

DEVELOPMENT AND OPTIMIZATION OF POLYMERIC NANOPARTICLES OF GLYCYRRHIZIN: PHYSICOCHEMICAL CHARACTERIZATION AND ANTIOXIDANT ACTIVITY

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ABSTRACT

Objective: The aim of the present study to develop, optimize and characterize Poly (D, L-lactic-co-glycolic) acid (PLGA) nanoparticles (NPs) loaded with isolated Glycyrrhizin (Glyc) and investigate for antioxidant activity.

Methods: PLGA nanoparticles loaded with Glycyrrhizin were synthesized by an adapted emulsion-evaporation method. Nanoparticles were evaluated for particle size, entrapment efficiency and Polydispersibility index (PDI). Further, Box Benken design was applied for optimization of the formulation parameters and the effect of three independent variables such as PLGA concentration, amount of glycyrrhizin, polyvinyl alcohol (PVA) concentration on particle size, polydispersity index and the entrapment efficiency (response variables) were investigated. The antioxidant capacity of optimized nanoparticle formulation loaded with glycyrrhizin was compared with free glycyrrhizin by DPPH assay.

Results: The particle size, entrapment efficiency and PDI of optimized Glyc-NPs was found to be 144.20 nm, 68.0% and 0.315, respectively. Optimized Glyc-NPs showed sustained release of drug 79.06% in 48 h with improved free radical scavenging activity than isolated Glycyrrhizin.

Conclusion: PLGA nanoparticles were found to be suitable carrier for Glycyrrhizin at lower levels than originally required for enhanced functional properties.

Keywords: Nanoparticles, Glycyrrhizin, Licorice

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INTRODUCTION

Glycyrrhizin, a triterpene glycoside and major active constituents of roots and rhizome of licorice *Glycyrrhiza glabra*. Glycyrrhizin reported to possess various pharmacological activities such as anti-bacterial [1], anti-tumor [2, 3], antiviral [4], antioxidative [5], anti-inflammatory [6], immunomodulatory [7], anti-allergen, anti-thrombotic [8] and cardioprotective effects [9].

Nanoparticulate drug delivery system is one of the best approaches to increase the solubility and therapeutic efficacy of poorly soluble bioactive compounds. Further, they are also utilized for targeted delivery, sustained drug release and to minimize the side effects [10, 11]. There are various polymers used for developing the nanoparticles like poly (lactic-co-glycolic acid), chitosan, polylactic acid, alginate, pectin etc. Among all these PLGA which is a synthetic polymer which has an advantage of high purity and reproducibility over natural polymers and it has been approved by US Food and Drug Administration for biomedical applications [12]. PLGA nanoparticles are utilized for their biocompatibility, biodegradability, controlled delivery properties [13]. Studies has been reported that encapsulating hydrophobic drugs on PLGA polymer is a promising method for sustained and controlled drug delivery with improved bioavailability [14]. The physicochemical properties of nanoparticles and active compound release are directly affected by the various parameters involved in nanoparticle synthesis, such as the amount and composition of polymer, synthesis method, surfactant, hydrophobicity, and particle size.

Glycyrrhizin has low aqueous solubility and has poor bioavailability after oral administration due to slow and incomplete absorption in GI tract [15]. Hence the present study is aimed to improve the solubility by nanoencapsulation of Glycyrrhizin using Poly (d l-lactic-co-glycolic) acid (PLGA) as a polymer and investigate the effect on antioxidant activity.

MATERIALS AND METHODS

Preparation of aqueous extract from licorice root

100g of licorice powder (from Eucca) treated with 1000 ml distilled water and boiled for 20 min. Solution was filtered.

Isolation of glycyrrhizin from licorice and identification of isolated glycyrrhizin

Glycyrrhizin was isolated from licorice aqueous solution by acid precipitation method. Aqueous solution of licorice was brought to pH to 2.8 by the addition of 50% sulphuric acid. The Glycyrrhizin precipitates out as brown mass. The Precipitate was collected by filtration and the precipitate was washed with water to remove excess acid and dried to obtain shiny brown powder of glycyrrhizin [16].

Preparation of glycyrrhizin-loaded PLGA nanoparticles

Glycyrrhizin Nanoparticles (Glyc-NPs) were prepared by emulsion evaporation method with using PLGA as polymer [17]. PLGA and glycyrrhizin were dissolved in 2 ml dichloromethane which constitutes the organic phase. Polyvinyl alcohol (PVA) was added to the aqueous phase. An organic phase is added to the aqueous phase with stirring at 2800 rpm for 30 min to form a primary oil-in-water emulsion. This emulsion is added to second PVA solution and stirred using magnetic stirrer at 1000 rpm at room temperature. This oil-in-water-in-water emulsion (o/w/w) is sonicated for 30 min in an ice bath (2 °C) at 70 W. Organic solvent was evaporated by stirring at room temperature for 4 h at 500 rpm. The resulting suspension was kept overnight at 4 °C and later ultracentrifuged at 13,400 rpm for 30 min to collect nanoparticles. The nanoparticle sediments were washed twice with Milli-Q water to remove the free drug and excess surfactant and then lyophilized for 2 d.

Experimental design

Box behnken design (BBD) was applied for the optimization of different variables affecting the properties of Glyc-NPs using Design Expert®

ver.9.0 software [18]. The independent variables selected includes the amount of PLGA, amount of Glycyrrhizin and PVA concentration.

The independent and dependent variables chosen at three different levels viz. low (-1), medium (0) and high (+1) are listed in table 1.

Table 1: Independent and dependent variables levels in box-behnken design

Code	Independent variables	Level		
		-1	0	+1
A	PLGA (mg)	25.00	37.50	50.00
B	Glycyrrhizin (mg)	10.00	30.00	50.00
C	PVA (%)	0.3	0.5	0.7
	Dependent variables	Constraints		
	Particle Size (z-average mm)	Minimize		
	Entrapment efficiency (%)	Maximize		
	Polydispersity Index	Minimize		

Selected design recommended 14 experimental trials to be done, which is given in table 2. After incorporating the data to BBD, analysis of results was done by performing mathematical modeling. Quadratic second-order model was selected and ANOVA was mainly used for the analysis of data fitting the model along with other parameters like the coefficient of correlation (r^2), adjusted and predicted r^2 values. Numerical desirability function and graphical optimization techniques are used to identify optimized parameters for Glyc-NPs formation.

Estimation of glycyrrhizin by UV-visible spectrophotometer

Stock solution of 100 μ g/ml was prepared by dissolving glycyrrhizin in phosphate buffer (pH 6.8). The λ_{max} was determined, which was found to be 256 nm. A calibration plot was prepared by taking the absorbance of serial diluted samples in the concentration range of 10-60 μ g/ml.

Characterization of polymeric nanoparticles

Nanoparticles were characterized by the determination of particle size (z-average), PDI, zeta potential and entrapment efficiency. Scanning electron microscopy was used for the determination of morphology of nanoparticles.

Measurement of particle size of the nanoparticles

Malvern Zeta sizer (Nano ZS, Malvern Instruments, UK) was used to determine particle size and PDI of Glyc-NPS by laser dynamic light scattering method.

Drug entrapment efficiency and percentage drug loading

Glyc-NPs suspension was ultra-centrifuged (Remi Mumbai, India) at 13,000rpm at 4 °C for 30 min and the supernatant was collected. Amount of untrapped drug in the supernatant was determined by UV spectroscopy. Equation used to calculate the percentage drug entrapment and drug loading is given below:

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Total amount of Drug added} - \text{amount of Drug is supernatant}}{\text{Total amount of Drug added}} \times 100$$

Drug Loading (%) = (Amount of drug-untrapped drug)/weight of Glycyrrhizin loaded PLGA-NPs \times 100.

Scanning electron microscopy (SEM)

SEM (JEOL, JSM 50A, Tokyo, Japan) was used for understanding the shape and surface morphology of Glyc-NPs

Fourier transform infrared (FTIR) analysis

FTIR analysis of isolated Glycyrrhizin and Glyc-NPs was carried out using Perkin Elmer BX II (PerkinElmer, Massachusetts, USA) by scanning the samples in the range of 400 to 4000 cm^{-1} . FTIR study gives information about the chemical interaction between drug and polymer.

In vitro release study

Optimized formulation of Glyc-NPs and isolated glycyrrhizin were placed in dialysis bag. After closing the dialysis bag, it was immersed

in 150 ml of phosphate buffer (pH 6.8) in a conical flask. This flask was placed in a shaking incubator at 100rpm at 37 °C. Samples are withdrawn at fixed intervals and analyzed using UV-Visible spectrophotometer to calculate the drug release from the nanoparticles.

Determination of antioxidant activity

Antioxidant activity of glycyrrhizin and Glyc-NPs were determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [19]. This assay is based on the scavenging capacity of test compound towards DPPH free radical. Dilution with methanol was carried by taking Glycyrrhizin-loaded nanoparticles (100 mg) and the isolated glycyrrhizin (concentration 10, 50, 100, 150, 200, 250 μ g/ml). DPPH (3.9 ml) was added to each test sample dilution (0.1 ml) followed by incubation in dark for the period of 30 min. Absorbance of test samples were determined at 517 nm using a UV-Visible spectrophotometer. Ascorbic acid was used as standard. IC 50 values were calculated, which indicates the concentration of antioxidant compound required to scavenge the free radical by 50% and values are expressed as grams of glycyrrhizin/gram of DPPH.

RESULTS AND DISCUSSION

Isolation of glycyrrhizin

Glycyrrhizin was isolated from an aqueous extract of licorice root by Acid precipitation method. Isolated glycyrrhizin was obtained as shining brown powder and the yield was found to be 12%.

Optimization of synthesis of glycyrrhizin-loaded PLGA nanoparticles

Box Behnken design suggested total 14 runs for the optimization of Glyc-NPs using three independent and dependent variables. All the prepared nanoparticle formulations were characterized for average particle size, PDI and entrapment efficiency and results are shown in table 2.

Effect of independent formulation variables on particle size

Particle size is the critical factor determining the physicochemical properties such as drug release, drug loading, bioavailability as well as the therapeutic efficacy of the nanoparticles. The effect of amount of PLGA, Glycyrrhizin and PVA concentration is shown in table 2 and fig. 1. The model created for particle size was found to be significant based on the F value which was 19.32. P value is less than 0.05, indicating that model terms are significant. Accordingly, significant model terms were A, B, BC, A² and B², which shows that amount of PLGA and Glycyrrhizin has a greater effect while the concentration of PVA has a negligible effect on particle size. The lack of fit value 0.63 indicates that it is non-significant which implies that this model is acceptable to determine the effect on particle size. Predicted R² value (0.7336) is close to the Adjusted R² value (0.9269) with a difference less than 0.2, showing reasonable agreement with both values.

The analysis finding arrived at the following quadratic equation:

$$\text{Particle size} = 190.30 + 91.00A^* - 58.70B^* - 21.97C - 22.22AB - 20.18AC + 60.53BC^* + 142.19A^2 + 97.54B^2 - 37.06C^2$$

Table 2: Effect of independent variables on dependent variables

Formulation code	A: PLGA (mg)	B: Glycyrrhizin (mg)	C: PVA %	Particle size (nm)	EE %	PDI
1	25	30	0.7	180.5±1.5	65±2.5	0.310±0.05
2	25	30	0.3	192.6±1.0	63.4±2.1	0.330±0.01
3	37.5	30	0.5	160.9±1.5	70±1.9	0.331±0.02
4	50	50	0.5	430.6±2.1	73±2.5	0.471±0.01
5	50	30	0.3	450.7±1.6	72±3.0	0.417±0.03
6	25	50	0.5	328.8±2.1	62±1.9	0.338±0.02
7	37.5	10	0.7	239.6±1.9	70±2.2	0.341±0.02
8	37.5	30	0.5	219.7±1.4	71±2.1	0.345±0.02
9	37.5	50	0.7	226.5±1.5	72±1.9	0.362±0.01
10	50	10	0.5	575.7±1.1	73±2.1	0.421±0.03
11	37.5	50	0.3	140.9±1.3	68±3.0	0.315±0.01
12	37.5	10	0.3	396.1±1.2	67±1.2	0.390±0.02
13	25	10	0.5	385.0±2.3	61±1.9	0.413±0.01
14	50	30	0.7	357.9±1.5	74.5±1.5	0.421±0.02

Values are expressed as mean±SD, n=3

The equation states that the amount of PLGA (A) has a positive effect on the particle size, whereas the two variables amount of glycyrrhizin (B) and PVA concentration (C) has a negative effect on particle size (fig. 1). As the PLGA amount was increased from 25 to 50 mg the particle size increased correspondingly. During emulsification, as the concentration of polymer increases, there is an increased frequency of collision between particles which results in the fusion of semi-formed particles, resulting in a collective increase in particle size. Reports state that smaller particle size results due to the spontaneous diffusion of the organic phase to the external aqueous phase [20]. Increased in viscosity of organic phase due to higher amount of polymer in turn reduces the rate of diffusion into the aqueous phase, which initiates the larger nanodroplet formation at the interface [21, 22]. During nanoparticle synthesis, surfactant helps in the uniform dispersion of organic and aqueous phase and they form a protective film around the droplet, thereby acts as

stabilizing agent [23, 24]. At lower concentration of glycyrrhizin (10 mg) resulted in higher particle size and as the concentration increased from 10 mg to 30 mg particle size decreased, at the concentration of 50 mg, particle size was maximum.

In the current study, PVA had a negative effect on the particle size. At low concentrations of PVA, it was not sufficient to form a film around particles, resulting in aggregation, hence leading to increased particle size whereas higher PVA concentration reduced the interfacial tension between the aqueous and organic phases leading to reduced size of nanoparticles.

The term AB, AC, BC, A^2 , B^2 , C^2 shows the interaction effect, which indicates the influence of any two factors simultaneously on the particle size. Optimizing the particle size to a minimum is necessary to enhance internalization and achieve higher cellular concentration, therefore enhancing therapeutic efficacy.

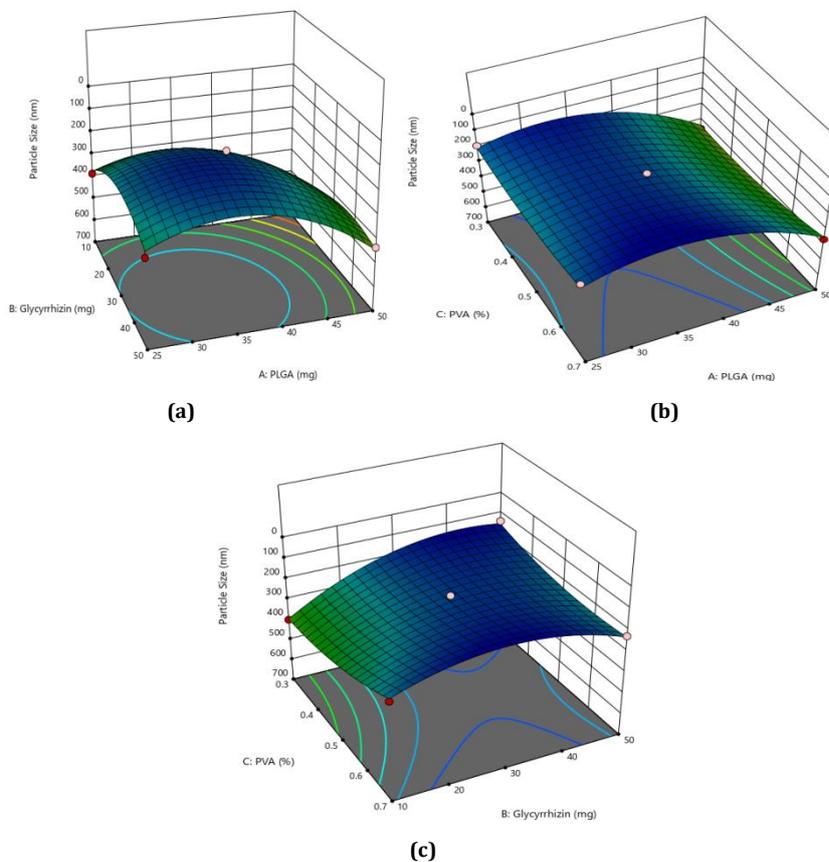


Fig. 1: Response surface plots showing effect on particle size of a) Amount of PLGA (A) and Glycyrrhizin (B); b) amount of PLGA and PVA concentration (C); c) PVA concentration and amount of glycyrrhizin

Effect of formulation variables on entrapment efficiency

Entrapment efficiency showed linear relationship with the variables and F value was 34.86, indicating that the model is significant. The model term a have p-value less than 0.001, which is significant. Lack of fit is non-significant, which is indicated by lack of fit F value 4.75. The Predicted R² value of 0.8384 is in reasonable agreement with the Adjusted R² value of 0.8865; i.e. the difference is less than 0.2.

$$\text{Entrapment efficiency} = +68.71 + 5.14A^* + 0.5000B + 1.39C$$

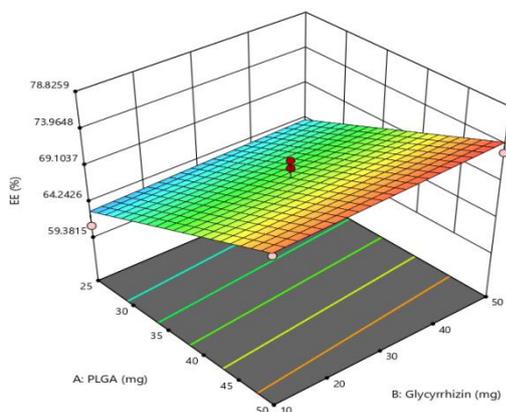


Fig. 2: Response surface plots showing an effect on entrapment efficiency of amount of PLGA (A) and glycyrrhizin (B)

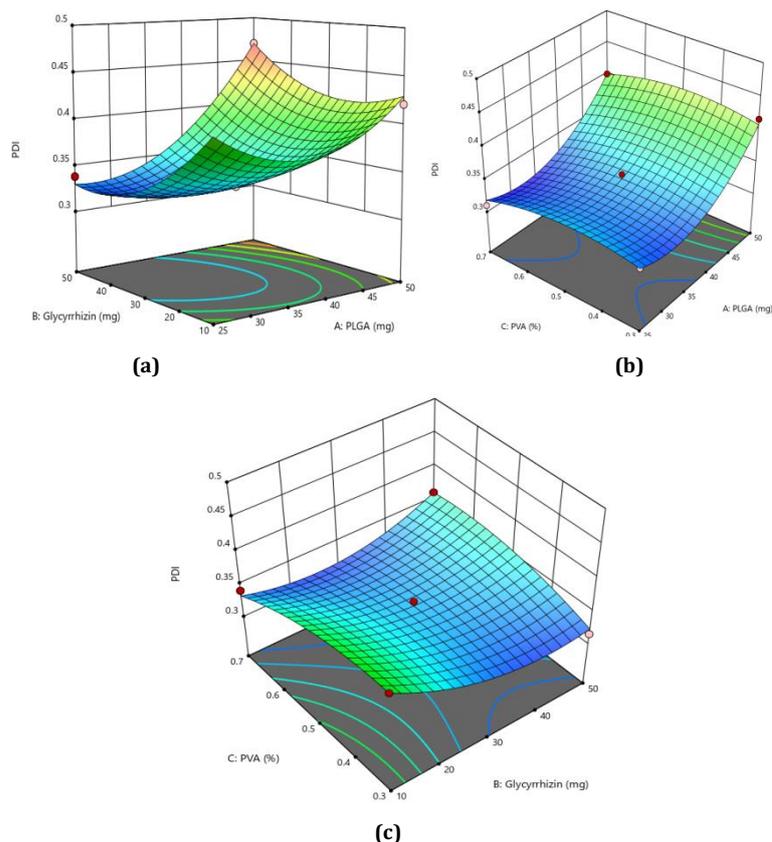


Fig. 3: Response surface plots showing the effect on PDI of a) Amount of PLGA (A) and glycyrrhizin (B); b) amount of PLGA and PVA concentration (C); c) PVA concentration and amount of glycyrrhizin

Data analysis and optimization

The optimum Glyc-NPs formulation was selected by applying

Entrapment efficiency is predominantly affected by factor a (amount of PLGA), whereas effect of factor B and C is negligible. As the polymer concentration is increased the entrapment efficiency increases. Since glycyrrhizin is a hydrophobic drug, will have higher miscibility and interactions with polymer solution, which causes more partitioning towards the polymeric phase, leading to higher entrapment within the nanoparticles. Same reports have been found for other hydrophobic drugs such as dexamethasone [25], lorazepam [26] and noscapine [27].

Effect of Formulation Variables on PDI

Quadratic model was observed for the effect of variables on PDI and F value was 25.50, indicating that the model was significant. Model terms such as A, AB, BC, A², B² were found to be significant since their p-value is less than 0.1000. Lack of fit is non-significant implicated by the lack of fit F-value of 1.42.

Even though the predicted R² values (0.7650) is as not so close to the adjusted R² value (0.9443), the signal-to-noise ratio of 15.828 implies an adequate signal for this model.

The polynomial equation for this model

$$\text{PDI} = +0.3380 + 0.0400A^* - 0.0099B - 0.0051C + 0.0302AB^* + 0.0007AC + 0.0235BC^* + 0.0423A^2 + 0.0295B^2 - 0.0160C^2$$

The stability of the formulation depends on homogeneity in the size of nanoparticles in the formulation. The above equation shows that the amount of polymer (A) had a positive impact on PDI whereas glycyrrhizin (B) and PVA (C) concentration had a negative effect.

constraint. On the basis of closeness to desirability factor close to 1, the point prediction of Design expert software determines the optimized nanoparticle formulation, which predicted the optimized

process parameter to be A 38.82 mg, B 28.55 mg, C 0.7% with predicted values of response of particle size 140.90 nm, Entrapment efficiency 70.60% and PDI 0.321. The optimized formulation was developed and characterized for particle size and drug loading. The experimental value for responses of particle size 144.20 nm, Entrapment efficiency 68.0% and PDI 0.315 of optimized formulation was found in good agreement with the predicted values. The percentage loading of optimized glycyrrhizin-loaded PLGA nanoparticles was found to be 10%.

UV-visible spectroscopy

Glycyrrhizin was dissolved phosphate buffer (pH 6.8) and scanned in the UV range of 200-400 nm showed the maximum absorption at a wavelength of 256 nm and at this wavelength was used for all the analysis. The serially diluted glycyrrhizin exhibited the absorbance values, which showed the linearity in the concentration range of 10-60 µg/ml and their regression was found to be 0.995.

FTIR analysis

The absorption band was observed in the range of 3200-3500 cm⁻¹ suggested the presence of O-H stretch hydroxyl group (fig. 6 and 7) Absorption band in the range of 2850-3000 cm⁻¹(C-H stretch),1720-

1740 cm⁻¹ (C=O stretch of saturated aliphatic compounds), 1640-1680 cm⁻¹ (-C=C stretch alkenes) and 1400-1500 cm⁻¹ (-C-C stretch in aromatics). 1000-1320 cm⁻¹ suggested the presence of C-O stretch (alcohols, carboxylic acids and esters) in glycyrrhizin.

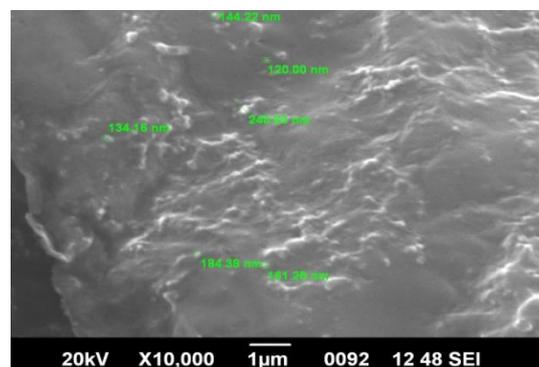


Fig. 4: SEM image of glycyrrhizin-loaded PLGA nanoparticles

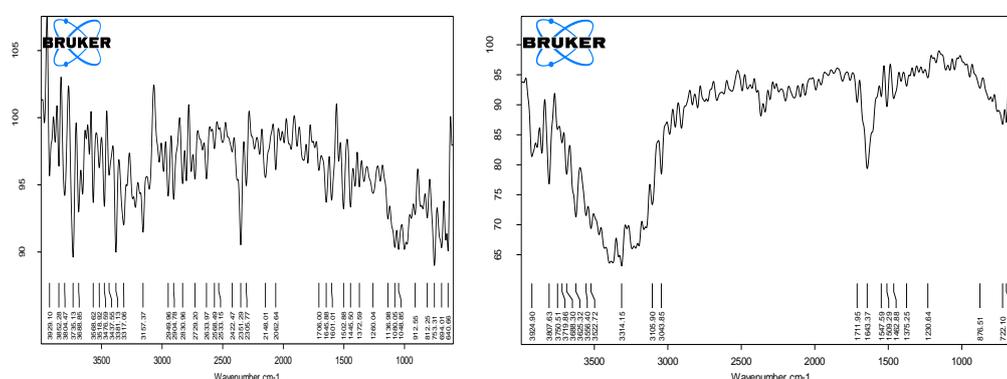


Fig. 5: FTIR spectrum of isolated glycyrrhizin and glycyrrhizin-loaded PLGA nanoparticles

In vitro drug release study

Optimized formulation of glycyrrhizin-loaded PLGA nanoparticles were subjected to drug release study for 48 h using phosphate-

buffer (pH 6.8) as media and result showed that 60% of drug released within 15h and 79.06 % drug released at the end of 48 h. This shows that there was an initial burst release followed by sustained release of drug after 15h to 48h.

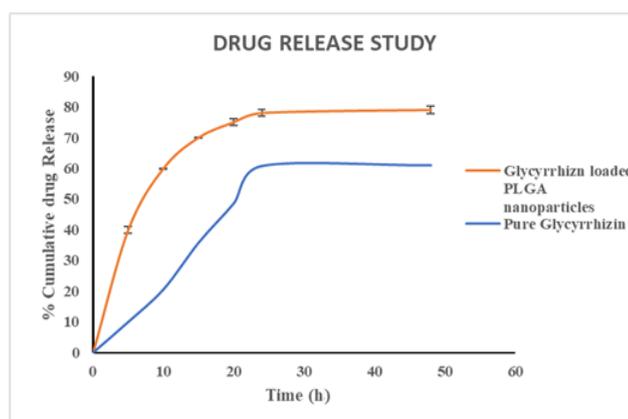


Fig. 6: In vitro drug release study of pure glycyrrhizin and glycyrrhizin PLGA-loaded nanoparticles

Antioxidant activity

Antioxidant activity was determined by DPPH scavenging assay. IC₅₀ value of isolated glycyrrhizin was found to be 102.55µg/ml with the maximum percentage of free radical scavenging activity was found to be 65.17%. Glycyrrhizin-loaded PLGA nanoparticles

showed 85.06 % free radical scavenging activity, which was significantly improved due to improved solubility of glycyrrhizin when loaded onto PLGA nanoparticles. Glycyrrhizin loaded onto PLGA nanoparticles will show maximum antioxidant activity due to burst release and prolonged antioxidant effect due to sustained release of drug. Standard Ascorbic acid showed IC₅₀ value 20.80

µg/ml. Hence Glycyrrhizin loaded onto PLGA nanoparticles will be a potential candidate for delivery of glycyrrhizin to provide efficient and prolonged antioxidant effect. Similar studies have been reported where results showed that PLGA is a proven candidate for delivery of quercetin and curcumin in the form PLGA nanoparticles based on the *in vitro* and *in vivo* activity studies.

CONCLUSION

Encapsulating of glycyrrhizin by using PLGA polymer will be a promising method for enhancing solubility, improving bioavailability and for producing the sustained release of the drug.

Oxidative stress has been implicated as one of several mechanisms that have induced toxic effects in different organs due to enhanced production of oxygen-free radicals. Glycyrrhizin has antioxidant activity which is related to its use as an anti-inflammatory, anticancer agent. Hence, the antioxidant status of glycyrrhizin after encapsulation will be beneficial for providing the long-term therapeutic effect due to sustained release of drug.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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