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Optimization Of Extraction Parameters For Folic Acid And Antioxidant Compounds From An Edible Plant (*Polygonum Cognatum Meissn*) Using Pressurized Liquid Extraction (PLE) System

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Abstract. Madimak *(Polygonum cognatum Meissn.)* plant is a wild edible plant with a cosmopolitan distribution and also traditionally consumed as food in anatolia. This study aims to optimize the extraction parameters in the pressurized liquid extraction (PLE) system and to search some beneficial properties of this plant. After the optimal extraction conditions was determined, the effect of PLE conditions on antioxidant properties was studied by using plant extracts in terms of total phenolic contents, free radical scavenging capacities and reducing power. A solvent including 1 M NaOH and water (1:99), 60 min of extraction time, 40 ^{o}C of extraction temperature and 1500 psi of pressure were optimized and found as the most productive operating conditions for PLE in the folic acid determination. Mainly, optimization of PLE conditions were carried out by considering folic acid amounts. Although antioxidant activity levels differ depending on solvent components, the similar results were obtained such in the other optimization parameters.

***Keywords*:** Folic acid, Pressurized Liquid Extraction, Polygonum cognatum Maissn, Antioxidant activity.

Yenilebilir Bir Bitki Olan *Polygonum Cognatum Meissn* dan Folik Asit ve Antioksidan Bileşiklerin Basınçlı Sıvı Ekstraksiyon (PLE) sistemi ile Ekstraksiyonu için Optimal Koşulların Belirlenmesi

Özet. Madımak **(***Polygonum cognatum Meissn)* bitkisi geleneksel olarak Anadolu'da gıda olarak tüketilen, kozmopolitan bir dağılıma sahip kendiliğinden yetişenbir bitkidir. Bu çalışma temelde bu bitkinin yararlı özelliklerini ortaya çıkarmak adına basınçlı sıvı ekstraksiyon sisteminin optimizasyonunu amaçlar. Optimal koşullar belirlendikten sonra, ekstraksiyon koşullarının antioksidan özellikler üzerine etkisi, toplam fenolik içerik, serbest radikal süpürücü etki ve indirgeme gücü özelinde değerlendirildi. İdeal optimizasyon koşullarının 1 M NaOH ve suyun (1:99) çözegen karışımı ile, 60 dk boyunca , 40 ^{o}C sıcalıkta, 1500 psi basınçta folik asit için en ideal olduğu belirlendi. Temelde, antioksidan aktivite için gerekli koşullar folik asit için yapılan optmizasyonlar dikkate alınarak belirlenmiştir. Elde edilen sonuçlara göre antioksidan aktivite seviyelerinin çözücü bileşiminden etkilendiği aynı şekilde diğer optimizasyon parametrelerin de değişkenlik gösterdiği gözlenmiştir.

***Anahtar Kelimeler*:** Folik Asit, Basınçlı Sıvı Ekstraksiyon Sistemi, Polygonum cognatum Maissn, Antioksidan aktivite.

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1. INTRODUCTION

Vitamins are important micronutrients in trace levels and are biologically active organic compounds that play a role in the metabolic and physiological functions of the human body. Folate term is a general identification for a mixture of folic acid (pteroylglutamate, PteGln) and conjugates. Folates are group B vitamins that are essential for DNA and RNA synthesis, the primary events for the cell. The main drawbacks in their deficiencies can be observed such as neural defects, coronary heart diseases, some types of cancer [1]. Folic acid is also known as Vitamin B9 and a very important vitamin in especially the pregnancy period. People need to take at least 0.4 mg of folic acid as average for per day [2–4]. Recent studies have shown that the best sources of folates are natural sources as vegetables, fruits and berries with folate content of 15–200 mg /100 g sample [5].

However, as a result of the literature reviews, it has been observed that complete and reliable data on the folate content are still lacking, especially in vegetable based foodstuffs. Particularly, there are still deficiencies in data on the content differences relative to food matrices, the growth area of food, the storage conditions and the effects on edible processing [6, 7]. Due to the inconsistencies of existing data on dietary folates in food databases and their wide variety of forms, the quantitative identification of folates and the difficulties associated with their characterization as instruments it is still contradictory [8-10]. Generally, high performance liquid chromatography (HPLC) is widely used which can be used with different detector systems for the detection of folates [11, 12].

As a source of vitamins and the other useful components, wild edible plants play a significant role in conventional diets. *Polygonum Cognatum Meissn* is a wild edible plant called ‘madimak’ in Turkish. It is perennial compare with similar plants with slender woody hull. It can be seen some species of this plant on slopes, roadsides, and cliffs [13, 14]. It is collected with their leaves in spring period. In Turkish folk medicine, this plant is used both as food and alternative medicine by considering some positive effects on diuretic, pain reliever, wound healing, and diabetes mellitus [15].

As a result of a detailed literature searching, it is considered that the extraction procedures have the greatest influence on the analytical results obtained from the analyzes to be made. Conventional extraction method for a wide variety of compounds of a matrix may be sometimes inefficient, slow, risk of oxidation, low yield and consume large of organic solvents. So, fast and useful extraction methods have been studying in the various research laboratories, every year. Among them, pressurized fluid extraction (PLE), supercritical fluid extraction and microwave assisted extraction are reported to have a very high recovery, efficiency and reliability, which are used extensively in an effective extraction process in recent years [16].

PLE is an easy, fast, and effective sample preparation approache that combines good properties of a few versatile methods. Some parameters can be optimized and adjusted in this technique such as temperature, time, solvent type, pressure, etc. So, it is achieved fast and efficient extraction of the analytes from the solid matrix. PLE has been shown to have significant advantages over competing techniques. PLE is an important sample preparation technique to enable the rapid and efficient extraction of solids from solid matrix analytes with high temperature capability, time programming, gradient solvent program, nitrogen inert medium and different pressure options. The most important advantages of PLE technique by compare with conventional ones are fast procedure, high pressure, minimal solvent using, automation, efficient recovery for target species [17]. The PLE method has great prominence in the extraction of organic compounds, which are particularly affected by atmospheric and light conditions.

A significant proportion of antioxidant activity in plants is phenolic constituents. Therefore, the determination of phenolic level and antioxidant properties during extraction with folic acid PLE is

an important parameter for the efficacy of PLE [18].

In this report, we have developed a convenient HPLC method coupled with a PLE for determination of folic acid of madimak plant. The first optimization of extraction conditions with PLE was aimed. These include extraction temperature, pressure, time, and determination of solvent and / or solvent mixtures. The second focus contains determination of the effect of PLE conditions on antioxidant properties of madimak plant in terms of total phenolic contents, free radical scavenging capacities and reducing power.

2. MATERIALS AND METHODS

2.1. Instrumentation

The HPLC system used is equipped with a pump, thermostatic column oven, an autosampler, DAD detector (Shimadzu). It was equipped with a C18 analytical column (Inertsil ODS-3 250 mm x 4.6 mm and 5 µm particle size). A pH meter (Selecta, Spain) was used to measure the pH values in sample matrix. A vacuum freezer drier was used for lyophilization of samples. The ASE 200 system (Dionex) was used for the extraction of folic acid from the madimak samples. A Shimadzu 2000S Model UV/VIS spectrophotometer (Kyoto, Japan) was used for detection of total polyphenol, antioxidant activity tests and calibration graphs in the samples. Ultra-pure water with a resistivity of 18.2 MΩ was obtained by a Milli-Q system (France).

2.2. Reagents and standard solutions

All reagents used were of analytical grade. Folic acid analytical standarts (vitamin B9) was purchased from Sigma (St. Louis, MO, USA) and methanol, isopropyl alcohol, Folin&Ciocaltue phenol reagent, DPPH and ABTS reagent, acids sodium carbonate and other chemicals were purchased from Merck (Darmstadt, Germany).

Stock solution of folic acid (100 mg L^{-1}) were prepared with methanol and standard solutions for calibration were freshly prepared at the following concentrations: 1.0, 5.0, 10, 20, 30 and 50 µg mL^{-1} from these stock solutions in every week and the pH adjusted in between 7.0 to 8.0 using of ammonium hydroxide and then the solutions was transferred into the amber volumetric flask and stored in dark at 4 °C.

2.3. Sample Preparation procedure

Madimak plants were collected from Sivas on June, 2016. Epiphytes, salt and sand were removed using tap water and then the samples were rinsed with pure water. The lyophilization of plant samples were carried out using proposed method by Erdogan et al. [17]). Reducing power assay was used as proposed by Oyaizu [19], radical scavenging power assay was studied as proposed by Re et al. [20], assay for total phenolic content was studied published by Singleton and Rossi [21] with some minor modifications.

Absorbance measurments was carried on BioTek Eon Eliza Microplate spectrophotometer at 700 for reducing power, at 734 nm for ABTS. The experimental results were calculated as mg trolox equivalent (TEAC) 100 g^{-1} for reducing power, ABTS radical scavenging activity. Then, absorbance measurments for the phenolic contents were performed by UV-VIS spectrophotometer at 755 nm, the data was calculated as mg gallic acid equivalent (GAE) 100 g^{-1} for total phenolic content [22].

2.4. Chromatographic analysis of folic acid in the extracts

For the HPLC analysis, separation and determination of folic acid was performed on an analytical column. Isocratic elution was performed with a mobile phase mixture containing 30 % Methanol and 70 % phosphate buffer (pH 3) containing 0.001 mol L^{-1} sodium hexane sulfonate. The other chromatographic conditions were applied like this: Column temperature 40 °C, flow rate 1.0 ml min^{-1}, injection volume 10 µL and detection performed at 284 nm. Concentrations of the folic acid were calculated from integrated areas.

3. RESULTS AND DISCUSSIONS

3.1. PLE extraction parameters

As a first step of the optimization, extraction conditions of folic acid from plant samples was studied for a sensitive determination. In order to obtain maximum transporting of target species from plant matrix to solvent, all parameters should be optimized in a extraction system as time of extraction, temperature, particle size of sample (pulverization of sample), solvents, sample/solvent ratio. Stability of folic acid depends on light, pH, and heat. Because it works in closed system and protects samples from light and O_2 [23], PLE technique also helps to protection of samples from degregation. Because, these parameters mainly determine the succession of extraction by means of sensitivity and selectivity.

In PLE systems, optimization studies generally start with determining of suitable solvent or solvent mixture and is usually followed by the same solvent in subsequent extraction operations. Buffers, diluted acids, diluted alkali, water, phosphate buffer (pH 8-9), ammonium acetate, ammonium hydroxide or other polar solvents are used often for recovery of a wide range of folic acid from diverse sample types including multivitamin, wheat flour, fortified cereal products, fruit juice, baby foods, infant milk, blood and vegetables [24-26].

Table 1 summarizes all optimized parameters in PLE. Various solvents were studied in order to find the most suitable solvents for effective extraction of target molecules at 40 ^{o}C and at 1500 psi for 15 min of static extraction time. Various extraction solvents such as water: Methanol: 1 M NaOH (85:14:1) (A); water:methanol:1 M NaOH (50:49:1) (B); water: 1 M NaOH (99:1) (C); 0.1 M phosphate buffer including 2% sodium ascorbate + 0.1 % mercaptoethanol) (D); Water: Acetonitrile: 1 M NaOH (50:49:1) (E) were studied and results evaluated. As can be seen in Figure 1A, higher extraction yield were achieved with a mixture of water:NaOH (99:1). This selection also favored the later evaporation of the extract.

Table 1. The studied parameters in the PLE procedure in order to optimize system.

Parameter	Test value
Solvent A (Water:Methanol:1 M NaOH)	85:14:1 v/v
Solvent B (Water:Methanol:1 M NaOH)	50:49:1 v/v
Solvent C (Water: 1 M NaOH)	99:1 v/v
Solvent D (0.1 M phosphate buffer (in 2% sodium ascorbate + 0.1% 2-mercaptoethanol)	100 v/v
Solvent E (Water: Acetonitrile:1 M NaOH)	50:49:1 v/v
Temperature (^{o}C)	20, 30, 40, 50 and 60
Time (min.)	5, 15, 45 and 60
Pressure (psi)	500, 750, 1000, 1250, and 1500

Several static extraction time extraction time (5, 15, 45 and 60 min) were examined using the water: 1 M NaOH mixture (99:1) at 40^{o}C and 1500 psi. It was found that the extraction efficiency increased with static extraction time increasing up to 60 minutes (Fig 1-B).

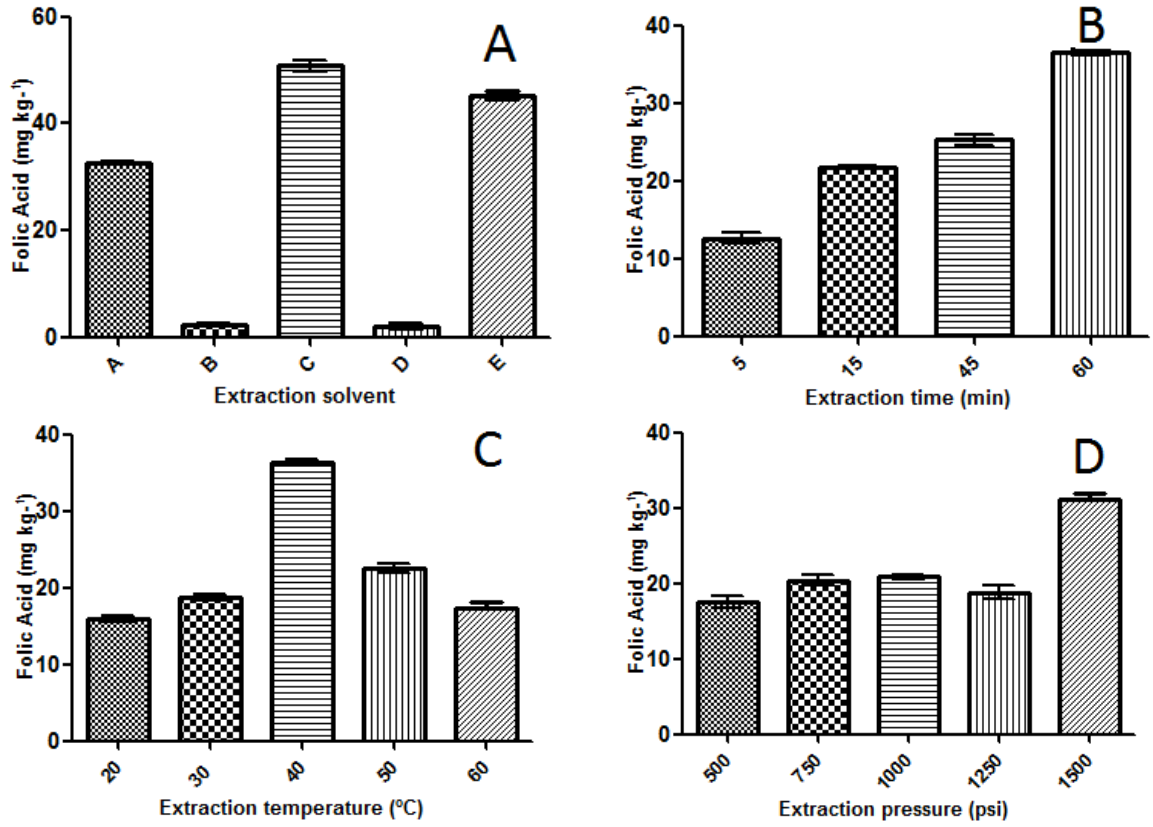

Figure 1. Effect of extraction solvents (A), time (B), temperature (C) and pressure (D) on the Folic acid extraction by PLE.

Thus, 60 minutes was considered the optimum time of extraction and used for further extraction tests. In general, longer extraction time was not preferred due to possible thermal decomposition of organic contents at high temperatures. Moreover, the duration of extraction and temperature should be kept low if possible in order to avoid oxidation or decomposition of the analytes in the matrix under specified operating conditions.

The another parameter needing to optimize in PLE is temperature which directly effects extraction efficiency. For this purpose, all experiments were repeated in five different temperatures. Diffusion rates, solubility of the analytes, mass transfer in the samples matrix, and kinetic energy of molecules increase by temperature while surface tension of the solvents and viscosity of sample decreases. The contact of target molecules with solvent system is changed with temperature and more efficient and fast extraction is carried out in PLE system. For optimization of this parameter, a various temperature is studies by applying various temperature (20, 30, 40, 50 and 60 ^{o}C). It was observed that the most efficient extraction temperature is 40 ^{o}C which also allowed better penetration of the solvent into a matrix (Fig 1C). The amount of folic acid in the extracts decreased beyond 40 ^{o}C. The decrease in the extraction yield could be due to a possible degradation of folic acid at high temperatures. As a result of the degradation, it was observed with low recoveries and by highest peak splitting on HPLC chromatograms[27,28]. Although there are a number of studies in the literature on the effect of the extraction processes on the folate content of foods, there is almost any data on the effect of extraction temperature on the folic acid content of the plant breeding extract [27]. As in comparative studies on pressure and temperature stability of 5-methyl-tetrahydrofolic acid in model systems and food products [29] .

In addition to the temperature, solvent, and extraction time that affect the performance of the PLE procedure, static pressure is one of the most important parameters and is usually increased by

the increase in the matrix pressure of the analytical matrix. High pressure helps the solubiliser reach the sample matrix pores more easily and allows the analytes to pass through the solvent. So, extraction pressure as an experimental variable was studied by using these values: 500, 750, 1000, 1250, and 1500 psi. Operation pressure directly influenced the amount of folic acid extracted which significantly increased up to 1500 psi (Fig 1-D). It is well known by means of the published articles that high pressures can cause to some changes on cell membrane permeability and intracellular organelle structural(30). The resulting solubilization of intracellular substances (metal ions, proteins *etc*.) and permeation of extra-cellular substances may increase interactions between folylpoly-c-glutamates [31]. Release of folylpoly-c-glutamates from endogenous plant folate binding proteins (FBPs) under pressure could have made them available for folate c-glutamyl hydrolase (FGGH) [32]. It was explained targetted application of high-pressure thermal extractions processing may lead to small folate losses that meet by chance with an accumulation of folylmonoglutamates in broccoli e.g. treatments at 25 ^{o}C, 400 MPa (25 min) resulted in ±10% total folate loss and 5-CH_3H_4PteGlu accumulation from 1.5% up to 21% of the total residual folate content in broccoli samples [33].

Furthermore, to prevent pressure losses of folic acid, and during operation, a pressure of 1500 psi was selected as the optimum pressure and this value was used in all subsequent tests.

Consequently, water: 1 M NaOH (99:1 v/v) as solvent, 40 ^{o}C as temperature, 1500 psi as pressure and 60 min as extraction time were determined as the operational parameters to be used for all subsequent extractions studies (Fig 1D). All optimized paratemetrs can be seen in Table 2.

Table 2. Optimized PLE conditions for extraction of Folic acid from madimak samples.

Extraction solvent	**Water / NaOH (99:1%)**
Temperature (°C)	40
Pressure (psi)	1500
Heat-up time (min)	5
Extraction time (min)	60
Flush volume (%)	60
Number of cycles	2
Purge time (min)	1
Cell volume (mL)	22
Total extraction time (min)[a]	126
Total solvent used (mL)[a]	33

[a] Per sample.

3.2. Determination Parameters for folic acid and analysis results

The calibration concentration range was determined by taking into account the range of values specific to each sample studied in order to obtain linear results. The calibration graph from experimental results were plotted by using peak area versus concentration of folic acid standarts. The obtained lineer range was acceptable and relative standart deviations were lower than 3.5 %. The linear range of the quantitation was observed in the range of 1.0-50.0 µg / mL with a correlation coefficient as 0.9998.

Figures 2 illustrates the obtained chromatogram and other HPLC conditions of the folic acid in madimak samples. The retention time (10 replicates) for folic acid was RT 2.96 ± 0.1 min. The folic acid levels determined by the present method were determined between 13.98 and 49.42 ppm after optimization of the different solvent mixture, temperature, extraction time and pressure parameters for folic acid extraction from solid madimak samples. Folic acid concentration in madimak sample level found to be 176, 25, 41, 28 and 10 times higher than different commercial fruit juices, spinach, broccoli, green beans, brussels sprouts, a nd soya bean tissue, respectively [34, 35]).

As a result, the folic acid composition in the madimak samples provides the folic acid amounts expected by the consumers. Our data shows that it is slightly higher than the total folate content of some processed products, commercially.

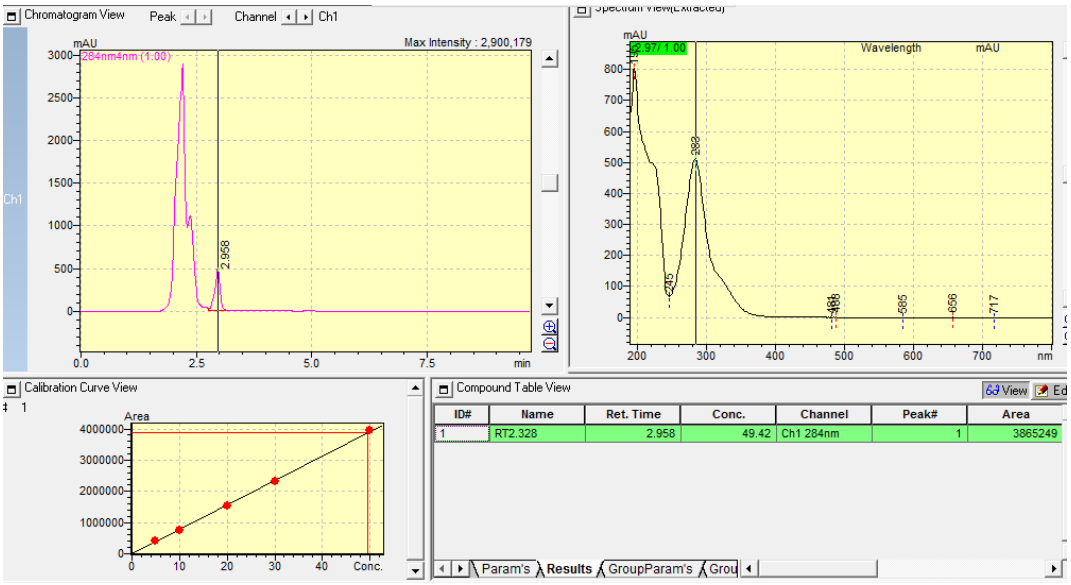

Figure 2. Chromatogram of the folic acid in fortified madimak sample folic acid (Rt 2.8 min). DAD detector sensitivity 1 µA. LC 18 column 5 µm (4.6 mm× 25 cm) and a mobile phase, consisting of 40 mM sodium phosphate dibasic, and 50:50% methanol: acetonitrile (v/v), pH 3.)

3.3. Effect of PLE conditions on antioxidant properties of Madimak extract

In the study, it was also determined how the antioxidant contents of madimak plant are affected by extraction parameters (solvent, temperature, time, pressure) during extraction of folic acid by PLE. In this context, it has been shown how the antioxidant properties of extracts obtained from different solvent components are changed by keeping the parameters of temperature (at 40 ° C), pressure (at 1500 psi) and time (15 min) constant. Depending on experimental conditions, the changing of total phenolic content, the ABTS radical scavenging activity and the reduction power of the extract were examined by the change of the experimental variables.

The total phenolic content of the extract according to the solvent types was shown in Figure 3A. The total amount of phenolic component of madimak plant was found as 6.167±0.223 mg GAE/g dry mass to be highest in the solvent D system and 2.137 ± 0.071 mg GAE/g dry mass to be lowest in solvent A system. According to ABTS radical scavenging method, the highest activity of Madimak plant extracts was obtained with solvent B system with 24.584 ± 0.447 mg trolax equivalent/g dry mass (Figure 4A). However, the highest antioxidant activity, according to the reduction method, was measured as 9.707±0.588 mg trolox equivalent/g dry mass in the solvent D system and was parallel to the total phenolic substance (Fig. 5A). The reason for the high activity in Solven D in the total phenolic content and reducing power tests can be explained by the fact that both the extraction system and the reaction mixtures in the methods are involved in the water-based systems. The high activity of the Solvent B system in the ABTS test is also related to the fact that both the solvent B and the ABTS method are alcohol-based systems. The similarity of the components of the antioxidant test system with the extraction solvent mixture has also been shown to influence antioxidant activity [36].

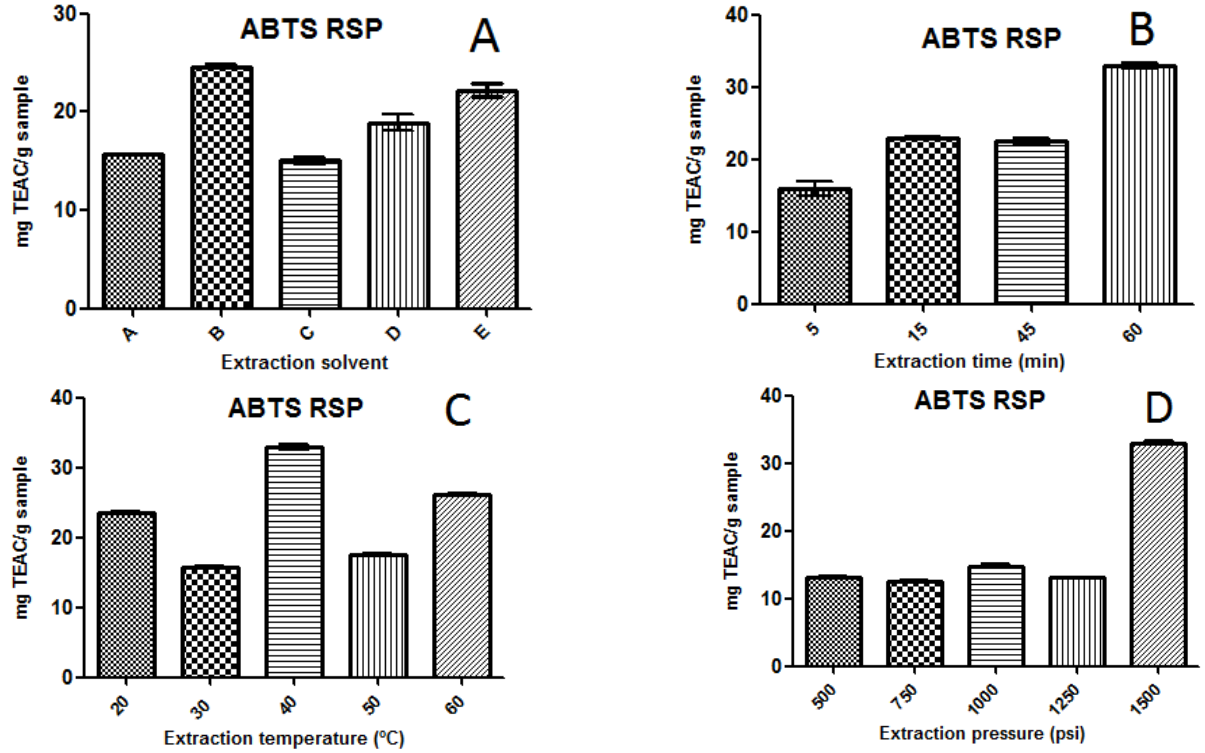

Figure 3. Effect of extraction solvents (A), time (B), temperature (C) and pressure (D) on the ABTS radical scavenging power as Trolox equivalent of Madimak. Each value is the average of triplicate experiments in with error bars indicating STDEVs (σ_{n-1}).

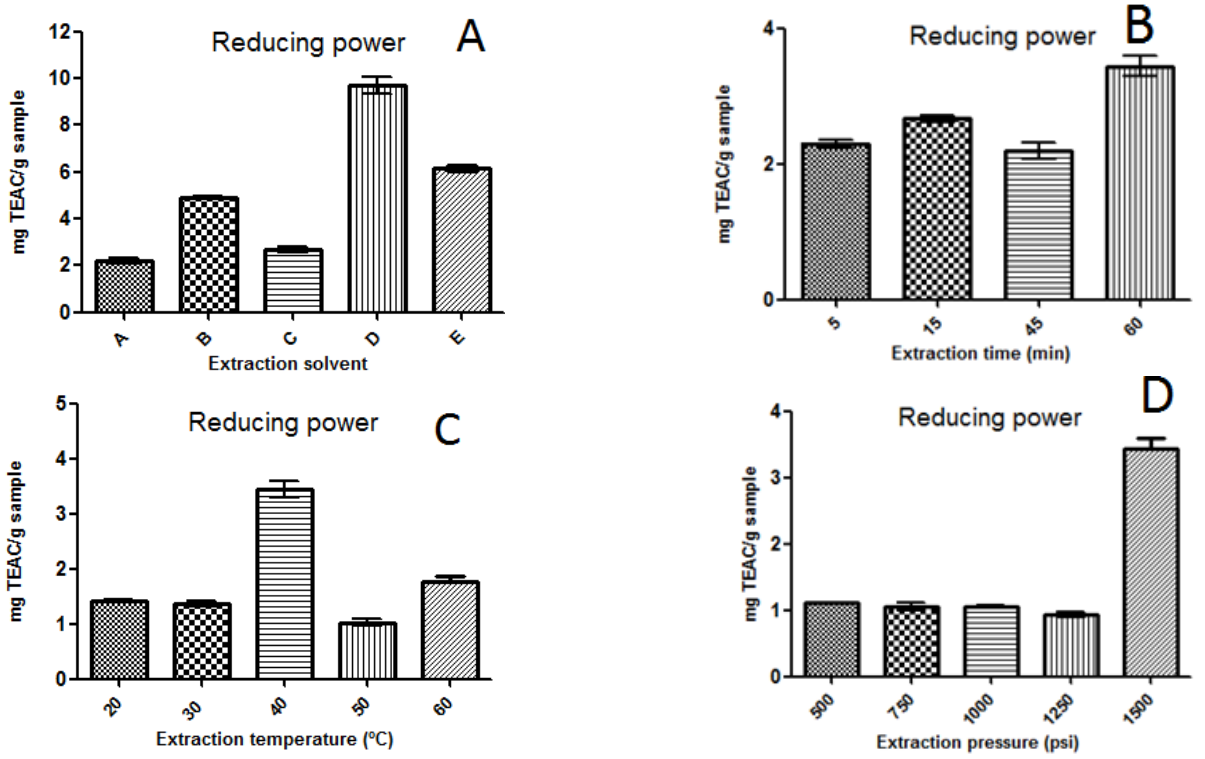

Figure 4. Effect of extraction solvents (A), time (B), temperature (C) and pressure (D) on the Ferric-reducing power as Trolox equivalent of madimak. Each value is the average of triplicate experiments in with error bars indicating STDEVs (σ_{n-1}).

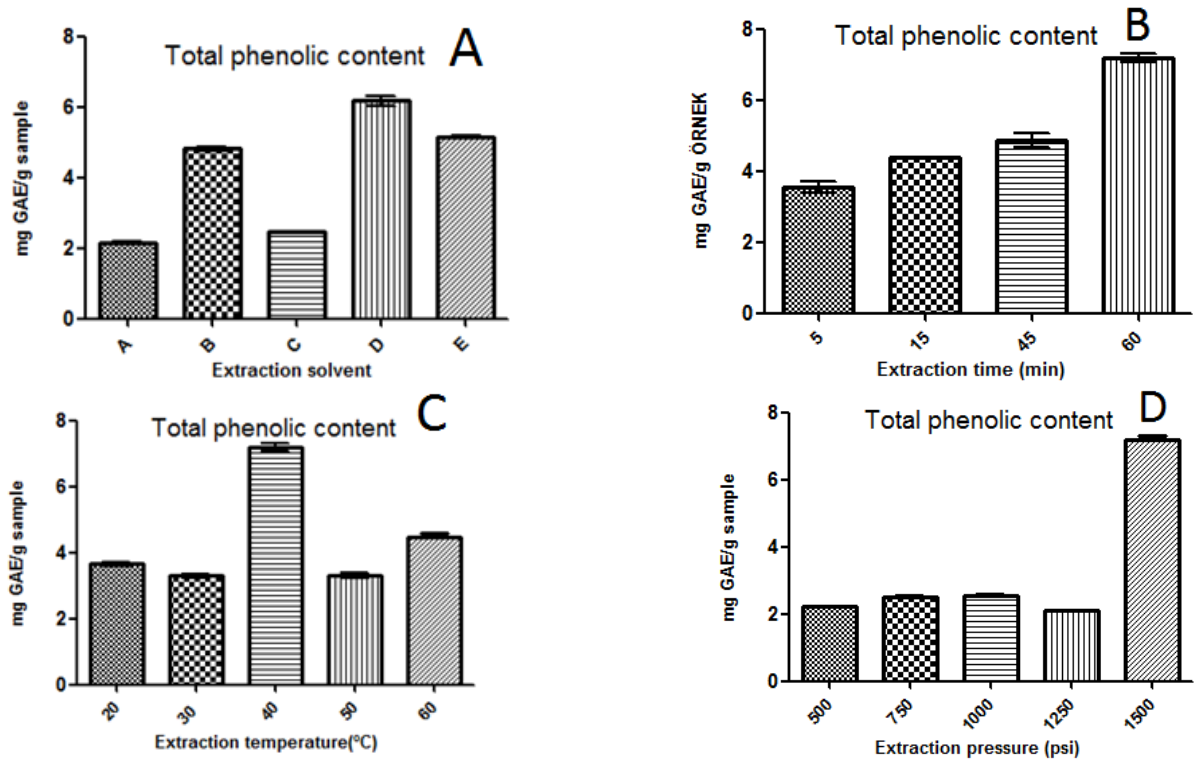

Figure 5. Effect of extraction solvents (A), time (B), temperature (C) and pressure (D) on the Total phenolic content as Gallic Acid equivalent of Madimak. Each value is the average of triplicate experiments in with error bars indicating STDEVs (σ_{n-1}).

Another important factor affecting the extraction yield of Madimak plant is the determination of the contact times of the compounds to be extracted with the solvent. The effect of the extraction time was investigated in four different time periods, 5, 15, 30 and 60 min. There was a linear increase in folic acid extraction with time. However, activity values obtained from antioxidant activity tests were close to each other for 5, 15 and 45 min. At 60 min of extraction time, the antioxidant activity values increased by approximately 40% (Figs. 3B, 4B and 5B). Generally, studies have also reported that high extraction time increases antioxidant activity [36].

The effects of temperature at 20, 30, 40, 50 and 60 ^{o}C, which are also used for PLE optimization of folic acid, on antioxidant components were also determined by these conditions. Antioxidant activity tests also showed that the optimum temperature was 40 °C as in folic acid extraction (Figures. 3C, 4C and 5C). This result can be explained with degradation of phenolic compounds during extraction at high temperatures. Moreover, it has been stated that the degradation of antioxidant compound increases with temperatures at over 50 ^{0}C and 40 ^{0}C temperature is extensively used in extractions of antioxidant components from plants [37, 38].

Finally, the effect of pressures from PLE conditions on the antioxidant activity was also investigated in Madimak plant extraction. It has been determined that antioxidant activity values are quite similar at 500-1250 psi. However, a dramatic increase was measured at 1500 psi (Figure 3D, 4D and 5D). These results are parallel to the optimum pressure values in the extraction of folic acid.

4. CONCLUSION

In the fist step, all parameters affecting extraction conditions were studied for folic acid extraction in madimak samples. The concentration of folic acid in real samples was determined by using optimal conditions. The concentration ranges for madimak samples were as follows: 16.12 and 49.42 ppm for folic acid. Folic acid concentration in madimak plant was 2-4 times higher than some edible vegetables. In the second step, the affecting parameters on antioxidant capacity of plant extracts were studied in detail. It was observed that the

antioxidant properties of extract effected from extraction conditions.

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