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Original Research

Biosynthesis of silver nanoparticles from leaf extract of *Litchi chinensis* and its dynamic biological impact on microbial cells and human cancer cell lines

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Abstract: Green synthesis of metallic nanoparticles has attracted a great deal of attention from scientific community due to its biocompatibility and environment friendly nature. In the present study, silver nanoparticles were biologically synthesized using leave extracts of *Litchi chinensis.* Biosynthesized silver nanoparticles were characterized and their applications were observed by different methodologies. Bio-reduction reaction was confirmed by the surface plasmon resonance of silver nanoparticles at 417 nm through UV-VIS spectrophotometer. FTIR analysis revealed that the amine groups present in the leaf extracts were responsible for the reduction of silver ions to silver nanoparticles. X-ray diffraction analysis was used to determine the crystalline nature of silver nanoparticles and their diameter was noted in the range of 41-55 nm by scanning electron microscopy. Antibacterial activity was observed against gram positive and gram negative strains of bacteria. Furthermore, human epithelial type 2 cancer cells (HEp-2) and Human breast adenocarcinoma cells lines (MCF-7) were treated with the biosynthesized silver nanoparticles using MTT assay. The resulting cell death rate was noted up to 40.91±1.99%. This study concludes that plant mediated biosynthesis of nanoparticles is the superior alternative compared to chemical and physical approaches, to utilize them as drug delivery tool and need to conjugate apoptosis inducing biological agents with silver nanoparticles to suppress the uncontrolled division of cancer cells.

Key words: Anticancer activity; Biosynthesized silver nanoparticles; Litchi chinensis; Human epithelial type 2 cancer cells (HEp-2); Human breast adenocarcinoma cells lines (MCF-7).

Introduction

Emerging technologies have a pivotal impact on the health sector advancement to meet the desired pace required to understand the complex architecture of the biological cell. Now a days, biomedical researchers emphasize on these technologies to conquer the unbeatable fort of progressive cancer and other commonly reported inflammations caused by microbial infection in humans. In recent findings, the researchers are utilizing nano-sized molecules and biological tools in molecular drug delivery system. In parallel, they have successfully synthesized nano-based materials to strengthen the clinical, disease diagnosis, textile, agriculture and veterinary related processes (1-4). Chemical, physical and biological methods have recently been used to synthesize the desired targeted metallic nanoparticles. It has been observed that chemical and physical methods are quite expensive and potentially harmful to the environment due to their toxic reducing agents (5). At the same time, the used chemicals cannot be easily degraded (6). A number of reports have been published that explain the biosynthesis of metallic nanoparticles from microorganisms. Microbial handling requires expensive culture media, has a slow reduction rate and only a limited size can be achieved. Moreover, their waste products may be harmful for the environment depending upon the type of microorganism (7). However, plant mediated biosynthesis of nanoparticles is an environment friendly technique, economically feasible, safe for therapeutic use and can be used for large scale production.

Nanoparticles are stable, target specific and reactive due to their large surface area and are practically proved to be functionally superior over the bulk materials. A large number of metallic nanoparticles have been synthesized by the scientific community such as silver, gold, platinum, palladium, aluminum, selenium, copper, cobalt, cadmium and nickel (8). Among these, silver nanoparticles have unique chemical, physical and electrical properties as compared to macro-clusters (9). They are chemically stable, catalytically active, show high conductivity and surface enhanced raman scattering (10). The Physical and biological properties of silver nanoparticles depend upon their size distribution. They are excellent antimicrobial agents and it is believed that silver nanoparticles are attached to the microbial cell membrane and disturb the cellular respiration by ion exchange mechanism that lead to the destruction of microbial cells (11).

Cotton is a natural fiber having cellulose with 1,4-D-glucopyranose and it provides a suitable environment for the growth of microorganisms. Numerous antibac-

terial finishes are applied on cotton products to control odor, deterioration and growth of pathogenic bacteria. Nano-silver based antibacterial textile products have become the challenge of present time. Silver has the ability to kill hundreds of disease causing microorganisms and its toxicity toward mammalian cells remains low (12). This remarkable characteristics make silver nanoparticles worthwhile for antibacterial finishing in textile industry. The Medical field always remain thought-provoking, as patients and medical practitioners are frequently exposed to disease causing microorganisms. Protective silver nanoparticles coated bandages can help them to control the pathogens. For the first time, in this particular work, the leave extracts of *Litchi chinensis* were used for the biosynthesis of silver nanoparticles.

"Feed your genes right" is the practical approach that reflect the importance of nutraceuticals in the treatment of various disorders including cancer. Many phytochemicals derived from medicinal plants have already made their way into various phases of clinical trials (13). Natural products represent a premium reservoir of high-quality molecules having ability to modulate wide ranging signaling pathways in different cancers (14-16).

Materials and Methods

Silver Nitrate ≥99% (Sigma Aldrich), Streptomycin (Chongqing P.R. China) and all other reagents were available in the Biochemistry laboratory, University of Gujrat. Fresh leaves of Litchi chinensis were collected from Lychee gardens located at Sharaqpur Road, Lahore. Bacterial strains and facility to perform XRD, FTIR, SEM were provided by laboratory at Institute of Industrial Biotechnology, Government College University Lahore. Cancer cell lines were obtained from Institute of Biomedical and Genetic Engineering, Islamabad.

Preparation of leaf extract

Fresh leaves of *Litchi chinensis* were collected from Lychee Gardens, Sharaqpur Road, Lahore, Pakistan. The obtained leaves were washed twice with hot water to remove contaminations and dried in the incubator. The completely dried leaves were crushed into fine powder form. Leaf extract was prepared by boiling the leaf powder (5 g) in 100 mL of distilled water for 15 min and leaf broth was cooled at room temperature. Finally, the leaf extract was filtered in 250 mL Erlenmeyer flask by using Whatman filter paper No. 1.

Biosynthesis of silver nanoparticles

Silver nanoparticles were synthesized by a green approach. The leaf extract (5 mL) was mixed with 100 mL of 1mM aqueous $AgNO_3$ solution. Bio-reduction reaction was carried out in 250 mL conical flask. The leaf extract was added drop by drop in $AgNO_3$ solution. The flask was wrapped with aluminum foil to minimize the photo-active reaction (17). The color of solution turn brown by the addition by leaf extract in silver nitrate solution and is the visual indicator of the synthesis of nanoparticles. Incubate it for 1-2 hours and adjust the PH at 8 with the help of PH meter.

Characterization of biosynthesized silver nanoparticles

Absorption spectrum of the silver nanoparticles was analyzed by UV-visible spectrophotometer (Shimadzu UV-1700 PharmaSpec). The Crystalline nature of the particles was determined by X-ray diffraction analysis using X'Pert Pro, PAN analytical 7602 X-ray diffractometer operated at 40 kV with Cu anode (Kα 1.5405 Å) in the range of 20-80°. FTIR spectrum was recorded to determine the functional groups of the stabilizing agents involved in bio-reduction reaction. Morphology and particle size distribution was studied through Jeol JSM-6480 scanning electron microscope (SEM).

Antibacterial analysis of silver nanoparticles

The biosynthesized silver nanoparticles obtained from leave extracts of *L. chinensis* were studied for antibacterial activity against Gram positive (*Bacillus subtilis and Staphylococcus aureus)* and Gram negative (*Escherichia coli and Pseudomonas sp*.) using disk diffusion method. The bacterial strains were dispensed in saline water and their concentration was compared with McFarland 0.5 standard solution. Each strain was swabbed by sterile cotton buds onto Muller Hinton Agar (MHA) media poured in sterilized petri plates. 5 µL of each of four solutions including silver nitrate, silver nanoparticles, leaf extract and streptomycin (10 µg/mL) was added by the use of micropipette on filter paper discs. The discs were placed on agar plates containing bacterial strains and incubated at 37°C for 17 hrs. Finally, the zone of inhibition was measured in millimeter "mm" (18).

Antibacterial activity of silver nanoparticles coated cotton cloths and its characterization by SEM

Silver nanoparticle solution was applied on cotton cloths of $1cm^2$ and incubated overnight. Next day, the cotton pieces were placed in sterilized petri plates at 50°C to evaporate the water content. The completely dried cotton pieces were analyzed by SEM. Afterwards, silver nanoparticles coated cotton pieces were subjected to antibacterial analysis. The autoclaved non-coated cotton cloths were used as negative control. After incubation for 17 hrs, zone of inhibitions were observed.

MTT assay against HEp-2 and MCF-7 cell lines

Biosynthesized silver nanoparticles were analyzed for anticancer activity against HEp-2 and MCF-7 cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide) colorimetric technique. Normal human corneal epithelial cells (HCEC) Cancerous cell lines were cultured in RPMI media containing 10% FCS (Fetal calf serum) and GPPS (4 antibiotics). The cells were incubated in 5% CO_2 incubator at 37˚C for 2-3 days. Further experiment were performed when above 70% confluency of cells was noted by inverted microscope. Four different concentrations of biosynthesized silver nanoparticles (8, 16, 24, 32 ppm) were prepared in fresh deionized water. HEp-2 and MCF-7 cells were seeded (100 µL) in 96 well plates. The silver nanoparticles solution (10 µL) was added in each well. The 96 well plates were incubated in the 5% CO_2 incubator at 37°C for 24 hrs. Then 10 µL of MTT assay (5 mg/mL) was added in the reaction solution containing cell lines

and silver nanoparticles. It was subjected to 5% CO_2 incubator at 37°C for 24 hrs. On next day, 100 µL of solubilization buffer was added in the 96 well plates and were incubated for 2 hrs at 37°C, in order to dissolve formazan crystals. The solution was mixed to ensure complete solubilization. The 96 well plates were subjected to ELISA plate reader and rate of cell viability was recorded by observing the absorbance at 570 nm. A control test was performed at the same time in the absence of silver nanoparticles. The cell viability in percentage was calculated by following formula.

Cell viability (%) = $[A]_{test}/[A]_{control} \times 100$

Where, $[A]_{test}$ is the absorbance of sample solution. While, $[A]_{control}$ is the absorbance of control.

Cell death rate was calculated by following formula.

Cell death rate (%) = Cell viability - 100

Results and Discussion

Aqueous leave extracts of *L. chinensis* was used to reduce the silver ions present in 1 mM $AgNO_3$ solution. Chemical constituents present in the leave extracts were responsible for bioreduction reaction. A visible dark brown color was observed after 30 min of mixing the leaf extract in aqueous silver nitrate solution "Figure 1". The intensity of the colour was directly linked with the time of incubation (19). In has been previously explained that a color change from yellow to dark brown is an indication of biosynthesis of silver nanoparticles (20). The surface plasmon resonance property of biosynthesized silver nanoparticles is responsible for the change of color in the solution (21).

UV-visible spectroscopy

UV-visible spectroscopic analysis helps in determining the size and shape of the silver nanoparticles in aqueous solution (22). In the present study, silver nitrate (1 mM), the leaf extract and biosynthesized silver nanoparticles were subjected to analyze the ultraviolet visible spectrum in the rage of 200-800 nm "Figure 2". Aqueous silver nitrate did not show any characteristic peak, due to the absence of any particle in the solution. In case of leaf extract, some peaks were shown representing the phytochemicals. The silver nanoparticles solution showed maximum absorption (λ_{max}) at 417 nm. This was a characteristic peak for biosynthesized silver nanoparticles (23). A literature survey revealed that biogenic silver nanoparticles exhibited maximum absorption in the range of 400 to 475 nm (21, 24).

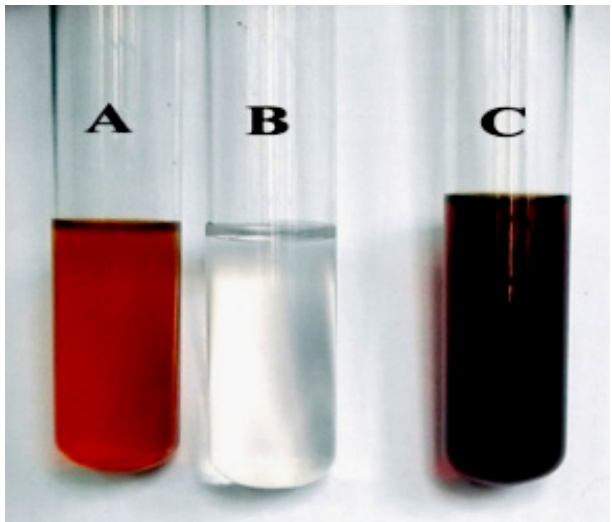

Figure 1. Change in color showing the biosynthesis of silver nanoparticles. (A) Leaf extract, (B) 1mM silver nitrate, (C) Biosynthesized silver nanoparticles.

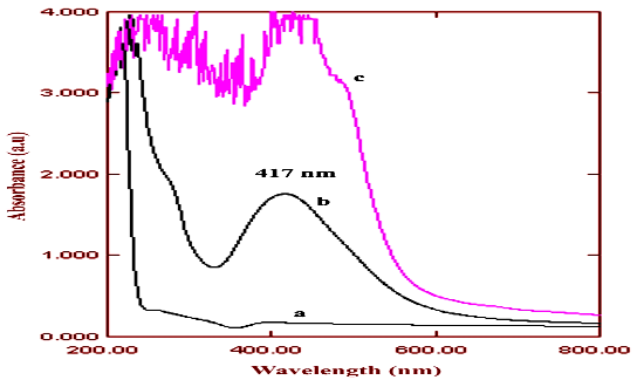

Figure 2. UV-VIS spectrum of biosynthesized of silver nanoparticles. (**a)** Silver nitrate solution, (**b)** Biosynthesized silver nanoparticles solution, (**c)** Leaf extract.

FTIR analysis

Fourier transforms infrared analysis (FTIR) was carried out to determine the functional groups of phytochemicals that were responsible for capping and stabilization of silver nanoparticles. The absorption band at 1053 cm^{-1} was representing the C–N stretching vibrations of aliphatic amines, commonly found in proteins (25). The other strong peak that was observed at 1384 cm^{-1} corresponds to C=C stretching of aromatic amine group (26). The absorption band at 1581 cm^{-1} was representing to the aromatic groups, while a strong peak at 2351 cm^{-1} was observed due to the N–H stretching of amines "Figure 3". On the whole, it was noted that amines groups present in the proteins were responsible for the biosynthesis of silver nanoparticles from leave extracts of *L. chinensis.*

X-Ray diffraction analysis

Crystalline nature of the silver nanoparticles was determined by the characteristic peaks at 2θ degrees of 38.20, 44.02, 64.29 and 77.22 which were attributed to the (111), (200), (220) and (311) planes of nano-crystalline silver, respectively "Figure 4". These peaks were indexed to face centered cubic crystalline structures (Ag XRD Ref. No. 00-087-0719). The whole spectrum ranging from 20-80° (2 theta) was used to fully understand the nature of silver nanoparticles. Two small peaks at 32° and 46° were observed due to the presence of Ag_2O

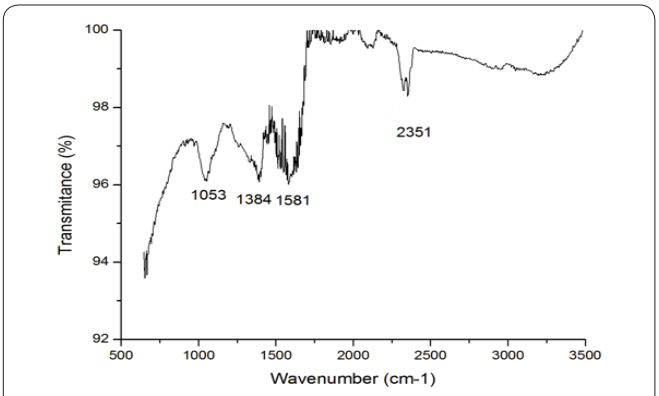

Figure 3. FTIR analysis of biosynthesized silver nanopaticules.

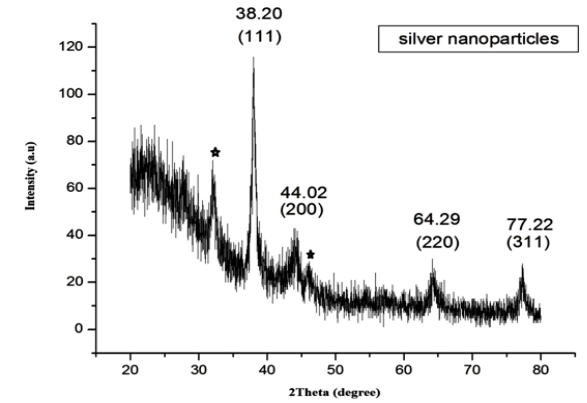

Figure 4. X-Ray diffraction pattern of biosynthesized silver nanopaticules.

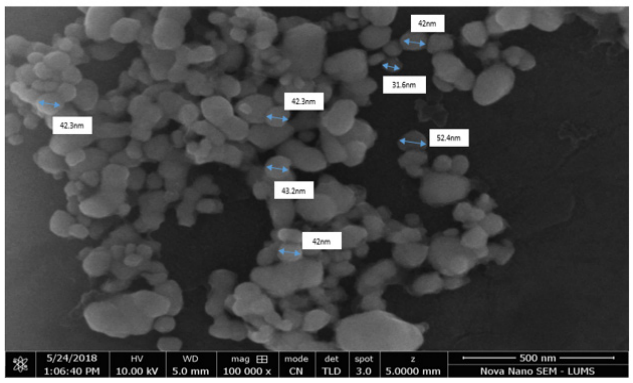

Figure 5. Scanning electron microscope (SEM) images of biosynthesized silver nanopaticules.

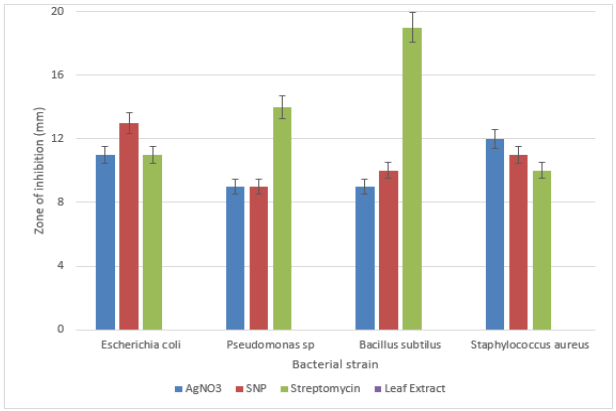

Figure 6. Antibacterial analysis of biosynthesized silver nanoparticules. Peaks showing zone of inhibition by using $AgNO_3$ biosynthesized silver nanoparticules and Streptomycin. While no prominent antimicrobial activity was observed by using litchi chinensis leaf extract.

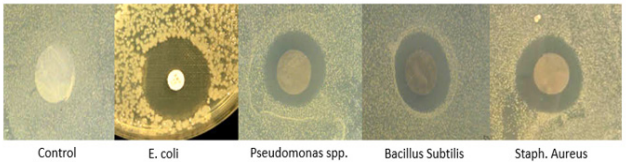

Figure 7. Antibacterial analysis of biosynthesized silver nanoparticules by diffusion method.

as impurity, while other small peaks were indicating to noise. The present results were in a good agreement with the results reported results (27, 28).

Scanning electron microscopy

Scanning electron microscopic analysis (SEM) was performed to determine the size and shape of the silver

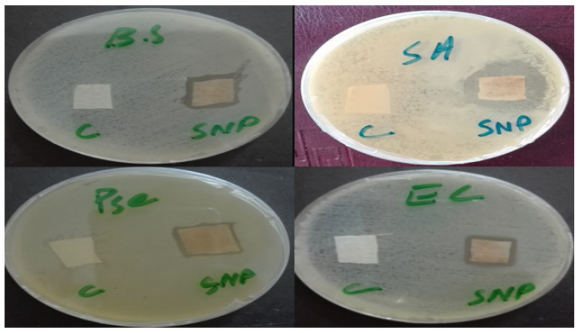

Figure 8. Antibacterial analysis of biosynthesized silver nanoparticules coted cotton fabrics. (C) Negative control, (B.S) *Bacillus subtilis*, (SA) *Staphylococcus aureus*, (EC) *Escherichia coli*, (Pse) *Pseudomonas sp.*

nanoparticles. Well dispersed spherical nanoparticles having diameter in the range of 41-55 nm were observed "Figure 5". The large sized nanoparticles were formed by agglomeration of the smaller ones (21, 29).

Antibacterial activity of biosynthesized silver nanoparticles

Four different bacterial strains, Gram positive *Bacillus subtilis, Staphylococcus aureus* and Gram negative *Escherichia coli, Pseudomonas sp.* were subjected to antibacterial analysis using disc diffusion method. Culture plates having sample discs were kept in static incubator at 37°C. Zone of inhibition were noted after 17 hrs of incubation. The maximum zone of inhibition was noted against *E. coli* as 13±0.65 mm. While, zone of inhibition against *Pseudomonas sp., B. subtilis* and *S. aureus* were observed as 9±0.45 mm,10±0.5 mm and 11±0.55 mm, respectively "Figure 6" and "Figure 7". It was noted that there was not much difference between the zone of inhibitions of silver nitrate and silver nanoparticles solution while streptomycin showed larger zones. The leave extracts of *L. chinenesis* did not show any antibacterial activity. Bacterial strains have the ability to develop resistance against antibiotics. Therefore, silver nanoparticles provide an alternative tool to control the bacterial growth. They interact with nuclear content of bacteria and destroy the bacterial cells.

Antibacterial activity of silver nanoparticles coated cotton cloths

Cotton cloths loaded with silver nanoparticles were tested against gram positive and gram negative bacterial strains. A well-defined area of inhibition was seen at the margins of silver nanoparticles coated cotton cloths while there was not any sign of inhibition around fresh autoclaved cotton cloths "Figure 8". Silver nanoparticles coated cotton cloths were analyzed under SEM. Crystallized spherical nanoparticles were clearly visible at cotton fibers "Figure 9". The present results have resemblance with the reported results of Balashanmugam (20).

Anticancer activity of silver nanoparticles by MTT assay

HEp-2 and MCF-7 cells were treated with four dilutions of silver nanoparticles (8, 16, 24 and 32 ppm). Silver nanoparticles showed considerable cytotoxicity. Maximum cell death rate was 43.71±1.99% at 16ppm

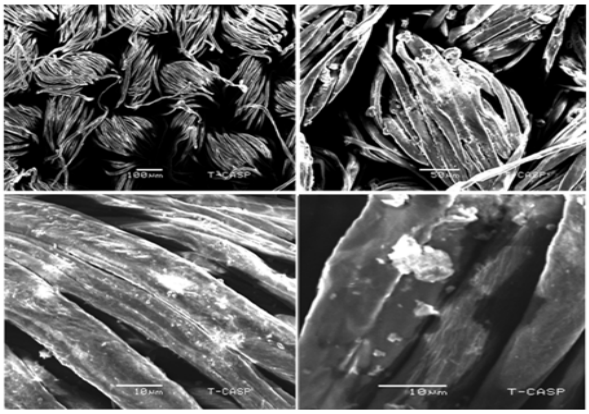

Figure 9. Scanning electron micrographs of silver nanoparticules coated cotton cloths.

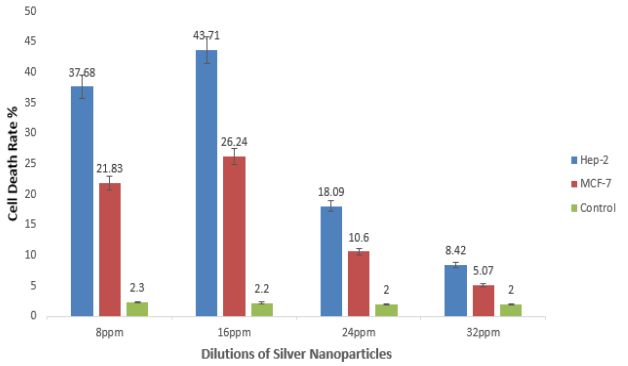

Figure 10. Anticancer activity of silver nanoparticles against HEp-2 and MCF-7 cell lines. HCEC cells were seeded as control.

dilution that indicated the optimum concentration of silver nanoparticles as prodrug to inhibit cancerous cell division. While, cell death rate was low in case of 8 ppm, 24 ppm and 32 ppm solutions as 37.83±5.26%, 18.09±2.63%, 8.42±4.89%, respectively "Figure 10". A negative control experiment was also conducted in the absence of silver nanoparticles showing the cell death rate of 2.16±0.06%. Couple of studies have been reported to examine the anticancer activity of different green extracts. Melia dubia leaf extract was used to prepare silver nanoparticles and to evaluate its anticancer activity against KB (breast cancer) cell lines. Silver nanoparticles showed remarkable cytotoxicity activity against KB cell line with evidence of high therapeutic index (30). The present results revealed that cancerous cell death can be achieved by the plant mediated silver nanoparticles. It has been reported that the cytotoxic effect increased with the concentration of nanoparticles. However, this particular study showed that cell death rate was linked with an optimum concentration of silver nanoparticles.

Plant mediated biosynthesis of metallic nanoparticles is inexpensive and environment friendly approach. In the present study, aqueous leave extracts of *Litchi chinensis* was successfully used to synthesize the silver nanoparticles in the absence of any toxic capping agent. Biogenic silver nanoparticles were fully characterized by UV-VIS spectroscopy, FTIR, XRD and SEM. Cytotoxic activity of silver nanoparticles revealed their biomedical application. It was concluded that cotton cloths impregnated with silver nanoparticles can be used to develop antibacterial textile products. The encouraging results of MTT assay provided a clue to couple these nanoparticles with prodrugs or miR34a to stop cancerous cell development. By gaining benefits from the emerging nanotechnology, plant derived nanoparticles provided a potential platform to conquer the unbeatable fort of cancer progression.

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Conflict of Interests

The authors declare that they have no conflict of interests.

Authors Contributions

It is to certify that all persons (co-authors) mentioned below participated sufficiently in the work to take responsibility of the content. The name of each author and contribution given below

Muhammad Javed Iqbal (Conception and Design of Study, Experimental performance), Sikander Ali (Analysis and interpretation of data), Umer Rashid (Conception and Design of Study, Analysis and interpretation of data), Muhammad Kamran (Sample collection and extract preparation), Muhammad Faheem Malik (Drafting of manuscript), Kalsoom Sughra (Data Analysis and Drafting of manuscript), Nadia Zeeshan (Experimental performance, Nanoparticle synthesis), Amber Afroz (Critical revision and drafting), Javaria Saleem (Sample collection), Mariam Saghir (Antimicrobial activity).

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