A COMPARATIVE ANTICANCER STUDY OF BIOSYNTHESIZED NANOPARTICLES, DOXORUBICIN & NANO-DOX CONJUGATE AGAINST CERVICAL CANCER HELA CELL LINE

Oncology

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ABSTRACT

Doxorubicin is the most common chemotherapy drug used in cancer therapy. Its usage is associated with various side-effects. In order to overcome the challenges in Doxorubicin administration, the present study has focussed on synthesizing a drug conjugate with biosynthesized gold nanoparticles and doxorubicin. The gold nanoparticles were biosynthesized using green extracts of medicinal plants with potential anticancer activities. The nanoparticle that possesses anticancer activity was conjugated with the drug for a combinatorial effect of the nanoparticles and the drug. The *in vitro* cytotoxicity was checked in Vero cell line through MTT assay. The *in vitro* anti proliferative effects were screened against cervical cancer in HeLa cell line. Fluorescence activated cell sorting analysis was carried out to detect the difference between live and dead cell populations. The preliminary confirmation was carried out in UV-VIS spectrophotometer. The morphological characterization was carried out by SEM and stability by Zeta potential. The IC_{50} of the nanocompounds demonstrated anti-proliferative activity against cervical cancer similar to the chemotherapeutic drug, Doxorubicin; additionally in a much lesser concentration of the drug. The *in vitro* cytotoxicity exhibited high viability of cells in Vero cell line with minimum viability of 80% in all the tested concentrations. There was a synergistic effect of the nanoparticles along with the drug; thereby an enhanced therapeutic efficiency was achieved. FACS analysis showed 36% of cell death in Dox treated HeLa cells whereas 96% of cell death in Nano-Dox treated HeLa cells. Nano-Dox conjugate has demonstrated high anticancer effects than drug alone Doxorubicin. Further biosynthesized nanomaterials based drug formulation can be developed as a potential strategy in cancer therapy.

KEYWORDS

Nanoparticles, Doxorubicin, Nano-Dox, Cervical cancer

INTRODUCTION

The World Cancer Report presented a widespread global view of cancer to grow by 50% over the next 20 years and to reach 15 million by 2020 (Kumar *et al.*, 2014) Nearly 70% of the cancer deaths occur in low- and middle income countries. (Casper 2011) Gold nanoparticles are reported as novel photo-thermal agents that induce cell death by conversion of light energy into thermal energy. The nanoparticles are easily bioconjugated with a range of delivery vectors. (Sayed *et al.*, 2006) The gold nanocages has been utilised in diagnostic imaging and *in vitro* therapeutics. (Dickerson *et al.*, 2008) Gold nanoparticles can be employed to amplify the biorecognition of anticancer drugs. (Shen *et al.*, 2008) Gold nanoparticles have advantages over other metal nanoparticles due to their biocompatibility and non-cytotoxicity and used in chemotherapy and diagnosis of cancer cell. (Cai and Chen, 2007). Gold nanoparticles have remarkable potential not only in biosensing drugs but also in drug, gene and protein delivery. (Li *et al.*, 2006) Doxorubicin is one of the widely employed chemotherapeutic drugs in the treatment of various cancers within all age groups. The list of side effects includes cardiotoxicity, myelosuppression, vomiting and diarrhea. (Du *et al.*, 2018) The onset of the side effects varies between individuals. (Carvalho *et al.*, 2009) Cardiotoxicity is the major adverse effects in the usage of Doxorubicin. (Thorn *et al.*, 2011) Delivery of Doxorubicin with carrier molecules and combination of Doxorubicin with other antimicrobial compounds has resulted in enhanced Dox activity with improved apoptotic index. (Pugazhendhi *et al.*, 2018)

With this background, the study was aimed at the biosynthesis of gold nanoparticles using the plant extract of *Guazuma ulmifolia* and to conjugate the AuNPs with chemotherapeutic drug Doxorubicin. Further to check the *in vitro* anticancer effect of Nanoparticles (A), Doxorubicin (B) and Nano-Dox combination (C) against cervical cancer HeLa cell line. The combinatorial effect of AuNPs along with Doxorubicin will result in an improved therapeutic index of the drug.

MATERIALS AND METHODS:

Collection of the plant material:

The bark of the plant *Guazuma ulmifolia* was collected from the campus of Women's Christian College, Chennai. The formal identification of the plant specimen used in this study was identified by Dr. G. Jeya Jothi, Taxonomist, Department of Plant Biology & Biotechnology, Loyola College, Chennai. The bark was dried, powdered and boiled for 10 minutes in double distilled water. Then it was cooled, filtered through Whatman filter paper and stored at -18°C for further synthesis.

BIOSYNTHESIS OF GOLD NANOPARTICLES:

The gold nanoparticles (AuNPs) were biosynthesized using bark extracts of plant *Guazuma ulmifolia*. Chloroauric acid was used as the precursor solution for the synthesis of gold nanoparticles. It was purchased from Loba Chemie which was used without further purification. The stock solution was prepared in double distilled water and adjusted for pH for further synthesis. Chloroaurate was reduced using the green extract as the reducing and stabilizing agent. The pale yellow color of the precursor solution was turned into wine red indicating the formation of gold nanoparticles.

SYNTHESIS OF NANO-DRUG CONJUGATE:

An aqueous solution of sodium tripolyphosphate (1mg/ml) was used as a gelating agent. Equal volumes of AuNPs and Doxorubicin (1mg/ml) were taken. This drug solution was taken and homogenized for 30 minutes at 400 rpm with drop wise addition of STPP. The prepared nano-drug conjugate was further characterized and studied for anticancer activity.

CHARACTERIZATION OF NANOPARTICLES USING UV-VIS SPECTRA

The bioreduction of gold chloride in aqueous base was observed by regular sampling of the aliquots of the solution. 1 part of the sample was diluted with 30 parts of double distilled water and was analyzed for the formation of nanoparticles. The UV-vis spectra was recorded using a Shimadzu UV 1800 - UV Visible spectrophotometer operating between the wavelengths of 200 to 800 nm.

SEM ANALYSIS (SCANNING ELECTRON MICROSCOPE)

The AuNPs were diluted with Milli-Q water in the ratio of 1:20. About 10 µl of sample was placed on the aluminum foil and allowed to air dry. The foil was placed on the SEM grid for analysis. The Scanning Electron Micrograph of the samples was carried out at Department of Nanoscience and Nanotechnology, Anna University, Chennai.

ZETA POTENTIAL

The stability of the synthesized NPs was elucidated on analysis of Zeta potential. It is the measure of the charged species present at the NPs surface that prevents from aggregation and ensuring highly stable nanoformulations. The more the positive or the more the negative, the Zeta measure possess the strongest stability. The Zeta potential analysis was carried out in Zetasizer, Translational Research Platform for Veterinary Biologicals (TRPVB) TANUVAS, Chennai.

PHYTOCHEMICAL ANALYSIS OF THE GREEN EXTRACTS

The plant extract was tested for the presence of various phytochemicals such as terpenoids, flavonoids, saponins, tannins, phenols, Coumarins, Triterpenoids and the results were recorded. These phytochemicals act as the reducing and stabilizing agents for the plant mediated synthesis of nanoparticles.

IN VITRO CYTOTOXICITY THROUGH MTT ASSAY

Vero cell line was used to check the cytotoxicity of the synthesized nanoparticles and nano-drug conjugates. The cell line used was procured from Translational Research Platform for Veterinary Biologicals (TRPVB) TANUVAS, Chennai. About 1×10^4 cells (10,000 total cells) were added into each well of 96 well plates and incubated overnight. To this different concentration of AuNPs were added. The treated cells were incubated for 24 hours at 37°C in the CO_2 incubator. After incubation, $10 \mu\text{l}$ (5 mg/ml) of MTT was added to each well. The cells were incubated for about 3-4 hours for the formation of formazan crystals. About $100 \mu\text{l}$ of DMSO solvent was added to dissolve the formazan crystals and incubated for 1-2 hours. The absorbance was read at 540 nm with a reference filter of 620 nm through Multimode plate reader, TRPVB, TANUVAS, Chennai. Percentage of cell viability was calculated. Formula for cell inhibition = $(\text{O.D of treated cells} / \text{O.D of control}) \times 100$. Formula to check cell viability = $(100 - \text{cell inhibition})$

ANTICANCER ACTIVITY OF THE SYNTHESIZED COMPOUNDS

The Cervical cancer HeLa cell line was used to check the anti-proliferative activity of the synthesized nanoparticles and nano-drug conjugate. The cell line used was procured from Translational Research Platform for Veterinary Biologicals (TRPVB) TANUVAS, Chennai. About 1×10^4 cells (10,000 total cells) were added into each well of 96 well plates and incubated overnight. To this different concentration of NPs were added. The treated cells were incubated for 24 hours at 37°C in the CO_2 incubator. After incubation, $10 \mu\text{l}$ (5 mg/ml) of MTT was added to each well. The cells were incubated for about 3-4 hours for the formation of formazan crystals. About $100 \mu\text{l}$ of DMSO solvent was added to dissolve the formazan crystals and incubated for 1-2 hours. The absorbance was read at 540 nm with a reference filter of 620 nm through Multimode plate reader, TRPVB, TANUVAS, Chennai. Percentage of cell viability was calculated. Formula for cell inhibition = $(\text{O.D of treated cells} / \text{O.D of control}) \times 100$. Formula to check cell viability = $(100 - \text{cell inhibition})$. The IC_{50} values were calculated using OriginPro 9.0 software.

FLOURESCENCE ASSISTED CELL SORTING – FACS ANALYSIS

The Cervical cancer HeLa cell line and Vero cell line were seeded into T25 flasks and incubated overnight. These cells were treated with Doxorubicin and Nano-Dox conjugate. The treated cells were incubated for 24 hours at 37°C in the CO_2 incubator. Then the cells were harvested in PBS and centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in PBS and centrifuged as before. To this cell pellet, 1 ml of sheath buffer was added and mixed well. From this $500 \mu\text{l}$ was filtered through $70 \mu\text{m}$ cell strainer. $10 \mu\text{l}$ of PI (Propidium Iodide) and $20 \mu\text{l}$ DAPI (4' 6-diamidino-2-phenylindole) was added to the strained cells. The cells were incubated for 10 minutes in dark. Then the cells were placed in FACS for analysis. The analysis was carried out through FACS analyzer equipped with CyClone custom plate and slide sorting, TRPVB, TANUVAS, Chennai.

RESULTS & DISCUSSION:

BIOSYNTHESIS OF GOLD NANOPARTICLES

The gold nanoparticles were synthesized using the bark extract of the medicinal plant, *Guazuma ulmifolia* upon reducing with Chloroauric acid. The biosynthesized AuNPs were deep wine red in colour. A Nano-Drug conjugate that was prepared in combination of AuNPs and the chemotherapeutic drug, Doxorubicin through STPP as the gelating agent. Fig.1 a & b represented the plant, *Guazuma ulmifolia* and its bark used for the synthesis. The biosynthesized gold nanoparticles and nano-dox conjugate was represented in Fig. 1 c & d.

Fig 1 a, b, c: *Guazuma ulmifolia* tree, Bark, Biosynthesized AuNPs, Nano-Drug conjugate

CHARACTERIZATION – UV- VISIBLE SPECTROMETER ANALYSIS

The UV absorption peak corresponds to the wavelength at which the optical absorption spectra of metal nanoparticles were dominated by surface plasmon resonance (SPR), which shifts to longer wavelength with increasing particle size. The preliminary confirmation of the nanoconjugate was carried out through UV visible spectrophotometer, which showed the characteristic peak for the synthesized nanoparticles. The UV – Vis absorbance spectrum of Nano-Dox conjugate was represented in Fig.2 which showed SPR peak at λ_{max} - 770, 459 nm.

Fig. 2: UV visible absorbance spectrum of AuNPs-Dox Nanoconjugate The peaks confirm the formation of nano-drug conjugate

ZETA POTENTIAL

The stability of the nanomaterials was measured through Zeta potential analysis. The Zeta potential of the synthesized nanoparticle was -34.6 mV and nano-drug conjugate was -39.8 mV. The results observed were in concordance with the standard reference values demonstrating highly stable nanoformulations. The synthesized nanoparticles were highly stable and mono dispersed. Fig. 3 demonstrated the graphical representation of zeta potential analysis. The results observed were in concordance to that reported by Bhumkar *et al.*, 2007.

Fig.3 Zeta Potential Analysis

SEM (SCANNING ELECTRON MICROSCOPY)

Fig 4a, b: SEM micrographs of AuNPs, c: Size measurement in SEM of Nano-Dox conjugates

The dimension of the Nano-Drug conjugate was 80 nm upwards, on an average of 100nm in size. It's a noteworthy nano dimension attained using a green chemistry approach. The nanoparticles were spherical in morphology and evenly dispersed. From the above images, it could be observed that the nano-drug conjugate was well developed in spherical structure.

PHYTOCHEMICAL ANALYSIS OF THE GREEN EXTRACTS:

The bark extract of the plant was screened for the presence of various secondary metabolites like Coumarins, Saponins, Flavanoids, Terpenoids, Triterpenoids, Carbohydrates, Phenols, Tannins, Proteins and Alkaloids. The plant extract showed the presence of 8 compounds such as Coumarins, Terpenoids, Triterpenoids, Carbohydrates, Phenols, Tannins, Proteins, Alkaloids and absence of 2 compounds such as Saponins and Flavanoids. The intensity of the observed results was indicated with the increasing number of + signs.

Table 1: Phyto chemicals present in the plant extract

S. No.	Compounds	<i>Guazuma ulmifolia</i> Bark (GUB)
1.	Coumarins	++
2.	Saponins	-
3.	Flavanoids	-
4.	Terpenoids	++
5.	Triterpenoids	+
6.	Carbohydrates	+
7.	Phenols	Brown color
8.	Tannins	++
9.	Alkaloids	++
10.	Proteins	+

*+ = presence of the compound, * = absence of the compound
The number of signs indicate the intensity of results observed

IN VITRO CYTOTOXICITY IN VERO CELL LINE

The *in vitro* cytotoxicity of the Nano-Drug conjugate was tested in an epithelial cell lineage, Vero cell line through MTT assay. Cytotoxicity was determined for varying concentrations of the nano-drug compounds. The results implied that the Nano-Drug conjugate indicated very minimal cytotoxicity at all the assessed concentrations. It could be noted that the minimum cell viability was determined to be more than 80% at all the tested concentrations. The graph represented the % of cell viability upon treatment with varying concentrations of Nano-Drug conjugate.

Fig. 7: Percent viability in treated Vero cell line

ANTICANCER ACTIVITY OF THE NANO-DRUG CONJUGATE

The anticancer activity was checked in 3 segments. Initially, the biosynthesized AuNPs were checked for potential anti proliferative effects. Followed by, screening for anticancer activity of Doxorubicin and Nano-Dox conjugate. Both the GUB AuNPs and Nano-Dox conjugate proved to be efficient in most of the concentrations with significant Inhibitory Concentrations, IC_{50} of 0.919ng/ml for GUB-AuNPs, 0.069 μ g/ml for drug alone Doxorubicin and 0.507ng/ml for Nano-Dox conjugate.

Fig. 8: a, b, c, d: Control HeLa cell line, GUB-AuNPs (A) treated HeLa cell line, Doxorubicin (B) treated HeLa cell line, Nano-Dox conjugate (C) treated HeLa cell line

From the above images, the differences between the untreated and treated cells could be comparatively observed. Though the GUB-AuNPs treated cells showed varying degrees of cell death, the Nano-Dox conjugate treated cells showed further degenerated cells emphasizing on the combinatorial effect of the AuNPs and Doxorubicin together.

Fig. 9a: IC_{50} of GUB-AuNPs

Fig. 9b: IC_{50} of Doxorubicin

Fig. 9c: IC_{50} of Nano-Dox conjugate

From the above graphs, the inhibitory concentration IC_{50} of the compounds tested has been represented. The biosynthesized GUB-AuNPs has shown good inhibitory concentration against cervical cancer similar to the chemotherapeutic drug, Doxorubicin and additionally in a much lesser concentration of the drug. A further extended study on these AuNPs will lead towards development of biosynthesized drugs in the treatment of the disease.

Fig. 9 a: % of Inhibition of Doxorubicin

b: % of Inhibition of Nano-Dox conjugate

From the above graph, it could be noted that there was a significant increase in the percentage of inhibition against cervical cancer between the Doxorubicin (B) and Nano-Doxorubicin conjugate ©.

FLUORESCENCE ASSISTED CELL SORTING – FACS ANALYSIS

Fluorescence activated cell sorting (FACS) differentiated between the live and dead cells in the given population.

Fig. 10 a, b, c treated Vero cells, Dox treated HeLa cells, Nano-Dox treated HeLa cells

The cells were labeled with fluorescence labels such as PI (Propidium Iodide) and DAPI (4',6-diamidino-2-phenylindole). DAPI was taken up by live cells whereas PI stained the dead cells. In Fig.10a, the percentage of live cells was highest in the treated Vero cells indicated by the DAPI log. In Fig.10c, the percentage of dead cells was highest in the Nano-Dox conjugate treated HeLa cells indicated by PI log compared to Fig.10b which represented the drug alone Doxorubicin treated HeLa cells. Lai *et al.* 2019 have reported on the similar method of FACS analysis in prostate cancer cell lines.

CONCLUSION

Gold nanoparticles (AuNPs) and nano-dox conjugate were successfully biosynthesized using green extracts of *Guazuma ulmifolia* and Doxorubicin. The preliminary confirmation was carried out in UV-VIS spectrophotometer which showed the precise peak indicating the formation of nanoparticles. The characterization was carried out by SEM, which revealed the size and distribution of the nanoparticles. The results of *in vitro* cytotoxicity in Vero cell line signified the non-toxicity of the nanoparticles with minimum viability of 80% in all the different concentrations. The anticancer activity was checked in cervical cancer HeLa cell line which indicated an increased effect of the nano-drug conjugate compared to the native drug alone compound.

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