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ORIGINAL ARTICLE

Biofabrication of Bifunctional Cerium Oxide Nanoparticles using *Phaseolus vulgaris* with Enhanced Antioxidant and Carbonic Anhydrase Class 1 Inhibitory Activity

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ABSTRACT

A new biogenic eco-friendly and simple method of synthesizing cerium oxide nanoparticles (CeO_2NPs) using aqueous seed extract of the food plant *Phaseolus vulgaris* (common bean) is described. The NPs were analyzed by Transmission Electron Microscopy and Fourier Transform Infrared Spectroscopy. Their size was in the range of 97 to 103 nm, and they exhibited enhanced antioxidant and carbonic anhydrase class 1 inhibitory activities *in vitro*. This method is very cost-effective and simple. These bifunctional CeO_2NPs might find potential applications in various domains of biomedical research and in fabricating targeted pharmaceuticals.

Keywords: Antioxidant, Biosynthesis, Carbonic anhydrase inhibition, Cerium oxide nanoparticles, *Phaseolus vulgaris*.

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Conflict of interest: None

INTRODUCTION

Cerium, which is the first element in the lanthanide group with 4f electrons, has attracted much attention from researchers in physics, chemistry, biology, and materials science.[1,2] Although cerium oxide nanoparticle (CeO_2NP) has emerged as a fascinating and lucrative material in biological fields, such as bioanalysis, biomedicine, drug delivery, and bioscaffolding,[3-7] the synthesis of multifunctional CeO_2NP is of great interest in research for future applications. The CeO_2NPs are generally synthesized by physical and chemical methods, such as hydrothermal,[8] solvothermal,[9,10] aqueous precipitation,[11] reversed micelles,[12] thermal decomposition,[13] flame spray[14] methods, etc. However, most of the techniques are complex, time-consuming, expensive, and hazardous. Biosynthesis production of CeO_2NPs using biological system is more desirable than physical and chemical methods due to its eco-friendliness. Biological synthesis of CeO_2NPs using plant system is not well explored. In recent years, there has been increasing attention toward eco-friendly synthesis of all types of metal oxide NPs using plants. This is because green synthesized NPs generally do not produce any toxic by-products and also they have been reported to be more stable compared with chemically synthesized ones.[1,2] In the past, antioxidant activity of CeO_2NPs has been established[15] and these CeO_2NPs (nanoceria) have also been used as a potential delivery device for human carbonic anhydrase II (hCAII) inhibitors, i.e., carboxybenzenesulfonamide.[22,23]

In the present investigation, bifunctional cerium NPs are synthesized in mild environment with enhanced antioxidant activity with carbonic anhydrase inhibitory activity using *Phaseolus vulgaris* seed extract. We have studied the optical, structural, antioxidant, and carbonic anhydrase class 1 (CAH1) inhibitory activity of CeO_2NPs. This process offers a plenty of advantages, such as simple protocol, mild environment operation, cost-effectiveness, and potential for large-scale commercial production. The CeO_2NPs are widely known for their antioxidant property[15]; therefore, enhancement of antioxidant activity along with CAH1 inhibitory activity of synthesized NPs will be of great interest in multitarget advanced biomedical applications.

MATERIALS AND METHODS

Preparation of *P. vulgaris* Extract

The *P. vulgaris L* seeds were purchased from local market and authenticated (Auth-16-025) by Plant Drug Authentication Service, Botany group, Plant Sciences Division, Agarkar Research Institute, Pune, Maharashtra, India. The extract was prepared by taking 10 gm of *P. vulgaris* crushed seeds in 50 mL distilled water and

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kept for 6 hours at room temperature (28°C). The mixture was then centrifuged at 3,000 rpm for 5 minutes and then filtered with Whatman filter paper No 1. The extract was finally sterilized with syringe filter and stored at room temperature for further use.

Synthesis of CeO_2NP

Cerium (IV) oxide salt (3 mM) was made partially soluble in distilled water at room temperature (28°C, pH 2.35) using concentrated HCl (37%) 1 µL/mL of water; 10 mL of *P. vulgaris* seed extract was added with 10 mL of 3 mm CeO_2 solution (interestingly, it has been observed that cerium (IV) oxide salt was completely soluble within a few minutes). This reaction mixture was allowed to react at 37°C for 48 hours. Color change of reaction mixture was observed after 48 hours. The mixture was then centrifuged at 3,000 rpm for 5 minutes and supernatant was collected for further analysis. The confirmation of formation of the cerium NPs in the reaction was monitored by using UV–Visible (UV–Vis) spectral analysis and transmission electron microscopy (TEM) analysis.

UV–Visible Spectroscopy Analysis

The color change during CeO_2NP synthesis was recorded through visual observation. The reduction of cerium ions into CeO_2NP samples was subjected to UV–Vis spectral analysis with the help of Shimadzu UV–Vis spectrophotometer (Model 1601).

TEM Analysis

The morphology of the synthesized CeO_2NP was examined using TEM. Samples for TEM analysis was performed on a PHILIPS instrument of IIT, Bombay, Model No CM200; operating voltages: 20 to 200 kV. Before the sample preparation for TEM analysis, sonication of sample was done for 10 minutes. The sample was prepared on a small copper grid.

Fourier Transform Infra-red Spectroscopy Measurement

Moreover, Fourier transform infra-red spectroscopy (FT-IR) analysis was carried out in the range of 400 to 4,000 cm^{-1} using Bruker, Germany, Model-3000 Hyperion Microscope with Vertex 80 FT-IR System. Sample was prepared on KBr pellet and it was allowed to dry.

Superoxide Scavenging Activity using NBT Assay

Superoxide scavenging effect of CeO_2 salt solution, *P. vulgaris* seed extract, and synthesized CeO_2NP was determined by the method described by Beauchamp and Fridovich,[16] with slight modification. The assay was based on the capacity of the samples to enhance the aerobic photochemical reduction of nitrobluetetrazolium (NBT) in the presence of riboflavin. For all assays, 2 mL of the reaction mixture contained ethylenediaminetetraacetic acid (2 mm), NBT (55 µM), and phosphate buffer (50 mm, pH 7.8). Add 1 mm riboflavin 40 µL/assay. The volume of tested sample was of 60 µL/assay. Control was also taken. Absorbance was recorded at 560 nm at 0 hour and the reaction was initiated by placing it under the UV light for 30 minutes. Absorbance was once again taken at 560 nm. The scavenging % was calculated using below formula, i.e.,

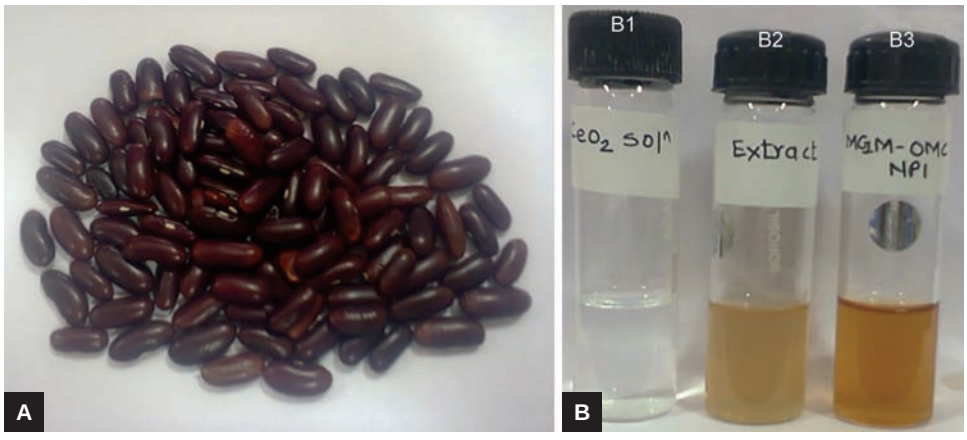
Scavenging % = (Absorbance of control – Absorbance of sample)/Absorbance of control × 100.

2,2-diphenyl-1-picrylhydrazyl Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed using the method described by Mensor et al with slight modification.[17] The DPPH, which forms a deep purple solution, reacts with antioxidant, and color loss at 517 nm is directly proportional to the antioxidant content. The sample (60 µL) was added to 1.5 mL of methanolic solution of DPPH (0.3 mm in methanol) and 1.44 mL of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the sample served as the positive control. After 30 minutes of incubation, the discoloration of the purple color was measured at 517 nm in a spectrophotometer (Shimadzu UV–Vis spectrophotometer 1601) and the radical scavenging activity was calculated.

Carbonic Anhydrase Inhibitory Activity

Carbonic anhydrase activity was performed using the method described by Armstrong et al with slight modification[18]; 3 mm p-nitrophenyl acetate was used as a substrate. Enzyme source (CA-I from human erythrocytes, Sigma) was prepared by mixing culture filtrate with Tris–HCl buffer, pH 8.0, in the reaction mixture; 10 µL enzyme source and 40 µL of CeO_2NPs along with 100 µL substrate prepared in same buffer made the final volume to 300 µL with Tris–HCl buffer and incubated at 37°C. The substrate breakdown leads to the formation of yellow color solution due to hydrolysis of p-nitrophenyl acetate to p-nitrophenol. The amount of p-nitrophenol released in the reaction was measured at 405 nm using an Elisa plate reader (Robonik Pvt Ltd). Carbonic anhydrase unit: 1 nm of free phenol released from the substrate (p-nitrophenyl acetate) per mL per minute under standard assay conditions. Percentage of carbonic anhydrase inhibition was calculated.



Figs 1A and B: (A) Seed of *P. vulgaris*; (B) B1 3 mm CeO_2 solution, B2 seed extract, B3 reaction mixture of 3 mm CeO_2 and *P. vulgaris* seed extract

RESULTS AND DISCUSSION

Synthesis of CeO_2NPs: Visual Observation

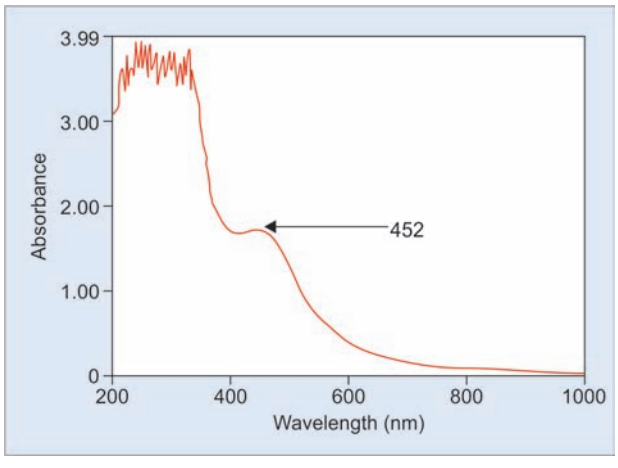
A distinct color change from light color to a dark color was observed on mixing of *P. vulgaris* aqueous seed extract with 3 mm CeO_2 solution (final pH of reaction mixture was –5.62) after 48 hours at 37°C. It indicates the formation of CeO_2NPs. It may be due to reduction of CeO_2. No precipitation was observed and color change was stable even after completion of reaction; 3 mm cerium (IV) oxide salt was made partially soluble in distilled water at room temperature using small amount of concentrated HCl (pH 2.35). Interestingly, it has been observed that cerium (IV) oxide salt was completely soluble within a few minutes, on addition of *P. vulgaris* aqueous seed extract (pH 6.56), indicating the presence of some CeO_2 solubilizing agent in the extract. Change in color on mixing of plant extract with CeO_2NPs solution is generally observed by that system, which is able to synthesize NP.

UV–Visible Spectrophotometer Analysis

The CeO_2NPs synthesized by this method were characterized initially under UV–Vis spectrophotometer. Cerium NPs generally display unique optical properties in relation to their size. The addition of seed extract to CeO_2 solution resulted in a slow color change of the reaction mixture. The color change exhibited by the samples is due to the excitation of electrons of CeO_2, which also affects the absorbance. The formed NPs exhibited dark coloration (Figs 1A and B), with peak at 452 nm (Graph 1). Quantitative analysis about the type of oxidation state and rate of reaction of cerium was studied by UV–Vis spectrophotometry.[19] Girija et al[20] also mentioned that Ce (IV) absorbs in the visible region, while Ce (III) absorbs both in the UV and the visible regions.

Transmission Electron Microscopy Analysis

Transmission electron microscopy was further used to confirm the synthesis of CeO_2NPs. The unique morphology and size distribution of the prepared NPs were elucidated from the TEMs. Generated CeO_2NPs in this process were magnified and recoded at 200 nm scales. Figure 2 exhibits morphological images of the prepared CeO_2NPs. Heterogeneous synthesis of CeO_2NPs was observed as cluster with varied size ranging from 97 to 103 nm in the reaction system. The TEM images of



Graph 1: UV–visible absorption spectra of synthesized cerium NPs showing peak at 452 nm

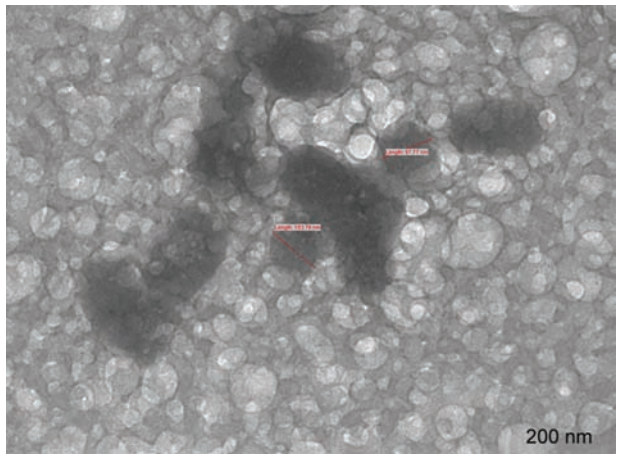


Fig. 2: The TEM micrograph of cerium NPs synthesized by seed extract of *P. vulgaris*. The CeO_2NPs shown in the micrograph are in the range of 97 to 103 nm in size (scale at 200 nm)

synthesized CeO_2NPs at different magnifications revealed that the CeO_2NPs were predominantly spherical in shape and mostly particles appeared in cluster. Although the present system is unable to generate very small size NPs, these generated NPs were able to represent distinct absorbance peak at 452 nm in the UV–Vis spectrophotometer, indicating the presence of functional NPs in reaction system with enhancement of antioxidant property and with the introduction of CAH1 inhibitory activity.

Biologically synthesized CeO_2NPs are generally spherical in shape. The morphology of the green synthesized CeO_2NPs was also studied using TEM by Priya et al[1] where they have shown that the growth of CeO_2NPs was spherical in shape.

FT-IR Analysis

The FT-IR spectroscopic studies were carried out to identify the possible group/molecules responsible for the reduction of CeO_2 to CeO_2NPs. The FT-IR spectra of the prepared CeO_2NPs are shown in Graph 2 using the KBr pellet method in the wave number range of 400 to 4,000 cm^{-1}. Representative spectra of generated CeO_2NPs manifest the various absorption peaks. The two prominent bands seen at 3,458.00 and 1,596.12 cm^{-1} were assigned to the stretching vibrations respectively (Graph 2). The band at 3,458.00 cm^{-1} represents the O–H stretching vibration. The band at 1,596.12 cm^{-1} represents the phenol ring. The short stretch peaks are also observed at 620.56, 1,725.15, and 2,924.45 cm^{-1}. The FT-IR results indicate that phenolic compound might be responsible for the formation of CeO_2NPs. Dos Santos et al[21] have also demonstrated the FT-IR spectrum of ceria also exhibits intense bands at 3,435 and 1,589 cm^{-1} that correspond to the ν (O–H) mode of (H-bonded) water molecules and (OH) respectively.

Superoxide Scavenging Activity

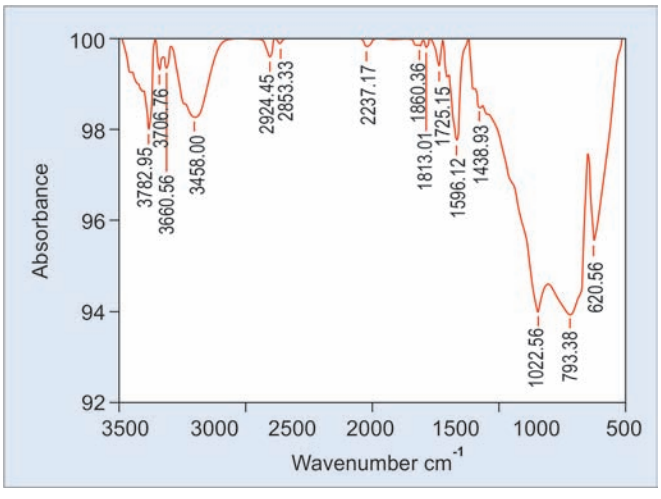
Cerium oxide is known for antioxidant activity. In our experiment, it was observed that CeO_2 salt solution at the concentration of 3 mM was able to scavenge UV-induced superoxide radicals with 18.25% ± 0.76 inhibition (Graph 3) using NBT assay. Superoxide scavenging activity of generated CeO_2NPs was also determined and compared with 3 mM CeO_2 salt solution. Interestingly, it was observed that CeO_2NPs generated by *P. vulgaris* seed extract was able to inhibit 75.19% ± 4.10 with 4.12 times enhancement of superoxide scavenging activity. This enhancement is significant in terms of overall activity. Seed extract of *P. vulgaris* is unable to scavenge UV-induced superoxide radicals with zero inhibition under the same.

2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity

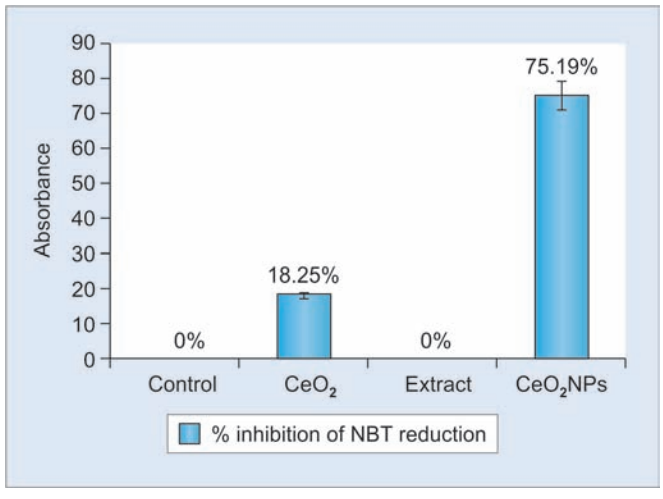
Cerium oxide salt solution, *P. vulgaris* seed extract, and synthesized CeO_2NPs by extract of *P. vulgaris* were investigated for their free radical scavenging activity using DPPH in well-defined assay system. It was observed that 3 mM CeO_2 salt solution was able to display 21.93% ± 0.21 DPPH radical scavenging activity, indicating its mild antioxidant property. Interestingly, it was observed that CeO_2NPs generated by seed extract displayed 69.56% ± 0.12, with significant enhancement of 3.17 times DPPH-based free radical scavenging antioxidant activity. It was also observed that seed extract of *P. vulgaris* was able to display only 8.56% ± 0.12 DPPH radical scavenging antioxidant activity (Graph 4).

Carbonic Anhydrase Activity

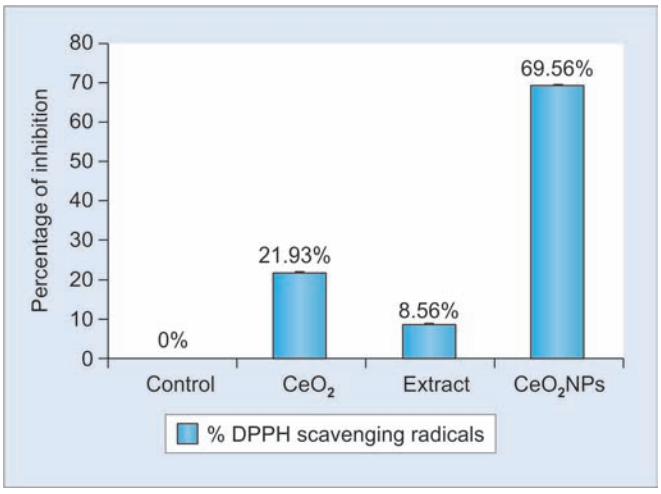
Inhibition of CAH1 of human erythrocytes by CeO_2NPs generated by *P. vulgaris* seed extract was evaluated.



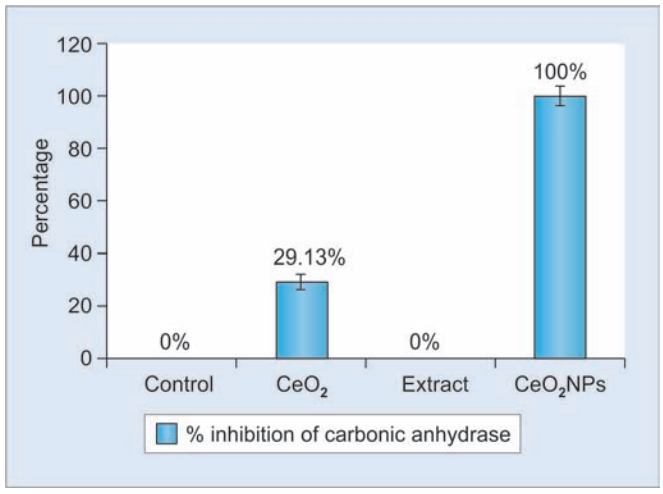
Graph 2: Fourier transform infra-red spectra of generated CeO_2NPs by *P. vulgaris* seed extract



Graph 3: Superoxide scavenging activity (% inhibition of NBT reduction) of CeO_2 solution, seed extract of *P. vulgaris*, and generated CeO_2NPs, where CeO_2: 3 mm CeO_2 solution; Extract: seed extract of *P. vulgaris*; and CeO_2NPs: Synthesized CeO_2NPs



Graph 4: Free radical scavenging activity (% DPPH scavenging activity) of CeO_2 solution, seed extract of *P. vulgaris*, and generated CeO_2NPs, where CeO_2: 3 mM CeO_2 solution; Extract: Seed extract of *P. vulgaris*; and CeO_2NPs: Synthesized CeO_2NPs



Graph 5: Carbonic anhydrase activity of CeO_2 solution, seed extract of *P. vulgaris*, and generated CeO_2NPs, where CeO_2: 3 mM CeO_2 solution; Extract: Seed extract of *P. vulgaris*; and CeO_2NPs: Synthesized CeO_2NPs

Interestingly, it was observed that seed extract of *P. vulgaris* extract was unable to inhibit carbonic anhydrase. Cerium oxide solution at the concentration of 3 mM was able to inhibit carbonic anhydrase with 29.13 ± 3.12% inhibition. Interestingly, it was observed that CeO_2NPs generated according to the invention was able to inhibit 100% ± 3.78 at the same concentration, which indicates the enhanced adaptation of carbonic anhydrase inhibitory activity in synthesized CeO_2NPs (Graph 5). Earlier CeO_2NPs (nanoceria) have also been used as a potential delivery device for hCAII inhibitors, i.e., carboxybenzenesulfonamide.[22,23]

CONCLUSION

Bifunctional CeO_2NPs were successfully synthesized using aqueous seed extract of *P. vulgaris*. The UV–Vis spectroscopy study and FTIR revealed the formation of CeO_2NPs. The TEM images clearly showed that CeO_2NPs possessed spherical-shaped morphology. Therefore, the work carried out in the current article can show the way toward the solution and it gives new prospective acceleration to fulfill the demands of newer ecological, green chemistry-based method for the synthesis of CeO_2NPs. These generated CeO_2NPs had shown enhanced antioxidant activity with CAH1 inhibitory activity. Bifunctional behavior of CeO_2NPs was found probably due to defined size of the synthesized NPs. This bifunctional activity of CeO_2NPs can be explored in many research domain and disease management where there is depletion of antioxidant activity and role of carbonic anhydrase during disease progression.

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