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Research Article

OPTIMIZATION OF THE ULTRASONIC ASSISTED EXTRACTION PROCESS TO OBTAIN PHENOLIC COMPOUNDS FROM POMEGRANATE (*Punica granatum*) PEELS USING RESPONSE SURFACE METHODOLOGY

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Abstract: Pomegranate peels are a potential source of bioactive compounds which are known for their antioxidant and antimicrobial activities. The present work aims to study, the optimization of ultrasonic assisted extraction (UAE) method to obtain phenolic compounds from pomegranate peels using ethanol as a solvent. It further aims to investigate the effect of three independent variables such as solvent–solid ratio, extraction temperature and extraction time on the response variables such as total yield (TY), total phenolic content (TPC) and total flavonoid content (TFC) using response surface methodology (RSM). The results of RSM revealed the optimized conditions of the solvent-solid ratio, extraction temperature and extraction time as 16.09, 30°C and 17.8 minutes respectively, with the maximum yield of 14.20%, TPC of 140.61 mg GAE/ml extract and TFC of 2.96 mg RE/ml extract. The results were further validated under the same conditions using UAE and then compared with the thermostatic water bath extraction method (TWE).

Keywords: *Ultrasonic extraction, Pomegranate peel, Phenolic, Flavonoid compounds*

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Introduction

A large amount of waste material is produced during the processing of fruits and vegetables. These waste residues, traditionally considered as an environmental problem, however, are being increasingly recognized as sources of bioactive compounds. The bioactive compounds such as polyphenols from waste residues may be used as natural antioxidants to substitute synthetic antioxidants that have posed health problems [1]. Recently, the waste coming from pomegranate, orange, apple, carrot, peach, onion and cherry have been used as sources of dietary fibre supplements with processed food. Pomegranate (*Punica granatum* L.) has high antioxidant activity because of which it is considered as one of the healthiest fruits [2, 3]. A pomegranate is a seeded or granular apple which has been cultivated for thousands of years [4]. According to Government of India, Ministry of Agriculture & Farmers Welfare, India produced approximately 2442 thousand MT of pomegranate in the year 2016-17. The nutrient values per100 g fruit of raw pomegranate are: moisture, 77.93 g; protein, 1.67 g; total lipid,1.17 g; carbohydrate 18.70 g; fibre 4.0 g; sugars, 13.67 g; vitamin C, 10.2 mg; vitamin E, 0.60 mg; vitamin K, 16.4µg; and niacin, 0.293 mg (https://ndb.nal.usda.gov/ndb/search/list). About 50% of the total fruit weight corresponds to the peel which is generally discarded as waste [5]. Studies showed that peel contains maximum amounts of bioactive compounds and higher antioxidant capacity as compared to juice against scavenging of hydroxyl radical, superoxide anion and copper sulphate inhabitation assays [6]. The Studies on pomegranate peel extracts also proved that it possesses a wide range of biological actions including anticancer activity, antidiarrheal activity, anti-inflammatory activity, and antimicrobial activity. Thus, the waste like peel generated during processing has emerged as an ideal substrate for the extraction of valuable bioactive compounds [7,8]. The extraction of antioxidants from industrial and agricultural by-products has gained great popularity in recent years [9]. The extraction of compounds is done using ultrasound, which is an important extraction technique that results in less operation time, reduced solvent usage and temperature and lowers energy input.

Therefore, ultrasound-assisted extraction (UAE) is an "environment-friendly" technique [10] which has many advantages over conventional Soxhlet extraction such as increased cavitation, increased the extraction efficiency, and shorter operating time. Ultrasound assisted extraction also has more advantages over other extraction techniques such as supercritical fluid extraction, microwave assisted extraction. In this study, ultrasonic assisted extraction of pomegranate peel using pure ethanol as the solvent was performed and the Response Surface Methodology was used for the experimental design. The ultrasonic water bath was used for the extraction that operates at a frequency of 40 KHz and 100 W. The experiments were performed with the different solvent-solid ratio (10:1 to 20:1) for different extraction time (10-40 minutes) and at different temperature (30-50°C). The optimized condition for total yield (TY), total phenolic content (TPC), and total flavonoid content (TFC) of the pomegranate peel were determined.

Materials and Methods

Fresh pomegranates were purchased from the local market of Aligarh, U.P., India. The peel was taken out with a sharp knife and dried on tray dryer at a temperature of 40°C until constant weight was attained. The dried peel was then grounded using Kitchen grinder. The grounded peel was then sieved using a sieve shaker, the particle size of 500µm was separated and packed in plastic pouches and stored in the deep freezer (- 20°C) till further use.

Extraction Procedure

The Sample mixture for the extraction process was prepared by mixing 2gm of dried pomegranate peel powder with pure ethanol as a solvent as per the designed solvent-solid ratio. The sample was treated in an ultrasonic water bath (40 KHz) at a different solvent-solid ratio (10:1 to 20:1), extraction temperature (30°C to 50°C) and extraction time (10 to 40 min). After the treatment, the sample was centrifuged at 5000 rpm for 5 minutes and then filtered using Whatman filter paper 1. The extract was then dried at 40°C and its weight was measured to calculate yield. Further analysis like TPC and TFC were also carried out.

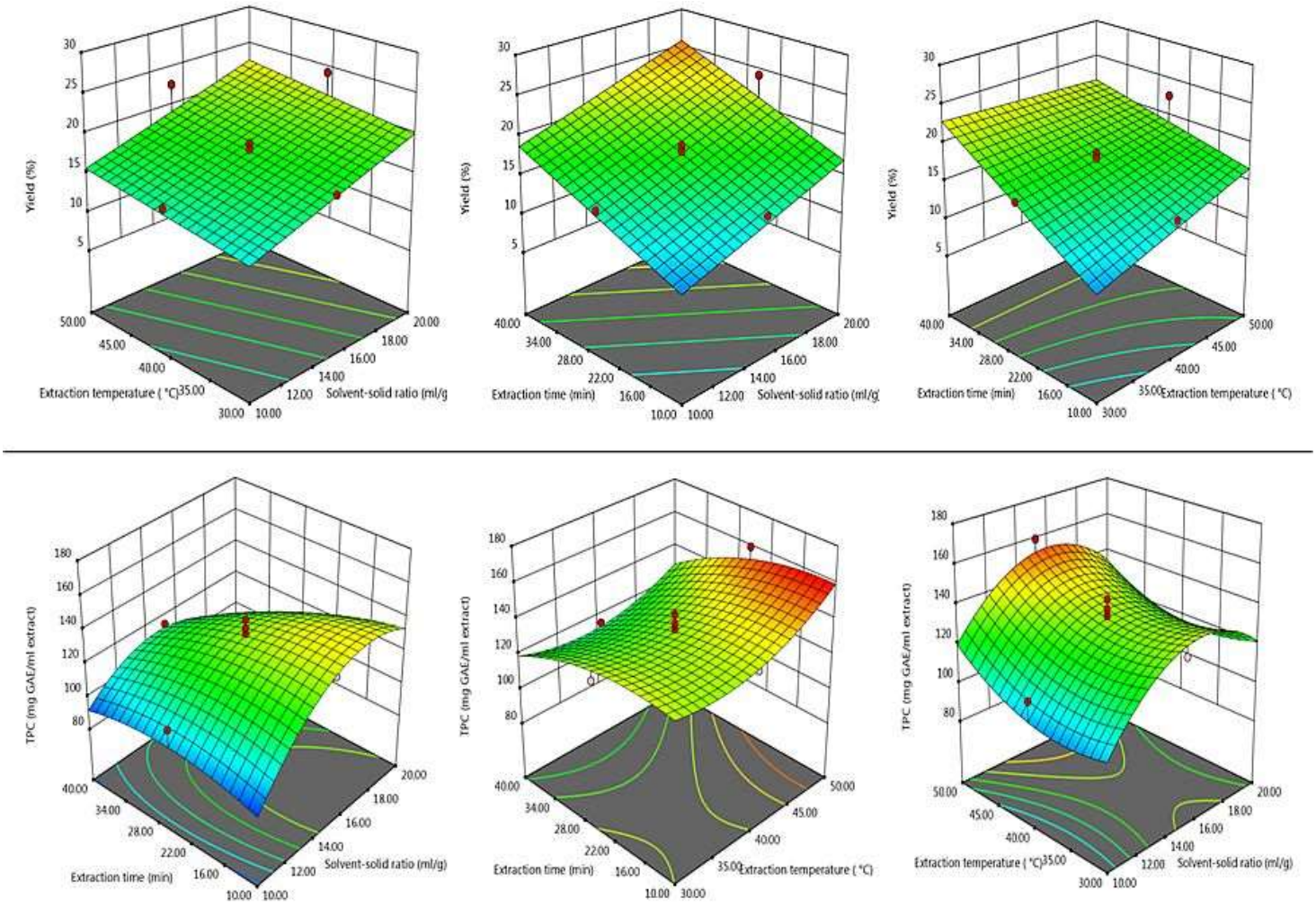

Fig-1 Response surface curves of total yield and total phenolic content as a function of solvent-solid ratio, extraction temperature and time for UAE of pomegranate peel

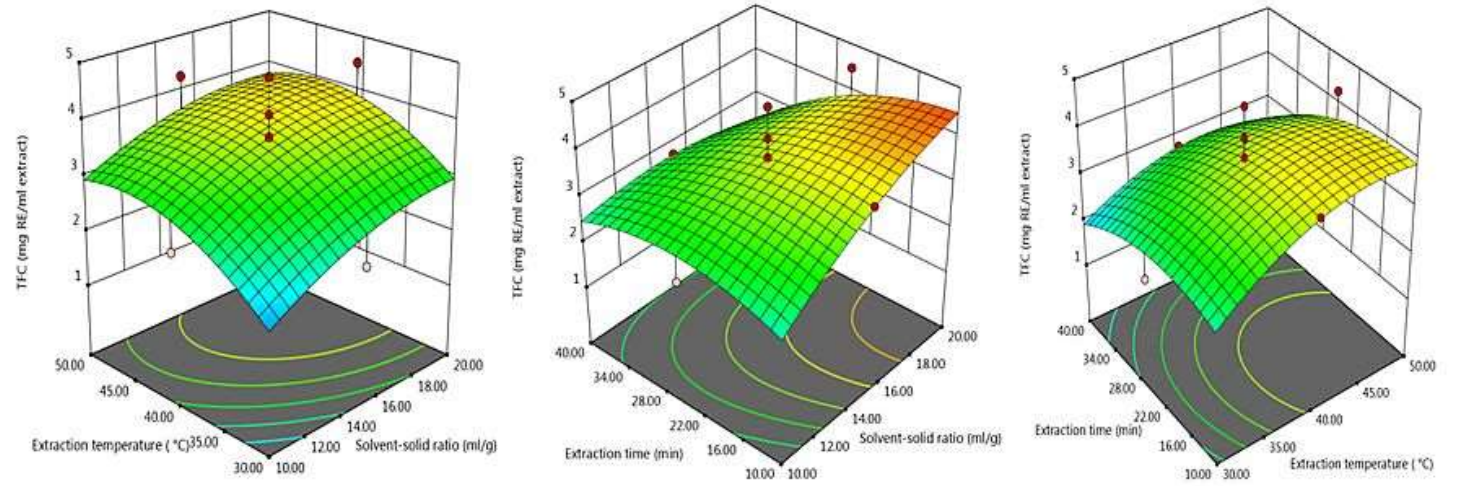

Fig-2 Response surface curves of TFC as a function of solvent-solid ratio, extraction temperature and time for UAE of pomegranate peel

Confirmation test for Phenolic and Flavonoid Compounds

The positive results of ferric chloride and alkaline reagent test were obtained which confirmed the presence of phenolic and flavonoid compounds in pomegranate peel. The tests were performed using the method suggested by Banu and Cathrine (2015) [11].

Determination of Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu's reagent method [12]. The extract was diluted 250 times. 0.1 ml of diluted extract (0.1 ml of distilled water for blank) was taken in a test tube and 7 ml of distilled water was added along with 0.5 ml FC reagent. It was properly mixed by shaking and incubated for 1-8 minutes at room temperature. 1.5 ml of Na_2CO_3 solution was added to the mixture and the mixture was made up to 10 ml. It was then incubated for 2 hours at a room temperature. The absorbance was taken at 765 nm against reagent as a blank using double beam UV-VIS spectrophotometer (UV5704SS). The standard curve for TPC was made using gallic acid solution (50 to 750 mg/l) under the same procedure as described above. The total phenolic content was expressed as mg GAE/ml extract. Each sample was carried out in triplicate.

Determination of Total Flavonoid Content

The Total flavonoid content was determined by using the method explained by saeed *et al*. [13].

The extract was diluted 250 times. In a 10 ml test tube, 0.3 ml of diluted extract, 3.4 ml of 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M) and 0.15 ml of $AlCl_3.6H_2O$ (0.3 M) were mixed. The mixture was incubated for 5 minutes at a room temperature and then 1 ml of NaOH (1 M) was added, the solution was mixed well and the absorbance was measured against the reagent blank at 506 nm. The standard curve for total flavonoids was made using rutin standard solution (0 to 100 mg/l) under the same procedure as described above. The total flavonoids were expressed as mg RE/g of dry sample. Each sample was carried out in triplicate.

Experimental Design

The ultrasonic assisted extraction process was optimized using the response surface methodology. It is a mathematical tool which is widely accepted for the quality of the optimization process [14]. The RSM was developed by Box and Wilson in the year 1951 [15]. The central composite design (CCD) was used to predict the responses.

Table-1 Values of independent variables

Independent variables	Symbol	Original values	Coded value
Solvent-Solid Ratio (ml/g)	X_1	10:1 15:1 20:1	-1 0 1
Extraction Temperature (°C)	X_2	30 40 50	-1 0 1
Extraction Time (minutes)	X_3	10 25 40	-1 0 1

The design of the experiment gave 20 runs out of which 8 factorial runs, 6 axial runs, and 6 centre runs were formed [Table-2]. The three independent variables were considered for the experiment *i.e.* solvent-solid ratio (X_1), extraction temperature (X_2) and extraction time (X_3) and its values range are given in [Table-1]. The levels of each variable were chosen on the bases of the available literature review and preliminary trials. The effect of three independent variables such as solvent-solid ratio, extraction temperature and extraction time was studied on responses such as Total yield, TPC, and TFC. The Analysis of variance (ANOVA) was conducted to determine the level of significance. The function was supposed to be obtained for prediction of the results in RSM. The general equation of this function is as follows [8].

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i<j}^{k} \beta_i X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \cdots$$

Where Y represents the response, βi represents regression coefficients, Xi represents the independent variables. If the response is defined by a linear function of independent variables, then it is a first order function that can be expressed as

$$Y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3$$

If the response is defined by a quadratic function of independent variables, then the second order function is used and can be expressed as

$$Y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Where Y is the response, X_1 is the solvent-solid ratio, X_2 is the extraction temperature, X_3 is the extraction time, β_o is the intercepts, β_1, β_2, β_3 are linear, β_{11}, β_{22}, β_{33} are quadratic and β_{12}, β_{13}, β_{23} are interaction regression coefficient terms respectively. The coefficient of determination (R_2) for each response was computed. For each response, response surface plots were produced from the equations. The optimization was done numerically.

Results and Discussion

In the present study, ultrasonic assisted extraction of phenolic compounds from pomegranate peel was performed using pure ethanol as solvent. The solubility of the phenolic and flavonoid compounds was studied by varying solvent-solid ratio (10:1, 15:1 and 20:1), extraction temperature (30, 40 and 50°C) and extraction time (10, 25 and 40 min). The experimental design was developed using RSM as shown in [Table-2]. The quadratic model was fit successfully for all the responses.

The value of the regression coefficient for linear, quadratic and interaction term obtained by RSM are shown in [Table-3]. Table-2 Design of experiment in terms of coded values and actual values

Run	Coded Values			Actual Values		
	X_1	X_2	X_3	X_1	X_2	X_3
1	1	0	0	20	40	25
2	0	0	0	15	40	25
3	0	0	-1	15	40	10
4	-1	1	-1	10	50	10
5	1	1	-1	20	50	10
6	1	1	1	20	50	40
7	0	0	0	15	40	25
8	0	1	0	15	50	25
9	0	0	0	15	40	25
10	0	0	0	15	40	25
11	0	0	0	15	40	25
12	-1	0	0	10	40	25
13	0	-1	0	15	30	25
14	-1	-1	1	10	30	40
15	-1	1	1	10	50	40
16	0	0	0	15	40	25
17	1	-1	-1	20	30	10
18	0	0	1	15	40	40
19	1	-1	1	20	30	40
20	-1	-1	-1	10	30	10

X_1: Solvent-solid ratio (ml/g); X_2: Extraction temperature (°C); X_3: Extraction time (min)

To test the fitness of the model and its adequacy, the values of the coefficient of determination (R_2) and lack of fit non significance was used. The respective values of R_2 for total yield, TPC, and TFC was found to be 0.902, 0.923, and 0.802 respectively which implies that the model equation has good prediction capability. The value of ANOVA *viz.* the sum of square, mean square, F-values, and P-values are shown in [Table-4].

Table-3 Regression coefficients of the RSM

Parameters	Total yield	Total Phenolic Content	Total Flavonoid Content
Constant	+ 17.815	133.152	3.682
X_1	+ 3.54	9.33	0.506
X_2	+ 1.36	8.35	0.495
X_3	+ 4.415	-11.75	-0.49
X_1X_2	-0.584	-1.625	-0.075
X_1X_3	+ 0.997	-13.075	-0.538
X_2X_3	-1.928	-3.4	-0.2
X_1^2	-	-25.155	-0.283
X_2^2	-	11.145	-0.528
X_3^2	-	-8.255	-0.453
R^2	0.902	0.923	0.802

Table-4 ANOVA results of process variables against each response of model

Responses	Model	Sum of square	Mean square	F-value	P-value
Total yield	Quadratic	379.16	63.19	19.91	<0.0001
TPC	Quadratic	7566.82	840.76	13.35	0.0002
TFC	Quadratic	16.01	1.75	4.49	0.0140

Total Yield

The average experimental values of total yield of the extract from dry pomegranate peel are in between 7.13% (s/s-10:1; T-30°C; t -10 min) to 28.15% (s/s-20:1; T-30°C; t-40 min). This suggests that more solvent is required for greater diffusion of compounds from the sample matrix to increase the total yield. The analysis of variance suggests that the developed regression model for total yield was significant ($P < 0.001$) and insignificant lack of fit ($p>0.05$). A second order polynomial equation in terms of coded factors with only significant terms was developed to study the relationship between total yield and independent process variables. A positive sign of the coefficient indicates that the total yield increases as the magnitude of variables increase and the negative sign of the coefficient indicate that the total yield decreases as the magnitude of variables increase.

$$Total\ Yield\ (\%) = 17.815 + 3.54X_1 + 1.36X_2 + 4.415X_3 - 1.92813X_2X_3$$

In the above equation, the sign and magnitude of the coefficients indicate the effect of independent variables on the total yield. The effect of solvent-solid ratio, extraction temperature and extraction time on total yield has been shown in [Fig-1]. It was found that yield increases as the solvent-solid ratio increases. The extraction temperature and extraction time were also found and it indicates that they have a positive effect on total yield. The interaction model of extraction time and the temperature had a negative effect on the yield. Sahin and Samli (2013) [8] reported that yield increases with the extraction time of ultrasound assisted extraction of olive leaf. Sood and Gupta (2015) [7] also reported that yield increases with a solvent-solid ratio of extraction of bioactive compounds from pomegranate peel.

Total Phenolic Content

The average experimental values of total phenolic content (TPC) of the extract from dry pomegranate peel are in between 85.7 (s/s-10:1; T-30°C; t-10 min) to 159.6mg GAE/ml extract (s/s-15:1; T-50°C; t-25 min). This suggests that there is a direct correlation of extraction of the total phenolic compound with the solvent-solid ratio, extraction temperature and time. The analysis of variance suggests that the regression model developed for the total phenolic content was significant ($P < 0.001$) and insignificant lack of fit ($p>0.05$). A second order polynomial equation in terms of coded factors with only significant terms was developed to study the relationship between total phenolic content and independent variables.

$$Total\ Phenolic\ Content\ (mg\ GAE/ml\ extract) = 132.12 + 9.33X_1 + 8.35X_2 - 11.75X_3 - 13.075X_1X_3 - 28.25X_1^2 + 8.05X_2^2$$

In the above equation, the sign and magnitude of the coefficients indicate the effect of independent variables on the total phenolic content of the extract. The effect of solvent-solid ratio, extraction temperature and extraction time on total phenolic content has been shown in [Fig-1]. It was found that the TPC increases as solvent-solid ratio and extraction temperature increases but TPC decreases with extraction time. The interaction model of solvent-solid ratio and extraction time had a negative effect on TPC. Sahin and Samli (2013) [8] also reported a negative coefficient of interaction model of solvent-solid ratio and extraction time on total polyphenol content of olive leaf extract by ultrasound-assisted extraction. Mohamed and Chang (2009) [16] also reported that the TPC increases as the solvent-solid ratio increases.

Total Flavonoid Content

The average experimental values of total flavonoid content (TFC) of the extract from dry pomegranate peel are in between 1.30 (s/s-10:1; T-30°C; t-10 min) to 4.75 mg RE/ml extract (s/s-15:1; T-40°C; t-25 min). This suggests that a higher value of the solvent solid ratio, extraction temperature and extraction time will result in a larger value of total flavonoid content. The analysis of variance suggests that the regression model developed for TFC was significant ($P < 0.05$) and insignificant lack of fit ($p>0.05$). A second order polynomial equation in terms of coded factors with only significant terms was developed to study the relationship between total flavonoid content and independent variable.

$$Total\ Flavonoid\ Content\ (mg\ RE/ml\ extract) = 3.05 + 0.506X_1 + 0.491X_2 - 0.49X_3 - 0.5375X_1X_3$$

The effect of solvent-solid ratio, extraction temperature and extraction time on TFC has been shown in [Fig-2]. It was found that the TFC values increases as the solvent-solid ratio and extraction temperature increases but the extraction time had a negative effect on the TFC. The interaction model of solvent-solid ratio and extraction time had a negative effect on the TFC.

Predicted and actual values of ethanol extraction

The optimal conditions of solvent-solid ratio, extraction temperature and time for extraction of bioactive compounds from pomegranate peels were found as 16.09 ml/g, 30°C, 17.8 minutes respectively and the predicted values of TY, TPC and TFC under this optimal condition were found as 14.20 %, 140.61 mg GAE/ml extract and 2.96 mg RE/ml extract respectively. Under the same optimal condition, the experiment was performed to validate the optimised value of UAE along with conventional thermostatic water bath extraction (TWE) to compare its result with UAE. The respective values of each response of UAE and TWE and its comparison are shown in [Table-5]. The actual total yield of UAE was found to be little less than its predicted value but higher than thermostatic water bath extraction. This was due to the cavitational effect of ultrasound which enhanced the sonoporation and sonocapillarity of the sample matrix which ultimately increased the diffusion rate of bioactive compounds from the sample matrix into the solvent [17]. The Total phenolic content was found higher with ultrasound assisted extraction as compared to thermostatic water bath extraction. A similar trend was also observed for the total flavonoid content.

Statistical Analysis

All experiments and measurements of ultrasound assisted extraction and the thermostatic water bath was performed in triplicate and was reported as a mean and standard deviation in [Table-5]. The Data was analysed by one-way analysis of variance (ANOVA) and independent sample t-test, using RSM and SPSS software [Table-5]. The level of significance was defined as $p \leq 0.05$.

Table-5 One-way ANOVA and Independent sample t-test for TY, TPC and TFC for the actual values of UAE and TWE at optimal condition of X_1 = 16.09 ml/g, X_2 = 30°C, X_3 = 17.8 minutes. Each value is expressed as mean ± standard deviation (n =3)

Extraction Method	*Actual Values*		
	TY (%)	*TPC (mg GAE/ml extract)*	*TFC (mg RE/ml extract)*
Ultrasound assisted extraction	13.90 ± 2.10[a]	90.50 ± 4.29[a]	2.75 ± 1.73[a]
Thermostatic water bath extraction	11.30 ± 2.17[b]	84.50 ± 4.73[b]	2.00 ± 1.20[b]

TY: Total Yield; TPC: Total Phenolic Content; TFC: Total Flavonoid Content. The values followed by the same letter (ab) in the columns are not significantly different according to one-way ANOVA and Independent sample t-test

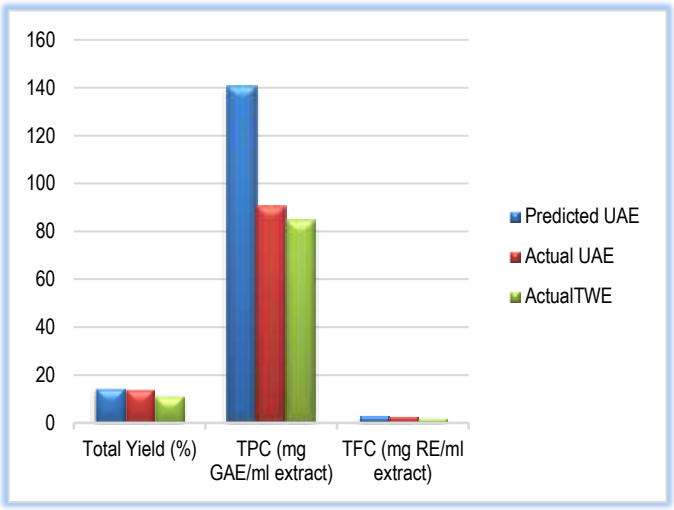

Fig-3 Comparison of predicted UAE values with actual UAE and TWE values

Conclusion

The present study confirmed the importance of RSM in optimizing the extraction conditions for total yield, total phenolic content and total flavonoid content, The results of RSM showed that the pomegranate peels are the potential source of antioxidant compounds, therefore, the extract can be used in nutraceutical and pharmaceutical industry and can also be used as natural antioxidants which can replace the current use of synthetic antioxidants. The results showed that all the response variables were affected by the extraction parameters. The ultrasound assisted extraction gave higher yield, total phenolic content and total flavonoid content than conventional thermostatic water bath extraction method. Also, the results of independent sample t-test [Table-5] showed that the values of all the responses obtained from an ultrasound assisted extraction method were

significantly different from the values obtained from the thermostatic water bath method.

Application of research: To extract the bioactive compounds from the by-products of pomegranate

Research Category: Extraction of compounds from agricultural waste

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Ethical Committee Approval Number:

References

[1] Zhou S.H., Fang Z.X., Lü Y., Chen J.C., Liu D.H. and Ye X.Q. (2009) *Food Chemistry*, 112(2), 394-399.
[2] Chidambara Murthy K.N., Jayaprakasha G.K. and Singh R.P. (2002) *J Agric Food Chem* 50(17), 4791-4795.
[3] Mertens-Talcott S.U., Jilma-Stohlawetz P., Rios J., Hingorani L. and Derendorf H. (2006) *Journal of Agricultural and Food Chemistry*, 54(23), 8956-8961.
[4] Jaiswal V., DerMarderosian A. and Porter J.R. (2010) *Food Chemistry*, 118(1), 11-16.
[5] Sreekumar S., Sithul H., Muraleedharan P., Azeez J.M. and Sreeharshan S. (2014) *J BioMed Research International*, 2014, 12.
[6] Li Y., Guo C., Yang J., Wei J., Xu J. and Cheng S. (2006) *Food Chemistry*, 96, 254-260.
[7] Sood A. and Gupta M. (2015) *Food Science*, 12, 100-106.
[8] Sahin S. and Samli R. (2013) *Ultrason Sonochem* 20(1), 595-602.
[9] Balasundram N., Sundram K. and Samman S. (2006) *Food Chemistry*, 99(1), 191-203.
[10] Khan M.K., Abert-Vian M., Fabiano-Tixier A.S., Dangles O. and Chemat F. (2010) *Food Chemistry*, 119 (2), 851-858.
[11] Banu K. S. and Cathrine L. (2015) *International Journal of Advanced Research in Chemical Science*, 2 (4), 25-32.
[12] Singleton V.L. and Rossi J.A. (1965) *American Journal of Enol. Vitic*., 16(3), 144-158.
[13] Saeed N., Khan M.R., Shabbir M. (2012) *BMC Complementary and Alternative Medicine*, 12(1), 221.
[14] Nazni P. and Karunathara D. (2011) *International Journal of Current Research*, 3(8), 27-32.
[15] Box G.E.P. and Wilson K.B. (1951) *Journal of the Royal Statistical Society. Series B (Methodological),* 13(1), 1-45.
[16] Mohamed A. S., Chang Y. L. (2009) *Food Chemistry*, 108, 977–985.
[17] Chemat F., Zille H. and Khan M.K. (2011) *Ultrasonics Sonochemistry*, 18(4), 813-835.