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EXTRACTION OF BIOACTIVE COMPOUNDS FROM FENUGREEK LEAVES BY MACERATION WITH D-OPTIMAL DESIGN

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ABSTRACT
In this study, optimum extraction conditions of bioactive compounds from fenugreek leaves (*Trigonella-foenum graecum* L.) were investigated using response surface methodology and the extracts having the highest total phenolic content, total flavonoid compounds, antioxidant activity and total saponin content were achieved. The independent process variables were solvent mixture ratio (water and ethanol, 0-100%), temperature (25-65°C) and sample–solvent ratio (10-50 g/L), and a constant extraction time of 120 minutes was used for all the design points. The experimental study was arranged according to D-optimal combined design and the process conditions were optimized using desirability function approach. Results showed that the extraction of phenolic compounds and antioxidant activity were increased at increasing water ratios and temperature and decreasing sample-solvent ratio. Saponins were extracted better when 25% ethanol was used as solvent. The optimum extraction conditions were determined as 100% of water, 49.71°C of temperature, and 10 g/L of sample–solvent ratio.
Key words: Fenugreek leaves, phenolic compounds, extraction, optimization, saponin

D-OPTİMAL TASARIM İLE ÇEMEN OTU YAPRAKLARINDAN BİYOAKTİF BİLEŞENLERİN MASERASYON YOLUYLA EKSTRAKSİYONU

ÖZ
Bu çalışmada çemen otu (*Trigonella-foenum graecum* L.) yapraklarından biyoaktif bileşenlerin optimum ekstraksiyon koşulları yanıt yüzey yöntemi kullanılarak belirlenmiş ve en yüksek toplam fenolik madde, toplam flavonoid, antioksidan aktivite ve toplam saponin içeriğine sahip ekstraktlar elde edilmiştir. Çözgen karışım oranı (su ve etanol, %0-100), sıcaklık (25-65°C) ve örnek–çözgen oranı (10-50 g/L) bağımsız işlem değişkenleri olarak seçilmiş ve tasarımın tüm noktalarında 120 dakikalık ekstraksiyon süresi sabit olarak uygulanmıştır. Deneysel çalışma D-optimal birleşik tasarıma göre düzenlenmiş ve istenirlik fonksiyonu yaklaşımı kullanılarak işlem koşulları optimize edilmiştir. Sonuçlara göre çözgende bulunan suyun yüzdesi ve ekstraksiyon sıcaklığı arttıkça, örnek–çözgen oranı azaldıkça fenolik bileşenlerin miktarı artış göstermiştir. Saponinler ise çözgen olarak %25 etanol kullanıldığında daha iyi ekstrakte edilmişlerdir. Optimum ekstraksiyon koşulları %100 saf su, 49.71°C ekstraksiyon sıcaklığı ve 10 g/L örnek–çözgen oranı olarak belirlenmiştir.
Anahtar kelimeler: Çemen otu yaprağı, fenolik bileşenler, ekstraksiyon, optimizasyon, saponin

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INTRODUCTION

Fenugreek (*Trigonella-foenum graecum* L.) is a medicinal plant and originates from Western Asia and Southeastern Europe (Khoja et al., 2021). Fenugreek seeds are used as spices and the leaves of the plant consumed as a green vegetable in the diet. Both fresh and dried fenugreek leaves are edible, and fenugreek leaves are reported as a good source of various minerals and vitamins, especially they are rich in choline (Srinivasan, 2006). In literature, the beneficial effects of fenugreek on hyperglycemia, hyperinsulinemia, and glycosylated hemoglobin were presented and it was also reported that the consumption of fenugreek could help controlling body weight, liver glycogen and have a significant effect on carbohydrate metabolic enzymes (Devi et al., 2003; Srinivasan, 2006). The fenugreek can also show antidiabetic effects. Moreover, fenugreek can lower the serum cholesterol, can raise HDL-cholesterol, can have lipid lowering effects in mellitus diet, and can improve the amount of the fecal excretion (Chaturvedi and Pant, 1987; Chaturvedi and Pant, 1988; Annida et al., 2004). Because of its health benefits, fenugreek leaves are freshly consumed; however, most of the fenugreeks have been discarded, the seeds of the plant are mostly used for spice production or medicinal purposes. Fenugreek leaves have a great potential for obtaining biomaterials and simple extraction methods can be used for this purpose.

Extraction is a unit operation used to separate or produce bioactive materials from the plants or foods (Chouhan et al., 2019). Extraction conditions extensively affect the extraction yield or activity of the bioactive compounds, and the important parameters for an extraction process are generally defined as the solvent type, sample–solvent ratio and the temperature (Goli et al., 2005; Belguith-Hadriche et al., 2013; Zuorro et al., 2016). In literature, there are several studies reporting that the extraction yield of phenolic materials can be enhanced by increasing the temperature (Yang et al., 2009; Mokrani et al., 2016). Temperature increment may lead to get a softer plant texture, so that phenol-protein and phenol-polysaccharide interactions get weakened. This phenomenon can ensure obtaining higher amounts of phenolics in the extract (Mokrani et al., 2016). On the other hand, the extraction yields of phenolic compounds may get lowered at increasing temperatures, therefore it is important to determine the optimum extraction temperature for an individual plant or the food (Silva et al., 2007; Ballard et al., 2009; Yim et al., 2012). In literature, maceration has been used extensively for the extraction of biomaterials from different parts of the plants. It is a cheap and a consistent method to produce phenolic-rich extracts. For this reason, it is vital to determine the optimum extraction conditions to produce fenugreek leaf extracts having the maximum amount of phenolics and saponins. To the best of our knowledge, there is no study in the literature presenting the optimum extraction conditions of biomaterials from the fenugreek leaves.

In this study, the extraction conditions namely solvent mixture ratio, sample–solvent ratio and temperature were evaluated to obtain phenolic compounds from the fenugreek leaves and were optimized to achieve the highest amount of the bioactive materials.

MATERIALS AND METHODS

Plant Material

Fenugreek leaves were provided from a local farmer at the harvest time in August 2021, and the plants were provided from Kayseri Province. The area where the plants were harvested located in the central Anatolia region with the geographical coordinates of 38° 44' north and 35° 29' east, and the elevation above sea level was 1054 m. Fenugreeks were harvested when the plants reached their peak of vegetative growth (2 months after planting, before flowering). Prior to extraction processes, the fresh leaves were pulled out from the plant stems and then the leaves were dried in the shade for 72 hours until their moisture contents were lower than 10% (8.42±0.22%, wet basis). After that, the dry leaves were crumbled by hand and the samples were sieved through a sieve having 630 μm pore diameter to remove the dust and soil residuals. The dried and cleaned samples were stored in dark at room temperature (25°C) for further analysis.

Chemicals

Diosgenin was purchased from Cayman Chemical (Michigan, USA). Quercetin was purchased from BLD Pharmatech Ltd. (Shanghai, China). Sodium nitrite ($NaNO_2$) and analytical grade ethanol were purchased from Tekkim Chemicals (Istanbul, Turkey). Folin-Ciocalteau reagent, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), potassium persulfate ($K_2S_2O_8$), sodium acetate (CH_3COONa) and sulfuric acid (H_2SO_4) were purchased from Merck Chemicals (Darmstadt, Germany). Gallic acid, sodium hydroxide (NaOH), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Trolox and vanillin were purchased from Sigma-Aldrich Co. (Steinheim, Germany). The chemicals used in this study were of analytical grade.

Extraction process and experimental design

Maceration was applied using water and ethanol as solvents to obtain biochemicals from the fenugreek leaves. The dried fenugreek leaves and water-ethanol mixture were agitated at 400 rpm for 120 minutes using a magnetic stirrer having a heating unit. Beakers with a volume of 100 ml were used as the extraction containers. The temperature of the magnetic stirrer was adjusted to the temperature value specified in the experimental design for each run. The lids of the beakers were kept close during the extraction process. After that, the samples were centrifuged at 4637 g (Hettich 320 R, Germany) for five minutes and then were filtered using a coarse filter paper. Filtered samples were used for the chemical analyses. The percentage of water in the solvent mixture (0-100%), percentage of ethanol in the solvent mixture (0-100%), temperature (25-65°C) and sample–solvent ratio (10-50 g/L) were chosen as independent variables. The experimental D-optimal combined design is shown in Table 1. Response surface methodology was used to optimize the extraction conditions of biomaterials from the fenugreek leaves ensuring the highest total phenolic compounds (TPC), total flavonoid compounds (TFL) and antioxidant activity (AA) (both DPPH and ABTS methods) according to the desirability function approach.

Table 1. The experimental design and analysis results

Run	Water (%) C_1	Ethanol (%) C_2	Temperature (°C) X_1	Sample–solvent (g/L) X_2	TPC	TFL	AA (DPPH)	AA (ABTS)	TSC
1	50	50	45	30	19.20±0.16	9.68±0.13	0.87±0.00	7.86±0.07	20.78±0.17
2	0	100	35	20	5.80±0.10	2.98±0.09	0.23±0.02	2.07±0.04	7.72±0.40
3	75	25	25	50	23.02±0.16	11.64±0.06	1.10±0.03	9.27±0.32	20.84±0.21
4	100	0	65	10	25.65±0.45	13.06±0.03	1.20±0.01	10.97±0.22	20.72±0.64
5	100	0	35	20	25.47±0.24	12.63±0.13	1.25±0.03	10.50±0.42	17.65±0.32
6	50	50	45	30	18.84±0.24	9.52±0.11	0.88±0.03	7.30±0.33	21.08±0.09
7	0	100	65	50	8.06±0.80	4.01±0.06	0.35±0.01	3.30±0.09	13.51±0.13
8	75	25	25	10	23.53±0.38	11.81±0.03	1.10±0.01	9.65±0.13	21.36±0.26
9	75	25	25	10	22.99±0.13	11.48±0.03	1.08±0.02	8.96±0.13	24.09±0.26
10	100	0	65	50	22.59±0.32	10.94±0.08	1.05±0.01	9.36±0.09	17.26±0.13
11	0	100	65	10	10.17±0.80	4.99±0.02	0.48±0.00	3.88±0.27	11.62±0.64
12	50	50	45	30	19.53±0.20	9.46±0.03	0.90±0.01	7.35±0.04	21.02±0.60
13	0	100	25	50	2.58±0.76	1.51±0.01	0.14±0.00	0.82±0.21	7.19±0.05
14	25	75	65	10	14.45±1.12	6.90±0.20	0.75±0.01	5.99±0.49	16.90±0.64
15	66.67	33.33	55	20	21.71±0.18	10.43±0.12	1.04±0.04	8.98±0.02	21.29±0.32
16	25	75	25	50	12.38±0.22	6.25±0.06	0.64±0.00	5.06±0.04	15.78±0.15
17	75	25	55	40	23.52±0.28	11.48±0.09	1.08±0.00	9.90±0.14	21.18±0.39
18	0	100	65	50	7.79±0.15	4.10±0.13	0.41±0.01	2.98±0.12	12.29±0.05
19	25	75	65	10	14.72±0.29	7.18±0.15	0.69±0.01	5.87±0.22	18.27±0.52
20	100	0	65	50	22.88±0.41	11.33±0.12	1.07±0.02	9.14±0.11	17.15±0.13
21	75	25	65	10	22.52±0.92	11.13±0.05	1.03±0.01	8.51±0.58	22.55±0.64
22	100	0	25	50	24.98±1.02	12.62±0.12	1.19±0.01	10.06±0.30	13.38±0.05
23	25	75	25	10	12.49±0.06	6.23±0.03	0.67±0.02	5.39±0.62	15.17±0.52
24	25	75	25	10	12.72±0.89	6.54±0.04	0.69±0.00	5.96±0.36	14.44±0.26

TPC: Total Phenolic Content (mg gallic acid/g dry sample), TFL: Total Flavonoid Compounds (mg quercetin/g dry sample), AA: Antioxidant Activity (mg Trolox/g dry sample), TSC: Total Saponin Content (mg diosgenin/g dry sample), ± values represent the standard deviations between two values of the related analysis results.

Analysis

Total phenolic compounds

Folin-Ciocalteau method described by Singleton and Rossi (1965) was used for the determination of TPC. 250 μL of sample was mixed with 250 μL of Folin-Ciocalteau reagent diluted with ultrapure (Milli-Q) water (1:1, v/v). 500 μL of Na_2CO_3 (210 g/L) solution and 4 mL of ultrapure water were mixed with this mixture. The samples were centrifuged at 1456 g (Hettich Eba 21, Germany) for 10 minutes after incubation at 25°C for 25 minutes. The absorbance of the supernatants was measured at 760 nm (T80+, PG Instruments, United Kingdom) and gallic acid was used as a standard. The TPC were presented as mg gallic acid/g dry sample.

Total flavonoid compounds

Spectrophotometric aluminium chloride method was used to determine TFL of the fenugreek leaf extracts (Belguith-Hadriche et al., 2013). The diluted samples (1:10, 1 mL) were mixed with 4 mL of ultrapure water and 0.3 mL of 5% $NaNO_2$ in a 15 mL test tube. This mixture was incubated for 5 minutes at 25°C. Following that, 0.3 mL of 10% $AlCl_3$ was added to the test tube and incubated for 6 minutes. After incubation, 2 mL of 1 M NaOH solution was added, and the volume of the solution reached 10 mL with ultrapure water. The absorbance of the samples was read at 510 nm, and total flavonoid compounds (TFL) were expressed as mg quercetin/g dry sample.

Antioxidant activity

DPPH and ABTS methods were used for the determination of the antioxidant activity (AA) of the samples. Slightly modified version of DPPH method was performed following to the method of Pająk et al. (2019). We mixed 50 μL of sample with 1.95 ml of 0.1 mM DPPH and this mixture was incubated for 30 minutes in dark at 25°C. We determined the absorbance of the samples at 515 nm wavelength. The AA of the fenugreek leaf extracts was expressed as mg Trolox/g dry sample.

Slightly modified version of ABTS-reducing AA analysis was performed according to the procedure of Pająk et al. (2019). ABTS-$K_2S_2O_8$ solution was prepared using 7 mM ABTS stock solution and 2.45 mM $K_2S_2O_8$ solution. These solutions were mixed on an equal basis and incubated for at least 16 hours at room temperature and in the dark. Following to incubation, 1 mL of this solution was diluted with 54 mL of buffer solution (20 mM sodium acetate, pH 4.5), and a 0.700 absorbance value was set at 734 nm wavelength. We mixed 150 μL of extract and 2850 μL of adjusted solution of ABTS and incubated it for 30 minutes at room temperature and in the dark. The AA (ABTS) of samples was presented as mg Trolox/g dry sample.

Total saponin content

The method of Akbari et al. (2019) with slight modifications was used for the determination of TSC of the extracts. 0.2 mL of the extract was mixed with 0.35 mL of 0.8% vanillin prepared in ethanol and 0.8 mL of distilled water. After that, 1.25 ml of H_2SO_4 (72%, v/v) was added to the tube and the cap of the tube was closed, and this mixture was gently mixed. Then the cap was loosened, and the samples were placed into a water bath adjusted to 60°C and were incubated for 10 minutes. When incubation time ended, all the samples were taken rapidly into an ice bath. The absorbance values of the samples were measured at 544 nm, and TSC values of the samples were expressed as mg diosgenin/g dry sample.

Statistical analysis

One-sample t-test for the optimum point validation was carried out using the SPSS 22.0 (IBM, USA) package program. The regression analysis which was used to determine the effects of the independent process variables on the responses, and the Design Expert 7.0 (Stat-Ease Inc., USA) package program was used for the optimization study.

RESULTS AND DISCUSSION

The total phenolic compounds (TPC), total flavonoid compounds (TFL), total saponin (TSC) and antioxidant activity (AA) of the extracts are shown in Table 1. In all conditions, when the solvent was used as only water (100%), the TPC,

TFL and AA values were increased (Table 1). Bioactive compounds of the plant materials are generally extracted with only water and organic solvents (or their mixtures) (Spigno et al., 2007). Our results can be correlated to the polarity of the extraction solvent, and more active compounds' solubility in water than that of in ethanol (Liu et al., 2012). Water and ethanol can be considered as the best solvents for the phenolic compounds extraction from the plant materials (Akbari et al., 2019; Isleroglu and Turker, 2022). However, it is vital to determine the solvent type and percentage of the solvent mixtures for the extraction of bioactive compounds to obtain the highest yield. Our results showed that only water can be used as the extraction solvent to obtain phenolic-rich extracts from the fenugreek leaves which supported the findings of Isleroglu and Turker (2022). Similarly, Fernández-Agulló et al. (2013) reported that only distilled water was the best solvent for the extraction of polyphenols from walnut green husks. Vuong et al. (2011) also reported that the preparation of plant extracts such as papaya leaf extracts, water is the most accessible, the safest and the lowest polluting solvent.

Sample–solvent ratio is another important parameter for the extraction of biomaterials. In general, lower ratios of sample–solvent ratio generate a higher concentration gradient, and this phenomenon increases the driving force of the mass transfer between the sample and the solvent. Hence, the extraction of biomaterials from the samples can be enhanced owing to the increased contact area between the solvent and the sample's surface area (Yancheshmeh et al., 2022). In this study, the highest results were obtained at the conditions having the lowest sample–solvent ratio (10 g/L). Optimization of the temperature is critical for the extraction processes. It was observed that TPC, TFL and AA values were decreased at the highest temperature of the design (65°C). However, TSC values behaved differently from the TPC, TFL and AA values. At lower temperature (25°C), TSC extraction could not be done efficiently, and 75% water percentage–25% ethanol percentage gave the highest TSC values. Same as our study, better saponin extraction was determined at the presence of ethanol in the solvent mixture, and the ethanol percentage of 25 to 100% affected positively the amount of total saponins (Kwon et al., 2003; Heng et al., 2006). In literature, there are many studies about the fenugreek seed extracts; however, there are only a few studies about phenolic extraction from the fenugreek leaves and none of them are optimization study. Mashkor (2014) used three different solvents to extract phenolics from the fenugreek seeds, and they reported 0.259 mg gallic acid/g dry sample of TPC, which was significantly lower than that of our findings. They also reported that acetone was the most suitable solvent for the phenolic extraction. Higher values of TPC for the fenugreek leaves were found by Hussain et al. (2016) (4.25-4.34 mg gallic acid/g dry sample), but these values were still lower than that of our findings. Only Khan et al. (2022) reported similar results (14.01 mg gallic acid/g sample) in their recent study. More studies should be presented to compare the TPC, TFL and AA values of the fenugreek leaf extracts.

The effects of the process variables on the TPC, TFL, AA and TSC of fenugreek leaf extracts were shown in the analysis of variance (ANOVA) table (Table 2). All the generated models were significant (P <0.05), and lack of fit values were not significant statistically (P >0.05). One of the most important criteria required for the generated models to be able to explain the experimental data with high accuracy is the lack of fit data found to be statistically insignificant (Myers and Montgomery, 1995). In this study, the statistically insignificant values of lack of fit (P >0.05) demonstrated that the success of the generated models for the TPC, TFL, AA and TSC of the fenugreek leaf extracts. Results also showed that the linear mixture effect was significant on the TPC, TFL, AA and TSC values (P <0.05) (Table 2). Interaction of the water percentage-ethanol percentage had the most significant effect for all responses (P <0.05). On the other hand, the interaction of ethanol percentage-temperature-sample–solvent ratio did not exhibit a significant effect on any of the responses (P >0.05). The determined model parameters significantly affected the responses (P <0.05) (Table 2).

Statistical results of the modelling of the TPC, TFL, AA (DPPH) and AA (ABTS) values were also shown in Table 2. The coefficient of determination (R^2) values of all different responses were >0.988 which indicated that the generated models were adequate and high proportion of variability was explained by the data. Moreover, the closeness of the R^2 and adjusted R^2 values showed the goodness of the models, and it was a proof that only statistically significant terms were included in the generated models (Table 2).

Table 2. ANOVA table and statistical parameters

Source	DF	Sum of Squares				
		TPC	TFL	DPPH	ABTS	TSC
Model	11	1112.25	269.83	2.35	188.60	473.62
Linear mixture	1	1013.04	246.91	2.13	172.41	197.74
C_1C_2	1	39.36	8.47	0.077	6.22	164.82
C_1X_1	1	1.39	0.32	$3.84x10^{-3}$	9.1x10-3	5.35
C_1X_2	1	3.78	1.01	$5.74x10^{-3}$	0.62	17.60
C_2X_1	1	22.98	5.37	0.044	3.45	35.11
C_2X_2	1	4.49	0.80	0.010	1.24	2.15
$C_1C_2X_1$	1	0.21	0.23	$5.09x10^{-3}$	0.16	6.45
$C_1C_2X_2$	1	4.55	1.01	$2.38x10^{-3}$	0.88	0.75
$C_1X_1X_2$	1	0.53	0.52	$2.42x10^{-3}$	0.32	0.78
$C_2X_1X_2$	1	$1.09x10^{-3}$	$1.11x10^{-5}$	$3.10x10^{-3}$	0.044	0.096
$C_1C_2X_1X_2$	1	0.75	0.28	$5.64x10^{-3}$	0.30	0.044
Residual	12	0.93	0.36	0.021	2.18	9.42
Lack of Fit	5	0.41	0.11	0.016	1.51	3.69
Pure Error	7	0.52	0.25	$4.39x10^{-3}$	0.67	5.73
Total	23	1113.18	270.19	2.37	190.78	483.03

	R^2	*adj- R^2*	*Adequate Precision*	*PRESS*	*C.V. (%)*
TSC	0.9992	0.9984	116.53	5.34	1.60
TFL	0.9987	0.9974	93.86	1.47	2.00
DPPH	0.9961	0.9926	57.57	0.05	3.33
ABTS	0.9886	0.9781	33.00	10.53	6.05
TSC	0.9781	0.9663	30.39	30.88	4.88

Source	DF	F Value				
		TPC	TFL	DPPH	ABTS	TSC
Model	11	1298.67	817.79	123.30	94.32	54.87
Linear mixture	1	13011.15	8231.72	1228.54	948.46	251.99
C_1C_2	1	505.48	282.33	44.21	34.24	210.04
C_1X_1	1	17.83	10.72	2.22	0.050	6.82
C_1X_2	1	48.52	33.62	3.31	3.43	22.43
C_2X_1	1	295.19	179.04	25.62	18.99	44.74
C_2X_2	1	57.64	26.77	5.91	6.84	2.74
$C_1C_2X_1$	1	2.70	7.65	2.94	0.90	8.23
$C_1C_2X_2$	1	58.42	33.68	1.38	4.83	0.96
$C_1X_1X_2$	1	6.78	17.21	1.39	1.76	1.00
$C_2X_1X_2$	1	0.014	$3.69x10^{-4}$	0.018	0.24	0.12
$C_1C_2X_1X_2$	1	9.58	9.40	0.033	1.65	0.056
Residual	12					
Lack of Fit	5	1.11	0.65	5.22	3.13	0.90
Pure Error	7					
Total	23					

Table 2. devam

Source	DF	p - Value				
		TPC	TFL	DPPH	ABTS	TSC
Model	11	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Linear mixture	1	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
C_1C_2	1	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
C_1X_1	1	0.0012	0.0067	0.1623	0.8264	0.0227
C_1X_2	1	< 0.0001	< 0.0001	0.0937	0.0890	0.0005
C_2X_1	1	< 0.0001	< 0.0001	0.0003	0.0009	< 0.0001
C_2X_2	1	< 0.0001	0.0002	0.0317	0.0226	0.1238
$C_1C_2X_1$	1	0.1265	0.0171	0.1121	0.3627	0.0141
$C_1C_2X_2$	1	< 0.0001	< 0.0001	0.2636	0.0484	0.3475
$C_1X_1X_2$	1	0.0231	0.0014	0.2605	0.2089	0.3374
$C_2X_1X_2$	1	0.9077	0.9850	0.8957	0.6323	0.7324
$C_1C_2X_1X_2$	1	0.0093	0.0098	0.8598	0.2235	0.8163
Residual	12					
Lack of Fit	5	0.4334	0.6747	0.0557	0.0847	0.5296
Pure Error	7					
Total	23					

C_1: Water (%), C_2: Ethanol (%), X_1: Temperature (°C), X_2: Sample-solvent ratio (g/L), TPC: Total Phenolic Compounds, TFL: Total Flavonoid Compounds, TSC: Total Saponin Content, DF: Degrees of Freedom, Adj- R^2: Adjusted R^2, PRESS: Predicted residual error sum of squares, C.V. (%): Coefficient of variation

The effect of the different process variables on the TPC, TFL, AA and TSC was shown with the response surface graphs in Figure 1. Response surface graphs visually supported our findings, and the higher percentage of water usage for the extraction increased the TPC, TFL, and AA values. The red regions on the Figure 1 show the highest values obtained in the experimental design. For the Figure 1 (a-d), at 10 g/L of sample–solvent ratio where the highest results were obtained, the temperature increment positively affected the extraction of the phenolics, flavonoids and antioxidant activity of the extracts. Vuong et al. (2013) reported that the optimum extraction temperature of papaya leaf polyphenols (using only distilled water as the solvent) was between at 50 and 70°C. They also reported that the extraction efficiency of the polyphenols decreased when the temperature was lower or higher than this temperature range. Irakli et al. (2018) extracted bioactive compounds (25-60°C) from the olive leaves such as oleuropein, phenolic acids and flavonoids. Researchers reported that it is vital to determine the optimum extraction temperature, because the temperatures beyond a certain value may decompose some phenolic materials, and the low temperatures than that of the optimum value may result with having lower extraction yields. In our study, similar graphs were obtained for the TPC, TFL, and AA values which indicated that AA values were mainly depended on TPC and TFL for the fenugreek leaf extracts. On the other hand, different trend of the TSC is observed in the Figure 1(e). TSC had the higher values at the water percentage of 75%.

Five different second-order polynomial models were generated for the extraction of bioactive compounds from fenugreek leaves, and the TPC, TFL, AA (DPPH), AA (ABTS), and TSC values were maximized to obtain the optimum extraction conditions (Equations 1-5). The relationships between the experimental and predicted data are shown in Figure 2, it was clearly observed that these values were close to each other.

$$\text{TPC (mg gallic acid/g dry sample)} = 0.26C_1+0.02C_2+1.81x10^{-3}C_1C_2-2.97x10^{-6}C_1X_1-5.27x10^{-5}C_1X_2+1.28x10^{-3}C_2X_1-5.91x10^{-4}C_2X_2 +9.95x10^{-7}C_1C_2X_2-1.05x10^{-5}C_1X_1X_2+7.77x10^{-7}C_1C_2X_1X_2 \quad (1)$$

$$\text{TFL (mg quercetin/g dry sample)} = 0.13C_1+1.21x10^{-3}C_2+1.19x10^{-3}C_1C_2+1.59x10^{-4}C_1X_1+1.96x10^{-4}C_1X_2+6.22x10^{-4}C_2X_1 -2.43x10^{-4}C_2X_2-2.25x10^{-5}C_1C_2X_1-4.55x10^{-6}C_1C_2X_2-1.03x10^{-5}C_1X_1X_2 +4.78x10^{-8}C_1C_2X_1X_2 \quad (2)$$

DPPH (mg Trolox/g dry sample) $= 1.43x10^{-2}C_1+6.79x10^{-4}C_2+7.54x10^{-5}C_1C_2+6.45x10^{-5}C_2X_1-1.94x10^{-5}C_2X_2$ (3)

ABTS (mg Trolox/g dry sample) $= 9.77x10^{-2}C_1+1.65x10^{-2}C_2+1.09x10^{-3}C_1C_2+4.09x10^{-4}C_2X_1-4.36x10^{-4}C_2X_2-6.36x10^{-6}C_1C_2X_2$ (4)

TSC (mg diosgenin/g dry sample) $= 1.76x10^{-1}C_1+7.60x10^{-3}C_2+4.75x10^{-3}C_1C_2+7.08x10^{-4}C_1X_1-1.04x10^{-3}C_1X_2 +1.59x10^{-3}C_2X_1-4.56x10^{-5}C_1C_2X_1$ (5)

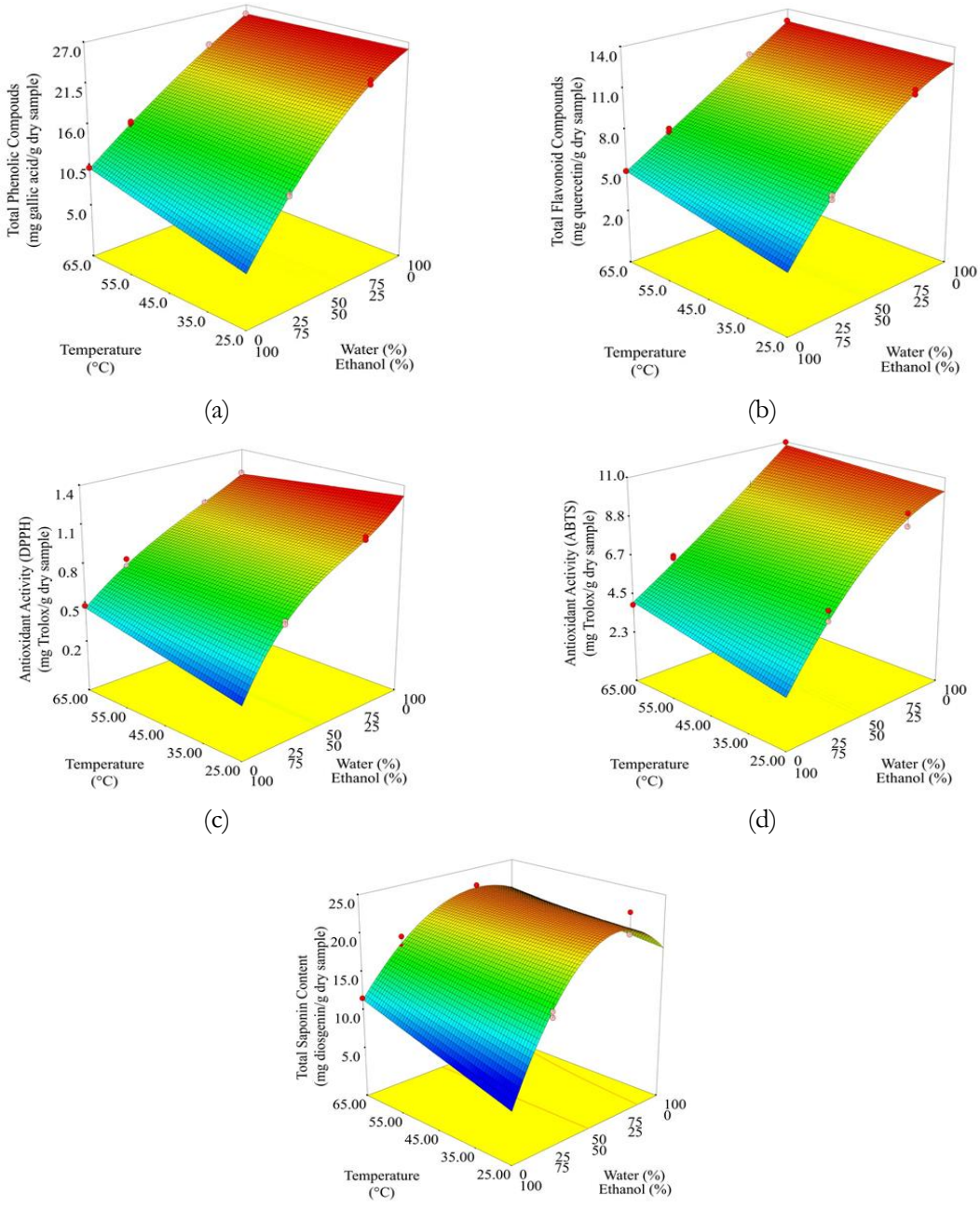

Figure 1. Response surface graphs for (sample–solvent ratio level of 10 g/L): (a) total phenolic compounds, (b) total flavonoid compounds, (c) antioxidant activity-DPPH, (d) antioxidant activity-ABTS, and (e) total saponin content of fenugreek leaf extracts

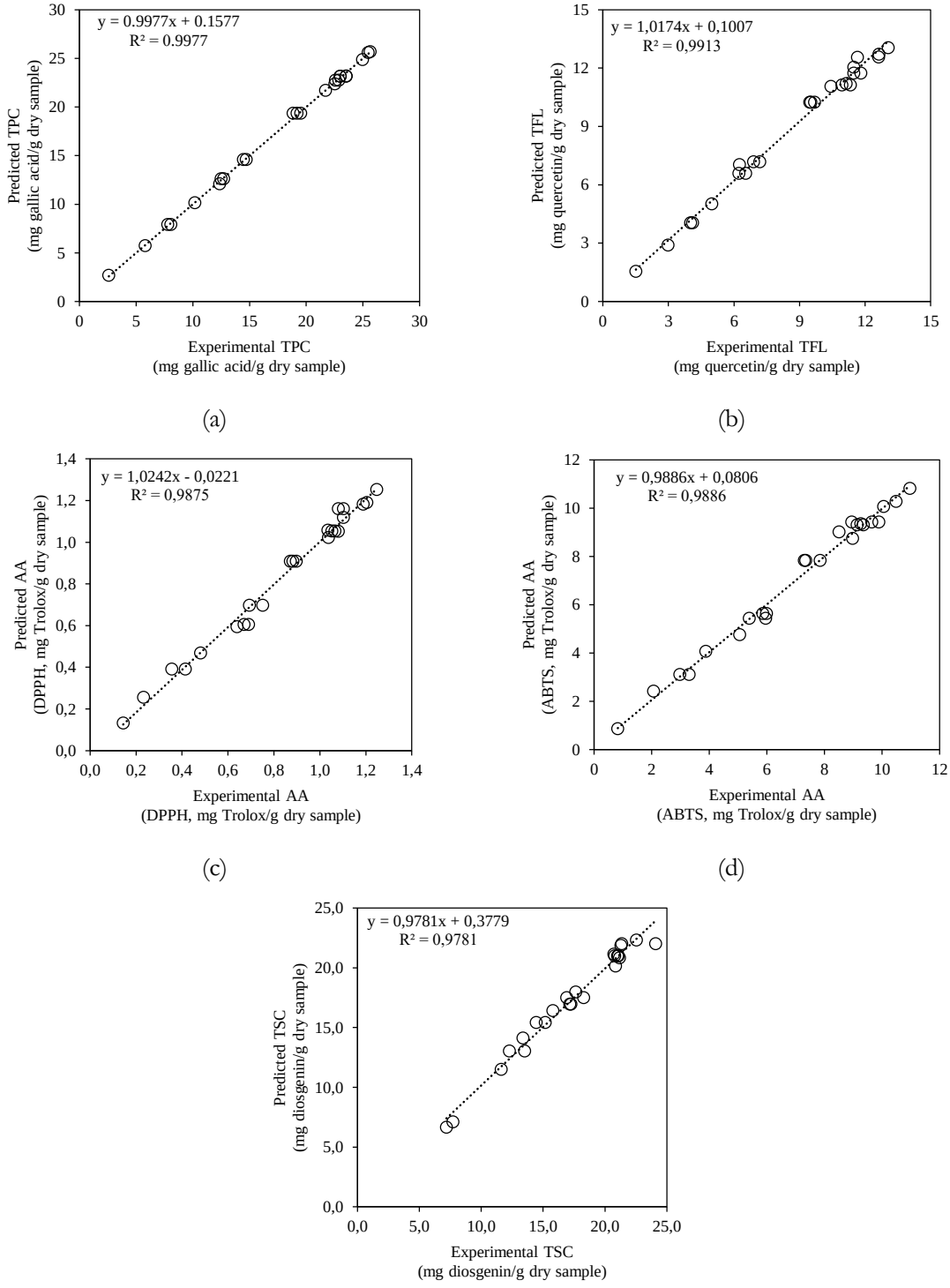

Figure 2. The relation between predicted and experimental data for: (a) total phenolic compounds, (b) total flavonoid compounds, (c) antioxidant activity-DPPH, (d) antioxidant activity-ABTS, and (e) total saponin content of fenugreek leaf extracts

Extraction process was subjected to numerical optimization to determine the optimum process conditions. We found out four different but quite similar optimization results and one of these results having the highest desirability (0.989) was chosen as the optimum point. The predicted optimum extraction conditions were determined as follows: 100% of water, 49.71°C of temperature, and 10.00 g/L of sample–solvent ratio. At this point, TPC was 25.869 mg gallic acid/g dry sample, TFL was 12.968 mg quercetin/g dry sample, AA (DPPH) was 1.247 mg Trolox/g dry sample and AA (ABTS) was 10.607 mg Trolox/g dry sample. The optimum point verification tests were done in thrice. According to the single sample t-test, there was no significant difference between experimental and predicted values at the optimum point (P >0.05).

In this study, phenolic compounds of the fenugreek leaves were extracted and the optimum process conditions ensuring the highest phenolic content, flavonoid content and antioxidant activity were determined by the response surface methodology. Our results showed that water percentage of 100% had the best extraction performance. Other extraction parameters namely extraction temperature and sample–solvent ratio also affected the responses. Our research showed that fenugreek leaves are worth to be investigated because of its high bioactive compounds, and the fenugreek leaf extracts can be produced by cheap and effective methods. Fenugreek leaf extracts produced using only water can be used in the model foods for the future researches.

CONFLICT OF INTEREST

As authors we declare there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

Hilal Isleroglu: Project administration, supervision, conceptualization, methodology, writing - review & editing. Izzet Turker: Investigation, formal analysis, writing-review & editing. The final version of the manuscripts was read by all authors.

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