

# Quantitative and Optimization of Phenolic Acid Extracted from Pomegranate by High Performance Liquid Chromatography (HPLC)

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## ABSTRACT

**Objective:** Pomegranate is scientifically known as *Punica granatum* L. which is a nutrient dense fruit rich in phytochemical compounds. Phenolic content is the main compound attribute for the most of the functional properties in pomegranate. The aim of this study is to quantify and optimize the composition of phenolic acids extracted from pomegranate extract by using High-performance Liquid Chromatography (HPLC). **Method:** The pomegranate extracted with three different methods by using two different solvents which is 50% ethanol and water. The methods were blended (aril+ seed), Soaking (aril+ seed) and soaking + squeezed manually. HPLC-PDA was used as equipment to quantify and optimize the phenolic acids extracted from pomegranate. **Result:** Validation method of HPLC was analysed according to the percentage of recovery, LOD, LOQ and coefficient correlation. Result showed that GA was detected in all sample from different method of extraction applied while CA compound not detected in any of extraction method applied. FA compound was only detected in blended method by 50% ethanol and water as solvent while EA compound was detected only in water extraction of all three methods applied. **Conclusion:** As a conclusion, according to the standard calibration data curve showed that this method proved to detect and quantify the targeted compounds. By comparing the data obtained from this study, it showed that water blended extract method is significantly higher content of targeted compound except for the CA compound. To the best of our knowledge, this sample can be a valuable source of antioxidant for better used in health benefits.

**Key words:** Pomegranate extract, HPLC, Gallic acid, Ferulic acid, Ellagic acid, Caffeic acid.

## INTRODUCTION

Pomegranate is scientifically known as *Punica granatum* L. which is a nutrient dense fruit rich in phytochemical compounds.<sup>1</sup> The fruit can be divided into (i) the seeds which constitute ~3% of the fruit weight, (ii) the juice which is roughly 30% and finally (iii) the peel which includes the interior network of membranes present inside the fruit.<sup>2</sup> According to the Sreekumar *et al.*<sup>3</sup> pomegranate divided into two part which is edible and non-edible part; edible part of the pomegranate fruit (50%) consists of 40% arils and 10% seeds. The Arils of pomegranate contain 85% water, 10% total sugars, mainly fructose and glucose, and 1.5% pectin, organic acid, such as ascorbic acid, citric acid, and malic acid, and bioactive compounds such as phenolic and flavonoids. According to Rahmani *et al.*<sup>4</sup> pomegranate peel and juice are rich source of tannins, flavonoids, phenolic compound, anthocyanin and punicalagin.

Phenolic content is the main compound attribute for the most of the functional properties of many fruits<sup>5</sup> and model study demonstrated the antioxidant activities

of the phenolic fruits.<sup>6</sup> According to Karakaplan and Özcan<sup>7</sup> pomegranate is an important source of bioactive compounds which are primarily phenolic or polyphenolic, flavonoids (flavonols, flavone flavanones, catechins and isoflavones) and related compounds (phenolic acids, chalcones and isoflavones). Phenolic acid mostly found in three types of derivative which is benzoic acid derivative, hydroxycinnamic acid derivative and depsides.<sup>8</sup>

The objective of this study is to quantify and optimize the composition of phenolic acids extracted from pomegranate extract by using High-performance liquid chromatography (HPLC). The analytical separation and determination of phenolic compounds were performed using reversed-phase HPLC with photodiode array detector.

## MATERIALS AND METHODS

### Materials

Trans-Ferulic Acid from sigma, Co. Chemical, St Louis (Switzerland), Caffeic Acid from sigma, Co.

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Chemical, St Louis (China), Ellagic Acid from Sigma, Co. Chemical, St Louis (United Kingdom), Gallic Acid from Sigma, Co. Chemical, St Louis (United Kingdom).

### Sample collection

Fresh Pomegranate fruits (*Punica granatum* L.) were purchased from Pasar Payang, Kuala Terengganu, Terengganu Darul Iman, Malaysia. The samples were submitted to Faculty of Bio-resources and Food Industry (FBIM), Tembila Campus, Universiti Sultan Zainal Abidin (UniSZA) for botanical features. After that, the fruits were washed and stored at 4°C until required for extraction.

### Pomegranate Juice Extraction Procedures

Method for the extraction of juice was applied Norhaslinda *et al.*<sup>9</sup> with slight modification. Each pomegranate was washed in cold Water and drained. Approximately two whole of pomegranate fruits were cut into two halves, to obtain aril juice and whole pomegranate juice from the same fruits. The arils were separated manually, and the inner white parts of the fruit were removed. All the arils of pomegranate were extracted with 50% of ethanol and water by using four different methods of extraction.

### Identification Phenolic Acid by HPLC-DAD Analysis

Specific method development was used to detect the phenolic acid compounds in pomegranate juice by using HPLC-PDA. 10mg of each extract sample was diluted in 1mL methanol and analysed by using Shimadzu HPLC (High-Performance Liquid Chromatography) System (Shimadzu, Japan). Separation was performed on reverse phase Hypersil Gold C-18 Column (250x4.6mm) (Thermo Fisher Scientific, USA).

The Column was maintained at 30°C. Before use, solvent was filtered over a 0.45 µm membrane filter. The mobile phase consists of two type of eluent which is A and B. Eluent (A) composed of 100% methanol while eluent (B) composed of water and Acetonitrile (50:50, v/v) at flow rate 0.8mL/min. The elution program of the solvent (B) were used as follow: 0-5 min, 80%; 7-10, 95%. The chromatogram monitored at 229nm – 310nm, with spectra taken continuously throughout the elution.

The UV spectra of the different compounds were recorded with a Diode Array Detector (DAD). The calculation of concentration was based on the standard external method. The external dilution standard was started at 10ppm, 15ppm, 20ppm, 25ppm, 30ppm and 35ppm of each phenolic acid standards (Caffeic acid, Ellagic acid, Gallic acid, and Ferulic acid) to fit a standard curve (peak area versus concentration in mg/L) with linear regression for each compound.

### Statistical Analysis

The SPSS version 22.0 was used for statistical analysis. The results were expressed as means ± SEM using oneway ANOVA followed by Dunnett's test for multiple comparisons and Kruskal-Wallis test followed by Man-Whitne test for multiple comparisons.

## RESULTS AND DISCUSSION

The method for determination of phenolic acid by using High-Performance Liquid Chromatography- photodiode array detector (HPLC-PDA) was developed and able to detect the targeted compounds based on the calibration of the external standard at optimum isocratic HPLC condition.<sup>10</sup> Reverse Phase HPLC methods reported as a simple, accurate and rapid technique for determination of phenolic acid in the sample.

### Validation Method of Phenolic Acid by using HPLC analysis

The effectiveness of the HPLC method was tested with the standard compound solution of Caffeic Acid (CA), Ellagic Acid (EA), Ferulic Acid (FA) and Gallic Acid (GA). External standard for each compound were detected at different concentration with the range 5 – 30 µg/ml. Table 1 show that each standard compound were detected at different wavelength 310nm, 253nm, 326nm and 229nm for CA, EA, FA and GA, respectively. The standard compound also been detected at slightly different retention time which is 7.366 min, 7.443 min, 7.520 min and 7.211 min for CA, EA, FA and GA, respectively.

Limits of detection (LOD) and of quantification (LOQ) are the most important values that researchers look for when considering method validity.<sup>11</sup> Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated according to Seal (2016). In this study, the recovery percentage was calculated as follow as Recovery (%) = (Concentration found – Concentration Applied) divided with 100% and result showed that recovery percentage (%) for standard compounds is more than 99%. According to Seal<sup>8</sup> the high recovery rate of sample to indicate the efficacy and consistency is 96 - 103%. The calibration curve obtained by plotting peak area versus the concentration of each standards solution and the square of the correlation coefficient  $R^2 > 0.99$  is indicative of the measure of linearity.

### Phenolic Acid of Pomegranate Extracts Quantification

Flesh of pomegranate was used in quantification of phenolic acid compounds because it is an edible part of fruit which is rich in the range bioactive compound. As mention by Anahita *et al.*<sup>5</sup> combination of seed and juice of pomegranate have higher antioxidant compare to other fruit part. Thus, three different methods applied in extraction of pomegranate; in order to determine which method, give the higher content of CA, EA, FA and GA compound. There was two solvent extraction used which is 50% ethanol and water. Both solvents were categorized as a low toxicity solvent for extraction. Each sample was analysed based on the optimized extraction procedure which described in the method. Identification of the peaks for sample carried out by comparing the retention time and wavelength of each component. The content of each compound was analysed according to the calibration curve.

In this work, the study was conducted to quantify the CA, EA, FA and GA compound of phenolic acid. The HPLC method of 50% etha-

**Table 1: The parameter calibration curve of external standard compound by using HPLC.**

Name of the Standard	Range (µg/ml)	Detected at wavelength (nm)	Retention Time (min)	Standard Error for Intercept	Regression Coefficient, $r^2$	LOD (µg/ml)	LOQ (µg/ml)	Percentage of Recovery (%)
Caffeic Acid	5 – 30	310	7.366	0.0030	0.9988	0.0277	0.0840	99.991 ± 0.025
Ellagic Acid	5 – 30	253	7.443	0.0027	0.9988	0.0128	0.0389	100.002 ± 0.011
Ferulic Acid	5 – 30	326	7.520	0.0042	0.9999	0.0157	0.0476	99.997 ± 0.010
Gallic Acid	5 – 30	229	7.211	0.0030	0.9967	0.1287	0.3899	99.993 ± 0.153

\*Standard concentration for precision = 20µg/mL.

**Table 2: Quantification of phenolic acid in pomegranate extracts (mg/100 g e.p).**

Phenolic acid Standard	Method					
	Blended		Soaking		Soaking + Squeeze	
	50% ethanol	water	50% ethanol	water	50% ethanol	water
Caffeic Acid (mg)	ND	ND	ND	ND	ND	ND
Ellagic acid (mg)	ND	0.85±0.06	ND	0.46±0.03	ND	0.66±0.01
Ferulic acid (mg)	0.47±0.03	0.95±0.04	ND	ND	ND	ND
Gallic Acid (mg)	42.90±4.06	57.93±2.66	23.75±3.26	23.96±1.72	32.07±0.28	41.20±3.75

\*ND= Not Detectable.

Each value in the table was obtained by calculating the average of triplicate data and data were presented as Mean ± SEM.

Statistical analysis was carried out using a One-Way ANOVA.

Different letters in the same column means a significant difference ( $p \leq 0.05$ )

nol and water extract of Pomegranate showed the presence of GA in all three different methods applied while FA compound was only detected in blended method by 50% ethanol and water as solvent (Table 2). EA compound was detected only in water extraction of all three method applied. Unfortunately, CA compound not detectable in any of method applied or solvent used for extraction. Study done by Karakaplan and Özcan<sup>7</sup> mentioned that CA in pomegranate extract is higher compare to other compound which is about 422.8mg/L.

There are a few factors which can affect the undetectable of CA compound in pomegranate extract. According to Nursyukriah *et al.*<sup>12</sup> the solvent used for extraction will influence the quantity and quality of the targeted bioactive compound. Dent *et al.*<sup>13</sup> mention that several factor influence the content of the compound such as solvent composition, time of extraction, temperature, pH, solvent ratio and solvent polarities. All those factors might be a reason CA compound undetectable.

As result showed in Table 2, High content of EA, FA and GA was detected in water blended method compare to the other method applied which amount of  $0.85 \pm 0.06$  mg/100 g e.p,  $0.95 \pm 0.04$  mg/100 g and  $57.93 \pm 2.66$  mg/100 g, respectively. Water is a polar solvent which potentially to extract most of compound while according to Dent *et al.*<sup>13</sup> aqueous are possibly the most suitable solvent systems for the extraction of polyphenols due to the different polarities of the bioactive constituents.

## CONCLUSION

As a conclusion, according to the standard calibration data curve showed that this method proved to detect and quantify CA, EA, FA and GA compound in pomegranate extract by using specific method development of HPLC-PDA. By comparing the data obtained from this study, it showed that water blended extract method is significantly higher content of targeted compound except for the CA compound. To the best of our knowledge, this sample can be a valuable source of antioxidant for better used in health benefits.

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## CONFLICT OF INTEREST

No conflict of interests exists.

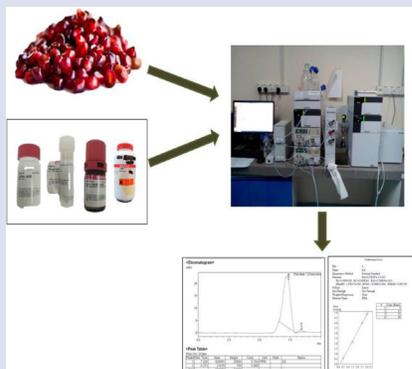
## ABBREVIATIONS

**HPLC:** High-Performance Liquid Chromatography; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **PDA:** Photodiode array; **CA:** Caffeic Acid; **EA:** Ellagic Acid; **FA:** Ferulic Acid; **GA:** Gallic Acid; **SPSS:** Statistical Package for the Social Sciences; **SEM:** Standard Error Mean; **%:** Percentage; **ug/ml:** microgram per milliliter; **mg/ml:** milligram/milliliter; **°C:** Degree Celcius; **mg/100 g e.p:** milligram per 100 gram edible portion.

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### GRAPHICAL ABSTRACT



### SUMMARY

- All samples using different extraction method and solvent in identifying phenolic acid compounds of pomegranate.
- Selected standard phenolic acids were optimized and quantify using developed method of High-performance Liquid Chromatography (HPLC).
- Calibration Curve of optimization method shows linear and accurate result as validation in determined standards compound.
- Comparatively the composition of each phenolic acid detected in water extraction of blended method is significantly higher in value of selected compound than the other method applied.

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