The Antifungal Activities of Multi-Walled Carbon Nanotubes Decorated with Silver, Copper and Zinc Oxide Particles

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***Abstract*—** The *Aspergillus* species is a pathogenic fungus that can lead to life-threatening pulmonary infections most notably within immune suppressed individuals. Invasive aspergillosis is mainly caused by *Aspergillus fumigatus* whereas *Aspergillus ochraceus* is known as the most prominent producer of the mycotoxin named ochratoxin A (OTA). With ineffective antifungal treatment currently in use by municipal systems (chlorination), individuals are easily exposed to *A. fumigatus* and food producers can unknowingly spread OTA when *A. ochraceus* is spread through their irrigation system. In this paper, we reinforced the antifungal activity of silver and zinc oxide nanoparticles, by combining them with pristine multi-walled carbon nanotubes (MWCNTs) to form a decorated antifungal compound. The ratio of MWCNTs to silver and zinc oxide was varied to find the optimal ratio and functionalisation of the MWCNTs was achieved by mild acid treatment, using nitric acid. To validate the potential of the new antifungal compound on *A. fumigatus* and *A. ochraceus*, the compounds were tested to find the inhibition of each compound using the dry weight estimation. On the basis of results, it can be shown that MWCNTs decorated with zinc, copper and silver nanoparticles can inhibit the growth of both *Aspergillus* species. It is recommended that these antifungal agents be tested in future studies, at large scale water purification operations.

***Index Terms*—** Antifungal activity, multi-walled carbon nanotubes, silver nanoparticles, copper oxide, zinc oxide.

I. Introduction

Throughout the world, the accessibility to clean, safe water remains a major concern. This was evident from the United Nations' (UN) 2015 goal to increase access to safe water up to 50% [1]. The challenges hindering the UN to meet that target highlights the difficulty in delivering sustainable clean water, especially in developing countries. The contamination of ambient water bodies by pathogenic fungi is particularly credited with restraining efforts to deliver safe water. This is due to pathogens exhibiting resistance to traditional wastewater treatment methods, such as chlorination, as claimed by Warris *et al*. [2]. In spite of the extensive existence, slight attention has been given to the existence of fungal sources in aquatic environments. Anaissie and co-workers (2002) first brought attention to the *Aspergillus* species in water after they had found the species within the water system of a hospital and further found 33% of municipal water, 55% of hospital storage tanks and 21% of water in patient care areas tested positive for an *Aspergillus* species [3]. *Aspergillus fumigatus* is a fungus that causes established lung diseases and can lead to tuberculosis, especially in individuals with compromised immune systems [2]. *Aspergillus ochraceus*, causes similar infections, however, its danger lies in Ochratoxin A (OTA), a mycotoxin that it produces and which is found in foods, most often in grain [4]. Recently, nanoparticle materials have gathered interest in breaching the gap with regards to resistant pathogenic fungi and has become a strong alternative to conventional water treatment technology for effective removal of microbial contaminants [5]. The unique physical and chemical properties provide an alternative compound to the established fungi control strategies [6]. Studies have already shown the potential of using silver (Ag), copper oxide (CuO), and zinc oxide (ZnO) to inhibit fungal growth [7-10]. Silver nanoparticles (Ag NPs) is the most widely used nano-metal for antimicrobial applications as it has a broad spectrum of antimicrobial properties and activities as well as low toxicity to humans. Therefore, it is an appealing choice for wastewater purification and microbial control [11, 12]. Multi-walled carbon nanotubes (MWCNTs) have been found to possess strong antimicrobial activity and hybrid complexes that consist of metal NPs and CNTs are more effective. MWCNTs were selected since it known for its high permeability, enhanced adsorption capabilities and lower economical cost [13]. Research further indicated that CNTs have produced strong antimicrobial effects due to their unique size and structure as well as the large specific area, since the increase in surface is directly correlated to the efficiency [14, 15].

In this study, we investigated the potency of these compounds decorated onto MWCNTs against the opportunistic fungi namely *Aspergillus Fumigatus* and *Aspergillus Ochraceus.*

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II. METHODOLOGY

A. Chemicals and Cultures

Zinc oxide powder, copper oxide powder, silver nitrate, nitric acid, N,N-dimethylformamide (DMF), and trisodium citrate dehydrate (>99 % purity) was purchased from Associated Chemical Enterprises (ACE, Johannesburg, South Africa). Sabouraud dextrose nutrient broth and agar powder were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The multi-walled carbon nanotubes were purchased from Graphene Laboratories Inc. (New York, USA) with a diameter range of 50-85 nm and a length of 10-15 µm.

Fungal strains (*Aspergillus fumigatus* and *Aspergillus ochraceus)* were supplied by the Department of Microbiology, Johannesburg University (South Africa).

B. Method

B.1 Mild acid treatment of carbon nanotubes

The MWCNTs were purified by a mild acid treatment process. A mass of 1 g MWCNTs was suspended in 50 mL nitric acid (30%) and stirred for 2 hours at 24°C in order to remove most of the impurities and increase the functional groups on the nanotube surface. The solution was filtered and rinsed with distilled water until the pH was increased to 7.0. The product was dried at 120°C for 24 hours.

B.2 Silver nanoparticle preparation

Silver nanoparticles (Ag NPs) were synthesized by the chemical reduction method of silver nitrate by sodium citrate. 100 mL $AgNO_3$ aqueous solution (0.01M) was boiled and 10 mL of 35 mM sodium citrate were added to the solution. The solution was left to cool to 25°C followed by centrifugation for 15 minutes at 15000 rpm. The supernatant was removed and the remaining solution was dried overnight at 50°C.

B.3 Composite material

DMF was used as the binding agent for the MWCNTs and metal nanoparticles. The DMF suspension of MWCNTs and NPs was sonicated for 20 minutes at 35 kHz. The decorated NPs were centrifuged at 4000 rpm and the supernatant was discarded and replaced with acetone that was allowed to evaporate at 25°C. The precipitate was collected and stored for further analysis and applications. This process was repeated with metal NPs to CNTs ratios of 2:1 and 5:1.

B.4 Characterization.

The MWCNT-composite morphology was characterized by scanning electron microscopy (SEM, FEI Quanta Quanta 250 FEG ESEM, Czech Republic).

B.5 Antifungal assessments

Radial fungal growth: Broth that contained varied concentration of the synthesized compounds (2000, 1000, and 500 µg/mL) was poor in the centre of the dish. The dishes were incubated at 37°C and the relative growth of the fungus colony was observed by measuring the radial growth. The inhibition rate (%) was calculated by comparing the controlled growth of the fungal mycelia with that of the *in vitro* assay compound growth.

Dry weight estimation: Broth (40 mL) inoculated with fungi was incubated for 24 hours at 37°C. Various concentrations of compounds were separately added to the broth to make final concentrations of 2000, 1000, and 500 µg/mL. The mixtures were further incubated for 7 days. After the incubation period, the inoculum was filtered and dried at 60°C for 24 hours after which it was weighed and compared with the positive control (with no antimicrobial compound added).

III. RESULTS AND DISCUSSION

A. Characterization

From the characterization of the CNTs, it can be seen that the diameter range correlates with that stated by the distributor (Figure 1). The effect of sonication can also be observed from Figure 2. The effect is already observed after 15 minutes, where the agglomerated tubes have started to disperse and the available area has increased. After 30 minutes of sonication, the tubes are more dispersed and functional areas are more available for nanoparticle binding to occur.

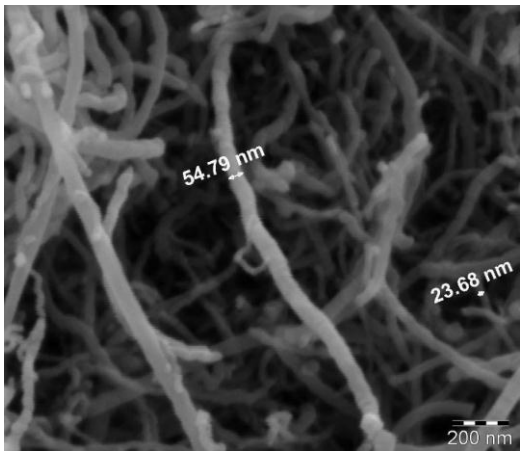

Fig. 1 Micrograph of MWCNTs that has been mild acid treated and sonicated.

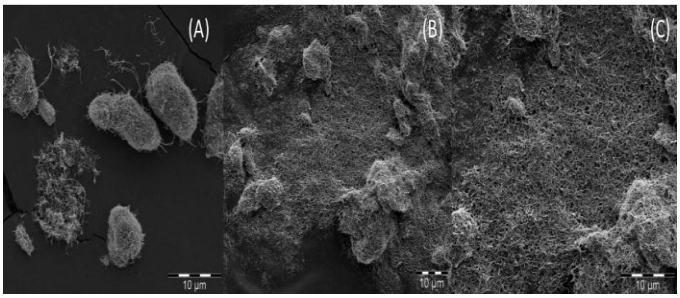

Fig. 2 The dispersion effect of sonication on MWCNTs. (A) No sonication, (B) 15-minute sonication, (C) 30-minute sonication.

B. Antifungal assays

B.1 Aspergillus fumigatus

Table 1 and Table 2 below represent the antifungal activity of different compounds on *A. fumigatus* with the biomass dry weight and radial growth tests.

TABLE I
DRY WEIGHT ESTIMATION OF *A. FUMIGATUS*. DATA IS PRESENTED AS THE MEAN ± STD ERROR. N=3

Complex	Dry fungal weight (g)			
	0 µg/mL	500 µg/mL	1000 µg/mL	2000 µg/mL
ZnO	0.1142 ± 0.0142	0.1097 ± 0.0094	0.0421 ± 0.0085	0.0290 ± 0.0189
Ag	0.1142 ± 0.0142	0.0914 ± 0.0127	0.0551 ± 0.0082	0.0425 ± 0.0108
CuO	0.1142 ± 0.0142	0.1018 ± 0.0130	0.0728 ± 0.0091	0.0176 ± 0.0091
CNTs	0.1142 ± 0.0142	0.1153 ± 0.0346	0.0977 ± 0.0109	0.0810 ± 0.0179
ZnO-CNTs	0.1142 ± 0.0142	0.1024 ± 0.0145	0.0309 ± 0.0092	0.0248 ± 0.0060
Ag-CNTs	0.1142 ± 0.0142	0.0998 ± 0.0107	0.0549 ± 0.0040	0.0175 ± 0.0032
CuO-CNTs	0.1142 ± 0.0142	0.1085 ± 0.0224	0.0940 ± 0.0129	0.0408 ± 0.0072

TABLE II
RADIAL FUNGUS GROWTH OF *A. FUMIGATUS*. DATA IS PRESENTED AS THE MEAN ± STD ERROR, N=3

Complex	Radial growth (mm) after 4 days			
	0 µg/mL	500 µg/mL	1000 µg µg/mL	2000 µg/mL
ZnO	85 ± 0.7	84 ± 0.9	82 ± 2.4	71 ± 7.4
Ag	85 ± 0.7	76 ± 2.5	70 ± 2.1	66 ± 1.1
CuO	85 ± 0.7	83 ± 1.6	82 ± 2.7	78 ± 1.6
CNTs	85 ± 0.7	85 ± 1.5	85 ± 1.2	82 ± 2.3
ZnO-CNTs	85 ± 0.7	84 ± 1.1	82 ± 2.8	70 ± 7.5
Ag-CNTs	85 ± 0.7	82 ± 3.3	71 ± 2.1	67 ± 2.4
CuO-CNTs	85 ± 0.7	85 ± 1.3	83 ± 2.5	82 ± 1.1

ZnO and ZnO-CNTs: From Table 1 it can be seen that the effect of ZnO, as well as the ZnO-CNTs compound, was more potent as the concentration was increased. The ZnO-CNTs compound was more effective at lower concentrations (500 and 1000 µg /mL) and only a slight increase was recorded at the highest concentration where the addition of MWCNTs didn't produce a significant improvement on the inhibition of the *A. fumigatus* species. The results of the radial growth test in Table 2 also confirmed the inhibition of the fungus with increasing concentration of the antimicrobial compounds.

Ag and Ag-CNTs: The result of the fungus weight after treatment with Ag and Ag-CNTs showed above in Table 1, showed that the lower concentration (500 and 1000 µg /mL) of Ag-CNTs composite material was on par with Ag. At the highest concentration (2000 µg /mL) the composite compound showed a notable increase in inhibition. The toxicity and physical combination of the Ag-CNTs compound were further confirmed by the results in Table 2 that showed the best reduction in radial growth for Ag and Ag-CNTs.

CuO and CuO-CNTs: The increase in concentration, as seen in Table 1, corroborated with stronger inhibition of fungus growth. The CuO-only compound was more effective than the composite across all concentrations.

CNTs and composites: From Figure 1, it follows that an increase in composite concentration will lead to reduced fungal growth. CNTs alone showed the least antifungal effect, with a minimal increase in inhibition despite a considerable increase in concentration. Ag-CNTs showed the highest inhibition at 500 and 2000 µg/mL with ZnO-CNTs showing more inhibition at 1000 µg /mL.

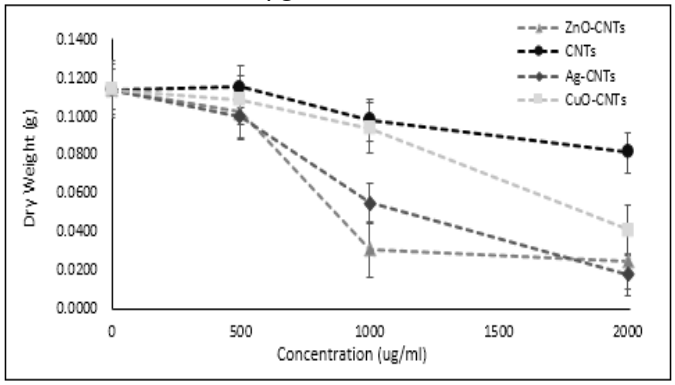

Fig. 3 Dry weight of *A. fumigatus* treated with CNTs, ZnO-CNTs, Ag-CNTs and CuO-CNTs

B.2 Aspergillus ochraceus

Table 3 and Table 4 follow below with data for the antifungal activity of different compounds on *A. Ochraceus* from the biomass dry weight and radial growth tests.

TABLE III
DRY WEIGHT ESTIMATION OF *A. OCHRACEUS*. DATA IS PRESENTED AS THE MEAN ± STD ERROR, N=3

Complex	Dry fungal weight (g)			
	0 µg/mL	500 µg/mL	1000 µg/mL	2000 µg/mL
ZnO	0.1379 ± 0.0576	0.1323 ± 0.0558	0.0756 ± 0.0313	0.0304 ± 0.0105
Ag	0.1379 ± 0.0576	0.0588 ± 0.0139	0.0361 ± 0.0162	0.0018 ± 0.0171
CuO	0.1379 ± 0.0576	0.1240 ± 0.0484	0.0332 ± 0.0151	0.0026 ± 0.0019
CNTs	0.1379 ± 0.0576	0.0972 ± 0.0474	0.0926 ± 0.0512	0.0864 ± 0.0556
ZnO-CNTs	0.1379 ± 0.0576	0.1305 ± 0.0514	0.0856 ± 0.0701	0.0530 ± 0.0205
Ag-CNTs	0.1379 ± 0.0576	0.1208 ± 0.0487	0.0830 ± 0.0124	0.0092 ± 0.0071
CuO-CNTs	0.1379 ± 0.0576	0.1038 ± 0.0203	0.0443 ± 0.0144	0.0270 ± 0.0152

TABLE IV
RADIAL FUNGUS GROWTH OF *A. OCHRACEUS*. DATA IS PRESENTED AS THE MEAN ± STD ERROR, N=3

Compound	Radial growth (mm) after 4 days			
	0 µg/mL	500 µg/mL	1000 µg/mL	2000 µg/mL
ZnO	85 ±	85 ± 0.5	81 ± 2.0	71 ± 7.7
Ag	85 ±	80 ± 1.3	75 ± 2.4	69 ± 1.6
CuO	85	83 ± 0.9	82 ± 0.7	82 ± 2.2
CNTs	85	85 ± 0.7	83 ± 1.9	82 ± 3.8
ZnO-CNTs	85	82 ± 2.6	81 ± 2.9	77 ± 2.9
Ag-CNTs	85	80 ± 1.9	76 ± 2.5	71 ± 1.9
CuO-CNTs	85	83 ± 1.8	82 ± 2.5	82 ± 0.8

ZnO and ZnO-CNTs: The combination of CNTs with ZnO did not result to more inhibition than ZnO alone at all concentrations. The discrepancy widened as the concentration was increased, as can be seen in Table 3 and confirmed by the radial growth measurements in Table 4.

Ag and Ag-CNTs: Ag was more effective than Ag-CNTs across all concentrations, but as can be seen in Table 3, with an increase in concentration, the discrepancy between the compounds narrowed considerably. This trend was also deduced from the results of Table 4.

CuO and CuO-CNTs: The CuO-CNTs compound showed more inhibition than CuO at 500 µg/mL. At higher concentrations, CuO was more effective and the difference in activity widened slightly. The close proximity of results in Table 3 was further confirmed by the relative radial growth of CuO and CuO-CNTs in Table 4.

CNTs and composites: From Figure 2 it can be seen that the effect of CNTs on *A. ochraceus* reached a plateau despite an increase in concentration. The use of CNTs alone, however, was the best compound at 500 µg/mL. At 1000 µg/mL ZnO-CNTs proved to be optimal while Ag-CNTs was more effective at 2000 µg/mL.

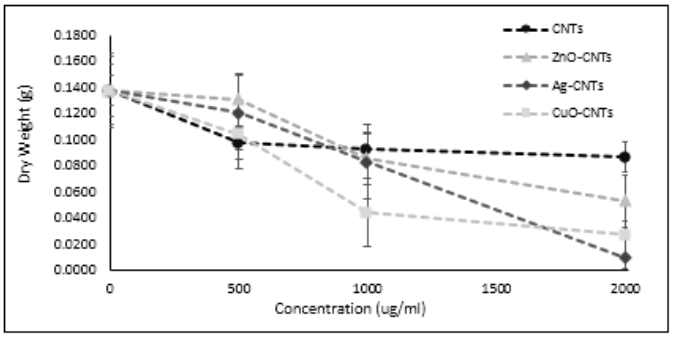

Fig. 4 Dry weight of *A. fumigatus* treated with CNTs, ZnO-CNTs, Ag-CNTs and CuO-CNTs**.**

IV. CONCLUSION

In conclusion, we have described the decoration of MWCNTs with ZnO, Ag and CuO NPs using a simple chemical evaporation technique and tested the compounds against various fungal species. The characterization has indicated that sonication successfully dispersed the nanotubes that resulted in more available surface area for enhanced antifungal activity. Overall, the control compound (AmB) had a better antifungal effect than the synthesized complexes, however the results showed that the metal NPs and CNTs composites were active against *Aspergillus fumigatus* and *Aspergillus ochraceus*. This correlates to similar findings from Najafzedah and co-workers [9]. The results reinforced the exceptional antifungal ability of NPs but also proved that the CNTs-based compounds could be viable alternatives to traditional wastewater treatment additives. Our findings also highlights the different effects of the various metal particles that has good antifungal properties. Against *A. fumigatus*, Ag-CNTs, ZnO-CNTs and Ag-CNTs showed the highest inhibition at 500, 1000 and 2000 µg/mL respectively. A number of antimicrobial mechanisms has been proposed to interpret the cytotoxicity of ZnO powders and NPs. These include the release of toxic ions, production of (Reactive Oxygen Species) ROS due to the presence of the NPs, stress caused on the surface, the shape and size of the particle, cell membrane damage by the adhesion of the particles, and the penetration through the membrane. Against *A. ochraceus*, CNTs, CuO-CNTs and Ag-CNTs showed the highest inhibition at 500, 1000 and 2000 µg/mL respectively. The strong antifungal effect is due to membrane damage that is caused due to oxidative stress from the carbon compound [14]. It is also proposed that the CNTs absorb the nutrients from the environment, leading the fungi to starve in the nutrient-deprived environment.

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