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OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF TOTAL FLAVONOIDS AND ANTIOXIDANT PROPERTIES FROM *TRIGONELLA FOENUM-GRAECUM* SEEDS WITH RESPONSE SURFACE METHODOLOGY

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Response surface methodology, total flavonoids, ultrasound-assisted extraction, antioxidant, *Trigonella foenum-graecum*

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ABSTRACT: *Trigonella foenum-graecum* is an annual plant in the family Fabaceae. The distinctive cuboid-shaped, yellow to amber coloured fenugreek seeds are frequently encountered in the cuisines of the Indian subcontinent. In this study, *Trigonella foenum-graecum* is used as an experimental matrix. Ultrasound-assisted extraction (UAE) of total flavonoids from *Trigonella foenum-graecum* is studied with dual wavelength UV-VIS spectrophotometer. Effects of various factors including ratio of material to liquid, ultrasonic time, methanol concentration and extraction times on extraction yield of total flavonoids are evaluated. Then, optimization of total flavonoid compound (TFC) extraction from *Trigonella foenum-graecum* seed is investigated using response surface methodology (RSM) in this paper. Statistical analysis of the experiments indicated that Ratio of material to liquid and methanol concentration significantly affected TFC extraction ($p < 0.01$). The Box-Behnken experiment design shows that polynomial regression models are in good agreement with the experimental results, with the coefficients of multiple determination of 0.9758 for TFC yield. The optimal conditions for maximum TFC yield are 70% methanol, 50min and 30 (v/w) liquid to solid ratios with a 2 time extraction time. Extracts from these conditions showed a moderate antioxidant value from 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, Ferric chloride reducing assay and hydrogen peroxide reducing assay. Fenugreek flavonoids have direct and potent antioxidant activities, might be developed and utilized as natural antioxidant.

INTRODUCTION: Fenugreek is an herb, scientific name *Trigonella foenum-graecum*, commonly found growing in the Mediterranean region of the world. The seeds and leaves are primarily used as a culinary spice. It is also used to treat a variety of health problems in Egypt, Greece, Italy, and South Asia [4].

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Due to its oestrogens-like properties, fenugreek helps to increase libido and lessen the effect of hot flashes, mood fluctuations which are common symptoms of menopause and Premenstrual syndrome (PMS).

In India, it has also been used to treat arthritis, bronchitis, improve digestion, maintain a healthy metabolism, increase libido and male potency, cure skin problems like wounds, rashes and boils, treat sore throat, and cure acid reflux. Fenugreek also use for the treatment of reproductive disorders, to induce labour, treat hormonal disorders, breast enlargement, and menstrual pain.

Recent studies mentioned that Fenugreek helps lowering of blood glucose level [5], cholesterol levels [6] and an effective treatment for both type1 and type 2 diabetes. It is also being studied for its cardiovascular benefits.

Exploring new bioactive compound from medicinal plants has becoming a hot spot. The extraction is the most important step in isolation, identification and analysis of bioactive compounds from medicinal plants. Thus, it is essential to choose optimize extraction conditions.

Ultrasound-assisted extraction (UAE), as an efficient extraction, is used for extraction of substances from plant because of shortening the time required and improving extraction efficiency [7]. Recently, UAE has been widely used for extraction of sesquiterpene, flavonoid, phenol and polysaccharide etc. Flavonoids are commonly contained in seeds, roots, barks and fruits of medicinal herbs. Flavonoid compounds are known to be responsible for antioxidant. Some research shows that flavonoids could protect against coronary heart disease, ageing, cancer and Diabetes.

Response surface methodology (RSM) is an effective statistical technique for optimizing the complex processes. It has been successfully determined that RSM can be used to optimize the TFC from many medicine plants. In the present study, the influence of some extraction variables on TFC is conducted. A Box-Behnken experimental design is performed in order to optimize the optimal extraction conditions of TFC from Trigonella foenum-graecum.

MATERIALS AND METHODS:

Materials: Fenugreek seeds are purchased from a grocery shop at Sambazar, Kolkata and powdered to a particle size less than 0.5 mm before the experiment.

Instruments and equipment: The volumetric flask is put at the centre of the water bath in the process of the experiment; the temperature is controlled and maintained at 25℃ by circulating external water from a thermo stated water bath.

Methods:

Ultrasound-assisted extraction: 1g dried fenugreek seedling powder are accurately weighed and then soaked in methanol (AR) solution (varying methanol volume fraction from 20% to 95%; varying liquid/solid ratio from 1:7 to 1:50) for 2.0 h, and then placed in ultrasound bath and sonicated at 25°C for certain time(varying extraction time from 10 to 60 min) [4].

a) Centrifugation

Flavonoids extraction is carried out according to experimental design. The extracts are centrifuged (15 min, 3000 rpm), and then filtered through what-man filter paper grade 4 and analyzed for flavonoids content by UV-VIS spectrophotometer.

b) **Determination of total flavonoids content:** The yield of total flavonoids is determined with the aluminium nitrate methods by colorimetry procedure [8, 9]. Two methods are mainly used to quantify the content of flavonoids:

1. A total of 1 ml of each sample extraction is transferred into a 25-ml volumetric flask and mixed with 1.00 ml of the 5% (w/v) sodium nitrite for 6 min. Then, 1.00 ml of the 10% (w/v) aluminium nitrate solution is added and shacked up. Then after 6 min, 10 ml of the 4% (w/v) sodium hydroxide is added to it. The mixture is further diluted with methanol solution up to 25 ml. The absorbance of the mixture against the blank solution is measured at 510 nm. The total flavonoids content is calculated.

2. Rutin standard solution (0.2 ml, 600.0 mg/l) is added to 1 ml each sample extraction. Other operations are performed as like previously. Rutin as an internal standard is used for the determination of flavonoids. The flavonoids content of sample is adjusted by rutin.

c) **Calibration and linearity:** A total of 600.0 mg/l of the rutin standard solution was prepared by dissolving rutin in methanol solution. Rutin solution (0.2 ml, 0.4 ml, 0.6 ml, 1.0 ml, 1.5 ml) is accurately taken and put in 25-ml volumetric flasks, respectively. The following steps are similar to those sample treatment.

The calibration curve (Y=0.0112X−0.007, whereY is absorbance value, X sample concentration) ranged 4.8-36 µg/mL (R^2=0.9995).

d) Optimization of flavonoids extraction

The TFC extraction from Fenugreek seed is further optimized through the RSM approach. A fixed extraction time (2) is chosen. The coded and actual levels of the three variables in **Table 1** are selected to maximize the TFC. 15 experiments are designated [10, 11, 12].

TABLE 1: CODED AND ACTUAL LEVELS OF THREE VARIABLES

Influence factors	Level		
	1	2	3
Ratio of material to liquid (X1)	1:20	1:30	1:50
Ultrasonic time/min (X2)	40	50	60
Methanol concentration (X3)	60%	70%	80%

e) **Statistical analysis:** All the experiments were performed in triplicate and the data are expressed as the mean ± SD (standard deviation). The results are analyzed by analysis of variance (ANOVA) [10, 11, 12].

f) **DPPH Free Radical Scavenging Activity:** The anti-oxidant potential of any compound can be determined on the basis of its scavenging activity of the stable 1, 1 –diphenyl-2-picrylhydrazyl (DPPH) free radical [13, 14, 15, 16]. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis.

The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as:

(DPPH) + (H-A) ⟶ DPPH-H + (A)
(Purple) (Yellow)

Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The absorption maximum of a stable DPPH radical in methanol is at 517nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules anti radical progresses, which results in the scavenging of the radical by hydrogen donation.

Preparation of DPPH solution: Solution of DPPH (0.1 mM) in methanol is prepared by dissolving 1.9mg of DPPH in methanol and volume is made up to 100ml with methanol. The solution is kept in darkness for 30 minutes to complete the reaction. After that the solution is kept in refrigerator at 4°c for further work.

Protocol for estimation of DPPH scavenging activity: Different concentrations of test sample are taken at 3 ml each. Mixed with 5 ml of methanolic solution of DPPH (0.1mM) and allowed to stand at room temperature for 30mins. After 30mins, the absorbance is recorded at 517nm. Similarly 3 ml of different concentration of L-ascorbic acid are added to 5 ml of DPPH solution and the absorbance is measured at same nm in a spectrophotometer.

Decrease in the absorbance in the presence of test sample solution and standard at different concentration is noted. A blank reading is taken using methanol instead of test sample solution.IC_{50} (Inhibitory concentration to scavenge 50% free radicals) is also determined.

Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. IC_{50} value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals. The capability to scavenge the DPPH radical is calculated using the following equation:

$$\text{DPPH scavenged (\%)} = \frac{A_{control} - A_{sample}}{A_{sample}} \times 100$$

Where; $A_{control}$ = absorbance of DPPH along with L-ascorbic acid, A_{sample} = Absorbance of DPPH along with different concentrations of test samples.

IC_{50} is calculated from equation of line obtained by plotting a graph of concentration versus % inhibition.

g) Ferric reducing antioxidant power (FRAP) assay:

Preparation of using reagents: Phosphate buffer (pH 6.6): 1.361gm potassium di – hydrogen phosphate (0.1M) is dissolved in 100ml distilled water (solution -1) and 1.78gm di-sodium hydrogen phosphate (0.1 M) in 100ml water (solution-2). Then 60ml of solution -1 and 40ml of solution -2 are mixed together made up to 100ml buffer solution and adjust the pH 6.6 [13, 14, 15, 16].

Potassium ferricyanide 1%: Dissolve 1gm potassium ferricyanide in 100ml water.

TCA 10%: Dissolve 10gm Trichloro acetic acid in 100ml.

$FeCl_3$ 0.1%: Dissolve 10mg ferric chloride in 100ml water.

Potassium ferricyanide + Ferric chloride ⟶ Potassium ferrocyanide + Ferrous chloride

Protocol for estimation of free reducing antioxidant power activity: The reducing power of the MEGA is determined according to the method of Oyaizu (1986). 3ml of different concentrations of the ethanolic solution of the test samples (5, 10, 20.40,50 µg/ml) are mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% potassium ferricyanide $K_3Fe(CN)6$. Then the mixture is incubated at 50°c for 30mins. Aliquots of 2.5ml of (10%) TCA are added to the mixture. There is no need of centrifugation due to that solution is transparent yellow colored.

Then, 2.5ml of that mixture is mixed with 2.5ml distilled water and a freshly prepared 0.5ml of (0.1%) ferric chloride solution. The absorbance is measured at 700nm in UV-visible spectrometer. A blank is prepared without adding extract. L-ascorbic acid at various concentrations (5-50 µm/ml is used as standard. As illustrated in figures Fe^{+3} was transformed to Fe^{+2} in the presence of products derivative solution. These results indicate that increase in absorbance of the reaction mixture indicates increase in reducing power.

% increase in Reducing Power = $\left(\frac{Atest}{Ablank} - 1\right) \times 100$

A_{test} = absorbance of test solution, A_{blank} = absorbance of blank.

The antioxidant activity of the product derivatives (test compounds) are expressed as EC_{50} and compared with standard.

h) Determination of H_2O_2 radical scavenging activity:

Preparation of using reagents: Phosphate buffer (pH – 7.4): according to IP to prepare (0.2M) potassium dihydrogen phosphate; 27.218gm of that solute is dissolve in 100ml water [13, 14, 15, 16]. And (0.2M) sodium chloride; 4gm of NaCl is dissolve in 500ml of water. Now 50ml of potassium dihydrogen phosphate solution is placed in a 200ml volumetric flask and 39.1ml of (0.2M) sodium hydroxide solution is added in this and finally volume is made up to 200ml with distilled water to prepare phosphate buffer (pH-7.4).

H_2O_2 – phosphate buffer: 50ml of phosphate buffer solution is taken and equal amount of H_2O_2 is added in this to generate free radicals and solution is kept a side for 10mins.

Protocol for estimation of H_2O_2 radical scavenging activity:
The ability of product derivative to scavenge H_2O_2 is determined using H_2O_2 – phosphate buffer. 3ml of different concentration of sample derivative (5-50 µg/ml) are taken and mixed with 0.7ml of H_2O_2 – phosphate buffer. Keep aside for 30mins. Absorbance of H_2O_2 at 230nm is determined against a blank solution containing the phosphate buffer without H_2O_2 scavenging of both the sample solution and standard compounds are calculated:

H_2O_2 scavenged (%) = $\frac{(Acont - Atest)}{Acont} \times 100$

RESULTS AND DISCUSSION

Experimental design: The extraction parameters are optimized using response surface methodology (RSM).

A Box-Behnken experiment is employed in this regard. Liquid-to-solid ratio (X1), extraction time (X2) and methanol concentration (X3) are chosen for independent variables. The range and centre point values of three independent variables presented in **Table 1** are based on the results of preliminary single factor experiments. The experimental design consists of 12 factorial experiments and three replicates of the central point (Table 2).

TFC is selected as the responses for the combination of the independent variables given in Table 2. Three triplicate experiments are carried out at each experimental design point and the mean values were stated as observed responses. Experimental runs are randomized, to minimize the effects of unexpected variability in the observed responses. The variables are coded according to the following equation:

$$x = (X_i - X_0)/\Delta X$$

Where x is the coded value, X_i is the corresponding actual value, X_0 is the actual value in the centre of the domain, and ΔX is the increment of *Xi* corresponding to a variation of 1 unit of *x*. The mathematical model corresponding to the Box-Behnken design is:

$$Y = \beta o + \sum_i^n \beta i Xi + \sum_i^n \beta ii\, Xi^2 + \sum_i^n \sum_j^n \beta ij Xi Xj$$

Where Y is the dependent variable (TFC), *β*0 is the model constant, *βi*, *βii* and *βij* are the model coefficients, and *ε* is the error. They represent the linear, quadratic and interaction effects of the variables. Analysis of the experimental design data and calculation of predicted responses are carried out using Design Expert software (Version 8.0.7.1., Stat-Ease, Inc., Minneapolis, MN)

Effect of methanol concentration on total flavonoids yield: Five gram of fine powder is extracted with 50 ml of different concentration methanol (20%, 30%, 40%, 50%, 60%, 70%, 80% and 95%) in ultrasonic bath at 25°C, respectively. All the time of ultrasonic was 30 min. The result is shown in **Figure 1**. It is noticed that the total flavonoids yield reached maximum when methanol concentration is 70%, while either too high or too low degree will suppress the yield. This can be due to the fact that the total flavonoids in Fenugreek seeds are moderate polar components, and 70% methanol may contribute to the extraction of flavonoids.

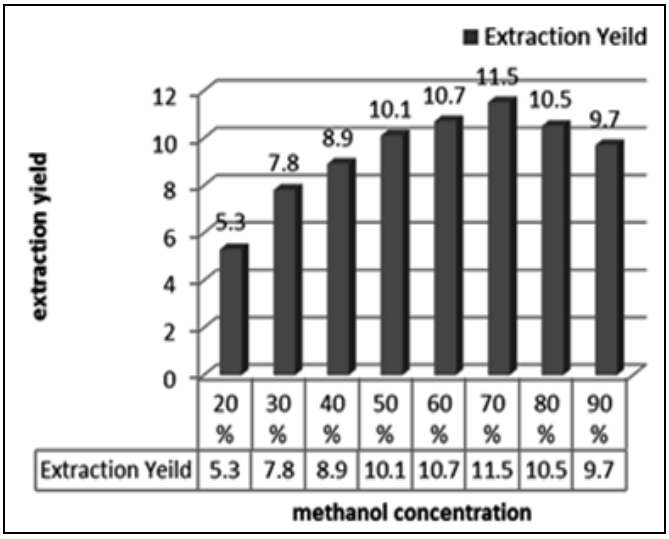


FIG. 1: EFFECT OF METHANOL CONCENTRATION ON EXTRACTION YIELD

Effect of ultrasonic time on total flavonoids yield: Different time of sonication (10, 20, 30, 40, 50, 60 min) is used for get the maximum yield of flavonoids in the experiment. Total flavonoids yield under different ultrasonic time are shown in **Figure 2**.

The extraction yield of total flavonoids increased sharply within 50 min, but after 50 min, its yield had no difference. Therefore, in the optimization process, 50 min is chosen as the extraction time.

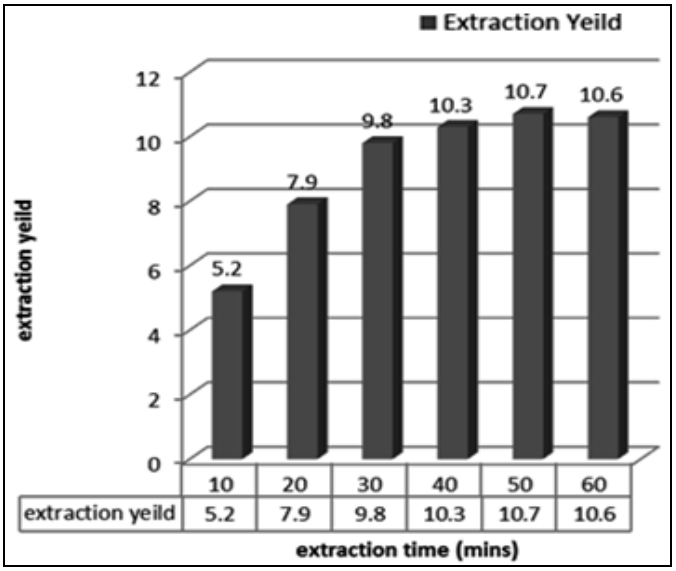


FIGURE 2: EFFECT OF EXTRACTION TIME ON EXTRACTION YIELD

Effect of the ratio of material to liquid on total flavonoids yield: Solvent volume is another important factor for efficient extraction. In this work, effect of solid/solvent ratio on the flavonoids yield is investigated. First, other extraction conditions such as methanol concentration, ultrasonic time, and number of extractions are fixed at 70%, 50 min, and only once, respectively, and the ratio of material to liquid is changed from 1:7 to 1:50.

Figure 3 showed the results. It is found that the extraction yield of total flavonoids rise as the ratio of solvent to material is increased and reached maximum (14.3 mg/g) when the ratio is 1:30. However, there are few changes when the ratio improved. This meant that more time and energy are required to condense the extraction solution in later separation process. For this reason, the solid/ liquid ratio of 1:30 is suitable to reach the high yield of flavonoids.

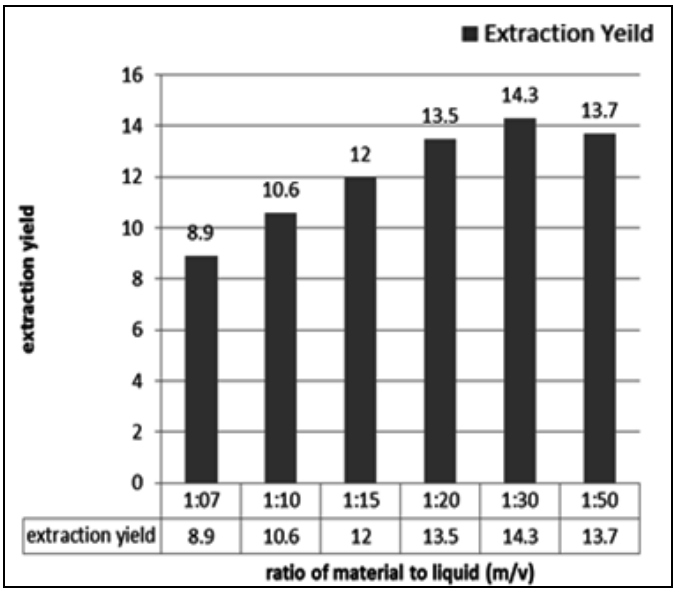


FIGURE 3: EFFECT OF MATERIAL TO SOLVENT RATIO ON EXTRACTION YIELD

Effect of extract times on total flavonoids yield: To determine the influence of number of extraction times on the yields of flavonoids, three groups of samples are extracted three times(3×50 min) under the above optimal conditions, that is, 70% methanol, 50 min of ultrasonic time, and the ratio of material to solvent at 1:30. The results are displayed in **Figure 4.** The yield of flavonoids is 14.2 mg/g at the first extraction step, decrease at the second (4.5 mg/g) and became very low (only 1.7 mg/g) at the third step.

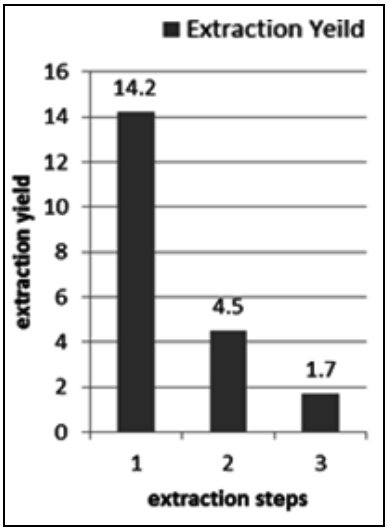


FIGURE 4: EFFECT OF EXTRACTION STEP ON EXTRACTION YIELD

Optimization of the extraction condition of flavonoids from fenugreek seeds: In 15 experiments 1–12 are factorial experiments and 13–15 are zero-point tests performed in triplicate to estimate the errors. **Table 2** shows the treatments with coded levels and the experimental results of TFC in Fenugreek seeds. The TFC yield ranged from 11.66 to 16.22 mg/g DM. The maximum yield of TFC is recorded under the experimental conditions of X1 = 1:30, X2 = 50 min and X3 = 70%. By applying multiple regression analysis on the experimental data, the response variable (TFC) and the test variables are related by the following second-order polynomial equation:

$$Y = +15.97 + 0.38 * X1 + 0.65 * X2 + 1.15 * X3 + 0.47 * X1X2 + 0.095 * X1X3 + 0.15 * X2X3 - 2.06 * X1^2 - 0.80 * X2^2 - 0.52 * X3^2$$

TABLE 2: EXPERIMENTAL DESIGNS USING BOX-BEHNKEN AND RESULTS

Run	X1	X2	X3	TFC mg/g
1	0.00	0.00	0.00	15.86
2	0.00	1.00	-1.00	14.24
3	-1.00	0.00	1.00	14.39
4	0.00	-1.00	-1.00	13.36
5	0.00	0.00	0.00	15.91
6	1.00	-1.00	0.00	12.32
7	1.00	0.00	1.00	15.29
8	-1.00	1.00	0.00	12.94
9	0.00	1.00	1.00	16.22
10	0.00	-1.00	1.00	14.75
11	1.00	0.00	-1.00	12.18
12	0.00	0.00	0.00	16.13
13	-1.00	-1.00	0.00	12.46
14	-1.00	0.00	-1.00	11.66
15	1.00	1.00	0.00	14.68

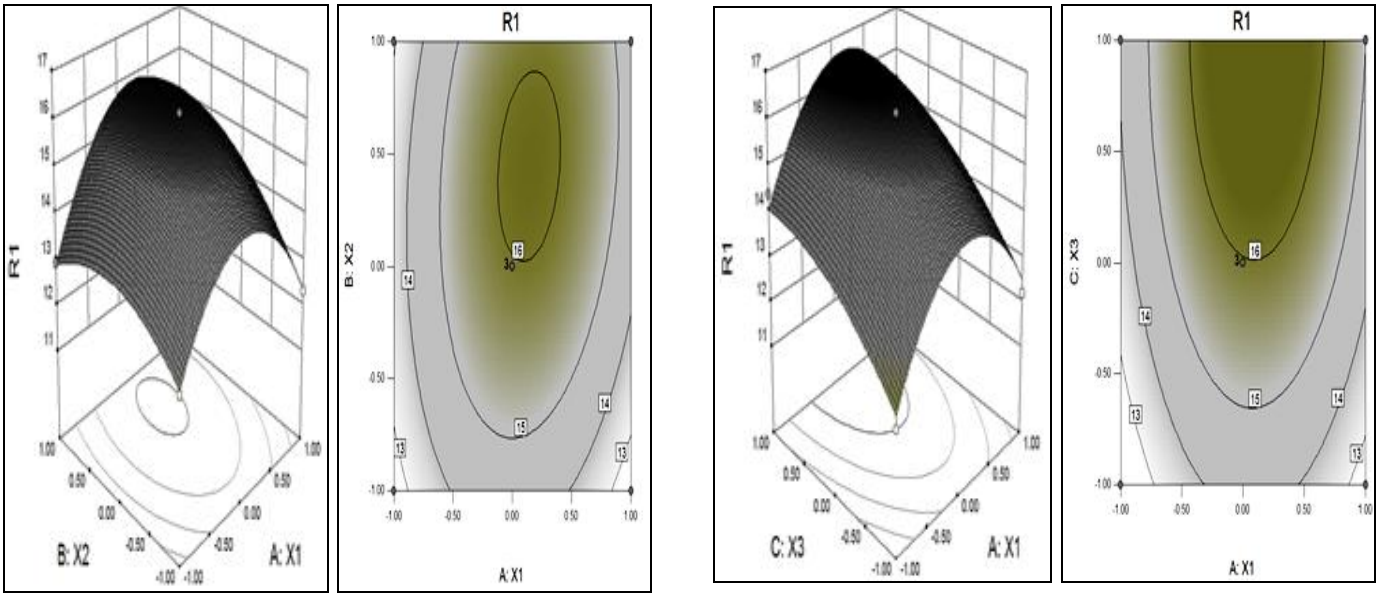
Table 3 shows the analysis of variance (ANOVA) for the regression equation. The linear term and quadratic term (except for $X3^2$) are significant ($p > 0.05$), while the interaction is not significant, indicating that the relationship between response variable (TFC) and the test variables is not simply a linear one. The lack of fit is used to verify the adequacy of the model. ANOVA for the lack of fit is not significant ($p > 0.05$) for the model, indicating that the model could adequately fit the experiment data.

The Model F-value of 22.39 implies the model is significant. There is only a 0.16% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case X1, X2, X3, $X1^2$, $X2^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The "Lack of Fit F-value" of 12.87 implies there is a 7.30% chance that a "Lack of Fit F-value" this large could occur due to noise.

Lack of fit is bad -- we want the model to fit. This relatively low probability (<10%) is troubling. The value of $R_{(Adj)}2$ (0.9322) for the equation is reasonably close to 1, indicated a high degree of correlation between the observed and predicted values, therefore the model is suitable. A very low value of coefficient of the variance (C.V.) (2.89) clearly indicated a very high degree of precision and reliability of the experimental values. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 13.893 indicates an adequate signal. This model can be used to navigate the design space.

TABLE 3: ANOVA ANALYSIS OF FOUR PARAMETERS FOR UAE: SD: SOURCES OF DEVIATION; DF: DEGREE OF FREEDOM; SS: SUM OF SQUARES; S: SIGNIFICANT; *P< 0.05, **P< 0.01

SD	DF	SS	F value	Prob >F	S
Model	33.77	9	3.75	22.39	**
X1	1.14	1	1.14	6.80	*
X2	3.37	1	3.37	20.09	**
X3	10.60	1	10.60	63.27	**
X1X2	0.88	1	0.88	5.27	
X1X3	0.036	1	0.036	0.22	
X2X3	0.087	1	0.087	0.52	
$X1^2$	15.74	1	15.74	93.91	**
$X2^2$	2.38	1	2.38	14.17	*
$X3^2$	1.01	1	1.01	6.01	
Lack of fit	0.80	3	0.27	12.87	



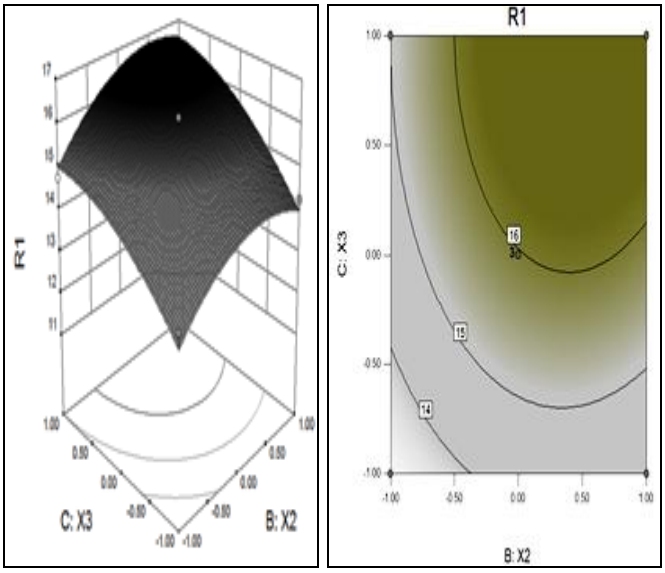


FIGURE 5: THREE – DIMENSIONAL PLOT OF TFC. (A) RESPONSE PLOT OF SOLID RATIO IN LIQUID (X1) VS. ULTRASONIC TIME (X2); (B) RESPONSE PLOT OF SOLID RATIO IN LIQUID (X1) VS. METHANOL CONCENTRATION (X3); RESPONSE PLOT OF ULTRASONIC TIME (X2) VS. METHANOL CONCENTRATION(X3)

Using RSM, the optimal conditions of TFC are an extraction of 70% methanol, 50min and 30 (v/w) liquid-to-solid ratio with 2 times extraction time. To confirm these results, three triplicate tests were performed under optimized conditions. The TFC yield value is 15.96 ± 0.409 (n = 1), which clearly showed that the model fitted the experimental data and therefore optimized the TFC extraction procedure from Fenugreek seeds.

DPPH Free Radical Scavenging Activity: DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517nm induced by antioxidants. The ethanolic solution of flavonoid is able to reduce the stable radical DPPH to the yellow colored diphenyl picrylhydrazine.

TABLE 4: ABSORBANCE AND % INHIBITION BY DPPH METOD

Concentration (µg/ml)	Absorbance	% Inhibition
5	0.186	75.36
10	0.149	80.26
20	0.0793	89.49
40	0.0472	93.74
50	0.0317	95.80

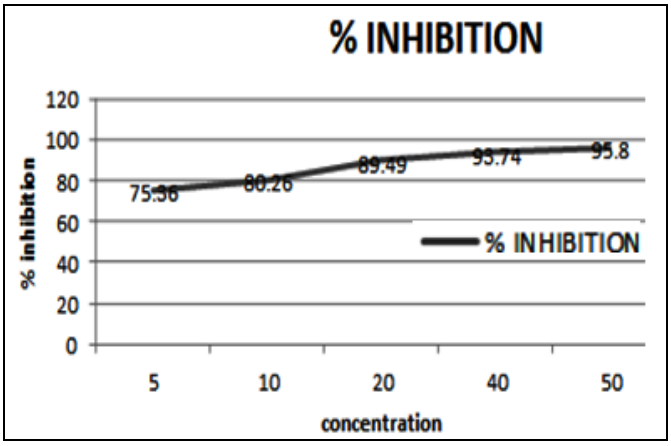


FIGURE 6: % OF INHIBITION OF FLAVONOID IN DPPH SCAVENGING ACTIVITY

From **Figure 7,** it was observed that a dose-response relationship is found in the DPPH radical scavenging activity; the activity increased as the concentration increased for each individual of ethanolic solution of flavonoid.

TABLE 5: ABSORBANCE OF STANDARD ASCORBIC ACID AND ETHANOLIC SOLUTION OF FLAVONOID IN DPPH METHOD

Concentration	Absorbance	
	Std. Ascorbic acid	Experimental solution
5	0.301	0.186
10	0.243	0.149
20	0.146	0.0793
40	0.093	0.0472
50	0.075	0.0317

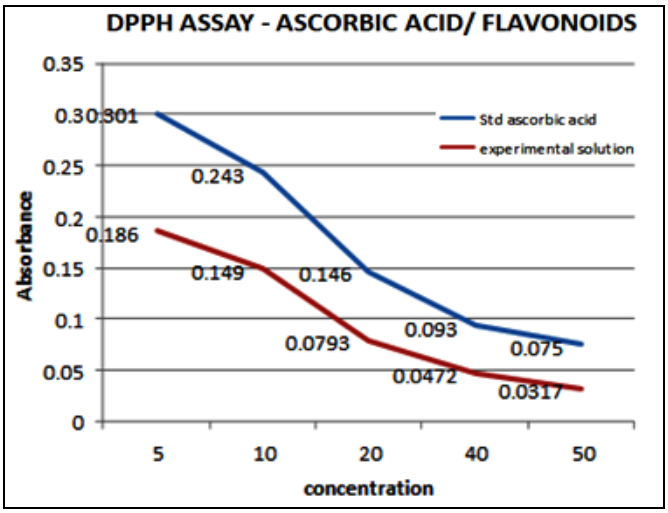


FIGURE 7: ABSORBANCE OF TEST COMPOUND AND ASCORBIC ACID IN DPPH RADICAL SCAVENGING ACTIVITY

From the present result it may be postulated that the experimental solution reduces the radicals to the corresponding hydrazine when it reacts with the hydrogen donor in the antioxidant principles.

DPPH radicals react with suitable reducing agent, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up.

Reducing power ability:

TABLE 6: SHOWS THE ABSORBANCE OF STANDARD (ASCORBIC ACID) AT VARIOUS CONCENTRATIONS IN FERRIC REDUCING POWER ASSSAY

CONCENTRATION	ABSORBANCE
5	0.131
10	0.166
20	0.176
40	0.240
50	0.255

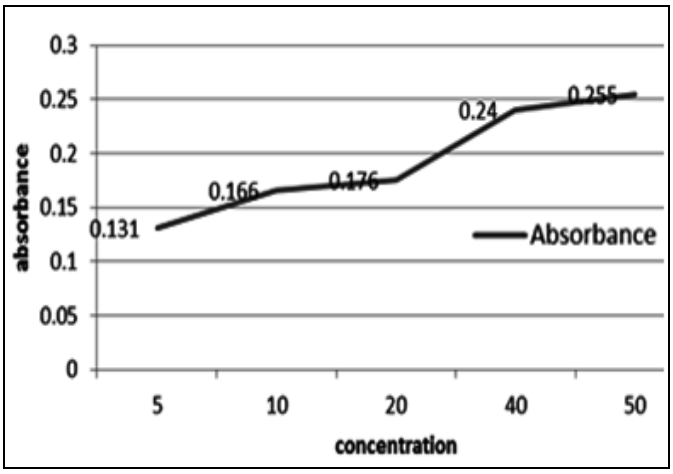


FIGURE 8: FERRIC REDUCING POWER ASSAY OF STANDARD ASCORBIC ACID

Figure 9 shows the reductive capabilities of the ethanolic solution of flavonoid when compared to the standard ascorbic acid. Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has as absorption maximum at 700 nm.

The reducing power of the ethanolic solution of flavonoid and standards increases with the increase in amount of sample and standard concentrations. The reducing power shows good linear relation in both standard as well as sample solution. The reducing capability of a compound may serve as a significant indicator of its potential antioxidant activity.

However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition- metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.

TABLE 7: SHOWS THE ABSORBANCE OF ETHANOLIC SOLUTION OF FLAVONOID AT VARIOUS CONCENTRATIONS IN FERRIC REDUCING POWER ASSAY

Concentration	Absorbance
5	0.182
10	0.194
20	0.204
40	0.256
50	0.271

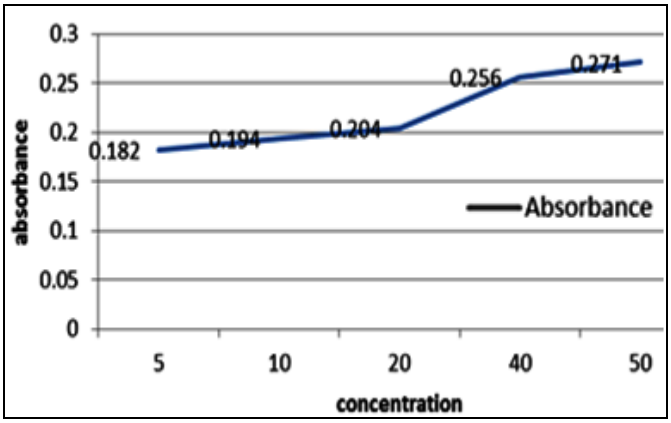


FIGURE 9: FERRIC REDUCING POWER ASSAY OF ETHANOLIC SOLUTION OF FLAVONOIDS

Scavenging of hydrogen peroxide: Hydrogen peroxide itself is not very reactive, but can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Scavenging of H_2O_2 by ethanolic solutions may be attributed which can denote electrons to H_2O_2, thus neutralizing it to water. The ethanolic solution was capable of scavenging hydrogen peroxide in a concentration dependent manner. From **Figure 11** shows that the ethanolic solution of flavonoid shows less scavenging activity than that of standard ascorbic acid.

TABLE 8: ABSORBANCE AND % INHIBITION OF ETHANOLIC SOLUTION OF FLAVONOID BY SCAVENGING OF HYDROGEN PEROXIDE

Concentration	Absorbance	% inhibition
5	0.274	35.07
10	0.276	34.59
20	0.337	20.14
40	0.379	10.18
50	0.417	1.184

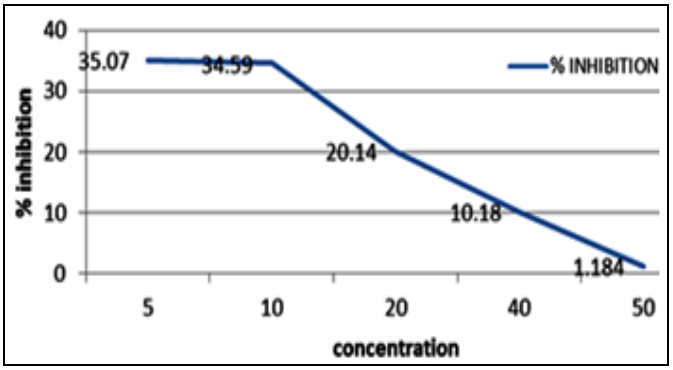


FIGURE 10: % INHIBITION OF ETHANOLIC SOLUTION OF FLAVONOID BY H_2O_2 SCAVENGING METHOD

TABLE 9: ABSORBANCE OF STANDARD ASCORBIC ACID AND ETHANOLIC SOLUTION OF FLAVONOID

Concentration	Absorbance	
	Ascorbic acid	Test compound
5	0.109	0.274
10	0.112	0.276
20	0.119	0.337
40	0.134	0.379
50	0.158	0.417

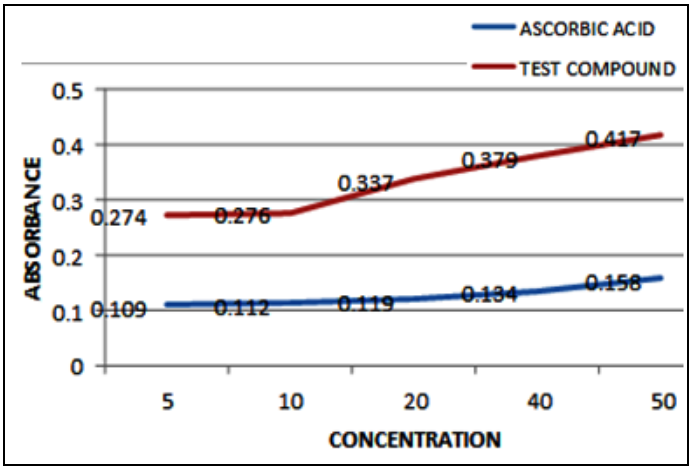


FIGURE 11: ABSORBANCE OF STANDARD ASCORBIC ACID AND ETHANOLIC SOLUTION OF FLAVONOID

CONCLUSIONS: This paper is first attempted to evaluate the effects of four experimental factors of namely ratio of material to liquid, ultrasonic time, methanol concentration and extraction times on the yields of total flavonoids from the seed of Fenugreek. The response surface methodology (RSM) is successfully employed to optimize the extraction of flavonoid compounds from Fenugreek seeds. Ratio of material to liquid and methanol concentration significantly affected TFC extraction ($p < 0.01$). The optimal conditions for maximum TFC yield were 50% methanol, 30min and 30 (v/w) liquid-to-solid ratios with a 2 time extraction time. Extracts from these conditions shows a moderate antioxidant value from 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay, Ferric chloride reducing assay and hydrogen peroxide reducing assay. Fenugreek flavonoids have direct and potent antioxidant activities, might be developed and utilized as natural antioxidant. This study has done the preliminary research of the flavonoids from Fenugreek seeds, and the results are useful for the further utilization of Fenugreek seeds.

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