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Research article

Development and optimization of Liquorice crude protein nanoparticles from *Glycyrrhiza glabra L.*

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Keywords: Liquorice crude protein nanoparticles, simple coacervation method, Design Expert -12® software design, desirability function, optimization, particle size.

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Abstract

This study is focused on extraction, development and optimization of Liquorice crude protein nanoparticles from the root powder of *Glycyrrhiza glabra L.* Liquorice decoction has been used for various ailments from the olden times itself besides its flavouring action. Extraction of protein was done in phosphate buffer at -4ºC by salting out method. The precipitated protein (Liquorice crude protein) was purified by dialysis bag method with MWCO (12-14kDa) to remove the excess salt. The main approach in designing nanoparticles is to control particle size, surface properties and drug release. Liquorice crude protein was formulated into nanoparticles by simple coacervation/desolvation method using ethanol, water and tween-80 (2%v/v) by thermal cross linking. Optimization was done by using 2 factors and one response in Design Expert -12® software. Based on desirability function the optimized formula was selected and was characterized for various physical and chemical properties of nanoparticles. The particle size of nanoparticle was found to be 326.9 nm with PDI 0.271 and zeta potential of -10.9 mV. The potential of LCP nanoparticle formulation with desirability function in optimizing nanoparticle formulation has made it possible to identify the impact of various independent variables on optimization of the formulation for better responses. The finished products of liquorice protein nanoparticles are high in safety as it exists in various decoctions. These Liquorice Crude Protein nanoparticles can be applied to *in-vivo* delivery of various drugs.

Introduction

Protein nanoparticles as drug carriers offer enhancement in the delivery of drugs, especially targeted delivery to site-specific tissue, cells or organs. This can be attained through ligand attachment, sustained and controlled release of the pharmacologically active drug.
Drugs from plant origin have been used for the treatment of various types of diseases for generations [1]. About 80% of the world population relies on medicines from natural sources, primarily plants.
Liquorice (*Glycyrrhiza glabra L.* of Family Fabaceae) is one such herb known to mankind since ancient ages [2]. Currently many research works are based on liquorice, due to its presence of many constituents which are having cytotoxic, antitussive, antimicrobial properties and many other therapeutic properties.
Nanotechnology offers a promise for the targeted delivery of drugs [3], genes and proteins to tumour tissue there by alleviating the toxicity of anticancer drug in healthy tissue. Therapeutic proteins and peptides have a

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significant role in every field of medicine, especially in targeted drug delivery system [4-7].

The present research work aims to extract liquorice crude protein (LCP) from the powdered liquorice root and to study the different variables for the formulation and characterization of nanoparticles by desolvation method.

Simple method for the preparation of albumin nanoparticles is by desolvation, which involves continuous drop wise addition (at a rate of 1ml/min from a syringe needle) of anti solvents like ethanol or acetone into aqueous solution of albumin under magnetic stirring. Continue stirring until turbidity appears.

The objective of the study is to extract liquorice crude protein from liquorice root powder by salting out method, formulation into nanoparticles by desolvation/simple coacervation method followed by optimization of formulation variables and characterization of optimized nanoparticles.

Materials and methods

Materials

Freeze dried liquorice crude protein extract in phosphate buffer, Ethanol (90%), Tween 80 (2%v/v) and all other reagents and solvents used were of analytical grade. Equipments used were: magnetic stirrer, sonicator, centrifuge, freeze drier, and zeta sizer (Malvern).

Preparation of liquorice crude protein (LCP) extract

Finely powdered liquorice root was extracted in phosphate buffer (pH 7.2) for 24 hrs under refrigeration (4°C), after coarse filtration and centrifugation the proteins were precipitated by salting out method. Impurities were removed by changing pH and dialyzed to remove the excess salt for 2 days with intermittent changing of buffer. The dialyzed liquid was freeze dried to get crude freeze dried liquorice protein extract (LCP) [8-11]. (Sherif EA Badr *et. al.* states nearly 10% of protein in the raw herb, and in the extract was confirmed by analytical reports).

Characterization of liquorice crude protein extract

Total protein content

Crude protein content in the solid extract was determined by Kjeldahl method.

Protein identification test

Identification was done using chemical reaction test for proteins.

Surface morphology

The surface morphology of LCP extract was examined using SEM.

Fourier Transform Infrared Spectroscopy (FTIR)

Crude liquorice protein extract was mixed with IR grade KBr and made into a transparent and homogenous pellet using pressed pellet technique. FTIR spectra were taken at 4cm^{-1} resolution averaged over 32 scans in range of 400-4000 cm^{-1}.

Formulation of LCP nanoparticles

10 mg of Liquorice crude protein (LCP) was dissolved in 4 ml of water, adjusted the pH to 7.4, followed by the addition of 2 drops of Tween 80 (2%w/v). Afterwards, the solution was desolvated by addition of ethanol at the rate of 1mL per minute with constant stirring at a speed of 600 rpm. Ethanol was evaporated and nanoparticles formed were heated at 60ºC for thermal cross linking followed by sonication for 40 mins (ice bath) and homogenization in high pressure homogenizer. The nanoparticles were then separated by centrifugation (10,000 x 2 minutes) and lyophilized to get the crude protein extract of liquorice as shown in Figure 1.

The effect of parameters were optimized in reducing the particle size of the formulation like concentration of LCP extract, stirring speed, sonication time, etc. and optimal conditions were selected [12-14].

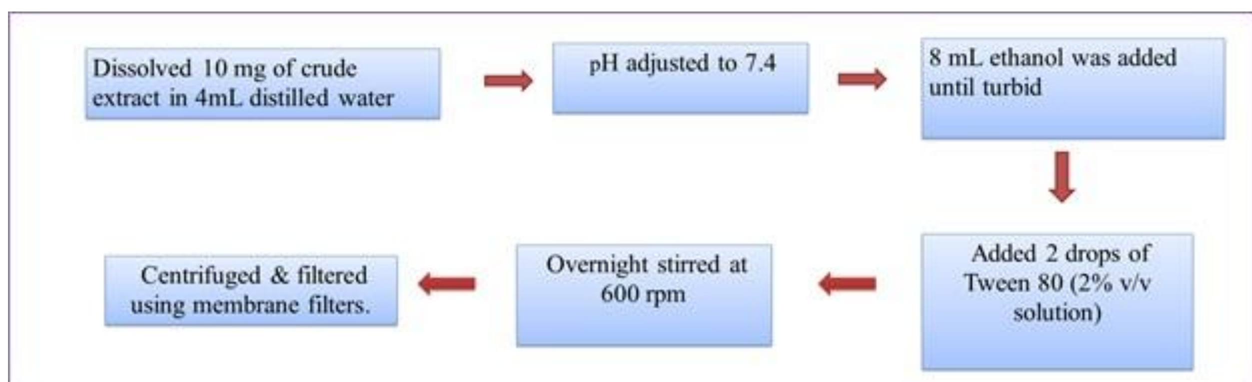


Figure 1. Flow chart of formulation of LCP nanoparticles by desolvation method.

Optimization for the formulation of blank LCP Nanoparticles

Application of Central Composite Design: Based on the information obtained from the literature review, two formulation parameters were selected for their effect on the preparation of LCP nanoparticles. These independent variables were; concentration of LCP (A) and stirring speed (B) with response as particle size (R).

Results and discussion

Characterization of liquorice crude protein extract

Total Protein content

Crude protein content in the solid extract was determined by Kjeldahl method and was found to be 33% as per analysis.
Same was also confirmed by Lowry assay method for total protein content.

Protein Identification test

Identification was done using chemical reaction test for proteins. Slight purple colour obtained with Biuret reagent (after recrystallisation of extract in ethanol) which confirms the presence of proteins.

Surface morphology

The morphology of LCP extract was examined using Scanning Electron Microscopy. The surface morphology of LCP extract shows irregular surface as shown in SEM image (Figure 2a).

Fourier Transform Infrared Spectroscopy (FTIR)

Crude liquorice protein extract was mixed with IR grade 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 taken at 4cm^{-1} resolution averaged over 32 scans in range of 400-4000 cm^{-1}.
FTIR spectra of Liquorice Crude Protein extract was obtained and shown in Figure 2(b) confirms the relevant peaks for proteins at 2923.56 for carboxylic OH group and at 1548.56 for NH group (thus confirms –CONH peptide linkage) as per standard spectra for albumin.

Optimization of synthesis of LCP nanoparticles [15]

The data obtained for experimental design are shown in Table 1. Liquorice crude protein nanoparticles were prepared by desolvation method by different trials. The ratio of water to ethanol was adjusted to 1:2 to obtain small sized particles. The required quantity of LCP was dissolved in distilled water with stirring, pH was adjusted and ethanol was added drop wise. This was followed by glutaraldehyde for cross linking and stirred for four hours. This was then sonicated for ten minutes, centrifuged and lyophilized. The Predicted R^2 of 0.9988 is in reasonable agreement with the Adjusted R^2 of 0.9993; i.e. the difference is less than 0.2.

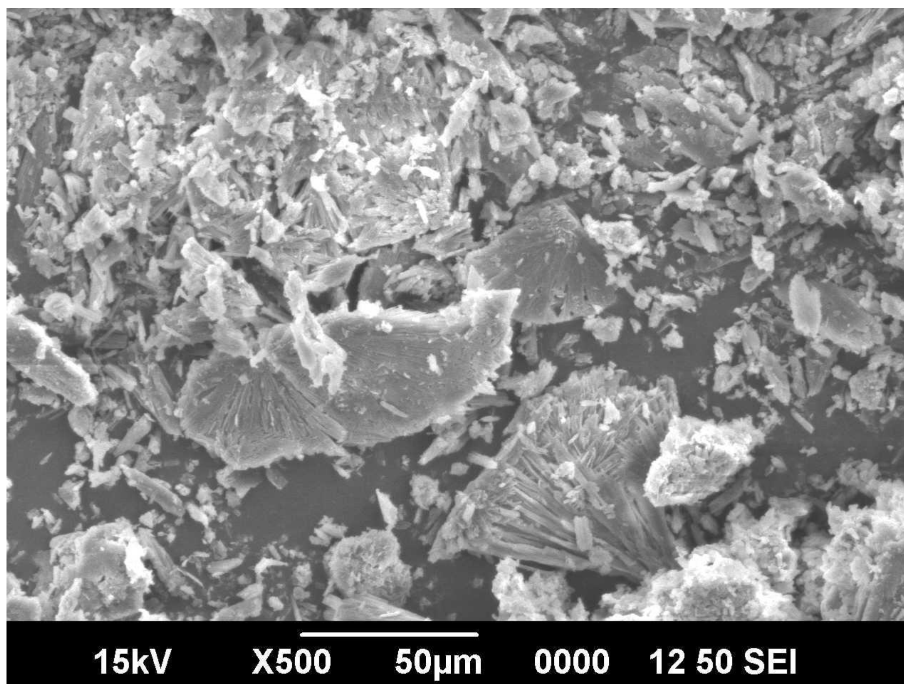


Figure 2. (a) SEM image of LCP extract.

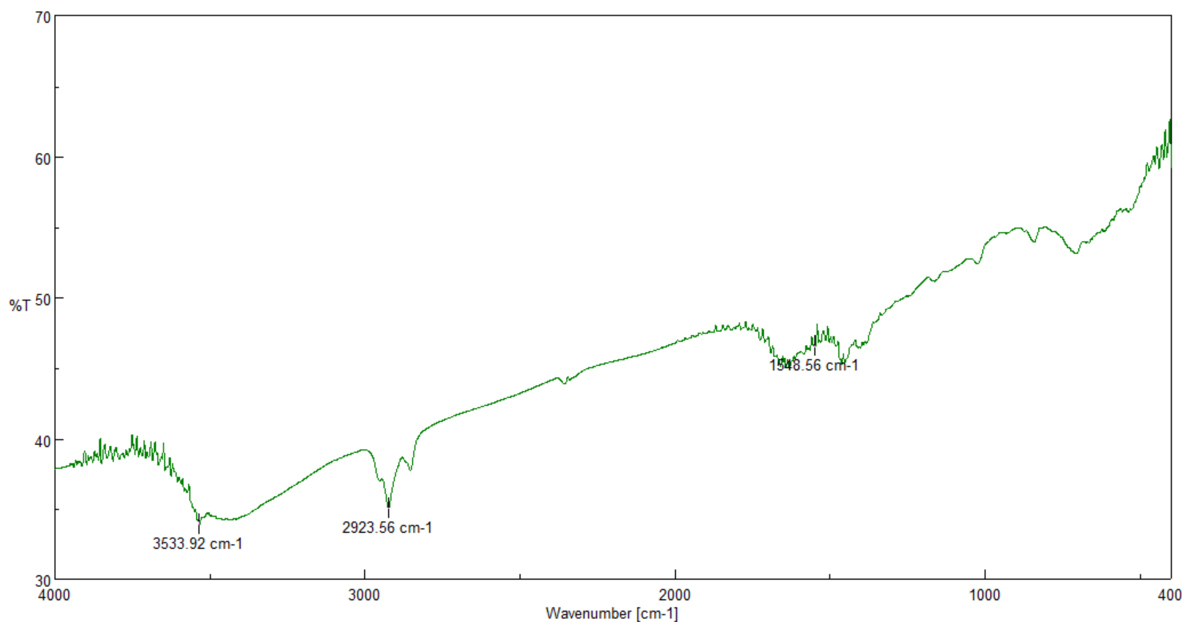


Figure 2. (b) FTIR spectrum of LCP extract.

Table 1. Central composite experimental design data for optimization of LCP nanoparticle synthesis.

Experimental run	Independent variables		Dependent variables
	A: Concentration (mg)	B: Stirring Speed (rpm)	Particle Size (nm)
1	10	900	264.2
2	12	900	505.2
3	10	900	263.2
4	10	900	264.8
5	10	1041	272.1
6	7	900	243.8
7	10	900	270.3
8	8	800	292.6
9	12	800	456.3
10	12	1000	417.3
11	8	1000	216.5
12	10	900	256
13	10	758	354.8

Figure 3 shows the graph for plot of predicted vs actual values for particle size.

The model proposed the following polynomial equation in terms of coded factors for particle size of LCP nanoparticles:

$$R1 = 265.50 + 91.77A - 29.01B + 9.28AB + 54.92A^2 + 24.40B^2$$

where R1 is particle size, A is concentration of protein and B is stirring speed.

The contour and 3D plot of effect of independent factors on particle size is shown in Figure 4.

The plots show that increasing crude protein concentration and decreasing stirring speed increased particle size. Probably this might be due to the formation of aggregates with larger diameter at high crude protein concentration and low stirring speed. From the plots it can be observed that in the region with low crude protein concentration and high stirring speed minimum particle size is obtained indicating the selected factors are significant.

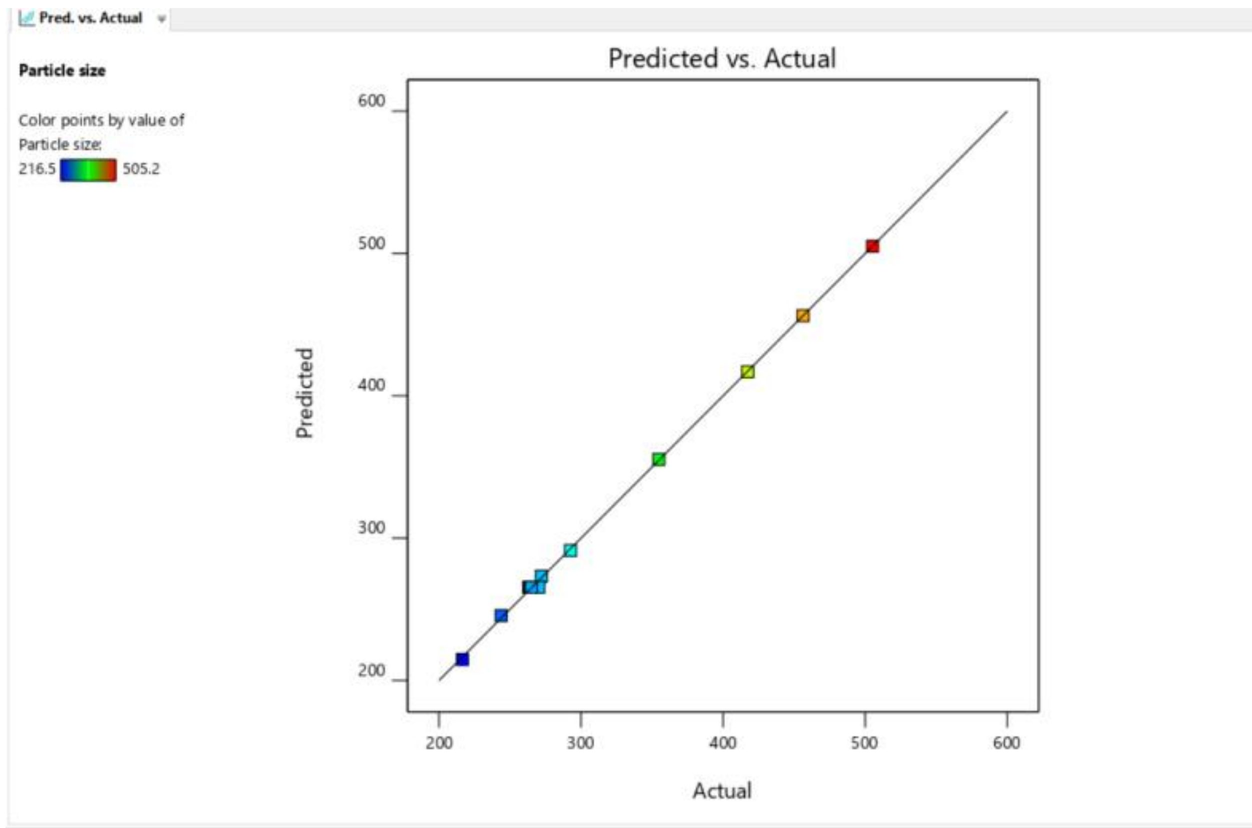
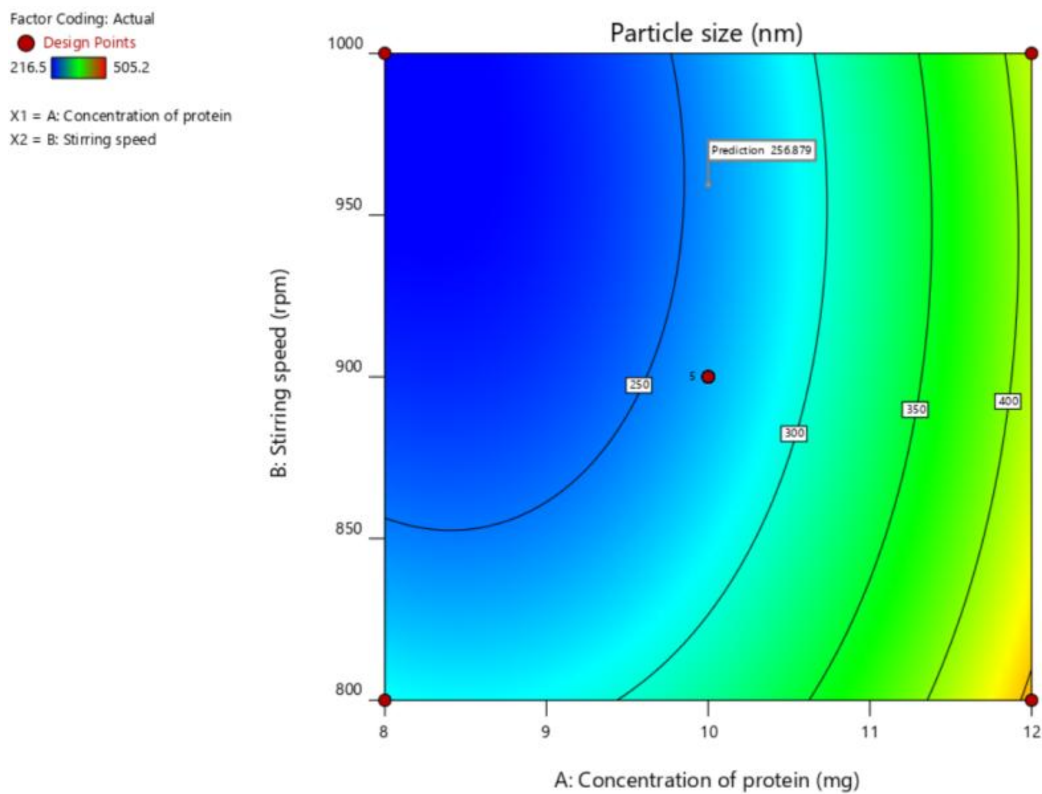


Figure 3. Plot of actual vs predicted values for particle size.



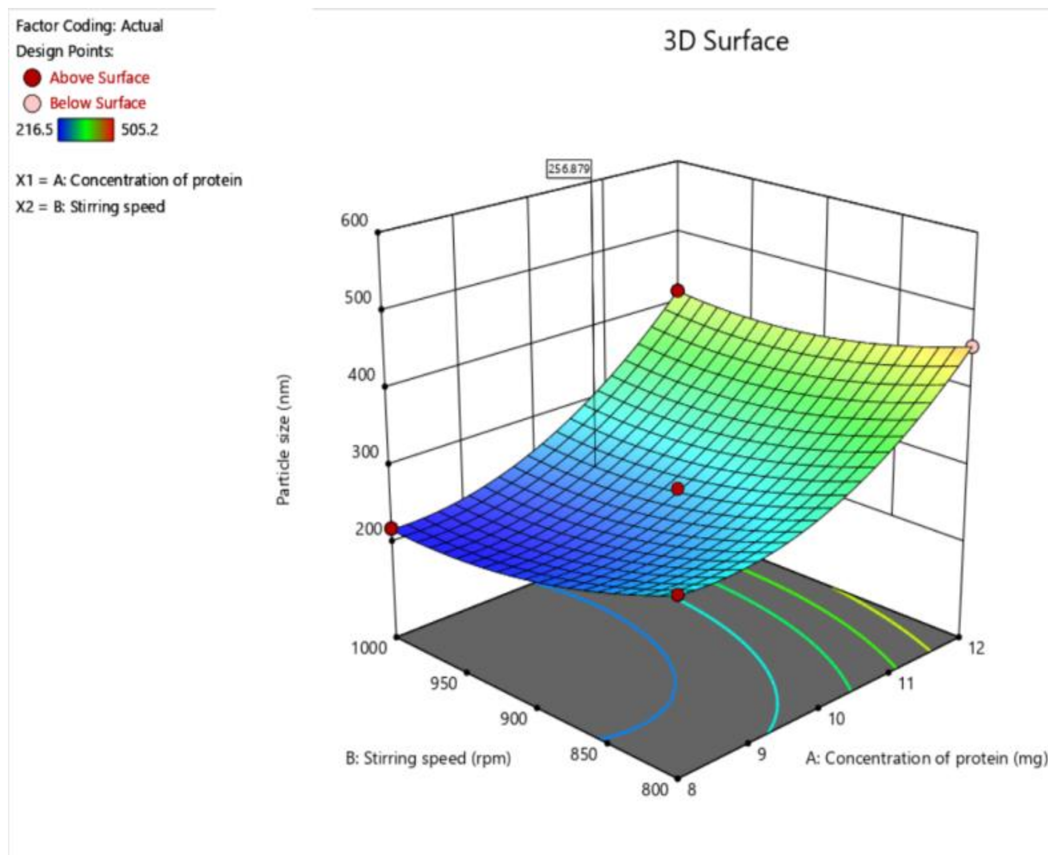


Figure 4. Contour plot and 3D surface plot of effect of independent factors on particle size.

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Optimized formula

The numerical optimization method was employed for the process optimization where the optimized formula was determined after studying the effect of independent variables on responses. Table 2 gives the optimization constraints selected for each variable. After defining the constraint for each variable, Design Expert -12® software automatically generated the optimized formula. Table 3 gives the optimized formula generated by Design Expert -12® software showing the predicted and experimental values obtained. It was observed that the percentage prediction error (0.342%) was low, which indicates the accuracy of the prediction by the software and the utility of the experimental design for process optimization [15].

Characterization of liquorice crude protein nanoparticles

Particle size

The average particle size and polydispersity index was 326.9 nm and 0.271 respectively (Figure 5) for optimized liquorice crude protein nanoparticles with signified good stability and uniform size distribution of nanoparticles [15].

Zeta potential

As presented in Figure 6, zeta potential of -10.9 mV denoted the high negative charge on particles that cause repulsion and thus inhibition of aggregation [15].

Surface morphology

Surface morphology of LCP nanoparticles was further studied by scanning electron microscope. The images captured by microscope revealed the spherical and smooth surface of LCP nanoparticles (Figure 7) [15].

FTIR spectroscopy of liquorice crude protein nanoparticles

FTIR spectroscopy was performed to examine the conformational changes occurred during the formation of nanoparticles and the spectra were examined for the presence of any characteristic peaks. The result obtained was in close agreement with the reported data. Presence of –CONH (at 2928.54 and 1660.01) of LCP nanoparticles confirm the peaks for proteins as per official data for albumin (Figure 8).

Table 2. Optimization constraints selected for optimization of LCP nanoparticle synthesis.

Variables	Constraints		
Independent variables	**Lower limit**	**Upper limit**	**Goal**
A: Concentration of Crude Protein (mg)	8	12	In range
B: Stirring Speed (rpm)	800	1000	In range
Dependent variables			
R1: Particle size (nm)	216.5	505.2	Target 300 nm

Table 3. Predicted and experimental values obtained based on optimized formula for LCP nanoparticle synthesis.

Predicted values based on optimized formula		
Factors		Response
Concentration of crude protein(mg)	Stirring speed (rpm)	Particle size (nm)
10	959.424	256.879
Experimental values based on optimized formula		
Factors		Response
Concentration of crude protein(mg)	Stirring speed (rpm)	Particle size (nm)
10	900	256
Percentage prediction error		0.342

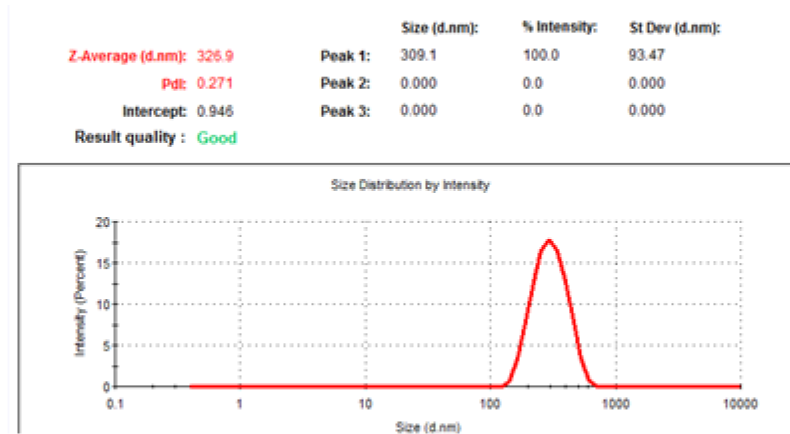

Figure 5. Particle size liquorice crude protein nanoparticles (nm).

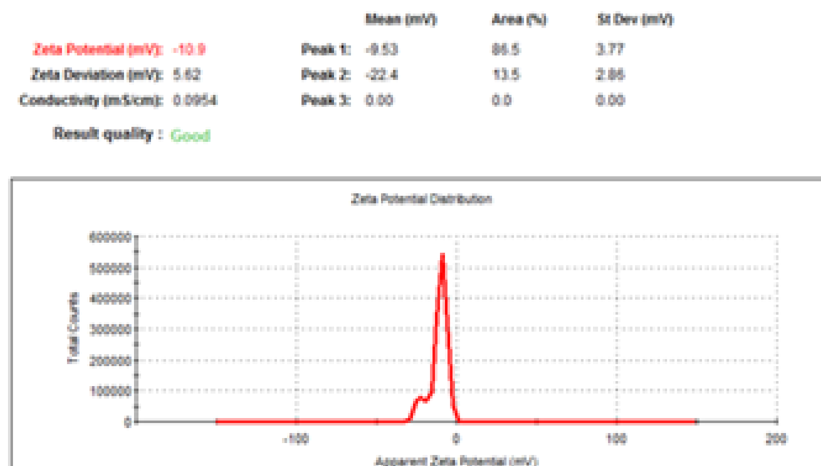

Figure 6. Zeta Potential liquorice crude protein nanoparticles (mV).

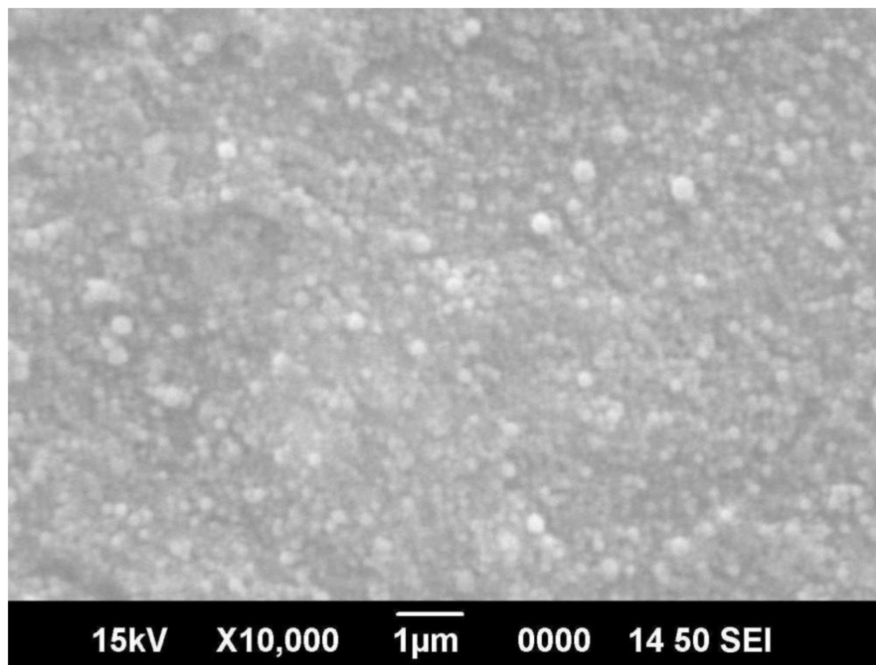

Figure 7. SEM image of LCP nanoparticles.

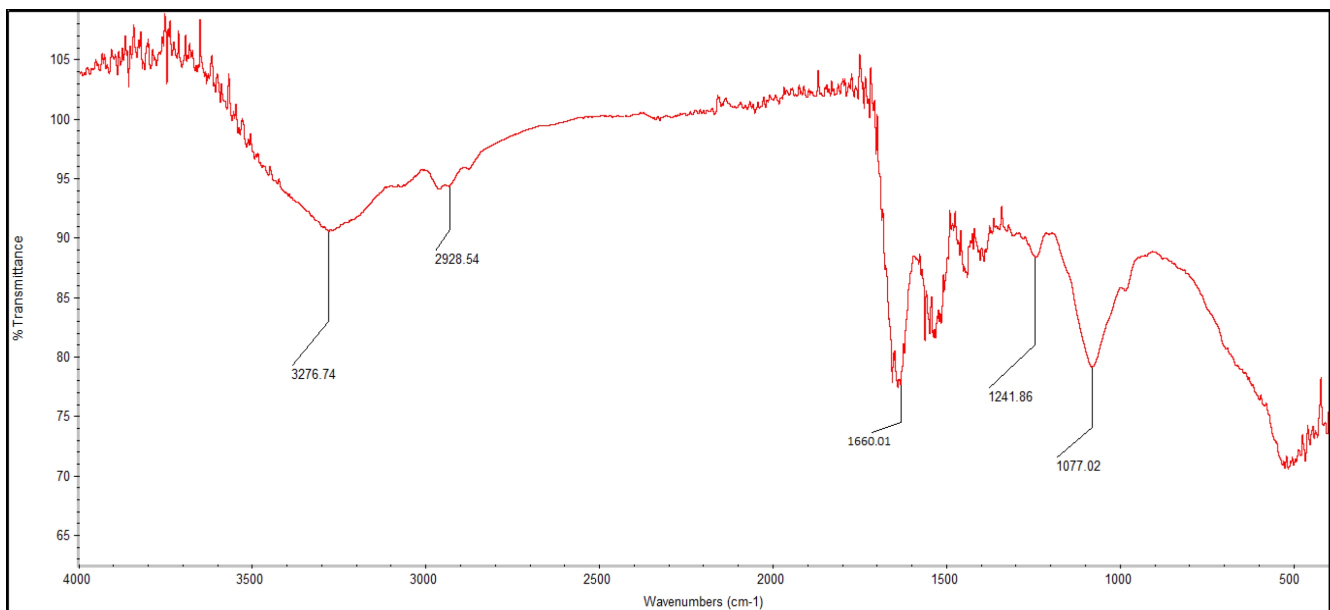

Figure 8. FTIR spectra of LCP nanoparticles.

Conclusion

A novel and simple process has been developed for the extraction of a natural crude protein from the magical herb liquorice, which has been used as decoction for many years for various ailments. Plant protein based biopolymers/carriers have very high potential as materials for developing in vivo drug delivery carriers due to their unique properties and proven safety.

In the present research work our first goal was to extract liquorice crude protein from the finely powdered root of liquorice (*Glycyrrhiza glabra L.)* by salting out method. After the removal of excess salts through dialysis, liquorice crude protein nanoparticles were formulated by desolvation method. Optimization was done through various process parameters like concentration of crude protein extract and stirring speed as independent variables to get minimum particle size as response. Characterization study of liquorice crude protein reveals that it contains protein as per analytical report about 33% and was confirmed by protein test. Further isolation of proteins can be done through electrophoresis after chromatographic separation of the crude protein extract. The characterization of nanoparticles shows the particle size ranging from 216.5 nm to 505.2 nm with optimized particle size as 326.9 nm. The nanoparticles had a uniform size distribution as evidenced from the low PDI 0.271 and with a zeta potential of –10.9 mV depending on nanoparticle composition. Scanning Electron Microscopy

results also confirm spherical particles of uniform size as shown in Fig 6a. Overall, this study gives a boon to the in vivo drug delivery system by introducing a new plant protein which is non toxic and economical from liquorice where much work has not been done before as per literature survey. This protein can be used as carrier for various targeted drug delivery. More studies are needed for the isolation and purification of different proteins present in the extract.

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