.0 statistical package (SPSS Incorporated. USA). One-way analysis of variance was done to determine significant difference as $p < 0.05$.

3. RESULTS

3.1 Isolation, Microscopic and Macroscopic Identification of Isolates

Three pure cultures of microorganisms were obtained via the successful isolation from each sample purchased at the three studied markets (Keffi market), (Orange market) and (Masaka market) respectively. Examination of these five isolates revealed a diverse colour range of cinnamon-brown, pink, greenish-brown, dark brown, and black with mycelia (Fig. 1, Table 1). The isolates were observed under (40X) microscope (Table 1). Analysis of the most frequently occurring isolate revealed that the organism suspected to be aspergillus was the most prevalent amongst others in all studied samples (Fig. 2). The least most isolated organisms were suspected to be fusarium (22%).

3.2 Molecular Identification

Fig. 3 was obtained from the analysis of amplified DNA fragments, approximately 1500bp in size, from the yeast isolates. The partial nucleotide sequences obtained were subjected to BLAST analysis and the identity was established based on sequence similarity and closest neighbour (Figs. 4-6). The blast and phylogenetic sequence query showed that *Aspergillus terreus* strain A2S4, *Meyerozyma guilliermondii* strain VV12, and *Fusarium oxysporum* strain E-225 shared the closest sequence homologies with the genomic DNA sequences of the three respective strains isolated at both ITS regions (Table 2).

3.3 Antifungal Susceptibility Test

Variations in response to different over-the-counter anti-fungal agents for incubation duration in the agar plate samples at their respective concentrations were examined (Table 3). The different zones of inhibition measured reflected each organism's predisposition to internationally recognized concentrations of universally accepted anti-fungal compounds.

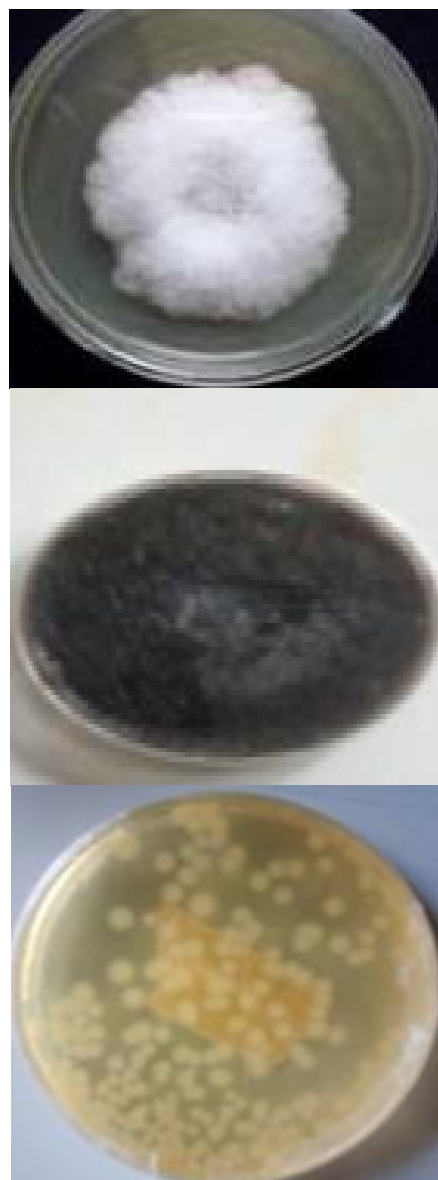


Fig. 1. Pictures of pure fungal isolates on SDA medium

4. DISCUSSION

The presence of fungi causes spoilage of *Tympanotonus fuscatus* var. *radula* by breaking down their parts and producing different acids and gas resulting in spoilage and deterioration. The taste, odour, flavor, and colour of the periwinkles are also affected. In the present study, fungi isolated from the raw periwinkle samples with shells based on macroscopic and microscopic features revealed that they harbor fungi suspected to be *Aspergillus* sp, *Fusarium* sp, and *Candida* sp. Most times, the

accumulation and concentration of pathogenic microorganisms and other toxic materials are usually from untreated human waste and industrial effluents that find their way into the water bodies that are inhabited by these shellfishes [14,15]. Omenwa et al., reported isolating *Saccharomyces cerevisiae* and *Fusarium sp* from raw farm reared periwinkles [16]. On boiling the periwinkles at 100°C for 5 minutes, the fungal load dropped to zero and this is in agreement with Omenwa et al., who reported zero fungal load for processed periwinkle samples. The morphological

examination and identification of fungi are useful for the identification of isolates up to the family or genus level [16]. However, this identification is not enough to identify the isolated fungi up to the species level [17]. Also, the characteristics of fungi are subject to the environment and conditions to which they are exposed to. Hence, the need for molecular identification makes it possible for the fungi to be identified to species level. The most frequently occurring fungi were subjected to molecular identification using recombinant DNA technique.

Table 1. Morphological Characteristics of Fungal Isolates from *Tympanotonus fuscatus* var. *radula*

S/N	Macroscopy	Microscopy	Probable organism
1	Cinnamon – brown colony, small, filamentous mycelia on the surface, grey on reverse	Compact Conidial heads, smooth and globose shaped conidiophores, unbranched sporangiophores	<i>Aspergillus</i> sp
2	Moist, flat, smooth, cream and yeast-like on surface, pink on reverse	Spherical	<i>Candida</i> sp
3	Dark brown cotton-like mycelia	Nonseptate, irregular branching, thick. Canoe shaped conidia	<i>Fusarium</i> sp

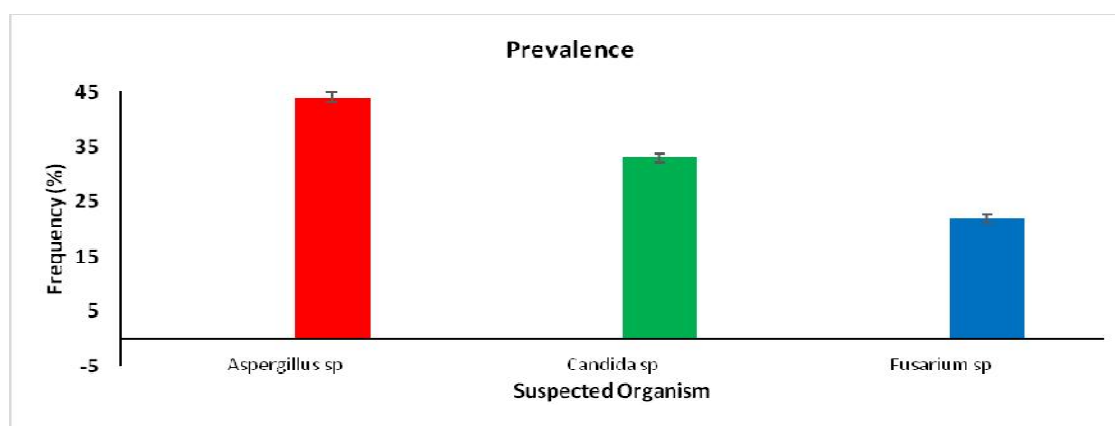


Fig. 2. Frequency of fungal isolates from *Tympanotonus fuscatus* var. *radula* sold in markets

Key: 1= *Aspergillus sp* (44%), 2= *Candida sp* (33%), 3= *Fusarium sp* (22%)

Table 2. Identification of yeast isolate based on sequence alignment (NCBI BLAST)

Nearest phylogenetic relative	Strain	Accession number	Sequence similarity (%)	Organism previously suspected
<i>Aspergillus terreus</i>	A2S4_D50	JX501387	96.23	<i>Aspergillus sp</i>
<i>Fusarium oxysporum</i>	E-225	KU059870	99.38	<i>Fusarium sp</i>
<i>Meyerozyma guilliermondii</i>	VV12	KT157518	99.65	<i>Candida sp</i>

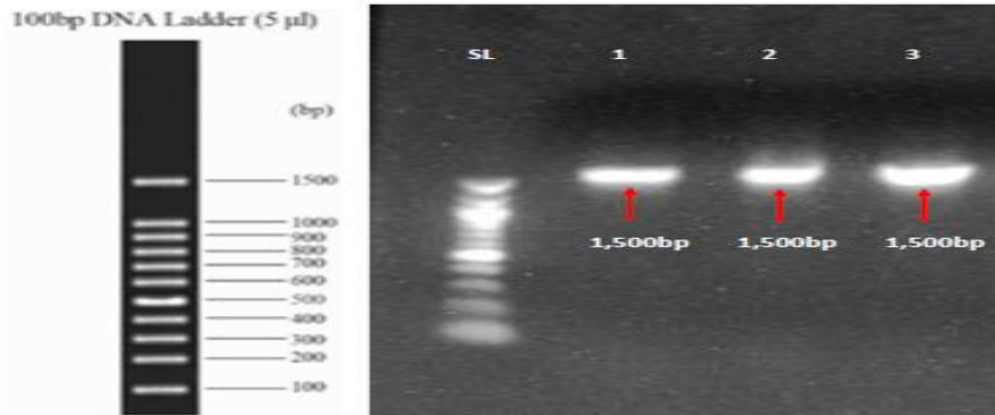


Fig. 3. Gel electrophoresis (1.2% Agarose) micrograph of amplified DNA for suspected organisms



Fig. 4. Phylogenetic tree of isolated presumed to *Aspergillus sp*

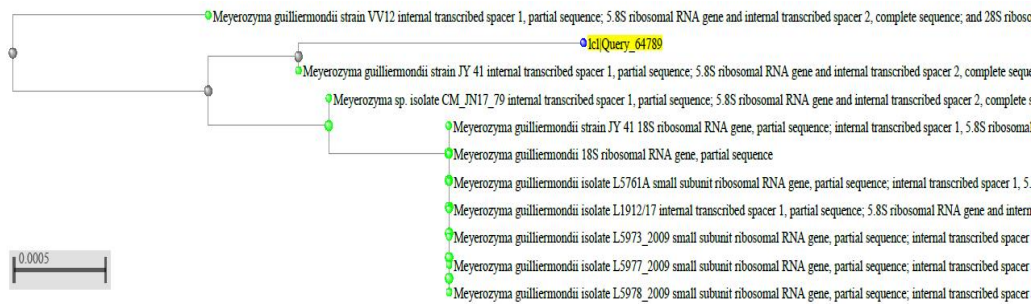


Fig. 5. Phylogenetic tree of isolated presumed to *Candida sp*

Table 3. Antifungal susceptibility of fungal isolates using disc diffusion method

Antifungals	Zone of Inhibition (mm)					
	<i>Aspergillus terreus</i>	<i>Meyerozyma guilliermondii</i>	<i>Fusarium oxysporum</i>	S	I	R
Voriconazole (1 µg)	35.0	20.0	19.0	5 (100)	0(0)	0(0)
Fluconazole (25 µg)	6.0	20.0	7.6	1(20)	0(0)	4(80)
Itraconazole (10 µg)	33.0	20.0	30.0	5 (100)	0(0)	0(0)
Ketoconazole (10 µg)	3.0	40.0	7.5	1(20)	0(0)	4(80)
%Susceptible	2(50)	4(100)	2(50)			
% Intermediate	0(0)	0(0)	0(0)			
%Resistant	2(50)	0(0)	2(50)			

(According to CLSI standard Interpretative for breaking points for *Candida* and *Filamentous fungi*)

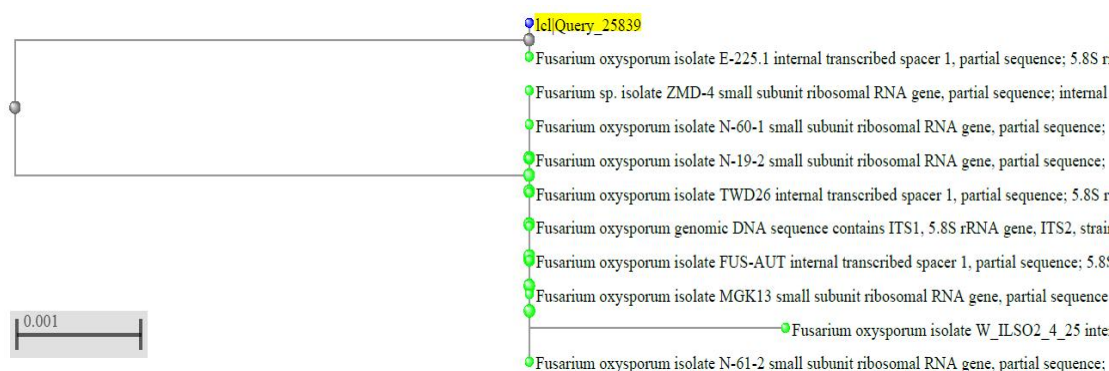


Fig. 6. Phylogenetic tree of isolated presumed to *Fusarium sp*

The fungus, *Candida sp* on subjection to molecular identification had a DNA sequence BLAST analysis indicating a high similarity of the obtained sequence corresponding 99.65% to *Meyerozyma guilliermondii*.

Meyerozyma guilliermondii is a highly anamorphous microorganism which shares close homology with other fungi such as *Candida fermenti*, *Candida carpophila*, and *Candida xestobii*, all of which are phenotypically undifferentiated from each other [18].

M. guilliermondii has been reportedly found in the human gut as part of the microflora in addition to being widely distributed in the soil [19]. Also, this microorganism has been associated with a range of disease conditions including but not limited to Candidemia, which can result in death [20].

M. guilliermondii have also been classified as flavogenic yeasts; the latter which is recognized for their characteristic flavour in fermented drinks and foods such as sweet wine, food additives, etc [21]. A study reported that *M. guilliermondii* possessed industrial significance as it produced isoflavone aglycone; an active principle widely sort-after and employed in a wide range of pharmacological activities [22]. In another study, the essential vitamins such as riboflavin, commonly known as vitamin B2 was easily produced from the same organism [23]. *M. guilliermondii* has been shown to exhibit great potential in the biological control of fungi responsible for post-harvest spoilage of fruits and vegetables [24,25].

Fusarium sp on subjection to molecular identification had a DNA sequence BLAST analysis indicating a high similarity of the obtained sequence corresponding 99.38% to *Fusarium oxysporum isolate E-225 1*.

Aspergillus sp on subjection to molecular identification had a DNA sequence BLAST analysis indicating a high similarity of the obtained sequence corresponding 96.23% to *Aspergillus terreus isolate A2S4_D50*.

Antifungal susceptibility was done using the disc diffusion method. The early diagnosis and initiation of treatments in fungal infections go a long way in aiding therapeutic success. In the present study, Voriconazole (VOR) (2mm) and Fluconazole (FLU) (10mm) exhibited weak antifungal activity against *Candida sp* while Itraconazole (ITR) and ketoconazole (KET) exhibited strong antifungal activity against the organism. KET is therefore the most efficient drug against *M. guilliermondii* species with a high zone of inhibition of 40.0mm. It also indicates that the *M. guilliermondii* species isolated from *Tyimpanotonus fuscatus* var. radula can be treated using KET. All the other fungal isolates show marked resistance to KET and FLU. *Aspergillus terreus* was susceptible to only VOR and ITR. However, Singh et al, reported that ketoconazole is the most effective antifungal agent against *Aspergillus terreus* [26].

5. CONCLUSION

It can therefore be concluded that in addition to morphological examination of fungi to the genus level, molecular techniques should also be employed for accurate and reliable identification of fungi to species level to aid in the best choice of antifungal agent to reduce chemotherapeutic failure and drug resistance.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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