GIDA
THE JOURNAL OF FOOD
E-ISSN 1309-6273, ISSN 1300-3070

Research/ Araştırma
GIDA (2021) 46 (4) 1040-1052
doi: 10.15237/gida.GD21047

OPTIMIZATION OF THE EXTRACTION CONDITIONS OF PHENOLIC COMPOUNDS FROM *ALCHEMILLA VULGARIS* USING RESPONSE SURFACE METHODOLOGY

Sercan Özbek Yazıcı*
Burdur Mehmet Akif Ersoy, University, Health Science Faculty, Nutrition and Dietetics Department, Burdur, Turkey

Received / Geliş: 28.02.2021; *Accepted*/ Kabul: 07.06.2021; *Published online*/ Online baskı: 17.07.2021

Özbek-Yazıcı, S. (2021). Optimization of the extraction conditions of phenolic compounds from Alchemilla vulgaris using response surface methodology. GIDA (2021) 46 (4) 1040-1052 doi: 10.15237/gida.GD21047

Özbek-Yazıcı, S. (2021). *Alchemilla vulgaris*'ten fenolik bileşiklerin ekstraksiyon koşullarinin yanıt yüzey yöntemi kullanilarak optimizasyonu. GIDA (2021) 46 (4) 1040-1052 doi: 10.15237/gida.GD21047

ABSTRACT
The object of this study is to determine the best solvent and optimum extraction conditions for extraction of maximum phenolic compounds from *Alchemilla vulgaris* leaves. Extractions were carried out using solvents with different polarities. Box-Behnken Design was used to optimize extraction conditions including extraction time, temperature, and liquid/solid ratio. In the study, extract obtained with acetone-water indicated the highest total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity. Optimum extraction conditions for both responses were found as 168 min, 48.5 °C, and liquid/solid ratio of 41:1. The maximum TPC and TFC values were determined as 7.17 mg GAE/gdw and 3.63 mg QE/gdw under optimum extraction conditions. Concentrations of five phenolic compounds analyzed by HPLC increased significantl after optimization. The results indicated that optimizing extraction conditions is critical for quantification of phenolic compounds. The present model can contribute to food industry where phenolic compounds have potential use as biopreservatives.
Keywords: *Alchemilla vulgaris*, antioxidant activity, Box-Behnken design, extraction optimization, phenolic compounds

ALCHEMILLA VULGARIS'TEN FENOLİK BİLEŞİKLERİN EKSTRAKSİYON KOŞULLARININ YANIT YÜZEY YÖNTEMİ KULLANILARAK OPTİMİZASYONU

ÖZ
Bu çalışmanın amacı, *Alchemilla vulgaris* bitkisinin yapraklarından maksimum fenolik bileşik ekstraksiyonu için en iyi çözücünün ve optimum ekstraksiyon koşullarının belirlenmesidir. Ekstraksiyonlar, farklı polaritelere sahip çözücüler kullanılarak gerçekleştirildi. Box-Behnken tasarımı (BBD), ekstraksiyon süresi, ekstraksiyon sıcaklığı ve sıvı / katı oranı içeren ekstraksiyon koşullarını optimize etmek için kullanıldı. Çalışmada aseton-su ile elde edilen ekstrakt, en yüksek toplam fenolik içerik (TPC), toplam flavonoid içerik (TFC) ve antioksidan aktivite göstermiştir. Her iki yanıt için optimum ekstraksiyon koşulları, 168 dakika, 48.5 °C ve 41:1 sıvı / katı oranı olarak bulundu. Optimum ekstraksiyon koşulları altında maksimum TPC ve TFC değerleri 7.17 mg GAE / gdw ve

*Corresponding author / Yazışmalardan sorumlu yazar:
✉: sozbekyazici@hotmail.com ✆ (+90) 248 213 3554 🖷: (+90) 248 213 3503
Sercan Özbek Yazıcı; ORCID no: 0000-0003-3406-4291

3.63 mg QE / gdw olarak belirlendi. HPLC ile analiz edilen beş fenolik bileşiğin konsantrasyonlarının, optimizasyondan sonra önemli ölçüde arttığı bulundu. Sonuçlar, ekstraksiyon koşullarının optimize edilmesinin fenolik bileşiklerin miktarının belirlenmesi için önemli olduğunu göstermektedir. Mevcut model, fenolik bileşiklerin biyo-koruyucu olarak potansiyel kullanıma sahip olduğu gıda endüstrisine katkıda bulunabilir.

Anahtar kelimeler: *Alchemilla vulgaris*, antioksidan aktivite, Box-Behnken tasarımı, ekstraksiyon optimizasyonu, fenolik bileşikler

INTRODUCTION

Today, plants are becoming increasingly popular related to their potential as a source of bioactive compounds. These compounds promote various health benefits and inhibit or reduce the risk of human diseases due to their antioxidant activities (Sepahpour et al., 2020). Among the phytochemical substances, many polyphenols with antioxidant properties exist in plants have been shown to be antibacterial, anti-inflammatory, and anti-tumor agents (Pham et al. 2019). In addition, polyphenols, which are natural antioxidants, have increased their use in the food and pharmaceutical industry instead of synthetic antioxidants (Sousa et al. 2019).

Extraction is a crucial step to obtain biologically active compounds (Pham et al. 2019). However, there is no standard method for the extraction of phenolics. This problem is due to the great diversity of these compounds, which give them different physicochemical properties (Sousa et al. 2019). For extraction, there are different extraction methods including traditional methods such as maceration and modern methods such as microwave extraction, ultrasound extraction. Although modern methods have ensured efficient extractions, they are expensive or/and have certain disadvantages (Cacique et al., 2020). For instance, the main disadvantages of microwave extraction method are: unbalanced heating and/or overheating of extracts cause thermal degradation of phenolic acids. In addition, it has been reported that ultrasonic waves may cause the degradation of some phenolic acids and the formation of reactive hydroxyl radicals (Al Jitan et al. 2018). Maceration which is one of the oldest traditional extraction methods is used commonly due to its simple methodology and low cost (Uysal et al., 2019). The disadvantages of this method are the large volume of solvents, the long processing time (Cacique et al., 2020). The extraction of phenolic compounds from plants are affected by various factors including extraction time, extraction temperature, extraction solvent and solvent concentration (Ahmed et al., 2020). Therefore, it seems necessary to optimize extraction conditions using the appropriate solvent type for each plant sample to obtain the maximum yield.

Response Surface Methodology (RSM) has been broadly employed to design, analyze, and predict extraction conditions. The conventional one-factor-at-a-time approach of optimization is neglected the combined interaction of variables and does not guarantee obtaining optimal conditions. To solve this problem, statistical approaches such as Box-Behnken Design (BBD), which is one of RSM, have offered the opportunity to predict optimum extraction conditions and understand the interactions among extraction parameters (Belwal et al., 2016: Pham et al., 2019).

Alchemilla L. genus that belongs to the Rosaceae family is a perennial herbaceous plant and a popular garden herb commonly known as Lady's Mantle. It is reported that *Alchemilla* species are traditionally used to treat gynecological and gastrointestinal diseases and have diuretic, anti-inflammatory, antioxidant and anti-influenza properties (Acet and Özcan 2018; Hwang et al. 2018; Tasić-Kostov et al. 2019). A previous study demonstrated wound-healing properties of *A. vulgaris* associated with promitotic activity in epithelial cells and myofibroblasts (Shrivastava et al. 2007). Neagu et al. (2015) found that the *A. vulgaris* extract showed high acetylcholinesterase and high tyrosinase inhibitory effects: They suggested that it could be extremely useful in the treatment of degenerative diseases due to these properties of *A. vulgaris* extract. In one of the recent studies, *A. mollis* was found to protect from

UVB. Therefore, it has been suggested that *A. mollis* may be useful as a functional food for preventing photoaging in human skin caused by UVB (Hwang et al., 2018).

Alchemilla species are rich in flavonoids, phenolic acids, and tannins, which are proven to be responsible for some pharmacologic activities (Renda et al., 2017). Previous studies have revealed that *Alchemilla* sp. have high phenolic content and good antioxidant properties (Acet and Özcan 2018; Hwang et al. 2018; Tasić-Kostov et al. 2019). However, there is a deficiency in the studies focusing on the optimization of extraction conditions for maximum phenolic compounds extraction from *A. vulgaris*. Therefore,the aim of the present study is to determine the best solvent and to optimize extraction conditions for extraction of maximum phenolic compounds from *A. vulgaris* leaves by using maceration extraction method. The BBD was employed to predict the model and to optimize the extraction conditions (temperature, time, liquid/solvent ratio) based on total phenolic and flavonoids content. In addition, the variation of phenolic compounds in the extracts obtained before and after optimization was analyzed using HPLC.

MATERIAL AND METHODS

Plant Material and Extraction

A. vulgaris was obtained in June 2020 from the local market. The leaves of *A. vulgaris* were ground in a domestic blender and sieved to yield a powder with average particle diameter of 0.423 mm.

Maceration method was used for extracting phenolic compounds of *A. vulgaris* leaves. To define the best extraction solvent, extracts were prepared using pure methanol, pure ethanol, pure acetone, water, and aqueous mixtures of acetone, methanol, or ethanol (50%). The ground plant samples (1 g) were extracted with the appropriate solvent (10 mL) in a water bath at 30 ºC and for 120 min after the mixture was vortexed. After the extraction procedure, the mixture was then centrifuged at 2000 x g for 5 min, and the supernatant was collected for analysis. The supernatant was stored at -18 °C until analyzed. The best solvent was determined based on maximum TPC, TFC yields, and antioxidant activities (DPPH radical scaevining activity, ferric reducing power).

Total Polyphenol Content Determination

In the extracts, TPC was determined by using the Folin–Ciocalteu method (Mouratoglou et al. 2016). Gallic acid was used as standard, and the results are expressed in milligrams of gallic acid equivalent (GAE) per gram dry plant weight (mg GAE/g dw).

Flavonoid Content Determination

A previously published methodology was employed (Mouratoglou et al. 2016). The total flavonoid concentration was calculated from a quercetin calibration curve. Total flavonoid content was indicated as mg quercetin equivalents (QE) per g of dry plant weight (dw) (mg QE/g dw).

DPPH Free Radical Scavenging Assay

The free radical scavenging activity of extracts was assumed by DPPH·(Sánchez-Moreno et al., 2003). The percentage of DPPH radical remaining against extract concentration was then plotted to obtain the amount necessary to decrease the initial DPPH radical concentration by 50% (IC_{50}). IC_{50} value was defined as extract concentration, providing 50% inhibition of mg dry plant sample per mL (mg (dw)/mL).

Ferric Reducing Power Assay

The Oyaizu method (1986) was used to determine ferric reducing power. The antioxidant capacity of extracts was expressed as EC_{50}. The EC_{50} value (the effective concentration at which the absorbance was 0.5) was calculated from the graph of absorbance at 700 nm against extract concentration (mg (dw)/mL).

Optimization of Extraction Process using a Statistical Approach

In the study, firstly, the best solvent for the maximum phenolic yield was determined. After that, optimization of extraction conditions to increase phenolic yield was aimed using a statistical approach. The solvent defined to be the

best for extraction was used for extraction in the optimization step.

For optimization of extraction conditions, three independent variables were evaluated based on total phenolic compound and total flavonoid compound of extracts from *A. vulgaris* by using Box Behnken Design. The independent variables and their intervals were determined according to the literature research (Eruygur et al. 2018, Uysal et al. 2019, Ahmed et al., 2020, Cacique et al. 2020). The variables were extraction time (A: 60-180 minutes), incubation temperature (B: 30-70 °C), and liquid/solid ratio (C: between 10:1 and 70:1). For BBD analyses, three levels as high (+1), medium (0) and, low (-1) were described. The code and the real value of each variable are presented in Table 1.

Table 1. Levels of independent variables tested in Box-Behnken design and their experimental designs levels for optimization of extraction conditions

Symbol	Variable	Units	Coded levels		
			-1	0	-1
A	Time	min	60	120	180
B	Temperature	^{0}C	30	50	70
C	Liquid / Solid ratio		10	40	70

BBD experiments were conducted for optimization. Experimental design method was carried out by 17 experiments with five center points to test the reproducibility of the test results. Table 2 represents the designs, and results of the experiment runs.

Table 2. Experimental design based Box-Behnken design for optimization of extraction conditions

Run order	Extraction Time (min)	Extraction Temperature (^{o}C)	Liquid/Solid Ratio	TPC* (mgGAE/gdw)	TFC* (mgQE/gdw)
1	120.00	30.00	70.00	3.99	2.07
2	120.00	50.00	40.00	7.02	3.01
3	60.00	50.00	70.00	3.11	1.11
4	120.00	50.00	40.00	6.83	3.39
5	120.00	50.00	40.00	7.12	3.27
6	180.00	50.00	70.00	6.23	3.01
7	60.00	30.00	40.00	2.47	1.11
8	60.00	50.00	10.00	2.81	1.11
9	180.00	70.00	40.00	7.25	2.61
10	120.00	50.00	40.00	6.82	3.11
11	180.00	50.00	10.00	5.54	3.04
12	60.00	70.00	40.00	4.57	1.75
13	180.00	30.00	40.00	5.95	3.76
14	120.00	70.00	10.00	5.43	1.97
15	120.00	30.00	10.00	4.91	2.01
16	120.00	50.00	40.00	7.11	3.17
17	120.00	70.00	70.00	6.63	1.61

*Experimental results

The response function coefficients were defined by regression using the experimental data and Design-Expert V7 trial version. For three variable systems, the model equation is as follows:

$$Y = \beta0 + \beta1A + \beta2B + \beta3C + \beta11A^2 + \beta22B^2 + \beta33C^2 + \beta12AB + \beta13AC + \beta23BC \quad (1)$$

Where Y is the predicted response (TPC and TFC), β0 is the model constant; A, B, and C are independent variables; β1, β2, and β3 are linear coefficients, β11, β22, and β33 are the quadratic coefficients and β12, β13, β23 are the cross-product coefficients. A, B, C are the independent variables. The coefficients of determination R^2, adjusted R^2, and predicted R^2 were used to determine the suitability of the developed mathematical polynomial equation. In addition, the three-dimensional (3-D) response surface graphs were plotted to illustrate the interaction of two variables (Figure 1). Lastly, experiments were made to confirm TPC and TFC values under the optimum extraction conditions predicted by BBD.

HPLC Analyses

Phenolic compounds (ellagic acid, catechin, chlorogenic acid, gallic acid, p-hydroxybenzoic acid) in the extracts were analyzed using high performance liquid chromatography (HPLC). This process was carried out as described in our previous study (Yazici et al., 2020). The results were expressed as μg/g dry weight (dw) of of *A. vulgaris* leaves.

Statistical Analyses

Statistical analyses were carried out with SPSS 20. Data are expressed as the mean ±SD. One-way ANOVA was used to analyze the differences among extracts obtained with different solvents. The differences shown by data were significant (*P<0.05*). Correlations among variables were performed with Pearson's correlation test.

RESULTS AND DISCUSSION

Solvent Selection for Extraction

The phenolic compounds have various chemical properties due to variations in type, number, and position of the functional groups. Therefore the solubility of these compounds can be affected by various solutions (Sepahpour et al., 2020). It is also reported that the polarity of solvents has a crucial role in the solubility of phenolic compounds (Haminiuk et al., 2014; Sepahpour et al., 2020). Therefore, the choice of solvent for extraction is essential as it affects the type and amount of phenolic compound recovered.

Ethanol, methanol, and acetone which are polar organic solvents are used mostly for extraction (Coklar and Akbulut 2017; Park et al., 2019). However, using water for bioactive compounds extraction may be a safer option for pharmaceutical and food applications (Shelembe et al., 2012). This study investigates the impact of solvent type on the extraction of the phenolic compounds, using seven solvents in the process: acetone, ethanol, methanol, water, and aqueous mixtures of acetone methanol, or ethanol (1:1 v/v). Table 3 shows the TPC, TFC, and antioxidant activities of the extracts obtained with different solvents. All extracts were found to have significant total phenolic contents ranged from 1.08 mg to 4.83 mg GAE/g dw. There were significant differences in TPC and TFC of extracts obtained with different solvents (*P<0.05*). Flavonoids and phenolics contents had similar extraction patterns, acetone-water extract exhibited the highest amount of TPC and TFC (4.83 mg GAE/g dw; 1.91mg QE/gdw respectively). However, water extract demonstrated the lowest amount of TPC and TFC (1.08 mg GAE/g dw; 0.68 mg QE/gdw, respectively) (*P<0.05*). Polyphenols effectively dissolved in organic solvents which are less polar than water (Haminiuk et al., 2014). In the study, the differences in the phenolic content of the extracts obtained with different solvents could be attributed to solvents' polarities. In this work, the solvents used in terms of polarity can be put in order as water> methanol> ethanol>acetone (Coklar and Akbulut 2017). This study shows that the most efficient solvent for extraction of TPC and TFC from *A. vulgaris* is acetone-water, followed by ethanol-water and methanol-water. Similar results were reported by Al-Farsi and Lee (2008). In that, they reported that flavonoid and phenolic contents from date seeds were extracted highest with 50% acetone followed by ethanol, methanol, and their aqueous 50% solutions. In addition, aqueous solvent mixtures were found to be better than mono-component solvent for phenolic extraction. Polar solvents are mostly

utilized for recovering phenolic compounds from plant material. The most suitable solvents are aqueous mixtures containing ethanol, methanol, and acetone (Do et al., 2014). It was reported that addition of water to solvent may cause to increase the polarity and swell plant materials by allowing the solvent to easily penetrate the solid matrix (Park et al., 2019). Similary, Yılmaz et al., (2006) reported that aqueous mixtures of acetone, methanol, or ethanol were more effective than a mono-component solvent (water, acetone, methanol, or ethanol) for the extraction of phenolic compounds.

Table 3. Effect of solvent type on total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities of extracts from *A. vulgaris***

Solvents	TPC * mgGAE/g dw	TFC* mg QE/gdw	IC_{50}* mg (dw)/mL	EC_{50}* mg(dw)/mL
Acetone	3.04^{cd}	1.49 ^{b}	0.19^{cd}	3.15^{d}
Methanol	2.21^{e}	0.91 ^{c}	0.59^{a}	5.98^{a}
Ethanol	2.56^{de}	0.89 ^{c}	0.43 ^{b}	4.33^{c}
Water	1.08^{f}	0.68^{c}	0.62^{a}	6.23^{a}
Acetone-water	4.83^{a}	1.91^{a}	0.11^{d}	2.13 ^{e}
Methanol-water	3.44^{bc}	1.32^{b}	0.31^{c}	5.35^{b}
Ethanol-water	3.94^{b}	1.47^{b}	0.24^{cd}	3.34^{d}

*Values are mean ±SD (n=3). Different letter within the same column show differences of means among the extracts obtained with different solvent (*P<0.05*).
** Extracts was obtained at 30 °C and 10:1 of liquid/solid ratio for 120 min in water bath.

Alchemilla species are rich in phenolic compounds, which are responsible for some of the pharmacological activities (Renda et al., 2017). Vlaisavljević et al. (2019) evaluated the phytochemical profile and antioxidant properties of extracts from *A. vulgaris* obtained with methanol, ethanol, ethyl-acetate, and water. They obtained the highest TPC (9.65 mg GAE/g dw) with ethyl acetate. Murathan (2018) found that the total phenolic content in *A. sericata* was 427.2 mg/100g, while the total flavonoid content was 29.52 mg/100g. Denev et al. (2014) found that the total flavonoid content of the methanol extracts from *A. sericata* was 1831 mg/100g. Acet and Özkan (2018) reported that the higher phenolic content from *A. ellenbergiana* was found in methanol extract (777.2 mg/g extract GAE) and ethanol extract (750 mg/g extract GAE) than ethyl-acetate and hexane extracts. The change in the phenolic content of *Alchemilla* species can be explained by several factors such as environmental and genetic effects, plant organ, extraction method, and storage conditions (Rebey et al., 2012).

The phenolic compounds can play an important role in neutralizing and absorbing free radicals (Borra et al., 2013). The effects of solvents on antioxidant activity of extracts were mostly attributed to the content change of total phenolic (Zhu et al., 2018). In this study, the antioxidant activities of extracts were evaluated with reducing power and DPPH assays. The lowest EC_{50} or IC_{50} means the highest antioxidant capacity. The DPPH IC_{50} values in extracts varied from 0.11 to 0.62 mg (dw)/mL. Acetone-water extract showed higher radical scavenging activity, followed by acetone extract. EC_{50} values of reducing power varied from 2.13 to 6.23 mg (dw)/mL. Similarly, ferric reducing power of acetone-water extract was the highest and significantly different from other extracts (*P<0.05*), followed by acetone extract. The extracts obtained with water and methanol exhibited significantly lesser antioxidant activity (*P<0.05*). Solvents dissolve substances with similar polarities (Wakeel et al., 2019). These differences in the antioxidant activity of the extracts obtained with solvents of different polarities may be related to with the variety of antioxidant compounds extracted. Similarly, in another study, the water extract of *A. vulgaris* showed significantly lower anti-radical activity compared to other extracts (Vlaisavljević et al., 2019).

Acetone–water mixture is reported as an effective solvent for the antioxidants (Yılmaz et al, 2006; Nasr et al., 2019). In this study, acetone-water extract with the highest TPC and TFC showed the best antioxidant activity. In contrast to our finding, methanol extracts of *Alchemilla* species are reported to have high antioxidant activities (Usta et al., 2013; Denev et al., 2014; Boroja et al., 2018; Murathan, 2018). These different results may be attributed to the extraction method and conditions that affect the antioxidant activities of the extract (Do et al., 2014).

In addition, Pearson's correlation coefficient indicated that TPC and TFC had a negative correlation with IC_{50} of DPPH scavenging activities (r = -0.883, -0.946; respectively, *P<0.01*) and EC_{50} of reducing power capacities (r = -0.816,-862, respectively; *P<0.01*). Previous studies found the correlation between TPC with DPPH scavenging activity and reducing power capacity (Tusevski et al. 2014). Phenolic compounds are majorly responsible for the antioxidant activity of plant (Zhao et al., 2014). The results of the study indicate that phenolic compounds may be the responsible for the antioxidant activity of extracts.As a result, phenolic content and antioxidants activity were observed to be affected by the solvent type. Acetone-water extract with the highest TPC and TFC showed the best antioxidant activity in the study. Therefore, optimization of phenolic compound extraction from *A. vulgaris* was carried out using acetone-water as solvent.

Optimization of Extraction Conditions using BBD

Maceration is the easy and simple method for the recovery of bioactive compounds (Uysal et al. 2019). Many factors have been established to influence the extraction efficiency, such as solvent type, and solid to solvent ratio, extraction temperature, and time (Eruygur et al. 2018, Uysal et al. 2019, Ahmed et al., 2020, Cacique et al. 2020). In this study, 50% acetone-water was used for extraction of phenolic compounds from *A. vulgaris* in optimization. Extraction parameters including extraction time, extraction temperature, and liquid/solid ratio were improved extraction in the next step for optimal TPC and TFC yield by BBD (Table 2). BBD was employed to define the optimum level of parameters that provided maximum total phenolic and total flavonoid extractions and to understand the relationships between the extraction parameters with TPC, and TFC.

Table 4. ANOVA results for quadratic model obtained by BBD

Source	Df	TPC		TFC	
		F-Value	Prob > F	F-Value	Prob > F
Model	9	191.96	< 0.0001*	88.63	< 0.0001*
A-Extraction Time	1	749.35	< 0.0001*	438.42	< 0.0001*
B-ExtractionTemperature	1	223.57	< 0.0001*	8.30	0.0236*
C- Liquid/Solid Ratio	1	8.38	0.0232*	0.89	0.3779
AB	1	6.65	0.0365*	52.15	0.0002*
AC	1	1.58	0.2490	0.015	0.9071
BC	1	46.70	0.0002*	2.87	0.1340
A2	1	327.85	< 0.0001*	36.52	0.0005*
B2	1	53.18	0.0002*	73.41	< 0.0001*
C2	1	247.29	< 0.0001*	157.29	< 0.0001*
Lack of Fit	3	1.27	0.3970	0.34	0.7980

* Significant parameter.

The variance analysis of the quadratic regression models designed for optimum TPC and TFC suggested that the models were statistically significant, as shown in Table 4 ($P< 0.05$). The fitted second-order polynomial equation

illustrating the TPC and TFC yields by using response surface analysis is given in Eq. (2 and 3).

$$TPC=+6.98+1.50*A+0.82*B+0.16*C-0.20*A*B+0.098*A*C+0.53*B*C-1.37*A2-0.55*B2-1.19*C2 \quad (2)$$

$$TFC=+3.19+0.92*A-0.13*B-0.041*C-0.45*A*B-7.500E-003*A*C-0.10*B*C-0.36*A2-0.52*B2-0.76*C2 \quad (3)$$

The statistical significance of model terms is assumed by their respective ***P**-value*. Also, "The Lack of Fit F-value" is a special diagnostic test for adequacy of a model. Non-significant lack of fit is desirable. In the study, the predicted R^2 (0.96 and 0.96 respectively) and adjusted R^2 values for TPC and TFC (0.99 and 0.99, respectively) were in Figure 1.

reasonable agreement with the value of R^2 (0.99 and 0.98, respectively). The values of R^2 imply a correlation between the experimental results and predicted values (Uysal et al., 2019).

The statistical analysis indicates that the evaluated variables have a significant effect on TPC and TFC. Values of "Prob > F" less than 0.05 indicate that model terms are remarkable. In this case A, B, C, AB, BC, A2, B2, C2 are significant model terms for TPC. In addition, A, B, AB, A2, B2, C2 are significant model terms for TFC (Table 4). The effects of extraction time, extraction temperature, and liquid/solid ratio on TPC and TFC were illustrated in 3-D response surface graphs. The 3-D plot graphical representations are shown in Figure 1.

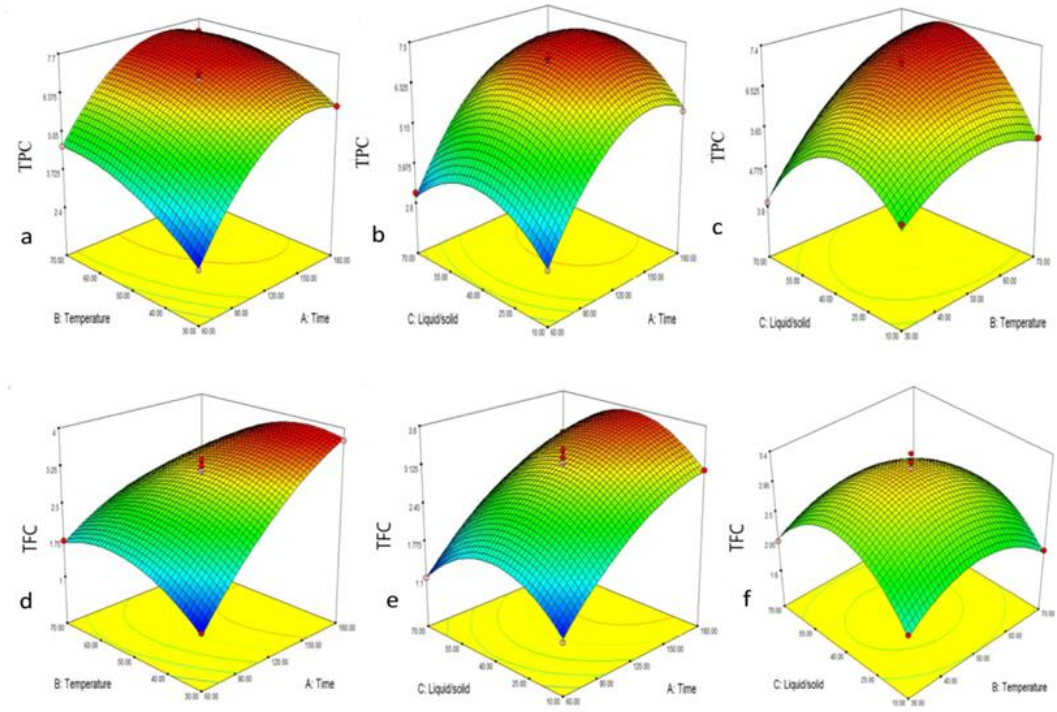

Figure 1. Response surface plots (3D) showing the interaction effect of variables on total phenolic content (a,b,c), and total favonoid content (d,e,f).

The TPC results obtained under different conditions were in the range from 2.47 to 7.25 mg/g GAE (Table 2). The lowest TPC was obtained at 30 °C in 40:1 liquid/solid ratio for 60 min (Run 7), while the parameters for the highest TPC were 70 °C, 40:1 liquid/solid ratio and 180 min (Run 9). As seen in Figure 1a, the effect of extraction temperature is positive on

TPC. Considering the interactive effect of variables, the interaction between time and temperature (A*B) showed a remarkable effect on TPC ($P<0.05$). The TPC significantly increased with increasing extraction temperature and time. As seen in Figure 1a, and c, TPC increased in the range of about 55 to 65 ºC; however, the amount of TPC tended to remain constant after 65 ºC. In line with our results, Shi et al. (2003) found extraction temperature of 65 °C to be the best for extracting phenolic compounds from grape seeds. The increase in the amount of TPC in parallel with the temperature is corresponded with literature (Silva et al., 2007; Belwal et al. 2016). According to Figure 1a, TPC increased until 150 min. After this duration, the level of the phenolic content begined to decline. The results show that extending the time of extraction might lead to a decline in TPC due to the applied extraction temperature, which may degrade the phenolic with the extended extraction time (Ahmadian-Kouchaksaraie et al., 2016).

In the study, the TPC increases significantly increasing until 40:1 liquid/solid ratio ($P< 0.05$) (Figure 1c). The liquid/solid ratio which gave the highest TPC was 40:1 mL/g. In addition, the interaction between extraction temperature and liquid/solid ratio (B*C) showed a remarkably positive effect on TPC ($P< 0.05$), but there was no significant interaction between liquid/solid ratio and extraction time (A*C) ($P>0.05$) (Table 4).

Similary, extraction time (A), and extraction temperature (B) profoundly influenced TFC ($P<0.05$), but the liquid/solid ratio had no significant effect on TFC ($P>0.05$) (Table 4). Figure 1d shows that the TFC increased in parallel to the extraction time. The maximum TFC was observed at about 45 °C. An increase in the yield of flavonoid was be achieved with the increase of extraction time at lower temperatures. The prolonged extraction time increases the chances of decomposition of phenolics (Belwal et al. 2016). The highest TFC (3.76 mgQE/gdw) was obtained at 30°C for 180 min (Run 13, Table 2). The increase in extraction temperature might be increased mass transfer improved with penetration of solvent into the plant matrix (Belwal et al. 2016) Interestingly, a negative interaction between extraction time and temperature (A*B) was obtained for the TFC. It may be attributed that the long extraction time, and sustained exposure to the high temperature increase the loss of phenolic compounds by oxidation (Al-Farsi and Lee, 2008).

After optimization, an improvement in antioxidant capacities was expected. The DPPH scavenging activity and ferric reducing power capacity were found as 0.073 mg (dw)/mL and 1.09 mg (dw)/mL, respectively. The finding implies that optimization of extraction conditions increases phenolic compounds, which have high antioxidant capacity.

Validation and Determination of Optimal Conditions for Extraction of Phenolic Compounds

The optimal conditions were determined by maximizing the desirability of the responses (TPC and TFC yields) using Design Expert software, and the validity of models was evaluated. These optimal conditions were used for the extraction process. The optimum conditions for TPC and TFC in a single experiment were extraction time (168 min), extraction temperature (48.5 °C), and liquid/solid ratio (41:1). The predicted TPC and TFC values under optimal conditions were 7.25 mg GAE/gdw and 3.72 mg QE/gdw, respectively. These predictions were validated by experiments in which 7.17 mg GAE/gdw of TPC and 3.63 mg QE/gdw of TFC were obtained. Experimental results for TPC and TFC values are in agreement with the predicted values.

HPLC Analyses of Phenolic Compounds

A. vulgaris mostly comprise phenolic acids (ellagic acid, gallic and chlorogenic acid, *p*-Hydroxybenzoic acid), flavonoids (catechin, quercetin) and flavonoid glycosides (rutin, avicularin, and tiliroside), as reported in the previous studies (Duckstein *et al.*, 2012; Boroja et al, 2018; Vlaisavljević et al., 2019). In this study, five phenolic compounds in the extracts obtained before and after optimization were analyzed using HPLC. The concentration of all phenolic

compounds evaluated in this study increased significantly after optimizing the extraction conditions (Table 5). Especially ellagic acid increased from 171.92 to 3724.65 µg/g dw. Zhang et al., (2010) found that the extraction conditions have a significant effect on the extraction yield of ellagic acid from *Platycarya strobilacea*. They found that the ellagic acid yield increased in parallel to extraction time, liquid/solid ratio and extraction temperature. Similary, the present study showed that extraction time, temperature and liquid/solid ratio positively influenced TPC.

The order of phenolic compounds were; ellagic acid> catechin>chlorogenic acid>gallic acid > p-hydroxybenzoic acid (Table 5). Our finding is in line with the previous studies showing that ellagic acid is the main phenolic component in leaves of *A. vulgaris* (Møller et al., 2009; Neagu et al., 2015; Ilić-Stojanović et al., 2017; Boroja et al, 2018). On the contrary, the 80% acetone extract from aerial parts of *A. glabra* was found to contain gallic acid (63mg/100g), 3,4-hydroxybenzoic acid (135mg/100g), chlorogenic acid (80mg/100g), catechin (250 mg/100 g), epicatechin (524 mg/100 g) and rutin (1057 mg/100 g) but not ellagic acid (Denev et al.2014).

Table 5. The concentrations of selected phenolic compounds in *A. vulgaris* extracts obtained after and before optimization of extraction conditions

Compound	Retention time (min)	UV band (nm)	Concentration* (µg/g dw)	Concentration ** (µg/g dw)
Gallic acid	6,8	280	0.65	11.89
Chlorogenic acid	15,7	280	78.65	109.6
p-hydroxybenzoic acid	18,2	320	2.98	8.41
Ellagic acid	47,7	240	171.92	3724.65
Catechin	15	280	88.77	137.64

*Concentrations of phenolic compounds in extract obtained before optimization
** Concentrations of phenolic compounds in extract obtained after optimization

As a result, the study shows that different extraction conditions affect the concentrations of phenolic compounds. In addition, *A. vulgaris* extracts seem to have high and rich phenolic content. The biological activity of *A. vulgaris* extracts may be attributed to their phenolic profile.

Conclusion

The results of the study indicate that solvents play an important role in the extraction, and acetone-water (50%) was the most efficient solvent for phenolic extraction from *A. vulgaris* leaves. The response surface methodology was successfully employed to optimize the extraction conditions (extraction temperature, time, and liquid/solid-ratio). It was observed that TPC yields significantly affected by liquid/solid ratio, extraction time, and temperature, while the most important factors affecting TFC yield were extraction time and temperature. The optimal extraction conditions were found to be extraction temperature of 48.5 °C, time of 168 min, and liquid/solid ratio of 41:1. The TPC and TFC values under optimal conditions were 7.17 mg GAE/gdw and 3.63 mg QE/gdw, respectively. In addition, five phenolic compounds were quantified in extracts by HPLC. Concentrations of all phenolic compounds evaluated in this study are found to increase after optimization of extraction conditions. The study confirmed the high phenolic content of extracts from *A. vulgaris* parallel with high antioxidant activity.

The results suggest that optimizing the extraction conditions is critical for accurate quantification of phenolic compounds in *A. vulgaris*. This study can be useful to the development of industrial extraction processes to enhance the efficacy of a large-scale extraction system.

CONFLICT OF INTEREST: The author expresses no conflict of interest associated with this work.

REFERENCES

Acet, T., Özcan, K. (2018). Aslanpençesi (*Alchemilla ellenbergiana*) Ekstrelerinin Antioksidan ve Antimikrobiyal Özelliklerinin Belirlenmesi. *GUFBED*, 8(1): 113-121.

Ahmadian-Kouchaksaraie, Z., Niazmand, R., Najafi, M.N. (2016). Optimization of the subcritical water extraction of phenolic antioxidants from *Crocus sativus* petals of saffron industry residues: Box-Behnken design and principal component analysis. *Innov Food Sci Emerg Technol*, 36: 234-244, doi: 10.1016/j.ifset.2016.07.005.

Ahmed, M.I., Xu, X., Sulieman, A.A., Mahdi, A.A., Na, Y. (2019). Effect of extraction conditions on phenolic compounds and antioxidant properties of koreeb (*Dactyloctenium aegyptium*) seeds flour. *J Food Meas Charact*, 1-10, doi.org/10.1007/s11694-019-00328-9.

Al-Farsi, M.A., Lee, C.Y. (2008). Optimization of phenolics and dietary fibre extraction from date seeds. *Food Chem*, 108 (3): 977-985, doi: 10.1016/j.foodchem.2007.12.009.

Al Jitan, S., Alkhoori, S.A., Yousef, L.F. (2018). Phenolic acids from plants: extraction and application to human health. *Stud. Nat Pro Chem*, 58, 389-417.

Belwal, T., Dhyani, P., Bhatt, I.D., Rawal, R.S., Pande, V. (2016). Optimization extraction conditions for improving phenolic content and antioxidant activity in *Berberis asiatica* fruits using response surface methodology (RSM). *Food Chem*, 207:115-124, doi: 10.1016/j.foodchem.2016.03.081.

Boroja, T., Mihailović, V., Katanić, J., Pan, S.P., Nikles, S., Imbimbo, P., Bauer, R. (2018).The biological activities of roots and aerial parts of *Alchemilla vulgaris* L. *S Afr J Bot*, 116: 175-184, doi: 10.1016/j.sajb.2018.03.007.

Borra, S.K., Gurumurthy, P., Mahendra, J. (2013). Antioxidant and free radical scavenging activity of curcumin determined by using different in vitro and ex vivo models. *J Med Plant Res*, 7(36): 2680-2690, doi: 10.5897/JMPR2013.5094.

Cacique, A. P., Barbosa, É. S., Pinho, G. P. D., Silvério, F. O. (2020). Maceration extraction conditions for determining the phenolic compounds and the antioxidant activity of *Catharanthus roseus* (L.) G. Don. *Ciência e Agrotecnologia*, 44: e017420, doi.org/10.1590/1413-7054202044017420.

Coklar, H., Akbulut, M. (2017). Anthocyanins and phenolic compounds of *Mahonia aquifolium* berries and their contributions to antioxidant activity. *J Funct Foods*, 35: 166-174.

Denev, P., Kratchanova, M., Ciz, M., Lojek, A., Vasicek, O., Blazheva, D., Nedelcheva, P., Vojtek, L., Hyrsl, P. (2014). Antioxidant, antimicrobial and neutrophil-modulating activities of herb extracts. *Acta Biochimica Plonica*, 61: 359–367.

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., Ju, Y.H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila* aromatica. *J Food Drug Anal*, 22(3): 296-302.

Duckstein, S.M., Lotter, E.M., Meyer, U., Lindequist, U. (2012). Phenolic Constituents from *Alchemilla vulgaris* L. and *Alchemilla mollis* (Buser) Rothm. at Different Dates of Harvest. *Z. Naturforsch., C. J. Biosci*, 67(11–12): 529–540, doi: 10.1515/znc-2012-11-1201.

Eruygur, N., Dincel, N. G. K., Kutuk, N. (2018). Modeling of Total Phenolic contents in Various Tea samples by Experimental Design Methods. *Open Chem J*, 16(1), 738-744, doi.org/10.1515/chem-2018-0082, doi.org/10.1515/chem-2018-0082.

Haminiuk, C.W.I., Plata-Oviedo, M.S.V., de Mattos, G., Carpes, S.T., Branco, I.G. (2014). Extraction and quantification of phenolic acids and flavonols from *Eugenia pyriformis* using different solvents. *J Food Sci Technol*, 51(10): 2862-2866, doi: 10.1007/s13197-012-0759-z.

Hwang, E., Ngo, H.T., Seo, S.A., Park, B., Zhang, M., Yi, T.H. (2018). Protective effect of dietary *Alchemilla mollis* on UVB-irradiated premature skin aging through regulation of transcription factor NFATc1 and Nrf2/ARE pathways. *Phytomedicine*, 39: 125-136.

Ilić-Stojanović, S., Nikolić, V., Kundaković, T., Savić, I., Savić-Gajić, I., Jocić, E., Nikolić, L.J. (2017). Thermosensitive hydrogels for modified release of ellagic acid obtained from *Alchemilla vulgaris* L. extract. *Int J Poly Mater*, 67(9): 553-563, doi: 10.1080/00914037.2017.1354202.

Møller, C., Hansen, S.H., Cornett, C. (2009). Characterisation of tannin-containing herbal drugs by HPLC. *Phytochem Anal*, 20: 231–239, doi: 10.1002/pca.1119.

Mouratoglou, E., Malliou, V., Makris, D.P. (2016). Novel glycerol-based natural eutectic mixtures and their efficiency in the ultrasound-assisted extraction of antioxidant polyphenols from agri-food waste biomass. *Waste Biomass Valorization*, 7(6): 1377-1387.

Murathan, Z.T. (2018). Kuzeydoğu Anadolu Bölgesi ekolojik koşullarında yetişen bazı tıbbi bitkilerin biyokimyasal içeriği ve antioksidan özelliklerinin belirlenmesi. *Balıkesir Üniv Fen Bilim Enst Derg*, 20(2): 51-60.

Nasr, A., Zhou, X., Liu, T., Yang, J., Zhu, G.P. (2019). Acetone-water mixture is a competent solvent to extract phenolics and antioxidants from four organs of *Eucalyptus camaldulensis*. *Turkish J Biochem*, 44(3): 231-239, doi: 10.1515/tjb-2018-0438.

Neagu, E., Paun, G., Albu, C., Radu, G.L. (2015). Assessment of acetylcholinesterase and tyrosinase inhibitory and antioxidant activity of *Alchemilla vulgaris* and *Filipendula ulmaria* extracts. *J Taiwan Inst Chem Eng*, 52: 1–6, doi: 10.1016/j.jtice.2015.01.026.

Oyaizu, M. (1986). Studies on Product of Browning Reaction: Antioxidative Activities of Products of Browning Reaction Prepared from Glucoseamine. *Japanese J Nutr Diet*, 44(6): 307-315.

Park, B. I., Kim, J., Lee, K., Lim, T., Hwang, K. T. (2019). Flavonoids in common and tartary buckwheat hull extracts and antioxidant activity of the extracts against lipids in mayonnaise. J *Food Sci Technol*, 56(5): 2712-2720.

Pham, N.M.Q., Vuong, Q.V., Bowyer, M.C., Scarlett, C.J. (2019). Optimization of ultrasound-assisted extraction conditions for phenolic compounds and antioxidant capacity from Tuckeroo (*Cupaniopsis anacardioides*) fruit. *Sep Sci Tech*, 55(17): 3151-3160, doi: 10.1080/01496395.2019.1673413.

Rebey, I.B., Bourgou, S., Debez, I.B.S., Karoui, I.J., Sellami, I.H., Msaada, K., Marzouk, B. (2012). Effects of extraction solvents and provenances on phenolic contents and antioxidant activities of cumin (*Cuminum cyminum* L.) seeds. *Food Bioprocess Tech*, 5(7): 2827-2836.

Renda, G., Özel, A., Barut, B., Korkmaz, B., Šoral, M., Kandemir, Ü., Liptaj, T. (2017). Bioassay Guided Isolation of Active Compounds from *Alchemilla barbatiflora* Juz. *Rec Nat Prod*, 12: 1-7.

Sánchez-Moren, C., Plaza, L., de Ancos, B., Cano, M.P. (2003). Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. *J Sci Food Agric*, 83: 430–439.

Shrivastava, R., Cucuat, N., John, G. W. (2007). Effects of *Alchemilla vulgaris* and glycerine on epithelial and myofibroblast cell growth and cutaneous lesion healing in rats. *Phytother Res*, 21(4): 369-373.

Sepahpour, S., Selamat, J., Abdul Manap, M.Y., Khatib, A., Abdull Razis, A.F. (2018). Comparative analysis of chemical composition, antioxidant activity and quantitative characterization of some phenolic compounds in selected herbs and spices in different solvent extraction systems. *Molecules*, 23(2): 402, doi: 10.3390/molecules23020402.

Shelembe, J.S., Cromarty, D., Bester, M.J., Minnaar, A., Duodu, K.G. (2012). Characterisation of phenolic acids, flavonoids, proanthocyanidins and antioxidant activity of water extracts from seed coats of marama bean [*Tylosema esculentum*]–an underutilised food legume. *Int J Food Sci Tech*, 47(3): 648-655.

Shi, J., Yu, J., Pohorly, J., Young, J.C., Bryan, M., Wu, Y. (2003). Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution. *J Food Agric Environ*, 1(2): 42-7.

Silva, E.M., Rogez, H., Larondelle, Y. (2007). Optimization of extraction of phenolics from Inga edulis leaves using response surface methodology. *Sep Pur Tech*, 55(3): 381-387.

Sousa, M.S.B., Júnior, J.M.L., de Souza Buarque, D. (2019). Optimization of the extraction of polyphenols and antioxidant capacity from *Byrsonima crassifolia* (L.) kunth fruit by response surface methodology. In Plant Physiological Aspects of Phenolic Compounds. *IntechOpen*, doi:10.5772/intechopen.83457.

Tasić-Kostov, M., Arsić, I., Pavlović, D., Stojanović, S., Najman, S., Naumović, S., Tadić, V. (2019). Towards a modern approach to traditional use: in vitro and in vivo evaluation of *Alchemilla vulgaris* L. gel wound healing potential. *J Ethnopharmacol*, 238: 111789, doi: 10.1016/j.jep.2019.03.016.

Tusevski, O., Kostovska, A., Iloska, A., Trajkovska, L., Simic, S.G. (2014). Phenolic production and antioxidant properties of some Macedonian medicinal plants. *Cent Eur J Biol*, 9(9): 888-900, doi: 10.2478/s11535-014-0322-1.

Usta, C., Yildirim, A.B., Turker, A.U. (2014). Antibacterial and antitumour activities of some plants grown in Turkey. *Biotech & Biotech Equip*, 28(2): 306-315.

Uysal, S., Cvetanović, A., Zengin, G., Zeković, Z., Mahomoodally, M.F., Bera, O. (2019). Optimization of maceration conditions for improving the extraction of phenolic compounds and antioxidant effects of *Momordica Charantia* L. leaves through response surface methodology (RSM) and artificial neural networks (ANNs). *Anal Lett*, 52(13): 2150-2163. doi:10.1080/00032719.2019.1599007.

Vlaisavljević, S., Jelača, S., Zengin, G., Mimica-Dukić, N., Berežni, S., Miljić, M., Stevanović, Z.D. (2019). *Alchemilla vulgaris* agg.(Lady's mantle) from central Balkan: antioxidant, anticancer and enzyme inhibition properties. *RSC Advances*, 9(64): 37474-37483.

Yazici Özbek, S., Ozmen, I., Yildirim, B., Genc, H., Ozeloglu, B., Gülsün, M., Ozcaka, S. (2020). Biochemical Composition of *Lathyrus* L. Seeds: Antioxidant Activities, Phenolic Profiles, *β-ODAP* and Protein Contents. *Legum Res*, 43: 723-727, doi: 10.18805/LR-516.

Yılmaz, Y., Toledo, R.T. (2006). Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. *J Food Comp Anal*, 19(1): 41-48, doi: 10.1016/j.jfca.2004.10.009.

Zhang, L.L., Xu, M., Wang, Y.M., Wu, D.M., Chen, J.H. (2010). Optimizing ultrasonic ellagic acid extraction conditions from infructescence of *Platycarya strobilacea* using response surface methodology. *Molecules*, 15(11):7923-7932, doi: 10.3390/molecules15117923.

Zhao, H.X., Zhang, H.S., Yang, S.F. (2014). Phenolic compounds and its antioxidant activities in ethanolic extracts from seven cultivars of Chinese jujube. *Food Sci Humn Well*, *3*(3-4), 183-190.

Zhu, D., Wang, C., Zhang, Y., Yang, Y., Shang, Y., Niu, X., Wei, Z. (2018). Insight into solvent effects on phenolic content and antioxidant activity of bamboo leaves extracts by HPLC analysis. *J. Food Meas Charact*, 12(3): 2240-2246, doi: 10.1007/s11694-018-9840-2