BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e19801

Article

In vitro release mechanism and cytotoxic behavior of curcumin loaded casein nanoparticles

Josphine Jenifer[1], Ravi Theaj Prakash Upputuri[1]*

[1]*PG Department of Bioscience, CMR Institute of Management Studies, Karnataka, India*

In the recent past, drug delivery through nanoparticles is considered an effective tool to treat various diseases. Biopolymeric nanoparticles such as protein based nanoparticles have vital role as drug carrier as it is non-antigenic, and easily biodegradable. Curcumin, plant polyphenolic anticancerous compound was loaded into the casein nanoparticles by coacervation method. Particle size and surface charge of spherical casein nanoparticles as observed to be 201.4 nm and -86.9 mV. The loading efficiency of curcumin loaded casein nanoparticles was found to 85.05 %. *In vitro* drug release was performed at different pH (7.4 and 3.0), and the cumulative release was observed to be 24.8 and 20.13% and at different temperatures (25°C and 37°C), the cumulative release was observed to be 24.8 and 28.60 % respectively in 48 h. Curcumin release from casein nanoparticles was shown to be in a steady, and prolonged rate. The nanoparticles were observed to have an effective antimocrobial activity than curcumin in free form. The drug loaded casein nanoparticles were found to be potent particles to protect cells from hydrogen peroxide and UV light damage. The cytotoxic activity of nanoparticles on MCF7 and A549 cells were assayed and was observed to have an IC50 value of 609 and 825.2µg/ml. Cell death was observed to be through apoptosis, accompanied by DNA fragmentation.

KEYWORDS: Curcumin. Casein nanoparticles. Loading Efficiency. MTT assay. Apoptosis**.**

INTRODUCTION

Nanoparticle based drug delivery systems is being established as a novel method for treating various diseases. These nanoparticles have role in controlled drug delivery and in eliminating unwanted side effect caused by drug, breakdown and clearance of drug by enzymes (Nevozhay, *et al.*, 2007; Wilczewska, *et al.*,2012). Biopolymeric nanoparticles such as proteins as nanocarriers are referred to as GRAS (generally regarded as safe) drug delivery system since their bioavailibilty is high, they are easily biodegradable, biocompatibility, less toxic, they escape from protease digestion, target delivery, have role in sustain / control delivery of drug at the target site of the body, high nutritional value and having excellent binding capacity with the drugs (Langer,2000; Soppimath, *et al.*, 2001; Elzoghby, Samy, Elgindy, 2012). Nanoparticles prepared from protein are found to be easily liable to surface modifications, which allow the drug to bind to it and have potent ability to target ligands ligands (Weber, *et al.*, 2000; Chen, Remondetto, Subirade, 2006; Elzoghby, Samy, Elgindy, 2012). Among biopolymeric nanoparticles, casein could be formulated in nanoform as the calcium-phosphate bridge in casein forms a bridge with drug molecules, and aids in excellent target release of drug. Casein is a bioactive compound, with high bioavailability, non-antigenity, and aid in prolong release of drug (Livney,2010).

Curcumin (diferuloymethane) is a polyphenolic, natural yellow pigment molecule occurring in turmeric, used in treating many pathological indications. They are primarily used to treat various infections due to their biological effects like anti-tumor, antioxidant, anti-microbial, anti-inflammatory, anti-diabetic, anti-Alzheimer, and anti-rheumatic (Maheshwari, *et al.*, 2006; Aggarwal, *et al.*, 2007; Yallapu, *et al.*, 2015). Curcumin is also known to suppress thrombosis, and acts as a neuro-

***Correspondence:** R. T. P. Upputuri. PG Department of Bioscience. CMR Institute of Management Studies. Bangalore-560043, Karnataka, India. theaj_ravi@yahoo.com. ORCID: https://orcid.org/0000-0002-4567-6579

protecant, hepato-protectant, and cardio-protectant molecule. It is a potential therapeutic molecule, which has multi targeting ability in some pathological conditions (Yallapu, *et al*., 2015). Curcumin is formulated in different forms like native, micro or nanoforms and modified form to attain the optimum result in disease conditions (Gupta, *et al*., 2013). The nanocurcumin formulations enhances the sustain release of curcumin at the specific target site, to increase the therapeutic benefits (Anand, *et al*., 2007). Curcumin and their nanoformulation have potent role in improving the radiosensitization/ chemosensitization, and also known to reduce the proliferation of tumor (Yallapu, *et al*., 2014; Nagahama, Sano, Kumano, 2015).

Curcumin loaded mPEG-PLGA nanoparticles and its encapsulation efficiency was studied using artificial neural networks (ANN). Under Low curcumin concentation, the maximum and minimum encapsulation efficiency (EE) was found to 97% and 72%. Higher concentration of cucumin led to higher EE (Malekpour, *et al*., 2018). Methotrexate (MTX) and curcumin (CUR) were used as model drugs to study their anticancer activities in various phases of the cell cycle on Glioma cells. The drug loading efficiency for CUR-loaded PLGA nanoparticles was 8.0%, whereas for MTX-loaded PLGA nanoparticles was 13.5% and the EE was found to 87.9% and 81.4% for CUR-loaded PLGA nanoparticles and MTX-loaded PLGA nanoparticles. The study indicated that CUR- and MTX-loaded PLGA nanoparticles both worked additively in S and sub-G1 phases in cell cycle, in which co-delivery of drug lead to apoptosis and mono-delivery of drug lead to necrosis in the Glioma cells (Mujokoro, *et al*., 2020). Curcumin loaded poly (lactic-co-glycolic acid) nanoparticles (CUR-PLGA-NPs) were prepared by single emulsion method to overcome and reduce the hydrophobic nature of CUR, and increase the bioavailability on the cancer cells. Cytotoxicity study was carried out between free CUR and Curcumin loaded poly (lactic-co-glycolic acid) nanoparticles (CUR-PLGA-NPs) on human glioblastoma U87MG cells and found that high cytotoxicity effects of curcumin loaded poly (lactic-co-glycolic acid) nanoparticles (CUR-PLGA-NPs) was observed compared to free curcumin (Arzani, *et al*., 2019).

The main objective of our work was to study the encapsulation of curcumin into casein nanoparticles, *in vitro* drug release of curcumin to assess the sustain release of drug from the nanoparticle and also to study the *in vitro* anti cancerous property of curcumin loaded casein nanoparticles on cancer cell lines.

MATERIAL AND METHODS

Casein, Glutaraldehyde, Sodium hydroxide (NaOH), Disodium hydrogen orthophosphate (Na2HPO4), Potassium chloride (KCl), Potassium dihydrogen phosphate (KH2PO4), Methanol, Ethanol, Curcumin, Hydrochloric acid were purchased from SD fine Private limited, Mumbai. Nutrient broth, Potato dextrose agar (PDA), Dialysis membrane (molecular cut off = 3000 Da) were purchased from Himedia, India.

Synthesis of Curcumin loaded Casein Nanoparticles

Curcumin loaded casein nanoparticles were prepared by coacervation/desolvation method (Lohcharoenkal, *et al*.,2014). About 200 mg of casein was made upto 10% solution with 1N sodium hydroxide and continuously stirred in magnetic stirrer. The desolvation step was followed by the addition of 8 ml of absolute ethanol intermittently to the casein solution at a rate of 1 ml/min. About 5 mg/ml of curcumin was added to the mixture. After 10 min of incubation on a magnetic stirrer, 235µl of 8% glutaraldehyde was added drop wise and was incubated on a magnetic stirrer overnight to obtain cross-linked nanoparticle. Different concentrations of curcumin were loaded to casein nanoparticles (1, 2.5, 7.5, and 10 mg/ml) to study the maximum loading efficiency.

Characterization of casein nanoparticles

Hydrodynamic diameter and surface charge of the nanoparticles was determined with NanoZS/ZEN3600 Zetasizer (Malvern, Instruments Ltd Malvern, UK). Size and morphology of the nanoparticles were studied through Atomic Force Microscopy (AFM, Nano Surf Easy Scan2, Switzerland) and Scanning Electron Microscopy (HR-SEM, FEI Quanta FEG 200).

Drug Loading Efficiency

Curcumin loaded casein nanoparticles were centrifuged at 5000 rpm for 30 min and the supernatant was collected. The amount of free curcumin in the supernatant was measured by UV- visible spectrophotometer at 421 nm (Sharma, Agrawal, Gupta, 2012). The absorbance was interpolated in standard graph to determine the concentration of curcumin. The amount of loaded curcumin was estimated using the mathematical expression (Raj, Uppuluri., 2015; Upputuri, *et al.*,2016).

$$\text{Loading efficiency (\%)} = \frac{\text{(Total amount of curcumin - Free curcumin)}}{\text{Total amount of curcumin}} \times 100$$

In-vitro drug release studies of curcumin loaded casein nanoparticles

Drug release studies were carried out by placing 0.5 ml of curcumin entrapped casein nanoparticles in a dialysis membrane (molecular weight cut-off 3000 Da) immersed in a beaker containing 50 ml of phosphate buffer saline (PBS), pH 7.4, and stirred constantly at 25°C. About 1 ml of the sample was collected every 1 h till 48 h and the amount of curcumin released was estimated using a UV-visible spectrophotometer at 421 nm. Release studies were carried out for samples incubated at different pH (3.0) and temperature (37°C), and the cumulative percentage release was calculated (Raj, Uppuluri, 2015; Upputuri, *et al.*, 2016).

Mathematical modeling and drug release kinetics

The data obtained from *in-vitro* drug release studies with different pH and temperature were evaluated to various mathematical models such as Zero order, First order, Higuchi, Hixson–Crowell and Korsemeyer-Peppas models. Pearson product-moment Correlation coefficient (R^2) was used to find the best fit model and release exponent (n) was used as the empirical parameter portraying the release mechanism (Shaikh, Kshirsagar, 2015; Upputuri, *et al.*,2016).

Antimicrobial activity

Antimicrobial activity was studied by agar well diffusion method. Three bacterial organisms *E. coli, Pseudomonas, Staphylococcus* were screened for their sensitivity towards samples including casein nanoparticles, curcumin loaded casein nanoparticles, free curcumin and a standard antibiotic, ciprofloxacin. Two fungal species *Penicillium,* and *Fusarium* were screened for their susceptibility against the samples and amphotericin B (standard). All antimicrobial activity was performed in triplicates (Buszewski, *et al.*, 2016; Jourghanian, *et al.*, 2016).

Genotoxicity studies on *Allium cepa* root tips

Allium cepa were used as a test to study the genotoxicity of curcumin loaded casein nanoparticles. Healthy onion root tips were used for the study. The root tips were treated with different samples like 1N HCl (Sample 1 – negative control), incubated in hydrogen peroxide for 1 h and treated in UV light for 30 minutes (Sample 2 – positive control), treated with curcumin loaded casein nanoparticles for 1 h (Sample 3), with curcumin loaded casein nanoparticles for 1 h, followed by 1 h incubation with hydrogen peroxide and 30 min under UV light (Sample 4), and incubated with hydrogen peroxide and UV light, followed by 1 h incubation with curcumin loaded casein nanoparticles in the separate eppendoff tubes (Sample 5). The tips were then observed for any chromosomal aberrations under a compound microscope (Kumari, Mukherjee, Chandrasekaran, 2009; Kumari, *et al.*, 2011).

In-vitro cytotoxicity assay – MTT Assay

In vitro cytotoxicity assay was performed through MTT assay for curcumin loaded casein nanoparticles against A549 (lung cancer cells) and MCF7 (breast cancer cells) cells. Briefly, cells were seeded to a 96-well plate at $1x10^4$ cells/well, plates were incubated at 37ºC in a humidified atmosphere supplied with 5% CO2. Curcumin loaded casein nanoparticles were added at different concentrations (25, 50, 100, 250, and 500

µg/ml) to the wells and plates were incubated. Around 10 µl MTT solution was added to each well to achieve a final concentration of 0.45 mg/ml, and incubated for 4 h at 37ºC. The medium was removed, and 100 µl of DMSO was added to each well. The amount of purple product formazan formed was determined by measuring the absorbance at 570 nm, using a multi-well plate reader. The cell viability was calculated using the equation and IC50 value was further determined (Balaji, Gothandam, 2016).

Cell viability (%) = (Absorbance of treated cells / Absorbance of control cells) ×100.

Apoptotic assay/Dual AO/EB fluorescent staining

Apoptotic assay was carried out using a dual stain method (Acridine orange (AO) and Ethidium bromide (EB)). A549 cells were treated with 500µg/ml of curcumin loaded casein nanoparticles and incubated at 37°C for 24 h in a CO2 incubator. The cells were harvested by trypsination and collected by centrifugation. The cells pellet was resuspended in the medium and placed on a microscopic glass slide. About 1 µl of dual fluorescent staining solution (100 µg/ml AO and 100 µg/ml EB) was added to the slide. The samples were incubated and observed under a fluorescent microscope. The difference between the live and dead cells and the morphology of the apoptotic cells, were observed within 20 min (Balaji, Gothandam, 2016).

DNA Fragmentation Assay

DNA fragmentation assay was performed on A549 cells. Briefly, the cells were seeded to the microtitre plate and were treated with 500µg/ml of curcumin loaded casein nanoparticles for 24 h. After treatment, the cells were harvested and lysed with 50 µl lysis buffer (100 mMNaCl, 1 mM EDTA, 50 mM Tris–Cl (pH 8.0), 0.5 % SDS, and 1 mg/ml proteinase-K) for 2 h at 54°C with gentle agitation. The fragmented DNA from the cells were extracted with 24:1 phenol:chloroform, followed by ethanol precipitation. DNA pellets were air dried and resuspended in TE buffer (20 µl which contains 50 µg/ml of RNase), and incubated at 37°C for 30 min. The DNA content was analysed, with equal amount of DNA being loaded onto 1.5% agarose gel. The DNA bands were visualized under UV light (Balaji, Gothandam, 2016).

Statistical Analysis

All the experiments were performed in triplicates and the results are presented as Mean±S.D. The graphs were plotted using the GraphPad Prism 5 software, version-5.01.

RESULTS

Curcumin loaded casein nanoparticles were prepared by desolvation method. The nanoparticles formed were crosslinked by addition of glutataldehyde. Morphology, size distribution, charge and shape of the casein nanoparticles were analysed. Hydrodynamic diameter, determined through size distribution revealved that casein nanoparticles exhibited an average particle diameter of 201.4 nm with a polydispersity index of about 0.874. Through zeta potential analysis, the particles were observed to be negatively charged with a zeta potential value of -86.9 mV (Figure 1 A and B). AFM and SEM images of casein nanoparticles is shown in Figure 1 C and D. The nanoparticles were found to aggregated when viewed in AFM. The particles as observed in SEM were found to be uniformly spherical in shape with a size of about 183.4 nm.

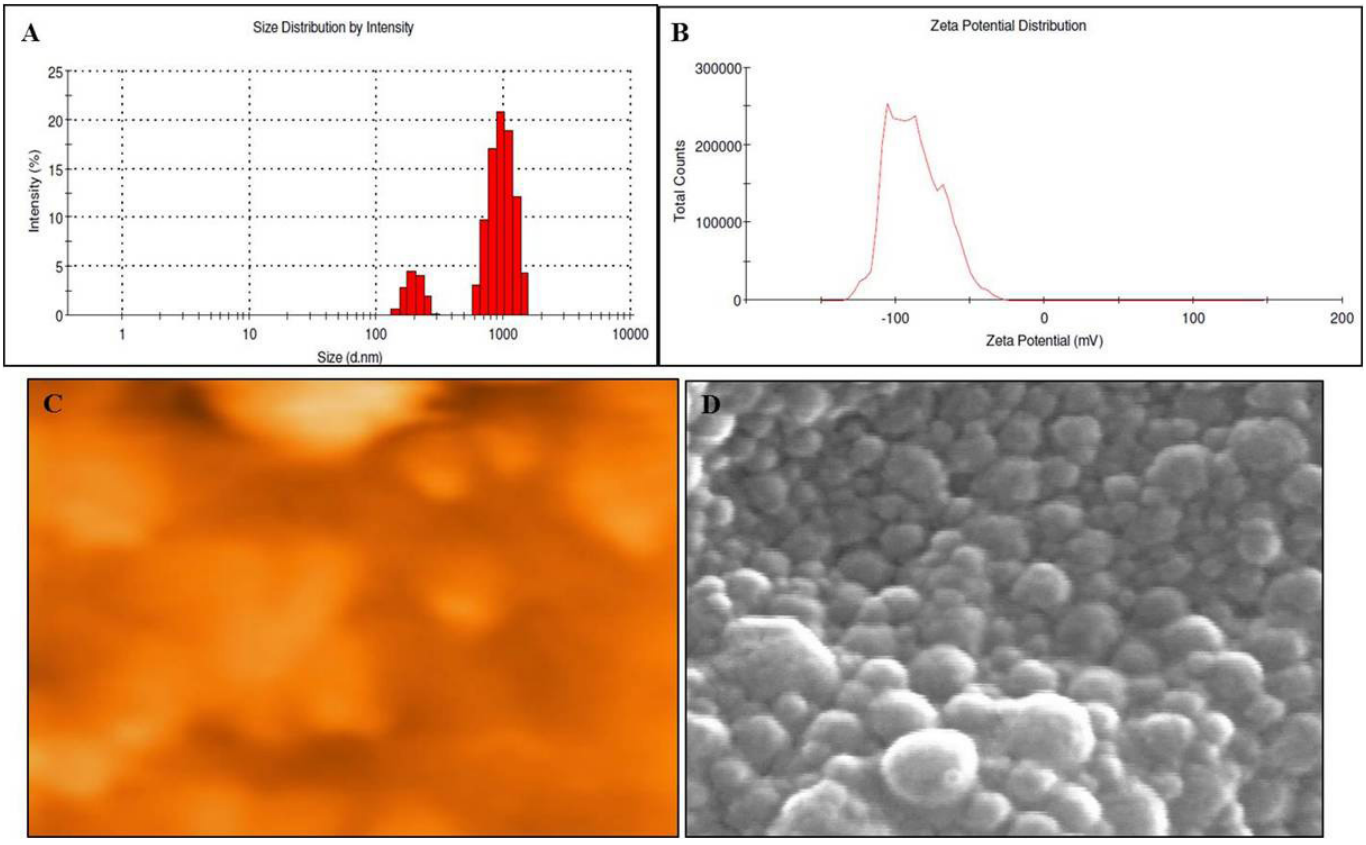

FIGURE 1 - Hydrodynamic diameter of the particle (A) and Surface charge of the nanoparticles which was shown to exhibit -86.9 Mv(B). Nanoparticles which are aggregated together were observed under AFM (C) and spherical nanoparticles in uniform manner with size of 183.4 nm observed using SEM (D).

Drug loading efficiency

Loading efficiency of curcumin loaded (5 mg/ml) casein nanoparticles was obsreved to be 87.05%. Loading efficiency was also calculated for curcumin loaded at different concentrations 1, 2.5, 5, 7.5 and 10 mg/ml into casein nanoparticles, their loading efficiency were found to be 22.9%,58.79%, 87.05%, 79.31% and 74.34%. The loading efficiency was observed to be gradually increasing with increase in curcumin concentration and decrease at its saturation point. Amaximum of 87.05% of loading efficiency were observed at a concentration of 5 mg/ml.All experiments were carried out in triplicates and the data are presented as mean±S.D.

In-vitro drug release

In-vitro drug release was performed in PBS at pH 7.4 for 48 h. Iniatilly about 19.28% of curcumin was released at 6 h, and a maximum of about 24.8% release was observed after a period of 48 h. Release of curcumin from casein nanoparticles was observed to be at a slower rate, followed by sustain and a prolonged release. Release studies was also performed in different parameters such as pH (7.4 and 3) and temperatures (25 and 37°C) for curcumin loaded casein nanoparticles,with 20.13% release at pH 3 (pH of gastrointestinal tract), and 24.81% release at pH 7.4 (Figure 2A**).** Release pattern of drug at 25°C and 37°C was studied and was observed that as temperature increased, curcumin release was observed to be at a higher rate, with a maximum of 24.8% and 28.60% release at temperatures of 25 and 37°C respectively (Figure 2B). The drug release was observed to decrease with increase in pH and increase with increase in temperature.

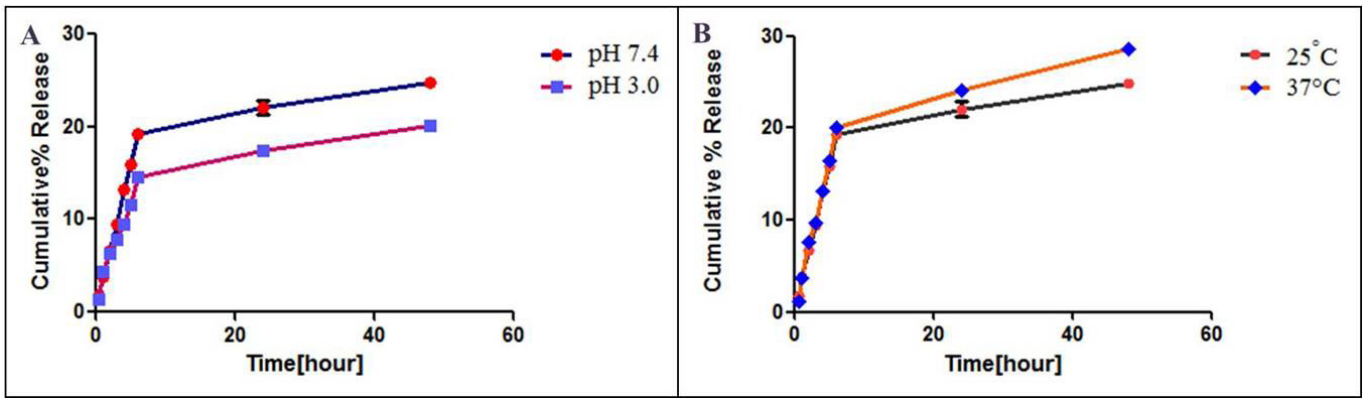

FIGURE 2 - Drug release at pH 7.4 and pH 3 at different time intervals showing that as the pH increased from 3 to 7.4, an increases in the percentage drug release was observed (A) and release at different temperatures of 25°C and 37°C showing a similar pattern of increasing percentage of drug release with increase in temperature (B).

Mathematical modelling and drug release kinetics

Mathematical modelling and the mechanism of drug release was studied by fitting the *in vitro* release data into various kinetic models and parameters such as regression coefficient (R^2) and release exponent (n) was analyzed and presented in table I. Release pattern of drug for all the parameters was observed to follow Korsmeyer-Peppas model. Release exponent (n) values was observed to be in a range of 0.53-0.65 for all the parameters studied, which was shown to follow an anomalous mode of drug release, involving a combination of diffusion and erosion mechanisms.

TABLE I - Release kinetics and drug release mechanism of curcumin from casein nanoparticles at different parameters

Parameters	Zero order	First order	Higuchi model	Hixson-Crowell model	Korsmeyer-Peppas model		Drug transport mechanism
	R^2	R^2	R^2	R^2	R^2	n	
pH 3.0	0.6752	0.7004	0.8373	0.6921	0.8348	0.5304	Anomalous
pH 7.4	0.6011	0.6309	0.7748	0.6248	0.8408	0.5636	Anomalous
25°C	0.6011	0.6309	0.7748	0.6248	0.8408	0.5636	Anomalous
37°C	0.6692	0.709	0.8315	0.6958	0.8167	0.6584	Anomalous

Antimicrobial activity

Effect of antibiotics, casein nanoparticles, curcumin loaded casein nanoparticles and curcumin samples on bacterial and fungal species was observed by measuring the zone of inhibition. The activity of curcumin loaded casein nanoparticles show higher synergistic activity against the *E. coli*, *Pseudomonas, Staphylococcus, Penicillium* and *Fusarium*. The zone of inhibition formed due the sensitivity of bacterial and fungal species towards the samples are illustrated in the Table II.

TABLE II - Zone of inhibition of microorganisms towards the standards (Ciprofloxacin-Bacteria and Amphotrecin B-Fungi) and other samples

	Zone of inhibition in mm			
Microorganisms	Standard	Casein nanoparticles	Curcumin loaded casein nanoparticles	Curcumin (mm)
Escherichia coli	29.33±1.154	19.66±0.577	19.33±1.154	18.66±0.577
Pseudomonas	39.0±1.0	22.33±1.154	18.33±1.154	17.66±0.577
Staphylococcus	33.33±0.577	14.33±0.577	19.66±1.154	13.0±0
Penicillium	12.166±0.3055	26.133±0.3214	14.966±0.0577	12.33±0.5773
Fusarium	19.333±0.577	11.666±1.154	15.333±1.154	14.333±0.577

The values are measured in mm and the data expressed as mean ± S.D.

Genotoxicity on onion root tips

The effect of curcumin loaded casein nanoparticles on DNA damage is shown in the Figure 3. No chromosomal aberrations were observed in negative control (Sample 1), with an intact metaphase and anaphase(Figure 3A). Chromosomal aberration was observed in metaphase stage chromosome after treatment with H2O2 and UV light in positive control (Sample 2) (Figure 3B). Intact metaphase and anaphase chromosome were observed in root tips when they are treated with samples (Sample 3, 4, 5) (Figure 3C, D, E).

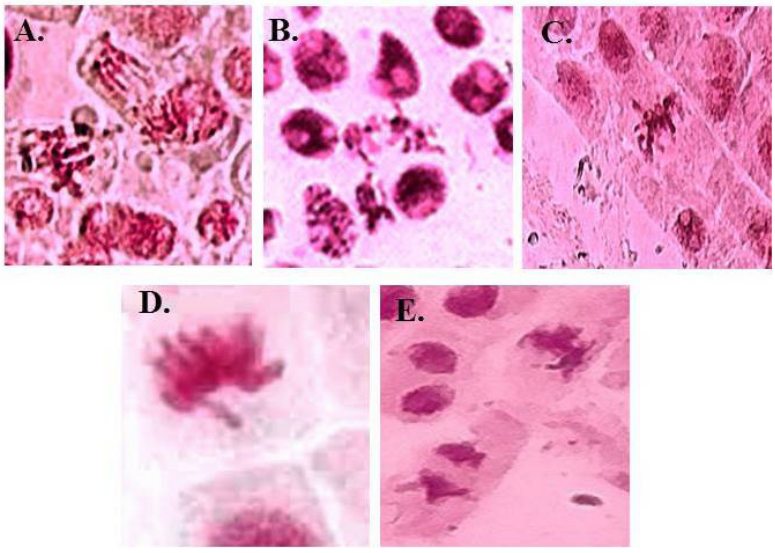

FIGURE 3 - Intact metaphase and anaphase was observed in negative control (A); Disrupted chromosome was observed in metaphase and anaphase chromosome in positive control (B);Intact metaphase was observed in root tips after treating with curcumin loaded casein nanoparticles in sample 3 (C); Undamage metaphase chromosome after treating with nanoparticles, H2O2 and UV light in sample 4 (D); Undamaged chromosome of metaphase and anaphase was observed, after the treatment with H2O2 under UV light, followed by treatment with nanoparticles in sample 5 (E).

In-vitro cytotoxic assay - MTT assay

Cytotoxic activity on two cell lines (MCF7 and A549) was observed to be dose dependent (25-500 µg/ml) as shown in Figure 4B and D, with a maximum cytotoxic activity being observed at 500 µg/ml for both the cell lines, with a cell viability of about 64.0 and 74.2% for MCF7 and A549 respectively. The IC50 values were determined and were observed to be 609 and 825.2 µg/ml for MCF 7 and A549 cells respectively. The number of cell count and size found to be decreased compared to the control cells. Morphology of the MCF7 and A549 3cells treated with increasing concentrations of curcumin loaded nanoparticles are shown in Figure 4A and 4C.

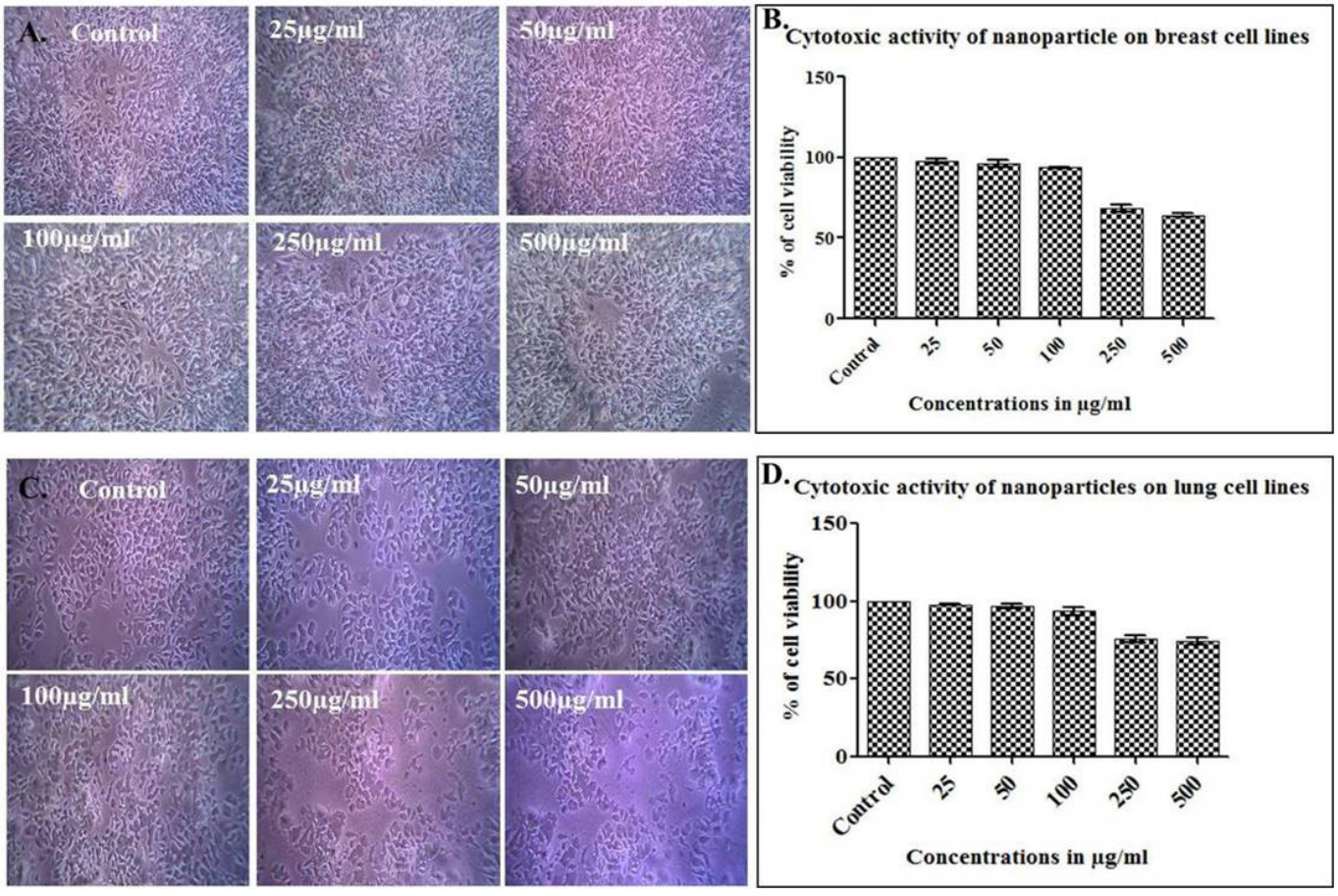

FIGURE 4 - Morphological changes observed at different concentrations of curcumin loaded casein nanoparticles (A) and the percentage viability of MCF7 treated curcumin loaded nanoparticles (B); Morphological changes observed at different concentrations of curcumin loaded casein nanoparticles (C) and the percentage viability of A549 treated curcumin loaded nanoparticles (D).

Apoptotic assay/Dual staining

Apoptotic assay was carried out for curcumin loaded casein nanoparticle (500 µg/ml) treated A549 cells to visualize the apoptotic cells. The morphological changes of the cancer cells were observed under fluorescent microscope. The green stained cells were characterized to be viable and early apoptotic cells with condensed chromatin and red stained cells are characterized as late apoptotic and death cells. The control cells showed intact chromosome, while nanoparticles treated cells showed condensed nuclei and apoptotic bodies, as shown in Figure 5 A and B.

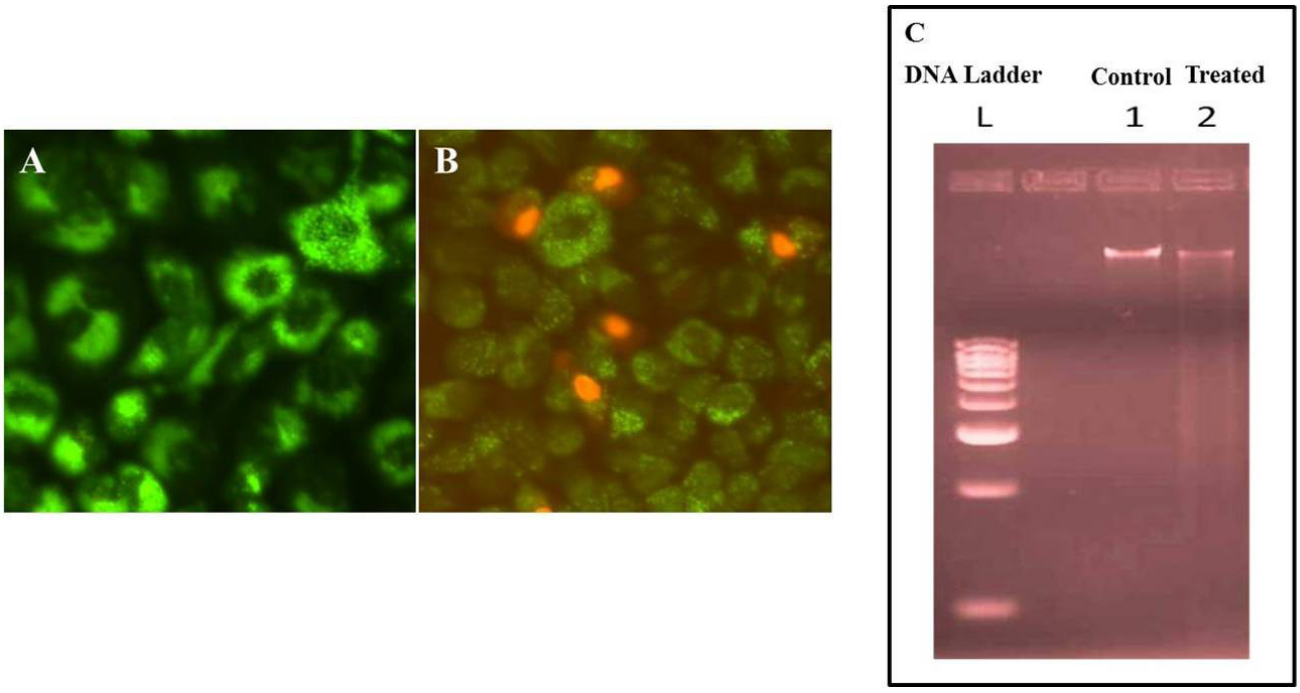

FIGURE 5 - A549 cells showing all the cells to be viable (A) and cells treated with 500 µg/ml of curcumin loaded casein nanoparticles showing apoptotic cells (B). (C) DNA fragmentation assay of curcumin loaded nanoparticle (500 µg/ml) treated A549 cellsshowing control DNA ladder (L), A549 cells as a control (1), and A549 cells treated with curcumin loaded nanoparticle (500 µg/ml) (2).

DNA Fragmentation assay

DNA fragmentation assay was performed on A549 cells treated with curcumin loaded casein nanoparticles (500 µg/ml). DNA band in control was observed to be intact and the test sample (cells treate d with nanoparticles) showed a light band with decreased intensity showing fragmented DNA (DNA smear was observed) as shown in Figure 5C.

DISCUSSION

Desolvation technique was used in the synthesis of curcumin loaded casein nanoparticles as it is easy and less expensive. Hydrodynamic diameter of nanoparticles was obtained from first peak with an average size of about 201.4 nm and a second peak was observed with average size of 995.8 nm, which might be due to clumping or aggregation of nanoparticles within the dispersion media. Zeta potential value of nanoparticle was found to be -86.9 mV. (Ye, Flanagan, Singh, 2006) studied zeta potential of sodium caseinare-gum Arabic nanparticles and was found to be +20.7 mV. The nanoparticles synthesized in our study was found to be more stable compared to the previous work (Ye, Flanagan, Singh, 2006)

The loading efficiency (LE) of curcumin loaded casein nanoparticle was observed to be 87.05%, much higher compared to the work carried out on curcumin loaded sodium caseinate (83.1%) (Pan, Zhong, Baek,2013). Loading efficiency of different concentration (1, 2.5, 5, 7.5, and 10 mg/ml) of curcumin was found to be 22.9, 58.79, 87.05, 79.31 and 74.34%. The efficiency seem to increase with increase in curcumin concentration, but at certain concentration, there was drop in percentage of loading efficiency. The higher loading efficiency was obtained at 5 mg/ml curcumin loaded casein nanoparticles. *In vitro* release study carried out in PBS showed a cumulative release percentage of about 24.8 % at 48 h at pH 7.4, but the cumulative percentage release of curcumin seemed to be 54.43% in 48 h, when encapsulated into Poly-(lactic acid) (PLA) (Rachmawati, *et al.*, 2016). Therefore, our study showed a sustained and a prolonged release of

curcumin up to 48 h. *In vitro* release was also carried out at pH 3 where about 20% of drug was released at 48 h. As the casein nanoparticles are intended as oral formulations, drug release study simulating the gastrointestinal tract was performed. The results from our study showed that maximum amount of drug was retained within the nanoparticle even after 48 h (67%). Drug release was also carried out at a higher temperature (37°C) simulating any infectious condition and was observed that a higher amount of drug was released at a higher temperature (28.60%). Drug release mechanism from polymeric nanoparticles was in a controlled manner. Several transport process are involved for controlled release of drug from matrices such as diffusion, erosion, degradation and in combination of these mechanism. Mathematical modeling was performed to study the basic mechanism of drug release and the significant transport process involved (El-Nashar, *et al.*, 2016). An ideal model followed for release of drug from nanoparticles system is the zero order. Data obtained from *in vitro* release of curcumin fitted into various models and by comparing the R^2 values of all the models, Korsmeyer-Peppas model was observed to be the best fit model. From Korsmeyer-Peppas model, release exponent, n value was calculated. The n value was found to be 0.43 in case of spherical and 0.45 in case of cylindrical nanoparticles. In our study, as the particles were observed to be spherical, the cut off value for the release exponent, n value was set at 0.43. If the n value is less than 0.43, the drug mechanism is Fickian transport, n value ranging between 0.43 and 0.89, it is said to be anomalous transport, and that more than 0.89 is said to be Supercase II transport. The curcumin release data fitted into various model and by comparing the R^2 values of all the models, Korsmeyer-Peppas model was observed to be the best fit model, and n was found between 0.53-0.65, so the transport was anomalous transport involving a combination of diffusion and erosion mechanisms (Upputuri, et a l., 2016).

Antimicrobial activity of casein nanoparticles was observed to be higher towards the *E. coli*, *Pseudomonas, Staphylococcus, Penicilium* and *Fusarium*. Onion root tips were treated with curcumin loaded casein nanoparticles to study its genotoxicity. The drug loaded casein nanoparticles was observed to be potent to protect the cell/chromosome from any damage, and no toxicity was observed in cells treated with drug loaded nanoparticles.

Cytotoxic assay of curcumin loaded nanoparticles was evaluated on MCF7 and A549 cells, and the IC50 value was observed to be 609 and 825.2 µg/ml respectively. Few studies showed that curcumin have increased cytotoxic activity on cells such as HeLa (cervical cancer cell lines), HepG2 (liver cancer cell line), K-562 (blood cancer cells) and PANC-1 (human pancreatic cell lines) (Sahu, Kasoju, Bora, 2008; Zhang, *et al.*, 2012). In a study on curcumin loaded β-casein, IC50 against MCF-7 cells was found to be 24 µM concentration (Mehranfar, Bordbar, Amiri,2015). In our study, since the concentration within the casein nanoparticles was less, more amount of drug loaded nanoparticles would be required to achieve a lower IC50 concentration. Therefore, increasing the concentration of curcumin loaded casein nanoparticles to treat cancer cells, could enhance its anti cancer property at a lesser time.

Apoptotic mechanism is a tightly regulated mechanism, in which many genes (p53, Bax, Bcl-2) are involved as a marker to induce cell death. The cytotoxic activity of curcumin loaded casein nanoparticles was assayed through dual staining method to study the cell death mechanism. Acrindine orange/Ethidium Bromide (AO/EB) staining was used where AO would be taken up by the viable and early apoptotic cells and emits green florescence, while EB is taken up by the non-viable and late apoptotic cells and emits orange florescence. At 500 µg/ml concentration of nanoparticles treated A549 cells, the dead cells were stained orange and the green color intensity was decreased due the activity of nanoparticles. The activity of curcumin released from casein nanoparticles was observed to cause more cell death compared to study carried out in previous work (Anand, *et al.*, 2016). Biochemical feature of apoptosis is the DNA fragmentation by specific nuclease. The cleavage at inter-nucleosomal site of DNA generated fragments known as DNA ladder. In A549 cells treated with curcumin loaded casein nanoparticles, DNA of cancer cells were fragmented and was observed as a smear under the UV light. Therefore, from our study,

curcumin loaded casein nanoparticles have shown to have potent anti-cancerous activity towards the cancer cells.

CONCLUSION

Curcumin loaded casein nanoparticles was evaluated *in-vitro* and its activity was found to be more potent on cancer cells. On oral formulation of curcumin loaded casein nanoparticles, curcumin release was found to be in steady and prolonged manner with more amounts of drug being entrapped within the nanoparticles even in acidic conditions. The anticancer property of curcumin loaded casein nanoparticles showed a major impact on cancer cells resulting in apoptosis and DNA fragmentation. Therefore, through our study, it could be shown that curcumin loaded casein nanoparticles could be used as a potent anticancer drug for targeting tumors.

ACKNOWLEDGEMENTS

The author would like to thank CMR Institute of Management Studies, Bangalore for providing the required sources to conduct this research article, and Pondicherry Center for Biological Science, Pondicherry, Karunya University, Coimbatore and VIT University, Vellore for carrying out the sample analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in the publication.

REFERENCES:

Aggarwal BB, Sundaram C, Malani N, Ichikawa H. Curcumin: the Indian solid gold. Adv Exp Med Biol. 2007;595:1–75.

Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Mol Pharm. 2007;4(6):807–818.

Anand P, Nair HB, Sung B, Kunnumakkara AB, Yadav VR, Tekmal RR, *et.al.* Design of Curcumin Loaded PLGA Nanoparticles Formulation with Enhanced Cellular Uptake, and Increased Bioactivity in vitro and Superior Bioavailability in vivo. Biochem Pharmacol. 2016;102(143):330-338.

Arzani H, Adabi M, Mosafer J, Dorkoosh F, Khosravani D, Maleki H et al. Preparation of curcumin-loaded PLGA nanoparticles and investigation of its cytotoxicity effects on human glioblastoma U87MG cells. Biointerface Res Appl Chem. 2019;9(5):4225-4231.

Balaji K, Gothandam K M. Cytotoxic Effect on Cancerous Cell Lines by Biologically Synthesized Silver Nanoparticles. Braz Arch Biol Technol. 2016;59:1-8.

Buszewski B, Railean-Plugaru V, Pomastowski P, Rafinska K, Szultka-Mlynska M, Golinska P, et al. Antimicrobial activity of biosilver nanoparticles produced by a novel Streptacidiphilus durhamensis strain. J Microbiol Immunol Infect. 2016;51(1):45-54.

Chen L, Remondetto GE, Subirade M. Food protein-based materials as nutraceutical delivery systems. Trends Food Sci Technol. 2006;17(5):272–283.

El-Nashar DE, Rozik NN, Soliman AM, Helaly F. Study the release kinetics of curcumin released from PVA/curcumin composites and its evaluation towards hepatocarcinoma. J Appl Pharm Sci. 2016;6(07):67-72.

Elzoghby AO, Samy WM, Elgindy NA. Protein-based nanocarriers as promising drug and gene delivery systems. J Controlled Rel. 2012;16(1):38-49.

Gupta SC, Sung B, Kim JH, Prasad S, Li S, Aggarwal BB. Multitargeting by turmeric, the golden spice: from kitchen to clinic. Mol Nutr Food Res. 2013;57(9):1510–1528.

Jourghanian P, Ghaffari S, Ardjmand M, Haghighat S, Mohammad NM. Sustained release Curcumin loaded Solid Lipid Nanoparticles. Adv Pharm Bull. 2016;6(1):17-21.

Kumari M, Khan SS, Pakrashi S, Mukherjee A, Chandrasekaran N. Cytogenetic and genotoxic effects of zinc oxide nanoparticles on root cells of *Allium cepa*. J Hazard Mater. 2011;190(1-3):613–621

Kumari M, Mukherjee A, Chandrasekaran N. Genotoxicity of silver nanoparticles in *Allium cepa*. Sci. Total Environ. 2009;407(19):5243–5246.

Langer R. Biomaterials in drug delivery and tissue engineering: One laboratory's experience. Acc Chem Res. 2000;33(2):94–101.

Livney YD. Milk proteins as vehicles for bioactives. Curr Opin Colloid Interface Sci. 2010;15(1-2):73–83.

Lohcharoenkal W, Wang L, Chen YC, Rojanasakul Y. Protein nanoparticles as drug delivery carriers for cancer therapy. Biomed Res Int. 2014;2014:1-12.

Maheshwari RK, Singh AK, Gaddipati J, Srimal RC. Multiple biological activities of curcumin: a short review. Life Sci. 2006;78(18):2081–2087.

Malekpour RM, Naghibzadeh R, Najafabadi MR, Esnaashari SS, Adabi M; Mujokoro B et.al. Effect of various parameters on encapsulation efficiency of mPEG-PLGA nanoparticles: artificial neural network. Biointerface Res Appl Chem. 2018;8(3):1-7.

Mehranfar F,Bordbar AK,Amiri R. *In Vitro* cytotoxic activity and binding properties of curcumin in the presence of β-casein micelle nanoparticles. Biomacromol J. 2015;1(1):69-79.

Mujokoro B, Madani F, Esnaashari SS, Khosravani M, Adabi M. Combination and co-delivery of methotrexate and curcumin: preparation and in vitro cytotoxic investigation on glioma cells. J Pharm Innovation. 2020;15:617-626.

Nagahama K, Sano Y, Kumano T. Anticancer drug-based multifunctional nanogels through self-assembly of dextran curcumin conjugates toward cancer theranostics. Bioorg Med Chem Lett. 2015;25(12):2519-2522.

Nevozhay D, Kañska U, Budzyñska R, Boratyñski J. Current status of research on conjugates and related drug delivery systems in the treatment of cancer and other diseases (Polish). Postepy Hig Med Dosw (Online). 2007;61:350–360.

Pan K, Zhong Q, Baek SJ. Enhanced Dispersibility and Bioactivity of Curcumin by Encapsulation in Casein Nanocapsules. J Agric Food Chem. 2013;61(25):6036−6043.

Rachmawati H, Yanda YL, Rahma A, Mase N. Curcumin-Loaded PLA nanoparticles: formulation and physical evaluation. Sci Pharm. 2016;84(1):191-202.

Raj J, Uppuluri KB. Metformin loaded casein micelles for sustained delivery: formulation, characterization and in-vitro evaluation. Biomed Pharmacol J. 2015;8(1):83-89.

Sahu A, Kasoju N, Bora U. Fluorescence study of the curcumin-casein micelle complexation and its application as a drug nanocarrier to cancer cells. Biomacromolecules. 2008;9(10):2905–2912.

Shaikh HK, Kshirsagar RV, Patil SG. Mathematical Models for drug release characterization: A review. World J Pharm Res. 2015;4(4):324-338.

Sharma K, Agrawal SS, Gupta M. Development and Validation of UV spectrophotometric method for the estimation of curcumin in Bulk Drug and Pharmaceutical Dosage Forms. Int J Drug Dev Res. 2012;4(2):375-380.

Soppimath SS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. J Controlled Rel. 2001;70(1-2):1–20.

Upputuri RTP, Kumar S, Kulandaivelu K, Mandal AKA. Encapsulation Efficiency and Release of Green Tea Polyphenols from Poly (Lactic Acid)-Poly (Ethylene Glycol) Nanoparticles are Controlled by the ratio of Poly (Lactic Acid)/Poly (Ethylene Glycol). Indian J Pharm Educ. 2016;60(2):301-309.

Weber C, Coester C, Kreuter J, Langer K.Desolvation process and surface characterisation of protein nanoparticles. Int J Pharm. 2000;194(1):91–102.

Wilczewska AZ, Niemirowicz K, Markiewicz KH, Car H. Nanoparticles as drug delivery systems. Pharmacol Rep. 2012;64(5):1020-1140.

Yallapu MM, Khan S, Maher DM, Ebeling MC, Sundram V, Chauhan N, et al. Anti-cancer activity of curcumin loaded nanoparticles in prostate cancer. Biomaterials. 2014;35(30):8635–8648.

Yallapu MM, Nagesh PHB, Jaggi M, Chauhan SC. Therapeutic Applications of Curcumin Nanoformulations. AAPS J. 2015;17(6):1341-1356.

Ye A, Flanagan J, Singh H. Formation of stable nanoparticles via electrostatic complexation between sodium caseinate and gum arabic. Biopolymers. 2006;82(2):121-133.

Zhang L, Zhu W, Yang C,Guo H, Yu A,Ji J, et al. A novel folate-modified self-microemulsifying drug delivery system of curcumin for colon targeting. Int J Nanomed. 2012;7:151–162.

Received for publication on 15th October 2019
Accepted for publication on 03rd February 2021