World Journal of Pharmaceutical research

Volume 3, Issue 1, 1403-1416. Research Article ISSN 2277 – 7105

SIMULTANEOUS QUANTITATIVE HPLC ANALYSIS OF ASCORBIC ACID, GALLIC ACID, AND CATECHIN IN *PUNICA GRANATUM, TAMARINDUS INDICA* AND *PRUNUS DOMESTICA* USING BOX-BEHNKEN STATISTICAL DESIGN

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Article Received on
22 October2013
Revised on 26 November 2013,
Accepted on 30 December 2013

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ABSTRACT

A reversed-phase HPLC method has been developed and validated for simultaneous estimation of ascorbic acid, gallic acid and catechin in *P. granatum, T. indica* and *Prunus domestica*. A C18 column was used with isocratic elution of 0.1% glacial acetic acid: acetonitrile (70:30; *v/v*) as mobile phase at a flow rate of 1.0 mL/min with 15 minute run time. Validation of the method was performed in order to demonstrate its selectivity, linearity, precision, robustness, accuracy, limits of detection and quantification. Calibration plots were linear over the concentration ranges 1-500µg/mL for ascorbic acid, 5-500 µg/mL for gallic acid, and 50-500 µg/mL for catechin with correlation coefficients 0.9953, 0.9951 and 0.9976, respectively. Precision RSDs of peak areas were less than 2.41%. The recovery of ascorbic acid, gallic acid and catechin in sample was found in the range from 98.88 to 102.50%. Limits of detection were 0.31, 1.53, 15.16 µg/mL and limits of quantification were 1.03, 5.12, 50.31 µg/mL for ascorbic acid, gallic acid and catechin, respectively. The box-behnken statistical design was used for the validation of robustness. It showed that proposed method was simple, precise and accurate and can be used for the quality control of the raw materials as well as formulations.

Keywords: RP-HPLC; *Punica granatum; Tamarindus indica; Prunus domestica,* Simultaneous, Box-behnken; Antioxidant.

INTRODUCTION

Ascorbic acid, gallic acid, and catechin (Figure 1) are naturally occurring organic compounds. The interest in these compounds is due to their pharmacological activity as radical scavengers.[1-2] The awareness of harmful effects of free radicals for human health has recently increased. Free radicals are very unstable molecules arising physiologically during cellular aerobic metabolism (2–3% of oxygen consumed by a cell is converted into free radicals).[3] They react quickly with other compounds, beginning chain reactions. Once the process is started, it can cascade, finally resulting in disruption of a living cell or in molecular and cellular DNA damage.[4]

Normally, the human body can handle these compounds, but if their amount becomes excessive, damage can occur. The development of chronic diseases, such as cardiovascular diseases (CVD), cancer, hypertension, type-2 diabetes and hepatic disorder involves large production of free radicals leading to oxidative stress.[4-5] A diet rich in fruits and vegetables has been associated with lower risk of chronic diseases, because in addition to its vitamin and mineral composition, it will also contain other compounds with protective effects, in particular antioxidants.[6-7] For their potential role in preventing such diseases, natural compounds with antioxidant activity have gained the attention of researchers and nutritionists. A large number of fresh fruits and vegetables are primary sources of antioxidants.

Pomegranate (*Punica granatum* L., Lythraceae); Tamarind (*Tamarindus indica* L., Leguminosae); and Plum (*Prunus domestica* L., Rosaceae), commonly used as nutritional supplements, are a major source of antioxidants [8-10] and contribute to the daily intake of a significant amount of these molecules.

It was found that no attempt has been made to develop a simultaneous method for quantification of these components in *P. granatum, T. indica* and *P. domestica*. Keeping in view of their biological importance, a sensitive method for rapid estimation of these markers has become a necessity to develop an analytical method for simultaneous estimation of these components to manage the man power, time and consumption of solvents. HPLC is the method of choice for analysis of these compounds, because of its extremely high versatility,

precision and relatively low cost.[11-13] The proposed method for simultaneous estimation of ascorbic acid, gallic acid, catechin in *P. granatum, T. indica* and *P. domestica* was validated as per the ICH guidelines [14] similar to the other methods reported by laboratory, [15-18] which are in use for the quality control of herbal drugs.

MATERIALS AND METHOD

Plant collection and identification

The fresh fruits of *P. granatum, T. indica* and *P. domestica* were collected from Delhi, India and were authenticated at Department of Botany, Jamia Hamdard, New Delhi, India, with a voucher specimen (PG/FP-367, TI/FP-368, PD/FP-369) which were deposited in the herbarium.

Chemicals and materials

Ascorbic acid (99%), gallic acid (98%) and catechin (98%) were procured from Sigma-Aldrich (USA), HPLC grade acetonitrile and water was purchased from J.T. Baker (NJ, USA). Glacial acetic acid was purchased from Merck (Germany).

Instrument conditions

The analysis was carried out on a Waters Alliance e2695 separating module (Waters Co., MA, USA) using photo diode array detector (Waters 2998) with auto sampler and column oven. The instrument was controlled by use of Empower software installed with equipment for data collection and acquisition. Compounds were separated on a C18 reverse phase column (25 x 4.6mm, particle size 5 µm, Merck, Germany) maintained at room temperature. The mobile phase used was 0.1 % acetic acid: acetonitrile (70:30 *v/v*) with a flow rate of 1 mL/ minute. Analysis was performed at room temperature. Detection was carried out at a wave length of 254 nm with PDA visible detector.

Standard preparation

Standard stock solutions of three reference standards, each of 500 µg/mL concentrations (ascorbic acid, gallic acid and catechin) were prepared by dissolving them in HPLC grade water. The stock solutions were then diluted to different concentrations and working standard solutions were stored at 4°C till it was applied on HPLC after filtration using 0.22 µm syringe filter before injecting for construction of calibration plots.

Sample preparation

The fruits of *P. granatum, T. indica and P. domestica* were dried in hot air oven at 40 -50ºC for a week. The dried fruits were powdered using mixer grinder. The accurately weighed samples (3g) were dissolved in 25 mL of extraction solvent (HPLC grade water) and sonicated for 30 min at 40±3°C in ultra sonicator water bath. The solution was filtered through a 0.22 µm membrane prior to injection into the HPLC system for quantification

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Method validation

The described method was validated according to the ICH guidelines. The following validation characteristics were evaluated: selectivity, linearity, precision, robustness, accuracy, limits of detection and quantification (LOD and LOQ).

Selectivity

Selectivity is the ability to assess unequivocally the marker in presence of other chemical constituent of plant. It is indicated by ascorbic acid, gallic acid and catechin peaks getting well resolved from peaks of other chemical constituents.

Linearity

Linearity was determined by injecting the standard mixture in a concentration range to get 1-500µg/mL for ascorbic acid, 5-500 µg/mL for gallic acid, and 50-500 µg/mL for catechin. The calibration curves were obtained for each individual compound by plotting the peak area versus the concentration. Regression analysis was performed in order to determine the linearity, in terms of r^2, of the calibration graphs.

Precision

Inter-day and Intra-day Precision were evaluated by analyzing standard solutions, six times and % RSD value was calculated to determine any intra-day and Inter-day variation.

Robustness

Robustness of the method was performed by introducing very small changes in the analytical methodology at a single concentration level (100µg/mL). Robustness of the proposed method was determined by changing in three parameters, i.e., temperature (A), flow rate (B), and wavelength (C). In the present study, the robustness was evaluated by using the box-behnken response surface design.[19] The design simultaneously evaluated the effects of the three important parameters on peak area: temperature of the column oven, flow rate of the mobile

phase and detection wave length and temperature of the column oven. Design-Expert 8.0 software, (Stat Ease Stat-Ease, Inc., Minneapolis, MN, USA) was used to evaluate the result. Three-dimensional graphs represented peak area dependence on temperature of the column, flow rate of the mobile phase and detection wavelength. Effects of the selected factors were evaluated over a range of conditions by determining the maximum area response of the ascorbic acid, gallic acid and catechin peaks.

Accuracy

To check accuracy of the method, recovery studies were carried out by addition of standard drug solution to sample solution at three different levels 50, 100 and 150 %. Mean percentage recovery was determined.

Limits of detection and quantification

The LOD and LOQ were calculated through the slope and standard deviation method, using the following formula: LOD = (3.3 × δ) / S, and LOQ = (10 × δ) / S, Where: δ: is the standard deviation of the Y intercept of the linear regression equations. S: is the slope of the linear regression equations.

RESULTS AND DISCUSSION

Optimization of HPLC chromatographic conditions

Optimum chromatographic conditions were obtained after running different mobile phase with a reverse phase C18 column. Acetonitrile was preferred over methanol as mobile phase because its use resulted in improved separation. The presence of acid in a mobile phase system gave a much better separation for ascorbic acid, gallic acid and catechin. The mobile phase consisted of a mixture of 0.1% glacial acetic acid and acetonitrile (70:30; *v/v*) was run in isocratic elution mode. The flow rate of the mobile phase was 1.0 mL/min, injection volume was 10µL, and detection wavelength was set to 254 nm. It can be seen a good separation can be achieved within 15 min using the condition described (Figure 2). Symmetrical, sharp and well-resolved peaks were observed for ascorbic acid, gallic acid and catechin. The elution order and the retention times for ascorbic acid, gallic acid and catechin were 2.163, 3.269 and 6.392 min respectively. Selecting 254 nm as the detection wavelength resulted in an acceptable responses and enable the detection of compounds used in this study.

Method validation for HPLC fingerprinting

The HPLC method was validated by defining the selectivity, linearity, accuracy, precision, robustness, limits of detection and quantification. As it was found that ascorbic acid, gallic acid and catechin peaks gets well resolved from peaks of other chemical constituents, hence we conclude that method is selective. For qualitative purposes, the method was evaluated by taking into account the precision in the retention time and selectivity of marker compounds eluted. LOD and LOQ value for ascorbic acid was found to be 0.31µg/mL and 1.03µg/mL; gallic acid was 1.53 µg/mL and 5.12 µg/mL; and catechin was 15.16 µg/mL and 50.31 µg/mL. Linear correlation was obtained between peak area and concentration of three markers. The proposed method was found to be linear over a wide range of concentrations, 1-500 µg/mL for ascorbic acid; 5-500 µg/mL for gallic acid and 50-500 µg/mL for catechin with regression coefficients (r2) of 0.995, 0.995 and 0.997 respectively (Table 1). Relative standard deviation of all the parameter was found less than 2.44% for the degree of repeatability, indicating the high repeatability of the proposed method. The relative standard deviation of intra-day, inter-day and inter-analyst precision revealed that the proposed method is precise (Table 2). The high recovery values (98.88-102.50 %.) indicated a adequate accuracy (Table 3).

The robustness was evaluated by using the Box-Behnken response surface design (Figure 3). The Design Expert software proposed the following polynomial equation for peak area of ascorbic acid, gallic acid and catechin.

Ascorbic acid

Peak area +3.137E+006+13361.75*A+31826.62*B+1.255E+005*C-1034.25*A*B

-10664.25*A* C+15720.00*B*C-21582.50*A^2-4324.25*B^2-23607.75*C^2

where A is the temperature (K), B is the flow rate (mL/min) and C is the wavelength (ºC). According to the above equation, flow rate and wavelength appeared to have more effect on the peak area than temperature. As the flow rate and detection wavelength increases the peak area was also found to increase the peak area, as indicated by the positive coefficient value. However, the lower magnitude of the coefficient indicated that the effect of temperature is less than the flow rate on peak area. The temperature affected the peak area in the opposite direction to that of observed for the detection wavelength and flow rate. The negative coefficient value of the temperature indicated that the peak area decreased with an increase in temperature.

Table 1. Linear regression data for the calibration plots (n = 3) for ascorbic acid, gallic acid and catechin.

Components	Concentration (µg/mL)	Regression equation	r^2
Ascorbic acid	1-500	y = 38373x - 37799	0.995
Gallic acid	5-500	76866x - 99604	0.995
Catechin	50-500	551.7x + 7773	0.997

Table 2. Repeatability, Inter-day, intra-day and inter-analyst precision of the HPLC method (n = 6) for ascorbic acid, gallic acid and catechin.

Conc. (µg/ml)	Repeatability		Inter-day precision		Intra-day precision		Inter-analyst precision	
	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD
Ascorbic acid								
50	1652202± 33163	2.00	1692188± 24644	1.45	1668360± 12296	0.73	1585019± 27941	1.76
100	3191050± 54764	1.71	3357702± 27526	0.81	3451702± 27529	0.79	3251722± 27474	0.84
200	6427919± 41208	0.64	6463714± 101080	1.56	6463714± 101080	1.56	6530361± 151242	2.31
Gallic acid								
50	2883502± 63921	2.21	2950166± 13442	0.45	3096835± 9339	0.30	2846833± 18093	0.63
100	5680097± 40379	0.71	585009± 42021	0.71	5923580± 61561	1.03	5943432± 143245	2.41
200	13007604± 157202	1.20	13274539± 174366	1.31	12540034 ± 52890	0.42	14141235± 150613	1.06
Catechin								
50	57392± 601	1.04	58396± 903	1.54	60393± 398	0.66	56848± 728	1.28
100	127103± 3101	2.44	129139± 650	0.50	139139± 650	0.46	129469± 2170	1.67
200	171129± 731	0.42	172665± 2967	1.71	175296± 3793	2.16	171821± 932	0.54

SD, standard deviation; RSD, relative standard deviation

Table 3. Accuracy of the HPLC method (n=3) for ascorbic acid, gallic acid and catechin

% of standard spiked to the sample	Theoretical content (µg/ml)	Amount of drug recovered µg/ml ±SD[a])	% of drug recovered	% RSD[b]
Ascorbic acid				
0	3	3.04± 0.06	101.44	2.13
50	4.5	4.47± 0.08	99.41	1.88
100	6	6.05± 0.10	100.95	1.76
150	7.5	7.42± 0.10	98.99	1.39
Gallic acid				
0	12	12.13± 0.21	101.11	1.71
50	18	17.80± 0.36	98.88	2.02
100	24	24.60± 0.20	102.50	0.81
150	30	29.96± 0.40	99.88	1.34
Catechin				
0	7	7.06± 0.15	100.95	2.16
50	10.5	10.73± 0.15	102.22	1.42
100	14	14.26± 0.25	101.90	1.76
150	17.5	17.4± 0.10	99.42	0.57

SD, standard deviation; RSD, relative standard deviation.

Table 4. Quantification of ascorbic acid, gallic acid and catechin in tested herbs by HPLC

Samples	Ascorbic acid (% w/w)	Gallic acid (% w/w)	Catechin (% w/w)
Punica granatum	0.003%	0.012 %	0.007 %
Tamarindus indica	0.0024%	0.004%	0.0018%
Prunus domestica	0.007%	0.0019%	0.0013%

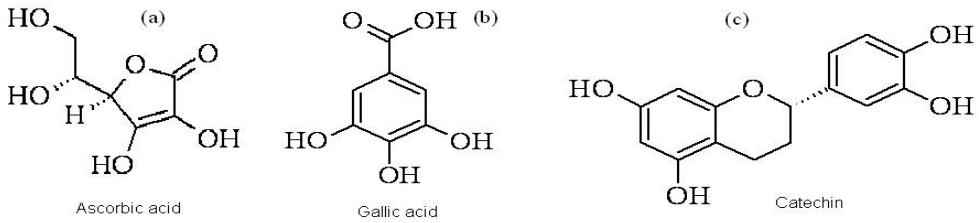

Figure 1: Chemical structure of ascorbic acid, gallic acid and catechin

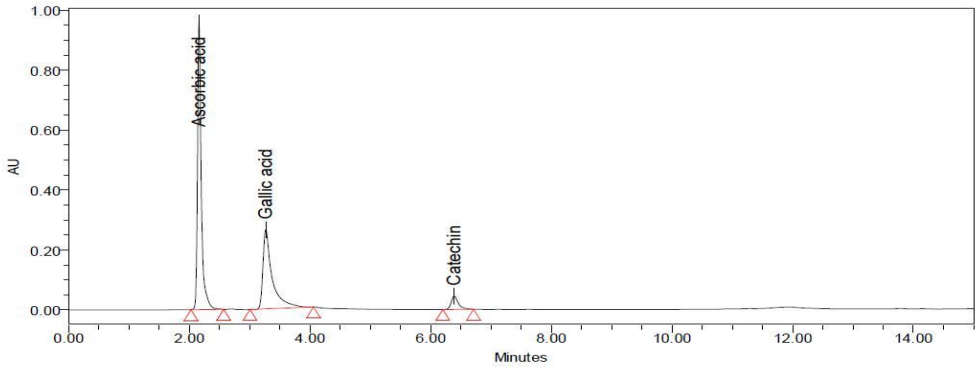

Figure 2: A typical HPLC chromatogram of ascorbic acid (R_t =2.163), gallic acid (R_t =3.269), catechin (R_t =6.392), at 254 nm

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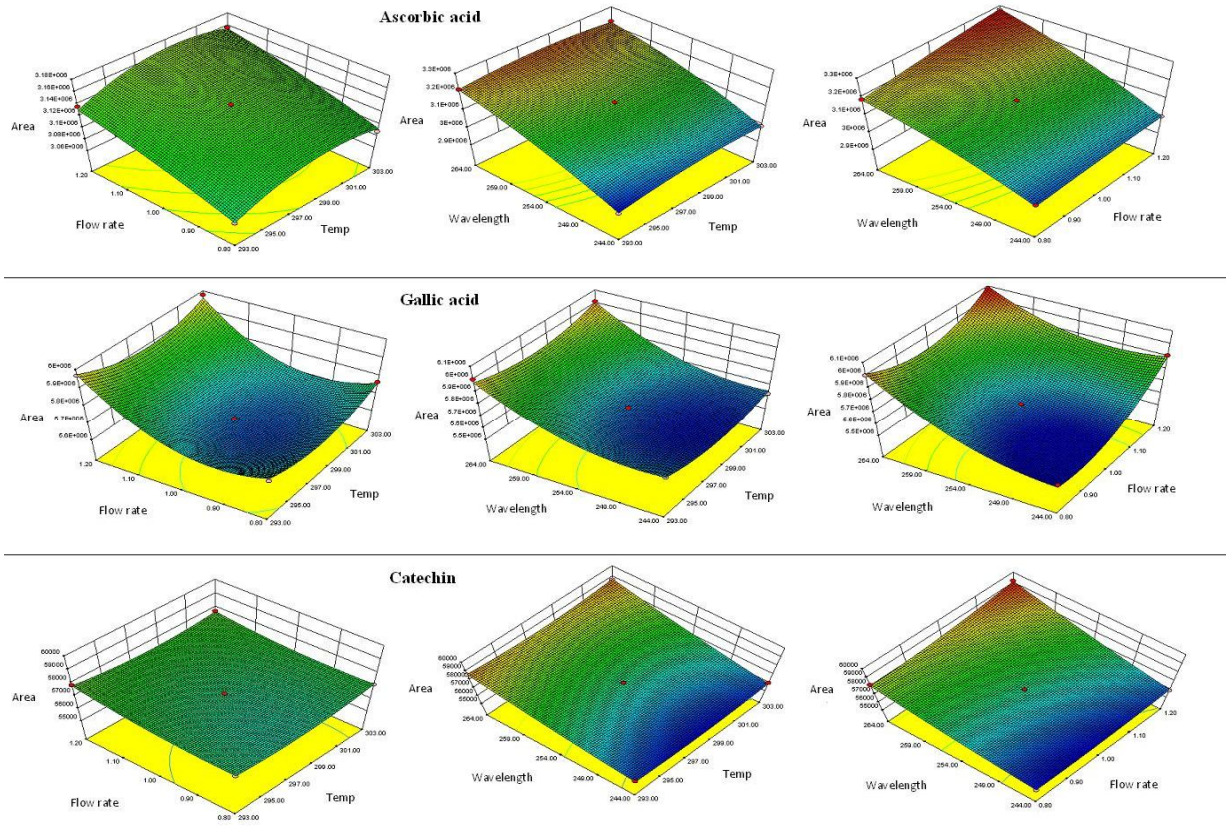

Figure 3: Three-dimensional graphs of ascorbic acid, gallic acid and catechin by using Box-Behnken experimental design

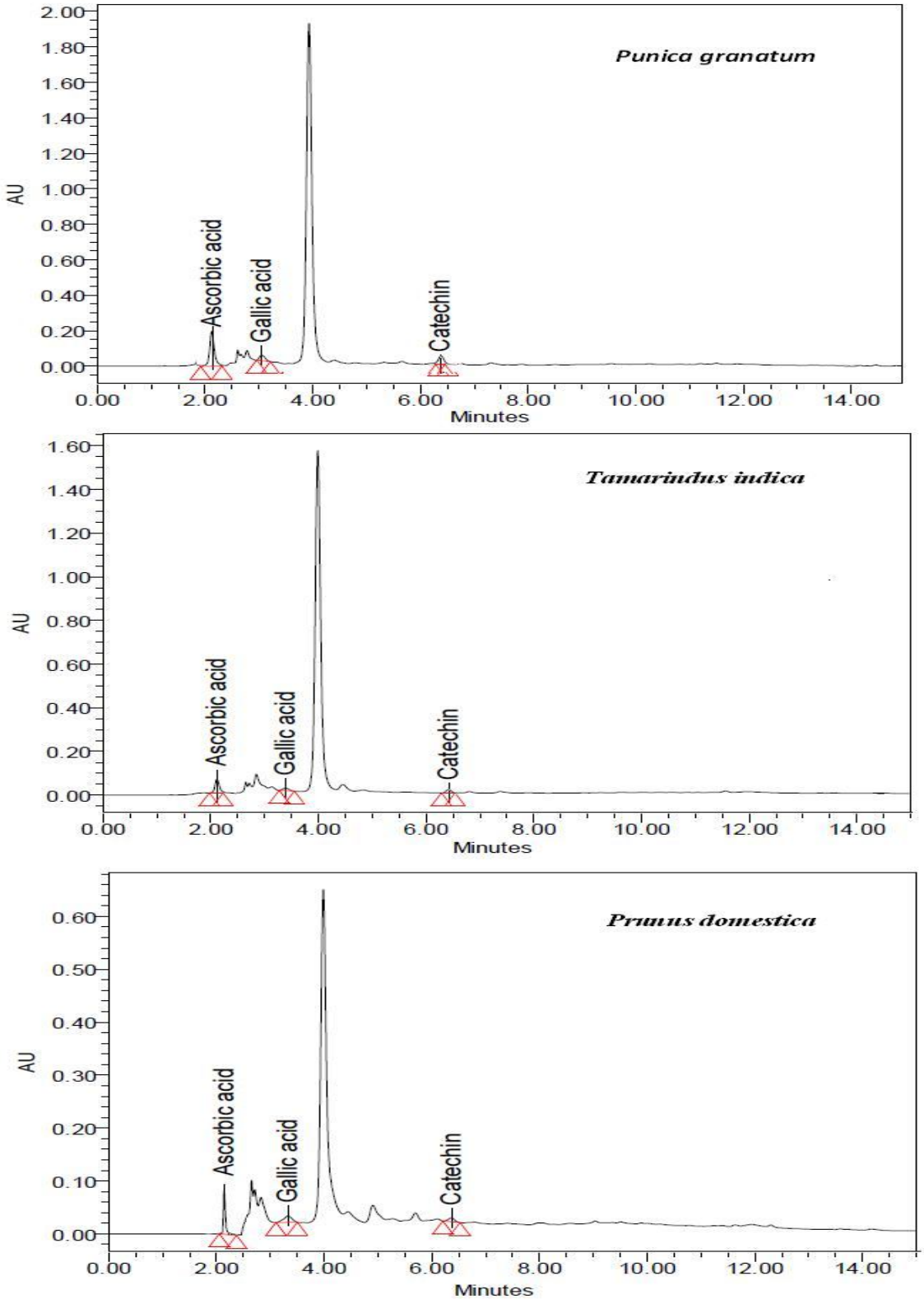

Figure 4: Quantification of ascorbic acid, gallic acid and catechin in tested herbs

Gallic acid

Peak area +5.638E+006+5820.50*A+1.119E+005*B+1.669E+005*C-11502.00*A*B +10411.00*A*C-68368.25*B*C+78103.04*A^2+1.415E+005*B^2+1.099E+005*C^2

The above polynomial equation of gallic acid shows that, temperature, wavelength and flow rate appeared to have more effect on the peak area. As the temperature, flow rate and detection wavelength increases the peak area was also found to increase the peak area, as indicated by the positive coefficient value. However, the lower magnitude of the coefficient indicated that the effect of temperature is less than the all factors on peak area.

Catechin

Peak area +56810.00+52.00*A+384.75*B+1442.50*C - 106.25*A*B+5.25*A*C+166.75*B*C +343.62*A^2+240.12*B^2+139.63*C^2

The equation, for catechin shows that temperature and flow rate appeared to have more significant effect on the peak area than detection wavelength. As the flow rate increases, peak area also increases. The temperature was also found to increase the peak area, as indicated by the positive coefficient value. However, the lower magnitude of the coefficient indicated that the effect of detection wavelength is less than the flow rate on peak area. The temperature affected the peak area in the opposite direction to that of observed for the detection wavelength and flow rate. The negative coefficient value of the wavelength indicated that the peak area decreased with an increase in temperature.

Although the software predicted that the different factors could affect the peak area, the values of peak area were in an acceptable range to demonstrate sufficient robustness of the analytical method. Therefore, this HPLC method can be regarded as selective, accurate and precise for the quantification of ascorbic acid, gallic acid, and catechin in *P. granatum, T. indica and P. domestica.*

Determination of ascorbic acid, gallic acid and catechin in samples

The amount of ascorbic acid, gallic acid and catechin in *P. granatum, T. indica* and *P. domestica* were analyzed using developed and validated chromatographic method. The samples were injected in triplicates in HPLC column and peak area of all the triplicate samples were used for analysis of content by regression equation. The developed mobile phase gave optimal separation, with well defined and well resolved sharp peaks in samples at

retention times for ascorbic acid, gallic acid and catechin were 2.163, 3.269 and 6.392 min respectively (Figure 4). The results of the analysis are given in the table 4.

One of the main problems associated with the standardization of traditional herbal drugs are the complexity in identification and quantification of the chemical components. The proper identification and quantification of markers needs fully optimized and validated analytical methods, otherwise it may lead to erroneous results. The multiconstituent herbal drugs need special attention and any incompatible sample preparation method can lead to significance errors in the recovery. Taking all these factors in consideration, we report here a novel HPLC method which can be employed for the proper, identification and simultaneous quantification of ascorbic acid, gallic acid and catechin in *P. granatum*, *T. indica* and *P. domestica*. The proposed method was optimized in such a way to give maximum resolutions between the components, which are critical to avoid merging/overlapping components and makes the method superior to other existing methods.[20-23]

CONCLUSION

A validated reverse phase HPLC analytical method has been developed for the simultaneous determination of three antioxidant compound ascorbic acid, gallic acid and catechin in *P. granatum*, *T. indica* and *P. domestica*. The proposed method is simple, precise, specific, accurate, less time consuming, cost effective and has the ability to separate the drug from its other constituents. This method has been properly optimized and well validated. It also has better linearity range and limits of determination. Application of design expert using Box-Behnken for validation of robustness testing showed that a slight change in temperature, flow rate and wavelength has a direct effect on peak area. Hence, special attention is required for strict monitoring of the aforementioned two factors during chromatographic testing. Statistical analysis proved that the method is suitable for the routine analysis of ascorbic acid, gallic acid and catechin. Since the antioxidant compounds have been of interest of health benefits, the present analytical study could be a potential application to identify and quantify the antioxidant compounds in other extracts.

ACKNOWLEDGEMENTS

The authors are thankful to the Jamia Hamdard and CCRUM, Department of AYUSH, Ministry of health and family welfare, Government of India, New Delhi, India for providing financial assistance to carry out the research work.

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