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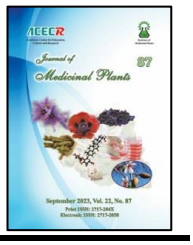

Research Article

The optimization of Fenugreek seeds (*Trigonella foenum-graecum* L.) extraction by response surface methodology based on *β*-Sitosterol

Zahra Tofighi[1,2], Mohammad Reza Khoshayand[3], Masoud Besati[4], Mostafa Pirali Hamedani[2], Abbas Hadjiakhoondi[1,2], Ali Khorshidi[2], Arian Salimi[1], Saied Goodarzi[1,5,*]

[1] *Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Iran*

[4] *Department of Medicinal Chemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran*

[5] *The Institute of Pharmaceutical Sciences (TIPS), Tehran University of Medical Science, Tehran, Iran*

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ABSTRACT

Background: Fenugreek is one of the most widely used medicinal plants in terms of therapeutic properties, and numerous pharmacological effects have been found in various studies. Fenugreek seed extract contains many effective substances, including phytosterols (mainly *β*-Sitosterol). **Objective**: This investigation was conducted to determine the optimal extraction method for fenugreek seeds based on the amount of *β*-Sitosterol detected on HPLC using a response surface methodology (RSM). **Methods**: At first, the appropriate solvent was selected. The main variables affecting the extraction efficiency, including temperature, time, solvent percentage, and the ratio of solvent to powder, were investigated to optimize the best method. Optimizing the number of 29 experiments determined that extraction was done using the dynamic maceration method. After finding the optimized method, the extracts were injected into the HPLC device three times to determine the amount of total *β*-Sitosterol. Then it was modelled, and the final formula was obtained. **Results**: The analysis of results were shown that the optimal extract (based on the amount of total *β*-Sitosterol and weight), using the dynamic maceration extraction method with 96 % ethanol at a temperature of 44 ˚C, a duration of 30 minutes, a solvent percentage of 70 % and a ratio of solvent to powder of 7:1 was obtained. **Conclusion**: The findings suggest the, this method seams the most efficient for maximum extraction of the *β*-Sitosterol compound from fenugreek seeds.

Abbreviations: RSM, Response surface methodology; HPLC, High Performance Liquid Chromatography; *T. foenum-graecum, Trigonella foenum-graecum*

*Corresponding author: goodarzi_s@sina.tums.ac.ir

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1. Introduction

Fenugreek (*Trigonella foenum-graecum*) is an herbaceous and annual plant belonging to the Fabaceae (Leguminosae) family, whose leaves and seeds have medicinal value. In Iran, fenugreek leaves are consumed fresh or dried. The origin of this plant is the Mediterranean area, which spreads in Western Asia, Ukraine, and also from India to China. Pollination of this self-fertile plant is done by wind and sometimes insects. Fenugreek has a long history of cultivation in Iran [1, 2]. Fenugreek seeds are egg-shaped, have a bitter taste and pungent smell, and change from fawn yellow to brown as they mature [3]. (Figure 1).

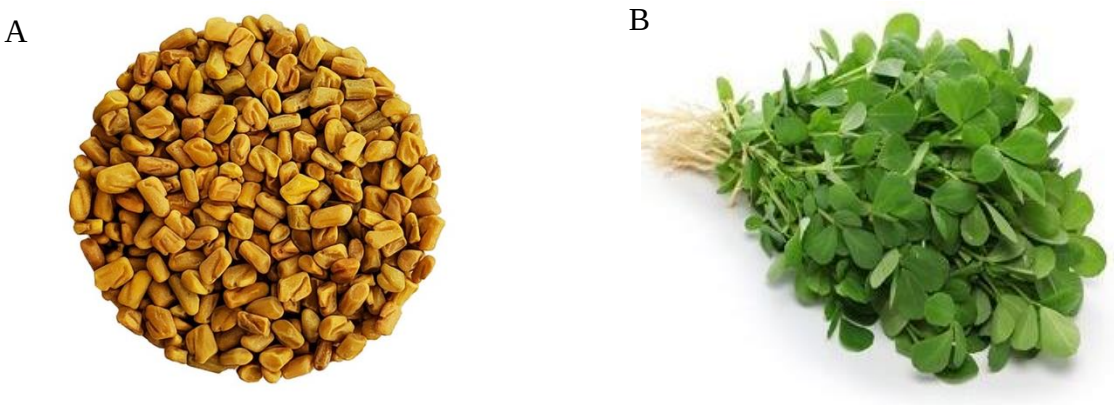

Figure 1. (A) Fenugreek seeds, and (B) Fenugreek plant.

The oil content in fenugreek seeds is about 7.8 %, with a golden-yellow color. The oil has an unpleasant smell and a bitter taste. A study on Egyptian fenugreek oil was demonstrated it consists 33.7 % linoleic, 35.1 % oleic, and 13.8 % linolenic acids [4]. According to Badami & Kalburgi (1969), hexadecanoic acid was found in fenugreek fatty acids [5]. According to Baccou *et al.* (1978), various countries have different compositions of fatty acids in their fenugreek oil. They found that the percentage of linoleic and linolenic acids differ according to the place and conditions of cultivation of the plant, and the oils had marked drying properties [6]. In evaluating the nutritional quality of oil, fatty acid composition occupies a special place. Several intervention studies have demonstrated a substantial reduction in cardiovascular disease risk due to dietary fatty acids, particularly polyunsaturated and monounsaturated fatty acids [7-10]. Phytosterols were found in fenugreek seeds in large amounts and *β*-Sitosterol was the major steroids in it. The amount of protein in this plant is rich in amino acids such as lysine, arginine, and tryptophan. There is a small percentage of histidine and sulfur-containing amino acids like as threonine, valine, and methionine. The carbohydrate part of this plant has less starch but more salts. Also, fenugreek seeds contain proteinase inhibitor compounds. Mineral salts of zinc, manganese, calcium, iron, phosphate, and vitamins such as nicotinic acid, B_1, C, A, and D were reported. The seeds of fenugreek were contained other compounds including alkaloids with trigonelline index, sugar-free or glycosidic flavonoids such as quercetin, vitexin (*C*-glycosidic flavonoid), orientin (arabinoside flavonoid) and coumarin compounds such as scopoletin. Other

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substances, such as tannin and carotenoid compounds, have also been reported [11, 12].

The response surface method (RSM), also is known as the response procedure method, was considered one of the methods of experimental modeling and design of experiments. Many phenomena were modelled based on their theories. Although many phenomena have not a suitable mathematical model, this method is due to many controlling factors, unknown mechanisms, or computational complexity. In such cases, the use of experimental modeling methods is effective; in the response surface method, an attempt is made to find a way to estimate interactions, quadratic effects, and even the three-dimensional shape of the studied response surface by using a suitable experimental design. Response surface methods can be presented differently depending on their application in the experiment. Among the methods of Central Composite Design, D-Optimal and Box-Behnken, etc., can be mentioned [13].

β-Sitosterol has a molecular formula of $C_{29}H_{50}O$ and a weight of 414.7, and its EC number is 201-480-6. Its physical form is solid, its melting temperature is 143.5˚C, and its solubility is 10 mg/ml in water [14]. (Figure 2).

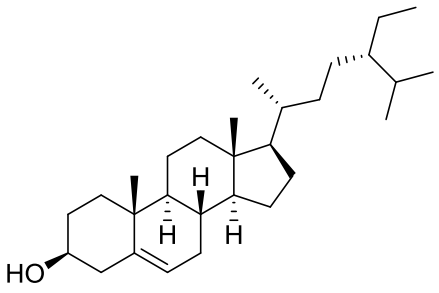

Figure 2. Chemical structure of *β*-Sitosterol from *Trigonella foenum-graecum* L.

Accordingly, this study aimed to optimize the conditions of the extraction process to obtain the highest possible extract based on *β*-Sitosterol concentration from fenugreek seed.

2. Materials and methods

2.1. Plant material

For this study, fenugreek seeds of *Trigonella foenum-graecum* L. were obtained from the market (Tehran) and voucher specimen was registered with herbarium code PMP-1755 in the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The seeds were washed carefully with distilled water after purchase to remove dust and contaminants from the natural habitat. To remove residual washing water from the leaves, absorbent paper was used, and the leaves were weighed on a digital scale (Ohaus Adventurer Pro, USA).

2.2. Reagents and practical approaches for HPLC analysis

HPLC-grade methanol and *β*-Sitosterol analytical standard (≥ 95.0 %) was purchased from Merck (Darmstadt, Germany), and in this work, water was purified by the Milli-Q system. Analyzes were performed on a KNAUER Smartline (Berlin, Germany) HPLC instrument coupled to a DAD. Analyzes were assumed at a wavelength of 245 nm.

Chromatographic analysis was carried out using an RP–18 reversed-phase column (SunFire C18 Column, 5 µm, 4.6 mm × 300 mm, 1/pk, San Francisco, United States) carried at room temperature. The eluents were water 75 % (A) and methanol 25 % (B). This solvent was used as the isocratic method, with an analysis time of 10 minutes. The flow rate was 1 ml/min, and the injection volume was 20 µl.

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2.3. Extraction using dynamic maceration method

The dried fenugreek seeds were sieved with a mesh size 20 using an industrial powder mill. A scale was weighed and powder was sifted and 1 g was used for each test.

To experiment, 1 g of plant seed powder was poured into a suitable flask, and ethanol was added with a specified percentage and volume (specified in the Box-Behnken test design method for each experiment). The top of sample was covered with aluminum foil and place it on the heater stirrer with the specified temperature in the Box-Behnken design method and then a magnet was put into Erlenmeyer, and the speed of the stirrer was set about 700 rpm. After the time specified in the Box-Behnken design, removed from the device and pass the resulting liquid through the filter paper and washed the filter paper with 20 ml of the solvent used for the test. Then, the obtained solution was poured into the petri dish, which already weighed with a scale, and was put under the laboratory fume hood for 24 hrs. to dry. These steps were performed for all 29 trials specified in the design expert software.

The extracts were stored to determine the amount of *β*-Sitosterol by HPLC.

2.4. Determination of β-Sitosterol by high-performance liquid chromatography (HPLC)

Since the *β*-Sitosterol was used in commercial studies of standardization of fenugreek, this phytosterol was selected for determination and optimization process. The calibration curve was drawn according to the pure *β*-Sitosterol, and a formula based on the quadratic relationship was reached. At this stage, the amount of the desired effective substance (*β*-Sitosterol in fenugreek seeds) should be tested in the calibration curve.

Since to optimize a method of determining the amount to make the study more accessible and convenient, according to the Box-Behnken method, 29 samples were added to the dynamic maceration method in their own petri dishes and were placed under the laboratory fume hood to dry. Then, each sample was injected into the HPLC.

One mg of dried extract from each petri dish was poured into a vial and mixed with 1 ml of methanol, and placed in a sonicator for 10 min. Before injection, the solution was filtered with a needle filter (0.22 μm).

2.5. Optimizing parameters based on a screening design

Conventional optimization relies on the one-factor-at-a-time (OFAT) method, which is time-consuming, laborious, and expensive. A conventional method can also not provide information regarding how different factors interact to influence the outcome. Design of experiments (DOE) is a statistical method of studying the appropriate operating variables that are used to optimize technological processes, most commonly orthogonal arrays, central composite designs (CCD), and Box-Behnken designs (BBD) [15-17]. To examine the influence of extraction parameters on effective substance of the seeds of this plant, i.e., *β*-Sitosterol, a four-factor, four-level BBD was used as a subset of RSM. To studying the extraction efficiency in the effective constituent's extraction, the following parameters were determined to be the most influential: time (X1), temperature (X2), solvent concentration (X3), and solvent-to-powder ratio (X4). Table 1 shows the suitable ranges of all four process variables, determined from preliminary experiments and the central point values.

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Table 1. Extraction process optimization ranges and factors

Factors	Coding symbols*	Range and levels		
		-1	0	1
Temperature (˚C)	X_1	40	50	60
Time (Min)	X_2	30	60	90
Solvent concentration (V/V %)	X_3	40	60	80
Solvent-to-powder ratio (ml/g)	X_4	7:1	14:1	21:1

* Coding symbols: X1: time, X2: temperature, X3: solvent concentration, X4: solvent-to-powder ratio.

2.6. Designing experiments with optimization

In this study, the investigated variables were temperature, heating time, solvent concentration, and solvent-to-powder ratio, according to the previous studies on the extraction and optimization of these extracts based on the effective substance of the seeds of this plant, i.e., *β*-Sitosterol. Four variables were considered the main and significant variables that have the most effect on the extraction process and are also controllable. Other factors, such as particle size, can be ignored due to passing the fenugreek seeds through a size 20 sieve before pulverizing them and completely pulverizing them by the grinding machine or the presence of a magnet during extraction, which did not affect the work process.

In this study, the Box–Behnken method was used, which increased the prediction accuracy of the study despite the number of trials being less than the rest of the statistical model methods and considering three levels for the variables.

To optimize the test conditions, four parameters of temperature, time, solvent concentration, and solvent-to-powder ratio were considered using the surface-response test design and the Box–Behnken method, as described in table 1. At this stage, the design was done by the Box-Behnken method based on the considered levels for each of the factors in the process to optimize the test model. The used model follows the quadratic relationship.

The following table shows the number of trials based on four factors with the Box-Behnken method with a total of 29 trials (Table 2).

By default, the Design Expert software (State-Ease Inc., version 7.0.0) [18] shows the order of experiments in the first column, and the side column (randomness of these experiments) is included to confirm the correctness of the working method when we want the order of experiments not to have an influencing factor.

Generally, the model used in the surface-response method is a quadratic polynomial relationship. In the response-level method, a model is defined for each independent variable that examines the main and mutual effects of the factors on each dependent variable. The multivariate model is in the form of the following equation:

$$Y = b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2$$

In the mentioned equation, Y is the predicted response, b_0 is a constant coefficient, $b_1/b_2/b_3$ are linear coefficients, $b_{11}/b_{22}/b_{33}$ are quadratic effects, and $b_{12}/b_{13}/b_{23}$ are common effects. $X_1/X_2/X_3$ are also independent variables [19].

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Table 2. Experiment design for the optimization process

Run	Std	Coding symbols*			
		X_1	X_2	X_3	X_4
1	29	40	30	60	14
2	14	60	30	60	14
3	18	40	90	60	14
4	20	60	90	60	14
5	10	50	60	40	7
6	23	50	60	80	7
7	8	50	60	40	21
8	24	50	60	80	21
9	25	40	60	60	7
10	28	60	60	60	7
11	12	40	60	60	21
12	19	60	60	60	21
13	2	50	30	40	14
14	27	50	90	40	14
15	21	50	30	80	14
16	3	50	90	80	14
17	16	40	60	40	14
18	11	60	60	40	14
19	13	40	60	80	14
20	9	60	60	80	14
21	17	50	30	60	7
22	15	50	90	60	7
23	6	50	30	60	21
24	4	50	90	60	21
25	26	50	60	60	14
26	1	50	60	60	14
27	7	50	60	60	14
28	5	50	60	60	14
29	22	50	60	60	14

* Coding symbols: X1: time, X2: temperature, X3: solvent concentration, X4: solvent-to-powder ratio.

2.7. Statistical analysis

The mathematical relationship between the response and the independent variables can be the model used in a second-order polynomial function. This equation can evaluate the linear or quadratic relationship and any relationship between the independent variables and the response. The regression method and the response surface diagram were used for the specialized statistical analysis of the data. The significance of the effect of independent variables on the response was evaluated using the one-way ANOVA test and to comparison between groups post-hoc Fisher was performed

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and p-value less than 0.05 was considered as significant difference. R^2 or multivariate correlation coefficient is an index that helps us confirm the study model's quality. Three-dimensional diagrams were also used for better interpretation of the considered factors and the logical relationship between the interactions of the factors.

3. Results

3.1. Statistical analysis and optimization of extraction

The design model was generalized to determine the optimal method for determining the amount of fenugreek seeds extract. This model is approved by Design Expert Software what degree the desired model is (first, second or third) is shown by data matching and analysis of variance. The data were created in the form of tables that could be analyzed. In Table 3, the model's validity can be evaluated by lack of fit. To estimate the lack of fit, F-value isb3.78, which indicates that the lack of fit is not significant, compared to the net error and our model is adequate. The P-value was also 0.0275, which confirms the study model with a quadratic relationship according to Fisher's law (higher than 0.1). Accordingly, the quadratic relationship is the most suitable method.

The quadratic relationship was more valuable because it showed both the main effects and the effects of each variable and the interactions of the effects on each other. The suitable designed model is also shown in Table 4.

Table 3. Validation of the use of the quadratic relationship according to the effective data

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value	
Mean vs Total	224.40	1	224.40			N/A
Linear vs Mean	32.54	4	8.13	0.9633	0.4456	N/A
2FI vs Linear	10.00	6	1.67	0.1557	0.9853	N/A
Quadratic vs 2FI	**100.01**	**4**	**25.00**	**3.78**	**0.0275**	**Suggested**
Cubic vs Quadratic	82.34	8	10.29	6.00	0.0212	Aliased
Residual	10.30	6	1.72	N/A	N/A	N/A
Total	459.59	29	15.85	N/A	N/A	N/A

Table 4. The ANOVA analysis for the response surface quadratic model

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value Prob > F
Model	**138.07**	**9**	**15.34**	**3.00**	**0.0209 significant**
X_1 (Temperature (˚C))	10.25	1	10.25	2.01	0.1730
X_2 (Time (Min))	2.12	1	2.12	0.4141	0.5276
X_3 (Solvent concentration (v/v %))	2.26	1	2.26	0.4425	0.5139
X_4 (Solvent-to-powder ratio (ml/g))	17.91	1	17.91	3.50	0.0767
X_1X_2	5.52	1	5.52	1.08	0.3116
X_2X_3	0.1225	1	0.1225	0.0240	0.8786
X_2X_4	2.82	1	2.82	0.5522	0.4665
X_2^2	66.05	1	66.05	12.92	0.0019
X_3^2	19.37	1	19.37	3.79	0.0665
Residual	97.12	19	5.11	-	-
Lack of Fit	**96.93**	**15**	**6.46**	**137.49**	**0.0001 significant**
Pure Error	0.1880	4	0.0470	N/A	N/A
Cor Total	235.19	28	N/A	N/A	N/A

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The average obtained from the factors is 2.78, and the standard deviation is an index that describes the dispersion (changes) of the sample data around the average that 2.26 was calculated, and this closeness of the two numbers indicates the reliability and accuracy of the test. Therefore, it was concluded that a quadratic polynomial equation could be used in the optimization of the *β*-Sitosterol extract from fenugreek seeds against independent variables.

The appropriate equation that can obtain the optimal point by deriving the quadratic equation was reported in table 5:

In the coded equation, Y is the amount of *β*-Sitosterol in 1 mg of fenugreek seeds extract, $X_1$is temperature, X_2 is time, X_3 is a solvent concentration, and X_4 is the solvent-to-powder ratio. The presence of a negative sign behind the coefficients indicates that increasing these values increases the amount of total *β*-Sitosterol.

Three-dimensional response-surface diagrams were drawn, which are suitable tools for showing interactions between variables. In these diagrams, R_1 refers to the total *β*-Sitosterol of fenugreek seeds.

Table 5. Final equation for the essential oil value of *β*-Sitosterol in terms of coded or actual factors.

Response	Types	Equations
***β*-Sitosterol value**	Actual	- 13.92 + 0.13 × Temperature - 0.179 × Time + 0.57 × Solvent concentration + 0.11 × Solvent-to-powder ratio - 0.004 × Temperature × Time - 0.009 × Temperature × Solvent concentration + 0.003 × Temperature × Solvent-to-powder ratio + 0.0003 × Time × Solvent concentration - 0.004 × Time × Solvent-to-powder ratio - 0.004 × Solvent concentration × Solvent-to-powder ratio + 0.0002 × $Time^2$ - 0.004 × $(Solvent\ concentration)^2$ + 0.014 × $(Solvent\text{-}to\text{-}powder\ ratio)^2$
	Coded	$Y = 2.19-0.92X_1+0.42X_2+0.43X_3-1.22X_4-1.12X_1X_2+0.18X_2X_3-0.84X_2X_4+3.09X_2^2-1.68X_3^2$

In figure 3, the effect of the simultaneous change of two variables on each other is shown on the assumption that the other two variables are constant.

The figure 3-A shows the effect of two variables, temperature and time, on the amount of total *β*-Sitosterol where the concentration of solvent and the solvent-to-powder ratio are constant. The figure shows that when the time is at its lowest, i.e., 30˚C, the temperature does not have a significant effect, but when the time increases (between 40 and 70˚C), the response decreases first. But after 70˚C, we see an increase in the response until we reach the highest degree, i.e., 90˚C, and the highest response; on the other hand, increasing of the temperature, the response decreases so that in the highest temperature and the lowest response observed.

In figure 3-B, the effect of two variables, time and solvent concentration, on the amount of total *β*-Sitosterol in a state where the temperature and solvent-to-powder ratio are constant was shown. The response was increased in the corners of the desired figure and a decrease in the response in the middle parts of the factors. Whether time is at its highest value or at its lowest value, when the solvent concentration is at its highest and lowest, the greatest response is observed.

Figure 3-C shows the effect of the two variables of time and solvent-to-powder ratio on the amount of total *β*-Sitosterol in a state where the temperature and concentration of the solvent are constant. As seen in the figure, the lowest response rate was demonstrated when the lowest amount of solvent was used at any time of the test. However, with increasing the amount of

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solvent, an increase in the response was observed and the highest response was shown at the highest amount of solvent.

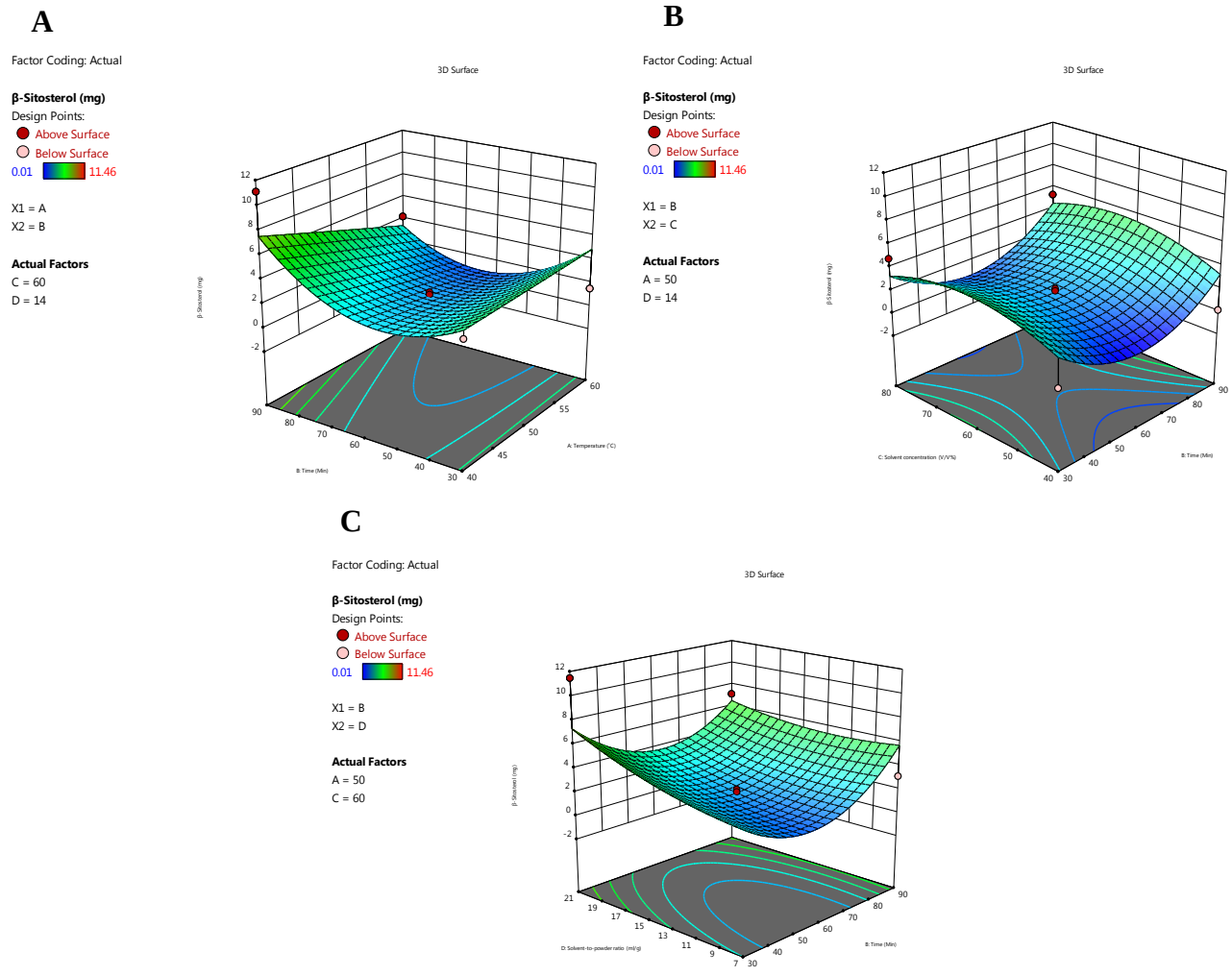

Figure 3. Response surface plots for the effect of (A) Temperature and time; (B) time and solvent concentration; (C) Time and solvent-to-powder ratio on the value of total *β*-Sitosterol of fenugreek seeds.

3.2. Determining the best solvent for extracting fenugreek seeds

To obtain the best solvent used in fenugreek seeds extraction process, two solvents, methanol and ethanol were used. The basis of comparing these two solvents is the better solubility of fenugreek extract and the percentage of extract obtained after drying it.

In general, extract solubility in methanol was not acceptable, it did not dissolve properly, and a clear solution was not obtained. In contrast, the extract was easily dissolved in ethanol.

The amount of weight obtained is also according to table 6:

Table 6. Results of the best solvent for extracting fenugreek seeds

Solvent	weight of extract obtained (g)
Ethanol	0.3
Methanol	0.16

As can be seen, if the ethanol solvent is used, the extract's weight is almost twice as much.

3.3. Analyzing of β-Sitosterol content with HPLC and optimizing the extraction method

The method and conditions of analysis were developed based on better separation and the amount of suitable UV absorption for identifying *β*-Sitosterol on the HPLC device. The solvents used in *β*-Sitosterol analysis were methanol, water, and acetonitrile.

The peak obtained from pure *β*-Sitosterol and the sample containing our extract is displayed in the third minute. According to the obtained observations for the study, both methods have sufficient accuracy. The methanol-water method creates less noise to show the desired peak than the acetonitrile-water method, and, as mentioned, it is better in terms of safety. Next, different percentages of water and methanol were tried to minimize the noise of the device, which was confirmed according to the trial-and-error ratio of water: methanol (75:25 (v/v)).

And the peak obtained from a selected sample with our method was obtained as shown in figure 4:

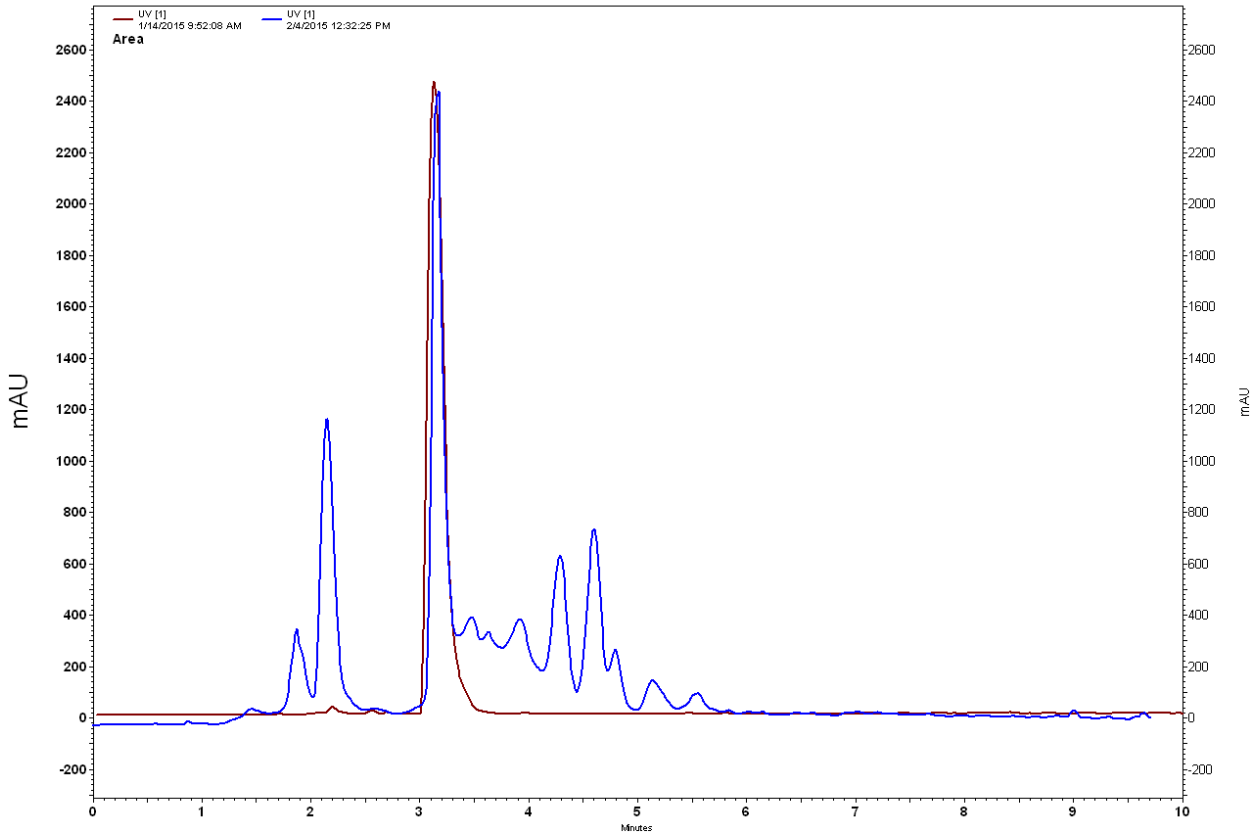

Figure 4. (-): The peak was obtained from a random sample of fenugreek seed extract using water-methanol method (75:25 (v/v)) and, (-): the peak was obtained from working standard of *β*-Sitosterol using water-methanol method (75:25 (v/v))

Based on the method of Swamy Gowda *et al.*, the mobile phase was acetonitrile: water (5:95 v/v), which was filtered through a 0.22 µm membrane filter and degassed in ultrasonic bath before use. The mobile phase was selected with a 2 ml/min flow rate in isocratic mode. The quantification of *β*-Sitosterol was done by injecting 20 µl of the active substance into the HPLC device and with a UV detector (at a wavelength of 202 nm) [20].

In a study, a method was discussed for isolating *Momordica charantia* components, i.e., Stigmasterol glucoside and *β*-Sitosterol glucoside by a simple extraction technique followed by HPLC. The identity of the isolated components was determined by chromatographic and spectroscopic techniques. Also, the reverse phase HPLC-DAD method was developed and validated for the estimation of stigmasterol glucoside and *β*-Sitosterol glucoside in *Momordica charantia* fruits. In this method, the C18 column (75 mm × 4.6 mm, 3.5 μm) was the stationary phase, and methanol: water (98:02 (v/v)) was used as the mobile phase. The inhibition time of STG and BSG were 10.707 minutes and 11.870 minutes, respectively. The approved method was used to evaluate the content of these compounds in different extracts and some commercial herbal formulations containing *Momordica charantia* fruit [21].

3.3. Mutual effect of extraction factors

Some operating variables could potentially modify the fenugreek seeds extraction process, and the extraction conditions for *β*-sitosterol from the seeds were investigated. An interactive independent variable and dependent variable are shown as a three-dimensional response surface model in Table 7.

Table 7. Design experiments by the Box-Behnken method, the results obtained from the injection of fenugreek seed extract into the HPLC device, and the corresponding results of each investigation.

Run	Factors				Response	
	X_1 Temperature (˚C)	**X_2 Time (Min)**	**X_3 Solvent concentration (v/v%)**	**X_4 Solvent-to-powder ratio (ml/g)**	**Predicted *β*-Sitosterol (mg/g $_{\text{of extract}}$)**	**Experimental *β*-Sitosterol (mg/g $_{\text{of extract}}$)**
1	40	30	60	14:1	4.4	3.7
2	60	30	60	14:1	4.9	1.5
3	40	90	60	14:1	7.5	11.1
4	60	90	60	14:1	3.3	4.2
5	50	60	40	7:1	-1.3	0.4
6	50	60	80	7:1	0.6	0.1
7	50	60	40	21:1	2.2	3
8	50	60	80	21:1	2.0	0.5
9	40	60	60	7:1	2.4	1.9
10	60	60	60	7:1	0.1	1.3
11	40	60	60	21:1	4.4	1.6
12	60	60	60	21:1	3.0	1.9
13	50	30	40	14:1	2.8	0.3
14	50	90	40	14:1	3.3	0.1
15	50	30	80	14:1	3.3	4.8
16	50	90	80	14:1	4.4	5.3
17	40	60	40	14:1	0.6	1.7
18	60	60	40	14:1	-0.9	1.2
19	40	60	80	14:1	1.8	1.2
20	60	60	80	14:1	-0.4	0.01
21	50	30	60	7:1	3.2	4.1
22	50	90	60	7:1	5.7	3.1
23	50	30	60	21:1	7.3	11.46
24	50	90	60	21:1	6.5	7.1
25	50	60	60	14:1	1.8	1.8
26	50	60	60	14:1	1.8	2.1
27	50	60	60	14:1	1.8	1.9
28	50	60	60	14:1	1.8	1.8
29	50	60	60	14:1	1.8	1.5

The solubility of *β*-Sitosterol in solvents increases with increasing temperature, and at a constant temperature, the solubility decreases with increasing polarity, except for *n*-hexane [22]. *β*-Sitosterol is more stable at lower temperatures, and following efforts to extract it better, attention should be paid to its stability [23].

For this reason, the extraction conditions are critical to optimizing extraction yields and enriching the effective constituents. It is essential to consider several factors before employing extraction techniques to ensure complete extraction without causing chemical modification, including solvent types and ratios, extraction temperatures, extraction times, and solid-to-liquid ratios [24-27].

3.4. Model evaluation

To validate the experimental model, which was obtained using surface-response methodology and Design Expert software, the best response was considered at a temperature of 44 ˚C, a time of 30 min, a solvent concentration of 70 %, and a ratio of solvent to powder of 7:1. In this case, the best response was predicted as 3.25 mg. To confirm the study's accuracy, the same values for the factors of temperature, time, solvent concentration, and the ratio of solvent to powder, which were prepared in the laboratory, were obtained by the dynamic maceration method of the dried extract. According to the stated method, the extraction and analysis was repeated three times.

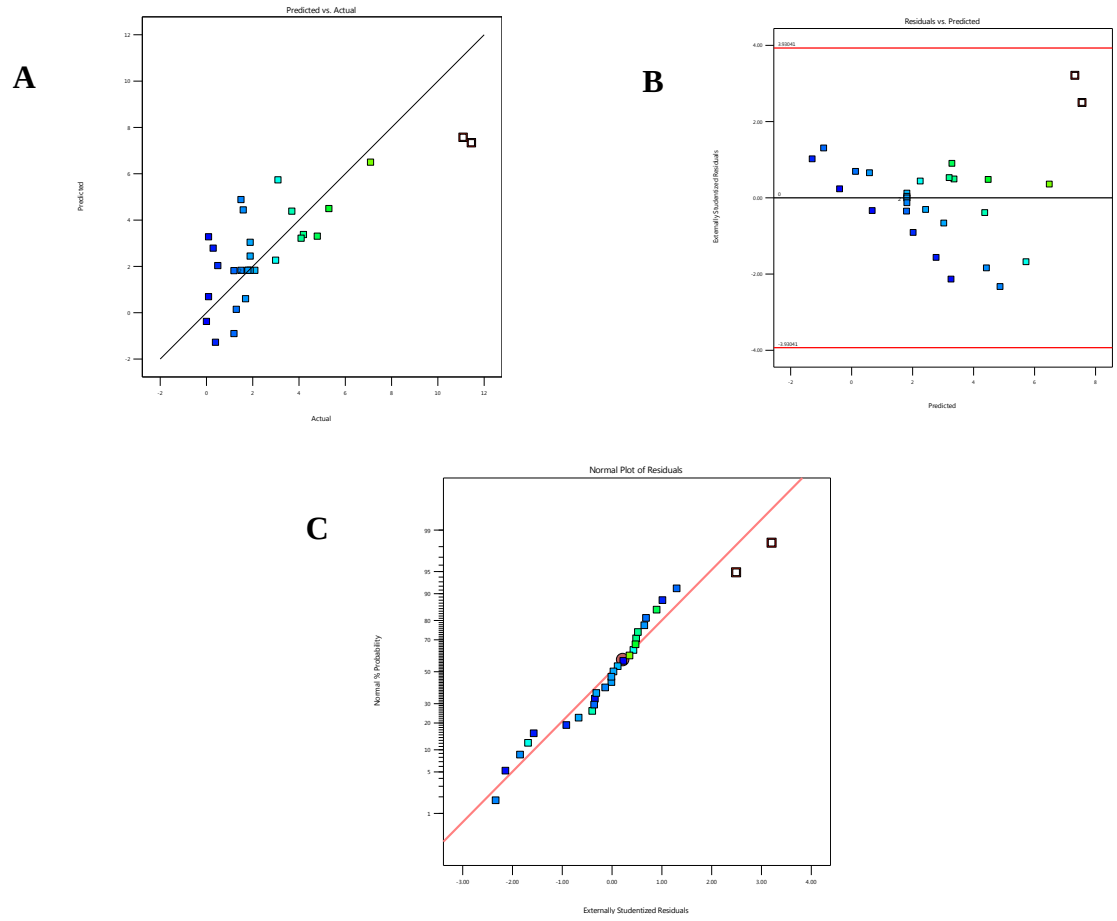

Figure 5. Evaluation of RSM proposed correlation between predicted and experimental *β*-Sitosterol values from fenugreek seeds; A) Predicted versus actual values plot, B) Residual versus predicted plot, C) Normal plot of residuals.

The samples were injected into the HPLC device, and their concentration was calculated according to the calibration curve (Figure 5) formula (Y = 3225X + 116.91, R^2 = 0.99). In this case, the average value of *β*-Sitosterol was 3.46 ± 1.48 mg, and the comparison of the experimental values with the predicted value by the mathematical model obtained from the Design Expert software shows that these two numbers match and the adequacy of the predicted model in the answers in the laboratory.

4. Discussion

The seeds of fenugreek vary in fat content between 5.8 % and 15.2 %. Among the main fatty acids are linoleic acid (45.1-47.5 %), also known as linolenic acid (18.3-22.8 %), oleic (12.4-17.0 %), palmitic (9.8-11.2 %), and stearic acid (3.8-4.2 %). There was a large variation in the percentage of *β*-Sitosterol in all samples, which ranged from 14.203 to 18.833 mg/kg of lipid. Sterols such as campesterol and cycloartenol, which together represented 56-72 % of total sterols, were also important. In fenugreek seeds lipids, unsaturated triglycerides (32.2-41.6 %) predominate over saturated triglycerides (56.9-66.5 %). Using fenugreek seeds for food applications may provide a source of nutrient-rich lipids and fats [28].

The most influential factors in the extraction process of fenugreek seed extract with the highest amount of *β*-Sitosterol were temperature, time, solvent concentration, and solvent-to-powder ratio, respectively. The temperature has an essential effect on the extraction of the extract due to its direct effect on the solvent and soluble structure. Temperature affects solubility, surface tension, diffusivity, and viscosity. The temperature is critical because the effective substance may not dissolve at a low temperature, and at a high temperature, there is a possibility of destroying the effective substance. The time also depends directly on the temperature. Different responses can be obtained if the effective substance is close to the solvent for a short or long time with different temperatures. In the conventional methods of extraction, the more each solvent, the amount of the effective substance is also higher. However, due to the increase in energy consumption in solvent removal, the possibility of destroying the environment, and not being economical, the ratio of solvent to plant should be held as low as possible. The solvent should be such that the effective substance can be dissolved and converted into a suitable extract [29].

According to Iranmanesh *et al*. study, the best method used for extraction was dynamic maceration [30]. A study investigated the toxicity of fenugreek seed extract; an aqueous extract was prepared using the modified method. The obtained aqueous extract was centrifuged, and the supernatant was lyophilized. The yield of the extract was 20 % (weight on weight in terms of powder) [31]. Also, in a study that was performed by Shailajan *et al.*, to investigate the development of an efficient and effective HPLC method for estimating trigonelline (a significant plant substance found in fenugreek seeds) from *T. foenum-graecum* seeds from Ayurvedic herbal formulations and other herbal formulations [32]. The diosgenin content of fenugreek was also studied by using biologically synthesized nanoparticles. A biosynthesized silver nanoparticle (Ag-NP) treatment of fenugreek seedlings significantly altered their growth parameters, such as leaf number, root length, stem length, and wet weight. The phytochemical enhancement effect of Ag-NP was highly significant compared to an untreated control. Consequently, the findings of this study can

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lead to the development of new methods for increasing the biosynthesis of natural medicinal products in plants based on nano-extractors [33]. In the study conducted to optimize fenugreek seeds extract by SFE method using different pressure and temperature, the performance of the extract, as well as its composition for using different solvents to extract each amount of dry fenugreek seeds was analyzed. To determine the optimal extraction conditions, a central composite design (CCD) and the response surface method (RSM) were used [34]. In another study, the thin-layer chromatography method was used to determine the properties of *β*-Sitosterol and stigmasterol from the *T. foenum-graecum*. Based on total flavonoids content, the ethanol concentration (72 %), solvent-to-material ratio (35 ml/g), in 41 min with 500 W of power ultrasound vibration, the optimum condition of extraction was achieved [35]. The method was performed using chloroform and methanol solvent (99:1) and silica gel G plates. Finally, a mixture of stigmasterol and *β*-Sitosterol was found isolated from the callus of cotyledon explants of *T. foenum-graecum*. In that study, it was found that fenugreek has the potential to show a diverse set of biological activities. However, only one part of these plants has been investigated so far, and there is an urgent need to quickly evaluate the extracts of this plant [36].

As it is known, many studies have been done on fenugreek from the effective substances of the plant in different ways. However, until now, statistical modeling for the effective substance *β*-Sitosterol has not been done with an HPLC device.

5. Conclusion

In this study, it was determined that the most optimal method of extracting fenugreek seeds for the existing factors is temperature, time, concentration, and solvent-to-powder ratio. The reason for choosing fenugreek seeds to determine the amount of *β*-Sitosterol was the presence of large amounts of various fats in the plant seeds.

According to the studies and experiments conducted in this research, the surface-response method can create an optimal method for the maximum extraction of fenugreek seeds extract, even on an industrial scale. In this experiment, extracting with dynamic maceration was done well. The effect of different parameters on extraction process was specified. Solvent and temperature had highest effect on extraction process. The relation between solvent polarity and chosen analyte polarity was played an important role in it. Also, increasing temperature will increase the extraction efficiency [37]. Thus, the solubility of *β*-Sitosterol was acceptable in ethanol (as safe solvent in pharmaceutical industry). So, determination of the amount of total *β*-Sitosterol of each extract was done with an HPLC device, which is the most optimal condition to achieve the highest amount of total *β*-Sitosterol considering the weight of fenugreek seeds, temperature 44 ˚C, time 30 min, the solvent concentration of ethanol to water is 70 % and the ratio of solvent-to-powder is 7:1.

Conflicts of interest

We wish to confirm that there are no known conflicts of interests associated with this publication**.**

Author contributions

Z. T. was contributed to the interpretation of the results and was revised the manuscript. M. R. Kh. was analyzed the results and was advisor. M. B. was wrote this manuscript and was analyzed data. M. P. H. was accomplished the experiments and was

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revised this article. A. H. was contributed to the interpretation of the results and was advisor. A. Kh. and A. S. was accomplished the experiments. S. G. was designed and was the supervisor of this study.

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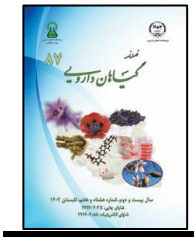

مقاله تحقیقاتی

بهینه‌سازی عصاره‌گیری دانه شنبلیله (*Trigonella foenum-graecum* L.) به روش سطح-پاسخ بر اساس بتاسیتوسترول

زهرا توفیقی[۱،۲]، محمدرضا خوشایند[۳]، مسعود بساطی[۴]، مصطفی پیرعلی‌همدانی[۲]، عباس حاجی‌آخوندی[۱،۲]، علی خورشیدی[۲]، آرین سلیمی[۱]، سعید گودرزی[۱،۵]*

[۱] مرکز تحقیقات گیاهان دارویی، دانشکده داروسازی، دانشگاه علوم پزشکی و خدمات درمانی تهران، تهران، ایران
[۲] گروه فارماکوگنوزی، دانشکده داروسازی، دانشگاه علوم پزشکی و خدمات درمانی تهران، تهران، ایران
[۳] گروه کنترل دارو و غذا، دانشکده داروسازی، دانشگاه علوم پزشکی و خدمات درمانی تهران، تهران ایران
[۴] گروه شیمی دارویی، پژوهشکده گیاهان و مواد اولیه دارویی، دانشگاه علوم پزشکی و خدمات درمانی شهید بهشتی، تهران، ایران
[۵] مرکز تحقیقات علوم دارویی، دانشگاه علوم پزشکی و خدمات درمانی تهران، تهران، ایران

اطلاعات مقاله

گل‌واژگان:
شنبلیله
روش سطح-پاسخ
بتا سیتوسترول
HPLC

چکیده

مقدمه: شنبلیله از نظر خواص درمانی یکی از پرمصرف ترین گیاهان دارویی است و در مطالعات مختلف اثرات دارویی متعددی از آن مشاهده شده است. عصاره دانه شنبلیله حاوی بسیاری از مواد موثره از جمله استرول‌ها (عمدتا بتا سیتوسترول) است. **هدف:** این تحقیق به منظور تعیین روش استخراج بهینه برای بذر شنبلیله بر اساس میزان بتا سیتوسترول ردیابی شده در HPLC با استفاده از روش سطح-پاسخ (RSM) انجام شد. **روش بررسی:** ابتدا حلال مناسب انتخاب شد. متغیرهای اصلی مؤثر بر بازده استخراج شامل دما، زمان، غلظت حلال و نسبت حلال به پودر برای بهینه‌سازی بهترین روش بررسی شدند. برای بهینه سازی تعداد ۲۹ آزمایش مشخص شد که استخراج با استفاده از روش خیساندن دینامیکی انجام شده است. پس از یافتن روش مناسب، عصاره‌ها سه بار به دستگاه HPLC تزریق شد تا میزان بتا سیتوسترول تام آنها مشخص شود. سپس مدل‌سازی شد و فرمول نهایی بدست آمد. **نتایج:** آنالیز نتایج نشان داد که بهترین عصاره (بر اساس میزان بتا سیتوسترول کل و وزن)، با استفاده از روش استخراج خیساندن دینامیکی با اتانول ۹۶ درصد در دمای ۴۴ درجه سانتی‌گراد، مدت زمان ۳۰ دقیقه، غلظت حلال ۷۰ درصد و نسبت حلال به پودر ۷:۱ به دست آمد. **نتیجه‌گیری:** یافته‌ها نشان می‌دهد که این روش برای حداکثر استخراج ترکیبات بر اساس بتا سیتوسترول از دانه‌های شنبلیله کارآمدترین حالت به نظر می‌رسد.

مخففها: RSM، روش‌شناسی سطح-پاسخ؛ HPLC، کروماتوگرافی مایع با کارایی بالا؛ *T. foenum-graecum*، شنبلیله

* نویسنده مسؤول: goodarzi_s@sina.tums.ac.ir

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