Determination of phenolics and saponins in fenugreek seed extracted via microwave-assisted extraction method at the optimal condition

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Abstract. Fenugreek an ancient plant also known as *Trigonella foenum-graecum* L. This plant has achieved a great interest due to its valuable medicinal properties. The current research is targeted to extract phenolic and saponin bioactive compounds from seeds of fenugreek via microwave-assisted extraction (MAE) technique. The seed extraction was carried out at irradiation time of (2-4 min), microwave oven power (500-700 W), ethanol concentration (40-80 %), ratio of seed-to-extraction solvent (1:8-1:12 g/mL) and a constant extraction temperature (70°C). The statistical process of response surface methodology (RSM) under software of Design-Expert was used to optimize the recovery of total saponin content (TSC) and total phenolic content (TPC) in extract of fenugreek seed. One-factor-at-one-time (OFAT) was used to evaluate the levels of independent factors, and the suitable levels (-1, 0, and +1) were selected for the optimization process. Results indicated that the optimal conditions of extraction process to achieve the highest recoveries were 2.84 min, 572.50 W, 63.68% and 1:9 g/mL, respectively. Analysis of variance (ANOVA) indicated that TSC and TPC were significantly influenced by the time of irradiation, microwave oven power, solvent concentration, and ratio of seed-to-extraction solvent. FTIR results confirmed the presence of specific peaks belonging to saponins and phenolic compounds.

Keywords: Microwave-assisted extraction; fenugreek seed; phenolic; saponin; optimization

1. Introduction
Fenugreek (*Trigonella foenum-graecum* L.) a very known medicinal plant belongs to the family of leguminous has been used for a long time in most countries of the world as food and natural medicine. The great nutritional value of this herb has made it more attractive plant to researchers. The whole parts of this plant have shown anti-microbial, anti-diabetic, anticancer, anti-inflammation and antioxidant activities [1–4]. Studies revealed that chronic ailments such as cancer, diabetes, heart diseases, infections, inflammations, and many other human diseases can be averted and prevented by using the natural sources of compounds with bioactivity such as phenolics, saponins, flavonoids, alkaloids, and other natural antioxidants [5–7]. It is also reported that these compounds have shown great advantages as natural antioxidants [8,9]. The synthetically produced antioxidants namely butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) and are very known to have harmful effects on human body. The consumption of synthetic antioxidants may cause liver problems as well as carcinogenic effects to the body [10]. Saponins and phenolics as natural antioxidants are...
able to scavenge the free radicals produced in human body as a result of environmental pollution or the normal metabolism in our body [11,12].

Extractions of bioactive compounds have been carried out via different method of extraction such as maceration, ultrasound-assisted extraction (UAE) [13], Soxhlet, and microwave-assisted extraction (MAE). Among these extraction methods, MAE has demonstrated potential benefits in terms of extraction yield, solvent consumption as well as qualitative analysis [14–17]. Therefore, this study is aimed to extract total saponin compounds (TSC) and total phenolic compounds (TPC) from seeds of fenugreek via MAE technique by using response surface methodology (RSM) for optimizing the extraction processes.

2. Materials and Methods

2.1. Material preparation and MAE extraction process
Methanol, ethanol, vanillin, diosgenin, gallic acid, Folin-Ciocateu reagent, acetonitrile, sodium carbonate and sulfuric acid were obtained from Sigma-Aldrich, Malaysia. Fenugreek seed was obtained from local markets in Kuantan, Pahang, Malaysia. Before extraction, some pretreatment of the seed such as cleaning, drying at 50°C and gridding in a powdered shape were carried out. The treated seed powder was stored at fridge under 4–5°C to avoid the degradation. The MAE process begun by measuring 10g of powdered fenugreek seed and exposing it under microwave power of (500–700 W), irradiation time (2–4 min), ratio of seed to extraction solvent (1:8–1:12 g/mL), ethanol concentration (40–80 %) and a fixed extraction temperature of 70°C. Prior to MAE using microwave extractor (ethos, Frequency 2450, 1000 W, Milestone, Italy), OFAT experiment was used to select the factor levels for the optimization process via RSM under Design-Expert 7.0 (DOE) software® (Stat-Ease, USA). Then MAE was applied to extract. After MAE process, the extract of fenugreek seed was filtered via a filter paper and the solvents evaporated by using a rotary evaporator (Büchi, R-200, Germany) at 50°C. All the experiments were triplicated.

2.2. Determination of total saponin content of the extract
The TSC of the extract was measured by the methods followed by Venegas-Calérón et al. [18], Hu et al. [19] and Moyo et al. [20]. Initially, 200 µL of the extract was mixed with 800 µL of methanol and then 350 µL of 8% vanillin in ethanol was also added into a glass test tube. Next, 1250 µL of 72% sulphuric acid was added and thoroughly mixed by hand for few seconds. Afterward, the mixture was heated for 10 min in an electrical water bath and cooled again with ice crystals for 5 min. The determination of TSC in the extract was performed using UV-Vis spectrophotometer device (Hitachi, U-1800, Japan) at a wavelength of 544 nm. A standard curve of diosgenin at different concentrations (100-600 mg/mL) was prepared to measure the TSC in fenugreek seed extract based on diosgenin equivalent (DE) per gram of dry weight (mg DE/g d.w). The measurements were triplicated, and methanol was used as the blank. Finally, TSC was calculated using Eq. (1).

$$TSC = V \times \frac{C}{m}$$

Where V indicates the volume of extraction solvent (mL), C shows the concentration obtained from diosgenin standard curve (mg/mL), and the dry weight of sample (g) used for extraction indicated by m.

2.3. Total phenolic content measurement
The measurement of TPC in fenugreek seed extract was performed by the technique described by Nickel et al. [21] and Sookjitsumran et al. [22] with little changes. Concisely, 0.2 mL of the extract and 0.2 mL of Folin-Ciocalteu was added in a test tube and the mixture was stored for 5 min at room temperature in a dark place. Afterward, 0.6 mL of Na₂CO₃ solution (20%) was added into the test tube and stored for another 2 h at the same condition. The TPC was then measured at 765 nm via UV–vis Spectrophotometer against methanol blank. The standard curve of gallic acid at concentrations of
(100-500 mg/mL) was prepared to measure the TPC content of the sample. The result was explicated as mg of gallic acid equivalent (GAE) per gram of the extract (mg GAE/g d.w). Eq. (2) was used to calculate the amount of TPC in the extract.

\[
TPC = V \times \frac{C}{m}
\]

(2)

Where \( V \) indicates the volume of extraction solvent (mL), \( C \) shows the sample concentration taken from GA standard curve (mg/mL), and \( m \) represents the dry weight of sample (g) used for extraction.

2.4. Optimization and experimental design

The optimization process was performed using face centered-central composite design (FCCCD) under RSM at three different levels and four input factors including time of extraction (\( X_1 \)), microwave power (\( X_2 \)), ethanol concentration (\( X_3 \)), and ratio of feed-to-solvent (\( X_4 \)). The factor ranges for optimization process were selected by a pre-screening process via OFAT method. Design Expert (DOE) software® was applied to design the experiment. The factor levels have been described in Table 1. The responses of \( Y_{TSC} \) and \( Y_{TPC} \) were obtained via 30 experimental runs at 6 center points. The second order polynomial equation is as follow (Eq. 3):

\[
Y (response) = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j
\]

(3)

Where \( Y \) shows the response, \( \beta_0, \beta_i, \beta_{ii}, \) and \( \beta_{ij} \) indicate the regression coefficients for the intercept, linear, square, and interaction, respectively. \( X_i \) and \( X_j \) represent the independent factors and \( n \) shows the number of factors involved.

Table 1. Experimental factors with their levels used in the experiment.

| Factors                          | Factor levels |
|----------------------------------|---------------|
|                                  | -1 | 0   | +1 |
| Extraction time, \( X_1 \) (min) | 2  | 3   | 4  |
| Ethanol concentration, \( X_3 \) (%) | 40 | 60  | 80 |
| Microwave oven power, \( X_2 \) (W) | 500 | 600 | 700 |
| Feed-to-solvent-ratio, \( X_4 \) (g/mL) | 1:8 | 1:10 | 1:12 |

To find the interaction between responses and independent variables analysis of variance (ANOVA) was applied. The significant terms of the model were also obtained by ANOVA. The model adequacy was determined based on \( R^2 \), adj-R\(^2 \), predicted-R\(^2 \), F-value and lack-of-fit. The ranges of predicted values to the average prediction errors were compared using adequate precision.

To validate the model the experimental run at the optimal conditions of TSC and TPC was repeated trice. The obtained results were then validated by comparing the predicted value with the actual values obtained from the experimental work.

2.5. FTIR analysis of the extract

The Fourier transform infrared (FTIR) analysis of fenugreek seed extract at the optimum condition was conducted using Thermo Scientific Nicolet iS5 spectrometer. The IR spectra was taken in the ranges of 400 to 4000 cm\(^{-1}\) with a constant resolution of 4 cm\(^{-1}\) at room temperature. The dry extract was placed on the surface of plate equipped with diamond crystal and after closing the lid the bonding structures were measured directly.
3. Results and Discussion

3.1. One-factor-at-a-time experiment

The OFAT experiment was applied prior to optimization process to discover the most significant factors in terms of high extraction yield. As it can be seen in Figure 1 the parameters of irradiation time (2-12 min), microwave power (300-700 W), ethanol concentration (20-100 %), feed-to-solvent ratio (1:8-1:16 g/mL) were studied in different 6 levels. The results revealed that 3 min, 600 W, 60% and 1:10 g/mL were selected as the maximum extraction points to be proceeded with the optimization process. Previous studies also claimed that higher levels of extraction parameters in a MAE will produce more heat in the system and hence cause to the degradation of bioactive compounds in a MAE method [14,15].

![Figure 1](image1.jpg)

**Figure 1.** Experimental result of OFAT carried out prior to optimization process; (a) time of irradiation, (b) proposed microwave oven power, (c) ethanol concentration, (d) ratio of feed-to-solvent.

3.2. Influences of extraction parameters

3.2.1. Influence of irradiation time and microwave power

In present study, the effect of different extraction time from 2-4 min was evaluated on the recovery yield of TSC and TPC. Results showed that increasing the extraction time from 2 min to 3 min enhanced the recovery of TSC and TPC from 194.72 (mg DE/g.d.w) and 75.96 (mg GAE/g d.w) to 195.32 (mg DE/g.d.w) and 81.55 (mg GAE/g d.w), respectively Figure 2a However, when the extraction time reached to 4 min, the recovery of TSC and TPC declined to 189.02 (mg DE/g.d.w) and 74.4 (mg GAE/g d.w), respectively. This could be as a result of bioactive compounds degradation when exposed for longer time. The results found by this study is in a good agreement with those found
by Xu et al. [14] and Maeng et al. [23] as they also claimed the 3 min as an optimum irradiation time for extraction of saponins, phenolics and antioxidant capacities of *Pulsatilla turczaninovii* and *Coriolus versicolor* mushroom, respectively, in a MAE.

![Three dimensional plots of fenugreek seed extract showing the influence of irradiation time and microwave power on recovery of TSC (a) and TPC (b).](image)

**Figure 2.** Three dimensional plots of fenugreek seed extract showing the influence of irradiation time and microwave power on recovery of TSC (a) and TPC (b).

The effects of microwave power from 500-700 W were evaluated on the recovery of TSC and TPC in fenugreek seed extract. As it can be seen in Figure 2b that at 500 W the recoveries of TSC and TPC
were 180 (mg DE/g.d.w) and 79.06 (mg GAE/g d.w), respectively. Increasing the microwave power to 600 W a significant enhancement of recovery was observed at 195.32 (mg DE/g.d.w) and 81.55 (mg GAE/g d.w), respectively. However, when the power of microwave further increased to 700 W, the recoveries were declined significantly to 178.12 (mg DE/g.d.w) and 77.40 (mg GAE/g d.w), respectively. Decline in recovery of bioactive compounds beyond 600 W was also reported by Stojic et al. [24], Ling et al. [25] and Shao et al. [26] from MAE of Prunus laurocerasus leaves, Radix astragali and Perilla Frutescens leaves, respectively. They also stated that further enhancement of microwave power results to the thermal degradation of plant matrix and hence to a declined yield.

3.2.2. Influence of ethanol concentration and ratio of feed-to-solvent

Among the extraction solvents, ethanol as a less toxic solvent has been widely used to extract phytochemicals and bioactive compounds from different types of plants. Commonly, to enhance the extraction efficiency in a MAE, water is mixed at different concentrations with ethanol. The high dielectric property of water turned it to a good co-solvent for extraction of phytochemicals in MAE. The effects of 40 to 80% ethanol on recoveries of TSC and TPC are shown in Figure 3 and Figure 4. It can be seen that, the recoveries of TSC and TPC at 40% ethanol were 167.72 (mg DE/g.d.w), 74.30 (mg GAE/g d.w), respectively. Further enhancement of ethanol concentration to 60% increased the yields of recovery to 195.32 (mg DE/g.d.w), 81.55 (mg GAE/g d.w), respectively. However, when the concentration of ethanol reached to 80% in the extraction medium the yields began to decrease to 184.02 (mg DE/g.d.w), 76.33 (mg GAE/g d.w), respectively.

Figure 3. Three dimensional plots of fenugreek seed extract showing the influence of ethanol concentration and ratio of feed-to-solvent on recovery of TSC.

The polarity of water is higher than ethanol, the mixture of these two solvents increases the recovery yield of bioactive compounds, while ethanol or water alone is not able to bear such efficiency. On the other hand, more than 45% of water in ethanol also declines the yield of TSC and TPC [27]. This result was in good agreement with those found by Ling et al. [25], they studied the MAE of polyphenols and saponins from Radix astragali and reported that there is no significant difference between 60-70% ethanol concentration of bioactive compounds in a MAE.
Feed-to-solvent ratio is also one of the important factors in the extraction process. This study indicated that increasing of feed-to-solvent ratio from 1:8 g/mL to 1:10 g/mL resulted in increasing of TSC and TPC recovery from 187.38 (mg DE/g.d.w) and 77.92 (mg GAE/g.d.w) to 195.32 (mg DE/g.d.w) and 81.55 (mg GAE/g.d.w), respectively. But, further increment in ratio of feed-to-solvent to 1:12 g/mL declined the recoveries of TSC and TPC to 164.66 (mg DE/g.d.w) and 75.36 (mg GAE/g.d.w), respectively (Figure 3 and Figure 4). During MAE an increase in the ratio from 1:8 to 1:10 g/mL provides a better wettability and microwave adsorption of plant matrix. On the other hand, further increase in the ratio to 1:12 g/mL results to the absorption of more energy and hence overheating of the sample, which leads to low extraction efficiency. The finding of this study was in correlation with those found by Alara et al. [28] on extraction of some phytochemicals from Vernonia amygdalina leaf at a F:S ratio of 1:10 g/mL as an optimized condition in MAE.

**Figure 4.** Three dimensional plots of fenugreek seed extract showing the influence of ethanol concentration and ratio of feed-to-solvent on recovery of TPC.

### 3.3. Model validation

The model validations were obtained based on the optimum condition extraction via RSM at 2.84 min, 572.50 W, 63.68% and 0.09 g/mL. In these optimum conditions, the responses of TSC and TPC were 196.48 (mg DE/g.d.w), 81.01 (mg GAE/g.d.w), respectively. Based on the suggested optimum conditions, the experimental runs were carried out thrice and the obtained results were found to be 195.89 ± 1.07 (mg DE/g.d.w) and 81.85 ± 0.61 (mg GAE/g.d.w), respectively. The results suggest that there is a desirable agreement between predicted and experimental values, means that the design fits the model.

### 3.4. FTIR

FTIR is a useful tool for analyzing the structural bonding and identifying the functional groups of a compounds. FTIR spectra of dry fenugreek seed extract depicted bond stretching ranged from 3284 cm⁻¹ to 466 cm⁻¹ (Figure. 5). The peak at 3284 cm⁻¹ has been attributed to the presence of O – H group which shows the existence of phenolic compounds in the extract. The peaks at 2923.34 cm⁻¹ could be associated to –CH stretching corresponds the presence of saponin glucosides, where 2114.23
cm$^{-1}$ is an au-identified spectrum. Peaks at 1637.95-1252.44 cm$^{-1}$ correspond to C=C, C=O, CH$_2$ and CH$_3$ which can be attributed to the presence of aromatic ring, aldehyde, carboxyl, phenols, flavonoids, saponin glycosides, and amino acids [29,30]. The peaks at 1034.29-839.81 cm$^{-1}$ also represents the presence of glycosidic bonds which indicates the existence of sugar chains belonging to saponins [31].

4. Conclusions
In the present study, the contents of total saponin and phenolic in the extract of fenugreek seed exposed under MAE at different parameters were determined. The optimal conditions of parameters obtained at 2.84 min irradiation time, microwave oven power 572.50 W, ethanol concentration 63.68% and 0.09 g/mL feed-to-solvent ratio. Based on the suggested optimum conditions the obtained responses of TSC and TPC were 195.89 ± 1.07 (mg DE/g d.w), 81.85 ± 0.61 (mg GAE/g d.w), respectively, (n=3). The results indicated no significance different between predicted and actual values. demonstrated no significant observation between actual and predicted values obtained from the experiment. FTIR results confirmed the presence of specific peaks belonging to saponins and phenolic compounds. This study recommends that the obtained model in this study can be applied for extraction of bioactive compounds from different types of plants.

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