**Zizyphus mauritiana** Fruit Extract-Mediated Synthesized Silver/Silver Chloride Nanoparticles Retain Antimicrobial Activity and Induce Apoptosis in MCF-7 Cells through the Fas Pathway

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**ABSTRACT:** Recently, green synthesis of silver/silver chloride nanoparticles (Ag/AgCl-NPs) has gained a lot of interest because of the usage of natural resources, rapidness, eco-friendliness, and benignancy. Several researchers reported that silver-based biogenic NPs have both antimicrobial and anticancer properties. In the present study, Ag/AgCl-NPs were synthesized from *Zizyphus mauritiana* fruit extract, and their antibacterial, antifungal, and antiproliferative mechanisms against human MCF-7 cell lines were evaluated. Synthesis of Ag/AgCl-NPs from the *Z. mauritiana* fruit extract was confirmed by the changes of color and a peak of the UV–visible spectrum at 428 nm. The nanoparticles were characterized by transmission electron microscopy, energy dispersive X-ray, X-ray powder diffraction, thermal gravimetric analysis, atomic force microscope, and Fourier transform infrared. Antibacterial activity was checked against four pathogenic bacteria and two fungi. Cytotoxicity was checked against human breast cancer cell line (MCF-7) and mice Ehrlich ascites carcinoma (EAC) cells by MTS assay and clonogenicity assay. Cell morphology of the control and nanoparticle-treated MCF-7 cells were checked by Hoechst 33342, YF488-Annexin V, and caspase-3 substrates. The level of reactive oxygen species (ROS) was studied by using 2′,7′-dichlorodihydrofluorescein-diacetate staining. Real-time polymerase chain reaction was used for gene expression. Synthesized nanoparticles were heat stable cubic crystals with an average size of 16 nm that contain silver and chlorine with various functional groups. The synthesized Ag/AgCl-NPs inhibited the growth of three pathogenic bacteria (*Bacillus subtilis*, *Shigella boydii*, and *Escherichia coli*) and two fungi (*Aspergillus niger* and *Trichoderma* spp.). Ag/AgCl-NPs inhibited the growth of MCF-7 and EAC cells with the IC50 values of 28 and 84 μg/mL, respectively. No colony was formed in MCF-7 cells in the presence of these nanoparticles as compared with control. Ag/AgCl-NPs induced apoptosis and generated ROS in MCF-7 cells. The expression level of FAS, FADD, and caspase-8 genes increased several folds with the decrease of PARP gene expression. These results demonstrated that the anti-proliferation activity of Ag/AgCl-NPs against MCF-7 cells resulted through ROS generation and induction of apoptosis through the Fas-mediated pathway.

**INTRODUCTION**

Cancer and microbial infections are serious problems worldwide. To solve these problems, researchers are trying to use nanoparticles in biomedical and pharmaceutical sectors as alternative anti-microbial and anti-cancer agents. Various metals, for example, silver, gold, and platinum, are being used for the preparation of effective nanoparticles. Recently, it was reported that silver has disinfecing nature and shows anticancer effects against different cancer cell lines. Synthesis of biogenic silver nanoparticles is a faster and cheaper process as compared with physical and chemical processes. Moreover, large-scale synthesis of nanoparticles is now possible without producing any toxic byproduct. For the synthesis of plant-mediated silver nanoparticles, different parts of plants, such as fruits, barks, roots, seeds, rhizomes, and leaves, are used, and the biomolecules such as proteins, enzymes, terpenoids, cofactors, and flavonoids present in the extract act as reducing and capping agents of silver in the nanoparticles. Different fruits were reported as sources of synthesized silver nano-
particles, but Zizyphus mauritiana fruit extract-mediated silver nanoparticles had not been reported in the literature so far.

Z. mauritiana Lam. is an important medicinal plant that belongs to the family of Rhamnaceae. Anticancer effects of different parts of Z. mauritiana against HeLa and MCF-7 cell line in vitro had been reported.\textsuperscript{13,14} Different varieties of Z. mauritiana are cultivated in different parts of Bangladesh among which BAU Kul was reported to contain polyphenols, flavonoids, tannin, steroids, saponin, triterpenoids, ascorbic acid, protein, alkaloids, glycosides, and reducing sugars.\textsuperscript{15–17} In this work, Ag/AgCl-NPs were biosynthesized by using Z. mauritiana fruit extract. The biogenic Ag/AgCl-NPs were characterized by UV−visible spectroscopy, X-ray powder diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, energy dispersive X-ray (EDX), transmission electron microscopy (TEM), and atomic force microscopy (AFM), whereas thermal characterization was performed by thermal gravimetric analysis (TGA). Antibacterial and antifungal activities were checked against four pathogenic bacteria and two fungi. Cytotoxicity was checked against human breast cancer cell line (MCF-7) and mice Ehrlich ascites carcinoma (EAC) cells. The anticancer mechanism of Ag/AgCl-NPs against the MCF-7 cell was elucidated in the present investigation.

### RESULTS

**Ag/AgCl-NP Synthesis and Morphological Characterization.** AgNO\textsubscript{3} was added at different concentrations to the Z. mauritiana fruit extract prepared with Tris-HCl, and the brown color was developed gradually with the increase of AgNO\textsubscript{3} concentrations indicating the formation of Ag/AgCl-NPs, as shown in Figure 1A. The deepest brown color was monitored at 5 mM of AgNO\textsubscript{3} and the maximum absorbance peak was measured at 428 nm (Figure 1A). Around 700 mg (7 mg/mL) of silver/silver chloride nanoparticles was obtained from 1 kg of fruits. Most of the solution was dried to perform various characterizations. Highly monodispersed and spherical nanoparticles were determined by TEM, and the average diameter was calculated to be around 16 nm, as presented in Figure 1B. The result of EDX analysis was shown in Figure 1C where strong signals for silver and chlorine ions were detected.

**Structural and Thermal Characterization of Ag/AgCl-NPs.** The reflection peaks of the XRD pattern were at 27.88, 32.32, 46.28, 54.82, 57.54, and 67.42\textdegree corresponding to the crystallographic planes (111), (200), (220), (311), (222), and (400), respectively, which indicates the formation of AgCl-NPs (card no. 00-901-1666) formation. On the other hand, peaks at 38.21, 44.28, and 64.71\textdegree that correspond to the planes (111), (200), and (220), respectively, were designated for AgNPs (card no. 00-150-9146) (Figure 2A). The crystal systems for both cases were cubic. Three weight loss steps of Ag/AgCl-NPs depicted in the TGA plot/profile (Figure 2B) occurred in the temperature range from 30 to 100 °C, 101 to 718 °C and 719 to 843 °C corresponding to 3, 36.2, and 4.08% weight loss, respectively.

**Surface Properties of Ag/AgCl-NPs.** An atomic force microscope was used to determine the dimensions (including size, shape, and dispersion) of the nanoparticles. The presence of bright spots on the surface as found in AFM is indicating the presence of Ag/AgCl-NPs, and the inverse showed the dark spots indicating size and dispersion of the nanoparticles. The 3D image showed the sharp peaks with lower to higher sizes of nanoparticles (Figure 3).

**Antifungal Activity.** Antifungal activities were checked against Aspergillus niger and Trichoderma spp. at the concentrations of 7.5, 15, 30, and 60 μg/mL of Ag/AgCl-NPs. A. niger growth was not affected at 7.5 μg/mL concentration of Ag/AgCl-NPs, whereas 9.6% growth of Trichoderma spp. was inhibited at the same concentration. Growth inhibition was increased with the rise of concentration of nanoparticles, and finally, 100% growth inhibition of Trichoderma spp. and A. niger growth inhibition was observed at a concentration of 30 and 60 μg/mL of Ag/AgCl-NPs, respectively, as shown in Figure 5.

![Figure 1. Synthesis of Ag/AgCl-NPs by treating AgNO\textsubscript{3}, TEM, and EDX. (A) UV−visible spectra of the reaction mixture at different concentrations of AgNO\textsubscript{3} during the synthesis of Z. mauritiana fruit extract-mediated Ag/AgCl-NPs. Inset: color formation after treatment of Z. mauritiana fruit extracts with 0 to 5 mM of AgNO\textsubscript{3}. (B) TEM micrograph showing the formation of Ag/AgCl-NPs by Z. mauritiana fruit extract. Inset: black solid bar indicates 50 nm. (C) Energy dispersive X-ray spectrum of Ag/AgCl-NPs.](https://dx.doi.org/10.1021/acsomega.0c02878)
Determination of Antiproliferative Activity by MTS Assay. To assess the cytotoxicity of Ag/AgCl-NPs and 5-fluorouracil against MCF-7 cell line, MTS assay was employed. Ag/AgCl-NPs and 5-fluorouracil induced the death of MCF-7 cells in a dose-dependent way, as shown in Figure 6A,B, and the IC_{50} value was 28 and 290 μg/mL, respectively. Ag/AgCl-NP-induced EAC cell death was in a dose-dependent manner, as shown in Figure 6C, and the IC_{50} value was calculated to be 84 μg/mL.

Effects of Ag/AgCl-NPs on the Colony Formation of MCF-7 Cells. It was found that Ag/AgCl-NPs completely inhibited the formation of colonies of MCF-7 cells. After two weeks, 120 ± 20 colonies were counted in control wells, while Ag/AgCl-NP-treated wells were colony less (Figure 6D).

Induction of Apoptosis by Different Fluorometric Assays. Ag/AgCl-NP-treated and -untreated MCF-7 cells were labeled with YF488-Annexin V. Regular shape was observed for untreated MCF-7 cells (Figure 7A1) which was compared to the irregular shapes of treated cells, as shown in bright field microscopic images (Figure 7B1). Induction of apoptosis in Ag/AgCl-NP-treated cells were confirmed after incubation with annexin V (Figure 7B2), whereas no change was observed in control MCF-7 cells (Figure 7A2). Expression of caspase-3 in the MCF-7 cell was observed using a fluorescence microscope after staining with the FITC-labeled caspase-3 substrate. The substrate bound to caspase-3 and green color of FITC was clearly visible in the Ag/AgCl-NP-treated MCF-7 cells (Figure 7B3), whereas no expression was observed in control MCF-7 cells (Figure 7A3). The expression of caspase-3 might be an indication of apoptosis in the treated MCF-7 cells. Bright field microscopic images of untreated and treated MCF-7 cells were shown in Figure 7B4 and 7B5, respectively.

Table 1. Zone of Bacterial Growth Inhibition by Z. mauritiana Fruit Extract-Mediated Ag/AgCl-NPs

| name of bacteria | Ag/AgCl-NPs (μg/mL) | streptomycin (unit) |
|------------------|----------------------|---------------------|
| B. subtilis      | 40                   | 20                  |
|                  | 20                   | 10                  |
|                  | 10                   | 20                  |

Figure 2. (A) XRD pattern of Z. mauritiana fruit extract-mediated Ag/AgCl-NPs. (+) and (★) indicating Ag-NPs and Ag/AgCl-NPs, respectively. (B) TGA micrograph showing the weight loss of Ag/AgCl-NPs with temperature.

Figure 3. AFM topography of Z. mauritiana pulp extract-mediated Ag/AgCl-NPs. (A) View in 2-dimension and (B) 3-dimension.

Figure 4. FTIR spectrum of Z. mauritiana fruit extract- and fruit extract-mediated Ag/AgCl-NPs.

Figure 5. Antifungal activity of Ag/AgCl-NPs against Trichoderma spp. and A. niger.
treated MCF-7 after staining with Hoechst 33342 are shown in Figure 7A4,B4, respectively. Condensed nucleus was observed in Ag/AgCl-NP-treated cells after incubation with Hoechst 33342 (Figure 7B5). On the other hand, condensed nucleus was absent in control cells (Figure 7A5). Intracellular reactive oxygen species (ROS) generation was evaluated using the fluorescent probe dichlorodihydrofluorescein diacetate (DCFH-DA). After treatment of MCF-7 cells, ROS was generated, as shown in Figure 7B6. No ROS generation was observed in control MCF-7 cells (Figure 7A6).

**Gene Expression.** To observe the expression level of apoptosis-related genes, MCF-7 cells were treated with *Z. mauritiana* fruit extract-mediated Ag/AgCl-NPs for 48 h, RNAs were purified, cDNAs were synthesized, and finally expressions levels of eight genes were examined by real-time polymerase chain reaction (PCR). Human 18s gene was used as references to normalize the real time PCR data. Several folds increase in the expression of FAS, caspase-8, and FADD were observed, while the expression level of PARP was decreased (Figure 8). Expressions of BID, p53, MLKL, and TNFα genes...
were not observed in control cells and after treatment with nanoparticles (data not shown).

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Percentages of relative mRNA expression after treatment of MCF7 cells with Ag/AgCl-NPs. Dashed line indicates 1.0 expression level.

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**DISCUSSION**

In the present study, *Z. mauritiana* fruit extract-mediated Ag/AgCl-NPs was biologically synthesized that was preliminarily verified by the change of color from transparent to deep brown. The extract contains various biomolecules which reduced Ag⁺ to Ag⁰ causing the color change. Because of the surface plasmon resonance, the absorbance peak between 400 and 450 nm is another indicator for the formation of silver nanoparticles. The maximum absorbance peak was observed at 428 nm.

The size of nanoparticles was found to be highly monodispersed and spherical with an average diameter approximately of 16 nm as determined by TEM. Most of the plant-mediated Ag/AgCl-NPs were found to be spherical, and several nanoparticles were reported to be around the size of 16 nm. The EDX spectrum exhibited the presence of elemental silver and chlorine ions along with signals of carbon and copper from the carbon-coated copper (Cu) grid. Because of the binding of biomolecules to the surface of Ag/AgCl-NPs, weak signals were formed. The first weight loss (3%) that appeared within temperature 100 °C, indicated the removal of water, and the second and third weight loss (40.28%) suggested the elimination or decomposition of the capping biomolecules as observed by TGA. This result indicated that the synthesized nanoparticles possessed high thermal stability. Crystalline character of the synthesized nanoparticles was proved by XRD, and the presence of Ag ions in the form of silver chloride was also found. A peak of silver chloride (AgCl) in the biologically synthesized AgCl-NPs was also reported. During biosynthesis, chlorine ions of the *Cissus quadrangularis* Linn leaf extract reacted with AgNO₃ to form AgCl-NPs. It was observed that the synthesis of AgCl-NPs from oligomeric chitosan was a two-step process where Ag ions reacted with Cl ions in the first step and then AgCl-NPs became stabilized with amino and hydroxyl groups. In the present study, silver ions reacted with chlorine ions of the Tris-HCl buffer to form AgCl and finally stabilized as AgCl-NPs with different materials of the extract.

Many researchers determined the size and shape of the nanoparticles by using the AFM image. In the present study, we were unable to determine the size of the single Ag/AgCl-NPs by using the AFM image because of the particle’s agglutinating nature on the glass surface, but we observed the topography of the agglutinated Ag/AgCl-NPs. Such type of agglutination is common and was previously reported by other researchers.

Various functional groups those presented in the extract and also in the synthesized nanoparticles were determined by FTIR. The peaks at 3399.25 cm⁻¹ might be due to the bending and stretching of hydrogen-bonded alcohols or phenols or −OH stretching of alcohols and phenols, or bending and stretching of hydrogen-bonded alcohols or phenols or could be due to the stretching of −OH in proteins, enzymes, or polysaccharides present in the extract. Peaks at 2923.72 and 2921.24 cm⁻¹ were present because of the −H stretching of alkanes, whereas 1635.45 and 1614.35 for the bending vibration of the amide 1 group, 1384.23 and 1384.48 for N-O groups, and 1077.33 and 1059.29 cm⁻¹ were present for the stretching of esters. The comparative analysis suggested that *Z. mauritiana* fruit extract and the Ag/AgCl-NPs shared certain common functional groups, and −OH groups were probably utilized in the reduction of Ag⁺ to Ag⁰.

Antibacterial activities of green AgNPs was reported at a very low dose. AgNPs were found to be more effective toward Gram negative bacteria than Gram positive bacteria because of the presence of their thinner outer membrane, the peptidoglycan layer. In the present study, we have checked the antibacterial activities of the newly synthesized Ag/AgCl-NPs against pathogenic bacteria. The nanoparticles inhibited both Gram positive (*B. subtilis*) and Gram-negative bacteria (*S. boydii* and *E. coli*). Among the bacteria, *B. subtilis* (+) was the most sensitive. The result is different to that reported earlier. The mechanism of action of Ag/AgCl-NPs against different bacteria is still unknown. It has been reported that AgNPs cause the formation of pores/pits in the bacterial cell wall that might depend on their particle size. Smaller-sized nanoparticles on bacterial surfaces increase the permeability and bind the functional groups of DNA and proteins to destroy the cells. The synthesized nanoparticles were found less effective when compared to commercial antibiotics.

Although several experiments were done on antibacterial potential of green AgNPs against bacteria, only a few were reported against fungi. *Turnip* (*Brassica rapa* L.) leaf extract-mediated AgNPs inhibited the growth of wood-degrading fungi *Gloeophyllum abietinum, Gloeophyllum trabeum, Chaetomium globosum,* and *Phanerochaete sordida.* A AgNP-incorporated reverse osmosis membrane exhibited good antifungal activities against pathogenic fungal strains such as *Candida tropicalis,* *Candida krusei,* *Candida glabrata,* and *Candida albicans.* Antifungal activity against *C. albicans* was also reported for reishi mushroom (*Ganoderma lucidum*) extract-mediated silver nanoparticles. The silver nanoparticles prepared by the modified Tollens process also showed antifungal activities against pathogenic *Candida* sp. In our present study, we found that Ag/AgCl-NPs inhibited the growth of *A. niger* and *Trichoderma* spp. Mode of action of silver nanoparticles against *C. albicans* was reported by Kim et al., and the results suggested that AgNPs exert antifungal activity by disrupting the structure of the cell membrane and inhibiting the normal budding process through the destruction of the membrane integrity.

Cancer is one of the main causes of death in the world, and more than 70% of total cancer deaths occur in Asia, America, and South and Central Africa. There are several kinds of cancer worldwide; one of the commonest and most frequent ones is the breast cancer which ranks fifth among the causes of...
Death from cancers. Chemotherapy and radiotherapy are commonly used for the treatment of breast cancer. These treatment methods not only are costly but also have side effects on normal cells. Many natural resources have already been used as chemotherapeutic agents. Medicinal herbal extracts have been widely used in traditional medicine since a long time ago. Recently, several plant-mediated biogenic AgNPs demonstrated antiproliferative activity toward MCF-7 breast cancer cell line, but the mechanisms of their anti-cancer activity were reported only in a few literatures.

The synthesized Ag/AgCl-NPs and 5-fluorouracil inhibited the proliferation of MCF-7 cell line with an IC_{50} value of 28 and 290 μg/mL. Cytotoxicity of Ag/AgCl-NPs was also checked against EAC cells, and the IC_{50} value was 84 μg/mL. These results indicated Ag/AgCl-NPs to be more effective against MCF-7 cell line compared to 5-fluorouracil. The cytotoxicity of AgNPs against MCF-7 cells was also reported in several articles. However, the long-term effect of Ag/AgCl-NPs on MCF-7 cell line was not reported. In the present study, the colony of MCF-7 cells was not observed in the synthesized Ag/AgCl-NP-treated wells after two weeks as compared with the non-treated wells. To find the molecular mechanism of the antiproliferative activity of Ag/AgCl-NPs, MCF-7 cells were stained with YF488-Annexin V. Treated cells exhibited the induction of apoptosis in MCF-7 cells which was also confirmed by monitoring of the binding of the SuperView 488 caspase-3 substrate inside the cells. This result was further verified by the staining with Hoechst 33342 where irregular shaped and condensed nuclei were observed. Induction of apoptosis in MCF-7 cell was reported by other scientists as well. Apoptosis is related with different cellular processes such as caspase up-regulation, ROS generation, and death-inducing signals. Several studies reported that cellular uptake of AgNPs leads to the generation of ROS in MCF-7 cells which provokes the oxidative stress causing apoptosis. ROS generation was also observed in this study after treatment of MCF-7 cells with DCFH-DA. Cytotoxicity of the AgNPs increased as the neutral silver (Ag^{+}) transformed to Ag, Ag−O and Ag−S which may cause production of ROS by chain effect. Our study revealed that the synthesized Ag/AgCl-NPs were capable of inducing cytosolic oxidative stresses and promoting cell death.

A group of genes are related to mitochondrial and death receptor pathways of cell signaling involved in apoptosis. These genes can be grouped as pro-apoptotic and anti-apoptotic. The pro-apoptotic genes include FAS, caspases, BAX and so forth, and anti-apoptotic genes include Bcl-2, Bcl-X, PARP, and so forth. After checking the expressions of eight such genes, no expression was observed for BID, p53, MLKL, and TNFα genes. Our results clearly demonstrated that Ag/AgCl-NPs activated death receptor FAS on the cell surface. As a result, the expression level of FADD known as FAS-associated protein with the death domain had also been increased causing the activation of caspase-3 through the caspase-8. In this investigation, Hoechst 33342 staining of the treated cells confirmed the damage of DNA. PARP plays a role for the repair of damaged DNA, while caspase-3 mediates the cleavage of PARP. Here, the expression level of DNA repairing protein PARP became decreased because of the over expression of caspase-3. As a result, apoptosis was induced in MCF-7 cell. From the above discussion, it can be hypothesized that FAS was possibly activated by Ag/AgCl-NPs that consequently activated caspase-3 via the activation of FADD and caspase-8.

Up-regulation of the above genes along with the down regulation of PARP and ROS generation caused apoptotic cell death in MCF-7 cells (pictorially represented in Figure 9).

![Figure 9. Pictorial representation of synthesized Ag/AgCl-NP-induced apoptosis in MCF-7 cells.](https://dx.doi.org/10.1021/acsomega.0c02878)

Previously, it was reported that *Achillea Biebersteinii* extract-mediated AgNPs inhibited the growth of MCF-7 cells via caspase activation and regulation of Bax and Bcl-2 gene expression, and *R. fairholmiamus* extract-mediated AgNPs induced apoptosis in MCF-7 cells through the mitochondria-mediated intrinsic pathway. Our present study reported that biogenic Ag/AgCl-NPs inhibited MCF-7 cell line by the induction of apoptosis, possibly in the FAS-mediated pathway.

**CONCLUSIONS**

Applying eco-friendly and rapid strategies such as using fruit extracts as reducing and stabilizing agents to synthesize metal-based nanoparticles is becoming usual day by day. In the present study, Ag/AgCl-NPs were synthesized from the extract of *Z. mauritiana* fruit and characterized by a number of spectroscopic methods. The nanoparticles showed marked antibacterial and antifungal activities against four pathogenic bacteria and two fungi, respectively. Growth of human (MCF-7) and mice (EAC) cancer cells also became inhibited by these nanoparticles. Mechanism of this growth inhibitory activity of the synthesized biogenic Ag/AgCl-NPs against MCF-7 cancer cells is being reported for the first time, and it became evident that it occurs through the induction of apoptosis possibly in the FAS-mediated pathway. The biogenic Ag/AgCl-NPs can be effective for the breast cancer treatment though further research is required to find out its efficacy and adverse reaction. The structure–activity relationship of these nanoparticles should also be studied in future to check their potential as anticancer drugs.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Hoechst 33342 and 5-fluorouracil were purchased from Sigma (USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco; fetal bovine serum (FBS) and Tripins-EDTA were purchased from HiClone (USA); Tris-HCl and silver nitrate were purchased from Carl Roth (Germany); YF488-Annexin V and SuperView 488 caspase-3 substrate were purchased from ABM (Canada). Streptomyecin and neomycin were purchased from Amresco, RNA isolation kit was purchased from Tiangen (China), and CYBR green master mix was purchased from...
Applied Biosystem (USA). Primers were purchased from TsingKe Biological Technology, China.

**Sample Preparation.** BAU Kul (Z. mauritiana Lamk) was bought from the local market (Voucher specimen number 22 identified by Prof. A. H. M. Mahbubur Rahman, Dept. of Botany, University of Rajshahi, Bangladesh) and washed by deionized water, slashed into small pieces, homogenized with deionized water at 1:5 ratios (w/v), and centrifuged at 10,000 g for 20 min. The supernatant (around pH 4.6) was collected, and pH was adjusted around 7.0 by the addition of Tris-HCL (10–20 mM). Finally, it was centrifuged again at 10,000g for 10 min, and supernatant was collected and subsequently used for silver/silver chloride nanoparticle synthesis.

**Silver Nanoparticles Synthesis and UV–visible Spectra Analysis.** Z. mauritiana supernatant was taken in 4 test tubes (2 mL/tube), and 1 M of silver nitrate was added to the clear supernatant to make final concentrations of 2, 3, 4, and 5 mM and kept in the sunlight for around 2 h and then subjected to UV–visible spectroscopic analysis (Hitachi U-1800, Japan) at the wavelength range from 250 to 700 nm. The best result was obtained in the presence of 5 mM of silver nitrate. For the synthesis of nanoparticles in large scale, 1 M silver nitrate was added to 500 mL of Z. mauritiana clear supernatant to the final concentration of 5 mM and kept at sunlight for 2 h and then centrifuged at 10,000 g for 30 min at 4 °C. Afterward, the pellet was rinsed three times by using deionized water and a part of the colloidal nanoparticles was lyophilized for the purpose of concentration measurement, TEM, TGA, FTIR, AFM, and XRD analysis. Rest of the colloidal solution was used for biological application. About 1.5 kg of fruit was used for the synthesis of nanoparticles.

**Characterization of Ag/AgCl-NPs.** TEM (2100F, JEOl, Japan) was utilized for the morphological analysis (size and shape) of the synthesized nanoparticles. At first, nanoparticles were dropped in the carbon-coated copper (Cu) grid, then dried in air, and finally, TEM observation was carried out with an accelerating voltage of 80 kV. Elemental analysis was carried out using an EDX spectrometer equipped with TEM.

Ultima IV (Rigaku, Japan) X-ray diffractometer with Cu Kα1 radiation was operated at 40 kV and 40 mA at a 2θ angle pattern which was used for the XRD analysis of the synthesized Ag/AgCl-NPs. Data were analyzed by using QualX2 software.

Thermal analysis of Ag/AgCl-NPs was measured by using TGA (PerkinElmer STA 8000, USA) at a heating rate of 10 °C/min under a nitrogen atmosphere.

Liquid/colloidal Ag/AgCl-NPs was taken in a glass slide and dried in air. Then surface properties and size of the synthesized silver nanoparticles were analyzed by using an atomic force microscope (AFM, Park system XE 70, Korea) where titanium coated nitrate tips were in tapping mode.

Lyophilized Ag/AgCl-NPs and crude extracts were mixed (1:100) with potassium bromide (KBr), and FTIR (PerkinElmer, Spectrum 100, USA) spectroscopic measurements were performed at the frequency range from 4000 to 225 cm⁻¹, with a resolution of 1 cm⁻¹.

**Antibacterial Assay by Disc Diffusion Methods.** Disc diffusion method was used to determine the antibacterial activities of the synthesized Ag/AgCl-NPs. B. subtilis, S. boydii, E. coli, and Salmonella enteritidis were used for this experiment. Ag/AgCl-NPs (10–40 μg/mL) and streptomycin (20 unit) applied on the paper disc with 5 mm in diameter were placed at the sterilized solidified nutrient agar plate where bacterial suspension was previously spread out. After incubation at 37 °C for 24 h, the diameter of each inhibitory zone (mm) was measured.

**Determination of Antifungal Activity by MTT Assay.** Antifungal activity was checked by MTT colorimetric assay by using RPMI-1640 media as described by Kabir et al. A. niger and Trichoderma spp. (8 × 10⁵ cells) were plated in the 96-well flat bottom culture plates containing serially diluted Ag/AgCl-NPs (7.5–60 μg/mL) and without nanoparticles in 100 μL of RPMI-1640 media. Then, the cells were incubated for 24 h at 32 °C, and MTT assay was performed.

**Cell Culture.** DMEM medium with 10% FBS, neomycin, and streptomycin in a 25 cm² cell culture flask was used for the culture of MCF-7 cells at 37 °C in 5% CO₂ incubator, and subcultures were carried out at 80–90% confluence.

**Antiproliferative Activity of Ag/AgCl-NPs by MTS Assay.** MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] colorimetric assay was carried out according to the manufacturer’s guideline (Promega, USA) to detect proliferation of MCF-7 cells. Briefly, 1 × 10⁴ MCF-7 cells in 150 μL of DMEM medium were seeded in each well of the 96-well flat-bottom culture plate and incubated at 37 °C in a CO₂ incubator for 24 h. Then, serially diluted Ag/AgCl-NPs and 5-fluorouracil in 50 μL of DMEM medium were added to each well of the culture plate to a final concentration of 8–32 and 40–320 μg/mL, respectively, and incubated at 37 °C in a CO₂ incubator for 48 h. EAC cells were collected from mice, and around 1 × 10⁵ EAC cells in DMEM media were seeded in each well of another 96 well cell culture plate and incubated at 37 °C in a CO₂ incubator for 24 h. Wells containing only MCF-7 and EAC cells without Ag/AgCl-NPs were used as controls. Then, aliquot of each well was removed, and 100 μL of DMEM medium was added. After that, 10 μL of MTS with phenazine ethosulfate was added to each well and incubated for 2 h. Finally, a micro plate reader with 490 nm filter was used for measuring the absorbance. Three wells were employed for each concentration, and by the following equation, cell proliferation inhibition was estimated

\[ \text{Proliferation inhibition ratio (%) = } (A - B) \times 100/A \]

where A and B designates OD₄₉₀ nm of the cellular homogenate (control) without Ag/AgCl-NPs and with Ag/AgCl-NPs, respectively.

**Colony Formation Assay.** To determine of the effect of Ag/AgCl-NPs on the colony formation, around 1.6 × 10⁵ MCF-7 cells were seeded in each well of the 6-well cell culture plate with complete DMEM medium. After 24 h, three wells of cells were treated with 28 μg/mL of Ag/AgCl-NPs and the remaining three wells were kept as controls. The cells were kept in the CO₂ incubator at 37 °C to grow and form colonies. Visible colonies were observed two weeks later, and then, the wells were washed by phosphate buffered saline (PBS). Afterward, fixation of cells was performed with 70% cold ethanol for 15 min and stained by incubation with 0.5% crystal violet at 25 °C for 2 h and washed with water. Finally, images were captured after air-drying the wells, and the numbers of colonies were counted.

**Observation of Morphologic Changes of MCF-7 Cells by YF488-Annexin V.** After treating MCF-7 cells with 28 μg/mL of Ag/AgCl-NPs in a 96-well cell culture plate as described above, cells were incubated with YF488-Annexin V according to the instruction of the producer (US Ever Bright). Finally, a fluorescence microscope (Olympus IX71, Korea) was used to observe the morphologic changes of the treated cells.
observe the morphological changes of the cells following a method as described by Kabir et al.\textsuperscript{62}

**Study of Cell Nuclei Change by Hoechst-33342 Staining.** Around 10,000 MCF-7 cells were seeded in each well of a 96-well cell culture plate and then treated with 28 μg/mL concentration of Ag/AgCl-NPs for 48 h as described above. After that, cells were rinsed with PBS and stained with Hoechst 33342 according to Kabir et al.\textsuperscript{63} Finally, cell morphology was observed under both dark and bright field using a fluorescence microscope.

**Observation of Changes of the ROS.** Changes of the ROS level in the MCF-7 cells after treatment with 28 μg/mL of Ag/AgCl-NPs were detected by using 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) staining. Cells were cultured in a 96-well plate and treated with Ag/AgCl-NPs for 48 h as described above. Cells were washed once by the serum free medium and incubated with diluted DCFH-DA (1:1000) at 37°C for 20 min, and finally, cells were examined by a fluorescence microscope.

**Detection of Caspase-3 Expression.** For the detection of caspase-3 expression, MCF-7 cells were cultured in 96-wells plate and treated with 28 μg/mL concentration of the synthesized Ag/AgCl-NPs for 48 h as described above and the treated and untreated MCF-7 cells was incubated with US. Ever Bright super view-488 caspase-3 substrate for 30 min according to the manufacturer’s instructions. Finally, morphological alteration of the cells was viewed using a fluorescence microscope as described by Kabir et al.\textsuperscript{62}

**Expression of Apoptosis-Related Genes by Real Time PCR.** For the isolation of RNA, MCF-7 cells were seeded (16 × 10⁴/well) in a 6-well cell culture plate and treated with 28 μg/mL concentration of Ag/AgCl-NPs, and MCF-7 cells seeded without Ag/AgCl-NPs were used as control. After incubation for 48 h, the medium was removed, cells were washed by PBS and dissolved with RZ buffer, and RNA was isolated according to the manufacturer’s guideline (Tiangen, China). cDNA was synthesized from an equal amount of RNA by reverse transcriptase enzyme as described by the manufacturer (ABM, Canada). Reaction mixture for real time PCR was prepared by the addition of cDNA, forward and reverse primers mentioned in Table 2, water, and 2X SYBR green master mix, which was used as described by the manufacturer (Applied Biosystems). Bio Rad thermo cycler (CFX96) was used for gene expression. The PCR condition was set to 50°C for 2 min and 95°C for 2 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Finally, real time PCR data were analyzed by double delta CT methods using Excel software.

**Statistical Analysis.** Results were expressed as the mean ± S.D. (standard deviation). For the calculation of data, one way ANOVA of the SPSS software (version 16) was used.

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A.A. and A.T.H. responsible for TEM and EDX related data and B.-S.C. provided lab facilities; A.A. also helped FTIR related work; M.M.R and J.I. synthesized silver nanoparticles; R.A. supported the first author for cell culture; T.D. and M.K.R.K. helped first author for the XRD related works; M.T.A. supported the first author for the real time PCR instrumental facility; X.Z. provided MCF-7 cell line; M.B.A.,

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**Table 2. List of Primers**

| Gene   | Forward Primer   | Reverse Primer   |
|--------|------------------|------------------|
| FAS    | AGCTTGTCATAGGTGAAA | RAGGCAAGATCATGAGATAT |
| TNFα   | CCTCTGCTCTCGGAGTGG   | GCTGTATCTCTACGCTCA |
| 18s    | GTACCCCGTTAAGCCCGTTT | RCCATCAATTCGATGAGCT |
| p53    | GCCCAAAACACAGCCTCT   | CCTTGAGACCATCGTTCCT |
| PARP   | GCAGCTGGTGGAAATG    | GCAAACTTACCGGATGCTCT |
| BID    | AGCGCAAACTTATAGCTGCT | TCCGGGATGCTAGCTCCT |
| MLKL   | TTAGGCCCAGCTATCCTGACCA | TGCACACGTTCTCTAGCACG |
| caspase-8 | ACAAGCCTGAGTAGACTCTCCTAA | AGGAAGTATGCTGTCCTCAGA |
| FADD   | GCTGCGTTGCTGACGCTCAAA | ACTGTTGGCTTCTCCCTCTT |

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R.G. and I.H. provided technical supports; first author did rest of the works and wrote the manuscript.

**Notes**
The authors declare no competing financial interest.

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