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Green synthesis, characterization and evaluation of biological activity of cerium oxide nano-particles synthesized from Croton sparsiflorus

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Abstract

Cerium oxide nanoparticles are potential Nano sized compounds established to have several biomedical applications. *Croton sparsiflorus* is a wild shrub considered to have various medicinal applications. In current study green synthesis of cerium oxide nanoparticles is carried out from *Croton sparsiflorus* extract. CeO_2-NPs are characterized by UV-visible spectroscopy, SEM, EDX and particle size analysis. UV-visible spectroscopy analysis reported presence of cerium oxide nanoparticles, which was reported by change in colour to brown and with a λmax at 330nm. SEM analysis showed presence of round and spherical nanoparticles of 20nm. Particle size analysis carried out using Dynamic light scattering reported an average diameter of 126.2nm of nanoparticles. EDX analysis confirmed the elemental composition of nanoparticles with presence of oxygen and cerium. Cerium oxide nanoparticles were also investigated for antioxidant activity and antimicrobial activity. Antioxidant activity was carried using DPPH assay, FRAP assay, H_2O_2 and Phosphomolybdenum assay. FRAP assay showed highest antioxidant activity with IC50 value 533.78µg/ml. CeO-NPs showed antimicrobial activity against gram positive bacteria *Bacillus cereus* with zone of inhibition of 9mm and 10mm. Therefore cerium oxide nanoparticles synthesized from *Croton sparsiflorus* are thus potential compounds that can be used in various biomedical applications.

Key words: Cerium oxide, nanoparticles, *Croton sparsiflorus*, antibacterial and antioxidant.

I. Introduction:

Nanotechnology has gained large amount of attention in recent years because of wide range of applications as anticancer agents (1), antibacterial (2), imaging (3) etc. The technology makes use of nano size of the compound which ranges from 1-100nm and processes unique physiochemical properties (4). Among many nanoparticles cerium oxide nanoparticles have explored widely because of unique surface chemistry, biocompatibility and high stability (5). Nanoparticles are synthesized by various Physico-chemical methods; however such methods require high temperature, pressure and toxic solvents which pose several disadvantages and threats to environment (6). Therefore green synthesis of nanoparticles has gain attention due to their safe and efficient source. Green synthesis of cerium oxide nanoparticles have been reported using plant extracts (7), microbes (8) and other biological derivatives (9). In current study cerium oxide nanoparticles are synthesized from *Croton sparsiflorus* extract. *Croton* genus belongs to Euphorbiaceae which consist of 1300 species and mainly grows and shrubs, trees and herbs in tropical and subtropical regions (10). The plant mainly grows in sandy clay soil in various parts of Asia and South America (11). It is one of the exotic weeds mostly available in wastelands of Southern part of India also. It possesses antibacterial and antifungal properties (12). As this herbal plant exhibits different pharmacological applications, it has been selected for the synthesis of cerium oxide nanoparticles in current study. Perilously green synthesis of sliver nanoparticles are synthesized from *Croton sparsiflorus* leaf extract, these nanoparticles showed cubic crystal shape and particle size of 16nm, which was analyzed using SEM and EDX, the nanoparticles also showed antibacterial and antifungal properties (13). Similarly gold nanoparticles synthesized from *Croton sparsiflorus* extract reported UV- protection property and antibacterial ability of gold nanoparticles. They also reported *in-vitro* anticancer property against HepG2 cell line which was studied via MTT assay (14). Current works reports green synthesis of cerium oxide nanoparticles from *Croton sparsiflorus* extract, characterization of nanoparticles using UV-vis spectrophotometer, EDX, particle size analysis and SEM. Nanoparticles will also be studied for biological activity including antioxidant and antimicrobial property.

II. Materials and methods:

2.1.Materials

Cerium nitrate (Ce (NO3)2.6H2O; 432.2 g/mole; 99.9% purity) and Sodium hydroxide (NaOH; 40 g/mole; 99.9% purity) were purchased from Sigma Aldrich Chemicals. These chemicals were used without further purification.

2.2.Plant Material

Croton sparsiflorus plant was collected from Karnataka University campus, Dharwad, India. Plant material was washed with water and air dried, then homogenized to fine powder. The powder was stored at -20˚c for further use.

2.3.Crude Extraction of Phytochemicals

100g of plant leaf material was extracted with distilled water using soxhlet apparatus for 4 to 6hrs at 40˚-50˚c. The solvent was evaporated with Rota evaporator and the crude yield was weighed. The crude extract thus obtained was used as reducing agent in the synthesis of nanoparticles.

2.4.Synthesis of cerium oxide nano-particles

Aqueous leaf extract of *Croton sparsiflorus* was used for synthesis of cerium oxide nanoparticles by green synthesis method. For preparation of CeO_2 NPs, aqueous extract of *Croton sparsiflorus* was added to of Ce $(NO_3)_3$ solution (0.001M in 1:10) dilution at room temperature and incubated for 10 minutes for reduction of cerium ions. In the next step, the CeO–O. after 6hr, finally, the purified green-synthesized CeO_2-NPs were obtained.

2.5.Characterization Techniques

2.5.1. UV-visible spectrophotometer:

Green synthesized CeO_2-NPs were visually detected done by observing color change in comparison with control samples. UV-visible spectrophotometric analysis of CeO_2-NPs solution was determined at room temperature using UV-visible spectrophotometer with a resolution of 0.5nm. Absorbances of samples were read at start weave length of 1100nm and end wave length of 200nm and double distilled water was used as blank (15).

2.5.2. Scanning electron microscopy:

CeO_2-NPs were studied to investigate their molecular structure using scanning electron microscope (SEM). SEM is a multipurpose instrument which is able to provide qualitative information of the material including its morphology, topography, composition, and crystallographic information (16). To prepare the sample for SEM, a double sided carbon adhesive tape was cut for the required shape and the side with exposed adhesive was adhered to the specimen holder and the other side has a thin layer of liner on the top. A small amount of CeO_2-NPs were dissolved with ethanol and sonicated for uniform dispersion of nanoparticles in ethanol. Immediately after sonication, the top layer of liner in the tape was removed and 10 µL of the sonicated solution was pipetted on the adhesive part of the specimen holder. The sample was dried and was loaded in the equipment to start the SEM analysis using SEM analyzer S-3400.

2.5.3. Particle size analysis:

HORIBA –scientific SZ-100 particle analyzer was used to investigate particle size distribution and surface charges of CeO_2-NPs. The analysis was done at 25˚c with 90˚ detection angle and 633nm. Sterile water was used as dispersion medium for CeO_2-NPs. Hydrodynamic diameter and polydispersity index were measured. The surface area and particle size of the CeO2nanoparticles have exceptional impact oversensitivity, conductivity and catalytic activity (17).

2.5.4. Energy Dispersive X-Ray Analysis (EDX):

Element composition of CeO_2-NPs was investigated using EDX. Dried powder of CeO_2-NPs was used for analysis. All measurements were performed at a voltage of 10KV. The results were analyzed using EDX software (18).

2.6.Evaluation of Biological activity

2.6.1. Antioxidant activity:

2.6.1.1. DPPH Assay: In eppendorf tube, 1.0 mL DPPH working solution (0.2 mM) was mixed with 0.5 ml of different concentrations (50, 100, 150, 200 and 250ul) directly from the given stock of test samples and the standard solution and incubated for 30 minutes in dark at room temperature. The absorbance was measured at 517 nm (Labman UV Visible Spectrophotometer).The percent antioxidant or radical scavenging activity was calculated using the following formula: %Antioxidant activity = [(Ac − As)/Ac] × 100 where, Ac and As are the absorbance of control and sample, respectively. Ascorbic acid was used as standard (19, 20).

2.6.1.2. FRAP Assay: The ferric reducing capacity of extracts was investigated by using the potassium ferricyanide method. Briefly, 0.2mL of nanoparticles at different concentrations, 2.5mL of phosphate buffer (0.2 M, pH 6.6), and 2mL of potassium ferricyanide K_3 Fe $(CN)_6$ (1%) were mixed and incubated at 50˚C for 300min, to reduce ferricyanide into ferricyanide. The reaction was stopped by adding 2.5mL of 10% (w/v) trichloroacetic acid. Finally 0.5mL of $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. The sample concentration absorbance (IC50) was calculated by plotting absorbance against the corresponding sample concentration (21).

2.6.1.3. H_2O_2 scavenging assay:

A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). Nanoparticles (100,200,300,400,500µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40mM) and final volume was 1.1 ml with distilled water. Absorbance of hydrogen peroxide at 230 nm (Labman UV Visible Spectrophotometer) was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of nanoparticles and standard compounds were calculated: % Scavenged $[H_2O_2]$ = $[(A_C - A_S)/A_C]$ x 100. Where A_C is the absorbance of the control and A_S is the absorbance in the presence of the sample of extracts or standards. Ascorbic acid was used as standard (21).

2.6.1.4. Phosphomolybdenum Assay:

The total antioxidant capacity of the nanoparticles was evaluated by the Phosphomolybdenum reduction assay method according to the procedure described by Prieto et al. 1999 (22). 0.5 mL of various concentrations (20, 40, 60, 80 and 100 µg/mL) of extract was combined with 1.5 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer.

2.6.2. Antimicrobial Activity

2.6.2.1.Antibacterial activity

Agar well diffusion method: Petri plates containing 25ml NA agar were seeded using glass rod with 24hr (old) culture of different bacterial strains separately. Spread plate method was followed. Wells were made using well-borer. Stock concentration of 1mg in 1ml and standard drug (cifrofloxacin) 30µl. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was confirmed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993) (23, 24).

2.6.2.2.Antifungal activity

Petri plates containing 25ml Optimized media were seeded using glass rod with 24hr (old) culture of *Candida albicans* strains separately. Spread plate method was followed. Wells were made using well-borer. Stock concentration of 1mg in 1ml and standard drug (Itracanozole) 30 ul. The plates were then incubated at 37°C for 24 hours. The antifungal activity was confirmed by measuring the diameter of the inhibition zone formed around the well.

2.7.Statistical analysis

All experiments were performed three independent replicates and the data are presented as average with standard deviation. The percent inhibition was calculated with respect to control. The statistical significance was calculated by t test with respect to control. * indicates p-value <.05 with respect to control. # indicates p-value <.01 with respect to control. A p-value ≤.05 was considered as statistically significant.

III. Results:

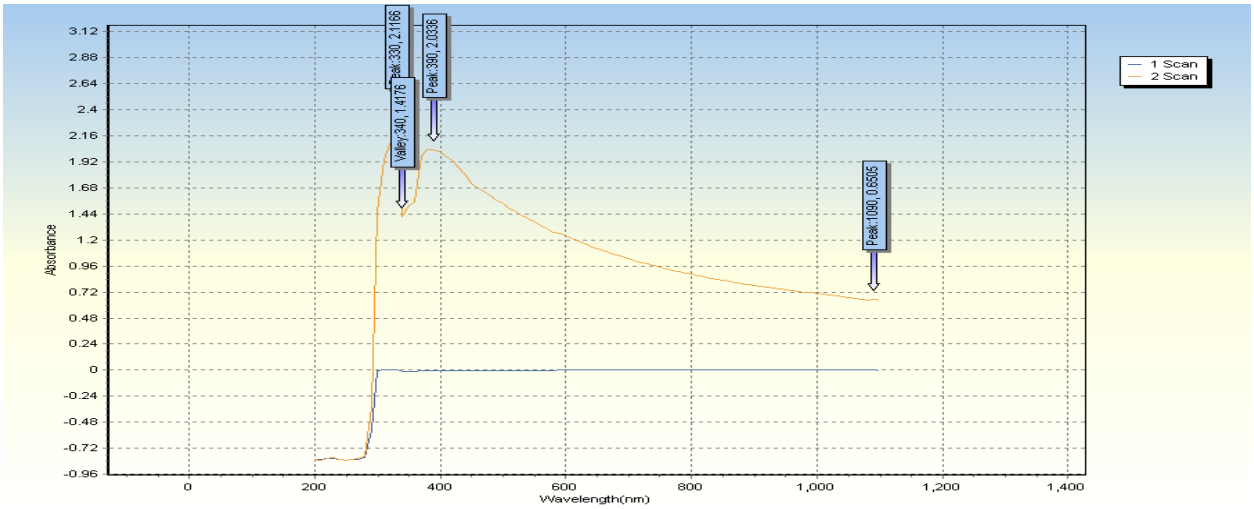
Cerium oxide nano-particles were successfully synthesized by green synthesis method and change in color from green to brown confirmed formation of CeO_2NPs.

Characterization

UV-visible spectrophotometer

The preliminary characterization of CeO_2-NPs was carried out by recording the UV–vis absorption spectrum (**Figure.1**). CeO_2-NPs exhibited an absorption maximum (λmax) at 330 nm indicating that synthesized CeO_2-NPs have a better optical property. The recorded λmax at 330 nm represents the fluorite cubic structure of CeO_2-NPs due to the quantum size effect of the blue shift in UV–Vis spectrum. Since optical properties of CeO_2-NPs can be detected between 300-600nm, current study confirms synthesis of CeO_2-NPs at 330nm (25).

Figure1. UV-Visible spectra of CeO_2-NPs



Scanning Electron Microscopy analysis

The cerium oxide nanoparticles were analyzed through Scanning Electron Microscope and as a result, the image with cluster of uniformly distributed particles was obtained as shown in the **Figure.2**. On measuring the nanoparticles using the image J software, the average size of the particles obtained was in the range of 40 nm to 50 nm thereby confirming the existence of nanoparticles.

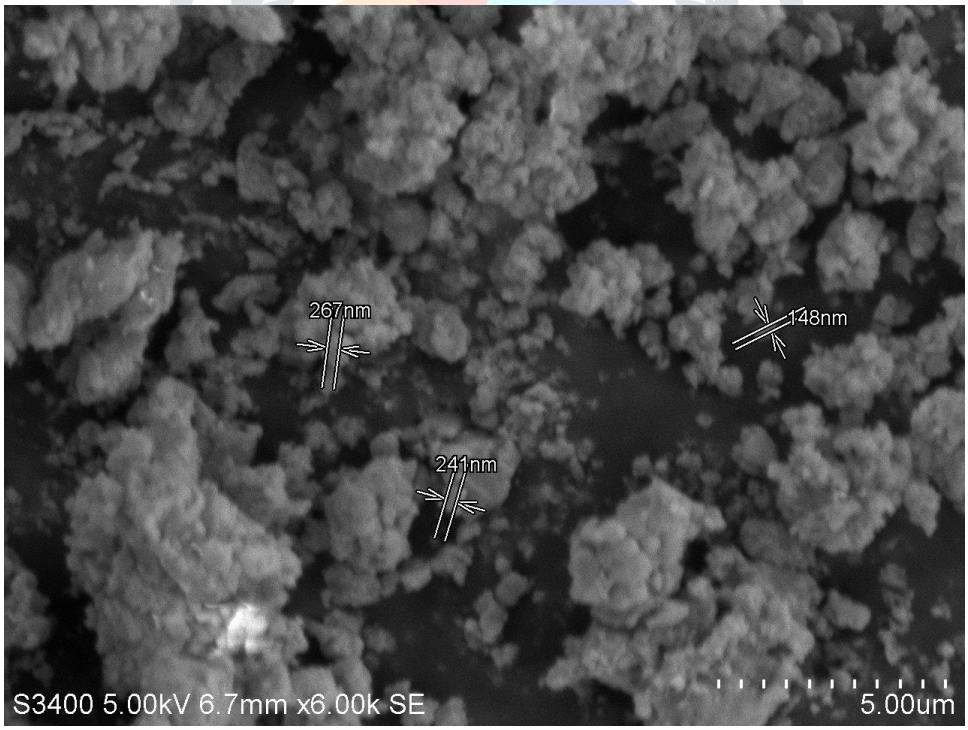


Figure.2. SEM image of CeO_2 nanoparticles

SEM analysis was used for the morphological study of nanoparticles of CeO_2. These analyses show that high homogeneity emerged in the samples surface by increasing annealing temperature. The results show that the morphology of the particles changes to the spherical shape with less agglomeration by increasing temperature.

Figure 2 shows the SEM image CeO_2 nanoparticles prepared by green synthesis method. In this figure, the particles prepared with formation of clusters.

Particle size analysis:

DLS (Dynamic light scattering) is used to determine particle size distribution of CeO_2 nanoparticles dispersed in the liquid. It determines size of the nanoparticles and their distribution in physiological solution[6]. The size distribution histogram of DLS analysis of CeO_2 nanoparticles shows particle diameter average of 126.2nm and PI index of 2.925 (**Figure.3**).

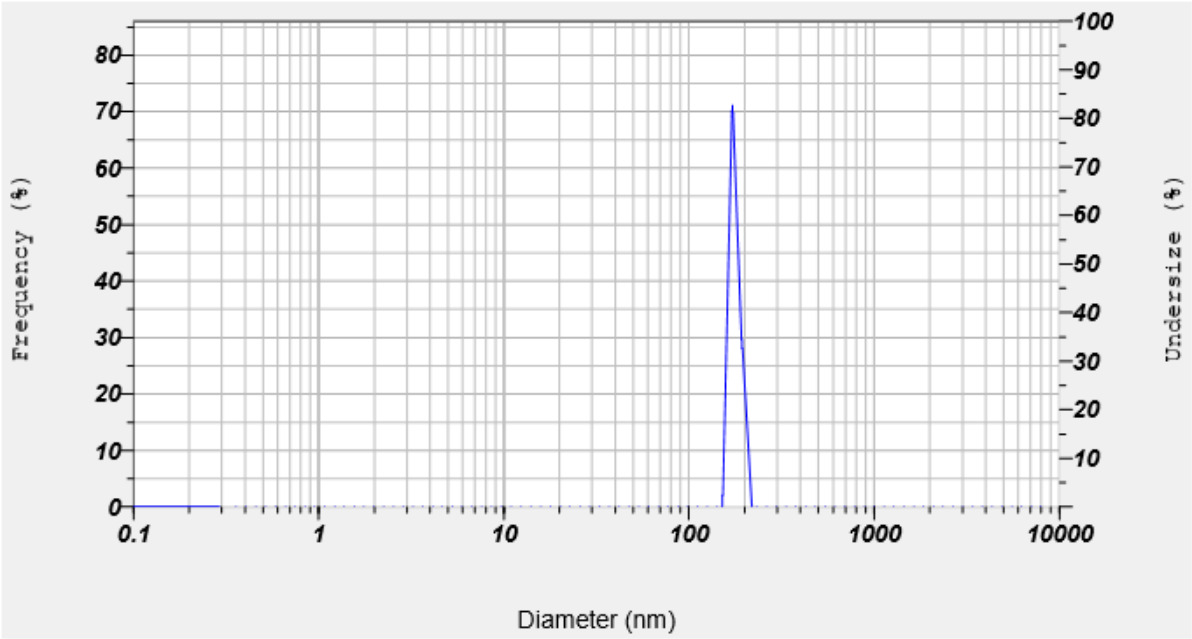


Figure.3. Histogram of particle size analysis of CeO_2 nanoparticles

EDX Analysis:

CeO_2 nanoparticles were subjected to Energy Dispersive X-ray Spectroscopy where the elemental composition of the cerium oxide nanoparticles along with their weight concentration was determined as shown in the **Figure 4**. It is evident from the EDX spectrum that cerium and oxygen are present making sure that the obtained cerium oxide nanoparticles are almost pure which is assured by the weight concentration of cerium and oxygen which accounts to a total of 100% (**Table1**).

Table.1. Element weight calculation

Element Line	*Weight %*	*Weight % Error*	*Atom %*
C K	22.28	± 0.89	41.96
O K	34.25	± 0.79	48.43
Cl K	5.45	± 0.38	3.48
Cl L	---	---	---
Ce L	38.02	± 1.88	6.14
Ce M	---	---	---
Total	100.00		100.00

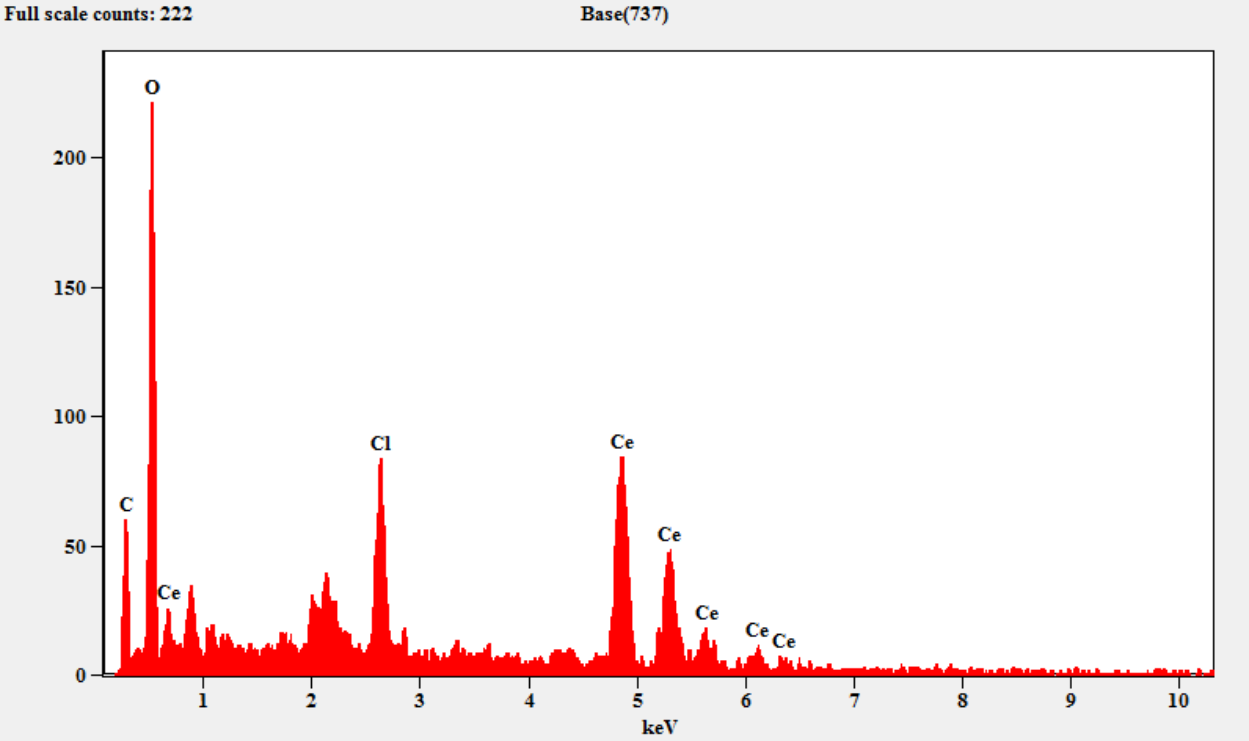


Figure.4. EDX analysis of CeO_2 nanoparticles

Evaluation of Biological activity

Antioxidant activity:

Antioxidant activity reported by each tests was performed with a standard ascorbic acid and is represented as IC50 values which represent inhibition constant of the CeO_2-NPs towards the test reaction. CeO_2-NPs subjected to DPPH assay reported IC50 value of >250µg/ml (**Figure.5.A**), while standard ascorbic acid reported IC50 value of 55.92µg/ml. FRAP assay reported IC50 value of 365.09µg/ml for standard ascorbic acid and CeO_2-NPs with 533.78µg/ml (**Figure.5.B**), H_2O_2 scavenging activity of CeO_2-NPs reported IC50 value of 367.14µg/ml, while ascorbic acid reported 355.99µg/ml (**Figure.5.C**), and finally Phosphomolybdenum assay reported 30.56µg/ml IC50 value for ascorbic acid and CeO_2-NPs with 153.63µg/ml (**Figure.5.D**). Highest IC50 value was reported by FRAP assay and also proving that CeO_2-NPs synthesized from *Croton sparsiflorus* to be having potential antioxidants. All the antioxidant assays showed increase in activity with increase in concentration of CeO_2-NPs.

Figure5.A DPPH Assay of CeO_2-NPs

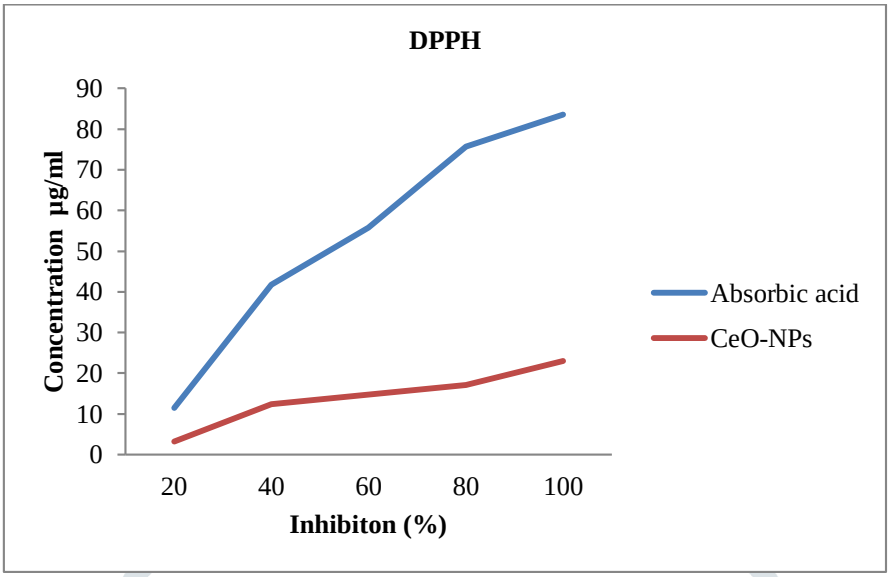


Figure.5.B FRAP Assay of CeO_2-NPs

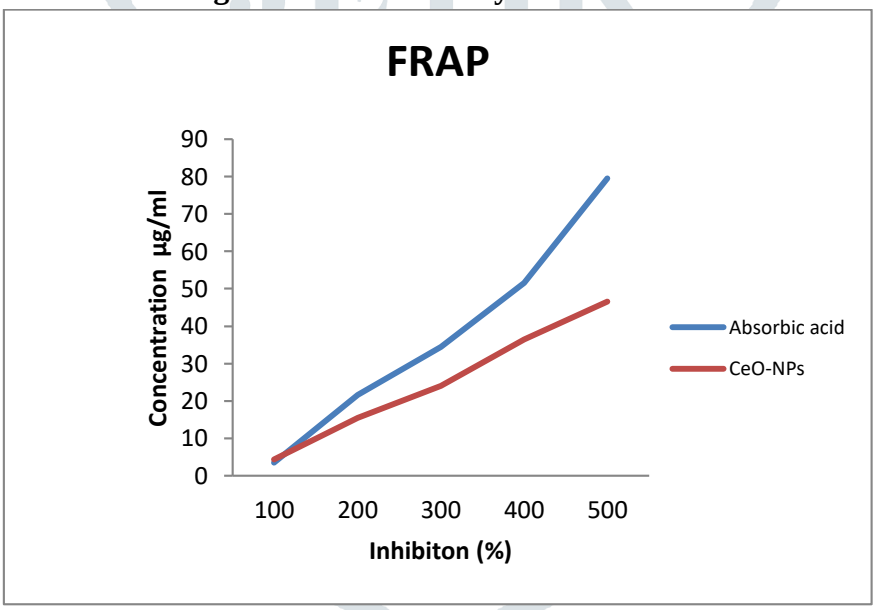


Figure.5.C. H_2O_2Assay of CeO_2-NPs

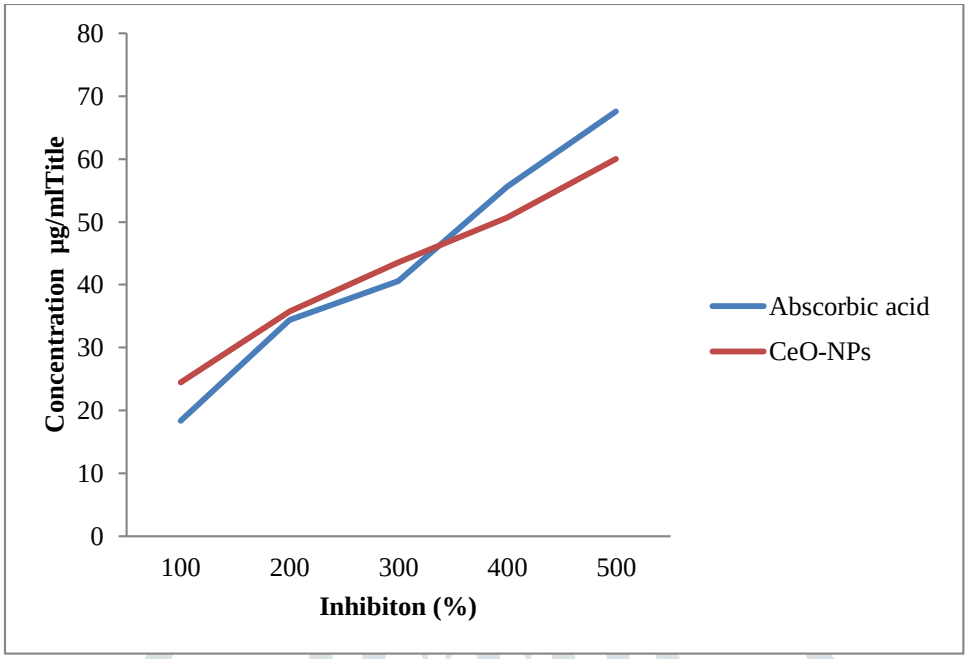
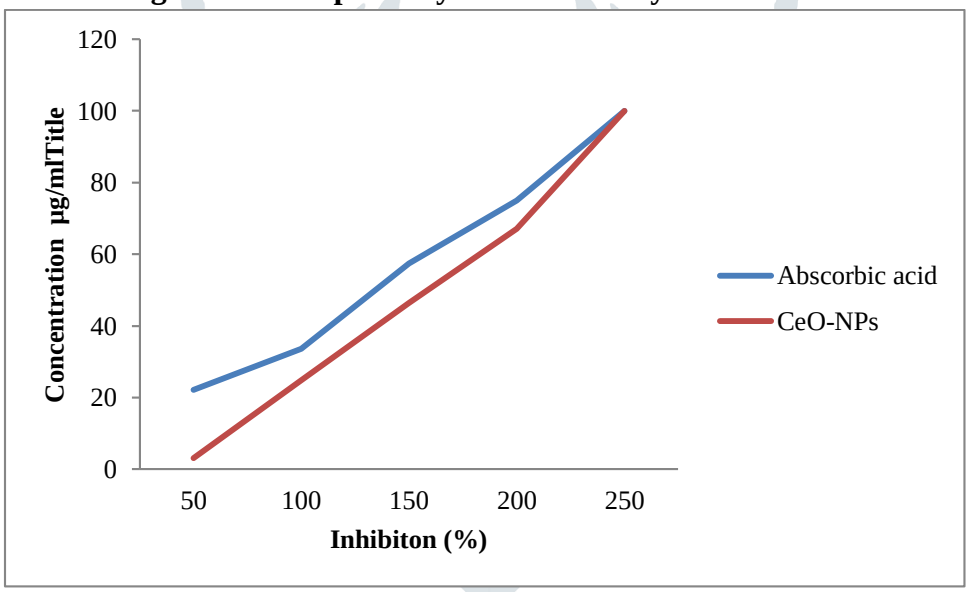


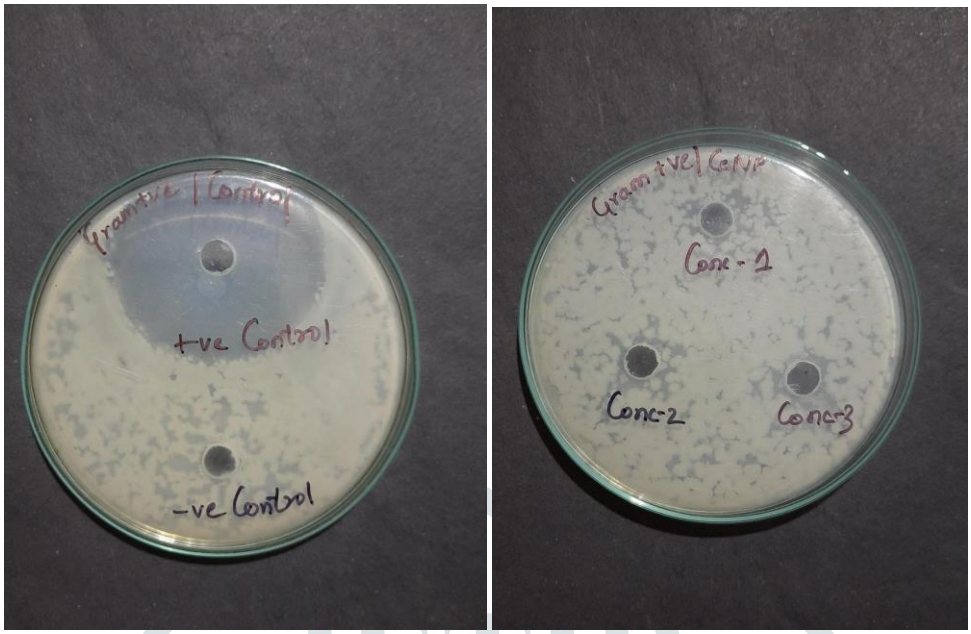
Figure5.D.Phosphomolybedenum Assay of CeO_2-NPs



Antimicrobial activity: Antibacterial activity of CeO_2-NPs was examined against two bacterial strains *Escherichia coli (*Gram-negative) and *Bacillus cereus (*Grams-positive), standard drug Ciprofloxacin was used to compare the activity. CeO-NPs of croton *sparsiflorus* showed zone of inhibition of 9mm and 10mm against *Bacillus cereus* and standard drug showed 28mm of zone of inhibition (**Figure.6**). Therefore CeO_2-NPs thus exhibit antibacterial activity.

Antifungal activity was examined against *Candida albicans* and standard drug Itraconazole was used to compare the activity. CeO_2-NPs did not report zone of inhibition against the fungal strain thus not exhibiting antifungal activity.

Figure.6. Antibacterial activity of CeO-NPs



DISCUSSION

Cerium oxide nanoparticles have gained much interest in recent years due to their application in biological systems as antioxidants, fuel cells and catalysts (26). In current study CeO_2-NPs were green synthesized from *Croton sparsiflorus* extract. Preliminary characterization of CeO_2-NPs was carried out using UV-visible spectrophotometer and its absorption was recorded at λmax 330nm (**Figure 1**). The obtained result is consistent with previous findings where cerium oxide nanoparticles synthesized using *Aspergillus niger* showed UV–visible absorbance maximum at this wavelength (27). Scanning electron microscopy determines effect of reaction time on size and shape of nanostructures, therefore the effect of these parameters is studied in the synthesis of CeO_2-NPs (28). **Figure 2** shows SEM analysis of CeO_2-NPs synthesized in the current study. The results showed when the reaction time was increased, the CeO2 nanoparticles grew rapidly and the aggregation particles were obtained. Particle size distribution is another important parameter studied for nanoparticles, it determines diameter of nanoparticles dispersed in physiological solution (29). In current study **Figure 3** shows particle size analysis of CeO2 nanoparticles, these findings are in coordination with cerium oxide synthesized previously by another study from *Origanum majoran* leaf extract (17). EDX or energy dispersion X-ray spectroscopy analysis of nanoparticles will explore their element composition. EDX is a powerful tool combined with spectroscopy used to analyze or confirm presence of desired elements in nanoparticles (18). In current study EDX analysis of cerium oxide nanoparticles synthesized from *Croton sparsiflorus* showed presence of oxygen and cerium (**Figure 4**).

Cerium oxide nanoparticles are widely studied for various biomedical applications because of their unique pharmaceutical abilities. Antioxidant activity is ability of the compounds to scavenge free radicals. Several studies have reported antioxidant activity of green synthesized CeO_2-NPs (20, 21). In current study highest antioxidant activity of CeO_2-NPs was reported by FRAP assay with IC50 value of 533.78µg/ml (Figure 5B). Previous

investigations have also reported antioxidant activity of CeO_2-NPs synthesized from gel extract of *Aloe Barbadensis* (23). Similarly CeO_2-NPs synthesized from *Origanum majorana* and *Ceratonia siliqua have* also showed high antioxidant activity (24). In another study performed (24) the CeO_2-NPs illustrated the scavenging activity of nitric oxide free radicals revealing the probable anti-inflammatory of this NP. Antimicrobial activity is an important therapeutic attribute that makes the compounds potential in pharmaceuticals. Several studies have reported cerium oxide nanoparticles to have potential to be used as antimicrobial agents, due to their low toxicity, absorption of UV light and heat resistance (27). Many studies have reported antibacterial activity of CeO_2-NPs and exhibited significant inhibition of both gram positive and gram negative bacteria (28). In current study green synthesized cerium oxide nanoparticles from *Croton sparsiflorus* showed zone of inhibition of 9mm and 10mm against *Bacillus cereus* (figure 6). Studies investigated previously on CeO_2-NPs synthesized via wet-chemical synthesis route have also showed antibacterial activity against *Bacillus cereus* (29). Another study has also reported inhibition of the same bacteria by ultrasound mediated CeO_2-NPs (29).

IV. Conclusion:

Green synthesis of nanoparticles has gained much attention in recent years due to increase in biomedical applications including antioxidant, antimicrobial, and anticancer. Therefore in current study cerium oxide nanoparticles were synthesized from *Croton sparsiflorus* extract and were studied for potential pharmaceutical properties. Synthesized nanoparticles appeared to processes potential antioxidant activity. Antimicrobial activity was investigated against gram positive and gram negative bacteria and CeO_2-NPs showed significant inhibition against *Bacillus cereus* gram positive bacteria. Therefore cerium oxide nanoparticles synthesized from *Croton sparsiflorus* extract are thus potential pharmaceutical agents.

Conflict of interest:

We wish to confirm that there are no known conflicts of interest associated with this publication.

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