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Adenia hondala-derived Biopolymer Nanoparticles Cause G2/M Cell Cycle Arrest in Breast Cancer Cells

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Silver nanoparticles (AgNPs) are a type of nanomaterials known for their antimicrobial and disinfectant properties due to their detrimental effects on targeted cells. This study investigated the delivery of a novel therapeutic system based on chitosan-coated silver nanoparticles (Ch-Ag NPs) for Tamoxifen to MCF-7 human breast cancer cells. The Ag NPs were synthesized from *Adenia hondala* tuber extract and coated with chitosan to form Ch-Ag NPs. Tamoxifen was loaded onto these nanoparticles to create Tam-Ch-AgNPs, functioning as a drug delivery system. Apoptosis induction was confirmed through annexin V staining and analysis of mitochondrial membrane

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potential. Cell cycle analysis showed G2/M phase arrest in Tam-Ch-AgNPs-treated cells, indicating apoptosis induction. The inhibition of cancer cell growth by Tam-Ch-AgNPs suggests potential for developing eco-friendly therapeutic agents in nano medicine, particularly for cancer treatment, prompting further research in this field.

Keywords: Nanoparticles; cell cycle; Adenia hondala; tamoxifen; breast cancer.

1. INTRODUCTION

Cancer represents the leading cause of death worldwide, with breast cancer being the most prevalent among women [1]. Initial responses to chemotherapy often diminish due to the development of resistance, necessitating the development of biocompatible and cost-effective treatments to mitigate the severe side effects associated with current chemotherapeutic agents [2]. The field of nanoscience and nanobiotechnology has emerged as a promising avenue, offering versatile applications across agriculture, medicine, and industry [3]. Nanoparticles (NPs) possess unique physical, surface, and chemical properties ideal for delivering drugs and biologically active molecules [4]. Among these, Silver Nanoparticles (AgNps) have garnered attention for their demonstrated antiviral, antibacterial, and anticancer properties [5].

AgNps can be synthesized and tailored using stabilizing agents such as cellulose and chitosan, biologically active polymers that enhance their functionality [6]. “Chitosan, a biocompatible polysaccharide, degrades in the human body into non-toxic byproducts. Its crystalline nature enhances adsorption efficiency and exhibits significant antimicrobial activity by binding to negatively charged bacterial cell walls and inhibiting replication” [7]. The bioactivity of chitosan can be further enhanced by combining it with other bioactive materials, including drugs, making it suitable for applications like biosensors and cancer treatment [8].

This study explores a novel drug delivery system based on chitosan-coated silver nanoparticles synthesized from *Adenia hondala*, known for their non-cytotoxic, environmentally friendly, and chemically stable properties. This system aims to enable controlled release of drugs and enhance chemotherapeutic efficiency. Specifically, the study focuses on delivering tamoxifen using chitosan-coated AgNps, referred to as Tam-Ch-AgNps, which were synthesized and characterized using scanning electron microscopy, Fourier transform infrared spectroscopy, and UV-Vis spectroscopy. The efficacy of Tam-Ch-AgNps was evaluated on MCF-7 breast cancer cells, demonstrating G2/M phase cell cycle arrest and investigating their impact on mitochondrial membrane potential (MMP) to understand their apoptotic mechanisms.

Overall, this research underscores the potential of chitosan-coated silver nanoparticles as a promising platform for targeted drug delivery in cancer therapy, offering enhanced therapeutic outcomes while minimizing adverse effects on healthy cells.

2. MATERIALS AND METHODS

The human breast cancer cell line (MCF-7) was procured from National Centre for Cell Science (NCCS, Pune). Analytical grade reagents were procured from Sigma Aldrich (Bangalore). Milli-Q water was used to prepare all samples.

2.1 Preparation of *Adenia hondala* Tuber Extract

The tubers of the plant *Adenia hondala* were collected from Pothigai hills, Southern part of Western Ghats, India.0.1 g of *Adenia hondala* tuber powder was added to a 100ml Erlenmeyer flask with 20ml sterile distilled water and then mixed for 4 hours at room temperature and filtered through Whatman No.1 filter paper (pore size 25µm). The filtrate was further filtered through 0.6µm sized filters and used for further studies.

2.2 Synthesis of AgNps from *Adenia hondala* Tuber Extract

“500 ml of a silver nitrate solution (1 mM) was mixed with 5ml of tuber extract. The reaction mixture was kept for 24 hrs at room temperature with mild stirring until the colorless solution converted into a reddish-brown color, which indicated the formation of AgNps. The particles were then purified by centrifugation at 15000 rpm for 20 minutes. The silver colloids were rinsed with deionized water at least three times to get rid of extra silver ions” [9].

2.3 Drug Loaded Polymer CoatedAg-Nps

"Chitosan solution (100µL, 10 mg/ml) was mixed with Ag NP solution (4.5 ml) and 40µL of 5 M NaOH at room temperature, followed by vigorous stirring to precipitate the Ch-AgNps. Centrifugation of the obtained Ch-AgNps at 6,000 rpm for 10 min was performed. A UV visible spectrometer (JASCO V-630, Tokyo, Japan) was used to analyze the supernatant in order to determine the unreacted Ag-Nps. Centrifuged composites were washed with 1 ml PBS, followed by centrifugation at 6,000 rpm for 10 min. The washing process was repeated twice. The washed Ch-AgNps were suspended in 250µl PBS. Synthesis of the Ch-AgNps composites was carried out in a laminar flow cabinet to prevent biological contamination. Tamoxifen loaded Chitosan coated Ag- Nps were prepared by incubating 2.5mg of Ch-AgNps overnight in 1 ml of Tamoxifen solution (0.2mg/ml)" [10].

2.4 Characterization of Drug Loaded Polymer Coated Silver Nanoparticles

Microscopic observations: The Surface morphology of Ch-AgNps was observed using a HITACHI-SU 6600 FESEM. Scanning electron microscopy (SEM) specimens of the composites were prepared by casting 5µl of a water dispersion of the Ch-AgNps, followed by drying at room temperature. Osmium plasma coating was conducted to enhance the conductivity of the specimens. Dried samples were coated with the help of a plasma multi-coater PMC-5000 (Meiwafosis Co., Ltd., Tokyo, Japan)" [10].

FT-IR Studies: "FTIR spectra of Ag-Nps, chitosan, Ch-AgNps were recorded on a Jasco, FTIR-430 (Japan). About 2 mg of the samples were ground thoroughly with KBr and the pellets were prepared using a hydraulic press under a pressure of 600 kg/cm^2. Spectra were scanned between 800 and 4000 cm^{-1}" [10].

2.5 Cell Cycle study

Annexin V-FITC Assay: The breast cancer cell line MCF-7 were grown in DMEM supplemented with 10% (v/v) FBS, 2% (v/v) Penicillin-Streptomycin and 2.5 µg/ml Amphotericin-B solution and incubated at 37°C in a humidified atmosphere of 95% (v/v) air, 5% (v/v) CO_2 for 3-4 days. Following 3 days of the incubation period, the adherent cells were detached from the medium using Trypsin (1X) and 0.25 % (v/v) EDTA solution. Then the cell count was carried out using Hemocytometer.

"Apoptosis was detected with an annexin V-FITC kit (Sigma Aldrich, USA) as per the instructions of the manufacturer without any modification. Briefly, MCF-7 cells at a concentration of 1×10^6 cells/mL were exposed to AgNps, Ch-AgNps and Tam-Ch-AgNps for 24 hours. After that, the cells were retrieved and centrifuged for five minutes at 1500 rpm to remove the medium. Then, the cell pellets were washed twice with 1 mL of PBS and centrifuged. Subsequently, the pellets were resuspended in 500μL ice-cold1x binding buffer, to which 5μL of annexin V-FITC conjugate and 10μL of propidium iodide (PI) were added. The cells were gently vortexed and left in the dark for 15minutes. Flow cytometric analysis was immediately conducted using laser emitting excitation light at 488nm and a BD flow cytometer equipped with an Argon laser (Cyan ADP, DAKO, Glostrup, Denmark). Lastly,the analysis was carried out using Summit V4.3 software" [11].

Cell Cycle Assay: The flow cytometer was used to support the cytotoxicity of AgNps, Ch-AgNps, and Tam-Ch-AgNps towards MCF-7 cells. Briefly, cells at a density of 2.5×10^6 cells/mL were cultured with the AgNps, Ch-AgNps, and Tam-Ch-AgNps and incubated for 24. "The cells were harvested by centrifugation at 1500 rpm/5 minutes and washed with 1 mL PBS. Subsequently, 600μL of 80% ice-cold ethanol was added to the cell pellets drop by drop with continuous vortexing to prevent clumping and aggregation of cells and then kept at 20°Cfor five days. Then 1mL PBS was added and spun down at 1500 rpm/5 minutes. After that, the cell pellets were harvested again and washed twice with 1 mL of PBS. Finally, collected cells were stained with a PBS staining buffer that contained 0.1% Triton X-100, 10 mM EDTA, 50μg/mL RNAase A, and 3μg/mL PI and incubated in the dark on ice for 20minutes.Flow cytometric analysis was conducted using laser emitting excitation light at 488 nm using a BD FACS Caliber flow cytometer equipped with an Argon laser (BD, USA). Data analysis was performed using Cell Quest Pro software" [11].

MMP assay: In brief, cells (5×10^4cells/well) treated with AgNps, Ch-AgNps, and Tam-Ch-AgNpsfor 24 hours, were harvested and washed twice with PBS. Cells were further exposed with 10 µg/mL of Rh-123 fluorescent dye for 1 hour at 37°C in dark. Again, cells were washed twice

with PBS then the fluorescence intensity of Rh-123 dye was measured using the Olympus CKX41 upright fluorescence microscope by capturing images at 20×magnification.

3. RESULTS AND DISCUSSION

The polymer Chitosan coated AgNps were prepared by mixing the acidic aqueous solution of Chitosan with an AgNps suspension. Due to the protonation of primary amines present in the Chitosan chains, it is water-soluble in acidic conditions. As the AgNps suspension is acidic (pH 5.23 to 6.25) in nature [12] the polymer-coated silver nanoparticles were partially precipitated. The solubility of the Chitosan chains was decreased by the binding of AgNps to the amino and hydroxyl groups of Chitosan [13]. The precipitation of the composite is completed by adding excess NaOH. The UV visible spectra of the AgNps suspension and the post-reaction mixture of AgNps and Chitosan is shown in Fig. 1. AgNps provide a peak near 400nm, and the same the peak lack in the supernatant of the post-reaction mixture which shows that the AgNps was completely bound to the chitosan and the AgNps are not present in the supernatant of the post-reaction mixture. Using FESEM the morphological surface of AgNp coated with Chitosan was analyzed. The AgNp shows cubical shaped particles (Fig. 2a) with almost uniform size and Chitosan coated Ag-Np (Fig. 2b) shows an assortment of Chitosan and Ag in which the silver nanoparticles are enveloped by the chitosan polymer. The synthesized nanoparticles are in aggregated form with low dispersion and highly stable form.

The FTIR spectra of AgNps, Chitosan and Chitosan coated AgNps are compared in Fig. 3(a-c). "AgNps and Chitosan coated AgNps showed the overlapped spectra. The characteristic amide I (due to C=O stretching) and amide II bands (out of phase combination in NH in-plane bending and CN stretching) of AgNps are observed as peak around 1670.35 $cm^{-1}$and 1448.54 cm^{-1} respectively. In the case of Chitosan (dry powder) the peaks at around 1560.44 cm^{-1} and 1458.16 cm^{-1} are assigned as amide I and amide II bands. The presence of these two amide bands of both Ag-Nps and Chitosan is expected in the FTIR spectra of Chitosan coated Ag-Nps, which are observed at around 1571.99 $cm^{-1}$and 1421.54 cm^{-1}. Hence, apart from electrostatic interaction, a covalent bond contributes to the stability and assembly. The peak at around 1448.54 cm^{-1} corresponding to CH_2 bending in AgNps is preserved in the FTIR spectra of polymer-coated AgNps. The band at around 1024.26 cm^{-1} is from the saccharide structure of Chitosan, which is observed at around 1026.13 cm^{-1} in the case of Chitosan coated AgNps" [10]. This FTIR spectra comparison between Chitosan coated AgNps and Ag-Nps and Chitosan confirm the occurrence of both constituents in the self-assemble polymer-coated Ag-Nps.

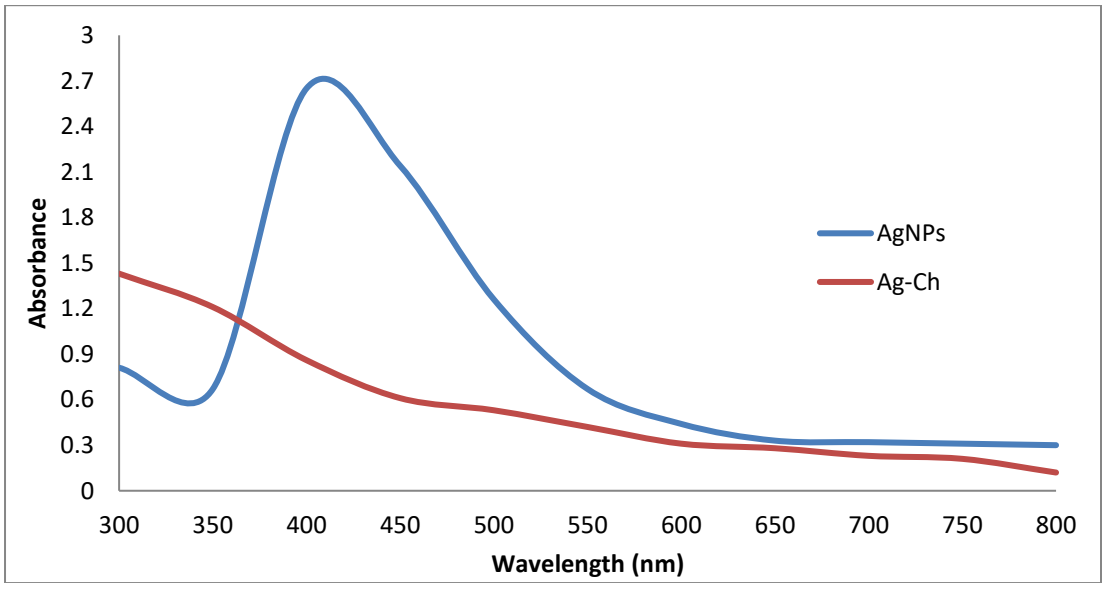

Fig. 1. UV visible spectra of the AgNps and the post reaction mixture AgNps with Chitosan)

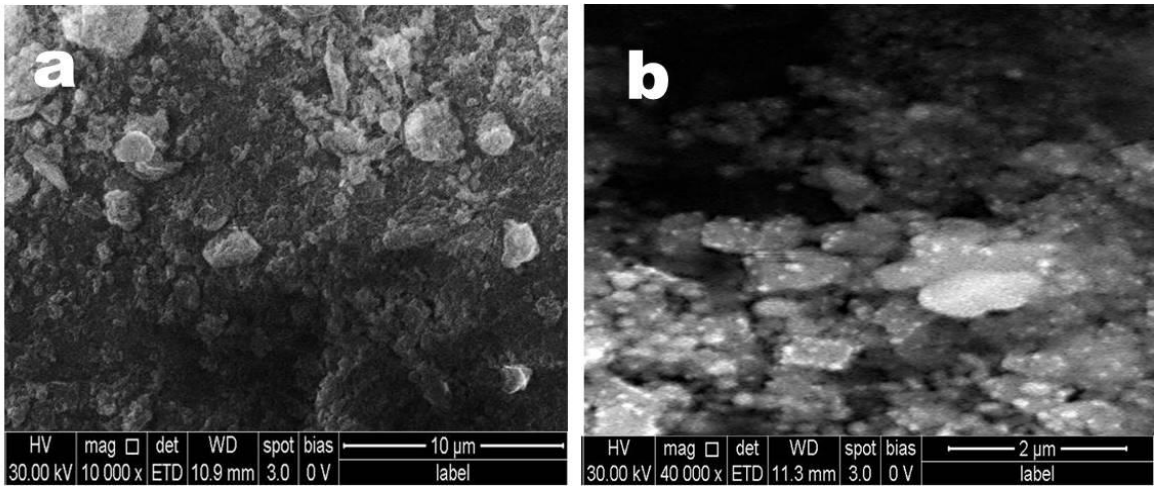

Fig. 2. Field Emission Scanning Electron Microscopy image of silver nanoparticles (a) and Chitosan coated AgNps (b)

To measure apoptosis, we evaluated the externalization of phosphatidyl serine on the cell surface using an annexin V-FITC/PI staining assay. The analysis revealed a significant and progressive increase in the percentage of cells stained with annexin V-FITC, indicating both early and late stages of apoptosis ($P < 0.05$) across all experimental groups over time, while the proportion of viable cells decreased accordingly (Fig. 4). In cells treated with AgNps, the percentage of apoptotic cells was relatively low, with 14.60% showing early apoptosis and 13.96% in late apoptosis. Conversely, cells treated with Ch-AgNps displayed a notable rise in apoptosis, with 41.35% of cells in early apoptosis and 28.65% in late apoptosis. No apoptotic cells were observed in the control MCF-7 group. Remarkably, cells treated with Tam-Ch-AgNps exhibited the highest percentage of late apoptotic cells (50.68%), likely due to the activation of apoptotic signaling pathways triggered by nanoparticle internalization. The increased apoptosis observed with Tam-Ch-AgNps suggests that targeted drug delivery enhances receptor-mediated endocytosis, resulting in higher intracellular drug concentrations and more effective apoptosis induction. Furthermore, the controlled and sustained release of Tam from AgNps may improve therapeutic efficacy by promoting apoptosis [14]. These findings support the potential of Ch-AgNps as a promising drug delivery system for targeting breast cancer cells. It is also well-documented that anti-tumor drugs can induce cell cycle arrest, which can be assessed by examining their effects on cell cycle phase distribution [15].

As an additional measure of apoptosis, FACS analysis of AgNps, Ch-AgNps, Tam-Ch-AgNps treated MCF-7 cells was carried out (Fig. 5) The results showed that the untreated cells showed normal DNA content and cell cycle distribution. Moreover, cell cycle arrest induced by AgNps in the S phase and G2/M phase with values of 23.60% and 27.90 % respectively. When compared to the untreated cells, the cells treated with Chi-AgNps shows a significant decrease in the accumulation of cells in S phase and G2/M phase. In Tam-Ch-AgNps, the cycle was arrested in the S phase and G2/M phase with values of 08.45% and 5.60% respectively. AgNps induced the up-regulation of cell cycle regulators associated with BAX gene expression and the cell numbers in the G0/G1/S phases and G2/M phase was markedly decreased [16]. Through mitochondrial disruption, an antiproliferative effect was induced by AgNps on blood cancer cells, which leads to the arrest of the cell cycle at the G2/M phase and followed by apoptotic cell death [11]. The apoptotic gene p53 can arrest cells at the G2/M phase transition in cell-type specifically and it's over expression blocks entry into mitosis phase [17] and G2/M phase arrest occur by p53 dependent and p53 independent pathways [18]. It is well known this study also described that increasing cellular stress and DNA damage arrest the MCF-7 cells at the G2/M phase transition mediated via the caspase-dependent pathway.

Microtubules are vital cytoskeletal proteins that play essential roles in cellular motility, transport, and mitosis [19]. They are critical for maintaining

cell shape, cell division, cell signaling, and the cellular transport system. Elevated concentrations of AgNps disrupt the organization of microtubules and the actin cytoskeleton, leading to reduced cellular motility, impaired transport, and disturbances in cell shape, signaling, and adhesion [20]. The study findings reveal that MCF-7 cells treated with Tam-Ch-AgNps experienced G2/M phase cell cycle arrest. This arrest is associated with the disruption of microtubule dynamics caused by Tam-Ch-AgNps, which leads to the depolymerization of microtubules and decreased polymerization. Consequently, this affects cellular transport, clonogenicity, cell-cell interactions, and adhesion to surfaces.

Mitochondria undergo two primary changes that contribute to mitochondria-mediated apoptosis: the permeabilization of the outer mitochondrial membrane and the loss of the electrochemical gradient [21]. The outer mitochondrial membrane's permeability is tightly regulated by members of the Bcl-2 family, while membrane depolarization is controlled by the mitochondrial permeability transition pore. Prolonged opening of this pore leads to a compromised outer mitochondrial membrane. Normally, mitochondria maintain a negative charge across their inner membrane, creating a negative electric potential [22]. In this study, cells were exposed to AgNps, Ch-AgNps, and Tam-Ch-AgNps for 24 hours and then assessed for rhodamine 123 uptake. Mitochondrial membrane potential was qualitatively evaluated using fluorescence microscopy with rhodamine 123 staining (Fig. 6). The results demonstrated a significant decrease in mitochondrial membrane potential. Specifically, cells exposed to Tam-Ch-AgNps showed a notable reduction in fluorescent intensity compared to those exposed to AgNps or Ch-AgNps, indicating a substantial loss of mitochondrial membrane potential.

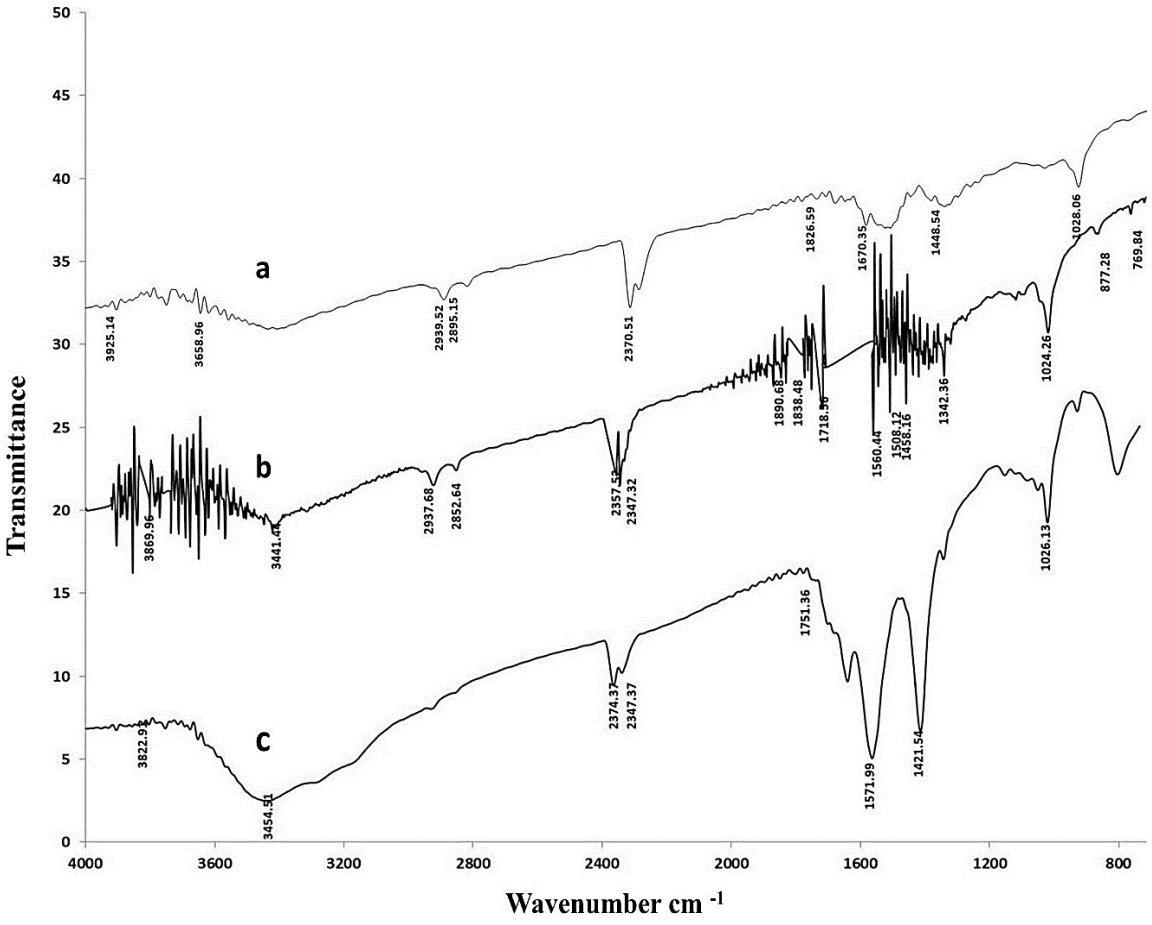

Fig. 3. The FTIR spectra of Ag-Nps (a), Chitosan (b) and Chitosan coated Ag-Nps (c)

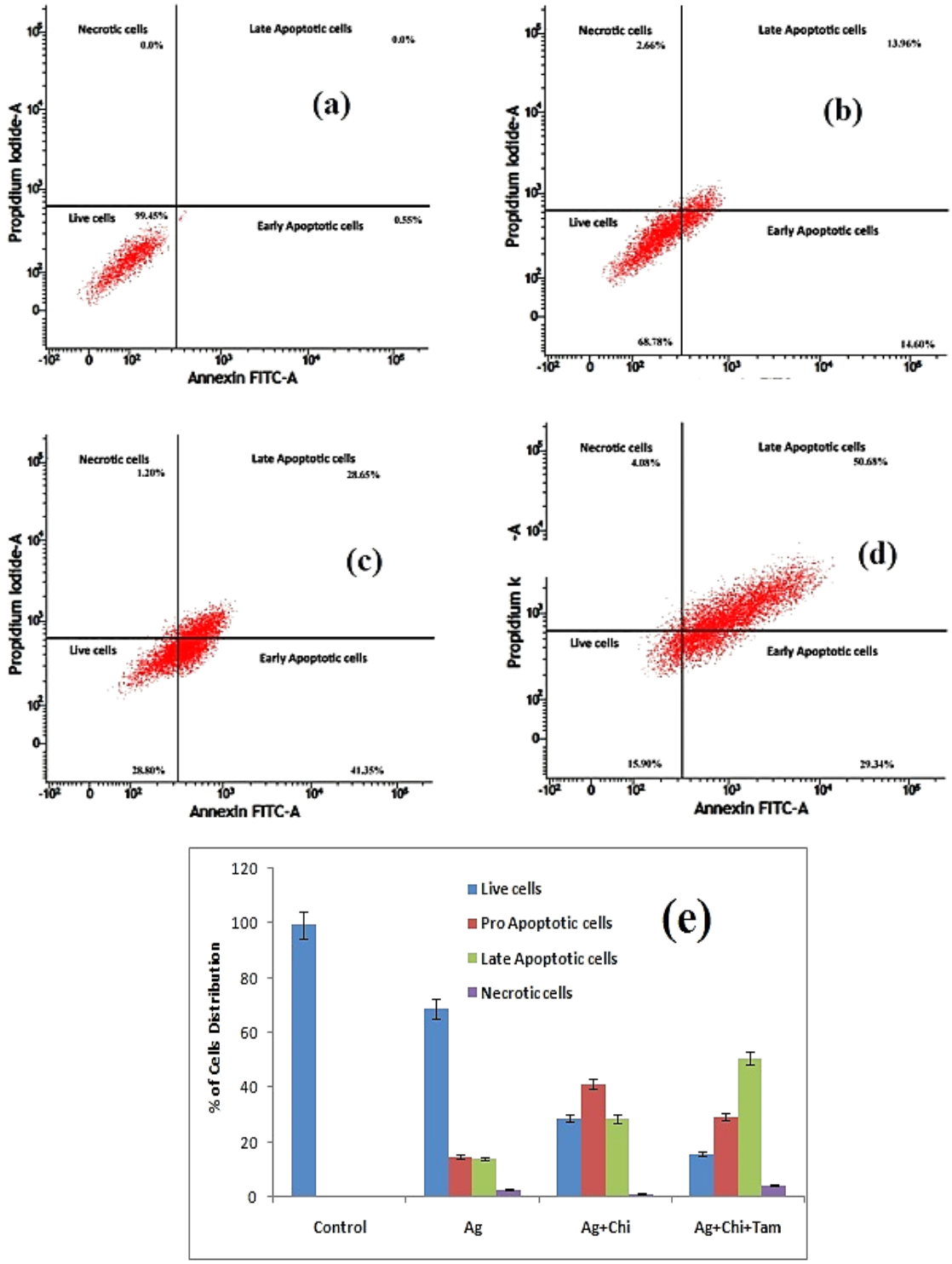

Fig. 4. Flow cytometric analysis of apoptosis induction in MCF-7 cells after staining with FITC-conjugated annexin V and PI. (a) Untreated (control) MCF-7 cells (b) AgNps (c) Ch-AgNps and (d) Tam-Ch-AgNps (e) Percentage of cells distribution and the values are mean±SD of three independent experiments. Significant differences (P< 0.05)

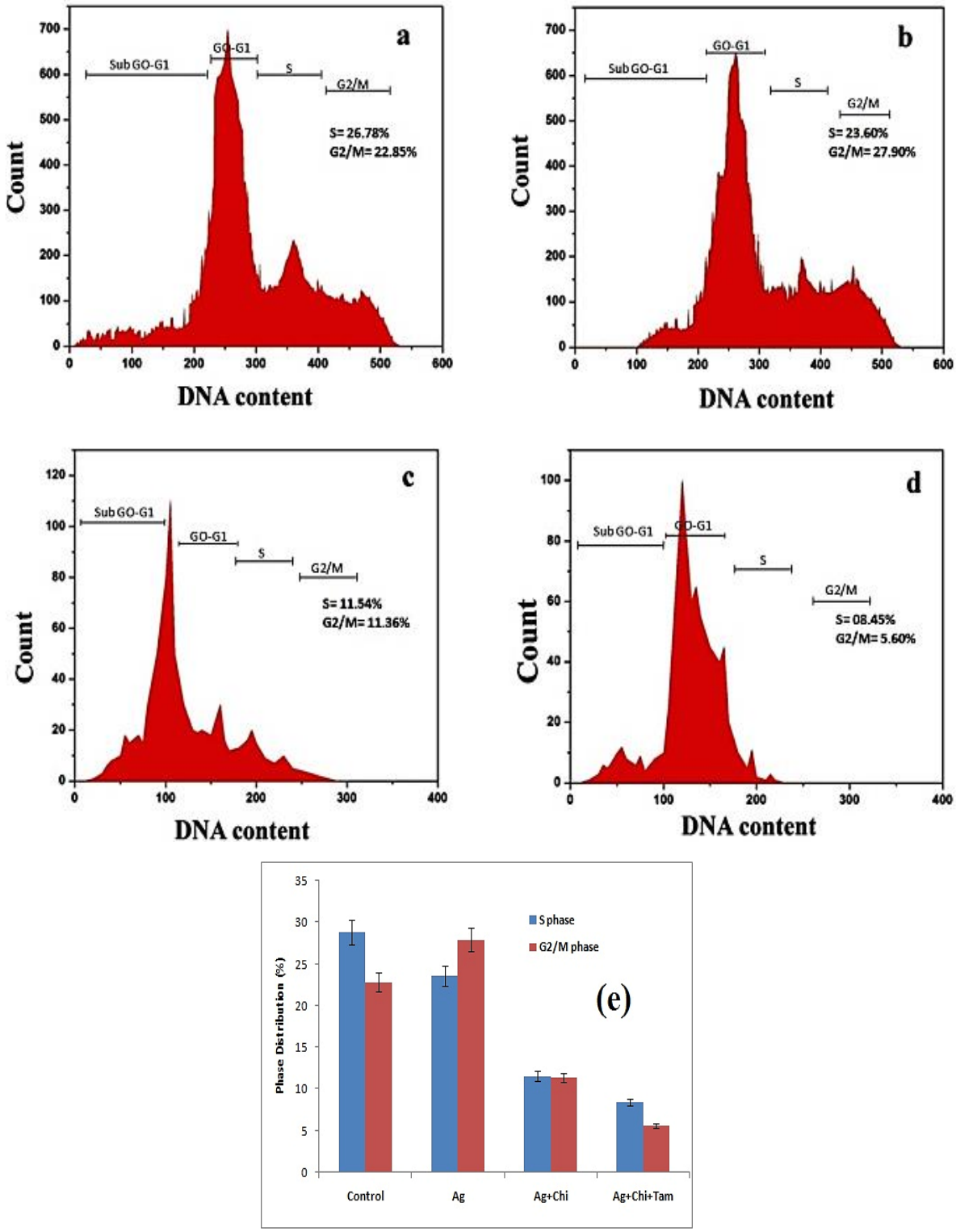

Fig. 5. Cell cycle analysis of MCF-7 cells treated after staining with PI. (a) Untreated (control) MCF-7 cells. (b) AgNps (c) Ch-AgNps and (d) Tam-Ch-AgNps G0/G1, G2/M, and S indicate the cell phase, and sub-G0-G1 refers to the portion of apoptotic cells. (e) Percentage of phase distribution and the values are mean±SD of three independent experiments. Significant differences (P< 0.05)

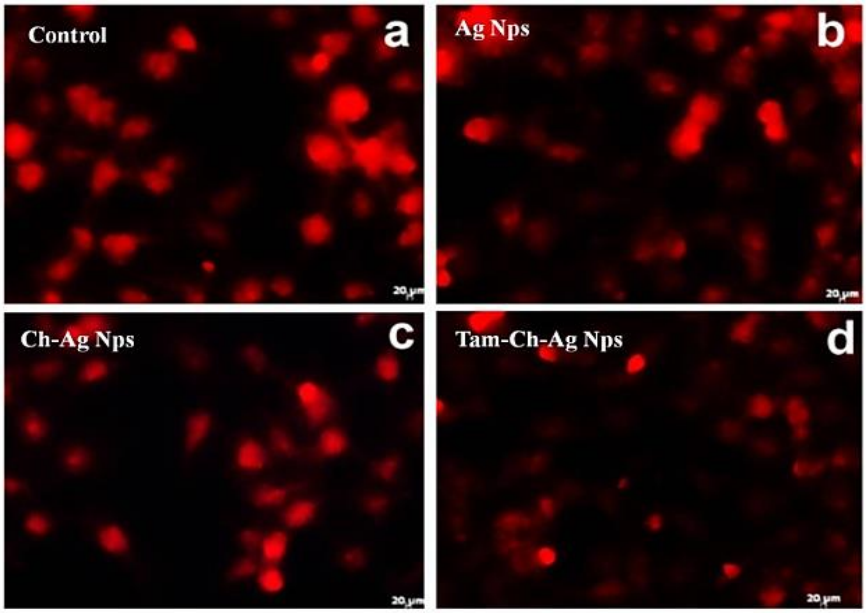

Fig. 6. Mitochondrial membrane potential (MMP) in McF-7 cells after AgNps, Ch-AgNps and Tam-Ch-AgNps exposure. Images were captured with an Olympus CKX41 fluorescence microscope (Olympus Corporation, Toyko, Japan) a) Control, b) Silver Nanoparticles, c) Chitosan coated AgNP, d) Tamoxifen loaded chitosan coated silver nanoparticles

The loss of mitochondrial membrane potential (MMP) is commonly observed following exposure to toxicants, leading to cell death through apoptosis, necrosis, or autophagy [23]. In HepG2 cells, exposure to AgNps, whether dispersed in deionized water or cell culture medium, resulted in a decrease in MMP and subsequent generation of reactive oxygen species (ROS). This suggests that AgNps may induce early cell death through mitochondrial membrane damage [24]. Elevated ROS levels are known to cause cellular damage, which can further lead to mitochondrial membrane disruption and toxicity. Utilizing the cationic fluorescent probe Rh123 dye to assess MMP levels revealed that ROS generation and oxidative stress play a significant role in AgNps-induced cell death in MCF-7 cells due to free radical production [25]. Overall, the results indicate that tamoxifen-loaded chitosan-coated silver nanoparticles, derived from the medicinal plant *Adenia hondala*, exhibit antiproliferative activity by inducing apoptosis in breast cancer cells. This suggests that these nanoparticles hold promise as an alternative therapeutic agent for treating human breast cancer.

4. CONCLUSION

Silver nanoparticles synthesized from the medicinal plant *Adenia hondala* were used in this study to enhance the efficiency of Tamoxifen drug delivery. The results demonstrated that Tamoxifen-loaded chitosan-coated silver nanoparticles exhibited superior antiproliferative activity compared to the drug alone, effectively inducing apoptosis in breast cancer cells. This suggests that these nanoparticles hold significant promise as a delivery system for breast cancer therapy. The study highlights the potential of Tamoxifen-loaded chitosan-coated silver nanoparticles to inhibit tumor cell growth and their cytotoxic effects as a treatment option. Additionally, their biocompatibility and ease of modification make these nanoparticles promising candidates for future biomedical applications.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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