



Selective Toxicity of Biosynthesised Silver Nanoparticles on MCF-7 and MDA MB-231 Breast Cancer Cell Lines

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Abstract : Biosynthesized nanoparticles have many applications in the field of biopharmaceutics due to its high surface to volume ratio, less toxicity and synergistic effects with conjugated biomolecules. This study reports the selective cytotoxicity effect of biosynthesized silver nanoparticles (AgNPs) on breast cancer cell lines MCF-7 and MDA MB-231. AgNPs were synthesized using an aqueous extract of *Mollugo cerviana* species of plant and characterized using UV-visible spectroscopy, zeta potential analyser, SEM, FT-IR, XRD, and EDX. An UV-visible spectrum of the extract shows the surface plasmon resonance peak of AgNPs at 420 nm. SEM analysis results confirm the sphericity of the AgNPs whose size is in the range of 50 – 100 nm. The zeta potential value of -27 mV indicates the stability of the biosynthesized AgNPs. Dose-Dependent cytotoxicity was observed against human breast cancer cells lines MCF-7 and MDA MB-231. The inhibitory concentrations (IC₅₀) are 21.53 µg/mL and 25.52 µg/mL respectively. There was no significant toxicity against Vero cells below 100 µg/mL concentration of AgNPs. The data obtained in the study reveal the potential therapeutic value of biogenic silver nanoparticles in cancer treatment and further studies are required to elucidate the mechanism of selective activity of biosynthesized AgNPs on cancer cells.

Key words: Silver nanoparticles, *Mollugo cerviana*, cytotoxic effect, Inhibitory concentration, Breast cancer cells.

I. Introduction

Breast cancer is one of the leading causes of death worldwide [1]. Approximately 60% of cancer related deaths in developing countries is attributable to breast cancer and around 40,000 deaths happen every year in India [2]. The estimated survival rate of a breast cancer patient is below 40% for developing countries [3]. Nanotechnology is an emerging technology in the field of targeted therapeutics. Nanomedicine is a branch of nanotechnology where a nanoparticle (<100 nm scale) is used for delivery of drugs, enzymes, proteins, and other therapeutic agents. Nanoparticles can be engineered to possess unique physical, chemical, and biological

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properties [4]. Usage of nanoparticles shows a new route towards tumor detection, prevention, and treatment. Nanoparticles eradicate cancer cells by penetration to different regions of tumors through blood vessels and then through interstitial space to reach the target cells [5]. Among other nanoparticles, silver nanoparticles (AgNPs) have unique properties[6]. Recent years, many attempts were made to synthesize AgNPs using biological sources like bacteria, fungi, yeasts, and plants as reducing agents. Also, biosynthesized AgNPs has antimicrobial, antiviral, antiangiogenic, and anti-inflammatory activities. Anti-cancer effects of biosynthesized silver nanoparticles against human breast cancer cells, human lung cancer cells, and prostate cancer cells are also reported [7]. Using plant and its extract for nanoparticle synthesis is a better alternative due to its less toxicity and easy scale up[8].

Mollugo cerviana is a species of flowering plant found on most continents growing as a weed in many types of dry and sandy habitat types. *M. cerviana* (L.) has been widely used as a potherb, also enhances eyesight, reduces body odour, acts as a good antiseptic, stabilizes blood pressure, has wound healing property, reduces fever, and it is used in the treatment of cough [9]. Several studies reveal the presence of flavonoids, tannins, saponins, triterpenoids, alkaloids, glycosides, phenolic groups and glycosides in methanol extract, ethyl acetate and n-butanol fractions. Saponin is known for its medicinal properties like expectorant, a natural blood cleanser and an antibiotic[10]. The plant extracts exhibited antibacterial activity [11,12], anti-fungal activity [13], anti-inflammatory activity [14], antioxidant activity [15], and hepatoprotective property [16]. In the present study, an effort was made to biosynthesize AgNPs using the aqueous extract of *Mollugo cerviana*. The optical property, surface charge, shape, and size of the AgNPs were evaluated. Thereafter, the cytotoxic effect of AgNPs against breast cancer cell lines MCF-7 and MDA MB231 has also been evaluated using cytotoxicity assay.

II. Materials And Methods

A. Sample collection

Mollugo cerviana plant was collected from Kanyakumari district, Tamilnadu, India. The collected plant was identified taxonomically and then used for further analysis. Thereafter, the aerial plant parts were shade dried and blended in an electric blender and stored at 4 °C for further use.

B. Biosynthesis of AgNPs

For biosynthesis of AgNPs, 2 g of plant powder was mixed with 50 mL of nanopure water and aqueous extract was prepared using Ultrasonic Assisted Extraction method. To 1 mL of 1 % silver nitrate solution, 9 mL of aqueous extract was added and continuously stirred using magnetic stirrer for 72 h at 37 °C. The reduction of Ag⁺ ions changes the color from green to yellowish brown which indicates the formation of AgNPs.

C. Characterization of AgNPs

The physical characterization of biosynthesized silver nanoparticles was performed using UV-visible spectroscopy, zeta potential, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), and energy dispersive X-ray (EDX).

UV-Vis spectroscopy analysis: By performing a wavelength scan between 400 and 800 nm, the optical properties of silver nanoparticles was analysed using 10 mm path length quartz cuvettes with Hitachi U-2900 UV-Visible double beam spectrophotometer (Tokyo, Japan).

X-Ray Diffraction Analysis (XRD): The formation and quality of compounds were checked by X-ray diffraction (XRD) spectrum. The XRD pattern was measured by drop coated films of AgNO₃ on glass plate and employed with X-ray diffractometer (INEL X-ray diffractometer, Stratham, USA) of characteristic Co- α 1 radiation ($\lambda = 1.78 \text{ \AA}$) in the range of 20° to 90° at a scan rate of 0.05°/min with a time constant of 2 sec. The powdered sample was loaded onto a glass sample holder and analysed with X-ray.

Fourier Transform Infrared Spectroscopy Analysis (FTIR): To estimate the presence of phytochemicals in the aqueous extract of *Mollugo cerviana*, a Perkin-Elmer FTIR spectrum-RX-1 (USA) was used and peaks were obtained in the range of 4000 - 500 cm⁻¹ at a resolution of 4 cm⁻¹.

Zeta potential analysis: Zeta potential (surface charge) analysis of biosynthesized AgNPs were measured using Dynamic Light Scattering methodology (DLS) (Zetasizer Nano ZS, ZEN3600 and Malvern, UK).

Scanning Electron Microscopy Analysis (SEM): Size and shape of bio-reduced AgNPs were characterized by SEM experiments (Hitachi, 2009 model, Tokyo, Japan). The sample was loaded on carbon-coated copper grids and the solvent was added. The solvent was allowed to evaporate under infrared light for 30 min. SEM measurements were performed on Icon Analytical, FEI Quanta 200 instrument.

Energy Dispersive X-ray (EDX): EDX spectra were acquired with a Link AN 10,000-system attached to the SEM (Carl Zeiss microscopy Ltd, UK). The spectra were evaluated using the RTSy2-FLS program taking into account the sensitivity factors for the different elements (k-factor correction). The beam spot sizes between 50 and 100 nm were used depending on the size of the particles.

D. Cytotoxicity of biosynthesized AgNPs

Vero, MDA MB-231, MCF-7 cell lines were obtained from NCCS, Pune, India. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with D-glucose, 2% L-Glutamine, hypoxanthine monosodium salt, linoleic acid, lipoic acid, putrescine dihydrochloride, phenol red indicator, sodium pyruvate, thymidine, amino acids, vitamins, 2% sodium bicarbonate supplemented with 5% FBS, 100 u/mL penicillin and 100 µg/mL streptomycin purchased from Sigma chemical company (St. Louis, MO. USA). Cytotoxicity of the biosynthesized silver nanoparticles was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated at a density of 1×10^5 cells/mL into 96-well tissue culture plate in DMEM medium without serum for 24-48 hour at 37°C and 5% CO₂. The wells were washed with sterile PBS and treated with various concentrations of the biosynthesised silver nanoparticles (20 – 100 µg/µL) in a serum-free DMEM medium for 24 hr. The analysis was performed in triplicates. After the incubation period, MTT (5 mg/mL) was added into each well and the cells incubated for another 2-4 hours until purple precipitates were clearly visible under an inverted microscope. MTT was aspirated off the wells and washed with 1X PBS (200 µL). To dissolve formazan crystals, 100 µL of DMSO was added and the plate and shaken for 5 min. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC₅₀ value was calculated using GraphPad Prism 6.0 software (USA). The percentage viability was calculated using the following formula.

$$\text{Viability \%} = \frac{\text{OD}_{\text{Sample}}}{\text{OD}_{\text{Control}}} \times 100$$

where OD_{sample} is mean OD value of the experimental sample (AgNPs), OD_{control} is the mean OD value of experimental control (untreated). The morphology changes of cells were observed using an inverted microscope [17].

E. Statistical Analysis:

All the concentration of AgNPs for cytotoxicity study were performed in triplicates. The results were represented as mean ± SD value.

III. Results and Discussion

A. Synthesis of silver nanoparticles

Aqueous extract of *Mollugo cerviana* was utilized for the green synthesis of silver nanoparticles. The reduction of the silver nitrate to silver nanoparticles was confirmed by the color change from yellowish brown to reddish brown [18] as shown in Figure 1.

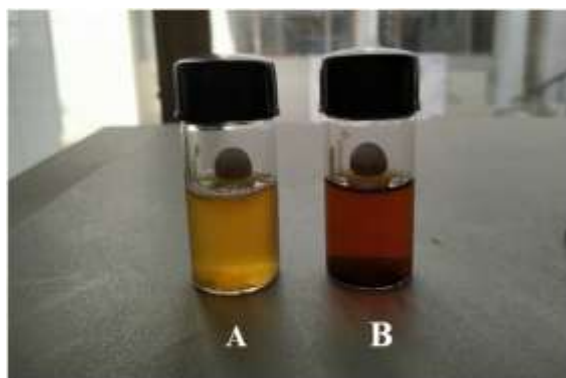


Figure 1: Synthesis of silver nanoparticles using the aqueous extract of *Mollugo cerviana*: (A) Aqueous extract of *Mollugo cerviana* (B) aqueous extract with silver nitrate (AgNO_3) after 24 hours of incubation.

This may be due to surface plasmon resonances vibration and polarization effect of light. Reduction of Ag^{2+} to Ag^0 may be due to the phytochemicals present in the aqueous plant extract.

B. Characterization of AgNPs

UV-vis absorption analysis

Usage of biosynthesized nanoparticles is essential in nanobiomedical research. The color change indicates the formation of silver nanoparticles, where there was no color change in the control. Plant extract of *Mollugo cerviana* was considered as control. The UV-vis absorption spectrum of biosynthesized AgNPs is shown in Figure 2.

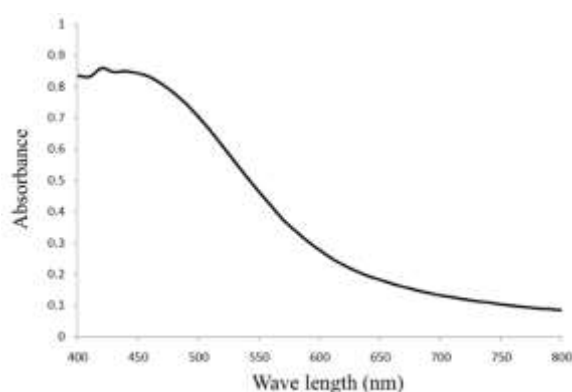


Figure 2: UV-vis absorbance spectrum of (a) *Mollugo cerviana* extract, and (b) biosynthesized AgNPs using *Mollugo cerviana* extract.

Absorbance peak at 420 nm was observed. Surface plasmon resonance absorbance is raised due to resonance coincidence of free electrons in the metal nanoparticles and light waves. It is reported that the absorption spectrum of spherical AgNPs present a maximum between 400 nm to 450 nm with a blue redshift when particle size diminishes or increase respectively[19].

X-ray diffraction studies

In XRD four prominent diffraction peaks were observed at $2\theta = 32.2247^\circ$, 46.2854° , 54.8654° and 76.7699° , which correspond to (111), (200), (220) and (311) Bragg's reflections of the face-centered cubic (FCC) structure of metallic silver, respectively (Figure 3).

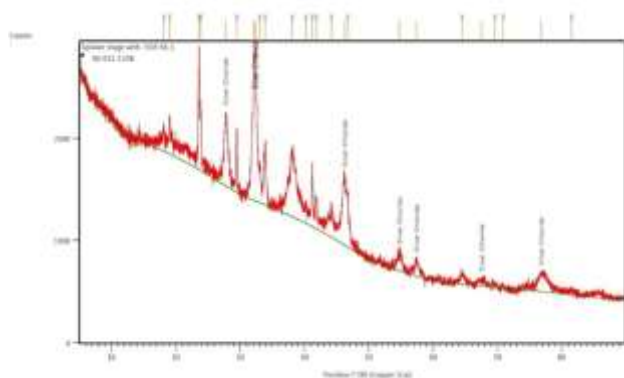


Figure 3: X-ray diffraction pattern of the biosynthesized silver nanoparticles using the aqueous extract of *Mollugo cerviana*

Peaks observed in the pattern agreed well with the previously reported values (JCPDS card no. 04-0783). The sharp, as well as broad diffraction pattern, infers that the synthesized system possess nano-dimensional state [20]. The multiple peaks represent the growth of particles from multiple faces. It is also presumed that the broadness in the peak may also arise from the local crystal defects (elongation strain/compressional stress) in the nanocrystals[21].

Fourier Transform Infrared Spectroscopic analysis (FTIR)

In the spectrum of plant extract, major bands at 1597 cm^{-1} , 1020 cm^{-1} , and 559 cm^{-1} correspond to C-C, O-H, and C-Br stretching vibrations modes of aromatic group, carboxylic acid, and carbon halogen, respectively. The minor peaks at 3333 cm^{-1} , 3233 cm^{-1} , 3136 cm^{-1} , 2921 cm^{-1} , and 605 cm^{-1} corresponds to O-H and C-H stretching and bending vibration of alcohol and alkane which has a strong role in the reduction of silver ions[19]. The absorption peak at 1600 cm^{-1} , 1379 cm^{-1} and 1019 cm^{-1} in the spectrum of AgNPs indicate C=C, C-H and O-C stretching and bending vibrations of aromatics group, alkane, and Carboxylic acid groups, respectively. The peak at 542 cm^{-1} shows the stretch for AgNPs [22]. FTIR spectrum of plant extract and AgNPs show a major shift in hydroxyl groups and carboxyl groups involving in reduction and capping of AgNPs (Figure 4) which avoids agglomeration in solution[20].

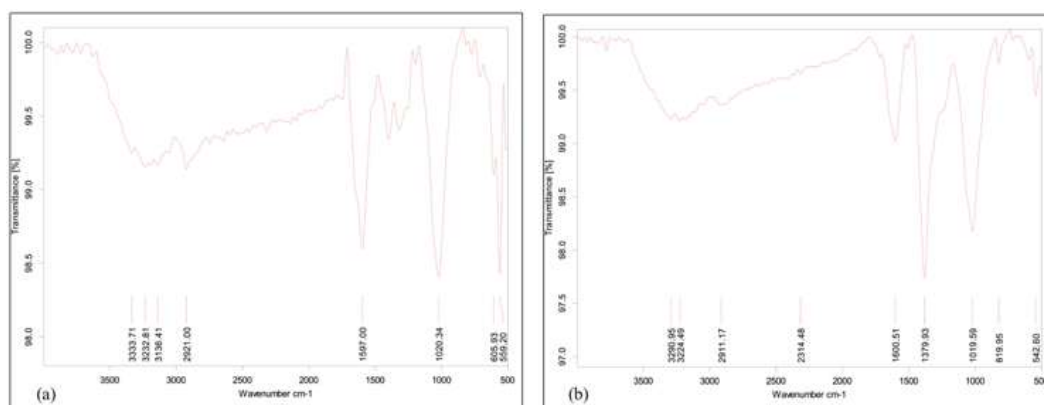


Figure 4: FTIR spectrum of (a) *Mollugo cerviana* extract and (b) biosynthesized AgNPs.

Zeta potential studies

The stability of AgNPs is represented by the parameter called zeta potential. Zeta potential indicates the electrostatic potential at the electrical double layer surrounding a nanoparticle in solution. Zeta potential is shown in Figure 5.

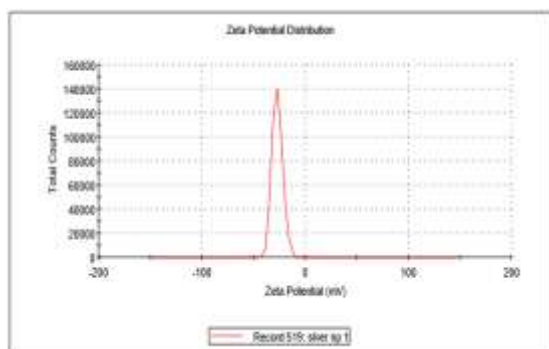


Figure 5:Zeta Potential of AgNP -27.0 mV.

If the zeta potential value is above +25 mV and below -25 mV, the particle is considered as adequately stable[23]. The value -27 mV shows that the biosynthesized AgNPs are strongly anionic. The electrostatic force between AgNPs and cells has a major role in the cytotoxicity effect[24].

SEM studies

SEM analysis was used to detect the size and shape of the AgNPs formed. The image of the SEM visualization is shown in Figure 6.

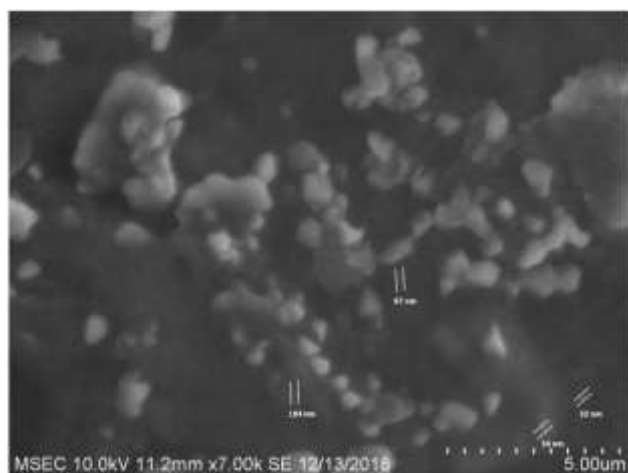


Figure 6: SEM micrograph of the biogenically synthesized silver nanoparticles.

Synthesized nanoparticles were in size ranging from 50 to 100 nm. Mixed shapes of AgNPs were also observed [25].

Energy Dispersive X-ray (EDX)

EDX was used to analyze the elements of biosynthesized AgNPs. Figure 7 shows the EDX pattern of AgNPs.

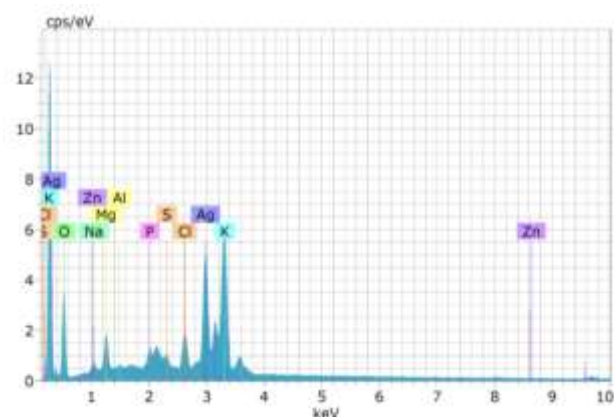


Figure 7:EDX pattern of nanoparticles confirming the presence of elemental silver as a major constituent.

The appearance of a strong peak around 3 KeV designate the position of elemental silver which arises from the localized surface plasmon resonance in AgNPs. Overall EDX result exhibited the presence of elemental silver as major constituents with the small number of other signals[26]. These weak signals may be originated from the surface-bound macromolecules and the copper grids used as supporting filament (Cu signal).

C. Cytotoxicity studies

The anti-proliferation assay at different concentrations of AgNPs on breast cancer cell lines MCF-7 and MDA MB-231 and Vero cells as normal cell control demonstrate that AgNPs have selective toxicity on both the breast cancer cell lines when it's not toxic to Vero cells. Figure 8 shows the morphological changes of cells depends on the dose.

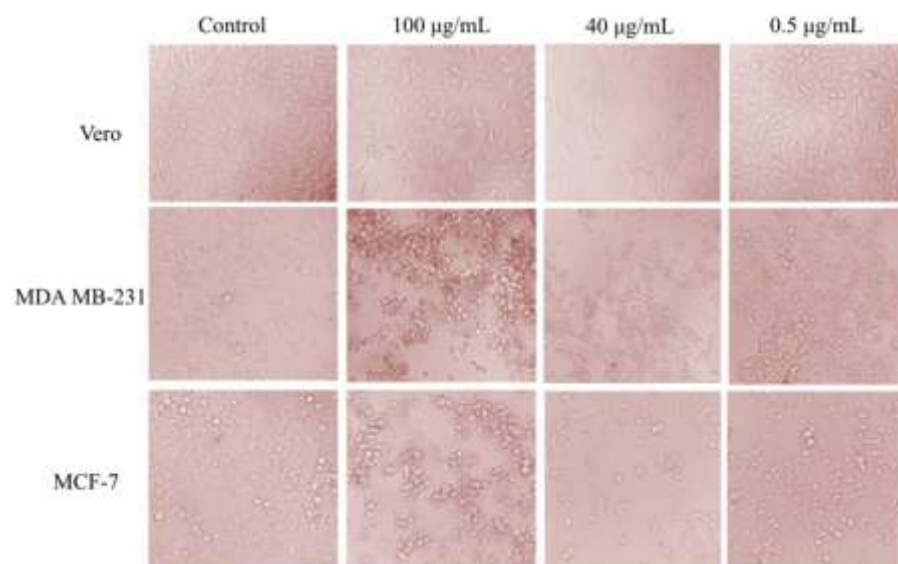


Figure 8: Morphological changes of cells at different concentration of AgNPs.

The data were analyzed using GraphPad Prism 6.0 software. The apoptosis rate of MDA MB-231 and MCF-7 cell lines increases with increase in the concentration of silver nanoparticles. A dose-dependent increase in cell inhibition was seen after 24 h exposure. The IC_{50} of silver nanoparticles against MDA MB-231 and MCF-7 was observed at 25.52 µg/mL and 21.53µg/mL respectively. The IC_{50} value predicts that the plant-mediated nanosilver will have a promising role in chemotherapeutic treatment (Figure 9), owing to its non-toxicity to normal cells.

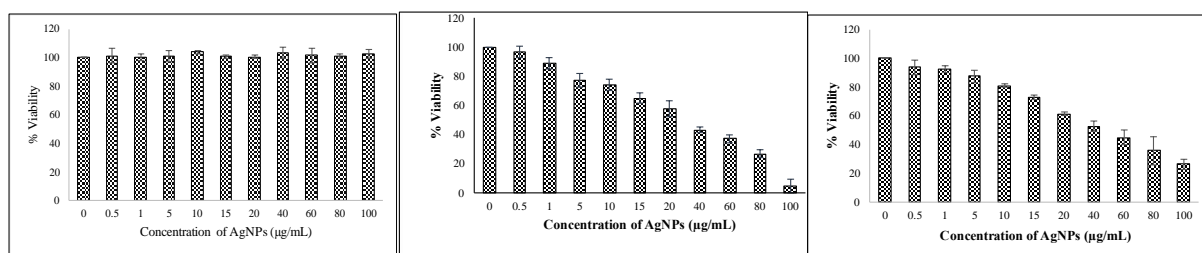


Figure 9: Dose dependent cytotoxicity effect of AgNP over (a) Vero (b) MDA MB-231 and (c) MCF-7 cells.

This selectivity may be due to the presence of phytochemicals present in the aqueous extract of *Mollusk cerviana* like phenols and flavonoids. On the other hand, due to the smaller size of the silver nanoparticles, they could easily enter the cells through the process called endocytosis. These nanoparticles increase the production of reactive oxygen species (ROS) when it is exposed to the acidic nature of the organelle's lysosome and endosomes. Highly reactive ROS like O^{2-} , $\bullet OH$ and hydrogen peroxide elevates oxidative stress of proteins and DNA which leads to the destruction of the cells [27]. Also, silver nanoparticles may interact with the cell membrane or mitochondrial membrane or outer membrane of lysosomes to release lipid peroxide. It makes the membrane leaky and leads to cell damage. Few studies reported the upregulation of metabolic and oxidative stress genes using RT-PCR analysis [28]. The elevated anti-proliferative activity of AgNPs due to the increased cell permeability and proliferation rate of cancerous cells which makes it more vulnerable to AgNPs [19].

VI. Conclusion

The increased usage of nanoparticles in various fields and the green synthesis of AgNPs are promoted to avoid drawbacks related to costs and toxicity effects. Biosynthesis of AgNPs using microorganisms has a low synthesis rate and the morphology of the nanoparticle is also limited. The plant or plant extracts show stable product production. Thus, in this present study, the aqueous extract of *Mollugo cerviana* is used to synthesize silver nanoparticle and the selective cytotoxicity against breast cancer cell lines like MDA MB-231 and MCF-7 was reported. Even though the exact mechanism of cytotoxicity activity is not clear, further research can be done towards the application of AgNPs in cancer therapy.

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