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Enhancing the yield of antitubercular compounds from fenugreek [*Trigonella foenum-graecum* (Methi)] seeds using response surface methodology

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ABSTRACT

The Plackett–Burman design was applied to determine the factors significant for the enhancement of antitubercular compound yield from fenugreek [*Trigonella foenum-graecum* (Methi)] seeds crude extract. By optimizing various conditions like solid-to-solvent ratio, time, shaking rate, temperature, seed particle size, and light yield of antitubercular compounds from fenugreek seeds studied in a batch system, a mathematical model was constructed showing the influence of each variable. The optimal level of each variable and its interaction with each other were determined by response surface methodology. A highly significant model was generated, with correlation coefficients (R^2) of 0.9109 and 0.8307 for adjusted correlation coefficients (R^2). Using this model, it was possible to achieve more than 90% of anti-TB compound yield from fenugreek seed crude extract, which could be produced when solid-to-solvent ratio, temperature, and time were set at 15 ml, 30°C, and 36 hours, respectively. Resazurin microtiter assay was used to determine the *in-vitro* antitubercular activity of the crude seed extract against *Mycobacterium tuberculosis*. The model accurately predicted the percentage yield and antitubercular effect of the crude extract.

INTRODUCTION

Tuberculous meningitis (TBM) infection affects the central nervous system and is considered the most fatal form of tuberculosis (TB), particularly among those infected with HIV and young children. The disease most frequently manifests as a pulmonary disease; however, it also affects other parts of the body, resulting in extrapulmonary TB. After inhalation, infectious droplet nuclei containing *M. tuberculosis* begin to spread hematogenously throughout the body or by seeding oxygen-rich tissues, including the brain and other parts of the central nervous system (CNS). The proportion of TBM among TB cases varies across studies, depending on the prevalence of TB in the respective settings (between 1% and 10%). Experts have estimated that 100,000 people get TBM each year. The global mortality rate from tuberculosis (TB) is higher, ranking second on a global scale after COVID-19. The advent of multidrug-resistant *M. tuberculosis* strains over the last couple of years has placed devastating effects on the socioeconomic state of our country. According to the World Health Organization's global TB report for 2020, about 10 million people contracted TB in 2019. Of these, 56% of men, 32% of women, and 12% of children (aged >15 years) contracted the disease. Almost two-thirds of these cases occurred in eight countries—India (26%), Indonesia (8.5%), China (8.4%), the Philippines (6.0%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%), and South Africa (3.6%). Even after adequate treatment,

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the survivors of TBM often suffer residual neurological sequelae, for which the estimated rate is more than 28% of cases. Mortality tends to be higher in early childhood because young children are at a higher risk of developing severe forms of the disease.

The current treatment is optimized for pulmonary tuberculosis but not for TBM (Horwitz *et al*., 2014; Nieuwenhuizen and Kaufmann 2018). As it is well known, CNS has multiple compartments [e.g., brain parenchyma and cerebrospinal fluid (CSF)] that are separated from systemic circulation by the blood–brain barrier (BBB) and blood–CSF barrier, and key antimicrobials, including rifampin (Cresswell *et al*., 2019), do not penetrate into the brain adequately. Aside from being difficult to diagnose and treat, it also causes significant morbidity and mortality even when treated with an appropriate and prolonged (at least 12 months) multidrug regimen of higher doses of antibiotics. The increasing number of cases of multidrug-resistant and extensively drug-resistant *M. tuberculosis* (MTB) strains complicates TB control and may lead to the Ineffectiveness of most of the current anti-TB drugs (Faksri *et al*., 2011; Ghajavand *et al*., 2019; Singh *et al*., 2019; Soria *et al*., 2019). In a study, antitubercular peptides were identified, and chimeric vaccine was prepared *in silico* to target multidrug-resistant MTB. The studies are being carried out to find the potent cure for tuberculosis (TB) (Batta *et al*., 2022). Thus, there arises the need for safe, cost-effective, and less toxic antitubercular compounds that can easily pass through the BBB and cope with disease resistance. There are several studies on the bioactive phytocompounds to have ethanomedicinal uses against *M. tuberculosis*. Araujo *et al*., (2021) studied the *in-vitro* antitubercular activity of plants from Rio, Brazil, from which the dichloromethane extracts of *Eremanthus crotonoides* and *Kielmeyera membranacea* showed to have lowest MIC_{50} against *M. tuberculosis* H37Rv (15.28 ± 1.21 and 4.38 ± 1.19 µg/ml, respectively) and *Mycobacterium bovis* BCG (2.17 ± 1.11 and 0.95 ± 1.08 µg/ml, respectively).

Trigonella foenum-graecum, a perennial plant belonging to the family Fabaceae, is used by people in Asia, Africa, and the Mediterranean as an ingredient in daily diets. It has been used in numerous fields such as medicine, nutrition, beverages, fragrances, and cosmetics, as well as other industrial uses (Gu *et al*., 2017; Munshi *et al*., 2020). Fenugreek is known to have several pharmacological effects including hypoglycemia, hypocholesterolemia, gastroprotective, chemopreventive, antioxidant, anti-inflammatory, antipyretic, and appetite stimulating effects (Gu *et al*., 2017; Norziah *et al*., 2015). The neuroprotective effects of fenugreek seeds were revealed in a study in which hypercholesterolemia and lipid peroxidation were controlled as bioactive antioxidant compounds which passed through the BBB (Im *et al*., 2015).

Phytochemical analysis has revealed that fenugreek contains many active ingredients, including alkaloids (such as trigonelline, gentianine, and choline), flavonoids (such as luteolin, apigenin, quercetin, and vitexin), steroids (such as cholesterol and sitosterol), saponins (such as diosgenin, gitogenin, and tigogenin) (Akbari *et al*., 2019; Assam *et al*., 2020; Norziah *et al*., 2015; Sethi *et al*., 2018; Wang *et al*., 2019), proteins, volatile oils, polysaccharides, triterpenoids, and nicotinic acid, which are proven to have therapeutic value. These phytochemicals can be extracted using different polar and nonpolar solvents. The quality and quantity of pharmaceutically active phytocompound extracts are determined by several factors, such as solvent type, temperature, pH, the number of extraction steps, liquid-to-solid ratios, as well as particle size of the solute, which affect extraction efficiency. Response surface methodology (RSM) is a useful extraction optimization method for evaluating the effects of multiple factors and their interactions on one or more response variables and is used instead of classical optimization experiments where only one factor is variable at a time, which is a tedious, time-consuming, expensive approach that fails to elaborate on interaction effects between variables (Bogdanovic *et al*., 2016; Jumeri and Kim 2011; Ong, 2011; Wang *et al*., 2019).

The main purpose of this research was to determine the factors that can enhance the yield of antitubercular compounds from crude extract of fenugreek [*T. foenum-graecum* (Methi)] seeds. Various extraction factors, such as temperature, time of exposure, solid-to-solvent ratios, etc., were studied using RSM and the Plackett–Burman design (PBD) approach.

MATERIALS AND METHODS

Plant extract preparation

Trigonella foenum-graecum (Methi) seeds were collected from local Aminabad market (26.8449°N, 80.9249°E). They were washed and shade-dried. The seed sample was then crushed to form powder. Three solvents (ethyl acetate, chloroform, and methanol) were used for the extracts' preparation. Phytochemical analysis using different methods was carried out to know about active constituents in seed extract (Table 1).

Mycobacterium tuberculosis sample collection

Clinical isolates of tuberculosis meningitis (TBM, extrapulmonary TB) and pulmonary TB were collected from the Department of Microbiology at SGPGIMS, Lucknow, UP, India. Acid-fast (AFB) microscopy was also carried out to test their growth (Table 2).

Table 1. Phytochemical Analysis of Plant Extract Samples in solvents of different polarity.

S. No.	Plant extract solvent	Phytochemicals						
		Fla	Phe	Tan	Ter	Car	Sap	Red Sug
1	Seed in chloroform	−	−	−	+	+	−	−
2	Seed in ethyl acetate	−	−	−	−	+	−	−
3	Seed in methanol	+	+	−	+	−	−	−

(+): Phytochemical Present (−): Phytochemical Absent
Fla: Flavanoids; Phe: Phenols; Tan: Tannins; Ter: Terapinoids; Car: Carbohydrates; Sap: Saponins; Res Sug: Reducing Sugar.

Screening of process variables using PBD

Fenugreek [*T.* foenum-graecum (Methi)] seeds' crude extract was analyzed using the PBD (Ong, 2011) to optimize the relative factors that affect the yield of antitubercular compounds. In this study, five determinative variables (solvent, compound concentration, temperature, shaking rate, and time) and two dummy variables (seed particle size and light) were tested in eight experimental designs. Table 3 shows the PBD of the factors studied with high and low levels. A series of duplicate experiments were carried out, and the yield of the anti-TB compound was taken as a response. Resazurin microtiter assay (REMA) was used to determine the *in vitro* antitubercular activity of the crude extract on *M. tuberculosis* (Helal *et al*., 2019; Katawera *et al*., 2014).

Antitubercular activity REMA

REMA was used to screen all the extracts of experimental design for antitubercular activity (Coban *et al*., 2014; Helal *et al*., 2019; Katawera *et al*., 2014; Tuyiringire *et al*., 2022). Briefly, in 96-well plate inoculums were dispensed in 100 µl Middlebrook 7H9 broth medium, single *Mycobacterium* samples per plate. Furthermore, 100 µl of plant extract was added in duplicate in the wells. Isoniazid and rifampicin were used as positive controls, while *Mycobacterium* culture was used as a negative control, as shown in Figure 1. The plate was then incubated for 24 hours at 37°C after loading. Following the incubation period, 30 µl of resazurin (0.02% w/v) was added to each well and reincubated for 24 hours. The optical density of each well was recorded using an enzyme-linked immunosorbent assay reader at 450 and 620 nm.

Optimization of process variables by response surface methodology

The optimization of variables for extraction of antitubercular compounds from fenugreek was carried out by using two statistical models, i.e., the PBD and the central composite design (CCD) of response surface methodology (Akbari *et al*., 2019). The CCD model was used in this study. Table 6 shows the variables for crude fenugreek extract at five coded levels (–2, –1, 0, +1, and +2). Various combinations of the independent variables within the central composite design matrix and the observed response are presented in Tables 4, 5, and 7. The experiments were repeated twice, and the average results of the duplicates were taken as the response (yield of antitubercular compound of fenugreek).

Cytotoxicity of fenugreek to Hek293T cells

For screening of compounds, cell-based assays are used to determine the cytotoxic effects of the test compound that can lead to cell death. In this colorimetric assay, reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mitra *et al*., 2016) by mitochondrial succinate dehydrogenase is measured. Hek293T cells (human embryonic kidney 293 cells containing the SV40 T-antigen) were cultured for 24 hours till they reached confluency. The cells were then seeded on a 96-well plate with a volume of 100 µg/well. Different concentrations of the extract (10, 50, 100, and 200 µg/ml) were dissolved in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and added to each well in triplicate. The plate was incubated for 24 hours. Positive control (1% SDS and 1% Trition X-100) and negative control (Hek293T cells in DMEM) were used. After incubation, 0.5 mg/ml MTT in phenol red free DMEM without FBS was added in each well and the plate was incubated for 4 hours. After incubation, the media was discarded and 100 µg/well DMSO was added. The absorbance at 570 nm was determined using an automated microplate reader (Bio-Rad). The MTT data represent the mean + SD of the experiment carried out in triplicate.

Statistical analysis

Statistical significance was assessed by the two-sample Student's *t*-test using GraphPad Prism 7 (Mitteer *et al*., 2018); *p*-values <0.05 were considered statistically significant. Furthermore, Design Expert Version 8.0 (Elsayed, 2018) and TIBCO Statistica® v13.0 (Tibco Software 2018) were used to develop the experimental design and analyze the data. One-way

Table 2. Number of *Mycobacterial* samples (*n*).

Samples	Positive and unaffected		
	Sensitive TB	MDR TB	Not affected
Sputum ($n = 100$)	69	14	17
CSF ($n = 40$)	21	7	12

Table 3. Plackett–Burman design.

Factor	Units	Code	Lower level (−1)	Higher level (+1)
Solvent	ml	X_1	5	10
Compound concentration	mg	X_2	0.5	1
Temperature	°C	X_3	25	35
Shaking rate	rpm	X_4	0	100
Time	hour	X_5	24	48
Seed particle size	µm	X_6	75–150	300–500
Light	-	X_7	Dark	Light

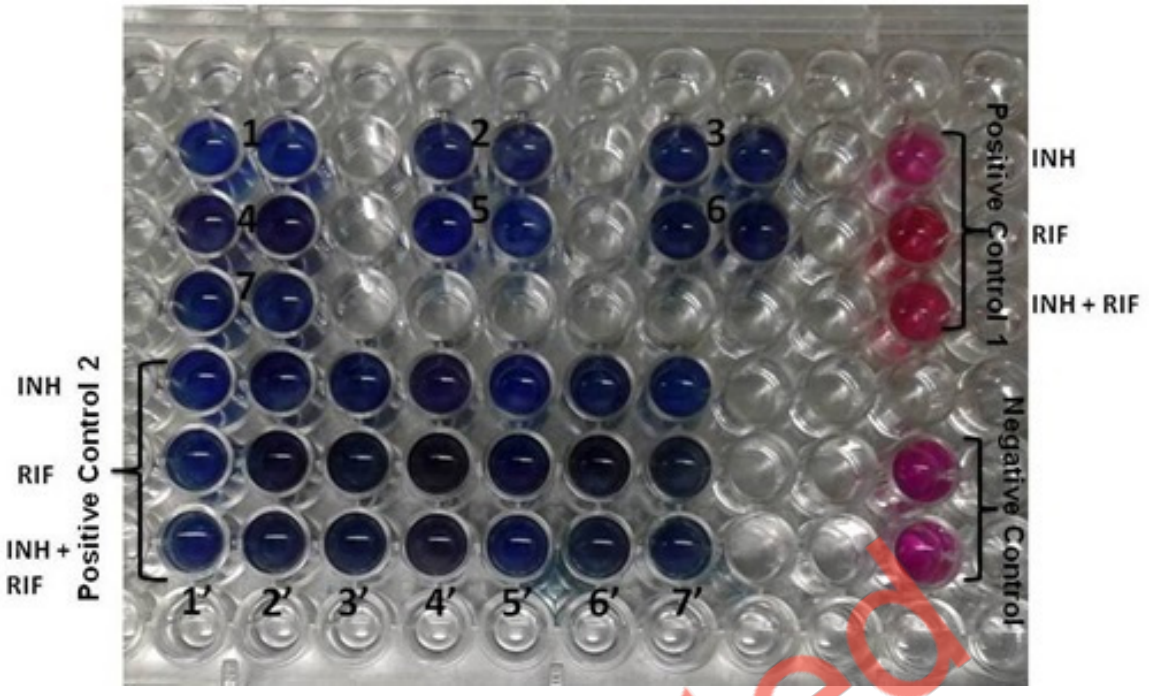

Figure 1. In 96-well plate, well 1 and 1' contains blank dye in media; from 2 to 7 were different solvent extracts of fenugreek in duplicate. Negative control contains mycobacteria in media only. Positive control 1 contains first line drugs (INH: Isonizid; RIF: Rifampicin; & Combination), in Positive control 2 from 2' to 7' contain extracts, drugs and mycobacteria. REMA assay was then performed.

Table 4. Screening design study.

Run order	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Yield (mg)	Anti-tuber activity (% reduction in bacterial growth)
1	+1	+1	+1	+1	+1	+1	+1	163.2	12.42
2	−1	+1	+1	−1	−1	+1	−1	140	10.18
3	−1	−1	+1	+1	+1	−1	+1	102.3	17.78
4	+1	−1	−1	+1	+1	+1	−1	112.1	31.68
5	−1	+1	−1	−1	+1	+1	+1	103.1	5.27
6	+1	−1	+1	−1	−1	−1	−1	202.5	52.02
7	+1	+1	−1	+1	−1	−1	+1	164.9	13.65
8	−1	−1	−1	−1	−1	−1	−1	96	1.40

Table 5. Screening design study effect and *p*-values.

Analysis	Coded factors						
	X_1	X_2	X_3	X_4	X_5	X_6	X_7
Σ (H)	160.68	142.80	152.00	129.83	120.18	139.43	143.20
Σ(L)	110.35	128.23	119.03	141.20	150.85	131.60	127.83
Difference	50.33	14.58	32.98	−11.38	−30.68	7.83	15.38
Mean square	316.58	26.55	135.92	16.17	117.62	7.65	29.55
Mean error dummy variables	18.60						
p-value	0.05	0.23	0.09	0.28	0.10	0.34	0.22
Effect	17.02	1.43	7.31	0.87	6.32		

Table 6. RSM.

				Coded levels				
Variables	**Recode**	**Code**	**Unit**	**(−2)**	**(−1)**	**(0)**	**(+1)**	**(+2)**
Solid: Solvent ratio	X_1	X_{1-2}	Ml	5	10	15	20	25
Temperature	X_2	X_3	°C	10	20	30	40	50
Time	X_3	X_5	Hours	12	24	36	48	60

analysis of variance (ANOVA) was carried out for the fitness of the model.

RESULTS AND DISCUSSION

Phytochemical analysis

As evident from various studies, fenugreek seed crude extract in different solvents contained many therapeutic phytochemical compounds that were analyzed in this study using different methods (Adhikari and Rai 2021; Aylanc *et al*., 2020; Hasan Khan *et al*., 2019). Table 1 shows that out of three extracts, those in methanol gave positive results for flavonoids, phenols, and terpinoids in phytochemical testing. These phytochemicals are known to have antitubercular activity. In a study, Shabbir *et al*., (2020) utilized *Maytenus royleanus* of the Celastraceae family methanolic leaf extract to reduce liver injury caused by anti-TB drugs. The study was designed by inducing liver injury in mice by Myrin®-p Forte. It was seen that the antioxidants of *M. royleanus* methanolic leaf extract exerted an hepatoprotective effect in liver injured mice (Shabbir *et al*., 2020). Thus, methanolic extracts were selected for further studies and *in-vitro* tested on MTB using REMA. Table 2 presents the number of clinical isolates of MTB culture acquired from pulmonary sputum (n = 100) and extrapulmonary CSF (40). The cultures were of three categories: first, drug-sensitive where the tubercular bacteria is not resistant against the anti-TB drugs; second, drug-resistant where the tubercular bacteria is resistant against the anti-TB drugs; and third were control samples, which are of other disease than TB. For pulmonary isolates, 69 were drug-sensitive, 14 were drug-resistant, and 17 were control samples. Similarly, the number of CSF isolates was 21, 7, and 12, respectively. Figure 1 shows the REMA where the calorimetric assay was performed using Resazurin dye. *Mycobacterium tuberculosis* sample used here is resistant to the first line of antituberculosis therapy (ATT) drugs, i.e., isoniazid (INH) and rifampicin (RIF) as shown as pink color in positive control 1 (drug + *M. tuberculosis*). The wells were compared to negative control (media + *M. tuberculosis*) and to blank dye in well 1 and 1′. In wells 2–7 are plant extracts and *M. tuberculosis*. In wells 2′–7′ are positive control 2 (drug + plant extract + *M. tuberculosis*).

Screening of process variables using the PBD

Using the PBD, various factors influencing the extraction yield of antitubercular therapeutic compounds from *T. foenum-graecum* seeds were identified. Extraction yield values are shown in Table 4 for the high (+1) and low (+1) levels of the eight run screen experiments. The study results in Table 5 indicate that the significant factor effect (Sf) and critical difference (CD) were computed at 17.02 and 316.58, respectively. X_1–X_5 were independent variables and X_6 and X_7 were dummy variables. According to the p-values and effect calculated by the PBD (Table 5 and Fig. 2), factors solvent, compound concentration, temperature, and time showed significant values of 0.05, 0.23, 0.09, 0.010 and 17.02, 1.43, 7.31, 6.32, respectively. In a recent study, Rizi *et al*., (2021) utilized the PDB and response surface methodology approach for the optimization of DNA biosensor which is PCR-free and can detect MTB in as low as 0.141 nm concentration (Rizi *et al*., 2021). Hence, three variables of the present study solid-to-solvent ratio, extraction temperature, and extraction time were statistically significant at a 90% confidence level and were further optimized using response surface methodology.

Optimization of the selected process variables by response surface methodology

The selected variables were recoded and further optimized using five levels of screening as shown in Table 6 where solid-to-solvent ratio, temperature, and time were studied using 20 combinational experiments. The crude fenugreek methanolic extract yield was tested *in vitro* on *M. tuberculosis* (MTB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was further carried out to test the toxicity of the extract on human embryonic kidney 293 cells (Hek293T cells) (Fig. 6). The extract was nontoxic to normal cells even to the 100 µg/ml concentration. The experimental combination factors were carried out in 20 runs as shown in Table 7. ANOVA for the response surface mathematical model is shown in Table 8. Figure 3 shows the normal probability plot of randomized observed and expected yield values. A below regression equation was developed using the RSM analysis which shows an empirical relationship between the logarithmic values of extracted metabolites in terms of yield and the coded units of the process variables.

$$Y = +169.73 + 15.87*X_1 + 0.62*X_2 - 5.63*X_3 + 0.75*X_1*X_2 + 23.25*X_1*X_3 - 12.25*X_2*X_3 - 16.14*X_1^2 - 10.39*X_2^2 - 8.64*X_3^2$$

Here is a quadratic polynomial equation where Y is the yield and X_1, X_2, and X_3 are variables with values for the model. With a model F-value of 11.36 and a probability value of 0.0004, it is evident that the model is highly significant. The value of R^2 (0.9109) indicates that the sample variation of 91% for the extraction yield is caused by the independent parameters and indicates that the model equation is significant (Singh *et al*., 2008).

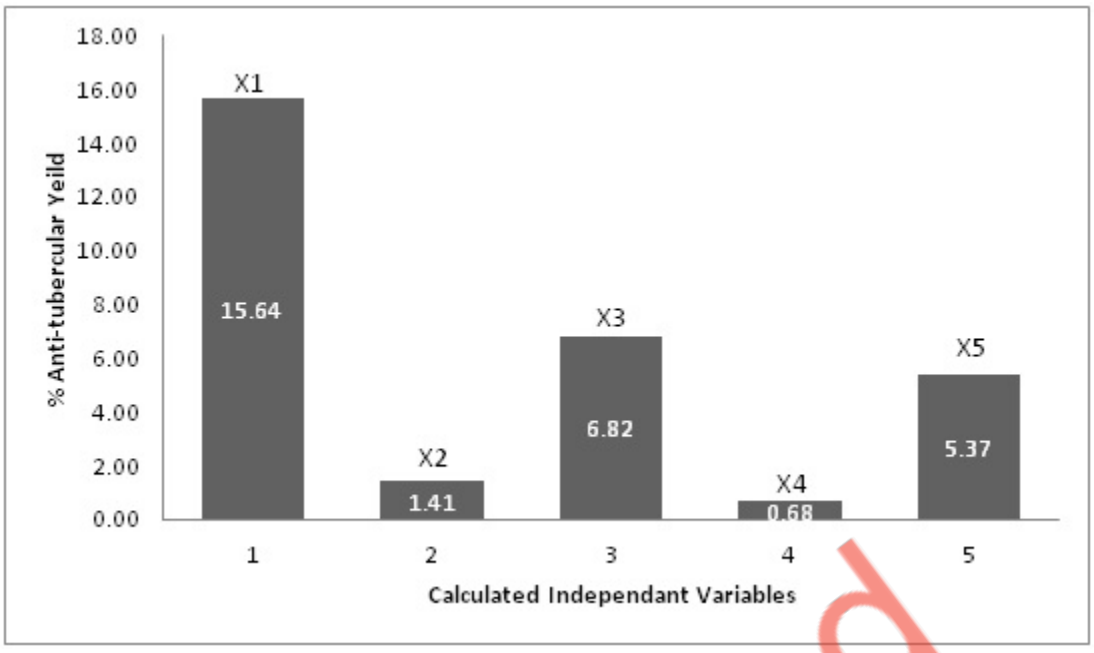

Figure 2. Plackett–Burman design calculated effects for variables. X1= Solvent, X2= Compound Concentration, X3= Temperature, X4= Shaking rate, X5= Time.

Table 7. Optimization study.

Run order	Coded values			Recoded values			Yield (mg)	Anti-tubercular activity (% reduction in bacterial growth per 100 µl from 1 mg/ml stock crude solution)
	X_1	X_2	X_3	X_1 (ml)	X_2 (°C)	X_3 (hour)		
1	−1	−1	−1	10	20	24	130	68
2	+1	−1	−1	20	20	24	124	69
3	−1	+1	−1	10	40	24	164	68
4	+1	+1	−1	20	40	24	137	69
5	−1	−1	+1	10	20	48	99	66
6	+1	−1	+1	20	20	48	162	67
7	−1	+1	+1	10	40	48	60	68
8	+1	+1	+1	20	40	48	150	65
9	−2	0	0	5	30	36	78	66
10	+2	0	0	25	30	36	145	66
11	0	−2	0	15	10	36	131	63
12	0	+2	0	15	50	36	138	68
13	0	0	−2	15	30	12	143	66
14	0	0	+2	15	30	60	140	66
15	0	0	0	15	30	36	167	66
16	0	0	0	15	30	36	155	67
17	0	0	0	15	30	36	175	67
18	0	0	0	15	30	36	185	66
19	0	0	0	15	30	36	175	65
20	0	0	0	15	30	36	174	66

Table 8. ANOVA.

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*p*-value (Prob>F)	
Model	18,311.73	9	2,034.64	11.36	0.0004	Significant
A—Solid solvent ratio	4,032.25	1	4,032.25	22.51	0.0008	
B—Temperature	6.25	1	6.25	0.035	0.8556	
C—Time	506.25	1	506.25	2.83	0.1236	
AB	4.5	1	4.5	0.025	0.8772	
AC	4,324.5	1	4,324.5	24.14	0.0006	
BC	1,200.5	1	1,200.5	6.7	0.027	
A^2	6,546.75	1	6,546.75	36.55	0.0001	
B^2	2,712.32	1	2,712.32	15.14	0.003	
C^2	1,875.32	1	1,875.32	10.47	0.0089	
Residual	1,791.07	10	179.11			
Lack of fit	1,286.23	5	257.25	2.55	0.1639	Not significant

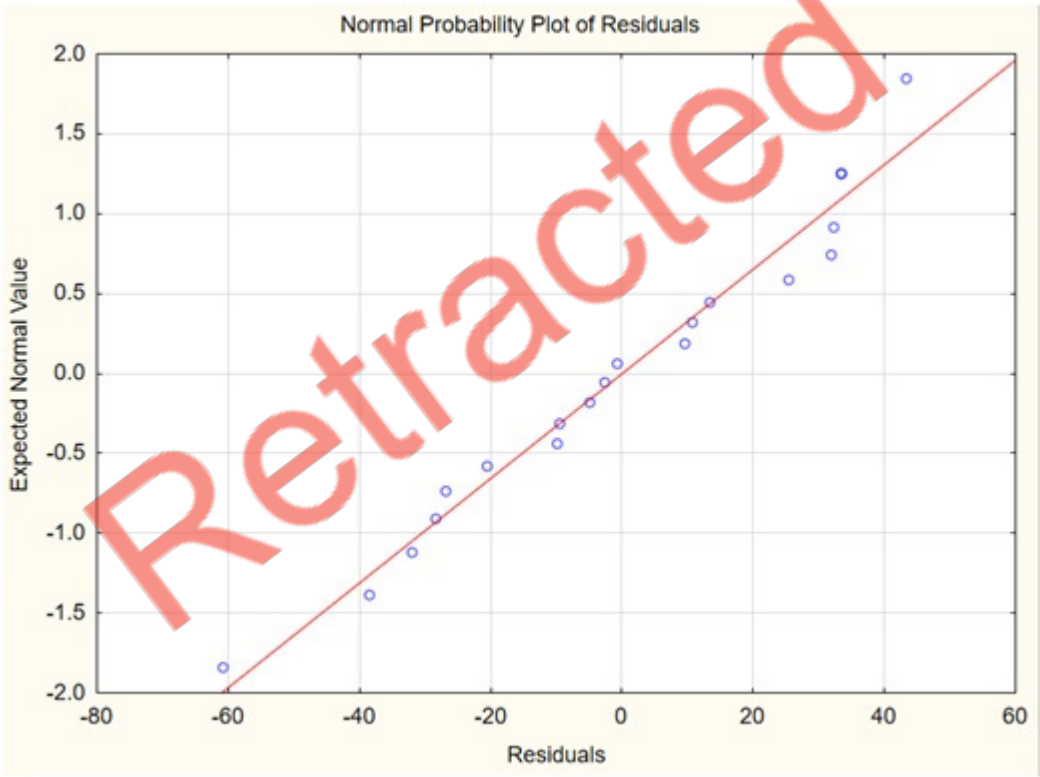

Figure 3. Normal probability plot.

There is a close correlation between the experimental values and the predicted values, as the multiple correlation coefficient R^2 (0.8307) is near 1. The signal-to-noise ratio for the analysis gives a precision of 11.15 (ratios >4 are desirable) and shows the polynomial quadratic model to be adequate. The contour and response surface plots from Figure 4 show that the variables solid-to-solvent ratio, temperature, and time coordinate with the mathematical model and the interaction between them shows synergy in enhancing the yield (extraction of antituberculosis compound). Figure 5 (in supplementary files) shows that by using this model it is possible to achieve more than 90% of anti-TB compound yield (169.727 mg) from fenugreek seed crude extract, which could be produced when solid-to-solvent ratio, temperature, and time were set at 15 ml, 30°C, and 36 hours, respectively.

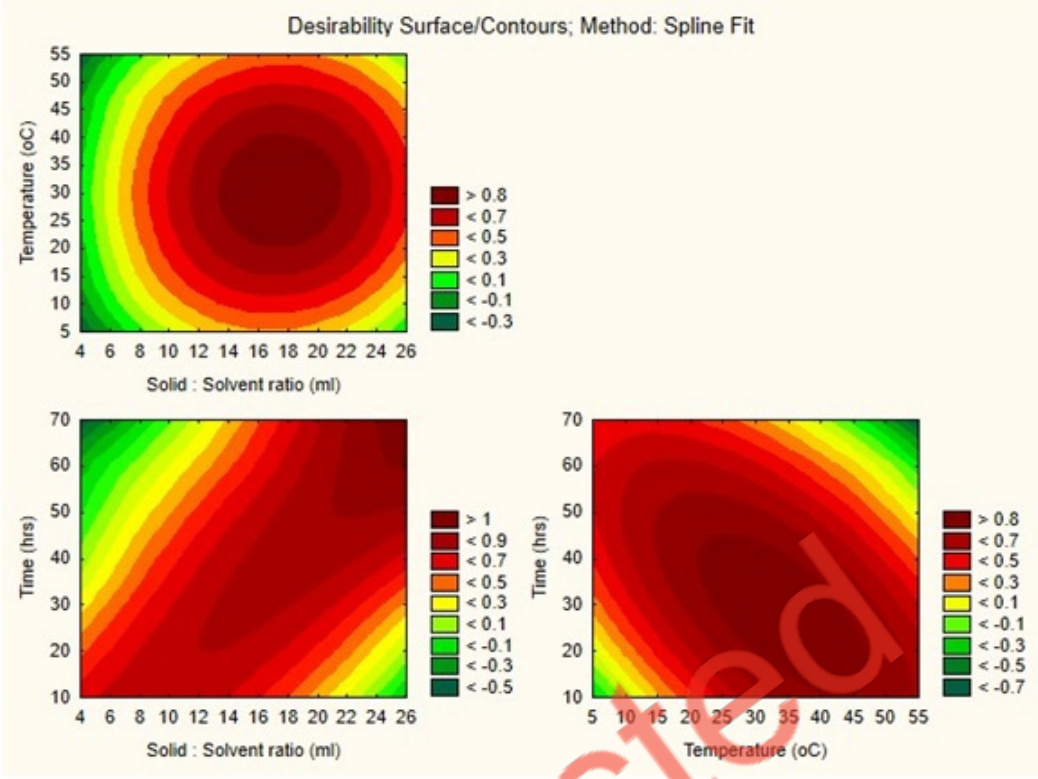

Figure 4. Contour response surface plot a: Solid-solvent ratio to Temperature; b: Solid-solvent ratio to Time; c: Temperature to Time all with respect to anti- tubercular compound yield from Fenugreek.

Figure 5. Model Desirability plots a: Solid-solvent ratio; b: Temperature; c: Time all with respect to anti- tubercular compound yield.

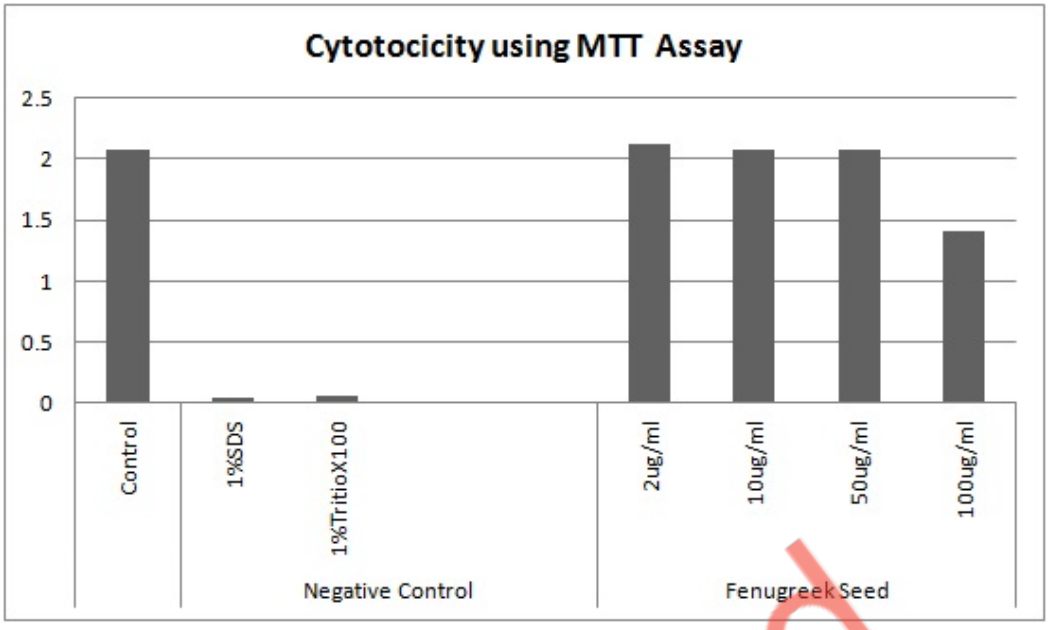

Figure 6. Cytotoxicity test of crude fenugreek extract was done on human embryonic kidney 293 cells (Hek293T cells) using MTT assay.

CONCLUSION

From this study, it is evident that the PBD can be successfully used for enhancing the yield of antituberculosis therapeutic compounds from *T. foenum-graecum* seeds. The response surface and the mathematical relationship between the screened factors were used to refine the best combination of solid-to-solvent ratio, temperature, and extraction time. Face-centered central composite design provided fairly accurate predictions for a wide area around the center point of the model. From MTT assay, it is evident that this study complements previous studies and the studied compounds are nontoxic and easy to use to combat tuberculosis infection. In the future, the prospective compounds can be isolated and studied *in vivo* in animal models.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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