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Quercetin loaded PLGA microspheres induce apoptosis in breast cancer cells

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ABSTRACT

Quercetin is well known for its effective inhibition of cancer cell proliferation by inducing the apoptosis. In the present study, quercetin isolated from *Allium cepa* were loaded on a biodegradable polymer, poly(lactic-*co*-glycolic acid) (PLGA) microspheres and its apoptotic potential in Michigan Cancer Foundation (MCF-7) cell was investigated. PLGA microspheres before and after loading indicated no significant difference in size as the average size of the microspheres were 100 µm. The encapsulation efficiency of quercetin on PLGA was found to be 74 ± 1.2%. Cytotoxicity and flow cytometric analysis were used to evaluate the capacity of quercetin-PLGA microspheres (PLGAq) on anticancer activity. PLGAq were assessed at two different concentrations (1.5 µg and 3.0 µg) and indicated that at higher concentrations it showed effective inhibition of cancer cells. The results indicated that the PLGAq microspheres efficiently inhibit cell proliferation by inducing the apoptosis demonstrating that the biodegradable PLGAq microspheres will provide a promising therapeutic approach for the treatment of breast cancer.

1. Introduction

Biodegradable polymers are primarily used in targeted drug delivery system as vehicles and tissue engineering scaffolds for comfortable growth of cells [1]. Depending on the function, polymers are fabricated by varying chain compositions, mechanical strength, and morphology [2]. Controlled release is an effective methodology to optimize the dosage of drug released to a particular site in a sustained way [3,4] and this could be achieved by using various polymers. Due to various side-effects associated with the currently available anti-cancer therapies, a plethora of attention has been focused on developing drugs that directly inhibit cancer cells without affecting the normal cells. Poly (lactic-*co*-glycolic acid) (PLGA) is one such polymer prepared either as scaffolds or microspheres for medical resorbable sutures and delivery vehicles [5]. Several techniques have been developed for the preparation of PLGA including water-oil-water [6], phase separation [7] and nano precipitation [8]. PLGA was found to be an effective alternative as it physically interacts with hormones and facilitates the transport of drug [9]. PLGA loaded with sulforaphane have been used as an injectable delivery system for treating osteoarthritis by delaying the progression of surgically-induced osteoarthritis in rats [10]. Encapsulation of drug such as curcumin in PLGA nanoparticles increases the bioavailability which is very crucial in targeted drug delivery systems [11,12]. Further, PLGA have been used to study its response on treating brain glioma by conjugating Transferrin-magnetic silica PLGA nanoparticles loaded with doxorubicin and paclitaxel [9]. These studies clearly indicate that PLGA has a spectrum of use in biomedical field which attracts researchers in drug delivery studies. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) belongs to the family of poly phenolic flavonoid compound and is present in all edible fruits and vegetables [13]. It has a wide range of biological activities such as anticancer, anti-oxidant, anti-inflammatory and anti-diabetic activity [14]. Despite its potential, the efficacy is limited primarily due to its water insolubility and chemical instability that ultimately lead to poor bioavailability of this compound [15,16]. Therefore, it is vital to develop alternative solutions for improving its bioavailability [17]. Almedia and co-workers have studied the bioavailability of quercetin and summed up the importance in a detailed work as the concentration of quercetin

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play a crucial role in human metabolism [18]. Previous studies have indicated that quercetin at low concentration induces mitochondria-mediated apoptosis in HeLa cells by activating the p53 gene [19] and reduces the cytotoxic side effects of doxorubicin in non-tumor cells [20]. Quercetin-loaded PLGA-D-α-tocopheryl polyethylene glycol 1000 succinate particles have been used to study targeted drug delivery against liver cancer and the authors found that it suppress tumor growth by 59.07% [21]. Quercetin also down-regulate the phosphatidylinositol and phosphatidylinositol phosphate kinase activities in human carcinoma cells that lead to a significant reduction in inositol triphosphate concentration and cell death [22]. It is also used to treat carcinomas by down-regulating signal transduction capacity [23]. Cellular uptake of PLGA loaded quercetin were studied using human triple negative breast (MDA-MB-231) and larynx epidermoid carcinoma (HEp-2) cell lines decreased G2/M population demonstrating the apoptotic potential and cellular arrest of cancer cells [24]. These examples clearly indicate the presence of potent anti-cancer properties in quercetin.

In the present investigation, we have studied the anti-cancer activity of quercetin loaded PLGA particles on Michigan Cancer Foundation cells (MCF-7). We have demonstrated that the quercetin-PLGA microspheres (PLGAq) efficiently inhibit cell proliferation by inducing the apoptosis, which shows that the biodegradable PLGAq microspheres will be a promising therapeutic approach for the treatment of breast cancer.

2. Materials and methods

2.1. Chemicals

Poly(lactic-*co*-glycolic acid) (PLGA) (50:50), polyvinyl alcohol (PVA) was obtained from Sigma Aldrich, US. Fluorescein isothiocyanate (FITC)-labeled Annexin V, propidium iodide (PI) kit were obtained from Novobiochem, US. Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were purchased from Invitrogen, US. All other reagents and solvents used in the reaction were of analytical grade and obtained from Merck, India.

2.2. Isolation and purification of the compound

Allium cepa leaves were dried and finely powdered to obtain extract using soxhlet apparatus. The crude residue was filtered, evaporated and treated with hexane to remove fat and oil. The hexane extract was resolved using silica gel column chromatography (100–200 mesh, Merck, toluene, ethyl acetate, methanol gradients) and 30 fractions were collected. Thin layer chromatography (TLC) was prepared using Silica gel 60 F 254 (Merck, Darmstadt, Germany) precoated plates ($10 * 10$ cm) with the solvent system toluene: ethyl acetate: methanol in the ratio of 5:3:2 (v/v/v). From 1000 µg/mL of crude quercetin samples around 10 µL and 20 µL of the samples were applied to the precoated thin layer chromatography (TLC) plates. The movement of the samples was found to be ascending and moved up to 6 cm. The developed plates were scanned under UV Transilluminator at 264 nm. Column chromatography was performed using different concentrations of elution solution and the column was eluted in the increasing order of polarity of solvent from 100% of extract, 25% of ethyl acetate in hexane, 50% of ethyl acetate in hexane, 75% of ethyl acetate in hexane and 100% of ethyl acetate. Then the polarity was increased using 2% methanol in ethyl acetate to 50% methanol in ethyl acetate. 8% of methanol and 92% of ethyl acetate concentration was found to be the optimal concentration after concentrating all fractions. Purified quercetin was separated and stored for further use.

2.3. Synthesis of the PLGA microspheres

Quercetin loaded microspheres were prepared using an oil-in-water emulsion process following the standard procedure [25]. Briefly, PLGA-Quercetin (PLGAq) microspheres were prepared by dissolving PLGA (50 mg) and quercetin (10 wt% polymer) in ethyl acetate (500 µL) and the mixture allowed to dissolve for 45 min under stirring was considered as the dispersed phase. PVA aqueous solution (5%) was considered as continuous phase (2 mL). The dispersed phase was added dropwise to the continuous phase under constant stirring. The resulted emulsion was mixed into 0.3% aqueous PVA solution (25 mL) and kept undisturbed for 4 h. At the end of the reaction, PLGAq microspheres were collected by centrifugation at 8000 rpm for 15 min and washed three times with distilled water. After complete removal of the solvent, the beads were freeze-dried and stored for further use. The aforementioned procedure was used for preparing bare PLGA microspheres without quercetin.

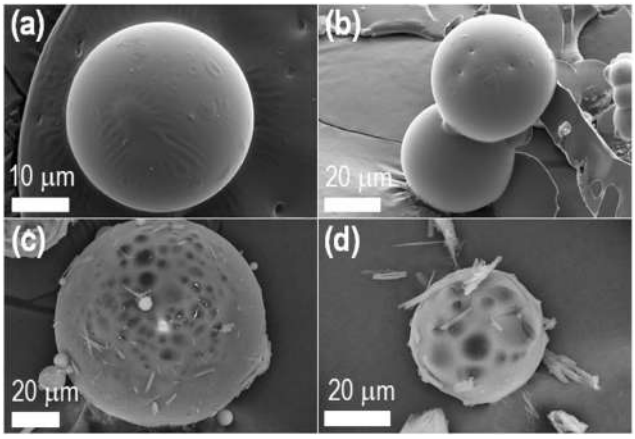

Fig. 1. Scanning electron micrographs obtained for PLGA (a–b) and quercetin loaded PLGA (PLGAq) microspheres (c–d).

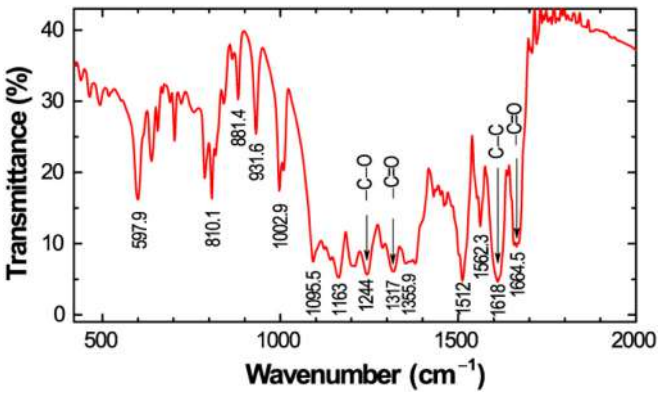

Fig. 2. FTIR spectra obtained for quercetin isolated from *Allium cepa*.

2.4. Characterization of microspheres

The surface morphology of PLGA and PLGAq was assessed using Field Emission Scanning Electron Microscope (FESEM-SUPRA 55) Carl Zeiss, Germany. PLGA and PLGAq microspheres were further characterized using IR Affinity-1s Fourier transform infrared spectroscopy (FTIR) Shimadzu, Japan. To ascertain the structure, ^{1}H and ^{13}C NMR spectra were measured by mixing with dimethyl sulfoxide (DMSO-d_6) and recorded at 800 MHz Bruker Spectrometer (Avance III, US[2]).

2.5. Encapsulation efficiency (EE%)

Encapsulation efficiency of the drug loaded microspheres was tested by centrifugation of PLGAq microspheres at 12,000 rpm for 20 min. The free quercetin in the supernatant was measured by UV 1800

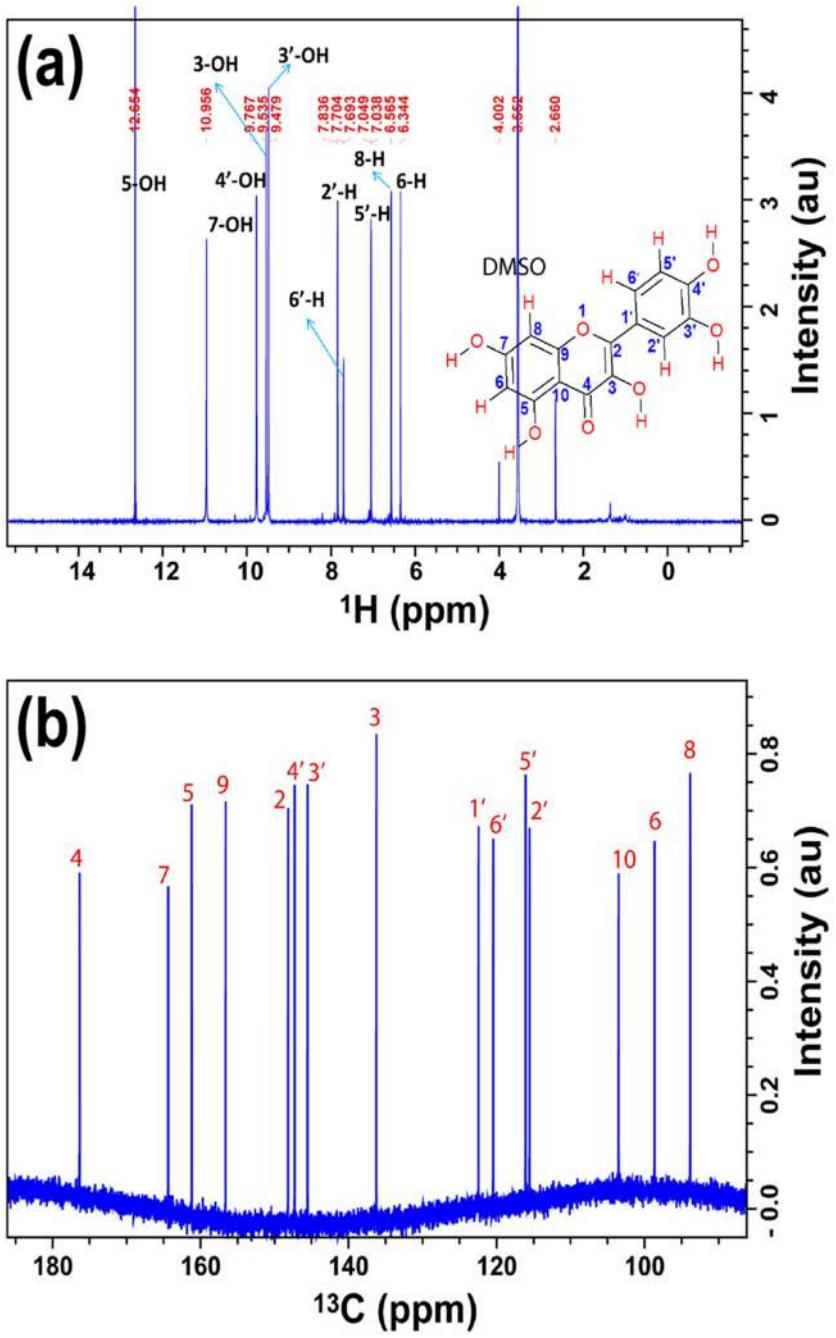

Fig. 3. 800 MHz 1D ^{1}H (A) and ^{13}C (B) NMR spectra of quercetin.

spectrophotometer (Shimadzu, Japan) at 372 nm. Encapsulation efficiency of microspheres was calculated using the formula

%Encapsulation Efficiency (EE%) = (Total amount of quercetin added − Amount of free quercetin in supernatant) /Total amount of quercetin added × 100

The quercetin content was calculated by using a linear regression equation against the standard curve of quercetin [26].

2.6. In vitro drug release profile

To study the drug release kinetics, PLGAq was investigated at normal physiological condition (pH 7.4) and tumor cell condition (pH 5.3, *i.e.* acidic environment). Briefly, PLGAq were incubated in 10 mL of medium containing 10% polyethylene glycol (PEG 400) (*v*/v) in phosphate buffer saline (PBS) and kept on an orbital shaker (Lark, India) at 100 rpm. The supernatant was measured at 372 nm and the amount of quercetin released from PLGA microspheres were calculated using a calibration plot.

2.7. In vitro cytotoxicity of PLGA microspheres.

2.7.1. Cell line and culture medium

The human monocytic cell lines (THP-1) were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were cultured under standard Rosewell Park Memorial Institute medium (RPMI), containing 10% FBS, 100 U/mL of penicillin and streptomycin at 37 °C with 5% of CO_2 incubator. The cells were sub-cultured after attainment of confluence.

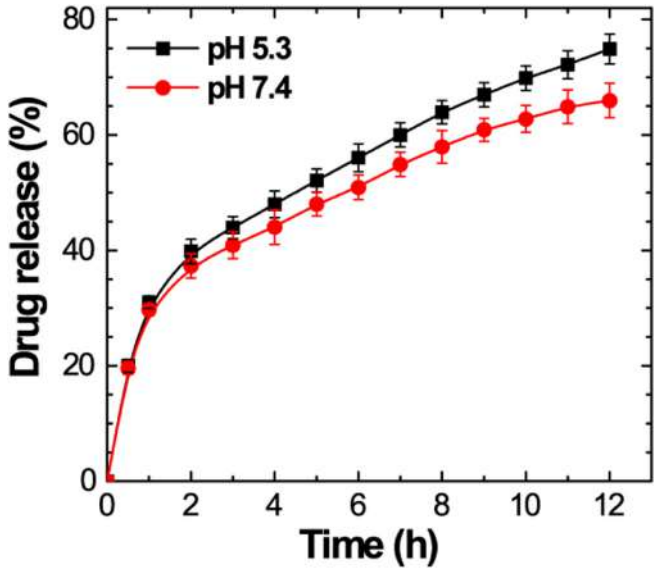

Fig. 4. *In vitro* drug release kinetics of quercetin release from PLGA microspheres under normal and tumor micro environment.

2.7.2. Cytotoxicity assay in THP-1 cell lines

The cytotoxic effect of PLGAq on THP-1 cells was tested by assessing the mitochondrial dehydrogenase activity using standard method [27]. Briefly, cells (5×10^3 cells/well) were incubated with different concentrations of PLGAq (0.625–10 µg/mL) in 96-well plates for 24 h in a humidified 5% CO_2 at 37 °C. After that, the culture media were removed and incubated with 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in fresh medium at 37 °C for an additional 4 h. After this, the supernatants were removed and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The plates were read on a microplate reader using MultiSkan GO, Thermo Scientific, US at a test wavelength of 540 nm and a reference wavelength of 650 nm [27].

2.8. In vitro anti-cancer activity

2.8.1. Cell lines and cell culture conditions

Michigan cancer foundation cell lines (MCF-7) were obtained from NCCS, Pune, India. The cells were cultured under standard conditions in Dulbecco's Modified Eagle's Medium (DMEM) medium, supplemented with 10% of fetal bovine serum, 100 U/mL of streptomycin and 100 U/mL of penicillin in a humidified incubator set at 37 °C with 5% of CO_2. The cells were subcultured after formation of monolayer and were removed by treating with trypsin (0.25% trypsin containing 0.01% EDTA) for 2 min and washed with PBS for further analysis [28].

2.8.2. Anti-proliferation activity using MCF-7 cell lines

The anti-proliferation activity of PLGA and PLGAq on MCF-7 cells was assessed by measuring the activity of mitochondrial dehydrogenase as described previously [28]. Briefly, cells (5×10^3 cells/well) were incubated with various concentrations (0.6–10 µg/mL) of PLGA and PLGAq, the culture media were removed and cells were incubated with 5 mg/mL MTT in fresh medium at 37 °C for an additional 4 h. After that, the supernatant was removed and 100 µL DMSO was added to each well to dissolve the formazan crystals. The plates were read on a microplate reader using (Thermoscientific, US) at a test wavelength of 540 nm and a reference wavelength of 650 nm [28].

2.9. Annexin V/propidium iodide double staining assay

Annexin V-FITC apoptosis detection kit (Calbiochem, CA, US) was used to determine the number of apoptotic cells. According to the manufacturer instruction, the treated (MCF-7) cells ($1–2 \times 10^5$ cells/well) were incubated at 37 °C with 5% CO_2 for 24 h in the presence of PLGA and PLGAq (1.5 and 3.0 µg/mL). Cells were trypsinized, washed twice with HBSS, suspended in 100 µL Annexin V binding buffer and then incubated for 20 min in 5 µL of Annexin V fluorescein isothiocyanate (FITC) 1 µL of 1 mg/mL propidium iodide solution was added and run immediately in flow cytometer (FACS Jazz Becton Dickinson, US).

3. Results and discussion

3.1. Scanning electron microscopy

Fig. 1(a–d) shows the SEM micrographs of PLGA and PLGAq. It is evident that spherical shaped particles were observed for PLGA and particles with a high degree of porosity were seen on PLGAq microspheres. Similar results have been obtained in a study where periyl alcohol-bearing PLGA microparticles were prepared by oil-in-water-based emulsion solvent evaporation technique [29]. Porous and non-porous PLGA microspheres prepared by oil-in-water emulsion technique showed that the porous microspheres were bigger whereas the non-porogen microspheres were found to be smaller in size [30,31]. Studies on that PLGA particle containing paclitaxel showed spherical shaped particles with varying degrees of smoothness on the surface [32].

3.2. Infrared and nuclear magnetic resonance spectroscopy

Fig. 2 illustrates the FTIR spectra obtained for quercetin. Peaks at 1664 cm^{-1} indicate the C=O absorption and 1618 cm^{-1} band indicate C—C stretching. The peak at 1244 cm^{-1} can be attributed to the –C–O stretching of the oxygen in the ring. Likewise, the –C–OH– deformation vibrations were seen at 1317 cm^{-1} and the peaks observed in the region 1163 to 1002 cm^{-1} accounts for the C—O stretching of the molecule. These observations comply with the previously published spectra by various researchers indicate its purity [33]. In order to ascertain the 5,7,3′,4′-tetrahydoxyflavonol structure of quercetin, the ^1H and ^{13}C NMR spectra of the isolated and purified compound were recorded in DMSO-d_6 and compared with the previously reported ^1H and ^{13}C spectra in terms of chemical shifts, coupling constant and overall spectral patterns. Importantly, the ^1H and ^{13}C NMR spectral features were completely matched with the ones previously reported for the quercetin [34]. The resulted NMR assignments of all the protons and carbon chemical shifts are shown in Fig. 3a and b, respectively. Further, the presence of a single set of peaks corresponding to quercetin resonances reflected that the compound had been isolated in a highly purified form.

3.3. Encapsulation efficiency (EE) and drug release kinetics

Encapsulation efficiency was assessed to check the loading of quercetin on PLGA and it was found that the percentage of encapsulation is 74 ± 1.2. From the results, it is evident that the quercetin has been loaded efficiently onto PLGA microspheres. *In-vitro* release profile of PLGAq was assessed and it was found that there was an initial burst followed by sustained release of drug was observed which is usual in the case of biopolymers. After the initial burst, there was a sustained release of drugs which could be attributed to the exposing of core microspheres after a period of time which will help in prolonged delivery of drug at the cancer site. We found that at normal pH (7.4) the amount of quercetin release was 67.81% in 12 h with an initial burst release of 29.36% within 1 h (Fig. 4). Similar results have been obtained when paclitaxel was loaded onto PLGA and used for sustained release of vitamin E [32]. When tested at pH 5.3, quercetin release was more efficient when compared with normal physiological pH with a cumulative drug release percentage of 72.21% in 12 h.

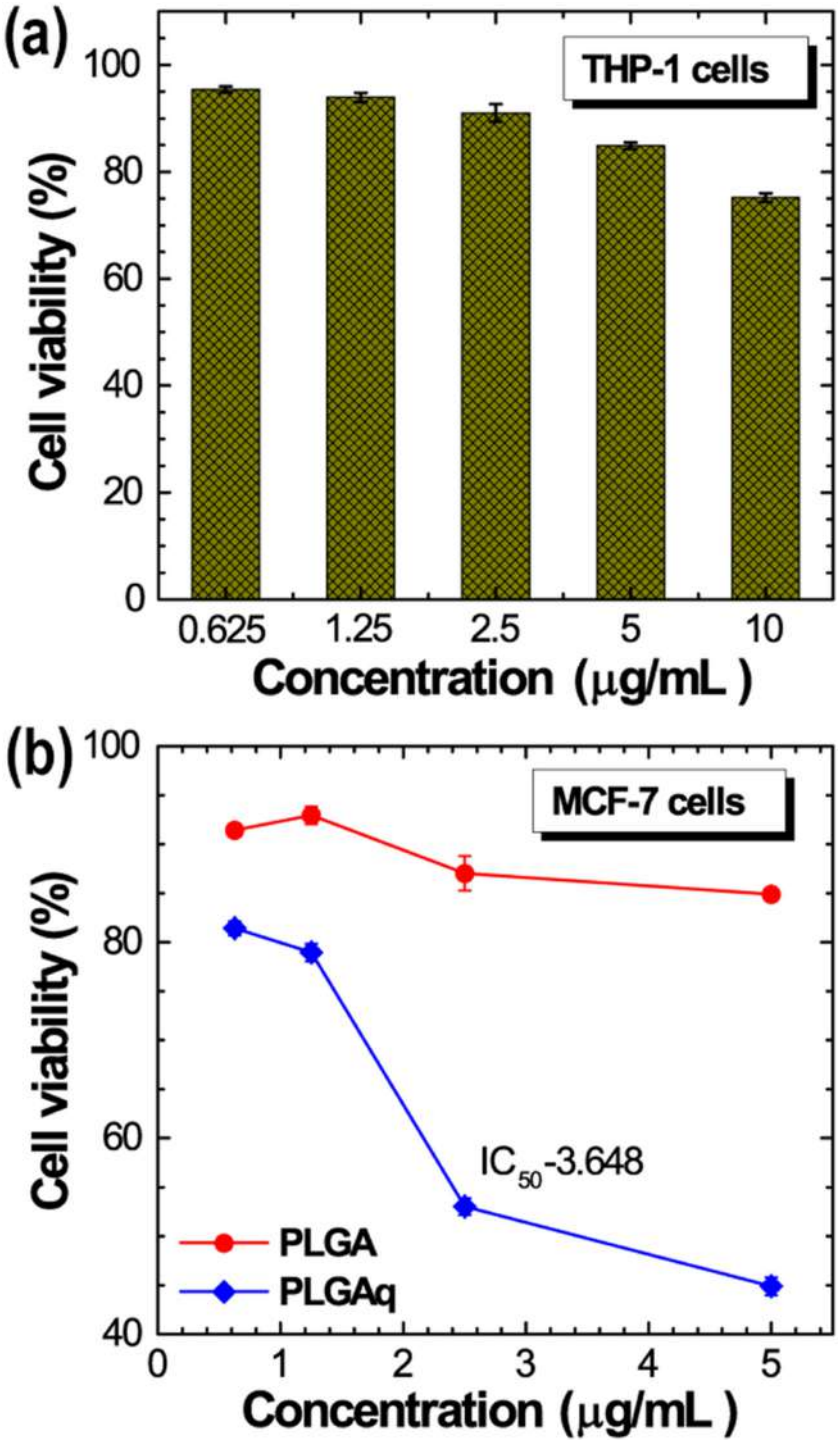

Fig. 5. (a) Cytotoxicity assay performed using THP-1 cell lines and (b) cytotoxicity of PLGA and PLGAq on MCF-7 cell lines.

3.4. Cytotoxicity assay

PLGAq microspheres were tested for its cytotoxicity against THP-1 cell lines and from the results, we could assess that PLGAq did not affect the normal cells (Fig. 5a). Further, the PLGA and PLGAq were tested for their anti-cancer activities against the MCF-7 lines (Fig. 5b). It was found that the carrier has very less or a negligible amount of anti-cancer activity in comparison to the drug-loaded carrier displayed excellent anti-cancer activity by decreasing the cell viability to 40% in the range of concentration of 0–2 μg/mL. The inhibition concentration (IC_{50}) value was found in the concentration of 3.648 μg. So comparing the above, cytotoxicity of PLGA in normal cell lines and cytotoxicity of the PLGAq in the cancer cell lines the range of 1.5 μg and 3.0 μg concentrations was found to be optimum for anti-cancer activity. Studies revealed that cell viability assay in pancreatic cell lines treated with various concentrations of free curcumin and nanocurcumin [35]. It is well known that the quercetin enhances anti-cancer activity of trichostatin A up-regulating p53 protein [36]. Similarly, a study carried on PLGA-curcumin nano formulation on MCF-7 breast cancer cell lines indicated high toxicity of particles to cancer cells [11]. Other studies have also indicated that cell proliferation has decreased significantly in PLGA-curcumin formulation when subjected to human colon cancer cell

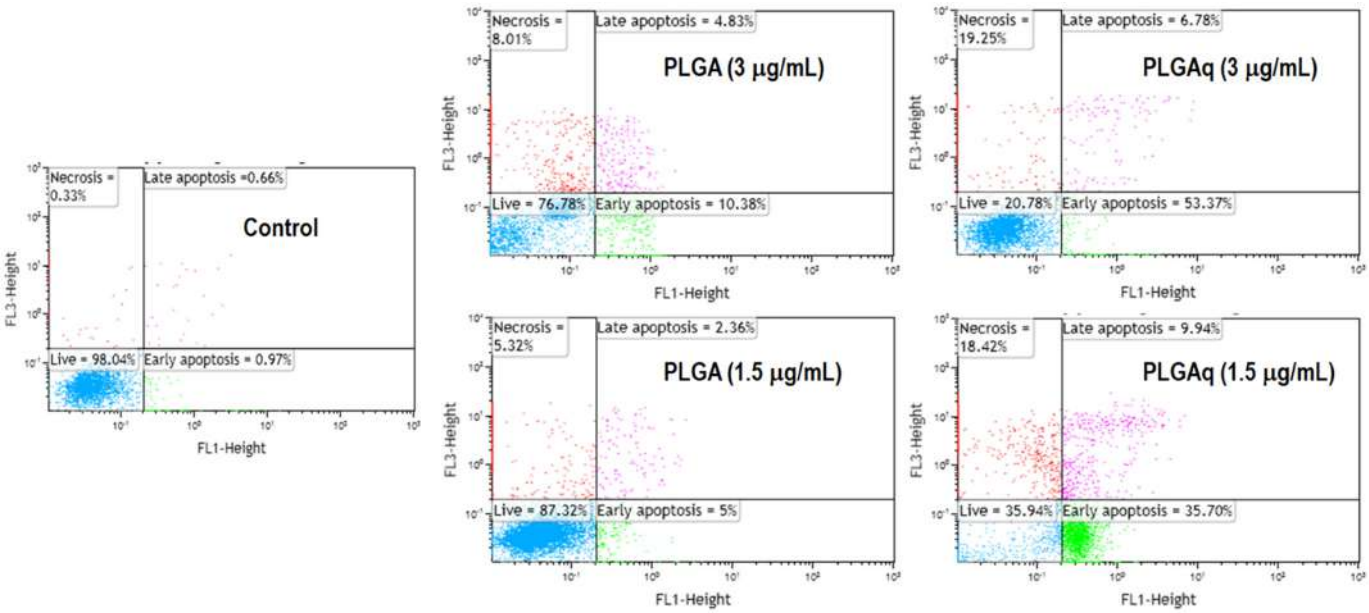

Fig. 6. Effect of PLGAq on induced apoptosis in MCF-7 cells using Annexin V-FITC and PI double staining method.

Table 1
Quercetin loaded with various carrier material for anticancer studies.

S. no	Drug	Cancer cell	Reference
1	PEG conjugated quercetin-PLGA	Cervical-tumor-derived cell line HeLa	[48]
2	Quercetin loaded chitosan	Human lung cancer cell line A549 and breast cancer cell line MDA-MB 468	[26]
3	Quercetin-loaded PLGA-D-a-tocopheryl polyethylene glycol 1000 succinate	Hepatocellular carcinoma cell line HepG2	[21]
4	PLGA-quercetin	Lung adenocarcinoma cell line A549	[49]

lines [37].

3.5. Annexin V/propidium iodide double staining assay

Apoptosis is the mechanism of programmed cell death which occurs in eukaryotic organisms [38] has enhanced research on detecting subcellular lesions at the single-cell level [39,40]. During early apoptosis, membrane blebbing of cells occur and phosphatidyl serine comes out from the inner side of the plasma membrane to the outer membrane leaflet [41]. It is then exposed to the available fluorochrome-labeled recombinant soluble Annexin V which is a useful tool for detecting and quantifying early apoptotic cells by flow cytometry [42,43]. Fig. 6 indicates the effect of bare and drug-loaded carrier microspheres on the rate of apoptosis in the in MCF-7 cell lines where cancer cell lines without PLGA were considered as control. PLGA was tested in the assay in two different concentrations which were mentioned above 1.5 µg and 3.0 µg. The summation of early and late apoptosis was just 5% and 2.36% in the concentration of 1.5 µg and in the concentration of the 3.0 µg it was found to be 10.38% and 4.83% in PLGA microspheres. In case of PLGAq at 1.5 µg concentration, 35.70% and 9.94% of apoptosis (early, late) cells were observed. However, when the concentration was increased to the 3.0 µg the apoptosis was (early, late) found to be 53.37% and 6.78%. The necrotic cell population was also observed with an increase in the concentrations of PLGAq and live cells significantly decreased after treatment. So it is concluded that PLGA in various concentration (1.5 µg and 3.0 µg) does not show any significant apoptosis but PLGAq showed elevated apoptotic activity by increasing the concentration of drug from 1.5 µg to 3.0 µg. We believe that the activity of PLGAq could be due to the inhibition of cell cycle progression as quercetin as an excellent inhibitor of the cell cycle and thereby cell death [44]. It is well known that quercetin induces apoptosis by activating caspase-3 and regulating B cell lymphoma-2 (Bcl-2) and cyclooxygenase-2 pathways as Niu and co-workers have studied its effects on human leukemia cell lines [45,46]. These results suggest that apoptosis induction by PLGAq confirms its direct antitumor effect on breast cancer cells. Studies indicate that the viability of cancer cell decreased when it was treated with nanoparticles in different concentration and different stages of apoptosis were also observed [28,47]. Similarly, low concentration of curcumin PLGA formulation is far more effective than native curcumin and showed enhanced anti-cancer activity by initiating apoptosis on human colon cancer lines [37]. Effects of quercetin on various *in vitro* and *in vivo* conditions have been indicated in Table 1. These studies and our current work suggest that the enhanced action of PLGAq could help in developing novel agents using PLGA in anticancer studies.

4. Conclusion

In this study, the effect of quercetin-loaded PLGA microspheres on cancer cell lines has been evaluated. Quercetin isolated from *Allium cepa* were purified and used as a drug for loading onto PLGA microspheres. The electron micrographs of bare and loaded microspheres suggest that the particles were found to have spherical shaped structures for bare microspheres and loaded PLGA microspheres with pores on the surface suitable for drug delivery studies. The quercetin loaded PLGA showed excellent anti-cancer activities on MCF-7 cells when tested using Annexin-V FITC/PI staining. These results suggest that quercetin loaded microspheres can be a potent anticancer agent.

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