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ANTIPROLIFERATIVE EFFECT OF SILVER NANOPARTICLES SYNTHESIZED USING VITEX NEGUNDO ON HEPG2 CELL LINE

Amudha Murugan and Krishnakumari Shanmugasundaram*

Department of Biochemistry, Kongunadu Arts and Science College
Coimbatore -641029, Tamilnadu, India.

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***Correspondence for Author**
Dr. Krishnakumari Shanmugasundaram
Department of Biochemistry, Kongunadu Arts and Science College Coimbatore Tamilnadu, India.

ABSTRACT

The environmental friendly synthesis of nanoparticles process is a revolutionary step in the field of nanotechnology. In this study, the biosynthesis of silver nanoparticles was carried out using *Vitex negundo* leaf extract as reducing agent. UV–visible spectroscopy was used for quantification of silver nanoparticle synthesis. The synthesized silver nanoparticles were characterized with Scanning electron microscopy (SEM), Energy dispersive X- ray analysis (EDX), X-ray Diffraction (XRD) and Fourier transform Infrared Spectroscopy (FTIR). The anticancer effects of silver nanoparticles were challenged with HepG2 cancerous cells in dose and duration dependent manner.

KEYWORDS: *Vitex negundo*, Silver nanoparticles, anticancer activity, cell line.

1. INTRODUCTION

Cancer is considered as one of the most deadly disease in the world high mortality. Since there are many cancer therapies available, chemotherapy has become an integral component of cancer treatment for most cancers. In the area of oncology drug discovery, conventional chemotherapeutic agents still exhibit poor specificity in reaching tumor tissue and are often restricted by dose-limiting toxicity .The combination of developing controlled-release technology and targeted drug delivery may provide a more efficient and less harmful solution to overcome the limitations in conventional chemotherapy. Recent interest has been focused On developing Nano scale delivery vehicles, which are capable of controlling the release of chemotherapeutic agents directly inside cancer cells [1]. Nano materials are expected hopefully to revolutionize the cancer diagnosis and therapy. Nano materials are expected

hopefully to target and visualize tumor site via an imaging technology, thereby allowing for the early detection of cancer. Furthermore, intelligent nanosystems can be constructed as controlled delivery vehicles which are capable of delivering anticancer drugs to a predetermined site and then releasing them with a programmed rate, which can improve therapeutic efficacy [2].

In inorganic nanoparticles, metal nanoparticles have received considerable attention in recent years because there unique properties and potential application in catalysis photonics, optoelectronics, biological tagging and pharmaceutical applications. A number of approaches are available for the synthesis of silver nanoparticles. For eg silver ions are reduced by chemical, electrochemical, radiation, photochemical method, Langmuir-Blodgett and biological techniques [3]. Among these methods, biological synthesis is a good way to fabricate benign nanostructures materials. Biological technique is less toxic and ecofriendly in the synthesis of nanoparticles capping agents are used.

In the present study, we have made an attempt to investigate the anticancer effect of *Vitex negundo* – silver nanoparicles and drug delivery efficacy of silver nanoparticles on HepG2 (Hepatocarcinoma) cell line which has not been previously studied.

2. MATERIALS AND METHODS

2.1 Synthesis of Silver Nanoparticles

The leaf of *vitex negundo* were collected from the Coimbatore district and authenticated in Botanical survey of India, Tamilnadu, India. The leaves were washed under running tap water, dried and cut into small pieces. The leaves were shade dried for 20 days. Then homogenized to get coarse powder.5g of the leaf powder was boiled for 10 min in 100ml sterile distilled water and filtered through What man No.1 filter paper (pore size 25µm).The filtrate was further filtered through 0.6µm size filters, stored in refrigerated condition and used for study.

2.2 Cytotoxicity Assay

To determine the cytotoxic effect of the aqueous extract, MTT (3-(4, 5-dimethyl –thiazole -2-yl) -2, 5 - diphenyl tetrazolium bromide assay was performed using HepG2 cells. To determine the cytotoxic effect of the aqueous extract, MTT (3-(4, 5-dimethyl –thiazole -2-yl)-2, 5 –diphenyl tetrazolium bromide assay was performed using HepG2 cells.

GroupI - Control

GroupII -HepG2 + 30µg/ml *Vitex negundo*

GroupIII -HepG2 + 20µg/ml *Vitex negundo* – silver nanoparticles

GroupIV -HepG2 + 30µg/ml 5-Fluorouracil

Cytotoxicity was evaluated using MTT Survival Assay. The cleavage of the tetrazolium salt MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide] into a blue colored formazan by the mitochondrial enzyme succinate dehydrogenase is potentially very useful for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of cells present. Thus, the MTT assay detects living but not dead cells and the signal generated is dependent on the degree of activation of the cells [4].

Sub confluent monolayer culture of HepG2 cells was trypsinized and the cells were collected in growth medium. The suspension was centrifuged at 1400 rpm for 5 mins and the cell pellet was resuspended in growth medium. Viable cells were quantitated using Tryphan Blue Exclusion. The cells were diluted to 5×10^4 cells/ ml and made up to 20 ml of cell suspension per microtitre plate. 200 ul of cell suspension to each wells including control were plated at a cell density of 1×10^4 cells/ well and was incubated in the 5% Co_2 incubator for 24 hrs to enable them to adhere properly to 96 well polystyrene micro plates.

After 24 hrs, media was removed. Extracts was prepared at 1 mg/ ml concentration and mixed with fresh medium to achieve the final working concentration (10 to 100 µg / ml). Each concentration of extracts was repeated in 3 wells. After incubation at 72 hrs at 37˚C in a humidified incubator, fresh media was added. 20 ul of MTT (5mg/ml in phosphate buffer saline) was added to each well and incubated for 4 hr at 37˚C. After this, viability was assessed by the ability of cells to convert the soluble salt of MTT into an insoluble formazan precipitate which was quantitated spectrophotometric ally following solubilization in DMSO. The absorbance was recorded on a microtiterplate recorder (Bio Rad Co.) at the wavelength of 570 nm and with the reference wavelength at 630 nm. The % of inhibition of each concentration was calculated by the following formula:

% of inhibition = Control-Sample / Control x 100

2.3lactate Dehydrogenase (LDH) Leakage Assay

Principle

The lactate is acted upon by lactate Dehydrogenase to form pyruvate in the presence of NAD. The pyruvate forms pyruvate phenyl hydrazone with 2,4dinitrophenylhydrazine. The color developed is reading a spectrophotometer at 440nm.

Reagents

1. 0.4N NaOH Glycine buffer, 0.1M, pH 10: 7.505g of sodium chloride was dissolved in 1 litre of water.
2. Buffered substrate: 125ml of glycine and 75ml of 0.1N NaOH were added to 4g of lithium lactate and mixed well.
3. Nicotinamide adenine Dinucleotide: 10mg of NAD was dissolved in 10ml of water.
4. 2,4- Dinitrophenyl hydrazine: 20mg of DNPH was dissolved in 100ml of 1N HCl
5. 0.4N NaOH.
6. Standard pyruvate, 1µmol/ml: 11mg of sodium pyruvate was dissolved in 100ml of buffered substrate (1 mole of pyruvate /ml).
7. NADH solution, (1µmol/ml:8.5mg/10ml buffered substrate.

Procedure

Placed 1.0ml buffered substrate and 0.1ml sample in to each of two tubes. Added 0.2ml water to the blank. Then to the test added 0.2ml of NAD. Mixed incubated at 37˚C for 15mins. Exactly after 15mins, 1.0ml of dinitrophenyl hydrazinewas added to each (test and control).Left for further 15mins.Then added 10ml of 0.4n sodium hydroxide and the color developed was read immediately at 440nm. A standard curve with sodium pyruvate solution with the concentration range 0.1-1.0µmole was taken.

2.4 DNA Fragmentation Assay

DNA fragmentation has long been used to distinguish apoptosis from necrosis, and is among the most reliable methods for detection of apoptotic cells. When DNA strands are cleaved or nicked by nucleases, 3'-hydroxyl ends are exposed with 27ug/ml concentration of silver nanoparticles. 1×10^6 cells were lysed in 250 µl cell lysis buffer containing 50 mM Tris HCl, pH 8.0, 10 mM EDTA, 0.1M NaCl, and 0.5% sodium dodecyl sulfate. The lysate was incubated with 0.5 mg/ml RNase A at 37°C for one hour, and then with 0.2 mg/ml proteinase K at 50°C overnight. Phenol extraction of this mixture was carried out, and DNA in the aqueous phase was precipitated by 25 µl (1/10 volume) of 7.5 M ammonium acetate and 250

µl (1/1 volume) isopropanol. DNA electrophoresis was performed in a 1% agarose gel containing 1 µg/ml ethidium bromide at 70 V, and the DNA fragments were visualized by exposing the gel to ultraviolet light, followed by photography.

2.5 Genotoxicity Assay-Comet Assays

The comet assay is based on the microscopic detection of damaged DNA fragments of individual cells, appearing as "comets" upon cell lysis, subsequent DNA denaturation and electrophoresis. The alkaline version (pH> 13) is mostly used for the detection of single and double DNA strand breaks, DNA cross-links, and alkali labile sites (ALSs). The comet assay is widely used to investigate genotoxicity of nanomaterials. Hep G2 cells were seeded in 24 well plates and exposed to 10 µg/ml AgNPs dispersions for 4 and 24 h. The dose was selected based on the cytotoxicity results. Cells were harvested and approximately 10^4 cells per exposure were embedded into 0.75% low melting agarose (on 0.3% agarose pre-coated glass slides) and lysed with a freshly prepared 1% Triton lysis buffer (pH 10) for 1 h on ice at dark conditions. Alkaline unwinding was performed for 40 min on ice at dark conditions using 0.3 M NaOH (pH > 13) followed by DNA electrophoresis in the same alkaline solution for 30 min at 29 V. The slides were neutralized in 0.4 Tris Buffer for 5 min twice, dipped in deionized water and left to dry overnight. Fixation was performed in methanol for 5 min. The slides were stained with ethidium bromide and scored using a fluorescence microscope. The alkaline comet assay was used to determine the DNA damage associated with exposure to non-cytotoxic concentrations (27µg/ml) of AgNPs in HepG2 cells. However, a statistically significant increase in overall DNA damage was observed after 24 h for all AgNPs, independent of size and coating.

3. RESULTS AND DISCUSSION

Silver nanoparticles characterization was recorded from UV-Vis spectrum, scanning electron microscopy (SEM), Transmission electron microscopy (TEM), X-ray (XRD), Energy dispersive spectroscopy (EDS) and (Fourier – transform IR) resulted in the formation of 10-50nm particle size of nanoparticles. Medicinal plants have been used as remedies for human diseases for centuries. The reason for using them as medicine lies in the fact that they contain chemical components of therapeutic value [8].The medicinal value of plants lies in some chemical substances (usually secondary metabolites) , that produce a definite physiological on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolics [9]

3.1Cytotoxicity Assay

Invitro cytotoxic activity of silver nanoparticles against Hepatocarcinoma cell line HepG2 was evaluated at different concentration (5µg, 10µg, 25µg, 30µg, 40µg, 50µg, 75µg, 100µg) by MTT assay. The effect was compared with VERO cell lines (normal cell lines).Results shows that at higher concentrations there is significant cell mortality. In relation to cell death a minimum of 25µg /ml of silver nanoparticles is well enough to induce 50% of cell mortality.

Table.1. *Invitro* cytotoxic effect of nanoparticles on Hepatocellular carcinoma cell line (HepG2) and VERO cell lines

S. NO	Concentration (µg/ml)	HepG2	VERO cell lines
1	5	82.97±1.15	95.30±1.33
2	10	63.95±0.97	90.82±0.27
3	25	52.12±1.80	87.10±0.40
4	30	49.37±0.60	58.47±0.38
5	40	35.67±0.64	58.22±0.78
6	50	29.23±0.33	55.09±0.56
7	75	25.04±1.30	45.83±0.55
8	100	12.89±0.92	47.95±1.64
9	Control	100	100

3.2 Lactate Dehydrogenase (LDH) Leakage Assay

LDH is a soluble cytosolic enzyme, which is released into extracellular medium because membrane damage consequently leads to apotosis. It is widely accepted as an indicator of lytic cell death. According to the level of Lactate dehydrogenase (LDH) released into the medium of control and synthesized silver nanoparticles treated (20, 40, 60, 80 and 100µg/ml).HepG2 cells are presented in Table 2. From this table it was observed that LDH activities found to be significantly elevated after 48h of exposure in the medium containing silver nanoparticles when compared to control.

Table.2. Cell viability and LDH leakage in control and silver nanoparticle treated HepG2 cells after 48h of exposure.

Concentration µg/ml	% of Inhibition	LDH activity (µmol of NADH / per well / min)
Control	0	0.11±0.005
20	0	0.13±0.006*
40	20.97±1.45*	0.15±0.003*
60	48.12±1.20*	0.21±0.002*
80	65.60±1.40*	0.36±0.002*
100	92.82±1.30*	0.50±0.002*

3.3 DNA Fragmentation Assay

The DNA laddering technique is used to visualize the endonuclease cleavage products of apotosis [10]. This assay involves extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis. Apoptosis of AgNP a treated cell was accompanied by a reduction in the percentage of cells was accompanied by a reduction in the percentage of cells in G0/G1 phase and an increase in the percentage of G2/M phase cells. Indicating cell cycle arrest at G2/M [11].DNA fragmentation is broadly considered as a characteristic of apotosis [12].Induction of apoptosis can be confirmed by two factors such as irregular reduction in the size of the cells, in which the cells are reduced and shrunken and lastly dna fragmentation.

The DNA fragmentation in the present study was verified by extracting DNA from HepG2cells treated with 25µg /ml of biosynthesized silver nanoparticles of *Vitex negundo* followed by detection in the agarose gel. Fig1 Clearly indicates that the deposition of metal nano particles inside the nucleus could affect the DNA and cell division.

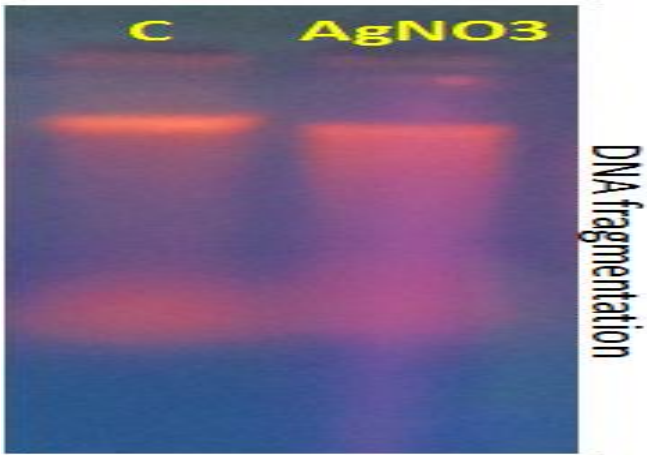
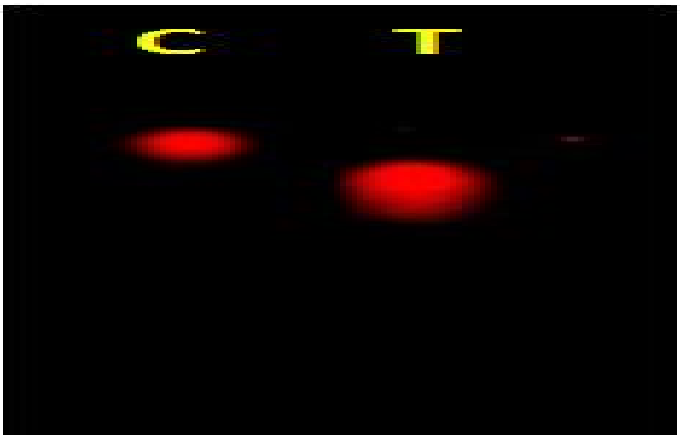


Fig1. dna Fragmentation Analysis

3.4 Genotoxicity Assay – Comet Assay

The potential of silver nanoparticles to induce DNA damage was investigated with comet assay. The comet assay assay was used to determine the DNA damage associated with exposure to non-cytotoxic concentration (25µg/ml) of silver nanoparticles in HepG2. No significant increase in the percentage of DNA in the comet tail was observed after 4h exposure to any of the silver nanoparticles. However, a statistically significant increase in overall DNA damage was observed after 24h for all silver nanoparticles, independent of size and coating. Fig 2 depicts Genotoxic studies of silver nanoparticles reveals the production of micronuclei.



Control-10µm; Test - 90µm

Fig2 Genotoxicity assay

CONCLUSION

In conclusion, plant based silver nanoparticles possess considerable anticancer effects compared with other methods. Use of silver silver nanoparticles should emerge as one of the novel approaches in cancer therapy and the molecular mechanism of targeting is better understood. The applications of silver nanopaticles are likely to expand further.

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