Methods

Preparation of the Extract

Limonia acidissima L. Fruit pulp was finely chopped into small pieces and boiled in (60g Fruit pulp /100 ml H_2O) 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 ^oC for further studies.

Preparation of gold nanoparticles

In a typical experiment, an aqueous solution of $HAuCl_4$ ($1x10^{-4}$, 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.

2.3. Characterization of AuNPs and EPI-FA-AuNPs

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 CuKα (λ=1.54 Å) source in the region of 2θ 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.

2.9. In-vitro cytotoxicity studies

Cell viability was measured by MTT assay described by Mossman et al, (1983). Approximately (5×10^3 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).

2.9. Apoptosis study

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 [2]. In brief, 5 × 105 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 μg/ml). After 36 h incubation, the cover slip was removed and stained with AO/EB (10 μl) for thirty minutes and washed with PBS for removing the excess staining dye. Images were captured by Nikon Eclipse inverted fluorescence microscope.

3.0. Flow cytometric analysis and western blotting

Flow cytometric analysis

To investigate the effect of the drug on the cell cycle distribution, the cells (1×10^5 cells/ml) were treated with IC_{50} 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 40μg/ml propidium iodide (PI) and 0.1mg/ml RNase A, followed by shaking at 37˚C for 30min [3]. 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

Western blotting was carried out as described in another paper [4]. Briefly, the cells (1.5×10^6) seeded onto 100-mm culture dishes in the presence or absence of different concentration of

compounds (IC_{50} 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 5min 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 24hrs 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.