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BIOSYNTHESIS OF NANOCERIA FROM *BACILLUS SUBTILIS*: CHARACTERIZATION AND ANTIOXIDANT POTENTIAL

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ABSTRACT: Antioxidants are inhibitors may prevent or delay some types of cell death from progressive oxidative stress causing Reactive Oxygen Species (ROS). The main aim of this study was to determine the antioxidant property of Cerium Oxide Nanoparticles. Best of our knowledge, for the first time CeO_2 Nanoparticles synthesized by means of bacteria, assisted with the extracellular supernatant of *Bacillus subtilis*. The formation of Nanoparticles was monitored by UV-Vis Spectrophotometer in the range of 200-400nm at different time intervals. The FTIR results confirmed the Ce-O-Ce stretching band of CeO_2 nanoparticles. Crystalline nature and particle average size of Cerium oxide NPs were about 8.022 nm determined by XRD. The morphology and elemental composition were studied by SEM and EDAX which confirmed the presence of cerium oxide Nanoparticles. The antioxidant property of CeO_2 Nps studied out in the method of DPPH Assay, Total Antioxidant assay, and reducing power assay. The results showed the different concentration of bio-nanoceria has probable antioxidant property. Since this work clearly proved that the nanoceria from bacteria could be used for effective antioxidant for the disorders associated with oxidative stress like Parkinson's disease, Atherosclerosis, Cancer, Diabetics, Rheumatoid arthritis, Myocardial infraction, Cardiovascular diseases, Chronic inflammation, Stroke.

KEYWORDS: Cerium Oxide, Antioxidant, DPPH, Oxidative Stress, Reducing power assay.

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1.INTRODUCTION

Nanotechnology involves measuring, imaging, and modeling and manipulating matter at dimension between 1 and 100 nanometers, unique phenomena enable novel applications. The

synthesis of nanoscale materials vital in nanotechnology because they changing the world amazingly from sunscreen to chemical catalysts to antibacterial agents--from the mundane to the lifesaving. Amongst, Metal Oxide nanoparticles highly used in efficient applications, because of their limited size on the surface corners, shape, chemical composition and its valence state. Between them, TiO_2, FeO_2, CeO_2 are highly reactive oxides and deeply interact with cells of metabolic networks [1]. The rare earth element of Cerium Oxide nanoparticles has a cubic fluorite structure and numerous industrial applications like catalyst [2], biodiesel blends [3], gas sensor [4], corrosion protection [5], solar cell [6], sunscreen creams [7] and biomedical applications like of biosensor [8], Antibacterial [9], Anti-inflammation [10], drug delivery [11] result of cyclic oxidation states of Ce^{3+} and Ce^{4+} based on the environmental condition [12]. CeO_2 nanoparticles have shown promising approaches as therapeutic agents in medical science since of physicochemical properties such as agglomeration status in liquid, surface charge, size, and ultimate interactions with target cells. Different methods were followed for the synthesis of nanoparticles such as physical, chemical, irradiation, and biological methods. Here in this study CeO_2-NPs were synthesized from microorganism Bacillus *subtilis*. This method also simple, ecofriendly and low cost. Based on the location where the NPs is synthesized it is classified into Intracellular and Extracellular. Extracellular supernatant of the bacteria acting a reducing agent for the reduction of Cerium III nitrate to cerium oxide NPs. *Bacillus subtilis* is a gram-positive, aerobic bacterium. It is rod-shaped and catalase-positive. This can form a tough, protective endospore and can withstand extreme environmental conditions. *B. subtilis* is found in soil and the gastrointestinal tract of ruminants and humans. In *Bacillus subtilis* single catalase and second catalase enzymes are found orderly vegetative and sporulation stages [13]. An earlier study proved that *B. subtilis* was used in the synthesis of gold and silver nanoparticles [14, 15]. The imbalance between free radicals and antioxidants leads to Oxidative stress, which causes several diseases in human beings. Antioxidants neutralize free radicals and are effective in preventing these diseases. Cerium Oxide PEG-coated 3 nm CeO_2-NPs significantly decreased oxidative stress, enhanced hippocampus neuronal survival and promoted neurogenesis [16]. Biosynthesis CeO_2-NPs was found to be a prospective antioxidant drug for oxidative stress-induced mouse neural cells [17]. Cerium Oxide consumes enzyme mimic activities of superoxide dismutase [18], catalase [19] Phosphatase [20] and ROS scavenging activities of hydroxyl radicals [21], Nitric acid and Peroxynitrite radicals [22].

2. MATERIALS AND METHODS

Chemicals

Cerium III nitrate hexa hydrate Ce $(NO_3)_3.6H_2O$, 2, 2-Diphenyl picrylhydrazyl, Ethanol, Ascorbic acid, Sulfuric acid, Ammonium molybdate, Sodium dihydrogen phosphate, Di sodium mono hydrogen phosphate, Potassium ferricyanide, Trichloroacetic acid, Ferric Chloride were procured

from standard vendors.

2.1 Preparation of Bacterial Culture

For this work, the MTCC Culture of *Bacillus Subtilis* (MTCC No. 441**)** was used as biological nano-factory for the synthesis of cerium oxide nanoparticles. The culture was maintained in the nutrient as slat in the Laboratory. 100ml of nutrient broth was prepared and one loop of slant culture was added and incubated at 37^0C in the incubator. After 24 hours, the turbidity nature of medium confirmed the bacterial growth. The broth was centrifuged at 8000 rpm for 15min. to remove the bacterial cells. The procedure was repeated until a clear solution was obtained. The supernatant was collected and filtered via whatmann No.1 filter papers twice. Now the extracellular supernatant was ready to use for the synthesis of nanoparticles.

2.2 Biosynthesis of Cerium Oxide Nanoparticles

Cerium III nitrate hexa hydrate (Ce $(NO_3)_3.6H_2O$) was purchased from HI media, Ltd. 80ml of 10mM Cerium nitrate solution was prepared and added with 20ml of the above bacterial supernatant solution dropwise under stirring condition at room temperature. The clear solution will be changed into turbid and pale yellow colour which indicates the Reduction of Nitrate and formation of oxide particles. After 24 hours, the solution was centrifuged at 8000 rpm for 20mins. The collected pellet was air dried in an oven at 80^0C for 4-5 hours. The Particles were calcinated at 450^0C for 4hours in a muffle furnace. The particles were collected and stored carefully for future use.

2.3 Physicochemical Characterization of Nanoceria

Ultraviolet-Visible Spectroscopy Analysis

UV-Vis Spectrophotometer, Lab man was used to record the optical absorption of the biosynthesized Cerium Oxide nanoparticles in the spectral range of 200 to 500 nm at different time intervals by taking 2ml of the sample, compared with 2ml of distilled water used as blank respectively initial, 1hour, 3hours, 5 hours and 24 hours at room temperature.

FT-IR Analysis

Fourier Transform infra-red spectroscopy (FT-IR) analysis was carried out in the range of $400cm^{-1}$ to $4000cm^{-1}$ in Perkin Elmer.

X-Ray Diffraction Studies

The biosynthesized CeO_2 NP samples of XRD pattern was recorded on Powder XRD (PANalytical X'PERT PRO system) using Cu Kα radiation (λ = 1.54060 Å) in the range of 2Θ from 10^0 to 80^0. The average crystallite size of synthesized CeO_2 NPs as calculated using Debye – Scherrer's formula.

$[D = 0.9\lambda/\beta\cos\theta]$

Where, D is the average particle size, k is the shape factor (constant 0.9), λ is the X-ray wavelength (1.5406 Å), β is the full width at half maximum of the peak (FWHM) and θ is the

diffraction angle.

SEM and EDAX

The **S**canning Electron Microscopic analysis was done using Carl Zeiss Evo 18 Secondary Electron Microscope with EDS machine with the magnification up to 50K – 100K depends on the sample. Thin films of the samples were prepared on a carbon-coated copper grid by just dropping a very small amount of sample on the grid. The Excess solution was removed using blotting paper and then the films on the SEM grid were allowed to dry for 1 hour.

The Energy Dispersive X-ray analysis attachment AMETEK EDAX octane series was used to carry out semi-quantitative elemental analysis of the samples.

2.4 Antioxidant Studies

DPPH Free Radical Scavenging Assay: Different concentration of cerium Oxide nanoparticles make up into 1ml with ethanol. 1ml of 0.3mM 2, 2-Diphenyl picrylhydrazyl (DPPH) in ethanol, was added to the above samples. The reaction mixture was well shaken and incubated in dark for 30 mins. Absorbance was checked at 517 nm against a blank (ethanol). Ascorbic acid was taken as the standard. Lower the absorbance of the reaction mixture indicates a higher percentage of scavenging activity [23].

The percentage of inhibition or scavenging of free radicals was determined by the formulae; % Inhibition = [(Absorbance Control – Absorbance Sample)/ Absorbance Control] x 100,

Where, control was prepared as above without sample.

Total antioxidant assay: 0.1 ml of different concentrations of cerium Oxide nanoparticles were mixed in separate Eppendorf with 1 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate; mixed in 1:1:1 ratio) respectively. The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as the standard and the total antioxidant capacity was expressed by increased absorbance [24].

Reducing power: Different concentration cerium Oxide nanoparticles were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (10g/L), then the mixture was incubated at 50º C for 20 minutes. 2.5 ml of trichloroacetic acid (100g/L) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1g/L) and absorbance measured at 700nm in UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used a blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power [25, 26].

Statistical analysis

All antioxidant experiments were done by analysis of variance and the results are presented as the

mean standard error of the mean. Data points were obtained from the mean of at least three triplicates.

3. RESULTS AND DISCUSSION

3.1 Synthesis Process of Nanoceria from Bacteria

Characterization, application of biosynthesized cerium oxide nanoparticles was important in the field of nanotechnology. Numerous microbial flora bacteria, fungi, and yeast used either extracellular or intracellular for the synthesis of metal nanoparticles because of bioreduction [27], eco-friendly and low cost. In Intracellular synthesis, the electrostatic interaction of bacterial cell wall with metal ions plays a significant role. In Extracellular synthesis basically mediated through Nitrate reductase enzymes. *Bacillus* species were found to be a metal reducing agent for gold [28], silver, cadmium, and lead [29]. So here, the extracellular products of bacillus act as a reductant for the synthesis of cerium nanoparticles.

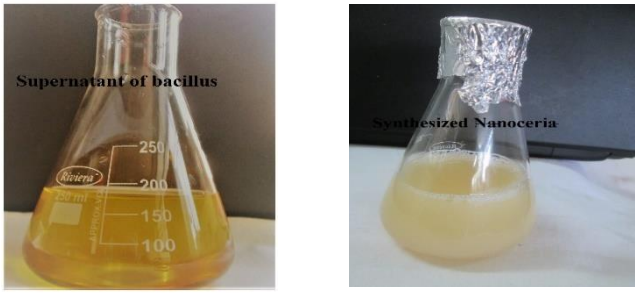

Fig. 1 Nanoceria synthesized from *Bacillus subtilis*

i) Extracellular Supernatant of bacteria ii) Synthesized nanoceria in 24hours.

3.2 Characterization studies of Nanoceria

UV-Visible Spectrophotometer

Fig.2 showed the UV absorption spectrum of bacteria-mediated biosynthesized Nanoceria. Nanoceria has two oxidation states Ce (III) was colorless, while Ce (IV) was yellow to red in color [30, 31]. Both oxidation states have two different UV adsorption peaks – Ce (III) in the range 230-260nm. Ce (IV) possessed in the range 300-400nm [32]. Now, in our method, the maximum adsorption of sharp peak found at 300 nm with yellow colour [33, 34], which indicates the presence of Ce (IV) oxidation states. The sharp peak spectrum showed within a 5 hours interval at 300nm of wavelength. Since, five hours was sufficient for the synthesis of cerium nanoparticles. In 24 hours the Peak spectrum showed broad it might be the aggregation of nanoparticles.

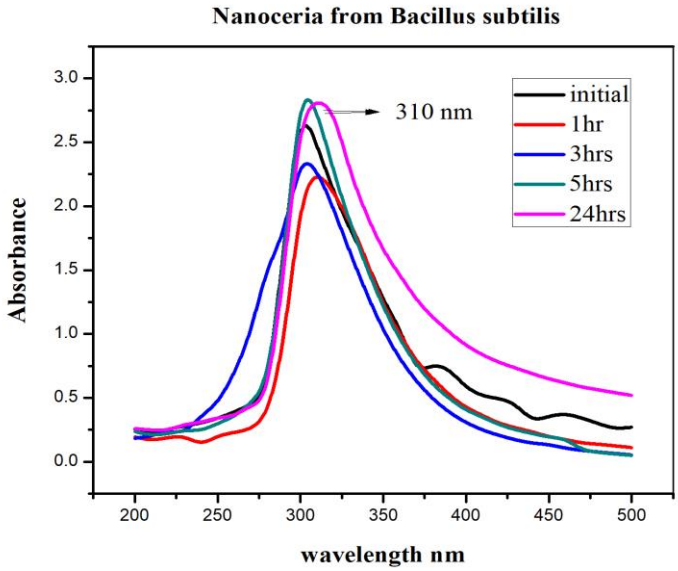

Fig. 2 UV-Vis spectrum of biosynthesized Nanoceria

X-Ray Diffractometer Analysis for Nanoceria

In XRD pattern, peaks were observed at 28.61, 33.23, 47.98, 56.41, 60.16, 69.29, 76.74 correspondent to planes 111, 200, 220, 311, 222, 400, 331, 420 respectively. The obtained Nanoceria XRD graph exactly matched with the report of Cerium (IV) oxide Nanoparticles [35]. The average crystallite size of the nanoparticles is 8.022nm was calculated by Debye-Scherrer's equation.

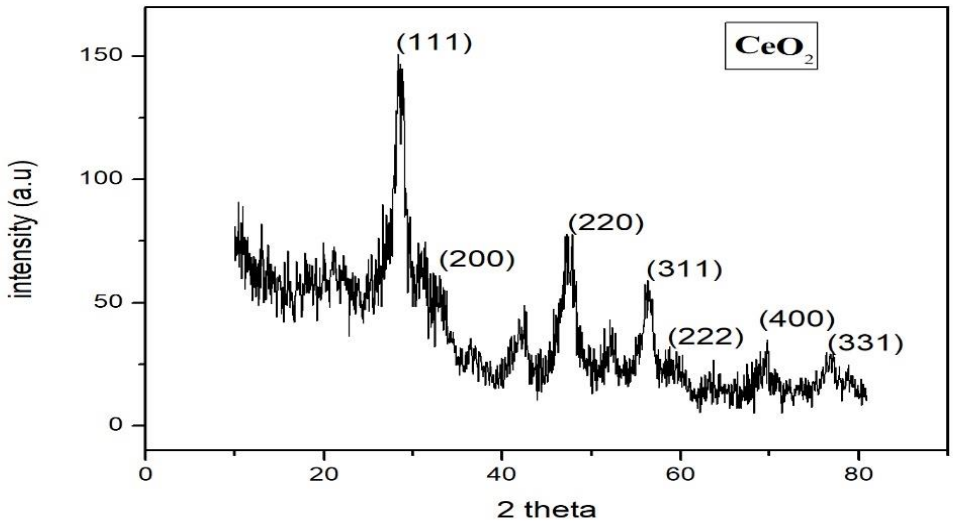

Fig. 3 XRD pattern of biosynthesized Nanoceria

Fourier Transform Infrared Spectrophotometer

In FT-IR spectroscopy study was done for bio nanoceria within a range of 400 cm^{-1} to 4000 cm^{-1} the peaks were showed at 433.90, 538.04, 607.46 and 997.01. The FT-IR spectrum of the CeO_2-NPs also exhibits a band below 700 cm^{-1} which is due to the δ (Ce–O–C) mode [36]. The Ce–O

stretching frequency was expected below 400 cm^{-1} but in this study it was observed at 433 cm^{-1}, indicating the formation of CeO_2 [37] Similar reports were predicted by Goharshadi *et al*., [38]. Where, the Ce–O stretching band appeared in 433 cm^{-1}. The peak at 997.01 cm^{-1} is responsible for the overtone band of the trace of Ce-OH [39]. The CO_2 peaks are observed at 2341 cm^{-1} and 1423 cm^{-1} for CeO_2 Nanoparticles [40].

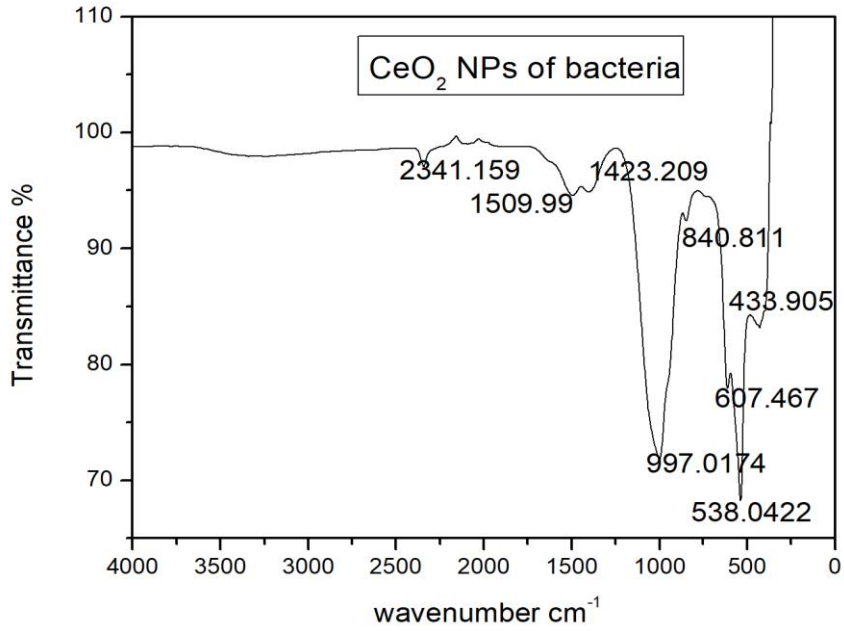

Fig.4 FT-IR spectrum of Biosynthesized Nanoceria

SEM and EDAX

Fig.5 showed the morphology of bacteria-mediated synthesized nanoceria with Scanning Electron Microscope. Cerium oxide nanoparticles were in high density with relatively spherical in nature. EDAX results showed the presence of Cerium Nanoparticles.

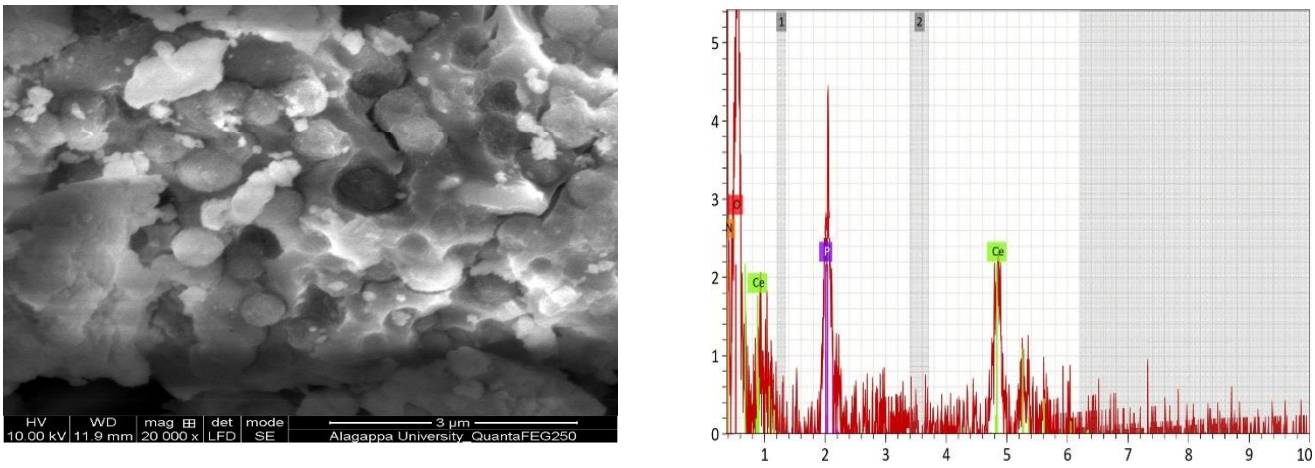

Fig. 5 SEM and EDAX Analysis of biosynthesized Nanoceria

3.3 Antioxidant Assay

In the DPPH, Free Radical Scavenging Assay of biosynthesized nanoceria (CeO_2) where, ascorbic acid (AA) was taken as standard. A freshly prepared DPPH solution showed a deep purple color with maximum absorption at 517 nm. This purple color vanishes when an antioxidant is existing in the medium. Thus, antioxidants molecules can reduce DPPH free radicals and convert them to a colorless product, resulting in a decrease in absorbance at 517 nm [41]. The DPPH free radical scavenging assay showed 48.58% inhibition efficiency of 50µg/500µl nanoceria. This specified the potent inhibitory capacity of nanoceria when compared with ascorbic acid. The percentage of inhibition of free radicals increased with increase in the concentration of samples.

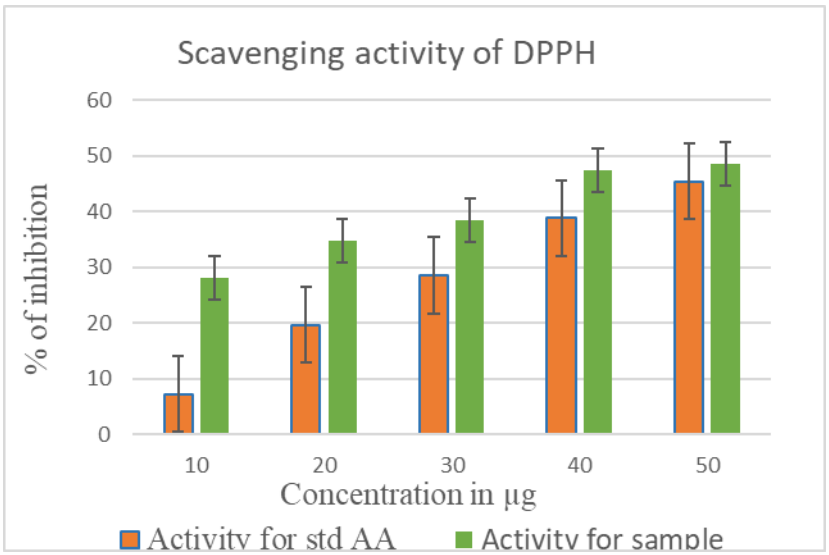

Fig. 6 Scavenging activity of DPPH (Di-Phenyl picryl hydrazyl) Orange Bar - Activity for Ascorbic acid, Green Bar– Activity of the standard.

Fig **7** showed the Total antioxidant capacity of ascorbic acid (AA) and biosynthesized nanoceria. The Total antioxidant ability was estimated based on the formation of the phosphomolybdenum complex where the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm [42]. The cerium oxide nanoparticles were found to have very high total antioxidant capacity as compared to the standard.

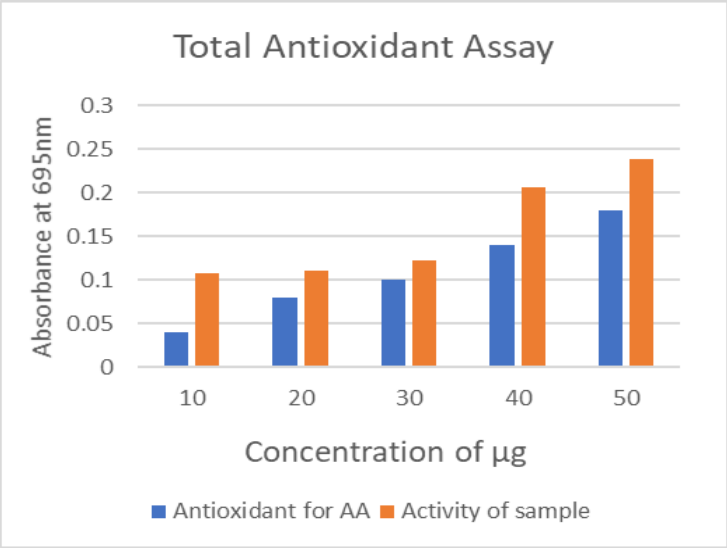

Fig. 7 Total Antioxidant assay for biosynthesized Nanoceria (Red bar) and Standard Ascorbic Acid (Blue bar)

Figure 8 showed the Reducing Power Assay of biosynthesized nanoceria where ascorbic acid (AA) was taken as standard. The reducing aptitude of a compound depends on the presence of reductants [43] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [44]. Presence of reducers causes the conversion of the Fe3+/ferricyanide complex used in this method to the ferrous form. By measuring the development of Perl's Prussian blue at 700 nm, it is possible to determine the Fe2+ concentration [45]. Nanoceria was found to have very high reducing capacity when compared to the standard and increased with increasing sample concentration.

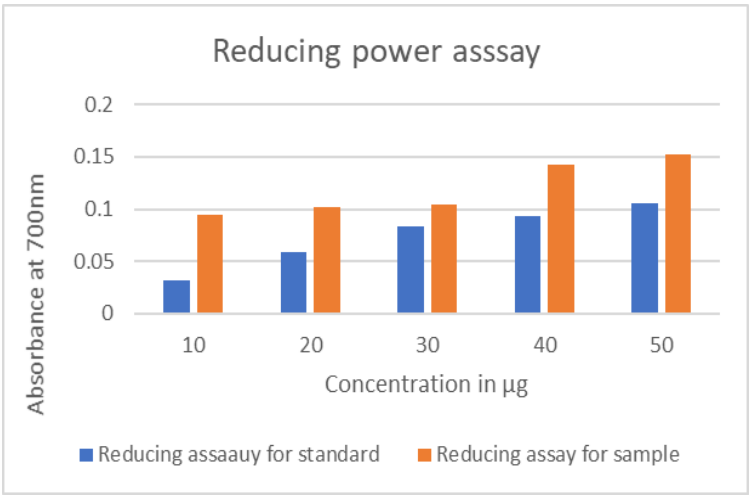

Fig. 8 Reducing Power assay for biosynthesized Nanoceria (Red bar) and Standard Ascorbic Acid (Blue bar).

4. CONCLUSION

In this process, cerium oxide nanoparticles were successfully synthesized in two important ways: there was no addition of any harmful or hazardous chemical reagents and other was synthesis at

room temperature. Synthesizing with a room temperature of Nanoceria has small, has beneficial anti-oxidant property compared with the nanoparticles produced at high temperature [46]. The synthesized nanoceria confirmed by various characterization techniques and antioxidant property also proved by chemical assays. The antioxidant is very important because they are capable of slowing or preventing the oxidation process. By this, the synthesized nanoceria could be acting as antioxidant agent for oxidative-stress related diseases like cancer, stroke, aging, arthritis, etc.

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CONFLICT OF INTEREST

There is no Conflict of Interest.

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