Original Article

Standardization of *Trigonella foenum-graecum* L. Seeds: A Quality by Design Approach

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ABSTRACT

Aim: To standardize *Trigonella foenum-graecum* L. Seeds by developing QbD based HPLC method for identification and quantification of trigonelline in *T. foenum graecum* L. seeds, along with evaluation of various quality control parameters. **Methods:** The Analytical Target Profile and Critical Quality Attributes were determined followed by optimization of HPLC method by using 2^2 factorial design for designing the experiments for selected independent factors. Method Operable Design Region was developed for finding out the optimized chromatographic conditions. Further quality control parameters such as macroscopic and microscopic characters, physicochemical and phytochemical characterization including determination of toxic elements were carried out on the herb. **Results:** By application of QbD approach the optimized mobile phase was identified as water with 0.01% Hydrochloric acid and Methanol in the ratio of 70:30, with the flow rate of 1 mL/ min and UV detection at 263 nm. The linear model was established in the range of 2-10μg/mL with R^2 value 0.998. The retention time of Trigonelline was found to be 2.877 min and the amount of Trigonelline in *T. foenum-graecum* L. Seeds was found to be 0.58%. The inter-day and intra-day precision were less than 2%, with accuracies between 96.6-110% of the true values. The quality control parameters showed the results within specified limits and the seeds showed absence of toxic elements in it. **Conclusion:** From the above finding we can conclude that the application of QbD approach for standardization of herbal drug can serve as an important tool for development of herbal drugs with desired quality.

Key words: Quality by Design, *Trigonella foenum-graecum* L., Trigonelline, Standardization, HPLC.

INTRODUCTION

Herbal drugs have been employed in the prevention and treatment of innumerable health ailments since ancient times.[1] According to an estimate of the World Health Organization (WHO), "about 80% of the world population uses herbs and other traditional medicines". They are known for their safety, efficacy, cultural acceptability and lesser side effects. This has engendered remarkable upsurge in the demand for herbal medicines and a necessity has been arisen for safeguarding the quality, safety and efficacy of herbal drugs.[2]

Quality control of herbal drugs is of paramount importance. The quality standards for herbal drugs rests on a clear scientific definition of the raw material. Depending on the type of crude drug, sensory properties, physical constants, adulterants, microbiological contamination and foreign materials, such as heavy metals, pesticide residues and aflatoxins, have to be checked to prove identity and purity. To substantiate the constant composition of herbal preparations, adoption of appropriate analytical methods and suitable concepts is of utmost importance in order to establish relevant criteria for uniformity.[3] Recently, the concept of "marker-based standardization" of herbal drugs is gaining

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impetus. Marker- based standardization is acknowledged as the widely accepted methods which is based on the principle of analyses of phytochemical markers by means of sophisticated chromatographic techniques such as HPLC, HPTLC etc.[4]

Along with the use of modern sophisticated instruments utilization and application of novel quality approaches are essential for developments of quality standardization parameters for herbal drugs. According to the recent literature the Quality by (QbD) concept can serve as novel approach for quality standardization of herbal drug. In recent times, pharmaceutical companies adopting QbD as a fundamental pharmaceutical quality model.[5]

Application of QbD approach in analytics is one of the alternatives which reduces the experimental time and cost for drug analysis. QbD approach indicates exploring the quality of analytical process during the development stage itself.[6,7]

Trigonella foenum-graceum also is known as Fenugreek or methi belongs to the Fabaceae family, the plant is cultivated in India and North African countries. The seeds of the plant have a long history of usage as potent antidiabetic agent in Ayurvedic and folklore medicine.[8] The main chemical constituents of seeds are alkaloids approximately 36%, steroidal saponins, mucilage, fibers.[9] Among alkaloid content of fenugreek seed trigonelline is major phytoconstituents which is responsible for most of the activity of the herb.[10]

In the present research work, an endeavor has been made to accomplish some standardization parameters for the quality control of *T. foenum-graecum* seeds with special emphasis on analysis of phytoconstituents by application of QbD approach. Hence, the primary objective of this research work was to develop a QbD based HPLC method for identification and quantification of trigonelline in *T. foenum-graecum* seeds, along with evaluation of various Quality control parameters such as macroscopic and microscopic characters, physicochemical and phytochemical characterization including determination of toxic elements in the herb.

MATERIALS AND METHODS

Plant material and Chemicals

T. foenum-graecum seeds were procured and authenticated from Shri B. M. Kankanwadi Ayurveda Mahavidyalaya, Belagavi-Karnataka. The seeds were cleaned thoroughly with water, shade dried, converted to fine powder with the help of a blender and stored in air tight bottles. Standard Trigonelline Hydrochloride was provided as a free gift sample by Himalaya drug company, Bengaluru India. Other chemicals and reagents used in the research work were of analytical grade.

Macroscopic and microscopic study

The macroscopic and microscopic characters of *T. foenum-graecum* seeds were studied by following standard procedure as specified in WHO guidelines.[11] Macroscopic characters of the seeds was studied based on shape, size, colour, odour, taste, surface characteristics and texture. Powder microscopy was performed on the seeds for the determination of microscopic characters. The photographs of specimens were captured by using the Trinocular microscope (Metzer) with Capture Pro software (4.6).

Physico-chemical analysis

The powdered seeds were subjected for analysis of physicochemical parameters such as Moisture content, extractive value, total ash value, acid insoluble ash value and water soluble ash value. All the physicochemical parameters were carried out according to the standard official methods described in WHO guidelines.[11]

Phytochemical analysis

The powdered seeds were subjected for preliminary phytochemical analysis. In order to assess the existence of secondary metabolites such as carbohydrates, Alkaloids, glycosides, tannins, flavonoids, phenols and, proteins.[12,13]

Analysis of toxic substances

The crude *T. foenum graecum* seeds were analyzed for the presence of toxic substances such as Aflatoxins, pesticide residues and heavy metals. Evaluation of Aflatoxins was carried out on Agilent HPLC instrument as per the standard procedure.[14] Aflatoxins B1, B2, G1 and G2 were analyzed in the powdered sample. The pesticide residue in the sample were determined by using Gas Chromatography-Mass spectra (GC-MS) Instrument. The presence of total 17 pesticide contaminants were analyzed in crude seed powder. The presence of heavy metals were analyzed by Atomic Absorption Spectroscopy by following standard method. Presence of Heavy metals namely lead, cadmium, arsenic, mercury and chromium were tested in the crude powdered sample.

Quality by Design based HPLC Method development

Analytical conditions

HPLC system (Agilent technologies 1220 Infinity II LC) used for the analysis consisted of a system controller, low pressure gradient pump, solvent delivery system, degasser, manual sample injector (injection volume: 5- 20µL) and UV-Vis detector. Reversed phase C_{18}

column (5µm, 4.6mmx 250mm, ZORBAX) was used for chromatographic analysis. Mobile phase comprised of acidic Water adjusted with Hydrochloric acid and Methanol in different ratio. For the analysis of the samples the flow rate was kept as 1 mL/min and the wavelength was set at 263nm. For the analysis 20µL sample was injected into the HPLC column.

Defining of Analytical Target Profile (ATP) and Critical Quality Attributes (CQA)

The first step in Analytical QbD approach is to define the analytical target profile (ATP). For designing the ATP, the necessary characteristics that are considered to be the indicators of method performance were determined.[15] The CQA's were defined from ATP to identify satisfactory performance of the developed method and to give reliable results.

Design of Experiments (DoE)

The optimization of analytical method was performed by employing design of experiments (DoE) using statistical software's. For performing the optimization a 2^2 full factorial design involving 2 factors and 2 levels, resulting in 4 experimental runs was employed in order to ascertain the critical parameters and to set their levels for designing the experiment. The design of the study was developed using Design Expert software version 12.0, (Stat-Ease Inc., Minneapolis, MN, USA).

Two independent variables i.e., % concentration of acid in aqueous phase (X1) and mobile phase ratio (X2) were varied at two different levels that were coded for low and high (-1 and +1 respectively). The response variables i.e. tailing factor (R1) and peak width (R2) were selected for performing the experiments. The DoE software was used to gain information on the critical values required to achieve the desired response of the selected independent variables.

Establishment of Method Operable Design Region (MODR)

Method Operable Design Region (MODR) was generated based on the regression models and an estimation of the probability for failure. Further, the prediction of the optimized mobile phase was carried out using the overlay plotting showing MODR. Within the design space, all the qualifications described in the ATP are accomplished at a specified risk level. On the basis of the criteria of selected CQA's, the optimized run was chosen.

Validation of the optimized method

The optimized RP-HPLC method was validated as per ICH Q2 (R1) guidelines.[16] The described method was extensively validated with reference to linearity, LOD, LOQ, precision and accuracy.

RESULTS AND DISCUSSION

Macroscopic and microscopic characteristics of seeds

The macroscopic study of *T. foenum graecum* seeds showed that the seeds were yellow in color, they were oblong in shape, 0.2- 0.5 cm long and 0.15-0.35 cm broad (Figure 1). Texture is smooth. The seeds have a pleasant odour and are bitter in taste. The microscopic characters of the seeds are depicted in Figure 2 which

Figure 1: Seeds of *T. foenum graecum* L.

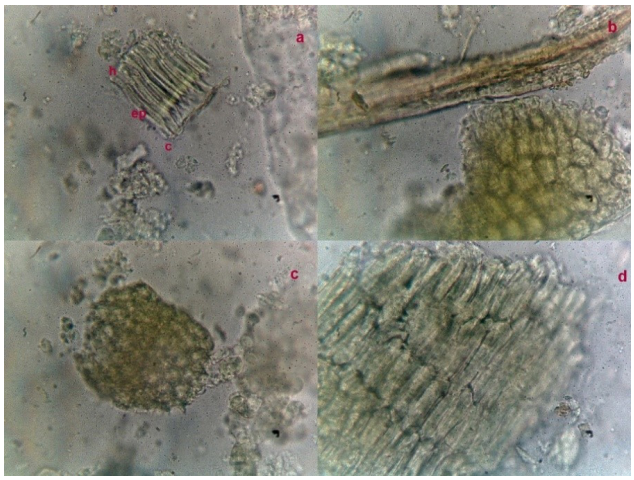

Figure 2: Microscopic characters of seed powder of *T. foenum graecum* L. showing a) Cuticle 'c', epidermis 'ep' and hypodermis 'h', of the testa, b)epidermis of the testa, c) hypodermis of the testa, d) parenchyma cells.

resembles in presence of microscopic characters as mentioned in previous literature.[17] The powdered seeds showed the presence of cuticle, epidermis, hypodermis, epidermis and parenchyma cells.

Physico-chemical analysis

The results of physico-chemical analysis of the powdered seed is shown in Table 1 along with the standard limits.[18,19] All the physicochemical parameters for crude methi seed powder showed the results within the standard limit.

Phytochemical analysis

The preliminary phytochemical analysis of *T. foenum graecum* seeds revealed the occurance of secondary metabolites such as alkaloids, flavonoids, tannins, phenols, saponins, triterpenes and steroids.

Analysis of toxic substances

The results for the analysis of toxic substances such as Aflatoxins, pesticide residues and heavy metals are depicted in Table 2.

Defining of Analytical target profile (ATP) and Critical Quality Attributes (CQA)

ATP of the proposed analytical method is to attain a good separation for quantification of Trigonelline HCl, with lesser tailing factor and peak width along with acceptable analysis time. Based on the above mentioned Analytical target profile CQA's were identified as Tailing factor (NMT 2) and Peak width (NMT 2).

Optimization of method

By performing the experiments as per the design, responses R1 and R2 were obtained for each trial and are summarized in Table 3. Further statistical optimization of analytical method was performed by comparison

Table 1: Physico-chemical parameters for *T. foenum-graecum* seeds.

Parameters	% values w/w	Standard limit[17,18]
Moisture content	7.3±0.35	NMT 9%
Aqueous soluble extractive value	33.57±1.29	NLT 30%
Alcohol soluble extractive value	21.67±0.58	NLT 5%
Petroleum ether soluble extractive value	4.40±0.53	-
Total Ash Value	3.5±0.5	NMT 4%
Acid Insoluble Ash Value	0.45±0.03	NMT 0.5%
Water Soluble Ash Value	2.83±0.29	-

NMT- Not More Than NLT-Not Less Than

Table 2: Analysis of Aflatoxins, Pesticide residues and Heavy metals of seeds of *T. foenum-graecum*.

Sr No.	Parameter	Results
1.	Determination of Aflatoxins	
	Aflatoxin B1+B2+G1+G2	BLQ (LQ: 0.2 ppb)
2.	Determination of Pesticide residue	
	DDT (Dichlora-Diphenyl-Trichloraethane)	BLQ(LOQ 0.01)
	Lindane (γ Hexachloracyclohexane)	
	α-HCH	
	β-HCH	
	δ-HCH	
	2, 4-dichlorophenoxyacetic acid	
	Endosulphon	
	Manocrotophos	
	Ethion	
	Chlorpyrifos	
	Phorate	
	Butalchlor	
	Alachlor	
	Atrazine	
	Methyl Parathion	
	Malathion	
	Aldrin	
3.	Determination of Heavy metals	
	Lead	BLQ (LOQ 1.1 mg/kg)
	Cadmium	BLQ (LOQ 0.5 mg/kg)
	Mercury	BLQ (LOQ 0.1 mg/kg)
	Arsenic	BLQ (LOQ 0.1 mg/kg)
	Chromium	BLQ (LOQ 0.5 mg/kg)

BLQ- Below Limit of Quantification LQ- Limit of Quantification

Table 3: Selected factor combinations for Trigonelline as per 2^2 full factorial design.

Code	Coded levels		Actual values		Responses	
	X_1	X_2	X_1	X_2	R_1	R_2
T1	-1	-1	0.01%	60:40	1.31	0.41
T2	-1	+1	0.01%	70:30	1.13	0.24
T3	+1	+1	0.02%	70:30	1.19	0.30
T4	+1	-1	0.02%	60:40	1.36	0.48

X_1: Conc. of HCl (%) X_2: Mobile phase ratio R_1: Tailing factor R_2: Peak width

of several statistical parameters, provided by Design-Expert® Software, Version 12. The statistical data of the applied design for Trigonelline is represented in Table 4. The independent variables and response were correlated using polynomial equations and statistical analysis through Design-Expert® Software. The coefficients X1, X2, their interaction and quadratic-terms are linked to the effect of these variables on the response.

If the coefficient is associated with a positive sign it indicates a synergistic effect whereas negative term besides the coefficient signifies an antagonistic effect upon the response. The higher coefficient indicates the potent impact of the independent variable on the response. To demonstrate graphically the influence of each independent variable on dependent variable (responses), the response surface plots were established. (Figure 3)

Establishment of the method operable design region (MODR)

The MODR for analytical method was established. MODR (Overlay plot) shown in Figure 4 had yellow color shaded region which indicates the region of successful operating ranges. From the MODR; analytical trial T2 (Conc. Of HCl 0.01% and Mobile phase ratio 70:30) and T3 (Conc. Of HCl 0.02% and Mobile phase ratio 70:30) falls under the region of successful operating ranges and fulfils the criteria of ATP and CQA for HPLC method. Among both the trials T2 having Conc. Of HCl 0.01%

Table 4: Summary of statistical parameters and polynomial equation.

Response		*P*-value	Model Significance	Polynomial equation
Trigonelline	R1	0.0272	Significant	+1.2+-0.0275*X_1-0.0875*X_2
	R	0.0268	Significant	+0.3575+0.0325*X_1-0.0875*X_2

X_1 and X_2 are independent variables where, X_1 – Conc. of Acid in aqueous phase X_2 – Mobile phase ratio

Table 5: Optimized chromatographic conditions.

Parameters	Chromatographic conditions
Stationary Phase	ZORBAX C18 (250mm x 4.6 mm, 5µ) column
Mobile phase	Water (0.01% HCl): Methanol
Mobile phase ratio	70:30
Flow rate	1ml/min
Detection wavelength	263nm
Injection volume	20µl
Retention time	2.877 min

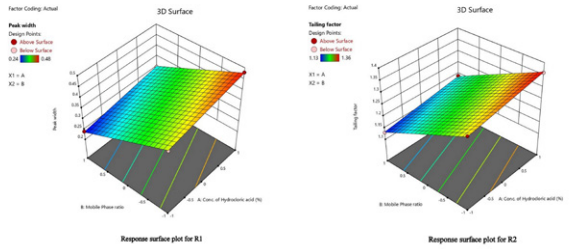

Figure 3: Response surface plot for optimization of HPLC method for Trigonelline.

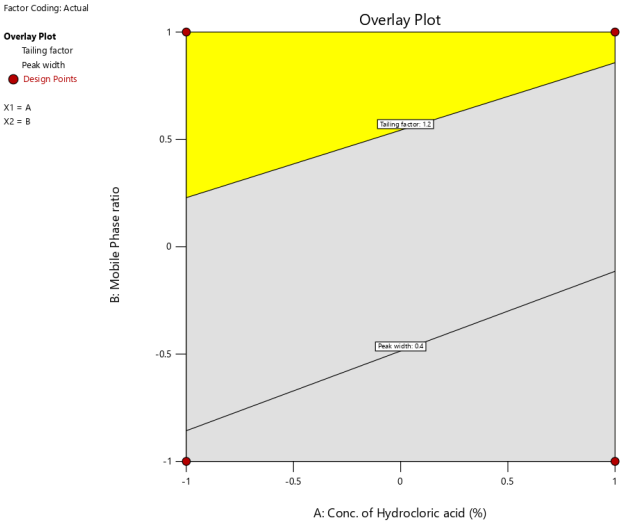

Figure 4: MODR for optimization of HPL C method for Trigonelline.

Table 6: HPLC quantification data for Trigonelline.

Marker compound	Raw material taken (mg)	Amount of marker obtained (mg)	Content of marker (%)
Trigonelline	1000	5.835	0.583

Table 7: Summary of validation parameters.

Validation Parameters		Results
Linearity range (µg/ml)		2-10
R^2		0.9981
Regression Equation		y = 307733x + 50248
LOD(µg/ml)		0.58
LOQ(µg/ml)		1.77
Precision (% RSD)	Intra-Day	1.56
	Inter-Day	1.66
Accuracy (% Recovery)		96.6-110%

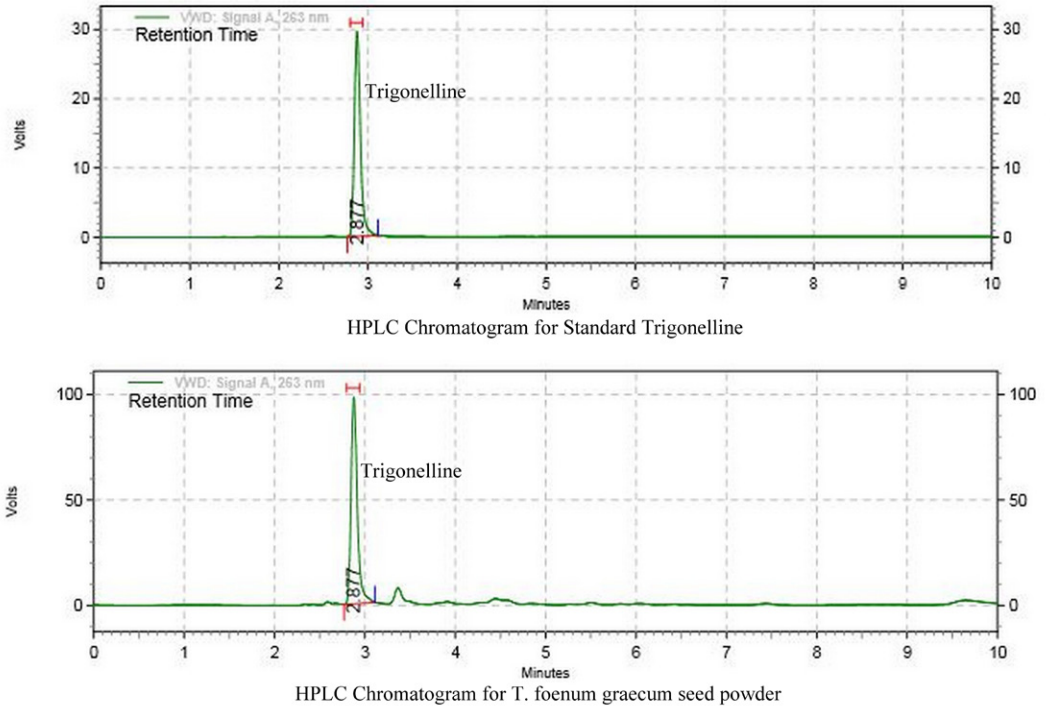

Figure 5: HPLC chromatograms of standard Trigonelline and *T. foenum graecum* L. seed.

and Mobile phase ratio 70:30 was selected as optimized HPLC method due to its ability to give lesser tailing factor and peak width Table 5.

The optimized method was further used for quantification of trigonelline in *T. foenum-graecum* seeds. The quantification data and HPLC chromatograms for trigonelline has been depicted in Table 6 and Figure 5 respectively.

Method validation

The validation of the developed RP-HPLC method was performed in order to confirm it's suitability for its intended purpose as described in ICH Q2 (R1) guidelines. The validation parameters are summarized in Table 7.

CONCLUSION

The present research work is a successful example of the implementation of QbD concept for marker-based standardization of herbal drug along with the evaluation of important quality control parameters. *T. foenum-graecum* is a valuable medicinal plant known for its usage in traditional medicine, consequently, it is imperative to standardize the herb for assessing its quality and its subsequent usage as a drug. Marker-based standardization of herbal drugs is an important technique for the assessment of the quality of herbal drugs and marked herbal products due to its ability to give an account on the phytoconstituents present in a particular plant and also to monitor batch to batch uniformity of phytoconstituents in the finished herbal products. In the present research work Trigonelline an important alkaloidal phytoconstituents has been used for standardization of *T. foenum-greacum* seeds along with the application of QbD approach. Utilization of QbD approach has assisted in developing chromatographic method which has given reproducible and reliable results along with reduction in analysis time and cost. By application of QbD approach the predefined Analytical target profile was achieved by monitoring the critical quality attributes. Further the quality control parameters for *T. foenum-greacum* seeds was accomplished as the morphological, physicochemical and phytochemical evaluation demonstrated results within the standard limits. In our study with seeds of *T. foenum-graecum*, the toxic contaminates profile was found to be satisfactory with amounts of aflatoxins, pesticide residues and heavy metals being below limit of detection.

From the above finding we can conclude that the application of QbD approach for standardization of herbal drug can serve as an important tool for development of herbal drugs with desired quality. Further the standard parameters studied in this research work will be beneficial for confirming the authenticity

of this valuable herb and also will pave a way for ensuring and maintaining the quality of crude drug.

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CONFLICT OF INTEREST

The authors declare no Conflict of Interest.

ABBREVIATIONS

QbD: Quality by Design; **HPLC:** High Performance Liquid Chromatography; **ATP:** Analytical Target Profile; **CQA:** Critical Quality Attributes; **MODR:** Method Operable Design Region; **DoE:** Design of Experiments.

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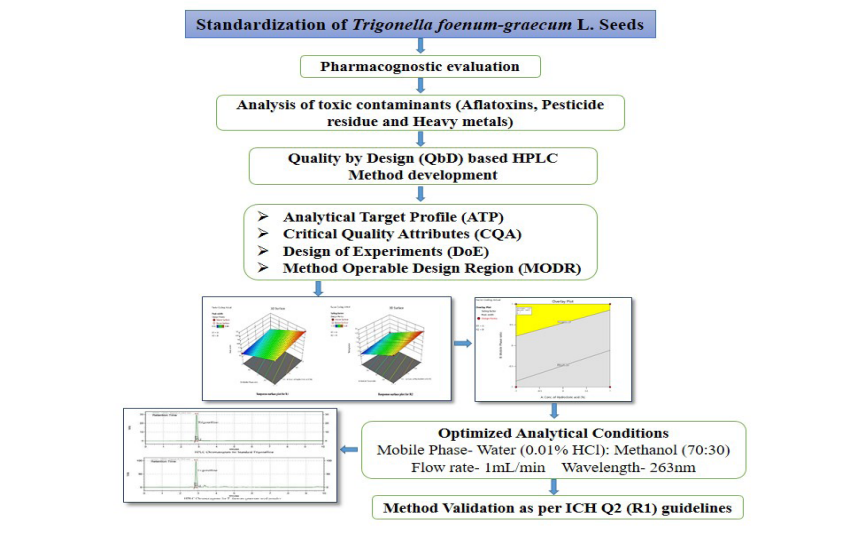
PICTORIAL ABSTRACT



SUMMARY

In the present research work standardization of *Trigonella foenum-graecum* L. Seeds was carried out by developing QbD based RP-HPLC method. Initially, the powdered seeds were subjected to Quality control evaluation and examination of toxic contaminants such as Aflatoxins, pesticide residues and heavy metals. Further, by using QbD principles ATP and CQA's were defined for the development of the RP-HPLC method. Depending on the developed Design of Experiments (DoE) analytical trials were performed and the optimized chromatographic conditions were derived from the Method Operable Design Region (MODR). Results revealed that all the Quality control parameters were within the standard limit and the toxic contaminates profile was found to be satisfactory with amounts of aflatoxins, pesticide residues and heavy metals being below the limit of detection. By application of the QbD approach, the optimized mobile phase was identified as water with 0.01% Hydrochloric acid and Methanol in the ratio of 70:30, with the flow rate of 1 mL/ min and detection wavelength 263 nm. The retention time of Trigonelline was found to be 2.877 min and the amount of Trigonelline in *T. foenum-graecum* L. Seeds was found to be 0.58%. Hence, it can be concluded that the QbD approach can serve as an important quality tool for marker-based standardization of Herbal drugs.

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