Optimization of Extraction Conditions by Ultrasound-Assist on the Ratio of Flavonoids, Anthocyanins Content and Antioxidant and Antimicrobial Activity of *Punica granatum Var. Pleniflora (Persian Golnar)* Extract

Maleki Kahaki, Ali Reza; Nateghi, Leila*[+]; Rajaei, Peyman

Department of Food Science and Technology, Varamin-Pishva Branch, Islamic Azad University, Varamin, I.R. IRAN

ABSTRACT: *This study aimed to investigate the effect of type of solvent (1: ethanol and 2: water), time (3, 6, 9 min), and power (100, 200, 300 W) of ultrasound assist in the extraction rate of flavonoids, anthocyanins, Ic50 as well as the antimicrobial effect of the extract of Punica granatum. Var. Pleniflora (PGP). In order to design treatments, analysis, and optimization of dependent variables the full factorial design is used. The result of multiple optimizations of independent variables revealed that the highest amount of flavonoids in PG) extract (9.0502 mg/mL) and the amount of anthocyanin (5.3669 Mmol/g) and the lowest Ic50 value or the highest rate of free radical scavenging in (PGP) (8.0452 mg/mL) with 88.97% desirability were observed at 300 w for 9 min by using methanol as a solvent. The highest mean values of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of PGP extract obtained by ultrasound pretreatment were 625 and 3750 µg/ml respectively against Clostridium perfringens. The optimized predicted treatment by ultrasound pretreatment had a greater antimicrobial effect on Staphylococcus aureus with the largest diameter of the growth inhibition zone (14 mm) compared to E. coli and C. perfringens. The results showed that the extract obtained from PGP could be introduced as an antioxidant source in marketable foods.*

KEYWORDS: *Anthocyanins; Flavonoid; MBC; MIC; Punica granatum Var. Pleniflora.*

INTRODUCTION

Natural matters obtained from medicinal herbs are among the most important raw materials for manufacturing cosmetics, perfumes, flavorings, colorings as well as medicines. Plants are known as natural compounds. Today the term "natural compounds" refers to herbs, herbal potions, herbal supplements, traditional medicines, and traditional medicine in general. According to the World Health Organization (WHO), today more than 80% of the world's population still uses herbal medicines to treat diseases. Nearly a quarter of the medicines made

*** To whom correspondence should be addressed.*
+ E-mail: leylanateghi@iauvaramin.ac.ir & Leylanateghi@yahoo.com
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in the world are extracted directly from plants or produced based on herbal compounds [1]. Phenolic compounds are a large group of natural plant materials including flavonoids, tannins, anthocyanins, etc. commonly found in fruits, vegetables, leaves, nuts, seeds, roots, and other parts of the plants. About a quarter of the medicines worldwide that contain these compounds have found many applications in foods, chemistry, pharmaceutics, and medicine because of their desirable biological effects such as antioxidant activity [2].

Replacement of synthetic compounds with natural antioxidants has attracted attention in the food industry due to their healthiness, dissolution in water and oil as well as the production of proper emulsions. The effect of such antioxidants on a wide range of microorganisms has been demonstrated in extensive studies and they have been used as food preservatives as long as they do not impair the flavor and smell of the product [3]. Conventional methods of extraction are based on placing the plant in an appropriate solvent which is accelerated by stirring or heating. These methods include soxhlet, distillation, and percolation [4]. Traditional extraction methods used to obtain natural plant compounds such as water or vapor distillation as well as extraction with organic solvent have disadvantages including loss of volatile compounds, low efficiency, long extraction time, degradation of unsaturated compounds, and residual toxic solvent [5]. The growing demand for greener alternatives and natural materials that contain no toxic compounds, pose no threat to human health and the environment, and do not use high amounts of chemical solvents has attracted the attention of industries to non-toxic and reliable extraction methods [6]. The continuous need of humans for the extraction of bioactive compounds from plants has resulted in extensive studies on the introduction of more efficient and cost-effective extraction techniques. There are various methods to extract plant active compounds [4]. Thus, various extraction methods have been commercially developed to extract such invaluable plant materials. They are mainly focused on novel solutions to reduce or even eliminate the use of solvents in the extraction process as well as obtain purer products and more extensive use in different applications [7]. *Mozdastan et al*. [8] examined the solvents of hexane, ethyl acetate, methanol, water, chloroform, and butanol for the extraction of phenolic and antioxidant compounds and stated that water and methanol showed the highest extraction of phenolic compounds and the lowest IC50. In this regard, *Ghorbani et al.* [4] obtained the extract from fennel, *Rezaei Payandeh et al.* [9] from the skin of 10 varieties of Persian pomegranate, *Mehdinia Lichani et al.* [10] from Ferula persica, *Le-Floch et al.* [11] from olive leaves, and *Khademi* and *Mardaninejad* [12] from spiny oleaster by ultrasound process.

Punica granatum var. pleni flora belongs to the Puniaceae family. Fruitless pomegranate flowers are used as an important medicinal plant in Fars province and some of the northern regions of Iran [13, 14].

Punica granatum Var. Pleniflora tree is the male pomegranate tree whose flowers are not fruit-bearing. The leaves appear on the first days of spring being oblong in shape, 5 cm long, and 0.5 cm wide. They are dark green with their upper surface being shiny and their underside being dull. Like a pomegranate tree, it has thorny branches [13].

Pavankumar and *Shalini* [15] evaluated the intensity of bioactive extraction from pomegranate peel using Pulse ultrasound was performed and expressed the optimal process conditions of 2.17 g/100 mL S/S ratio at 116 W power with 80% duty cycle for 6 min resulted in 0.48 g/g yield, 177.54 mg GAE/g total phenolics content, 35.71 mg QE/g total flavonoids, 160.54 mg GAE/g antioxidant capacity, 21.65 mg cyn-3-glc/100 g anthocyanin content with 54.92 browning index in dry pomegranate peel.

PGP extract has great medicinal potential. In addition, it has natural antioxidant and potent antibacterial activity. In food products, it can render antioxidant and medicinal properties, increase nutritional value and storage time, enhance quality and color, prevent many diseases, and be used as a replacement for synthetic additives. Pomegranate skin extract was studied as a natural preservative for decontaminating and preserving the quality of steak and it was shown effective in surface disinfection and increasing the quality [16].

Ultrasonic waves are one of the novel methods for extracting phenolic and other valuable compounds from foods at laboratory and industrial scales [17]. Ultrasonic waves destroy the cell wall within a short time. The advantages of using ultrasound in the extraction process include shorter extraction time compared with traditional extraction using the Soxhlet method, high efficiency, and simple equipment compared with extraction using supercritical fluid and microwaves, low cost, and low extraction temperature, which provides the possibility

of extracting heat-sensitive compounds and using different solvents for extracting a wide range of natural plant compounds [18].

By extracting bioactive compounds from PGP (Persian Golnar) as a natural source of antioxidant compounds and optimizing its extraction conditions can be obtained a new source of natural antioxidants. The main goal of the present study was to optimize extraction conditions by ultrasound-assist on the amount of flavonoids and anthocyanins as well as antioxidant and antimicrobial properties of PGP.

EXPERIMENTAL SECTION

PGP was purchased from a local market in Saveh, Iran, and then ground into a fine powder. The chemicals including methanol (Purity 80%), potassium chloride 0.2 M, chloric acid 0.2 M, Sodium acetate 1 M, Hydrochloric acid 1 M, Aluminum chloride, potassium acetate 1 M, DPPH (2,2-diphenyl-1-picrylhydrazyl), and Mueller Hinton broth were obtained from Merck. Co (Germany).

Extraction by ultrasound

Initially, *PGP* was rinsed, peeled, and cut into small pieces. Next, it was dried in the oven (UFSS/UN, Momment company Germany) at 45°C for 24 h to reach a constant weight. It was then ground into floury granules by using a mill (ML-320p, Pars Khazar Co., Iran) and sieved with a mesh No. 16. The obtained powder was stored in polyethylene containers at 25°C in a dry place for further use.

The results of research by other researchers have shown that the highest amount of phenolic compounds is extracted by polar solvents such as water and methanol [8].

The extraction of pigments from PGP was performed in a complete factorial design with tree independent variables including the type of solvent (water and methanol), time (3, 6, 9 min), and power (100, 200, 300 W) as shown in Table 1. 20 g of dried samples of PGP were mixed with each solvent (water and methanol) separately in the ratio of 10:5 [solvent (ml): plant matter (g)] and then sonicated at frequencies of 28-34 kHz in an ultrasonic bath (Tecno-Gas .S.P.A., Italy) at 100, 200, and 300 W for 3, 6 and 9 minutes. The extract, then was separated with Whatman filter paper No.1. The filtered extract was concentrated by a rotary evaporator at 50°c to get a brix value of 60 brix and dried in an oven at 40 brix for complete drying [19].

Table 1: Design of treatments by full factorial method.

Treatment	Solvent type	Power (w)	Time (min)
1	2	100	6
2	1	300	9
3	2	200	3
4	1	200	9
5	2	200	9
6	1	300	3
7	2	200	6
8	2	300	3
9	1	300	6
10	1	200	6
11	1	100	6
12	1	100	9
13	2	100	9
14	2	100	3
15	2	300	6
16	1	200	3
17	1	100	3
18	2	300	9

1. Methanol 2. Water

Tests performed on samples measurement of total flavonoid content

Total flavonoid content was measured through calorimetry. 0.5 mL of the extract was dissolved in 1.5 mL of methanol in a test tube to which 0.1 mL of 10% aluminum chloride and 1 M 0.1 ml of potassium acetate were added. Finally, 2.8 ml of distilled ml water was added, and stored at ambient temperature for 30 min, and then the absorbance was read at 415 nm wavelength by spectrophotometry. Quercetin (Merck Co., Germany) was used as the standard for drawing the calibration curves. The flavonoid content was expressed as quercetin equivalent per g of dried sample [20].

Measurement of total anthocyanins content

Anthocyanins content was determined by pH differential method. The first 2 mL of the extract reached the desired volume by using 25 mL of a butter solution at pH 1 consisting of a mixture of 0.2 M potassium chloride

and 0.2 M chloric acid and then another 2 ml of the extract reach a 25 ml volume by a buffer solution at pH 4.5 (the lowest amount of anthocyanins hydrolysis is at pH 4.5) containing 1 M hydrochloric acid and the absorbance was read at 0.5 nm. The concentration of anthocyanin was calculated by using the following Eq. (1):

$$\text{Anthocyanin} = \frac{(\text{Abs pH } 1 - \text{Abs pH } 4.5) \times 484.82 \times 1000}{24825} \times \text{DF} \quad (1)$$

Where 82.484 and 24825 are molecular weight and molar absorption coefficient of Cyaniding-3-giucoside, respectively, at 510 nm in the buffer solution. DF is the dilution factor [21].

Measurement of free radical scavenging activity of DPPH

The ability of donating hydrogen atom or electron by *(PGP)* was measured by discoloration of ethanolic DPPH solution. 2, 2,-diphenyl-1-pycrylhydrazyl is a purple color stable radical which is converted into yellow color diphenyl-1- pycrylhydrazine via reducing by electron- or hydrogen- donating elements (antioxidants). To do so DPPH was used as stable radical compounds. 50 µL of different concentrations of the ethanolic essence were added to 5 mL of 0.004% DPPH ethanol. After incubation for 30 min at room temperature in order to supply hydrogen or electrons by using ethanol DPPH solution, the absorbance was read at 517nm against control and IC50 or the concentration required for scavenging 50% of the radicals was calculated by Eq. (2) [22].

$$\text{DPPH} = \frac{\text{Control absorbance}(\%) - \text{Sample ansorbance}(\%)}{\text{Control absorbance}(\%)} \times 100 \quad (2)$$

The concentration at which the PGP extract was able to scavenge 50% of DPPH free radicals was expressed as IC50. To calculate IC50, the diagram of free radical scavenging percent against antioxidants (0.5, 0.25, 0.125, 0.625, 0.312, 0.0156, 0.0078 mg/mL) were drawn.

Microbial tests

The optimized predicted treatments by all three pretreatments (maceration, microware, ultrasound) with the highest antioxidant activity was produced, their antioxidant property was confirmed and then their antimicrobial effect was examined via growth inhibition well and disc diffusion methods on *E.coli*, *S.aureus* and *C.perfringens*. 96-well microplates were used to investigate the antimicrobial properties of the extracts. First, 95 µl of Meuller Hinton broth and 5 ml of the bacterial suspension equivalent to 0.5 McFarlad tube were added to each ELISA plate well and then 100 mL of the serial dilution of the extract were added. The samples were mixed by the shaker (300 rpm, 20 s) and incubated at 37 °C for 24 h. Next the turbidity was read at 540 nm by ELISA system (Model ELX 800, biotech Co. USA) and in the case of lack of turbidity MIC was determined. The sample from wells without turbidity were placed on Mueller Hinton broth medium and the colonies were counted. The first tube in which the reduction in bacterial growth rate greater than one thousandth occurred as compared to the zero time of control was determined as Minimum Bactericidal Concentration (MBC) [23].

Statistical analysis

In order to designs of treatments, analysis, and optimization of dependent variable the full factorial design was performed in Minitab 16 software. To compare and evaluate the antimicrobial properties of the optimal sample, one-way analysis of variance Duncan's in Minitab 16 software was used.

RESULTS AND DISCUSSION

The efficiency of the extraction of phenolic compounds depends on factors including time, temperature, particle size, matrix porosity, type of solvent, concentration of solvent, PH, ratio of sample to solvent and solvent diffusion coefficient [24]. Also for the ultrasonic process, in addition to the mentioned factors, the power of ultrasound also affects the extraction efficiency.

Flavonoid contents of PGP extract obtained by ultrasound extraction

Flavonoid content of PGP extract obtained by ultrasound methods under different conditions is presented in Table 2. As shown in the Table, the conditions of extraction (ultrasound power, time and the type of solvent) had significant effect on the flavonoid content of PGP extract extracted by ultrasound pretreatment. Flavonoid content ranged from 5.06 to 9.81 mg/mL. The results showed that increase in ultrasound power (from 100 to 300 W), time of extraction (from 3 to 9 min) and use of methanol instead

of water had significant ($p \leq 0.05$). The highest flavonoid content (9.81 mg/ mL) was observed at 300 W for 9 min by using methanol and the lowest content (5.056 mg/mL) was found at 100 W for 3 min by using water as solvent. The type of solvent and ultrasound power had a significant effect on flavonoid content but the type of solvent with a higher coefficient had the greatest effect with a positive slope. Higher flavonoid content resulting from longer ultrasound time may attributed to the cavitation phenomenon in which propagation of sound waves in the solid-liquid phase generates contraction and expansion cycles in the environment that produce bubbles which grow and finally collapse. It causes solid and liquid particles to oscillate which is accelerated by ultrasound. As a result, solutes from solid phase are rapidly despersed in the solvent. The power of ultrasound leads to more mechanical oscillations in the liquid and the greater effect of such oscillations causes more solvent to penetrate into the cellular material improving mass transfer [25]. The effects of cavitation and mechanical mixing are the most important ultrasound extraction mechanisms that increase the extraction efficiency and decrease the time of extraction that has been demonstrated in this study. In addition, thermal degradation of heat-sensitive compounds is avoided because no thermal processing is applied [26]. In agreement with our result, *Guandalini et al.* [27] studied the successive extraction of phenolics and pectin from mango skin by ultrasound. The result revealed that ultrasound methods did not have a significant effect on extraction efficiency. The highest efficiency was obtained when the solvent containing 50% ethanol in water was used and ultrasound was not applied. The residues from this extraction were used for pectin extraction by ultrasound. The results showed that the use of water instead of methanol had a significant effect on the extraction of flavonoids. The extraction of phenolics from artichoke wastes by successive processes using ultrasound and membrane technology was investigated by *Rabelo et al.* [28]. Their results showed that the application of ultrasound power higher than 240 W did not have a significant effect on the efficacy of the process and that the most important factor was the solvent composition.

Anthocyanin content of PGP extract obtained by ultrasound

The anthocyanin content of PGP extract extracted by ultrasound pretreatment under different conditions by tested and predicted methods is presented in Table 2. As illustrated in Table 2, different extraction parameters (ultrasound power, extraction time, and type of solvent) had a significant effect on the amount of anthocyanin of PGP extract obtained by ultrasound pretreatment. The result showed that an increase in ultrasound power (from 100 to 300 W), time of extraction (from 3 to 9 min) and the use of water instead of methanol had significant ($p \leq 0.05$) effect on increased anthocyanin content. Anthocyanin content ranged from 3.03 to 5.818 mmol/g. The highest anthocyanin content (5.818 mmol/g) was observed at 300 W for 9 min by using water and the lowest amount of anthocyanin (3.03 mmol/g) was obtained at 100 W for 3 min by using methanol solvent.

Type of solvent, ultrasound power and time had significant effect on the amount of anthocyanin with higher ultrasound power with higher coefficient exerting the greatest effect with a positive slope. Ultrasound waves are sinusoidal so they generate bubbles in the environment which are filled with solvent vapor. During the pressure cycle, these bubbles with the gas inside them are compressed and exploded increasing the pressure and temperature of the environment resulting in better mixing of the solvent and plant matter. In addition, ultrasound also produces a mechanical force and increases the penetration of solvent into the plant tissue thereby increasing mass transfer and breakdown of cell wall. Ultrasound also may cause chemical changes because of producing free radicals during cavitation phenomenon promoting the penetration of solvents into cells as well as mass transfer. The rapid changes in temperature and pressure caused by cavitation lead to shear breakage and cell membrane thinning which allow ultrasound to change the environment [7].

Saifullah et al. [29] reported the time and temperature of extraction had a positive effect on the efficiency of the extraction of phenolics, flavonoids, and anthocyanins from lemon verbena stated that total flavonoid content increased as the time of extraction increased, The highest amount of flavonoids was obtained at 50 ºC for 60 min. *Gunjevic et al.* [30] studied the extraction technologies for extraction of anthocyanins from grape pulp by use of deep eutectic (relating to or denoting a mixture of substances (in fixed proportions) that melts and solidifies at a single temperature that is lower than the melting points of the separate constituents or of any other mixture of them) solvent and reported that the highest extraction efficiency

Table 2: Comparison of flavonoid and anthocyanin content and IC50 value of PGP extract obtained by ultrasound pretreatment under different conditions by tested and predicted method.

Treatment	Flavonoids (mg/g)		Anthocyanin (µmol/g)		IC50 (mg/mL)	
	Experiment concentration	Predicted concentration	Experiment concentration	Predicted concentration	Experiment concentration	Predicted concentration
1	5.675	5.620	3.506	3.414	15.074	15.206
2	9.810	9.305	5.152	5.298	8.102	8.150
3	5.985	5.900	3.939	3.981	14.475	14.766
4	8.089	8.441	4.636	4.572	9.517	9.455
5	6.477	6.419	4.885	4.893	13.852	13.451
6	8.171	8.428	4.515	4.435	9.006	9.103
7	6.638	6.169	4.424	4.388	14.203	14.056
8	6.559	6.338	4.566	4.573	13.694	13.413
9	8.799	8.876	4.879	4.818	8.848	8.574
10	7.979	8.090	4.182	4.185	9.813	9.859
11	7.324	7.273	3.212	3.263	10.807	10.921
12	7.460	7.545	3.636	3.557	10.466	10.537
13	5.958	5.792	3.688	3.825	14.560	14.622
14	5.056	5.430	3.152	3.100	15.962	15.896
15	6.586	6.868	4.939	5.073	12.556	12.683
16	7.843	7.721	3.848	3.895	10.096	10.368
17	7.187	6.983	3.030	3.066	11.724	11.410
18	6.723	7.015	5.818	5.671	11.77	12.058

was obtained at 50 W for 10 min and with a solvent with 30% V /V water.

IC50 value of PGP extract obtained by ultrasound method

The procedure of extracting phenolics is an important factor affecting the determination of antioxidant properties of the extract obtained by ultrasound pretreatment under different conditions by using the tested method and predicted values are presented in Table 2. As shown in the Table, different extraction parameters (ultrasound power, time, and type of solvent) had a significant effect on IC50 of the PGP extract. IC50 value ranged from 8.102 to 15.962 mg/mL. The results showed that the highest rate of free radical scavenging or the lowest IC50 value (8.102 mg/ mL) in the PGP extract was observed at the of 300 W for 9 min by using methanol and the highest Ic50 value or the lowest rate of free radical scavenging (15.962 mg/ mL) was found at the ultrasound power of 100 W for 3 min by using water as solvent. PGP extract is rich in phenolic compounds which are capable of scavenging free radicals due to the presence of hydroxyl groups and acts as donors of electron or hydrogen. The presence of more antioxidant compounds leads to more inhibition of free radicals and thus reduces the amount of IC50 which was observed in more ultrasound power and longer time and the use of methanol solvent, which can be due to the presence of higher phenolic compounds in the extract.

Mozdastan et al. [31] evaluated the effect of the ultrasonic extraction method by using methanol on the antioxidant activity of myrtle leaves and stated that the highest rate of free radical scavenging was 1.355 µg/mL. *Saifullah et al.* [29] reported the Ic50 of lemon verbena extract obtained by DPPH under optimal extraction conditions (50°C, 60 min, 200 W) as 29.889 mg/mL. The use of the ultrasound method showed the highest efficiency in the extraction of phenolic compounds and detection of antioxidant properties.

Table 3: Polynomial model for prediction of flavonoid, anthocyanin and IC50 in PGP extract obtained by ultrasound.

Source	Model	R^2	R^2-adj
Flavonoids (mg/mL)	$Y= 7.12948- 0.96075A+ 0.66706B+ 0.30963C -0.01594B^2-0.00911C^2 -0.13432AB-0.05009AC+ 0.07854BC$	96.33	93.06
Anthocyanin (µmol/g)	$Y=4.28687+ 0.10152A+ 0.80369B+ 0.39707C -0.14470B^2+0.04848C^2+ 0.02591AB+0.05869AC+0.09318BC$	98.81	97.75
IC50 (mg/mL)	$Y= 11.9576 +2.0986A -1.2176B-0.5569C -0.1114B^2+0.0525C^2 -0.0440AB-0.1002AC-0.0201BC$	99.26	98.61

A. Type of solvent B. ultrasound power C. ultrasound time

Pan et al. [32] investigated the extraction of tanshinones from Salvia miltiorrhiza by using soxhlet, microwave, and ultrasonic methods and reported that the highest rate of extraction was obtained by microwave technique for 60 s. In all the above-mentioned methods, a high ratio of water solvent could extract high flavonoid content. *Aghajani et al*. [33] stated that in the DPPH test the use of the microwave method resulted in a higher amount of extracts with higher free radical scavenging activity as compared to the maceration method. *Wang et al*. [24] studied the effect of high-power ultrasound on the antioxidant capacity of kiwifruit extract. The results showed that the ultrasound at 400 W and 25 kHz for 16 min increased the antioxidant capacity significantly compared to control samples. The microscopic images revealed that there were canals or cavities in the tissue of the ultrasound-treated samples and the cells were destroyed resulting in the tissue rupture. Phenolic compounds are commonly soluble in plant tissues. Ultrasound with high pressure destroys the cell wall and vacuoles increasing the phenolic content. The increase in flavonoids may also be the result of cell destruction which causes the release of flavonoids in Kiwifruit extract. The antioxidant capacity of Kiwifruit extract increased by the application of ultrasound.

Polynomial model of PGP extract obtained under different conditions

The results of variance analysis on flavonoids in this quadratic polynomial model are shown in Table 3. As shown in the Table, the coefficient of determination of this model (R^2) was 96.33% and its adjusted coefficient of determination (R^2-adj) was 93.06% indicating the good fit of the model to the experimental data. Variance analysis was performed on anthocyanins in this quadratic polynomial model and the results are presented in Table 3. As shown in the Table, the coefficient of determination (R^2) of this model was 98.81% and its adjusted coefficient of determination (R^2-adj) was 97.75 suggesting the good fit of the model to the experimental data.

Analysis of variance was performed on Ic50 in this quadratic polynomial model and the results are illustrated in Table 3. As shown in the Table, the coefficient of determination (R^2) of this model was 99.26% and its adjusted coefficient of determination (R^2-adj) was 98.61% showing the good fit of the model to the experimental data.

Results of analysis of variance

As shown in Table 4, the linear effect of all three variables of ultrasound power, extraction time, and type of solvent on the flavonoid content of PGP extract was significant ($p\leq0.05$). The quadratic effects of ultrasound power, type of solvent, and extraction time as well as interaction effects of ultrasound power, extraction time, and type of solvent on flavonoid content were not significant ($p>0.05$). Given the value of F factor, the linear effect of the type of solvent had the greatest influence on flavonoid content in the PGP extract obtained by the ultrasound method followed by the linear effects of ultrasound power.

In Table 4, the linear effect of all three variables of ultrasound power, extraction time, and type of solvent and the quadratic effect of ultrasound power (B^2) on anthocyanin content of PGP extract were significant ($p\leq0.05$). however, the quadratic effects of type of solvent and extraction time as well as the interaction effects of ultrasound power, type of solvent, and extraction time on anthocyanin content in PGP were not significant ($p>0.05$).

Considering the value of F factor, the linear effect of ultrasound power had the greatest influence on anthocyanin content in PGP extract followed by the linear effect of extraction time.

As shown in Table 4, the linear extraction effect of ultrasound power, extraction time and the type of solvent on IC50 (mg/mL) in PGP extract were significant ($p\geq0.05$). While the quadratic effects of ultrasound power,

Table 4: Results of analysis of variance for extraction of PGP extract by ultrasound assist.

Source of Changes	Flavonoids (mg/mL)		Anthocyanins (μmol/g)		IC50 (mg/mL)	
	F-value	P-value	F-value	P-value	F-value	P-value
Constant	29.51	0.000*	93.53	0.000*	151.45	0.000*
Linear effects	77.69	0.000*	244.14	0.000*	403.03	0.000*
Solvent Type (A)	167.60	0.000*	13.82	0.005*	951.01	0.000*
Power (B)	53.86	0.000*	577.61	0.000*	213.44	0.000*
Time (C)	11.60	0.008*	140.99	0.000*	44.66	0.000*
Quadratic effects	0.01	0.993	3.47	0.076	0.36	0.705
Power ×Power (B^2)	0.01	0.922	6.24	0.034*	0.60	0.460
Time × Time (C^2)	0.00	0.955	0.70	0.424	0.13	0.725
Interaction	1.00	0.438	2.95	0.091	0.59	0.638
Solvent × power (A×B)	2.18	0.174	0.60	0.458	0.28	0.611
Solvent × Time (A × C)	0.30	0.595	3.08	0.113	1.45	0.260
Power × Time (B × C)	0.50	0.498	5.18	0.049*	0.04	0.848
Lack of fit	0.1	0.2	0.4	0.5	0.1	0.3

type of solvent and extraction time as well as interaction effects of ultrasound power, extraction time, and type of solvent on Ic50 (mg/mL) were not significant ($p>0.05$). Given the value of F factor, the linear effect of the type of solvent had the greatest influence on antioxidant content followed by the linear effect of ultrasound power.

Single optimization conditions for flavonoid, anthocyanin, and IC50 in PGP extract obtained by ultrasound method

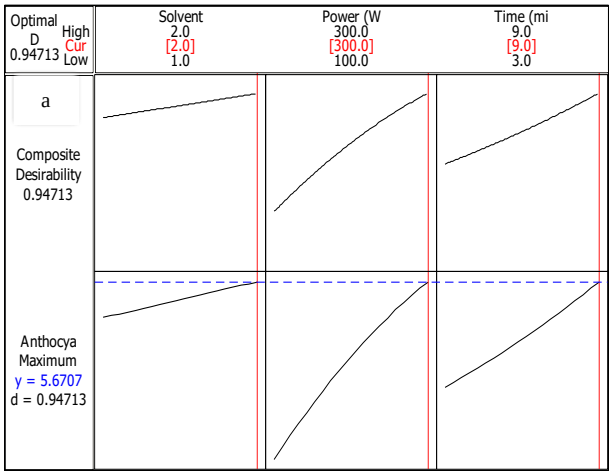
Fig. 1 shows the optimized conditions for obtaining the maximum amount of anthocyanin in PGP extract by ultrasound. As illustrated in the Figure, the maximum amount of anthocyanin was obtained as 5.6707 Mmol/g with the desirability of 94.713% Mmol/g at 300 W for 9 min by use of methanol as solvent. The in vitro predicted maximum amount of anthocyanin (5.6709 Mmol/g) was obtained showing no significant ($p>0.05$) from the anthocyanin content predicted by the full factorial model.

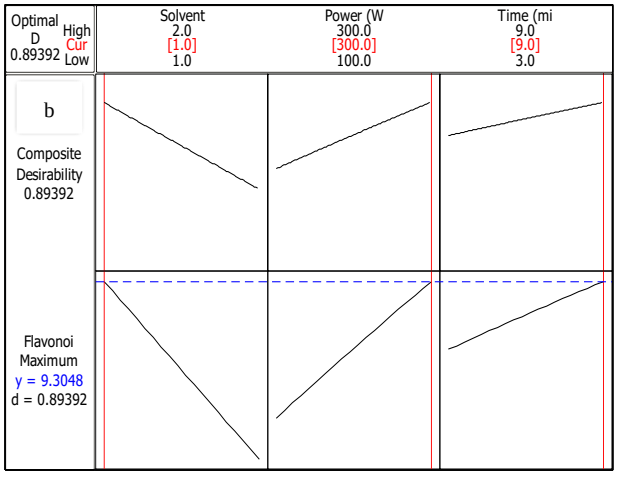
Fig. 1 b shows the optimized conditions for obtaining the maximum amount of flavonoids in PGP extract obtained by ultrasound. As shown in the Figure, the maximum amount of flavonoid was obtained as 9.3048 mg/mL with desirability of 89.392 % at 300 W for 9 minutes by use of water. The in vitro predicted maximum amount of flavonoid (9.3094 mg/mL) was obtained showing no significant ($p>0.05$) difference from the flavonoid content predicted by the full factorial.

Fig. 1C shows the optimized conditions for obtaining the maximum value of IC50 in PGP extract by ultrasound. As shown in the Figure, the lowest IC50 value, namely the highest amount of antioxidants was obtained as 8.0425 mg/mL with a desirability of 100 at 300 W for 9 min by use of water as solvent. The in vitro predicted maximum amount of antioxidant or the lowest IC50 (8.0224 mg/mL) was obtained showing no significant ($p>0.05$) difference from the IC50 (mg/mL) value predicted by the full factorial model.

Simultaneous optimization of flavonoid, anthocyanin, and IC50 values in PGP extract obtained by ultrasound under different conditions

Fig. 2 shows the diagram of simultaneous optimization of flavonoid, anthocyanin, and IC50 values in PGP extract obtained by ultrasound under different conditions. As indicated in the Figure, the optimization of the extraction conditions for obtaining PGP extract with the highest flavonoid and anthocyanin content and the lowest IC50(mg/mL) simultaneously with the desirability of 88.974% at 300W for 9 min by use of water as solvent





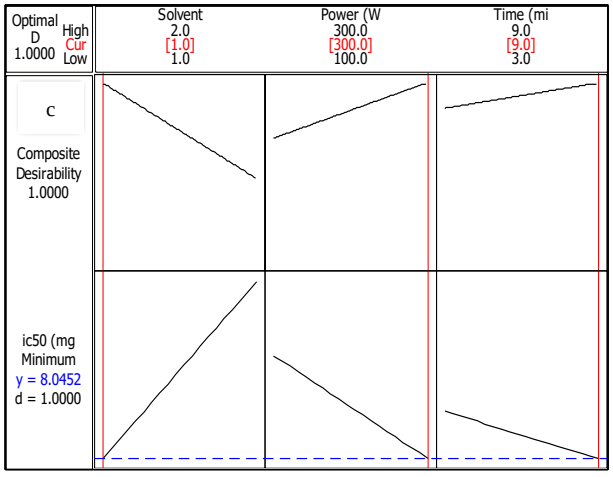


Fig. 1: Diagram of singe optimization of flavonoid, anthocyanin, and IC50 values in PGPextract obtained by ultrasound (a) flavonoid content, (b) anthocyanin content, (c) IC50.

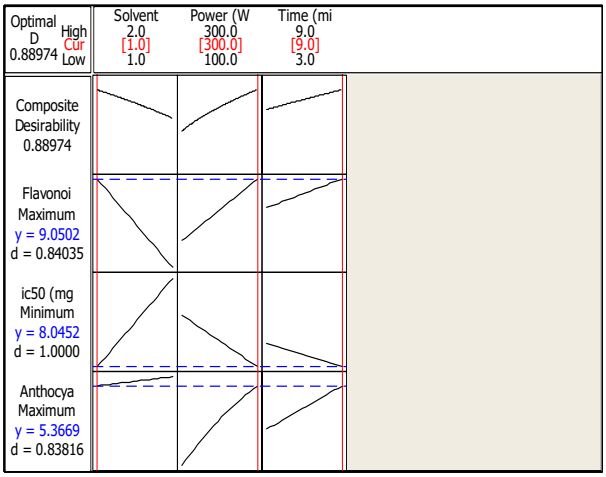


Fig. 2: Multiple optimized conditions for flavonoid, IC50 and anthocyanin in PGP extract obtained by ultrasound.

was predicted. As shown in Fig. 7 flavonoid content of 9.0502 mg/mL, anthocyanin content of 5.3669 Mmol/g and IC50 of 8.0452 mg/mL were predicted. The in vitro amounts of anthocyanin, flavonoid, and IC50 were 5.512 Mmol/g, 9.11 mg/mL, and 8.05 mg/mL, respectively that were not significantly ($p>0.05$) different from the values predicted by the full factorial model.

Antimicrobial effect of PGP extract obtained by ultrasound

Multiple optimization conditions under which the highest amounts of flavonoid, antioxidants, and anthocyanin were selected for extraction of PGP extract, and the antimicrobial effects on *S. aureus*, *E. coli*, *C. perfringens* were examined by well and disc methods as illustrated in Fig. 3.

Table 5 shows the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against *S. aureus*, *E. coli* and *C. perfringens* for the predicted treatment by ultrasound method. The results revealed that there was no significant ($P<0.05$) difference in MIC of *PGP* extracted obtained by ultrasound method between *C. perfringens* and *E.coli*, however, MIC of this extract showed significant ($P\leq0.05$) difference among *C. perfringens*, *E.coli* and *S. aureus*. The lowest mean MIC and MBC of PGP extract obtained by ultrasound method were 312.5 µg/mL and 1250, respectively against *S. aureus* and the highest mean MIC and MBC were 625 µg/mL and 3750 respectively against *C. perfringens*. The optimum

Table 5. Results of mean MBC and MIC in PGP extract obtained by ultrasound pretreatment against S. aureus, E.coli and C. perfringens

Microorganism	Well (mm)	Disc (mm)	MIC (µg/mL)	MBC (µg/mL)
Staphylococcus aureus	32	14	312.5[b]	1250[c]
E.coli	18	8	625[a]	2500[b]
Clostridium perfringens	17	7	625[a]	3750[a]

Similar letters in each column are not significantly (P>0.05) different.

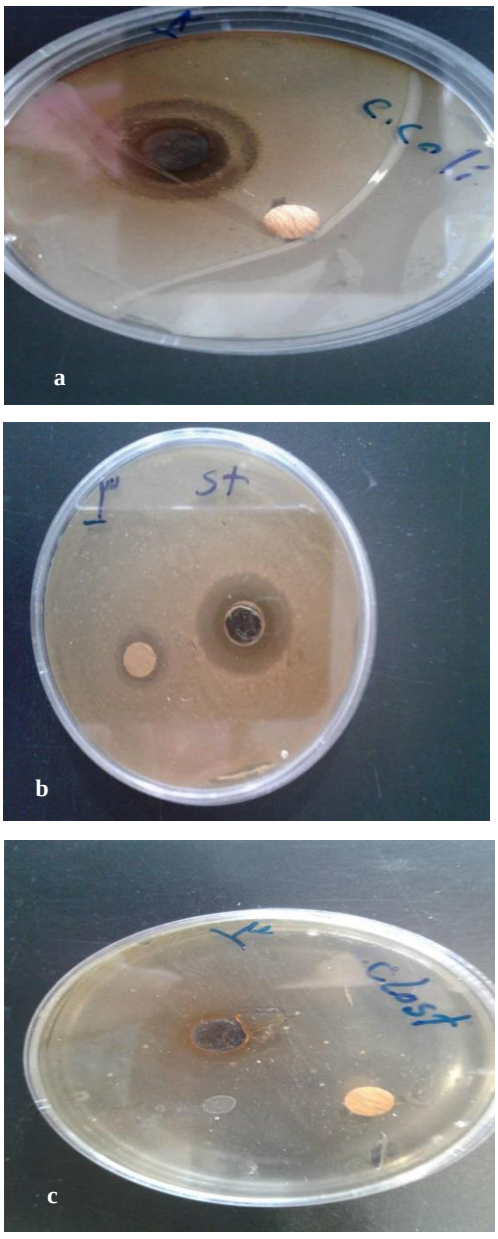


Fig. 3: Growth inhibition halo diameter generated by PGP extract obtained by ultrasound pretreatment against (a) S. aureus, (b) E.coli (c) C. perfringens.

predicted treatment had a superior antimicrobial effect against *S. aureus* with the largest growth inhibition halo (14 mm) being found for *E.coli* and *C. perfringens*. Antimicrobial compounds in medicinal herbs and spices are typically attributed to phenolics with - OH groups and thymol and carvacrol are the responsible for antimicrobial properties of medicinal herbs [34].

The hydroxyl group of phenolic compounds binds to the active site of enzymes preventing their metabolism. There is an important synergism between carvacrol and its precursor, para-cymene, as para-cymene first swells the cell membrane which facilitates the influx of more carvacrol and finally the effect of carvacrol kills the microorganism [35]. By another likely mechanism, these compounds bind to phospholipids of the cell membrane reducing the selective permeability and increasing the membrane permeability so that the cellular constituents exit from the cell impairing energy metabolism. The uptake of nutrients by microbial cells as well as electron transfer and the synthesis of genetic materials is changed [35]. *Shanmugapriya et al.* [36] studied the antioxidant and antimicrobial effects and the chemical composition of black pepper and demonstrated that black pepper extract containing phenolic compounds could exert inhibitory effects on important pathogenic bacteria, yeasts and some fungal strains.

Shirmohammadi and *Zaringhalami* [37] studied the effect of extraction methods and ultrasound on the antimicrobial activity of ethanolic extract of linseed powder and reported that the extract obtained by ultrasound process for 30 min had the highest antioxidant activity. This extract could control the growth of *E.coli* and *S. aureus* with halo diameter of 9 and 6 mm.

CONCLUSIONS

Extraction assisted by ultrasound has been demonstrated as a simple, inexpensive, fast, environment-friendly and high- efficiency method. However, in order

to obtain the best and the most effective extract, parameters including the characteristics of plant material, time and temperature of extraction, appropriate solvent, method of extraction, and accuracy of extraction steps need to be considered. The results of the present study suggest that PGP extract has flavonoids, anthocyanins as well as antioxidants and therefore it is a good source of antioxidant compounds. Thus, IC50 of 8.05 mg/mL, anthocyanin content of 5.512 µmol/g, and flavonoid content of 9.11 mg/mL were obtained under in vitro predicted optimized conditions. Ultrasound waves could increase the amount of soluble flavonoids thereby increasing the antioxidant activity. This finding is important because the extracts have no harmful effects of toxic and dangerous solvents such as methanol and are easily applicable in the food, pharmaceutical, and drink industries.

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