

Synthesis and Characterization of Silver Nanoparticles Conjugated with Folate and Curcumin for Their Anti-Cancer Activity

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Nanoparticles are a wide range of small particles having a size of between 1-100 nanometers. Similarly, silver nanoparticles are nanoparticles made of silver having the same size as nanoparticles. They have been widely used in different branches as well as medicine, health maintenance, food, commercial use, etc. Silver nanoparticles could be harmful to normal cells in the body regulated by different concentrations and the time-span of exposure. Besides its harmful effects, it is best for wound healing and killing the bacteria that cause infection. In the past, silver was used as a natural antibiotic. In this study, silver nanoparticles are conjugated with curcumin and folic acid by the glutaraldehyde method having anti-cancer properties. Curcumin is an active compound to kill cancer cells and folic acid is an organic form of vitamin B9 that will create and preserve the new cells from cancer. Silver nanoparticles are interacted by PEG (Polyethylene Glycol) and are further conjugated by Folic acid ($C_{19}H_{19}N_7O_6$) and Curcumin. Curcumin is attached to the NH_2^- ion connecting with PEG and folic acid is attached to the carbonyl group connecting with PEG. Average crystalline size is calculated by XRD and FTIR analysis was done to identify functional groups and bonds made by nanoparticles. Silver Nanoparticles are more beneficial as compared to chemotherapy and many other radio-therapy cells to kill tumor cells.

Keywords: Silver nanoparticles (Ag NPs), Polyethylene Glycol (PEG), Chemotherapy, Cancer, Targeted Drug Delivery.



Introduction:

There is a great future challenge in addressing the rising death rate associated with cancer, the most prevalent disease [1][2]. One of the most common ways to treat cancer is using chemotherapy, and among the many chemotherapy medicines used for solid tumor management, cisplatin (CP) ranks high [3][4]. Among the many challenges faced by oncologists when treating cancer with chemotherapy is the development of cell resistance to cisplatin (CP). Multidrug resistance, ineffectiveness, and cytotoxicity are the main problems with traditional cancer chemotherapy that lead to treatment failure. There may be restrictions on the efficacy and safety of current therapeutic approaches. Therefore, these problems and the need for safer, more precise alternatives are the focus of continuing research efforts [5][6]. Specifically, numerous nanoparticles engineered for tumor targeting in cancer therapy have recently emerged in response to the increasing interest in using nanotechnology for cancer treatment. Consequently, nanoparticles (NPs) offer a tailored method for cancer treatment detection, targeting, and management; this strategy has the potential to reduce multidrug resistance and lessen the likelihood of unwanted side effects [7]. Extensive research has been conducted on nanoparticle (NP)-based techniques to improve the anti-tumor efficacy of chemotherapeutic drugs by targeting cancer cells specifically, while simultaneously reducing the numerous adverse effects that are associated with these agents [8]. This is because malignant cells have a vascular structure that is distinct, disorganized, and loaded with many pores. Additionally, lymphatic outflow is impeded, leading to enhanced permeability and retention effects. To improve chemotherapy drug accumulation within tumors while decreasing absorption by healthy cells, a nanotechnology-based drug delivery approach can take advantage of these features [9]. Nanoparticle therapy using metals, such as gold and silver, has tremendous therapeutic and diagnostic potential compared to more conventional methods of treatment [10]. Nanoparticles absorb light and transform it into heat, which kills cancer cells [11].

Diagnostics, treatment, and targeted tumor therapy have all seen significant increases in the use of nanotechnology in the medical field in the last several decades, thanks to the technology's ability to improve both safety and effectiveness. Significant advantages in cancer treatment have been shown by drug delivery systems based on nano-carriers, including improved pharmacokinetics, precise targeting of tumor cells, reduced side effects, and fewer cases of drug resistance [12]. Nanoparticles (NPs) are chosen for these systems based on their size and characteristics that are specific to tumor pathogenesis. Nanoparticles (NPs) and the targeted delivery of drugs are key components in cancer treatment, with nano-carriers being custom-made to bind to tumor cells. Then, they kill tumor cells by releasing therapeutic medicines into the tumor tissue. These nano-carriers can work as both gene and cytotoxic therapy due to the medications they contain, which can comprise nucleic acids and traditional chemotherapeutic agents [13]. Finally, NPs provide a viable option for the encapsulation and systemic delivery of certain drugs that have poor solubility [14]. NPs enhance drug permeability and retention due to their size and surface features, which prolongs pharmaceutical half-life and makes it easier for medicines to accumulate within tumors [15]. In addition, these systems' targeting mechanisms are critical for preventing cytotoxic effects on healthy cells, which in turn lessens the adverse effects associated with cancer treatments.

Silver nanoparticles (AgNPs) have shown great promise in cancer therapy studies [16][17][18], in addition to their antibacterial applications. Many of the cancer drugs used today are extremely harmful, leading to unpleasant side effects and even drug resistance [23]. Research indicates that AgNPs have the potential to target cancer cells specifically by causing ultrastructural disruption, which in turn generates reactive oxygen species (ROS), causes DNA damage, and finally triggers cell death and necrosis [19]. The main curcuminoid extracted from *Curcuma longa* rhizomes is curcumin. Antioxidant, hypotensive, anti-inflammatory, anticoagulant, antifertility, antiulcer, antimicrobial, antivenomous, antifibrotic, antimutagenic,

antidiabetic, anticarcinogenic, and most significantly, anticancer activities are among the numerous biological and pharmacological characteristics possessed by this bioactive component [20]. The crystalline structure, limited water solubility, and bioavailability (~1%) of curcumin have prevented its full utilization in biomedical applications, despite the well-established health benefits of curcumin [21]. Current studies are focused on increasing the bioavailability of curcumin [22], which can be achieved by reducing its size, which enhances its solubility and, by extension, its bioavailability. Because of this, hybrid NPs encased in curcumin were discovered, which can include either organic or inorganic NPs [23].

The anti-cancer and improved physical properties of silver nanoparticles encased in curcumin and folate were the subjects of this investigation. The study provides further details on its anti-cancer properties and offers targeted drug delivery to tumor cells by taking advantage of the overexpression of folate receptors. The resultant AgNPs were studied for their antibacterial and anti-cancer bioactivities, and this method demonstrates the promise of using sophisticated targeted delivery systems to enhance the effectiveness of cancer treatments while reducing adverse effects.

Objectives:

The objective of the study is to synthesize silver nanoparticles conjugated with folate and curcumin by green synthesis and assess their anticancer activity,

Experiments (Methodology):

Formation of Silver NPs:

In this study, lemon juice was extracted and filtered. A 0.1 M silver nitrate solution was prepared by dissolving 1.6987 g of silver nitrate in 100 ml of distilled water. Then, lemon juice and silver nitrate solution were mixed in a 1:4 ratio in a falcon tube and stirred for 2 hours at 150 RPM. [24]

Synthesis of Silver-curcumin NPs in Polyethylene Glycol (PEG):

10 of 0.1 M (0.16g silver nitrate dissolved in 10 water) silver nitrate solution was added to 1 g of curcumin and 20 ml of 0.1M PEG solution with 0.1 g of PVP (Polyvinyl pyrrolidone) at a pH of 4, followed by the addition of 10 ml of lemon juice. The mixture was stirred for 10 minutes at room temperature at 150 RPM. [25]

Folic acid conjugation to the nanocomposite by glutaraldehyde method:

We took 5ml of Ag-Cur NPs in a falcon tube and added 25ml of coupling buffer which is 0.01 M of Pyridine made by adding 79 μ l of Pyridine into 100ml of distilled water. Then, the mixture was shaken by hand for 10 minutes. Afterward, 1.25 ml of glutaraldehyde (5% of the solution) was added, and the mixture was incubated for 3 hours in a shaking incubator.

In the end, we added 5mg of Folic Acid to the solution collected 3-4 ml as pre-coupling, and kept the mixture in a shaking incubator for 24 hours.

The solution was transferred into Eppendorf tubes and centrifuged at 7000 rpm for 10 minutes. The supernatant was then collected and discarded. Next, 1 ml of 1 M glycine was added, and the mixture was kept at room temperature for 30 minutes and then centrifuged again under the same conditions. We washed the pallet with a tris-buffer solution that is made by adding 0.01M Tris-base salt and 0.15M of NaCl (0.12-gram trisma base and 0.87-gram NaCl in 100ml water) and repeated the process of centrifugation one more time. At the last, the pallet was stored at 4°C.

Synthesis of drug conjugated Silver-curcumin NPs by Glutaraldehyde Method:

Five milligrams of folic acid-conjugated Ag-Cur nanoparticles were placed in a falcon tube, followed by the addition of 25 ml of 0.01 M pyridine coupling buffer. Then it was stirred vigorously for 10 minutes. Next, 1.25 ml of glutaraldehyde (5% of the solution) was added, and the mixture was incubated for 3 hours in a shaking incubator.

Finally, 5 mg of curcumin was added to the solution, and 3–4 ml of the mixture was collected as pre-coupling before being incubated in a shaking incubator for 24 hours. Transfer the solution into Eppendorf and the sit was centrifuged at 7000 rpm for 10 min. Afterward, the supernatant was collected and discarded. Then added 1ml of 1M glycine, kept it at room temperature for 30 minutes, and re-centrifuge the solution under the same conditions. We washed the pallet with tris-buffer solution prepared by mixing 0.01M Tris-base salt and 0.15M of NaCl followed by a repeat of the centrifugation process. At the last, the pallet was stored at 4°C.

MTT Assay:

1000 tumor tissue cells were collected in 24 well plates and incubated in one well as a control and some other wells with the composite NPs for the next 24 hours. Then, included 10µl of Methylthiazol Tetrazolium (MTT) reagent (12mM) in both (controlled and test) wells and incubated for 2-4 hours. We added 100 µl of the detergent reagent that is 10 ml of 0.01M HCl and 1g of sodium dodecyl sulfate (SDS) in both wells and again incubated for 2 hours, in dark, at room temperature. We checked the absorbance of both control and sample cells at 570nm.

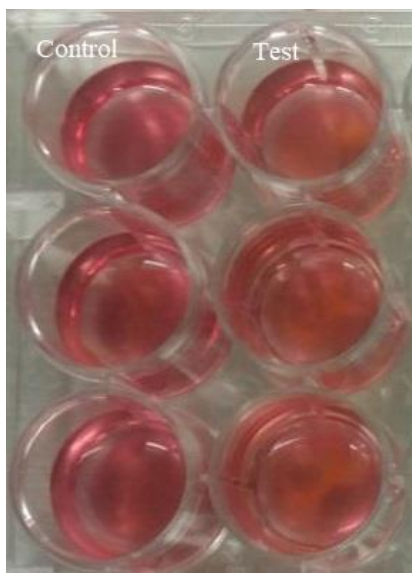


Figure 1. MTT Assay Analysis

The absorbance of control at 570nm = A570
= 1.61

The absorbance of the test at 570nm = A570
= 1.34

% age Cell Viability = Absorbance of test/Absorbance of Control*100
= 1.34/1.61*100

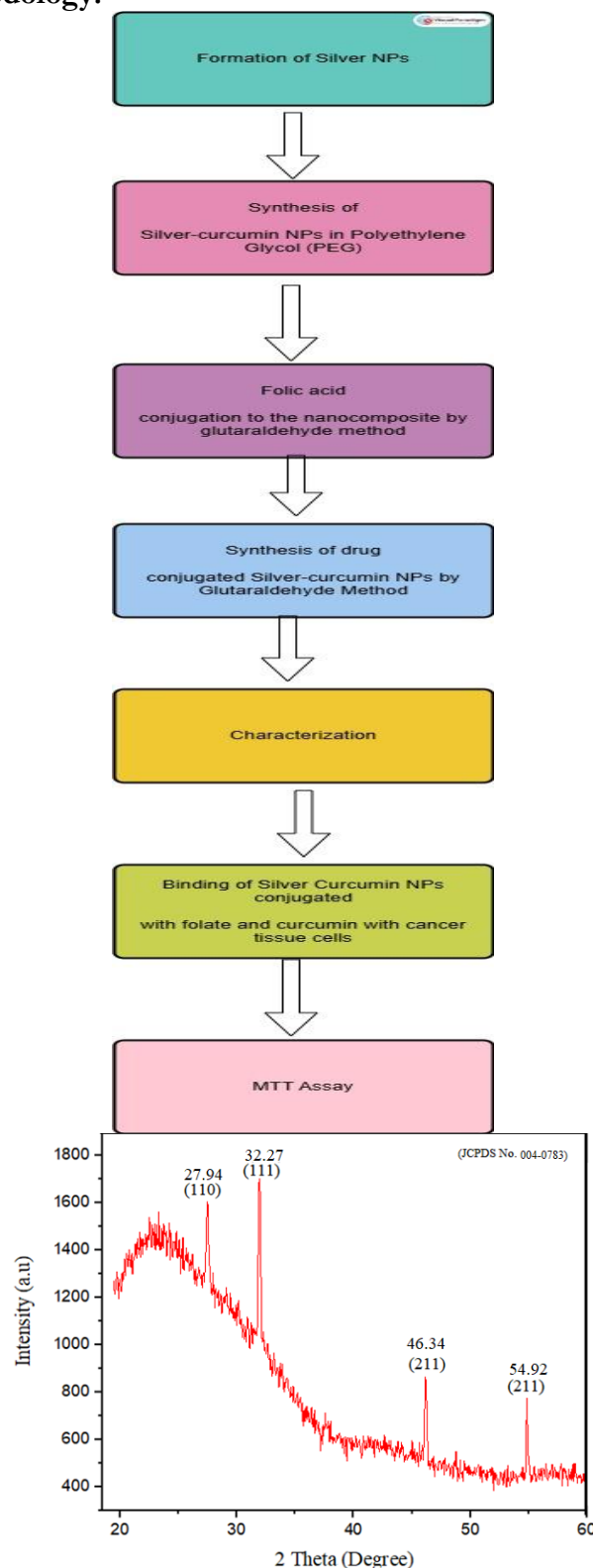
% age Cell Viability = 83%

Results and Discussions:

Characterization of Ag NPs:

The synthesized silver NPs were in a diluted solution; they were filtered and subsequently incubated at 37°C to dry and form granular-sized particles. We processed them under X-ray diffraction (XRD) with a 2θ range of 20°-60°. In the results, peaks were shown on (110), (111), (211), and (211) diffraction planes with the position 2θ 27.94°, 32.27°, 46.34° and 54.92° respectively with JCPDS Card No. 004-0783 that shows it has Face-Centered Cubic Ag Nanoparticles. The average Crystalline size of Ag NPs is 20 nm calculated by the Scherrer formula.

Flow chart of methodology:

**Figure 2.** XRD spectrum of Ag NPs

FTIR analysis of Ag NPs is shown in Fig. 2 illustrating that two peaks in FTIR analysis at 2357.5 cm^{-1} and $1620\text{--}1680\text{ cm}^{-1}$ which corresponds to Nitrogen bonds originated from AgNO_3 and the peak intensity in the presence of alkenes ($\text{C} = \text{C}$) respectively. The peak

observed at 3333.2 cm^{-1} indicated the presence of oxygen bond vibrations characterized by strong peak intensity and broad presence of alcohol.

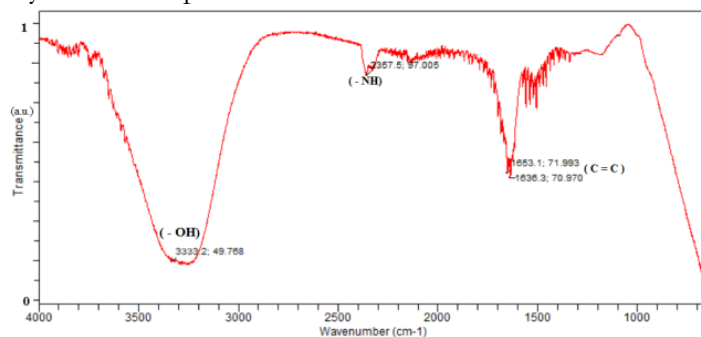


Figure 3. FTIR Analysis of Ag NPs

Figure 3 illustrates two additional peaks in the FTIR analysis of folate- and curcumin-conjugated Ag nanoparticles at 2359.4 cm^{-1} the carboxyl group of curcumin is giving peak value and 1636.3 cm^{-1} , corresponding to bonds associated with NH groups of the folate as well as alkenes (C=C). The presence of oxygen bond vibrations showed a strong peak intensity and the broad presence of alcohol can be seen at the indicated peak of 3311.7 cm^{-1} .

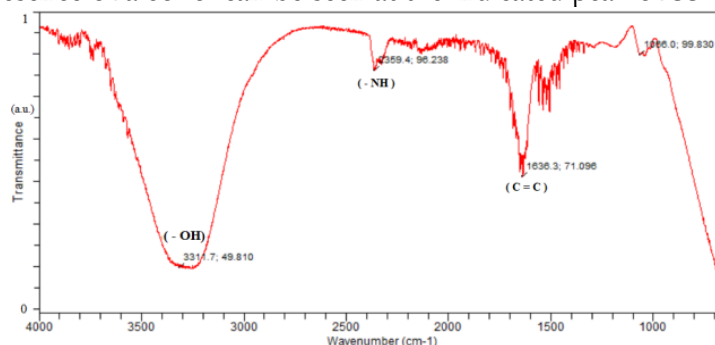


Figure 4. FTIR Analysis of Conjugated Ag NPs with Folate and Curcumin

Spectrophotometric analysis of Silver NPs was performed at various wavelengths to carry out the absorption spectrum and identify the wavelength at which the NPs discharged the fluorescence. Silver NPs showed high absorbance and fluorescence at the wavelength of 420 nm . The observed peak at 420 nm exhibits a redshift of 20 nm compared to the original Surface Plasmon Resonance (SPR) peak at 400 nm . The spectrophotometric analysis graph of Ag NPs can be seen in Figure. 4.

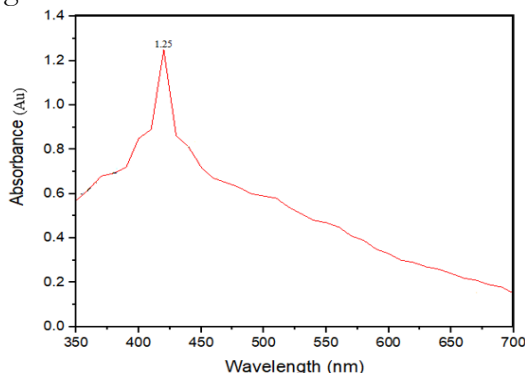


Figure 5. Spectrophotometric analysis of Silver NPs

Silver-Curcumin conjugated Ag NPs showed high absorbance and fluorescence at the wavelength of 430 nm , which can be shown in Figure. 5. The peak detected at 430 nm is the sign of diarylheptanoid chromophore group of curcumin and that is blue-shifted by 10 nm from Ag-Cur absorption peak at 430 nm from its diarylheptanoid chromophore group. The presence of this peak plays a significant role in anticancer applications.

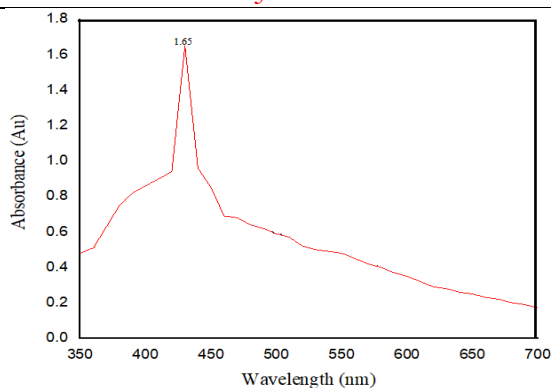


Figure 6. Spectrophotometric analysis of Conjugated Ag NPs with folic acid and curcumin
Binding of Silver Curcumin NPs conjugated with folate and curcumin with cancer tissue cells:

Firstly, we sliced the epithelial tumor cell of a mouse and dropped the proposed particles by dropper on the cells. Then let the particles rest for 10-15 minutes. After that, washed the sliced tumor cell and checked it under the microscope. Figure. 6 represents the binding of conjugated Ag NPs with the mouse epithelial tumor cells by giving a low dosage of the produced NPs.

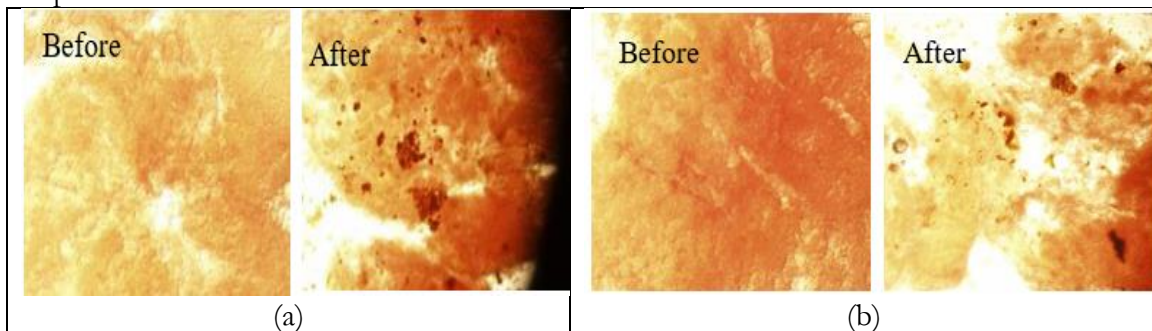


Figure 7. (a) & (b): Overview of Cancer tissue cells bound with conjugated Ag NPs

When cancer tumor cells are disclosed, nanoparticles deliver the drug to the location of cancer cells in the body. A drug could be encapsulated in nanoparticles or may be connected to the top of the capsule. Tumors exhibited highly dense deviant blood vessels such as distended and badly transformed. After the particles have reached the tumor cells, they steadily start to deliver the drug and demolish the cancer cells in a day or hours.

Discussion:

This research shows that Ag NPs can be greenly synthesized with the help of lemon juice (as a reducing and capping agent) and then conjugated with folic acid and curcumin using the glutaraldehyde cross-linking process. All things considered, the MTT assay, XRD, FTIR, and UV-Vis spectra prove that the produced nanocomposite has the necessary structural, chemical, and biological properties for specific anticancer uses.

The XRD pattern of Ag NPs showed distinct peaks that are indicative of their face-centered cubic (FCC) structure. According to the calculations, the crystallite size is 20 nm, which means that nanoscale particles have formed. These particles are ideal for biological interactions, especially when it comes to medication delivery and cellular uptake. It is confirmed that the Ag NPs are pure and crystalline since there are strong peaks at 2θ values that match the JCPDS Card No. 004-0783. Similar diffraction patterns and average crystallite sizes were previously observed in Ag NPs synthesized in a greener way; thus these results are in line with that.

To further understand the functionalization process, FTIR analysis was conducted. Conjugation with curcumin and folic acid added to the peaks that correspond to alkene,

nitrogen, and hydroxyl groups in the unaltered Ag NPs, confirming that the nanoparticles' surfaces were successfully changed. The presence of carbonyl and NH functional groups suggests that PEG, curcumin, and folic acid bind effectively. To improve biocompatibility and tailored medication distribution, this functionalization is essential. If the glutaraldehyde method of conjugation is successful, then the change in vibrational frequencies indicates the production of new bonds.

The structural findings are further supported by the UV-Vis spectrophotometric investigation. Conjugation with curcumin and folic acid causes a redshift to 430 nm in the surface plasmon resonance (SPR) spectrum, which is indicative of the creation of larger or more complex nanostructures, as compared to the bare Ag NPs' peak at 420 nm. Further evidence that the curcumin chromophore has integrated into the surface of the nanoparticles is the blue shift that has been seen. This kind of optical change has been described before and lends credence to the idea that the curcumin and folate interact with the Ag NP core in a meaningful way.

Using the MTT assay, we checked if the produced nanocomposite has any biological activity. Treatment with the synthetic NPs resulted in an 83% survival rate of cancer cells. Despite the relatively high survivability, it suggests that the Ag-Cur-Fol NPs elicit considerable cytotoxicity, which could make them suitable anticancer agents. Using bio-conjugated Ag NPs as a safer substitute for traditional treatments is emphasized by the moderate cell death seen at low dosages, which lends credence to the idea of dose-dependent behavior.

Microscopic examination revealed that the folate-conjugation enabled receptor-mediated endocytosis since conjugated Ag NPs interacted and bound with epithelial tumor cells. In line with previous findings, folic acid is a great targeting ligand for nanoparticle delivery because folate receptors are overexpressed in numerous cancer cell types.

All things considered, the synthesized Ag NPs showed impressive multifunctionality, with successful synthesis, conjugation, optical response, and early anticancer activity. As an alternative to traditional chemical processes, a green synthesis technique utilizing lemon juice is both economical and environmentally beneficial. The therapeutic potential and specificity of the nanoparticles are further enhanced by conjugation with curcumin and folic acid.

Conclusion:

Nanotechnology is encouraging extraordinary advancements in different fields, e.g., IT and electronics, transportation, and medicine. "Nano" word in nanotechnology means the material has a size between 1-100 nm. We can imagine that 1 nm is equal to the billionth of one meter and the thickness of a human hair is approximately 80,000-100,000 nm. Nanoparticles can be passed through the human body to treat various diseases like cancer or tumor cells. Silver Nanoparticles allowed a favorable platform for the detection and medication of cancer. Silver Nanoparticles conjugated with folate and curcumin by the glutaraldehyde method could be used for the cure of different cancer types as well as epithelial tumors. It is a cost-friendly and environment-friendly method as compared to chemotherapy or radiotherapy and it has fewer complications than other cancer treatment methods.

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