Research article

Efficacy of green synthesized gold nanoparticles conjugated with 5-fluorouracil in targeting breast cancer cell - An *in vitro* study

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ABSTRACT

Introduction and Aim: Presently, research and development in nanomaterials is gaining hypersonic reach in various areas of applications. Biological way of preparing such nanomaterials is acquiring noteworthiness in the view of affordability and environment-friendly approach. In this current work, gold nanoparticles(AuNPs) were prepared using leaves extract of *Chloroxylon swietenia* having anticancer behaviour with the focus to blend the therapeutic activity among the nanoparticles was studied by conjugating with 5 Fluorouracil (5-FU) to target breast cancer cells.

Materials and Methods: *Chloroxylon swietenia* leaf extract was prepared and chloroauric acid (HAuCl4) was added to synthesis AuNPs. As prepared AuNPs were characterized using UV-visible spectrophotometry, TEM and EDAX. The antioxidant tests were done by DPPH assay. Further, the AuNPs were conjugated with5-fluorouracil. The *in vitro* cytotoxicity of the synthesized gold nanoparticles and conjugated AuNPs were assessed by MTT assay against normal and A549 breast cancer cell lines and the efficacy was noted.

Results: Preparation of AuNPsthrough the leaf extract of *C. swietenia* was carried out in an effective way.The preliminary test for the confirmation of as synthesized NPs was done through spectroscopic analysis, resulting in a characteristic peak at 539nm. The TEM examination provided the information about size and the pattern of NPs. Cytotoxicity investigation of NPs with VERO cell lines confirmed its non-toxic nature towards the cells. Anticancer study with A549 breast cancer cell lines also proved the NPs with IC50 value at 25µl/ml.

Conclusion: The conjugated AuNPs with 5-FU revealed anticancer activity in A549 breast cancer cell line but correspondingly it is proved to be safe with standard VERO cell line. The findings stand with a strong point to expand the studies through the evolution of potential drug molecules to deal with the disease of interest.

Keywords: Gold nanoparticles; conjugation; cytotoxicity; anticancer activity; 5-fluorouracil.

INTRODUCTION

World widely, cancer becomes the notable cause of death with almost 10 million casualties in 2020 within breast cancer alone leads to 6,85,000 deaths (1). Breast cancer evolves if any of the cells in breast (connective tissues, lobules, duct) becomes cancerous. There are various approaches towards managing the breast cancer ranging from surgery, radiation therapy, chemotherapy, gene therapy, immune-modulations, virus-mediated oncolysis etc., but overwhelming responses were given to the field of nanotechnology towards breast cancer for as much as nearly 250 clinical trials were in progress looking into their potency as drug delivery carriers targeting cancer(2). The nano drug delivery systems are convenient with additional metabolic durability, improved membrane permeability, augmented bioavailability and prolonged activity. Anti cancer agents can be equipped with these nano transporters for their specific target action towards the tissues. Nanoparticle size contributes towards higher transcellular uptake when compared with larger carrier molecules which in turn enhances the efficiency of therapeutic agents (3,4). Among the metal NPs, gold nanoparticles (AuNPs) with different sizes and configurations were proven to be a multifunctional nano carrier or vehicle for cancer therapy. Also their various characteristics like multiple surface functionalities promotes highly sufficient to carry and deliver high drug concentration even with multiple drugs on single particle (5-7).

Amid the various synthesis procedures, green synthesis of nanoparticles from biological sources has been focused nowadays because of their non-toxic nature, low production cost and eco-friendly attributes. By nature most of the plants are abundant with amino acids, enzymes, protein, vitamins, alkaloids, flavonoids, phenolic acids, terpenoids, etc., which serve like capping and stabilizing factors these in turn help in the bio-reduction of metals to metal ions as NPs with diverse proportions and structures (8). In this current work, AuNPs were synthesized

through leaves extracted from *Chloroxylon swietenia* - an indigenous plant of the Indian sub-continent where various parts of this plant have been known for their medicinal values (9).

In order to increase the efficacy of green synthesized AuNPs, 5-fluorouracil (5-FU) a well-documented antimetabolite drug prescribed for cancer treatment for the past 30 years, has been conjugated using PEG (10). Finally, the conjugated AuNp + 5-FU were aimed to target the breast cancer cells and its effectiveness was recorded.

MATERIALS AND METHODS

Green synthesis of AuNPs from *Chloroxylon swietenia* leaf extract

Chloroxylon swietenia leaves were gathered, cleaned, dried up and ground into fine powder. Leaf extract was prepared by boiling 2g of powder with 50 ml of $D.H_20$ at 60°C for 20 mins. A clear extract solution was obtained after cooling and this crude aqueous extract was used for preparing AuNPs. To 1 ml of the leaf extract, 20 µl of 10mM Chloroauric acid ($HAuCl_4$) was added and mixed with 4ml of $D.H_20$. The above suspension was incubated at room temperature for 24 hrs under static conditions indicating AuNPs production which was assured by the colour change to intense purple (11). Thus prepared AuNPs emulsion was maintained at 4°C until further use.

Characterization of AuNPs

The constancy of as prepared AuNPs was confirmed by UV-Vis spectrophotometer among the range 300-700 nm at regular time interval (1-23 days).Whereas the size, shape and morphology of nanoparticles was analysed through Transmission Electron Microscope (TEM) using SC1000 Orius CCD camera. For this purpose, AuNPs were kept on a copper grid layered with carbon further it was dried using vacuum desiccators and preserved overnight. Following overnight incubation, synthesized AuNPs were placed on carbon layered copper grid with a specimen holder. The micrograph of as prepared AuNPs was captured using JOEL JSM 100CX TEM equipment functioning at an accelerating voltage of 200 kV. The elemental composition of AuNPs in powder form was determined by energy-dispersive X-ray (EDX) spectroscopy on a Horiba EMAX Energy EX-400 analyzer.

Antioxidant activity

Antioxidant activity of green synthesised AuNPs was studied through its efficiency of scavenging 1, 1-diphenyl 2-picrylhydrazyl (DPPH)free radicals.In brief, 1.6 mL aliquots with various concentrations of AuNPs (100-500 µg/mL) prepared in Milli-Q water were added with 0.4 mL of 0.1 mM DPPH in methanol. The above assortment was kept for incubation at 37° C in dark for particular durations up to 30 min with shaking at 100 rpm. Absorbance was read against a control (Methanol) at 517 nm using a UV–vis spectrophotometer. Decrease in absorbance indicated increased radical scavenging activity (12).

The inhibition percentage of DPPH oxidation was calculated with the equation as follows:

Percentage of Inhibition = (Control value– sample value / control value) × 100.

Conjugation of AuNPs with 5 fluorouracil (5-FU)

AuNPs were conjugated with 5 - fluorouracil (5FU) through polyethylene glycol following the method where the thiol group of the polymer binds to the gold nanoparticles. Briefly, 5 ml of AuNPs solutionwas added with 1 ml of PEGand stir mixed for 6 hours at room temperature. To the PEGylatedAuNPs,0.005g of 5 - fluorouracil (5FU) was added and stirred for 4hours. Finally the stability of conjugated AuNPs was measured on UV-spectrophotometer between the range 500–700 nm (13).

Assessment of *in-vitro* cytotoxicity of conjugated AuNPs

To assess the *in-vitro* cytotoxicity of conjugated AuNPs as well as AuNPs, human breast cancer cell line A549 and VERO cell line were used which were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Initially the cells were maintained in DMEM medium enriched with 10% foetal bovine serum (FBS) and 1% antibiotic solution at room temperature in a humidified incubator with 5% CO_2. 96 h later, the culture wassubjugated with about 80% confluence further the cells were extracted using 1 ml of trypsin-EDTA solution. As prepared cell's proliferation and cytotoxic nature was assessed through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (14). Briefly, the cells were placed in 96-well microplates (1 x 10^6 cells/well) and subjected to incubation in 5% CO_2 incubator for 48 h at 37°C till it acquires70-80% confluence state. Following that themedium was substituted with different concentrations (6.25,12.5, 25, 50, 100 µg/mL) of samples and incubated for 24 h. At the end of 24 h, morphological changes of untreated (control) and the treated cells were recorded under digital inverted microscope with 40X magnification. Using phosphate-buffer saline (PBS, pH-7.4), the cells were then washed and to each well 20 µL of MTT solution with concentration of 5mg/mL in PBS was added. The well microplates were left undisturbed for 2h at 37 ºC in the dark. Using 100 µL DMSO the formazan crystals were dissolved and the absorbance was recorded spectrophotometrically at 570 nm. The percentage of cell viability was estimated using the following formula,

Percentage of Cell viability = (Absorbance value of sample/Absorbance value of control)*100.

Anticancer activity of AuNPs

Antiproliferative activity of prepared AuNPs was assessed using A549 breast cancer cell line. This experiment was performed by adding 1 × 10^4 cells (10,000 total cells) into every well of 96-well plates and kept for overnight incubation. To this, varying concentrations of AuNPs were added and the treated cells were incubated at 37°C in the CO_2 incubator for 24 h. Following that, 10µl (5 mg/ml) of MTT was supplied to each well and the formazan crystals were formed by incubating the cells for about 3 h. DMSO solvent (100 µl) was added for dissolving the formazan crystals and incubated for about an hour. Using a spectrophotometer, the absorbance was read at 540 nm and the readings were recorded (15). Percentage of cell viability and the percentage to assess cell inhibition was given as follows,

Cell inhibition = (O.D of treated cells/O.D of control) × 100. Cell viability = (100 – cell inhibition).

Apoptosis

Using Acridine orange/Ethidium bromide (AO/EB) dual staining method, the apoptosis-associated modifications in A549 cells were assessed (16). Viable andnonviable cells equally take up AO stain and give off green fluorescence, whereas only nonviable cells can uptake EO stain and transmit red fluorescence by losing theirmembrane integrity. In a 6-well plate (1 × 10^5cells/well), A549 cells were cultured and treated with IC_{50} concentration of sample for 24 h keeping the untreated A549 cells as control. Later, the treated cells were washed using PBS and stained with 20 µl of AO/EB staining solution (100 µg/ml AO and 100 µg/ml EB) for 5 min. The stained cells were observed under a fluorescence microscope (Invitrogen EVOS FL Cell Imaging; 40X magnification).

RESULTS

Most of the research studies found that diversified compounds isolated from different plant extracts perform as reducing agents for the generation of metal nanoparticles. It is found that, nanoparticles generated through plant extracts possess medicinal properties with major biological importance like antibacterial, antioxidant and cytotoxic properties while comparing with efficiency of nanoparticles generated from other methods. Various bioactive compounds from plant materials can easily interact with inorganic substances which can be effectively exploited in the area of nanotechnology while generating nanoparticles (8). In the present study, leaf extract was prepared from *Chloroxylon swietenia,* AuNPs were generated using *Chloroxylon swietenia* leaf extract and extracted compounds function as a way of capping and reducing agent. In addition, as prepared AuNPs were further conjugated with 5-FU through PEGylation process (Fig.1).

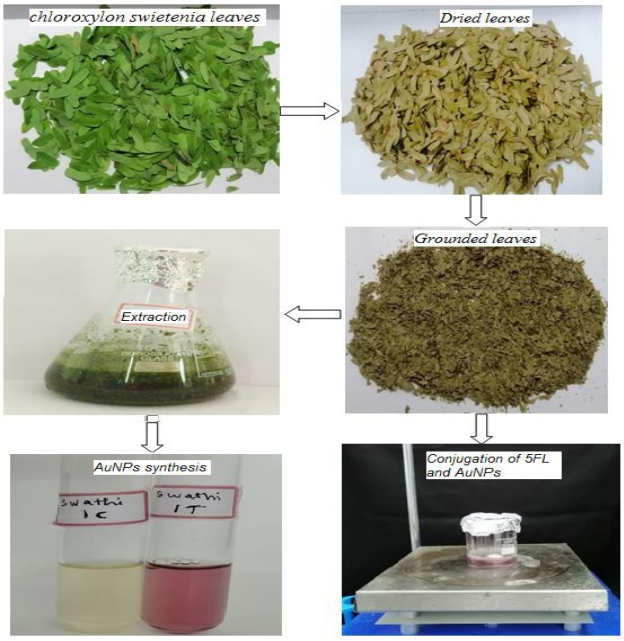

Fig. 1: Extraction of *Chloroxylon swietenia* leaf extract, AuNP synthesis and conjugation of AuNP with 5-FU.

Fig. 2 reveals about UV-visible spectrum analysis of Au coated *Chloroxylon swietenia* nanocomposite in the wavelength in the range of 300-700 cm^1. Between the range 530–550 nm a clear absorption peak resembling the formation of AuNPs was noted. Transformation of Au^+ to Au^0 is clearly understood by examining the absorption spectrum of a prepared nanoparticle solution. A peak at 539 nm wavelength resembles the transition of the d-d region of Au^+ ion which was found to disappear completely following the method of green synthesis. This dispossession was treated as a proof of complete reduction of Au^+ cation. It was already reported in the work that surface Plasmon resonance of AuNPs derived from green synthesis through plants with medicinal values confers absorption from 510 to 550 nm.

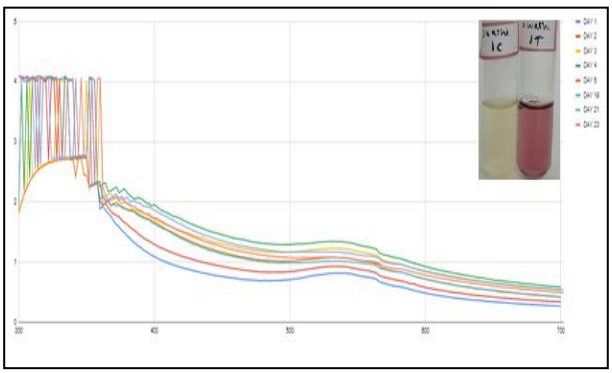

Fig. 2: UV- visible spectrophotometer analysis of green synthesis of Gold nanoparticles (AuNPs) by *Chloroxylon swietenia* through Day 1 - 23.

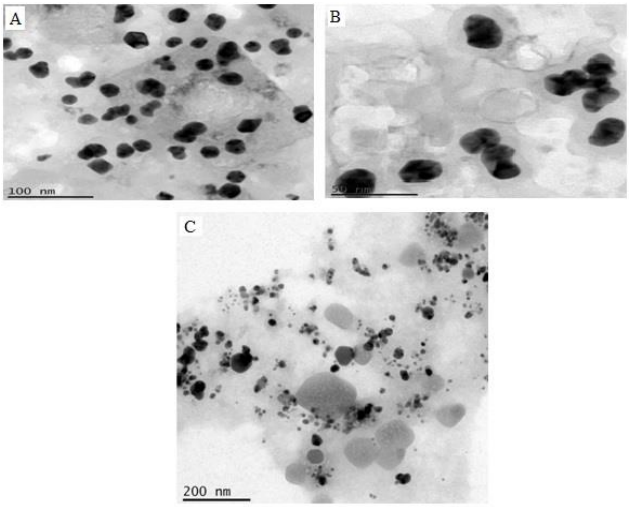

Fig.3: A & B shows TEM image of green synthesized AuNPs by *C. swietenia* leaf extract in the scale of 100 and 50 nm respectively. C shows TEM image of AuNP conjugated with 5-FU in the range of 200 nm.

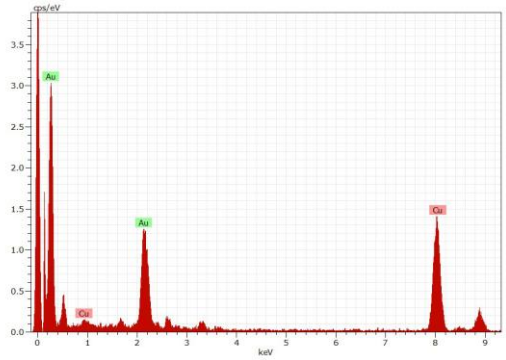

Fig. 4: EDAX graph of green synthesized AuNPs

Based on the results of TEM images (Fig. 3 A & B), it is clear that the size of Au nanoparticles exists in the range of 20-40 nm. This also shows that the spherical shaped Au nanoparticles were well evenly distributed. In more detailed examination, the tiny globular Au particles were observed. Fig. 3C shows that AuNps were well bound with 5-FU with the help of polyethylene glycol which is evenly distributed. The elemental compositions of prepared AuNPs are shown in Fig. 4. This illustrates the successful synthesis of AuNPs by showing the apparent peak at Au region. Other peaks like carbon and oxygen pertain to the organic fractions of *C. swietenia* aqueous leaf extract. It was revealed that the metallic Au nano crystals display an optical absorption peak at almost 2.1 keV as a consequence of their Surface Plasmon Resonance characteristics.

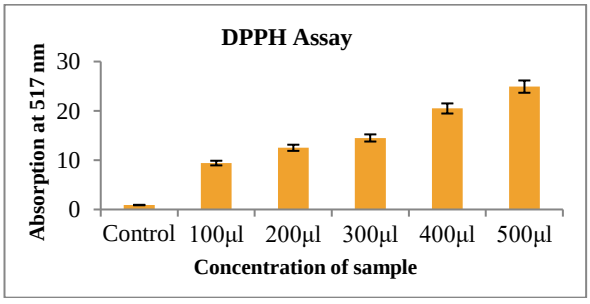

Fig. 5: Antioxidant assay using DPPH

Fig.5 shows the effect of green synthesized AuNPs in quenching the free radicals by reacting with DPPH. The result reveals that prepared nanoparticles have increased antioxidant activity with increased concentration.

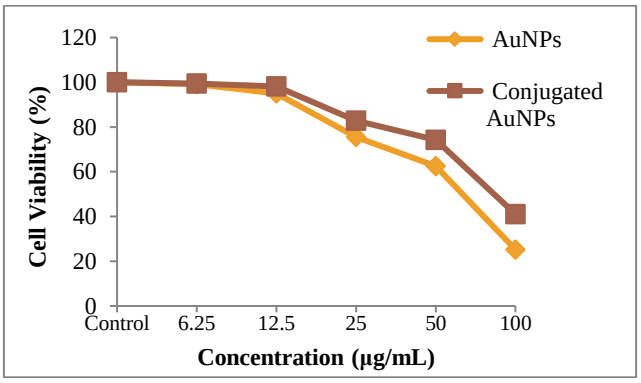

Fig. 6:MTT assay comparing the effect of AuNps and conjugated AuNPs against Vero cells

Fig.6 indicates that the MTT assay results of both green synthesized gold nanoparticles as well as conjugated AuNps with 5-FU against the Vero cell line at different concentrations. In case of AuNPs alone, the cell viability is decreased with increase in the concentration of gold nanoparticles. The concentration at 6.25µl and 12.5µl is not that much toxic at the Vero cell. The concentration above 25 µl shows considerable harm to the cell. Further the concentration at 50µl and 100 µl, it causes cell damage. It was understood from the result of *in vitro* cytotoxicity assay done with VERO cell line AuNPs possess enhanced cell viability with various concentrations tested. Thereby it is clear that the synthesized AuNPs are safe over the treated VERO cell line. Whereas in case of conjugated AuNPs with 5-FU, the cell viability is decreases with increase in the concentration. The conjugated AuNPs with 5-FU are not toxic at the concentration of 6.25 and 12.5 µl. When the concentration is increased it causes cell damage. The viability ratio of conjugated AuNps was well established through MTT assay in the VERO cell line, proving that the conjugated AuNPs presented in this study possess excellent cell viability ratio with all tested concentrations. Consequently the synthesized conjugated AuNPs can be counted for further extensive studies.

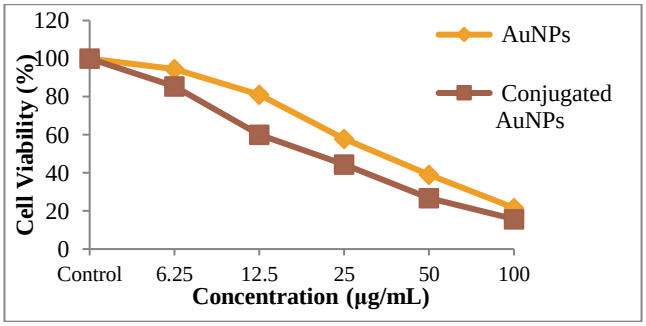

Fig. 7:MTT assay of AuNps and conjugated AuNPs against A549 breast cancer cell line

Fig. 7 indicates that the MTT assay result of both green synthesized gold nanoparticles as well as conjugated AuNps with 5-FU against breast cancer cell line. With only green synthesized AuNPs, the cell viability decreases with increase in AuNPs concentration. The gold nanoparticles fight against the cancer cell A549 which reveals the higher anticancer activity of AuNps synthesized using *Chloroxylon swietenia* leaf extract.

Whereas in case of conjugated AuNPs with 5-FU, the viability of cell decreases with increased concentration of conjugated AuNPs with 5-FU. The conjugated nanoparticles fight well against the cancer cell A549 than mere AuNPs and it is clear from the Fig. 6.

Fig. 8 and 9 reveal the effect of cytotoxicity of conjugated AuNPs with 5-FU against Vero cell lines and breast cancer cell lines respectively. Cytotoxicity tests showed that the conjugated AuNPs with 5-FU were toxic to breast cancer A549 cells but not toxic to the normal cell line, the cytotoxicity was dependent on dosage value. Out of these results, it can be hypothesized that the cytotoxicity of green-synthesized gold nanoparticles and conjugated 5-FU + AuNPs are dose dependent. Hence, *Chloroxylon swietenia* gold nanoparticles may be an ideal anticancer material for biological study applications.

The outcome of the anticancer assay with the breast cancerA549 cell line revealed that the *Chloroxylon swietenia* regulated generation of AuNPs using its leaf extracts, have well validated the anti-proliferative activity over the cancer cell line and verified as efficient in most of the concentrations tested recorded out of the study. According to the study, it was found that the AuNPs and conjugated AuNPs generated from *Chloroxylon swietenia* leaf extract possess enhanced anticancer activity. Fig. 9 shows apoptosis results of dead cells and live cells. In that we can show that some of the cells are dead. So the synthesised AuNPs and conjugated AuNPs have higher anticancer activity.

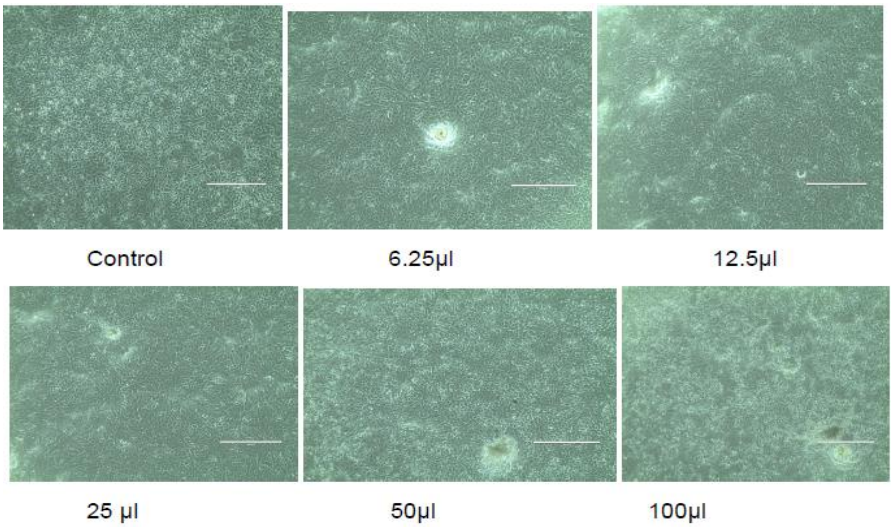

Fig. 8: Effect of conjugated AuNPs on Vero cell lines at various concentrations

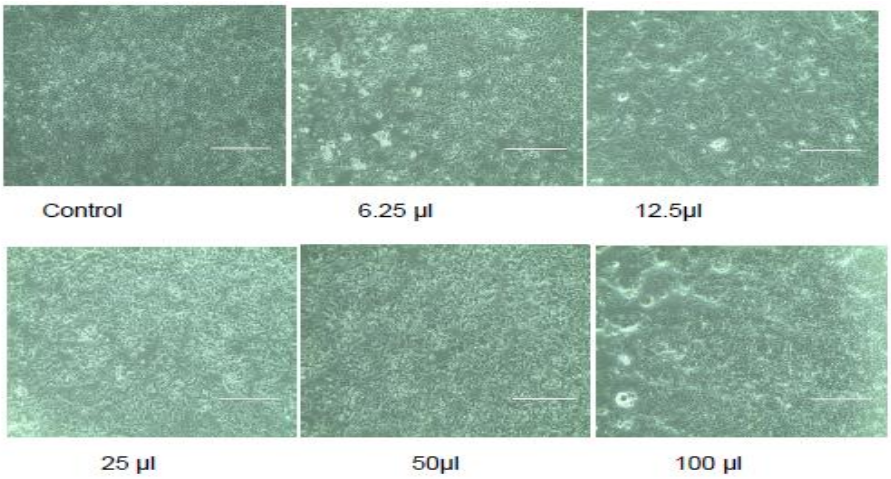

Fig.9: Effect of conjugated AuNPs on breast cancer cell lines at various concentrations

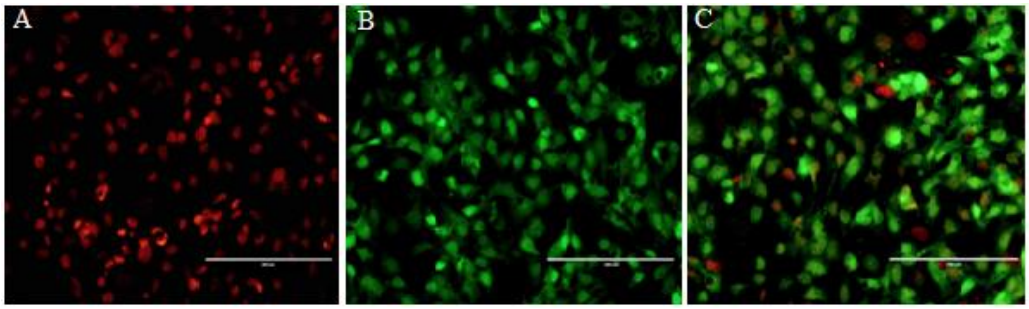

Fig.10: Apoptosis study, Untreated A549 cells – control (A), Treated A549 cells with AuNps (B), Treated A549 cells with conjugated AuNps with 5-FU (C).

DISCUSSION

Nano-medicine has evolved as a desirable diagnostic medium and an innovative therapeutic approach in treating cancer. Recent advancements incorporating the nanoparticle usage provide promising attainability to achieve more efficient and safer route to deliver anticancer drugs to their tissue's destiny (17-19). Synthesis of gold nanoparticles is done through *Chloroxylon swietenia* leaf extract under normal room temperature, at neutral pH and lacking the need of external factors like shaking. This approach is uncomplicated, more economical, safer and efficient compared to chemical synthesis methods. The TEM images revealed that the synthesized AuNPs possess consistent morphology with size ranging from 20-40nm. The AuNPs with more density and sphere-shaped have been well distributed. The plant extract were tested for various phytochemical analysis such as carbohydrates, alkaloids, saponins, glycosides, amino acids, proteins, flavonoids, phenols, terpenoids, steroids moreover these phytochemical agents perform as reducing and stabilizing agents for generating gold nanoparticles through plant extracts. Phenols present in leaf extract have carboxyl and hydroxyl groups which are capable of binding with heavy metals. Besides, other research studies have shown that the proteins with free amine groups can attach to nanoparticles and therefore have the possibility of obtaining well stabilized gold nanoparticles. Hence, it can be inferred that the biological molecules can involve in either functions of generation and stabilization of gold nanoparticles in aqueous medium. Following surface tailoring of gold nanoparticles, they can perform as a better drug delivery system to carry the drugs to targeted sites. Most of the research studies have employed AuNp at low concentration for enhanced activity.The cytotoxic efficacy of AuNPs was also evaluated. The current study shows that Au nanoparticles of *C. Swietenia* and the percentage cell viability was found to decrease with increase in the concentration i.e. concentration dependent cytotoxic effects. The results are in accordance with cytotoxic effect of gold nanoparticle synthesis with leaf extract of *C. Swietenia* showed similar cytotoxic effects.Cytotoxicity tests showed that the AuNPs and conjugated AuNPs with 5FU were toxic to Vero A549 cells and not toxic to normal cell lines. The cytotoxicity was dose dependent. It was reported that although there were various levels of inhibition among cell lines, AuNPs can provoke a concentration dependent constraint of growth in every tested cell line (20).

The outcome of this work furnishes a thriving importance of an ethno medically important plant in cancer treatment. Since *Chloroxylon swietenia* is extensively acknowledged for its antifeedant, anti-inflammatory, antimicrobial and antioxidant activity, in this study it has been exploited for nanoparticle synthesis and further its anticancer activity are well evidenced. In addition, the bioactive materials which are responsible for this activity ought to be confirmed. In order to identify its mode of action against the disease, the compounds have to be extracted and purified. Also its efficacy could be checked for other type of cancer cells (21).

CONCLUSION

The current findings from this study support the practice of using plant extracts sustaining the traditional way of treating the disease. *Chloroxylon swietenia* has been exploited in the ethnic medicine and its therapeutic usage was seen high in history. Recently, ethnomedicine is attaining its lost magnificence and notably applied to various medications. In this circumstance, the healing properties applicable among the medicinal plants, *Chloroxylon swietenia* was capitalized in this study. The AuNPs was synthesized through the leaf extract of *Chloroxylon swietenia* and characterized for their stability. Further the gold nanoparticles were conjugated with 5 fluorouracil (5FU) and their cyto-toxicity was assessed. The *in vitro* cytotoxicity showed that the synthesized gold NPs and conjugated AuNPs are safer on VERO cell lines and anticancer studies opposing A549 cell lines confirmed that the AuNPs have excellent antiproliferative impact over the breast cancer cell line. Relatively, the conjugated AuNPs have higher anticancer activity than green synthesized AuNPs. In future, the ability of prepared AuNP conjugated with 5-FU would be assessed against the other cancer cells.

CONFLICT OF INTEREST

Authors have no conflicts of interest.

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