Asian Journal of Chemistry; Vol. 27, No. 12 (2015), 4320-4326

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.19051

Extraction of Polyphenols from Fresh Pomegranate Peel Using Response Surface Methodology

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Received: 23 January 2015; *Accepted*: 25 February 2015; *Published online*: 29 August 2015; AJC-17466

Pomegranate (*Punica granatum* L.) peel, as industrial waste is a rich source of phenolic compounds like hydrolyzable ellagitannin, which possess strong antioxidant activities. The aim of the present study was to develop a suitable method for extraction of ellagitannin from fresh pomegranate peel using physical, chemical and enzymatic treatment. Response surface methodology involving central composite design was used to optimize the independent variables *i.e.*, temperature (range 19.6-90.4 °C) and time (range 17.6-102.4 min) to maximize the total hydrolyzable ellagitannin content as GAE and antioxidant activity as DPPH in fresh pomegranate peel extract. The experimental data were fitted to a quadratic model and appropriate statistical analysis was used. From RSM based study, it has been concluded that there is a significant effect of temperature and time on the extraction of ellagitannin from fresh pomegranate peel. Ellagitannin content was high in pomegranate peel extract obtained by enzymatic method followed by physical and chemical methods. Temperature and time combination of 91.2 °C and 18 min in enzymatic method yields highest ellagitannin content of 45.01 % as GAE was obtained showing 63.74 % antioxidant activity as DPPH.

Keywords: Pomegranate, Ellagitannin, Enzymatic treatment, Antioxidant activity, Optimization.

INTRODUCTION

Pomegranate (*Punica granatum* L.) fruit is widely consumed fresh and in processed form as juice, jam and wine. Pomegranate fruit peel, a byproduct of pomegranate juice industry is rich source of hydrolyzable tannin called ellagitannin[1] (ETs). Pomegranate ellagitannin *i.e.* polyphenols is composed of punicalagin and its isomers (2,3-hexahydroxydiphenyl-1-4,6-gallogyl glucose) as well as lesser amount of punicalin (4,6-gallagyl glucose), gallagic acid, ellagic acid and ellagic acid glycosides (hexoside, pentoside, rhamnoside *etc*.)[2,3].

Pomegranate peel extracts are currently used for treatment of respiratory diseases and in the preparation of tincture, cosmetics and other therapeutic formula[4,5]. Pomegranate extracts are also being investigated for their potential use as food biopreservatives and for the formulation of products in the neutraceutical industry[4].

Recovery of tannin/phenolic compounds is commonly performed through a solvent extraction procedure but at present an ambiguous data on the method and conditions for extraction are available. Sometimes the reports are contradictory particularly when different raw materials are compared. The results are difficult to compare because different estimation method for phenols are used. Sometimes only the total phenol concentration of final extract is reported, but not the total yield. The aim of an extraction process should be maximize the yield of target substances with highest quality[6]. The effect of solvent-sample ratio has been investigated by Pinelo *et al.*[7] and by other authors for different raw materials[8,9]. Higher total solids are obtained with higher solvent-sample ratio. It was independent of the solvent used according to mass transfer principle[6].

Alcoholic solvent is commonly employed to extract phenolics from natural sources as they give quite high yield of total extract, even though they are not highly selective for phenolics[7,10]. Time and temperature of extraction are important parameters to be optimized, even minimize energy cost of the process. Many authors agree to the fact that an increase in the working temperature favours extraction, enhancing both solubility of the solute and the diffusion coefficient. However beyond a certain temperature, phenolic compounds can be degraded[6]. Also the effect of drying before extraction and processing parameters on the extraction kinetics and product properties were systematically studied using water as an environment friendly solvent for the extraction. The yield and content of antioxidants increased with reduced particle size and increased water sample ratio and temperature but antioxidant activity was low where extraction temperature was high[11].

Total hydrolyzable polyphenols of pomegranate peel were degraded during the first 72 h of solid state fermentation by *Aspergillus niger* culture (GH1and PSH strains), probably

by tannin acyl hydrolaze activity and produced pure ellagic acid in fermented mass which was extracted by simple extraction process. Pomegranate husk is a good support and an excellent substrate in the production of high commercial interest metabolites like ellagic acid due to the degradation of ellagitannin content[12]. Production of ellagic acid may be due to biodegradation (microbial enzymes activity) of ellagitannin[13].

Several methods have been developed to evaluate the antioxidant activities of pure compounds and plant extracts. In those methods, stable free radical species such as 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2-azinobis(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) are often used. DPPH, a paramagnetic compound with an odd electron shows strong absorption at 517 nm in methanol. The absorbance decreases with colour change from purple to yellow due to the scavenging of free radical by antioxidants through donation of hydrogen to form the stable DPPH-H molecule[14].

The present study was conducted to develop an effective extraction process for extraction of ellagitannin from fresh pomegranate peel. Effects of three extraction techniques like physical, chemical and enzymatic methods were used. Response surface methodology (RSM) was used to find out optimal time temperature combination for ellagitannin extraction in all three methods.

EXPERIMENTAL

Fresh pomegranate peel: Pomegranate peels were obtained from fresh pomegranate fruit of Baghwa variety by manual peeling of pomegranate fruit and separating the peels from aril.

Sample preparation: The obtained fresh pomegranate peels (FPP) were crushed in mixer grinder (Maharaja White lines, India). The ground peel was then passed through 2 mm pore size sieve to ensure uniform particle size of pomegranate peel. The grounded peel was stored at -18 °C in deep freezer until utilization.

Folin Denis ciocalteu reagent, sodium carbonate, methanol, hydrochloric acid (Merck, India), gallic acid, DPPH (CDH, India), pectinase and cellulase enzyme were procured from Sigma Aldrich (USA).

Estimation of total ellagitannin content: Total ellagitannin content (TEC) in the extract was determined by using Folin-Denis ciocalteu reagent as described in AOAC method 952.03 with slight modification. 250 µL of extract was diluted with distilled water to 10 mL. Aliquots of 1 mL of diluted extract were mixed with 5 mL of 10 fold diluted Folin-Denis Ciocalteu reagent. After 3 min, 4 mL of 7.5% sodium carbonate was added. The mixtures were allowed to stand for 0.5 h at 30 °C in incubator before the absorbance was measured at 734 nm in UV-visible spectrophotometer (Shimadzu UV-Vis-2600, Singapore). The total polyphenol content in the extract was calculated and expressed as gallic acid equivalent (GAE; g/100 g on dry basis) using a gallic acid (0-120 mg/L) standard curve. All samples were prepared in triplicate[15].

Measurement of the antioxidant activity by DPPH radical-scavenging method: The antioxidant activity of different extracts was measured in term of hydrogen donating or radical scavenging ability using stable DPPH method according to the method proposed by Brand-Williams *et al.*[16]. Aliquot of 250 µL of the extract was diluted with distilled water to 10 mL. Aliquot of 200 µL of samples were mixed with 2 mL of 100 µM DPPH methanolic solution. The mixture was placed in dark at room temperature for 1 h. the absorbance of resulting solution was then read at 520 nm in UV-visible spectrophotometer (Shimadzu UV-Vis-2600, Singapore). The antiradical activity was expressed in terms of percentage reduction of the DPPH. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Antioxidant activity as DPPH (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Extraction of ellagitannin

Physical method: Initially an experiment was conducted to know the appropriate sample solvent ratio to produce higher content of total ellagitannin. For that 50 mL of distilled water (used as solvent) was percolated on 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g of grounded FPP to produce the sample/solvent ratio of 0.5/50, 1/50, 1.5/50, 2/50, 2.5/50 and 3/50 in 250 mL conical flask. The flasks were shaken at 30 °C temperature, at 180 rpm for 0.5 h in a shaker incubator (Kuhner, Switzerland). The content of each conical flask was centrifuged at 3000 rpm for 5 min by a centrifuge machine (Sigma-vsi, Germany) to get a clear supernatant. Supernatant liquids were then tested for its total ellagitannin content. The process was performed in triplicate and the average of three values was reported as data point.

Chemical method: In hydrochloric acid based hydrolysis process, the low pH enhances the release of hydrolyzable tannins from FPP. In this method, we have taken HCl in distilled water as solvent with the composition of 0.5, 1, 5, 10, 15 and 20 % to find an effective composition of HCl in water to get a higher extractability of ellagitannin from pomegranate peel. Here only HCl in water is used as solvent because HCl is a low cost commercially available chemical where as other solvents *e.g.,* ethanol, methanol, acetone, *etc.* are not preferable due to higher cost at commercial level and environmental issues. Apart from this, the organic solvents may not favour purification of ellagitannin through column chromatography because these solvents are being used for the elution of ellagitannin which is bound in resin packed in column. About 1 g of grounded FPP was taken for each concentration of solvent in to 250 mL conical flasks and added 50 mL solvent (HCl in water) of 0.5, 1, 5, 10, 15 and 20 %, shaken in shaker incubator at 30 °C, 180 rpm for 0.5 h. The content of each conical flask was centrifuged at 3000 rpm for 5 min by a laboratory centrifuge to get a clear supernatant. Each supernatant liquid was tested for its total ellagitannin content (TEC) and antioxidant activity (AOA) as DPPH reduction. The process was performed in triplicate and the average of three values was reported as data point.

Enzymatic method

***Aspergilus niger* strain and preparation of spore inoculums:** *Aspergilus niger strain* MTCC 281 was procured

from Institute of Microbial Technology (CSIR), Chandigarh, India and used for the study. Potato dextrose agar slants were used for the maintenance of *A. niger*. Fully sporulated slants were stored at 4 °C in refrigerator for further use, followed by sub culturing. Fungal spore inoculum was prepared by adding 2.5 mL sterilized water containing 0.1 % Tween 80 to a fully sporulated culture. The spores were dislodged by using a sterile inoculum loop under laminar air flow (Labtech, Korea). The number of viable spores in the suspension was determined using the plate count method. 1 mL of the prepared spore suspension contained concentration of 3.8×10^{10} spores. This concentration was used in further studies of ellagitannin extraction.

Enzymatic treatment: In enzymatic method of ellagitannin extraction, pectinase and cellulase were used at concentration of 0.1 mL and *Aspergillus niger* spore inoculum (3.8×10^{10} spores per mL of inoculum) was employed as enzyme source. About 1 g of grounded fresh pomegranate peel (FPP) taken in three conical flasks was mixed with 50 mL acetate buffer of pH 4.5 followed with 0.1 mL pectinase enzyme, in second conical flask 0.1 mL cellulase and third flask kept without any enzyme as control and shaken in shaker incubator at 30 °C, 180 rpm for 0.5 h. The flask's content was centrifuged at 3000 rpm for 10 min to get the clear supernatant extract containing ellagitannin compound and analyzed for TEC. Simultaneously a study also carried out to know the suitable volume of *A. niger* spore inoculums for one gram of grounded FPP to obtain higher amount of ellagitannin extract. In each of seven conical flasks 1 g grounded FPP was taken and added with 0.05, 0.10, 0.15, 0.20, 0.25 and 0.3 mL of *A. niger* spore inoculums (3.8×10^{10} spores per mL of inoculum) and one flask with sample kept without spore inoculums as a control and the contents were thoroughly mixed and incubated at 30 ºC for 7 days. This process termed as solid state fermentation (SSF) in which mycelium of *A. niger strain* grows on the surface of peel. The organism releases tannin hydrolaze enzyme, which hydrolyzes the complex compound of tannin into simpler compounds. After incubation, 50 mL distilled water was added in each flask and shaken in shaker incubator at 30 °C, 180 rpm for 0.5 h. Each flask's contents were centrifuged at 3000 rpm for 10 min to get the clear supernatant extract containing ellagitannin compound. These extracts were then analyzed for TEC. Above experiments were performed in triplicate and average value considered for graphical presentation (Fig. 1c and 1d).

In above study it was seen that *A. niger* is producing higher TEC, hence it is selected as enzyme source for extraction of ellagitannin from FPP at different time temperature combination. For this grounded FPP (100 g) fermented with 25 mL inoculums (3.8×10^{10} spores) of *Aspergillus niger* strain for 7 days at 30 °C in an incubator. After incubation, whole fermented pomegranate peel kept in deep freezer to inhibit the further fermentation. After thawing, it was utilized for extraction of ellagitannin and TEC and AOA of extract was estimated.

Experiment design: After determining the ellagitannin extraction by three different methods, further experiments were conducted to observe the effect of varying extraction temperature (19.6, 30.0, 55.0, 80.0, and 90.4 °C) and time (17.6, 30, 60, 90 and 102.4 min) by employing central composite design (CCD). Total ellagitannin content (TEC) and antioxidant activity (AOA) as DPPH were responses in physical, chemical and enzymatic method to see the effect of time and temperature on ellagitannin extraction by each method. The response parameters pTEC, cTEC and eTEC are total ellagitannin content where as pAOA, cAOA and eAOA are antioxidant activity for physical, chemical and enzymatic method respectively. All experiments were carried out randomly in order to minimize the effect of unexplained variability in the observed responses due to systematic errors.

RESULTS AND DISCUSSION

Effect of sample-solvent ratio on extractability of ellagitannin from fresh pomegranate peel (FPP): There was a significant difference between the extractions obtained from different sample-solvent ratios. Sample-solvent ratio of 0.5/50 and 1.0/50 (g/mL) showed uniform extraction of ellagitannin in water, while ratio of 1.5/50, 2.0/50, 2.5/50 and 3.0/50 (g/mL) showed gradual decreases in the extraction of ellagitannin (Fig. 1a). It means there was a saturation effect in solvent after leaching of a specific amount of ellagitannin, which restricts further release of ellagitannin in to water from tissues of pomegranate peel at time-temperature combination of 0.5 h and 30 °C respectively. Different ratios of water to raw material affect the extraction yield significantly. If ratio of water to raw material is too small or too high, compound of interest in raw material cannot be completely extracted[17]. From Fig. 1a, it can be observed that the ratio of 0.5/50 and 1.0/50 (g/mL) is giving higher extractability of ellagitannin *i.e.* 17.19 ± 0.5 and 17.37 ± 0.5, respectively. From above experiment it was found that a sample solvent ratio of 1/50 is appropriate ratio to get a higher extract of ellagitannin. Hence, 1/50 ratio of sample to water is selected for further extraction of total ellagitannin content (TEC) and antioxidant activity (AOA) as DPPH in fresh pomegranate peel (FPP) extract at different time and temperature combination.

Effect of hydrochloric acid water ratio on extractability of ellagitannin from FPP: There is a significant effect of concentration of HCl on ellagitannins extraction from FPP. From Fig. 1b, concentration of HCl in water (% v/v) of 0.5, 1, 5, 10, 15 and 20 % shows a descending values of TEC in FPP extract. Using 0.5, 1% and 5% HCl in water produced almost similar amount of ellagitannin *i.e.* 17.78, 18.41 and 17.87 % respectively. While 10, 15 and 20% HCl in water produced lower values of TEC *i.e.* 13.52, 7.19 and 6.78 % respectively. Higher concentration of HCl may be causing the destructive effect on ellagitannin molecules and possibility of change in chemical properties of elllagitannin. From the above experiment it was concluded that 1% HCl in water is an appropriate concentration to get a higher extract of ellagitannin. Hence, concentration of 1% HCl in water is selected for further extraction of ellagitannin from fresh pomegranate peel.

Effect of enzymatic treatment on extractability of ellagitannin from FPP: Enzymatic treatment with pectinase, cellulase and use of *A. niger* as enzyme source significantly enhances the extractability of ellagitannin in comparison with control. It can be observed from Fig. 1c that ellagitannin content was high when *A. niger* was used as enzyme source in

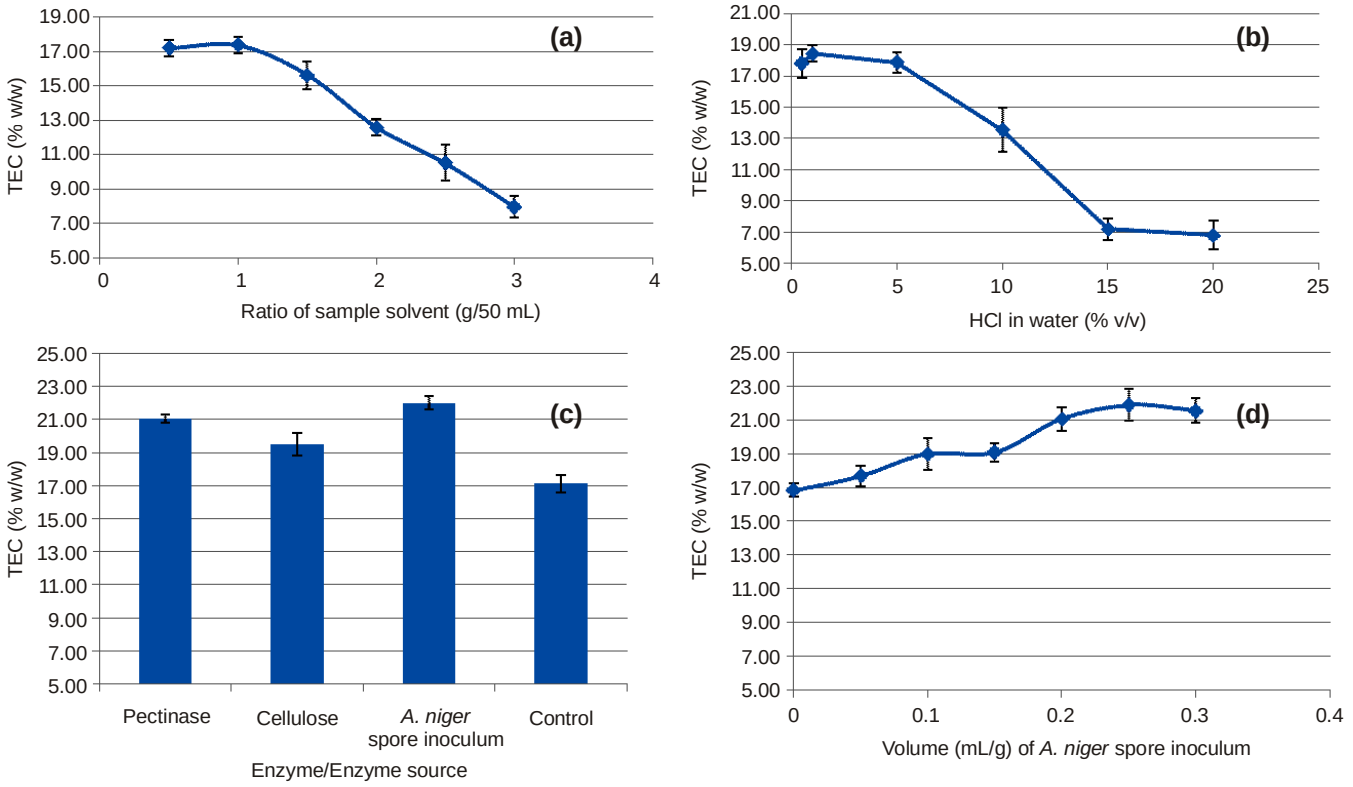

Fig. 1. Ellagitannin extraction from fresh pomegranate peel (FPP) as affected by (a) sample-solvent ratio (b) HCl concentration (c) enzyme and enzyme source and (d) *A. niger* spore inoculum volume

comparison with pectinase and cellulase. It has been observed that *Aspergillus niger* may produce tannase which is a hydrolytic enzyme acts on tannins during solid state fermentation of agro wastes. The optimum temperature for enzymatic activity was found to be 30-40 °C at which the enzymatic activity was the higher[18]. Fig. 1d showed that there is a range of *A. niger* spore inoculums of 0.20-0.30 mL which was producing higher extract of ellagitannins from FPP. Inoculums volume of 0.05 to 0.15 mL is not sufficient to ferment the 1 g mass of FPP in 7 days at 30 °C. Hence 0.25 mL of *A. niger* spore inoculum is sufficient to ferment the 1 g of FPP for release a optimum amount of ellagitannin and this *Aspergillus niger* spore inoculums is selected for further study of ellagitannin extraction from FPP. Consequently, it has been found that by using of *Aspergillus niger* strain as enzyme source can produce higher extraction of ellagitannin from fresh pomegranate peel than by using of pectinase and cellulase enzyme in terms of economical and physical feasibility. Hence *A. niger* was selected for further study of ellagitannin extraction at different time temperature combination for comparison with physical and chemical method of ellagitannin extraction.

Effect of temperature on TEC and AOA: To study the effect of temperature on extraction of ellagitannin from FPP, the extraction process was carried at different temperature *viz*; 19.6, 30, 55, 80 and 90.4 °C (Table-1). From Fig. 2, it can be observed that with the increase of temperature, pTEC, cTEC, eTEC, pAOA, cAOA and eAOA was increasing up to 80 °C *i.e.* the highest temperature for getting higher value of all six responses. From 80 to 90.4 °C, TEC and AOA were not increasing in all the three methods, which indicate higher temperature after a limit is not favourable for the extraction of ellagitannin with high AOA. Increasing temperature will also increase the cost for extraction process from the industrialization point of view. Therefore, suitable extraction temperature can be any temperature between 80 °C and 90.4 °C which is in agreement with the results of Zhu *et al.*[17].

Effect of extraction time on TEC and AOA: Extraction time is a factor that would influence the extraction efficiency. This might be due to the time requirement of the exposure of the ellagitannin molecules to the release medium where solvent penetrated in to the plant tissue, dissolved the compound of interest and subsequently diffused out from the plant tissue[19]. Effect of time on extraction of TEC is shown in Fig. 2, it can be observed that TEC increases with ascending of time while AOA may get slightly reduced with increasing in time. Longer exposure of extract to oxygen may be the cause of reduction of AOA. This trend was common for all the methods *i.e.* physical, chemical and enzymatic methods. In physical and chemical methods effect of time factor on pTEC, cTEC, pAOA and cAOA is almost similar while for eTEC and eAOA less time is required to produce higher ellagitannin. In enzymatic treatment solid plant parts may be hydrolyzed during incubation and facilitate extraction of ellagitannin from plant tissue into solvent is considerable.

Effect of time and temperature on extraction of TEC and AOA by chemical, physical and enzymatic methods and process optimization

Statistical analysis and model fitting: Response surface optimization is more advantageous than the traditional single parameter optimization as it saves time and raw material. There were a total of 13 runs of extraction for optimizing the ellagitannin

TABLE-1
EFFECT OF TIME-TEMPERATURE ON ELLAGITANNIN CONTENT AND ANTIOXIDANT ACTIVITY IN FRESH POMEGRANATE PEEL EXTRACT OBTAINED BY PHYSICAL, CHEMICAL AND ENZYMATIC TREATMENT

Run	Independent variables		Dependent variables					
	Temperature (°C)	Time (min)	pTEC	cTEC	eTEC	pAOA	cAOA	eAOA
1	55.0	60.0	26.87	24.46	25.99	49.07	47.49	48.57
2	80.0	90.0	39.89	35.59	38.83	56.99	54.53	56.98
3	55.0	60.0	26.76	24.26	26.37	49.26	47.75	49.09
4	55.0	17.6	26.68	24.68	26.21	48.83	46.64	49.63
5	55.0	60.0	26.74	24.70	25.99	48.74	48.68	49.16
6	55.0	60.0	26.93	24.57	26.09	50.10	48.49	48.81
7	30.0	30.0	18.74	18.10	21.86	38.02	37.17	43.71
8	80.0	30.0	40.33	38.38	41.34	61.68	57.92	59.55
9	55.0	60.0	26.62	24.47	26.21	49.26	47.75	49.99
10	55.0	102.4	26.54	23.77	26.63	48.16	41.74	47.14
11	90.4	60.0	38.51	36.94	38.23	58.38	54.53	58.23
12	30.0	90.0	19.91	19.16	24.19	37.35	36.50	46.64
13	19.6	60.0	13.60	13.70	15.15	34.72	33.02	36.94
R^2			0.9635	0.9682	0.9274	0.9773	0.9641	0.9253
Adj. R^2			0.9375	0.9455	0.8756	0.9611	0.9384	0.8719
SD			2.00	1.71	2.48	1.58	1.84	2.17
Sig.			< 0.0001	< 0.0001	0.0007	< 0.0001	< 0.0001	0.0008

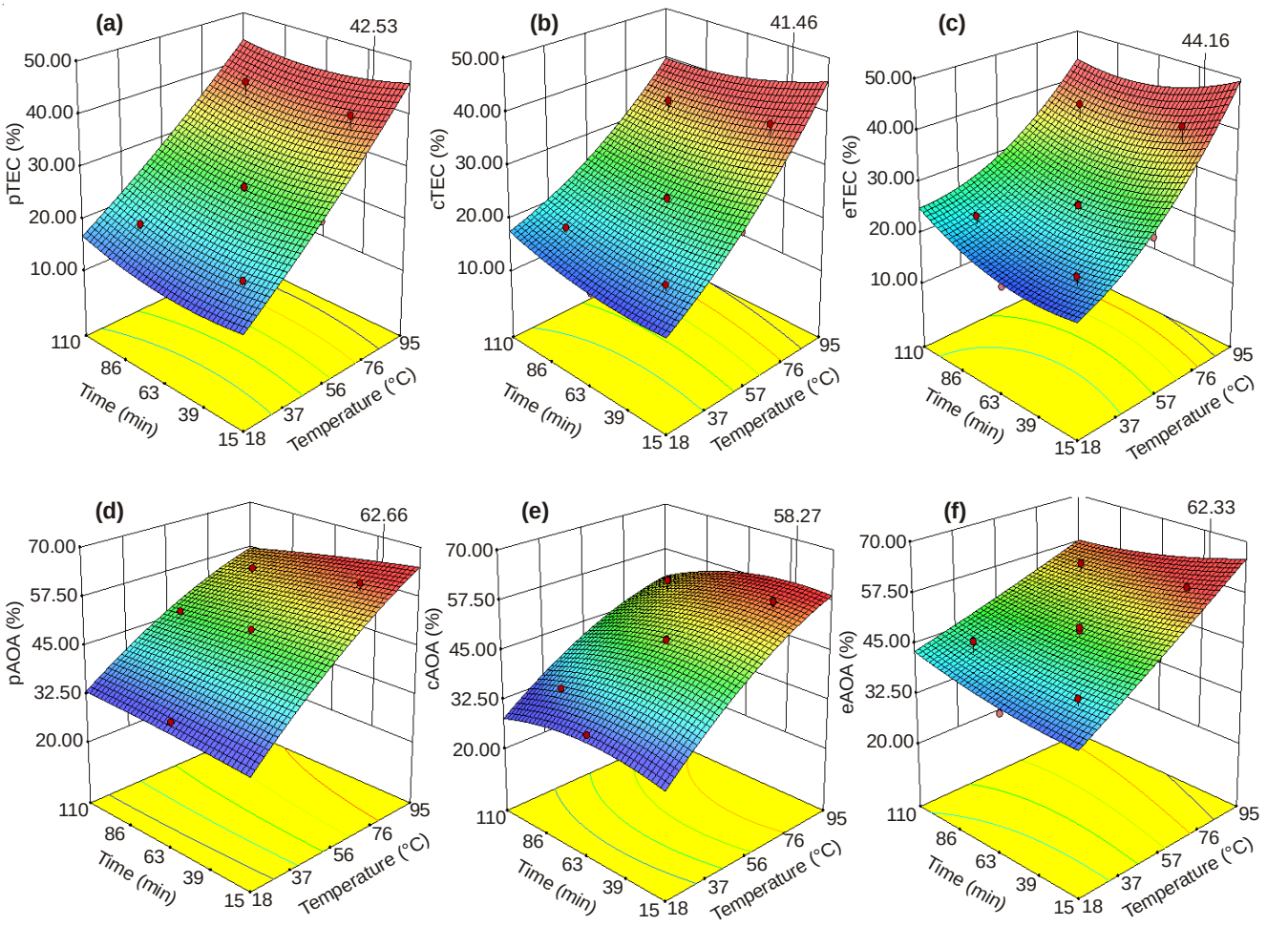

Fig. 2. 3D Response surface graph exhibiting effect of temperature and time on TEC and AOA in FPP extract obtained by physical, chemical and enzymatic method

extraction condition to obtain an optimum level of TEC and respective AOA by physical, chemical and enzymatic methods in Central Composite design. The obtained data were analyzed by multiple regression analysis using the Design-Expert 9.0.3.1 and the following polynomial equation (equation 1-6) was derived to represent the responses of pTEC, cTEC, eTEC, pAOA, cAOA and eAOA as the function of independent variable (temperature, T and time, t) tested. Table-1 shows the

TABLE-2
OPTIMUM CONDITION FOR TEC AND AOA PREDICTED BY RSM MODEL

Temperature (°C)	80	94.44	89.45	94.21	91.13	94.1	90.09	90.09	91.23	92.05	92.3	94.2	Maximum value (%)
Time (min)	30	33.56	34.61	48.13	47.21	47.35	29.01	29.01	18.18	26.71	21.73	53.19	
pTEC	37.96	**44.22**	41.89	43.37	42.02	43.35	42.53	42.53	43.90	43.59	44.09	43.19	44.22
cTEC	36.33	43.29	40.60	41.86	40.38	41.87	41.46	41.46	43.24	42.73	**43.41**	41.47	43.41
eTEC	38.45	46.10	43.04	44.28	42.61	44.29	44.16	44.16	46.55	45.65	**46.60**	43.83	46.60
pAOA	59.40	63.61	62.11	62.49	61.74	62.52	62.66	62.66	63.68	63.39	**63.78**	62.1	63.78
cAOA	55.96	**59.12**	58.16	58.69	58.19	58.71	58.27	58.27	58.23	58.64	58.57	58.42	59.12
eAOA	58.66	63.33	61.48	61.66	60.75	61.70	62.33	62.33	**64.09**	63.31	64.03	61.17	64.09

process variables and experimental data and the coefficients of determination (R^2), adjusted R^2, standard deviation (SD) and model significance (sig.). The models were found significant for all the six dependent variables.

$$pTEC = 9.1292 + 0.3314T-0.0695t – 0.0005Tt + 0.0008T^2 + 0.0008t^2 \quad (1)$$

$$cTEC = 10.0548 + 0.2184T-0.0253t – 0.001Tt + 0.0019T^2 + 0.0007t^2 \quad (2)$$

$$eTEC = 17.4215 + 0.0811T-0.0788t – 0.0016Tt + 0.0031T^2 + 0.0014t^2 \quad (3)$$

$$pAOA = 19.3110 + 0.6615T + 0.0672t – 0.0013Tt – 0.0018T^2 – 0.0002t^2 \quad (4)$$

$$cAOA = 16.4015 + 0.6629T + 0.1752t-0.0009Tt – 0.0024T^2 – 0.0014t^2 \quad (5)$$

$$eAOA = 31.4369 + 0.3625T + 0.0124t – 0.0018\ Tt + 0.0003T^2 + 0.0006t^2 \quad (6)$$

Effect of time temperature combination and process optimization: Fig. 2 shows the 3D response surface for extraction of ellagitannin by physical method (pTEC, 13.60-40.33 g GAE/100 g), chemical method (cTEC, 13.70-38.38 g GAE/100 g), enzymatic method (eTEC, 17.15-41.34 g GAE/100 g) and antioxidant activity as DPPH by physical method (pAOA, 34.72-61.68 %), chemical method (cAOA, 33.02-57.92 %) and enzymatic method (eAOA, 36.94-59.55 %) as a function of temperature and time. pTEC, cTEC and eTEC increased with increasing of temperature up to 80 °C and the extraction time up to 60 min. Long time might make phenolics dissolve and diffuse quickly and thoroughly[20]. A higher antioxidant value indicates good quality and quantity of ellagitannin content. From experimental result it has been observed that when ellagitannin extraction done at higher temperature for longer time the antioxidant activity is getting lowered which may be due to the effect of oxidative reduction of ellagitannin. Total ellagitannin content is higher when enzymatic treatment was done by *A. niger* than physical and chemical methods of ellagitannin extraction. In chemical method of ellagitannin extraction the TEC and AOA was lower than that in physical and enzymatic method, which was noticed at and above 55 °C. The lower value of cTEC and cAOA may be considered due to interference of other water soluble polysaccharides coming in extract at low pH. Higher value of eTEC, eAOA in comparison of pTEC, cTEC, pAOA and cAOA below 30 °C indicating that there is a quick and higher release of ellagitannin from peel to solvent due enzymatic degradation of complex ellagitannin compound in to monomer compound of ellagic acid, gallic acid *etc*. It has been observed that there is correlation between TEC and AOA at a specific temperature and time combination but not a directly proportional relation between TEC and AOA.

In the optimization study, RSM model predicted total twelve combination of time and temperature combination (Table-2) in which three optimum temperature time combinations *i.e.* 94.44 °C and 33.56 min.(pTEC = 44.22 % and cAOA = 59.12 %), 92.30 °C & 21.73 min (cTEC= 43.41 %, eTEC = 46.60 %, pAOA = 63.78 %) and 91.23 °C and 18.18 min (eAOA = 64.09 %) were unique. It is apparent that the optimal temperature is above the highest value of the temperature used in the CCD of experiments. To reduce the number of experiments and resource utilization the ranges of the independent variables (*i.e.*, temperature and time of extraction) were based on common experience of similar studies. Hence it is not surprising that the predicted optimal value is outside the range of experimental design value. A similar analysis has been given by Traynor *et al.*[21]. Also, from above responses it may be concluded that higher value of TEC may not produce higher AOA value at an optimum point of temperature and time combination. However our aim of the study is to get a suitable method producing a higher TEC with maximum AOA. In this context, AOA can be considered as the key parameter to decide optimum extraction condition predicted by model, which is 91.2 °C temperature and 18 min time combination for enzymatic method of ellagitannin extraction, at which maximum TEC, 46.55 % and AOA, 64.09 % can be achieved.

Validation of optimal point: Response surface methodology model generated a confirmation report containing two sided t-test with confidence (95 %, n = 1), at optimum temperature 91.23 °C and time 18.18 min, model predicted the maximum eTEC (46.55 %) and eAOA (64.09 %). Considering the predicted values, experiments were conducted at modified temperature (91.2 °C) and time (18 min), to validate the predicted result. The mean values of responses are shown in Table-3. The obtained values from validation experiments were close to the predicted values hence it confirms that the response surface methodology was successfully used to maximize the extraction of ellagitannin.

TABLE-3
VALIDATION OF OPTIMAL POINT (PREDICTED AND EXPERIMENTAL VALUE)

Dependent variables	Predicted value	Experimental value
eTEC	46.55	45.01 ± 0.85
eAOA	64.09	63.74 ± 1.28

Conclusion

From this study, it can be concluded that sample to solvent ratio, HCl to water ratio and inoculum level of *A. niger* as enzyme source affect the ellagitannin content of FPP extract in physical, chemical and enzymatic methods respectively. Further extraction temperature and time was important for extracting high ellagitannin content and antioxidant activity in fresh pomegranate peel extract by all the extraction method. At optimum temperature time combination of 91.2 °C and time 18 min maximum yield of ellagitannin *i.e.*, 45.01 g as GAE/ 100 g of dried equivalent of fresh pomegranate peel with 63.74 % of antioxidant activity as DPPH was obtained after a proper enzymatic treatment. Both physical and enzymatic method may be recommended as an economical method for the extraction of ellagitannin from fresh pomegranate peel except the enzymatic method is more time consuming.

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