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RESEARCH ARTICLE

Optimization of Extraction Process of Bioactive Compounds from Bene Hull Using Subcritical Water

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Abstract Bene hull contains antioxidant components. Optimum conditions for bioactive compound extraction processes from Bene hull using subcritical water with response surface methodology (RSM) were obtained. Temperature (110-200°C), processing time (30-60 min), and the water to Bene hull ratio (10:1-50:1) were the investigated factors. The optimal conditions for maximizing the antioxidant activity were 196.8°C for 52.6 min and a ratio of 43.6:1 for water to Bene hull. Under these conditions, the amount of polyphenolic compounds, the reduction power (RP) (EC_{50}), and the DPPH free radical scavenging activity (RSA) (EC_{50}) were predicted to be 2,284 mg of gallic acid/100 g of Bene hull, 0.2002 mg/mL, and 0.6284 mg/mL, respectively. HPLC analysis was used to identify the main phenolic compounds. The subcritical water extraction technique could be used as a beneficial method to obtain bioactive compounds from Bene hull.

Keywords: Bene hull, antioxidant, reduction power, radical scavenging, subcritical water

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Introduction

The positive effect of natural antioxidants on human health has been illustrated in recent years (1). On the other hand, the detrimental effects of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), on human health have led scientists to search for new natural antioxidant resources. The toxicity of synthetic antioxidants and suspected actions as promoters of carcinogenesis have been investigated (2). However, a demand for alternative natural and safe sources of food antioxidants has been created and research into natural antioxidants, particularly from plant origins, has developed in recent years (3).

Bene (*Pistacia atlantica* subsp. *mutica*) is a wild variety of pistachio and a native plant that grows in the Zagross region of Iran at 600-3,000 m above sea level (4). The fruit is round to oval, somewhat flat, and a dark green to yellow or brownish color and 0.5-0.7 cm in diameter. The Bene kernel is covered with a hard wooden shell that can easily be removed by hand. The soft hull comprises 24% of the whole fruit (25% of the kernel and 51% of the hard shell) and contains up to 30% oil (5). A few investigations have been carried out on the antioxidant properties of Bene (6-11).

Subcritical water is defined as a region of the condensed phase of water between 100°C (boiling point of water) and 374°C (critical point of water) with pressure proportional to maintain a liquid state (10). This condition is also called pressurized low polarity water (PLPW) or pressurized hot water (PHW) (11). The dielectric constant (eee) of water is 80 at 25°C and decreases to 27 when the temperature rises

to 250°C. Methanol has en eee value of 33 and ethanol has an eee value of 24 at 25°C. Subcritical water behaves like certain organic solvents that can dissolve a wide range of medium and low polarity analysts (12).

In recent years, several novel extraction methods, including pressurized-hot water extraction (PHWE), supercritical fluids extraction (SFE) mainly with CO_2, microwave assisted extraction (MWE), ultrasonication assisted extraction (USE), pulse electric field assisted extraction (PEFE), and high pressure assisted extraction (HPE) have been developed for extraction from different substances. These methods are green technologies that exhibit a number of advantages over conventional extraction techniques, including hydro-distillation and organic solvent extraction, simplicity, a higher selectivity, a high yield and productivity (time, cost and energy saving), higher extract quality, and environmental safety (13). SWE has been successfully applied to extraction of many antioxidants and functional compounds from natural resources (13-15). SWE has been used to extract nutraceutical compounds from citrus pomace (16) and from *Centella asiatica* (17), antioxidant compounds from Sea buckthorn leaves (18), onion (*Allium cepa L.*) peels (19), and rosemary plants (20), phenolic antioxidants from the fruit of *Terminaliachebula Retz* (21) and pomegranate seed residues (22), and even proanthocyanidins from wine-related products (23).

Optimization of extraction conditions to achieve stability and high value of antioxidant activities are important issues. Response surface methodology (RSM) is a common approach for optimization of complex and multi-variable research problems (24). Many researchers have used this method to optimize different processes (22,25-27). One of the advantages of RSM is the possibility of determining interactions among independent variables (28). The main goals of this research were first, to determine optimized conditions for Bene hull subcritical water extraction based on antioxidant activities and second, to compare the subcritical water and hot water extraction methods.

Materials and Methods

Samples and reagents Fruits of *Pistacia atlantica* were collected from the Marvdasht region in Fars province (Iran) in November of 2010 and stored at -18°C to minimize oxidation of compounds during experiments. The fruit hull was separated immediately before each treatment. Deionized water was used as a solvent during SCW operations. BHT, L-ascorbic acid, and gallic acid (GA) were purchased from a local agency of Sigma-Aldrich Chemicals (St. Louis, MO, USA). DPPH, Folin-Ciocalteu reagent (FCR), potassium ferricyanide, ferric chloride, and sodium carbonate were obtained from Merck (Darmstadt, Germany). Whatman No. 4 filter paper and trichloro acetic acid were obtained from VWR International (Mississauga, ON, Canada). All other chemicals and solvents (analytical grade) used were purchased from Beijing Chemical Co. (Beijing, China).

Subcritical water extraction Extraction of juice from Bene hull was carried out using a laboratory-built apparatus (Fig. 1). The system consisted of a deionized water feed tank, a high pressure metering pump (Comet type: MTP AX 2/70 m) to deliver water and solvent through the system, an extraction vessel (140 mL), ferro-nickel heating wires wrapped around the extraction vessel to supply the required temperature, and a temperature control unit. The extraction process was carried out after adjusting the temperature and pressure (110°C, 2 bars, 155°C, 5 bars, and 200°C, 15 bars). The minimum pressure was applied to maintain the condensed phase of water. The extraction vessel was heated before each experiment, then the temperature was changed according to the desired temperature for extraction. The duration of initial heating was automatically

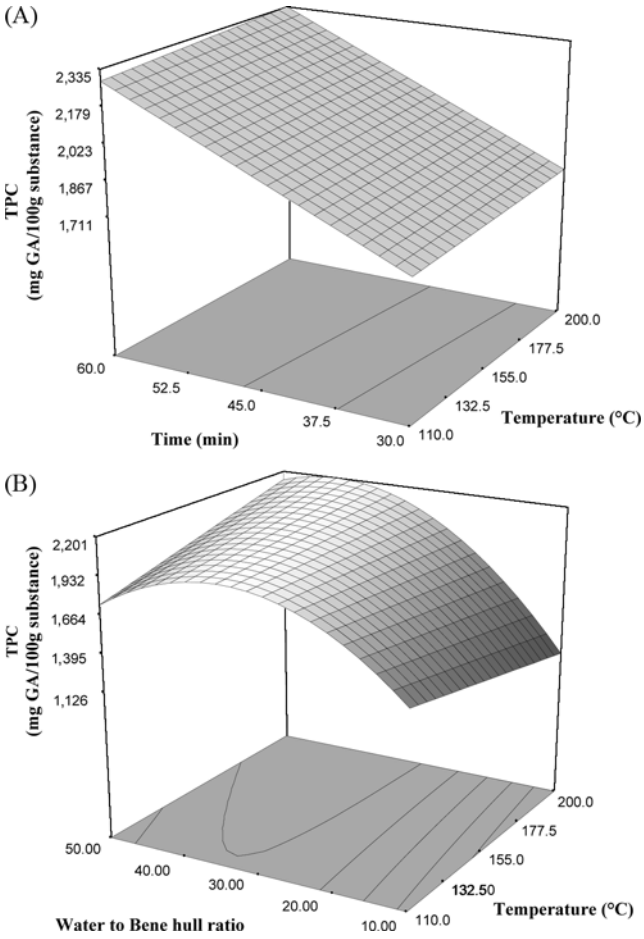

Fig. 1. Response surfaces and contour plots of the total phenolics of Bene hull SWE extracts as a function of (A) extraction time and temperature and (B) extraction temperature and water to the Bene hull ratio.

controlled by the thermo control device. Times of 5, 7 and 9 min of initial heating were used when the extraction temperature was set at 110, 155, and 200°C, respectively. After the extraction procedure, the obtained extract was filtered through Whatman No.4 filter paper, then measured for volume. The extract was concentrated at a constant weight by evaporation of water under a vacuum. Between each extraction process, the system was completely rinsed using ethanol and subsequently with deionized water to remove any residual products.

Hot water extraction (85°C) Hot water extraction was carried out using 5 g of Bene hull in 100 mL of water (1:20), at 85°C and 30 min on a platform shaker as a conventional extraction method for comparison. The extracted solution was filtered and lyophilized. Analysis of extracts was carried out immediately after freeze-drying (29).

Determination of the total phenolic content (TPC) The total phenolic content was determined using FCR as described by Singh *et al*. (30). An amount of 0.5 mL of each sample was mixed with 2.5 mL of FCR (diluted 1:10 using distilled water). Then, 2 mL of 7.5% Na_2CO_3 (w/v) was added to the mixture. The prepared solutions were incubated at room temperature in the dark for 30 min. The absorbance of the mixtures was measured at 765 nm. GA was used to produce a standard curve (0-200 mg/L). Measurements of the phenolic compounds were expressed as mg of gallic acid equivalents (GAE)/100 g of the dry weight of plant material.

Reduction power assay The reducing power was measured following the method of Barros *et al*. (31). According to this method, 2.5 mL of the test solution (a concentration of 0.1-1.0 mg/mL) was mixed with 2.5 mL of a 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferri cyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 min. Then, the mixture was acidified using 2.5 mL of 10% trichloro acetic acid (w/v), then centrifuged at 1,400 g for 8 min (E200 Labofuge centrifuge). The supernatant (5 mL) was mixed with 5 mL of distilled water and 1 mL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm using a UV spectrophotometer (Jenway 6105). The extract concentration that produced a median effective absorbance value (0.5 of absorbance (EC50) was calculated from the absorbance graph at 700 nm versus the extract concentration. Ascorbic acid was used for comparison.

DPPH free-radical scavenging assay The DPPH free-radical scavenging median effective value (IC_{50}) was estimated using the method of Rodriguez-Meizoso *et al*. (2), with some modification. BHT was used as a positive control (2). Briefly, 23.5 mg of DPPH was dissolved in 100 mL of methanol. This solution was diluted at a ratio of 1:10 using methanol. During the assay, 0.1 mL of a test solution (a concentration of 0.5-2.5 mg/mL) was mixed with 3.9 mL of a DPPH solution. The reaction was completed after 4 h at room temperature and the absorbance was measured against the blank reagent at 516 nm using a UV/VIS Spectrophotometer (Jenway). The purple-colored, stable free radical DPPH was reduced to yellow diphenyl picryl hydrazine when antioxidant was added. The scavenging capability of DPPH radicals was calculated using Eq. (1):

$$\text{Scavenging effect (\%)}=[1-(ABS_{sample}/ABS_{control})] \quad (1)$$

The percentage of scavenging capability (S.C%) was plotted against the extract concentrations and a polynomial regression curve was established in order to calculate the EC_{50} value (mg/mL), which was the concentration of the extract that inhibited DPPH by 50%. The lower the EC_{50} value, the higher the antioxidant power.

HPLC-DAD analysis of the extracts Identification of polyphenolic compounds was performed using high performance ternary gradient liquid chromatography (HPLC; Young lin, Korea), equipped with a C18 reverse-phase column (4.6 mm×25 cm), type Spherisorb ODS-25 mm, 100 A°, with a spectrophotometric UV detector at 280 nm and an integrator. Syringic acid was used as an internal standard. The mobile phase was (A) water and 0.2% H_3PO_4 (V/V), (B) methanol, and (C) acetonitrile. Elution solvents should be de-gassed. The gradient conditions were 0 min at 96% A and 2% B; 40 min at 50% A and 25% B; 45 min at 40% A and 30% B; 60 min at 0% A and 50% B; 70 min at 0% A and 50% B; 72 min at 96% A and 2% B; and 82 min at 96% A and 2% B. The total run time was 82 min and the injected volume was 20 μL. The flow rate was kept at 1 mL/min (32). A total of 6 phenolic compounds, including epicatechin, chlorogensaure, kaffesaure, flavanomarein, ethyl vanillin, and apigenin 7-glucoside were identified and quantified.

Statistical analysis Optimized experiments were carried out according to a central composite face centered design with the 3 independent variables extraction temperature (110-200°C), extraction time (30-60 min), and the water to Bene hull ratio (10:1-50:1). RSM was applied to the experimental data using the commercial statistical package of Design-Expert version 6.0.2 (Statease Inc., Minneapolis, MN, USA). The complete design consisted of 20 experimental points, including 6 replications of the center point, and the experiment was carried out randomly to minimize the effects of unexplained variability on the

observed responses. Decoded and coded values of the independent variables and the experimental data are shown in Table 1. In order to predict the optimal point, a second-order polynomial model was fitted to correlate relationships between independent variables and responses (polyphenolic compounds, reducing power, and DPPH free-radical scavenging). Relationships between the 3 factors are shown in Eq. (2):

$$Y_n=\beta_0+\beta_1X_1+\beta_2\cdot X_2+\beta_3\cdot X_3+\beta_{11}\cdot X_1^2+\beta_{22}\cdot X_2^2+\beta_{33}.X_3^2 +\beta_{12}\cdot X_1\cdot X_2+\beta_{13}\cdot X_1\cdot X_3+\beta_{23}\cdot X_2\cdot X_3 \quad (2)$$

where Y_n is one of the 3 responses; X_1, X_2 and X_3 represent the independent variables, β_0 is a constant; β_1, β_2, and β_3 are the linear-term coefficients, β_{11}, β_{22}, and β_{23} are the quadratic-term coefficients, and β_{12} and β_{13} are interaction coefficients.

An analysis of variance (ANOVA) was performed and the fitness of the polynomial model equation was evaluated using lack of fit (model error), coefficient of regression (R^2), adjusted-R^2, coefficient of variation (CV), and the Fisher test value (F value). Statistical significance of the model and model variables was determined based on an F test at a probability (p) of either 0.01 or 0.05. The effect of variables was displayed in 3-dimensional response surfaces and 2-dimensional contour plots.

Results and Discussion

Fitting the models To fit the explanatory models and the variation of the total phenolic content, the reducing power, and radical scavenging, the sum of squares of the sequential model was analyzed. A quadratic regression model was the most appropriate model for the 3 response variables (Table 2).

Regression coefficients obtained from the ANOVA by fitting experimental data to the second order polynomial response surface models for each of the response variables (Y_n), and corresponding coefficients of determination (R^2) values are shown in Table 3. In addition, adj-R^2 and coefficient of variation (CV) values were calculated to check the model adequacy. R^2 values for TPC, the reducing power (EC_{50}), and DPPH (EC_{50}) were 0.928, 0.902, and 0.987, respectively. These R^2 values indicated that the regression model was suitable to explain the behavior of the system. Adjusted R^2 values were also higher than 0.8, indicating that non-significant terms were not included in the model.

The coefficient of variation (CV) was lower than 10% for all responses (Table 3), representing good precision and reliability of the results. These models were adequate for predicting operating conditions within the ranges of the variables used.

Table 1. Coded and uncoded levels of independent variables for central composite design and, TPC, reducing power and radical scavenging of the superheated water extracts

Exp.	Coded level			Independent variables (actual values)			Dependent variables[1)] (experimental data)		
	X_1	X_2	X_3	Temperature (°C)	Time (min)	Ratio	TPC	RP	RSA
1	+1	+1	+1	200	60	50	2203	245.5	646.4
2	0	0	+1	155	45	50	2113	259.6	700.2
3	-1	+1	+1	110	60	50	1923	343.6	1461
4	0	0	0	155	45	30	1991	255.2	782.7
5	+1	-1	+1	200	30	50	2006	255.0	777.7
6	0	0	0	155	45	30	1991	223.7	740.6
7	0	0	0	155	45	30	1797	233.2	698.3
8	0	0	-1	155	45	10	1423	234.7	639.8
9	+1	+1	-1	200	60	10	1714	203.4	676.8
10	-1	+1	-1	110	60	10	1644	265.5	1445
11	0	+1	0	155	60	30	2283	302.8	782.0
12	0	0	0	155	45	30	2148	230.6	665.8
13	+1	-1	-1	200	30	10	489	381.0	1005
14	0	0	0	155	45	30	2138	253.8	723.9
15	0	-1	0	155	30	30	1842	336.4	886.0
16	-1	-1	-1	110	30	10	1118	210.5	1418
17	-1	-1	+1	110	30	50	1440	249.8	1426
18	0	0	0	155	45	30	1993	239.8	784.2
19	+1	0	0	200	45	30	2003	213.1	685.2
20	-1	0	0	110	45	30	2045	230.6	1450

[1)]TPC, total phenolic content (mg GAE/100 g of Bene hull); RP, reducing power (EC_{50}, mg/L); RSA, DPPH radical scavenging (EC_{50}, mg/L)

Table 2. Sequential model sum of squares for total phenolic content, reducing power, and radical scavenging

Source	DF	Total phenolic content		Reducing power		Radical scavenging	
		Sum of squares	*p*>*F*	Sum of squares	*p*>*F*	Sum of squares	*p*>*F*
Mean		6.59×10^{07}		1.33×10^{06}		1.69×10^{07}	
Linear	11	1.92×10^{06}	0.004	858.6	0.953	1.19×10^{06}	0.001
Interaction	8	4.12×10^{05}	0.250	2.45×10^{04}	0.007	4.91×10^{04}	0.822
Quadratic	5	9.02×10^{05}	0.001	1.28×10^{04}	0.002	6.74×10^{05}	<0.001
Cubic	1	1.38×10^{05}	0.242	2.61×10^{03}	0.146	1.45×10^{04}	0.216
Residual		1.13×10^{05}		1.54×10^{03}		1.09×10^{04}	
Total		6.94×10^{07}		1.378×10^{06}		1.87×10^{07}	

Table 3. Second order response model constants and regression analysis for TPC, Reduction Power and Radical Scavenging of the superheated water extracts

Term	Coefficient[1)]	TPC[2)]	RP	RSA
Intercept	β_0	-704.5**[3)]	+1.80**	+6095**
X_1 (Temperature)	β_1	+3.979	+8.57	-53.39**
X_2 (Time)	β_2	+38.49**	-19.61	-31.56*
X_3 (water to the Bene hull ratio)	β_3	+57.22**	+1.797	+10.72
X_1^2 (Temperature)	β_{11}	-0.041	-0.016*	+0.166**
X_2^2 (Time)	β_{22}	-0.198	+0.291**	+0.451**
X_3^2 (water to the Bene hull ratio)	β_{33}	-0.848**	-0.017	-0.156
X_1 (Temperature)×X_2 (Time)	β_{12}	+0.076	-0.062**	-0.096**
X_1 (Temperature)×X_3 (water to the Bene hull ratio)	β_{13}	+0.195*	-0.028**	-0.039
X_2 (Time)×X_3 (water to the Bene hull ratio)	β_{23}	-0.446*	+0.086**	+0.085
	R^2	0.928	0.902	0.987
	Adj-R^2	0.863	0.814	0.975
	F	14/31	10/23	83/52
	p	<0.001**	0.0006**	<0.001**
	Standard Error	0.222	0.075	0.383
	CV	8.73	7.88	5.49

[1)] $Y_n=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3$
[2)] TPC, total phenolic content (mg GAE/100 g of Bene hull); RP, reducing power (EC_{50}, mg/L); RSA, DPPH radical scavenging (EC_{50}, mg/L)
[3)] *Significant at $p<0.05$; **significant at $p<0.01$

The total phenolic compound contents The response surface quadratic model for the polyphenolic content was statistically significant ($p<0.01$), while the lack of fit test showed no significant difference ($p>0.05$) (Table 2). Therefore, the regression model was in good agreement with the experimental results. The linear effect of time (B) and water to the Bene hull ratio (C), and also the quadratic effect of water to the Bene hull ratio (C^2) were highly significant ($p<0.01$). Among the interaction terms, the extraction temperature and water to the Bene hull ratio (AC), and the extraction time and water to the Bene hull ratio (BC) were significant ($p<0.05$) for total phenolic compound contents (Fig. 2). The values of the coefficient of determination (R^2=0.928) and the adjusted coefficient of determination (Adjusted R^2=0.863) were reasonably close to 1, indicating a high degree of correlation between the observed and predicted values (Table 3). Equation (3) shows the reduced quadratic model for polyphenolic compound content prediction.

$$Y=-48.57-5.31X_1+32.54X_2+68.72X_3-1.04X_3^2+0.19X_1X_3-0.45X_2X_3 \quad (3)$$

Phenolic compounds modulate the occurrence and intensity of Millard and caramelization reactions (33). The 3-dimensional surfaces and contour plots of the effects of the extraction temperature, time, and ratio of water to Bene hull on the polyphenolic content of Bene hull conducted with subcritical water extracts are shown in Fig. 1. The purpose of optimization of this variable was to achieve a higher polyphenolic compound content. In other words, more polyphenolic compounds indicated a higher extract antioxidant capacity. The extraction time affected the polyphenolic compound content in a linear manner (Fig. 2A). However, there was no significant ($p>0.05$) effect for the temperature term. The polyphenolic compound content was increased with an increase in the extraction time. A significant ($p<0.01$) effect was observed for the extraction time ($p<0.05$). The effect of the extraction temperature and

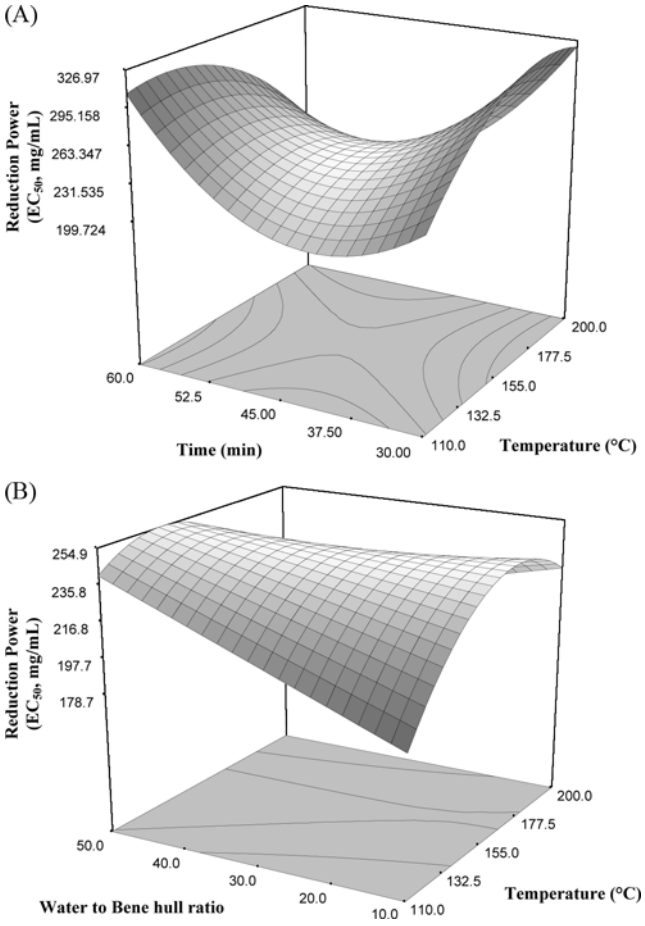

Fig. 2. Response surfaces and contour plots of the reducing power (EC_{50}) of Bene hull SWE extracts as a function of (A) extraction time and temperature and (B) extraction temperature and water to the Bene hull ratio.

water to the Bene hull ratio (AC) on TPC are shown in Fig. 1B. The extraction time had no significant ($p>0.05$) effect on the polyphenolic compound content, while the interaction between the extraction temperature and water to the Bene hull ratio was significant ($p<0.05$). The effect of the increase in water to the Bene hull ratio was non-linear. There was an increase in the polyphenolic compound content with an increase in the ratio of water to the Bene hull ratio at a specific level (30-1). The variables with the most effect were the linear and quadratic term of water to the Bene hull ratio (C and C^2), the linear term of the extraction time, followed by the interaction effect of the extraction temperature-water to the Bene hull ratio (AC) and the temperature-water to the Bene hull ratio (BC). The extraction procedure was better with an increased water to Bene hull ratio.

The solvent diffusivity increased at high ratio values of water to Bene hull and the solvent was also saturated with a higher amount of extracted material. The results of this study were in agreement with Plaza *et al*. (34) in which it was observed that various natural substances represent different polyphenolic compound contents. Although there was no statistical difference between the polyphenolic compound content obtained using *Thyme* and *Halopitys incurvus* at 100 and 200°C ($p>0.05$), other substances showed ND differences (34). Results of this study were also in agreement with reported results of Rodriguez-Meizoso *et al*. (2) for *Oregano* where it was observed that the polyphenolic compound content showed no differences between different extracts. The amount of extracted phenolic compounds was similar, but the type and structure of the phenolics were different.

The increase in the ionization constant (K_W) of water under subcritical conditions affects the Bene hull hydrolysis reaction. Lignin, a component of the plant cell wall, has been shown to decompose into phenols under subcritical conditions. This characteristic of water improves the extraction of phenols from fruits via a catalyzed hydrolytic degradation of the polysaccharide-lignin network of the cell wall matrix (21).

Reducing power of Fe^{+3} The response surface quadratic model for the reducing power was statistically significant ($p<0.05$) whereas the lack of fit test was not significant ($p>0.05$). The regression model was able to explain the behavior of the system (Table 2). None of the linear model terms were significant ($p>0.05$) by themselves. Among the quadratic terms, temperature (A^2) and time (B^2) were significant ($p<0.01$). All of the mutual interactions between independent variables, including temperature and time (AB), temperature and water to the Bene hull ratio (AC), and time and water to the Bene hull ratio (BC) were also found to be significant ($p<0.01$).

The R^2 and adj-R^2 values for the reduction power were 0.902 and 0.814, respectively. The reduction power (Table 3) demonstrated that the relationship between reducing power and extraction parameters was quadratic with a good regression coefficient (R^2=0.902). The relationship in a reduced quadratic model is shown in Eq. (4):

$$Y = -38.04 + 8.98X_1 - 18.56X_2 + 0.75X_3 - 0.02X_1^2 + 0.28X_2^2 - 0.062X_1X_2 - 0.028X_1X_3 + 0.086X_2X_3 \quad (4)$$

The response surface and contour plots between independent variables and the reducing power of Bene hull subcritical water extracts are shown in Fig. 2. The reducing power was calculated based on EC_{50} values. The low absorbance of the reaction mixture indicated a high reducing power, which indicated a high antioxidant capacity. Achieving a lower EC_{50} value was the purpose of this variable optimization. None of the linear model terms were significant ($p>0.05$) optimization of the reducing power.

The EC_{50} value was decreased when the extraction temperature and time were increased. This generally results in an increase in the reducing power. At short extraction

times, the value of EC_{50} increased when the temperature increased. On the other hand, the value of EC_{50} decreased with an increasing extraction time. This was due to the influence of the operating extraction time inside temperature. The second-degree temperature (A^2) and extraction time (B^2) terms, that relate to the mathematical model were significant ($p<0.05$). This might be the reason for the curvature of the surface. The effects of temperature and water to the Bene hull ratio on reduction power are shown in Fig. 2B. The EC_{50} value decreased with temperature and water to the Bene hull ratio. Curvature of the surface was due to the significance ($p<0.05$) of the quadratic temperature term (Table 3). According to the total sum of squares, the importance of the independent variables on reduction power could be ranked in the order of interaction between temperature and time (AB), the quadratic term of time (B^2), the interaction between temperature and water to the seed ratio (AC), and the quadratic term of temperature (A^2).

A similar trend was reported by Hassas-Rodsari *et al*. (35) where the effect of a temperature increase from 110 to 160°C as the only variable factor on canola meal subcritical water extracts was studied with the conclusion that there was no significant difference between the reducing power of the studied temperatures. Moreover, Hossain *et al.* (25) reported a significant interaction between temperature and the solvent concentration in rosemary, marjoram and oregano. hen the extraction temperature and time were increased, the dielectric constant of water was decreased due to increased solubility of less polar compounds, resulting in an increase in the extract reducing power.

DPPH scavenging ability The response surface quadratic model for the DPPH scavenging ability had a significant F-value ($p<0.01$), while the lack of fit test was not significant ($p>0.05$). As a result, the regression model was appropriate for predicting the DPPH scavenging ability. The linear and quadratic effects for extraction temperature and time were significant ($p<0.05$). Among the interaction terms, extraction time and temperature were significant ($p<0.05$). The R^2 and adj-R^2 values for this response variable were 0.987 and 0.975, respectively, indicating that the regression model was suitable to explain the behavior of the system. The relationship between the DPPH scavenging ability and the extraction parameters in a reduced quadratic model is shown in Eq. (5):

$$Y=+5779.74-50.98X_1-19.64X_2+0.15X_1^2+0.35X_2^2 -0.096X_1X_2 \quad (5)$$

To better understanding changes, contour plots and response surface plots for extraction variables against the DPPH scavenging ability were prepared (Fig. 3, 4). The DPPH scavenging ability was calculated based on EC_{50} values. In DPPH testing, antioxidants were able to reduce the stable purple DPPH radical to yellow diphenyl pricryl hydrazine. A low absorbance of the reaction mixture indicated a high free radical scavenging activity, which was interpreted from a graph of inhibition percentage against compound concentration. Achieving a low EC_{50} value was the purpose of this optimization. The temperature effect on the DPPH scavenging was significant ($p<0.05$). The DPPH scavenging ability increased when the temperature increased, resulting in a low EC_{50} value. For the effect of extraction time on the DPPH scavenging ability, an increase in the extraction time increased the DPPH scavenging ability (Fig. 4A). Based on the interaction effect of the temperature-time extraction (AB), the optimal point in the DPPH scavenging ability was near the surface center. The reasons for curvature of the surface were significant ($p<0.05$) second-degree temperature (A^2) and extraction time (B^2) terms.

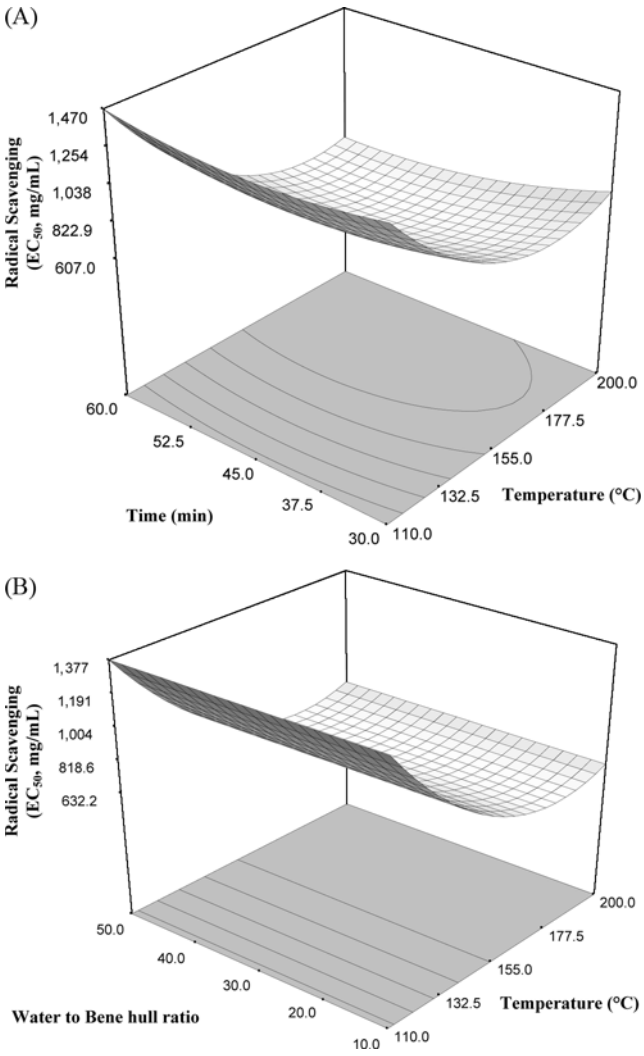

Fig. 3. Response surfaces and contour plots of the DPPH• scavenging ability (EC_{50}) of Bene hull SWE extracts as a function of (A) extraction time and temperature and (B) extraction temperature and water to the Bene hull ratio.

The effect of extraction temperature and water to the Bene hull ratio on the DPPH scavenging ability is shown in Fig. 3B. The temperature variation was similar to extraction temperature-time surface changes (Fig. 4A). Changes in the solvent mixture had no significant (p>0.05) effect on the DPPH scavenging ability. The scavenging ability remained constant at all of the mixing ratios (Fig. 4B). Generally, the temperature (A) was the most influential factor on the DPPH scavenging ability. However, other independent variables were ranked in the order of the quadratic terms of temperature (A^2), the interaction term between temperature-time (AB), the linear term of time (B), and the quadratic term of time (B^2). Rodriguez-Meizoso *et al*. (2) reported similar results.

The solubility of substances in water generally increases with temperature. A decrease in the dielectric constant of water at high temperatures and pressures is responsible for changes in the structure of electrolyte solutions. Compounds under these conditions result in an improvement in the DPPH scavenging ability, results of which were positively correlated with the reducing power. Extracts with a high DPPH scavenging ability also had a high reducing power. The obtained results were compatible with results reported by Farhoosh *et al*. (6) for Bene hull oil.

Optimization Optimum conditions for the subcritical water extraction procedure were determined in order to obtain a maximum polyphenolic content with a minimum EC_{50} value. The optimum conditions for extraction of Bene hull bioactive compounds were an extraction temperature of 196.8°C, time = 52.6 min, and water to the Bene hull ratio of 43.6:1. Under optimal conditions, the total phenolic content, the reducing power of Fe^{3+}, and the DPPH scavenging ability were 2,284 mg of GA/100 g of Bene hull, 200.161 mg/L (based on EC_{50} values), and 628.374 mg/L (based on EC_{50} values), respectively.

Farhoosh *et al*. (8) studied Bene hull oil antioxidative properties and unsaponified matters materials using traditional extraction methods with a hexane solvent, and the amount of Bene hull oil polyphenolic compounds obtained was 310 mg/kg. Furthermore, the DPPH scavenging ability (based on EC_{50} values) of Bene hull oil unsaponified material was 990 mg/L. Comparing these reported results with the report of Hassas-Roudsari *et al*. (35) showed that the amount of polyphenolic compounds and the reducing power of Bene hull subcritical water extracts were more than for canola meal subcritical water extracts, while canola meal subcritical water extracts had a higher DPPH scavenging ability.

In this study, the extraction yield of Bene hull subcritical

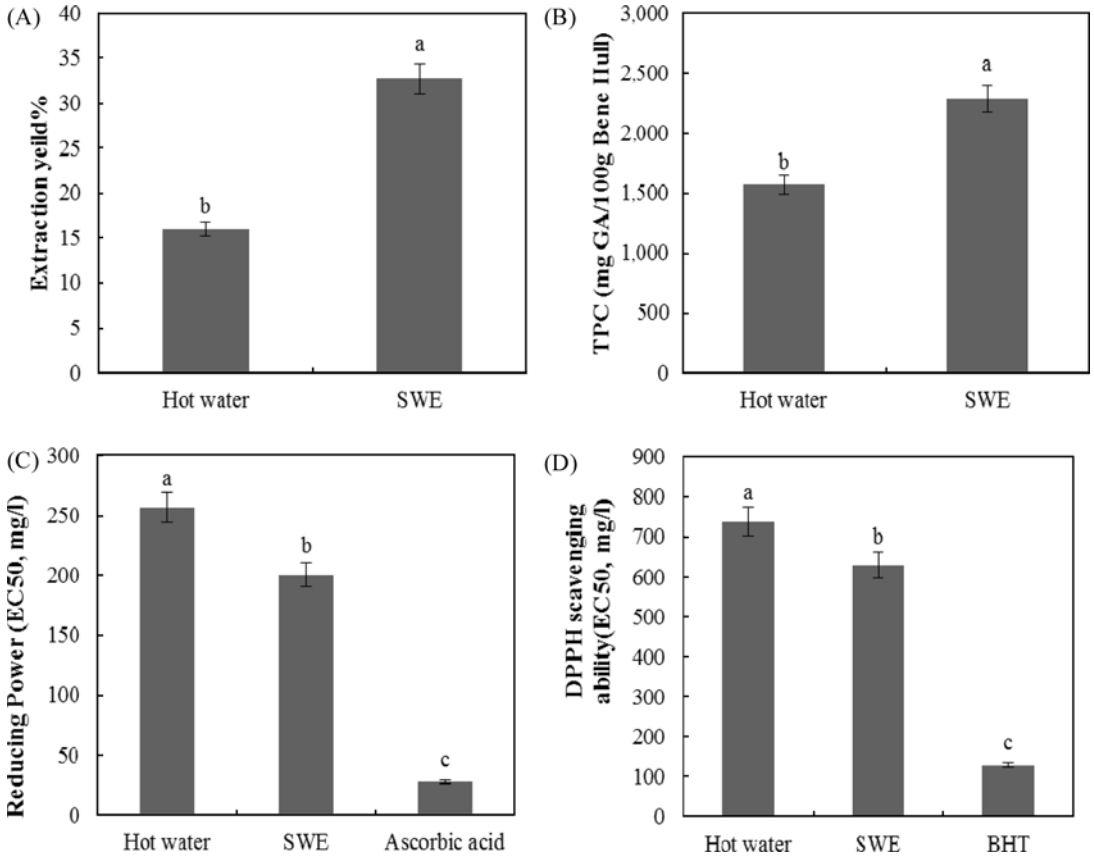

Fig. 4. Extraction yield (%) (A), polyphenolic compounds (mg/100 g of Bene hull) (B), reducing power of Fe^{3+} based on EC_{50} (mg L) (C), and DPPH scavenging ability (D) based on EC_{50} (mg/L) of hot water extracts (85°C) of *Pistacia atlantica* in comparison with SWE extracts and synthetic antioxidants. Duncan's test, p<0.01

water extracts was calculated as 32.72% at the optimal point, while the reducing power of ascorbic acid as an effective antioxidant was 27.454 mg/L (based on EC_{50} values). In other words, the reducing power of 200.161 mg/L of Bene hull subcritical water extracts was equal to the reducing power of 27.474 mg/L of ascorbic acid. The DPPH scavenging ability of the standard BHT was calculated to be 127.589 mg/L. Thus, the DPPH scavenging ability of 628.374 mg/L of Bene hull subcritical water extracts was equal to 127.859 mg/L of BHT. This result was expected because both ascorbic acid and BHT are pure antioxidants while Bene hull subcritical water extracts were not pure and contained other bioactive compounds and antioxidants. Yogendra Kumar *et al*. (18) reported that the ascorbic acid reducing power as a pure antioxidant was more than for sea buckthorn leaf subcritical water extracts.

Hot water extraction (85°C) Results for subcritical water extraction were compared with results obtained using a hot water extraction method at 85°C (Fig. 5). The average extraction yield, total phenolic content, and reducing power of both Fe^{3+} and DPPH for hot water extracts with 3 replicates were 15.95%, 1564.8 mg/100 g of Bene hull, 256.44 mg/L (based on EC_{50} values), and 737.233 mg/L (based on EC_{50} values), respectively. A significant difference ($p<0.01$) was observed between the 2 extraction methods. The subcritical water extraction method had a higher extraction yield and an antioxidant capacity than the traditional hot water extraction method.

HPLC analysis of the Bene hull SWE extracts Chromatograms of SWE extracts from Bene hull obtained under optimal conditions (temperature=196.8°C, time=52.6 min, and water to seed ratio=1:43.6) are shown in Fig. 5. By comparison of the retention times (R_t) of the experimental chromatograms with a standard chromatogram, that obtained from some standard compounds. the HPLC chromatogram of Bene hull SWE extracts under optimal conditions contained epicatechin (4.05%), chlorogensaure (5.86%), kaffesaure (0.51%), flavanomarein (0.28%), ethyl vanillin (0.49%), and apigenin 7-glucoside (0.55%). The Bene hull SWE extracts under optimal conditions included 51.81% of a component with R_t=4.2 min for the main phenolic component. Furthermore, the most important unknown compound obtained comprised 8.92% of the total components with R_t=1.97 min, followed by an unknown compound that comprised 8.71% of the total components with R_t=5.05 min, and a third unknown compound that comprised 7.24% of the total components with R_t=2.7 min.

Overall, the amount of polyphenolic compounds obtained from HPLC analysis was calculated to be 31,865.65 mg/kg using syringic acid as an internal standard. This amount was almost equal to the amount obtained using a chemical assay considering the conversion ratio of syringic acid to GA. The presence of phenolic compounds with a medium-high polarity was indicated by these results. Similar results have been reported by Rodriguez-Meizoso *et al*. (36). The presence of such compounds can partially explain the Bene hull SWE extract antioxidative activities.

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Disclosure The authors declare no conflict of interest.

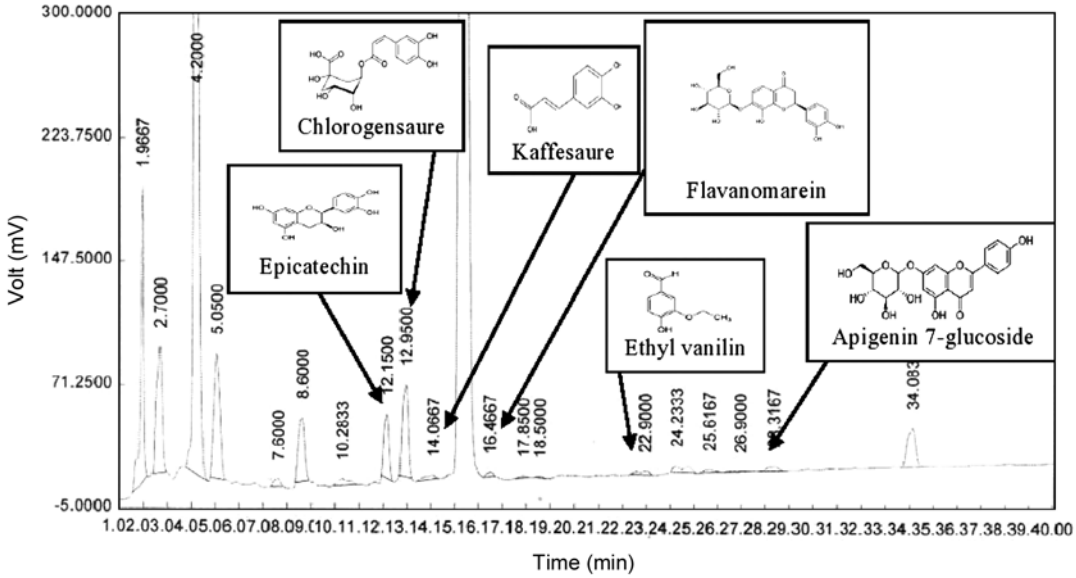

Fig. 5. HPLC chromatogram for subcritical water extracts of *Pistacia atlantica* for analysis of phenolic compounds.

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