

Research Article

Characterization and cytotoxicity of nanoceria phytosynthesized using *Eucalyptus camaldulensis* bark extract

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ABSTRACT

Cancer, a complex group of diseases characterized by uncontrolled cell growth, poses a significant global health risk. In the context of the high occurrence of breast cancer (BC) in women, conventional chemotherapeutic agents, although effective, frequently bring about challenges such as resistance and recurrence. Nanotechnology is emerging as a promising approach, with attention focused on cerium oxide nanoparticles (CNPs). CNPs possess distinctive characteristics such as antioxidant activity, the ability to transition between Ce³⁺ and Ce⁴⁺ oxidation states, and biocompatibility with living systems. This study introduces a novel phytosynthesis method using *Eucalyptus camaldulensis* bark extract to synthesize CNPs, emphasizing environmentally friendly methods. Characterization techniques including Fourier transform infrared spectroscopy (FTIR), Powder X-ray diffraction (XRD), Scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), Transmission electron microscopy (TEM), Diffuse Reflectance (DR) were used to elemental, structural, and morphological properties of synthesized CNPs. Results have shown the successful synthesis of CNPs having cubic fluorite structure with a space group of *Fm3m* (225) and a crystallite size of 11.2 nm based on XRD characterization. FTIR and EDX have confirmed the elemental analysis of CNPs and the presence of extracted biomolecules during the synthesis process. SEM and TEM images revealed the morphology of the phytosynthesized CNPs as sphere-like, with a size of 2 to 10 nm. Finally, cytotoxicity by MTT assay and uptake of these CNPs on MCF-7 cells demonstrates successful uptake and their potential to inhibit these cancerous cells. The protective effect of CNPs on normal cells was further investigated using a ROS assay on the MCF10A cell line. Furthermore, the catalase-mimic (CAT-mimic) activity of phytosynthesized CNPs was assessed by a commercial CAT-mimic kit assay.

1. Introduction

The term “cancer” is used to describe a diverse group of diseases, characterized by the rapid and uncontrolled growth of abnormal cells that can affect any part of the body [1]. When not detected in its early stages, cancer can metastasize, potentially damaging multiple organs and making it a formidable disease to treat, often leading to death worldwide [2]. Breast cancer (BC) is a malignant proliferation of epithelial cells lining the ducts or lobules of the breast and is the most

common form of cancer in women, excluding skin cancer. It accounts for approximately one-third of all female cancer cases, with one million new cases diagnosed globally each year, underscoring its prevalence [3,4].

Chemotherapy can be described as a treatment involving high levels of toxicity and several terrible side effects [5]. Despite all the advanced treatments and advanced management resulting in a 90 % cure rate, the healthcare system still endures significant problems, including treatment resistance and malignancy recurrence [6,7]. Based on studies among patients, more than 50 % of adults and 20 % of children

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experience recurrence after successful cancer treatment with chemotherapy [8,9]. Additionally, radiotherapy, while effective, may inadvertently harm adjacent organs. Knowing all the above facts, researchers are now focused on developing more efficient and safer tools using new technologies to help cancer patients. Nanotechnology is one of the most interesting branches of science that has received attention in recent decades [10,11]. Its diverse applications span various fields, including imaging, commercial industries, electronics, and healthcare. Nanotechnology involves the use of nanoparticles (NPs), which are defined as tiny particles with sizes between 1 and 100 nm. Because of their high surface-to-volume ratio and quantum confinement effects, NPs potentially increase efficacy and reduce biotoxicity [12,13].

In recent years, research on the anticancer potential of nanoceria has shown its ability to induce the death of cancer cells through increasing reactive oxygen species (ROS) while reducing tumor growth and protecting healthy tissues against oxidative stress with antioxidant properties [14–17]. Cerium is a member of the lanthanide group and is the most abundant rare earth (RE) metal with atomic number 58, found in the Earth's crust [18–20]. Nanoscale ceria can exhibit exceptional properties such as a smaller band gap than bulk ceria [21,22]. The property that makes nanoceria a rare and unique metal is that, unlike other rare earth metals, cerium can exist stably in both Ce^{+3} and Ce^{+4} states, while the others only exist in the stable RE^{+3} state. Cerium can switch reversibly between these two oxidation states and change with changing environmental conditions [23–26]. Nanoceria has a very reactive surface to neutralize free radicals, since the structure of nanoceria may be disturbed due to the change of physical and chemical properties; therefore, with the reduction of the size of NPs, oxygen deficiency occurs due to oxygen vacancies in the structure [27,28]. Nanoceria is believed to not only protect cells against ROS but also to protect normal cells against radiation damage [29,30]. Due to its radical-inhibiting properties, nanoceria can reduce oxidative stress damage and can help prevent and fight aging, nervous stress, and cell damage caused by toxins and cancer [31–34].

Research in this area has demonstrated that the synthesis of nanoceria involves various methods tailored for specific applications, but the main methods are the chemical and the green synthesis methods [35–38]. Chemical techniques, such as precipitation, hydrothermal, solvothermal, sol-gel, emulsion, thermal decomposition, electrochemical, microwave irradiation, and laser ablation, have been utilized for CNP synthesis [39,40]. In these methods, coating materials or additives, non-polar solvents, and toxic chemicals are used. According to the articles, the synthesis of nanoceria with chemicals causes toxicity and damage to cells, which has negative implications for medical applications, human health, and the environment [41–43]. Many researchers aim to develop a biocompatible and environmentally friendly process for the synthesis of NPs using biological resources such as plants and microbes [44,45]. Green synthesis of NPs, unlike conventional chemical methods, uses renewable and environmentally friendly sources, including plant extracts, microbes, and fungi. They offer cost-effective alternatives to chemical and physical techniques because they do not require complex equipment, high-pressure conditions, or toxic substances [46–48].

In past decades, the use of plant extracts for the phytosynthesis of CNPs has gained significant attention [49]. Plant extracts have emerged as an exciting resource for CNP synthesis since they are safe, environmentally friendly, renewable, and non-toxic, and they act as stabilizing and reducing agents [50–52]. Recent studies have reported the successful synthesis of CNPs by natural reducing agents like plant extracts (such as *Ferula gummosa* [53], *Aquilegia pubiflora* [54], *Caccinia macranthera* [48], *Portulaca oleracea* [55]). *Eucalyptus camaldulensis* (*E. camaldulensis*), a significant ethno-medicinal plant from the Myrtaceae family, has been traditionally used to treat bacterial infections such as sore throat and respiratory and urinary tract infections. The leaves of *E. camaldulensis* contain various phytochemical components, including cineol, cuminal, phellandrene, aromadendral, valeraldehyde, geraniol,

cymene, catechol, tannins, terpenes, isoprenoids, phenolics, cardiac glycosides, sterols, saponins, and flavonoids [51].

This study represents the first report on the phytosynthesis of CNPs using *E. camaldulensis* Bark Extract and its biomedical application. The phytosynthesized CNPs underwent elemental, structural, and morphological characterization through various techniques including Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), Transmission electron microscopy (TEM), and Diffuse reflectance spectroscopy (DR). Moreover, the anticancer activity of these CNPs against MCF-7 cancer cells by the MTT method demonstrates the potential of synthesized CNPs in cancer treatment. Finally, a cellular uptake test, ROS, and catalase-mimic (CAT-mimic) assays were utilized to evaluate the pathway that is responsible for the anticancer activity of phytosynthesized CNPs. Notably, no chemical substances were utilized in the synthesis process, making it suitable for biological applications where the purity of NPs is of utmost importance.

2. Materials & methods

2.1. Materials

Deionized water (DI) was used to prepare aqueous solutions. All chemicals including Gibco RPMI-1640 and fetal bovine serum (FBS, Fisher Scientific (U.S.)), Penicillin – streptomycin ($50 \times$), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), tetroxide (OsO_4 , Sigma-Aldrich), Trypsin/ethylenediaminetetraacetic acid (EDTA) solution ($10 \times$) (Biosera, France), Triton X-100, I, Cerium (III)nitrate hexahydrate ($\text{Ce}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$, Sigma-Aldrich), 3-aminopropyltriethoxysilane (APTES, $\text{NH}_2(\text{CH}_2)_3\text{Si}(\text{OC}_2\text{H}_5)_3$, 99 %, Sigma-Aldrich), fluorescein isothiocyanate isomer I (FITC, $\text{C}_{21}\text{H}_{11}\text{NO}_5\text{S}$, 90 %, Sigma-Aldrich), ethanol (EtOH, Fluka 99.8 %), acetone (Sigma-Aldrich 99.9 %) and N,N-dimethylformamide ($\text{C}_3\text{H}_7\text{NO}$, DMF, Sigma-Aldrich), Triton X-100 (Sigma-Aldrich), Dimethylformamide (DMF, 99.9 %, Sigma-Aldrich), Dimethyl sulfoxide (DMSO, 99.9 %, Sigma-Aldrich), 4',6-Diamidino-2-phenylindole dihydrochloride, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, 98 %, Sigma-Aldrich) were used without further purification.

2.2. Preparation of *E. Camaldulensis* bark extract

E. camaldulensis bark was sourced from its native habitat in Cape Town, South Africa. The collected bark was thoroughly cleaned 3 times using tap water and three times using DI to eliminate any accumulated dust and debris. Subsequently, 100 g of the tree's bark was carefully weighed and placed in a 500 ml container filled with 200 ml DI. The mixture was then heated on a heater stirrer to reach a temperature of 80°C and stirred for one hour. Then the solution was continuously stirred for the next 24 h at room temperature. After 24 h, a brown solution of *E. camaldulensis* bark extract was obtained. To ensure the removal of macromolecules and residual substances, the cooled bark extract was meticulously filtered three times using Whatman filter paper. Finally, the clarified extract was stored in amber glass bottles, intended for the biosynthesis of CNPs.

2.3. Synthesis of CNPs

The production of CNPs was carried out through an environmentally friendly synthesis method, using an aqueous extract derived from the bark of *E. camaldulensis* as a reducing and capping agent. To start the process, 2 g of $\text{Ce}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ powder was dissolved in 50 ml of *E. camaldulensis* bark extract in a glass flask. After thorough mixing, the solution turned from light brown to a darker brown. After an overnight (24 h) aging period, the solution was carefully transferred to a Falcon tube, centrifuged, and then washed three times with D and ethanol, respectively. The obtained precipitates were then transferred to a Petri

dish and dried in the oven at 60 °C for 6 h. To finalize the synthesis, the obtained powder was annealed at 400 °C for 2 h; as a result, the final product was obtained as a yellowish-white colored powder. This powder was stored in a vial for later identification and subsequent cytotoxicity and uptake tests. A detailed illustration of extract preparation and green synthesis of CNPs can be found in Fig. 1 [56].

2.4. Characterization of CNPs

CNPs were identified using different techniques. To identify functional groups in the samples, FTIR analysis was performed in the spectral range of 600–4000 cm^{-1} . The crystal structure of the synthesized NPs was carefully analyzed by powder X-ray diffraction (XRD). XRD patterns were recorded in the 2θ range from 10 to 80 degrees using a Bruker Advanced D8 diffractometer. The device was operated following the Bragg-Brentano geometry using monochromatic Cu K α radiation with a wavelength of 1.5406 Å, at a current of 40 mA and a voltage of 40 kV. The crystallite size was estimated using the Debye-Scherrer equation, which relies on the width at half maximum. In addition, to analyze the morphological characteristics and structural arrangements of CNPs, we employed SEM characterization with EDX analysis using a Tescan MIRA3 GMU Univac SEM. This machine is equipped with a secondary electron detector and Drawbeam advanced/offline software. For a detailed exploration of the morphology of nanoparticles, particle size, and size distribution, TEM was employed. TEM observations were conducted with an FEI Tecnai 20 transmission electron microscope at 200 kV (Expander Lab6). Particle sizes were precisely measured using Image J software (version 1.53v). Additionally, the DR of phytosynthesized CNPs was utilized using diffuse reflectance UV–vis spectroscopy.

2.5. Functionalization of CNPs using FITC

To investigate the uptake and distribution of biosynthesized CNPs in MCF-7 cells, a labeling process was conducted. Initially, the CNPs were functionalized using APTES. This involved combining 40 mg of sample 2 with 500 μl of APTES and 10 ml of ethanol at room temperature and stirring the mixture for 24 h. The resulting amino-functionalized CNPs were subsequently washed with ethanol and underwent centrifugation at 7000 rpm, repeated three times. The resulting precipitate was then dried for 24 h at room temperature. Next, 4 mg of the washed and dried amino-functionalized CNPs were suspended in 1 ml of a 1 mg/ml FITC solution in DMF. The suspension was stirred at room temperature for 4 h, followed by washing using centrifugation at 7000 rpm with acetone and PBS three times each. The resulting orange precipitate was diluted in PBS to achieve a concentration of 150 $\mu\text{g}/\text{ml}$, and this solution was refrigerated for the uptake study.

2.6. Cell Culture & MTT assay

The MCF-7 cell line was obtained from the National Cell Bank of Iran,

Pasteur Institute of Iran. Cells were cultured in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal calf serum, streptomycin (100 $\mu\text{g}/\text{ml}$), penicillin (100 IU/ml), and incubated at 37 °C in a humidified 5 % CO_2 atmosphere. All cells were subconfluently grown and passaged by treating them with 0.05 % trypsin-EDTA.

For the MTT assay, around 10^4 cells were seeded into pre-filled 96-well plates and incubated at 37 °C. Once the cells reached approximately 80 % confluency, they were subjected to different treatments or left untreated (as a control) for durations of 24, 48, and 72 h. The treatments involved various concentrations of CNPs, namely 10, 20, 40, 80, and 100 $\mu\text{g}/\text{ml}$. As a reference for complete the toxicity, cell cultures treated with 4 % Triton for 1 h were included. Next, an MTT solution in PBS was added to each well, resulting in a final concentration of 500 $\mu\text{g}/\text{ml}$, and the plates were incubated at 37 °C for 4 h. After carefully removing the supernatant, the remaining formazan product was dissolved in DMSO. The absorbance of the dissolved product was measured at 492 nm using a multiwell scanning spectrophotometer (ELISA reader). This measurement was used to assess the viability of the cells.

2.7. Cellular uptake

MCF-7 cells were plated in each well of a 6-well plate at a density of 10^5 cells and incubated with growth medium at 37 °C for 24 h. Subsequently, the cells were exposed to either 150 $\mu\text{g}/\text{ml}$ of FITC-labeled CNPs or non-labeled CNPs (as a control) for 24 h. Following the treatment, the growth medium was removed, and the cells were immediately washed with PBS. To detach the cells, a trypsin/EDTA solution (2X) was used, and the resulting cell suspension was centrifuged at 4000 rpm for 5 min. The cell pellets obtained were then re-suspended in PBS, fixed with 1.5 % PFA for 5 min, and washed with DI. The cell nuclei were stained with a 1 μM concentration of DAPI for 15 min. Finally, the cells were washed with DI and prepared for imaging using a Nikon Eclipse Ti-E inverted confocal laser microscope from Japan [57–60].

2.8. ROS & CAT-mimic assay

To assess the antioxidant effects of CNPs, a ROS assay was conducted to measure intracellular ROS levels using the oxidative product of DCFH-DA (2',7'-dichlorofluorescein diacetate) and fluorescence intensity readings ($\lambda_{\text{ex}} = 485 \text{ nm}/\lambda_{\text{em}} = 535 \text{ nm}$) [48]. MCF10A cells, seeded at approximately 0.8×10^4 cells per well in 96-well plates, were incubated at 37 °C for the designated duration. The cells were treated with various concentrations (25, 50, and 100 μM) of CNP suspended in complete Dulbecco's Modified Eagle Medium (DMEM). After 4 h of incubation, the cells were exposed to H_2O_2 (1 mM final concentration) for another 4 h. Following this, the cells were washed and incubated with 20 μM DCFH-DA reagents at 37 °C in the dark for 50 min. After incubation, the growth medium was removed, and the cells were washed with PBS for 2–3 min. Finally, the fluorescence emission from the samples was measured in PBS using the Synergy H4 hybrid microplate reader.

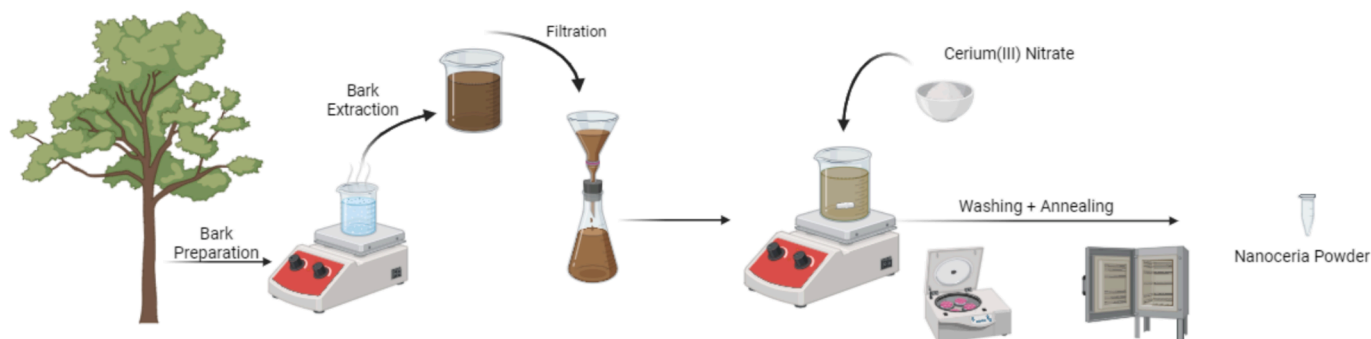


Fig. 1. Schematic diagram of phytosynthesis of CNPs.

[16,59].

The commercial CAT-mimic assay kit works by reacting CNPs with methanol (a hydrogen-donor reactant) in the presence of optimal levels of H_2O_2 . The resulting formaldehyde is then quantified. The reaction is terminated by adding KOH, and the formaldehyde concentration is measured spectrophotometrically using a chromogen within the 540–550 nm wavelength range. The CAT-mimic activity was assessed using a colorimetric assay kit, following this procedure: First, 100 μl of assay buffer, diluted with 30 μl of methanol, was added to the wells of a microplate and mixed. Next, 20 μl of the standard solution at varying concentrations was added to the wells. To begin the reaction, 20 μl of the prepared H_2O_2 solution was added, and the plates were shaken, covered, and incubated on a slow, constant shaker at temperatures below 20 °C for 20 min. Then, 30 μl of KOH was added to stop the reaction, and after mixing, 30 μl of the chromogen was added and mixed again. The plates were covered and shaken slowly for another 10 min at temperatures below 20 °C. After 10 min, 10 μl of the KIO_4 and KOH solution were added, and the final absorbance was measured using a microplate reader at a wavelength of 550 ± 20 nm [19,56,61].

3. Result and Discussion

3.1. FTIR

The FTIR spectrum of phytosynthesized CNPs using eucalyptus bark extract was obtained in the range of 600–4000 cm^{-1} (Fig. 2a). The observed broad and strong band at ~ 3250 cm^{-1} contributed to the hydroxyl (O–H) stretching of phenols and flavonoids in reported *E. camaldulensis* extract FTIR spectra. Significant bands were observed at ~ 1630 cm^{-1} and ~ 2100 cm^{-1} , which can be attributed to the presence of Carbonyl (C=O) and hydroxyl (O–H) stretch of carboxylic acids and esters derived from phytochemical compounds in the plant extract. The presence of peaks around 1630 cm^{-1} in this spectrum is consistent with other reports in which the FTIR spectrum shows the same characteristic peaks of CNPs [62,63]. The bands at ~ 1335 and ~ 1052 cm^{-1} are due to the characteristic vibrations of CeO_2 . Furthermore, the FTIR spectrum of CNPs shows distinct peaks at 649 cm^{-1} , which confirms the formation of CNPs through the Ce–O vibration [64]. In summary, these findings not only confirm the formation of CNPs but also reinforce the importance of biomolecules derived from plant extracts in the synthesis process.

3.2. XRD

The crystal structure and crystallite size of phytosynthesized CNPs were determined through XRD analysis, and the results are shown in Fig. 2b. Using Xpert HighScore Plus software, the average crystallite size was calculated based on the Debye-Scherrer equation as 11.2 nm. The peaks observed at 28.55° , 32.08° , 47.48° , 56.33° , 59.09° , 69.40° ,

76.70° , 79.07° , and 88.41° correspond to (111), (200), (220), (311), (222), (400), (311), (420) and (422) crystallographic planes, respectively. The XRD peaks corresponding to the respective planes represent the cubic fluorite structure of CNPs with a space group of $Fm\bar{3}m$ (225) as identified using JCPDS file 81–0792 and lattice parameters as $a = 5.41$ Å and $\alpha = 90^\circ$. Furthermore, there are no peaks associated with $\text{Ce}(\text{OH})_3$ or $\text{Ce}(\text{OH})\text{CO}_3$. Notably, the XRD diffraction patterns of CNPs are consistent with those reported by Ahmad et al [50]. presented similar XRD diffraction patterns for CNPs when incorporating Phoenix dactylifera plant extract as part of the synthesis process [65].

3.3. SEM & EDX

SEM coupled with EDX characterization was conducted to investigate the elemental, morphological, and structural characteristics and arrangements of CNPs. The SEM images and EDX spectra of the samples are shown in Fig. 3(a–e). According to SEM images, NPs accumulate next to each other as a mass, and this observed accumulation can be attributed to the reduction of particle size and, as a result, larger surface area and the presence of biomolecules. EDX spectrum confirmed the chemical composition and purity of the produced CNPs. According to research, the peaks of strong cerium (Ce) are between 1.0 and 6.0 eV. The presence of cerium and oxygen in the phytosynthesized CNPs is indicated by the spectrum peak. The increase in the elemental composition of Cerium is due to the oxidation state change from 3 to 4. This was consistent with the findings of Mary Isabella Sonali et al. [66] who synthesized CNPs using a bacterial strain. Another possible peak was caused by carbon (C) and phosphorus (P), with a very low percentage. The same finding was observed in the work reported by [53], in the synthesis of CNPs using *Elaeis guineensis* (oil palm) leaves, whereby the author reported the presence of C, P, and K in their NPs.

3.4. TEM and particle size

The images obtained by TEM (Fig. 4(a–e)) show that the synthesized CNPs are almost spherical, and several NPs are agglomerated to produce larger particles. Image J software (1.53v) was also used to identify the particle size by a histogram, as shown in Fig. 4(f), showing the distribution of CNPs. The obtained CNPs exhibited a very small size, which was 2–10 nm in size. Moreover, the obtained morphology of the phytosynthesized CNPs seems to be sphere-like. These observations indicate that the plant extract is rich in phytochemicals and plays an important role in the synthesis process rather than merely acting as a coating agent to control the size of the CNPs [58].

3.5. DR

Fig. 5 shows the reflectance spectra of the samples performed using UV–vis DR spectroscopy. Within the wavelength range of 350–400 nm, a

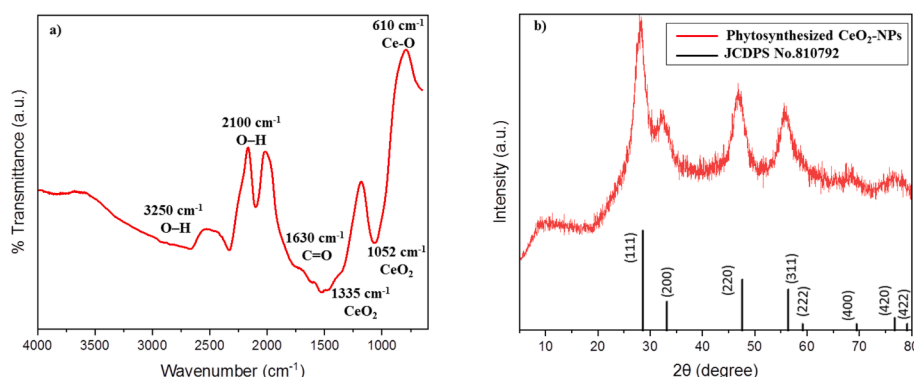


Fig. 2. A) ftir spectra of CNPs; b) XRD analysis of CNPs.

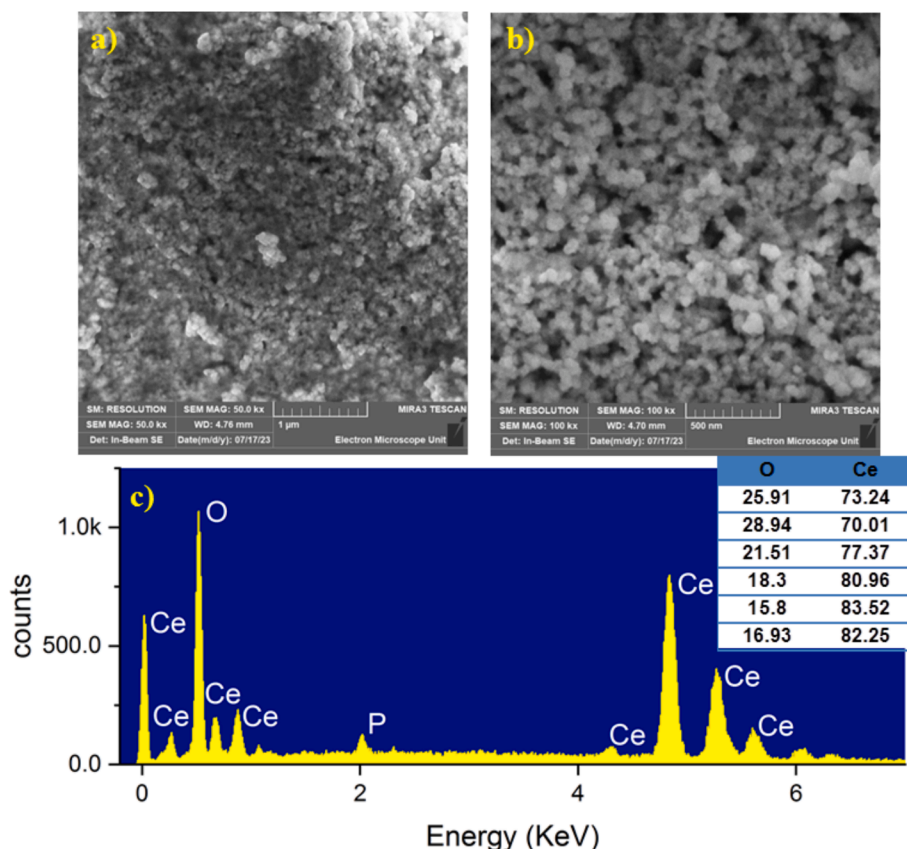


Fig. 3. (a, b) SEM images; (c) EDS table and spectra of phytosynthesized CNPs.

sharp band emerged, signifying light absorption at this specific wavelength. This notable absorption band is attributed to the charge transfer from the 2p valence band of O^{2-} to the 4f band of Ce^{4+} [59], thereby substantiating the optical and photocatalytic activity of CNPs. This band is a typical characteristic of nano-sized cerium oxide samples, confirming the successful synthesis of CNPs. Furthermore, the DR spectrum confirms that synthesized CNPs are photocatalytically active [60].

3.6. Cytotoxicity test by MTT assay

The anticancer activity of CNPs was evaluated in the MCF-7 cell line by the MTT assay. The result demonstrated that the CNPs with the lowest concentration in 24 h are the least toxic to MCF-7. The results on cell Viability and IC50 values are summarized in Fig. 6. Increasing the CNP concentration decreases the cell viability against MCF-7. Furthermore, the concentration of 400 $\mu\text{g}/\text{ml}$ showed comparable cytotoxicity in 24, 48, and 72 h (49 %, 45 %, and 69 %), indicating a significant increase in cytotoxicity over time with increasing concentration. The concentration at which 50 % (IC50) of cell death occurred was 200 $\mu\text{g}/\text{ml}$ after 72 h, 400 $\mu\text{g}/\text{ml}$ after 24 and 72 h. These findings provide evidence of substantial cytotoxicity and an inhibitory effect of phytosynthesized CNPs against the MCF-7 cancerous cells, due to their small size, large surface area, and presence of biomolecules on their surface. In a previous study, Lin et al. reported viabilities of 88 %, 78 %, and 72 % for CNPs with an average size of 10–100 nm after 24 h, 48 h, and 72 h, against the A549 lung cancer cell line [56]. In another study, CNPs were synthesized by Parvathy et al. using *Artabotrys hexapetalus* leaf extract; they also determined their cytotoxicity against the MCF-7 cell line [64]. Their results have shown the IC50 of $48 \pm 0.05 \mu\text{g}/\text{ml}$ against these cancerous cells. There is speculation that the enhanced activity could be ascribed to either the larger surface area resulting from a smaller size or the heightened enzyme-mimic activity of the surface, the presence of

ROS, or possibly all of them [30,67]. The cytotoxic efficiency of the CNPs generally depends on the presence of ROS, however, additional research, such as conducting extracellular enzyme-mimic tests, is necessary to investigate and gain a thorough understanding of the solution comprehensively.

3.7. Evaluation of CNP uptake

Confocal microscopy was utilized to investigate cellular uptake and assess whether the intracellular pathway contributes to the cytotoxicity of the synthesized CNPs. Fig. 7 presents a summary of the results, displaying confocal microscopic images of MCF-7 cells following a 24-hour incubation with 150 $\mu\text{g}/\text{ml}$ of both FITC-labeled and non-labeled CNPs. The images clearly demonstrate a substantial uptake of biosynthesized CNPs by MCF-7 cells. The confocal microscopic analysis confirms that the synthesized CNPs can penetrate the cell membrane of MCF-7 cells, suggesting that the inhibition of these cells may involve intracellular processes.

3.8. Evaluation of in-vitro ROS & CAT-mimic activity assays

Based on MTT findings, a concentration range of 25–100 $\mu\text{g}/\text{ml}$ was selected for evaluating (Fig. 8) the antioxidant effects of CNPs on MCF10A in the ROS assay [57]. The ROS assay results revealed the highest efficacy for the 100 $\mu\text{g}/\text{ml}$ concentration, indicating a direct concentration dependency. With the increase in the concentration of the CNPs, the ROS scavenging activity also increases.

The CAT-mimic activity of the CNPs was measured using colorimetric kits (Fig. 8) and calculated using Equation 1. As shown in Fig. 8, the CAT activity for CNPs followed the same trend: 100 $\mu\text{g}/\text{ml}$ > 50 $\mu\text{g}/\text{ml}$ > 25 $\mu\text{g}/\text{ml}$ (a concentration-dependent trend). It was found that the CAT-mimic activity of the 100 $\mu\text{g}/\text{ml}$ concentration of CNPs was 1.14

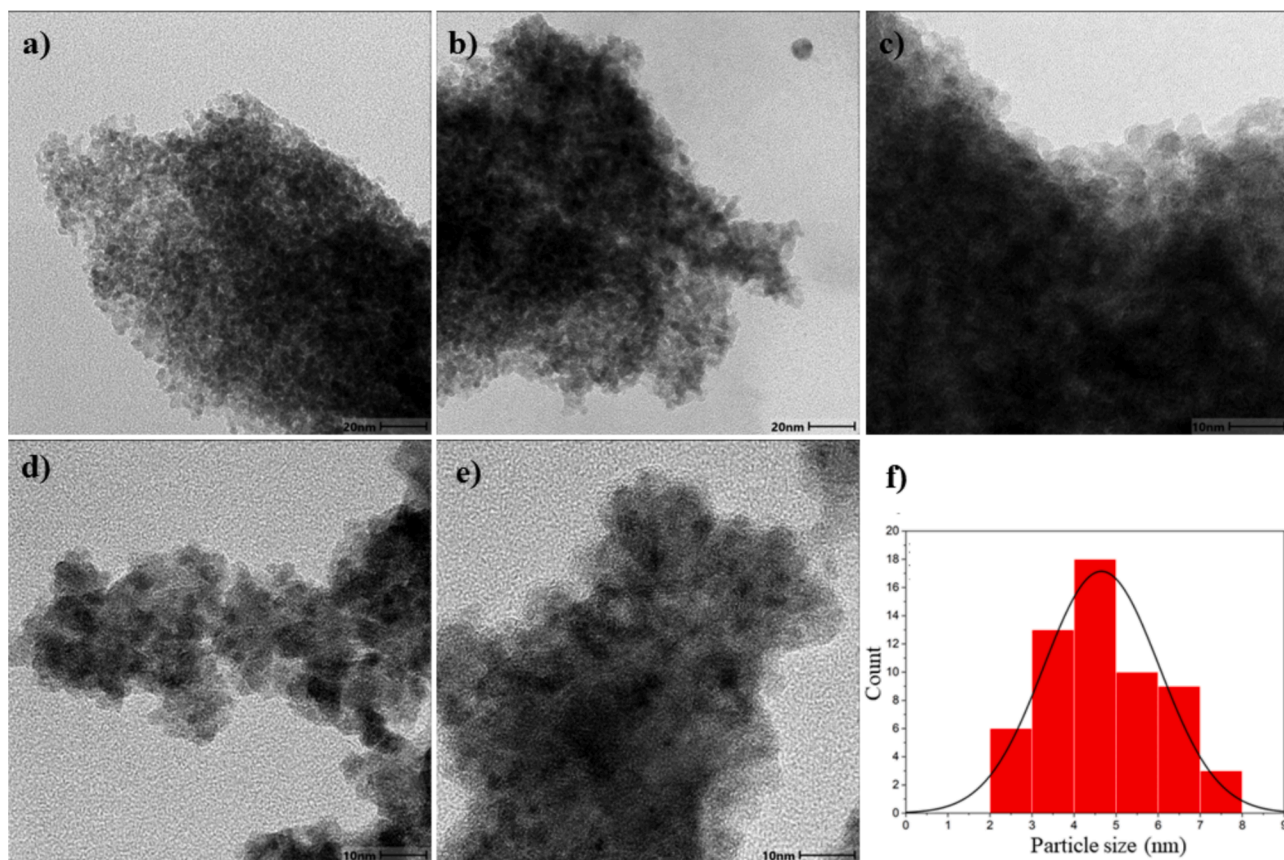


Fig. 4. (a, b) TEM images at a resolution of 20 nm, (c, d, and e) at a resolution of 10 nm of the phytosynthesized CNPs; (f) Histogram of Particle Size Distribution Curve of CNPs.

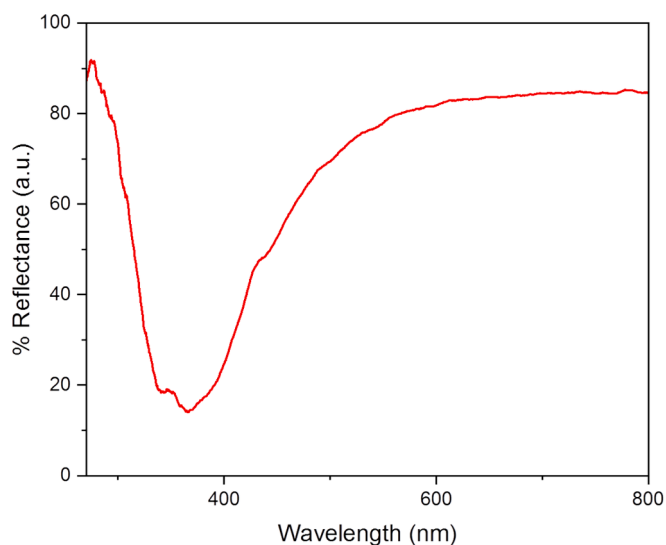


Fig. 5. DR spectra of the phytosynthesized CNPs.

times higher than 50 $\mu\text{g/ml}$, and 1.33 times higher than 25 $\mu\text{g/ml}$. Furthermore, the CAT mimic activity of phytosynthesized CNPs with a concentration of 100 $\mu\text{g/ml}$ has been higher than the control [68].

4. Discussion

4.1. Factors Contributing to the anticancer activity of biosynthesized CNPs

The anticancer effect of biosynthesized CNPs on MCF-7 cancer cell lines can largely be attributed to their small size, spherical substructure morphology, peak absorbance in the UV range, and prooxidant behavior within the cancer cell environment.

4.1.1. Morphology, Shape, and size

The morphology, shape, and size of CNPs are key factors influencing the behavior of cancer drugs, including circulation time, biodistribution, cellular uptake, and targeting efficiency. Most nanoparticles designed for anticancer applications are typically spherical. Spherical nanoparticles are less affected by shear forces and tend to interact more strongly with cell surfaces, improving cellular uptake and enhancing therapeutic effectiveness [69–71]. Previous studies have demonstrated that biosynthesized CNPs with nano-sized domains and a spherical shape exhibit significant cytotoxicity against various cancer cell lines. However, agglomeration can cause particle clustering, reducing cellular uptake and limiting the surface area available for interaction with cancer cells [72]. Therefore, it is crucial to maintain a small size and optimal morphology with minimal or no agglomeration to maximize the anticancer effectiveness of the nanoparticles. In a study by Panneerselvam et al., the effect of calcination temperature on particle agglomeration was examined at temperatures of 300 °C, 400 °C, 500 °C, and 600 °C. Lower calcination temperatures (300 °C, 400 °C, and 500 °C) led to increased agglomeration, with mean particle sizes of 17 nm, 21 nm, and 24 nm, respectively. However, CNPs calcined at 600 °C exhibited a mean particle size of 31 nm and a more uniform, spherical

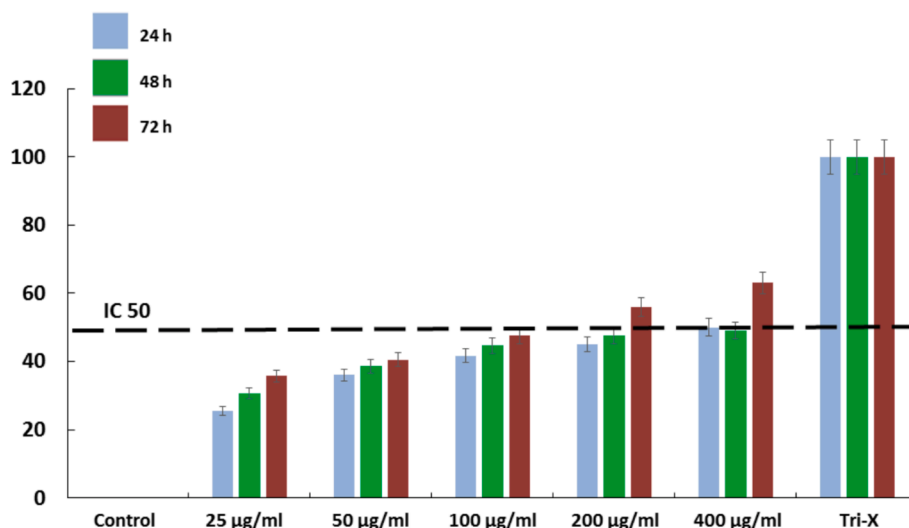


Fig. 6. The cellular toxicity effect of CNPs against MCF-7 BC cell lines was treated or not treated (Ctr) for 24 h, 48 h, and 72 h with different concentrations of 25, 50, 100, 200, 400 ($\mu\text{g/ml}$). The 1 h-treated cell cultures with 4 % Triton were the reference of 100 % toxicity (Tri-X). Data are represented as the mean $\pm$ SD. The test was done in triplicate ($p\text{-value} \leq 0.0001$).

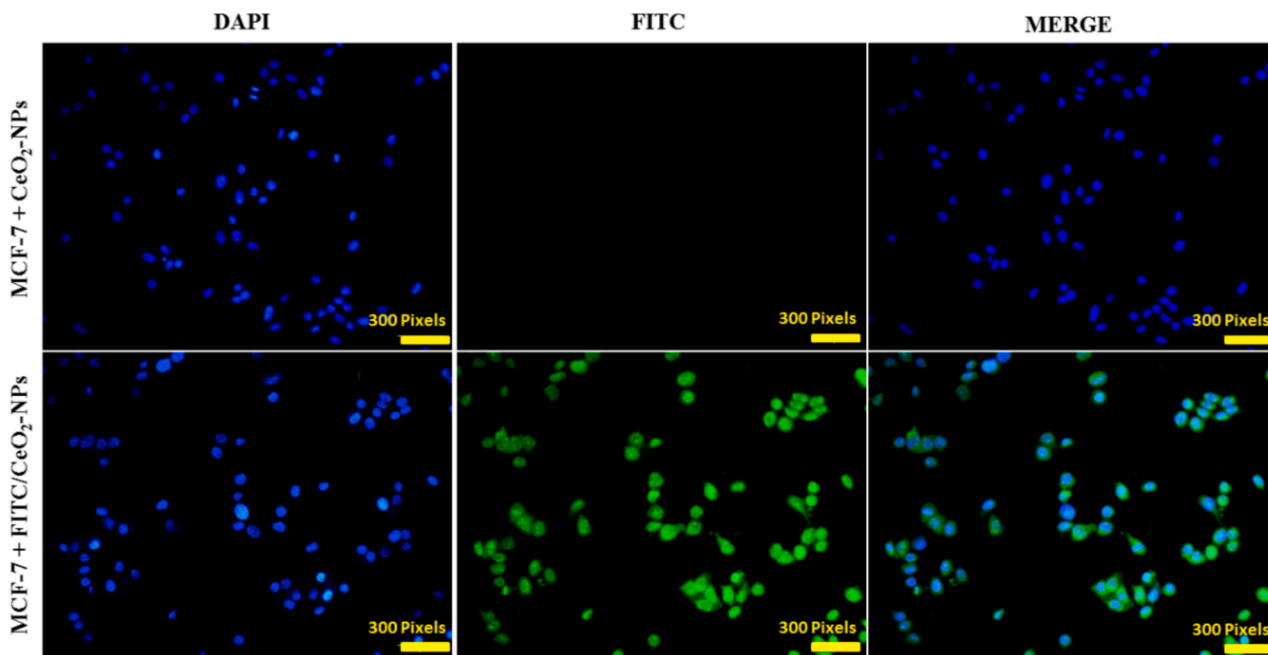


Fig. 7. Cellular uptake; confocal microscopic imaging of MCF-7 cells after incubation with 150 $\mu\text{g/ml}$ of the FITC-labeled CNPs and unlabeled CNPs for 24 h.

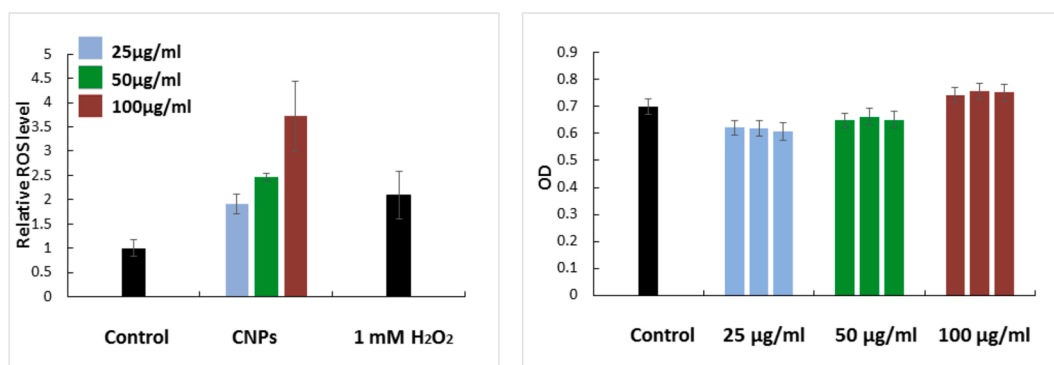


Fig. 8. Results of a) ROS assay and b) CAT-mimic activity assay.

morphology with reduced agglomeration. In this study, *E. camaldulensis* was used as a biomodulator, capping agent, and oxidizing agent to synthesize CNPs at a calcination temperature of 400 °C. The resulting CNPs exhibited spherical substructure morphology, as shown in TEM and SEM images [73–75]. To assess the effect of agglomeration on cellular uptake, an uptake study was conducted with MCF-7 cells, and the results indicated successful uptake of the CNPs, suggesting minimal or no agglomeration and an ideal morphology and size for anticancer applications.

4.1.2. Optical properties

The optical properties of CNPs provide important insights into their potential for light interactions and their role in cancer treatment. As shown in Fig. 5, biosynthesized CNPs exhibit maximum absorbance in the UV region, which enables the efficient generation of reactive oxygen species (ROS) when excited by light. Due to their high electronic excitation, CNPs can generate a higher number of free radicals, leading to an increase in intracellular ROS levels [59]. Upon photo-excitation, an electron from the valence band (VB) is promoted to the conduction band (CB) when the energy of the incident light exceeds the bandgap. This creates a hole (h^+) in the VB and an electron (e^-) in the CB, forming an exciton, a less stable, redox-active entity that initiates ROS production. In the VB, the hole oxidizes water molecules to produce hydroxyl radicals ($OH^\bullet$) and hydrogen ions (H^+), while in the CB, the excited electron reduces oxygen molecules (O_2) to generate superoxide radicals ($^{\bullet}O_2^-$), which then combine with H^+ to form $HO_2^\bullet$ radicals [64–66]. $HO_2^\bullet$ radicals can further react with h^+ ions to form hydrogen peroxide (H_2O_2), which reacts with electrons to produce additional $OH^\bullet$ radicals. This cascade of reactions results in the production of ROS in the cancer cell microenvironment. When ROS levels exceed the antioxidant defenses of the cell, oxidative damage to biomolecules such as proteins, lipids, and DNA occurs, potentially leading to cell death [73,76].

4.1.3. Prooxidant nature

The anticancer activity of biosynthesized CNPs in cancer cells is likely due to their prooxidant nature in the tumor microenvironment. Cells maintain a balanced redox state, with low levels of ROS, to ensure proper function and signaling. Normal cells possess antioxidant systems, such as SOD and CAT enzymes, to regulate ROS and maintain redox homeostasis. However, when this balance is disrupted, the antioxidant mechanisms become overwhelmed, leading to excessive ROS production and oxidative stress, which can result in irreversible damage to proteins, lipids, and DNA. In a neutral pH environment, CNPs exhibit antioxidant properties, functioning similarly to antioxidant enzymes by reducing ROS and protecting normal cells. In contrast, the acidic environment typical of cancer cells enhances the prooxidant behavior of CNPs due to their higher redox state (Ce^{3+}/Ce^{4+}) [16,17,77]. In this environment, CNPs promote oxidative stress by generating large amounts of ROS, which can trigger apoptosis in cancer cells. Therefore, the small size, spherical morphology, and beneficial optical properties of biosynthesized CNPs collectively contribute to their prooxidant activity against MCF-7 cancer cells, leading to ROS accumulation and apoptosis induction [77]. The current in vitro studies suggest that biosynthesized CNPs have significant anticancer potential against MCF-7 breast cancer cells. However, additional research, including ex vivo and in vivo studies, is required to fully understand the mechanisms of action and evaluate the clinical applicability of CNPs in cancer treatment.

4.2. Mechanism of anticancer activity of CNPs

Nanotechnology offers a promising, emerging approach to cancer treatment, particularly through the nanocatalytic generation of reactive oxygen species (ROS) via Fenton or Fenton-like reactions [73,76]. These ROS can damage key biomolecules, such as lipids, proteins, enzymes, and DNA, as well as cellular organelles like mitochondria, leading to cancer cell destruction. Recent studies have highlighted the potential of

ROS to exploit the differences between the normal physiological environment and the tumor microenvironment (TME) [78]. The TME is characterized by abnormalities such as acidosis, hypoxia, inflammation, excessive hydrogen peroxide (H_2O_2) production, and irregular blood vessel formation. While balanced ROS levels are necessary for normal biological processes like protein regulation, gene transcription, and immune defense, excessive ROS, referred to as oxidative stress, is associated with various diseases, including cancer, cardiovascular and neurological disorders, inflammation, and diabetes. The role of ceria nanoparticles (CNPs) in regulating ROS production is often linked to specific ROS species, such as superoxide ($^{\bullet}O_2^-$), and their key regulators, including superoxide dismutase (SOD) and catalase (CAT). SOD plays a critical role in protecting cells from damage caused by superoxide, and an increase in its expression signals the activation of the body's antioxidant defense mechanisms. SOD also helps prevent the oxidative inactivation of nitric oxide ($^{\bullet}NO$), a reactive nitrogen species (RNS), thus inhibiting the formation of peroxynitrite ($ONOO^-$), another harmful RNS. Interestingly, the enhanced production of SOD can be leveraged in cancer therapy to generate ROS and the highly reactive H_2O_2 , which contribute to ROS regulation and the selective destruction of cancer cells. The role of CNPs in ROS regulation can be summarized in three key concepts [74,77,78]. First, cancer cells, which already have elevated ROS levels, can be selectively targeted, as further ROS generation can push the levels beyond the cytotoxic threshold, leading to cell death. Second, normal cells can tolerate temporary increases in ROS levels without reaching the cytotoxic threshold, thus maintaining homeostasis. Finally, the $Ce^{3+} \leftrightarrow Ce^{4+}$ redox cycle, associated with ROS production, acts as an auto-regenerative antioxidant mechanism that is sensitive to the pH of the surrounding environment [16]. The acidic conditions of the cancer cell microenvironment (pH $\sim$ 6.4) favor the formation of Ce^{4+} as a prooxidant, while the slightly basic physiological environment (pH $\sim$ 7.4) favors Ce^{3+} , promoting antioxidant activity [79].

5. Conclusion

In the current study, for the first time, CNPs have been successfully synthesized by a green phytosynthesis method using *E. camaldulensis* bark extract. The initial indication of CNP formation was observed through a distinct color change in the solution during the synthesis process. The average sizes of the CNPs were determined to be 11.2 nm, and 4.7 nm using XRD and TEM. FTIR and EDX analyses confirmed the elemental composition of the CNPs and revealed the influence of plant extract biomolecules during the synthesis process. The phytosynthesized CNPs exhibited unique structural and morphological characteristics, featuring small sphere-like substructures that contributed to a high surface area, facilitated by these biomolecules. The revealed NPs exhibited unique structural and morphological characteristics with high surface area due to very small substructures. To assess the anticancer activity of the CNPs, a cytotoxicity test was conducted on the MCF-7 breast cancer cell line using the MTT assay. The results indicated that the synthesized CNPs effectively inhibited the growth of MCF-7 cancer cells, suggesting their potential as a promising anticancer agent. Confocal microscopy was employed to verify the uptake of the synthesized CNPs by MCF-7 cells and investigate the underlying cytotoxicity pathway. The results demonstrated a significant uptake of the nanoparticles by the MCF-7 cancer cells. While the eco-friendly synthesis of CNPs showed potential in inhibiting the growth of MCF-7 cells, further in vitro and in vivo research on biosynthesized CNPs, including assessing cytotoxicity against normal cells, is necessary to validate their anticancer activity.

CRedit authorship contribution statement

Fatemeh Abedi Tameh: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Hamza Elsayed Ahmed Mohamed: Visualization, Validation, Resources, Methodology, Formal analysis, Data curation. **Leila Aghababae:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Mahmood Akbari:** Writing – review & editing, Supervision, Resources, Investigation, Conceptualization. **Mohammad Hasan Javadi:** Validation, Software, Methodology. **Marique Aucamp:** Software, Data curation. **Karen Jacqueline Cloete:** Writing – review & editing, Investigation, Data curation. **Janet Solimannejad:** Supervision, Resources, Conceptualization. **Malik Maaza:** Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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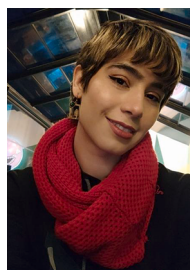
Data availability

Data will be made available on request.

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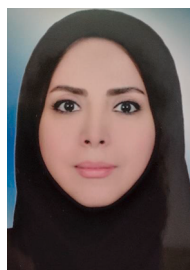
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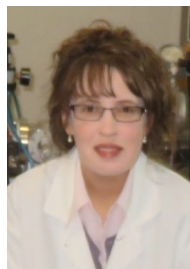
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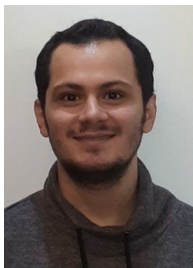
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