Microbial Synthesis of Platinum Nanoparticles and Evaluation of Their Anticancer Activity

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Abstract: Present work describes an eco-friendly method for the synthesis of platinum nanoparticles (PtNPs) using cell free extract (CFE) of *Saccharomyces boulardii*. Nanoparticle (NP) formation was confirmed by UV-Vis spectroscopy, primary size determination by zeta sizer analysis, elemental composition by EDX analysis, functional group detection by FTIR analysis and size and shape by electron microscopy (SEM & TEM). Parameters such as salt concentration, temperature, culture age, cellmass concentration, pH, and reaction time have been optimized to exercise a control over the yield of nanoparticles and their properties. Synthesized nanoparticles had the size range of 80-150 nm with negative zeta potential (−14.8±3 mV) indicating excellent stability. Role of proteins/peptides in NP formation and their stability were also elucidated. In vitro anticancer activity of PtNPs was evaluated using A431 cell line which showed the IC_{50} value of NPs >100 µg/ml and for MCF-7 cell lines IC_{50} values of 70 µg/ml was obtained after 24 h treatment. After 48 h, the IC_{50} values were found to be 57 and 65 µg/ml for A431 and MCF-7 cell lines, respectively. These results are promising for the use of biologically synthesized PtNPs as an anticancer agent.

Keywords: Anticancer activity, microbial synthesis, platinum nanoparticles.

I. INTRODUCTION

Microbial synthesis of nanoparticles has been emerged as a promising field of research as 'nanobiotechnology' interconnecting biotechnology and nanotechnology. Synthesis of monodisperse nanoparticles with different chemical composition, size and shape has been a challenge in nanotechnology [1]. Various physical and chemical methods are extensively used to synthesize monodispersed nanoparticles. The use of toxic chemicals, non-polar solvents, high temperature and high pressure in the synthesis procedure limit their applications. Therefore, it is necessary to develop clean, biocompatible, non-toxic and eco-friendly methods for nanoparticle synthesis. Although biological methods are regarded as safe, cost-effective, sustainable and eco-friendly processes, they also have some drawbacks in the generation of catalysts in the form cellmass and difficulties in providing better control over size distribution, shape and crystallinity. These problems limits the biological synthesis approaches. However these can be solved by strain selection, optimizing the conditions such as pH, incubation temperature, reaction time, concentration of metal ions and the amount of biological material [2]. Biosynthesis of silver, gold, gold–silver alloy, platinum, tellurium, magnetite and uranium nanoparticles by bacteria, plant, actinomycetes, fungi, yeast and viruses have been reported [3]. The industrial sectors most readily taking on nanotechnology are information and communication sector, including electronic and optoelectronic fields, food technology, energy technology, pharmaceuticals and drug delivery systems, diagnostics and medical technology.

Metal nanoparticles have potential applications in various areas such as electronics, cosmetics, coatings, packaging and biotechnology. Usually, nanoparticles possess a wavelength below the critical wavelength of light. This renders them transparent, a property that makes them very useful for applications in cosmetics, coatings and packaging. Platinum nanoparticles are highly significant in the industrial production of fuel cells and used as catalyst for the reduction reactions in organic synthesis, while gold particles have currently come under investigation in catalysis research [4]. Platinum nanoparticles also has application as affinity probe and matrix for direct analysis of small biomolecules and microwave digested proteins using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [5]. Nanowires and nanocantilever arrays are among the leading approaches under development for the early detection of precancerous and malignant lesions from biological fluids [6].

II. MATERIALS

Chloroplatinic acid hydrate (99.99%) was purchased from Sigma-Aldrich (Steinheim, Germany). Different media components were purchased from HiMedia (Mumbai, India) and Central Drug House (P) Ltd. (New Delhi, India). *Saccharomyces boulardii*, a probiotic strain was purchased from local market.

III. EXPERIMENTAL METHODOLOGY

A. Reaction of Chloroplatinic Acid with Whole Cells

The strain of *S. boulardii* was inoculated in 20 ml yeast peptone dextrose (YPD) medium. Culture was incubated at 35°C in incubator shaker (Innova) at 200 rpm for overnight. Inoculum (2 % v/v) was transferred to production medium and incubated in rotary shaker at 200 rpm for 24-48 h depending upon the growth requirement of organisms. The

cells were harvested by centrifugation at 7000×g for 10 min. Supernatant was discarded and cell pellet was washed thrice with deionized water to remove the remaining media components. Wet cellmass was suspended in 10 ml water to give a final cell concentration of 10 % (w/v). The reaction was carried out at 35°C and 200 rpm for 72 h by incubating cells with chloroplatinic acid (0.5 mM). After 72 h, cells were observed for color change and reaction mixture was subjected to wavelength scan in UV-Visible spectrophotometer in the range of 200-500 nm.

B. Reaction of Chloroplatinic Acid with Cell Free Extract (CFE) of Microorganisms

After harvesting the cells, 1 g wet cellmass was suspended in 10 ml deionized water. The cell suspension was kept in rotary shaker at 35°C and 200 rpm for 36 h. After 36 h, the supernatant (CFE) was collected by centrifugation at 7000×g and 15°C for 10 min. Chloroplatinic acid was added to CFE to give a final concentration of 0.5 mM and reaction was carried out at 35°C and 200 rpm for 72 h. After 72 h, reaction mixture was observed for the presence of platinum nanoparticles.

C. In-vitro Evaluation of Anticancer Activity of Platinum Nanoparticles

Anticancer activity of platinum nanoparticles was evaluated *in vitro* using different cancerous cell lines (A431 and MCF-7). MCF-7 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and A431 from National Centre for Cell Science (Pune, India). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. DMEM and FBS were obtained from Sigma Chemicals (USA). Antibiotics were purchased from Invitrogen (USA). Cell cultures were maintained in flasks under standard conditions, incubation at 37°C with 5% CO_2. The cells were treated with different concentrations (10, 50 and 100 µg/ml) of PtNPs for 24 and 48 h.

Cell viability was performed using MTT assay (MTT was obtained from Hi-Media). Cells were incubated in the presence of increasing concentration of PtNPs (10, 50, 100 µg/ml). After 24 h, cells were treated with MTT solution for 4 h at 37°C in a cell culture incubator at 37°C with 5% CO_2. MTT which is a tetrazolium salt is converted into insoluble formazan by succinate dehydrogenase in live cells. Formazan was dissolved in DMSO (Merck) and absorbance was measured at dual wavelength of 550 nm and 630 nm on an ELISA plate spectrophotometer (FlexStation 3 instruments). The total number of viable cells relative to viable cells in untreated control was calculated. The same process was repeated after 48 h for both the cell lines.

D. Optimization of Various Physicochemical Parameters For Nanoparticle Synthesis

1) Metal salt concentration

Platinum can be highly toxic depending on its concentration and speciation. Different concentrations of chloroplatinic acid (0.2, 0.5, 1, 1.5, and 2 mM) were used for optimization of the reaction conditions. The flasks were kept at 35°C and 200 rpm for 72 h.

2) Temperature

Temperature has direct effect on the rate of reaction and enzyme stability. To optimize reaction temperature for the platinum nanoparticle synthesis, reaction flasks with CFE and chloroplatinic acid (0.5 mM) were incubated at various temperatures (20, 25, 30, 35, and 40°C) and 200 rpm for 72 h.

3) Cellmass concentration

To determine the optimum cellmass concentration for the synthesis of platinum nanoparticles, different cellmass concentration (100, 200, 300, 400, and 500 mg/ml) were used to produce CFE. To this CFE chloroplatinic acid (0.5mM) was added and flasks were kept in incubator shaker at 35°C with 200 rpm for 72 h.

4) pH

For the optimization of pH, chloroplatinic acid (0.5 mM) reacted with CFE of *S. boulardii* adjusting pH of CFE at 3, 5, 7, 9, and 11 at 35°C and 200 rpm for 72 h.

5) Reaction time

To optimize the reaction time, CFE was incubated with chloroplatinic acid (0.5 mM) at 35ºC and 200 rpm in an incubator shaker and UV-Vis absorbance was measured at regular interval of time from 6 to 72 h.

IV. CHARACTERIZATION TECHNIQUES

Platinum nanoparticles absorb light in the range of 200-300 nm depending upon their size and shape. The UV-Visible spectra of reaction mixture were recorded on U-3010 spectrophotometer (Hitachi) in the range of 200-500 nm. Nanoparticle formation is accompanied by the corresponding decrease in absorption in wavelength region from 200-300 nm. Zeta potential, particle size distribution and polydispersity index (PDI) measurements were done using zeta sizer (Malvern Zeta Sizer, Nano ZS). Platinum nanoparticle colloidal solution mounted on glass slide, after drying sample was subjected for scanning in microscope chamber at 15 kV. The surface images of platinum nanoparticles were taken by SEM-S3400N (Hitachi). Platinum nanoparticles were visualized by transmission electron microscopy (HRTEM-FEI). Putting a drop of platinum nanoparticle solution on carbon coated copper TEM grids formed thin film and the micrograph of the samples was obtained. Platinum nanoparticle solution was subjected to fourier transform infrared spectroscopy (FTIR) using Synthesis Monitoring System (Perkin Elmer) to examine the presence of functional group on the outer surface of platinum nanoparticles. Energy-dispersive X-ray spectroscopy of platinum nanoparticles was performed at 200 kV (EDAX-FEI).

V. RESULTS AND DISCUSSION

A. Reaction of Chloroplatinic Acid with Whole Cells

PtNPs has been successfully syntheiszed in water by

addition of chloroplatinic acid to the whole cell suspension of microorganisms. The culture of *S. boulardii* showed change in color due to presence of PtNPs but the absorbance peak was not obtained when the reaction supernatant was subjected to the UV-Vis spectroscopy. This might be due to absence of nanoparticles in the supernatant while intracellular nanoparticles were present.

B. Reaction of Chloroplatinic Acid with CFE of S. boulardii

Cell suspension of *S. boulardii* showed dark/greyish brown colour upon reaction, indicating that the intracellular synthesis of platinum nanoparticles. Therefore, new strategy was applied for the synthesis of platinum nanoparticles. The CFE of *S. boulardii* was used for the reaction. Chloroplatinic acid was added to the CFE and the color changed from pale yellow to greyish brown was observed after 72 h of reaction confirming formation of PtNPs. λ_{max} of reaction mixture was obtained at 260 nm. Because of surface plasmon resonance, platinum nanoparticles showed greyish brown colour in the solution.

C. Optimization of Synthesis of Platinum Nanoparticles Using CFE of S. boulardii

Reaction of chloroplatinic acid with CFE of *S. boulardii* gave higher yield of platinum nanoparticles and furhter optimization studies were carried out with CFE only.

1) Effect of chloroplatinic acid concentration

To study the effect of concentration of chloroplatinic acid on platinum nanoparticle synthesis, CFE of *S. boulardii* was reacted with the increased metal salt concentration ranging from 0.2 to 2 mM. Then reaction flasks were incubated at 35°C (200 rpm). At every 6 h interval, reaction mixture was observed by UV-Vis spectroscopy for the presence of platinum nanoparticles. It was observed that absorbance decreased (indicating nanoparticle formation) most when 0.5 mM chloroplatinic acid was used in reacrion mixture. Increase or decrease in concentration of chloroplatinic acid showed higher absorbance indicating slower rate of nanoparticle formation. Fig. 1 shows the comparison of UV-Vis absorbance between chloroplatinic acid blank and various concentrations of chloroplatinic acid reacted with CFE.

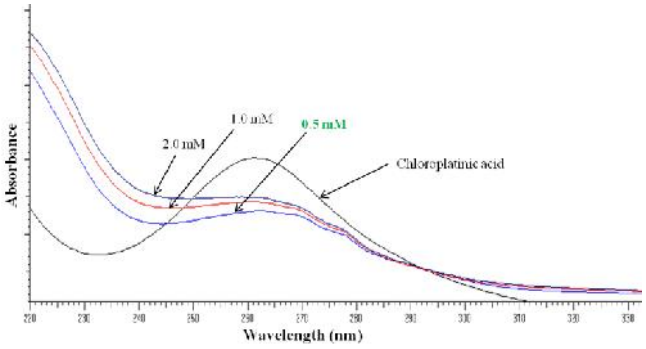

Fig. 1: UV-Visible absorbance spectra of PtNPs synthesized using different concentrations of chloroplatinic acid

2) Effect of temperature

Chloroplatinic acid was added to CFE to make its final concentration becomes 0.5 mM. The flasks were incubated at different temperatures (20°C, 25°C, 30°C, 35°C and 40°C) under the conditions mentioned above. At every 6 h interval, reaction mixture was observed by UV-Vis spectroscopy for the presence of platinum nanoparticles. UV-Vis absorbance data (Fig. 2) showed that increase in temperature increased the platinum nanoparticle synthesis upto 35°C beyond which it decreased.

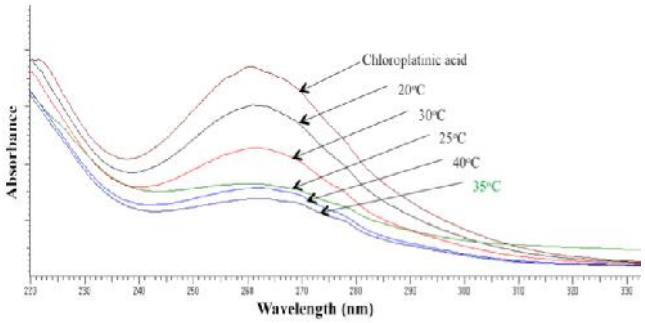

Fig. 2: UV-Visible absorbance spectra of PtNPs synthesized at different temperatures

3) Effect of cellmass concentration

Different cellmass concentrations were used for the preparation of CFE. Cells were suspended in deionised water making their final concentration as 100 mg, 200 mg, 300 mg, 400 mg, and 500 mg per ml of deionised water. The CFE obtained was used for studying the effect of cellmass concentration on platinum nanoparticle synthesis. Chloroplatinic acid was added to each flask to make final concentration of 0.5 mM and incubated at 35°C (200 rpm) for 72 h. Reaction mixture was observed for presence of platinum nanoparticles at regular interval. UV absorbance data (Fig. 3) revealed that increase in cellmass concentration from 100 mg/ml to 500 mg/ml resulted in increase in the PtNPs synthesis.

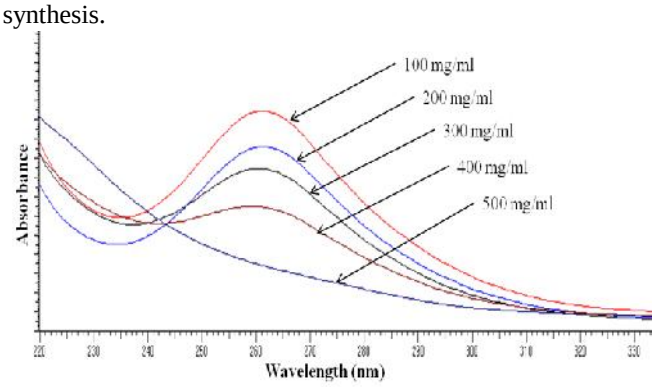

Fig. 3: UV-visible absorbance spectra of PtNPs synthesized by using various cellmass concentrations

4) Effect of pH

pH of the medium affects reduction of Platinum ion (Pt^{+4}) to elemental Platinum (Pt^{0}). To study the effect of pH on PtNPs synthesis, CFE prepared from *S. boulardii* cells adjusted to different pH (3, 5, 7, 9, and 11). Chloroplatinic acid was added making final concentration of 0.5 mM. Reaction flasks were incubated at 35°C (200 rpm) for 72 h. Absorbance was measured at regular interval for presence of

PtNPs. Fig. 4 showed UV-Visible absorbance spectra of PtNPs synthesized at different pH of reaction mixtures.

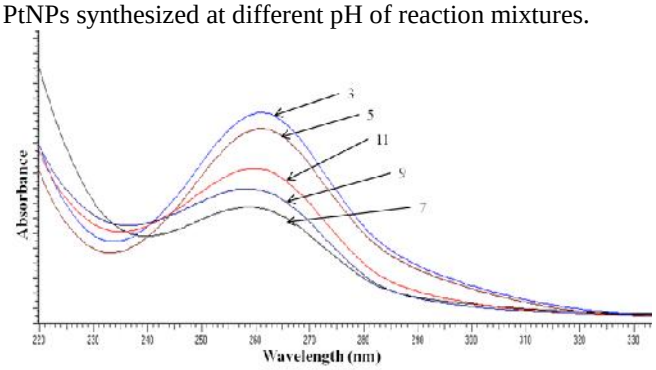

Fig. 4: UV-visible absorbance spectra of PtNPs synthesized at different pH conditions

5) Effect of reaction time

The reaction time directly affects size of nanoparticles [7]. To determine the optimum reaction time, harvested *S. boulardii* cells were incubated with chloroplatinic acid (0.5 mM) at 35°C (200 rpm). At 6 h interval, sample was withdrawn and absorbance was measured to test the formation of platinum nanoparticles. Microbial synthesis of PtNPs is a reduction step followed by nucleation and crystal growth. As incubation time increases, the size of the nanoparticles formed is also increased because of more deposition of the reduced elemental Pt on the already formed nanoparticles. Till 12 h there was no color change and very little change in absorbance. After 36 h of reaction greyish brown coloration appeared indicating the formation of PtNPs. As shown in the Fig. 5, absorbance decreased with time of reaction indicating the formation of nanoparticles.

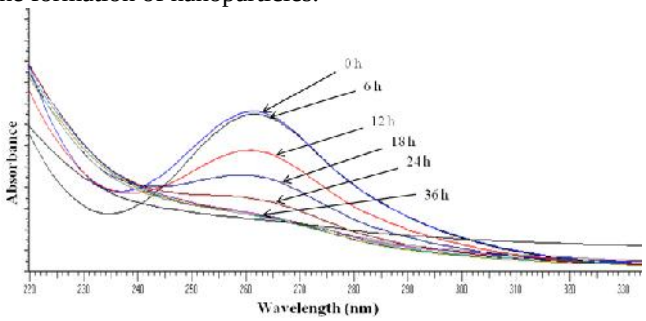

Fig. 5: UV-Visible absorbance spectra of PtNPs synthesized by CFE of *S. boulardii* at different time period

D. Characterization of Nanoparticles

1) Zeta sizer and zeta potential

Zeta potential and particle size distribution (obtained by zeta sizer) of PtNPs provided further details of size, shape and morphology of the synthesized nanoparticles. Fig. 6 (a) gives –ve zeta potential of a representative sample indicating the presence of negatively charged molecules on the particles that repel the particles from each other preventing their agglomeration. Fig. 6 (b) represents dynamic light scattering (particle size distribution) analysis of platinum nanoparticles formed showing average particle size of 115 nm with PDI of 0.24.

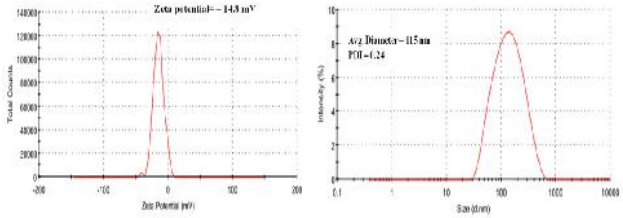

Fig. 6: (a) Zeta potential, (b) Particle size distribution of PtNPs synthesized by CFE of *S. boulardii*

2) SEM analysis

SEM analysis showed that PtNPs are spherical in shape and have average particle diameter of 90 nm. The representative SEM image of the sample is shown in Fig. 7.

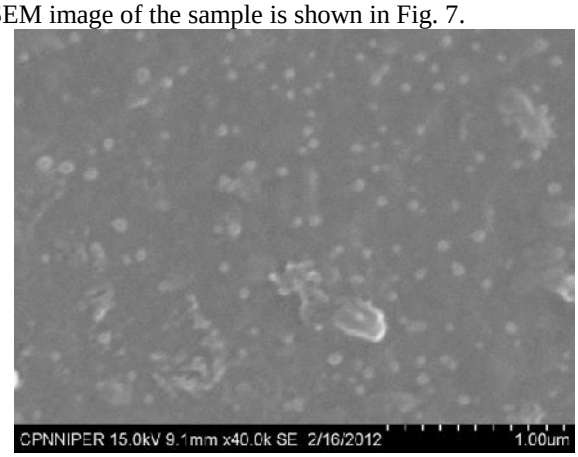

Fig. 7: SEM image of PtNPs synthesized by CFE of *S. boulardii*

3) TEM Analysis

Fig. 8 shows TEM image of PtNPs with average particle size of 120 nm and coated by protein layer. This protein/s may be responsible for reduction as well as capping of the synthesized nanoparticles.

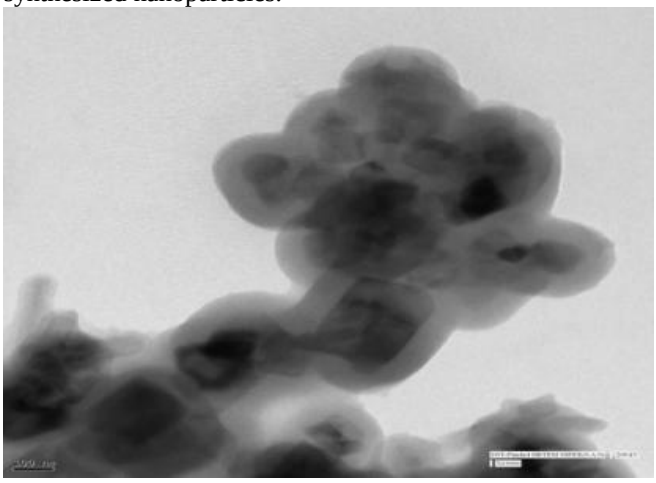

Fig. 8: TEM image of PtNPs (scale 100 nm) synthesized by CFE of *S. boulardii*

4) FTIR spectra analysis

Fig. 9 shows FTIR spectra of the platinum nanoparticles. Two bands at 1634 and 3434 cm^{-1} may be assigned to the amide and OH^- of the carboxyl group of amino acid of proteins respectively. It is well known that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins [8]. These capping agents might be

responsible for the stability of the biosynthesized metal nanoparticles [9].

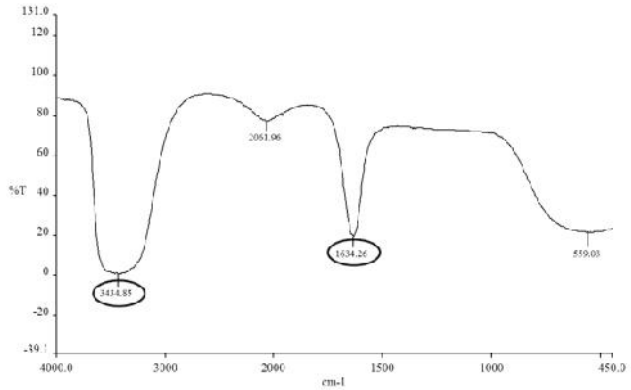

Fig. 9: FTIR spectra of PtNPs synthesized by CFE of *S. boulardii*

5) EDX/EDS analysis

Energy dispersive x-ray analysis showed peak at 2 keV indicating the presence of elemental platinum in the sample (Fig. 10).

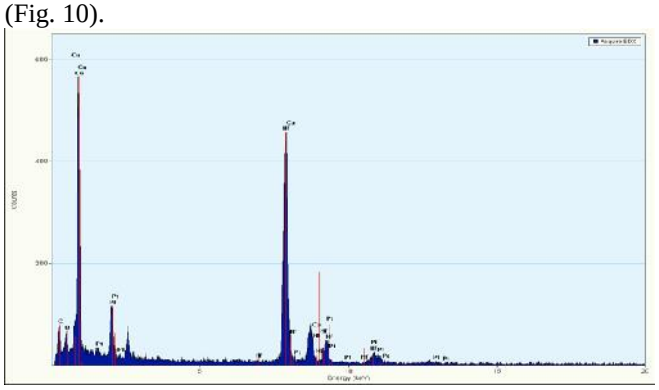

Fig. 10: Energy Dispersive X- ray analysis of PtNPs synthesized by CFE of *S. boulardii*

E. In-vitro Evaluation of Anticancer Activity of PtNPs

Cell lines on treating with different concentrations of PtNPs using different concentrations (10, 50, 100 µg/ml) showed promising anticancer effect at higher concentration (100 µg/ml). As the toxicity profile was not studied for the PtNPs, higher concentration more than 100 µg/ml was not used. Fig. 11 shows that there was 44 and 54% reduction in viability of A431 and MCF-7 cells, respectively after 24 h of PtNPs treatment. This percentage reduction in viability of cancerous cells indicates the IC_{50} value for A431 cells >100 µg/ml and that for MCF-7 is 70 µg/ml after 24 h treatment.

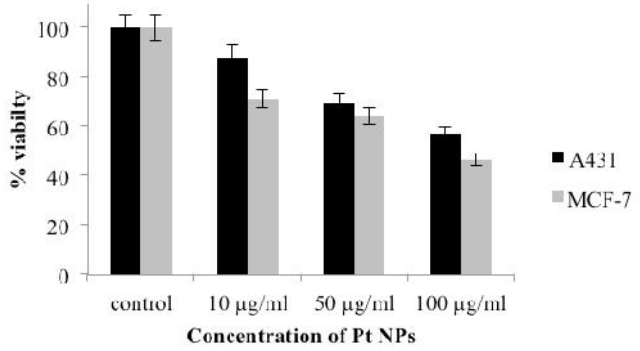

Fig. 11: *In vitro* anticancer activity of PtNPs (24 h) synthesized by CFE of *S. boulardii*

Fig. 12 showing that there was 79 and 60% reduction in viability of A431 and MCF-7 cells, respectively after 48 h of PtNPs treatment. This percentage reduction in viability of cancerous cells indicates the IC_{50} value for A431 cells was 57 µg/ml and that for MCF-7 cells is 65 µg/ml after 48 h treatment.

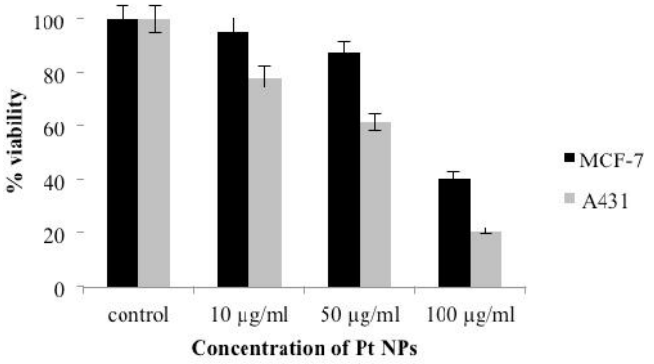

Fig. 12: *In vitro* anticancer activity of PtNPs (48 h) synthesized by CFE of *S. boulardii*

VI. CONCLUSION

With the growing importance of platinum nanomaterials both in health and engineering applications, the cost-effective route of nanoparticle synthesis using microorganisms becomes more and more important. There are limited studies on platinum naoparticle synthesis using microorganisms. Considering an importance in the areas of research in nanobiotechnology, which deals with the synthesis of nanoparticles of different chemical compositions, sizes, shapes and controlled monodispersity, we have synthesized PtNPs using CFE of *S. boulardii*.

In this study, it was found that CFE generated from a cellmass concentration of 500 mg/ml in presence of chloroplatinic acid (0.5 mM) at 35°C (pH 7, 200 rpm) on 36 h showed maximum PtNPs synthesis. These conditions were selected for future studies. Reactions carried out in optimized conditions gave platinum nanoparticle with the size range between 80-150 nm and -14.8±3 mV zeta potential. FTIR spectra shows the bands which confirm involvement of amide and hydroxyl group containing capping agent on the platinum nanoparticles. EDX analysis shows the presence of platinum in the NPs.

In vitro anticancer activity of PtNPs was evaluated using A431 cell lines which show the IC_{50} value for PtNPs >100 µg/ml while with MCF-7 cell lines the IC_{50} value was found to be 70 µg/ml after 24 h treatment. After 48 h, the IC_{50} value was found to be 57 µg/ml for A431 and 65 µg/ml for MCF-7 cell lines. These results are promising for the use of biologically synthesized PtNPs as an anticancer agent.

ACKNOWLEDGMENT

Authors would like to thank Department of Biotechnology, Government of India for providing the research funding. Authors also gratefully acknowledge to Central Instrumentation Laboratory, Centre for Pharmaceutical Nanotechnology, Department of Pharmacology of NIPER,

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Mohali for providing various characterization facilities and cell lines studies, respectively.

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Second Author Abhishek Kaler has completed his PhD from NIPER, Mohali. His area of research includes synthesis of metallic nanoparticles via biological reducing agents. He has published 12 research articles focusing on synthesis of silver nanoparticles and studying their free radical scavenging and antioxidant activity. He also has explored the anticancer activity of them. He has explained probable mechanism of metallic nanoparticle formation via biological media.

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