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Fabrication of Cerium Oxide Nanoparticles with Improved Antibacterial Potential and Antioxidant Activity

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Recent years have seen a dramatic uptick in both research into and practical application of nanoparticles (NPs). Many biomedical applications have found success with the use of nanoparticles due to their wide spectrum of significant biological effects, including antibacterial and antioxidant properties. Nanoparticles that aren't harmful are gaining traction as a promising new class of antioxidants. Cerium oxide is a lanthanide rare-earth element. Cerium oxide nanoparticles (CNPs) exhibit a large surface area and good catalytic activity, the result of the dual oxidation state of CNPs, Ce3+ and Ce4+, has good antibacterial and antioxidant activity. CNPs were characterised by using analytical techniques such as the UV-Visible spectrophotometer, scanning electron microscopy, X-ray diffraction, zeta potential, Fourier transform infrared spectroscopy, and dynamic light scattering (DLS). CNPs exhibited a strong zone of inhibition against S. aureus (15mm) and E. coli (14mm). In vitro antioxidant activity of CNPs was investigated using the DPPH and ABTS techniques, with 50% of their radical scavenging potential being observed at concentrations of 47.61μg/mL and 49.26μg/mL respectively. Thus, our study reports that CNPS could be used as a prominent and efficient antioxidant and antibacterial agent. However, further studies are needed to understand the possible mechanisms of toxicity assessment.

Keywords: Antioxidant; ABTS; Cerium oxide nanoparticles (CNPs); cell wall damage; DPPH.

Nanotechnology is one of the most exciting research frontiers in current materials science because of their unique physiochemical properties[1]. Synthesis of metallic nanoparticles with a size range of 1nm to 100 nm or less has a peculiar property, for example, size, shape, and high distribution, with enhanced applications such as the biomedical field[2]. In cancer therapy, nanoparticles worked with minimal side effects and improved pharmacokinetics[3].

The metal oxide nanoparticles are used in highly potential biological applications due to their controlled size, shapes, chemical constituents, and valence state. Among the metal oxide nanoparticles, TiO_2, FeO_2 and CeO_2 are extremely reactive oxides that acutely interconnect with the metabolic networks of cells[4]. Cerium oxide (CeO_2) is a rare earth metal in the lanthanide family. Among the researchers, cerium oxide nanoparticles are becoming the most significant material due to their two oxidation states, $Ce^{3+}$and Ce^{4+} as well as their greater oxygen mobility and loading capacity[5,6]. The cubic fluorite structure of cerium oxide nanoparticles (CNPs) is active

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in biomedical applications such as anticancer, antioxidant, antibacterial, bone implant material, device fabrication, and drug carrier[6,7] due to their topography, size, and biocompatibility[8]. Cerium oxide nanoparticles are active and play a significant role in toxicity against yeast, bacteria, and fungi[9] by interacting with the microbe's cell wall through electrostatics [10, 11]. The paired oxidation states Ce^{3+} and Ce^{4+} of cerium oxide nanoparticles are responsible for the enhanced antibacterial potential. Sufficient dispersibility makes them exist as a catalytic location for the attachment and long-chain phospholipid hydrolysis found on the surface of the bacterial membranes[12]. The Ce atom's ability to transition between the 3^+ and 4^+ states of oxidation consists of cerium oxide nanoparticles that exhibit autocatalytic activity, which results in a strong radical-scavenging potential whenever this state occurs in a biological system at pH 7.4 [13, 14].

In this present study, we demonstrate CNPs as good hybrid nanomaterials for bacterial growth inhibition *Staphylococcus aureus* and *Escherichia coli* also have good antioxidant potential for radical scavenging that was investigated by using the DPPH and ABTS assays. Further cerium oxide nanoparticles have been characterised using different techniques such as the UV-Visible spectrophotometer, morphology identification using SEM, functional groups of nanoparticles identification by FT-IR, DLS to determine hydrodynamic diameter, Zeta potential analyses to identify the surface charge of nanoparticles, and XRD for confirmation of the crystalline nature of nanoparticles.

MATERIALS AND METHODS

Cerium oxide nanoparticle Synthesis

Cerium oxide nanoparticles were synthesised according to the work of Pinna *et al.,* 2020[14] with some modifications. In 10 mL of 2-propanol, 3.5g of Ce(NO3)3.6H2O was dissolved, and then 1M of 0.25mL HCl was added and stirred until complete dissolution. In a separate vial, 1g of urea was dissolved in 10 mL of 2-propanol that contained 1M of 0.25mL HCl and stirred for 5 minutes. The solution of urea was added dropwise to the $Ce(NO_3)_3$ solution under stirring conditions, and NH_4OH 7mL was added to that mixture when the addition was complete. The samples were then microwaved four times at 600 W for 10 seconds before being washed with water and centrifuged at 10,000 rpm. After that, the discarded supernatant (5 mL of suspension) was allocated with a light yellow milky pellet (500mg of CeO_2).

Characterization

UV-visible spectroscopy measurements were performed at room temperature using a spectrophotometer UV-2450 (Shimadzu) and observed in the 200–800 nm range for cerium oxide nanoparticles (CNP). The topographical architecture of CNPs was studied using scanning electron microscopy (SEM) (JSM-6480 LV). The hydrodynamic size of CNPs is measured by DLS, and the surface charge of CNPs is determined by the zeta potential of (æ) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Fourier transform infrared spectroscopy (FT-IR) analysis was performed at a range of 4000-400cm^{-1} (Perkin Elmer, USA). X-ray diffractometer (XRD) analysis confirmed the crystalline morphology of the CNPs (SmartLab, Rigaku Corporation, Japan).

Investigation of antibacterial activity

Diffusion method in agar well

The antibacterial potential of CNPs was assessed using the agar well diffusion technique against *S. aureus* and *E. coli*. Bacterial culture was spread using cotton swabs on nutrient agar (HiMedia, India) plates. Wells were performed using gel puncture (the diameter of the wells was 6 mm) on nutrient agar plates. In each well added with different doses of CNPs (25-100ìg/mL), streptomycin (HiMedia, India) was used as a positive control (10ìg/mL) and 30ìL of autoclaved, double distilled water acted as a negative control. After that, the nutrient agar plates were incubated for 24 hours at 37°C. After the incubation period, the zone of inhibition diameter was measured in mm[15].

Analysis of bacterial Growth curve

Staphylococcus aureus and *Escherichia coli* were treated with different concentrations of CNPs 25, 50, 75, 100ìg/mL which were compared with the untreated bacteria as a control to assess the bacterial growth curve. Briefly, *a* 96-well microliter plate was filled with 250 ìL of LB and 20ìL of bacterial suspension (10^7 CFU/mL) and incubated at 37 °C for 24 hours. Every 3 hours for 24 hours, bacterial growth rate was measured at

580 nm using a Synergy HT Multimode Reader (Biotek, Winooski, USA). The experiments were performed in triplicates[16].

Evaluation of antioxidant activity in i*n vitro*

Radical scavenging activity by DPPH method

The scavenging activity of CNPs against 2, 2-diphenyl-1-picrylhydrazile (DPPH) (HiMedia, India) was calculated according to the[17]. In a 96 well plate, was added at increasing doses (20, 40, 60, 80 and 100ig/mL), and the control was ascorbic acid (Vitamin C). 1mM of DPPH 100iL solution was added to each well, and the samples were incubated in the dark for 30 minutes at room temperature. The solution colour was changed from violet to yellow colour indicate that reactive oxygen species have been scavenged, and it has been measured at 517 nm using the Synergy HT Multimode Reader (Biotek, Winooski, USA). Finally, the percentage of scavenging ability was calculated using the following equation:

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \times 100$$

Whereas, Ac – OD value of blank, As – OD value of CNPs treated.

Radical scavenging activity by ABTS method

The scavenging activity of CNPs at various concentrations (20 - 100ig/mL) against radicals was evaluated using the $ABTS^{+}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (HiMedia, India) assay, adapting a procedure from a previous study. In the ABTS assay, ascorbic acid was used as the standard antioxidant, and the ability of CNPs to scavenge the ABTS radical (ABTS.ABTS+) was compared. The ABTS solution was prepared by simply mixing 7mM of aqueous ABTS solution with 2.45mM of potassium persulfate in a dark medium at room temperature for 12 hours. After diluting the same standard solution with ethanol, the ABTS+ reaction mixture was added to each well, and the reaction was measured at 734nm after 10 minutes in the dark using the Synergy HT Multimode Reader (Biotek, Winooski, USA)[15].

RESULT AND DISCUSSION

Analytical characterization of CNPs

UV-Visible spectrophotometer of CNPs

The UV-Vis spectrophotometer investigations have been helpful for structural integrity, changes, and keeping track of the formation of nanoparticles determinination[18]. The absorption spectra of CNPs were observed at 300nm absorbance, as shown in (Fig.1). The stability of the CNPs was evaluated based on the time intervals from 0 to 60 days as shown in (Fig.2**)**, when the incubation time was increased, a hypochromic shift was observed, which indicates the CNPs are partially stable [19]. Strong absorption peaks at 300 nm were visible in the absorption spectra, which is the particular characteristic mentioned in earlier studies[20]. The interaction of Ce ions with cancer cells and microbes to exhibit anticancer and antibacterial activity, respectively, has been previously reported for CNPs[21].

SEM Analysis

We examined the topography of the spherical CNPs using a scanning electron

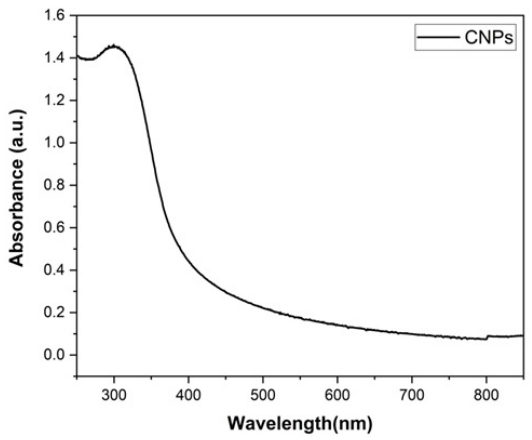

Fig. 1. UV-Vis spectra of CNPs

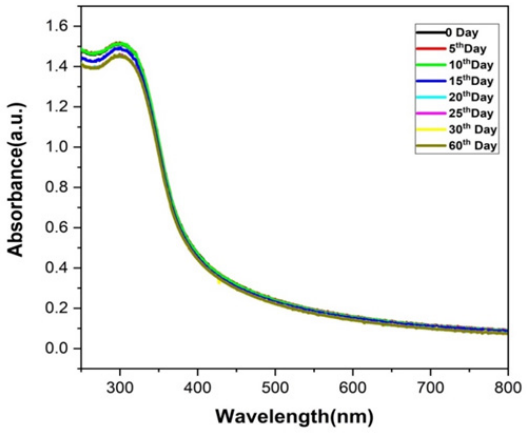

Fig. 2. UV-Vis spectra of CNPs for Stability analyse

microscope, as shown in (Fig.3). The CNPs' spherical appearance and restricted particle range imply that particle distributions are highly constant and that spherical agglomerates have formed. As mentioned in a previous article, synthesis duration, temperature, solvent, and calcination temperature[22] are all critical reaction parameters that may explain why this kind of reaction emerges. According to phenomenological evidence, CNPs have a spherical shape with smooth surfaces.

Dynamic light scattering (DLS) analysis was used to investigate the hydrodynamic size distribution of CNPs. The average size of dispersion CNPs was 35 ±0.4 nm, as shown in (Fig. 4). CNP zeta potential investigation revealed +24.3 mV as shown in (Fig. 5).

Fourier transforms infrared spectroscopy (FT-IR)

The Fourier transform infrared spectroscopy (FT-IR) approach has been shown to be helpful in determining the functional groupings of CNPs, as shown in (Fig.6).This particle absorption at a specific regain is observed as minor and major peaks for respective particles. The significant absorption at 3435cm^{-1} in the high-frequency region of the spectrum is due to physically absorbed water, an O-H stretching vibration, or a surface hydroxyl group. The bending vibration of linked structures shows a small shoulder peak at 2073cm^{-1} responsible for (H-O-H). The 1633cm^{-1} peaks are associated with O-C-O symmetric stretching, but the 666 cm^{-1}

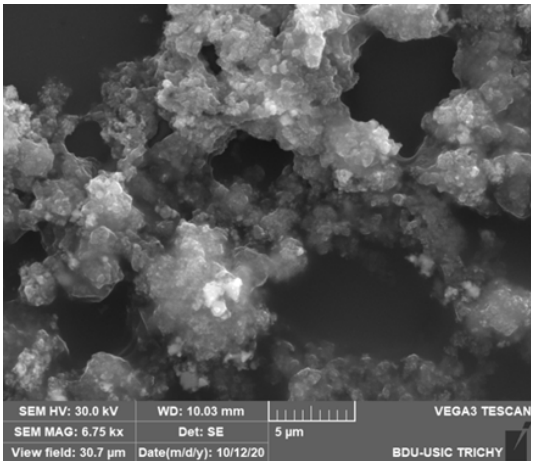

Fig. 3. SEM image of CNPs

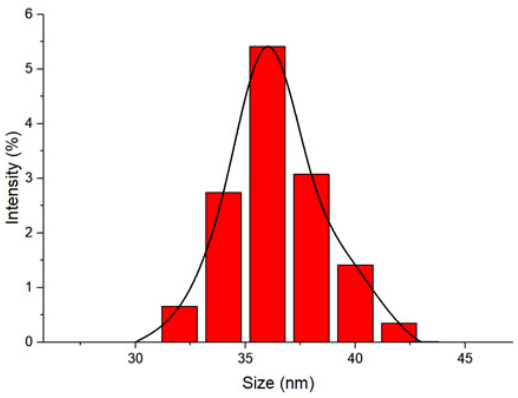

Fig. 4. DLS analysis of CNPs

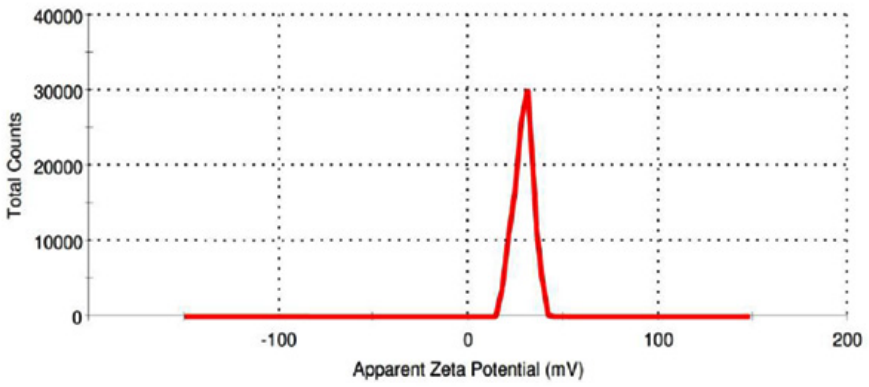

Fig. 5. Zeta potential analysis of CNPs

peaks are directly associated with the frequency of CeO stretching[8, 23].

XRD analysis of CNPs

CNPs are precipitated, as evidenced by the X-ray diffraction (XRD) pattern in (Fig.7). The XRD pattern of the nanoparticles reveals that they are made of cubic fluorite, CeO_2. For diffraction peaks with values around 28.41°, 32.87°, 47.54°, 56.38°, and 60.18°, the planes (111), (200), (220), (311), and (222) are chosen. This set of peak deflections agrees with the powder X-ray diffraction standard JCPDS No. 34-0394. The XRD pattern showed no peaks related to impurities or other phases, suggesting that the CNPs generated are made of a pure crystal of CeO_2[21].

Antimicrobial activity Study

CNPs have been tested for antibacterial potential against bacteria such as *S. aureus* and *E. coli.* According to the (Fig. 8) revealed a higher inhibition zone was observed. These findings show that the communication between CNPs and the bacteria's cell wall has efficiently promoted the toxicity of the bacteria and caused cell death. Among the four dosages of CNPs, 100µg/mL depicts a higher inhibition zone for *E. coli* (14 mm) and *S. aureus* (15 mm). The nanoparticles attached to the bacterial cell wall through ionic interactions between negatively charged organisms and positively charged nanoparticles. The binding of CNPs to peptidoglycan consists of gram positive bacteria, which are bound with teichoic acid; this may be a possible interaction with CNPs in antibacterial activity. More importantly, the possible mechanism of antibacterial activity of CNPs may interact with the bacterial cell membrane and bind with the mesosome, which is involved in cellular breathe, ability to cell divide, replicate their DNA, and, ultimately leading to cell death[24]. The observed antibacterial potential of CNPs at 100 µg/mL concentration exhibits significant efficacy against *S. aureus* and *E. coli*, due to the strong electrostatic forces the nanoparticles attach to the cell membrane to inhibit the growth of bacteria. In an early study, it was reported that CeO_2/GO nanocomposites are potentially active against wound-associated infectious pathogens such as *S. typhi*, *S. aureus, E. coli,* and *P. aeruginosa* and present an absence of visible condition[23]. The antibacterial activity of Ag-Au loaded CeO_2 nanoparticles against *E. coli* and *S. aureus* has potentially improved compared to other nanocomposite materials due to their

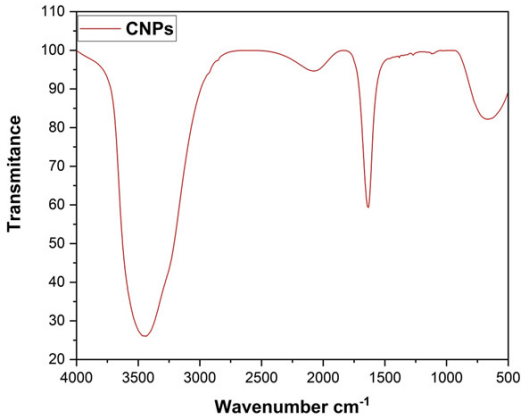

Fig. 6. FT-IR spectra of CNPs

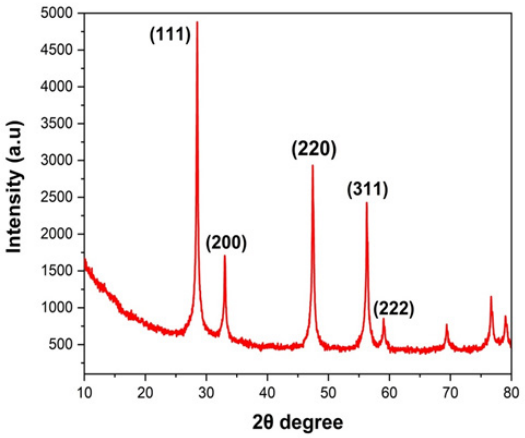

Fig. 7. XRD analyses of CNPs

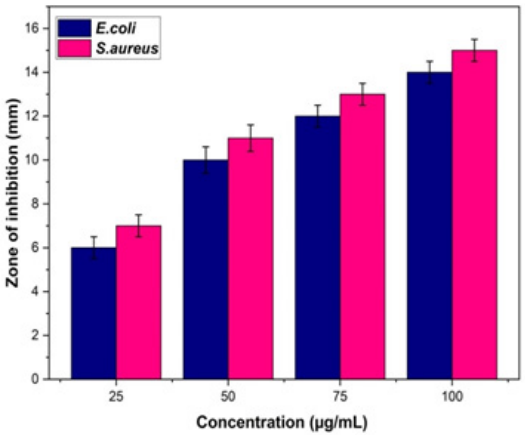

Fig. 8. Antibacterial activity of CNPs against *E.coli* and *S.aureus*

individual metals containing ions[25-27]. The possible mechanism of antibacterial activity has been electrostatic communication between bacterial cell walls and Ag-Au-loaded CeO_2 nanocomposites that influence inhibition of microbial growth and induce microbial death, as reported in past studies[1, 6, 28-30]. Another mechanism of antibacterial activity reported in previously small nanoparticles has been to easily penetrate inside the cell wall of bacteria and cause cell death[5,8]. In an early study[21], CNPs potentially damaged the cell wall of E. coli at a concentration of 0.06mg/mL when exposed to X-ray radiation.

Effect of CNPs on bacterial growth

The effect of CNPs on *E.coli* and *S.aureus* growth was demonstrated. The lag, log, stationary, and death phases of *E. coli* and *S. aureus* growth curves were clearly represented in (Fig. 9). While the effect of different doses of CNPs from 25µg/mL to 100µg/mL has been observed, the constriction of the log phase was visible, demonstrating that CNPs have a dose-dependent manner to exploit against *E. coli* and *S. aureus*. The findings could imply that the binding of CNPs to the bacterial cell membrane surface, due to their dual oxidation state of Ce^{3+} and Ce^{4+}, provided a catalytic site for the bonding and

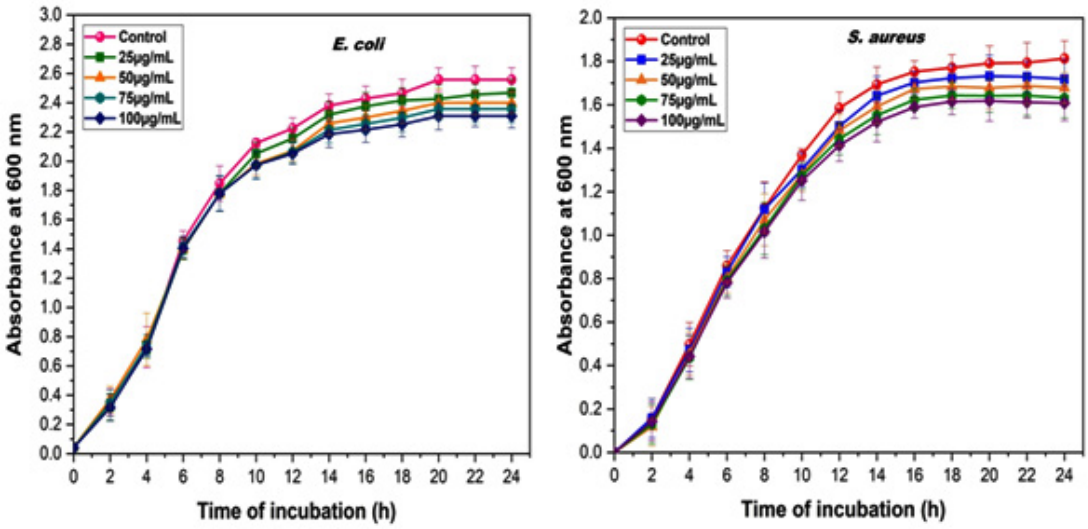

Fig. 9. Growth curve of *E. coli* and *S. aureus* under the CNPs treatment

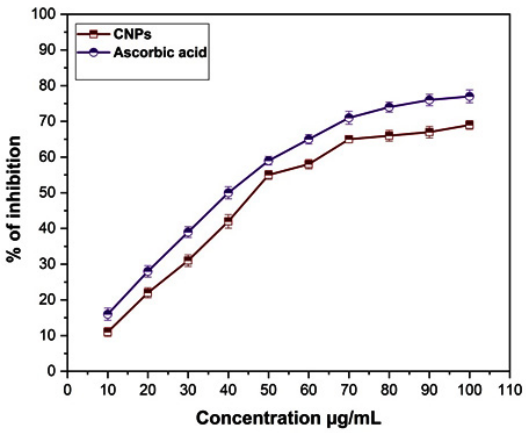

Fig. 10. DPPH radical scavenging activity of CNPs

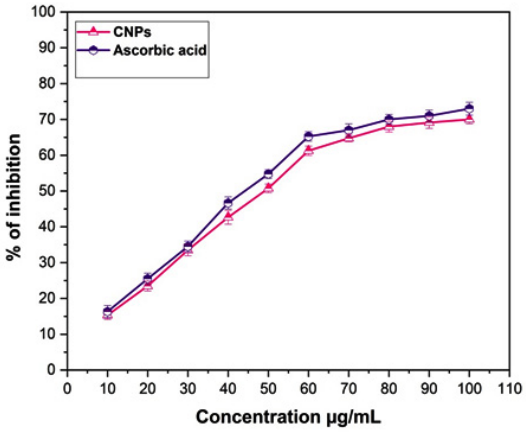

Fig. 11. Antioxidant activity of CNPs using ABTS method

hydrolysis of long-chain phospholipids found on bacteria's cell walls, resulting in cell wall damage and biomass reduction[31].

Radical Scavenging activity by DPPH method

The antioxidant activity of CNPs was determined using the (DPPH)-2, 2-diphenyl-1-picrylhydrazyl method. When DPPH combines with an antioxidant, it produces a stable free radical that can be converted into a non-radical state. The DPPH radical was initially purple, but an antioxidant converted it to yellow, the colour of the non-radical version. As compared to the industry standard (Vitamin-C), CNPs' free radical scavenging (DPPH) activity revealed significant antioxidant power, with an IC_{50} percentage of 47.61ìg/mL as shown in (Fig.10). A previous study found that the levan coated CNPs have higher antioxidant capacity because polysaccharides contain multiple hydroxyl groups that can react with free radicals, and reduced radical chain reactions are related to antioxidant[32,33]. The probable mechanism of the radical scavenging potential of cerium oxide nanoparticles, it has a dual oxidation state that converts together based on pH, generating ROS in acidic pH and scavenging the ROS in normal pH, as reported in early study[34,35]. In DPPH assay 50ìg/mL of CNPs has highly effective with 87.6% of scavenging potential compared with control á-tocopherol 76.3 % and BHA 52.9% has reported in earlier[36]. Free radical scavenging activity of nanoceria with a dose dependent manner, which is exposing the concentration of 50µg/500µl for 48.58% of radical inhibition in an early study, is reported at[32]. Early studies found that 9mg/mL of CeO_2NPs increased DPPH inhibition by up to 67%, while polysaccharide-coated CeO_2NPs were 85% effective at getting rid of DPPH[21].

Both solvothermal CeO_2 nanoparticles and hydrothermal CeO_2 nanoparticles were able to get rid of up to 55% and 30% of DPPH, respectively[37]. The IC50 value for the antioxidant activity of CeO_2 NPs is 4.38 mg/ml. CeO_2 nanoparticles displayed higher antioxidant activity (IC_{50} = 8-10 mg/mL) than ZnO nanoparticles, according to the literature[38-40]. According to past reports, the CNPs have been potentially active against radicals in the concentration range of 0.05g/L upto 0.06g/L when the activity has been low, below the concentration of 0.05g/L[41].

ABTS Radical Scavenging activity

The ABTS method was used to assess the antioxidant activity of CNPs, and the reduction of free radicals caused by the CNPs was seen at 734 nm. CNPs have scavenged free radicals in a dose-dependent manner, with the best scavenging ability being noted in (Fig.11). The obtained result has depicted that CNPs have a dose-dependent inhibitory effect on the generation of ABTS radicals with an IC_{50} percentage of 46.26ig/mL. It is reasonable to believe that antioxidant capabilities increase directly with nanoparticle concentration[15]. $ABTS^{\bullet+}$ scavenging activity of Ce_2O_3NPs is significantly high with 87.2% compared with standard á-tocopherol 74.9 % and BHA 50.1% reported in early study [36]. In previous report, it was shown that the CNPs have potentially active and scavenged free radicals and inhibit the production of ABTS+ radicals in a dose dependent manner compared with Trolox[15]. The scavenging potential of *Mentha royleana*-mediated CNPs is 46.7%, compared to 49.7% for control ascorbic acid at an IC_{50} value of 5.39 g/ml, whereas the IC_{50} value for *Mentha royleana* was found at a concentration of 5.57g/ml, as reported in an earlier study. The ABTS+ radical's species was reduced in proportion to the amount of greenly synthesised CeONPs. Other evidence suggests that CeONPs scavenge ROS from normal cells preferentially, protecting them from reactive oxygen species[42-44].

CONCLUSION

The current study demonstrated cerium oxide nanoparticles (CNPs) have been a considerable help in combating microbial pathogens with significantly formed zones of inhibition against *E.coli* and *S.areus* and reduced biomass with investigation of the bacterial growth curve, which shows considerably reduced growth of *E.coli* and *S,areus* in a dose dependent manner up to the incubation period of 24 hours. The ionic interaction between CNPs and bacterial cell membranes is crucial to their antibacterial activity. This is because the CNPs dual oxidation state of Ce^{3+} and Ce^{4+} surface provides a catalytic site for the attachment and hydrolysis of long-chain phospholipids found on the cell wall of bacteria, damaging the cell wall and decreasing

the biomass. The obtained results from the present study unequivocally proved CNPs have good antibacterial properties. DPPH and ABTS are the methods that were used to investigate the *in vitro* antioxidant activity of CNPs. In the DPPH assay, the CNPs have an IC_{50} concentration of 47.61ìg/mL, which yields a significant result when compared with the control. The result that was obtained from this study, which was the ABTS radical scavenging assay of CNPs, demonstrated that there was an inhibition of ABTS radical production in a dose-dependent manner, in addition to a significant IC_{50} concentration of 46.26ìg/mL. This finding was made possible by the fact that the ABTS radical scavenging assay of CNPs was conducted. According to the results that were obtained, CNPs have beneficial antioxidant properties, with the paired oxidation states Ce^{3+} and Ce^{4+} that contain CNPs having the most significant influence. The SEM, XRD, DLS, Zeta potential, FTIR and UV-Visible spectrophotometer characterizations studies are supported to the antibacterial and antioxidant activity properties of CNPs. Thus, the present study demonstrated that cerium oxide nanoparticles could be used as effective antimicrobial and antioxidant agents. In our subsequent research, we focused on the toxicity of CNPs that had been functionalized with phytochemicals in both *in vitro* and *in vivo* studies to better understand the potential mechanisms involved.

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Conflict of Interest

The authors declare no conflict of interest.

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