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A cytotoxic herbal Silver Nanoparticles as A Remedy for Mammary Carcinoma

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ABSTRACT

Mammary carcinoma is most common and progressively increased form of cancer mostly among women. It is developed in various stages and late diagnosed. Various therapies have been tried to cure this cancer but none of them is without side effect. These might be attributed to indiscriminate destruction of normal cells along with cancer cells or other systemic effects of chemotherapeutic agent. Theresearch designed to formulate the herbal silver nanoparticles by biological reduction method using the extract of leaves of curly kale and which will show a significant cytotoxic action than individual silver as well as curly kale leaves extract. The hydroalcoholic herbal extract of kale leaveswas prepared using maceration process. It further synthesizes and characterizes herbal extract mediated Silver Nanoparticles using biological reduction method. Further the cytotoxic study was performed on the mammary carcinoma. Extraction of plant material by maceration process followed by preparationand characterization of silver Nanoparticles by biological reduction method. Further the required parameters were performed to characterize and evaluate. The said herbal silver nanoparticles show considerable cytotoxic action in mammary carcinomaous cells by showing visible cell cycle arrest and apoptosis induction in T47D cell line and hence it provides best alternative to existing solution to Mammary carcinoma as well as it is cost effective, and affordable anticancer remedy.The present work demonstrated a new application of Herbal Silver Nanoparticle as a remedy for mammary carcinoma using curly kale leaf.

Keywords:*Curly Kale, Silver Nanoparticles, Mammary Carcinoma*

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INTRODUCTION

Mammary carcinoma is most common and progressively increased form of cancer mostly among women. It is developed in various stages and late diagnosed. Various therapies have been tried to cure this cancer but none of them is without side effect. These might be attributed to indiscriminate destruction of normal cells along with cancer cells or other systemic effects of chemotherapeutic agent. These difficulties initiates urge to develop targeted drug delivery systems. Nanodrug delivery systems are being used for targeting in treatment of various diseases, hence this concept is also applicable to treatment of mammary carcinoma.[3-6]. The serious adverse events associated with conventional mammary carcinoma therapy have led to the increased acceptance of herbs as an adjunct to the conventional therapies.[1-2] Combination of herbal medicine with nanotechnology has been shown to enhance the action of plant extracts and bioactives which include enhancement of solubility, bioavailability, protection from toxicity, enhancement of pharmacological activity, enhancement of stability, improved tissue macrophage distribution, sustained delivery, and protection from physical and chemical degradation.[11-12]. Metallic Nanoparticles prepared by using herbal extract is the most accepted method with the added advantage that the plants are readily available, free from toxic chemicals and widely distributed and safer to handle moreparticularly. Plant extracts not only act as reducing and stabilizing agents due to presences of various secondary metabolites for the bioreduction reaction to synthesize metallic nanoparticles but also shows added pharmacological potential. Among others, a vegetable like curly kale (botanical name- Brassica oleraceaevar.sabellica. belonging to family Brassicaceae) is assumed to affect the growth of numerous forms of cancers since it contains multiple chemical constituents such as, Lutein, β-Carotene, indole 3 carbinol, zeaxanthine, glucobrassisin etc. shows anticancer activity. Hence, the research designed to formulate the herbalsilver nanoparticles by biological reduction method using the extract of leaves of curly kale leaves and which will show a significant cytotoxic action than individual silver as well as curly kale leaves extract.[16-18]

MATERIAL AND METHODS

Experimental Materials

The curly kale leaves were received as gift from the local Farmer Shri. The plant was recognized and validated from Botanical Survey of India, Western Regional Center, Pune, Maharashtra, India. The chemicals of analytical or chromatographic grade were purchased from Loba ChemiePvt. Ltd., Mumbai and Merck Specialties Pvt. Ltd., Mumbai. The cancer cell line T-47D was procured from the National Centre for Cell Sciences, Ganeshkhind, Pune-411007, Maharashtra, India.

Methods

Preparation of Extract of curly kale leaves

1000 gm of leaves were cut in to the small pieces and allowed for maceration with solvent mixture having ethanol and water (8:2) ratio for 3 days. After maceration filtration was carried out. It wasthen allowed for evaporation of solvent. After the evaporation, extract (CKLE) was weighed for further biosynthesis of formulations.[9]

Synthesis of Silver Nanoparticles

CKLE Stock Solution (1%) is prepared by dissolving 1 gm of extract in 100 ml of distilled water. Resulted Stock Solution was further used for synthesis of herbal silver nanoparticles. [7]Stock Solution of Silver Nitrate is prepared by dissolving $AgNO_3$ (169.8 g) in 1000 ml of distilled water. Obtained stock solution is diluted further to get 1mM, Silver Nitrate ($AgNO_3$) solution. CKLE extract was taken into a beaker and placed on Magnetic stirrer. Further the silver nitrate ($AgNO_3$) solution was pored drop wise with continuous stirring.[7]The produced herbal silver nanoparticles were separated by centrifugation using of REMI centrifuge at 6000 rpm for period of 25 minutes. The supernatant liquid was thrown away and the pellets was collected and stored. The optical absorption of green synthesized silver nanoparticles was studied using UV-VIS (Shimadzu UV-VIS 2550, Japan) spectral analysis. The crystalline nature of green synthesized AgNPs was confirmed by XRD pattern. Morphology, size, and electron diffraction pattern were examined by FESEM (JSM-7600F, Japan) and TEM (JEM2100F, Japan) at a voltage 200 kV, respectively. EDX analysis was used to confirm the presence of elemental silver in green synthesized AgNPs.Zeta potential was measured in order to check stability of AgNPs. Particle size analysis was done to check average particle size of AgNPs.

Determination of cytotoxicity by MTT assay

During determination of cytotoxic activity by the CKLE and CK-AGNP's, cell viability by MTT assay is done. The MTT assay is a colorimetric assay for measuring cell metabolic activity. In typical procedure, the T-47D cells were maintained in complete growth medium RPMI-1640 (Thermo Inc, USA) supplemented with 10% heat-inactivated fetal bovine serum (Himedia, India), 0.2 units/mL bovine insulin, 1% penicillin and streptomycin.[8-10]

Determination of Apoptosis Induction: Detection of membrane proteins

During determination of cytotoxic activity by the CKLE and CK-AGNP's, Determination of apoptosis induction by CKLE is checked. A quantitative assessment of apoptosis was performed by using Annexin V-FITC Apoptosis Detection Kit (Thermo Inc. USA) as per the manufacturer's instructions. [13-15]

Determination of Cell Cycle Arrest

During determination of cytotoxic activity by the CKLE, mammary carcinoma cell cycle arrest by CKLE was checked. During this experiment approximately, $1x10^5$ cells of each cells were plated 500 µL of (RPMI-1640 or L-15) with 10 % v/v FBS in six-well plates and allowing to adhere overnight. After 24 hrs of serum-starvation, CKLE was added. The spent media was collected and spun at 1000 × g for 5 min to collect detached cells or floaters, which were combined with adherent cells for cell cycle distribution analyses by flow cytometry. The cells were washed twice with the PBS and fixed in 70% (v/v) ice-cold ethanol at 4^0 C for 24 hrs. 50 µL of RNase A solution (100 µg/mL in PBS)was added to the cells. The fixed cells were stained with propidium iodide (Thermo Inc. USA). The samples were then analysed in an Attune flow cytometer (Thermo Inc. USA The data generated from all the experiments was analysed by two tailed t tests followed by ANOVA. The difference was considered significant if $P<0.05$. The Graph pad Prism software was used for all statistical analyses.[17]

RESULT AND DISCUSSION

The extraction of plant material CKLE leave was done by maceration process. Further, the preparation and characterization of silver Nanoparticles using CKLE leaf extract was accomplished by biological reduction method. The UV-Vis spectra of herbal silver nanoparticles synthesized using the Kale leaves extract evince the blue shift of the absorption band and it appears at 210.5 nm (**Figure 1**). For 1mM $AgNO_3$ the absorption peak was centered around 435 nm. This information shows that the Ag nanoparticles have formed in the extract, where the Ag^+ has been reduced to Ag^0. The crystalline nature of Herbal Silver Nanoparticles was

confirmed by XRD. It shows the XRD pattern of Herbal Silver Nanoparticles. Diffraction peaks were observed in the 2Θ range 20° to 80°. It showed that Herbal Silver Nanoparticles were found to be crystalline. FESEM of Herbal Silver Nanoparticles is checked in order to observe its surface morphology. The obtained result has showed that the average diameter is found to be 42 nm, and the shape was found to be spherical. EDX of herbal extract shows presence of C,O,N,K,S,P, and Cl. EDX of Herbal Silver Nanoparticles shows presence of Ag. It means that Ag was successfully loaded in Herbal Silver Nanoparticles. The zeta potential of Herbal Silver Nanoparticles was found to be -27.2 mV which indicates that Herbal Silver Nanoparticles were moderately stable. Particle size analysis result was showed that the average particle size of the formulation is found to be 40.15 nm.

Determination of Cytotoxicity by MTT assay

During determination of cytotoxic activity by the CKLE and CK-AGNP's, cell viability by MTT assay was done. Human breast cancer cells, T-47 D were treated with CKLE (**Figure2**)or CK-AgNP's, and the antiproliferative effect was evaluated by MTT assay. A dose dependent decrease in viability of cancer cells was observed with increasing concentration of the CKLE and CK-AgNP's. After 16 h of exposure of cells to CKLE, IC_{50} value of CKLE in T-47 D was found low i.e. 42.12 ± 4.7 µg/mL (**Figure 2**). The cell viability was decreased after 16 h in T47D cell lines. After 24 h exposure to CKLE, $IC_{50}$was 15.19± 1.3 µg/mL for T-47D (**Figure 2**). The CK-AgNP's IC_{50} was found after 16 hours 38.4 ± 3.2 µg/mL against the T-47D cells.(**Figure 2**).The IC_{50} of CK-AgNP's after 24 hours was found 24.4 ± 1.2 µg/mL(Fig. 9b). CK-AgNP's was found effective against the human breast cancer cells.[25-26]This suggested that time and dose dependent treatment with CKLE and CK-AgNP's inhibited the growth and induced apoptosis in these cells. Significantly decreased cell viability was found in CKLE or CK-AgNP's treated T-47D ($P<0.0001$ compared to untreated control). Here, we find a significant effect of CKLE or CK-AgNP's on viability of breast cancer cells- T-47D.

Determination of Apoptosis Induction: Detection of membrane proteins

During determination of cytotoxic activity by the CKLE and CK-AGNP's, Determination of apoptosis induction by CKLE was checked. A quantitative assessment of apoptosis was performed by using Annexin V-FITC Apoptosis Detection Kit (Thermo Inc. USA) as per the manufacturer's instructions. Morphological changes clearly indicates the induction of apoptosis in the breast cancer cells–T-47D. Annexin-V binds to externalized phosphatidylserine (PS) of apoptotic cells membranes. The occurrence of early and late apoptosis is validated by the increase of Annexin-V positive cells in dose dependent manner. Annexin V, Alexa Fluor® 568 conjugate /PI flow cytometric assay was performed to confirm this. The majority of the untreated control cells were found to be viable (96%). In CKLE treated T-47D cells, we found viability of the cells decreases from 92 % (Untreated) to 62.7 % (10 ug/mL of CKLE) (**Figure 3a**), 56.7 % (20 ug/mL of CKLE) (**Figure 3b**) and 52 % (30 ug/mL of CKLE) (**Figure 3c**). Apoptosis induction was also found in CK-AGNP's treated T-47Dcells . The viability of the cells was decreased from 96% to 89 %(**Figure 3d**), 85.3% (**Figure 3e**) and 75.5% (**Figure 3f**) in 10 µg/mL, 20 µg/mL and 30 µg/mL CK-AgNP's treated cells, respectively. The early apoptosis was observed in 7.2% cells containing 10 µg/mL CK-AGNP's (**Figure 3**d). A dose dependent increase in early apoptotic cells 11.5% (**Figure 3**e) and 18.2% (**Figure 3**f) is found in 20 and 30 µg /mL CK-AGNP's treated cells, respectively. The growth inhibition leds to apoptosis in CK-AGNP's or CKLE treated T-47D cells. [16]

Determination of Cell Cycle Arrest

During determination of cytotoxic activity by the CKLE,mammary carcinoma cell cycle arrest by CKLE was checked. The effects of CKLE or CK-AgNP'son the cell cycle of T-47D cells were evaluated by using CKLE treated and untreated cells. To determine whether CKLE or CK-AgNP's induced apoptosis was related to arrest of cell cycle progression in breast cancer cells, flow cytometry was used to quantitate the cell cycle distribution under treatment with CKLE or CK-AgNP's at a concentration of 10 µg/mL. The number of cells in G2/M phase increased and that in the G0/G1 phase decreased in CKLE or CK-AgNP's treated cells (**Figure 4 a-d**). After confirmation of apoptosis in CKLE treated T-47D cells, the cell cycle was analysed. In untreated T-47D cells 62.4 % of the cell populations were found in G1 phase (**Figure 4** a). The G2/M phase population is 13.4% in untreated cells (**Figure 4** a). The cell population of the G1 phase was found to decrease to 13.31 % in CKLE treated T-47D cells (**Figure 4** b). The cell cycle arrest in the G2/M phase was also observed in CK-AgNP's treated cells. The population of G2/M phase was increased from 5.1 % to 12.3 % in CK-AgNP's treated cells (**Figure 4** c-d). This experimental finding implies that CKLE or CK-AgNP's induced apoptosis in breast cancer cells via G2/M phase arrest. The dose dependent cell cycle halt was observed in CKLE or CK-AgNP's treated T-47D cells. The G2/M arrest was associated with the intrinsic pathways of apoptosis induction in CKLE or CK-AgNP's treated breast cancer cells. In this report, the cell cycle arrest in G2/M phase confirmed apoptotic induction in invasive breast cancer cells T-47D cells.[17]

The data generated from all the experiments wasanalysed by two tailed t tests followed by ANOVA. The difference was considered significant if P<0.05The Graph pad Prism software is used for all statistical analyses.

FIGURE LEGENDS

Figure 1: Illustrates UV-Visible scan of of kale leaves extract (a) and Herbal Silver Nanoparticles of kale leaves (b)

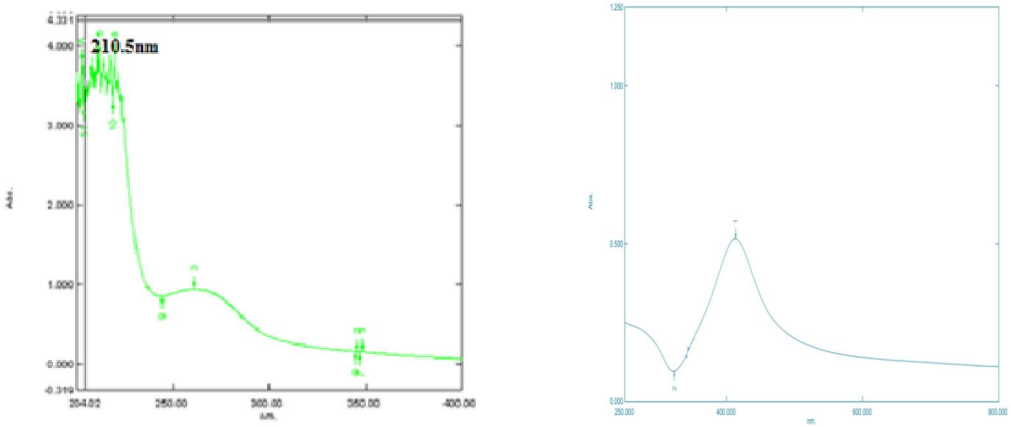
Figure 2: Illustrates graphical representation of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for determination of cell viability in T47D mammary carcinoma cells

Figure 3: Illustrates result of determination of apoptsis induction in T47D mammary carcinoma cells-Detection of membrane proteins on CKLE treated cells. Apoptosis in T-47D cells was assessed after 16 h of treatment with CKLE and CK-AGNP's by Annexin V, Alexa Fluor® 568 conjugate /PI binding and measured by flow cytometry.

a. T-47D cells with 20 ug/ml CKLE,
b. T-47D cells with 40 ug/ml CKLE
c. T-47D cells with 60 ug/ml CKLE
d. T-47D cells with 20 ug/ml CK-AGNP's,
e. T-47D cells with 40 ug/ml CK-AGNP's
f. T-47D cells with 60 ug/ml CK-AGNP's
g.

Figure 4: Illustrates result of determination of cell cycle arrest in T-47D mammary carcinoma cells-Cell cycle in T-47D was assessed after 16 h of treatment with CKLE by PI staining and measured by flow cytometry analysis. Numbers indicate the percentage of cells in each gate /phase.

a. T-47D cells with 20 ug/ml CKLE,
b. T-47D cells with 40 ug/ml CKLE,
c. T-47D cells with 20 ug/ml CK-AGNP's,
d. T-47D cells with 40 ug/ml CK-AGNP's

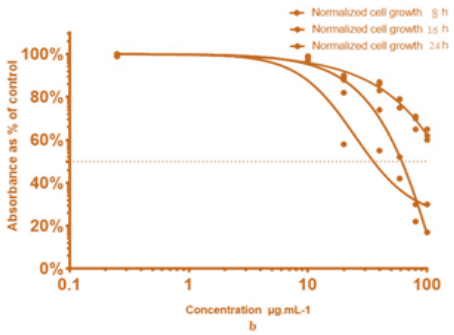


(a) **(b)**

e.

Figure 1

Figure 2



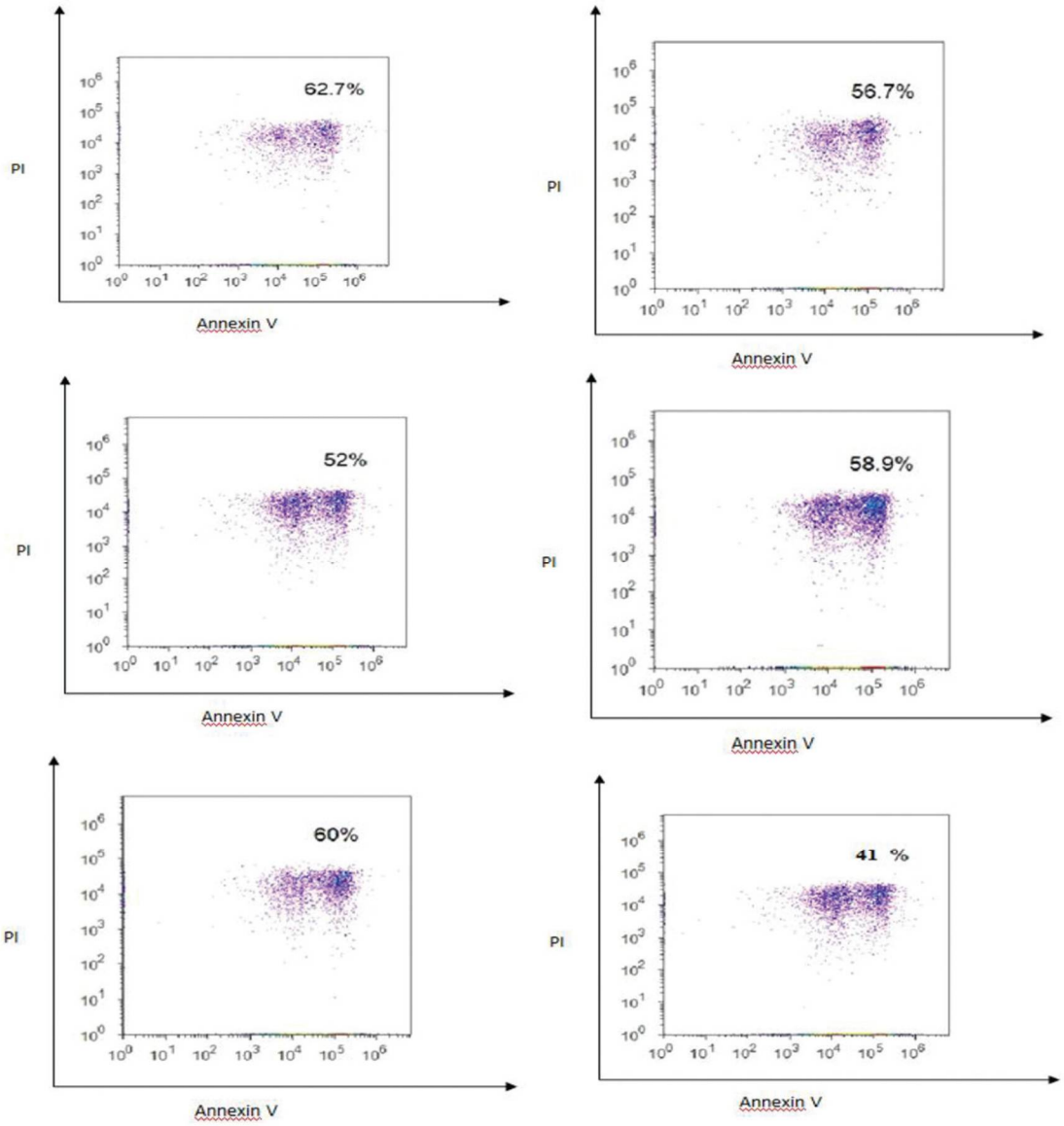


Figure 3

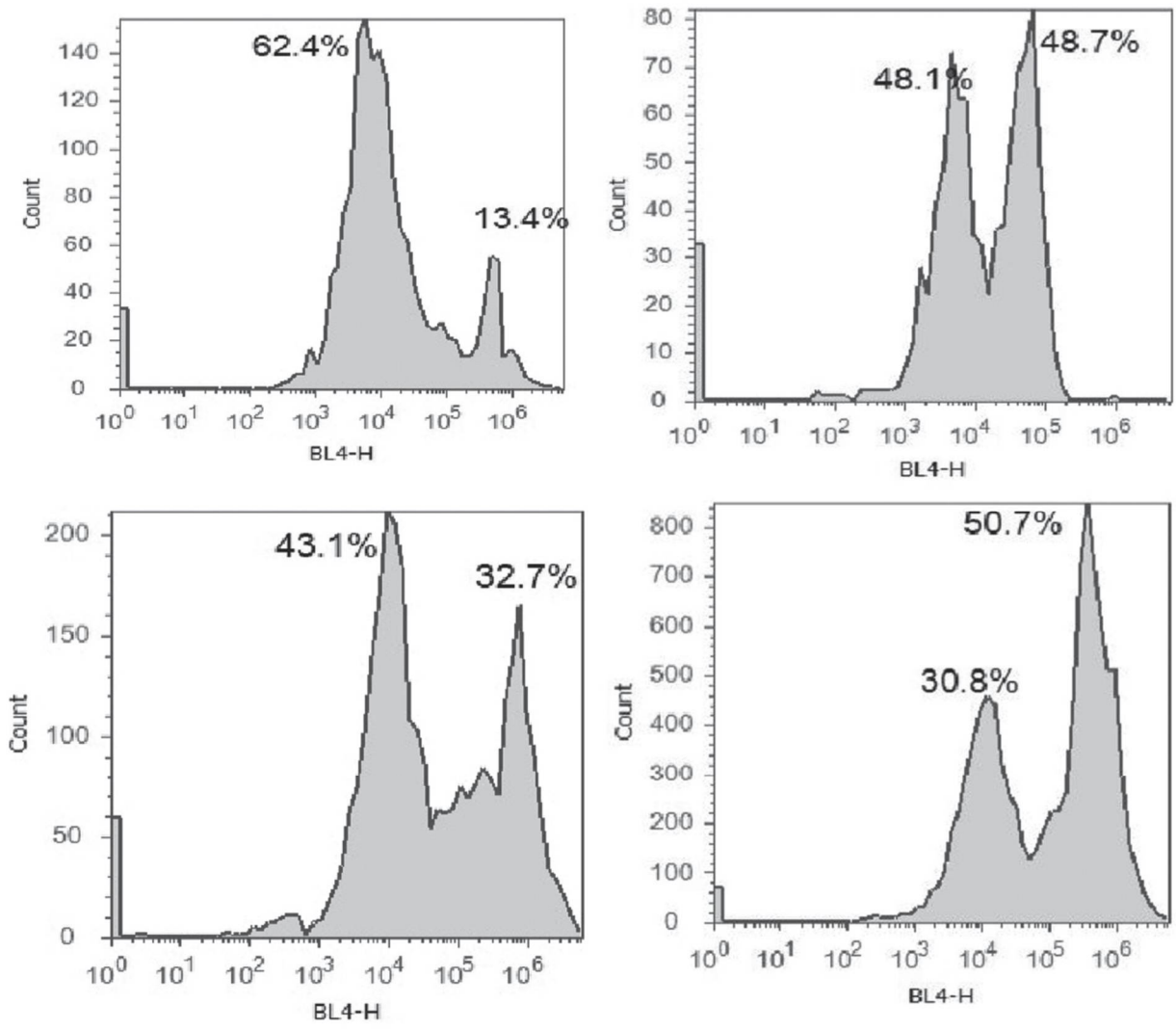


Figure 4

CONCLUSION

This investigation demonstrated the biosynthesis of silver nanoparticles (AgNPs) for the first time, via a single-step reduction of silver ions using curly kale leaves and its anticancer potential against breast cancer cells (T47D). Our results showed that biosynthesized AgNPs (CKAgNPs) induced a concentration-dependent cytotoxicity in T47D cells. This study showed that CKAgNPs have the capacity of inducing apoptosis cell death of T47D cells through G2M cell cycle arrest. Thus, our findings suggest the anticancer potential of biosynthesized CKAgNPs against human Breast cancer cells and could play an important role in the development of new therapeutic agent for the treatment of cancer.

DATA AVAILABILITY

The data used to support the findings of this study are included within the article.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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