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ASSESSMENT OF THE ANTIFUNGAL ACTIVITY OF ZINC OXIDE NANOPARTICLES AGAINST SOME SELECTED FUNGAL ISOLATES

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ABSTRACT

Over the years, economically important crop production and productivity worldwide have experienced a continuous decline due to diseases affecting plants induced by phytopathogens. This research aimed to investigate the antifungal effect of zinc oxide nanoparticles. Fungi were isolated from diseased *Cyperus esculentus* (Tiger nut) and were identified as *Aspergillus subflavus* TP-FLF5-OR073649*, Rhizopus spp.* and *Candida spp. Z*inc oxide nanoparticles (ZnONPs) were synthesised using *Moringa oleifera* leaf extract and characterised with a UV-VIS spectrophotometer, producing peaks at 350 nm. ZnONPs at all concentrations had an insignificant inhibitory effect on *Rhizopus* sp. and *Candida spp.* However, ZnONPs (50%) demonstrated significantly higher inhibitory activity on *Aspergillus subflavus*_TP-FLF5-OR073649 ($p<0.05$) compared to the control. *The* zones of inhibition ranged from (3.33 ± 0.94 to 14.00 ± 2.83 mm) and this was different from the control (6.67 ± 3.39mm). The study's findings indicate that ZnONPs demonstrated antifungal activity against the test fungi. More study should be carried out to explore in detail the applicability of these outcomes in a field trial.

Keywords: Phytopathogens, zinc oxide nanoparticles, *Moringa oleifera*, Agar Well diffusion, UV-VIS Spectrophotometer, Antimicrobial activity.

INTRODUCTION

The plant world is such that is filled with microorganisms. Both continue to grow in the same environment throughout their life cycle for a few or many years. Over the years, economically important crop production and productivity worldwide have experienced continuous reductions as a result of diseases that affect plants elicited by phytopathogens such as fungi, viruses, bacteria and nematodes (Chowdappa and Gowda, 2013; Sharma et al. 2017). A phytopathogen is any living organism that causes disease in or on a plant host (Allen, 2009). The act of survival of these plant pathogens inside the plant (hosts) imposes threats to the plant, which causes a reduction in the plant yield. Directly or indirectly, this affects food security with respect to the increasing population of humans in the present time, as well as affecting human health (Savary et al. 2012).

Man has devised different management processes to control phytopathogens and such processes include: physical, chemical, agricultural and biological methods, and millions of dollars have been channelled into controlling the threat posed by plant pathogens (Agrios, 2005; Sharma et al. 2017b). These control methods are aimed towards eradicating or reducing the pathogen inoculum; however, they are faced with several limitations, as they are labour-intensive, some method works best on some pathogens but not on others, resulting in environmental pollution as well as growing microbial resistance against agrochemicals (Chowdappa and Gowda, 2013; Sharma et al. 2017b). To break free from this problem that holds us back, it is imperative to explore new innovative solutions to protect our crops from diseases. Solutions that are gentle on the planet, cost-friendly, and effective. Nanotechnology, which is a powerful tool, has positioned itself as a game-changer in the pursuit of eco-friendly agriculture.

Nanotechnology is a cross-disciplinary field that involves altering materials at the atomic scale to develop or create innovative solutions for a wide range of challenges across multiple sectors. (Scrinis and Lyons, 2007; Koka et al. 2019). At the core of nanotechnology lies the remarkable potential of nanoparticles. (Chaudhary et al. 2018). Nanoparticles are extremely tiny particles, between 1-100 nanometers in size, which exhibit unique biological, physical and chemical characteristics because of the minute size they

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possess (Ahmed et al. 2016; Khan et al. 2017). The enhanced attributes of nanoparticles compared to large materials hold significant potential, particularly in reducing the reliance on toxic chemicals, such as pesticides, and enabling a more sustainable approach to plant protection. (Prasad et al. 2014). Also, nanoparticles can be used as good fertilisers (nano-fertilisers). These fertilisers allow for the controlled release of nutrients into the site where they are needed by plants. They can also be used to inhibit the growth of phytopathogens.

The production or synthesis of nanoparticles can be achieved via various methods such as biological, chemical and physical methods (Khan et al. 2017). Currently, there is a preference for the biological method (green synthesis) over the use of both physical and chemical methods (Rathore and Tarafdar, 2015; Ahmed et al. 2016; Dobrucka, 2018). The biological approach of synthesising nanoparticles utilises plant-based materials or plant extracts and has received great attention and offers additional advantages over the use of macromolecules and microorganisms (Shankar et al. 2004; Ahmed et al. 2016; Ali et al. 2016). The photo-assisted technique for producing nanoparticles is a simple and low-tech method, eliminating the need for sophisticated equipment (Gurunathan et al. 2009). It is cheaper and has environmental benefits, as there are no release of hazardous chemicals that cause harm to the environment (Prakash et al. 2013; Rajamanickam et al. 2013).

Zinc oxide nanostructures serve as highly efficient catalysts, thanks to their large surface area and exceptional reactivity, which makes them ideal for diverse catalytic uses (Chen and Tang, 2007). Zinc oxide (ZnO), which is a semiconductor with a wide band gap with a 3.37 eV energy gap at room temperature, is widely utilised because of its photochemical, electrical and catalytic attributes (Wang, 2004; Ashour et al. 2006). Zinc oxide nanoparticles (ZnO NPs) exhibit exceptional antibacterial, antimicrobial, and UV-blocking capabilities (Smijs, 2011).

Studies on ZnO as an antimicrobial agent have explored both microscale and nanoscale forms (Sirelkhatim et al. 2015). Reducing ZnO particle size alters its optical, electrical and chemical properties, potentially due to quantum confinement effects, thereby facilitating new applications (Ahmad, 2011). For example, reducing ZnO to the nanoscale enhances its antimicrobial effectiveness (Sirelkhatim et al. 2015). Specifically for antimicrobial uses, inorganic materials like ZnO offer advantages over organic materials, including lower toxicity, greater durability, reduced resistance, and high selectivity (Rana et al. 2006). The antifungal mechanism of ZnO nanoparticles involves their entry into cells via diffusion and endocytosis. Once inside, they interfere with normal mitochondrial activity, triggering Reactive Oxygen Species (ROS) and Zn^{2+} ions to be released, which can permeate the membranes of the cell and cause irreversible chromosomal damage, which leads cell mortality (Shoeb et al. 2013). Possessing antifungal effects against fungi, ZnO nanoparticles show promise for development as innovative biomedical solutions for human health (Abdulwahid *et al.* 2019).

MATERIALS AND METHODS

Collection of reagents

Zinc nitrate hexahydrate [$Zn(NO_3)_2.6H_20$] was obtained from Solutions Biotechnology Laboratory, Edo State, Nigeria.

Sterilisation of materials

With the use of detergent, laboratory glassware like conical flasks, test tubes, beakers, and measuring cylinders were soaked, washed and then rinsed using distilled water. After which, they were then wrapped in aluminium foil paper and autoclaved at 170°C for 45-50 minutes.

Preparation of potato dextrose agar

For this study, PDA was used as the nutrient medium, prepared following the manufacturer's guidelines. The medium was made by using 1L of distilled water to dissolve 39g of PDA powder, and then sterilised by autoclaving at 121°C for 15 minutes. Once cooled to 40°C, chloramphenicol (6mg) was added to inhibit bacterial growth. The prepared PDA medium was then aseptically dispensed into sterile Petri dishes (Klaus et al. 2021).

Isolation of fungi from Tigernut

Tiger nuts purchased from the market were segregated and tagged according to the purchase site. A measurement of 10g was placed in a sterile beaker. Then it was surface sterilised with 70% Ethanol for 5 minutes to remove surface

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contaminants. It was then macerated using a mortar and pestle and immersed in 10ml of normal saline for 30 minutes. The sample was homogenised by shaking vigorously. Afterwards, the saline was decanted and dispensed into sterile McCartney bottles labelled P1 as the stock and 9 ml of distilled water into a McCartney bottle labelled P2 and P3, respectively. For 15 minutes and 121°C, the bottles (P2 and P3) were autoclaved to ensure sterilisation. A sterile micro-pipette was used to transfer 1 ml of the stock solution to the first McCartney bottle labelled P2 to obtain a dilution of 10^{-1}. 1 ml was then transferred from that bottle labelled P2 serially into the last bottle labelled P3 to obtain a dilution of 10^{-2}. The spread plate inoculation method was used to isolate fungal plant pathogens from the tiger nut sample. 1 ml each of serially diluted solution from the bottle labelled 10^{-1} and $10^{-2,}$ respectively were poured into the Petri dishes, which had the PDA in them. It was then incubated at room temperature for 72 hours for fungal growth (Tomasiewicz et al. 1980).

Sub-culturing of fungi

Using a wire loop that was sterilised, fungal mycelia were picked. The mycelia were streaked on fresh potato dextrose agar medium. Incubation of the PDA plates was then done for 72 hours at a temperature of 28 ± 2°C to obtain pure cultures (Tomasiewicz et al. 1980).

Identification of fungi

Macroscopy and microscopy were carried out to identify the fungi isolates. By observing morphological and cultural characteristics of the colony, the macroscopy method was carried out. The isolates were stained with lactophenol blue to aid easy viewing (Barnett and Hunter, 2000).

DNA extraction

DNA extraction was initiated by combining 100 mg of fungal tissue (suspended in 200 µl isotonic PBS) with 750 µl of lysis buffer. The mixture was homogenised using a bead beater and cleared via centrifugation at 10,000 x g for one minute. A 400 µl aliquot of the supernatant was then passed through a Zymo-spin™ IV Spin Filter (7000 x g, 1 min). After discarding the filter's snap-off tip, 1,200 µl of fungal DNA binding buffer was integrated into the sample. We then transferred 800 µl of the mixture to a Zymo-spin™ IIC column and centrifuged at 10,000 x g for one minute. To recover the purified DNA, the column was moved to a 1.5 ml micro-centrifuge tube and eluted with DNA elution buffer via a final 30-second spin at 10,000 x g.

DNA amplification protocol

(1) The PCR master mixer (2x) was gently swirled for a short time and centrifuged after thawing.

(2) Individual 25 µl reactions were assembled on ice within PCR tubes, using the specific volumes and components detailed below.

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   a. One Taq One Step PCR master mix (2x) - (12.5µl) was quickly loaded.
   b. A primer (20µM) - (1.25µl) - forward
   c. A primer (20µM) - (1.25µl) - reverse.
   d. A Template DNA – (5µl)
   e. Nuclease-free water (5µl)

(3) The sample was gently swirled and spun.

(4) PCR was then done using the specific temperature and timing cycles

(5) PCR products (10 µl) were resolved by 1.0% agarose gel electrophoresis and visualised using ethidium bromide staining (Table 1).

Table 1: Thermal cycling conditions using PCR

Step	Temperature	Time	Cycle Fr equency
Start-up DNA separation	95°C	3mins	1
DNA separation	95°C	30sec	
Primer hybridization	55°C	30sec	35
Elongation	73°C	1min	
Final elongation	73°C	7mins	1
Cold storage	5°C	8	

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Preparation of agarose gel (1%)

A 1.0% (w/v) agarose suspension was created by adding 1 g of agarose to 100 ml of TBE buffer. The mixture was heated for approximately 3 minutes until the agarose fully dissolved, then allowed to cool to 56°C. At that point, ethidium bromide of 5 µl was added, and after ensuring the comb was properly aligned in the gel mould, the liquid agarose was poured and left to solidify at room temperature for about 45 minutes.

Agarose gel electrophoresis

10 µl of the DNA ladder was mixed with loading dye and loaded into the initial well. The gel was then placed in an electrophoresis tank filled with TBE buffer and subjected to a constant 90V for 60 minutes. Band migration was subsequently captured and analysed using a UV transilluminator within a gel documentation system.

Collection of plant sample

Moringa oleifera leaves were harvested from a *Moringa* tree in a garden in the Faculty of Agriculture, University of Benin, Nigeria.

Preparation of synthesis materials

Zinc nitrate functioned as the precursor in the reaction, while the Moringa oleifera extract acted as the natural reducing agent.

Preparation of Plant Extract

Moringa oleifera leaves were obtained from the Faculty of Agriculture, University of Benin, Nigeria, air-dried and pulverised. 10 g was weighed into 100 mL of distilled water and left to boil for 10 minutes at 80°C for optimal extraction of the phytochemical components. After boiling, the beaker was removed and left to cool. The resulting solution was sieved through a Whatman paper to obtain the plant extract (Seralathan et al. 2014).

Preparation of precursor solution

A zinc nitrate aqueous solution was prepared by dissolving 2.97g of zinc nitrate hexahydrate [$Zn(NO_3)_2 \cdot 6H_2O$] in 100 mL of double-distilled water. The reaction was carried out under constant agitation at room temperature, utilising a magnetic stirring bar for consistent mixing (Tokumoto *et al*., 2003).

Synthesis of zinc oxide nanoparticles

For the synthesis of ZnONP, to a flask containing 50 mL of the zinc nitrate aqueous solution, 50 mL of *Moringa oleifera* extract was added dropwise, and the mixture was continuously stirred for 3 hours at 80°C. A yellow colouration was observed indicating the formation of ZnONPs. (Dobrucka, 2018).

Characterisation of zinc oxide nanoparticles

The absorbance spectra of the nanoparticles were studied using a UV-Vis spectrophotometer at a range of 250 – 500 nm, and distilled water served as blank. (Abbas, 2019).

***In vitro* testing of the antifungal activity of phytosynthesized zinc oxide nanoparticles**

Following synthesis, the photosynthesized nanoparticles were subjected to antifungal screening using the agar well dilution. Different concentrations of nanoparticles (100%, 50% and 25%) were screened for their antifungal inhibition potential. A 9mm cork borer was used to create wells in the agar previously impregnated with fungal spore suspension. Wells without nanoparticles served as control. The zones of inhibition were measured and recorded. The experiment was performed in triplicate (Sindhu et al. 2020).

STATISTICAL ANALYSIS

Statistical significance was assessed using ANOVA via SPSS software, with data points representing the average of three independent replicates ± standard error. Descriptive metrics were also calculated to summarise the primary datasets. Means were separated using the Duncan multiple range test.

RESULTS

The results from the morphological description of fungi isolated from tiger nut in Table 2 reveal that the isolates were *Aspergillus subflavus, Rhizopus spp.* and *Candida spp.* Colonial features such as shape, size, margin, surface texture, optical activity and margination were recorded. The isolate identified as *Aspergillus* spp. was then subjected to phylogenetic analysis for further identification, which revealed the isolate was *Aspergillus subflavus*_TP-FLF5-OR073649 as shown in Figure 1.

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Table 2: Morphological description of fungal isolate from Tiger nut

MORPHOLOGY	ISOLATE 1	ISOLATE 2	ISOLATE 3
Margin	Entire	Undulate	Undulate
Elevation	Flat	Flat	Flat
Size	Large	Medium	Medium
Texture	Rough	Powdery colony	Mucoid
Pigmentation	Light green	Army green	Light yellow
Optical property	Opaque	Opaque	Translucent
Form	Circular	Circular	Irregular
Suspected organism	*Aspergillus spp.*	*Rhizopus* spp.	*Candida* spp.

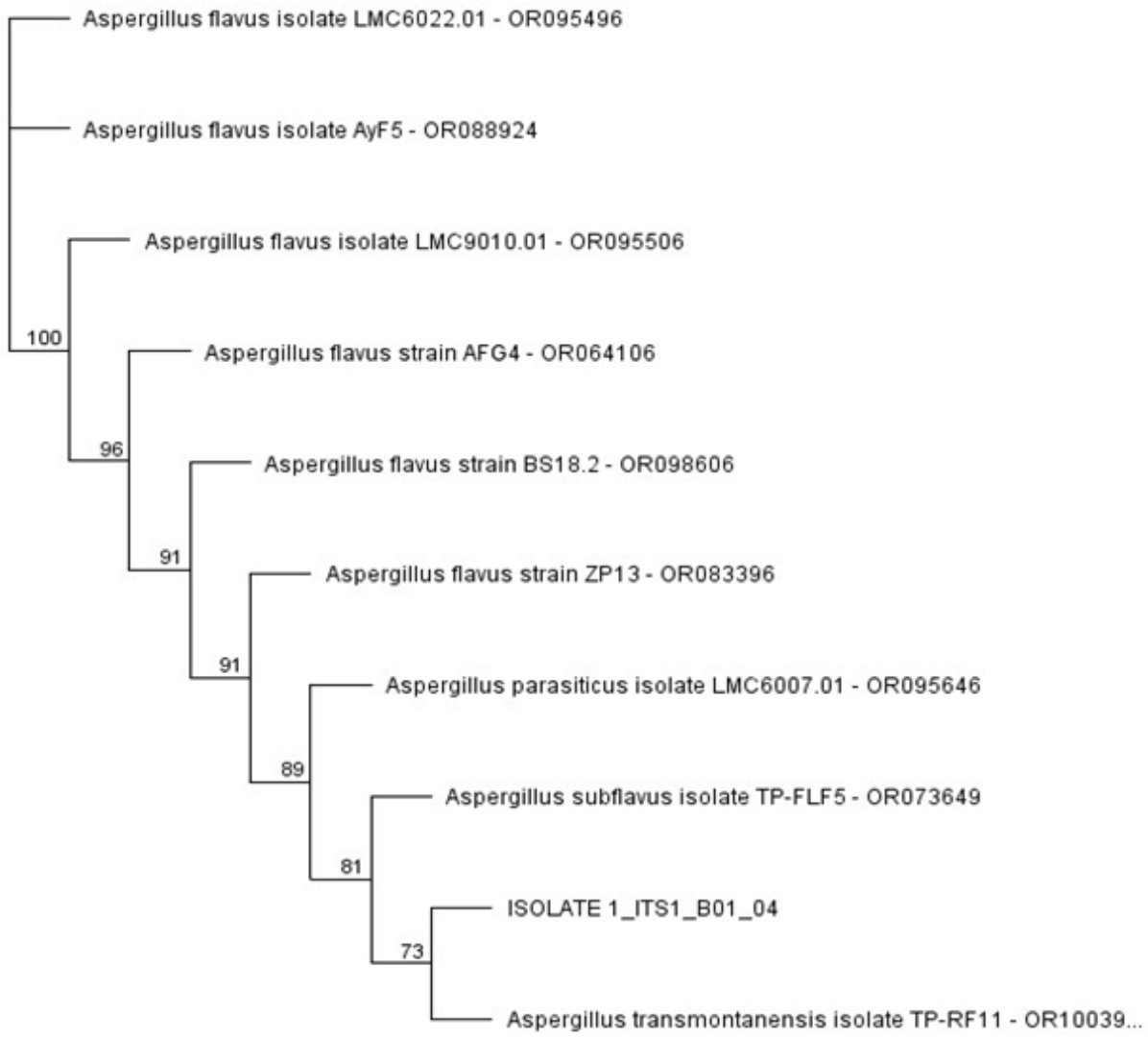

Figure 1: Phylogenetic characterization of the fungal isolates based on partial ITS region nucleotide sequences.

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Figure 2 shows the value of Absorbance of the biosynthesized ZnO nanoparticle recorded 24 hours after synthesis. The absorbance spectrum exhibited its maximum peak at 350 nm, indicating nanoparticle synthesis.

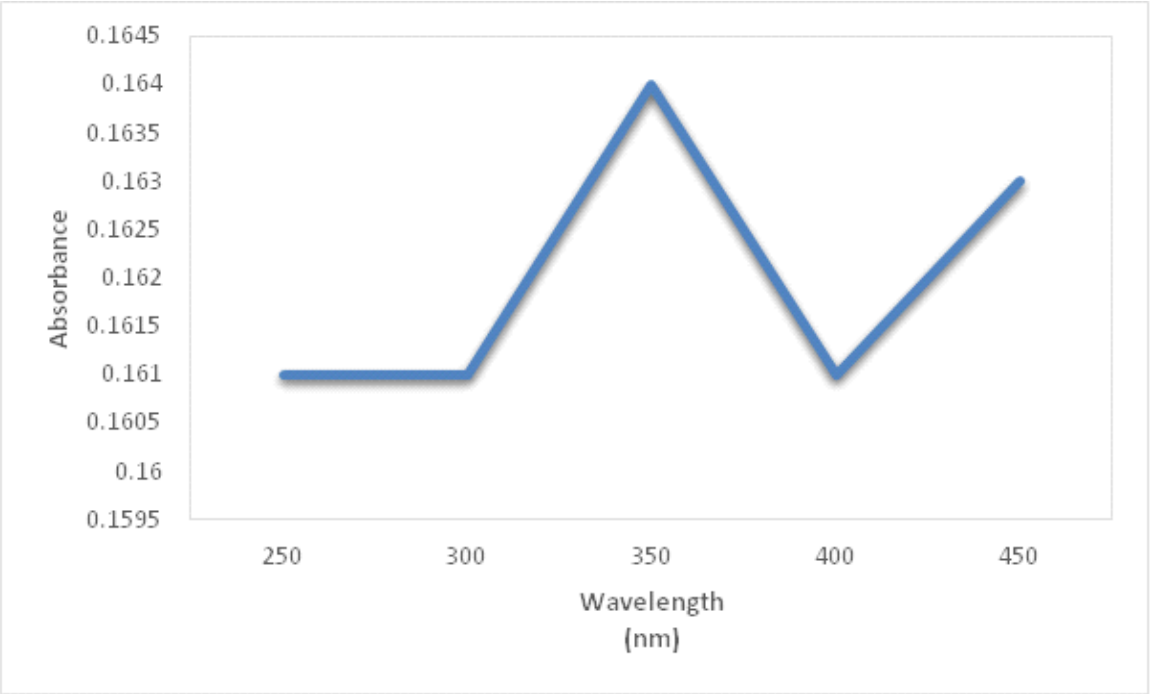

Figure 2: UV-VIS absorbance spectrum of the biosynthesized ZnONPs at 24 hours post-synthesis

Table 3 shows the *in vitro* antifungal efficacy of the synthesized ZnO nanoparticles on *Aspergillus subflavus*_TP-FLF5-OR073649 as represented by their zone of inhibition (mm) at various concentrations (%). Different concentrations of ZnO nanoparticles did not show antifungal activity against the *Rhizopus* spp and *Candida spp.* Isolates showed resistance to zinc oxide nanoparticles even at the highest concentration (Table 4 and 5).

Table 3: Antifungal effect of the bio-synthesized ZnONPs against *Aspergillus subflavus*_TP-FLF5-OR073649 represented by the inhibition zones (mm).

Concentration (%)	Zone of Inhibition (mm)
25	*3.33 ± 0.94 [a]
50	12.00 ± 2.83 [b]
100	14.00 ± 2.83 [b]
[+]Control	6.67 ± 3.39 [ab]

Legend: *Values are mean ± Standard errors; different superscript shows notable variation at $p < 0.05$ (Duncan multiple range test), [+]fungi culture inoculated with Ketoconazole alone.

Table 4: Antifungal effect of the bio-synthesized ZnONPs against *Rhizopus* spp. represented by the zones of inhibition (mm).

Concentration (%)	Zone of Inhibition (mm)
25	*0.00±0.00 [a]
50	0.00±0.00 [a]
100	0.00±0.00 [a]
[+]Control	16.67 ± 1.69 [b]

Legend: *Values are mean ± Standard errors; different superscript shows notable variation at $p < 0.05$ (Duncan multiple range test), [+]fungi culture inoculated with Ketoconazole alone.

Table 5: Antifungal effect of the bio-synthesized ZnONPs against *Candida spp.* represented by the zones of inhibition (mm).

Concentration (%)	Zone of Inhibition (mm)
25	*0.00±0.00 [a]
50	0.00±0.00 [a]
100	0.00±0.00 [a]
[+]Control	13.33 ± 1.25 [b]

Legend: *Values are mean ± Standard errors; different superscript shows notable variation at $p < 0.05$ (Duncan multiple range test), [+]fungi culture inoculated with Ketoconazole alone.

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DISCUSSION

This work aimed to determine the antifungal activity of ZnO nanoparticles on selected phytopathogen. Three fungi *Aspergillus subflavus*_TP-FLF-OR073649*, Rhizopus spp.* and *Candida spp.* were isolated from tiger nut and used in this study. These findings align with the observations of Chukwu *et al.* (2013) and Austin (2020), who also indicated the presence of *Aspergillus subflavus, Rhizopus spp.* and *Candida spp*. in both fresh and dry tiger nuts. Characterisation of ZnO nanoparticles was performed with the use of a UV-VIS spectrophotometer and exhibited peak absorbance at 350 nm. This aligns with a previous report by Hassan *et al.* (2021), where they reported that the absorption characteristics of the ZnO nanoparticles were recorded between 200 and 800 nm following the spectrophotometric parameters.

The inactivity of ZnONP in this study does not align with the findings of Janaki *et al*. (2015), who reported excellent antifungal activity against *Candida albicans* and *Penicillium notatum,* but aligns with the work of Abomuti *et al.* (2021), who reported that ZnONP was ineffective against *C. albicans* 5112 with a minimum fungicidal concentration of 31.25 µg/mL compared to fluconazole (64.00 µg/mL). However, the positive antifungal effect of ZnONP on *Aspergillus subflavus*_TP-FLF5-OR073649 used in this research at all concentrations aligns with the reports of Sohail *et al.* (2022), who reported that ZnONPs displayed excellent antifungal activities against *Aspergillus parasiticus* with a percentage inhibition of 77 ± 2 %, and this inhibition could be a result of ergosterol synthesis inhibition and membrane integrity disruption in fungi (Abomuti et al. 2021).

The inhibitory action of ZnO nanoparticles on the fungal isolate could also be attributed to their interaction with thiol groups containing vital enzymes, resulting in enzyme denaturation and death via oxidative stress. This aligns with the observation by Gurunathan *et al*. (2022), who indicated that the inhibitory activity is due to induced oxidative stress and released metal ions. Zinc oxide nanoparticles may have also induced the generation of Reactive Oxygen Species, leading to time- and concentration-dependent disruption of the cell membrane and cell wall in Aspergillus subflavus TP-FLF5-OR073649, which is similar to the report by Xu *et al.* (2013).

Different concentrations of zinc oxide nanoparticles (100%, 50% and 25%) synthesised using *Moringa oleifera* plant extract did not inhibit mycelia growth of *Rhizopus spp.* and *Candida spp.* except for the control ketoconazole. The large size of ZnONPs (30-150nm), mutation and the development of acquired resistance could account for the low *in-vitro* mycelial inhibition against *Candida* and *Rhizopus* spp.

CONCLUSION

The study's findings indicate that ZnO nanoparticles exhibit antifungal activity against *Aspergillus subflavus* _TP-FLF5-OR073649 in a concentration-dependent manner. More work should be carried out to establish the mode of antifungal action and the applicability of this treatment *in vivo*, thus contributing towards the attainment of sustainable agriculture and food security.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest.

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