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Antimicrobial studies of green-synthesised pure and mixed cerium–zirconium oxide nanoparticles

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Abstract

In the present study, we have proposed and tested a biogenic approach for synthesising ceria, zirconia and ceria–zirconia ($Ce_{0.5}Zr_{0.5}O_2$) mixed metal oxide (MMO) nanoparticles using *Melia dubia* leaf extract with the primary goal to investigate the antimicrobial efficacy. The structural and morphological properties and the elemental composition of the prepared nanoparticles were characterised. The powder X-ray diffraction patterns showed that the green-synthesised nanoparticles are single-phase and nano-crystalline. The lattice parameters and the crystallite size were calculated from the X-ray diffraction data. Scanning electron micrographs revealed agglomeration in the case of ceria and MMO nanoparticles, while the zirconia nanoparticles remained uniform in size without agglomeration. The energy dispersive spectra of the samples confirmed the surface elemental composition of the sample with a pronounced oxygen deficiency. The nanoparticles were screened for antibacterial and antifungal potential against the bacterial strains *Pseudomonas aeruginosa* and *Streptococcus mutans* and the fungal strain *Candida albicans*. The zirconia nanoparticles showed good antibacterial activity against gram-positive and gram-negative bacterial strains compared to the pristine ceria and MMO nanoparticles. In particular, the zirconia nanoparticles displayed excellent antibacterial activity compared to the positive control Gentamicin. However, the ceria nanoparticles exhibited superior antifungal activity compared to the positive control employed in the experiment, Amphotericin B.

Keywords: *antibacterial; antifungal; ceria; mixed metal oxide; nanoparticles; X-ray diffraction patterns; zirconia.*

1. Introduction

In recent years, the emergence of multi-drug resistant pathogens has become a serious concern to the global health care and scientific communities (Leibovici et al., 2016; Mingeot-Leclercq & Decout, 2016). Evolving antimicrobial resistance has led to the rise of potentially untreatable infections that pose serious challenges to existing drugs in conventional medicine for the prevention, control and eradication of diseases (Ding, Duan, Ding, Liu, & Xu, 2018; Zamani et al., 2021). Most of the common underlying factors for unresponsiveness to treatment are not merely due to the mutations driven by selection pressure and expression of efflux pumps but also the misuse and overuse of drugs in the clinical context (Laxminarayan et al., 2013). In this regard, nanotherapeutics may offer a solution that complements conventional medicine to combat drug resistance in microorganisms (Huhand, &

Kwon, 2011). Nanomedicine has intrinsic advantages, including the tunability of size, shape and surface properties to suit the specific target site. The reduced dimensionality and higher surface area, along with the manipulability of surface charge, have been effectively used to target the cellular membrane of microorganisms (Durán et al., 2016; Khan, Musarrat, & Al-Khedhairy, 2016; Nikzamir, Hanifehpour, Akbarzadeh, & Panahi, 2021).

Metal oxides constitute a novel class of materials known for their exotic phenomena and unique properties from both a fundamental science and a technological point of view. In a therapeutic context, the desirability of using metal oxides in biomedical applications is due to their biocompatibility, non-toxicity and cyto-friendliness. In addition, the switchable stoichiometry of metal oxides can be effectively harnessed to release reactive oxygen species (ROS) in the intracellular membrane of pathogens, eventually causing oxidative stress (Pachaiappan, Rajendran, Show, Manavalan, & Naushad, 2021).

Metal oxide nanoparticles can be synthesised using green as well as chemical methods. A green method is a desirable synthetic route due to its intrinsic advantages compared to other chemical methods generally employed in the scientific literature (Ovais et al., 2017). Several studies have reported that synthesising metal oxide nanoparticles through chemical methods led to the production of highly toxic by-products harmful to the environment and human health. In particular, when synthesised through the green method, such metal oxides often have better properties and are preferable to those prepared from conventional synthetic routes for biomedical applications (Alves et al., 2018; Akbaripoor Tafreshi Nejad et al., 2022). The key advantages of the green method include cost-effectiveness, large-scale production, environmental neutrality and sustainability (Ahmed, Saifullah, Ahmad, Swami, & Ikram, 2016; Bouafia & Laouini, 2021; Sathiyavimal et al., 2021). In particular, for plant-extract-mediated synthesis, the phytoconstituents present in the extract act as a capping agent to stabilise the nanoparticles (Ghenaatian, Honarmand, Seyedabadi, & Shakourian-Fard, 2021). Moreover, the yield obtained using plant-extract-mediated synthesis is relatively high compared to that from the microbe-mediated method (Bhuyan, Mishra, Khanuja, Prasad, & Varma, 2015; Kamble et al., 2016).

Among metal oxides, ceria and zirconia metal oxide nanoparticles are considered safe, non-toxic and biocompatible in higher organisms. Earlier studies on ceria and zirconia metal oxide nanoparticles have revealed that these materials have better antibacterial activities against gram-positive (Arumugam et al., 2015) as well as gram-negative bacteria (Annu, Sivasankari, & Krupasankar, 2020). Thema, Letsholathebe, and Mphale. (2021) have synthesised ceria nanoparticles using *Agathosma betulina* extract, and they argued that the green-synthesised ceria powder could be used as an efficient antibacterial agent. Also, the antimicrobial properties of zirconia nanoparticles have been studied by Goyal, Bhardwaj, Mehta, and Mehta (2021). They synthesised zirconia nanoparticles using *Helianthus annuus* seed. These zirconia nanoparticles had good antimicrobial activity and could be used for biomedical applications. Ceria/Zirconia core nanoparticles were synthesised by an ionic liquid-mediated extract of *Justicia adhatoda* leaves, and this extract had greater antibacterial, anti-biofilm and antioxidant abilities (Pandiyan, Murugesan, Sonamuthu, Samayanan, & Mahalingam, 2018).

Due to the advantages of the green method over the chemical route and the importance of metal oxide nanoparticles in biomedicine, we have synthesised pure and mixed metal oxides (MMOs) of ceria and zirconia nanoparticles using the green method and assessed their antimicrobial activity. The nanoparticles were synthesised using *Melia dubia* leaf extract and characterised by powder X-ray diffraction and scanning electron microscopy (SEM) with energy dispersive X-ray analysis (EDX) to study the structure, microstructure and elemental composition, respectively. The antimicrobial activity in terms of antibacterial and antifungal potential was measured against representative bacterial strains—gram-negative *Pseudomonas aeruginosa* and gram-positive *Streptococcus mutans*—and the fungal strain *Candida albicans*.

2. Objectives

Synthesise ceria, zirconia and ceria–zirconia MMO nanoparticles by the green method using *M. dubia* leaf extract as it has advantages over the chemical method.

Study the antimicrobial properties of pure and MMOs of ceria and zirconia nanoparticles produced using green synthesis.

3. Materials and methods

3.1 Synthesis of pure and MMOs of ceria and zirconia nanoparticles

Analytical reagent grade cerium nitrate hexahydrate (99.9%, LOBA, India), zirconyl nitrate (99.5%, LOBA, India), *M. dubia* leaves (Common Name: Malabar Neem) and deionised water were used for the preparation of ceria, zirconia and ceria–zirconia MMO nanoparticles. Ethanol was used for the purification of the as-synthesised nanoparticles.

In a typical synthesis, 100 g of *M. dubia* leaves were collected from Tirunelveli, India. The leaves were added to deionised water in a beaker and boiled to obtain the extract. After the extract was prepared, 0.1 M cerium nitrate hexahydrate was added to 100 mL of *M. dubia* leaf extract in the beaker. After 2 hours of constant stirring at 80°C, the nanoparticles were precipitated from the solution. Then the dispersion of nanoparticles was centrifuged, and the precipitate was washed twice with deionised water and ethanol. The precipitate was dried in a hot air oven for 1 hour at a temperature of 60°C. Finally, the precipitate was calcined in a muffle furnace at 800°C for 2 hours. The ceria nanoparticles obtained in this method appeared pale yellow in colour.

For the synthesis of zirconium oxide nanoparticles, 0.1 M zirconyl nitrate was mixed with 100 mL of *M. dubia* leaf extract. The above synthesis process was repeated to obtain the white zirconium oxide nanoparticles.

Zirconyl nitrate (0.05 M) and cerium nitrate hexahydrate (0.05 M) was used as precursors for the preparation of MMO nanoparticles, and the entire procedure was repeated. Finally, pale yellow MMO nanoparticles were obtained.

3.2 Characterisation techniques

Powder diffraction patterns were recorded for ceria, zirconia and ceria–zirconia MMO nanoparticles on an X-ray diffractometer (Bruker D2 Phaser) for 2θ from 20° to 90°. Using the Scherrer formula, the crystallite size was determined from the broadening of the X-ray diffraction peaks. Scanning electron micrographs **(**FEI-Quanta FEG 200F) were acquired to study the morphological properties of the nanoparticles. EDX spectroscopy was conducted to analyse the local elemental composition of the synthesised ceria, zirconia and ceria–zirconia MMO nanoparticles. The prepared nanoparticles were screened for antibacterial and antifungal potential using the agar well diffusion method.

3.3 Statistical analysis

Microcal Origin software (OriginPro 2018) was used for data processing and analysis. The particle size distribution was calculated using the software ImageJ (ij153-win-java8) from the obtained SEM images. The zone of inhibition was calculated using GraphPad Prism 6.0 software during the evaluation of the antibacterial and antifungal activities of the synthesised nanoparticles.

4. Results and discussion

4.1 Structural studies

The crystal structure and phase purity of ceria, zirconia and ceria–zirconia MMO nanoparticles were determined from the powder X-diffraction patterns. The XRD patterns revealed that all the synthesised nanoparticles are nano-crystalline. The major peaks of the zirconia, ceria–zirconia MMO and ceria nanoparticles were indexed in accordance with JCPDS cards No. 80-2155, 38-1436, and 43-1002, respectively (Figure 1). The ceria nanoparticles crystallised in thermodynamically stable cubic fluorite structure. The major peaks observed at 28.56°, 32.94°, 47.64°, 56.17°, 69.44° and 77.10° could be indexed to the (1 1 1), (2 0 0), (2 2 0), (3 1 1), (4 0 0) and (3 3 1) crystal planes, respectively. The unit cell volume and lattice constant were calculated to be 160.10 $Å^3$ and 5.43 Å, respectively. Using the Scherrer equation, $D = (0.94\lambda)/\beta \cos\theta$, where λ is the radiation wavelength, β is the peak width at half the maximum intensity (FWHM), and θ is the Bragg angle, the average crystallite size 'D' was estimated (Premkumar, 2020; Rajakani, Shajan, Arulgnanam, & Premkumar, 2022). The average crystallite size of the synthesised cerium oxide nanoparticles was 4 nm.

Pure zirconia exists in three polymorphic phases, monoclinic, tetragonal, and cubic as a function of temperature. The reflections obtained for the zirconia nanoparticles synthesised in the present study matched well with the JCPDS card

[80-2155], which is tetragonal crystal structure. Major peaks were seen at 30.63°, 35.08°, 50.88°, 60.58°, 63.25°, 75.08° and 82.31° were indexed as the (1 0 1), (1 1 0), (1 1 2), (1 0 3), (2 0 2), (0 0 4) and (2 1 3) planes, respectively. The lattice constants and unit cell volume of the synthesised zirconia nanoparticles were a = b = 3.67 Å and c = 5.12 Å and 68.96 Å^3, respectively. The average crystallite size of the synthesised zirconia nanoparticles was calculated to be 7 nm.

In the case of ceria–zirconia MMO nanoparticles, the diffraction peaks were shifted between those of the ceria and zirconia nanoparticles. The JCPDS data [80-2155] showed that the ceria-zirconia nanoparticles crystallised in tetragonal structure. The major peaks were seen at 29.68°, 34.36°, 49.67°, 58.94°, 72.70° and 80.54° can be readily indexed to the (1 0 1), (1 1 0), (2 0 0), (2 1 1), (2 2 0) and (3 0 1) lattice planes, respectively. The lattice constant and unit cell volume of the ceria-zirconia nanoparticles were calculated as a=b=3.69 Å and c=5.24 Å and 77.34 Å^3, respectively. This result shows that the zirconia lattice parameter was modified due to incorporation of ceric ion (Campos et al., 2022). The calculated average crystallite size of the synthesised ceria-zirconia nanoparticle was 8 nm.

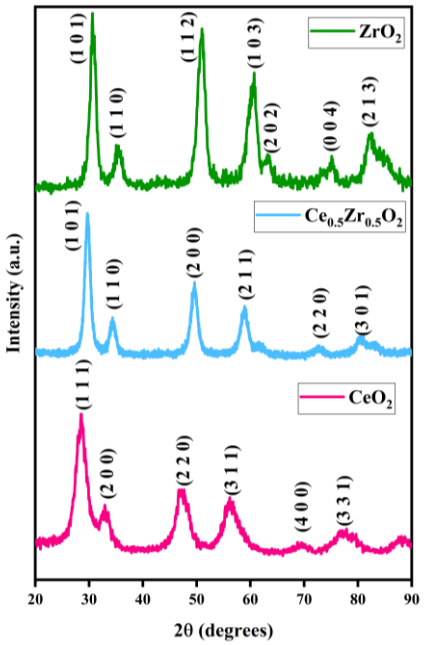

Figure 1 Powder X-ray diffraction patterns of pure and MMO ceria and zirconia nanoparticles

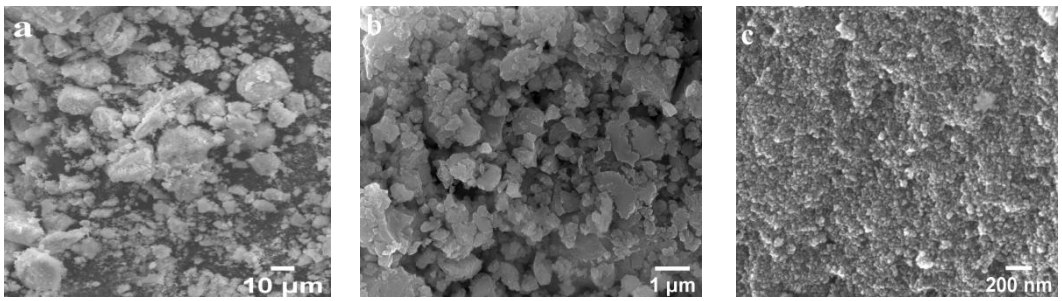

Figure 2 Scanning electron microscopy images of (a) ceria (b) ceria-zirconia and (c) zirconia nanoparticles

The morphology of the prepared nanoparticles was examined in the SEM images and is displayed in Figure 2. The SEM images of ceria and ceria–zirconia MMO nanoparticles

revealed that the particles were agglomerated, potentially due to their hygroscopic nature. The zirconia nanoparticles also had uniform size distributions with roughly spherical shapes (Chau, Kandasamy, Chinnathambi, Alahmadi, & Brindhadevi, 2021) compared to the ceria and ceria-zirconia nanoparticles. The average particle sizes of the ceria, ceria-zirconia and zirconia nanoparticles were around 160, 80 and 25 nm, respectively.

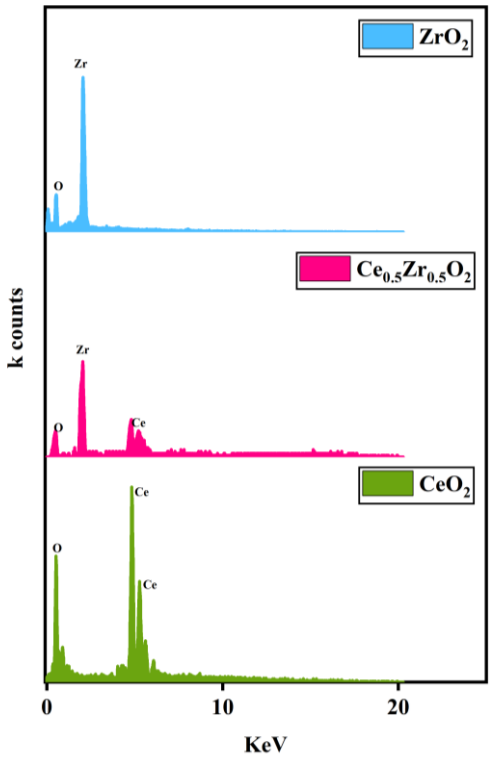

Figure 3 Energy dispersive spectra of the pure and MMO ceria and zirconia nanoparticles

The elemental composition of green-synthesised ceria, zirconia and ceria–zirconia MMO nanoparticles are presented in Figure 3. All the spectra show a characteristic peak at 0.5 keV that confirmed the presence of oxygen in the prepared nanoparticles. In the case of zirconia nanoparticles, the characteristic peak at 2.04 keV represents the zirconium, and for ceria nanoparticles, the characteristic peak at 4.8 keV represents the cerium, which confirmed the purity of the synthesised sample. In the ceria–zirconia MMO, that is, at an equimolar ratio, all the characteristic peaks were present and confirmed the presence of cerium and zirconium in the nanoparticles. The experimentally observed atomic percentage of oxygen in the prepared nanoparticles was less than the theoretical values, which confirmed the presence of oxygen vacancies in the sample.

4.2 Antibacterial activity

Generally, metal oxide nanoparticles are known for their antibacterial potential towards a wide range of bacterial species due to various interactions between the nanoparticles and the bacterial cytoplasmic membrane. In particular, reducing the size of the nanoparticles increases the effectiveness of cellular uptake as well as increasing the surface-to-volume ratio improves the extent of the cellular interaction. The size of the nanoparticles influences the interaction with the site where the plasma membrane and protein are bound (Rajeshkumar & Naik, 2018; Precious Ayanwale & Reyes-López, 2019). The nanoparticle–cell interaction further favours internalisation followed by the production of highly ROS, such as hydrogen peroxide, hydroxyl radicals, singlet oxygen, and superoxide anion radicals, to cause oxidative stress that damages the bacterial cell wall. (Anitha, Ramesh, Ravishankar,

Kumar, & Ramakrishnappa, 2018; Henam, Ahmad, Shah, Parveen & Wani, 2019; Tran et al., 2022). In general, bacteria are classified as gram positive and gram negative, depending on the structure of the cell envelope. Due to the presence of an outer membrane (OM) in addition to the peptidoglycan in gram-positive bacteria, gram-negative bacteria are immune to attack by therapeutic agents and other external stresses.

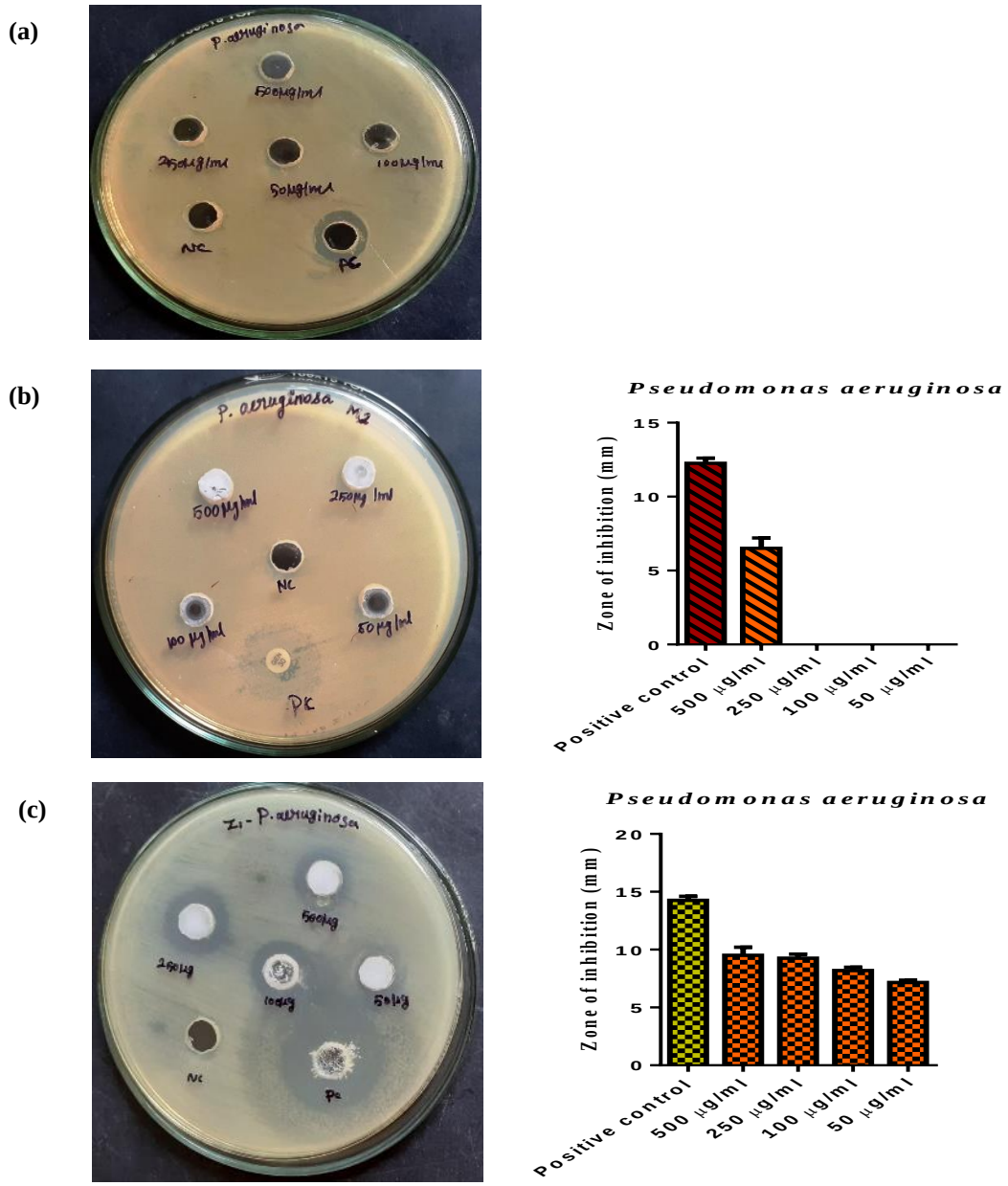

Figure 4 Effect of (a) ceria, (b) ceria-zirconia and (c) zirconia nanoparticles against *Pseudomonas aeruginosa*

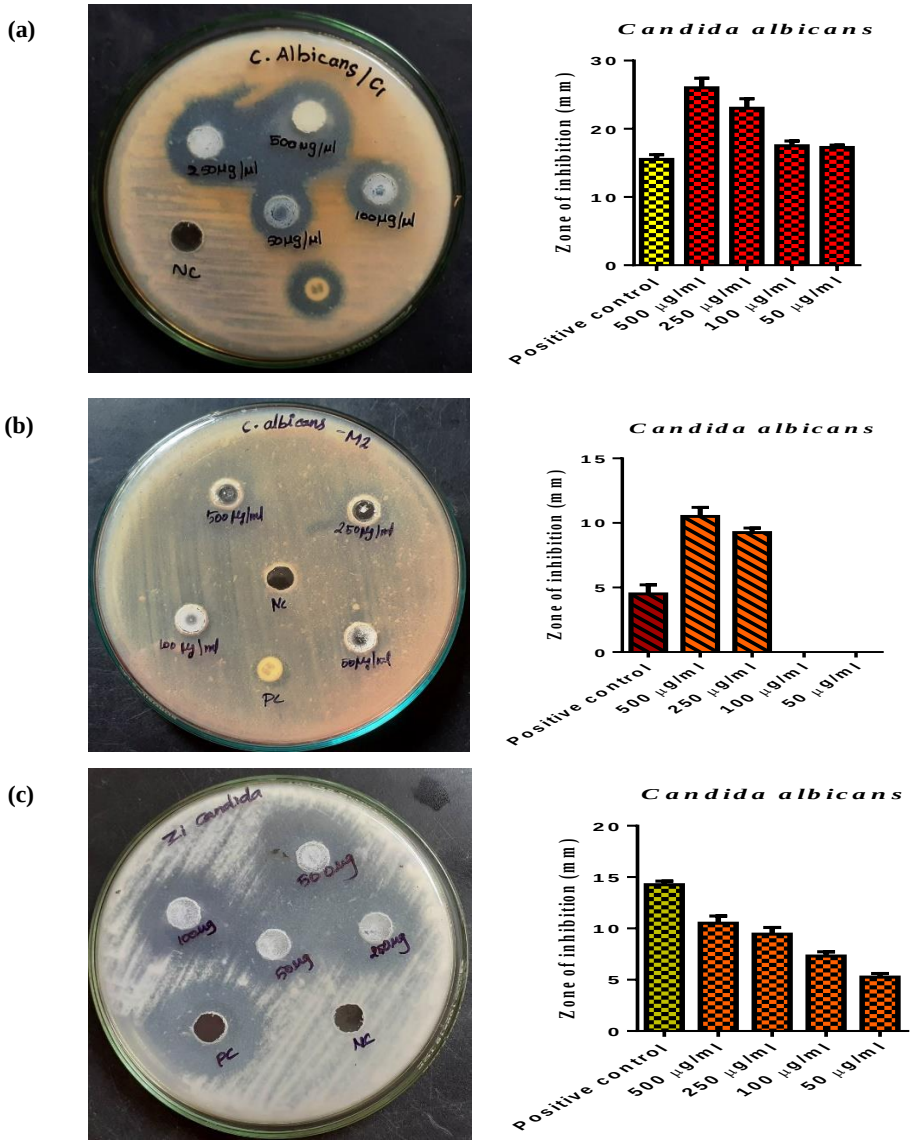

Figure 5 Effect of (a) ceria, (b) ceria-zirconia and (c) zirconia nanoparticles against *Streptococcus mutans*

To assess the *in vitro* antibacterial activity of the nanoparticles, the agar well diffusion method was used following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). In order to evaluate the antibacterial activity of the green-synthesised pure and mixed ceria–zirconia oxide nanoparticles, the antibiotic-resistant pathogenic species found most prevalently in biofilms, gram-positive *S. mutans* and gram-negative *P. aeruginosa*, were chosen as the representative species.

The nutrient agar medium and nutrient broth were prepared by dissolving 2.8 g of nutrient agar medium (HiMedia) in 100 mL of distilled water and autoclaved (15 psi) at 121°C for 15 minutes. It was then poured onto 100-mm diameter Petri plates (25–30 mL/plate) containing 20 mL of nutrient agar medium and seeded with 24-hour cultures of bacterial strains (*S. mutans* ATCC 25175 and *P. aeruginosa* MTCC 424). Wells were cut, and different concentrations of samples (50, 100, 250 and 500 µg/mL) were added. The plates were then incubated at 37°C for 24 hours. The antibacterial potential was evaluated by measuring the diameter of the inhibition zone formed around the wells, and the antibiotic Gentamicin was used as the positive control. The bactericidal effects of synthesised ceria, ceria–zirconia MMO and

zirconia nanoparticles against the bacteria *P. aeruginosa* and *S. mutans* are presented in Figures 4 and 5, respectively.

Table 1 shows the zone of inhibition resulting from ceria, ceria–zirconia and zirconia metal oxide nanoparticles against *P. aeruginosa* and *S. mutans.* Figure 4 shows that the pristine ceria nanoparticles did not show any antibacterial activity against the gram-negative bacteria *P. aeruginosa*. However, the ceria–zirconia MMO sample showed antibacterial activity against gram-negative bacteria *P. aeruginosa*, which could be due to the incorporation of zirconium in the ceria lattice. The pure zirconia nanoparticles showed better antibacterial activity than the ceria and ceria-zirconia nanoparticles.

Figure 5 shows that the ceria nanoparticles exhibited antibacterial activity against the gram-positive bacteria *S. mutans* but only at 500 µg/mL concentration. The antibacterial activity of the ceria–zirconia MMO sample against the gram-positive bacteria *S. mutans* increased compared to that of pure ceria. At the same time, the zirconia nanoparticles displayed high antibacterial activity against the gram-positive *S. mutans*. The antibacterial activity changes depending on the size, concentration, method of synthesis and shape of the nanoparticles (Hawar et al., 2022). A possible mechanism of the observed activity in zirconia incorporated ceria could be due to the release of zirconium ions, which destroy the cell wall and interact with the cell membrane. The protein present in the cell membrane or inside the cell that contains DNA (which contains sulfur) is the best coordination site for the zirconia nanoparticles. After binding, the zirconia ions inhibit DNA replication, leading to the death of the bacterial cells (Alyamani et al., 2021; Khane et al., 2022). In addition, the enhanced activity of the zirconia nanoparticles could be due to their smaller size, which increases the chances of cellular uptake. From the results, the zirconia nanoparticles showed better activity towards the gram-positive bacteria *S. mutans* compared to the gram-negative bacteria *P. aeruginosa*, which could be due to the presence of an OM in the latter. The antibacterial activity of the zirconia nanoparticles at a concentration of 500 µg/mL was even superior to the positive control, Gentamicin. The green-synthesised zirconia nanoparticles prepared in the present study showed better antibacterial activity against *S. mutans* compared to the zirconia nanoparticles synthesised through the chemical route, which could be considered an added advantage (Precious Ayanwale & Reyes-López, 2019).

Table 1 SD±means of the zone of inhibition against *Pseudomonas aeruginosa and Streptococcus mutans* obtained from samples

Name of the test organism	Composition of the test sample	Zone of inhibition (mm) SD ± Mean				
		500 µg/mL	250 µg/mL	100 µg/mL	50 µg/mL	PC
Pseudomonas aeruginosa	Ceria	0	0	0	0	6.5±0.7
	$Ce_{0.5}Zr_{0.5}O_2$	6.5±0.7	0	0	0	12.25±0.35
	Zirconia	9.5±0.7	9.25±0.35	8.2±0.28	7.15±0.21	14.25±0.35
Streptococcus mutans	Ceria	6.5±0.7	0	0	0	7.5±0.7
	$Ce_{0.5}Zr_{0.5}O_2$	7.25±0.35	6.2±0.28	0	0	13.75±1.06
	Zirconia	11±1.41	10.35±0.49	9.25±0.35	8.2±0.28	10.5±0.7

4.3 Antifungal activity

The antifungal property of metal oxide nanoparticles is also well-established in the literature. The most common mode of action is due to the electrostatic attraction between the positively charged protein corona of the metal oxide and the negatively charged cell wall of the microorganism. The metal oxide–cell interaction disrupts the cellular membrane, eventually leading to the death of the microorganisms (Arokiyaraj et al., 2017; Fathima, Pugazhendhi, & Venis, 2017; Kasithevar, Periakaruppan, Muthupandian, & Mohan, 2017). In addition, the size, shape and surface functionality also play a major role in the therapeutic activity of nanoparticles by improving cellular uptake.

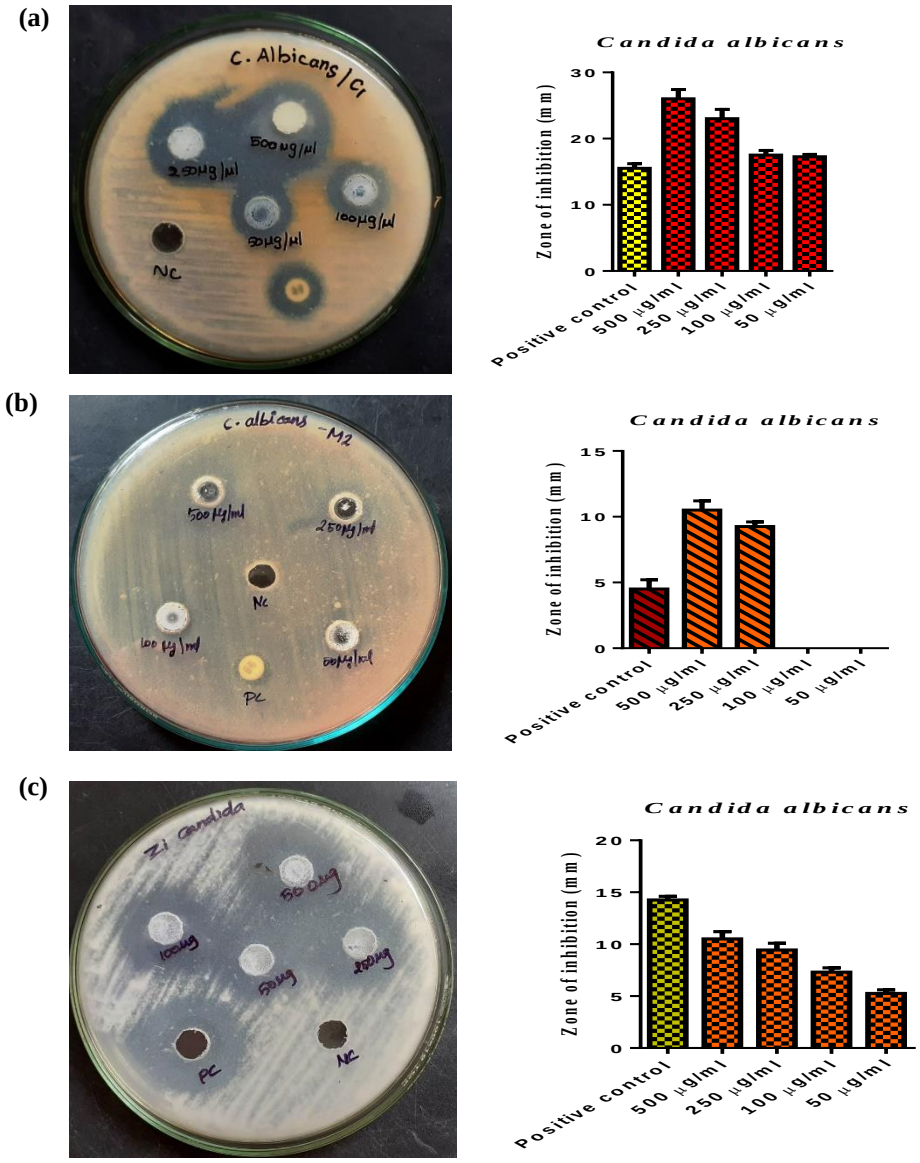

Figure 6 Effect of (a) ceria, (b) ceria-zirconia and (c) zirconia nanoparticles against *Candida albicans*

The antifungal activity of the synthesised nanoparticles was evaluated using the agar well diffusion method. The potato dextrose agar medium was prepared by dissolving 20 g of potato infusion, 2 g of dextrose and 1.5 g of agar in 100 mL of distilled water and autoclaved (15 psi) at 121°C for 15 minutes. Then the medium was thoroughly mixed and poured onto 100-mm Petri plates (25–30 mL/plate) while still liquid. Petri plates containing 20 mL of potato dextrose agar medium were seeded with 72-h cultures of the fungal strain *(C. albicans).* Wells were cut in the medium, and different concentrations of samples (50, 100, 250 and 500 50 µg/mL) were added. The plates were then incubated at 28°C for 72 hours. The antifungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B was used as the positive control. The effect of synthesised nanoparticles against the opportunistic pathogen *C. albicans*, the leading cause of fungal infections in human beings, was studied. The results are shown in Figure 6. Table 2 shows the zone of inhibition of the ceria, ceria–zirconia and zirconia metal oxide nanoparticles against the fungus *C. albicans.*

The metal oxide nanoparticles (ceria, zirconia and ceria–zirconia MMO) are positively charged, whereas the cell wall of fungus possesses a negative charge and hence there could be an electrostatic attraction between the metal oxide nanoparticles and the fungus. This interaction disrupts the cell wall and eventually causes the death of the fungus. In Figure 6, the ceria nanoparticles show better antifungal activity than the zirconia and ceria-zirconia nanoparticles. The concentration-dependent activity of ceria nanoparticles was also observed. The antifungal activity of the ceria nanoparticles is higher for all the concentrations compared to the positive control. Also, the green-synthesised ceria nanoparticle showed superior antifungal properties against *C. albicans* compared to earlier reports (Rajan, Rajan, Philip, & John, 2019; Elango, Deepa, Subramanian, & Saraswathy, 2020). For the equimolar ratio ($Ce_{0.5}Zr_{0.5}O_2$), the antifungal property increased compared to zirconia nanoparticles and was even better than the positive control. The antifungal property of zirconia nanoparticles was observed to be the lowest compared to the ceria and ceria-zirconia nanoparticles, which could be due to their lower binding affinity for the fungal cell wall.

Table 2 SD±means of the zone of inhibition against *Candida albicans* obtained from samples

Name of the test organism	Composition of the test sample	Zone of inhibition (mm) SD ± Mean				
		500 µg/mL	250 µg/mL	100 µg/mL	50 µg/mL	PC
Candida albicans	Ceria	26±1.41	23±1.41	17.5±0.7	17.25±0.35	15.5±0.7
	$Ce_{0.5}Zr_{0.5}O_2$	10.5±0.7	9.25±0.35	0	0	4.5±0.7
	Zirconia	9.5±0.7	9.25±0.35	8.2±0.28	7.15±0.21	14.25±0.35

5. Conclusion

Ceria, zirconia and ceria-zirconia MMO nanoparticles were synthesised using *M. dubia* leaf extract. The structural and microstructural properties of the synthesised nanoparticles were investigated before screening for antimicrobial activity. The powder X-ray diffraction pattern revealed that the ceria nanoparticles crystallised in a cubic fluorite structure, whereas the synthesised ceria-zirconia and zirconia nanoparticles showed tetragonal structures. The SEM images of the ceria and ceria-zirconia nanoparticles showed agglomeration. The energy dispersive spectra indicated the presence of oxygen vacancies in all nanoparticles. The antibacterial activity of the zirconia nanoparticles was observed to be better than that of the ceria and ceria-zirconia nanoparticles and showed superior antibacterial activity against the gram-positive *S. mutans* than the positive control. The synthesised ceria nanoparticles displayed good antifungal activity against *C. albicans* compared to the zirconia and ceria-zirconia nanoparticles and exhibited superior activity to that of the positive control.

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