International Journal of Modern Science and Technology, 2018;3(5):104-111.
ISSN: 2456-0235.
www.ijmst.co

Research Article

Synthesis and Characterization of Cerium Oxide Nanobiocomposite of Asparaginase for Curbing Lung Cancer

G. Baskar*, K. Lalitha

Department of Biotechnology, St. Joseph's College of Engineering,
Chennai – 600 119. India.

*Corresponding author's e-mail: basg2004@gmail.com

Abstract

Cerium oxide nanoparticles have numerous applications owing to their catalytic and auto-regenerative property. They can shift between ce^{3+} and ce^{4+} oxidation states making it a good free radical scavenger and a suitable candidate for biological applications. They are also known for their ability to inhibit SOD and CAT activity. Cerium oxide nanobiocomposite of L-asparaginase labeled with FITC were synthesized using simple co-precipitation method and was found to have size of 2.77 nm. Maximum absorption was shown in the UV spectrum between 400-500nm. The cubic shape of the cerium oxide nanobiocomposite was found using SEM. Anticancer activity was checked by performing MTT assay. The cell viability was found to be 52.62% for IC_{50} concentration of 125 (µg/ml) on A549 lung cancer cell line.

Keywords: Cerium oxide; Nanobiocomposite; Asparaginase; Anticancer activity.

Introduction

Lung cancer is known to be the leading cause of death in European countries. It is reported to occur due to long term tobacco smoking (85%) and about (15%) due to passive smoking. Also genetic factors, exposure to radon gas, asbestos can cause lung cancer [1]. Current treatments for lung cancer focus on surgery, chemotherapy and radiotherapy. All the above methods are temporary and provide palliative cure risking the healthy nearby cells. Hence a targeted drug delivery system should be designed for a promising future therapy [2]. Cerium oxide nanoparticles are known and have been widely used for chemical and mechanical planarization in semiconductor industry [3]. Studies have shown that cerium oxide nanoparticles prevent various cellular damages due to biological chemical and radiological effects making them suitable for biological applications [4]. They show promising effects when used as catalysts in solar panels, fuel cells, diesel fuel additive for glass and ceramic applications [5]. Nanoceria are known for their stability as they don't undergo crystallographic change at room temperature due to is high melting point [6].

Cerium oxide nanoparticles work in a unique way by protecting the surrounding tissue of the targeted cells/tissues from radiation induced damage and oxidative stress as they have capacity to kill only the irradiated cancer cells [7]. In another way they exhibit differential toxicity on cancer cells through SOD like activity. SOD enzyme act as a radio- sensitizing agent interfering with DNA damage response by delaying the G_2/M cell transition causing cell accumulation in G_2 phase of cell cycle allowing more cell death through DNA damage [8]. Nanoceria are known to display pro-oxidant or antioxidant redox activity, thereby increasing the ROS level in tumor cells resulting in apoptosis of cancer cells, while normal surrounding cells remain intact. All the above citing is in accordance with the fact that cerium oxide nanoparticles have antitumor property and can be used for targeted drug delivery applications. The present work is focused the synthesis of cerium oxide nanobiocomposite of L-asparaginase and testing their cytotoxicity on A549 lung cancer cell line.

Cerium oxide nanobiocomposite of L-asparaginase provide synergetic effect in killing cancer cells due to their inherent anticancer property of L-asparaginase.

Received: 06.05.2018; Received after Revision: 18.05.2018; Accepted: 20.05.2018; Published: 28.05.2018

Asparaginase is a hydrolase enzyme with enzyme commission (EC) number 3.5.1.1. It catalyses the deamidation reaction of L-asparagine present on the surface of cancer cells to L-aspartate and ammonia thereby reducing their concentration on the cell surface [9]. Though L-asparagine is also present on the surface of normal cells, they have the inherent mechanism and can synthesize intracellular L-asparagine from aspartic acid and glutamine using asparagine synthetase [10].

However neoplastic cells/tumor cells lack the cellular mechanism to re-synthesize L-asparagine due to damaged DNA mechanism and become depleted of the L-asparagine concentration on their cell surface concentration on their cell surface, leading to their death on treating with L-asparaginase as L- asparagine is crucial for activation and proliferation of tumor cell by acting as an amino acid exchange factor [11, 12]. Thus the present work was focused on synthesis of cerium oxide nanobiocomposite of L-asparaginase for use as a drug for lung cancer.

Materials and methods

Chemicals used

The metal precursor cerous nitrate for cerium oxide nanoparticles were purchased from LOBA CHEIME, India. The fungal strain *Aspergillus terreus* MTCC 1782 used for the production of L-asparaginase was obtained from CSIR-Institute of Microbial Technology, Chandigarh, India.

Preparation of inoculum culture for asparaginase synthesis

Aspergillus terreus strain was cultivated in Czepak agar slants at 37ºC for 4 days. Czepak-Dox Agar slant containing the following ingredients; Solution-A: 0.1 g L-asparagine, 0.4 g sodium nitrate. 0.1 g potassium chloride, 0.1 g magnesium sulphate, 0.002 g ferrous sulphate dissolved in 10 ml of distilled water and stored in refrigerator. Solution-B: 0.2 g of dipotassium hydrogen phosphate was dissolved in 10mlof distilled water and stored in refrigerator. Solution-C: 0.1 g zinc sulphate was dissolved in 10 ml distilled water and stored in refrigerator. 100 ml of Czepak-Dox medium, 5 ml of solution B, 0.1 ml of solution C, 89.9 ml, 3 g of glucose and 2 g of Agar was dissolved in 89.9 ml distilled water, autoclaved and used for slant preparation [11].

Chemical synthesis of cerium oxide nanoparticles

Cerous nitrate (0.2 M) was mixed with excess of 1M sodium hydroxide and stirred continuously at room temperature at 1150 rpm for 30 min light purple precipitate was formed in 10 min which later turned into creamy white colour in 50 min. The above solution was ultrasonicated followed by 24 hrs resting at room temperature. The above solution was centrifuged at 4800 rpm for 50min.The supernatant was discarded and the pellet was washed with deionized water and dried at 70℃ for 5 hrs [13]. The procedure was modified slightly.

Production of L-asparaginase

Aspergillus terreus was inoculated in 250ml Erlenmeyer flask containing 100mlof modified Czepak-Dox Liquid medium containing 2.0 g L-proline, 1.0 g L-asparaginase, 0.2 g glucose, 1.0 g sodium nitrate,0.052 g potassium chloride, 1.52 g dipotassium hydrogen phosphate, 0.001 g zinc sulphate, 0.001 g ferrous sulphate and 0.052 g magnesium sulphate maintained at pH 6.2.The fungus was grown aerobically by agitating in an orbital shaker at 160 rpm at 32°C for 3 days after the incubation period the culture was filtered using Whatman#1filter paper, which is rich with L-asparaginase [11].

Estimation of L-asparaginase activity and protein concentration

Crude enzyme (0.1 ml) was added to 0.9 ml of 0.1M phosphate buffer (pH 8.5) along with 1ml of 0.04 M of L-Asparaginase and incubated at 37℃ for 10 min. The reaction was stopped by adding 0.5 ml of 15% trichloro acetic acid, and centrifuged at 6000 rpm for 10 min at 4°C. 0.1 ml of supernatant was taken and diluted to 8 ml with distilled water.1ml of 2 M NaOH and 1 ml of Nessler's reagent were added and incubated for 10 min at room temperature. Then OD was taken at 480 nm [14]. The protein concentration was estimated using Bradford's method [15].

Synthesis of nanobiocomposite of cerium oxide nanoparticles and fungal L-asparaginase bound with FITC

Cerium oxide nanoparticles (0.4 g) were mixed with 400 ml of phosphate buffer pH (8.5). 4 ml of glutaraldehyde was added and stirred for 2 hrs at 30°C.0.004 g of FITC was dissolved in 4 ml of DMSO (1 mg/ml). 970 µl of FITC solution

was taken and mixed with 200 ml of asparaginase along with pre-treated nanoparticles solution and stirred for 30 min at 4℃. The so formed cerium oxide nanobiocomposite solution was stored at 4℃ for 6 hrs and later centrifuged at 5000 rpm for 30 min. The supernatant was discarded and the pellet was washed with de- ionized water and dried at 70℃ for 5hrs.

Anticancer activity of nanobiocomposite of cerium oxide nanoparticles and fungal L-asparaginase using MTT assay

The MTT assay [16] is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. The cells were maintained in MEM (Minimum Essential Media). Cells (1×10^5/well) were plated in 24-well plates and incubated in 37°C with 5% CO_2. After the cells reached confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the samples were removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 hrs. After incubation, 1ml of DMSO was added to the wells .The absorbance was measured at 570nm with UV- Spectrophotometer using DMSO as the blank. The concentration of the sample required to kill 50% of cell population (IC_{50}) was determined graphically. The % cell viability was calculated using equation 1.

$$\% \text{ cell viability} = (A570 \text{ of treated cells} / A570 \text{ of control cells} \times 100) \quad \text{.....(1)}$$

Graph was plotted taking % of Cell Viability in Y-axis and concentration of the sample in X-axis. Cell control and sample control was included in each assay to compare the full cell viability assessments.

Fluorescent microscopic study of cerium oxide nanocomposite of L-asparaginase bound with FITC

VERO and A549 cells were seeded with (one lakh cells per ml) on a cover slip placed in a 6 well plate and incubated for 24 hrs. After incubation, the mono layer of cells was treated with IC_{50} concentration of cerium oxide nanobiocomposite labeled with FITC and incubated for 24 hrs. The treated cells were washed with sterile PBS. 70% ethanol was used to fix the cells. 0.2 ml of propidium iodide (10 µg/ml) was added to the cells and left for 15 to 30 min. The cover slip containing the stained cells were removed from the 6 well plates and placed on a clean grease free glass slide. Fluorescent images of VERO and A549cells were taken.

Results and discussion

Asparaginase activity

The specific activity of L-asparaginase was reduced from 28.36 (IU/ml) to 23.41 (IU/ml) in cerium oxide nanobiocomposite. This is because the active site of the enzyme is involved in binding with cerium oxide nanoparticles and FITC. Binding efficiency of enzyme was found by calculating the ratio of "total protein bound to nanoparticles" to "total protein taken for binding". A = Protein bound to nanoparticles = (Protein concentration in nanoparticles (mg/ml)*Total volume of nanoparticles suspension). B = Total protein available for binding = (Protein concentration in crude enzyme (mg/ml)*volume of crude enzyme taken for binding).

$$\text{Binding efficiency} = (A/B)*100 \quad \text{.....(2)}$$

Using eq. (1) the A value was calculated as (0.328 mg/ml*60.49 ml=19.43 mg). B value was calculated as (0.120*200=24 mg). Now Binding efficiency = (A/B)*100 = (19.43)*100=82.6%

UV spectroscopic analysis of cerium oxide nanoparticles and nanobiocomposite

Characteristic peak at 497 nm in (Fig. 1(a)) indicated the formation of cerium oxide nanoparticles while peaks at 492 nm, 358 nm, 226 nm in (Fig. 1(b)) indicated the presence of cerium oxide nanoparticles, binding of asparaginase, FITC to cerium nanoparticles respectively confirming the formation of cerium oxide nanobiocomposite.

SEM analysis of cerium oxide nanoparticles and nanobiocomposite

Fig. 2 (a) and (b) depicts the SEM image of cerium oxide nanoparticles and nanobiocomposite. The SEM images were taken at 24 kV power setting, scanned between 5 to10 µm. these images were analysed at various magnifications and the following characteristics were observed. Both cerium oxide nanoparticles and nanobiocomposite were spherical in nature

having average size ranging between 2-3 nm and 2.77 nm respectively.

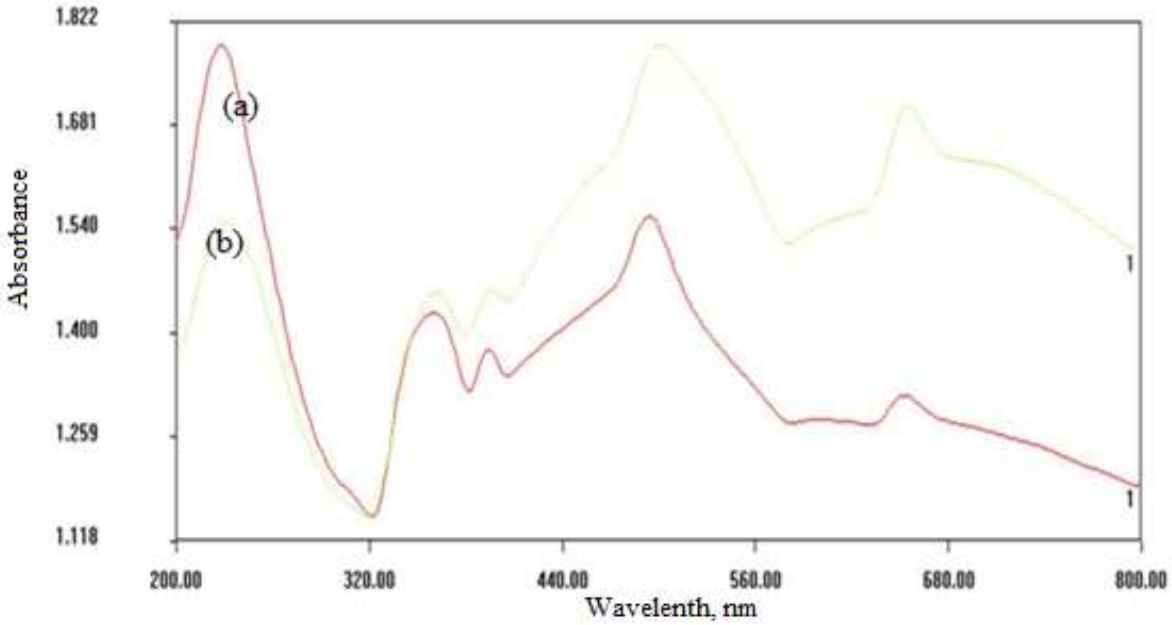

Fig. 1. UV spectrum of (a) cerium oxide nanoparticles (b) cerium oxide nanobiocomposite

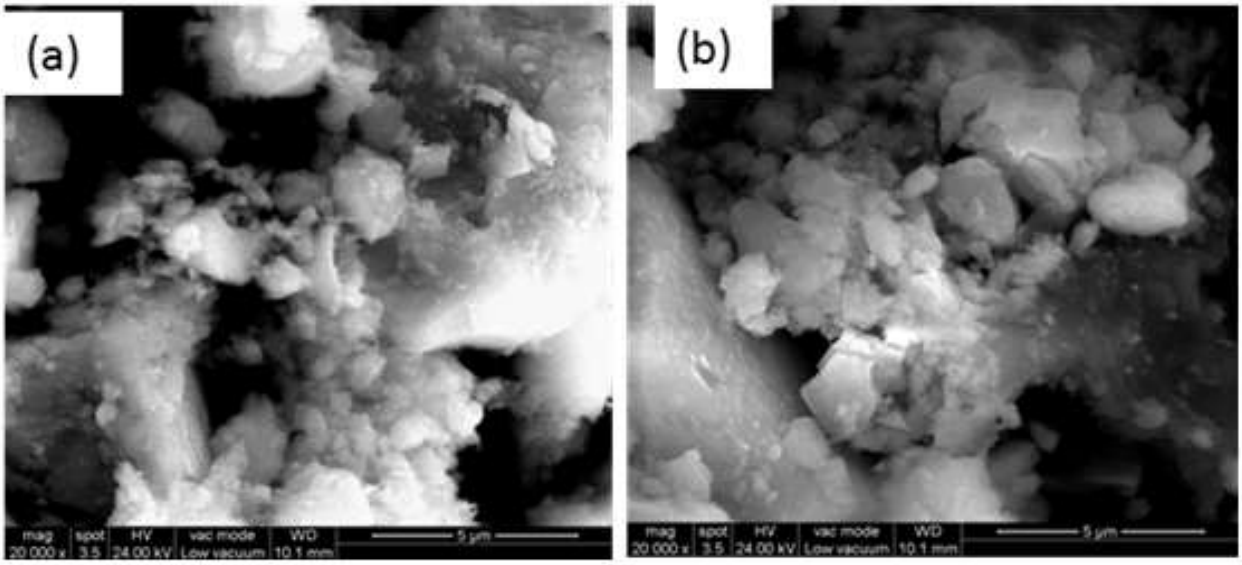

Fig. 2. SEM image of (a) cerium oxide nanoparticles at 20,000X (b) SEM image of cerium oxide nanobiocomposite at 20,000X

XRD analysis of cerium oxide nanobiocomposite

The crystal structure of cerium oxide nanobiocomposite was analyzed using X-RAY Diffraction (Fig. 3). The 2-theta values of the Peaks were indexed using JCPDS FILE (43-1002, 34-0394, 75-0076) and corresponded to following planes of cerium crystal. The indexed planes corresponded to phase centered cubic fluorite structure of Cerium asparaginase nanobiocomposite. The sharpness of the Peaks indicated that the cerium oxide nanobiocomposite was in pure crystalline phase.

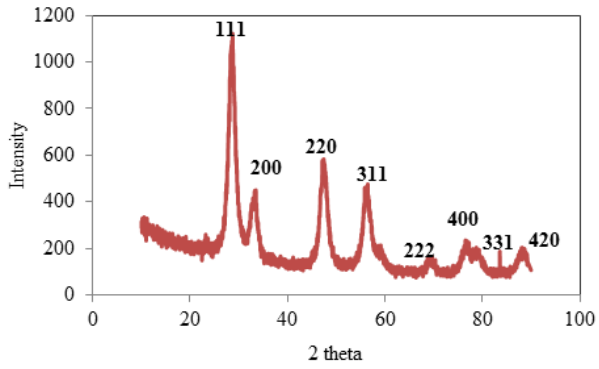

Fig. 3. XRD pattern of cerium oxide nanobiocomposite

The average particle size of nanobiocomposite was calculated using Scherrer

formula given in eq. (3).

$$D_p = (0.98 * \lambda / \beta * \cos\theta) \quad \ldots (3)$$

where D_p is the average crystalline size, λ is the wavelength of the Cu Kα line which is equal to 1.54 nm, θ is the Bragg angle (14.24˚) and β is the full width at half-maximum of the diffraction peak in radians (0.562). The size was calculated to be 2.77 nm.

H-NMR analysis Of FITC labeled asparaginase bound cerium oxide nanobiocomposite

H-NMR spectrum of cerium oxide nanobiocomposite in terms of chemical shift of various functional groups is represented in Fig. 4. A strong narrow peak ranging from 1.2-1.3 ppm showed the presence of singlet peak confirming the presence of secondary alkyl group. The quartets at 1.4 ppm and at 1.5 ppm showed the presence of secondary and tertiary alkyl groups. The multiplet 7 peaks ranging between 1.6-1.7 ppm confirms the presence of allylic carbon and tertiary alkyl group. The multiplet 7 peak at 1.8 ppm showed the presence of alkyl tertiary carbon. Further multiplet 7 peaks ranging between 1.9-2.0 ppm is indicative of the presence of allylic carbon. The multiplet and triplet peaks ranging between 2.0-2.1 and 2.1-2.2 ppm respectively showed the presence of methyl ketone group. From the above interpretation it can be confirmed that tertiary alkyl group is involved in binding of asparaginase to Cerium oxide nanoparticles.

FTIR analysis of FITC labeled asparaginase bound cerium oxide nanoparticles and cerium oxide nanobiocomposite

On comparing the FTIR spectrum of cerium oxide nanoparticles and nanobiocomposite the following peaks were found in common (Fig. 5): 3400 cm^{-1}, 1400 cm^{-1}, 1000 cm^{-1}, 1114 cm^{-1} indicating the presence of C-H medium stretch vibrations -N-H- stretch vibration at 3400 cm^{-1}, alkoxy C-O medium stretch vibration at 1400cm^{-1}, 1000 cm^{-1} and 1114 cm^{-1}. Additional peak at 667 $cm^{-1}$was found in cerium oxide nanobiocomposite corresponding to SP^2 hybridized C-H medium stretch vibration. Thus it can be inferred that SP^2 hybridized C-H bond is involve in binding of asparaginase to cerium oxide nanoparticles.

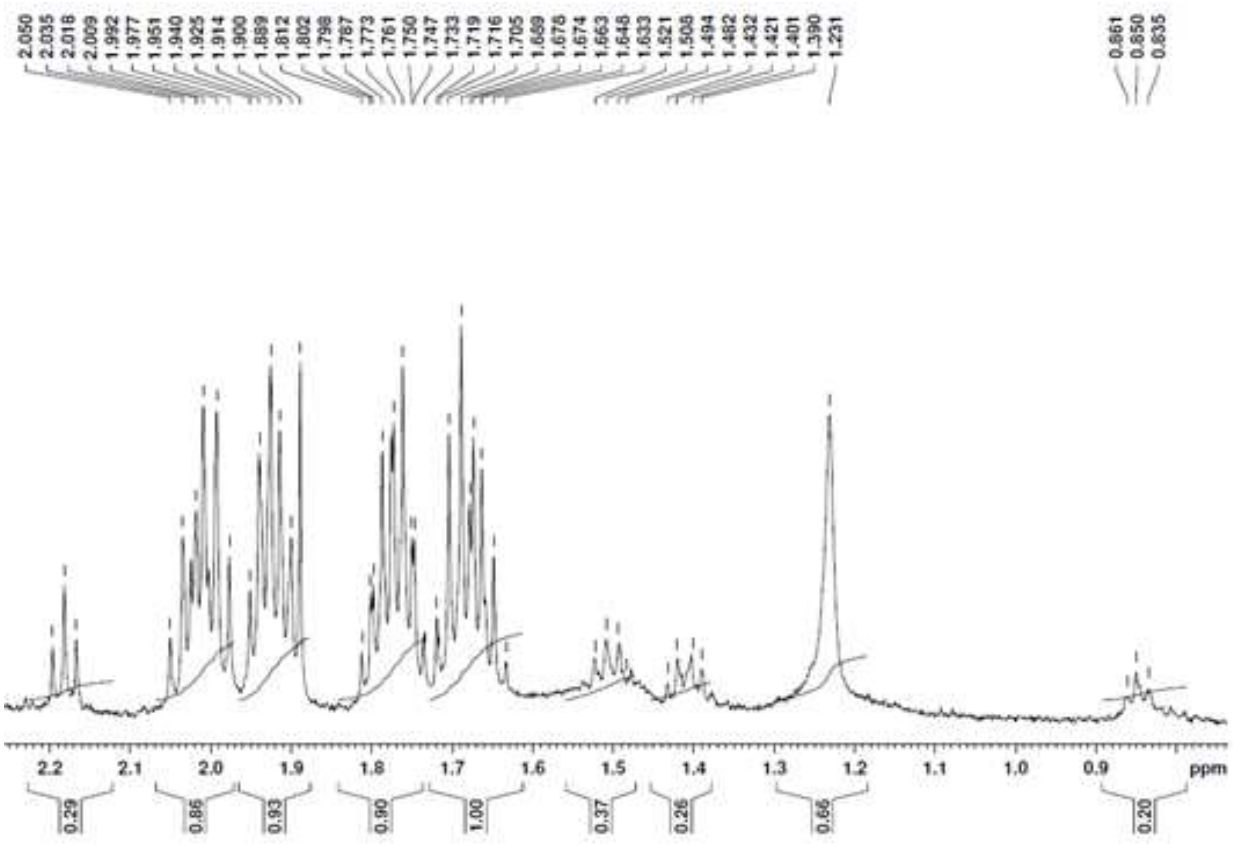

Fig. 4. H-NMR pattern of cerium oxide nanobiocomposite

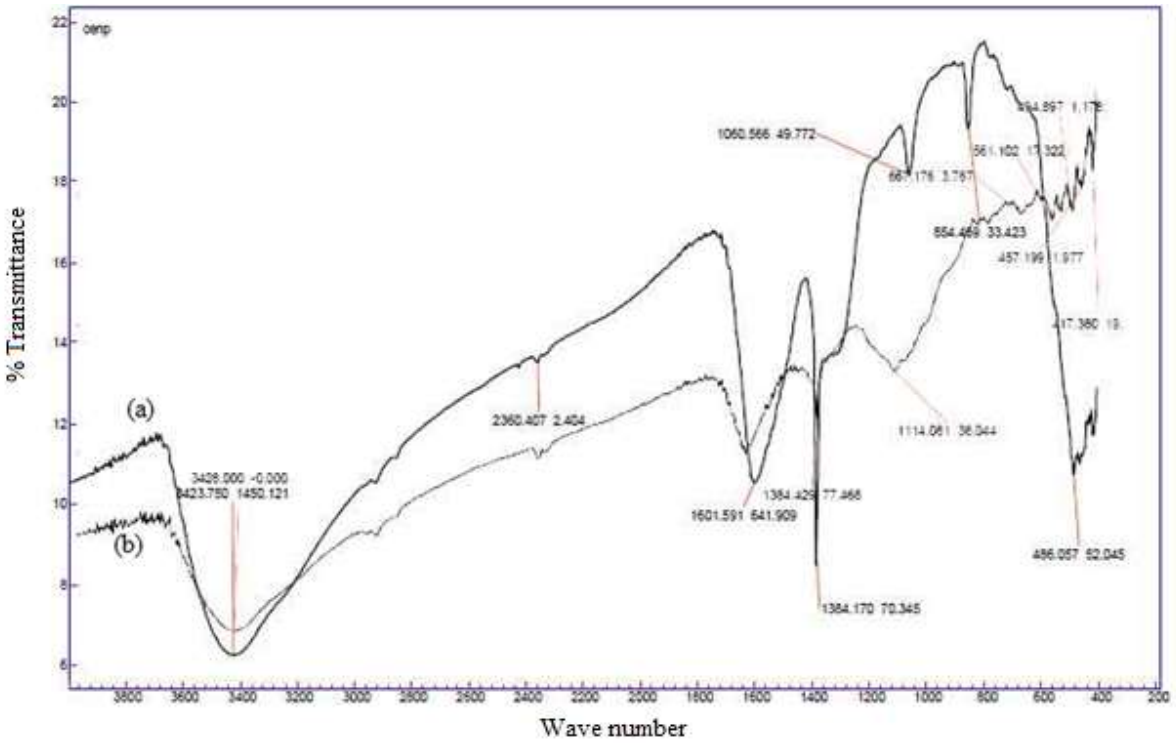

Fig. 5. FTIR pattern of (a) cerium oxide nanoparticles (b) cerium oxide nanobiocomposite

Anticancer effect of cerium oxide nanobiocomposite on A549 cell line

Cerium oxide nanobiocomposite was taken in various concentrations ranging from 1000-7.8 (µg/ml) and in various dilutions starting from 1:1-1:64. The IC_{50} value was found to be 125 (µg/ml), providing cell viability of 52.62% (Fig. 6). Increase in the concentration of cerium oxide nanobiocomposite (1000-7.8 (µg/ml) led to considerable decrease in viability of the cells to as low as 30.37% (Fig. 6 and 7). The absorbance values at 570 nm for each concentration were noted which is indicative of ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product which were found to be as high as 1.846 for 7.8 (µg/ml) due to higher % of live cells and low as 0.718 for 1000 (µg/ml) inversely due to higher % of dead cells supporting its efficacy in cancer cell apoptosis.

Fluorescent microscopic studies of cerium oxide nanobiocomposite on A549 cell line

The cerium oxide nanobiocomposite of asparaginase labeled with FITC was targeted efficiently and internalized through cooperative binding (endocytosis) with prolonged attachment to the cell membrane due to the small size (2.77 nm) of synthesized cerium oxide nanoparticles (Fig. 8). Due to their smaller size they form cluster of certain size to induce membrane invagination, with increase in time they were internalized in the intracellular membrane as clusters.

The strong columbic interactions between the positively charged surface of the nanoparticles and the negatively charged plasma membrane, spherical shape of cerium oxide nanobiocomposite aid in attachment and efficient penetration into the cell. The presence of adapter proteins attached to the clathrin coated pits help in the attachment of ligands (aldehyde and tertiary alkyl groups) on the surface of nanoparticles to the receptors of the adapter proteins providing sufficient membrane wrapping. The multidomain GTPase protein Dynamin forms a helix around the nanoparticles enclosed vesicle, facilitating pinching and release of the vesicle into the cytosolic compartment [17].

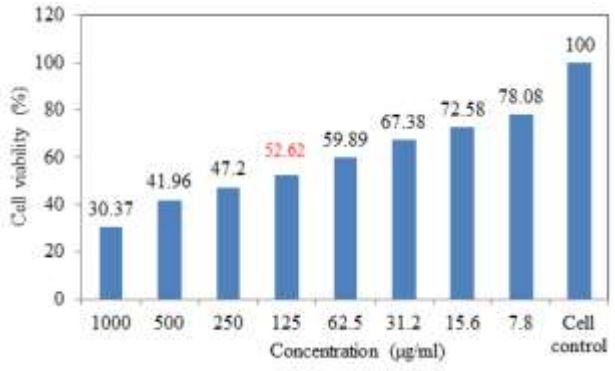

Fig. 6. Cell Viability (%) of cerium oxide nanobiocomposite on A549 cell line

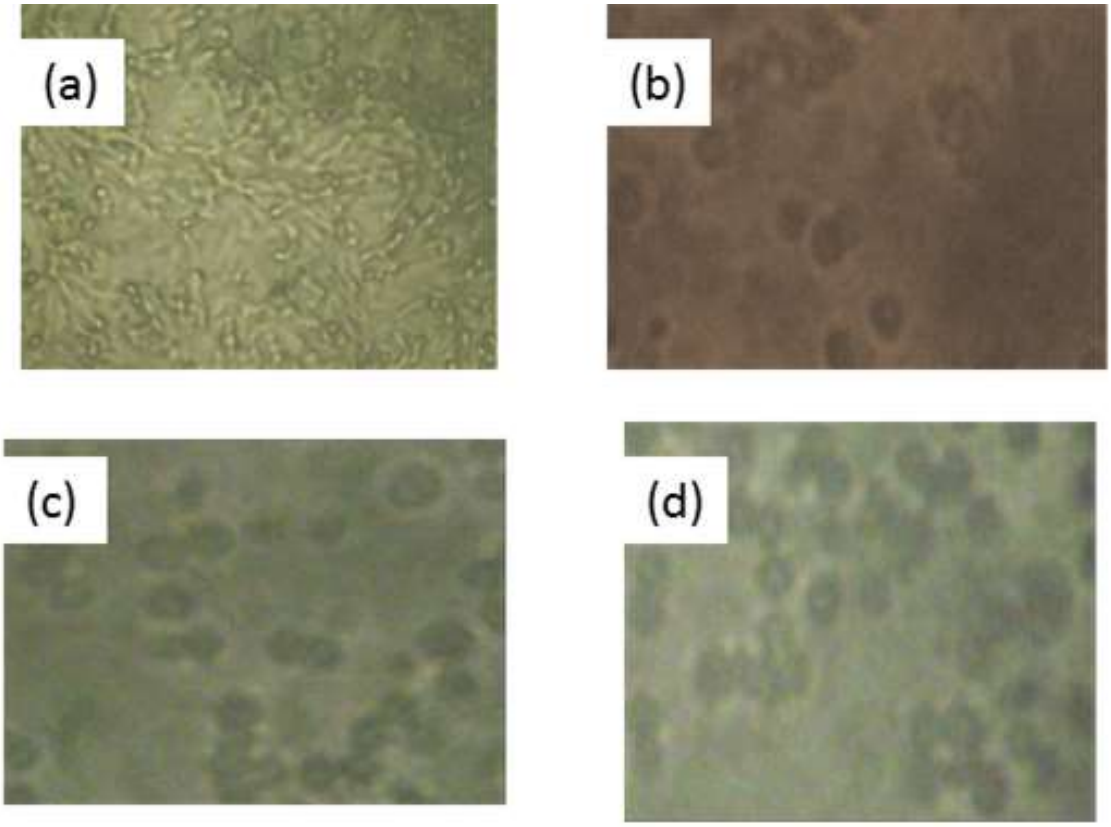

Fig. 7. Anticancer effect of cerium oxide nanobiocomposite on A549 cell line (a) Untreated A549 Cell (b) Toxicity 1000 µg/ml (c) Toxicity 125 µg/ml (d) Toxicity 7.8 µg/ml

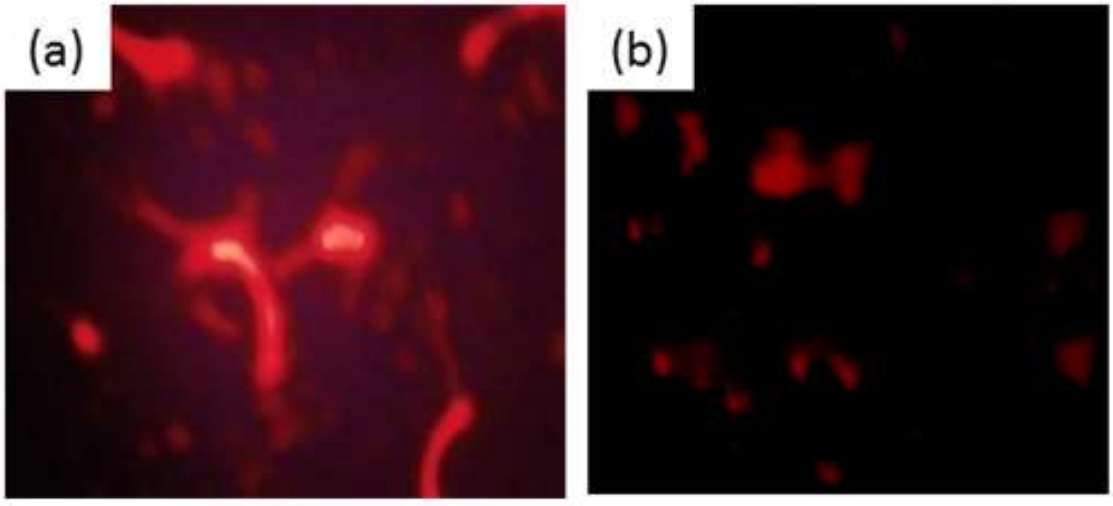

Fig. 8. Fluorescent microscopic images of (a) Control (A549 cell line) (b) A549 cells loaded with IC_{50} concentration of cerium oxide nanobiocomposite

Conclusions

The synthesized nanobiocomposite was found to be 2.77 nm in size with phase centered cubic fluorite structure which was confirmed from SEM and XRD analysis. From FTIR and H-NMR analysis it was confirmed that tertiary alkyl group and SP^2 hybridized C-H bonds were involved in binding of asparaginase to cerium oxide nanoparticles. Anticancer testing of IC_{50} concentration of cerium oxide nanobiocomposite on A549cell line confirmed the following results: It can be inferred that cerium oxide nanobiocomposite has % cell viability of 52.62% on A549 cell line confirming its higher efficacy against A549cell line. From fluorescent microscopic studies it was confirmed that cerium oxide nanobiocomposite has targeted efficiently and was incorporated into the cytoplasmic membrane proving its efficacy as a potential lung cancer drug in the near future.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Alberg AJ, Brock MV, Samet JM. Epidemiology of lung cancer looking to the future. J Clin Oncol. 2005;23:3175-85.

[2] Thangavelu M, Munusamy C, Sundaram P, Baskar G, Chandhuru J, Thotapalli PS. Carbon nanoparticle from a natural source fabricated for folate receptor targeting, imaging and drug delivery application in A549 lung cancer cells. Eur J Pharm Biopharm. 2014;88(3):730-36.

[3] Nelson BC, Johnson ME, Walker ML, Riley KR, Sims CM. Antioxidant cerium oxide nanoparticles in biology and medicine. Antioxidants. 2016;5:15.

[4] Celardo I, Traversa E, Ghibelli L. Cerium oxide nanoparticles: a promise for applications in therapy. J Exp Ther Oncol. 2011;9:47-51.

[5] Kavitha P, Rajan MR, Ramesh R, Stella, C. Synthesis and characterization of cerium oxide nanoparticles by using rapid precipitation method. Paripex-Indian Journal of Research. 2015;4:91-3.

[6] Farahmandjou M, Zarinkamar M, Firoozabadi TP. Synthesis of cerium oxide (CeO2) nanoparticles using simple co-precipitation method. Revista Mexicana de F´ısica. 2016;62:496-99.

[7] Vinardell MP, Mitjans M, Antitumor activities of metal oxide nanoparticles. Nanomaterials. 2015;5:1004-21.

[8] Ali D, Alarifi S, Alkahtani S, AlKahtane AA, Almalik A. Cerium oxide nanoparticles induce oxidative stress and genotoxicity in human skin melanoma cells. Cell Biochem Biophys. 2014;71:1643-51.

[9] Alili L, Sack M, Karakoti AS, Teuber S, Puschmann, K., Hirst SM, Reilly CM, Zanger K, Stahl W, Das S. Combined cytotoxic and anti-invasive propertiesredox-active nanoparticles in tumor-stroma interactions. Biomaterials. 2011;32:2918-29.

[10] Krall AS, Xu S, Graeber TG, Braas D, Christofk HR. Asparagine promotes cancer cell proliferation through use as an amino acid exchange factor. Nat Commun. 2016;7:11457.

[11] Baskar G, Renganathan S. Optimization of L-asparaginase production by *Aspergillus terreus* MTCC 1782 using response surface methodology and artificial neural network linked genetic algorithm. Asia-Pac J Chem Eng. 2012;7:212-20.

[12] Baskar G, Chandhuru J, Fahad KS, Praveen AS, Chamundeeswari M, Muthukumar T. Anticancer activity of fungal L-asparaginase conjugated with zinc oxide nanoparticles. J Mater Sci Mater Med. 2015;26:43.

[13] Zoriţa D, Lucian BT, Cristina C, Loredana L, Amalia MR, Oana P, Dumitrita R, Răzvan Ş, Flaviu T, Septimiu T, Carmen S. Cerium oxide nanoparticles and its cytotoxicity human lung cancer cells. Rom Biotech Lett. 2015;20:1-9.

[14] Wriston JCJr, Yellin TO. L-Asparaginase: a review. Adv Enzymol Relat Areas Mol Biol. 1973;39:185-8.

[15] Bradford MM. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.

[16] Mossman T. Rapid colorimetric assay for cellular growth and survival–application to proliferation and cytotoxicity assays. J Immunol Methods. 1983:65:55-63.

[17] Shang L, Nienhaus K, Jiang X, Yang L, Landfester K, Mailander V, Simmet, T, Nienhaus GU. Nanoparticle interactions with live cells: Quantitative fluorescence microscopy of nanoparticles size effects. Beilstein J Nanotechnol. 2014;5:2388-97.

****

