



Design of Lipid Nanoparticles Containing Fenugreek Seed Extract

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: The main purpose of this study was to maximize the efficacy of fenugreek seed extract by loading it in an optimised solid lipid nanoparticles (SLNs) formula.

Methods: To achieve an effective extraction method, preliminary studies were carried out to confirm the extract, and the extract was standardised using trigonelline. The influence of independent variables lipid concentration (X1), surfactant concentration (X2), and cosurfactant concentration (X3) on dependent variables particle size (Y1) and entrapment efficiency (Y2) was also studied and optimised using the Box–Behnken design. Melt emulsification followed by ultrasonication was used to prepare SLN formulations. To understand the effect of independent variables on the dependent quality parameters, response surface plots and mathematical equations were produced.

Results: The results confirmed that soxhalation was the most suitable method for extraction of fenugreek seeds, confirmed by standardization. Further optimization revealed that particle sizes ranged from 193.4 to 312.3 nm, with entrapment efficiencies ranging from 61.2 to 74.32 percent. This implies that the developed formulations can be used for further in vitro and in vivo characterizations.

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1. INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) is a dicotyledon leguminous annual crop popular as a spice and herb. It is native to a region that stretches from Iran to Northern India, but it is now grown in China, North and East Africa, Ukraine, and Greece.

Fenugreek has anti-diabetic, antioxidant, anti-neoplastic, gastroprotective, hepatoprotective, hypercholesterolemic, and hypoglycemic properties due to its high phytochemical content. Fenugreek seeds contain saponins, particularly diosgenin, yamogenin, and gitogenin derivatives, alkaloids (trigonelline), flavonoids, vitamins, and the fibre galactomannan [1]. Ayurvedic medicine also makes use of it [2].

Fenugreek seeds have been used and valued as a medicinal material since ancient times. Fenugreek, as a chemurgic crop, has a wide range of industrial applications. Its seeds are thought to be commercially valuable as a source of the steroid diosgenin, which is important in the pharmaceutical industry [3]. Fenugreek's biological and pharmacological actions are attributed to a variety of constituents, including steroids, N-compounds, polyphenolic substances, volatile constituents, amino acids, and so on [4].

Fenugreek seed contains 45-60% carbohydrates, primarily mucilaginous fibre (galactomannans), 20-30% proteins high in lysine and tryptophan, 5-10% fixed oils (lipids), pyridine alkaloids, primarily trigonelline (0.2 - 0.38 percent), choline (0.5 percent), gentianine and carpaine, the flavonoids apigenin, luteolin, orientin, quercetin (n-alkanes and sesquiterpenes) [5]. Hence on the basis of all these, fenugreek seeds are exploited for extraction.

Solid lipid nanoparticles (SLNs) are a viable option for delivering drugs that are both hydrophilic and hydrophobic. The lipid core of SLNs is responsible for their high drug-loading capacity, improved blood circulation time, control release kinetics, and increased overall therapeutic effectiveness of anti-cancer drugs [6,7]. Solid lipid nanoparticles have also been considered as novel nanoparticulate carrier systems due to improved drug delivery and stability [8].

Optimization is a technique used in the design and development of a wide range of controlled-release dosage forms that makes use of available resources to achieve the best possible results. The optimization technique has largely replaced the widely used trial-and-error method, and it can be found in a variety of applications in the chemistry and pharmaceutical industries. Such research entails the use of appropriate experimental designs, the generation of polynomial relationships, and the application of optimal research techniques using appropriate software [9].

We reasoned that encapsulating fenugreek seed extract in SLNs would increase their activity. As a result, the primary goal of this study was to develop, characterise, and optimise SLNs containing Fenugreek seed extract. The Box-Behnken design (BBD) was used with Design Expert® (version 11, Stat-Ease Inc., Minneapolis, Minnesota) to identify critical parameters that influence properties of prepared Fenugreek seed extract-SLNs in order to select the best formula.

2. MATERIALS AND METHODS

2.1 Materials

The seeds of *Trigonella foenum graecum* were obtained from local market. Standard Trigonelline was purchased for SIGMA ALDRICH. Glycerol monostearate was purchased from Loba chemie. Ethanol, Tween 80 and soyalecithin was procured from Hi Media. Ultra-pure deionized water was used throughout the experiments. All other solvents and chemicals used were of analytical grades or higher.

2.2 Collection and Identification of Plant Drug

Prof K G Bhat, from Udupi, identified and authenticated *Trigonella foenum graecum* (Fenugreek) seeds purchased from a local market. To obtain coarse powder, seeds were pulverised and sieved through 40 mesh [10].

2.3 Extraction of Fenugreek Seeds

Powdered fenugreek seeds were subjected to conventional extraction methods such as maceration, soxhlation and also by microwave assisted extraction (MAE) using ethanol (95%) as solvent.

2.3.1 Maceration method

About 500 g of powdered seeds were soaked in ethanol (95%) for three days with occasional stirring. After three days, the mixture was filtered and filtrate were concentrated using rotary vacuum evaporator.

2.3.2 Soxhlation method

Fresh seeds were collected and washed under running water followed by distilled water and dried for about 24h under sun light and later powdered in mixer grinder. About 50 g of fenugreek seed powder was weighed and filled in filter paper packet. It was placed in a thimble and 250 ml of 95% of ethanol was filled in a round bottomed flask (500 ml capacity) and extraction was carried out at 50°C for about 6-7 h to complete 4 cycles per day, for 6 days. The process is repeated for 4 times. The extract was collected and concentrated in a rotary flash evaporator.

2.3.3 Microwave assisted extraction (MAE)

It was carried out in a microwave oven manufactured by Catalyst Systems (Pune, India), which was outfitted with a magnetron of 2450MHz with a nominal maximum power of 700W, 10 power levels, a reflux unit, a time controller, an exhaust system, a beam reflector, and a stirring device. Accurately weighed 50g of the sample was placed in extraction vessel along with 250ml of solvent and placed inside the microwave cavity and MAE was carried out for 140 W, temperature of 60°C for 4 min [11].

Following the extraction, the solutions were filtered, and the filtrate was concentrated using a rotary vacuum evaporator. The extracts' physicochemical properties, such as colour, odour, and percentage yield, were reported.

2.4 Qualitative Analysis of the Extracts

The preliminary phytochemical studies were performed on the ethanolic extract obtained by refluxation, soxhlation and microwave extraction methods for determining the presence of different phytoconstituents [12]. Different chemical tests were done for alkaloids, carbohydrates, Flavanoids, saponins, steroids, Tannins, phenolic compounds, proteins and amino acids.

2.4.1 Estimation of trigonelline by UV-spectrophotometric method [13]

Trigonelline constitutes one of the important constituent present in fenugreek. The UV

spectrophotometric method was used with phosphate buffered saline (pH 7.4) as the solvent medium and trigonelline hydrochloride as the standard.

Standard trigonelline hydrochloride and fenugreek extract solution in phosphate buffer saline of (pH 7.4) was scanned in between the wavelength range of 200-400 nm. Trigonelline showed maximum absorption at 265nm (λ max).

2.4.2 Preparation of standard trigonelline solutions for calibration curve (Standard plot)

Twenty-five mg of standard trigonelline hydrochloride was weighed accurately and dissolved in 25.0 ml of distilled water (stock solution). Aliquots of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 ml of the solution were pipetted into a 10.0 ml volumetric flask and filled with phosphate buffer saline (pH 7.4) to give concentrations of 10, 20, 30, 40, 50, and 60g/ml. A UV spectrophotometer (Spectroquant Merck millipore) was used to measure absorbance at 263 nm against a blank solution (phosphate buffer saline (pH 7.4)). The experiment was repeated in triplicate and the average of three readings was taken to plot the calibration curve (concentration on x-axis and absorbance on y-axis).

2.4.3 Estimation of trigonelline in ethanolic extract of fenugreek

Ethanolic extract of about 50mg of fenugreek seed was dissolved in phosphate buffer saline of (pH 7.4) and volume was made upto 50.0 ml with buffer. These solutions were filtered through whatman filter paper number 40. From this 1ml solution was pipetted and volume was made to 50 ml with phosphate buffer. The absorbance was measured at 265 nm against blank solution in phosphate buffer saline of (pH 7.4) using a UV spectrophotometer.

2.5 Design of Experiments

The statistical tool Design Expert[®] (version 11, Stat-Ease Inc., Minneapolis, Minnesota), was used to implement the BBD three-level three-factor (3^3) in the current investigation.

Formulation variables were statistically examined to produce ideal particle size and high encapsulation efficiency percent when making Fenugreek seed extract SLNs. The cube's centre point was duplicated in the next five runs, which served as the mid-point to each edge of the

multidimensional cube. Particle size (Y1) and entrapment efficiency (EE) (Y2) were chosen as dependent variables, with lipid (stearic acid) concentration (X1), surfactant (tween 80) concentration (X2), and cosurfactant (lecithin) concentration (X3) as independent factors. Table 1 summarises the independent and dependent variables.

Table 2 shows the BBD-compliant composition of the produced Fenugreek seed extract SLNs.

The optimal formula was chosen based on its desirability, which was then exposed for further research [9].

2.6 Preparation of Fenugreek Seed Extract Loaded Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) of fenugreek seed extract were prepared from the melt

emulsification-probe sonication method. Previously, the SLN was optimized in terms of particle size, polydispersity index and entrapment efficiency (data not shown). The procedure for the optimized batch is briefly discussed here. Weighed quantity of Glyceryl monostearate was taken to prepare the lipid phase. Tween 80 (surfactant) and Soya lecithin (cosurfactant) were dissolved in Milli-Q water to make the aqueous phase. Both of these phases were kept at 70°C. Extract (2.5%) was introduced to the molten lipid mixture when stirring constantly, and then the aqueous phase was added drop by drop to the lipid phase under steady stirring at 2000 rpm for 30 minutes until a homogeneous emulsion was created. The resulting primary emulsion was ultrasonicated for 15 minutes at 75% amplitude under hot condition. After cooling the solution to room temperature, the SLN dispersion was obtained [14].

Table 1. Independent and dependent variables and their levels

Independent Variables	Levels		
	Low (-1)	Medium (0)	High (+1)
Lipid concentration,(g) (X ₁)	1.16	2.33	3.5
Amount of surfactant (ml) (X ₂)	0.67	1.335	2
Amount of co-surfactant (g) (X ₃)	0.2	0.4	0.6

Lipid= GMS, Surfactant=Tween 80, Co-surfactant=Soya Lecithin

Dependent Variables	Constraints
Particle size, (a)	Minimize
Entrapment efficiency, (b)	Maximize

Table 2. Fenugreek seed extract-loaded solid lipid nanoparticles: Box-behnken design responses

Formulation code	Lipid concentration (g)	Surfactant concentration (ml)	Co-Surfactant concentration (g)
F1	2.33	1.335	0.4
F2	3.5	0.67	0.4
F3	1.16	1.335	0.6
F4	2.33	1.335	0.4
F5	2.33	0.67	0.6
F6	2.33	1.335	0.4
F7	3.5	2	0.4
F8	2.33	0.67	0.2
F9	1.16	2	0.4
F10	3.5	1.335	0.6
F11	2.33	2	0.2
F12	2.33	2	0.6
F13	2.33	1.335	0.4
F14	1.16	1.335	0.2
F15	1.16	0.67	0.4
F16	2.33	1.335	0.4
F17	3.5	1.335	0.2

2.7 Characterisation of Fenugreek Seed Extract Loaded Solid Lipid Nanoparticles

2.7.1 Determination of particle size, polydispersity Index and zeta potential

The particle size, polydispersity index, and zeta potential of the SLN were determined by Dynamic light scattering, using Zetasizer Nano-Series (Nano-ZS 90, Malvern Instruments, U.K.) at 25°C, with a scattering angle of 90°. Samples were diluted with distilled water before analysis for better accuracy. Estimations were performed in triplicates, and the average was considered [15].

2.7.2 Entrapment efficiency

Entrapment efficiency was calculated by centrifugation of the known amount of SLN dispersion at 10000 RPM for 15 minutes. The free drug present in supernatant was analysed at 265 nm, using UV visible spectrophotometer after suitable dilution with phosphate buffer saline of pH 7.4. All estimations were performed in triplicate [16].

3. RESULTS AND DISCUSSIONS

3.1 Phytochemical Investigation

Ethanollic extracts of fenugreek seeds were subjected to various chemical tests and results of which is shown in Table 3.

Previous studies of various seed extracts of fenugreek reported that it contains alkaloids (trigonelline), amino acids, saponins, steroidal sapinogens (diosgenin, trigogenin), flavonoids, fibers, lipids, carbohydrates, and proteins. Studies have also shown that it contains mucilage, volatile oils and alkaloids such as choline and trigonelline, sotolone and pyrazines. The chemical tests of fenugreek seed extract showed that it contains all the reported chemical constituents.

3.2 Extraction of Fenugreek Seeds

Extraction of fenugreek seeds done by conventional methods and MAE, their results are given in Table 4. All the extracts obtained were sticky solid brown in colour with aromatic odour. Based upon the yield of extracts we can

Table 3. Chemical tests of fenugreek seed extracts

Sl. No	Tests	Extract from Soxhlation
1.	Alkaloids	
	a) Dragendorff's test	+
	b) Hager's test	+
	c) Wagner's test	+
	d) Mayer's test	+
2.	Carbohydrates	
	a) Benedict's test	+
	b) Fehling's test	+
	c) Molisch's test	+
3.	Proteins and Amino acids	
	(a) Biuret test	+
	(b) Million's test	+
4.	Flavanoids	
	a) Shinoda's test	+
5.	Saponins	+
6.	Steroids	
	a) Liebermann -Burchard's test	+
	b) Salkowski Test	+
7.	Tannins	+
8.	Starch	+
9.	Phenolic compounds	
	a) Ferric chloride test	+
	b) Lead Acetate test	+
	c) Gelatin test	+

Table 4. Yield of the extracts

Parameter	Conventional methods		MAE
	Maceration	Soxhlation	
Yield of the extracts	10%	21%	14%

conclude that soxhlation was the best method for extraction with most of the chemical constituents present in it.

Amount of trigonelline in fenugreek extract was calculated from the calibration curve equation $Y = 0.02x + 0.024$ which was found to be 1 mg /g of the extract [17].

3.3 Quantitative Analysis of Extract

3.3.1 Estimation of Trigonelline by UV-spectrophotometric method

The amount of trigonelline in fenugreek extracts was calculated using the UV spectrophotometric method. UV spectrum of standard trigonelline hydrochloride and fenugreek extract are given in Figs. 1 & 2.

3.4 Toxicity Profile of Fenugreek Seed Extract

The acute toxicity values (LD50) for fenugreek from alcoholic seed extract are 5 g/kg (in rats, oral) and 2 g/kg (in rabbit, dermal). Alcoholic seed extract is non-irritating and non-sensitizing to human skin.

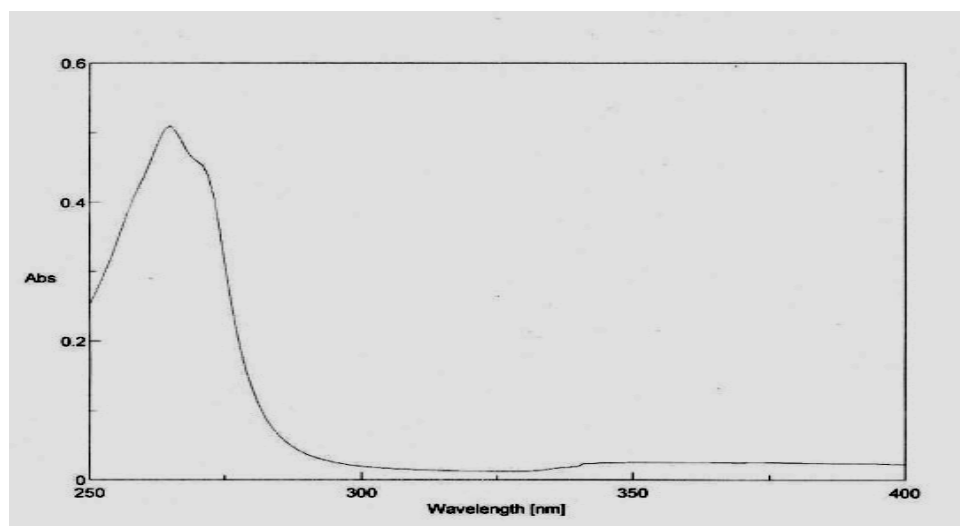


Fig. 1. UV Spectrum of standard trigonelline in Phosphate buffer saline of pH 7.4

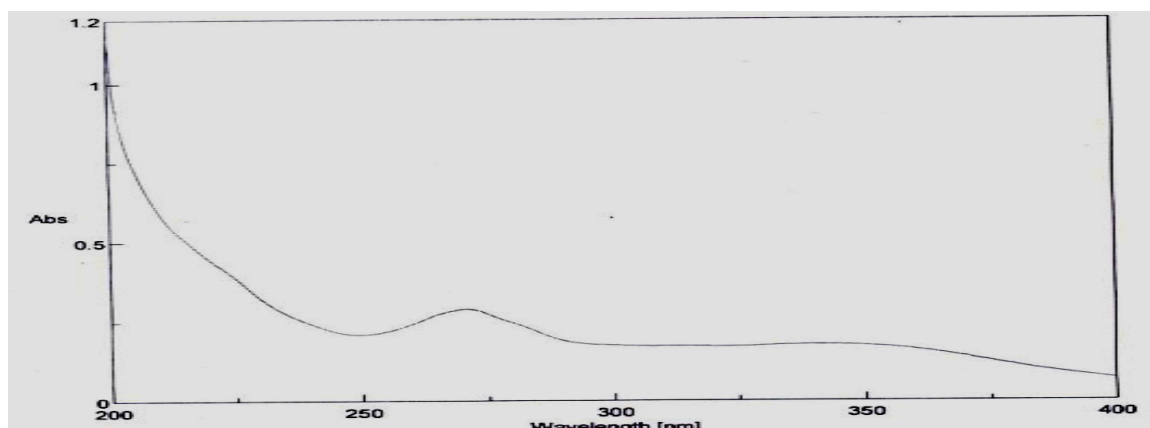


Fig. 2. UV Spectrum of fenugreek seed extract in Phosphate buffer saline of pH 7.4

3.5 Fourier Transform Infrared Analysis (FTIR)

Fourier transform infra red spectra of Standard trigonelline and Extract were assessed using FT-IR spectrophotometer (Bruker) in the range of 4000–500 cm⁻¹ and compared for any significant change Figs. 3, 4.

Based upon the results we can confirm that trigonelline is present in the extract as peaks of extract are matching the with standard trigonelline peaks.

Major IR peaks (wave numbers) are given in Table 5.

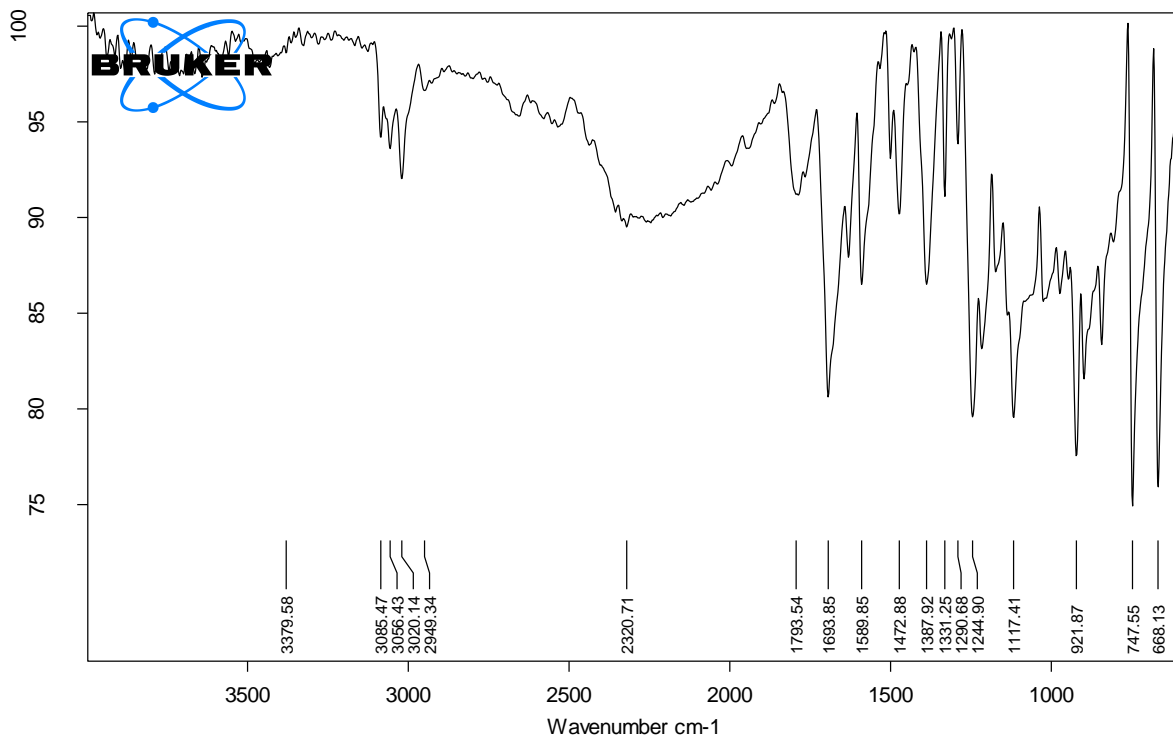
3.6 Design of Experiments

Table 3 shows that the mean particle size (Y1) ranged from 193.4 to 312.3 nm depending on the variables level used during manufacture. Due to the difference in factor combinations as shown in Table 6, the EE percent (Y2) ranged from 61.2 percent to 74.32 percent.

BBD was used in this work to optimise and assess the main, interaction, and quadratic effects of the independent factors on the dependent variables at various levels. Based on various combinations of variables and variable levels, significant variances in responses were produced.

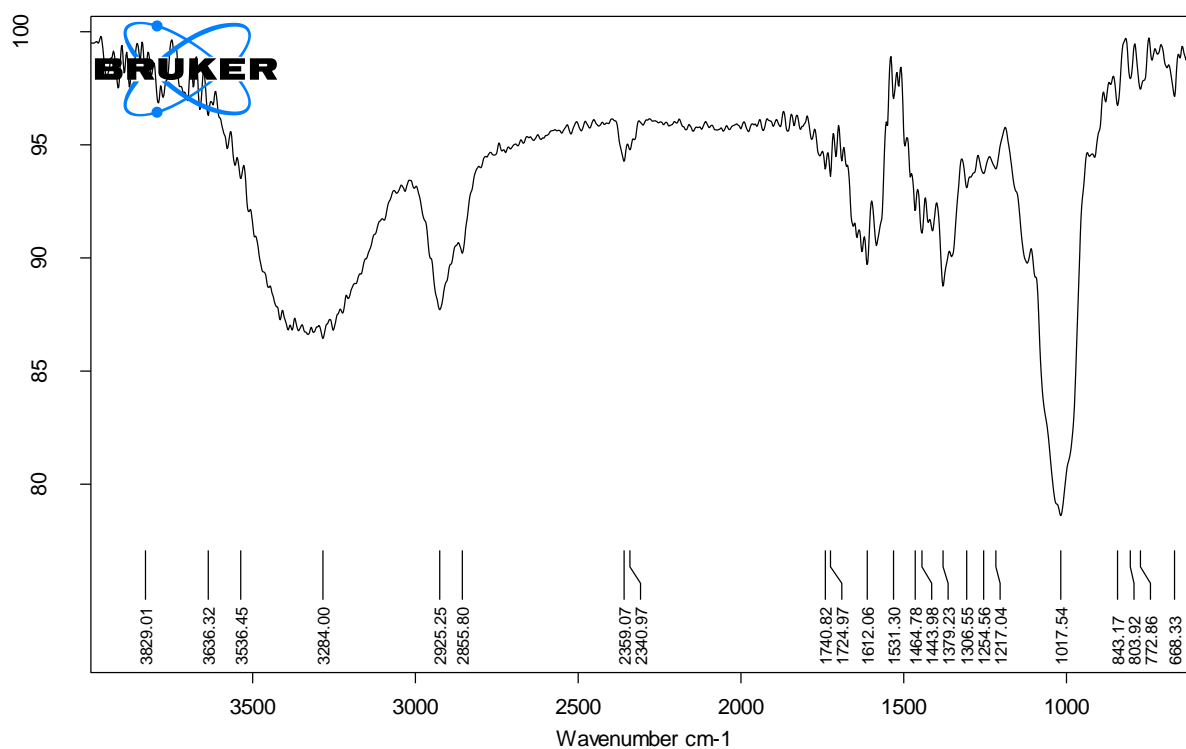
Table 5. Major IR peaks (wave numbers) of standard trigonelline hydrochloride, fenugreek seed extract and fenugreek seed extract formulation

Formulations	Major peaks (Wave numbers, cm ⁻¹)
Standard trigonelline hydrochloride	3379.58, 3020.14, 2949.34, 2320.71, 1793.54, 1693.85, 1472.88, 1387.92, 1244.90, 1117.41, 921.87, 747.55
Fenugreek seed extract	3829.01, 3638.32, 3538.45, 3284, 2855.80, 2340.97, 1740.82, 1464.78, 1379.23, 1254.56, 1217.04, 1017.54, 843.17



D:\FTIR\TRIGONELLINE STANADRD.1	TRIGONELLINE STANADRD	Instrument type and / or accessory	8/13/2020
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Fig. 3. IR spectra of standard trigonelline hydrochloride



D:\FTIR\FENUGREEK SEED EXTRACT.0	FENUGREEK SEED EXTRACT	Instrument type and / or accessory	8/13/2020
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Fig. 4. IR spectra of fenugreek seed extract

Table 6.Response values observed for 15 pomegranate extract solid lipid nanoparticle formulations

Formulation code	Response 1 PS nm	Response 2 EE %
F1	224.36	71.02
F2	193.46	61.2
F3	310.66	72.03
F4	196.66	70.73
F5	199.06	64.75
F6	218.36	69.72
F7	224.06	70.48
F8	201.36	62.6
F9	219.76	69.26
F10	312.36	70.11
F11	215.86	70.24
F12	208.36	62.84
F13	203.56	70.97
F14	307.66	68.86
F15	225.36	73.04
F16	295.66	74.32
F17	220.46	69.96

The lipid content and amount of surfactant have favourable impacts on the entrapment efficiency of fenugreek seed extract SLNs, whereas the

amount of co-surfactant has negative effects, according to the response surface plots for particle size and entrapment efficiency.

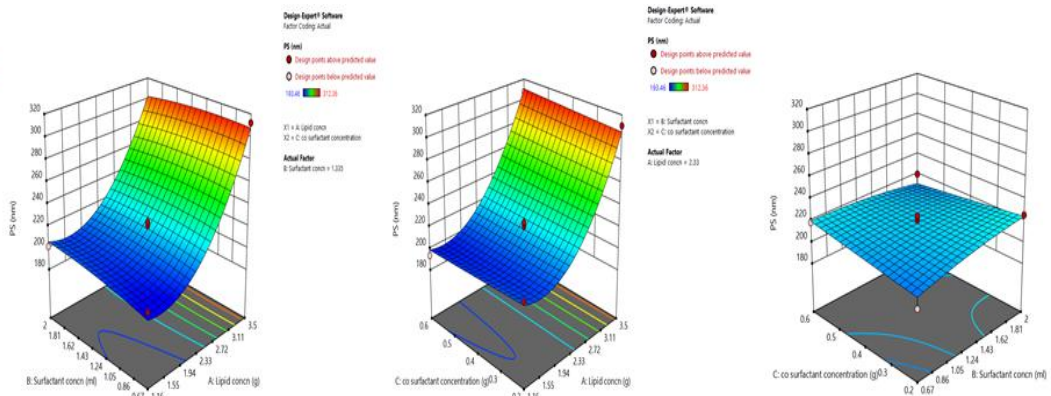


Fig. 5. Response Surface Plots for studying the effect of independent variable on Particle size

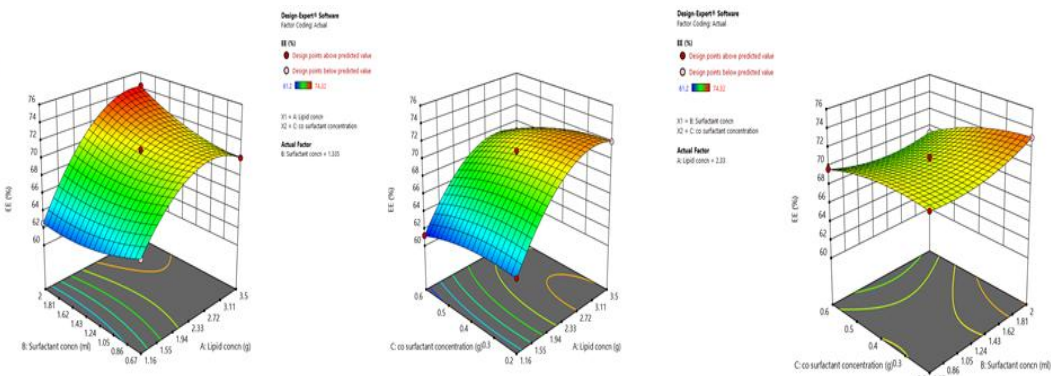


Fig. 6. Response Surface Plots for studying the effect of independent variable on entrapment efficiency

Table 7. Showing the expected and observed for the optimized formula of Fenugreek seed extract loaded SLNs

Variables	Optimum	Responses	Predicted	Observed
Lipid concentration (gm)	2.337	Particle size	221.54	223.36
Surfactant concentration (ml)	2.000	Entrapment efficiency	72.864	74.56
Co-surfactant concentration (gm)	0.876			

3.6.1 Mathematical equation

$$\text{Particle Size (a)} = +217.66+53.01A+1.96B-0.2250C-4.75AB+2.98AC-6.83BC+37.23 A^2-2.78B^2+0.1500C^2$$

$$\text{Entrapment efficiency (b)} = +70.53+4.24A+0.4887B-1.20C+1.59AB-0.3825AC-0.6925BC-3.52 A^2+0.9330B^2-0.7795C^2$$

3.6.2 Prediction of the optimized SLNs formulation

The experimental elements were analysed to assess the examined answers in order to find the

best combination of factors to maximise the desirability function. Using the numerical point prediction optimization approach of the Design expert® programme, the optimum formulation of the Fenugreek seed extract-loaded SLN system was determined based on the criteria of achieving the maximum value of EE and minimising particle size. The responses at the best possible combination of independent variables were tested to ensure that the computed optimal factors and predicted responses were correct. Table 7 shows the optimised formula's composition, the observed and projected responses, and the residual values

for the responses. Based on these findings, the optimal combination of independent factors validated the optimum particle size, PDI, and EE%.

4. CONCLUSION

Fenugreek seeds can be extracted using soxhalation as a best efficient method with maximum yield.

Formulations of Fenugreek seed extract SLNs were optimized and the optimized formula was developed using BBD which was successfully implemented to statistically optimize the formulation variables. It was found that the lipid and surfactant concentration had the main effect on the Particle size and Entrapment efficiency. Further these formulations can be exploited for in vitro and in vivo characterizations. Based on the results obtained these formulations can be scaled up for commercial production and no sophisticated instrument is required during fabrication.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

NOTE

The study highlights the efficacy of "Ayurvedic medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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