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Synergism of Zinc Oxide Quantum Dots with Antifungal Drugs: Potential Approach for Combination Therapy against Drug Resistant *Candida albicans*

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Candidiasis caused by *Candida albicans* is one of the most common microbial infections. Azoles, polyenes, allylamines, and echinocandins are classes of antifungals used for treating *Candida* infections. Standard drug doses often become ineffective due to the emergence of multidrug resistance (MDR). This leads to the use of higher drug doses for prolonged duration, resulting in severe toxicity (nephrotoxicity and liver damage) in humans. However, combination therapy using very low concentrations of two or more antifungal agents together, can lower such toxicity and limit evolution of drug resistance. Herein, 4–6 nm zinc oxide quantum dots (ZnO QDs) were synthesized and their *in vitro* antifungal activities were assessed against drug-susceptible (G1, F1, and GU4) and resistant (G5, F5, and GU5) isolates of *C. albicans*. In broth microdilution assay, ZnO QDs exhibited dose dependent growth inhibition between 0 – 200 μg/ml and almost 90% growth was inhibited in all *Candida* strains at 200 μg/ml of ZnO QDs. Synergy between ZnO QDs and antifungal drugs at sub-inhibitory concentrations of each was assessed by checkerboard analysis and expressed in terms of the fractional inhibitory concentration (FIC) index. ZnO QDs were used with two different classes of antifungals (azoles and polyenes) against *Candida* isolates: combination 1 (with fluconazole); combination 2 (with ketoconazole); combination 3 (with amphotericin B), and combination 4 (with nystatin). Results demonstrated that the potency of combinations of ZnO QDs with antifungal drugs even at very low concentrations of each was higher than their individual activities against the fungal isolates. The FIC index was found to be less than 0.5 for all combinations in the checkerboard assay, which confirmed synergism between sub-inhibitory concentrations of ZnO QDs (25 μg/ml) and individual antifungal drugs. Synergism was further confirmed by spot assay where cell viabilities of *Candida* strains were significantly reduced in all combinations, which was clearly evident from the disappearance of fungal cells on agar plates containing antifungal combinations. For safer clinical use, the *in vitro* cytotoxic activity of ZnO QDs was assessed against HeLa cell line and it was found that ZnO QDs were non-toxic at 25 μg/ml. Results suggested that the combination of ZnO QDs with drugs potentiate antimicrobial activity through multitargeted action. ZnO QDs

could therefore offer a versatile alternative in combination therapy against MDR fungal pathogens, wherein lowering drug concentrations could reduce toxicity and their multitargeted action could limit evolution of fungal drug resistance.

Keywords: ZnO QDs, *Candida albicans*, synergism, antifungals, multidrug resistance, fractional inhibitory concentration

INTRODUCTION

Recurrence of local and systemic fungal infections have posed a life-threatening public health problem globally for immunocompromised individuals, and treatment for fungal infections is estimated to cost around more than $7.2 billion (Benedict et al., 2019). *Candida* species are the most notorious, opportunistic fungal pathogens, known to cause oral and systemic candidiasis (Lamoth et al., 2018). Individuals with cancer treatment, organ transplantation surgeries, congenital immunodeficiency syndromes, acquired immunodeficiency syndrome (AIDS), and indwelling medical devices are frequently infected by *Candida*, resulting in morbidity and mortality due to invasive candidiasis (Prasad et al., 2011; Pal et al., 2017; Tso et al., 2018; Xiao et al., 2019). Antifungal drugs such as triazoles (fluconazole, itraconazole, and voriconazole) constitute the first line of standard treatment for candidiasis but due to their fungistatic action, fungal strains acquire resistance in due course toward the standard doses of these drugs (Watt et al., 2013). The next class of antifungal drugs comprises of polyenes (amphotericin B and nystatin) and next-generation echinocandins (caspofungin and micafungin). Although polyenes are effective fungicidal compounds, amphotericin B has toxic side effects such as renal impairment (Spampinato and Leonardi, 2013). Echinocandins are safer than amphotericin B and disrupt fungal cell wall integrity by inhibiting synthesis of β (1,3)-D-glucan (a fungal cell wall component). But echinocandins have a few drawbacks such as poor oral availability, high cost, and administration by intravenous injection only; which limit their use as standard treatment for invasive candidiasis (Spampinato and Leonardi, 2013).

Consequently, due to the limited antifungal arsenal, researchers have sought to improve treatment via different approaches. The approach of combination therapy consisting of two or more drugs in synergism has been considered as an alternative for treatment (Ahmed et al., 2014; Campitelli et al., 2017) to achieve broad spectrum antimicrobial activity and simultaneously reduce the risk of acquiring resistance during therapy. Combination therapy in place of monotherapy was therefore, introduced as a novel, safe, effective therapy, especially to target drug resistant strains and improve survival rate in high-risk life-threatening infections (Tamma et al., 2012). The objective behind this use of combination therapy was to lower the toxicity by reducing the standard administrative doses of drugs and improve the clinical efficacy of existing drugs (Campitelli et al., 2017). It is speculated that combination therapy affects multiple cellular/molecular targets in microbial pathogens, which prevents acquisition of MDR (Worthington and Melander, 2013; Campitelli et al., 2017). In combination therapy, the effect of interactions between two drugs is classified into three types: synergism, indifferent, and antagonism. The combination is called synergistic when the effect of combined drugs is greater than a single drug, indicating two different cellular targets for their action (Campitelli et al., 2017). While if the treatment shows no distinct difference, the combination is considered indifferent and if the effect is smaller than a single drug, then it is called antagonistic (Campitelli et al., 2017). Notably, the antagonistic effect might be due to the competition of both drugs for the same target, which might also be the reason for the antagonistic behavior of the amphotericin B and fluconazole combination against *Cryptococcus gattii*, where both compete for the same target, viz., ergosterol (Santos et al., 2012).

Use of two different classes of drugs targeting multiple components of fungal cells might be an effective strategy for treatment of fungal infections and in that context nanoparticles (NPs) might be an ideal choice for combination therapy because of their nano size, ease of surface modification, excellent antimicrobial activity, and cost-effective synthesis (Radhakrishnan et al., 2018a; Radhakrishnan et al., 2018b). Smaller, spherical NPs (within 50 nm) were highly permeable even in drug-resistant microbes which showed altered cell membrane/cell wall and overexpression of drug efflux pumps (Baptista et al., 2018). Moreover, the unique surface chemistry of NPs allows their conjugation with biomolecules for targeted drug delivery by facilitating controlled and sustained drug release (Wang et al., 2017). But toxicity due to NPs have been reported, which restricts the use of NPs as preferred antimicrobial agents for treatment. However, among various NPs, the ZnO NP, a semiconductor metal oxide, is extensively used in the medicinal industry and believed to be a biosafe, biocompatible, and non-toxic nanomaterial (Ali et al., 2018). Biocompatibility studies revealed no significant toxicity of ZnO NPs either in cell lines or during topical application for skin infection (Pati et al., 2014; Barman, 2015; Yusof et al., 2019). In addition to having excellent antimicrobial activity and preventing microbial biofilm deposition on implants, ZnO NPs also promoted proliferation of normal cells and angiogenesis (Memarzadeh et al., 2015; Oleshko et al., 2020). Hence, ZnO NPs were used for incorporation in implant and scaffold development (Pati et al., 2014; Barman, 2015; Yusof et al., 2019). Moreover, ZnO NPs displayed the potential for use as a supplement of Zn (essential micronutrient) in diet for regulation of innate and adaptive immune responses, enhancement of growth and development, prevention of local and systemic candidiasis, etc. (Swain et al., 2016; Yusof et al., 2019).

ZnO NPs mediate broad spectrum antimicrobial activity through targeting multiple cellular and molecular mechanisms,

which might prevent further acquisition of fungal drug resistance (Sirelkhatim et al., 2015). Therefore, it is likely that ZnO NPs in combination with other antifungal drugs might lead to a reduction in standard doses of antifungals, cost of treatment, and drug toxicity (Benedict et al., 2019). Furthermore, the size-dependent microbial toxicity of ZnO NPs was widely documented, wherein reduction in size increased antimicrobial activity (Preeti et al., 2020a; Jin et al., 2009; Joshi et al., 2009; Palanikumar et al., 2014; Sirelkhatim et al., 2015; da silva et al., 2019.

Spherical nanoparticles (commonly within 10 nm) with size comparable to or less than the Bohr radius of the material, where quantum confinement effects are prominent, are known as "quantum dots" (QDs) (Poulopoulos et al., 2012; Samanta et al., 2018). The quantum confinement in QDs create surface defects and produce active sites, which react with oxygen and hydroxyl ions to form highly reactive hydroxyl and superoxide radicals (Mudunkotuwa et al., 2012; Siddiqi et al., 2018). The smaller size in case of ZnO QDs facilitate higher dissolution of Zn^{2+} ions in solution, which could be responsible for the augmentation of reactive oxygen species (ROS) and thus resulting in peroxidation of biomolecules and cell death in microbial pathogens (Joshi et al., 2009; Asok et al., 2015). A recent study reported broad spectrum microbicidal activity of ZnO QDs (5–6 nm) against MDR pathogens (*E. coli* and *C. albicans*), implying that antimicrobial activity of nano ZnO can be tuned by controlling the size (Preeti et al., 2020b). Antimicrobial activity of ZnO QDs has been reported singly against various microbial pathogens. However, in this study, we have demonstrated the use of ZnO QDs in combination with antifungal drugs of different classes against drug-susceptible and drug-resistant *Candida albicans* strains, in order to develop a biosafe, nano-based versatile alternative of combination therapy against MDR fungal pathogens, wherein drug concentrations can be lowered to reduce toxicity with the simultaneous advantage of multitargeted action to limit evolution of fungal drug resistance.

MATERIALS AND METHODS

Materials

Yeast extract, peptone, dextrose, and agar for fungal cell culture were purchased from Fisher Scientific (India) and Hi-Media (India). Absolute ethanol was obtained from Alfa Aesar (MA, United States) and hexane from Merck (NJ, United States). Zinc acetate dihydrate, lithium hydroxide monohydrate, standard antifungal drugs (fluconazole, ketoconazole, amphotericin B, and nystatin), dimethyl sulfoxide (DMSO) solvent, and cell culture media DMEM (Dulbecco's Modified Eagle's medium) were procured from Sigma Aldrich (United States). Penicillin-streptomycin antibiotic mix and fetal bovine serum (FBS) were obtained from Fisher Scientific (India) and trypan blue was obtained from Hi-Media (India).

Fungal Strains

Three isogenic matched pairs of *Candida albicans* (*C. albicans*) strains (G1 and G5, F1 and F5, and GU4 and GU5) were used in this study. These strains were provided as a kind gift by Joachim Morschhäuser, University of Würzburg, Germany. The strain description is given in **Table 1**. These strains were originally isolated after regular intervals from three AIDS patients suffering from oropharyngeal candidiasis (OPC). During the course of azole therapy administered to these OPC patients, drug-susceptible G1, F1, and GU4 evolved after several months to highly drug-resistant G5, F5, and GU5, respectively due to acquisition of MDR (Franz et al., 1998; Franz et al., 1999).

Growth Media and Culture Conditions

The three isogenic matched pairs of *C. albicans* strains (G1 and G5, F1 and F5, and GU4 and GU5) were grown on (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose) (YEPD) media at 30°C for 14–16 h under continuous shaking at 140–150 rpm. Exponentially grown log phase microbial cells were used for all experiments. The cells were stored at 4°C on YEPD plates containing 2.5% agar.

Antifungal Compounds

Two different classes of standard antifungal drugs: azoles (fluconazole and ketoconazole) and polyenes (amphotericin B and nystatin) and ZnO QDs were used as test compounds. The stock solutions (2 mg/ml) of fluconazole were prepared in deionized (milli Q) water, while ketoconazole, amphotericin B, and nystatin were dissolved in DMSO. The stock solution (4 mg/ml) of ZnO QDs was prepared in deionized water (milli Q) and sonicated for 1 h before use.

Mammalian Cell Culture

Human cervical cancer cell line, HeLa was obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM media supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. Further, the cell line was incubated and maintained at 37°C under a humidified condition with 5% CO_2 (Gurung et al., 2019).

Synthesis and Characterization of Zinc Oxide Quantum Dots

ZnO QDs were synthesized using zinc acetate dihydrate as the precursor, as described earlier (Meulenkamp, 1999) with slight modifications. Briefly, the ethanolic solution of zinc acetate dihydrate (0.1 M) was prepared by refluxing the solution for 30 min. After that, the ethanolic solution of lithium hydroxide monohydrate (0.14 M) was added drop-wise on zinc acetate dihydrate solution kept under vigorous stirring for 1 h. Subsequently, ZnO QDs were precipitated by adding *n*-hexane and collected by centrifugation. After drying at 60°C under vacuum, ZnO QDs were obtained as white powder and their structural, optical, and functional properties were characterized using the following techniques.

The optical property of ZnO QDs was characterized using a UV-Vis spectrophotometer (Perkin Elmer, UV-Vis Spectrophotometer Lambda 35, Singapore). For structural characterization, X-ray diffraction (XRD) pattern was recorded in the scan range of 20°–80° diffraction angle and a step size of 0.016° using an X-ray powder diffractometer with Cu Kα radiation (λ = 1.5418Å) (PANalytical X'Pert PRO

TABLE 1 | Description of *C. albicans* strains used.

C. albicans strains	Description and molecular changes	References
G1	• Drug-susceptible	Franz et al. (1998)
G5	• Drug-resistant • Enhanced *MDR1* mRNA levels • Mutation in *ERG11* gene • Change from *ERG11* heterozygosity to homozygosity	Franz et al. (1998)
F1	• Drug-susceptible	Franz et al. (1998)
F5	• Drug-resistant • Enhanced *MDR1* and *ERG11* mRNA levels	Franz et al. (1998)
GU4	• Drug-susceptible	Franz et al. (1999)
GU5	• Drug-resistant • Enhanced *CDR1/2* mRNA levels	Franz et al. (1999)

diffractometer, Almelo Netherlands). The shape and size of ZnO QDs were analyzed by a transmission electron microscope (TEM) (JEM 2100F; JEOL, Tokyo, Japan) using Image Tool software (multipoint image database software for grain and particle analysis; Dietermann and Heuser Solution GmbH, Greifenstein, Germany). High resolution TEM (HRTEM) analysis was done to visualize planar lattice spacing (d spacing) and electron diffraction pattern. The elemental composition of ZnO QDs was determined by EDX-mapping and spectra was obtained from TEM equipped with an energy-dispersive X-ray (EDX) spectrometer (EDS; Bruker Nano GmbH, Berlin, Germany).

Assessment of *In Vitro* Antifungal Activity of Zinc Oxide Quantum Dots

In vitro antifungal activity of ZnO QDs against isogenic matched pairs of *C. albicans* strains (F1 and F5, G1 and G5, and GU4 and GU5) was assessed by the broth microdilution method, as described by the standard protocol of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2008; Prasad et al., 2005; Preeti et al., 2020a; Radhakrishnan et al., 2018a; Radhakrishnan et al., 2018b). The broth microdilution assay was performed in 96-well microtiter plates in triplicate. *Candida* strains were grown overnight, then resuspended in 0.9% saline in order to achieve an optical density (OD) of 0.1 at 600 nm, which corresponded to 0.5–1 x 10^6 CFU/ml. These cells were further diluted 100-fold in YEPD media to attain a final concentration of 10^4 CFU/ml. ZnO QDs ranging in concentration between 0–200 μg/ml were added to the YEPD media in plates containing cells of respective strains and incubated at 30°C for 48 h. Respective growth controls for the fungal strains were maintained without ZnO QDs. Minimum inhibitory concentration (MIC) was determined by measuring the OD at 600 nm at the lowest concentration of ZnO QDs that completely inhibited the growth of the fungal cells, and the percentage of growth inhibition was calculated from the differences in OD between the cells grown in the presence and absence of ZnO QDs.

Determination of Synergy Between Antifungal Agents by the Fractional Inhibitory Concentration Index in a Checkerboard Assay

The synergy between ZnO QDs and antifungal drugs was determined by calculating the fractional inhibitory concentration (FIC) index in a checkerboard assay. The individual potency of four different combinations comprising of ZnO QDs and antifungal drugs (fluconazole, ketoconazole, amphotericin B, and nystatin), where each was used at very low concentrations, was assessed by comparing with their individual activities against isogenic matched pairs of *C. albicans* strains (G1 and G5, F1 and F5, and GU4 and GU5).

FIC index was determined by a two-dimensional broth microdilution checkerboard assay, as described in the Clinical Microbiology Procedures Handbook (Moody, 2004). The assay was performed in 96-microtiter plates, where YEPD media was added aseptically in all wells and the concentration range was between 0.178–200 μg/ml for ZnO QDs, 0.125–64 μg/ml for fluconazole, and 0.015–8 μg/ml each for ketoconazole, amphotericin B, and nystatin. In each plate, the antifungal drug was serially diluted along the abscissa, while ZnO QDs were serially diluted along the ordinate. Subsequently, inoculum of 10^4 CFU/ml of each *Candida* strain was prepared separately in YEPD media. Then, 100 μl of the respective cell suspension was added into each well of the 96-well plates containing test compounds and incubated for 48 h at 30°C. The MICs of each antifungal drug alone, ZnO QDs alone, and their combinations were determined both visually and by measuring the ODs of cells at 600 nm using a microtiter plate reader. The inhibition in growth of fungal cells was calculated with respect to the growth control (absence of both drugs and ZnO QDs).

The ΣFIC index was calculated as follows:

$$\sum FIC\ Index = FIC_A + FIC_B$$

TABLE 2 | MIC values of antifungal drugs against *C. albicans* strains.

C. albicans strains	Fluconazole alone	Ketoconazole alone	Amphotericin B alone	Nystatin alone
	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)
G1	8	1	2	4
G5	≥50	2	2	4
F1	16	4	2	4
F5	≥50	4	2	4
GU4	16	0.5	4	2
GU5	≥100	2	4	4

Here

$$FIC_A = \frac{MIC\ of\ agent\ A\ in\ combination}{MIC\ of\ agent\ A\ alone}$$

$$FIC_B = \frac{MIC\ of\ agent\ B\ in\ combination}{MIC\ of\ agent\ B\ alone}$$

Where

A is the individual antifungal drug (fluconazole, ketoconazole, amphotericin B, and nystatin) and B is ZnO QDs.

Interpretation of interactions in combinations of A (antifungal drug) and B (ZnO QDs) is as follows:

When the FIC index is ≤0.5, the combination is synergistic, at >0.5 to <4.0, the combination is indifferent, and at ≥4.0, the combination is antagonistic.

Spot Assay

A spot assay was performed to confirm the synergism between ZnO QDs and the four different antifungal drugs (fluconazole, ketoconazole, amphotericin B, and nystatin) against isogenic matched pairs (G1 and G5, F1 and F5, and GU4 and GU5) of *C. albicans* (Mukhopadhyay et al., 2004; Prasad et al., 2005; Preeti et al., 2020b; Radhakrishnan et al., 2018a; Radhakrishnan et al., 2018b). In the spot assay, separate YEPD plates were prepared by adding ZnO QDs alone, antifungal drugs alone, and four combinations containing both ZnO QDs and individual drugs (fluconazole, ketoconazole, amphotericin B and nystatin). Overnight grown log phase cells of each strain were separately resuspended in 0.9% saline and OD of each cell suspension at 600 nm was adjusted to 0.1. Subsequently, CFU/ml was calculated and 5-fold serial dilutions containing 1×10^6 cells/ml cells were prepared. A total of 5 μl of each serially diluted cell suspension was spotted onto each YEPD plate containing ZnO QDs alone, antifungal drugs alone, and four combinations containing both ZnO QDs and individual drugs. The plates were incubated for 48 h at 30°C. A separate growth control plate was also maintained without ZnO QDs and drugs. The images were then recorded and growth differences were evaluated by comparison with the growth control.

In Vitro Cytotoxic Activity of Zinc Oxide Quantum Dots

The *in vitro* cytotoxic activity of ZnO QDs was evaluated on HeLa cell line using a trypan blue exclusion assay (Gurung et al., 2019). Approximately 4×10^4 cells were plated in a culture dish containing DMEM media and allowed to adhere for 12 h, then ZnO QDs (0 and 25 μg/ml) were added and incubated for 48 h in a humidified incubator (5% CO_2) at 37°C. After that, cells were trypsinized, harvested, and resuspended in equal volumes of phosphate buffer saline (PBS) and 0.4% trypan blue for cell counting. Thereafter, viable (unstained) and non-viable (blue-stained) cells were counted using a hemocytometer to find out the total numbers of living and dead cells after treatment with ZnO QDs.

Statistical Analysis

All experiments were performed at least three times and results are represented as the mean ± standard deviation (SD). A one-way ANOVA test was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA) for validating the reproducibility and significance of results. The difference between the means was considered statistically significant at $^*p \leq 0.05$.

RESULTS AND DISCUSSION

Physicochemical Characterization of Zinc Oxide Quantum Dots

Figure 1A shows the typical absorption peak of ZnO QDs at 365 nm (corresponding to band gap of 3.4 eV), indicating the formation of ZnO QDs. **Figure 1B** represents the X-ray diffraction (XRD) peaks at 31.89°, 35.45°, 36.46°, 47.82°, 56.77°, 62.90°, and 68.40°. XRD confirmed a single phase hexagonal wurtzite structure, which was in agreement with JCPDS (Joint Committee on Powder Diffraction Standards), file no. 36–1,451. Furthermore, the TEM image (**Figure 2A**) revealed the average size of ZnO QDs to be ~ 4–6 nm (**Figure 2B**), while HRTEM confirmed the d spacing as 0.201 nm between the atomic planes of ZnO QDs (**Figure 2C**), which was in accordance with XRD data. The corresponding selected area electron diffraction (SAED) pattern confirmed the crystalline nature of ZnO QDs and the obtained rings matched with the diffraction planes of the XRD spectra (**Figure 2D**). TEM-EDX mapping revealed the presence of only zinc and oxygen elements in ZnO QDs (**Figure 2E**), while EDX spectra displayed typical sharp peaks of zinc around 1 and 8.6 keV and a single peak of oxygen at 0.5 keV, indicating no trace of impurity in the sample (**Figure 2F**).

Antimicrobial Potential of Zinc Oxide Quantum Dots

Severe fungal infections occur as a consequence of several health problems in immunocompromised cases due to acquired

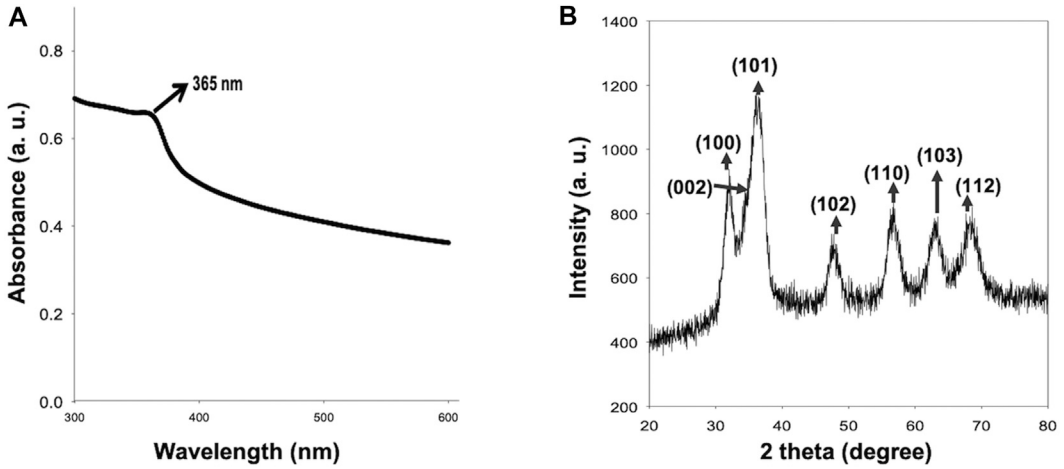


FIGURE 1 | (A) Absorbance spectrum of ZnO QDs and absorbance maxima observed at 365 nm; **(B)** X-ray diffraction of ZnO QDs.

immunodeficiency syndrome (AIDS), organ transplantation, chemotherapy, cancer, asthma, corticosteroid therapies, etc. Rampant use of antifungals in immunocompromised individuals have led to increasing incidence of fungal drug resistance. The pathogenesis and severity of fungal infection depend on various immunological and non-immunological factors. *C. albicans* is the most common, opportunistic fungal pathogen and forms a part of human microbiota, however, its pathogenesis depends on the immunity of the individual. Since the last few decades, several MDR strains of *Candida* spp. causing severe invasive candidiasis have emerged (Franz et al., 1998; Franz et al., 1999; Colombo et al., 2017). A study of *Candida* species in patients with AIDS showed 33% of late-stage patients with drug-resistant strains of *Candida* albicans in their oral cavities (White et al., 1998). Oropharyngeal candidiasis (OPC) is one of the most prevalent fungal infections occurring in HIV patients (occurrence range between 0.9 and 83%). Drug resistance in *C. albicans* is mainly responsible for failure of antifungal therapy when administered in standard doses (Patil et al., 2018). Interestingly, Franz et al. studied the mechanisms for acquired drug resistance in isogenic matched pairs of *C. albicans* strains (isolated from three AIDS patients, who were suffering from OPC and received azole standard therapy) and found that during the course of treatment, the drug-susceptible strains (G1, F1, and GU4) acquired drug resistance and evolved into drug-resistant G5, F5, and GU5. The proposed primary mechanisms for resistance toward azoles and polyenes include ergosterol gene mutation, alterations in membrane permeability, and efflux pump overexpression, which result in altered drug target (ergosterol), reduced drug uptake, and increased efflux of drugs from the cells (Franz et al., 1998; Franz et al., 1999; Srivastava et al., 2018).

In order to improve the treatment outcome of microbial infections, nano-based drug compositions might be ideal candidates to address the challenges of microbial drug resistance by favoring multitargeted action, broad spectrum activity, and lower host toxicity (Radhakrishnan et al., 2018a; Radhakrishnan et al., 2018b). In addition to standard susceptibility testing and appropriate drug dosing, one of the ways to prevent drug resistance is the use of combination antifungal therapy. Therefore, in order to develop a nano-based therapeutic agent, we first investigated the antimicrobial activity of ZnO QDs against three isogenic matched pairs of *C. albicans* strains (drug-susceptible G1, F1, GU4 and drug-resistant G5, F5, and GU5) using a broth microdilution assay. Subsequently, synergy of ZnO QDs in different combinations with four antifungal drugs was assessed against the *C. albicans* strains using a checkerboard assay. Individual potency of these four different combinations of ZnO QDs and antifungal drugs (fluconazole, ketoconazole, amphotericin B, and nystatin), which were used at very low concentrations of each, was assessed by comparing their individual activities against the isogenic matched pairs of *C. albicans* strains.

Broth Microdilution Assay

The broth microdilution assay was performed in the presence of different concentrations of ZnO QDs between 0–200 μg/ml. Results demonstrated that ZnO QDs exhibited dose-dependent growth inhibition of all *C. albicans* strains (G1 and G5, F1 and F5, and GU4 and GU5) (**Figure 3A**). The maximum growth of cells was observed in control (without ZnO QDs), and growth of cells gradually decreased with increasing concentration of ZnO QDs (25–200 μg/ml) (**Figure 3A**). Further, the percentage of growth inhibition was calculated from the differences in OD at 600 nm between the fungal strains grown in the absence and presence of different concentrations of ZnO QDs. Interestingly, our results revealed almost 90, 90, 87, 85, 75, and 90% growth inhibition, respectively for the strains G1, G5, F1, F5, GU4, and GU5 at 200 μg/ml, however, 25–100 μg/ml of ZnO QDs showed growth inhibition between 30 and 50% for the strains (**Figure 3B**).

Respective growth controls for the fungal strains were maintained without ZnO QDs.

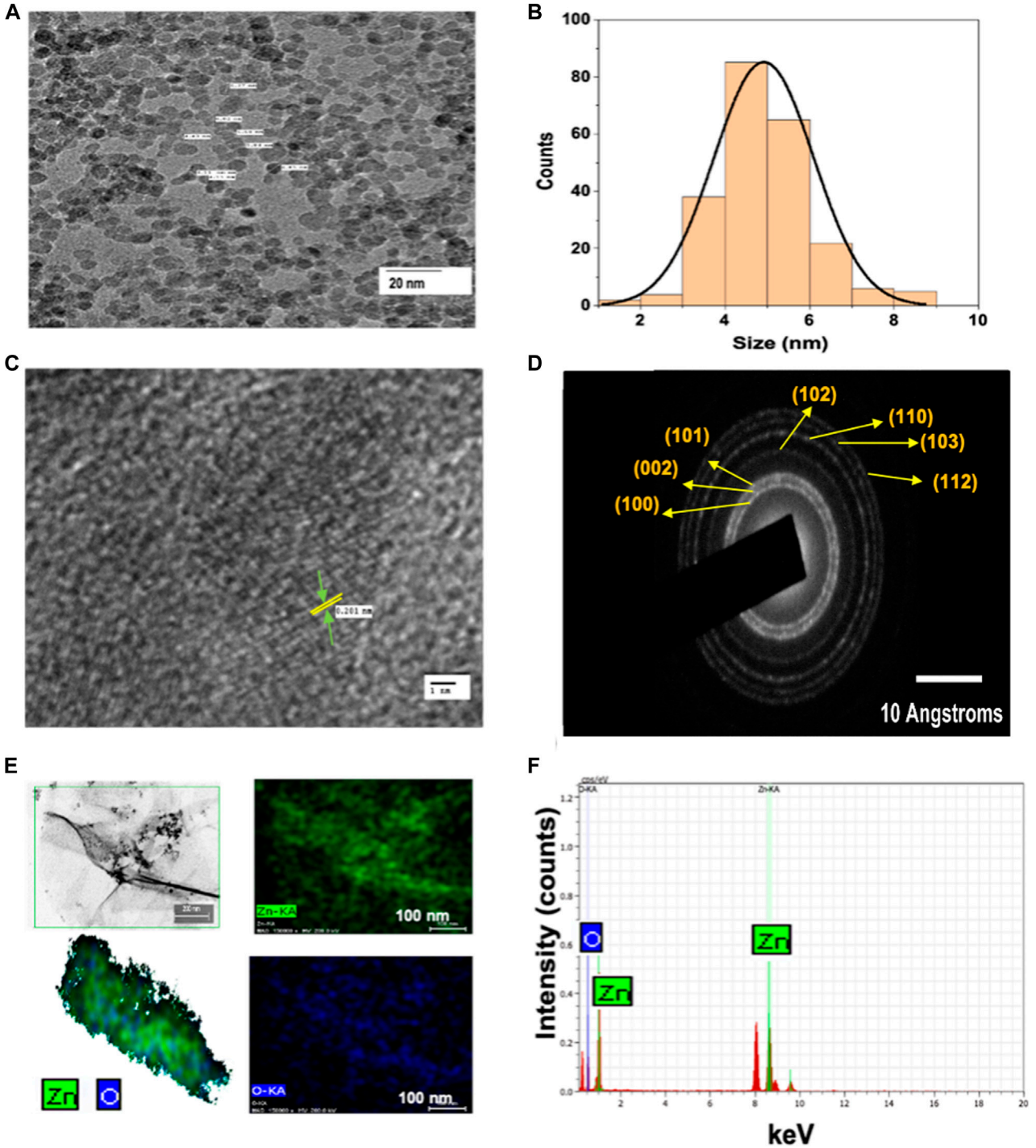


FIGURE 2 | (A) TEM image showing spherical ZnO QDs; **(B)** histogram depicting mean size (4–6 nm) of ZnO QDs; **(C)** high-resolution TEM image of ZnO QDs, where lattice spacing d = 0.201 nm (d represents the distance between two lattice fringes); **(D)** selected area electron diffraction (SAED) pattern of ZnO QDs; **(E)** TEM-EDX mapping of ZnO QDs; **(F)** TEM-EDX spectra of ZnO QDs with strong peaks at 0.5, 1.0, and 8.6 keV.

Fractional Inhibitory Concentration Index

The effect of interaction of drugs in combination is assessed by FIC index in the checkerboard assay (Doern, 2014). In this study, synergy between ZnO QDs and different antifungal drugs against *C. albicans* strains (G1 and G5, F1 and F5, and GU4 and GU5) was assessed by checkerboard assay and expressed in terms of FIC index. Firstly, MIC was determined for each drug (fluconazole, ketoconazole, amphotericin B, and nystatin)

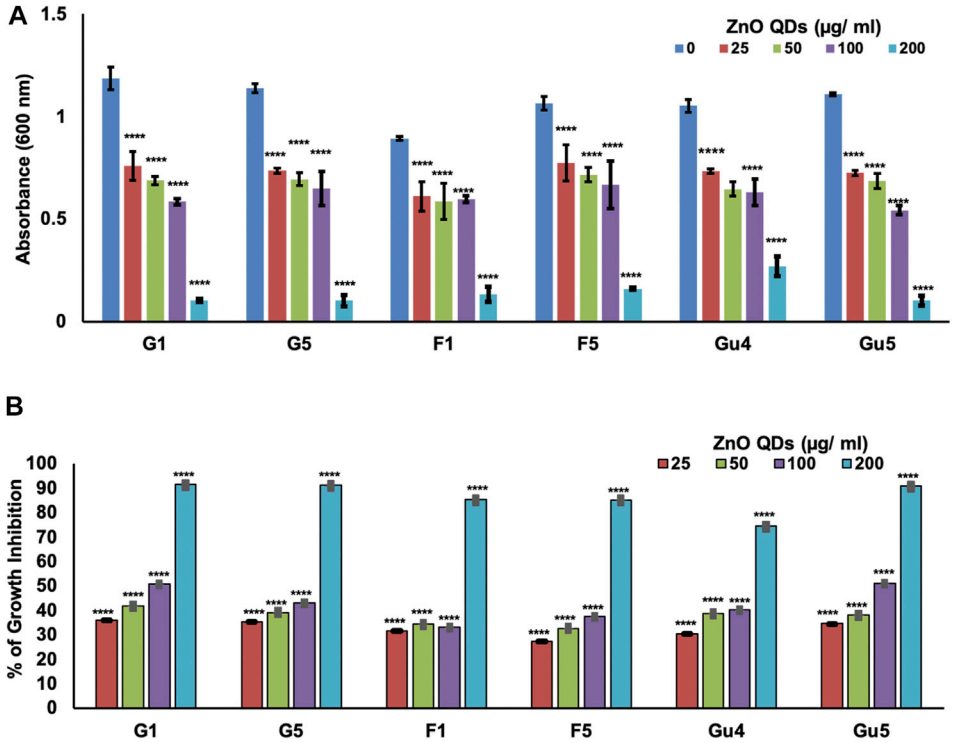


FIGURE 3 | Determination of dose-dependent antimicrobial activity of ZnO QDs against *C. albicans* strains (isogenic matched pairs G1 and G5, F1 and F5, and GU4 and GU5): **(A)** Determination of growth by measurement of OD_{600nm} using the broth microdilution method for *C. albicans* cells grown at 30°C in the presence of 0, 25, 50, 100, and 200 µg/ml of ZnO QDs, respectively. The values given are mean ± SD of three independent sets of experiments. **** represents $p < 0.0001$, calculated with respect to growth control (absence of ZnO QDs); **(B)** Percentage of growth inhibition of *C. albicans* strains (isogenic matched pairs) in the presence of ZnO QDs (0, 25, 50, 100, and 200 µg/ml). The values given are mean ± SD of three independent sets of experiments. **** represents $p < 0.0001$, calculated with respect to growth control (absence of ZnO QDs).

TABLE 3 | FIC index for different combinations of ZnO QDs and antifungal drugs tested against *C. albicans* strains.

	FIC index and interpretation for effect of QDs and drug combinations			
***C. albicans* strains**	**ZnO QDs + fluconazole**	**ZnO QDs + ketoconazole**	**ZnO QDs + amphotericin B**	**ZnO QDs + nystatin**
G1	0.37 **Synergistic**	0.13 **Synergistic**	0.32 **Synergistic**	0.25 **Synergistic**
G5	0.165 **Synergistic**	0.14 **Synergistic**	0.32 **Synergistic**	0.18 **Synergistic**
F1	0.25 **Synergistic**	0.12 **Synergistic**	0.32 **Synergistic**	0.12 **Synergistic**
F5	0.165 **Synergistic**	0.12 **Synergistic**	0.32 **Synergistic**	0.12 **Synergistic**
GU4	0.25 **Synergistic**	0.15 **Synergistic**	0.17 **Synergistic**	0.37 **Synergistic**
GU5	0.156 **Synergistic**	0.13 **Synergistic**	0.22 **Synergistic**	0.25 **Synergistic**

against *C. albicans* strains (G1 and G5, F1 and F5, and GU4 and GU5). **Table 2** lists the MIC for each drug against respective *Candida* strains. Subsequently, based on MIC obtained for ZnO QDs and each antifungal drug, singly and in combination, the FIC index for each combination was calculated as given in the Material and Methods section. Results demonstrated that the

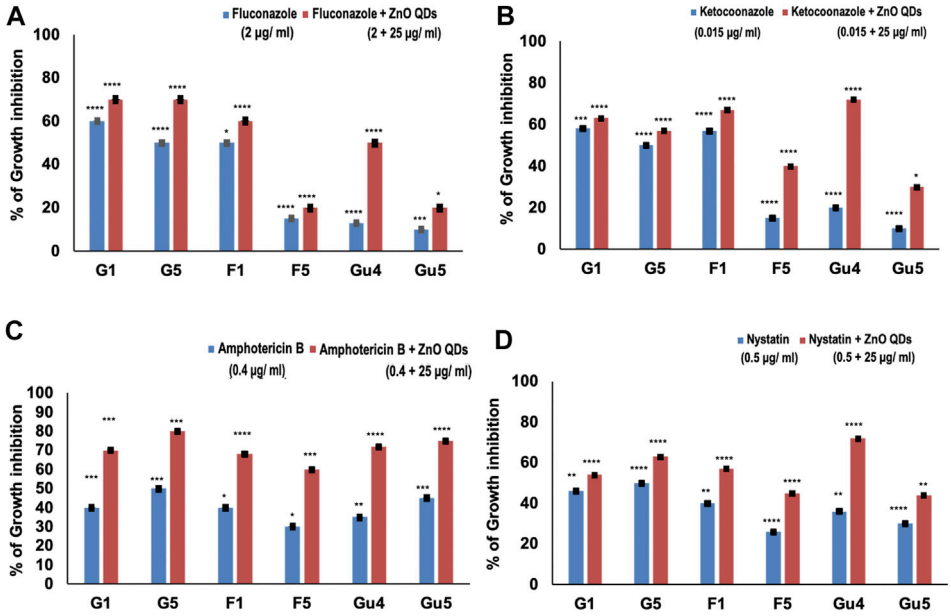


FIGURE 4 | | Percentage of growth inhibition in C. albicans strains (isogenic matched pairs G1 and G5, F1 and F5, and GU4 and GU5) in the presence of both drugs and ZnO QDs as compared to growth control (absence of drugs and ZnO QDs): **(A)** Fluconazole + ZnO QDs; **(B)** Ketoconazole + ZnO QDs; **(C)** Amphotericin B + ZnO QDs; **(D)** Nystatin + ZnO QDs. The values are mean ± SD of three independent sets of experiments. **** represents $p < 0.0001$, *** represents $p < 0.0002$, ** represents $p < 0.0046$, * represents $p < 0.0408$, calculated with respect to growth control.

potency of combinations of ZnO QDs and antifungal drugs even at very low concentrations of each was higher than their individual activities against the fungal isolates. FIC index obtained in the checkerboard assay was less than 0.5 for all combinations tested (ZnO QDs + fluconazole; ZnO QDs + ketoconazole; ZnO QDs + amphotericin B and ZnO QDs + nystatin), which confirmed synergism between sub-inhibitory concentrations of both ZnO QDs (25 µg/ml) and individual antifungal drugs (**Table 3**). ZnO QDs and fluconazole exhibited synergism in combination, at a concentration of fluconazole which was 2 to 8-fold lower than the respective MIC values for fluconazole alone against the fungal strains tested (**Figure 4A**). Similarly, ZnO QDs displayed good synergistic activity in combination with very low concentrations of 0.015 µg/ml of ketoconazole, 0.4 µg/ml of amphotericin B, and 0.5 µg/ml of nystatin, and the concentration of each antifungal drug used in combination was much lower than the respective individual MIC values obtained for each drug against *Candida* strains (**Figures 4B–D**). Moreover, an increase in growth inhibition by 10–20% was observed in all strains for the four combinations of ZnO QDs and antifungal drugs, as compared to the strains grown in the presence of either individual drugs or only ZnO QDs (**Figure 4**).

Synergism observed in combinations indicates that ZnO QDs and individual drugs (fluconazole, ketoconazole, amphotericin B, and nystatin) probably share a non-competitive cellular target, which potentiates the antifungal activity against both drug-susceptible (G1, G5, and F1) and resistant (G5, F5, and GU5) strains, even at concentrations much lower than their respective individual MICs. However, the FIC index for the checkerboard assay has certain limitations since it only tests antimicrobials for a fixed incubation time rather than a continuous time frame and is also not capable of testing more than two antimicrobials at a time (combinations of three and four antimicrobials cannot be tested), which can slow down the assessment of potential synergistic combinations appropriately (Rybak and McGrath, 1996; Doern, 2014). Therefore, spot assay was further performed in this study to validate the results.

Spot Assay

The spot assay (**Figure 5**) further confirmed the synergism exhibited between ZnO QDs and antifungal drugs in combinations (**Table 4**). As compared to growth control (without ZnO QDs or drugs) in **Figure 5A**, a slight growth inhibition and reduction in fungal colonies was observed for all strains in the presence of sub-inhibitory concentration of ZnO QDs (**Figure 5B**) and individual antifungals drugs (**Figure 5C**). However, this reduction in cell viabilities was further increased for all *C. albicans* strains in the presence of different combinations of ZnO QDs and antifungal drugs, which was clearly evident from the disappearance of fungal cells on agar plates (**Figure 5D**).

The FIC index (**Table 3**) and spot assay (**Figure 5**) results indicate that ZnO QDs might be a potential adjuvant for antifungal combination therapy. The standard antifungal classes of drugs, i.e., azoles and polyenes act by inhibiting ergosterol biosynthesis and targeting the ergosterol moiety, respectively in the fungal membrane, whereas multiple

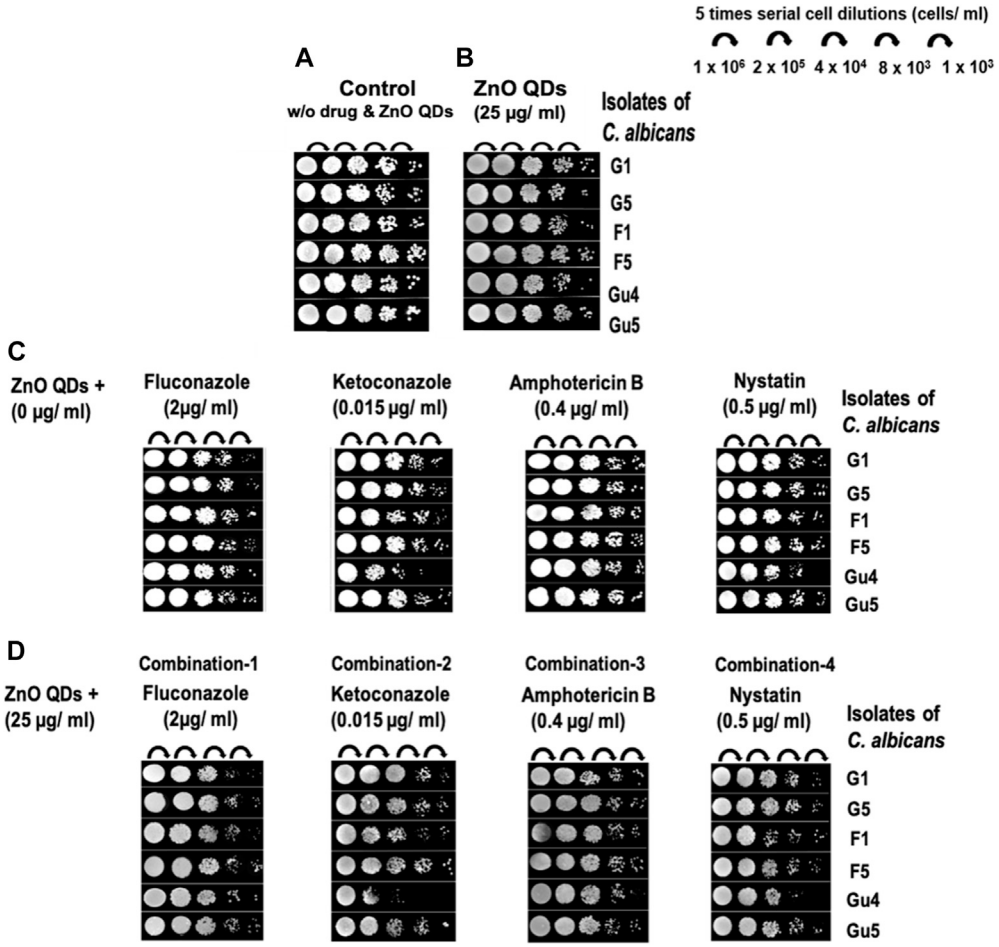


FIGURE 5 | Spot assay for *C. albicans* clinical isolates (G1 and G5, F1 and F5, and GU4 and GU5) in the presence of: **(A)** Growth control (absence of both ZnO QDs and drugs); **(B)** ZnO QDs (25 µg/ml) only; **(C)** drug alone: fluconazole (2 µg/ml), ketoconazole (0.015 µg/ml), amphotericin B (0.4 µg/ml), and nystatin (0.5 µg/ml); **(D)** different combinations containing both ZnO QDs (25 µg/ml) and antifungal drugs [(fluconazole (2 µg/ml) or ketoconazole (0.015 µg/ml) or amphotericin B (0.4 µg/ml) or nystatin (0.5 µg/ml)].

mechanisms of action are proposed for ZnO QDs such as high penetrability, greater retention, generation of oxidative stress, etc. When combined together, ZnO QDs and antifungal drugs might have exerted a concerted impact and were able to counter MDR in drug-resistant *Candida* strains (G5, F5, and GU5) (Asok et al., 2015; Sirelkhatim et al., 2015; da Silva et al., 2019; Gold et al., 2018; Tiwari et al., 2018).

This study demonstrates the promising potential of ZnO QDs for both single and combination antifungal therapy against MDR fungal pathogens. Since, ZnO QDs in combination with respective antifungal drugs exhibit synergism and can potentiate antifungal activity even at very low concentrations, use of ZnO QDs in combination therapy can reduce the toxicity of antifungal compounds. Additionally, simultaneous multitargeted action in combination therapy can prevent the development of fungal drug resistance.

However, for safer clinical use of ZnO QDs, we further investigated the *in vitro* cytotoxic activity of ZnO QDs against the HeLa cell line.

In Vitro Cytotoxic Activity

The *in vitro* cytotoxic activity of ZnO QDs (0, 5, and 25 µg/ml) was assessed by trypan blue assay in HeLa cell line and expressed as the percentage of viable cells after treatment with ZnO QDs, as compared to control cells without any treatment. Almost 93 and 86% cell viability was observed in the presence of 5 and 25 µg/ml of ZnO QDs, respectively, indicating that there was negligible toxicity to mammalian cells at 25 µg/ml of ZnO QDs (**Figure 6**) and hence, ZnO QDs at this concentration exhibited synergism with antifungal drugs and therefore, may be suitable for combination therapy.

CONCLUSION

Monotherapy against microbial infections often allow for the emergence of MDR in microbes with time and makes the standard drug doses ineffective leading to failure in treatment.

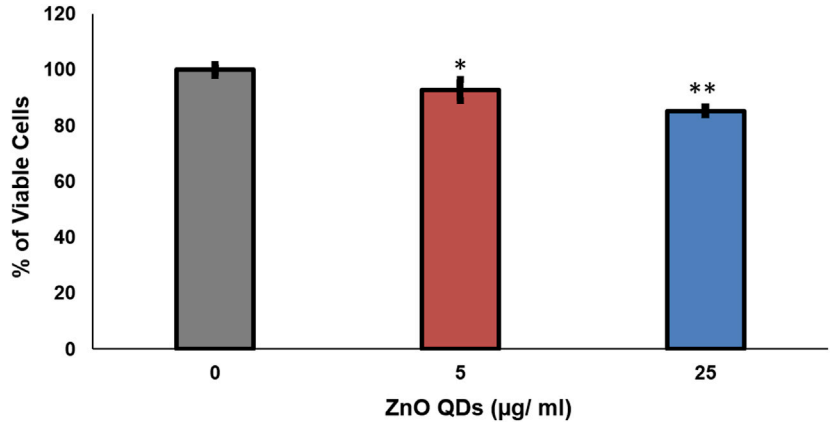
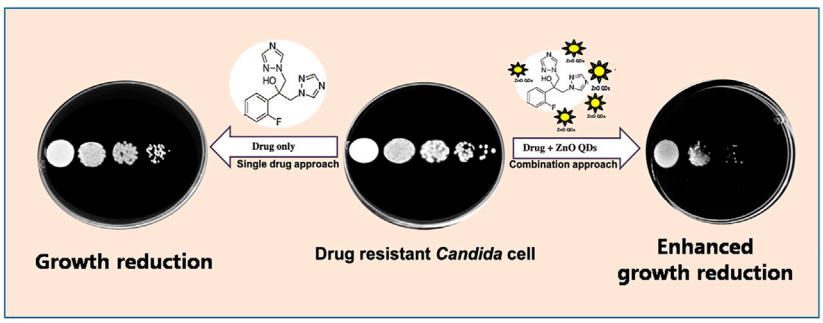


FIGURE 6 | *In vitro* cytotoxic activity of ZnO QDs against HeLa cell line. The values are mean ± SD of three independent sets of experiments. ** represents $p < 0.001$, * represents $p < 0.01$, calculated with respect to growth control.



SCHEME 1 | Graphical illustration of this study.

TABLE 4 | Concentrations of ZnO QDs and antifungal drugs used in different combinations for spot assays.

Combination	ZnO QDs + drug	ZnO QDs (concentration in µg/ml)	Drug used (concentration in µg/ml)
1	ZnO QDs + fluconazole	25	Fluconazole (2)
2	ZnO QDs + ketoconazole	25	Ketoconazole (0.015)
3	ZnO QDs + amphotericin B	25	Amphotericin B (0.4)
4	ZnO QDs + nystatin	25	Nystatin (0.5)

The bold represents a particular drug combination and the respective detail is provided in the next column only as ZnO QDs +Fluconazole , ZnO QDs +Ketoconazole, ZnO QDs +Amphotericin B And ZnO QDs +Nystatin. No further explanation is required.

Additionally, the high rates of morbidity and mortality caused by fungal infections are associated with availability of the limited antifungal arsenal and high toxicity of the compounds. Because of many similarities between fungal and human cells, identifying novel drug targets remains challenging. Owing to the limited availability of antifungal compounds, researchers have sought to improve treatment via different approaches. Therefore, to overcome these problems, combination therapy is considered for treatment (Ahmed et al., 2014; Campitelli et al., 2017). The combination therapy approach, however, is likely to offer a broader antimicrobial spectrum, lower toxicity of the antimicrobial compounds due to synergism at much lower concentrations in combination, and reduce the risk of acquiring resistance during therapy. Herein, we report synergism between sub-inhibitory concentrations of both ZnO QDs and individual antifungal drugs against all drug-susceptible and drug-resistant isolates of *C. albicans* tested. ZnO QDs thus appear to be a potential adjuvant in combination antifungal therapy against MDR fungal pathogens (**Scheme 1**), wherein drug toxicity can be reduced and their simultaneous multitargeted synergistic action can limit development of fungal drug resistance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICAL STATEMENT

The *Candida albicans* clinical isolates were kind gifts from Joachim Morschhauser, University of Wurzburg, Germany. These isolates have been extensively studied and their molecular characterization has been reported by various researchers all over the world, which is also mentioned in the materials and methods section of this study. The present study only involved *C. albicans* strains and did not require any samples from patients or involvement of any animal or human subject in the study. Therefore, no ethical approval was required.

AUTHOR CONTRIBUTIONS

PC, SPS, and TP contributed to the concept and design of the study. PC conducted all experiments. PC and SK performed cytotoxicity assay on HeLa cells. PC and TP participated in the acquisition of data and statistical analysis. PC, NM, SPS, and TP participated in interpretation of data and drafting of the manuscript. SPS and TP contributed to editing the manuscript and revised it critically for significant intellectual content. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnano.2021.624564/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.