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RESEARCH PAPER

Comparison of antifungal activities of zinc, copper, cerium oxide, silver, gold, and selenium nanoparticles against clinical isolates of *Aspergillus*

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ABSTRACT

Objective(s): *Aspergillus species* are found as opportunistic agents to cause a wide variety of clinical manifestations. Regarding the drug resistance emergence against *Aspergillus* species, new aspects of using nanoparticles (NPs) as antifungal agents are considerable. This study takes a new approach to biosynthesized NPs of zinc oxide, copper oxide, cerium oxide, silver, gold, and selenium influence on the clinical isolates of *Aspergillus* species.

Materials and Methods: The antifungal activities of six NPs were examined against a total of 12 clinical isolates of *Aspergillus* species, including *A. flavus* (n=4), A. *welwitschiae* (n= 4), and *A. fumigatus* (n=4) based on the M38-A2 guideline.

Results: According to minimum inhibitory concentration (MIC) values, NPs of ZnO, Ag, Au, and Se showed a significant antifungal effect. CuO-NPs and CeO_2-NPs didn't show an inhibitory effect against *Aspergillus* isolates. The MIC ranges of ZnO-NPs, Ag-NPs, Au-NPs, and Se-NPs were 128-512, 26-53, 21-85, and 6-26 µg/mL for *A. fumigatus*; and 512->512, 26-53, 85, and 1-13 µg/mL for *A. welwitschiae*, respectively. In addition, the MIC ranges of Ag-NPs and Se-NPs were 26-53 and 106-425 µg/mL for *A. flavus*, respectively. However, *A. flavus* were not inhibited by NPs of ZnO and Au.

Conclusion: Among the examined NPs, ZnO, Ag, Au, and Se showed a significant effect against *Aspergillus* isolates except for CuO and CeO_2. However, Ag-NPs seemed to be the most effective nanoparticle against the *Aspergillus* species. Compared to other *Aspergillus* species, *A. flavus* was not inhibited by NPs of ZnO and Au.

Keywords: *Antifungal agents, Aspergillus, Clinical, Nanoparticles*

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INTRODUCTION

Aspergillus species are known as opportunistic fungal agents that can cause a wide variety of clinical forms such as allergic bronchopulmonary aspergillosis, aspergilloma, and tissue invasion [1, 2]. However, *Aspergillus* species have significant clinical importance in immunosuppressed patients, especially those with leukemia and transplant recipients [3, 4]. Therefore, this infection will cause high mortality in these susceptible patients due to the critical conditions of the patients and recently due to the coronavirus disease of 2019 (COVID-19) pandemic [2]. The antifungal drugs used to treat aspergillosis can cause a wide range of side effects both acutely (e.g., hepatic toxicity) and chronically

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(e.g., neuropathy) [5]. Moreover, chronic use of some of these agents such as voriconazole has been linked to more severe conditions like skin cancers; thus, their therapeutic regimen needs expensive and complex monitoring [6]. Crucially multiple studies indicate a broad spectrum of resistance against conventional antifungal drugs [7-9]. These limitations demonstrate a need to develop novel or supplementary agents to overcome this problem [5]. Recently, nanoparticles (NPs) are widely advised as the most reliable alternative to traditional antibiotics/ antifungals [10, 11]. Generally, these particles are nanosized ranging from 10 to 100 nm. Various types of NPs can be categorized based on their microscopic shape. Due to their unique properties, metal NPs have gained a lot of attention in the field of medicine [12]. Zinc oxide (ZnO) NPs (ZnO-NPs) are one the most promising nanoscale agents with antimicrobial activity [13]. It has been recognized as a safe product by the Food and drug administration (FDA) of the United States, and there are no scientific data about its potential threat to public health [14, 15]. ZnO-NPs can be found in personal products such as sunscreens, and anti-dandruff shampoos [16]. Furthermore, they demonstrate anticancer activity and the potential to be used in drug delivery systems [10]. With numerous applications in the medical and industrial fields, copper oxide NPs (CuO-NPs) are among the most widely used NPs [17]. In multiple studies, CuO-NPs showed effective antimicrobial, antioxidant, and anticancer activities [18]. Cerium oxide exists as CeO_2 or Ce_2O_3 with catalytic activity as a result of cerium's redox behavior [19]. These NPs trigger oxidative stress in bacteria and fungi; therefore, they can be used as antimicrobial agents [20]. Due to their large surface area to volume ratio, silver NPs (Ag-NPs) have a wide variety of potential activities ranging from medical imaging techniques to other biomedical fields [21]. In addition, Ag-NPs are known to be effective disinfectants and antimicrobial agents [22]. Gold (Au) NPs (Au-NPs) are another nanosized metal particle that has gained a lot of attention because of their unique properties [12]. They exhibit antibacterial and antiviral activities. Selenium NPs (Se-NPs) are described as a new class of potential antimicrobial agents [23]. Various studies indicate that Se-NPs can show antifungal activities significantly, thus they are gaining more attention among scientists in medical fields [11, 23, 24].

This study aims to investigate the impact of six NPs of ZnO, CuO, CeO_2, Ag, Au, and Se against clinical isolates of *Aspergillus* species.

MATERIALS AND METHODS

Fungal strains

A total of 12 *Aspergillus* clinical isolates were obtained from patients (Bronchoalveolar lavage and sinus biopsies) with aspergillosis at University Hospitals in Mashhad, Northeastern Iran. The *Aspergillus* species included *A. flavus* (n=4), *A. welwitschiae* (n= 4), and *A. fumigatus* (n=4), that identified by using morphological characteristics, PCR sequencing (using partial calmodulin (*CaM*) and β-tubulin (*BenA*) gene sequences) (using the primers CMD5 and CMD6) as described previously [25-27].

Synthesis and purification of nanoparticles

The synthesis and purification of ZnO-NPs [28], CuO-NPs, CeO_2-NPs [29], Ag-NPs [30], Au-NPs [31], and Se-NPs [32] refer to the methods previously approved by our research group.

Moreover, the current study evaluated the antifungal effects of NPs of ZnO, CuO, CeO_2, Ag, Au, and Se according to the Clinical and Laboratory Standards Institute (CLSI) M38-A2 guideline [33].

Antifungal activity evaluation of NPs against clinical isolates of Aspergillus

The suspension of NPs was solved in distilled water with the stoke concentration of 2048 µg/mL for ZnO, CuO, and CeO_2, 1700 µg/mL for Ag and Se, and 340 µg/mL for Au.

To obtain fresh samples, all isolates were subcultured on sabouraud dextrose agar (SDA) (Merck, Germany) and incubated at 35 °C for 3 days. To prepare inoculum suspensions, the surface of *Aspergillus* colonies was scraped with a sterile cotton swab and dissolved in a sterile saline solution containing 0.05% polysorbate 20 (Tween®20)) (Sigma, Germany), to prevent the adhesion of the spores. The transmittance rate of the mold suspensions was set to 80 % - 82 % at a wavelength of 530 nm by a spectrophotometer. The optical density (OD) would range from 0.09 to 0.13 for *Aspergillus* spp. Afterward, suspensions were diluted 1:20 in RPMI 1640 medium to reach the final concentration of 0.4-5×10^4 CFU/ml. To prepare RPMI 1640 medium, 3-N-morpholinepropanesulfonic acid (MOPS, Bio basic, and Canada) was used as a buffer to obtain a pH of 3.8. Subsequently, the pH of the medium was brought to 7 by adding a few drops of NaOH (1.0 N). To avoid bacterial growth, a few drops of sterile chloramphenicol were added, either. Initially, all of the 96-well plates were filled with 0.10 mL of RPMI 1640 medium; then, the indicated NPs concentrations of ZnO, CuO, CeO_2, Ag, Au, and Se along with the fungal suspensions were added to them. The final concentrations of each well were 1-512 µg/mL for ZnO, CuO, and CeO_2, 0.5-425 µg/mL for NPs of Ag and Se, and 0.125-85 µg/mL for Au-NPs. All of the filled plates were placed in an incubator at 35 °C for two days. Eventually, the minimum inhibitory concentration (MIC) ranges were evaluated visually as the lowest concentration of each nanoparticle, which inhibited 100 % of the fungal growth, in comparison to the positive control well.

RESULTS

The MIC range of each nanoparticle was reported in comparison with the positive control in Table 1. Among the MIC results of AgNP for *A. fumigatus*, just two out of four cases were inhibited in 0.5 and 53 µg/mL; so, the remaining cases were inhibited in 26 µg/mL. Besides, the MIC of 2 and 21 µg/mL in AuNP has been shown in two cases and 85 µg/mL in residual cases of *A. fumigatus.* The MIC results of SeNP were reported 6-106 which was 6, 26, 106, and >425 µg/mL for four cases of *A. fumigatus*. The MIC results of SeNP were 425 µg/mL in one case out of four and 106 µg/mL in the remainders of *A. flavus*, and 13 µg/mL in one case out of four and 1 µg/mL in the remainders of *A. welwitschia* (Fig. 1). The MIC_{50} which is the MIC range of 50 % of each *Aspergillus* isolates and geomean of each species is gathered

Table 1. The antifungal susceptibility profiles for nanoparticles (NPs) of ZnO, CuO, CeO_2, Ag, Au, and Se among clinical isolates of *Aspergillus* species

Aspergillus species	No. (%)	NPs	MIC (µg/mL)	Negative control	Positive control
A. fumigatus	4 (33%)	ZnO	128-512	-	G
		CuO	128	-	G
		CeO_2	>512	-	G
		Ag	0.5-53	-	G
		Au	2-85	-	G
		Se	6-106	-	G
A. flavus	4 (33%)	ZnO	>425	-	G
		CuO	>512	-	G
		CeO_2	>512	-	G
		Ag	26-53	-	G
		Au	>85	-	G
		Se	106-425	-	G
A. welwitschia	4 (33%)	ZnO	512	-	G
		CuO	>512	-	G
		CeO_2	>512	-	G
		Ag	26-53	-	G
		Au	85	-	G
		Se	1-13	-	G
Total	12 (100%)				

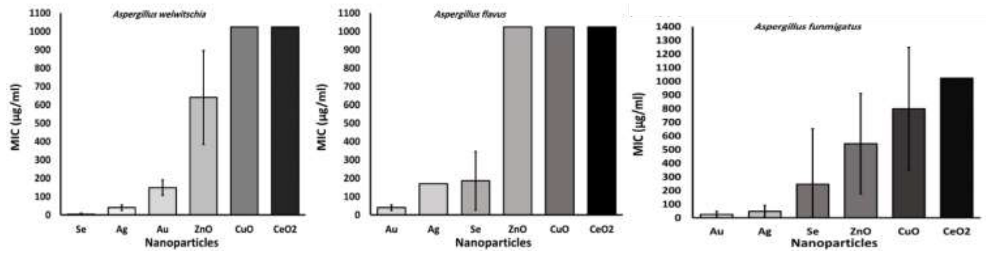

Fig. 1. The comparison of minimum inhibitory concentration (MIC) values of nanoparticles (NPs) in *A. flavus*, *A. welwitschiae*, and *A. fumigatus*

Table 2. MIC_{50} and G-Mean of nanoparticles (NPs) of ZnO, CuO, CeO_2, Ag, Au, and Se against clinical isolates of *Aspergillus* species

Aspergillus species	NPs	MIC_{50} (µg/mL)	G-Mean
	ZnO	512	322.53
	CuO	-	128
A. fumigatus	CeO_2	-	-
	Ag	26	11.56
	Au	85	23.47
	Se	-	25.47
	ZnO	-	-
	CuO	-	-
A. flavus	CeO_2	-	-
	Ag	53	37.12
	Au	-	-
	Se	106	149.99
	ZnO	512	512
	CuO	-	-
A. welwitschia	CeO_2	-	-
	Ag	53	37.12
	Au	-	-
	Se	1	1.89

in Table 2. Among the species, *A. fumigatus* was susceptible and *A. flavus* were highly resistant to nanoparticles. Also, among six nanoparticles, Ag-NPs showed a significant inhibition effect, and CeO_2-NPs did not exhibit a considerable effect.

The analysis of the statistical package for the social sciences (SPSS) 22 with a confidence interval of 95% and a power of 70%, and according to Fisher's exact test showed that the correlation between geometric mean (GM) results and various nanoparticles was not significant (p-value=0.830). On the other hand, the statistical analysis of GM results and the *Aspergillus* spp. have demonstrated that these variables were not significantly associated with specific species of *Aspergillus* (p-value =1.0).

DISCUSSION

One of the most difficult challenges in today's medicine is to develop highly specific novel antifungal agents since fungal agents are eukaryotes and more related to humans than bacteria. There are reports of broad-spectrum resistance against conventional antifungal drugs which can lead to adverse effects in patient management [7]. Various studies indicate that nanoparticles can be a promising replacement for traditional antimicrobial agents [34, 35]. This study aimed to evaluate the antifungal activities of NPs of ZnO, CuO, CeO_2, Ag, Au, and Se against the clinical isolates of *Aspergillus* species. Due to the distinct biological features of fungi, it is expectable to observe different sensitivity patterns against each metal NPs [13].

ZnO-NPs are considered safe by FDA, therefore can be used in many modern personal products such as sunscreens, and toothpaste [14, 15]. Several sources of the literature have demonstrated that ZnO-NPs can have inhibitory effects on various pathogenic fungi [13]. One of the first studies to investigate the effect of metal NPs in laboratory and field control of fungal contamination was done by Hassan and coworkers. They found that the addition of ZnO-NPs can inhibit the existence of aflatoxigenic fungi and aflatoxin production [36]. Although in the current study, we didn't examine the inhibition of aflatoxin production, however, the studied *A. flavus* is one of the main sources of aflatoxin production that could consider a new role for ZnO-NPs too.

In another study, Gunalan *et al.* described ZnO-NPs as an effective antifungal agent with broad-spectrum biocidal activities against different fungi [37]. CuO-NPs play a critical role in the field of medicine as an antioxidant with antimicrobial effects [18]. In 2017, Devipriya *et al.* evaluated the antifungal activity of *Cassia quadrangularis*-mediated CuO-NPs against *A. niger* and *A. flavus* which showed better results than standard fungicides [38]. In 2020, Velsankar *et al.* conducted a similar study, where they synthesized CuO-NPs via *Allium sativum* extract and tested the nanoscale agents against *A. fumigatus, A. flavus,* and *A. niger.* The results of this study indicated that CuO-NPs have an effective fungal growth inhibitor [39].

Depending on the synthesis method, Nanoceria (CeO_2-NPs) shows different properties. To produce antimicrobial and antifungal nanoparticles, the green synthesis route should be considered an environmentally friendly method of choice [20]. In an attempt to evaluate the antifungal effects of CeO_2-NPs against *A. fumigatus, A. flavus,* and, *A. niger,* Ahmed Mohamed *et al.* showed that green nanoceria had a significant effect against all of the examined strains, except *A. fumigatus*. Moreover, the inhibition zones related to *A. niger* indicated the most susceptible strain [40]. By disk diffusion method, Maqbool *et al.* determined the antifungal activity of CeO_2-NPs against *A. flavus,* and *A. niger.* The results of this study demonstrated that both strains were susceptible to nanoceria with a zone of inhibition of 19 mm [41].

Ag-NPs are among the most used nanosized agents with a wide variety of applications in the field of medicine [12]. Studies show that Ag-NPs can be also used as an effective disinfectant and antimicrobial agent with anticancer and anti-inflammatory activities [10, 22]. In 2021, Hosseini Bafghi *et al.* investigated the antifungal activity of biosynthesized Ag-NPs against standard strains of *A. flavus,* and *A. fumigatus* [42]. The results of this study indicated that Ag-NPs at varying concentrations showed considerable inhibitory impacts on the growth of the fungal strains.

Au-NPs are deeply related to chemistry with various applications in scientific fields like diagnosis, and cancer treatment or can be used as an antibacterial or antiviral agent [43, 44]. In 2014, Jebali *et al.* demonstrated that among nanocubes, nanospheres, and nanowires of Au, nanocubes had the highest antifungal effects against *Candida* species [45]. In another study, Khan *et al.* conducted research to evaluate the antifungal activity of Au-NPs conjugated with methylene blue (MB) against *Candida* species and concluded that Au-NPs had a minimum inhibitory effect on selected strains, whereas Au-NPs-MB showed a MIC of 31.2 µg/mL [44]. By the way, unfortunately, there are limited data about the possible effects of Au-NPs against *Aspergillus* species.

Due to their low toxicity and various properties such as antibacterial, antiviral, and antioxidant activities, Se-NPs have gained a lot of research attention [11]. In 2014, Kheradmand *et al.* studied the effect of Se-NPs enriched probiotics against *C. albicans* and observed a direct antifungal activity when *C. albicans* was co-cultured with Se-NPs-enriched *Lactobacilli* [46]. In 2015, Shakibaie *et al.* indicated that biogenic Se-NPs had an acceptable antifungal effect against *A. fumigatus* with a measured MIC of 100 µg/mL [47]. It is important to conduct clinical trials to observe evidence of any side effects of biosynthesized NPs in humans. Due to the rise of antifungal-resistant fungi, alternative methods are required to administer successful therapies and disinfectants. Our study was one of the first to evaluate the effectiveness of six NPs against clinical isolates of *Aspergillus* spp. However, there were some limitations, including the low number of clinical isolates of *Aspergillus* spp. and the restricted use of related species. Further studies are required, using a larger number of clinical isolates of predominant fungal agents such as *Candida* species.

CONCLUSION

Except for CeO_2-NPs, all tested NPs of ZnO, CuO, Ag, Au, and Se were effective against *Aspergillus* isolates, according to the findings of the current study. Ag-NPs were the most effective of these NPs. Additionally, *A. fumigatus* and *A. flavus* exhibited extreme susceptibility and resistance, respectively. Given the significance of nanoparticles as a potential future fungicide, additional research must be conducted to determine their effects on other *Aspergillus* species.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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