EX-VIVO ANALYSIS OF HUMAN SERUM ALBUMIN ACTS AS A DRUG DELIVERY AGAINST MCF-7 CELL LINE

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ABSTRACT

Researchers in the field of nanomedicine have recently paid more attention to drug delivery systems based on nanotechnology because they can achieve ideal drug release and biodistribution. Serum albumin-based nano vehicles have been extensively created and studied due to their notable biological properties and many other materials that are utilized to prepare drug delivery vehicles for efficient cancer treatment. One of these is human serum albumin that is a remarkable promising carrier as an anti-cancer agent. HSA enjoys advantages such as long half-life, repeated recycling, and specific accumulation. HSA includes a number of binding pockets where different ligands, including fatty acids and ions, can bind, giving medications the choice of non-covalent binding sites. Moreover, HSA has lengthened the half-life of medications, lessen renal drug clearance and aid in drug accumulation in particular tumor tissues. HSA offers a promising chance to distribute well-known drugs in a novel method. Brucine is a plant alkaloid compound that was extracted from a fresh leaf of *Wrightia tinctoria*. Silver nano particles were synthesized by adding silver nitrate with brucine solution. Characterization of nano particles were done by using UV-vis Spectrophotometer, SEM and FTIR. Bioconjugation of HSA with Phyto therapeutic agent capped Ag-NPs were performed and studied in this review. Human serum albumin as a drug delivery vehicle against MCF-7 breast cancer cell line was performed and analyzed in *ex-vivo* method. The results suggest that Phyto therapeutic agent capped Ag-NPs coated with HSA can be employed as a practical drug delivery system in cancer treatment.

KEYWORDS: Human serum albumin, Nanoparticles, Bioconjugation, Drug delivery.

INTRODUCTION

Small chemical compounds make up the majority of the medications that are now on the market and are used to treat a variety of human ailments. However, these small-molecule medications frequently have drawbacks, including quick breakdown, brief circulation times, quick renal clearance, non-specific distribution, and hazardous buildup in particular organs or in particular tissues (Wang *et al*., 2020). Serum albumin, a naturally occurring ligand carrier that circulates for a long time in the blood and is highly concentrated, has demonstrated incredible promise as a delivery system for anti-cancer drugs. Albumin has the ability to extend the half-life of medications that are typically removed quickly from circulation and, more crucially, to encourage their accumulation within tumors (Hoogenboezem & Duvall, 2018). One class of effective drug delivery systems uses serum albumin as a well-behaved carrier material to encapsulate or conjugate therapeutic compounds for delivery of drugs that are targeted to tumors (Elzoghby *et al*., 2012). The most prevalent protein in plasma, human serum albumin that regulates plasma colloidal osmotic pressure and transports endogenous substances. The primary reason for selecting albumin protein as drug

delivery cargo is its excellent biocompatibility, biodegradability, and non-immunogenicity (An & Zhang, 2017). Researchers attention and enthusiasm for developing novel applications of nanotechnology to cure various types of cancer have been captured by the effective therapeutic benefits of cancer treatment assisted by nanomaterials that have appeared in recent decades (Barreto *et al*., 2011, Nazir *et al*., 2014). In that, AgNPs are extensively used in *in-vitro* and *ex-vivo* studies using various cancer cell models due to their inherent anticancer effect, some recent scientific studies have sought to exploit AgNPs in conjunction with anticancer pharmaceuticals (Morais *et al*., 2020). The tremendous anticancer potential of AgNPs has led to a breakthrough in the metastatic cancer treatment. Also, we evaluated the most current studies on the toxicological action of AgNPs at the cellular level, which portrays it as a powerful anticancer agent with clear therapeutic efficacy (Jabeen *et al*., 2021). Analytical methods such as UV-visible spectrophotometer, FT-IR and SEM analysis are used to characterize chemical nature and morphology of the synthesized AgNPs (Revathy *et al*., 2022). HSA conjugates have the ability to localize pharmaceuticals at specific sites and regulate drug release. HSA is therefore viewed as a viable option for the delivery of drugs in the treatment of cancer (Taheri *et al*., 2011). AgNPs application is recommended in transportation of conjugated drug molecules as it has no adverse effect on serum proteins. Since HSA is present in the circulatory system, it may be possible to use AgNPs in a variety of biomedical applications (Hazarika & Jha, 2020). However, the fundamental cause−effect relationships are still ill-defined so that a better understanding of the NP−protein interactions is essential in order to develop new functional and safe NPs that is one of the most pressing areas of collaborative study in materials science and biology (Goy-López *et al*., 2012). After bioconjugation, cell line activities were studied. Breast cancer is a common malignancy that causes 14% of all cancer deaths and accounts for 23% of all cancer occurrences. For this study, Michigan Cancer Foundation-7 (MCF-7) cells are utilized (Rao & Deeba, 2020). The human breast cancer cell line Michigan Cancer Foundation-7 (MCF-7) is frequently employed in experimental investigations because the mammary epithelium of the breast cancer patient, like the MCF-7 cell line, has individual characteristics. Hence, this cell line is a widely used research tool in the study of cancer (Holliday & Speirs, 2011). With these evidences, here we investigated human serum albumin as a targeted drug delivery vehicle using Phyto therapeutic agent capped Ag-NPs and its anti- cancer activity against Michigan Cancer Foundation-7 cell line for *ex-vivo* studies.

MATERIALS AND METHODS

PREPARATION OF PLANT AQUEOUS EXTRACT

Fresh leaves of *Wrightia tinctoria* were collected from local surroundings of Madurai, India with the help of a field botanist. The plant leaves were collected and the plant leaf alkaloid compound (Brucine) was identified and extracted. Briefly, 5g of plant leaf alkaloid compound (Brucine) was weighed and mixed in 17ml of Millipore distilled water and 3 ml ethanol individually. This aqueous extract solution was used for further study.

PREPARATION OF $AgNO_3$ SOLUTION

For preparing silver nitrate solution 0.002g $AgNO_3$ weighed and dissolved in 100 ml of deionized water. This 100ml of 1mM aqueous silver nitrate solution was used for further studies.

SYNTHESIS OF AgNPs

Briefly, 5ml of prepared leaf extract aqueous solution was added to 95ml of 1mM aqueous silver nitrate solution separately for the reduction of Ag^+ ions. The effect of temperature on synthesis rate and particle size/ shape of the prepared AgNPs was studied by carrying out the reaction in a water bath at 95^0C for 20 mins. Thus, the solution obtained was purified by repeated centrifugation at 3500rpm for 30 mins at

room temperature, followed by resuspended of the pellet in distilled water to remove unwanted biological molecules. To ensure better separation of free entities from metal nanoparticles, the process of centrifugation and resuspension in sterile deionized water was repeated three times. By doing this, we can get rid of any uncoordinated biological compounds (Sukirtha *et al.*, 2012). After synthesis the Phyto therapeutic agent capped AgNPs were characterized.

CHARACTERIZATION OF SYNTHESIZED AgNPs

UV-VIS SPECTROSCOPY ANALYSIS

Color change was recorded as a preliminary confirmation of Phyto therapeutic agent capped AgNPs synthesis before proceeding into UV-vis spectroscopic analysis. Based on their surface plasmon resonance, shape, size and distribution, preliminary characterization studies of Phyto therapeutic agent capped AgNPs were carried out on a Hitachi double beam spectrophotometer, in the range of 200- 1200 nm to identify the bio active compound.

FT-IR ANALYSIS

The purified Phyto therapeutic agent capped AgNPs were examined for the presence of biomolecules using FT-IR analysis. Fourier transform infrared (FT-IR) spectra were done for the analysis of functional groups using an FT-IR spectrometer (Shimadzu) 400 cm^{-1} to 4000 cm^{-1} was used for the analysis to obtain an infrared spectrum of absorption of the sample. The result of FT-IR analysis confirms the presence of functional groups which plays a major role in bioconjugation of human serum albumin with silver nanoparticles.

SEM ANALYSIS

SEM is used to characterize and visualize surface morphology, particle size, distribution, particle/crystal shape, agglomeration of nanoparticles and surface functionalization and in single-particle analysis. Thus, the majority of studies apply this technique to characterize the morphological properties of nanoparticles. The synthesized AgNPs sample image was analyzed by VEGA3 TESCAN SEM instrument.

BIOCONJUGATION

Bioconjugation is a chemical technique used to couple two molecules together, at least one of which is a biomolecule, such as a carbohydrate, nucleic acid, or protein. Proteins are especially diverse biomolecules due to the variety of amino acids available and are thus important substrates in bioconjugation reactions. Bioconjugation reactions play a critical role in the modification of proteins. HSA was purified by liquid chromatography using a Superdex 75 column that had been pre-equilibrated with 0.01 M phosphate before use. In order to completely cover the surface of a specific volume of HSA coated Ag-NP solution, 1 ml of an HSA stock solution with a concentration 10 times in excess to a defined volume of Ag NP solution (10^{12} NPs/mL) was added.

Spectrophotometric analysis was used to measure the protein concentration using a molar absorption value of 35 219 M^{-1} cm^{-1} at 280nm (Pace *et al.*, 1995). In order to track the development of protein adsorption on the NP surfaces, protein-NP bioconjugates were incubated for varying lengths of time (between 0 and 48 h) with slow stirring. After incubation, the bioconjugate samples were centrifuged between 8000 and 16000rpm for 20 min (the larger the size, the lower the speed) and then the sample was resuspended in protein-free water solution, and then centrifuged again to remove any excess unbound or loosely bound protein molecules to the NP surfaces (Capule & Yang, 2012; Casals *et al.*, 2011).

Protein estimation done through Bradford method at 595 nm. Human serum albumin as a drug delivery vehicle against MCF-7 breast cancer cell line was performed and analyzed in *ex-vivo* method. The results suggest that Phyto therapeutic agent capped Ag-NPs coated with HSA can be employed as a practical drug delivery system in cancer treatment.

RESULTS AND DISCUSSION

PREPARATION OF PLANT AQUEOUS EXTRACT

Fresh leaves of *Wrightia tinctoria* were collected from local surroundings of Madurai, India with the help of a field botanist. The plant leaves were collected and the plant leaf alkaloid compound (Brucine) was identified and extracted. Briefly, 5g of plant leaf alkaloid compound (Brucine) was weighed and dissolved in 17ml of Millipore distilled water and 3 ml ethanol individually. The brucine solution got precipitated and used for the synthesis of Phyto therapeutic agent capped silver nanoparticles.

Brucine powder

Plant alkaloid (Brucine) solution

PREPARATION OF $AgNO_3$ SOLUTION

For preparing silver nitrate solution 0.002g $AgNO_3$ weighed and dissolved in 100 ml of deionized water. This 100ml of 1mM aqueous silver nitrate solution was used for synthesis of Phyto therapeutic compound capped silver nanoparticles.

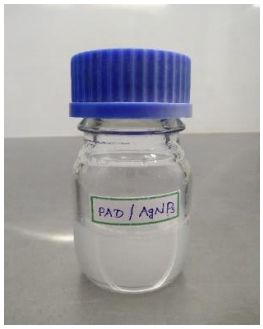
Silver Nitrate

1mM aqueous silver nitrate solution

SYNTHESIS OF AgNPs

Briefly, 5ml of prepared leaf extract aqueous solution was added to 95ml of 1mM aqueous silver nitrate solution separately for the reduction of Ag^+ ions. The effect of temperature on synthesis rate and particle size/ shape of the prepared Phyto therapeutic agent capped AgNPs was studied by carrying out the reaction in a water bath at 95^0C for 20 mins. Thus, the solution obtained was purified by repeated centrifugation at 3500 rpm for 30 mins at room temperature, followed by resuspended of the pellet in

distilled water to remove unwanted biological molecules. To ensure better separation of free entities from metal nanoparticles, the process of centrifugation and resuspension in sterile deionized water was repeated three times. By doing this, we can get rid of any uncoordinated biological compounds (Sukirtha *et al*.,2012). After synthesis the Phyto therapeutic agent capped AgNPs were characterized.



Phyto therapeutic agent capped AgNPs – Before synthesis

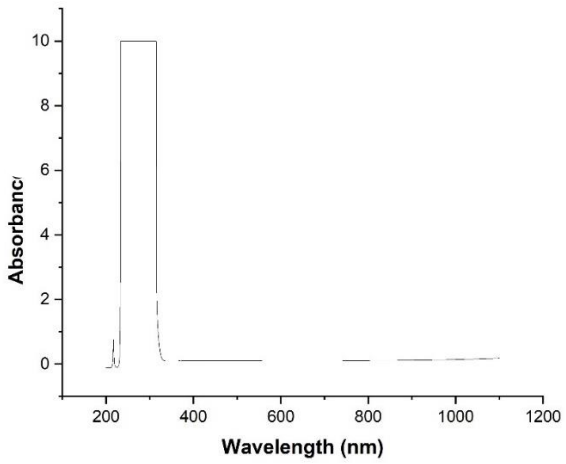
Phyto therapeutic agent capped AgNPs – After synthesis

Phyto therapeutic agent capped AgNPs – Pellet formation

CHARACTERIZATION OF SYNTHESIZED AgNPs

UV-VIS SPECTROSCOPY ANALYSIS

Color change was recorded as a preliminary confirmation of Phyto therapeutic agent capped AgNPs synthesis before proceeding into UV-vis spectroscopic analysis. Based on their surface plasmon resonance, shape, size and distribution, preliminary characterization studies of Phyto therapeutic agent capped AgNPs were carried out on a Hitachi double beam spectrophotometer, in the range of 200- 1200 nm to identify the bio active compound.



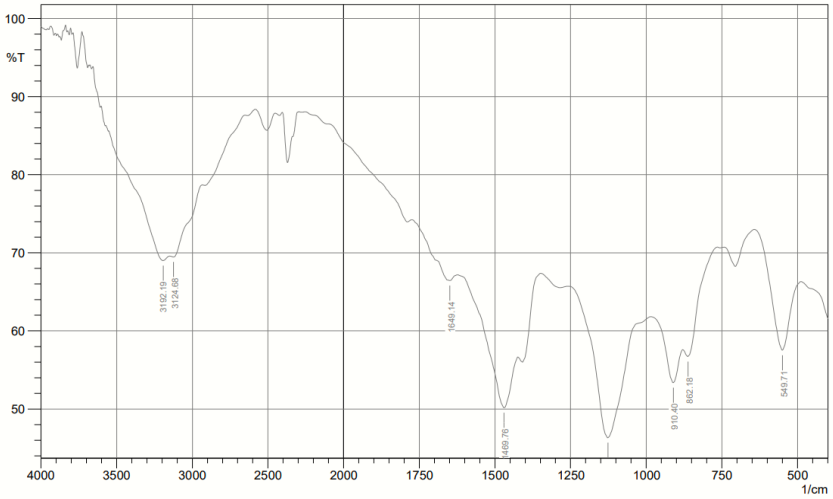
UV-Spectro conforming the SPR band of Brucine synthesized AgNPs

Ultraviolet-Visible spectra of as-prepared sample in range between 200-1200 nm. The plant sample was deposited on clean glass substrate using screen printing technique. UV-Vis results show the peaks of 225 nm and 330 nm wavelength shows high degree of 10 absorbance. From this UV-Vis spectroscopy result, found the confirmation of the broad of SPR band of brucine derived silver nanoparticles.

FT-IR ANALYSIS

FT-IR spectrometer, equipped with a deuterated triglycine sulphate (DTGS) as a detector and a germanium as beam splitter, interfaced to computer operating under Windows-based system, and connected to software of OPUS operating system (Version 7.0 Bruker optic), was used during FT-IR spectra acquisition. A few drops Phyto therapeutic agent capped AgNPs sample were positioned in contact with attenuated total reflectance (ATR) plate.

The purified Phyto therapeutic agent capped AgNPs were examined for the presence of biomolecules using FT-IR analysis. Fourier transform infrared (FT-IR) spectra were done for the analysis of functional groups using an FT-IR spectrometer (Shimadzu) 400 cm^{-1} to 4000 cm^{-1} was used for the analysis to obtain an infrared spectrum of absorption of the sample. The result of FT-IR analysis confirms the presence of functional groups which plays a major role in bioconjugation of human serum albumin with silver nanoparticles.

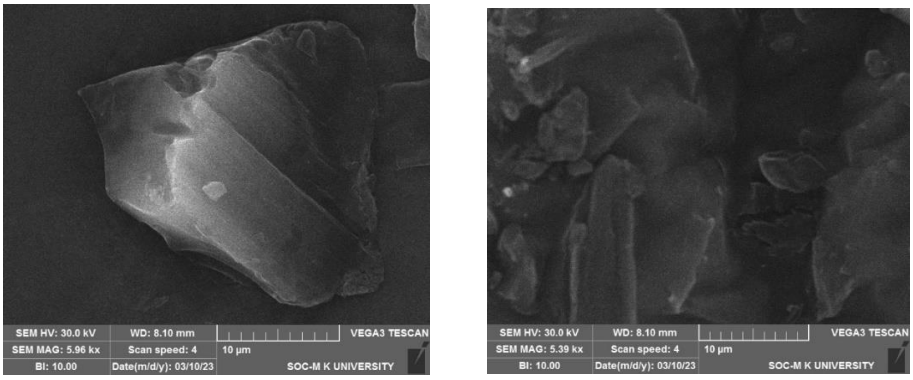


FT-IR spectra Acquisition of Brucine synthesized AgNPs

The results of FTIR analysis confirm the presence of functional groups Which shows the major peak values that determined the bonds relevant to amine N–H stretching (1126.43 cm^{-1}) and bending (1649.14 cm^{-1}), alkane C-H stretching (2954.95 cm^{-1}) and bending (1469.76 cm^{-1}), alkene C=H bending (910.4 cm^{-1}), carboxylic acids and derivatives –COOH stretching (3124.68 cm^{-1} & 3192.19 cm^{-1}) and halogen compound C–Cl (549.71 cm^{-1}). These results indicated that the carbonyl group of proteins adsorbed strongly to metals, indicating that proteins could have also formed a layer along with the bio-organics, securing nanoparticles. This implicit and proved the free energy to reduce the silver ions to AgNPs.

SEM ANALYSIS

SEM is used to characterize and visualize surface morphology, particle size, distribution, particle/crystal shape, agglomeration of nanoparticles and surface functionalization and in single-particle analysis. Thus, the majority of studies apply this technique to characterize the morphological properties of nanoparticles. The synthesized AgNPs sample image was analyzed by VEGA3 TESCAN SEM instrument.



SEM Characterization of synthesized AgNPs

The SEM images showed the presence of cubes structures of AgNPs. The formation of cubic shaped silver nanoparticle extracted. Scanning Electron Microscopic (SEM) analysis was done using VEGA3

TESCAN SEM instrument. Silver nanoparticle synthesized within 10 minutes has an absorbance at 430 nm and the broadening of the peak indicates the poly dispersion of the particle. The SEM shows that cubic shape nanoparticle was formed.

BIOCONJUGATION

Bioconjugation is a chemical technique used to couple two molecules together, at least one of which is a biomolecule, such as a carbohydrate, nucleic acid, or protein. HSA was purified by liquid chromatography using a Superdex 75 column that had been pre-equilibrated with 0.01 M phosphate before use. In order to completely cover the surface of a specific volume of HSA coated Ag-NP solution, 1 mL of an HSA stock solution with a concentration 10 times in excess to a defined volume of Ag NP solution (10^{12} NPs/mL) was added. Spectrophotometric analysis was used to measure the protein concentration using a molar absorption value of 35 219 M^{-1} cm^{-1} at 280nm(Pace et al., 1995).

In order to track the development of protein adsorption on the NP surfaces, protein-NP bioconjugates were incubated for varying lengths of time (between 0 and 48 h) with slow stirring. After incubation, the bioconjugate samples were centrifuged between 8000 and 16000rpm for 20 min (the larger the size, the lower the speed) and then the sample was resuspended in protein-free water solution, and then centrifuged again to remove any excess unbound or loosely bound protein molecules to the NP surfaces (Capule & Yang, 2012; Casals *et al*., 2011). Protein estimation done through Bradford method at 595 nm. The blue color was observed. Based on the OD value of 0.987, we confirmed the presence of HSA in nanomaterial after the synthesis.

HSA stock solution

Synthesized AgNPs solution

HSA coated Phyto therapeutic agent capped AgNPs

CELL LINE STUDIES

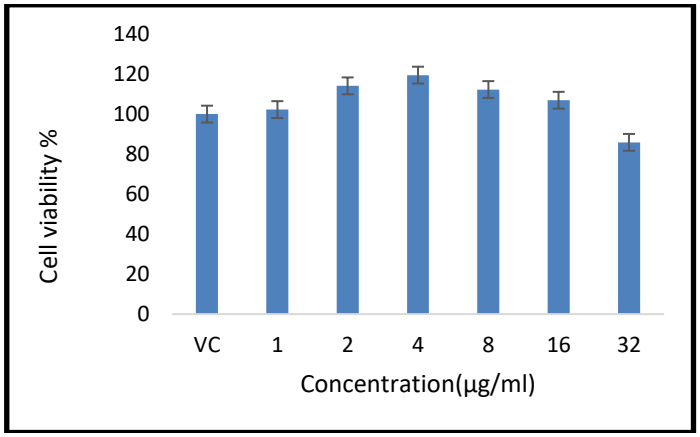
The cytotoxicity potential of the AgNPs on the human breast cancer cell line (MCF-7) was evaluated by the MTT assay. MCF-7 cells are useful for *in-vitro* breast studies because they retained several ideal characteristics particular to mammary epithelium, such as the processing of estrogen, in the form of estradiol, via estrogen receptors (ER) in the cell cytoplasm. It is the first hormone responding breast cancer cell line. The MCF-7 cell line was procured and it was maintained in the Minimal Essential Medium (MEM, GIBCO) as per the standard protocol (Al-Sheddi *et al*., 2018). The active MCF-7 cells (106 cells/well) were loaded in 96 well plates and incubated for 1 day at 37^0C and later, the cells were rinsed with 100 µl of serum free medium and kept starved at 37^0C for 1 h. Various concentrations (1, 2, 4, 8, 16, and 32 µg ml^{-1}) of the HSA coated synthesized AgNPs were administered to the starved MCF-7 cells and incubated at 37^0C for 24 h.

At the end of the treatment, the medium was replaced with serum free medium supplemented with MTT (0.05 mg ml^{-1}) and incubated at 37^0C for 4 h in a CO_2 incubator. Then, the medium was aspirated, and the cells were rinsed with Phosphate Buffered Saline (PBS) and then, the cells were dissolved with 100µl of Dimethyl Sulfoxide (DMSO) colored with formazan stain and blended well. The microplate reader (Bio-Rad Model: 680; USA) was used to read the 96 well plates at 570 nm. The percentage of cell viability was calculated using the following formula.

$$\text{Percentage of cell viability} = \frac{\text{Sample absorbance}}{\text{Control absorbance (Untreated)}} \text{ X } 100$$

Samples(µg/ml)				Average	Percentage			Average	stdvp
VC	0.272	0.231	0.205	0.236	115.2542	97.88136	86.86441	100	11.68652
1	0.213	0.286	0.225	-	90.25424	121.1864	95.33898	102.2599	13.54313
2	0.294	0.247	0.267	-	124.5763	104.661	113.1356	114.1243	8.160371
4	0.281	0.292	0.273	-	119.0678	123.7288	115.678	119.4915	3.300373
8	0.237	0.287	0.271	-	100.4237	121.6102	114.8305	112.2881	8.834178
16	0.241	0.251	0.265	-	102.1186	106.3559	112.2881	106.9209	4.170854
32	0.201	0.197	0.21	-	85.16949	83.47458	88.98305	85.87571	2.303603

HSA coated synthesized silver nanoparticles against MCF-7 cell line



MCF-7 cell viability Vs HSA coated synthesized silver nanoparticles

Human serum albumin as a drug delivery vehicle against MCF-7 breast cancer cell line was performed and analyzed in ex-vivo method. The results showed that the Phyto therapeutic agent capped Ag-NPs coated with HSA has anti- cancer potential at 32 µg/ml against MCF-7 breast cancer cell line and it suggest that Phyto therapeutic agent capped Ag-NPs coated with HSA can be employed as a practical drug delivery system in cancer treatment.

CONCLUSION

These predict that HSA could broadly serve as an excellent drug delivery vehicle via non-covalently binding, covalently binding and genetic fusion strategies. Indeed, some HSA-based or HSA-binding drugs such as Albiglutide, Semaglutide, Abraxane and Levemir have been successfully developed and clinically applied. Many more promising drug candidates are currently under investigation around the world. We are also constructing different HSA-based drugs in the fields of cancers and immune diseases. These drug candidates displayed their potent efficacy and are receptor-selective. Nowadays, albumin is gradually becoming the hot topic and is expected to be the next-generation drug R&D platform. However, there are still certain challenges or limits for albumin to act as a drug delivery vehicle. For example, albumin can be uploaded with different cargoes. Their interactions may impact the binding of albumin with endogenous ligands and their homeostasis. Thus, it is critical to optimize the albumin-based drug design. And also, albumin may not be suitable for all cancers and for all drugs to be coupled. Particularly, the drugs need to be of quick clearance.

The anti-cancer activity of human serum albumin as a drug delivery vehicle against MCF-7 breast cancer cell line was performed and analyzed in ex-vivo method. The results showed that the Phyto therapeutic agent capped Ag-NPs coated with HSA has anti- cancer potential at 32 µg/ml against MCF-7 breast cancer cell line and it showed more effectiveness when compared to other concentration levels. Further work is needed to carry out more concentrations from the Phyto therapeutic agent capped AgNPs coated with HSA in order to support anti-cancer activity against MCF-7 breast cancer cell line. Our study demonstrated that the Phyto therapeutic agent capped Ag-NPs coated with HSA shows anti-cancer potential.

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