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Research Article

Anticancer Activity of Iron oxide Nanobiocomposite of Fungal Asparaginase

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Abstract

Drug delivery using nanoparticles has been found to be more effective compared to normal drug delivery and has many advantages. Iron oxide nanoparticles synthesis is of greater importance because targeting the drug to the desired location is brought by applying magnetic field, which is of lesser cost. In the present work iron oxide nanobiocomposite was produced using L-asparaginase rich culture filtrate produced from *Aspergillus terreus*. Synthesized iron oxide nanobiocomposite was characterized using UV-Vis spectrum, X-Ray Diffraction (XRD), Fourier Transform Infra-Red (FTIR), Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray (EDX) analysis. An absorption peak at 330 nm confirmed the formation of iron oxide nanoparticles by fungal culture filtrate with asparaginase. The presence of functional groups like O-H, C=O, C-H and C=N on surface of the iron oxide nanobiocomposite was confirmed by FT-IR analysis. The XRD analysis proved that the synthesized nanobiocomposites are crystalline in nature. Produced nanobiocomposites were in the size range of 40-100 nm. The EDX analysis confirmed the presence of iron and oxygen in the synthesized nanobiocomposite. The MTT assay was carried to study the Anti-cancerous nature of the synthesized nanobiocomposites on MCF-7 cell line. MTT assay showed that decrease in cell viability, the viability of the cells decreased to 41.9% when it was treated with iron oxide nanobiocomposite of asparaginase. Thus it is proved that the iron oxide nanobiocomposite of asparaginase is a potential anticancer drug.

Keywords: Nanobiocomposite; Characterization; L-asparaginase; Anticancer activity; Drug delivery.

Introduction

Development in nanotechnology is responsible for synthesizing nanomaterials for various applications. Nanobiotechnology is the field where biotechnologists play a major role in production of nanomaterials using biological method for various biomedical applications [1,2]. Large surface area to volume of nanomaterials has given us an opportunity to coat the drug and use it as a drug carrier. Using nanomaterials as drug carriers has many advantages like increased bioavailability. Nanomaterials can easy penetrate the cell as a result dosage dependent toxicity can be avoided. Toxicity due to bioaccumulation can be avoided due to easy excretion nanobiomaterials and decreased amount of drug required to cause intended effect [3-5]. Researchers focus on biological method for synthesis of nanomaterials leaving mechanical, chemical and physical methods. Biological method has many advantages such as non-toxic, no harsh chemicals used, high stability and do not aggregate. This has made us to focus on biological method for synthesizing nanoparticles. Nature has provided us with a number of biological resources such as plants and plant products, algae, fungi, yeast, bacteria, and viruses for synthesis of nanoparticles [5-7].

Research reports on synthesis and applications of bifunctional and multifunctional nanomaterials in medicine field are very rare. Bifunctional nanomaterials are used for both therapeutic and diagnostic purpose, whereas multi-functional nanoparticles are used for therapeutic, diagnostic and imaging purposes [8-9]. The use of nanobiocomposites in the field of medicine is very less; few reports are available on the use of nanocomposites to target the cancer cells. Metalloproteins are nothing but metal nanoparticles coated with proteins on its surface which may have therapeutic value. Metalloproteins have many advantages over the metal nanoparticles in medicine field [10,11].

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Therapeutic drugs are becoming more tailored to specific diseases and patients in recent years [5,12]. Hydrogenase enzyme from *Fusarium oxysporum* was reported for synthesis of platinum nanoparticles [13]. Zinc oxide nanoparticle conjugated with L-asparaginase was reported for good antimicrobial and anticancer activity against MCF-7 cell lines [14,15]. Therefore the present work was focused on production of multifunctional therapeutic nano-metalloproteins (nanobiocomposite) using *Aspergillus terreus* for use as drug in anticancer applications.

Materials and methods

Fungi growth conditions for inoculum

The fungi *Aspergillus terreus* was obtained from IMTECH, Microbial Type Culture Collection (MTCC), Chandigarh, India. The fungus was sub-cultured in modified Czapek agar slants for 96 hrs at 32°C and refrigerated at 4°C for further use.

Biosynthesis iron oxide metalloproteins (nanobiocomposite) using Aspergillus terreus

Aspergillus terreus was used for the synthesis of metalloproteins as it has the ability to produce both L-asparaginase and nanoparticles. *A. terreus* was inoculated in three different 500 ml Erlenmeyer flask containing modified Czapek-Dox liquid medium containing (g/100ml of distilled): 2.0 L-Proline, 1.0 L-Asparagine, 0.2 glucose, 1.0 sodium nitrate, 0.052 pottasium chloride, 0.152 Di-pottasium hydrogen sulphate, 0.001 zinc sulphate, 0.001 copper sulphate, 0.001 ferrous sulphate and 0.052 magnesium sulphate, maintained at pH 6.2.L-Asparagine present in the media acts as an inducer for L-asparaginase. *A. terreus* was grown aerobically in an orbital shaker at 160 rpm at 32°C for 4 days. After the incubation period the culture was filtered under vacuum through Whatman #2 filter paper.

Filtrate contains rich L-Asparaginase and proteins required for reducing the metal ions to nanoparticles [15]. The filtrate obtained was mixed with $FeCl_3$ (1 mM concentration) in 500 ml Erlenmeyer flasks and kept in a shaking incubator at 150 rpm at 32°C for 24 hr [16,17]. Change in the colour of filtrate from pale yellow to dark brown colour in the flask confirmed the formation of iron oxide nanoparticles. The suspended particles in the flasks were separated by centrifugation at 10,000 rpm for 10 min and lyophilized for characterization.

Characterization of iron oxide nanoparticles and iron oxide metalloproteins

Optical properties of the iron oxide nanoparticles and iron oxide metalloproteins were analyzed using UV-Visible spectroscopy. The samples collected after the transformation process was analyzed using SYSTRONICS Double Beam UV-Visible spectrophotometer 2201. The spectrum values were obtained between the wavelength range 200 to 900 nm. The characteristic functional groups present in the iron oxide nanoparticles and iron oxide metalloproteins were analyzed using Fourier Transform-Infra Red (FT-IR) spectroscopy. FT-IR spectroscopy was measured on BRUKER α-T FT-IR Spectrometer. The samples were mixed with KBr (binding agent) and were made into discs at high pressure using hydraulic press. These discs were scanned in the range of 500 to 4000 cm^{-1} to obtain FT-IR spectra. Lyophilised iron oxide metalloproteins were examined by SEM on a QUANTA 200 with magnification range 35 to 30,000, equipped with Energy Dispersive Spectroscopy (EDS).

The structure of the synthesized nanoparticles was studied using XRD analysis. XRD patterns were recorded on a XPERT-PRO diffractometer. This diffractometer uses Cu-K as an anode, acts as a X-Ray source (wavelength = 1.54060 Å), operating with Cu- tube radiation at 40 KV and 30 mA. The scan step for 2θ was 0.0170° with a scan step time of 38.1s. Approximately 1 g of the lyophilized sample was taken and XRD analysis was performed.

Assay of L-asparaginase activity

On addition of L-asparagine to the cell free filtrate, crude L-Asparaginase catalyzes L-Asparagine to form aspartic acid and ammonia at 37°C. The concentration of ammonia was determined by using Nesslers reagent method. 0.1 ml of the cell free filtrate was taken. 900 µl of 0.1M phosphate buffer pH 8.5 and 1ml of 0.04M L-Asparagine solution was added to it and incubated at 37°C for 10 min. The reaction was stopped by the addition of 500 µl of 15% Trichloro acetic acid. Centrifugation was done at 6000 rpm for 15 min.

Then 0.1 ml of the supernatant was taken and diluted to 8ml with distilled water. 1ml of 2M NaOH and 1ml of Nessler's reagent was added. After 10 min the absorbance value was taken at 480 nm [18]. One unit (IU) of L-Asparaginase activity is defined as the amount of enzyme which liberates 1 µmole of ammonia per minute under the standard assay conditions.

Anticancer activity of the iron oxide metalloproteins

The MCF-7 cells were cultured to carryout *in vitro* anti-cancerous studies. The cells were grown in folate-free Dulbecco Modified Eagles Medium (FFDMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and100 µg/mL streptomycin and 4 mM L-glutamine, incubated at 37°C in a 5%CO_2/95% air humidified atmosphere. The cytotoxicity of the prepared samples were measured using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, ayellow tetrazole) assay [19].

The cells were grown in 96 wells tissue culture plates at a density of 0.2 X 10^6 cells/well. After 24 h the old medium from each well was discarded and replaced with filtrate containing increasing concentration of iron oxide metalloproteins. After 24 h incubation, 10 µl of MTT solution was added at a final concentration of 0.5mg/mL in phenol-red-free Roswell Park Memorial Institute (RPMI) medium containing 10% heat inactivated fetal bovine serum (FBS) in the dark at 37 °C. The cells were incubated for 4 h and the supernatant from each well were carefully removed without disturbing blue formazan crystals produced by the viable cells. The purple crystals were then dissolved completely using 100 µl of solubilising buffer (1:1dimethyl formamide/20% sodium dodecyl sulfate). Colorimetric measurements were performed at 595 nm with a scanning multi-well spectrometer (Thermo, USA).

The untreated cells absorbance was used as control reference. The cell viability was calculated using the equation 1.

% of cell viability = OD540 (sample)/ OD540 (control) X 100 (1)

where O.D. of the sample represents iron oxide metalloproteins treated MCF-7cells and O.D. of control refer to untreated MCF-7 cells.

Results and discussions

L-Asparaginase activity

The L-asparaginase activity of the free enzyme was found as 29.96 IU/ml. The asparaginase activity of the iron oxide nanobiocomposite was 37.86 IU/ml. The L-asparaginase activity of iron oxide nanobiocomposite was increased to 126%.

UV spectrum analysis of iron oxide nanobiocomposite of fungal asparaginase

The Iron oxide nanoparticles were produced using fungus *A. terreus* filtrate. The colour of the culture filtrate was changed from pale yellow to dark brown colour after 48 hrs of incubation. The colour change was might be due to the excitation of surface plasmon resonance (SPM), this indicates the reduction of ferric chloride ions by the proteins present in fungal culture filtrate which resulted in the formation of nanoparticle. Optical properties of the Iron oxide nanoparticles can be studied from UV spectrum analysis. Fig. 1 shows the absorption peaks for Iron oxide nanoparticles and metalloproteins.

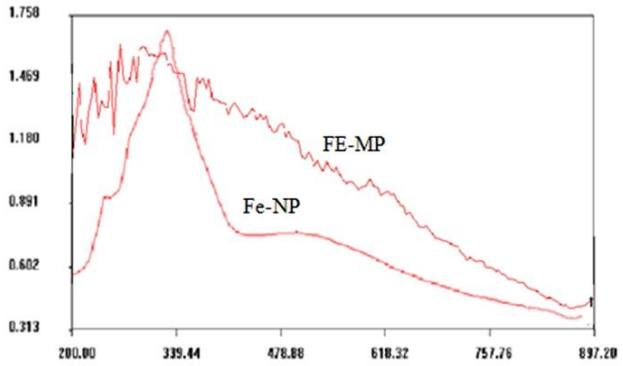

Fig. 1. UV Spectrum of synthesized iron oxide nanoparticles (Fe-NP) and iron oxide metalloprotein (Fe-MP)

An absorption peak at 330 nm in both the samples confirms the presence of iron oxide nanoparticles in the liquid sample obtained after 48 hours of incubation. The peaks at 220 and 270 nm were also observed, which confirmed the presence of protein in the sample along with the iron oxide nanoparticles. The protein peak formed is might be due to the presence of L-asparaginase in the sample along with nanoparticles. Formation of absorption peak at 330 nm is due to the excitation of Surface Plasmon Resonance (SPM).

FT-IR Spectrum analysis of iron oxide nanobiocomposite of fungal asparaginase

The synthesized iron oxide nanoparticle was analyzed using FT-IR spectroscopy to understand various functional groups associated with the synthesized nanoparticles. The peaks indicate the characteristics functional group present in the synthesized iron oxide nanoparticles as shown in Fig. 2(a). Absorption peaks in the range of 3422.75 cm^{-1}, 2928.31 cm^{-1}, 1661.06 cm^{-1}, 1543.82 cm^{-1}, 1268.93 cm^{-1}, 1041.20 $cm^{-1}$and 561.13 cm^{-1} were observed. The absorption peak at 561.13 cm^{-1} may corresponds to vibrational mode of metal-oxygen (iron-oxide).

The peak at 1041.20 cm^{-1} is ascribed to aliphatic fluro compounds, primary amine C-N stretch. The peak at 1268.93 $cm^{-1}$and 1384.72 cm^{-1} are ascribed to primary or secondary alcohol O-H in-plane bend, aromatic ethers, aryl-o- stretch and gem – Dimethyl or 'tert – butyl' group. The peak at 1543.82 cm^{-1} is ascribed to aliphatic and aromatic nitro compounds.The peak at 1661.06 cm^{-1} is ascribed to alkenyl C=C stretch and open chain imino (-C=N-).The peak at 2928.31 cm^{-1} is ascribed to methylene C-H asym./sym. stretch.The peak at 3422.73 cm^{-1} is ascribed to hydroxyl group, H- bonded O-H stretch.

The synthesized iron metalloprotein was also subjected to FT-IR analysis to understand various functional groups associated with the synthesized nanoparticles. The peaks indicate the characteristics functional group present in the synthesized iron metalloprotein as shown in Fig. 2(b).

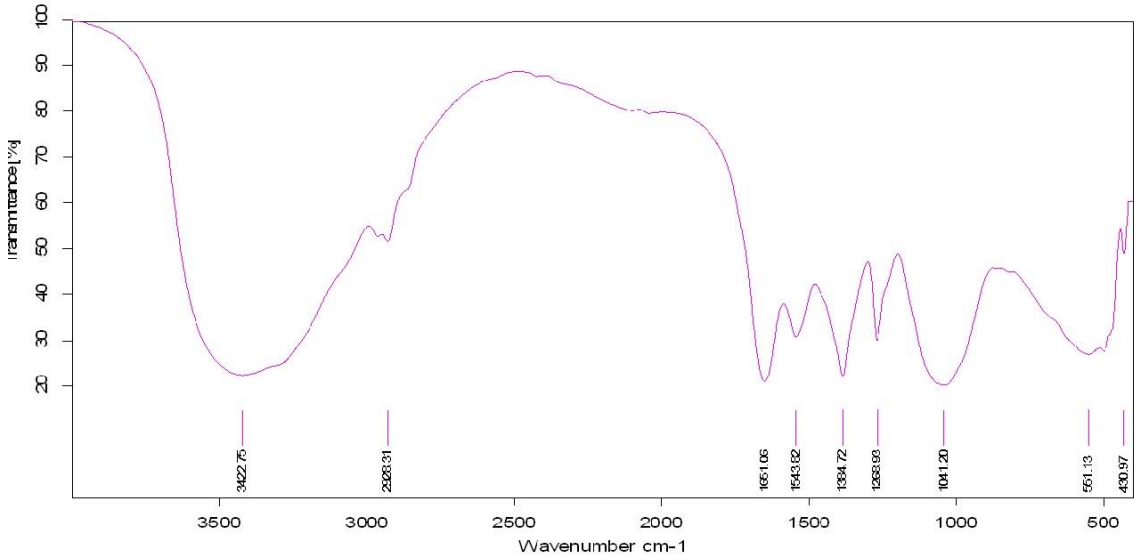

Fig. 2(a) FT-IR spectrum of synthesized iron oxide nanoparticles

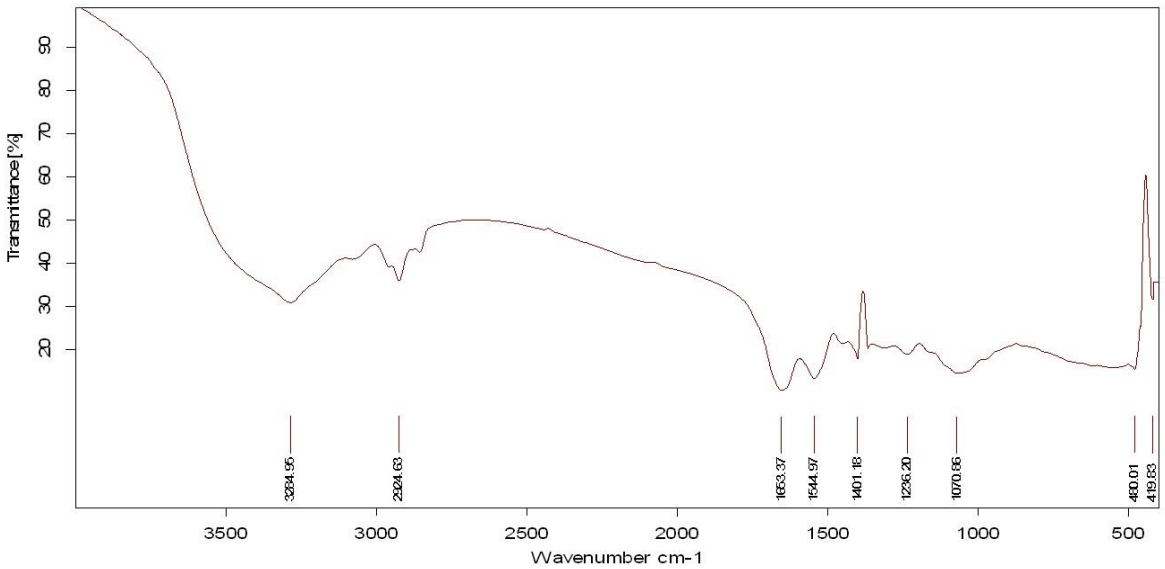

Fig. 2(b). FT-IR spectrum of synthesized iron-oxide nanobiocomposite

Absorption peaks were observed in the range of 3284.96 cm^{-1}, 2924.63 cm^{-1}, 1653.37 cm^{-1}, 1544.97 cm^{-1}, 1401.18 cm^{-1}, 1070.86 $cm^{-1}$and 480.01 cm^{-1}. The absorption peak at 480.01 cm^{-1} may corresponds to vibrational mode of metal-oxygen (iron-oxide). The peak at 1401.18 cm^{-1} is ascribed to carboxylate group and organic sulphates. The peak at 1544.97 $cm^{-1}$and 1653.37 cm^{-1} are ascribed to aliphatic or aromatic nitro compounds and alkenyl C=C stretch or open-chain aimino group. The peak at 2924.63 cm^{-1} is ascribed to methylene C-H asym./sym. stretch. The peak at 3284.96 cm^{-1} is ascribed to hydroxyl group or normal polymeric O-H stretch. Thus from the FT-IR analysis the protein conjugated to the iron-oxide nanoparticles was confirmed. The protein conjugated iron-oxide nanoparticle is known as iron oxide metalloprotein (iron oxide nanobiocomposite).

XRD analysis of iron oxide nanobiocomposite of fungal asparaginase

The XRD pattern obtained from iron oxide nanoparticles shows a number of Bragg reflections characteristic of Fe_3O_4. Sharper and stronger diffraction peaks were observed from Fig. 3 at 11.53°, 23.98°, 29.81°, 37.62° and 37.64°.The peaks 23.98 and 29.81 are assigned to the planes 012 and 211. The average crystallite size was calculated by the Debye Sherrer formula $D = K \lambda / \beta_{1/2}\cos\theta$ where K is the Sherrer constant (K=0.9 for spherical particle), λ is the X-ray wavelength (λ=1.54060 Å), $\beta_{1/2}$is the width of the XRD peak at half height, θ is the Bragg diffraction angle.

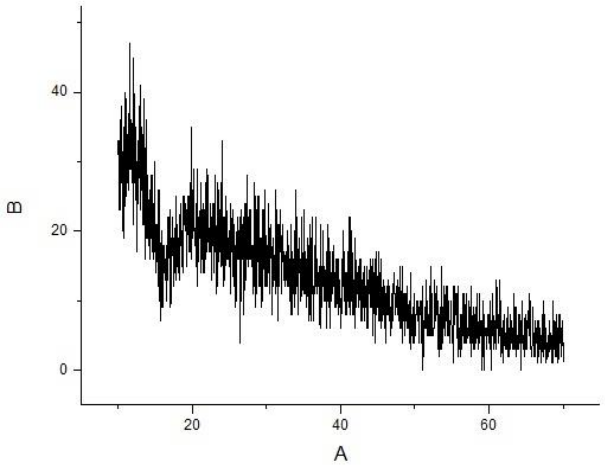

Fig. 3. XRD pattern of synthesized iron-oxide nanobiocomposite

Structural Characterization of iron oxide nanobiocomposite using SEM

The localization of iron oxide nanobiocomposite was done in SEM using a secondary electron detector. It can be clearly seen from the Fig. 4 that the nanobiocomposite in the samples were almost round shaped and compactly arranged. The size range of nanobiocomposites was in between 40-100 nm.

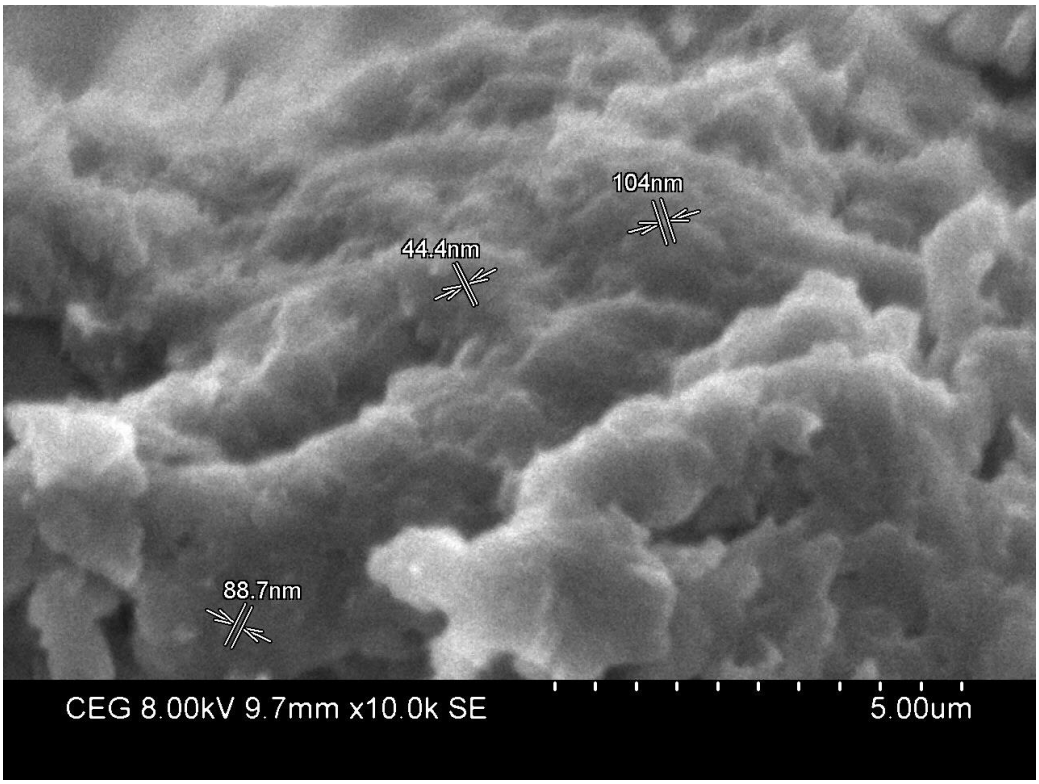

Fig. 4. SEM image of synthesized iron-oxide nanobiocomposite

Anticancer activity of the synthesized iron oxide nanobiocomposite

After culturing the MCF-7 cells the anti-cancerous activity of iron oxide nanobiocomposite was studied through MTT assay. The percentage of cell viability based on the optical value from the incubated control well was calculated. MCF-7 cells were grown in 96 wells tissue culture plates. Increasing volumes of culture filtrate containing iron oxide nanobiocomposite was added and incubated. The viability of the cell decreased to 41.9% in the wells treated with iron oxide nanobiocomposite (Fig. 5). Thus the iron oxide nanobiocomposite was found to have potential anticancer activity.

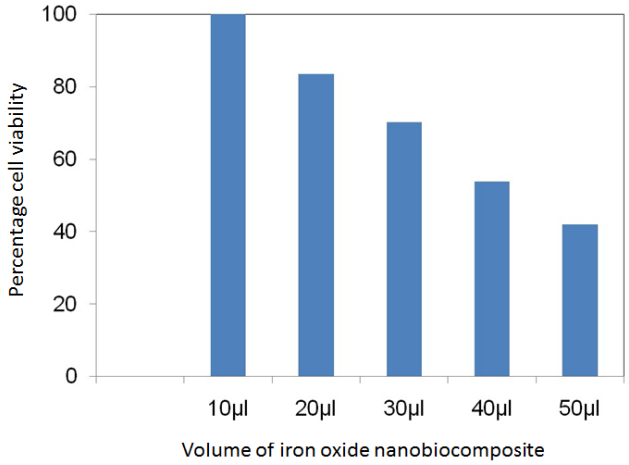

Fig. 6. Viability of MCF-7 cancer cells at varied concentration of iron oxide nanobiocomposite

Conclusions

Aspergillus terreus was effectively used to produce bi-functional iron oxide nanobiocomposite. The XRD results confirmed the formation of crystalline iron oxide nanoparticles. The UV absorption peak at 330 nm confirmed the formation of iron oxide nanobiocomposite. The functional groups present in the iron oxide nanoparticles and iron oxide metalloproteins were found by FT-IR analysis. The size range of iron oxide metalloproteins was found in the range of 40-100 nm using SEM analysis. Decrease in the cell viability of MCF-7 cell lines on treatment with iron oxide metalloprotein proves its anti-cancerous activity. Thus the synthesized bi-functional iron oxide nanobiocomposite can be used for treatment cancer.

Conflict of interest

Authors declare there are no conflicts of interest.

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