CHAPTER 5

ULTRAFAST SYNTHESIS OF STABILIZED GOLD NANOPARTICLES USING AQUEOUS FRUIT EXTRACT OF LIMONIA ACIDISSIMA L AND CONJUGATED EPIRUBICIN: TARGETED DRUG DELIVERY FOR TREATMENT OF BREAST CANCER

5.1 INTRODUCTION

While chemotherapy drugs are effective, they, unfortunately, have a lot of side effects, namely: (i) the conventional cancer drugs are lethal and cause death of healthy cells as well as cancerous cells; (ii) the drugs have a short lifetime in the body and up to 90% of intravenously delivered drugs may be gathered up by macrophages within the first 5 min of their entering the body; and (iii) the drugs have low solubility, and hence, it is necessary to deliver a larger dose of the conventional anticancer drugs in order to meet a good therapeutic index (Asadishad et al. 2010).

To overcome this problem, we have established a method to synthesize gold nanoparticles (AuNPs) functionalized with folic acid (FA) for targeted delivery of epirubicin (EPI), an anthracycline chemotherapy agent (Tian et al. 2010). EPI has been clinically used to treat various types of cancer such as breast cancer, lymphomas, sarcomas in soft-tissue, pancreatic cancer, gastric cancer, small-cell lung cancer, and acute leukemia. EPI shows less hematologic or myocardial toxicity at comparable doses
(Kumar et al. 2014). AuNPs are identified as favorable candidates for drug delivery applications due to their unique dimensions, tunable surface functionalities, nontoxicity, and controlled drug release capability (Joshi et al. 2012). Green chemistry-based eco-friendly methods are predominantly used for the synthesis of AuNPs instead of chemical synthesis (Devi et al. 2015). Plant extracts are used as reducing and stabilizing agents to synthesise the nanoparticles. Plant extracts contain different concentrations and combinations of organic reducing agents, which influence the characteristics of the nanoparticles produced (Mittal et al. 2013).

Flavonoids, the plant metabolites, contain various functional groups capable of triggering nanoparticle formation. It has been suggested that the tautomeric transformation of flavonoids from the enol-form to the keto-form releases a reactive hydrogen atom that can reduce the metal ions into nanoparticles (Makarov et al. 2014). Fruits of L. acidissima L, contain higher quantities of flavonoids, tyramines, tannins, phytosterols, saponins, glycosides, carbohydrates, vitamins, coumarins, triterpenoids, and amino acids as their chemical constituents (Priyadarsini et al. 2013). The targeted drug delivery system uses the anticancer drug for treatment of cancer cells alone, which reduces the effects of the drug on noncancerous cells and simultaneously increases its efficacy on cancer cells (Betancourt et al. 2007). The main aim of using the targeted drug delivery system is to deliver the anticancer drug to the cancerous cells without loss of the drug’s efficacy (Jain, 2005, Li et al. 2009).

The folate receptor (FR) is a meticulously studied ligand for the selective delivery of anticancer drugs on FR-positive tumor cells (Lu et al. 2012). In general, the FRs are highly up-regulated on the surface of different types of malignant cells (Zhang et al. 2010). In this study, the zebra fish embryo has been used as an in vivo prominent vertebrate model.
for assessing the toxicity of AuNPs. The zebra fish offers several advantages as a model for *in vivo* high-throughput drug screening. It is low cost, transparent, and the human genome and the zebra fish genome are highly comparable in terms of tissue types, fertilization, and the development of different systems and their active functions (Poggi et al. 2003). This article deals with the rapid green chemistry-based synthesis of AuNPs that can be used as a carrier for EPI, a drug used to treat breast cancer. Zebra fish embryos were used to examine the developmental toxicity of both normal and drug coupled AuNPs. The efficacy of both EPI and EPI-FA-AuNPs were tested on the MCF-7 cell line (breast cancer).

### 5.2 MATERIAL AND METHODS

#### 5.2.1 Material

*L. acidissima* L. fruit was used for this study. EPI HCl was obtained from SRL Limited, Mumbai (India). Hydrochloroauric acid (HAuCl₄), FA, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemicals (USA). MCF-7 cell lines were obtained from National Center for Cell Science (NCCS), Pune, (India). The antibody to actin, Bcl-2, caspases- 3 and caspases-8, Fas, FasL, and FADD bax were purchased from Santa Cruz Biotechnology, CA, USA and Neo Markers, USA. Millipore milli Q water was used for all the experiments. All other chemicals and reagents were of analytical grade.

#### 5.2.2 Methods

#### 5.2.2.1 Preparation of the extract

*Limonia acidissima* L. Fruit pulp was finely chopped into small pieces and boiled in (60g Fruit pulp /100 ml H₂O) of sterile double distilled...
water for 10 min. The extract was centrifuged at 10,000 × g for 10 min to remove any undesired impurities. This extract was filtered to get the pure extract was stored in the refrigerator at 4°C for further studies.

5.2.2.2 Preparation of gold nanoparticles

In a typical experiment, an aqueous solution of HAuCl₄ (1x10⁻⁴, 100µL) was reduced to gold nanoparticles (AuNPs) by plant extract was mixed using 75µL. It was observed that the yellow colored gold solution turned to wine red color within a 30 seconds. The yield of gold nanoparticles were purified by centrifugation at 15000 × g for 20 min followed by repeated water wash to remove any impurities from AuNPs.

5.2.3 Activation and Attachment of FA to AuNPs

Activation of FA has been reported earlier by Pandey and coworkers (Pandey et al. 2013). It was carried out by dissolving 0.25 g of FA in 20 ml of dimethyl sulfoxide (DMSO), and the mixture was then subjected to sonication for 45 min. The carboxylate group of FA was activated by the addition of 0.225 gm of n-hydroxy succinamide (NHS) and 0.125 gm of dicarboxy aminocarbodiimide (EDC). The reaction was allowed to take place in an inert environment created by argon gas at 28 °C for 12 h (FA/NHS/EDC molar ratio 2: 2: 1). The resultant mixture was filtered through Whatman filter paper and was used for further characterization. Attachment of FA was carried out by adding 9 ml of AuNPs to 1 ml of activated FA. The solution was purged continuously for 4 h under N₂ atmosphere and stirred constantly using a magnetic stirrer. After 4 h, both inlet and outlet valves were sealed, and the solution was allowed to rest for 24 h. After 24 h the compound was filtered using Whatman filter paper and stored at 20 °C. Free FA was removed using a 3000 KDa dialysis bag against
phosphate buffered saline (pH 7.2). The post-dialyzed samples were centrifuged at 6000 RPM for 15 min at 20 °C. The pellet was redialyzed with deionized water for 24 h under constant stirring. The samples were subjected to UV-Vis spectroscopy at systematic intervals for examination.

5.2.4 Synthesis of AuNPs-FA-EPI Complex

EPI was conjugated to the AuNPs-FA complex for the destruction of cancer cells. Equimolar concentrations of EPI (0.25mM) and AuNPs-FA (0.25mM) were subjected to reduction with trimethylamine, 0.5mM (TEA) using DMSO as a solvent. The molar ratio of AuNPs–FA/EPI/TEA was 1: 1: 2. The mixture was purged using argon gas with continuous stirring at 50 °C for 4 h. The resultant EPI-FA-AuNPs conjugate was refined using dialysis against nano-pure water for three days with a dialysis tube (MW cut-off of 3000 Da) to remove the excess amount of unbound EPI molecules and DMSO. The water was changed at 6-hour intervals. The entire compound was characterized using UV-Vis spectrophotometry and FTIR.

5.2.5 Characterization of AuNPs and EPI-FA-AuNPs

Preliminary characterization of AuNPs and EPI-FA-AuNPs was carried out by FTIR, XRD, HR-TEM, particle size and zeta potential analysis method. LC–MS analysis of the extract was also been carried out. The FTIR spectra of pure epirubicin and EPI-FA-AuNPs were recorded on IR Pestige-21 Shimadzu spectrometer at a resolution of 4 cm\(^{-1}\) in the range 400-4000 cm\(^{-1}\) on KBr pellets. The powder XRD studies were carried out on Bruker D8 ADVANCE X, ray powder diffractometer (Bruker AXS Inc.) using CuK\(\alpha\) (\(\lambda=1.54\) Å) source in the region of 20 from 30° to 75°. X-ray diffractometer, operating at a voltage of 40 kV and a
current of 20 mA. The High resolution-transmission electron microscopic (HRTEM) pictures were recorded in a JEOL-JEM-2100 HRTEM operated at 200 kV. For the HRTEM analysis of AuNPs was spread onto a carbon coated copper grid (300 meshes) and dried under the IR lamp.

Micrographs were taken both in the transmission mode and in the diffraction mode. The hydrodynamic particle size and the nanoparticle charge quantified as zeta potential, was determined on a Zetasizer 300 Nano ZS (Malvern, UK). Analysis (n=3) was carried out at room temperature by keeping angle of detection at 90°. DLS and nanoparticle charge measurements, were determined using the same instrument at 25 °C. The polydispersity index (PDI) was also quantified to determine the particle size distribution range. The phytocompounds existing in the optimized extract were identified by LC-ESI-MS/MS study using Time of Flight mass spectrometer (micrOTOF-QIII, Bruker Daltonics, German). The separation was carried out on ODS C18 column (2.1 mm x 150 mm, 1.9µ particle size). The composition of mobile phase were: (A) Methanol (B) Water. An isocratic elution was performed at the flow rate of 0.2 mL/min with the following run conditions; (i) 65% of solvent A, from 0 to 30 min, (ii) 55%, from 31 to 40 min (iv) 35%, at 41-60 min of total run time. 20 µL of the sample was injected, and identification of the substances was done under the conditions of negative and positive ion mode, mass range of 50 - 2000 m/z, spray electric potential 4 kV, gas temperature 325 °C, gas flow 8 L/min, Nebulizer 40 psi.

5.2.6 In Vitro Stability Studies

1 ml of AuNPs was incubated with 0.5 ml each of 0.9 % NaCl saline and phosphate buffer saline (PBS, pH 1.2, 4.5, 6.8 and 7.4),
respectively, at 37°C for 48 h and were analyzed spectrophotometrically by measuring at 480 nm.

5.2.7 In Vivo Toxicity Study of AuNPs in Zebra Fish Embryos

Fertilized eggs of zebra fish were obtained from the natural mating of adult zebra fish, and embryos were collected within 2 h of spawning. Six healthy embryos from fertilized eggs, approximately 2 h post fertilization (hpf), were transferred to each well of a 24-well plate containing 1 ml of the E3 medium. The embryos were exposed to different concentrations of AuNPs for 4 days. Duplicates were maintained without the AuNPs as control. Toxicity was assessed by studying the hatching rate, percentage survival rate, and morphology changes in the embryos.

5.2.8 Loading Efficiency

From the total concentration of EPI used for loading onto AuNPs, the unloaded drug was removed by separating the supernatant after centrifugation (Eppendorf centrifuge 5430R) at 15,000 × g for 15 min. Free EPI present in the supernatant was determined by UV-Vis spectrophotometry by measuring at 480 nm.

The percentage of drug loading efficiency was calculated using the following Formula 5.1.

\[
\text{Drug loading efficiency} = \frac{\text{Theoretical amount of drug loaded} - \text{Free drug}}{\text{Theoretical amount of drug loaded}} \times 100. \quad (5.1)
\]

5.2.9 In Vitro Drug Release Characteristics

EPI-conjugated AuNPs (equivalent to 1000 µg of EPI) were dialyzed against 100 ml of sodium phosphate buffer (pH 5.7 and 7.4), at
37 °C with continuous stirring at 100 rpm. One milliliter of the sample was withdrawn at specific time intervals and analyzed spectrophotometrically. The sink condition was maintained by replacing equal volumes of the buffer. The release studies were performed in triplicate, and the average was taken. The percentage of drug release was calculated by the using following Formula (5.2):

\[
\text{Release (\%)} = \frac{\text{Release EPI}}{\text{Total EPI}} \times 100
\]  

(5.2)

5.2.10 In Vitro Cytotoxicity Studies

Cell viability was measured by MTT assay described by Mossman et al. (1983) Cell viability was measured by MTT assay described by Mossman et al. (1983). Approximately \(5 \times 10^3\) cells/well) into a 96 well plates and incubated for 24 h for attachment. After incubation, supernatant media was replaced with maintenance medium an equal amount of fresh media containing different concentrations of EPI- FA-AuNPs. After incubation for 48 hours, MTT solution was added to the plate at a final concentration of 0.5 mg/ml and incubated for 4 hours in dark at 37°C. The resulting formazan crystals were dissolved in DMSO. Cell Viability was calculated by measuring optical density at 570 nm in ELISA reader (Bio-Rad Instruments Inc., USA).

5.2.11 Apoptosis Study

The influence of EPI-FA-AuNPs inducing apoptosis in the breast cancer cells was confirmed using staining methodology (Karthik et al. 2014). The influence of EPI- FA-AuNPs in inducing apoptosis in the breast cancer cells was confirmed using acridine orange (AO) and ethidium bromide (EB) [1 mg/ml for each AO and EB in PBS] staining
methodology (Tian et al. 2010). In brief, $5 \times 10^5$ cells/well were cultured on a cover slip in a 6-cell well plate and incubated. After that, the cells were treated with fresh medium containing pure-EPI and EPI-FA-AuNPs (100 $\mu$g/ml). After 36 h incubation, the cover slip was removed and stained with AO/EB (10 $\mu$l) for thirty minutes and washed with PBS for removing the excess staining dye. Images were captured by Nikon Eclipse inverted fluorescence microscope.

5.2.12 Flow Cytometric Analysis and Western Blotting

To investigate the effect of the drug on the cell cycle distribution analysis described by Krishnan 18, Western blotting was carried out as described in another paper (Tu et al. 2004). To investigate the effect of the drug on the cell cycle distribution, the cells ($1\times10^5$ cells/ml) were treated with IC50 concentrations of EPI-FA-AuNPs and cultured for 24 h. The treated cells were harvested, washed with PBS and fixed using 80 % ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40$\mu$g/ml propidium iodide (PI) and 0.1mg/ml RNase A, followed by shaking at 37°C for 30min (Kumar et al. 2014). Cells were analyzed by Becton-Dickinson FAC scan flow cytometer and the percentage of cells in different stages were calculated using Win MDI 2.9 software (TSRI, La Jolla, CA, USA). Cells were washed with cold PBS and Western blotting was carried out as described in another paper (Joshi et al. 2012).

Briefly, the cells ($1.5\times10^6$) seeded onto 100-mm culture dishes in the presence or absence of different concentration of compounds (IC50 concentrations) were treated for 24h. The medium was removed and the
cells were washed with PBS (0.01M, pH 7.2) several times and lysed on ice in lysis buffer containing 100 µg/ml phenyl methyl sulfonyl fluoride (PMSF), 50 mM Tris-base at pH 8.0, 150 mM NaCl, 0.02% NaNO3, 1% NP-40, 10µM aprotinin, 10µM pepstatin A and 10µM leupeptin. The supernatants were collected by centrifugation at 10,000×g for 5 min at 4°C, and were used as the cell protein extracts.

The protein concentration was measured using a protein assay kit (Bio-Rad). Equal amounts of proteins (50-100µg) were separated in 7.5%-12.5% SDS-PAGE gel and electro transferred onto PVDF membrane. Proteins were blocked 24 hrs with 5% non-fat dried milk in PBS-T at 2-8°C. After washing in PBS containing 0.1% Tween 20 three times, the membrane was incubated in 5% (w/v) skimmed milk in PBST. After overnight incubation at 4°C, the membrane was then washed three times with Tris-Buffered Saline (TBST), incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody at room temperature for 2 h, and then washed three times with TBST. After reaction with horseradish peroxidase-conjugated goat anti-mouse antibody, the immune complexes were visualized by using the chemiluminescence ECL PLUS detection reagents following the manufacturer’s procedure (Amersham Bioscience). The same nitrocellulose incubated with β-actin monoclonal antibody (Sigma) at a 1: 2000 dilution for 1 h, which acted as a control for loading and blotting.

5.2.13 High Content Imaging

Localization of EPI-FA-AuNPs and the cytotoxic effect were tested on MCF-7 cell line. Briefly, 2×104 cells/well of MCF-7 cancer cell line were seeded on 96-well Cell Carrier microplates (PerkinElmer,
When the cells reached 80% confluence, the medium was changed, and then the cells were treated with AuNPs, EPI, and EPI-FA-AuNPs, and the plate was incubated for 24 h in a humidified incubator at 37°C with 5% CO2. The cells were washed twice with ice-cooled PBS, and the drug was localized in live cells using the Operetta High Content Imaging System (PerkinElmer, US).

5.3 RESULTS AND DISCUSSION

5.3.1 Characterization of EPI-FA-AuNPs

In the present study, we explain the reducing, stabilizing, and biocompatible properties of *L. acidissima* L extract for synthesis of AuNPs. *Lacidissima* L has higher quantity of polyphenolic compounds that can actively chelate and reduce metal ions to nanoparticles. It was assumed that the tautomeric conversions of flavonoids from the enol-form to the keto-form may release an activated hydrogen atom that can reduce metal ions to form nanoparticles (yellow color converted to wine red color).

The Photochemical profiling of the extract was done by LC-MS analysis which revealed the presence of phytocompounds such as (i) kinetin, (ii) esculin, (iii) 3,4,2′,4′,6′- pentamethoxychalcone, (iv) N-desmethylpheniramine. The identification of compounds was verified by mass fragmentation analysis, and the LC-MS spectra of the compounds are shown in Figure 5.1. The combined action of these chemical components and others may be responsible for the observed reduction of the metal ions to form nanoparticles (Matsubara et al. 2005).
The AuNPs were formed by simple mixing without applying any external energy (heating, sun light, or microwaving). The resultant mixture produced a significant color change (to wine red) within a few seconds after the addition. Further, our study focused on using the synthesized AuNPs to enable conjugation of biomolecules for different applications in drug delivery (Scheme 5.1).
We observed that the solution containing gold ions (Au$^{3+}$) and *L. acidissima* L. turned into wine red (537 nm) within 30 seconds (The synthesis of AuNPs is given in the video still). We found that 75µL of *L. acidissima* L extract Figure 5.2 (A) was sufficient to reduce 1mM HAuCl4.
Figure 5.2 (A) Optimization of AuNPs by changing various concentrations of (L. acidissima L extract) while keeping the gold solution constant. (B) FT-IR study of: (i) L. acidissima L extract (ii) AuNPs (iii) FA (iv) EPI (v) EPI-FA-AuNPs

FTIR spectrum of L. acidissima L extract Figure 5.2 B (i) shows a peak at 3448 cm\(^{-1}\), which could be attributed to the phenolic hydroxyls (O-H bond) in extracts, the absorption peaks at 1048 and 1612 cm\(^{-1}\) representing costretching functional present in the extract. Further, carboxyl stretching vibration peak formed at 1730 cm\(^{-1}\).
However, in the FTIR spectrum of AuNPs Figure 5.2 B (ii), the peak at 3420 cm\(^{-1}\) becomes comparatively narrow, which could be attributed to the phenolic hydroxyls (O-H bond) confirming that the phenolic hydroxyls react with the AuNPs resulting in the partial destruction of the hydrogen bonds in the molecules of the L. acidissima L extract. A shift in the peak from 1048 cm\(^{-1}\) to 1017 cm\(^{-1}\) also indicates that L. acidissima L extract interacts with AuNPs through its adjacent phenolic hydroxyls and/or formed quinones. The comparison of FTIR spectra of bare AuNPs, FA, EPI, and EPI-FA-AuNPs shows that the spectrum of FA-AuNPs exhibits the characteristic IR absorption peaks of FA Figure 5.2 B (iii), showing a peak at 1697cm\(^{-1}\) (amide I stretching), 1603 cm\(^{-1}\) (amide II stretching), and 1481cm\(^{-1}\) (hetero-ring, conjugated double bond). FTIR spectra of EPI and EPI-FA-AuNPs are shown in Figure 5.2 B (iv) and (v). FTIR spectra of EPI clearly confirm the basic structural units such as OH, CH, and carbonyl groups carboxyl groups by the peaks that appear at 3300 cm\(^{-1}\), 2940 cm\(^{-1}\), and 1620 cm\(^{-1}\), respectively. The FTIR spectrum of EPI-FA-AuNPs shows the peaks at 3300 cm\(^{-1}\) and 2940 cm\(^{-1}\), demonstrating the OH and CH content of EPI-FA-AuNPs. Moreover, the disappearance of the peak at 1730 cm\(^{-1}\) describes the amide bond formation between the carboxyl functional of EPI and amine functional of FA.

These spectra confirm the formation of EPI-FA-AuNPs. Energy dispersive X-ray (EDX) spectrum analysis of AuNPs showed the presence of AuNPs in the sample. Figure 5.3 (A) reveals a strong and typical optical absorption peak at approximately 2.2 keV, which could be attributed to the SPR of the metallic Au nanocrystals. The crystalline structure of nanoparticles was determined based on X-ray diffraction (XRD) analysis. The crystalline peaks that were identified as AuNPs based on XRD analysis
showed that the intense peaks of reflected radiation (Bragg peaks) were at the points (1 1 1), (2 0 0), (2 2 0), and (3 1 1) and were the diffraction lines of the face-centered- cubic (fcc) gold shown in Figure 5.3 (B). The selected area of electron diffraction (SAED) outline of the AuNPs showing that the rings designated 1, 2, 3, and 4 arise because of the reflections from (1 1 1), (2 0 0), (2 2 0), and (3 1 1) shown in Figure 5.3(C).

Figure 5.3 (A) The EDAX spectrum, (B) X-ray diffraction (XRD) spectrum, (C) SAED pattern of L. acidissima L fruit extract reduced AuNPs
Figure 5.4  HRTEM image of (A) AuNPs (B) EPI-FA-AuNPs, (C) The UV–Vis spectra showing the in vitro stability of EPI-FA-AuNPs in the pH range 1.2–7.4 and PBS, (D) AuNPs conjugated EPI-FA-AuNPs

The morphological results (HRTEM) images Figure 5.4 (A) reveals that the AuNPs and EPI-FA-AuNPs appear to be nearly spherical in shape Figure 5.4 (B). The stability of EPI-FA-AuNPs in different buffers was examined Figure 5.4 (C), and only slight changes (< 15%) were observed in the SPR band at 537 nm when the AuNPs were exposed to different buffers for 48 h. However, at pH 1.2, about 20% decline in the absorbance at 537 nm was observed compared with the absorbance of EPI-FA-AuNPs at pH (7.4). The observed reduction in the absorption of EPI-FA-AuNPs in extremely acidic conditions could be attributed to the limited aggregation of EPI-FA-AuNPs as a result of the screening of the negative charge on the external of the AuNPs at pH (1.2). The results from these in vitro stability
studies established that the AuNPs were intact and demonstrated excellent in vitro stability in biological fluids at various pH (Ganesh Kumar et al. 2013). EPI binding onto the AuNPs was further confirmed by the shift of the SPR band in Figure 5.4 (D) towards a higher wavelength (from 537 nm to 543 nm). The percentage of EPI loaded onto the AuNPs was determined based on EPI content in the obtained pellet, and it was found that 52 ± 4% of the drug could be loaded. Inductively coupled plasma optical emission spectrophotometer (ICP-OES) was performed to quantify the amount of gold in the aqueous solutions of AuNPs, and the same was used in further studies.

Figure 5.5 (i) The particle size distribution of AuNPs (ii) EPI-FA-AuNPs (iii) The zeta potential distribution of AuNPs in an aqueous medium. (iv) The zeta potential of distribution EPI-FA-AuNPs in an aqueous medium.
5.3.2 Particle Size and Zeta Potential Measurements

The hydrodynamic particle size of the AuNPs was found to be 134 ± 4 nm with a polydispersity index of 0.219 shown in Figure 5.5 (i). The average hydrodynamic diameter of EPI-FA-AuNPs is 139 ± 3 nm with a polydispersity index of 0.249 shown in Figure 5.5 (ii). A low polydispersity index shows that the particle size distribution of the AuNPs and EPI-FA-AuNPs is uniform. Zeta potential is considered an essential parameter to study the surface charge of the nanoparticle surface and predict the long-term stability of the nanoparticles (Pooja et al. 2015). The zeta potential of the AuNPs was found to be $-27.9$ mV Figure 5.5 (iii) indicating that the AuNPs were properly capped with anionic (carboxyl group) FA. EPI-FA-AuNPs were isolated by centrifugation and then suspended in an aqueous solution to quantify the electrophoretic mobility. The calculated zeta potential of EPI-FA-AuNPs was $-16.0$ mV shown in Figure 5.5 (iv). Though the surface charge is reduced to some extent, EPI-FA-AuNPs has enough repulsive force to prevent aggregation during long-term storage.

5.3.3 Toxicity of AuNPs in Zebrafish Embryos

Zebra fish embryos, which are ideal organisms, were used to investigate the developmental toxicity of AuNPs (Sharma et al. 2014). The survival rate was better for the embryos that had hatched within 96 h post fertilization, as compared with the control group shown in Figure 5.6 (A) (i). The mortality level was indicated by the dead embryos 96 h post fertilization as compared to the control group Figure 5.6 (A) (ii). The survival rate and mortality rate examined shows no apparent toxicity at various concentrations ranging from 50 to 450 µg/ml (Sharma et al. 2014). The AuNPs-treated embryos were observed using a compound stereo microscope to find abnormalities such as pericardial edema, pigmentation, deformity of the pericardial sac of the larvae, and deformity of the tail. Exposure of
zebrafish egg embryos to AuNPs did not produce any seeming toxicity in the development of the zebrafish shown in Figure 5.6 (A) (iii). Hence, this investigation of results clearly indicated that the AuNPs might be carriers for drug delivery applications.

Figure 5.6(A) (i) Graph representing the toxicity of AuNPs in terms of survival rate (%) of larvae, (ii) Mortality rate (%) analysis of AuNPs toxicity, (iii) Examples of zebrafish larvae exposed to control and various concentrations of AuNPs. All assays were performed in triplicate and the mean ± standard deviations are shown. Significant differences compared to control calculated with the one-way ANOVA test and are indicated by (P= 0.0004) ***p<0.001
5.3.4 Release Profile of EPI-FA-AuNPs

The *in vitro* study of pH-controlled EPI release from the EPI-FA-AuNPs was performed in PBS (pH = 7.4) and acetate buffer (pH = 5.7) at 37°C, pH (7.4) and (5.7) being near the physiological and endosomal pH conditions, respectively, of a cancer cell. The results are presented in Figure 5.6 (B). The pH-dependent drug release is also considered to be an essential factor in cancer therapy (Kumar et al. 2015). The EPI-FA-AuNPs display the slow and controlled release of the drug, with the release rate calculated showing negligible release when compared to that of free EPI in acidic and neutral atmospheres, respectively. Furthermore, the release efficiency of the drug from the EPI-FA-AuNPs was rapid, higher at pH (5.7) than at pH (7.4). EPI from EPI-FA-AuNPs in pH (7.4) will help to reduce toxicity of EPI to the normal tissue since the physiological pH of the body is maintained at pH (7.4) (Rafiee et al. 2014).
Figure 5.7a Zero order kinetics

Figure 5.7b First order kinetics
Figure 5.7c Higuchi kinetics

Figure 5.7d Korsmeyer peppas kinetics
In order to determine the release model which best describes the drug release pattern of EPI, the obtained release data was fitted in various kinetics models like zero order (Figure 5.7a), first order (Figure 5.7b) and Higuchi equations (Figure 5.7c). The $R^2$ values were compared and it was found that high linearity was observed for zero order followed by higuchi kinetics. The corresponding plot for Korsmeyer peppas (Figure 5.7d) also showed good linearity. The release exponent “n” was found to be 0.713, which falls in range of 0.45 and 0.89. In general if the value of $n = 0.45$ it indicates Fickian (case I) release, $n > 0.45$ but $< 0.89$ indicates Non-Fickian (anomalous) release; and $n > 0.89$ indicates super case II type of release. By considering this it can be understood that EPI release from EPI-FA-AuNPs was controlled by both diffusion and erosion mechanism.

Figure 5.8  (A) *In vitro* cytotoxicity studies of drug EPI and EPI-FA-AuNPs in MCF-7 cell lines. All assays were performed in triplicate, and the mean ± standard deviations are shown. (B) Images of MCF-7 cell lines visualized under inverted microscope. (i) Control, (ii) Blank, (iii) EPI, and (iv) EPI-FA-AuNPs

5.3.5  In Vitro Cytotoxicity Studies

To determine the cytotoxic effect of EPI and EPI-FA-AuNPs, cell viability study was done by a standard MTT-reduction assay with slight modifications (Tariq et al. 2015). The results of the MTT assay for free
and EPI-FA-AuNPs on MCF-7 cell lines are shown in Figure 5.8 (A). The IC50 value for EPI-FA-AuNPs was found to be around 2 µg/ml, while that for free EPI ranged from 28 µg/ml on the MCF-7 cells. These results show that the EPI-FA-AuNPs exhibited better cytotoxic activity than free EPI. This could possibly be due to the result of the variations in the cellular uptake profile leading to better activity of EPI-FA-AuNPs as suggested by Ormrod and coworkers (Ormrod et al. 1999). The targeted drug delivery system uses the anticancer drug for treatment of cancer cells alone, which reduces the effects of the drug on noncancerous cells and simultaneously increases its efficacy on cancer cells (Betancourt et al. 2007). The main aim of using the targeted drug delivery system is to deliver the anticancer drug to the cancerous cells without loss of the drug’s efficacy (Jain 2005, Li et al. 2009). The folate receptor (FR) is a meticulously studied ligand for the selective delivery of anticancer drugs on FR-positive tumor cells (Lu et al. 2012). In general, the FRs are highly up-regulated on the surface of different types of malignant cells (Zhang et al. 2010). The results of this study suggests that FA functionalized AuNPs can effectively deliver the drug to MCF-7 cancer cells by means of active targeting.

5.3.6 Apoptosis Study

Apoptotic changes, cytotoxic effect caused by EPI-FA-AuNPs occurred in MCF-7 cells when treated using acridine orange/ethidium bromide differential staining method. The stained cells characterized to EPI-FA-AuNPs caused more effective cell death than free EPI. The number of nonviable cells increased dramatically after treatment with EPI-FA-AuNPs in MCF-7 cells shown in Figure 5.8 B (iv). The red color is due to the nonviable cells (dead cells), and it was revealed that EPI-FA-AuNPs treatment resulted in a significant increase in apoptosis compared to EPI treatment. The control and blank cells in Figure 5.8 B (i) and (ii), the
condensed nuclei, as well as the membrane blebbing fluoresced uniformly bright green indicating (early apoptotic) that they did not undergo any apoptotic changes.

Figure 5.9(A) Effects of EPI-FA-AuNPs on cell cycle analysis. MCF-7 non-small cell breast cancer cells were cultured for 36 h (i) Control (ii) EPI (iii) EPI-FA-AuNPs.

Figure 5.9(B) Western blot, illustrating the different protein expressions in apoptosis after treatment with EPI and EPI-FA-AuNPs. Lane 1: Control, Lane 2: AuNPs, Lane 3: EPI, Lane 4: EPI-FA-AuNPs.
5.3.7 Effect of EPI-FA-AuNPs on Cell Cycle Analysis

Generally, the flow cytometry study was performed to investigate whether apoptosis and the cell cycle arrest are closely related. In our study, EPI-FA-AuNPs-treated MCF-7 cells, In Figure 8 (A) shows the cell cycle analysis of (i) control, (ii) EPI and (iii) EPI-FA-AuNPs. Control cells after 36 h had 20.3% the proportion of cells in the G2/M phase in Figure 5.9 (A) (i), and the EPI and EPI-FA-AuNPs produced a dramatic increase of 28.95% in Figure 5.9 (A) (ii) and 43.21% shown in Figure 5.9 (A) (iii) in the G2/M population, respectively. These data clearly prove that the cell cycle is arrested significantly at the G2/M phase in EPI-FA-AuNPs-induced MCF-7 cells (Halloran et al. 2004). The anti-cancer therapy completely eradicates the cancer cells by triggering different caspase-mediated cell death pathways (Ghavami et al. 2009), which confirms the activation of the apoptotic pathway after EPI-FA-AuNPs’ exposure.

5.3.8 Western Blot Analysis

The Western blot results showed mechanisms of EPI-FA-AuNPs-mediated cell death; the apoptotic regulators have been measured using mRNA and protein expression patterns. Cyclin D1 regulates the proliferation process by adjusting its expression levels and, accordingly, regulates the cell cycle control machinery (PankajRoy et al. 2006). EPI controls the reduction of Cyclin D1 levels in tumors (Velázquez et al. 2011). The results as seen in Figure 5.9 (B) showed that EPI-FA-AuNPs inhibited the function of Cyclin D1, and its expression was dramatically diminished. Cyclin E showed a high intense band when AuNPs and native EPI-treated cells were compared. The functions of Cyclin-dependent kinase (CDK)4-, CDK6-, and CDK2-mediated phosphorylation of the retinoblastoma (Rb) family of tumor suppressor proteins during apoptosis
were also observed (Murphy et al. 2015). EPI-FA-AuNPs-induced apoptosis in cancer cells was evident from the reduced expression of anti-apoptotic CDK6, CDK4, and CDK2 in MCF-7 cells. In this study on the efficacy of CDK inhibition as a means to arrest the proliferation of MCF, β-actin was used as a loading control, and it showed similar expression in all the lanes. Intrinsic pathways of Western blot studies are shown in Figure 5.9 (B). The present study also investigated EPI-FA-AuNPs-induced up-regulation of Bax proteins and down-regulation of Bcl-2 proteins in EPI-FA-AuNPs cells. p53 is a leading switch that coordinates stress signals between apoptosis and cell cycle arrest (Hedenfalk et al. 1997) shown in Figure 5.9 (B). EPI-FA-AuNPs induced increase in the levels of p53, the tumor suppressor protein. Caspases, a family of cysteine acid proteases, can be regarded as key factors in apoptosis (Sun et al. 2011). Figure 5.9 (B) shows that EPI-FA-AuNPs-treated cells induced cleavage of caspase 3 and caspase 9. On the whole, these results clearly indicate that the molecular level apoptosis was induced when MCF-7 cells were treated with EPI-FA-AuNPs. Cell death was further studied based on PARP cleavage after treatment with EPI-FA-AuNPs, as shown in Figure 5.9 (B). Our studies showed that PARP proteins cleaved into fragments after treatment with EPI-FA-AuNPs.

5.3.9 High Content Imaging

The cellular uptake study was made in MCF-7 cell line for EPI, AuNPs, and EPI-FA- AuNPs using high content imaging methods, and the results are shown in Figure 5.10. EPI-FA-AuNPs’ localization into the cell was compared with EPI in which bright field and fluorescent images were taken. The merged images help to calculate the localization of EPI-FA-AuNPs. Control cells without the exposure to EPI and AuNPs indicated no fluorescence (Yordanov et al. 2012). The EPI-FA-AuNPs-treated cells
showed maximum localization of EPI-FA-AuNPs into cells compared to EPI-treated cells.

Figure 5.10 Drug localization studies of control, EPI, AuNPs, and EPI-FA-NPs in MCF-7 cell line

5.4 CONCLUSION

To summarize the present study, a rapid and green chemistry-based method, which relies on the reduction of gold ions by *L. acidissima* *L* extract acting as both reducing and capping agents, has been established to
synthesize AuNPs. The EPI-FA-AuNPs exhibits prominent anticancer activity against MCF-7 cells. Results of the toxicity study in the zebra fish embryo model revealed that no significant malformation occurred, ensuring that the AuNPs nanoparticles are highly compatible for drug-delivery applications. Furthermore, maximum localization into cells compared with EPI in the MCF-7 cell line was observed. The EPI-FA-AuNPs could be promising for targeting breast cancer with the enhanced therapeutic activity of EPI.