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Research Article

Optimization of Ultrasound-Assisted Phenolic Extraction from Red Pepper Seed by Response Surface Methodology

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Abstract

In this study, phenolic compounds of Kahramanmaraş red pepper (*Capsicum annuum* L.) seeds which are waste products from red pepper processing line were extracted by ultrasound-assisted (UAE) and conventional solvent extraction (SE) methods. Two different extraction methods were evaluated in terms of total phenolic contents (TPC) and antioxidant capacity. Response surface methodology (RSM) was used to optimize UAE conditions of phenolic extracts including extraction temperature (40, 50 and 60 °C), extraction time (20, 40 and 60 min) and solvent volume (50, 75 and 100 mL) for obtaining maximum total phenolics with higher antioxidant capacity. Effects of temperature, time and volume of solvent on extraction of phenolics were evaluated. As a result of RSM analysis, the optimum conditions determined as an extraction temperature of 51 °C, an extraction time of 60 min, and solvent volume of 50 mL. The results showed that ultrasonic treatment was more effective than conventional extraction method on phenolic extraction yield.

Key words: Antioxidant capacity, extraction, red pepper seed, response surface method, total phenolic content, ultrasonic.

Kırmızı Biber Tohumundan Ultrason Destekli Fenolik Ekstraksiyonunun Tepki Yüzey Metodu ile Optimizasyonu

Özet

Bu çalışmada, kırmızı biberlerin işlenmesi sonucu atık ürün olarak elde edilen Kahramanmaraş kırmızı biber (*Capsicum annuum* L.) çekirdeklerinin içerdiği fenolik bileşenler, ultrason-destekli ve geleneksel çözücü ekstraksiyon yöntemleri ile ekstrakte edilmiştir. İki farklı ekstraksiyon yöntemi, toplam fenolik içerik ve antioksidan kapasite yönünden değerlendirilmiştir. Daha yüksek antioksidan kapasite ile maksimum toplam fenolik içeriği elde etmek amacıyla, fenolik ekstrakların, ekstraksiyon sıcaklığı (40, 50 ve 60 °C), ekstraksiyon süresi (20, 40 ve 60 dk.) ve çözücü hacmi (50, 75 ve 100 mL) aralığını içeren ultrason-destekli ekstraksiyon koşullarını optimize etmek için tepki yüzey yöntemi kullanılmıştır. Fenoliklerin ekstraksiyonuna, çözücü hacmi, ekstraksiyon sıcaklığı ve süresinin etkileri değerlendirilmiştir. Tepki yüzey yöntemi sonuçlarına göre, optimum koşullar, 51 °C ekstraksiyon sıcaklığı, 60 dakika ekstraksiyon süresi ve 50 mL çözücü hacmi olarak belirlenmiştir. Fenolik bileşenlerin ekstraksiyonu üzerine ultrason uygulamasının, geleneksel yönteme göre daha etkili olduğu görülmüştür.

Anahtar Kelimeler: Antioksidan kapasite, ekstraksiyon, kırmızı biber çekirdeği, tepki yüzey yöntemi, toplam fenolik içerik, ultrason.

Introduction

In recent years, the assessment of food processing line wastes and by-products become a new area of interest with the growth and development of the food industry (Yılmaz, 2011; Arsunar, 2014). Red peppers grown in large quantities in Turkey and especially in Kahramanmaraş region are commercially important products. During the processing of red peppers, the seeds come out in high quantities. Red pepper seeds contain 9.30% moisture, 19.32% fat, 23.64% protein, 3.55% ash and 48.98% carbohydrate on dry basis. And also, red pepper seeds are rich in phenolic compounds as ellagic acid (2.001 mg g^{-1}), gallic acid (0.053 mg g^{-1}), 3,4-hydroxy benzoic acid (0.165 mg g^{-1}), epicatechin (0.886 mg g^{-1}) ve ferulic acid (0.208 mg g^{-1}). Red pepper seeds have a strong antioxidant activity (Fıratlıgil Durmuş, 2008). At this point, it is important to evaluate the seeds as a new, alternative and inexpensive source of the bioactive compounds and to be regained to food industry as a new way of using red peppers (Silva et al., 2013). In recent years, they are very important compounds due to their positive effects on health and their alternative use as natural antioxidants (Cavuldak et al., 2016). Extraction of polyphenols is complicated by insoluble structures such as vacuoles, cell walls and lipoprotein bilayers and polyphenols are enclosed in these structures (Corrales et al., 2008). Conventional extraction of secondary metabolites from seeds is difficult by these factors. To overcome these problems, ultrasonic treatment has gained increasing popularity. In the ultrasonic method, acoustic vibrations are applied to the sample with frequencies above 20 kHz and these vibrations cause cavitation effect in the liquid environment. This effect, also known as cavitation, leads to the formation of bubbles and mechanical shaking of the solids, thereby enabling the separation of particles (Büyüktuncel, 2012). Thus, the ultrasonic application mechanically breaks the cell walls and transfers material, since the cell wall is removed the extraction process is faster than other extraction methods (Bayraktaroğlu and Obuz, 2006). Ultrasonic-assisted extraction methods have benefits with industrial applications economically, and it is used to enhance extraction yield and to reduce the usage of organic solvent and extraction time (Tiwari, 2015). Response surface methodology (RSM) can be used for optimization of polyphenols yield from seeds. It contributes to reduce the number of experiments and provide mathematical and statistical models (Li et al., 2015).

However, there is no studies that focused on the optimization of polyphenolic content and antioxidant capacity of extracts from red pepper *(Capsicum annuum* L.) seeds. The objectives of this study were to (a) observe the effectiveness of UAE on the TPC and antioxidant capacity of extracts from red pepper seed and compare to the SE method, (b) to optimize UAE conditions of phenolic extracts to obtain maximum polyphenol yield and antioxidant capacity, (c) to determine the effects of UAE solvent volume, extraction temperature and time on total phenolic content and antioxidant capacity by Box-Behnken Design (BBD) of RSM.

Materials and Methods

Materials

For the analyses, the analytical grade chemicals were used and these were supplied from Sigma Chem. Co. (St. Louis, MO, USA). Red pepper (*Capsicum annuum* L.) seeds were obtained from a red pepper plant located in the Kahramanmaraş region during the processing period of 2015 as waste products. Sun dried red pepper seeds had moisture 5.57%. Before extraction the seeds were milled into powder for 1 min by using an electric grinder.

Methods

Ultrasonic-assisted extraction

The prepared seed powder (5 g) was extracted with methanol in a blue cap bottle. The UAE was performed in an ultrasonic bath (UC-10, Jeiotech, Seoul, Korea) with a fixed power (330 W) and 40 kHz frequency. Various experimental conditions such as solvent volume (50, 75, 100 mL), extraction temperatures (40, 50, 60 °C) and time (20, 40, 60 min) were carried out for extraction procedure. The extracts were filtered through filter paper (0.45 µm, Whatman) by vacuum. Filtrates were stored at 4 °C in the bottles that covered with aluminum foil until analysis (Teh and Birch, 2014).

Conventional extraction

Conventional extractions were carried out in a shaking water bath at 200 rpm at room temperature (25 °C) (SE25) and at 51 °C (SE51) for 60 min. Due to compare ultrasonic extraction, similar conditions were performed for conventional extractions.

Measurements of total phenolic content in seed extracts

The Folin–Ciocalteu reagent was used to determine the total phenol contents (TPC) of seed extracts spectrophotometrically by measuring the absorbance at 760 nm. TP was expressed as mg of gallic acid per 100 g of seed (Singleton and Rossi, 1965).

DPPH radical scavenging assay

The antioxidant activity of seed extracts was measured by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay that was based on the method of Brand-Williams et al. (1995) with some modifications. The variation of free radical scavenging activity by the time was carried out by reaction using DPPH in methanol at 515 nm by spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The antioxidant activity was calculated as the percent of inhibition by using the equation (1):

$$\% \text{ Inhibition} = \left[1 - \left(\frac{A_{sample}}{A_{control}}\right)\right] x100 \qquad (1)$$

Response surface optimization of UAE

In this study, RSM was used to determine the optimum conditions of extractions of polyphenols from seed extracts. The Box-Behnken trial design was performed at three levels with three independent variables by using the Design Expert software program (Version 10; Stat-Ease, Inc., Minneapolis, MN, USA). According to the polynomial equation obtained from RSM was given as follows (1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_2 + b_{23}X_2X_3 \qquad (1)$$

Where *y* is the response variable (TPC and % inhibition of DPPH); b_0, b_1, b_2, b_3… are the regression coefficients ; and X_1, X_2, X_3 are the non-coded values for solvent volume, temperature and time, respectively (Table 1).

17 experimental runs were carried out by Box-Behnken trial design and the responses of TPC and % inhibition of DPPH using UAE are given in Table 2. ANOVA was used to analyze the data due to determine the lack of fit and the effects of variables and their interactions on TPC and % inhibition of DPPH.

Statistical analysis

All measurements were repeated three times. The results were expressed as mean values and standard deviations. The data were statistically compared by Tukey's honestly significant difference Duncan test (SPSS v.23, IBM, USA). Statistical significance was accepted at a level of $p < 0.05$.

Table 1. Levels and ranges of independent variables used to measure TPC and % inhibition of DPPH**.**

Variables	Variable codes	Levels		
		-1	0	+1
Solvent volume (mL)	**X_1**	50	75	100
Extraction temperature (°C)	**X_2**	40	50	60
Extraction time (min)	**X_3**	20	40	60

Table 2. The responses of TPC and % inhibition of DPPH based on BBD model using UAE.

Run	Solvent volume (mL)	Extraction temperature (°C)	Extraction time (min)	Total phenol content (mg GAE 100 g^{-1})		DPPH %	
	X_1	X_2	X_3	Experimental	Predicted	Experimental	Predicted
1	100	40	40	147.9	140.25	21.5	21.09
2	100	50	20	193.11	203.17	25.55	24.49
3	50	50	60	363.67	353.61	47.89	48.95
4	75	60	20	165.3	154.30	34.41	33.74
5	50	40	40	225.81	224.87	44.67	42.94
6	100	60	40	141.53	142.47	27.16	28.89
7	100	50	60	215.59	212.23	29.38	29.17
8	50	60	40	249.92	257.57	48.52	48.98
9	50	50	20	258.14	261.50	46.48	46.75
10	75	40	20	136.11	134.66	28.19	29.71
11	75	40	60	176.42	187.42	29.58	30.25
12	75	50	40	213.95	223.67	38.43	40.13
13	75	50	40	262.01	223.67	39.07	40.13
14	75	50	40	207.85	223.67	40.64	40.13
15	75	50	40	230.09	223.67	44.67	40.13
16	75	50	40	204.47	223.67	37.83	40.13
17	75	60	60	199.33	201.74	41.63	40.11

Results and Discussion

Response surface analysis of total phenolic content

The ANOVA results of total phenolic content are represented in Table 3. The model was statistically significant with the model F-value (12.58) and P value ($p<0.05$). Values of "Prob > F" less than 0.05 revealed that the model terms as solvent volume (X_1), extraction time (X_3), square effect of solvent volume (X_1^2) and square effect of extraction temperature (X_2^2) were significant for TPC. The model correlation coefficient R^2 was 0.9418. In this case, the model was well matched with the experimental data. The "Lack of Fit p-value" of 0.7874 suggested that the Lack of Fit is not significant. The non-significant lack of fit value shows the fitness of the model. As a result of the experimental data subjected to regression analysis, the following second order polynomial model equation (Eq.(2)) was obtained for TPC.

$$Y_{TPC} = +223.67 - 49.93X_1 + 8.73X_2 + 25.29X_3 - 7.62X_1X_2 - 20.76X_1X_3 - 1.57X_2X_3 + 27.98X_1^2 - 60.36X_2^2 + 5.98X_3^2 \quad (2)$$

According to the absolute value of the coefficients, the order of factors affecting the response value of TPC was determined as solvent volume > extraction time > extraction temperature. The effect of solvent volume and extraction time on the TPC as a result of regression analysis was found to be significant ($p<0.05$). Three-dimensional (3D) response surface plots are useful for the determination of optimal point of responses and the identification of the binary interactions between the process variables.

Table 3. ANOVA statistics for TPC.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	45646.82	9	5071.87	12.58	0.0015*
X_1-solvent vol	19941.04	1	19941.04	49.46	0.0002*
X_2-temp.	609.70	1	609.70	1.51	0.2585
X_3-time	5118.19	1	5118.19	12.70	0.0092*
X_1X_2	232.26	1	232.26	0.58	0.4726
X_1X_3	1724.33	1	1724.33	4.28	0.0774
X_2X_3	9.86	1	9.86	0.024	0.8801
X_1^2	3295.57	1	3295.57	8.17	0.0244*
X_2^2	15340.72	1	15340.72	38.05	0.0005*
X_3^2	150.41	1	150.41	0.37	0.5606
Residual	2822.04	7	403.15		
Lack of fit	597.48	3	199.16	0.36	0.7874
R^2=0.9418					

*p<0.05 significant; p>0.05 not significant

The combined effects of three independent variables on the TPC as a result of the regression analysis was not statistically significant ($p>0.05$). Fig. 1a, Fig. 1b and Fig. 1c show the combined effects of extraction temperature-solvent volume, extraction temperature-time and solvent volume-extraction time on the TPC, respectively. The total effects of all binary interactions on TPC were found to be negative.

The experimental values for phenolic content (TPC) were ranged from 136.11 to 363.67 mg GAE 100 g^{-1}. Most of the phenolic compounds exhibit a wide range of biological activities such as antioxidant, antifungal, antibacterial, antitumor and antiviral. Sora et al. (2015) reported that the phenolic contents of the peppers (pulp and seed) of the genus *Capsicum* ranged from 119.97 ± 3.44 to 2060.12 ± 20.56 mg GAE 100 g^{-1}. Gurnani et al., (2016) reported that the amounts of the total phenolic content for *C. frutescens* seeds were in the ranges of 7.95–26.15 gallic acid equivalents (GAE mg/g) of (hexane-chloroform) extracts.

Response surface analysis of DPPH radical scavenging activity

The ANOVA results of DPPH radical scavenging activity are represented in Table 4. The model was statistically significant with the model F-value (19.63) and P value ($p<0.05$). Values of "Prob > F" less than 0.05 revealed that the model terms as solvent volume (X_1), extraction temperature (X_2), and square effect of extraction temperature (X_2^2) were significant for DPPH radical

scavenging activity. The second order polynomial model for DPPH radical scavenging activity was shown in equation (Eq.(3)) obtained as a result of regression analysis.

$$Y_{DPPH} = +40.13 - 10.50X_1 + 3.47X_2 + 1.73X_3 + 0.45X_1X_2 + 0.60X_1X_3 + 1.46X_2X_3 - 0.40X_1^2 - 4.27X_2^2 - 2.41X_3^2 \quad (3)$$

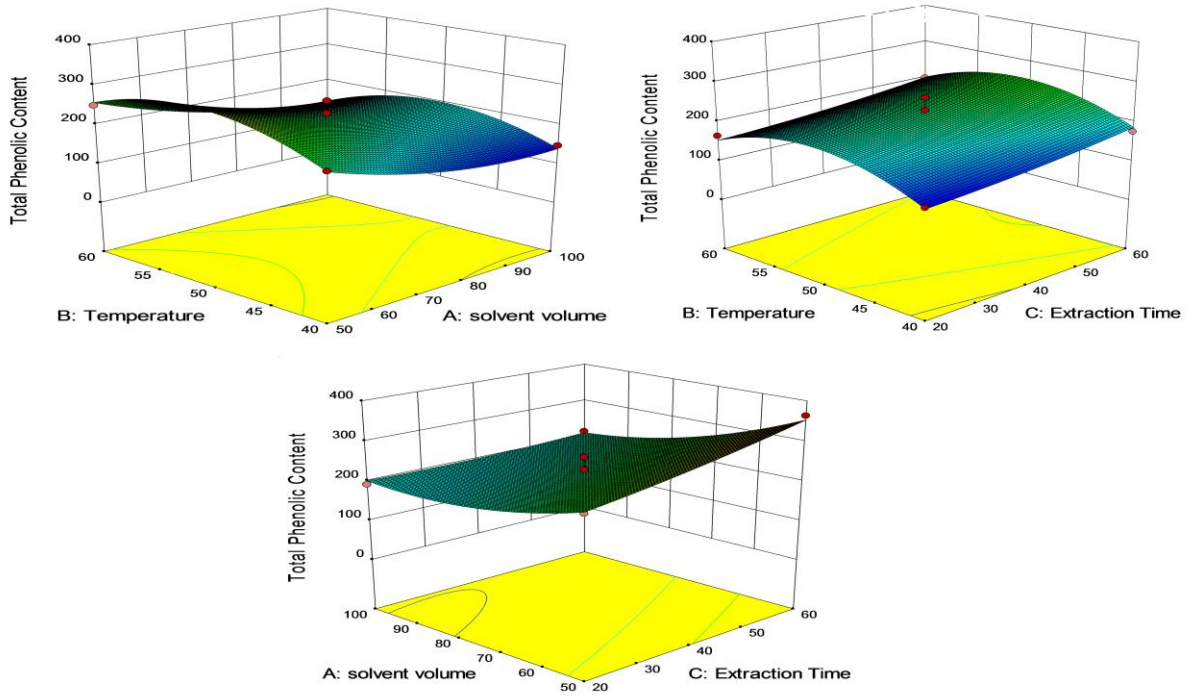

Figure 1. Response surface plot for TPC as a function of (a) solvent volume and temperature (b) extraction time and temperature (c) solvent volume and extraction time.

According to the absolute value of the coefficients, the order of factors affecting the response value of DPPH radical scavenging activity % was determined as solvent volume > extraction temperature > extraction time. The combined effects of three independent variables on the % inhibition of DPPH radical scavenging activity as a result of the regression analysis was not statistically significant ($p>0.05$) (Fig. 2a). Fig. 2b shows the combined effect of extraction temperature and time and Fig. 2c shows the combined effect of extraction time and solvent volume on the % inhibition of DPPH. The total effects of all binary interactions on % inhibition of DPPH were found to be positive.

Table 4. ANOVA statistics for DPPH scavenging activity.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	1121.05	9	124.56	19.63	0.0004
X_1-*solvent vol*	881.37	1	881.37	138.87	< 0.0001
X_2-*temp.*	96.47	1	96.47	15.20	0.0059
X_3-*time*	23.98	1	23.98	3.78	0.0930
X_1X_2	0.82	1	0.82	0.13	0.7300
X_1X_3	1.46	1	1.46	0.23	0.6457
X_2X_3	8.50	1	8.50	1.34	0.2852
X_1^2	0.66	1	0.66	0.10	0.7562
X_2^2	76.73	1	76.73	12.09	0.0103

X_3^2	24.38	1	24.38	3.84	0.0908
Residual	44.43	7	6.35		
Lack of fit	14.25	3	4.75	0.63	0.6332
R^2=0.9619					

*p<0.05 significant; p>0.05 not significant

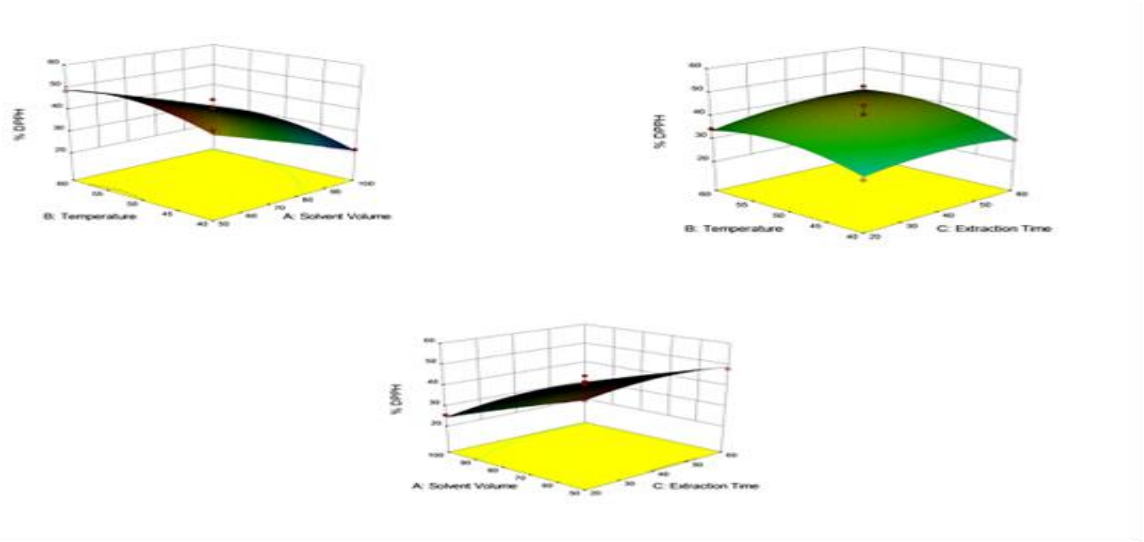

Figure 2. Response surface plot for DPPH scavenging activity as a function of (a) solvent volume and temperature (b) extraction time and temperature (c) solvent volume and extraction time.

The experimental values for DPPH scavenging activity were ranged from 21.5 to 48.52%. For evaluating radical scavenging effects of natural antioxidants, 1,1-Diphenyl-2-picrylhydrazyl that is a free radical donor is widely used (Matsukawa et al., 1997; Jao and Ko, 2002).The correlations between the contents of total phenolics and the antioxidant activities obtained in this study were highly similar to Sora et al. (2015). Gurnani et al., (2016) reported that the in vitro antioxidant activity of *C. frutescens* seeds via DPPH assay, n-hexane and chloroform extracts showed 26.9% and 30.9% free radical scavenging abilities, respectively, at the concentration of 1 mg/mL and considering these results, *C. frutescens* seeds can be used as a source of novel antimicrobial and antioxidant compounds. Sim and Sil, 2008 reported that the scavenging effect of red pepper seed was only 13% at 100 µg mL^{-1} concentration. Many studies reported that free radical scavenger effects of red pepper are because of red pepper seed.

Optimization of the conditions

The optimum conditions for TPC and % inhibition of DPPH values were determined as extraction temperature of 51 °C, extraction time of 60 min, and solvent volume of 50 mL The experimental value results were validated with the predictive values.

Comparison between ultrasonic-assisted and conventional solvent extraction of red pepper seed

The results showed that higher extraction yield of total phenolics was observed with UAE compared to SE (Fig. 3). Carrera et al., (2012), found same effects of ultrasound treatment on extraction of phenolic compounds from grapes. Total phenolics of ultrasonicated seed extract were 336.30±3.15 mg GAE 100 g^{-1}. Total phenolics of conventional seed extracts were 75.20±2.96 and 262±2.21 mg GAE 100 g^{-1}, respectively. TPC of seed extracts by UAE and SE were significantly different at p<0.01. Ultrasound treatment increased mass transfer and recovery yield of bioactive compounds. It was an alternative for conventional extraction techniques. In a study, optimum conditions for protein extraction were investigated for red pepper *(Capsicum frutescens)* seed meal by using RSM (Fıratlıgil Durmuş, 2008). In another study, the antimicrobial and antioxidant activities of *Capsicum frutescens* L. seeds were determined. In a study, the TPC of the crude extracts of *Capsicum frutescens* L. seeds was found to vary between 7.95 and 26.15 mg GAE g^{-1} of dry weight of extract (Gurnani et al., 2016). The differences from the previously reported values may be due to several factors such as differences in harvest year, climatic conditions, maturity stage of fruits, kind of

solvents, extraction methods, extraction conditions (time, temperature, solvent to solid ratio, etc.), locality, and cultivar of fruits (Menichini et al., 2009; Zhuang et al., 2012).

% inhibition of DPPH value for ultrasonication extracts was higher than the conventional ones. Ultrasonicated seed extract exhibited % inhibition of DPPH of 54.50% and the conventional seed extracts exhibited % inhibition of DPPH of 38.72% and 50.62%, respectively (Fig. 4). Silva et al. (2013), assessed the antioxidant activity of aqueous extracts from *C. annuum* seeds against DPPH*.''Sweet Italian'' seeds showed IC25 = 0.413 mg mL^{-1} and ''Reus long pairal'' seeds showed IC25 > 18.2 mg mL^{-1} capacity to scavenge DPPH. In this study, UAE optimization for maximum extraction of phenolic compounds with high antioxidant activities exhibited better results than previously reported studies on TPC (267.3 mg GAE 100 g^{-1}) and 47% radical scavenging activity from the ethanol extracts of red pepper (*Capsicum frutescens*) seed (Fıratlıgil Durmuş, 2008).

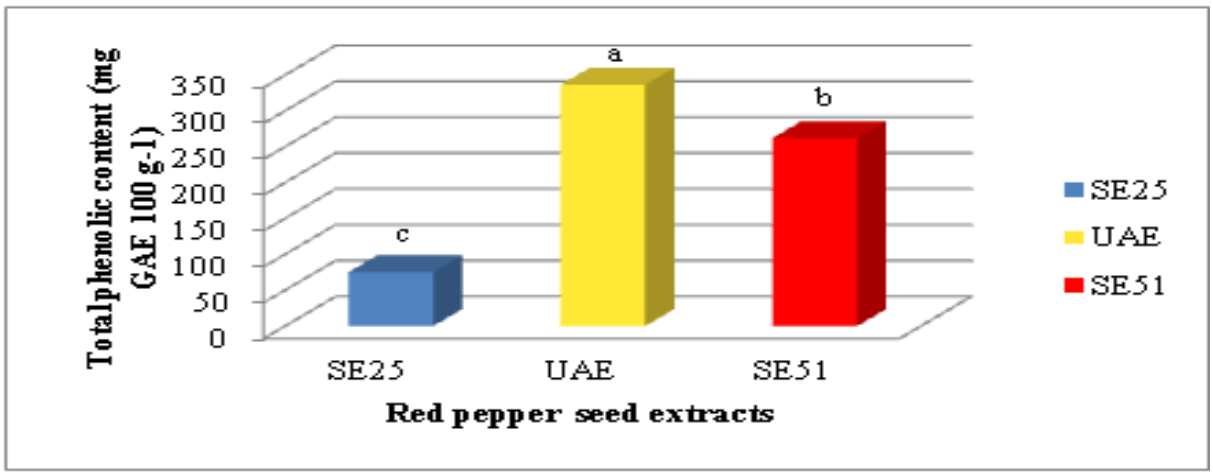

Figure 3. Total phenolic content of the seed extracts by ultrasonic treatment and the conventional extractions. (UAE : Ultrasonic-assisted extraction was carried out at optimum conditions (extraction temperature of 51°C), SE25 : Conventional extraction was carried out at room temperature (25 °C), SE51 : Conventional extraction was carried out at 51 °C). Values are expressed as mean±standard deviations for three (n=3) measurements. Lower case letters (a,b and c) within bars of the same sample with different extraction methods are highly significantly different at p<0.01.

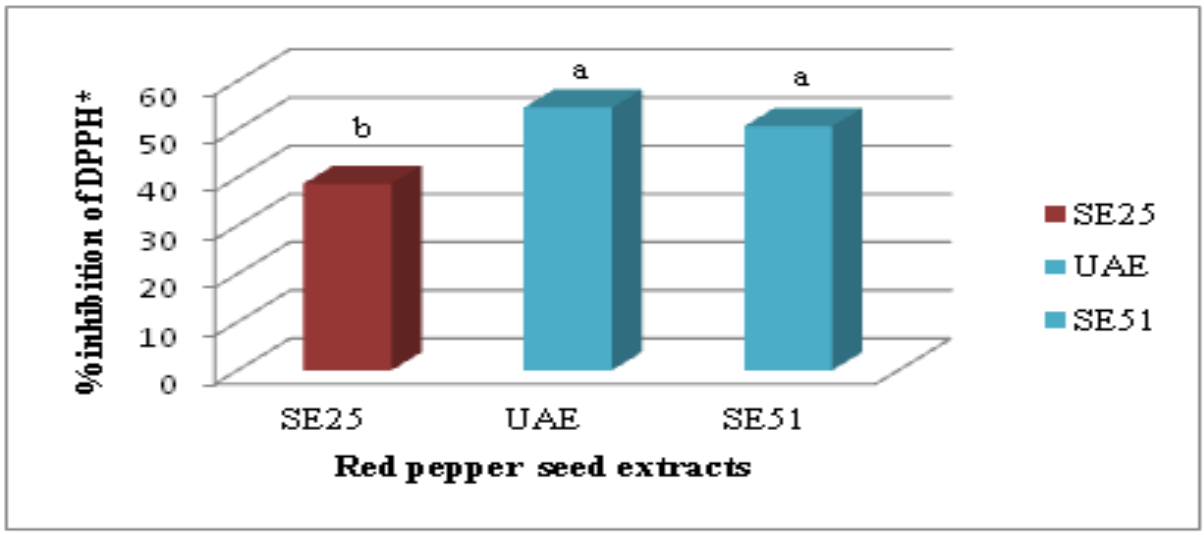

Figure 4. % inhibition DPPH* of the seed extracts by ultrasonic treatment and the conventional extractions. (UAE : Ultrasonic-assisted extraction was carried out at optimum conditions (extraction temperature of 51°C), SE25 : Conventional extraction was carried out at room temperature (25 °C), SE51 : Conventional extraction was carried out at 51 °C). Values are expressed as mean±standard deviations for three (n=3) measurements. Lower case letters (a-b) within bars of the same sample with different extraction methods are highly significantly different at p<0.01.

Conclusion

In this study, Maraş pepper seed can be a good potential food source due to its valuable content and chemical, biological, ecological importance. Further studies on seed's functional properties are needed. Conventional solvent and ultrasound-assisted solvent extraction methods have different advantages in terms of phenolic extraction yield and quality parameters. Total phenolic content at maximum yield is possible with shorter extraction time and less solvent usage by ultrasound-assisted solvent extraction. Ultrasound-assisted solvent extraction is a non-thermal modern technique that supplies consumer demands for greener alternatives and natural ingredients. This study is also of particular importance in the evaluation of waste products.

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