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RESEARCH ARTICLE

Comparison of *In Vitro* Anticancer Activity of Plumbagin Silver Nanoparticle and Monoclonal Antibody Labelled Plumbagin Silver Nanoparticle in SKBR3 Cell Lines by MTT Assay

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Abstract

Background: A combination of plumbagin with trastuzumab conjugated silver nanoparticle shows more inhibitory activity than plumbagin silver nanoparticle due to its target delivery. **Aim:** This study reports the effectiveness of monoclonal antibody labelled plumbagin nanoparticle over nanoparticle alone on targeting therapy against HER2-positive breast cancer cells. **Methods**: Plumbagin is encapsulated into silver nanoparticles, and these nanoparticles were conjugated with trastuzumab, a monoclonal antibody targeting HER2 receptors. Successful conjugation of trastuzumab is confirmed by SEM, TEM, FTIR and BCA assay. The result confirmed the successful conjugation of trastuzumab with plumbagin silver nanoparticle. Cytotoxicity of the conjugated nanoparticle was determined using MTT assay on SKBR3 cell lines and is compared with plumbagin nanoparticle. 100µg/ml of plumbagin was taken as the standard. **Results:** Plumbagin shows only 32 percent inhibitory growth with no damage to normal cells. By converting to silver nanoparticles and performing MTT assay, this shows an inhibitory growth of 45 per cent. Conjugated nanoparticle shows 72 percentage of inhibitory effect at a maximum concentration of 100 µg/ml. when up to 50 µg/ml of concentration, this shows a rapid increase in inhibitory effect. After this, there is a decrease in concentration. At a concentration of 20 µg/ml, it reaches IC_{50} value. **Conclusion:** These results demonstrate that conjugation with a monoclonal antibody enhances the inhibitory effect.

Major Findings: The findings of this study highlight the potential of plumbagin silver nanoparticle conjugated with monoclonal antibodies as a promising approach for the treatment of HER2-positive breast cancer.

Keywords: HER2 Receptor, MTT Assay, Plumbagin, SKBR3 Cell Lines, Trastuzumab

1. Introduction

Plumbagin is a naturally occurring naphthoquinone compound obtained from the *Plumbaginacea* family plant, such as *Plumbago zylanica*. This is recognised with many medical functions such as antibacterial, anti-inflammatory, antioxidant and anticancer activity[1]. Plumbagin shows more action on cytotoxic action against breast cancer, including triple-negative and HER2-positive cancer cell lines[2]. Clinical studies found that orally administered plumbagin has only 42% bioavailability due to its rapid metabolism, low melting point. The stability of plumbagin can be increased by converting it to a more stabilised form, such as a silver nanoparticle. The quinine moiety of naphthoquinone is showing anticancer activity by inhibiting cell mitosis. In large concentrations, it shows cytotoxicity[3]. Preclinical studies were conducted on HER2-positive breast cancer.

Plumbagin can downregulate HER2 expression in cancer cell lines by inhibiting phosphorylation, which leads to decreased cell proliferation and increased apoptosis in HER2-overexpressing cells. This can also produce reactive oxygen molecules, causing oxidative stress that selectively kills cancer cells[3].

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Trastuzumab is a monoclonal antibody that is the first drug in choice for targeting HER2-positive breast cancer cell lines as targeted cancer therapy. This acts by inhibiting HER2 signalling and promotes the antibody-dependent cellular cytotoxicity[4]. Trastuzumab is nowadays more commonly used as a combined therapy with other drugs as a targeted agent, which will enhance efficiency and delay resistance and improve survival outcome. It shows improved outcomes in the early stage of metastatic HER2-positive breast cancer[5,6]. When trastuzumab is combined with plumbagin will reduce HER2 blockage and overcome trastuzumab resistance will giving more stability to the antibody and plumbagin[6].

MTT assay is commonly used to measure the %cell inhibition or %cell cytotoxicity of Drug. Comparing the treated cell absorbance with that of the untreated, we can detect % cell inhibition[7]. The action of antibody antibody-conjugated drug over a drug nanoparticle can be detected by comparing the %cell inhibition of two samples[7].

SKBR3 cell lines are chosen for performing the MTT assay, which is a human breast adenocarcinoma used for the treatment of HER2-positive breast cancer detection. SKBR3 cells have amplifying and overexpressing action on the ERBB2 (HER2) gene, which is a specific feature of HER2-positive tumours. SKBR3 cell lines tend to activate the HER2-related signalling pathway and show rapid proliferation required for study[8,9].

The present study focused on the action of plumbagin silver nanoparticle and monoclonal antibody conjugated plumbagin nanoparticle effect on SKBR3 cell lines.

2. Materials and Methods

The investigation used only analytical-grade, very pure chemicals and biological ingredients. Plumbagin, trastuzumab and silver nitrate were purchased from Sigma Aldrich. Milli-Q water from India Mart. N-Hydroxy Succinimide (NHS) from Hemant Bioscience Hyderabad. 2-NMorpholino Ethane Sulphonic acid (MES), 1-ethyl-3-(3-dimethylaminopropyl carbodiimide (EDC) from India Mart. Cell lines used for the MTT assay are SKBR3, a Murine human breast cancer cell line used which was obtained from NCCI Pune. DMEM supplemented with 10% fetal bovine serum and antibiotics was employed to keep the cells alive in an incubator set at 37°C with 5 percentage carbon dioxide and 95 percentage humidity. BSA standard, BCA Reagent (1X), prepared by mixing I volume of copper reagent to 50 volumes of BCA solution.

2.1 Methodology

2.1.1 Preparation of Plumbagin Silver Nanoparticles

The chemical reduction process is used for producing silver nanoparticles. A synthesis mixture was first prepared by adding and dissolving $AgNO_3$ 0.1 g and PVP 0.2g in 50 ml of Milli-Q water. The mixture was taken and stirred at room temperature at 1000 RPM until the mixture fully dissolved. A fresh solution of 0.05 M $NaBH_4$ was first prepared by dissolving the required amount of $NaBH_4$ in cold Milli-Q water. $NaBH_4$ solution was added dropwise to PVP/ $AgNO_3$ solution, followed by continuous stirring for about 10 minutes. Reaction was permitted to proceed for about 30 minutes to ensure complete reduction of silver ions to silver nanoparticles[10]. Prepared silver nanoparticle is collected and lyophilised for storage.

2.1.2 Plumbagin Loaded to Silver Nanoparticles.

Silver nanoparticle was synthesised by the chemical reduction method, where the synthesis solution was first prepared by dissolving silver nitrate and PVP in distilled water. Sodium borohydride solution was added dropwise to the mixture for about 10 minutes by continuous stirring for about 1000 RPM. The prepared nanoparticle is separated and centrifuged. Plumbagin solution was added into the silver nanoparticle solution[11].

2.1.3 Conjugation with Trastuzumab

2.1.3.1 Preparation of Trastuzumab Solution

Reconstitute the trastuzumab solution in PBS to achieve a concentration of 1 mg/mL. Filter solution through a 0.22 µm membrane filter to ensure sterility and remove any particulate contaminants present in the solution.

2.1.3.2 Nanoparticle-Antibody Conjugation

Add the activated silver nanoparticles to the prepared trastuzumab solution in a ratio of 1:10 (nanoparticles to antibody). For example, if 1 mg of nanoparticles is used 10 mg of trastuzumab has to be added[12]. Incubate

the mixture at room temperature for about 2-4 hours with gentle continuous stirring to allow conjugation with silver nanoparticles. Alternatively, the mixture can be incubated at 4°C overnight to enhance conjugation efficiency and preserve antibody activity[13,14].

2.1.4 Purification of Conjugated Nanoparticles

Centrifuge the conjugated nanoparticles at 10,000 rpm for about 10 minutes to ensure the pellet formation of nanoparticles. Carefully decant the supernatant liquid and resuspend the pellet in PBS. Repeat the centrifugation and resuspension steps thrice to remove any unbound trastuzumab and any residual activation agents present. Perform dialysis against PBS solution using a dialysis membrane having an appropriate molecular weight of 50 kDa to further purify the conjugated nanoparticle[15,16]. Ensure the sterility of the final suspension by filtering through a 0.22 µm membrane filter.

2.1.5 Confirmation of Conjugation with Protein

2.1.5.1 Bicinchoninic Acid Assay (BCA)

This technique is a colourimetric method to determine the total protein concentration in a solution. When a nanoparticle gets conjugated with trastuzumab, it will produce a colour change since the protein present in trastuzumab will easily detect its presence. The principle behind the BCA assay is that the reduction of Cu^{2+} to Cu^{+} by protein present in an alkaline medium will turn to a pink colour whose concentration can be determined calorimetrically[17].

Bradford reagent was added to 96-well plate, and added with standard BSA (5 µl) (Bovine Serum Albumin) of different concentrations (200-1000 µg/ml) was added for standard curve preparation. A 4mg/ml concentration of the sample was prepared using 1mg sample in 250 µl in distilled water. 200 µl of BCA Reagent was added to the sample for the protein estimation in each well and mixed. The plate was taken and kept for incubation for 15 minutes, closed at room temperature. Absorbance was taken at 595 nm using an ELISA Microplate Reader (iMark Bio Rad). Standard curve was plotted, and the amount of protein was estimated as mg/ml[18,19].

2.1.6 Comparison of Cell Line Activities using MTT Assay

In vitro cell line activities of plumbagin, plumbagin nanoparticle and monoclonal antibody labelled nanoparticle were performed and compare the development in activities.

2.1.7 Culture Cell Lines

In 96-well plates, SKBR3 cell lines were obtained, seeded in full media, and incubated for around twenty-four hours. A series of sample dilutions in DMEM supplement solution, ranging from 0-200µg/ml, was made in a different 96-well plate. The medium used was removed first, and the resulting cells were twice rinsed with phosphate buffer solution before being combined with DMSO solution for nutrient supplementation once the cells were more densely packed. This solution was then kept for incubation for 72 hours. The medium was taken and washed twice using phosphate buffer solution as a buffer, and after that, the solution was discarded. 20 µl of MTT solution was added to each well and plate and incubated for 4 hours at 37°C. To this solution add Dimethylsulfoxide (DMSO) was added. Absorbance of the resulting solution can be measured at 532nm wavelength[20,21].

2.1.8 Cytotoxicity Assessment of Test Compound

After being chosen, the SKBR3 cell lines (5 X lA4 lmL DMEM) were sown in 24-well plates and given time to proliferate. A full medium containing 0-100 pg/ml of separate test materials (either plumbagin nanoparticle or plumbagin nanoparticle coupled monoclonal antibody) was added to the growth medium at 80% confluence, and the mixture was then allowed to incubate for 48 hours. After rinsing the residual medium once with sterile PBS at the conclusion of the incubation period, MTT was added to DMEM (20 µL of 5 percentage MTT). The cells were then incubated under the same conditions for four hours. DMSO was used to dissolve the generated Formosan crystal. An ELISA plate reader set to 532 nm was used to take and read the OD. The untreated well's optical density (OD) was regarded as 100 percentage viable, and the treated well's viability was assessed in relation to the control wells' OD, as explained below. The experiment was repeated, and the values are the average of four wells[22].

$$Percentage\ inhibition = \frac{OD\ of\ control\ well - OD\ of\ treated\ well}{OD\ of\ control\ well} \times 100$$

3. Results

The first step involved the preparation of silver nanoparticles by encapsulating plumbagin into the core region. According to this, the silver nanoparticle is encapsulated with plumbagin, where PVP act as a stabilising agent. Here, a plumbagin nanoparticle was formed with a core whose surface was modified to promote conjugation. COOH group is introduced to the surface using 11-mercaptoundecanoic acid, which will form a disulfide with the carbonyl group on its end. N - Hydroxy succinamide is added to the mixture, followed by 1-Ethyl-3(3-dimethylamino propyl carbodiamide as a stabiliser. This provides an amino group, which governs conjugation with monoclonal antibody Trastuzumab via peptide bond formation.

In order to determine the stability of the obtained nanoparticle and the conjugated nanoparticle, the zeta potential was compared. The zeta potential of plumbagin nanoparticle is -42.3, whereas the antibody conjugated nanoparticle is -38.0 mentioned in Table 1, which reveals a high stability of the synthesised nanoparticle and conjugated nanoparticle.

To confirm the conjugation TEM image before and after conjugation was compared to detect an increase in molecular weight and size after conjugation. Based on SEM analysis, the surface morphology of the bound nanoparticle can be detected. TEM reports before and after conjugation were checked. This shows that a shadow is seen after conjugation with a nanoparticle whose thickness is increased. The hydrodynamic diameter was increased when comparing the conjugated nanoparticle with that of the unconjugated one.

The images using SEM are obtained using a Zeiss SEM system, which provide an idea regarding the surface morphology and dispersion of both unconjugated and conjugated plumbagin-loaded nanoparticles. In figures 1 and 2, the unconjugated nanoparticle appears to be spherical with smooth and uniform size distribution whose size range from 150-170nm mentioned in the DLC result. The particles are well dispersed, showing minimal aggregation, which suggests that the efficiency of the PVP stabilising agent will prevent the clubbing during its synthesising procedure.

3.1 FTIR Studies

FTIR studies are performed to study any interaction during the conjugation reaction. This shows the presence of a primary amide linkage at the final product, that the presence of a conjugated nanoparticle.

3.1.1 FTIR Analysis and Interpretation for HER-2 Antibody Conjugated Silver Nanoparticles

The FTIR spectra fig 3 of plumbagin show a characteristic peak in - C=O stretching around 1600-1700 cm^{-1}, which is sharp and intense. It also has hydroxyl (O-H), as well as aromatic rings. - O-H stretching around 3200-3500 cm^{-1}, which can appear broad due to hydrogen bonding, and- C=C stretching in aromatic ring region, usually observed around 1450-1600 cm^{-1}. For the plain silver nanoparticles after they are stabilised by a capping agent like polyvinylpyrrolidone (PVP), characteristic peaks

Table 1. Particle size of plumbagin silver nanoparticle and antibody conjugate nanoparticle

Compound	Hydrodynamic diameter	PDI	Zeta potential
Plumbagin silver nanoparticle	38.6±0.5	0.16±0.22	-42.3±1.8
Monoclonal antibody conjugated nanoparticle	62±1.5	0.255±0.001	-38.0±4.0

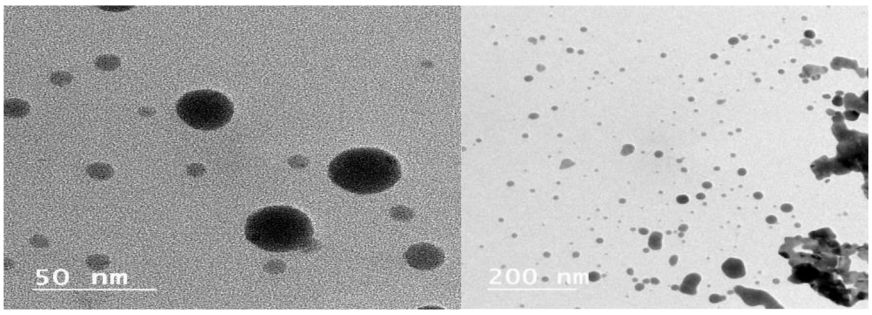


Figure 1. TEM analysis of conjugated nanoparticle.

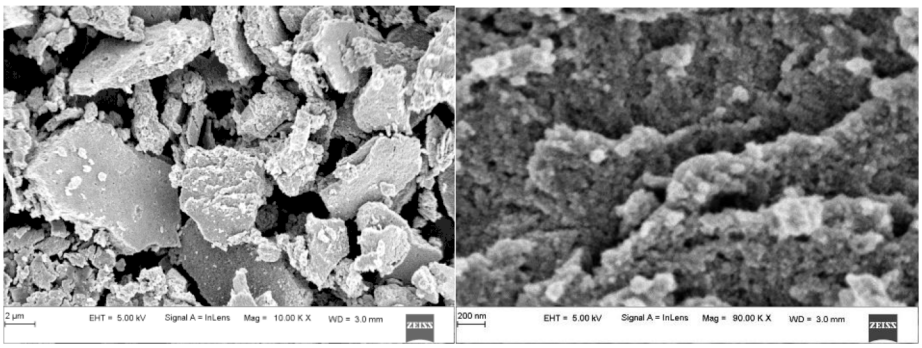


Figure 2. SEM analysis of conjugated nanoparticle.

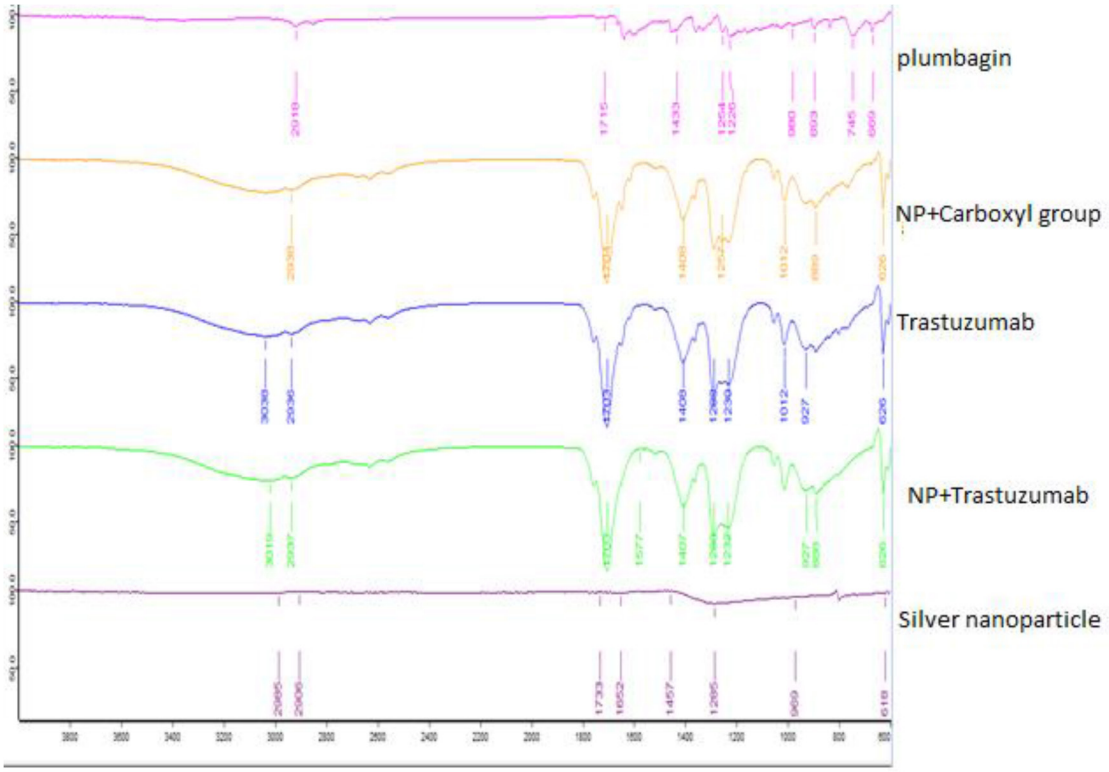


Figure 3. Characteristic peak of plumbagin, silver nanoparticle, nanoparticle combined with carboxyl group, individual peak of trastuzumab and its conjugation with nanoparticle.

of the capping agent would dominate the spectrum:- C=O stretching (PVP) around 1650 cm^{-1}. The FTIR spectra collectively demonstrate the progression from plain silver nanoparticles to surface-modified particles, and then to trastuzumab-conjugated nanoparticles. The indicator for identifying successful conjugation is the presence of amide I and II bands around 1650 cm^{-1} and 1550 cm^{-1} in the conjugated drug sample, which were only present in the spectrum of trastuzumab before conjugation. The presence of C=O stretching from the carboxyl-modified nanoparticles confirms the successful surface modification. This information provides clear spectral evidence that trastuzumab has been successfully conjugated to the silver nanoparticles[16,23].

3.2 Confirmation of Conjugation by BCA Assay

The presence of protein and its quantity of conjugation can be determined using the 3.3 BCA assay. –COOH group was introduced to the nanoparticle, which will enhance its binding to the monoclonal antibody. Before

conjugation and after conjugation, the protein present is calculated. Different concentration of 0-1000µg/ml was selected to determine the concentration of protein mentioned in fig 4. The obtained result was then compared with that of the blank result; from this, the protein concentration was found to be 4.9741mg/ml.

3.3 MTT Assay

MTT assay was performed over 48 hours in SKBR3 cell lines to determine its cytotoxicity. Seriel dilution of plumbagin was prepared, whose inhibition was determined is compared with that of the blank sample. The maximum concentration for the study was considered as 100µg/ml in Figure 5. Since the maximum concentration limit is 120µg/ml, 100µg/ml of plumbagin was taken its the standard. At this tested concentration range, it shows only 32% inhibitory growth with no damage to normal cells. By converting to silver nanoparticles and performing an MTT assay, this shows an inhibitory growth of 45%. When conjugated with a monoclonal antibody its inhibitory effect increases to 72%.

This prove that by target delivery, the drug will have more action on target breast cancer cell line when it is compared with others. Monoclonal antibody will promote the action of drug more in breast cancer cell lines.

MTT Assay of plumbagin alone shows a dose-independent type of response to MTT Assay. It shows a maximum inhibition of 32% in its higher concentration of 25 µg/ml. After this, when the dose increases, it shows low inhibitory action. So the IC_{50} value could not able to detected.

MTT Assay of plumbagin nanoparticle in Figure 6 shows that the percentage inhibition of 45% in maximum concentration of 100 µg/ml. This shows a dose dependent inhibitory effect. When concentration increases inhibitory effect also increases. Here 100 µg/ml was taken for study inhibitory effect. More than 100 µg/ml sample is required for plumbagin silvernanoparticle to show IC_{50} value.

From the results in Figure 7, the MTT Assay of plumbagin nanoparticle conjugated with monoclonal antibody shows a dose-dependent increase in inhibitory

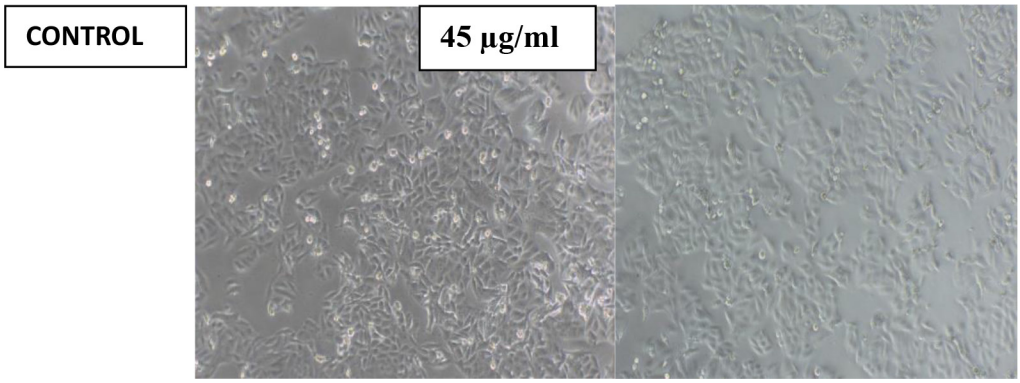


Figure 5. Control and maximum inhibition of plumbagin silver nanoparticle at 45 µg/ml.

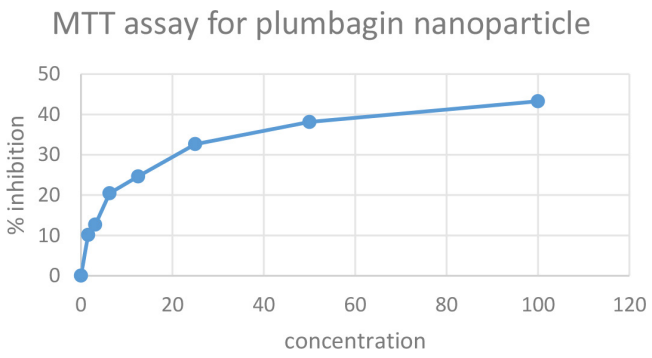


Figure 6. MTT Assay of plumbagin silver nanoparticle.

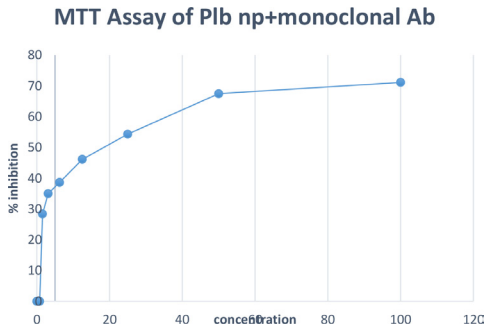


Figure 7. MTT Assay of plumbagin nanoparticle with monoclonal antibody.

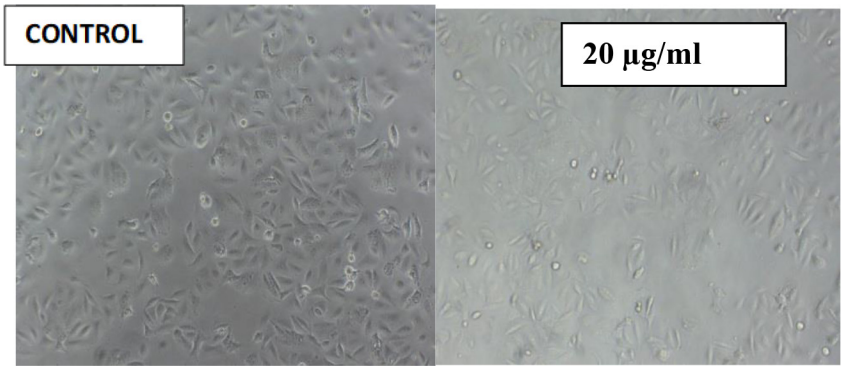


Figure 8. Control and LD50 of plumbagin nanoparticle with monoclonal antibody at 20 µg/ml.

action. when the dose increases inhibitory effect also increases.

It shows 72 percentage of inhibitory effect at the maximum concentration of 100 µg/ml. Here, up to 50 µg/ml concentration, there is a rapid increase in inhibitory effect. After this, shows a small effect in concentration. Up to 35 µg/ml, this shows a fast inhibitory effect, and also at a concentration of 20 µg/ml, it reaches the IC50 value shown in Figure 8. These results show that when conjugation with a monoclonal antibody, the effectiveness of the inhibitory effect is increasing.

4. Conclusion

The present study demonstrated that plumbagin, a naturally occurring naphthoquinone having a potential anticancer activity, exhibits limited bioavailability and cytotoxicity when administered alone. This will achieve only 32 per cent inhibition at higher concentrations. To overcome these limitations, plumbagin was encapsulated in silver nanoparticles, which enhanced drug stability and delivery efficiency. This will also improve cytotoxic activity to 45 per cent inhibition in SKBR3 HER2-positive breast cancer cell lines. Further functionalization by conjugating the plumbagin-loaded silver nanoparticles with the monoclonal antibody trastuzumab resulted in a significant improvement of activity. It reaches 72 per cent inhibition at 100 µg/ml concentration with a clear dose-dependent response. Especially, antibody conjugation also lowered the effective inhibitory concentration of IC50 observed at 20 µg/ml. This enhanced specificity and target-directed cytotoxicity to breast cancer. Particle characterisation confirmed the successful synthesis and conjugation, along with stable zeta potential, increased hydrodynamic size, and protein quantification through BCA analysis confirmed trastuzumab binding. FTIR spectra further confirmed the presence of amide linkages, supporting effective conjugation. Overall, this study concludes that antibody-mediated, nanoparticle-based delivery of plumbagin not only enhances drug stability and bioavailability but also improves tumour-specific cytotoxicity against HER2-positive breast cancer cells. Such a strategy can potentially overcome the limitations of free plumbagin and trastuzumab resistance, providing a promising targeted therapy approach for aggressive HER2-positive breast cancers.

5. Author Contribution

Author 1 conducted the experiments and performed the data analysis, while Author 2 developed the research concept and prepared the initial draft of the manuscript. Both authors have read and agreed to the published version of the manuscript.

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