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Formulation and evaluation of folic acid conjugated gliadin nanoparticles of curcumin for targeting colon cancer cells

Shipra Sonekar*, Manoj Kumar Mishra, Anil Kumar Patel, Suresh Kumar Nair, Chandra Shekhar Singh, Amit Kumar Singh

Shambhunath Institute of Pharmacy, Jhalwa, Allahabad, Uttar Pradesh, India.

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ABSTRACT

Preparation of folic acid (FA) conjugated (FA-CUR-GNPs) and non-conjugated (CUR-GNPs) gliadin nanoparticles of curcumin were successfully formulated by desolvation method for oral delivery of drug for targeting colon cancer cell. F1, F3, F5 (conjugated) and F2, F4, F6 (Non-conjugated) were formulated using various drug-polymer ratio (1:2). They were further characterized by FTIR, Mass spectroscopy, NMR, solubility studies, entrapment efficiency, TEM, particle size, surface charge, In-vitro release studies, In vivo toxicity studies and simultaneously evaluated. F3 (curcumin 10mg, gliadin 20mg and FA 5mg) and F4 (curcumin 10mg and gliadin 20mg) were found as the optimized formulation among both the categories. For F3 and F4 formulations; average particle size (168.1 and 195.7nm), zeta potential (-16.5 and -24.4mV), cumulative % drug release (92.92 and 94%) and In vivo toxicity studies were conducted and compared with the control (phosphate-buffer saline, pH 6.8) reveals no toxicity. From the characterization and evaluation studies it was identified that F4 (FA-CUR-GNPs) had better solubility, In vitro release profile and no specified In-vivo toxicity than F3 (CUR-GNPs) formulation with nano-range particle size throughout the experiment. Improved bioavailability and increase targeting capacity toward colon cancer tumor cells were successfully achieved.

INTRODUCTION

Prolonged exposure to almost any provocative agent in the environment (radiation, chemicals, viruses, inflammation, etc.) can potentially cause cancer (Seyfried and Shelton, 2010). Nano therapeutics are the most advanced field which are reduced the limitations of conventional drug delivery system such as lack of specified targeting, lower aqueous solubility, poor oral bioavailability and decreased therapeutic indices (Gelperina *et al*., 2005). As per literature, folate receptor has been explored to deliver protein toxins, low-molecular weight chemotherapeutic agents, radio-imaging agents, MRI contrast agents, radio therapeutic agents, liposomes containing chemotherapeutic drugs, genes, antisense oligonucleotides, ribozymes, and immunotherapeutic agents to cancer cells (Duthie, 1999).

* *Corresponding Author*
Shipra Sonekar, Shambhunath Institute of Pharmacy, Jhalwa, Allahabad, Uttar Pradesh, India. Email: shipra.qc123 @ gmail.com

The proliferancy of colon cancer is increasing day by day in developed countries (Pisani *et al*., 2002). Pre-clinical research studies using curcumin has proved reduction of various cancerous cells due to inhibition of NF-κB and COX-2, arachidonic acid metabolism via lipoxygenase, scavenging of free radicals, expression of inflammatory cytokines IL1b, IL-6, TNF-a and down-regulation of enzymes (Jobin *et al*., 1999; Huang *et al*., 1991; Cho *et al*., 2007; Liu *et al*., 1993). Curcumin (CUR), is a bis-R,-unsaturated-diketone (commonly called diferuloylmethane) which exhibits keto–enol tautomerism having a predominant keto form in acidic and neutral solutions and stable enol form in alkaline medium is obtained from the rhizome of the perennial herb *Curcuma longa*, can reverse the multidrug resistance phenomenon (Bansal *et al*., 2011). It inhibits proliferation in various cancer cells isolated from malignancies like leukemia, breast, lung, prostate and colon tumors (Anand *et al*., 2008). Studies were also done in various tumerogenetic models (Tuttle *et al*., 2012). Curcumin blocks the NFκB pathway responsible for tumor cell survival and metastasis (Srimal and Dhawan, 1973).

Folate receptor conjugate vitamin and drugs with a high affinity and carries these bound molecules into the tumor tissues (Xiang *et al*., 2008). Cellular uptake of folates are facilitated by a high affinity glycosylphosphatidylinositol-linked FR (K D ≈100 pM), which exhibits highly limited distribution. Gliadin (GNP) nanoparticles are suitable for both passive and active drug targeting offers a wide variety of possibilities for preparing better tolerated parenteral and oral formulations either maintaining the same characteristics engineered to pharmacokinetic and tissue distribution or enhancing the site specific delivery (Jahanshahi and Babaei, 2008).

Present study was to design and develop gliadin nanoparticles of curcumin for oral delivery of drug for targeting colon cancer cell.

MATERIAL AND METHODS

Materials

Curcumin, Gliadin, Pluronic F-68 and Folic Acid were obtained from Sigma Aldrich Corp., Switzerland as gift samples. Ethanol was obtained from Qualigens Fine Chemicals (P) Ltd, Mumbai, India; Chloroform, Dimethyl sulfoxide (DMSO), Ethylene Dichloride (EDC) and Dichloromethane were obtained from Merck Specialties Pvt. Ltd., New Delhi, India; Sodium hydrogen phosphate and Disodium Hydrogen Phosphate were obtained from Sarabhai M. Chemicals, Vadodara, India. Acetone and Benzene were obtained from Avantor Performance Materials Ltd., Gurgaon, India.

Identification and characterization of curcumin

Fourier transform infrared spectroscopy (FTIR)

The Infrared absorption spectrum of drug, polymer, folic acid conjugation (FA-CUR-GNP) and folic acid non- conjugation (CUR-GNP) were obtained by finely grounding and dispersing in KBr using FTIR spectroscopy was performed on a Spectrum 65 (Perkin Elmer). Spectra were recorded between 4000 and 600 cm^{-1} range. The IR absorption spectrum of drug, polymer, FA-CUR-GNP and CUR-GNP exhibits maxima only at the same wavelengths as that of a reference standard.

Mass spectroscopy

Electron Spray Ionization mass spectra (ESI-MS) of Curcumin and Gliadin were performed.

NMR spectrum of curcumin

^{1}H NMR spectra were taken on Bruker Advance DRX-500, Brucker Biosciences Corporation, Billerica, MA, USA at 500 MHz CD_3OD was taken as solvent. Pure drug curcumin (5 mg) was dissolved in 1 ml of CD_3OD and subjected to NMR analysis.

Solubility studies

Solubility profile of curcumin

The equilibrium solubility of CUR was determined at different solvents. Equivalent amount of the drug was added and shaken in separate flask containing different solvents using a mechanical shaker at room temperature (25˚C) for 24 h (n=3). The samples were then centrifuged at 2000 rpm, filtered through membrane filter (0.45µm) and analysed by HPLC to quantify dissolved drug content. The solubility of the drugs in water, methanol, ethanol and acetone were checked by High Performance Liquid Chromatography (HPLC) (Yanyu *et al*., 2006).

Solubility profile of gliadin

Gliadin is a prolamine, which is soluble only in suitable ratios of aqueous and organic phases. To find its solubility in different concentrations, 1% of the gliadin solutions were prepared by using different ratios of the ethanol and water and solubility were determined with the absorbance value from UV spectrophotometer at 283 nm.

Partition coefficient

The Partition coefficient between n-octanol/water was determined at room temperature (30 ˚C). 10 ml of n-octanol and 10 ml of distilled water was taken in a glass stoppers graduated tube and 5 mg of accurately weighted drug was added, the mixture was then shaken with the help of mechanical shaker for 24 h at room temperature the mixture was then transferred to a separating funnel and allowed to equilibrate for 6 h. The aqueous and octanol phase were separated and filtered through membrane and drug content in aqueous phase was analyzed by UV-Visible spectrophotometer.

Entrapment efficiency

Drug was loaded by adding the drug with the gliadin polymer in the 70% alcoholic mixture. Different ratios of drug and polymers were taken and different batches of nanoparticle were prepared.

Drug entrapment efficiency (Moradhaseli *et al*., 2013) was determined by dispersing the nanoparticle suspension in the 70% aqueous ethanol and drug was extracted after vigorous vortexing followed by centrifugation. The curcumin concentration in the supernatant was determined with the help of UV spectrophotometer (Perkin Elmer, USA) at 549 nm and measured with the help of the calibration curve describing the absorbance and concentration relationship.

Formulation development

Folic acid conjugation method

FA-CUR-GNP_S were prepared by three step method. Firstly, the phenolic group of CUR easily attached to the amino group of GNP forming stable CUR-NH_2 bond. Secondly, EDC was used to activate the carboxyl group of gliadin forming an intermediate EDC- carboxylate. Thirdly, activated carbonyl group react subsequently with the free amino group of folic acid resulting in the formation of FA-CUR-GNP_S.

For Folic acid conjugation method; Folic acid (5 mg) was dissolved in 20 % w/v aqueous solution of sodium hydroxide

(10ml) and the formed solution was dropped into 10ml of phosphate buffer suspension (pH 7.4) containing 20 mg of gliadin-NPs under oscillation for 30 min.

Loading efficiency of FA in gliadin-NPs was determined by measuring the difference between the initially added FA amount and FA in the supernatant. The collected products were washed 3-4 times with deionized water, centrifuged at 20000 rpm for 15 min at 4°C, and freeze dried to obtain powders.

Preparation methodology of nanoparticles

Gliadin nanoparticles were prepared by simple desolvation method (Coester *et al*., 2000). This method includes preparation of aqueous phase A and non-aqueous phase B. Phase-B containing different concentrations of gliadin solubilized at 25°C in 20 ml of 70% ethanol/water mixture.

Drug (curcumin) was added to this phase in required quantities. Phase-A contains 0.5% concentrations of surfactant PF-68 in 40ml of triple distilled water. These two phases were filtered (Millipore 0.45 µ). Then phase-B is added dropwise with the help of the disposable syringe for 1 h. The nanoparticle suspension was concentrated using Rota vapor and were further purified by centrifugation for 15 min at 20000 rpm at 4°C. The supernatant formed was removed and pellets were re-suspended in 2ml of triple distilled water. Finally lyophilized by using 5% mannitol as a cry protectant.

Optimization of folic acid conjugated and non-conjugated nanoparticle

The optimization was done by Zetasizer Nano-ZS (Malvern Instruments Inc., UK) and the formulation with better particle size and zeta potential were selected for the study. The samples were loaded onto 1cm^2 cuvettes in a thermostat chamber and particle size were determined (Table 1).

Characterization of nanoparticles

Particle morphology by TEM

The characterization of FA-CUR-GNP$_S$ and CUR-GNP$_S$ were done by using transmission electron microscopy (TEM, JEOL 1011, Japan). A drop each of FA-CUR-GNP$_S$ and CUR-GNP$_S$ were suspended in triple distilled water placed onto a carbon-coated copper grid, forming a thin liquid film negatively stained with a drop of 1% (w/v) phosphotungstic acid. Excessive volume of staining solution was removed by using filter paper and the sample was air-dried. These samples were visualized under TEM and photographed.

Particle size measurements

The particle size (Storp *et al*., 2012) of FA-CUR-GNP$_S$ and CUR-GNP$_S$ were measured by Zetasizer Nano-ZS 90 (Malvern Instruments Inc., UK).

Surface charge (zeta potential) studies (Weber et al., 2000)

In disperse systems, electrical charges are developed at the interface between the dispersed phase and the aqueous medium. Two most common mechanisms are the ionization of surface functional groups and the specific adsorption of ions. The electrical charge determines the interaction between the particles of the dispersed phase and physical stability of the system, particularly for those in the colloidal size range (emulsion).

For a system when the zeta potential is relatively high (25mV or more, absolute value) the repulsive forces exceed the attractive London forces and the system is deflocculated. On the other hand, when the zeta potential is low, the attractive forces exceed the repulsive forces and leads to flocculation. Thus, the magnitude of the zeta potential gives an indication of the potential stability of the colloidal system (Table 2).

***In-vitro* release studies**

In-vitro drug release studies were carried out for FA-CUR-GNP$_S$ and CUR-GNP$_S$ in colonic fluids of pH 6.8 ±0.5 for 48 h at 37°C. The release studies of drug from both conjugated and non-conjugated were performed using a US Pharmacopeia dissolution rate test apparatus (paddle apparatus, 100 rpm, 37 ± 0.1°C).

Samples (2 ml) were withdrawn at appropriate time intervals and estimation of curcumin was done using UV spectrophotometer at 424 nm. Sink conditions were maintained with the addition of an equal volume of fresh medium at the same temperature. The percentage of drug released was expressed with respect to the drug content of the nanoparticles.

***In-vivo* toxicity studies**

The animal experiments were carried out by following experimental protocol guidelines of council for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India. Experiments were conducted after ethical clearance by the Institutional animal ethical committee (IAEC approval no. 1632/PO/Re/S/12/CPCSEA).

Acute-toxicity study

10 albino mice were taken for the study. Calculated dose was suspended in 5 ml of the vehicle and were given in divided doses at time interval of 2 h, for 15 days followed-up by monitoring of the animals for any occurrence of signs and symptoms of toxicity or mortality.

Sub-acute toxicity

6 male and 6 female albino mice, were taken for four test groups. The animals were gavage the relevant doses suspended in 0.5 ml of the vehicle, once daily, for a period of 28 days. At the end of the treatment, the animals were bled from the orbital sinus for clinical pathology assessment which included analysis of various hematology parameters like Hemoglobin [Hgb (g/dl)], total red blood cell count (RBC), total white blood cell count (WBC), packed cell volume (PCV %) etc. The animals were sacrificed by cervical dislocation.

RERULTS AND DISCUSSION

Identification and characterization of Curcumin

Fourier transform infrared spectrum (FTIR)

The drug, polymer, FA-CUR-GNP and CUR-GNP were identified by IR spectroscopy (Fig. 1) and characteristic peaks obtained were compared with standard spectra of pure drug, polymer, folic acid conjugation and non-conjugation as reported in official monograph and were found in agreement with the standard.

Functional group frequencies for Curcumin shows (Carbonyl 1651.46 cm-^{1}, Alkenes 1649.8 cm-^{1}, Alcohol 1025.92 cm-^{1} and Benzene 1431.91 cm-^{1}); Gliadin (Amides 1676.84 cm-^{1}, Benzene 1559.92 cm-^{1}) Folic acid conjugation (carbonyl 1651.46 cm-^{1},hydroxyl 1225.92 cm-^{1}, amino3000.69 cm-^{1}); Folic acid non conjugation (phenolic 1227.42 cm^{-1}) indicating the purity of drug.

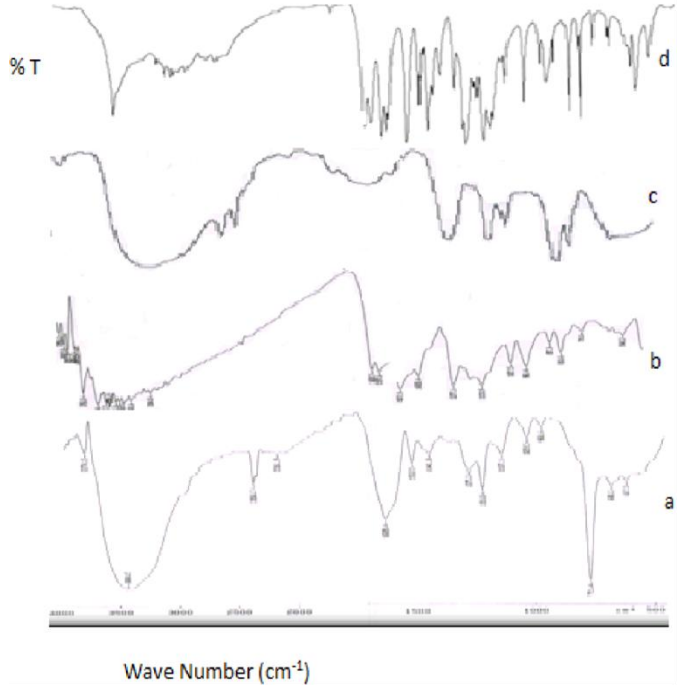

Fig. 1: FTIR spectra of curcumin, gliadin, FA-CUR-GNP and CUR-GNP.

Mass spectroscopy

The mass spectrum (Fig. 2A and 2B) clearly indicates the exact molecular weight result of Curcumin and Gliadin were of 369.1323 and 542.3 respectively as shown in base peak. The mass spectrum result of Gliadin (Fig. 3) clearly indicates the exact molecular weight 542.3 compared with the standards which was reported in the literature (Jasim and Ali, 1988).

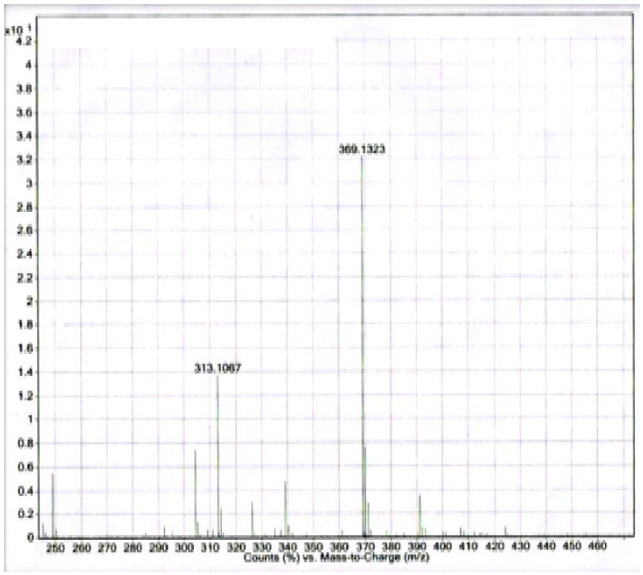

Fig. 2a: Mass spectrum of curcumin.

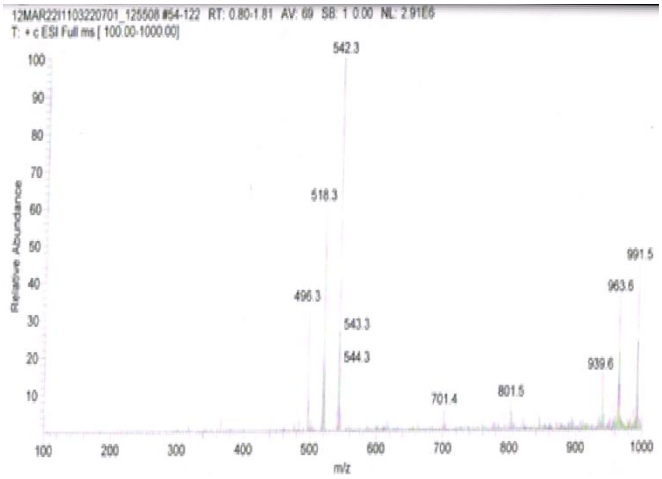

Fig. 2b: Mass spectrum of gliadin.

NMR spectrum of curcumin

The NMR spectra were recorded on a Bruker WM-600 (600 MHz) spectrometer. The data was processed using the Topspin software (Fig. 3).

The ^{1}H NMR spectrum of curcumin contains two singlets at 3.83 and 9.69 ppm due to the protons of the two methoxy groups and the protons of the two hydroxyl groups respectively which reflects its symmetric structure. The most important signals which give aid to elucidate the molecular structure of the compounds are those arise within the ranges 2.40-2.63, 2.80-3.08 and 4.66-5.08 ppm each of them has the integral of one proton (Wolinski *et al*., 1990).

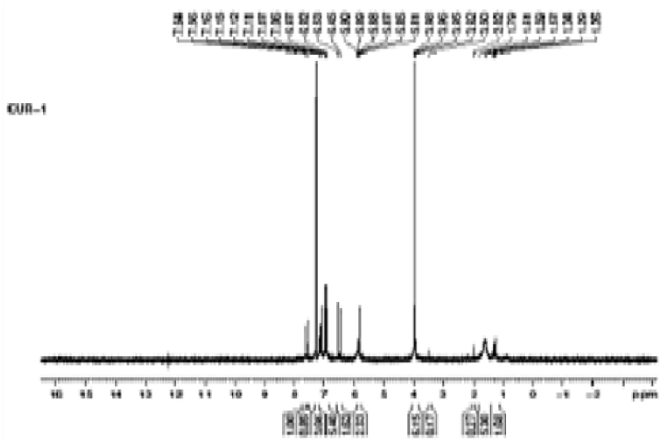

Fig. 3: NMR spectrum of curcumin.

Solubility studies

Solubility profile of curcumin

The solubility of curcumin were determined in aqueous and non-aqueous solvents. Curcumin is insoluble in water, slightly soluble in methanol, ethanol, soybean oil, oleic acid and vitamin E acetate but freely soluble in acetone.

Solubility profile of gliadin

The highest solubility of Gliadin was found in ethanol (70% v/v).

Partition coefficient

The partition coefficient was found to be 3.62 in n-octanol: water system. This concludes that drug is lipophilic in nature since the drug did not show any preference to the water and only soluble in the organic solvents. The octanol/water partition coefficient (log $P_{O/W}$) constitutes a quantitative and easily accessible, hydrophobicity measurement. Log P values are widely used in bio-accumulation studies, in drug absorption and toxicity predictions and lipophilicity of the drug. Partition coefficient values of 3.72 indicates the lipophilic chacteristic of the drug.

Entrapment efficiency

Size Distribution of various formulation of different batches were presented (Table 1). The entrapment efficiency of F5 and F6 were highest (52% and 56%) among CUR-GNP_S and FA-CUR GNPs respectively. Among the six formulation, F-3 and F-4 were selected as an optimized formulation due to its narrow size distribution as well as good entrapment efficiency and were further evaluated for drug release and *in vivo* uptake.

Preparation of nanoparticles

Preparation of folic acid conjugated Gliadin nanoparticles of curcumin were successfully formulated. FA-CUR-GNPs and CUR-GNPs were carefully envisaged. Folic acid is also conjugated with the polymer that helps in the targeted delivery of the drug towards the cancer cells since many cancer cells are expressing folic acid on its surface. Folic acid has received enormous consideration for biocompatibility or no immunogenicity, high stability and economic cost (Bhattacharya *et al*., 2011). By mean of receptor-mediated endocytosis, it has been reported that folate conjugates mediate faster internalization kinetics than other approaches through cellular membrane.

Characterization of nanoparticles

Particle morphology by TEM

The particle morphology by TEM (Fig. 4) for both F-3 and F-4 were found to be spherical. The characterization of FA-CUR-GNPs and CUR-GNPs nanoparticles obtained were of size range of 160–190 nm (Fig. 4). The folic acid conjugated cross-linked particles showed a round morphology with increased size. Transmission electron microscopy has been the preferred method of studying the cellular uptake of nanoparticles. Due to its high resolution TEM enables the imaging of membrane invaginations, vesicle formation and organelles (Song *et al*., 2010).

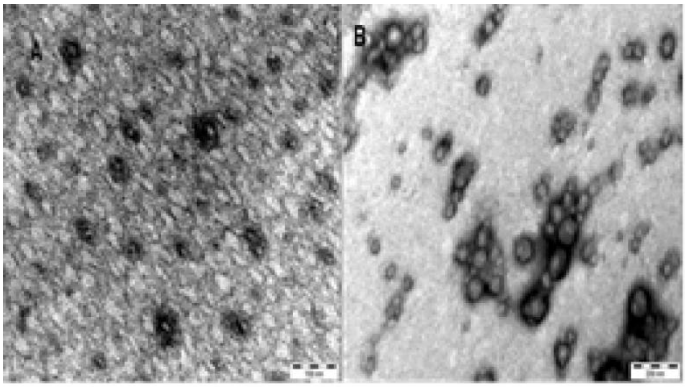

Fig. 4: Transmission electron micrographs of (A) CUR-GNP; (B) FA-CUR-GNP.

Particle size distribution

Particle size distribution of F-1, F-2, F-3, F-4, F-5 and F-6 were measured (Table 1) and shows that the particle size of CUR-GNP_S is smaller than FA-CUR-GNP_S. F-3 and F-4 formulations of CUR-GNP and FA-CUR-GNPs respectively were found satisfactory. Increased particle size (118.5 to 476.8 nm) was due to rise in polymer amount in ethanol (20 to 25 mg). Firstly modification of CUR and FA onto the surface of GNP_S increases the particle size secondly, the amine and carboxyl group of gliadin and FA attract counter ions from the solution to form electrical double layer. Increase in particle size could be significantly affected by the polymer amount when other formulation variables were kept constant.

Optimal particle size should be needed for a drug delivery, so that it will not easily leak out of the capillaries and up taken by the macrophages. We developed particle (190 nm) which is optimal for the cellular uptake and FA conjugation will help in the active targeted delivery of nanoparticle into the cancer site leads to faster accumulation and release of drug to that particular site (Khare and Peppas, 1995).

Table 1: Particle size optimization.

Formulation No.	Drug (curcumin) amount (mg)	Folic Acid (mg)	Polymer (gliadin) amount (mg)	Size (nm)	P.D.I.	Zeta potential (mV)	% E.E.
F-1	5	5	10	118.5±13.7	0.432	-1.58	18±0.9
F-3	10	5	20	168.1±13.6	0.339	-16.5	45±2.6
F-5	12.5	5	25	367.7±22.8	0.440	-12.9	52±3.1
F-2	5	-	10	202.2±16.2	0.469	-5.45	25±1.2
F-4	10	-	20	195.7±16.5	0.456	-24.4	48±2.4
F-6	12.5	-	25	476.8±25.3	0.392	-2.43	56±3.5

Results are expressed in terms of mean ± SD (n= 3), PDI: Polydispersity index, nm: nanometer, mV: millivolt, % EE: percent entrapment efficiency, SD: standard deviation.
F-1, F-3, F-5 CUR-GNP$_S$; F-2, F-4, F-6 FA-CUR-GNP$_S$
CUR-GNP$_S$ (Folic acid non-conjugated gliadin nanoparticles of curcumin)
FA-CUR-GNP$_S$ (Folic acid conjugated gliadin nanoparticles of curcumin)

Surface charge (zeta potential) studies

F-3 and F-4 formulations were found satisfactory because of their higher (-16.5 and -24.4 mV) zeta potential values as mentioned in classification of colloidal system according to zeta potential distribution (Table 2). Greater the zeta potential, greater the stability. Zeta potential was a reliable indicator in the prediction of stability of particles in liquid medium and the possible interactions with other materials. Previous work had shown that negatively charged lipid vesicles (especially with a charge around -30 mV) had good stability and might be optimized for drug delivery (Tan *et al*., 2010).

Table 2: Classification of colloidal system according to zeta potential distribution

Zeta potential (mV)	Stability behavior of the colloid
from 0 to ±5	Rapid coagulation or flocculation
from ±10 to ±30	Moderate stability
from ±30 to ±40	Good stability
mV: millivolt	

In-vitro release studies

The *in vitro* release profile shows how a delivery system works and gives a valuable insight into its *in vivo* behavior.

The release of CUR from the Gliadin nanoparticles was relatively faster than that from the FA-GNP nanoparticles. About 33% and 10% of CUR were released from the GNP and FA-GNP nanoparticles respectively. But after 12 h, the release of CUR from Gliadin nanoparticle was slower than that of FA-GNP nanoparticle. The drug release data obtained for the selected formulation F-3 and F-4 (Fig. 5) and their cumulative % release were found 92.92% and 94% respectively. The result reveals that the cumulative release of CUR from FA-GNP nanoparticle is more than of GNP nanoparticle. *In vitro* release profile of CUR appeared to be in different patterns. A burst release in the first period. The rapid initial release involved the diffusion of the bound or adsorbed drug at the surface of the nanoparticles. Release of CUR from the gliadin nanoparticles was relatively faster than that from the FA-GNP nanoparticles; this is due to the lower crosslinking ability of the FA–GNP nanoparticles. After 12 h the release of CUR from Gliadin nanoparticle was slower than that of FA-GNP nanoparticle that due to degradation of the polymer.

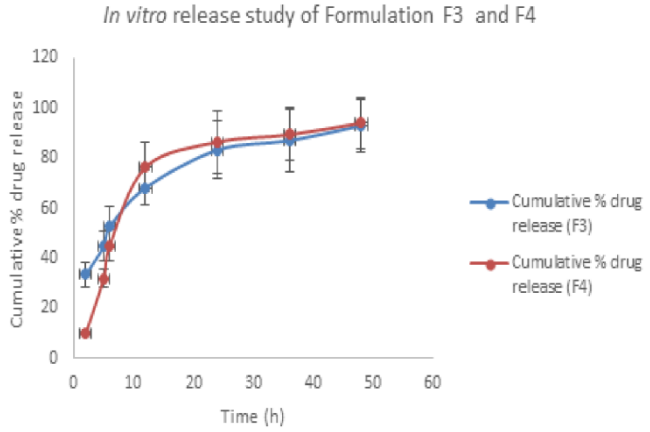

Fig. 5: *In vitro* release study of formulation F-3 (CUR-GNP) and F-4 (FA-CUR-GNP).

In-vivo toxicity study

The toxicity study were performed for both FA-CUR-GNPs, CUR-GNPs and then compared with the control and toxicity profile was shown in (Fig. 6). The above graph shows that both F-3 and F-4 formulations were safe, compatible and within the normal range. Clinical pathological testing of laboratory animals is a major safety assessment for new drugs in development for general health screening of animals considered for placement in toxicity studies (Leissing *et al*., 1985). Phosphate-buffered saline (pH 6.8) has been used as a vehicle for *in vivo* exposure and does not alter the biological activity of the test nanoparticle (Sager *et al*., 2007).

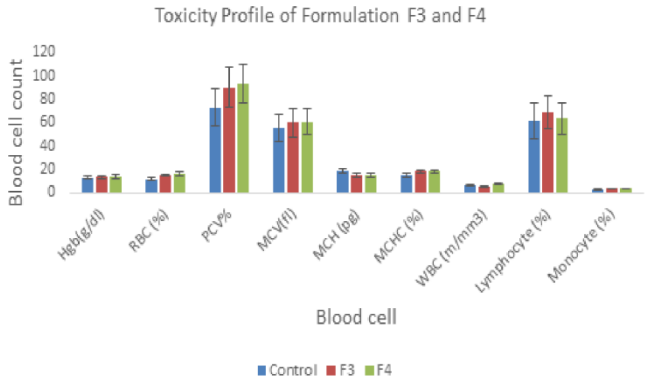

Fig. 6: Toxicity Profile of formulation F-3 (CUR-GNP) and F-4 (FA-CUR-GNP).

CONCLUSION

Colon cancer is the third most diagnosed and leading cause of death. The development of multidrug resistance in cancer

therapeutics results in a minimal response to conventional cytotoxic agents and targeted biological therapies. A great need for alternative treatment options of the disease has encouraged researchers. Curcumin, is a prominent candidate for treating colon cancer. Lower solubility overcome by improving its bioavailability, protect it from degradation and metabolism, and increase its targeting capacity toward cancer tumor(s). Therefore the development of efficient curcumin-gliadin- folate conjugate system is a current challenge in this era. The FA-CUR-GNP_S showed high *in vitro* drug release and the stability of the formulations was also maintained. The globule size was also found in the Nano-range, hence providing better bioavailability of the drug. The surface charge was also found to be in range thus, the stability of the formulations was also maintained throughout the experiment. Further *in vivo* toxicity study also revealed that the formulation was non-toxic.

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