Plant Archives Volume 20 No. 2, 2020 pp. 7127-7134 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

BIODEGRADABLE PLGA NANOPARTICLES CONJUGATED TO FOLIC ACID FOR TARGETED DELIVERY OF KAEMPFERITRIN

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Abstract

Kaempferitrin loaded biodegradable poly (D, L lactide-co-glycolide) acid (PLGA) nanoparticles were developed by solvent emulsion evaporation method. Folic acid with the % of PLGA successfully absorbed, and incorporation of drug content was performed for the formulated PLGA-Nps. The synthesized KM+FA+PLGA-Nps were characterized by XRD, FTIR, SEM, and TEM. *In vitro* cytotoxicity study on HT-29 cell lines using MTT assay, apoptotic study revealed that KM+FA+PLGA-Nps has high cytotoxic effect compared to kaempferitrin drug solution. These results evidenced that KM+FA+PLGA-Nps might be a promising anti-cancer pharmacotherapy for targeted drug delivery in HT-29 colon adenocarcinoma cells.

Key words: PLGA, Kaempferitrin, Folic acid, Nanoparticle, Cytotoxicity, Anticancer

Introduction

Despite widespread research efforts, colon cancer is in the fourth position for the cause of death, related to tumor in worldwide (You *et al*., 2016; Ahmadi *et al*., 2017). Chemotherapy is the commonly used effective treatment for colon cancer including 5-fluorouracil, irinotecan, oxaliplatin, and cepecitabine (Di Desidero *et al*., 2019). In chemotherapy, therapeutic concentration of an antitumor medicine in the cancerous tissue reaches at a huge concentration to the rest of the body. This low specificity leads some toxicological side effects and recently, tumors are able to making resistance to chemotherapy (Housman *et al*., 2014). Nowadays novel bioactive molecular therapy has the most important advantages in pharmaceutical industries.

Nano assembles like nanocompounds, nanocrystals, polymeric micelles, repetitively branched molecules and spherical vesicle having lipid bilayer possess novel strategies for the medication of carcinoma cells. Nano-drug delivery systems could improve drug bioavailability and reduce non-specific toxicity (Zhang *et al*., 2018).

In recent times nanomedicine based drug delivery has received widespread attention on the properties of nanotechnology for the identification and targeted drug delivery for many diseases (Yugui *et al*., 2019). For the fabrication of polymeric nanoparticles, poly (D, L lactide-co-glycolide) acid (PLGA) was used, due to its high biocompatibility and biodegradable features. Formulated Nps were developed from many chemical components (Luo *et al*., 2014). In addition, this type of carriers made of nano sized materials could modify the surface of a material with binding molecule to significantly increase the drug delivery and targeting efficacy (Baskararaj *et al*., 2020; Nagheh *et al*., 2017). Folate can be obtained from supplementary sources because it cannot be synthesized by mammals (Liu *et al*., 2017). The oxidized folic acid is taken as decoration ligand for folacin, because of its high stability and bioavailability when compared to folate. Folic acid has strong affinity ($Kd=10^{-9}$ M), specificity towards receptors, cost effective and also easy availability (Ji *et al*., 2017). The folic acid receptors are specifically over-expressed in number of tumors like squamous cell cancer, malignant lung tumor, breast cancer, as well as low or non-detectable expression in normal cells. In the present work, we have developed novel KM+FA+PLGA-Nps delivery through conjugation of PLGA and folic acid for delivery of anticancer drug kaempferitrin in colon cancer cells.

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Materials and Methods

Chemicals

Kaempferitrin (soluble, 250 mg/mL in dimethyl sulfoxide), PLGA (Poly lactic-co-glycolic acid; 50/50, M.W. 40 to 70 kDa), PVA (polyvinyl alcohol; M.W. 30 to 50 kDa), methanol, ethanol, dichloromethane, acetone, DMSO and Dulbecco's solu-tion.

Preparation of Kaempferitrin nanoparticles (KM+FA+PLGA-Nps)

KM+FA+PLGA-Nps were formulated by using combined emulsion solvent evaporation method with few modifications (Ji *et al*., 2017). 25 mg of kaempferitrin dissolved in 2 ml of dichloromethane (DCM) containing 250 mg of PLGA and 5 mg of folic acid to form an organic phase. This organic phase was stirred for 15 min at 250 rpm. Then, 1% polyvinyl alcohol was mixed with the organic phase and applying sound energy to stir particles at 35% power to form micro-emulsion for 40 s. The magnetic stirring was continued at 30°C for 3 h for the complete evaporation of DCM to progress the kaempferitrin encapsulation in the PMC of nanoparticles. The reaction mixture was stirred at 17,000 rpm for 12 h at 25°C. The formed Nps were washed with de-ionized water by ultracentrifugation at 15,000 rpm for 30 min. The same procedure was repeated for 3 times. The developed Nps were lyophilized to obtain dry powder. These Nps were stored at 4°C in dark till use.

Characterization of nanoparticles

The particle size and zeta potential of the KM+FA+PLGA-Nps was measured by dynamic light scanning (DLS, Malvern Instrument Ltd., UK). This instrument measures 0.4 nm to 6 µm particle diameter. Deionized water was used to place the nanoparticles again. Followed by homogenization at 20°C for 10 minute the Nps dimensions were analyzed. The size distribution and morphology of KM+FA+PLGA-Nps was measured by the scanning electron microscope (Zeiss EVO LS10, UK).

Infrared spectra of kaempferitrin, PLGA, Folic acid, KM+PLGA, and KM+FA+PLGA particles were collected by Fourier transform infrared spectrophotometer (Therma Nicolet corp, USA). The freeze-dried samples were analyzed and recorded.

XRD analysis of KM+FA+PLGA-Nps was recorded by using an automated X-ray diffractometer (Siemens 850, Germany). The peaks were compared with combination of powder diffraction standards for the identification of KM+FA+PLGA-Nps.

The surface morphology of KM+FA+PLGA-Nps was confirmed under the transmission electron microscope (TEM). Samples were prepared by dropping sonicated samples on a 400 mesh holey carbon coated copper grids.

Drug loading and release of kaempferitrin

The percentage of drug content and encapsulation efficiency was evaluated with KM+FA+PLGA-Nps. Kaempferitrin was taken as anticancer medicine to assess the loading content of drugs. The following equations were used to determine drug loading and encapsulation efficiency:

Drug loading (%) = (Drug weight in Nps/ weight of the Nps) ×100

Drug encapsulation efficiency = Drug weight in the Nps/weight of drug used in Nps formulation) ×100

The drug release was determined by suspending KM+FA+PLGA-Nps in 15 mL of PBS with Tween® 80 (0.05%) in triplicates. Followed by 30 min incubation of Nps, the supernatant was removed and the absorbance was recorded at 230 nm to 480 nm.

MTT assay

In vitro cytotoxicity detection of KM+FA+PLGA-Nps was evaluated by using MTT assay as mentioned in Mossman (1983). Approximately 1×10^6 HT-29 cells/well seeded in a 96 well flat-bottom culture plate and incubated for 24 hrs at 37°C in a 5% CO_2 incubator. Different concentrations 0, 7.5, 15.0, 30.0 and 100 µM of KM+FA+PLGA-Nps was added to the culture medium. Followed by 24 hr incubation MTT reagent was added to the growth medium and allowed to incubate under 5% CO_2 incubator. The supernatant was removed by adding 100 µL DMSO to each well. The absorbance was recorded at 540 nm with a microplate spectrophotometer.

Apoptosis

Annexin V-FITC/PI staining was used to evaluate the efficacy of KM+FA+PLGA-NPs on HT-29 cell lines. In brief, 1×10^{-6} cells were treated with standard 5-flurouracil and KM+FA+PLGA-NPs and incubated for 24 h. Media with 0.1% DMSO were added in the control group cells. Followed by 24 hours incubation, harvested the cells and cleaned twice with PBS and again placed in Annexin-V binding buffer. Then, apoptosis staining was done for cell staining conjugate and incubated for 15 minutes at 37°C in dark condition. Finally, the cells were stained with 5 µg/mL propidium iodide and analyzed by flow cytometry.

Statistical analysis

The data were expressed as mean ± standard deviation. The significance of differences were determined by the one way ANOVA.

Results

Synthesis, characterization - polymer nanoparticles

KM+FA+PLGA-Nps were synthesized using biodegradable and biocompatible polymer PLGA by emulsion solvent evaporation method. Kaempferitrin has

wide pharmacological activity but it has poor solubility in aqueous solution. In order to overcome this limitation kaempferitrin encapsulated with PLGA and KM+FA+PLGA-Nps were used as they are highly dispersible or soluble in aqueous solution.

Under FTIR energy, many chemical materials have their molecular bonds bent or stretched which yielded a special IR spectrum to categorize functional groups along with newly formed chemical bonds. FTIR spectrum of kaempferitrin (KM) revealed absorption bands at 3335 cm^{-1} (NH_2), 2927 cm^{-1} (C H), 2080 cm^{-1} (N=N=N), 1640 cm^{-1} (C=O), 1460 cm^{-1} (O H), 1355 cm^{-1} (NO_2), 1230 cm^{-1} (C C N), 1095 cm^{-1} (C O), 949 cm^{-1} (CH_2=CH_2) and 567 cm^{-1} (SO_2 rocking). The FTIR spectra of PLGA revealed absorption at 3447 cm^{-1} (NH), 2949 cm^{-1}(H OH), 2850 cm^{-1} (C H), 2340 cm^{-1} (P H), 1757 cm^{-1} (C=O), 1618 cm^{-1} (C=C), 1458 cm^{-1} (CH_2), 1425 cm^{-1} (OH), 1392 cm^{-1}(O=C=O), 1275 cm^{-1}(C N), 1182 cm^{-1}(SO_2) and 1128 cm^{-1}(C=S). The FTIR of folic acid (FA) showed 3450 cm^{-1} (NH), 3000 cm^{-1} (=C-H), 2936 cm^{-1} (CH), 1765cm^{-1} (C=O), 1635cm^{-1} (C=O), 1460cm^{-1} (CH_3), 1400cm^{-1} (C N), 1270cm^{-1} (C O), 1190cm^{-1} (C C N), 1094cm^{-1} (C N) and 620cm^{-1} (C CO C). The FTIR spectra of KM+PLGA showed 3438 cm^{-1} (NH), 2944 cm^{-1} (CH), 1758cm^{-1} (C=O), 1468cm^{-1}(CH_2), 1393cm^{-1} (O=C=O), 1107cm^{-1} (C O C), 1030cm^{-1} (C=O) and 748 cm^{-1} (C Cl). The FTIR spectra of KM+FA+PLGA nanoparticles showed 3447 cm^{-1} (NH), 2937 cm^{-1} (CH), 1758 cm^{-1} (C=O), 1633 cm^{-1} (C=O), 1447 cm^{-1} (CH_3), 1402 cm^{-1} (C N), 1268 cm^{-1} (C O C), 1188 cm^{-1} (SO_2), 1090 cm^{-1} (C N), 1020 cm^{-1} (=CH), 955 cm^{-1} (=CH), 851 cm^{-1} (NH_2), and 746 cm^{-1}(C Cl) Fig. 1.

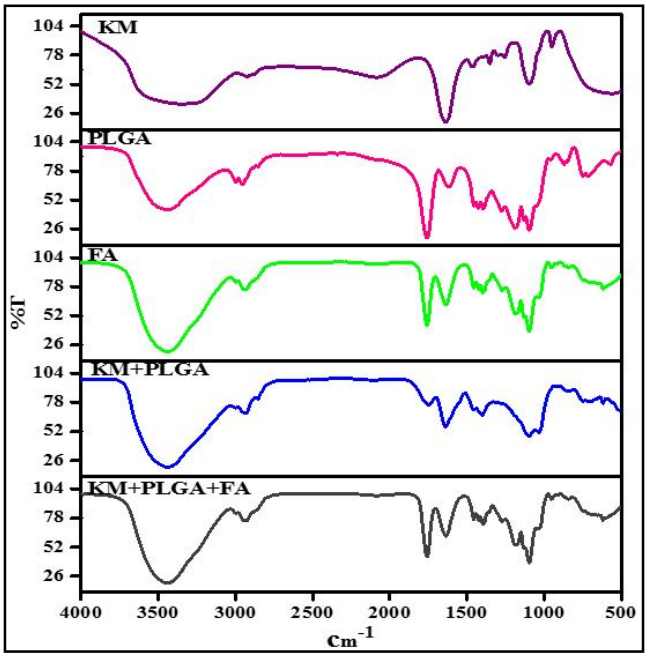

Fig. 1: FTIR spectra of KM (Kaempferitrin), PLGA, KM+PLGA and KM+PLGA+FA nanoparticles recorded in the range of 4000-500 cm^{-1}.

Table 1 depicted the diameter of the compounds, poly dispersity index, electrical potential, efficiency for drug encapsulation and loading capacity for drug. The mean particle size of KM+FA formulations was found to be 138.57 ± 6.8 and the mean particle size of KM+PLGA formulations was found to be 143.76 ± 7.4. The mean particle size of the KM+FA+ PLGA nanoparticles was found to be 151.21 ± 8.4. The favorable entrapment of kaempferitrin to the nanoparticle surface was determined by the increased size of nanoparticles. The poly dispersity index of KM+FA, KM+PLGA and KM+FA+PLGA formulations were determined as 0.068 ± 0.012, 0.085 ± 0.030 and 0.092 ± 0.021, respectively. The smaller size of nanoparticles can be developed for drug release carrier for anti-cancer medications.

The ZP value of KM+FA, KM+PLGA and KM+FA+PLGA formulations were determined as -6.28 ± 0.37, -15.0 ± 0.41 and -23.4 ± 0.56, respectively. The presence of carboxyl group in PLGA polymer will make negative surface on nanoparticles. The optimum size, encapsulation efficiency and drug loading capacity of nanomaterials are important to obtain the preferred reason of anti-cancer effect. The entrapment efficiency (%) for KM+FA, KM+PLGA and KM+FA+PLGA formulations were determined as 15.73 ± 0.06, 23.35 ± 0.18 and 68.57 ± 2.05, respectively. The drug loading capacity (DL %) for KM+FA, KM+PLGA and KM+FA+PLGA formulations were determined as 0.157 ± 0.009, 0.184 ± 0.015 and 0.256 ± 0.017, respectively.

The XRD patterns of KM, PLGA, FA, PLGA+FA,

Table 1: Physical and chemical features of combinatorial nanoparticles.

S.No.	Samples	Diameter of compounds (nm)	Poly dispersity index	Zeta potential of compounds	Encapsulation efficiency of particles	Drug loading capacity (DL (%)
1.	KM+ FA	138.57± 6.8	0.068±0.012	-6.28±0.37	15.73±0.06	0.157±0.009
2.	KM + PLGA	143.76± 7.4	0.085±0.030	-15.0±0.41	23.35±0.18	0.184±0.015
3.	KM+FA+ PLGA	151.21± 8.4	0.092±0.021	-23.4±0.56	68.57±2.05	0.256±0.017

Note: The various letters in the table column represents statistical variation - One way ANOVA ($p < 0.05$).

and KM+PLGA+FA are shown in Fig. 2. Generally, poly and FA were considered as non-crystalline solids

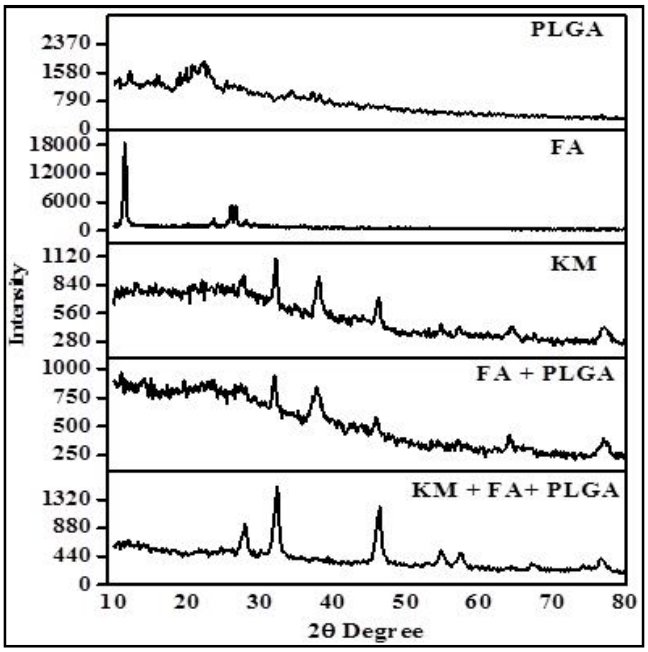

Fig. 2. XRD spectra of combinatorial nanoparticles, PLGA, FA (Folic acid), KM (Kaempferitrin), FA+PLGA and KM+FA+PLGA.

(Makadia & Siegel, 2011). The XRD of PLG+FA and KM+PLGA+FA were differing from KM, because of the full range of encapsulation of KM with nano compounds and composed of non crystalline solids.

The pure kaempferitrin shows 2θ values of diffraction peaks at 32.2°, 38.1°, 46.28°, 54.74°, 64.46°, and 77.08°. For PLGA there was no sharp peak observed, which shows amorphous nature of biodegradable polymer PLGA. In folic acid (FA), the diffraction peaks at 11.5°, 23.64° and 26.12° were observed. In PLGA+FA nanoparticles the 2è values at 32°, 37.8°, 46°, 64.14° and 77.12° were observed. PLGA+ FA+ KM nanoparticles showed diffraction peaks at 27.88°, 32.3°, 46.38°, 54.76°, 57.76°, 67.18° and 76.68° Fig. 2.

The selection of PLGA nanoparticle desirable formulation mainly depends on increased encapsulation, identical size distribution, spheroid morphology, high zeta potential value and high drug loading capacity. In the crrent study to choose finest formulation, we used kaempferirin (KM), folic acid (FA) and PLG in the following combination, KM+FA, KM+PLGA and KM+FA+PLGA. Ultimately, the KM+FA+PLGA combination emerged as the most desirable formulation.

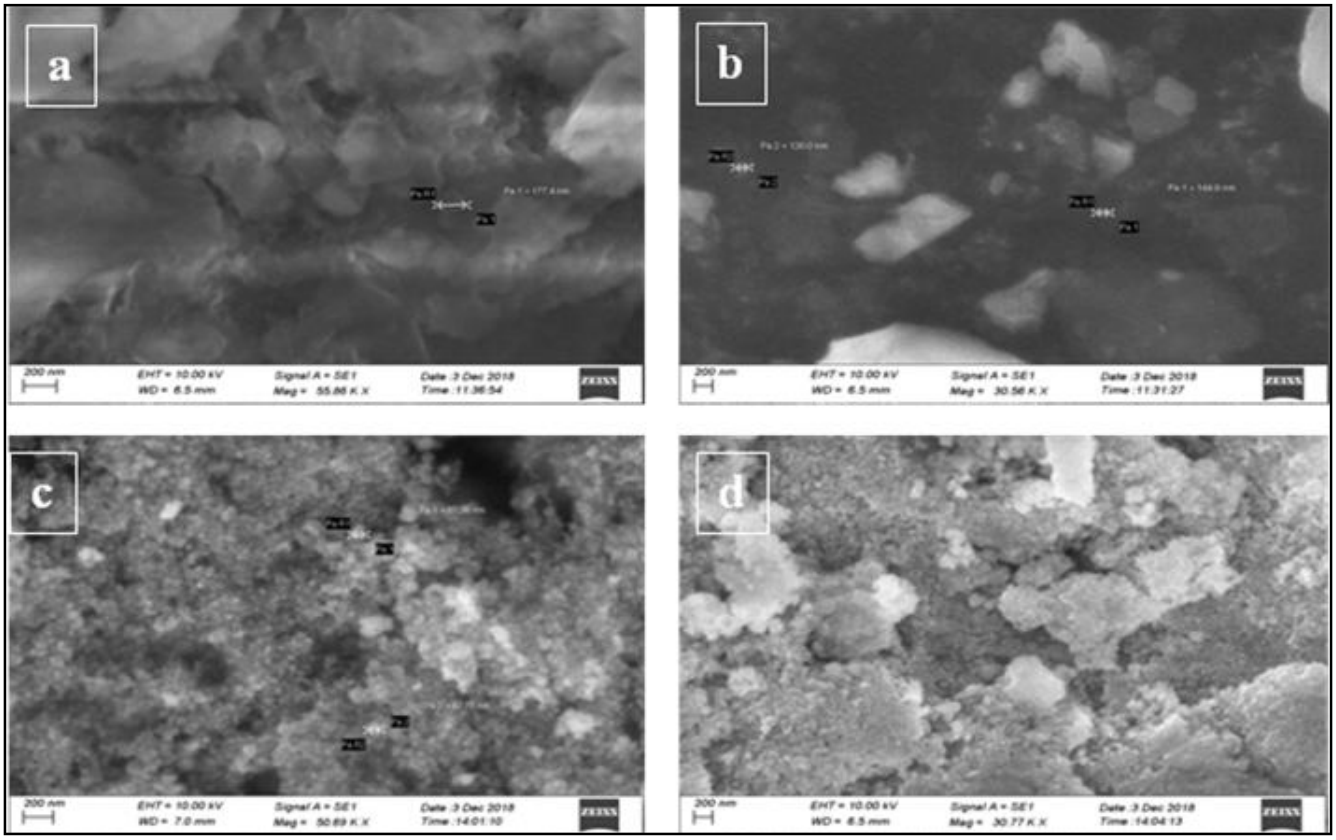

Fig. 3: Scanning electron microscopy images showing the surface morphology of (a) Kaempferitrin Nps prepared by nano-precipitation, (b) PLGA-loaded Nps prepared by nano-precipitation (c) Kaempferitrin-folic acid Nps (d) KM+FA+PLGA-NPs.

The formulation stability, drug release and cellular uptake were determined by the size of nanoparticles and zeta potential.

SEM

The shape of surface KM+FA+PLG-nanoparticles was characterized by scanning electron microscopy. The spherical morphology was found under the scanning electron microscopy which is shown in the Fig. 3. The KM+FA+PLGA-Nps showed spherical shaped particles with sizes ranging between 87.36 and 144.9 nm.

TEM

The structure of surface of KM+FA+PLG-nanoparticles was characterized by microscopic analysis. Different magnification of TEM images was depicted in Fig. 4. The KM+FA+PLGA-NPs were found to be spherical in morphology and average size of NPs ranged from 5.0 to 20.0 nm. The entrapment efficiency was found to be ~68.57% with % DL of ~0.256%.

MTT assay

The MTT assay of KM+FA+PLGA-NPs formulations treatment group is showed in Figure 5. Due to the robust structure, KM+FA+PLGA-Nps were chosen for the *in vitro* cytotoxicity study. To enhance the intracellular uptake of the particles, the surface of KM+FA+PLGA-Nps was functionalized by FA, cancer cell targeting. The existence of FA on the outermost layer of KM+FA+PLG-nanoparticles was confirmed by the decrease of absolute zeta potential of nanoparticles. This might be because of some carboxyl groups (-COO-) from kaempferitrin reacted with amino moieties of FA during

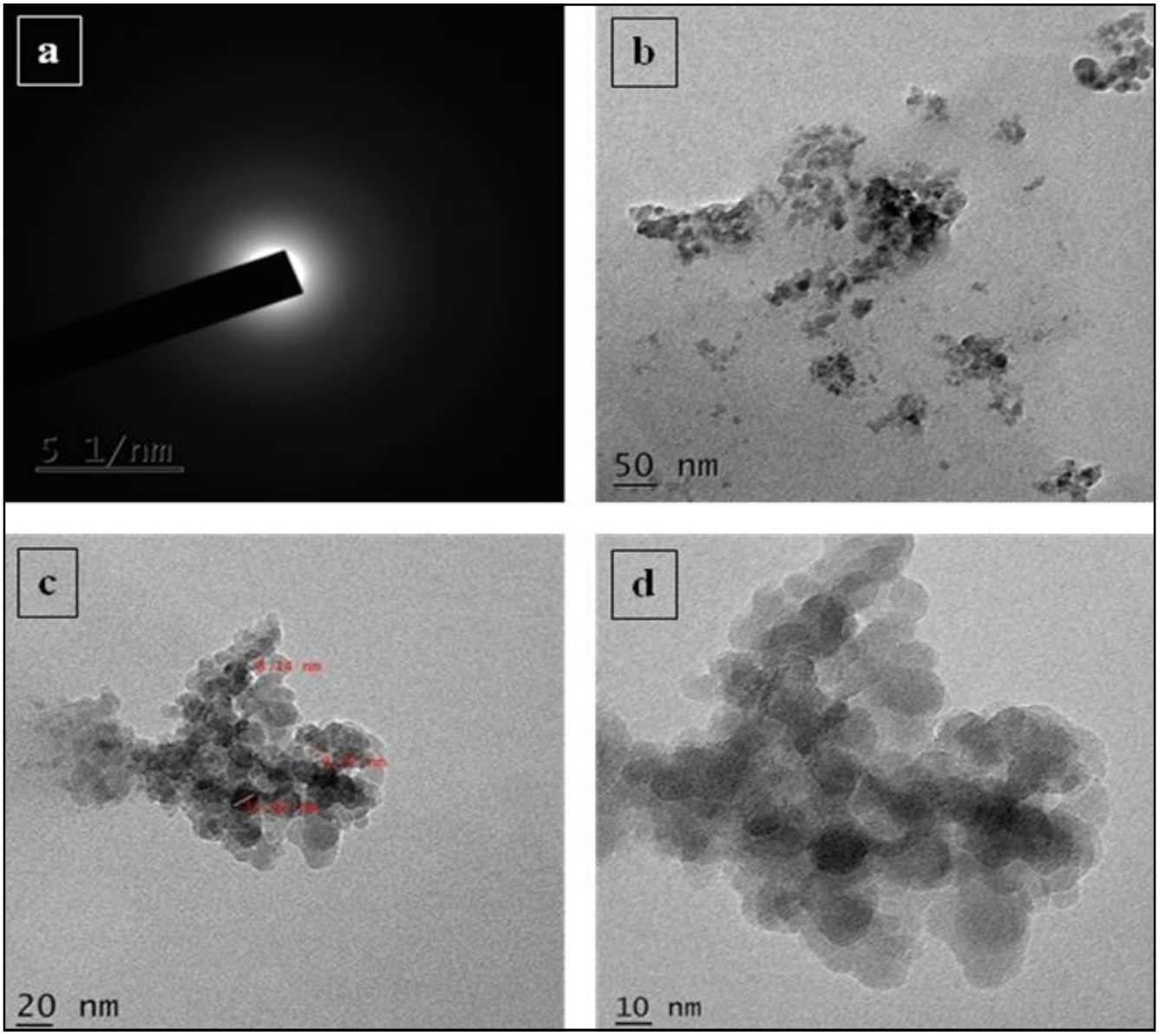

Fig. 4: TEM images of Kempferitrin-Folic acid- PLGA nanopartiles.

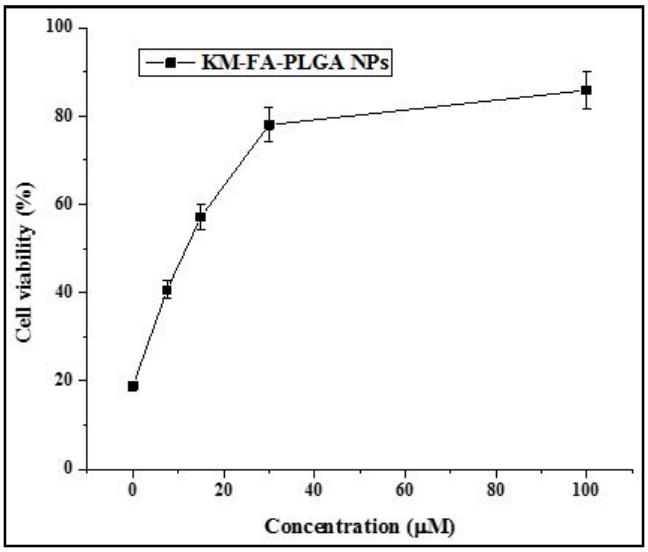

Fig. 5: MTT assay of HT-29 cells activated by KM+FA+PLGA nanoparticles.

conjugation. The number of viable HT-29 cells after incubating with KM+FA+PLGA-Nps was determined by the MTT assay, a bio-reduction of tetrazolium salt, as mammalian cells can generate a colored formazan product that strongly absorbs light at wavelength of 490 nm.

Apoptosis

Apoptosis (programmed cell death) is an important progression in cell toxicity induced by drugs. Apoptosis was detected by the Annexin V/PI double staining using flow cytometry. A significant increase of apoptotic cells were observed in KM+FA+PLGA-Nps exposed cells Fig. 6. The percentage of apoptotic cells significantly increased in KM+FA+PLGA-Nps exposed cells when compared to the control cells.

Discussion

KM+FA+PLGA-Nps were formulated with folic acid and PLGA was used as the major resource as the features of folic acid, PLGA, PVA and KM+FA+PLGA-Nps were biologically compatible and degradable (Fattahi *et al*., 2019). The particle size distribution of FA formulated PLGA nanoparticles were higher than non-formulated PLGA nanoparticles (Zhang *et al*., 2017). Zeta potential value -6.28 ± 0.37, -15.0 ± 0.41 and -23.4 ± 0.56 were observed. The negative zeta potential value may be the existence of ionized carboxyl groups of PLGA segments (Lince *et al*., 2008). The smaller particle size of >100 nm is advantageous to drug accumulation on cancer site. Formulated nanoparticles might be prohibited by diffusion process (Liu *et al*., 2018). Scanning electron microscope revealed that folic acid and PLGA were well dispersed into kaempferitrin samples. Our results revealed that KM+FA+PLGA-Nps were spherical in morphology.

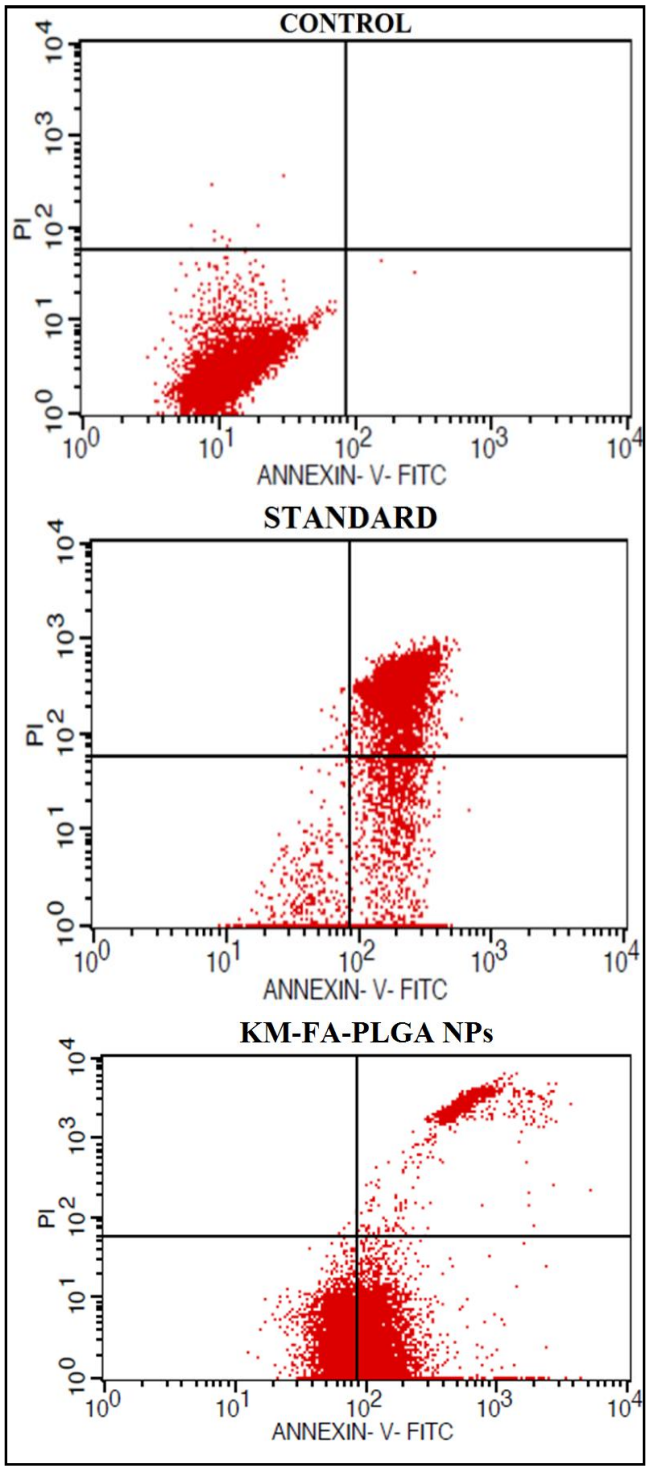

Fig. 6: Effect of KM+FA+PLGA-Nps in HT-29 cells. HT-29 cells were treated with control, standard (5-fluorouracil) and KM+FA+PLGA-Nps to induce apoptosis.

The drug encapsulation efficiency was increased with decreased cytotoxicity. The use of folic acid formulated (KM+FA+PLGA) nanoparticles may improve drug loading efficiency and cellular uptake (Chronopoulou *et al*., 2013; Chen *et al*., 2016). The cellular uptake effectiveness of KM+FA+PLGA-Nps, kaempferitrin was

used as a major molecule, which can be formulated into diverse nano-carriers. The results of cellular uptake indicate FA modified KM+PLGA-Nps, FA play a major part in the absorption of medicines in HT-29 cells *in vitro*. Increased incorporation of kaempferitrin formulated NPs towards the cells could increase the anti-tumor efficiency, which may lead to inhibition of cellular growth, initiation of cell death, and arrest cancerous cell pathways. *In vitro* toxicity of KM+FA+PLGA-Np was investigated by MTT assay. KM+FA+PLGA-Nps showed more active cancer cell inhibition than the other samples (Karthi *et al*., 2016). The folic acid formulation increased the capability to hold the cell membrane, and improve the intracellular drug accumulation and play a major role in cancer therapy.

Apoptosis is a major physiological process of cell death and it plays an important role in elimination of cancer cells. *In vitro* cell death was evaluated by FCM through Annexin V/FITC (Oguz *et al*., 2013). The apoptosis rates were observed after KM+FA+PLGA-NPs exposure at 24 h. The KM+FA+PLGA-NPs exposure induced an increased number of cell apoptosis when compared to standard. The results indicated that KM+FA+PLGA-NPs could induce apoptosis which shows that biologically modified biodegradable particles may be used as a potential nano-carrier system to improve the pharmaceutical activity of kaempferitrin.

Conclusion

In our current study, biologically degradable PLGA nanoparticles were formulated with folic acid and characterized to improve the anticancer drugs. Particle size distribution of formulated KM+FA+PLGA-Nps was advantageous (<100 nm) which also confirmed morphologically spherical particles under the TEM analysis. KM+FA+PLGA-Nps established extensively high cytotoxicity in HT-29 colon adenocarcinoma cell lines. The improved cytotoxicity activity was being mediated by high selectivity and thus considerably induced the cell apoptosis of colon adenocarcinoma HT-29 cell lines. Therefore, KM+FA+PLGA-Nps can be used as promising drug carrier to deliver drugs for cancer therapy thereby improving the anti-cancer efficacy.

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