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Original Article

FORMULATION, OPTIMIZATION AND *IN VITRO* EVALUATION OF 5-FLUOROURACIL LOADED LIQUORICE CRUDE PROTEIN NANOPARTICLES FOR SUSTAINED DRUG DELIVERY USING BOX-BEHNKEN DESIGN

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ABSTRACT

Objective: To formulate, optimize and evaluate 5-fluorouracil loaded liquorice crude protein nanoparticles for sustained drug delivery using Box-Behnken design.

Methods: 5-fluorouracil (5-FU) loaded liquorice crude protein (LCP) nanoparticles were prepared by desolvation method using ethanol-water (1:2 ratio), Tween-80 (2%v/v) as stabilizing agent and gluteraldehyde (8% v/v) as cross linking agent. The optimization of prepared nanoparticles was carried out using Box-Behnken design with 3 factors 2 levels and 3 responses. The independent variables were A)5-FU concentration B)LCP concentration and C) sonication time while the responses were R1) Drug entrapment efficiency R2) Drug loading efficiency and R3) Particle size. The correlation between factors and responses were studied through response surface plots and mathematical equations. The nanoparticles were evaluated for FTIR, physicochemical properties like particle size and zeta potential by Photon correlation spectroscopy (PCS) and surface morphology by TEM. The entrapment efficiency, drug loading efficiency and *in vitro* drug release studies in PBS pH 7.4 (24 h) were carried out. The observed values were found to be in close agreement with the predicted value obtained from the optimization process.

Results: 5-fluorouracil loaded LCP nanoparticles were prepared by desolvation method, the optimization was carried out by Box-Behnken design and the final formulation was evaluated for particle size (301.1 nm), zeta-potential (-25.8mV), PDI(0.226), with entrapment efficiency (64.07%), drug loading efficiency (28.54%), *in vitro* drug release (65.2% in 24 h) respectively. The formulated nanoparticles show Higuchi model drug release kinetics with sustained drug delivery for 24 h in pH7.4 buffer.

Conclusion: The results were proved to be the most valuable for the sustained delivery of 5-Fluorouracil using liquorice crude protein as carrier. 5-FU–LCP nanoparticles were prepared using Tween-80 as stabilizing agent and gluteraldehyde as cross-linking agent to possess ideal sustained drug release characteristics.

Keywords: Nanoparticle, Plant protein, Desolvation method, Particle size, Zeta potential, Entrapment efficiency, Drug release

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INTRODUCTION

The formulation of nanoparticle-based drug delivery systems is rapidly developing due to their great targeting potential. These materials are mainly used in oncology for early detection of malignancy and precise localization of cancer therapeutics without or with minimal adverse effects to the somatic tissues [1]. Drugs, vaccines, nutrients and cosmetics are protected by the use of properly designed carriers. Nanoparticles exert its site-specific drug delivery by bypassing the reticuloendothelial system, making use of enhanced permeability and retention effect and tumour specific targeting. The formation of nanoparticles and physicochemical parameters such as pH, monomer concentration, ionic strength as well as surface charge, particle size and molecular weight are important factors for drug delivery. A major problem in chemotherapy, which is multidrug resistance can be reversed by these nanoparticles.

Biodegradable protein nanoparticles have attained much interest in the past few years due to their wide variety of desirable properties like low toxicity [2]. Nanoparticles derived from green particles (natural proteins) are easily adaptive to surface modifications and targeting ligands. Biomolecule carrier-mediated drug delivery suggests a number of design opportunities for the delivery of a particular drug, with greater therapeutic effect [3]. It is proven that the encapsulation of anti-cancer agents in nanometer and micrometer particle size range can control their release, and nanotechnology could be a better alternative to increase the life expectancy of cancer patients.

5-Fluorouracil (initially 7-12 mg/kg IV for 4 d) [4] is a cell cycle–phase-specific broad-spectrum anti neoplasic agent. 5-FU exerts its cytotoxic activity by interfering with nucleoside metabolism thus competing for the enzyme that is essential for the synthesis of thymidine, an important substrate for DNA synthesis. Due to its high rate of metabolism in the body, a continuous administration of high dose is required for the maintenance of therapeutic serum concentration, which leads to severe toxic effects.

The present study aims to formulate, optimize and characterize 5-fluorouracil loaded liquorice crude protein nanoparticles for sustained drug delivery using Box-Behnken design.

Protein-based nanoparticles are notable due to their relative safety, easy preparation and size monitoring. They are also susceptible to various modifications to incorporate functional and targeting capabilities. A protein-based nanocarrier system that has made an impact in cancer therapy is the albumin-bound Paclitaxal (Ambraxane, ABI008) by FDA for metastatic breast cancer. A number of studies have given evidence that albumin accumulates in solid tumours making it a potential carrier for targeted delivery of antitumor drugs [5-7]. They are deemed as ideal compounds for nanoparticle preparation because of their amphiphilicity, allowing them to interact well with both the drug and solvent. Protein-based drug delivery systems have a great advantage of conventional drug delivery systems due to their biodegradability, non-antigenicity and excellent biocompatibility to improve the therapeutic properties of anti-cancer drugs [8].

In this present study, nanoparticles were prepared with crude protein extract obtained from Liquorice (*Glycerrhiza glabra L.*) roots (Fabaceae) as carrier molecule and with broad-spectrum anticancer drug 5–fluorouracil as a model drug [9-11].

The model drug used in the present study is 5-Fluorouracil, which is a hydrophilic pyrimidine analogue and is one of the broad spectrums anti-cancer drugs used in the treatment of various malignancies. Due to its short half-life and various side effects, its medical applicability is limited.

Many studies as per the literature review, shows 5-fluorouracil nanoparticles prepared with BSA (Bovine serum albumin) shows good targeting properties compared to free drugs. As an alternative to blood-derived albumin and recombinant human serum albumin, natural/green protein derived from liquorice crude protein (LCP), which are albuminoidal in nature, has been used in this study for the sustained delivery of 5-fluorouracil for the first time [12, 13].

MATERIALS AND METHODS

Drugs and chemicals

5-fluorouracil (5-FU, purity 99%) was obtained from Sigma–Aldrich Chemical Company Ltd. Liquorice fine powder was purchased from Indus valley Bioorganic (100% natural pure liquorice powder). Liquorice crude protein extract was prepared in phosphate buffer (pH 7.2) and all the reagents used in the present study were of analytical grade.

Preparation of liquorice crude protein (LCP) extract

Finely powdered liquorice root was extracted in phosphate buffer (pH 7.2) in 1:5 (w/v) ratio and 100Mm NaCl in order to stabilize the protein. After 24 h under refrigeration (4 °C), coarse filtration and centrifugation were done [14]. Then proteins were precipitated by salting out method. Impurities were removed by changing pH and dialyzed to remove the excess salt for 2 d with intermittent changing of buffer. The dialyzed liquid was freeze-dried to get crude freeze dried liquorice protein extract. Sherif EA Badr studied the protein content in different extracts of liquorice, which was confirmed by the analytical report of the present study, which showed 33.53% crude protein using Kjeldahl method in PBS pH 7.2. Lowry assay method was conducted to quantify the total protein content of the freeze-dried extract after dialysis [15].

Protein estimation

Protein was estimated by the method as described by Lowry. The blue colour developed by the reduction of the Phosphomolybdic-Phosphotungstic components in the Folin-ciocalteau reagent by the amino acids Tyrosine and Tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry method.

Materials used are 2% Sodium carbonate in 0.1N Sodium Hydroxide (Reagent A), 0.5% Copper sulphate ($CuSO_4 \cdot 5H_2O$) in 1% potassium sodium tartarate (Reagent B), Alkaline copper solution: About 50 ml of reagent A was mixed with 1 ml of reagent B prior to use (Reagent C), Folin Ciocalteau reagent (Reagent D): Phenol reagent is mixed with an equal volume of water before use, Protein Stock Solution–About 50 mg of Bovine serum albumin (BSA) was dissolved in 50 ml of standard flask, Working standard: About 10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask (One ml of this solution contains 200µg protein).

Sample preparation

About 0.5 mg of sample was taken and dissolved in 500 µl distilled Phosphate buffer

Procedure for estimation of protein: About 0.2 ml of sample extract was taken. Volume was made up to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank. 5 ml of reagent C was added to each tube, including blank. It was then mixed well and allowed to stand for 10 min. Then 0.5 ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 min. Blue colour developed was read at 660 nm. A standard graph was plotted using BSA as standard and amount of protein in the sample was calculated.

Formulation of 5–Fluorouracil loaded LCP nanoparticles

5FU loaded LCP nanoparticles were formulated using the desolvation method to yield the nanoparticles with lower particle size, more uniform size distribution and high entrapment of 5-FU. For the preparation of nanoparticles 5-FU was added to the LCP solution (1:2) in water, adjusted the pH to 8-8.4. Afterward, the solution was desolvated by the addition of ethanol at the rate of 1 ml per minute under stirring, followed by the addition of Tween 80 (2%w/v) with constant stirring in a magnetic stirrer of 600 rpm until the suspension becomes turbid. One drop of glutaraldehyde solution (8% v/v) was added as a cross-linking agent and continued stirring for another 4 h. Ethanol was evaporated and Nanoparticles formed were sonicated for 40 min (ice bath) and homogenized in a high-pressure homogenizer. The drug-loaded nanoparticles were then separated by centrifugation (10,000 x 2 min) and lyophilized to get the 5-FU loaded LCP nanoparticles [16-18].

Optimization of prepared nanoparticles

Box-behnken design

A Box-Behnken statistical design with 3 factors, 2 levels and 17 runs was selected for the study using Design-Expert 11.0.2 software trial version (Stat-Ease Inc, Minneapolis, USA). This design is suitable for exploring quadratic response surfaces and constructing second order polynomial models. The primary screening studies revealed that the LCP concentration, Drug concentration and sonication time as significant factors [19-21].

The polynomial equation generated by the experimental design is as follows:

$$R=C_0+C_1A+C_2B+C_3C+C_4AB+C_5AC+C_6BC+C_7A^2+C_8B^2+C_9C^2$$

Where R is the dependent variable, C_0 is the intercept C_1–C_9 are the regression coefficients, and A, B, C are the independent variables.

Evaluation of 5-fluorouracil loaded LCP nanoparticles

Particle size, surface charge and morphology

Particle size and size distribution of nanoparticles were determined by dynamic light scattering (DLS) using a particle size analyzer (Malvern Instrument Ltd, UK). Samples were diluted with double distilled water and measured at the temperature of 25 °C and a scattering angle of 90°. To determine the zeta potential, Nanoparticles were dispersed in de-ionized water and taken in disposable zeta cells and measured by laser Doppler anemometry using Zetasizer (Malvern Instrument Ltd, UK) [26]. The experiments were performed in triplicate and results are presented as mean±SEM. The morphological features of lyophilized 5-FU-LCP Nanoparticles were examined by Transmission electron microscopy (TEM) (JSM-7600F, JEOL, USA) [22-25].

Transmission electron microscopy-(TEM)

The morphology, structure and particle size of 5-FU LCP nanoparticles were examined by Transmission Electron Microscopy by JOEL model JSM-6390 LV an Electronic Transmission Microscope at 70 KV. In this method, nanoparticles were dispersed onto a surface of a copper grid sample holder. Upon drying the grids, which is then stained with 1% w/v phosphotungstic acid for 120 seconds and dried at room temperature. These samples were then placed in sample holders and probed with Transmission Electron Microscopy. The image was then photocopied, in which objects were visualized in order of angstroms.

Drug entrapment efficiency (% DEE)

For the determination of entrapment efficiency, accurately weighed 5-FU loaded nanoparticles (10 mg) were added to 10 ml of phosphate buffer and centrifuged at 5000 rpm for 1 hour using REMI R-8C centrifuge. The supernatant solution was filtered, diluted suitably and absorbance was measured using JASCO V-630 UV spectrophotometer at 266 nm. Concentration of drug in the supernatant was calculated using the standard calibration data. The entrapment efficiency was calculated using the formula.

$$\text{Entrapment efficiency (\%)} = \frac{\text{Entrapped drug in nanoparticles} \times 100}{\text{Amount of total drug}} \quad \text{-Equation 1}$$

Drug loading efficiency (% DLE)

Evaluation of drug loading efficiency of LCP nanoparticles were done by taking the UV-Visible absorbance of initial 5-FU content in the

solution and the residual 5-FU content in the collected supernatants at 266 nm. The amount of 5-FU was determined by a calibration curve method and the calibration plot was generated from a series of 5-FU solutions with different concentrations.

$$\text{Drug loading efficiency (\%)} = \frac{\text{Entrapped drug in the nanoparticles x 100}}{\text{Amount of nanoparticles}} \quad \text{-Equation 2}$$

Drug excipients compatibility study by FTIR spectroscopy

5 FU-LCP mixtures was mixed with IR grade Potassium bromide (KBr) and made into a transparent and homogenous pellet using pressed pellet technique. FTIR scanning was performed using Agilent Technologies CARY 630 FTIR. FTIR spectra were obtained in the range of 400-4000 cm^{-1} under operational conditions. The spectrum obtained and peaks were studied and compared with the reference spectrum.

***In vitro* drug release study**

To study the release kinetics, a sample of nanoparticles equivalent to 5 mg of drug was dissolved in 2 ml buffer solution and taken in a dialysis bag with both ends clipped (dialyzing membrane-150, molecular weight cut off 12000-14000 Dalton) were kept in 100 ml of phosphate buffer saline pH 7.4 under continuous stirring at 60 rpm at 37±0.5 °C. After different time intervals, 2 ml of sample volume were withdrawn from dissolution media and replaced with the fresh buffer to maintain sink conditions. The samples were filtered with a membrane filter (0.22µ) and the amount of drug released was quantified by using UV Spectrophotometer at a wavelength 266 nm against blank and cumulative drug release was calculated [27]

Release kinetics

The mechanism of 5-FU release from the nanoparticles was studied by fitting the *in vitro* release data into kinetic models such as zero order, first order, Higuchi and Korsmeyer–Peppas model.

Stability studies

The 5-FU loaded LCP nanoparticles suspension was stored at 4 °C, 25 °C, and 40 °C respectively for 6 mo and the effect on particle size, drug loading efficiency and drug release were studied.

RESULTS AND DISCUSSION

Estimation of protein by lowry assay method

As per Lowry assay method, total protein content of the freeze dried sample of LCP extract is shown in table 1.

Table 1: Protein content in LCP extract as per lowry assay method

Sample	Protein(µg/ml)	(µg/mg) or ppm	Percentage %
1	91.25	456.25	0.04
1a	92.25	460.12	0.04
2	89.5	447.5	0.04
2a	94.75	473.25	0.04

Optimization using box-behnken design

The independent variables were A) 5-FU Concentration (% w/v) B) LCP Concentration (%w/v) C) Sonication time (min) and the dependent variables were R1) Drug entrapment efficiency (%) R2) Drug loading efficiency (%) and R3) Particle size (nm). The goal of optimization was to maximize entrapment efficiency and drug loading efficiency and to minimize particle size (table 2). Each factor was tested at two levels, upper limit and lower limit. Based on optimization in Box-Behnken design, contour plots and response surface plots were studied for the effects of independent variables on dependent variables with maximum desirability.

Table 2: Variables and their constraints in the box-behnken design

Variables	Constraints	
Independent variables	**Lower limit**	**Upper limit**
A: 5FU Concentration (%w/v)	1	3
B: LCP Concentration (%w/v)	2	6
C: Sonication time (mins)	20	40
Dependent variables	**Goal**	
R1: Drug Entrapment Efficiency (DEE) (%)	Maximise	
R2: Drug Loading Efficiency (DLE) (%)	Maximise	
R3: Particle size (nm)	Minimise	

Therefore a 3 factor 2 levels based Box-Behnken design with 17 formulations was applied to understand the impact of independent variables on dependent variables (table 3).

Table 3: Formulations of 5FU-LCP nanoparticles

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
	A: 5FU conc	B: LCP conc	C: Sonication time	Drug entrapment efficiency (DEE)	Drug loading efficiency (DLE)	Particle size
	%w/v	%w/v	min	%	%	nm
1	2	6	20	74.76±0.41	34.47±0.95	293.77±4.23
2	3	2	30	68.13±0.32	36.23±0.88	321.85±1.66
3	1	2	30	75.07±0.44	38.86±0.67	286.84±3.06
4	1	6	30	72.31±0.39	37.28±0.79	309.01±1.25
5	2	4	30	62.47±0.21	20.56±1.61	346.65±3.74
6	2	6	40	71.27±0.37	34.53±1.32	299.56±2.14
7	1	4	20	78.82±0.32	33.83±0.54	270.89±3.62
8	2	2	40	74.19±0.40	35.86±0.61	277.89±7.59
9	2	4	30	63.61±0.35	21.38±1.49	346.65±4.50
10	3	4	20	71.22±0.49	31.08±1.38	307.98±3.11
11	2	2	20	77.33±0.67	36.16±0.24	271.78±6.85
12	1	4	40	76.59±0.42	35.89±2.05	275.48±3.64
13	2	4	30	63.19±0.39	21.32±1.74	348.72±4.73
14	2	4	30	63.14±0.40	20.56±1.32	346.56±1.44
15	3	4	40	67.87±0.36	30.89±1.95	310.55±2.56
16	2	4	30	61.29±0.43	22.13±1.22	346.65±3.82
17	3	6	30	64.69±0.56	34.07±0.64	344.01±1.32

*Data are expressed as mean±SD (n=3)

Statistical optimization by response surface methodology

The Quadratic model was chosen as the best fit model based on regression coefficient values (R^2) very close to 1 (table 4) and based on p-values<0.05, which deemed the model to be significant.

Table 4: Fit statistics showing R^2 values

Response	R^2	Adjusted R^2	Predicted R^2
R1: Drug entrapment efficiency (%)	0.9930	0.9839	0.9736
R2: Drug loading efficiency (%)	0.9961	0.9912	0.9726
R3: Particle size (nm)	0.9995	0.9989	0.9957

Response 1: Drug entrapment efficiency (%)

The effect of entrapment efficiency on 5-FU conc, LCP conc and sonication time were studied.

The EE (%) increases with an increase in 5-FU conc and sonication time were observed (fig. 1).

The predicted R^2 value of 0.9839 was found to be in reasonable agreement with an adjusted R^2 value of 0.9736, The Model F-value of 109.68 implies that the model is significant.

$$R1 = 62.74-3.86A-1.46125B-1.52625C-0.17AB-0.28AC-0.0875BC+3.27375A^2+4.03625B^2+7.61125C^2$$

After observing p-values of coefficients in the polynomial equation, it is seen that terms A, B, C, A^2, B^2, C^2 are significant.

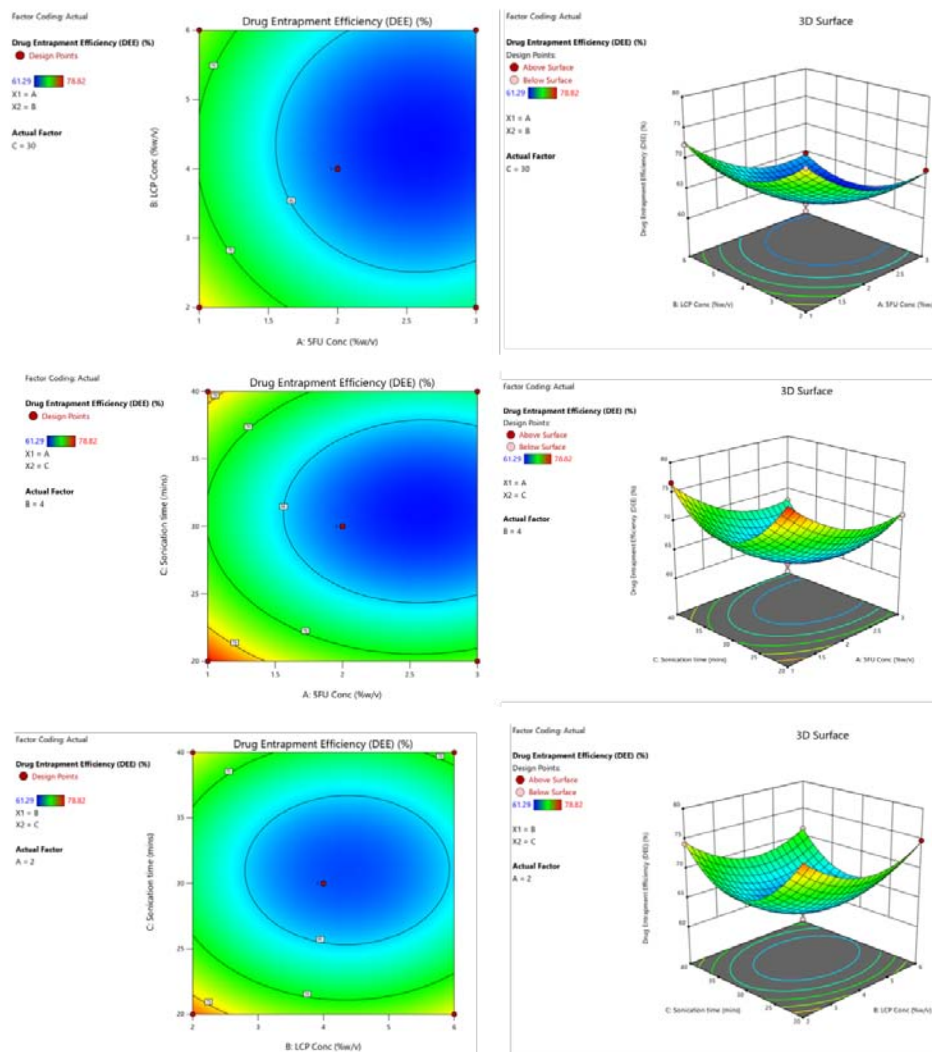

Fig. 1: Contour and response surface plot of factors against drug entrapment efficiency (R1)

Response 2: drug loading efficiency (%)

The effect Drug loading efficiency of on formulation factors was studied, the RSM plots shows direct relation between factors and responses (fig. 2).

The predicted R^2 value of 0.9726 was found to be in reasonable agreement with adjusted R^2 value of 0.9912, The Model F-value of 200.72 implies that the model is significant.

$$R2 = 21.19-1.69875A-0.845B+0.20375C-0.145AB-0.5625AC+0.09BC+6.54375A^2+8.87625B^2+5.18875C^2$$

After observing the p-values of coefficients in the polynomial equation, it is seen that terms A, B, A^2, B^2, C^2 are significant.

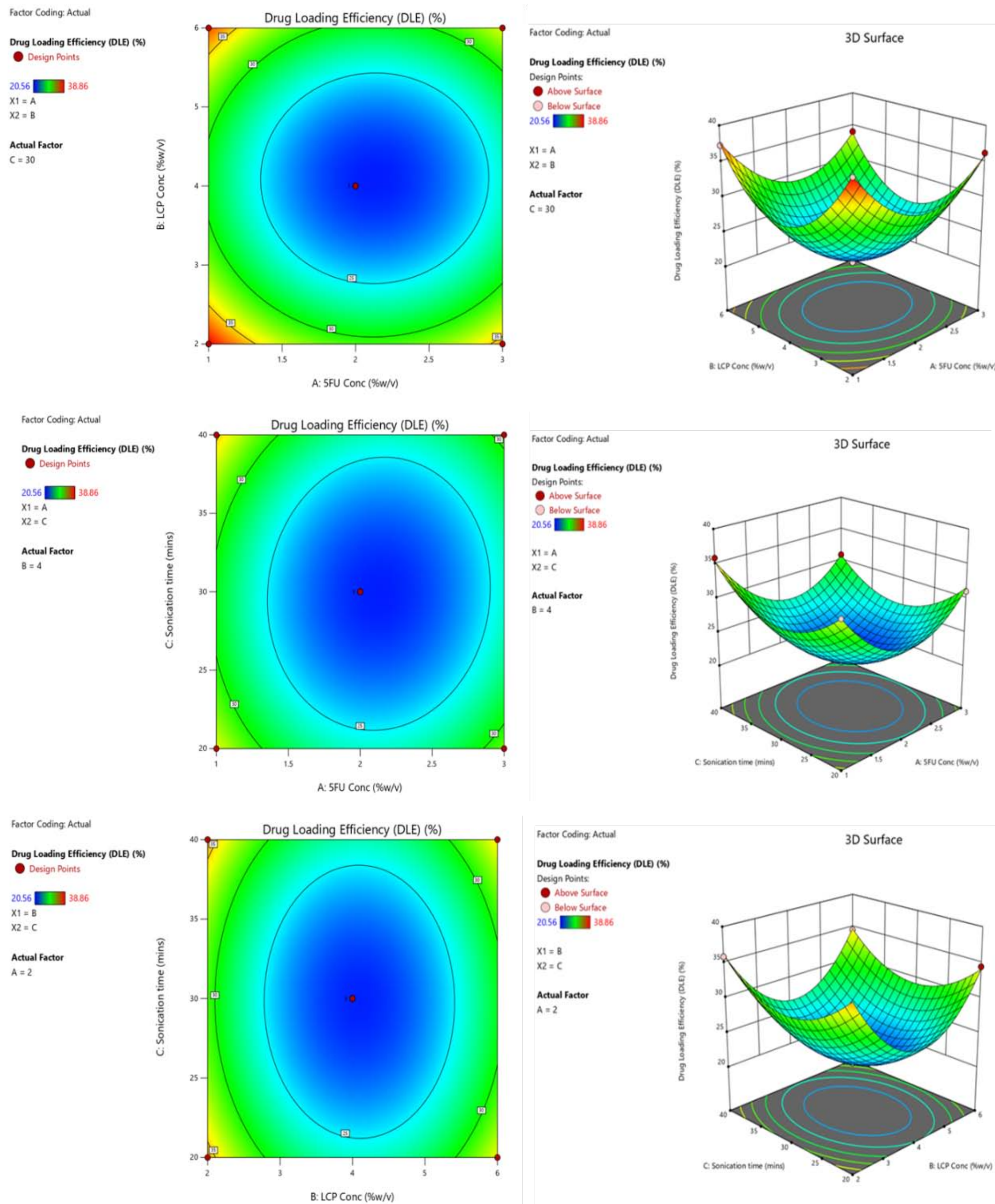

Fig. 2: Contour and response surface plot of factors against drug loading efficiency (R2)

Response 3: particle size (nm)

Effect of particle size on variables showed a direct relationship with 5-FU conc and LCP conc and an inverse relationship with sonication time as per fig. 3. The predicted R^2 value of 0.9989 was found to be in reasonable agreement with an adjusted R^2 value of 0.9957, The Model F-value of 1590.06 implies that the model is significant.

$R3 = 347.046+17.7713A+10.9988B+2.38253C-0.0025AB-0.55BC-0.0800592BC-13.0717A^2-18.5468B^2-42.7493C^2$

After observing the p-values of coefficients in the polynomial equation, it is seen that terms A, B, C, A^2, B^2, C^2 are significant.

Based on the above pre-optimization parameters the 5-FU loaded LCP nanoparticles were prepared.

The optimum levels of formulation factors for an optimized formulation based on the Box-Behnken design were 3% w/v of drug 5-FU, 4% w/v of LCP, 40 min sonication time with predicted values of 310.87 nm for particle size, 67.95% for DEE%, and 30.86% for DLE%.

Particle size, PDI, zeta potential

The mean particle size of nanoparticle formulation was in the range of nm. Formulation of 5-FU LCP nanoparticles showed relatively small particle size, i.e.270.89 nm to 348.72 nm. Poly dispersivity index (PDI) of 0.226 (fig. 4) and zeta potential of-33.2mV (fig. 5) were in the good range.

Fig. 3: Contour and response surface plot of factors against particle size (nm) (R3)

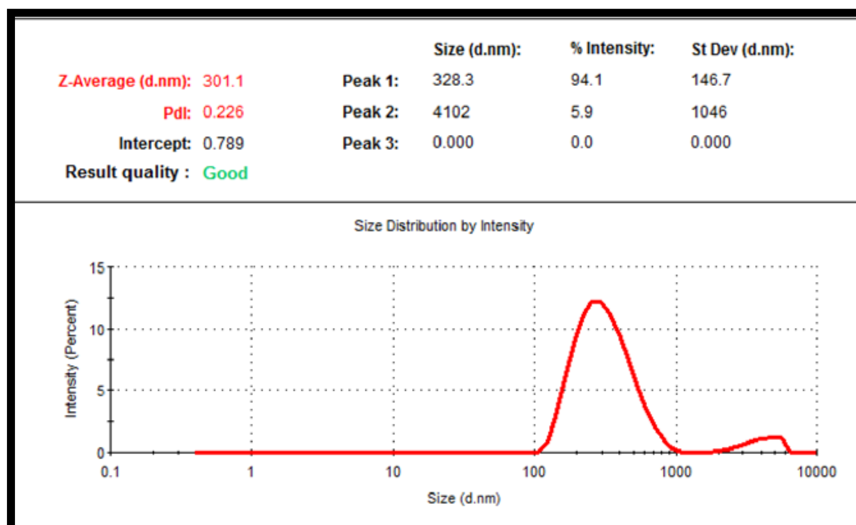

Fig. 4: Particle size analysis and polydispersity index of formulated 5-FU loaded LCP nanoparticles

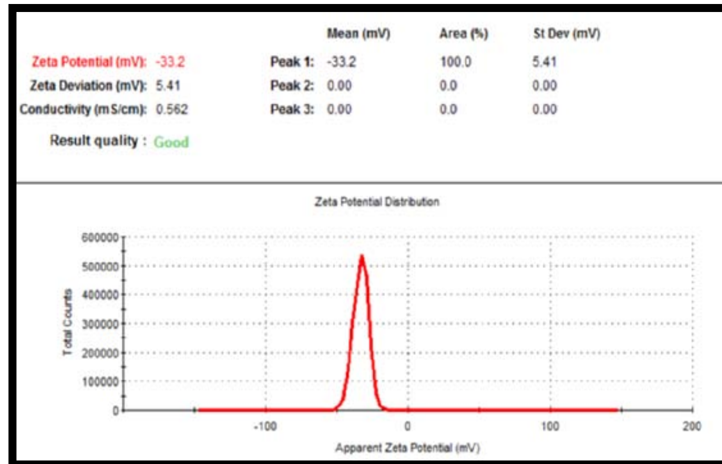

Fig. 5: Zeta potential of formulated 5-FU loaded LCP nanoparticles

TEM analysis of the prepared nanoparticles revealed a homogeneous solid matrix structure without any aggregation (fig. 6). Particle size distribution analysis by zetasizer showed that the mean average of the prepared nanoparticles was 301.1 nm and encapsulation efficiency of the prepared nanoparticles was found to be 64.07%.

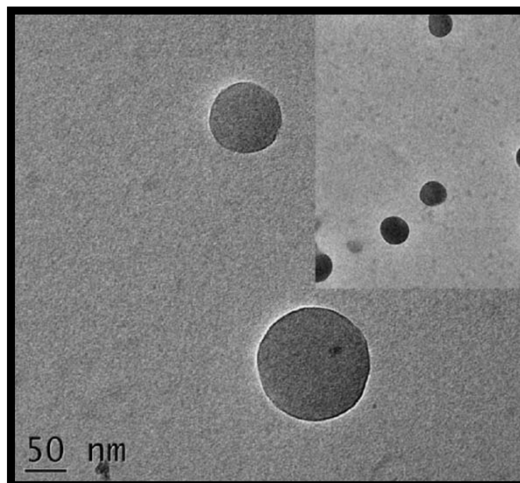

Fig. 6: TEM analysis of formulated 5-FU loaded LCP nanoparticles

Fourier transform infrared spectroscopy (FTIR) studies

FTIR spectra of pure 5-FU (fig. 7), LCP Extract (fig. 8) and the mixture of 5-FU and LCP Extract (fig. 9) are shown below. The principal IR absorption peaks of 5-FU were all observed in the spectra of 5-FU as well as 5-FU–LCP mixture. These observations indicated that no interaction between 5-FU and LCP were seen in the mixture.

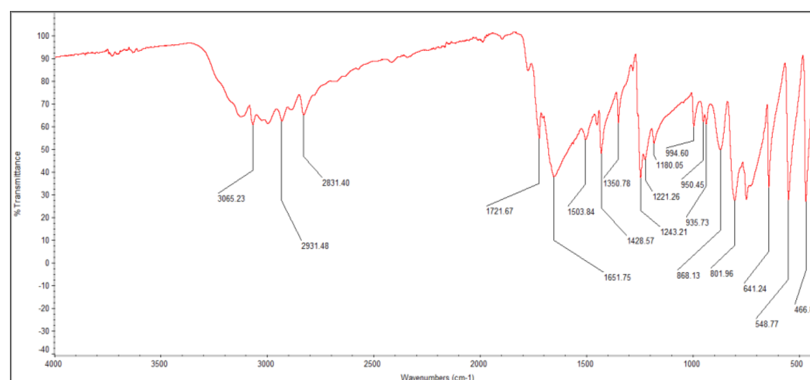

Fig. 7: FTIR spectrum of 5-FU

Table 5: FTIR data of 5-FU

Wave number (cm^{-1})	Functional group
3065.23	N-H stretching
1651.75	C=O stretching
1428.57	C-N stretching
1243.21	In-plane C-H bending

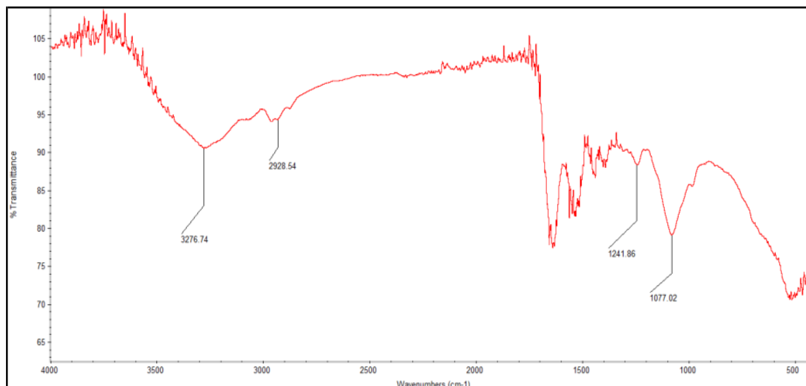

Fig. 8: FTIR spectrum of LCP extract

Table 6: FTIR data of LCP Extract

Wave number (cm^{-1})	Functional group
3276.74	-NH Stretch.
2928.54	-COOH stretch
1660	C=O bond stretching in peptide
1241.86	In-plane C-H bending

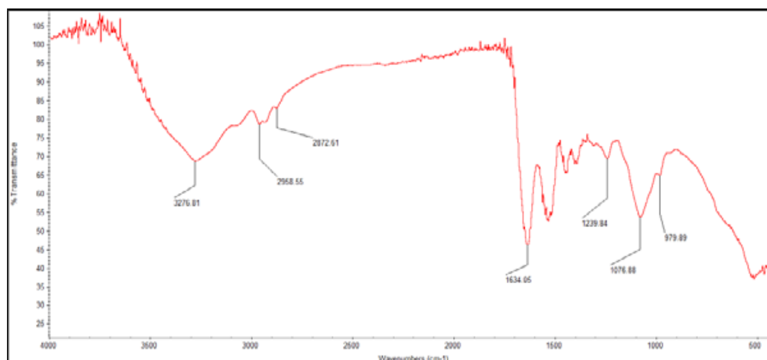

Fig. 9: FTIR spectrum of 5-FU and LCP extract

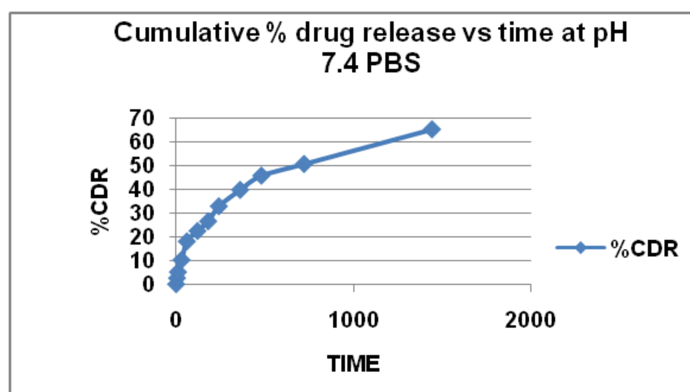

Fig. 10: Drug release profile of 5-FU from 5-FU loaded LCP nanoparticles

Table 7: FTIR data of 5-FU and LCP extract

Wavenumber (cm^{-1})	Functional group
3276.81	-NH Stretch.
2958.55	CH stretch
1634.05	-CO Stretch.
1239.84	Secondary amine NH bend

In vitro drug release from nanoparticles

The percentage release of the cumulative 5-fluorouracil from the optimized formula after a different time period is shown as a graph (fig. 10). After the initial burst release for 45 min, the release rate of 5-fluorouracil from the nanoparticles slowed down. After 24hours, the amount of accumulated 5-fluorouracil in PBS was calculated to be 65.2% of the entrapped drug [13].

Table 8: Data for drug release profile

Time in min	%CDR
0	0
5	2.54±1.21
10	5.08±0.87
30	10.18±1.42
60	18.01±2.74
120	22.41±1.34
180	26.54±2.37
240	32.89±3.11
360	39.76±0.97
480	45.72±1.97
720	50.68±2.01
1440	65.22±3.01

*Data are expressed as mean±SD (n=3)

In vitro kinetics studies of 5-FU LCP nanoparticles

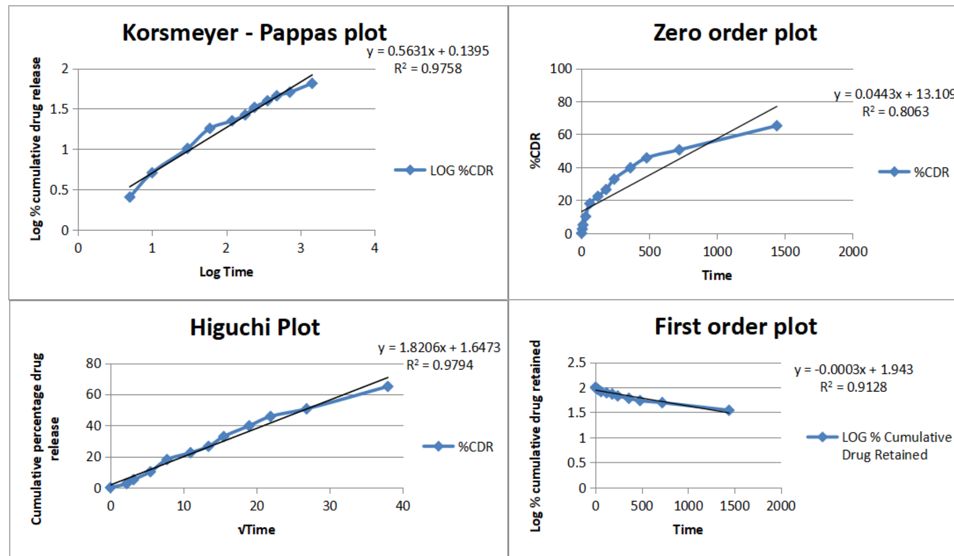

Fig. 11: *In vitro* drug release kinetics

Table 9: Release kinetics

pH	Zero-order	First-order	Higuchi	Korsmeyer–Peppas	
	R^2	R^2	R^2	R^2	N
7.4	0.806	0.912	0.979	0.975	0.562

Drug release kinetics

Drug release kinetics of the selected formulation was assessed by zero-order, first-order, Higuchi and Korsmeyer-Peppas mechanisms and the relevant plots are shown in fig. 11 and the corresponding data in table 9. It is evident that the *in vitro* drug release of 5-FU from LCP nanoparticles at pH 7.4 was best explained by Higuchi's model as the plot showed the highest linearity with the regression value of R^2 value 0.979. Korsmeyer-Peppas plot (R^2 value 0.975) with n value= 0.562 proved that the release follows Anomalous non-Fickian diffusion controlled mechanisms.

The optimized formulation was prepared using the desolvation method and the actual values of the responses were 301.1 nm for

particle size, 64.07% for drug entrapment efficiency, and 28.54% for drug loading efficiency. The actual values of responses were found to be in very close agreement to the predicted values which indicated the validity of the Box-Behnken design.

Stability studies

The 5-FU loaded LCP Nanoparticles suspension was stored at 4 °C, 25 °C, and 40 °C, respectively. The drug loading and encapsulation efficiency decreased sharply at 40 °C after 10 d. On the contrary, at 4 °C or at 25 °C, the drug loading and encapsulation efficiency decreased slowly during the test time. However, the nanoparticles at 25 °C were aggregated after 6 mo storage, while at 4 °C the nanoparticles were not. Moreover, at 4 °C the encapsulation efficiency and drug loading of nanoparticles decreased less than the other storage conditions (25 °C and 40 °C). The drug loading decreased only 1% after 6 mo storage at 4 °C. Therefore, the nanoparticles were suitable to be stored at 4 °C. Generally, proteins are best stored at ≤ 4 °C. Storage at room or higher temperature often leads to protein degradation and/or inactivity.

In recent years, the targeted delivery of anti-cancer drugs encapsulated in natural polymers, such as proteins of natural origin (green protein) has gained much attention as these proteins can act as carriers in targeting drugs to the particular sites. In the present study, an attempt was made to develop a nanoparticulate sustained delivery system for 5-Fluorouracil with LCP extract by simple coacervation/desolvation method. FTIR studies were carried out to find the possible interaction between the drug and the LCP extract and the fig. 9 shows there was no interaction between the drug and LCP extract. Optimization was done using Box Behnken design with 3 factors and 3 responses, which enables us to obtain spherical discrete spheres with a size ranging from 270.89 nm to 348.72 nm and the surface morphology was revealed by TEM analysis showed spherical particles with distinct borders (fig. 6). Among the different batches the formulation F15 was selected as ideal formulation, after considering the drug loading capacity, drug entrapment efficiency and *in-vitro* drug release with minimum particle size. The optimized formula was reformulated with 3%w/v 5-FU, 4%w/v LCP with sonication time of 40 min, after characterizing the particles we studied the *in vitro* drug release profile of formulated nanoparticles in PBS pH 7.4 (fig. 10) for 24 h with an drug entrapment efficiency of 64.07%, drug loading efficiency of 28.54% and particle size of 301.1 nm with very slight variations between optimized and observed values. The particle size was found to be reduced with sonication time due to the presence of Tween 80, which acts as a solubilizing agent. Release kinetics showed the drug release follows the Higuchi Non-Fickian diffusion-controlled (R^2= 0.979) with n value of Korsmeyer-Peppas equation 0.562 (R^2=0.975). Protein content in the LCP extract was confirmed by the Lowry assay method. The Study reveals the fact that Liquorice crude protein extract in phosphate buffer (7.2) contains albuminoidal protein as per the study by Li-jing Ke, for aconitine encapsulation with licorice protein. T. Mallamma studied the 5-Fluorouracil loaded HSA (Human Serum Albumin) nanoparticles for controlled drug delivery [23-30].

The *in vitro* drug release profile of the formulated 5-fluorouracil liquorice crude protein nanoparticles has two distinct phases-(i) Initial burst release phase (for about 45 min)–Must be due to the dissolution and diffusion of the drug that was poorly entrapped in the polymer (protein) matrix. (ii) Slower and continuous phase–After the initial burst release, this phase is attributed to the diffusion of the drug localized from the core of the nanoparticles. By comparing the *in vitro* drug release of 5-fluorouracil alone, and BSA 5-fluorouracil nanoparticles, this pattern of drug release has been reported before [5].

CONCLUSION

5–FU loaded Liquorice crude protein nanoparticles were formulated by desolvation/simple coacervation technique after extraction of LCP from the powdered liquorice root in PBS at pH 7.2. The present study provides a new approach and material for the application of active phytochemicals with much less safety concerns. By comparing the drug release from 5-fluorouracil nanoparticles alone, which is very fast, a sustained release of drug profile was obtained in the present study. With advanced techniques for purification and isolation of particular protein from the crude extract, and with proper selection of a dissolution medium at different pH, a sustained release pattern as that of albumin nanoparticles is attainable. The results of this study clearly indicate that there is a potential for nano particulate delivery of 5-fluorouracil liquorice crude protein (as carrier) from liquorice extract in minimizing drug induced toxicity. However extensive studies in terms of chronic toxicity, pharmacokinetic and pharmacodynamic are needed before establishing nanoparticle mediated green protein encapsulated delivery of this drug.

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AUTHORS CONTRIBUTIONS

All the author has contributed equally.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest associated with this work.

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