



Original article

HPTLC method for simultaneous determination of ascorbic acid and gallic acid biomarker from freeze dry pomegranate juice and herbal formulation

Prawez Alam ^{a,*}, Y.T. Kamal ^b, Saleh Ibrahim Alqasoumi ^c, Ahmed Ibrahim Foudah ^a, Mohammed Hamed Alqarni ^a, Hasan Soliman Yusufoglu ^{a,*}

^a Department of Pharmacognosy, College of Pharmacy, Prince Sattam Bin Abdulaziz University, P.O. Box 173, Al-Kharj 11942, Saudi Arabia

^b Department of Pharmacognosy, College of Pharmacy, King Khalid University, Abha 61471, Saudi Arabia

^c Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia

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ABSTRACT

A new rapid, simple, sensitive and high performance thin layer chromatography (HPTLC) has been established for the simultaneous determination of ascorbic acid and gallic acid in the freeze-dried pomegranate fruit juice and herbal formulation. HPTLC method was carried out using ethyl acetate: acetone: water: formic acid, 10:6:2:2 (% v/v/v/v) on 20 × 10 cm glass coated silica gel 60 F₂₅₄ plates and scanned at 254 nm for ascorbic acid and gallic acid. Ascorbic acid and gallic acid in the freeze-dried pomegranate fruit juice were identified by comparing their single spot at R_f = 0.54 ± 0.02 and R_f = 0.83 ± 0.01 respectively. The value of regression equation ($r^2 \geq 0.9992$) revealed a good linear relationship between peak area and amount of ascorbic acid and gallic acid in the range of 100–800 ng/band. The method was validated for precision, accuracy, robustness LOD and LOQ. The method proposed can be useful for routine determination of ascorbic acid and gallic acid in various crude as well as herbal formulations as a quality control tool.

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1. Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest recognized edible fruit belonging to Punicaceae family, planted widely from Europe, Southern Asia to Middle East regions. Since these plants have been cultivated in different geographical regions there are lot of differences observed in the appearance as well as the chemical compositions (Al-Maiman and Ahmad, 2002). The fruits gained a recent attention due to the awareness of the various antioxidant and other health beneficial compounds reported. These fruits are reported with high content of polyphenol compounds which gained the attention of nutraceutical, pharmaceutical and cosmeceutical companies (Herceg et al., 2016; Anonymous, 1999;

Ferrara et al., 2014). These circumstances lead to the commercialization of the various traditional formulations. More over the various pharmacological activities such as antioxidant, anti-atherosclerotic and cardio protective properties (Karasu et al., 2012; Shema-Didi et al., 2010) reported on pomegranate.

As per various literatures available, pomegranate fruit has been reported for hydrolysable tannins, anthocyanins, procyanidins, phenolic acids and flavonol glycosides (Viuda-Martos et al., 2010; Shema-Didi et al., 2010). Major hydrolysable tannins reported are gallotannins, ellagic acid tannins and gallagyl tannins, known as punicalagins. Apart from this pomegranate is also reported for its rich source of ascorbic acid.

The recent attraction gained by the pomegranate and its various value added products leads to the commercial booming, which ultimately led to the adulteration and absence. In a survey conducted in recent time on pomegranate fruit juices for its 'polyphenol contents' showed surprisingly that there were some of the product which even contains no traces of ellagitannins (Mullen et al., 2007). It became an extremely important issue to have some quality control methods to ensure the authenticity.

In this research we tried to develop a simultaneous method for the quantification of ascorbic acid and gallic acid as biomarker for

* Corresponding authors.

E-mail address: h.yusufoglu@psau.edu.sa (H.S. Yusufoglu).

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the freeze dried pomegranate juice and herbal capsule formulation. When analyzing the juice sample analyst used to face the problems in extraction methodology due to the high amount of sugar present in the sample. Here in this research we tried to develop an efficient solid phase extraction method which enables us ensure the maximum recovery of the marker constituents.

2. Experimental

2.1. Standard and chemicals

Standard ascorbic acid and gallic acid were purchased from Sigma-Aldrich, Delhi, India. The methanol, acetonitrile and water used for the experiment were HPLC grade purchased from Merck, USA (LiChrosolv®).

2.2. Preparation of standard solutions

Carefully weighed 25 mg of both standards (gallic acid and ascorbic acid) and initially dissolved in 10 mL of HPLC grade water and further the volume was made up to 25 mL with methanol (HPLC grade) in a volumetric flask to give concentration of 1000 µg/mL. From this stock solution 10 mL of the solution was transferred to a 100 mL volumetric flask and diluted to volume with methanol to get a concentration of 100 µg/mL. The solutions were applied on TLC plate to get the concentration range of 100–800 ng. Using the above concentrations linearity plots were plotted to obtain correlation coefficient.

2.3. Materials and methods

Four different commercially available pomegranate fruits and one herbal formulation were purchased from local markets of Al-Kharj, Riyadh. The fruit juice was prepared using Avance collection juicer HR1871/00 (Philips). The juice was freeze-dried in a Millrock LD85 tray type freeze dryer (Millrock Technology, USA) at –40 °C for 24 h and stored in airtight container at –18 °C until extraction, since it is highly hygroscopic in nature.

2.4. Extractions procedure

The freeze dried sample was extracted using matrix solid phase extraction technique. The weighed quantity (0.5 g) of the sample was mixed with same quantity of octadecylsilyl derivatized silica (C18). The mixture was transferred to a column and packed. Further the column was eluted using 100% methanol. The procedure was repeated thrice and the fraction were pooled and dried. Accurately weighed amount of 1 g from each dried extract was separately dissolved in methanol. The concentrate from each sample was separately reconstituted in accurately measured 50 mL of methanol using volumetric flasks.

Twenty herbal capsules were accurately weighed and calculated the average weight of each capsule. The shells were removed and checked for the powder weight to find out the average filled weight. The powder was triturated to get an uniform sample. From the above powder, 1 g was taken and dissolved in 25 mL methanol and made the sonication for 20 min. Then the volume was made up with methanol in a 50-mL volumetric flask after filtration. The resulting solution was used as test solutions

2.5. Chromatographic conditions

The samples were spotted as bands of width 6 mm on a 10 × 20 cm glass-backed plates coated with 0.2 mm layers of silica

gel 60 F254S (E-Merck, Germany) using the sample applicator. The sample application rate was kept as 150 nL/s. The slit dimension was kept at 4 × 0.45 mm and 20 mm/s scanning speed was employed. Fifteen mL of the mobile phase consisted of ethyl acetate:acetone:water:formic acid, 10:6:2:2 (% v/v/v/v) was used for per chromatography. Prior to the development, the TLC chamber was saturated with mobile phase for 15–20 min. After development the TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was achieved on Camag TLC scanner III operated by WinCats software at wavelength 254 nm.

2.6. Method validation

The newly developed method was validated (ICH guidelines) for different parameters like linearity, accuracy, precision, robustness and LOD and LOQ (Alam et al., 2016).

2.6.1. Linearity

The linearity of the method for ascorbic acid and gallic acid were checked between 100 and 800 ng/spot and the linearity curve was plotted.

2.6.2. Accuracy

For determining the accuracy of the method the pre-analyzed samples were spiked extra ascorbic acid and gallic acid standard and the mixtures were reanalyzed at three different concentration levels. The percentage recovery was calculated at 0, 50, 100 and 150%.

2.6.3. Precision

Precision of the method was evaluated by assessing the repeatability and intermediate precision (3 different levels). Intra-day variations were studied for assessing the reparability of the sample, whereas inter day variations were carried out in order to assess the intermediate precision.

2.6.4. Robustness

The robustness of the method was done by making deliberate changes in the experiment methodology i.e., by changing the mobile phase compositions. The %RSD of the area of the method was used for analyzing the robustness of the method.

2.6.5. Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) were determined by signal to noise ratio method. The standard deviation of the response was determined based on the standard deviation of y-intercepts of regression lines.

2.6.6. Specificity

Specificity of the suggested TLC densitometric was confirmed by examining and comparing the R_f values and spectra of the spot for ascorbic acid and gallic acid in the samples with that of the standards.

2.7. Quantification of ascorbic acid and gallic acid in freeze-dried pomegranate fruit juice

The standard and test solution were applied using the sample applicator and chromatograms were obtained for both ascorbic acid and gallic acid. The peaks were identified by the R_f value

and amount and the quantification were carried out using linearity curve and regression equation.

3. Results and discussion

3.1. Method development

The ratio of HPTLC mobile phase was optimized to accomplish accurate, sharp well resolved peaks for both ascorbic acid and gallic acid. The mobile phase ethyl acetate:acetone:water:formic acid, 10:6:2:2 (% v/v/v/v) resulted in good separated and symmetrical peaks at R_f value of 0.54 ± 0.02 and 0.83 ± 0.01 for ascorbic acid and gallic acid respectively (Fig. 1).

3.2. Method validation

The linearity plot of peak area against amount of ascorbic acid and gallic acid was found linear in the range 200–800 ng/spot (Figs. 3 and 4). Linear regression data for the plot confirmed the good linear relationship (Table 1). The recovery experiments are conducted at 3 different levels to establish the accuracy of the method (Table 2). The precision of the method was determined by conducting repeatability and intermediate precision. The % RSD results are depicted in Table 3. The robustness of the method was done by making deliberate changes in the experiment methodology i.e., by changing the mobile phase compositions (Table 4). LOD and LOQ of the method was carried out by Signal to noise ratio and as 8.22 and 15.21 as well as 7.43 and

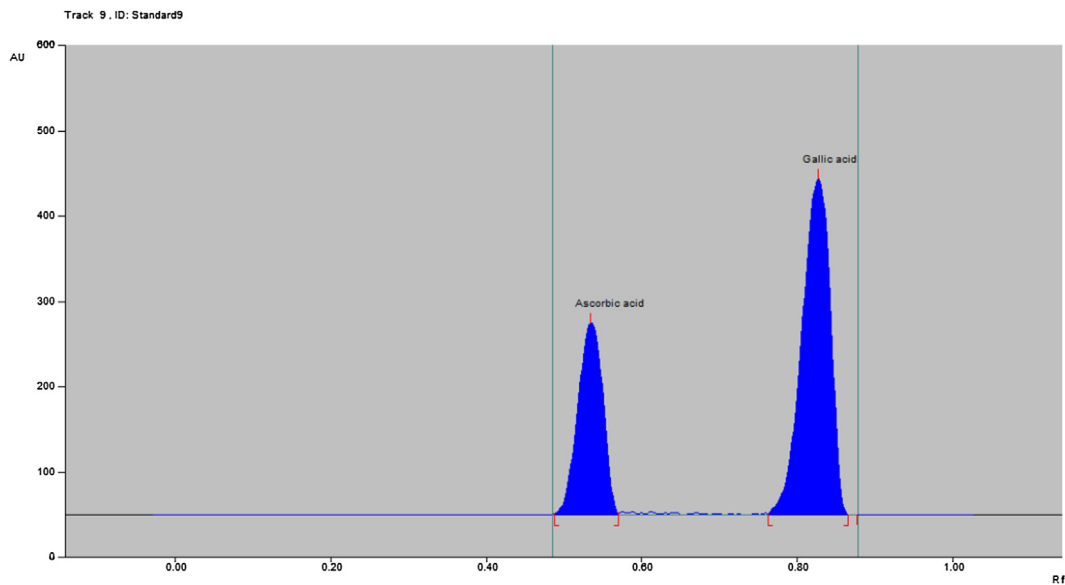


Fig. 1. HPTLC densitogram of standard Ascorbic acid and Gallic acid.

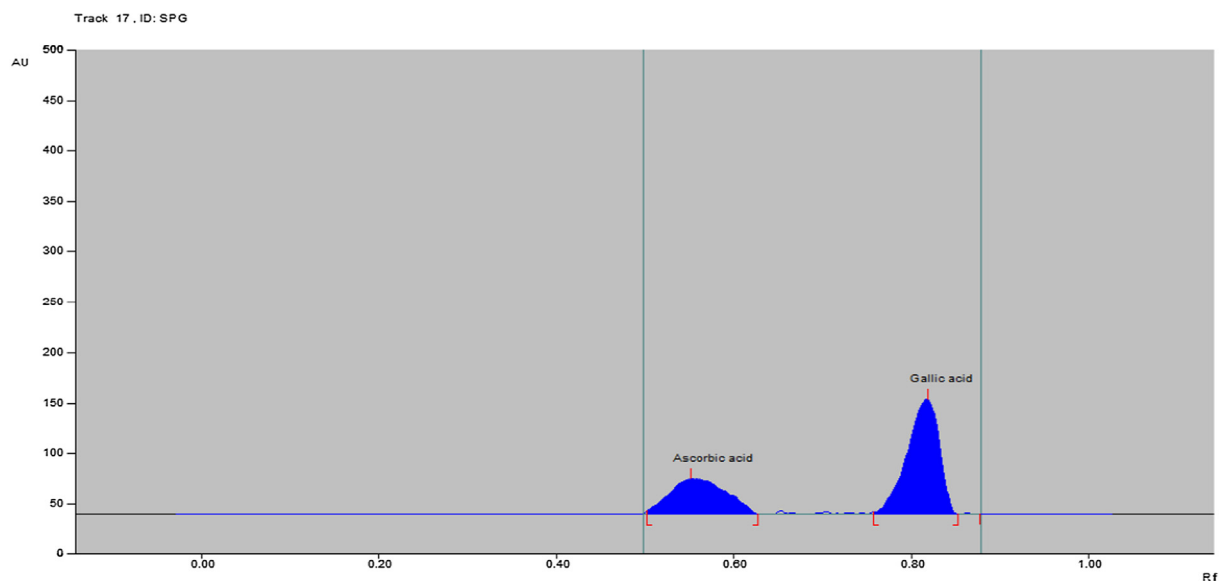


Fig. 2. HPTLC densitogram of the Syrian pomegranate (SPG) sample.

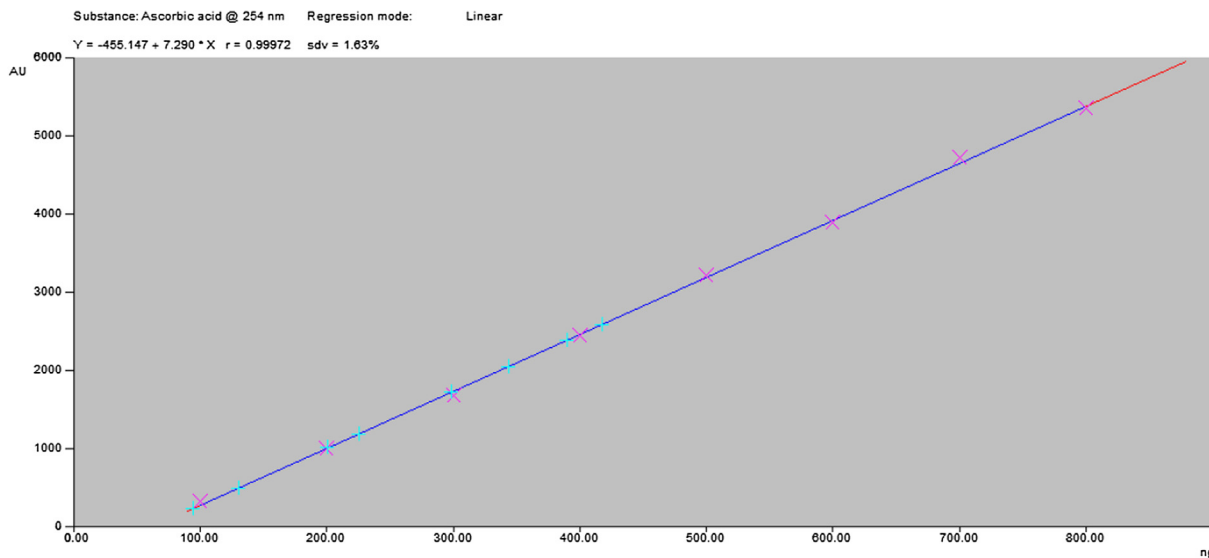


Fig. 3. The linearity graph of the standard Ascorbic acid.

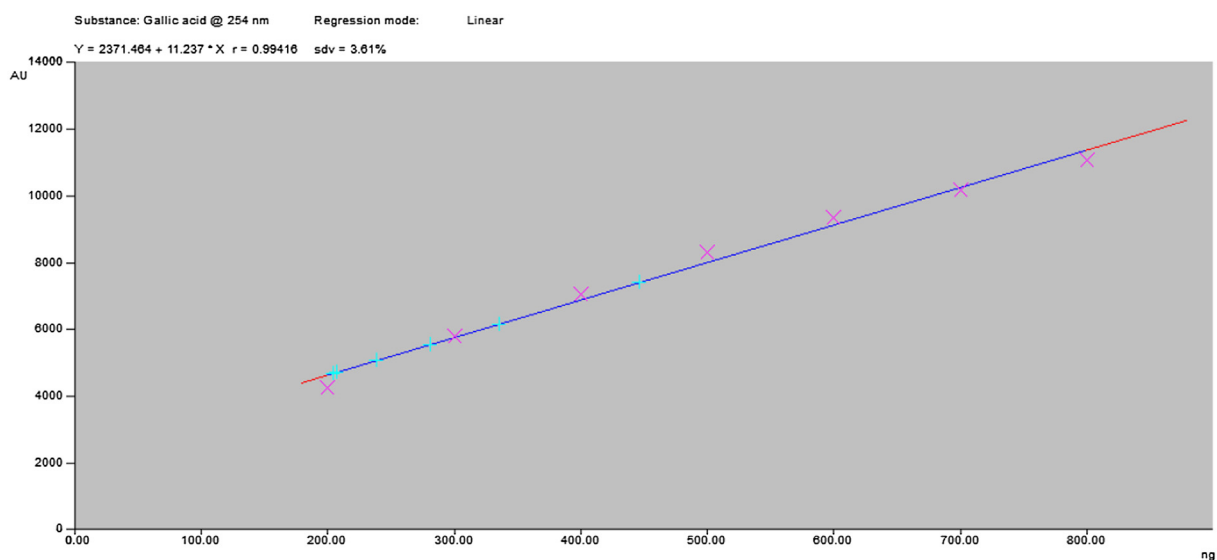


Fig. 4. The linearity graph of the standard Gallic acid.

Table 1

Linear regression data for the calibration curve of ascorbic acid and gallic acid (n = 6).

Parameters	Ascorbic acid	Gallic acid
Linearity range (ng/spot)	200–800	100–700
Regression equation	$Y = 7.1671x + 469.05$	$Y = 14.457x + 297.86$
Correlation coefficient	0.9994	0.9992
Slope \pm SD	7.1671 ± 0.1225	14.457 ± 0.1380
Intercept \pm SD	469.05 ± 71.61	297.86 ± 66.62
Standard error of slope	0.0510	0.0734
Standard error of intercept	29.31	37.01
95% confidence interval of slope	9.129–9.762	12.12–13.23
95% confidence interval of intercept	5034–5389	3010–3220
P value	<0.0001	<0.0001

18.20 ng/mL for ascorbic acid and gallic acid, respectively. The peak purity of ascorbic acid and gallic acid was assessed by matching the overlaid spectra at peak start, peak apex and peak end position of the spot. The overlaid spectra of ascorbic acid and gallic acid standards and freeze-dried pomegranate fruit juice and herbal formulation were given in Fig. 5.

3.3. Quantification of ascorbic acid and gallic acid in freeze-dried pomegranate fruit juice and herbal formulation

Ascorbic acid and gallic acid peaks from freeze-dried pomegranate fruit juice and herbal formulation were identified by comparing their single spot at $R_f = 0.54 \pm 0.02$ and 0.83 ± 0.01 values respectively with the peaks of standards (Fig. 2). The ascorbic acid

Table 2
Accuracy of the proposed method (n = 6).

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. Found (ng) ± SD	% Recovery	% RSD
Ascorbic acid				
0	200	196.50 ± 1.64	98.25	0.84
50	300	295.00 ± 2.00	98.33	0.68
100	400	396.50 ± 3.83	99.13	0.97
150	500	495.83 ± 3.31	99.17	0.67
Gallic acid				
0	200	195.33 ± 1.86	97.67	0.95
50	300	294.00 ± 2.45	98.00	0.83
100	400	396.83 ± 1.72	99.21	0.43
150	500	490.67 ± 4.59	98.13	0.94

Table 3
Precision of the proposed method of ascorbic acid and gallic acid.

Conc. (ng/spot)	Ascorbic acid Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Avg Conc. ± SD (n = 6)	Standard error	% RSD	Avg Conc. ± SD (n = 6)	Standard error	% RSD
300	1665.33 ± 15.12	6.17	0.91	1656.00 ± 21.63	8.83	1.30
400	2355.17 ± 22.82	9.32	0.97	2354.50 ± 28.24	11.53	1.20
500	3128.17 ± 18.63	7.61	0.60	3129.83 ± 29.65	12.11	0.95
Gallic acid						
300	4131.50 ± 25.88	10.57	0.63	4133.83 ± 30.77	12.56	0.74
400	5470.17 ± 25.49	10.41	0.47	5462.83 ± 30.04	12.27	0.55
500	7008.50 ± 18.13	7.40	0.26	7010.83 ± 23.28	9.50	0.33

Table 4
Robustness of the proposed HPTLC method of Ascorbic acid and Gallic acid.

Conc. (ng/spot)	Ascorbic acid Mobile phase composition (ethyl acetate: acetone: water: formic acid)		Area ± SD (n = 3)	% RSD	Rf	
	Original	Used				
400	5: 3: 1: 1	4.9:3.1:1:1	-0.1, +0.1	2361.83 ± 19.82	0.84	0.55
		5:3:1:1	0.0	2356.33 ± 25.79	1.09	0.54
		5.1:2.9:1:1	+0.1, -0.1	2359.33 ± 27.01	1.14	0.53
Gallic acid						
400	5:3:1:1	4.9:3.1:1:1	-0.1, +0.1	5470.50 ± 25.54	0.47	0.85
		5:3:1:1	0.0	5464.50 ± 29.73	0.54	0.83
		5.1:2.9:1:1	+0.1, -0.1	5462.83 ± 31.60	0.58	0.81

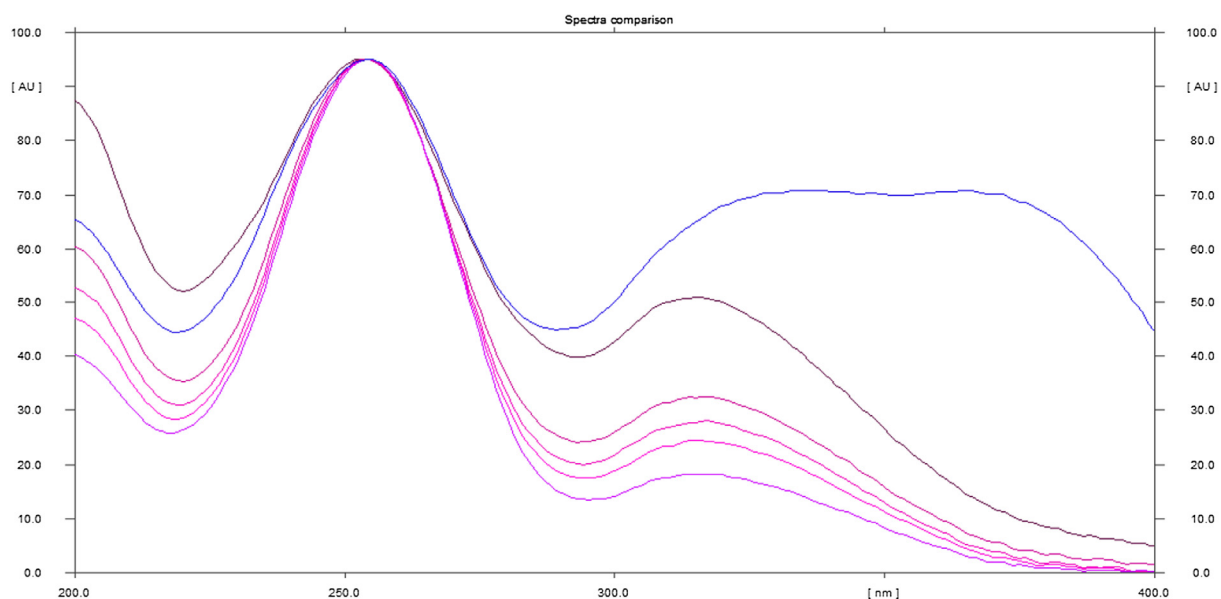
**Fig. 5.** Overlaid UV absorption spectra of standard Ascorbic acid and Gallic acid.

Table 5
Contents of ascorbic acid and gallic acid in pomegranate extract.

Samples	Ascorbic acid (Contents (% w/w))	Gallic acid (Contents (% w/w))
YPG	0.031	0.056
IPG	0.028	0.047
SPG	0.030	0.020
EPG	0.016	0.019
GNC	0.087	0.091

and gallic acid content in freeze-dried pomegranate fruit juice and herbal formulation were quantified using the linear regression equation and the amount were presented in Table 5.

4. Conclusion

The above newly developed and validated HPTLC method for the analysis of ascorbic acid and gallic acid in freeze-dried pomegranate fruit juice and herbal formulation was found to be simple, accurate, reproducible, and sensitive and can be adopted by any laboratories for the analysis ascorbic acid and gallic acid containing products. We believe that the proposed method is the first HPTLC method for quantification of ascorbic acid and gallic acid in freeze-dried pomegranate fruit juice and herbal formulation. The MSPD extraction was found as a successful tool for the extraction of complex nature samples. It helps to extract the targeted components based on their polarity, which helps to avoid the interferences between the peaks, which could possibly occur in conventional extraction methods. The newly developed method was found to be repeatable, accurate and precise for the analysis of ascorbic acid and gallic acid and can be adapted for the analysis various samples which contain gallic acid or ellagic acid.

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