

Original Article

APOPTOSIS INDUCING EFFECT OF SILVER NANOPARTICLES SYNTHESIZED USING *MAGNOLIA CHAMPACA* LEAF EXTRACT ON MCF-7 CELL LINE

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ABSTRACT

Objective: To synthesize silver nanoparticle (AgNP) using the ethanolic leaf extract of *Magnolia champaca* (MC-AgNP) and to evaluate its cytotoxicity and apoptotic activity in MCF-7 cell line, and to analyse active Caspase-3 expression in inducing apoptosis.

Methods: AgNP was green synthesized using ethanolic leaf extract of MC. Shape and size were analysed by SEM and particle size analyser. Cytotoxicity was tested to find IC₅₀ value. Apoptosis was explored by mitochondrial membrane potential ($\Delta\Psi_m$) assay and active Caspase-3 expression study using flow cytometry.

Results: AgNP synthesized was observed to be in spherical shape through SEM micrograph with 164.6 nm in size. IC₅₀ value of 15.90 $\mu\text{g/ml}$ was obtained after 24 h of drug treatment. Changes in $\Delta\Psi_m$ and positive active Caspase-3 protein expression were observed.

Conclusion: The present results suggest that MC-AgNP has potential anticancer property and also induced cell death via apoptosis through caspase dependent pathway in MCF-7 cells.

Keywords: *Magnolia champaca*, Green-synthesis, Anticancer, Apoptosis, Caspase-3

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INTRODUCTION

Cancer is one among the leading cause of human death across the world. Breast cancer is the most diagnosed of all type cancer with about 1 million new cases per year and accounts for about 23% of total cancer cases and 14% of cancer deaths. Natural products mostly plant products have been used in cancer treatment for more than 3,500 y. These play a key role in modern medicine as a natural source of cancer drugs with least side effects [1-3]. Plant products and their derivatives are dependent upon widely as effective chemotherapeutic drugs. Around 50% of the small molecules sanctioned for cancer chemotherapy in western medication throughout the most recent 70 y are either natural products that are unmodified structurally or synthetic derivatives of those products [4, 5].

Magnolia champaca (MC) is a member of family Magnoliaceae that is famous for its fragrant flowers. It is also known as Swarna Champa and originated in the temperate Himalayan region and distributed throughout the Indian, Chinese and Indonesian lands. MC is a tall woody tree species, which has high economic value for medicinal and fragrance products such as essential oils, cosmetics and perfumes [6-8]. Various parts of the tree including flower, seed, bark and leaves have been reported to possess various pharmacological activities such as anti-microbial, anti-oxidant, anti-diabetic, anti-cancerous, anti-inflammatory, leishmanicidal, anthelmintic, anti-ulcer and anti-uretic etc [9-15].

Green synthesis of nanoparticles ensures exclusion in the usage of the substances which are detrimental to human health and hazardous to environment. Nanoparticles ranges from 1 to 100 nm and are synthesized in variable shapes, sizes and chemical compositions, with potential properties for human benefits. Metal nanoparticles are essential tools in drug delivery due to special properties of bulk material formation and production of quantum effects due to their smaller size [16-18]. In the present study, cytotoxicity of silver nanoparticle synthesised from the ethanolic extract of leaves of *Magnolia champaca* (MC-AgNP) was investigated on human breast cancer cell line (MCF-7). Apoptosis-inducing effect and active caspase-3 expression of MC-AgNP was determined by flow cytometry to confirm the anticancer mechanism.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle Medium with High Glucose (DMEM-HG), Foetal Bovine Serum (FBS), Dulbecco's Phosphate Buffered Saline (DPBS), trypsin-EDTA solution, MTT reagent, dimethyl sulfoxide, paraformaldehyde solution, Bovine Serum Albumin (BSA), triton-X 100 were all purchased from HiMedia, India. Flow cytometry mitochondrial membrane potential detection kit BD™ MitoScreen (BD, Biosciences, catalog no. 551302) and FITC rabbit anti-human active caspase-3 IgG Antibody (BD Biosciences, catalog no. 559341) was bought from BD Biosciences, USA.

Plant material and preparation of extract

MC leaves were collected from a mature tree growing at the main campus of CHRIST (Deemed to be University), Bangalore, India. The leaves were identified and authenticated by Foundation for Revitalization of Local Health Traditions (FRLHT), Bangalore. The leaves were cut into small pieces, washed with distilled water, dried and powdered. The sample was extracted using Soxhlet apparatus with ethanol as the extracting solvent and 60 g powdered leaf. The extract was filtered using Whatman filter paper no. 1, weighed and stored at 4 °C.

Phytochemical analysis

Qualitative phytochemical analysis of the ethanolic extract of MC was performed following Liebermann's test for glycosides, Salkowski test for terpenoids and steroids, Wagner's test for Alkaloids, Ferric Chloride test for tannins, phenols and flavonoids [19-23].

Green synthesis of silver nanoparticles

MC leaf extract of 5 mg/ml was added to 50 ml of 20 mmol aqueous solution of AgNO₃. It was kept covered with an aluminium foil and was stored in dark at room temperature for 72 h. The silver nanoparticle was filtered with a microfilter of 0.2 μ , dried and stored in a vial covered with aluminium foil at 4 °C.

Characterization and size analysis of nanoparticles

About 25 μl of the MC-AgNP synthesized was loaded on a copper stub (1 mm diameter) attached to a double-sided carbon material, provided for SEM analysis. SEM observation was performed on a JEOL JSM-6360 electron microscope, with an accelerating voltage of 18 kV and the working distance was adjusted to around 18 mm. The particle size was established by particle size analyzer by Horiba scientific SZ-100. 5 mg/ml stock solution of nanoparticle was prepared in distilled water and sonicated for 15 min at 40 kHz using a bath type sonicator (GT-Sonic Model: VGT-1613GTD). It was diluted at 10:1000 with distilled water and analyzed.

Cell culture

MCF-7 (Michigan Cancer Foundation-7) cell line was obtained from NCCS Pune and matched with ATCC STR profile. The cells were maintained in Dulbecco's Modified Eagle Medium with High Glucose (DMEM-HG) supplemented with 10% Foetal Bovine Serum (FBS) and 2% penicillin-streptomycin. The cells were incubated at 37 °C and 5% CO₂ atmosphere.

Cytotoxicity assay by MTT assay

MCF-7 cells were plated to 96 well microtitre plate and the count was adjusted to 10,000 cells/well, using DMEM HG medium. To each well, 200 μl of the cell suspension was added and the plate was incubated for 24 h at 37 °C and 5% CO₂ atmosphere. The cells were treated with 200 μl of different concentrations (25, 12.5, 6.25, 3.12 and 1.56 $\mu\text{g/ml}$) of MC-AgNP for 24 h followed by addition of 10% MTT reagent to get a final concentration of 0.5 mg/ml and was incubated for 3 h at 37 °C. The culture medium was removed completely without disturbing the crystals formed. Then 100 μl of solubilisation solution (DMSO) was added to the wells and the plate was gently shaken in a gyratory shaker to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm and at 630 nm. The percentage growth inhibition was calculated and concentration of MC-AgNP needed to inhibit cell growth by 50% (IC₅₀) was obtained from the dose-response curve for MCF-7 cell line [24]. The experiment was performed in duplicates and the results were expressed as mean \pm standard error. Data is statistically analysed by one way ANOVA where means not sharing a common single letter found to be significantly different at $p \leq 0.05$.

Apoptosis study by mitochondrial membrane potential ($\Delta\Psi\text{m}$) assay

Flow cytometry was used in apoptotic study (BD™ MitoScreen Kit) according to the instructions of the manufacturer. MCF-7 cells at a density of 3×10^5 cells/2 ml were cultured in a 6-well plate followed by incubation in CO₂ incubator overnight at 37 °C for 24 h. The cells

were treated with (IC₂₅) of MC-AgNP, Cisplatin (80 $\mu\text{g/ml}$) as the positive control and one of the wells was left untreated to be used as the negative control. The cells undergone trypsin treatment with 200 μl of trypsin-EDTA solution after 24 h of incubation. All the cells were harvested and centrifuged for 5 min at 300 \times g (25 °C). After decanting the supernatant, pellet was re-suspended in PBS to which about 0.5 ml of freshly prepared JC-1 working solution was added followed by incubation for 15 min at 37 °C in a CO₂ incubator. The cells were washed twice with 1X assay buffer, re-suspended and centrifuged at 400 \times g for 5 min. The sample was analysed by Cytomics FC500 flow cytometer (Beckman Coulter, USA) after re-suspension of the cell pellet in 0.5 ml of 1X assay buffer.

Active caspase-3 expression study

A 6-well plate was used for seeding MCF-7 cells at a density of 3×10^5 cells/2 ml. And the cells were treated with (IC₂₅) of MC-AgNPs (experimental test compound), after 24 h of seeding. MCF-7 cells were treated with Cisplatin that served as the positive control while the untreated cells served as the negative control. The cells were provided with trypsin treatment after incubation of 24 h followed by centrifugation at 300 \times g at 25 °C for 5 min and the obtained pellet was re-suspended in PBS. This was followed by the addition of 0.5 ml of 2% paraformaldehyde solution and incubation for 20 min. The cells were then washed with 0.5% BSA in 1X PBS. Triton-X 100 (0.1%) in 0.5% BSA solution was added to the cells. After 10 min incubation, it was washed twice with 0.5% BSA in 1X PBS. Then 0.5% BSA in 1X PBS and 20 μl FITC rabbit anti-active caspase-3 IgG was added according to manufacturer's instructions. It was mixed thoroughly by pipetting and incubated for 30 min in the dark at room temperature (25 °C). 0.5 ml of PBS was added after washing the cells, mixed thoroughly and analysed using Cytomics FC500 flow cytometer (Beckman Coulter, USA).

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of MC leaf extract showed positive for glycosides, alkaloids, flavonoids, tannins, phenols, steroids and terpenoids. Silver nanoparticles were synthesised from ethanolic extract of MC leaf using silver nitrate. The colour change from green to brownish-yellow denoted the successful formation of AgNP after 72 h incubation in dark at room temperature. The MC-AgNPs were observed to be sticky. Fig. 1 shows SEM micrograph of AgNP aggregates. The SEM images showed the nanoparticles of various shapes, most of which were spherical. A mean size distribution of 164.6 nm was recorded by Particle size analyser as shown in fig. 2. Silver nanoparticle possess unique physical, chemical, biological and physicochemical properties such as chemical stability, high thermal and electrical conductivity etc. which makes it applicable in medicines [25].

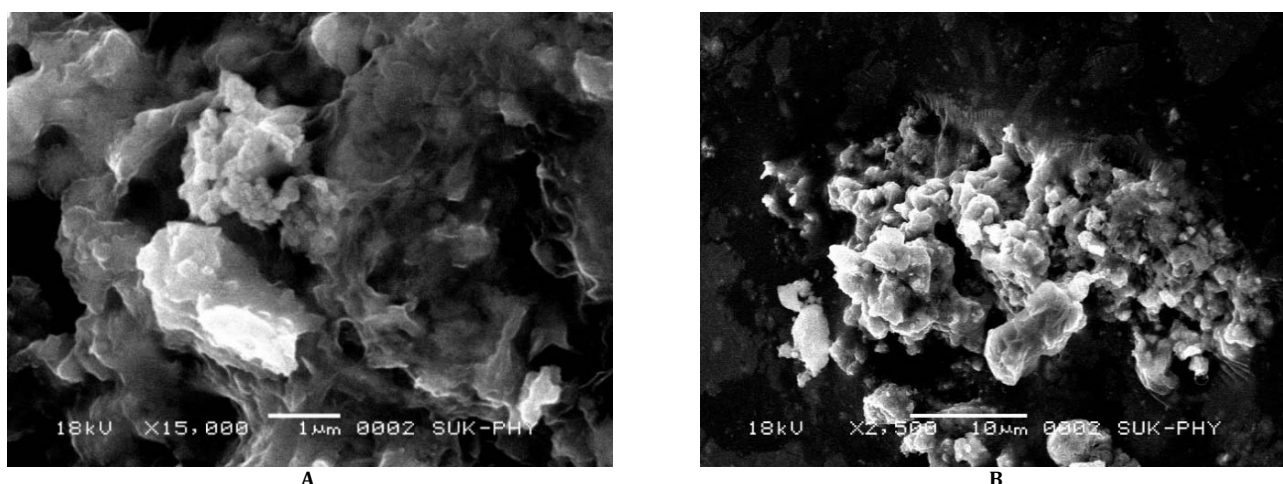


Fig. 1: Scanning tunnel electron microscope images of MC-AgNP leaf extract A) 15000X magnification and B) 2500X magnification, captured with JEOL JSM-6360 electron microscope

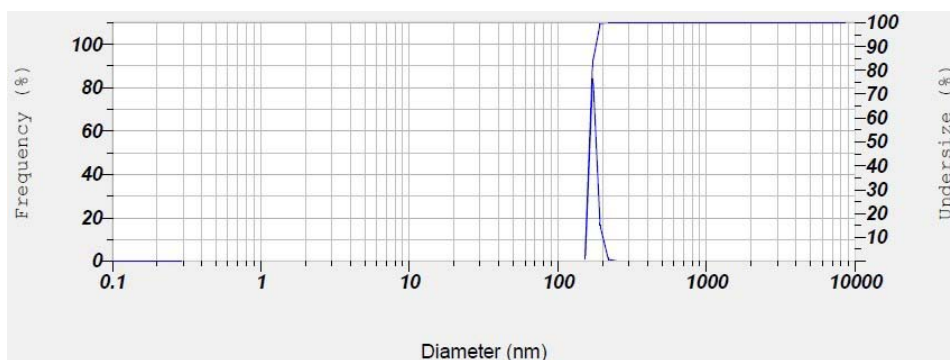


Fig. 2: Particle size distribution graph of MC-AgNP recorded by particle size analyzer (Horiba scientific SZ-100)

Anti-proliferative activity of MC-AgNPs on MCF-7 cell line was evaluated using MTT assay. MTT acts as a substrate which is reduced by mitochondrial dehydrogenase enzyme to form DMSO soluble formazan crystals [26]. MTT assay was performed for testing cytotoxicity by treating different concentration of the MC-AgNPs (3.125, 6.25, 12.5, 25 and 50 $\mu\text{g/ml}$) on MCF-7 cell line (fig. 3). Based on the IC_{50} value of MC-AgNPs that was observed to be 15.90 $\mu\text{g/ml}$ for 24 h, it could be concluded that the drug had an inhibitory effect on the growth of MCF-7 cells.

Apoptosis plays a critical role in immune response development, homeostatic control of tissue integrity and tumour regression where many morphological and biochemical changes occur in the cell, following specific signals [27]. Significantly, the events taking place

in mitochondria during apoptosis is the loss or collapse of mitochondrial membrane potential ($\Delta\Psi\text{m}$) [28]. The ability of MC-AgNPs to induce apoptosis on MCF-7 cell was studied by JC-1 uptake into mitochondria that can be used to detect change in $\Delta\Psi\text{m}$. Polarization of $\Delta\Psi\text{m}$ of normal, healthy mitochondria allows itself to take up the dye in aggregated form to give red fluorescence [29, 30]. As shown in table 1, Cisplatin (positive control) at a concentration of 80 $\mu\text{g/ml}$ caused 32.5% apoptotic cell death and the MC-AgNPs (test drug) at IC_{25} caused 77.1% apoptotic cell death, whereas in untreated cells, only 3.94% induction of cell death was observed via apoptosis. JC-1 green fluorescence was collected in the FL1 detector with 525 nm band pass filter while red fluorescence was collected in the FL3 detector using 620 nm band pass filter (fig. 4).

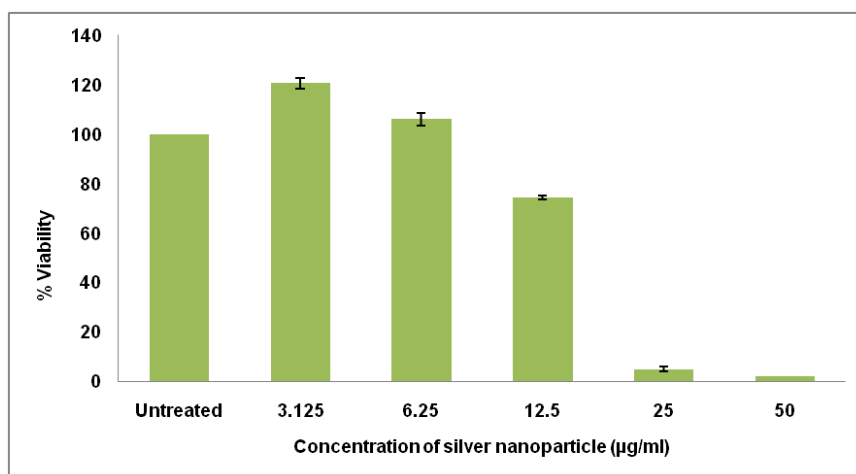


Fig. 3: Inhibition of MCF-7 cell growth by silver nanoparticle synthesised from *Magnolia champaca* leaf extract

Table 1: The values of apoptosis study by mitochondrial membrane potential assay through JC-1 staining using flow cytometry in MCF-7 cell line

	Geometric mean fluorescence intensity (MFI) of JC-1 red fluorescence (FL3-A parameter)	Percentage of cells	
		Healthy	Apoptotic
Untreated	46637	96.0	3.94
Cisplatin	23660	67.4	32.5
MC-AgNP	2819	21.3	77.1

Caspases are key protease that gets activated in both pathways of apoptosis [31]. Of all the caspases, Caspase-3 is the most important in the induction of apoptosis as it cleaves majority of caspase substrates [3, 27]. An increase in the active caspase-3 positive cell population was observed in MCF-7 cells treated with the test sample MC-AgNP and positive control (Cisplatin) as compared to the untreated cells from 7.21% positive caspase-3 cells in the untreated

to 66.7% in Cisplatin treated cells and 13.7% in the MC-AgNP treated cells (table 2). Enzyme assay showed about 7% increase in the active caspase-3 positive cell population in the cells treated with the MC-AgNP as compared to the untreated cells (fig. 5). Therefore, it was found that in MCF-7 cells induction of apoptosis was through caspase dependent pathway that contributed to its anti-cancerous property.

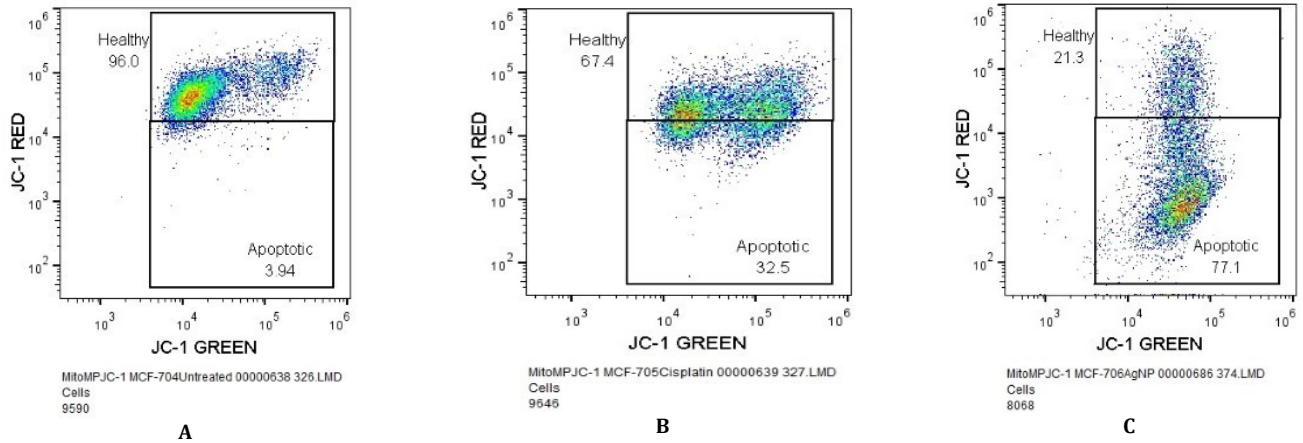


Fig. 4: The plot showing the green versus red fluorescence of JC-1 A) Untreated MCF-7 cells, B) Cisplatin treated MCF-7 cells and C) AgNP treated MCF-7 cells in apoptotic study by mitochondrial membrane potential assay using Flow cytometry

Table 2: The values of flow cytometry detection of activated caspase-3 in MCF-7 cell line

	Geometric mean fluorescence intensity (MFI) of FITC Caspase-3 (FL1-A parameter)	Percentage of cells	
		Caspase-3 negative	Caspase-3 positive
Untreated	613	92.8	7.21
Cisplatin	1709	33.3	66.7
MC-AgNP	623	86.3	13.7

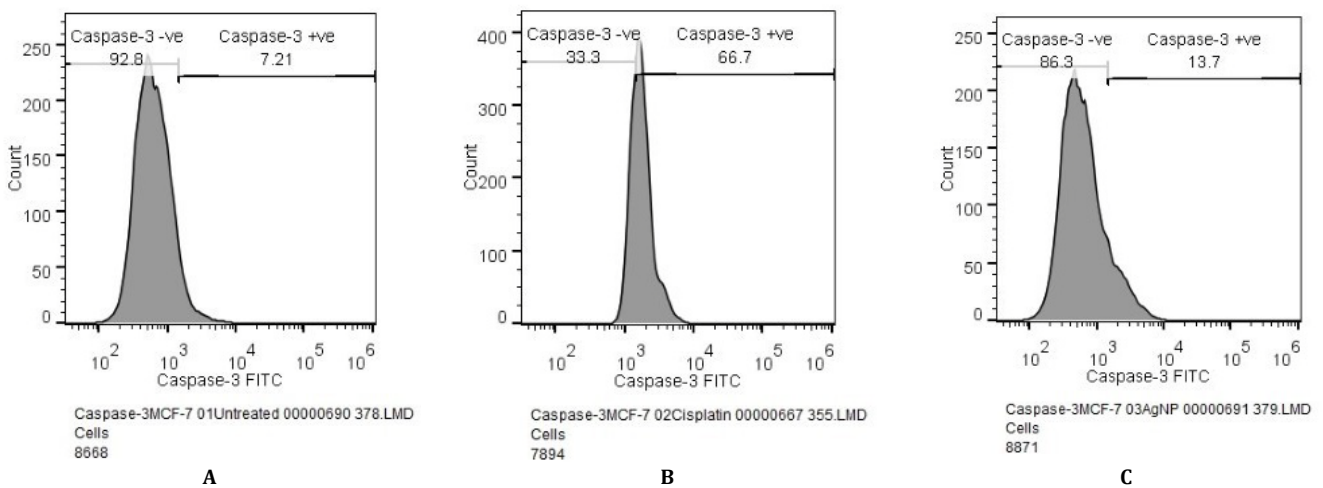


Fig. 5: The histogram plots showing the expression of active caspase-3 in x-axis versus count A) Untreated MCF-7 cells, B) Cisplatin treated MCF-7 cells and C) AgNP treated MCF-7 cells in Caspase 3 study using Flow cytometry

CONCLUSION

From the present study, it can be concluded that the MC-AgNPs can potentially induce cell death by inhibiting cell proliferation and by activating caspase dependent apoptotic pathway in MCF-7 cells. This was the first study assessing the apoptosis induction properties of silver nanoparticle synthesised from ethanolic extract of MC leaves on MCF-7 cell line. Advanced studies are crucial to interpret the appropriate mechanism by which MC-AgNP induces apoptosis.

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Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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