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Assessment of *in vitro* antitumor potential of luteolin loaded polymeric nanofromulations

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Abstract

Non-small cell lung cancer (NSCLC) is one of the fetal type of lung cancer worldwide with high resistant against current chemotherapeutic agents due to increase their aggressive features. Many plant derived natural compounds have been found to possess antimetastatic potential against NSCLC by inhibiting molecular product and correspondingly their growth. Luteolin is plant derived flavonoid with potential antioxidant, anti-inflammatory and anticancer potential against multiple malignancies but its bioavailability is low due to hydrophobic nature with less half-life. Current study was designed to increase bioavailability of luteolin by formulate in physiologically stable and biodegradable polymer Poly (lactic-co-glycolic) acid. Surface modified PLGA with PEG (PEG-PLGA NPs) was used as comparative formulation. Both formulations were made by single emulsion method. Physiochemical characteristics including surface morphology, size and charge and stability in NaCl and serum medium was done for both formulations. Invitro antitumor potential of free and formulated luteolin against Non-small cell lung cancer (NSCLC) was done using CCK-8 assay and % viability was determined. Results from physiochemical characterization showed 156nm particles of PEG-PLGA and slightly large 350nm sized particles of PLGA suspended in PBS whereas TEM images showed <100nm sized PEGylated NPs with high payload of luteolin drug with sustained release up to two days as determined by HPLC analysis. Invitro tumor growth inhibition assay showed cytotoxic potential of nanofromulation of luteolin as compared to free luteolin. Results of both PLGA and PEG-PLGA formulations of luteolin showed promising potential for further in vivo therapeutic approach against NSCLC.

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Introduction

Lung cancer is one of the most lethal type of cancers with higher mortality ration compared to other cancers worldwide. According to American cancer association (ACA, 2005), prevalence of lung cancer found to be equal among male and female cases diagnosed in which more than 80% cases reported be non-small cell lung cancer (NSCLC). Non-small lung cancer comprises Adenocarcinoma, squamous cell carcinoma and adenocarcinoma (Molina *et al.*, 2008). Despite of all advance diagnostic and treatment procedures with radiotherapy, chemotherapy and with combination therapy prevalence of NSCLC has been increased. Diagnosis at late stage of development further limits treatment options ultimately leads to short life span and increased mortality. Currently with early diagnosis, life span on average 44% cases is 1 year which further reduced to 25% survival rate with late diagnosis (ACA, 2005). Once diagnosed with small-non lung cancer, patients are being treated with combination therapy of chemo and radio followed by surgery (Jemal *et al.*, 2007).

Although chemotherapy showed less advantage for treatment of lung cancer due to relapse of cancer, high dose related offsite toxicity, and resistance developed against many chemotherapeutic drugs (Winton *et al.*, 2005). Also most chemotherapeutic drugs are highly hydrophobic and poorly solubility and low intravenous bioavailability are other challenges for the treatment of lung cancer (Schiller *et al.*, 2002). Recently naturally occurring compounds and dietary agents are in preclinical and clinical trials for chemoprevention due to reduce toxicity and high efficacy. An ideal chemotherapeutic agent is expected to possess high efficacy, reduced toxicity and enhanced bioavailability (Huang *et al.*, 2017).

Luteolin (3',4',5,7-tetrahydroxyflavone) along with other plant derived flavonoids present as natural antioxidant present in glycosylated form in green leafy vegetables such as cauliflower, spinach, broccoli, green chili (Kandaswami *et al.*, 2005) and possess anti-inflammatory and potent antitumor potential against different types of carcinomas due to its reduced toxicological profile compared to commercially available chemotherapeutic products. Anticancer potential of luteolin has been found against head and neck cancer, breast cancer, colon cancer, lung cancer and prostate cancer (Zhang *et al.*, 2008; Amin *et al.*, 2010; Majumdar *et al.*, 2014). Cellular apoptotic potential of luteolin is due to inhibition of specific tumor necrosis factor NF-kB pathway (Wang *et al.*, 2007), and induction of apoptosis and inhibition of G2/M phase by Akt-GSK-3b-cyclin D1 pathway (Lee *et al.*, 2010; Ju *et al.*, 2007), angiogenesis (Yang *et al.*, 2008). However, there is limitation of preclinical and clinical application of luteolin is due to short shelf life, oral mediated low bioavailability. Also high extent of hydrophobicity of luteolin leads to poor intravenous and intra peritoneal administration further limits its clinical application.

Nanotechnology is emerging field of drug delivery for treatment of cancer and other drug resistant diseases. Many properties such as stability in serum, high payload of drugs, sustained release and long circulation within blood, enhanced cellular bioavailability and permeability and retention (EPR) in tumor compared to normal cells make nanoparticles an ideal drug delivery system for different ailments (Peer *et al.*,2007). Due to sustained release of drug, nanoparticles also found to reduce offsite toxicity compared to free drug (Kawasaki *et al.*, 2007). In previous studies our group successfully formulated polymer based nanofromulations of <100nm with high payload of chemotherapeutic drugs and proved nanotechnology as promising approach for enhanced bioavailability and chemoprevention (Timbie *et al.*, 2017; Nance *et al.*, 2012).

Thus in present study we formulated polymeric poly lactic-co-glycolic acid (PLGA) nanofromulation of luteolin and further investigated whether this delivery system further stabilize and improve in vitro antitumor efficacy. For this purpose, A549 lung cancer cell line was used to assess free luteolin and its

polymeric nanofromulation and compared cellular toxicity and uptake.

Materials and methods

Synthesis of luteolin nanofromulation

Nanofromulation of luteolin was prepared using optimized single emulsion method as described by Nance *et al.*, 2014. PLGA (75:25) polymer (MW: 15 kDa; Jinan Daigang Biomaterials Co. Ltd., Jinan, China) and PEG-PLGA (75:25 with 25% (MW 5 kDa; Creative PEG Works). Briefly 20mg/mL of both PLGA and PEG-PLGA dissolved in DCM and 5mg/mL of luteolin was added drop wise under probe sonication (30% amplitude for 2 min) in ice water bath. To evaporate excess of organic solution, emulsified solution was added in 25 mL of 0.5% CHA solution and stirred for 2 hours followed by filtration using amicon ultra filters (MWCO: 50kDa) at 3600g with three repetitive wash with deionized water. Final solution was collected and stored at 4°C, 25°C and 37°C for further analysis.

Physiochemical characterization of nanofromulation

Size and charge determination: Both conventional and PEGylated nanoparticles (0.5% NPs solution in 10mM NaCl) was analyzed for their hydrodynamic diameter, Polydisparity index (PDI) and net surface charge (□- potential) was determined using dynamic light scattering principle using Zetasizer Nano.

Surface morphology using TEM

Surface morphology of both PLGA and PEG-PLGA NPs was determined using transmission electron microscopy (TEM: Hitachi). NPs solution was 100 folds diluted in ultrapure water and poured 10□L on Cu grids placed on parafilm. Samples were placed at room temperature for 2-4 hours. After drying, grids were placed in TEM holders and imaged at 30,000-70,000 resolution.

HPLC analysis of nanoparticles

Estimation of drug content

For HPLC analysis of PLGA and PEG-PLGA NPs, luteolin of different concentrations (50□g/ml, 25□g/ml, 12.5□g/ml, 6.25□g/ml, 3.125 □g/ml, 1.56□g/ml) were prepared in 100% methanol and ran at HPLC conditions of 75:25 ACN: water (0.1% TFA) and 350nm. Both standard drug samples and NPs solutions were analyzed and % loading (%LE) and % encapsulation efficiency (%EE) was determined using following formula.

% LE = mass of drug in NPs/ total mass of NPs X 100

%EE = mass of drug in NPs/ total mass of drug used initially X 100.

In vitro release kinetics

The in vitro release kinetics of luteolin loaded nanoparticles was done using dialysis method. Nanoparticles of known concentration of drug was placed in PBS (supplemented with 0.5% Tween 80) solution under sink conditions using dialysis membrane (MWCO: 1KDa), at 37 °C and 150 rpm and at specific time intervals whole suspended medium was replaced with fresh medium and analyzed by HPLC. Drug content in that specific interval and cumulative release of drug was calculated after end of experiment.

In vitro CCK-8 assay

In vitro toxicity of free drug and NPs was done on epithelial lung cancer cell lines A549 (ATCC®CCL-185) and sarcoma cell linesF98 (ATCC® CRL-2397™)using CCK-8 assay. Previously both F98 and A549 cell were cultured in F-12K- DMEM and F-12K EMEM medium respectively with 5% PENSTREP (penicillin: Streptomycin) under humidified conditions with 5% CO_2.For toxicity test, cell lines were cultured in 96 well plates at 5000cells/well in 100 □L medium. On day of treatment, cells were washed with PBS and treated with different concentrations of free luteolin and PLGA-PEG NPs loaded with luteolin. After 72 hours, cell media were aspirated, loaded with same volume of 10% dojindo solution and incubated further for 3 hours. Absorbance at 450 nm was determined using plate reader and % cell viability was determined using following formula.

% Cell viability = (Absorbance of treated group/ Absorbance of control group) X 100.

Statistical analysis

All experiments were done in triplicates and data was analyzed using statistical software Graphpad Prism. Student's t-test for unpaired data was used to evaluate statistical differences between mean values. All data are presented as the mean ± standard deviation (SD), and data were considered statistically significant at $P < 0.05$.

Results

Physiochemical characterization (Size and charge determination)

We have developed conventional (Non-PEG) and PEGylated nanofromulation of PLGA encapsulated with high content of luteolin by single emulsion method using low percentage of cholic acid (CHA) as surfactant.

Size distribution of both conventional PLGA-NPs and PEG-PLGA formulations was confirmed by dynamic light scattering with average diameter of 350 ± 25.87 and 156 ± 5.29 respectively (Table 1).

Table 1. Physiochemical analysis of luteolin loaded NPs.

Sr. no		NPs formulation	
		PLGA-NPs	PEG PLGA-NPs
1	Hydrodynamic diameter (nm)	350 ± 25.87	156 ± 5.29
2	Mean diameter (nm)	150 ± 6.8	80 ± 3.98
3	PDI	0.34±0.18	0.12±0.06
4	Z- potential (mV)	-40.6±5.8	-5.76±2.5
5	Loading efficiency (%)	3.08 ± 2.5	5.87 ± 1.5
6	Encapsulation efficiency (%)	68 ± 3.8	72± 8.6

†Hydrodynamic diameter, mean diameter and PDI were measured using zetasizer Nano whereas z-potentials were measured by laser Doppler anemometry in 10 mM NaCl at pH 7.0. Data represent the mean ± SEM (N ≥ 3 measurements).

Due to high negative COOH groups on PLGA surface charge (Z- potential) was found to be highly negative (-40.6±5.8) whereas PEG-PLGA NPs showed nearly neutral surface charge (-5.76±2.5).

This is due to high PEG density on PLGA NPs which prevent hydrophobic core molecules from aggregation and stabilize nanofromulation. Polydisparsity index (PDI) of PLGA-NPs found to be slightly higher (0.34±0.18) further indicated unstable formulation and aggregation of NPs residues in suspended medium whereas PEG-PLGA formulation with lower hence stable PDI of 0.12±0.06 revealed dense layer of PEG protection from aggregation.

Morphological properties and drug content determination

Mean diameter of both PLGA-NPs and PEG-PLGA-NPs loaded with luteolin as analyzed by TEM found to be slightly lower than hydrodynamic diameter mentioned in table 1 due to removal of hydrated layer from NPs. Surface morphology revealed by TEM showed regular spherical suspension of both NPs formulations (Fig. 2). Consequently, % luteolin loading and encapsulation efficiency of both conventional and PEGylated PLGA-NPs showed comparable results of 3.08 ± 2.5 and5.87 ± 1.5 loading and 68 ± 3.8 and 72± 8.6 encapsulation efficiency of PLGA-NPs and PEG-PLGA-NPs respectively as determined by HPLC (Fig. 3).

In vitro release Kinetics

In vitro release kinetics of luteolin (Figure 3) showed comparable release of luteolin from both formulations with initial burst release of 40% in first 20 hours and subsequently slow release in later time points. % cumulative release was found to be more than 90% of luteolin release from PLGA-NPs formulation and more almost 70% luteolin released from PEG-PLGA NPs (Figure 4).

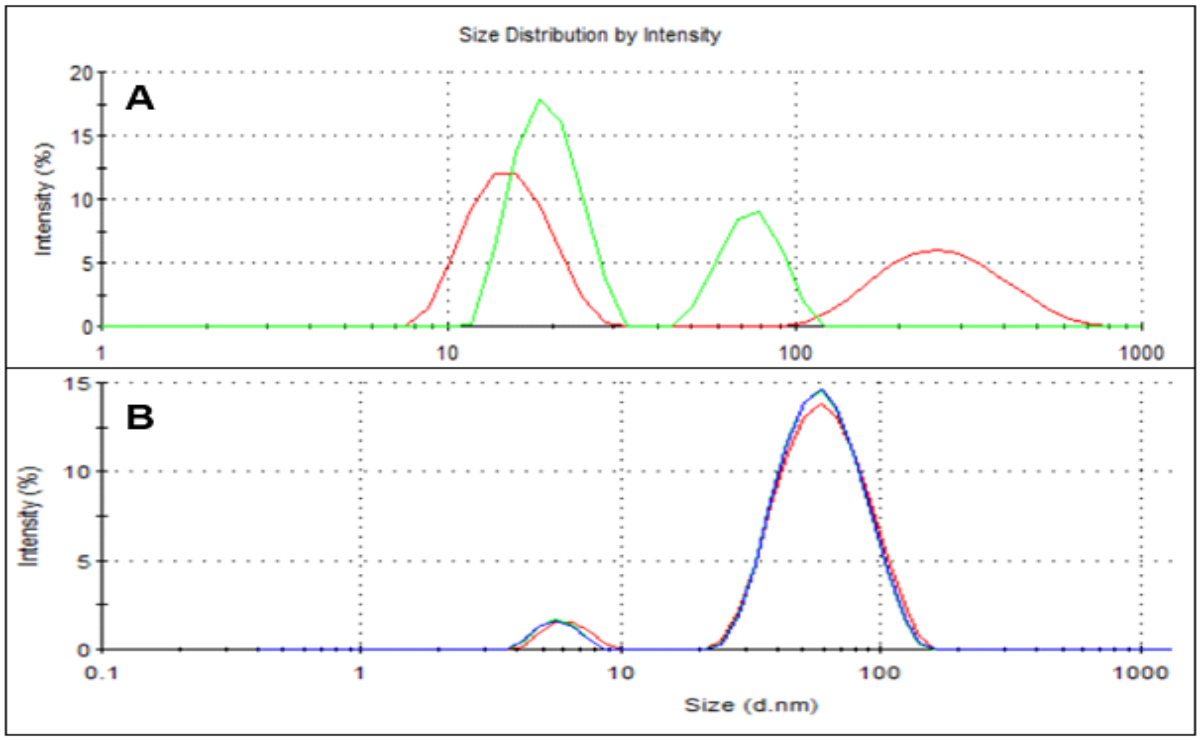

Fig. 1. Size determined by Zetasizer of PLGA NPs (A) and PEG-PLGA NPs (B).

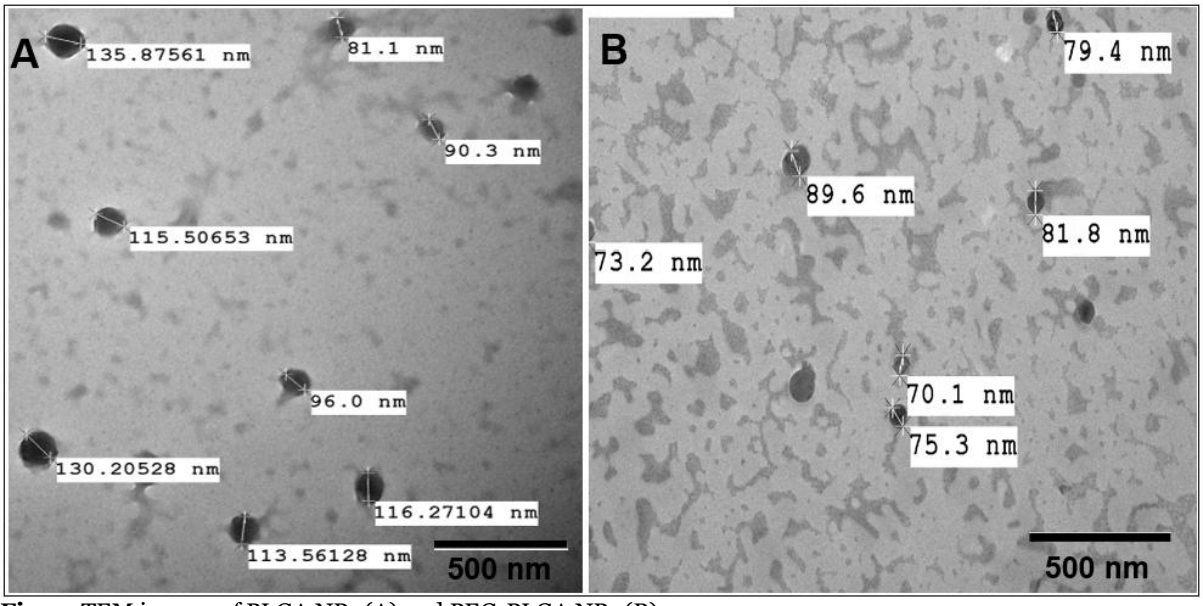

Fig. 2. TEM images of PLGA NPs (A) and PEG-PLGA NPs (B).

This comparatively slow cumulative release from PEG-PLGA-NPs further revealed stability of PEGylated formulation as compared to conventional PLGA.

In vitro cell viability assay

To evaluate antitumor potential of free and formulated luteolin we performed CCK8 assay and treated A549 lung cancer cell lines with free luteolin and both PLGA-NPs PEG-PLGA-NPs having same concentration of drug and determined viable cell density based on bound protein content with CCK8 solution. Results (Fig. 5) showed similar % inhibition of tumor cell growth by both free and formulated luteolin at higher concentration (12.5 □M and 25 □□) whereas NPs showed 2folds' lesser toxicity at lower concentration (2.5□M and 7.5 □M) inhibited 26% compared to 35% and 40% inhibition for same

concentration of free luteolin respectively. IC50 of both free and formulated drug calculated by Graph Pad Prism showed significantly lower IC50 of NPs (10.5□M PLGA and PEG-PLGA compared to free drug 15□□M free luteolin). Lower toxicity of NPs formulations further supported sustained drug released in medium and effects tumor growth compared to free drug. At higher concentration (>25 □□□□both free luteolin and NPs loaded luteolin eradicated more than 20% cells and no colonies showed up. Hence both free and formulated drug showed in vitro antitumor activity in dose dependent manner.

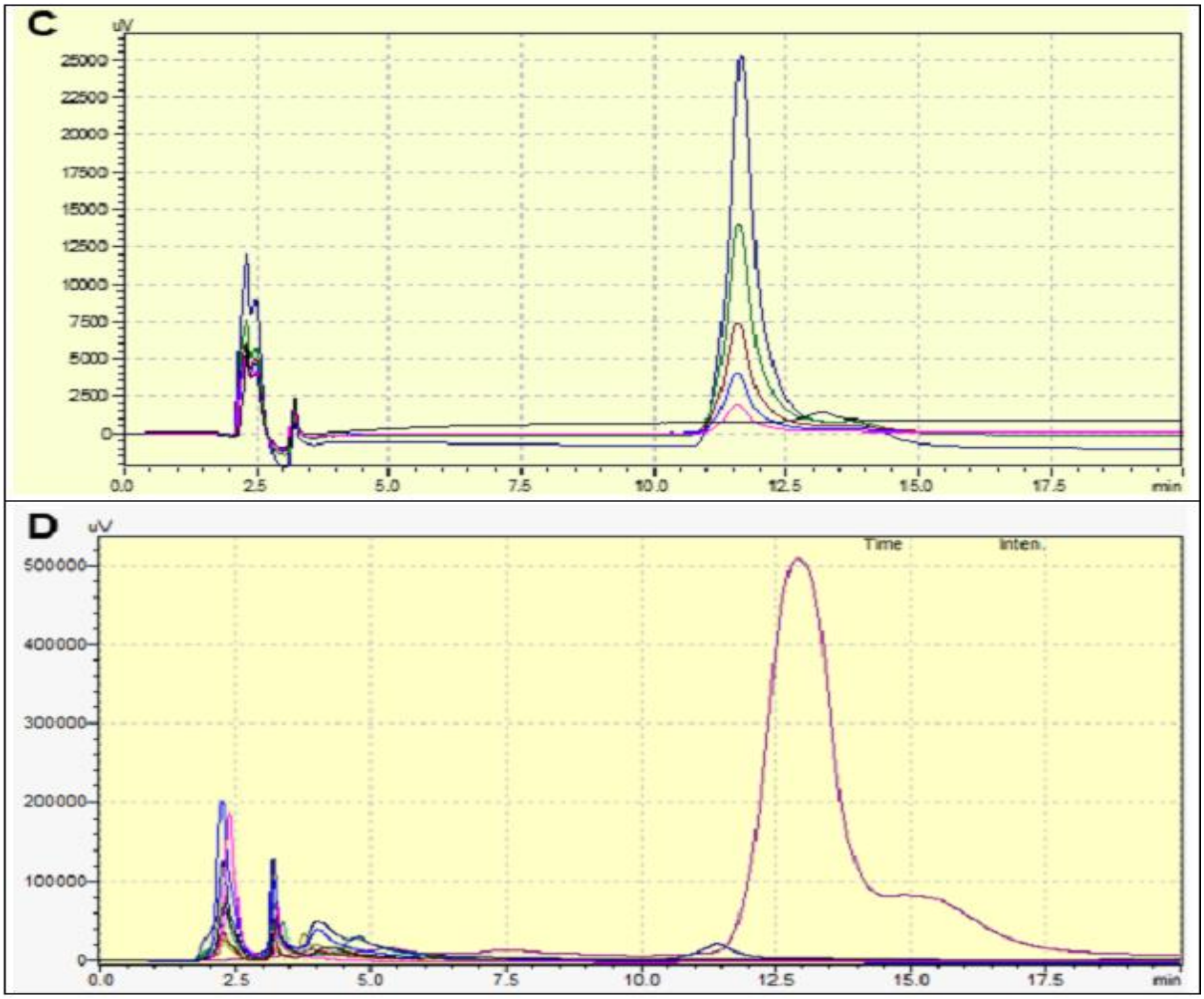

Fig. 3. HPLC analysis of luteolin for estimation of drug content. (C) Standard concentrations (25□g/ml, 12.5□g/ml, 6.25□g/ml, 3.125 □g/ml, 1.56□g/ml) of luteolin. (D) HPLC chromatogram of luteolin in NPs.

Discussion

The current study demonstrated enhanced antitumor activity of luteolin by polymeric nanoparticles formulations. We successfully prepared stable formulation of both poly (lactic-co-glycolic acid) PLGA and PEG-PLGA with high payload of luteolin (Figure 3) and released in sustained manner under normal serum conditions. This sustained release further confirms stability of formulations which incorporate hydrophobic drug molecules inside core region. Previously PEG-PLGA formulations has been rpoted to possess sustained drug release (Nance *et al.*, 2014) with standard hydrophobic chemotherapeutic drug paclitaxel. Current study reported comparable physiochemical characteristics to previous studies. Polymeric nanoparticles have been widely used as promising drug delivery system since last two decades for many potent drugs whose clinical applications were compromised due to high hydrophobic nature (You *et al.*, 2012; Sadat *et al.*, 2011).

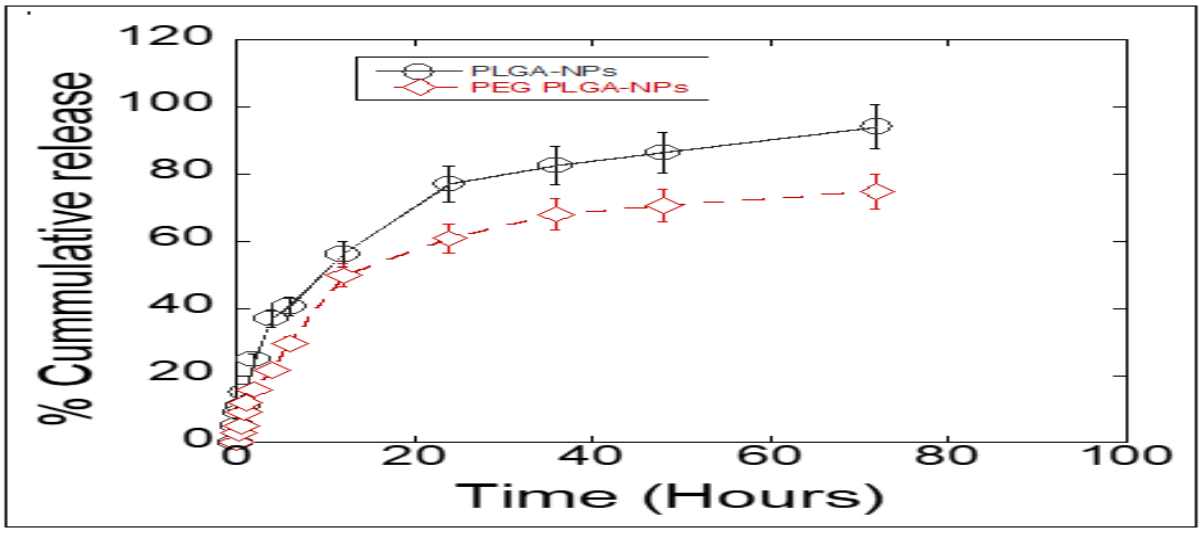

Fig. 4. % Cumulative release of luteolin from PLGA and PEG-PLGA-NPs in PBS (pH: 7.4) at 37°C.

Polymeric nanofromulation being water soluble in nature, improved bioavailability of those drugs by reducing hydrophobicity and dose dependent toxicity due to sustained release. PLGA is one of the most effective polymers for nanofromulations currently under investigations in clinical trials for different ailments. Non-PEG PLGA nanofromulation of luteolin showed large hydrodynamic diameter in suspended medium as due to aggregation of NPs in ionic solution.

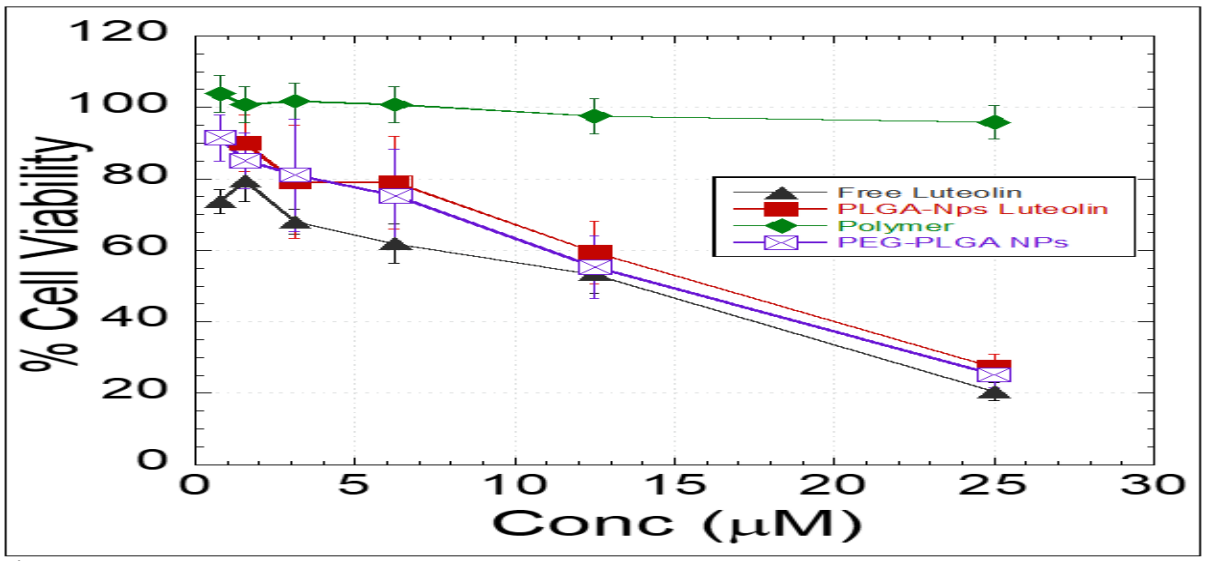

Fig. 5. *In vitro* % cell viability assay of free luteolin and its comparison to PLGA and PEG-PLGA-NPs in A549 cell lines.

This aggregation occurs due to hydrophobic interactions developed among hydrophobic lactic and glycolic acid residues and small populations were also found in suspension (Fig. 1). Biodegradable nature of PLGA is effective due to less immunogenic response and systemic toxicity since it decomposes in monomeric subunits lactic acid and glycolic acid which further used in metabolic processes in biological system (Kumari *et al.*, 2010). PLGA nanoparticles below 100nm size have been proven as optimal delivery system for antitumor drugs (Nance *et al.*, 2012). Non-small cell lung cancer ((NSCLC) with high mortality rate includes more than 80% cased reported with lung cancer Lung cancer cell metastasis including steps being identified in solid tumors, such as (i) detachment from extracellular

matrix (ECM), (ii) local migration and invasion, (iii) entry into blood or lymphatic systems (Van Meerbeeck *et al*., 2011; ACA,2005), (iv) survival in circulatory system, (v) extravasation at metastatic site and (vi) proliferation and formation of new tumor (Pithi *et al*., 2016).

Due to high ant proliferative and anti-inflammatory potential, plant based flavonoids and polyphenols have been proven to be potent against lung cancer (Ding *et al*., 2014; Cai *et al*., 2011).

Physiochemical characteristics such as size, charge and surface morphology significantly alter the interaction of colloidal solutions to other biological molecules and effect the efficacy (Cristina *et al*., 2002) Many strategies have been made for polymeric nanofromulation to show enhanced cellular uptake including surface modification of nanoparticles by PEG moiety, reduction of surface charge to prevent aggregation by using different surfactants such as F68, F127, cholic acid (CHA) and P80. Effects of surfactants have been widely studied for enhance permeability. A study reported by Kulkarni and Feng, 2011 has shown enhanced endocytosis of nanoparticles followed by uptake in BBB endothelial cells. Another study showed increase effect of F68 on nanofromulation tumor inhibition compared to conventional uncoated nanoparticles.

Surface modification of polymer with PEG further stabilize nanoparticles by preventing non-specific binding to RES increase circulation and cellular uptake due to leaky tumor vasculature (Siddiqui *et al*., 2009; Sahu *et al*., 2008). Thus nanoformulated luteolin showed more effective in tumor growth inhibition compared to free luteolin.

Conclusion

Results obtained from current study showed successful formulation of both conventional uncoated PLGA and PEGylate PLGA nanoparticles with high payload of antitumor flavonoid luteolin. Formulations further characterized for physiochemical and *in vitro* antitumor potential which revealed high stability, sustained release in physiological environment and enhanced *in vitro* antitumor efficacy compared to free unstable luteolin with shorter shelf life. In conclusion, this formulation showed promising preliminary *in vitro* results against small non lung cancer cell A549 and potential for further in vivo pharmacokinetic and efficacy studies.

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