ejpmr, 2017,4(1), 401-407

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

IN VITRO CYTOTOXICITY TESTING OF BIOSYNTHESISED SILVER NANOPARTICLE OF ANDREDERA CORDIFOLIA IN PROSTATE CANCER CELLS (PC-3)

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Article Received on 11/12/2016 **Article Revised on 01/12/2016** **Article Accepted on 21/12/2016**

ABSTRACT

Silver nanoparticles because of their interesting characteristics are currently the most widely used nanoparticles. One of the interesting properties is that they display antimicrobial activity. Concomitantly they exert cytoprotective effect on human cells. It is of interest to validate and analyse this property. In the present study, silver nanoparticles were synthesised with particle size between 40-60nm in diameter. These were then used to analyse their uptake and consequent cytotoxic effects on Human Prostate cancer cells (PC-3). The formation of Ag-NPs was confirmed by UV-Visible Spectroscopy, X-Ray Diffraction (XRD) pattern, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The synthesized Ag-NPs were predominately spherical in shape and polydispersed. Fourier Transform Infra-Red (FT-IR) spectroscopy analysis showed that the synthesized nano-Ag was capped with bimolecular compounds which are responsible for reduction of silver ions. The AgNPs showed potent cytotoxic activity against the human prostate cancer (PC-3) cell line at higher concentrations.

KEYWORDS: Cytotoxic effect, PC-3 cell line, silver nanoparticle,*Andredera cordifolia*,Cell viability,etc

INTRODUCTION

Carcinogenesis is a complex and multi-step process in which distinct molecular and cellular alterations occur. A cancer chemopreventive agent could be effective at any of these defined stages of carcinogenesis: initiation, promotion, progression.[1, 2] The preventive mechanisms of tumor promotion by natural phytochemicals range from the inhibition of genotoxic effects, increased antioxidant and anti-inflammatory activity, inhibition of proteases and cell proliferation, protection of intercellular communications to modulation of apoptosis and signal transduction pathways.[3]

Prostate cancer is the most common non-cutaneous solid cancer occurring among men in the USA. The most recent estimates from the American Cancer Society. [4] showed that about 240,890 new cases of prostate cancer would be diagnosed in 2011, with 33,720 deaths attributable to prostate cancer in the United States alone. About one of six males in the U.S. may be afflicted with this cancer, and the risk is increased drastically for older males. Moreover, the risk of death due to metastatic prostate cancer is 1 in 36. Genetics, age, race, diet, and family history, and even lifestyle may all contribute to prostate cancer risk.[5] The treatment options for prostate cancer are surgery, chemotherapy, cryotherapy, hormonal therapy and/or radiation, but all are only beneficial at the early stages, with no significant effects after metastasis. Therefore, there is a high need for treatments that will stop the metastasis and invasion of prostate cancer cells.

Rapidly developing field of nanoscience had raised the possibility of using therapeutic nanoparticles in the diagnosis and treatment of human cancers [6]. Nanoscale particles and molecules are a potential alternative for treatment of disease because they have unique biological effects based on the structure and size, which differ from traditional small molecule drugs.[7]

The role of silver nanoparticles as an anticancer agent should open new door in the field of medicine. Silver nanoparticles should serve as one of the best ways of treating diseases that involve cell proliferation and cell death.[8]

The cytotoxic effects of silver are the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA.[9,

10] The silver-based inorganic antimicrobial/ anticancer agents were produced in the forms of silver-supported inorganic powders, silver colloids, metal silver powders.[11] Biosynthesis of nanoparticles by plant extracts is currently under exploitation [12]. The green synthesis is more advantageous over chemical and physical method as it is cost effective and environment friendly.[13]

The use of plant extracts and derived products in the treatment of cancers is of exceptional value in the control of malignancies, due to the fact that most of the anticancer drugs severely affect the normal cells. It has been recommended that ethnopharmacological usages, such as immune and skin disorders, inflammatory, infectious, parasitic and viral diseases be taken into account when selecting plants used to treat cancer, since these reflect disease states bearing relevance to cancer or a cancer symptom. [14, 15]

In the present study, we report for the first time synthesis of silver nanoparticles, reducing the silver ions present in the solution of silver nitrate by the aqueous extract of *Andredera cordifolia*.Morphological characterizations are performed using X-ray diffractometer (XRD). The optical absorption spectrum of silver nano particles was recorded by using UV-visible spectrophotometer and furthers its toxicity against human prostate cancer cell line (PC-3)were evaluated.

MATERIALS AND METHODS

Biosynthesis of Silver Nanoparticles

For synthesis of silver nanoparticles, 15ml of *Andredera cordifolia linn* aqueous extract was added to the 250 ml Erlenmeyer flask containing 100ml of AgNO3 (1mM) and incubated at room temperature for 12 hr in a dark condition. The solution was centrifuged at 20,000rpm for 25 min. The collected pellets were stored at -4°C. Reduction of silver ion into silver particle during exposure to the plant extract could be followed by color change. Silver nanoparticle exhibited light green-dark brown in aqueous solution due to the surface Plasmon resonance phenomenonto monitor the silver nanoparticle synthesis.

Characterization of Silver Nanoparticles

The silver nanoparticles were characterized by UV-visible spectroscopy, Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR).

Cytotoxicity Assays

Chemicals

Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and TPVG were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai.

Cell lines and Culture medium

PC - 3 (Human prostate cancer) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 60 mm petriplates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For Cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with Ham's F-12 supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay.[16]

Principle

The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

PROCEDURE

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using Ham's F-12 containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the

following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right)$$

Determination of cell viability by SRB Assay[17]

Principle

SRB is a bright pink aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds to protein basic amino acid residues in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two orders of magnitude. Colour development in SRB assay is rapid, stable and visible. The developed colour can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96 well plate reader.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different extract concentrations were added to the cells in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere, microscopic examination was carried out and observations were recorded every 24 h. After 72 h, 25 µl of 50% trichloro acetic acid was added to the wells gently in such a way that it forms a thin layer over the extract to form an overall concentration of 10%. The plates were incubated at 4° C for 1 h. The plates were flicked and washed five times with water to remove traces of medium, extract and serum, and air-dried. They were stained with SRB for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. Tris base (10 mM, 100 µl) was then added to the wells to solubulise the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using micro plate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated as mentioned in the previous procedure.

$$\% \text{ Growth Inhibition} = 100 - \text{Mean} \left(\frac{\text{OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right)$$

LDH leakage assay.[18]

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere. After 72 h, the drug solutions in the wells were pooled separately and LDH levels were estimated as per manufacture instructions (ERBA diagnostics).

DNA fragmentation studies

PC - 3 Cells (3 x 10^6 /ml) were seeded into 60mm Petri dishes and incubated at 37°C with 5% CO_2 atmosphere for 24 h. The cells were washed with medium and were treated with extract, standard drug and incubated at 37ºC, 5% CO_2 for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with G Biosciences, USA apoptotic DNA ladder kit. The recovered DNA was loaded onto 2% agarose gel electrophoresis and run 50 V/cm for 3 hrs. The gel was visualized under UV transilluminator and photographed.

Determination of expression of Caspase 3 and apoptotic activity in PC – 3 cell line.[19]

RT-PCR

Procedure

The mRNA expression levels Caspase 3 carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the PC – 3 cells were cultured in 60 mm petridish and maintained in DMEM medium for 48 hrs. The DMEM medium was supplemented with FBS and amphotericin. To the dish was added the required concentration of Test substances (1000 and 500 µg/ml) and incubated for 24 hr. Total cellular RNA was isolated from the untreated (control) and treated cells using Tri Reagent according to manufacturer's protocol. cDNA was synthesized from total isolated RNA by reverse transcriptase kit according to manufactures instructions (Thermo scientific). Then 20µl of the reaction mixture was subjected to PCR for amplification of Caspase 3 cDNA usingspecifically designed primers procured from Eurofins India, as an internal control, the house keeping gene GAPDH was co-amplified with each reaction.

Amplification conditions for Caspase 3 gene

95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 minute , annealing at 58°C for 1 minute and extension at 72°C for 1 min. This was followed by final extension at 72°C for 10 min.

RESULTS AND DISCUSSION

Cytotoxic Activity

Cytotoxic effect of PC3 cell line by the aqueous extract of Biosynthesized AgNPs by MTT assay

In-vitro cytotoxicity effect of Biosynthesized silver nanoparticles were screened against Prostate Cancer cell

line (PC3) and viability of tumor cells was confirmed using MTT assay. Characterization of in vitro nanoparticles uptake and localization is intrinsically linked to cytotoxicological studies because uptake provides evidence of nanoparticle–cell interaction, wherein the delicate intracellular machinery is exposed to nanoparticles. Invitro cytotoxicity of silver nanoparticles synthesized from *Andredera cordifolia* was evaluated on PC-3 cell line.

The Biosynthesized silver nanoparticles were able to reduce viability of the PC3 cells in a dose-dependent manner as shown in table 1 and figure 1,2. Different concentrations (62.5µg/ml, to 1000µg/ml) of Biosynthesized AgNPs were used in MTT assay. CTC_{50} is>1000. As the concentration increased the cell proliferation percentage increased. The % toxicity increases with increase in concentration of silver nano particles suggests that biosynthesized silver nanoparticles could be of immense use in medical field to certain extent as anticancer agent. The result of MTT assay of Biosynthesized AgNPs confirms the cytotoxic activity against the PC3 cell line.

MTT assay measured the cell viability based on the reduction of yellow tetrazolium MTT to a purple formazan dye mitochondrial dehydrogenase enzyme. So, the amount of formazan produced reflected the number of metabolically active viable cells. MTT results showed that the extract possessed cytotoxic effect against PC3 cell line in a dose dependent manner. Such anti-proliferative activity of the extract was characterized by the dose-dependent and tumor-selective manner, as reflected by the comparatively low IC50 values.

Martins *et al.* (2010) reported that the cytotoxic effect of silver is the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins as well as with the nitrogen bases.[20] Sriram *et al.* (2010) demonstrated that AgNPs serve as antitumor agents by decreasing progressive development of tumor cells.[21]

Table: 1 Cytotoxic effect of PC3 cell line by the aqueous extract of Biosynthesized AgNPs by MTT assay

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	Biosynthesized AgNPs	1000 500 250 125 62.5	15.75±2.2 12.57±0.5 5.80±1.7 5.34±0.5 1.88±0.8	>1000±0.00

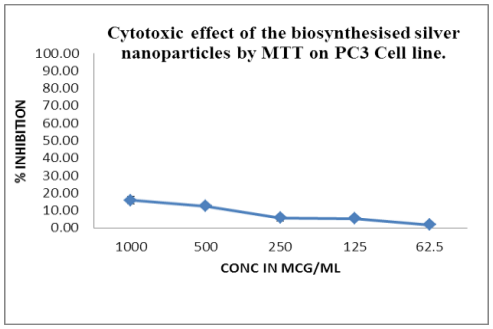


Figure 1: Graphical representation of MTT Assay on PC-3 Cell line

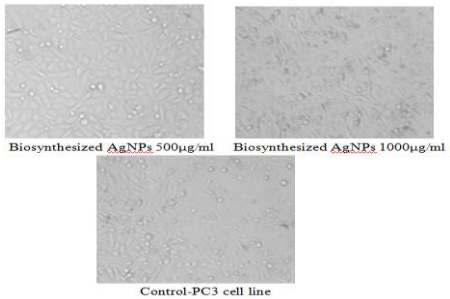


Figure 2: Cytotoxic effect of PC3 cell line by the aqueous extract of BiosynthesizedAgNPs

Cytotoxic effect of PC3 cell line by the aqueous extract of Biosynthesized AgNPs by SRB assay

In-vitro cytotoxicity effect of biosynthesized silver nanoparticles was studied against Prostate Cancer cell line (PC3) and viability of tumor cells was confirmed using SRB assay. The Biosynthesized silver nanoparticles were able to reduce viability of the PC3 cells in a dose-dependent manner as shown in table 2 and figure 3. Different concentrations (62.5µg/ml, to 1000µg/ml) of Biosynthesized AgNPs were used in SRB assay. CTC_{50} is>1000. The result of SRB assay of Biosynthesized AgNPs confirms the cytotoxic activity against the PC3 cell line.

Table 2: Cytotoxic Effect of Biosynthesised Silver nanoparticle by SRB on PC-3 Cell line

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	**Biosynthesised Silver Nanoparticle**	1000	22.85±1.6	>1000±0.00
		500	17.87±0.7	
		250	9.45±0.8	
		125	3.44±0.4	
		62.5	2.11±1.9	

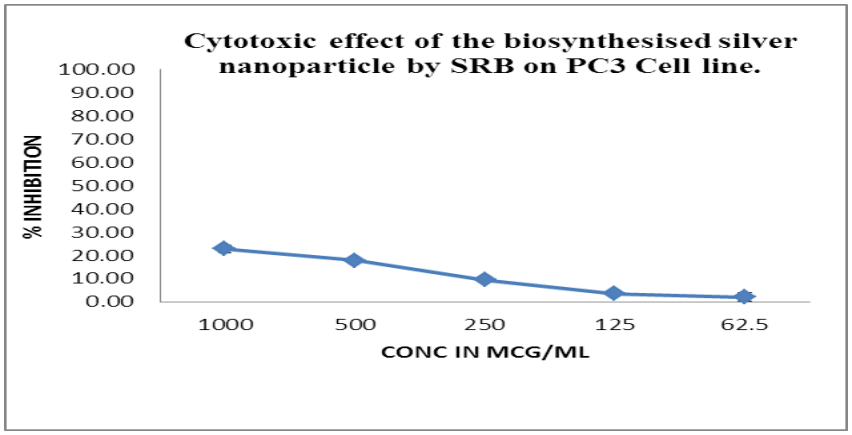


Figure 3: Graphical presentation of SRB Assay

Cytotoxic Effect of Biosynthesised Silver Nanoparticle on PC-3 Cell line by Tryphan Blue Dye Exclusion Method

Percentage cell viability of cells was carried out by using Trypan blue dye Exclusion technique (Table- 3 and Figure). The results show dose dependent response. The extract showed different antiproliferative profiles regarding extract concentrations. There were different inhibitions produced by different concentrations of at 24-hours incubation. On the other hand, there was no difference in the level of inhibition produced by different concentrations (62.5µg/ml, to 1000µg/ml).

Table 3: Cytotoxic properties of test drugs on PC3 cell line by Trypan blue dye exclusinon method.

Concentration	Viable cells	% Inhibition
1000	35	16.67
500	36	14.29
250	38	9.52
125	40	4.76
62.5	41	2.38
CC	42	0.00

Cytotoxic effect of PC3 cell line by the aqueous extract of Biosynthesized AgNPs by LDH leakage assay

The result of LDH leakage assay was presented in table 4. Different concentrations 500 and 1000 µg/ml of biosynthesized silver nanoparticles showed the LDH leakage of 99.6 IU/ml and 171.6 IU/ml. The standard showed the value of 117.7 IU/ml. Cell death or cytotoxicity is classically evaluated by the quantification of plasma membrane damage by the measurement of activity of lactate dehydrogenase (LDH) release. LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane.

TABLE-4 LDH Leakage Assay

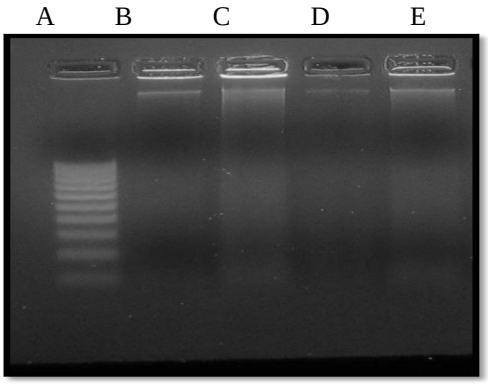
Sl.No	Name ofTest Sample	Test Conc.(µM)/ (µg/ml)	LDH IU/ml
1	Biosynthesized AgNPs	500	99.6
		1000	171.6
2	Standard	1.5	1044.2
3	Cell control	---	117.7

Cytotoxic effect of PC3 cell line by the aqueous extract of Biosynthesized AgNPs by DNA fragmentation assay

DNA fragmentation occurs in cells that produce intrinsic apoptosis activity when induced by a variety of agents. As a biochemical hallmark of intrinsic apoptotic cell death, DNA fragmentation was used to determine

whether the antiproliferative effect of silver nanoparticles on cells acts through apoptosis pathway. The results confirmed the induction of apoptosis in PC3 cells treated with aqueous extract of biosynthesized AgNPs from *Andredera cordifolia linn.* The treatment of PC3 cells treated with Biosynthesized silver nanoparticles (Lane B; Untreated PC3 cells, Lane C; standard, Lane D and E; various concentrations) showed typical features of DNA laddering an agarose gel, whereas untreated cells produced intact genomes (Figure-4). Therefore, the biosynthesized silver nanoparticles from aqueous extract of *Andredera cordifolia* can induce nucleosomal DNA fragmentation of PC3 cells.The cytotoxic effects of biosynthesized silver nanoparticle are the result of active physicochemical interaction of reduced silver atoms with the functional groups of intracellular proteins, as with the nitrogen bases and phosphate groups in DNA. The DNA fragments were visualized by exposing the gel to ultraviolet light, followed by photography.

Figure-4



Lane A: DNA ladder
Lane B: PC – 3 cells (Untreated)
Lane C: Standard (5 µg/ml)
Lane D: PC – 3 cells treated with Biosynthesized AgNPs(1000 µg/ml)
Lane E: PC – 3 cells treated with Biosynthesized AgNPs(2000µg/ml)

Cytotoxic effect of PC3 cell line caspase 3 gene amplified by the aqueous extract of Biosynthesized AgNPs by RT-PCR assay

Apoptosis or programmed cell death is a gene-regulated phenomenon, which is important in both physiological and pathological conditions. The important regulatory mechanisms of apoptosis include death receptors, activation of Caspases, mitochondrial responses, regulation of GAPDH gene expression. According to the gene expression 1000µg/ml of biosynthesized silver nanoparticles showed up regulationof Caspase 3 gene by 0.13 fold. GAPDH gene expression was significantly increased after treatment with AgNPs. As our results showed, the expression of GAPDH gene expression could be regulated by AgNPs.Our results showed that Caspase 3 activity increased in treated cells, as shown in Figure 5. The primer used for the first strand synthesis is OligodT primer.Forward sequence 5' TTCAGAGGGACGTCGTTGTAAGAAGC 3' and reverse sequence synthesis 5' CAAGCTTCTCGGGCTCTGTTTCAG 3' are used for the synthesis of second strand. The product size for 264 bp.

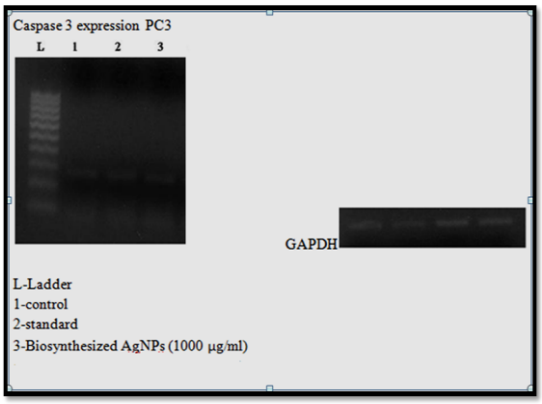


Figure-5 Caspase 3 expression on PC-3

Table-5 Cytotoxic effect of PC3 cell line by the aqueous extract of Biosynthesized AgNPs by RT-PCR assay

Test sample	Control	Standard	Biosynthesized AgNPs(1000 µg/ml)
Regulation in 1terms of Folds	1.00	1.20	1.07

CONCLUSION

The cytotoxic effects of silver are the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA. The Biosynthesised nanoparticles have been characterized by SEM, TEM, EDS, FT-IR XRD and UV-VIS spectroscopy. The AgNPs are crystalline in nature and the size of silver nanoparticles is 60 nm. The biologically synthesised AgNPs showed excellent antioxidant potential, antimicrobial activity and possessed considerable cytotoxic effect on PC-3 cell line. The biosynthesized silver nanoparticles proved to be high potential candidates for medical applications where antioxidant, antimicrobial and, cytotoxic activities are highly essential. Hence the biosynthesized nanoparticles

would be more efficient in the drug delivery process. Therefore further studies are needed to fully characterise the toxicity and the molecular mechanisms involved with the antimicrobial and anticancer activity of these nanoparticles.

ACKNOWLEDGEMENT

This research work was funded by University Grants commission. I would like to thank DST –FIST Laboratory,Dr.N.G.P Arts and Science college to carry out some of this work.

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