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Original article

Investigation of antioxidant compounds in commercial pomegranate molasses products using matrix-solid phase dispersion extraction coupled with HPLC

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ABSTRACT

Pomegranate is a well known fruit for its unique flavor, taste and health benefits. The medicinal properties of this fruits directly associated with the phenolic content present, with great anti-oxidant potential. The research is intended to develop matrix solid phase dispersion method (MSPD) and HPLC quantification of four major anti-oxidant marker constituents (vitamin C, gallic acid, rutin & ellagic acid) in pomegranate molasses samples. The effects of several important experimental parameters like type of dispersant, sample-dispersant ratio, solvents and its volume, time of extraction were investigated. A C_{18} column with the specification (5 μm, 250 × 4.0 mm) was used for the separation. A gradient flow of mobile phase was selected after many trials containing 0.1%, v/v solution of orthophosphoric acid and acetonitrile. The flow rate was 1.0 mL/min; and the chromatograms were recorded at 254 nm. The validation parameters, like linearity (r^2 = 0.9985, 0.9965, 0.9925 & 0.9986), accuracy (100.3, 99.5, 100.9 & 101.9%), intra-day precision (%RSD = 1.09, 1.02, 1.26 & 0.97), inter-day precision (%RSD = 1.32, 0.83, 1.07, & 1.15) LOD (0.07, 4.50, 0.45 & 0.40 μg/mL), LOQ (0.095, 9.50, 0.85 & 9.5 μg/mL) and robustness (% RSD = 0.92, 0.76, 0.81 & 0.83) respectively for vitamin C, gallic acid, rutin & ellagic acid, were found satisfactory as per ICH guidelines.

1. Introduction

Pomegranate (*Punica granatum* L.) is one of commonly used fruits which is cultivated in a wide geographical area from Europe (Spain, Italy, Turkey, Greece), Southern Asia (India, Pakistan, Bangladesh) to middle east regions (Saudi Arabia, Egypt, Yemen, Siriya). The increase of recent popularity of this fruit is mainly due to the scientific research outcome on high polyphenol content of this fruit which attracted the pharmaceutical and cosmetic industries. This scenario brought different forms of food and beverages stuffs in the market like juices, jelly, molasses, concentrate and aerated beverages, syrups, wine etc., as many consumers believe it to be a preferred food to be included in everyday nutrition. The presence of high content of polyphenols makes pomegranate a powerful astringent and anti-inflammatory agent which can be applied in the treatment for trauma haemorrhage, ulcers, infections, and disorders of the digestive tract such as diarrhoea and dysentery (Anonymous, 1999; Gil et al., 2000).

Phenolic compounds are secondary plant metabolites which provide defence to plants against oxidizing agents and free radicals (Brahmi et al., 2012). Polyphenols known for their anti-oxidant property (Fraga 2007). They transfer an electron to the free radicals, which thus become stable as their electrons are paired. This prevents damage to cells and tissues caused by oxidant stress. Studies also proved the antioxidant and anti-atherosclerotic properties which also attributed to its high content of polyphenols in its free and bound gallotannins, anthocyanins and other flavonoids (Yoon and Baek, 2005). The antioxidant activity of pomegranate is three times stronger than many other dietary sources of polyphenols, such as green tea and *Emblica officinalis* (Golbon

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et al., 2014). The daily intake of pomagaranate reduces the risk of various ailments associated with heart, kidney, skin and it also promotes general physical and mental health (Miguel et al., 2010; Viuda-Martos et al., 2010).

In many commonly consumed fruits like pomegranate, berries, emblica etc., phenolics were the main antioxidant components, and their total contents were directly proportional to their antioxidant activity (Shih-Chuan et al., 2009). That means the quantification of main phenolic components present in the samples will give a rough idea about the anti-oxidant potential. One of the main challenges which analyst face on establishing the HPLC assay of molasses samples are the high viscosity due to the sugar syrup. The high content of sugar syrup makes the molasses sample unique. A high efficient extraction technique needs to establish in order to overcome this problem. The sample extraction procedure for the HPLC analysis of herbal compounds were conventionally done by liquid–liquid and solid phase extraction.

Matrix solid-phase dispersion (MSPD) is an smart substitute for conservative liquid–solid extraction methods (Barker, 2007; Kristenson et al., 2006; Visnevschi-Necrasov et al., 2009). The MSPD extraction technique can successfully replace the conventional liquid–liquid and solid phase extractions and effectively overcome most of the complications associated with conventional extraction procedures. The cost, labour and time can be saved when compared to conventional extraction procedures by reducing clean up steps and quantity of solvents. MSPD method found compatible due to less interference of impurity peaks in the chromatographic run (Capriotti et al., 2010). So the aim of the study is to develop a simple, reliable and reproducible method that can be successfully used for the simultaneous quantification of multiple active anti-oxidant components present in these commonly used traditional molasses products for their quality control as well as to propose an optimised MSPD extraction procedure in order to improve the recovery of the marker constituents.

2. Materials and methods

2.1. Samples, chemicals, solvents and standards

Three different commercially available pomegranate molasses samples were purchased from the supermarkets, Riyadh, Saudi Arabia. All the solvents used for the experiments were of HPLC grade (Merck, Germany) and Milli-Q water was used (Millipore, USA). Ortho-phosphoric acid (H_3PO_4) 88% was purchased from Fisher Scientific Company (UK). All the standards (vitamin C, gallic acid, rutin & ellagic acid with $\leq$99% purity) were purchased from Sigma–Aldrich, USA. C_{18} Silica gel, end capped (230–400 mesh) and solid phase extraction tubes (SPE) were purchased from Sigma–Aldrich, USA.

2.2. Matrix solid-phase dispersion

0.5 g of pomegranate molasses samples were weighed and placed in a glass mortar which containing 0.5 g of previously weighed C_{18} silica as sorbent. The mixture was then filled into a column containing absorbent cotton at the bottom and slightly compressed. The column was first eluted with hexane (5 mL $\times$ 3) and the fractions collected were discarded to remove any non polar compounds. Further the components were eluted using 70% methanol. The fractions were collected and evaporated to dryness at low temperature. To this 10 mL of methanol is added to dissolve in volume flask and analyzed by HPLC. The extracts were filtered through a disposable 0.22 μm PTFE membrane prior to HPLC analysis.

2.3. Chromatographic conditions and instrumentation

A Waters Alliance separating module (Waters Co., MA, USA) with PDA detector and autosampler was used for the analysis, controlled by 'Empower' software. A RP-C_{18} column (25 $\times$ 4.6 mm, 5 μm) was used for the separation. A gradient flow of mobile phase was selected after many trials containing 0.1%, v/v solution of orthophosphoric acid and acetonitrile. The flow rate was 1.0 mL/min; and the chromatograms were recorded at 254 nm.

2.4. Method validation

The proposed method was validated for linearity, accuracy, precision, LOD, LOQ as per ICH guidelines.

2.5. Calibration curves, limits of detection and quantification

The linearity of vitamin C, gallic acid, rutin & ellagic acid was calculated using six concentrations of each compound (500 μg/mL). The calibration graph was drawn using area of the peaks on Y-axis versus corresponding concentration of the drug in X-axis. Further the least square regression equation and correlation coefficient (r^2) were calculated from the graph.

The LOD and LOQ of the method were determined by diluting and injecting the low concentrations of each compound in the calibration curve. The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively.

2.6. Precision and accuracy

The precision test was carried out by the intra-day and inter-day variability for Vitamin C, gallic acid, rutin & ellagic acid. The intra-day assay was determined at three concentrations levels on the same day for 6 times and inter-day assay was calculated by repeating the same procedure 3 consecutive days for 6 times.

The standard addition method was used for determining the accuracy of the method. For this a pre-analyzed quality control samples of all four components at three different known concentration levels to the standard i.e. 50, 100 and 150%. Further the mixture re-analyzed and calculated the recovery in percentage.

2.7. Robustness

Robustness was studied during method development by determining the effects of small variations done in a number of chromatographic parameters such as mobile phase flow and detecting wavelength. The% RSD of the experiment was calculated to assess the robustness of the method.

2.8. Specificity

The specificity of the method was assessed by comparing the chromatograms obtained from standards and from the sample. The chromatograms were examined for any of the impurity peaks which may co-elute with the targeted peaks. No impurity peaks were found co-eluted with the analytes, indicating the method is selective and specific.

3. Results and discussions

3.1. Optimization of chromatographic conditions

Many trials have been conducted to optimize the chromatographic conditions for attaining perfect peak shape and good

resolution between the compounds. The small differences between the compounds in their polarity made things worse. So many kind of solvent combinations were tried. Out of various combination of mobile phase tried, excellent separation was achieved on acetonitrile and 0.1% orthophosphoric (H_3PO_4) acid in gradient elution method starting acetonitrile percentage 10 to 100 in 20 min (Fig. 1).

3.2. Optimization of extraction procedure for sample preparation

The extraction methodology was optimized for maximum extraction efficiency without draining the compound of interest and to avoid chemical modification. Pomegranate molasses is a concentrated fruit extract and very viscous in nature. So the sample preparation plays an important role. Any small variations happens in the quantity/solvent/sorbent/time can have a huge impact on the results. So the right choice of extraction conditions is critical for the MSPD technique.

Out of two different types of sorbent tried (normal silica; 40-60mesh and C18; 200–300 mesh), C18 dispersing material found more attractive due to its good adsorption power, mechanical strength and good recovery of the targeted compounds with less impurities. For the choice of organic solvent to elute the components with minimum impurities different

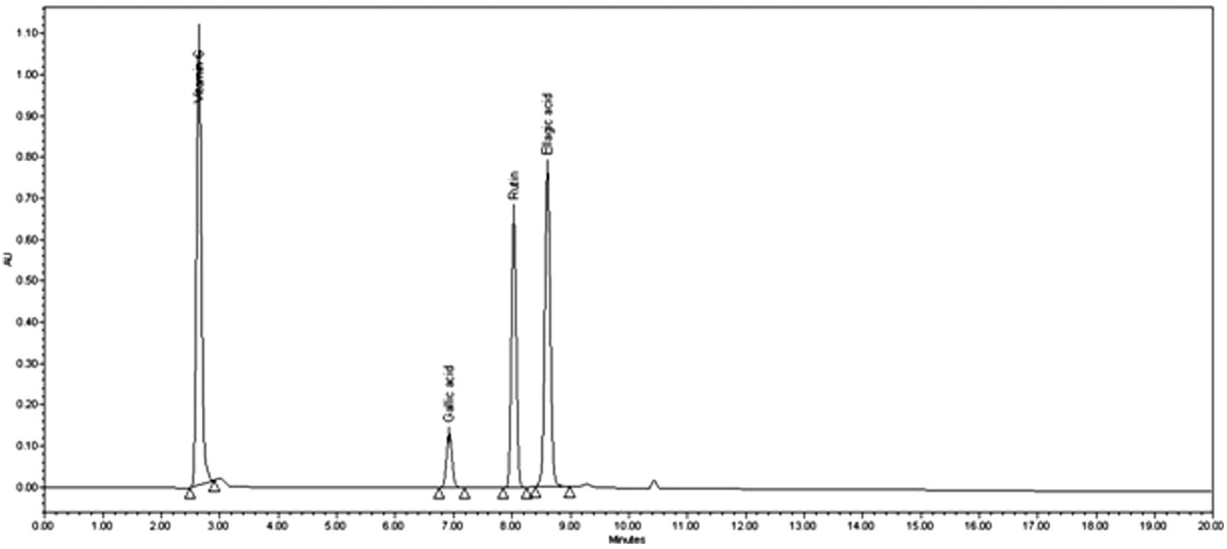

Fig. 1. HPLC chromatogram of four reference standards at 254 nm.

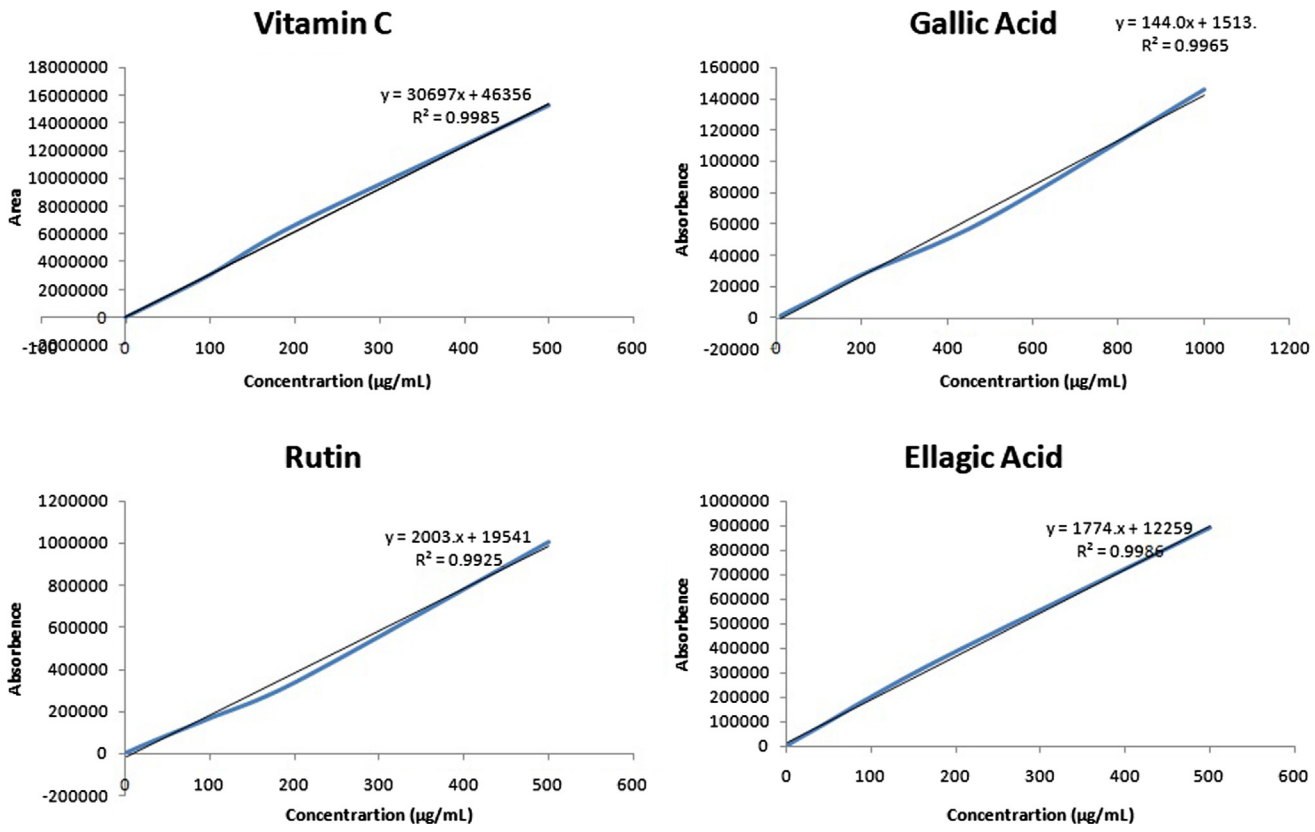

Fig. 2. Calibration graph of Vitamin C, gallic acid, rutin and ellagic acid in different concentration range.

trials have been made with different solvents and compositions.

The sorbent and the sample ratio were also studied for the appropriate concentration. Out different ratios of sample-sorbent (1:1, 1:2: 1:4, 1:5) tested, the appropriate one is found as 1:2.

From many trails made using different proportion of methanol and water, the concentrations of all four components in 70% methanol in water found to be maximum. The column was first eluted using hexane (5 mL × 3), which helped in removing the non polar fractions from the sample. It also helped to bring down the impurity percentage in the chromatograms due to the absence of non polar components.

3.3. Calibration curves, limits of detection and quantification

Calibration curves were linear in relatively wide ranges of concentrations (0.1–500 μg/mL for vitamin C, 10–1000 μg/mL for gallic acid and 1–500 μg/mL for rutin and ellagic acid with high correlation coefficient values ($r^2 \geq 0.9925$) between peak area (y) and amount of each compound (Fig. 2). The LOD & LOQ values along with results of linearity had depicted in Table 1.

3.4. Precision and accuracy

As shown in Table 2, the RSD of intra-day and inter-day variability was less than 2.23%, which demonstrated good reproducibility of the method. Accuracy of the method conducted as recovery found satisfactory as the average recoveries of investigated targets ranged from 97.0% to 104.3%, and R.S.D. values were all <4.5% (Table 3).

3.5. Robustness

Robustness of the proposed method was determined in two different ways, i.e. by changing the mobile phase flow (1 ± 0.2 mL/m in) rate and detecting wavelength (254 ± 4 nm). The% RSD of the experiment was calculated to assess the robustness of the method (Table 4).

3.6. Specificity

The specificity refers the ability of a method to distinguish between the peak of interest and the impurity peaks in the sample. The specificity of the method was determined by injecting a blank of 70% methanol which was previously run through MSPD column, into the HPLC system and the chromatogram was analyzed for any co-eluted peaks. The develop method was found to possess good resolution between the targeted and impurity peaks.

3.7. Application in real samples

The three different molasses samples were extracted using the optimized MSPD technique and analyzed by developed HPLC

Table 1
Linear relationships between peak area and sample concentration.

Compound	Linear range (μg/mL)	Regression equation	Slope ± SD	Intercept ± SD	Regression coefficient	LOD (μg/mL)	LOQ (μg/mL)
Vitamin C	0.10–500	y = 30697x + 46,356	31152 ± 421.2	46814 ± 628.1	0.9985	0.07	0.095
Gallic acid	10–1000	144.0x + 1513.	153.5 ± 8.6	1531 ± 56.9	0.9965	4.50	9.50
Rutin	1–500	y = 2003.x + 19541	2039.2 ± 54.7	19645 ± 170.5	0.9925	0.45	0.85
Ellagic acid	1–500	y = 1774.x + 12259	1790.3 ± 45.7	12434 ± 164.8	0.9986	0.40	0.95

Table 2
Intra and inter-day variations of the method (n = 6).

Compound	Actual retention time	Intra-day precision (%RSD)				Inter-day precision (%RSD)			
		Standard		Sample		Standard		Sample	
		Retention time	Area	Retention time	Area	Retention time	Area	Retention time	Area
Ascorbic acid	2.677	1.12	0.54	2.11	0.44	2.23	0.75	1.50	0.81
Gallic acid	6.987	0.51	1.58	0.21	1.78	0.65	0.70	1.27	0.69
Rutin	8.024	1.24	0.69	1.22	1.87	0.99	1.55	0.58	1.15
Ellagic acid	8.825	0.65	0.74	1.61	0.88	0.28	0.67	2.01	1.64

Table 3
Accuracy of the assay.

Compound	Mean of actual amount (μg/mL)	Mean amount spiked	Mean recovered amount	Mean% recovery	% RSD
Ascorbic acid					
1	76.12	38.06	113.1	99.1	0.27
2	75.81	37.905	115.0	101.1	0.31
3	75.11	37.555	113.6	100.8	1.21
Gallic acid					
1	177.4	87.88	258.2	97.0	2.33
2	176.0	174.2	264.5	100.2	1.01
3	174.9	263.15	265.9	101.4	0.67
Rutin					
1	27.27	17.68	41.2	100.7	0.56
2	26.95	35.87	40.6	100.4	0.33
3	27.82	54.11	42.5	101.8	1.12
Ellagic acid					
1	42.32	28.11	66.2	104.3	1.30
2	42.56	57.77	64.3	100.7	3.93
3	43.01	85.76	65.1	100.9	4.44

Table 4
Robustness of the method by changing flow rate of the mobile phase and detection wavelength (n = 6).

		Vitamin C		Gallic acid		Rutin		Ellagic acid	
		Retention time (RSD)%	Peak area RSD (%)	Retention time (RSD)%	Peak area RSD (%)	Retention time (RSD)%	Peak area RSD (%)	Retention time (RSD)%	Peak area RSD (%)
Flow rate (mL/min)	0.8	0.88	0.44	0.28	0.38	0.78	0.36	0.58	0.88
	1	1.02	0.53	0.87	0.58	1.25	2.11	0.40	0.53
	1.2	0.45	1.58	0.66	1.55	1.23	0.52	0.87	0.74
Detection wavelength (nm)	250	0.84	1.46	0.98	0.84	0.84	0.78	0.96	0.64
	254	0.71	1.28	1.11	0.57	0.56	0.56	0.28	0.66
	258	1.58	0.35	0.58	0.71	0.38	0.44	1.62	1.84

method. One of the main concern regarding the extraction and analysis was the high content of sugar in the products. But the optimized MSPD extraction technique was found efficient to overcome this problem. The content of vitamin C, gallic acid, rutin and ellagic acid found in the samples were depicted in Table 5. The MSPD extraction and clean up method found helps in removing impurity peaks in the chromatographic run. Moreover the use of suitable elution solvent has brought down the impurity percentage in the chromatograms, comparing the same with chromatogram obtained from liquid-liquid extraction, which leads to accurate and reproducible analysis (Fig. 3).

Recent days, due to the awareness on the health benefits of pomegranate and its various products, an increase in demand was found worldwide. By considering this fact in mind a highly efficient, less complicated and reproducible extraction technique and a simple, accurate and rapid HPLC method for the simultaneous determination of four components (vitamin C, gallic acid, rutin and ellagic acid). This was the first report on the simultaneous determination these 4 markers in a pomegranate molasses samples. The main advantage of the method includes the simplicity of extraction procedure, simultaneous detection and quantification of all four bioactive components in a single chromatographic run.

4. Conclusion

The MSPD extraction methodology and HPLC method successfully employed for the quantification of vitamin C, gallic acid, rutin and gallic acid in pomegranate molasses samples. Identification of the peaks in the sample chromatograms were carried out by comparing retention time of each component. From the calibration curve the quantity of compounds present in each sample were calculated. The HPLC method was efficient enough to separate all four marker components in various ratios.

In conclusion, the developed MSPD coupled HPLC method is useful as a trustworthy, rapid and cost effective tool can be used for the quality control of various formulations and beverages and for the standardization of different formulations containing pomegranate. This simple multi-component assay method will be helpful in quality control of a large no of formulations and can also be extended for the pharmacological, biopharmaceutical and pharmacokinetic studies.

Table 5
Amount of four active compounds found in the pomegranate molasses samples.

Mean contents (% w/w) ± SD (n = 3)				
	Ascorbic acid	Gallic acid	Rutin	Ellagic acid
Sample 1	0.154 ± 0.002	0.354 ± 0.013	0.054 ± 0.003	0.084 ± 0.0039
Sample 2	0.250 ± 0.002	0.054 ± 0.011	0.027 ± 0.001	0.139 ± 0.0033
Sample 3	0.214 ± 0.002	0.611 ± 0.014	0.012 ± 0.0015	0.058 ± 0.0031

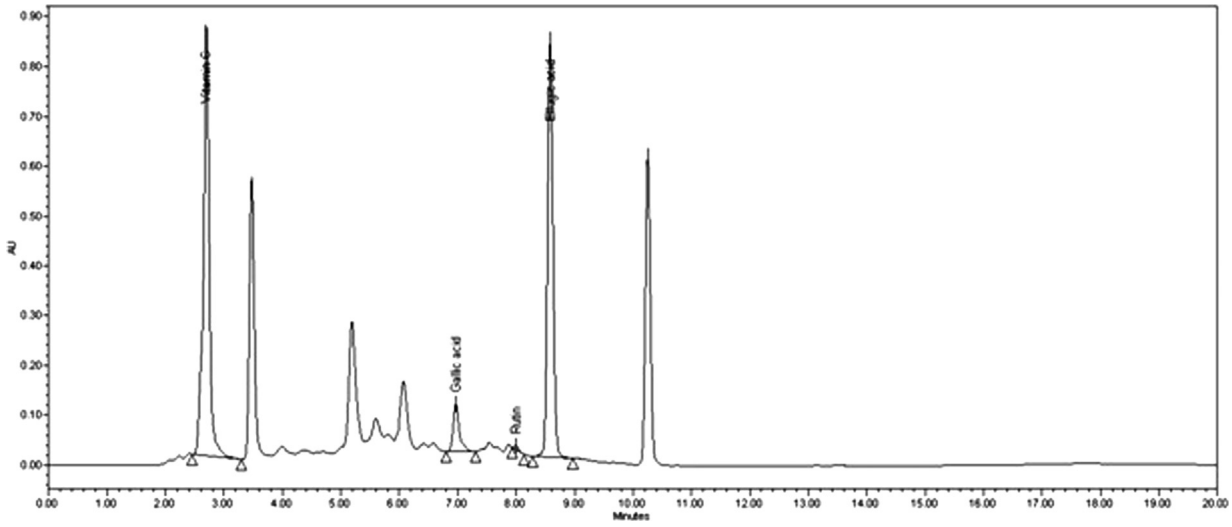

Fig. 3. HPLC chromatogram of pomegranate molasses sample at 254 nm.

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