

# Classification of pomegranate cultivars by multivariate analysis of biochemical constituents of HPLC

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## Classification of pomegranate cultivars by multivariate analysis of biochemical constituents of HPLC

**Abstract:** Pomegranate fruits are highly diverse and may be divided into geographical groupings based on their characteristics. Genetic research has verified these categories in recent years and further categorized variants into geographic-genetic groupings. This study aimed to assess the biochemical contents of eight varieties of pomegranate fruit seed and the categorization of pomegranate using multivariate statistical analysis. Polyphenolic chemicals are key secondary metabolites in pomegranate, and their presence influences the quality and sensory qualities of the fruit they produce. Fruit extracts from the Faqyan cultivar contained the highest total phenolic content of all studied cultivars. Pomegranate cultivars such as Shaqlawa, Halabja Sour, and Faqyan were shown to have the highest antioxidant activity. Gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, cinnamic acid, rutin, apigenin, rosmarinic acid, and quercetin were the most abundant phytochemical components in the study. According to the results of multivariate analysis, pomegranate cultivars were divided into four major groups. The pomegranate fruit seed is the most abundant source of antioxidants and beneficial phytochemical elements. Finally, the Sidakan Sweet and Shaqlawa cultivars included a significant content polyphenolic compounds.

**Key words:** antioxidant; gallic acid; chlorogenic acid; *Punica granatum*, phenolic compounds

## Razvrščanje sort granatnega jabolka z multivariatno analizo biokemičnih sestavin izmerjenih s HPLC

**Izvleček:** Plodovi granatnega jabolka so zelo raznoliki in jih lahko razdelimo v geografske skupine na osnovi njihovih lastnosti. Genetske raziskave so v zadnjih letih potrdile te skupine in jih nadalje razčlenile v geografsko genetske podskupine. Namen te raziskave je bil ugotoviti biokemično sestavo plodov osmih sort granatnega jabolka z multivariatno analizo. Polifenoli so glavna sestavina sekundarnih metabolitov v granatnem jabolku, njihova prisotnost vpliva na kakovost in senzorične lastnosti plodov. Izvlečki plodov iz sorte Faqyan so vsebovali največ polifenolov izmed vseh preučevanih sort. Sorte granatnega jabolka kot so Shaqlawa, Halabja Sour in Faqyan so se izkazale z največjo antioksidacijsko aktivnostjo. Galna, kavna, klorogenska, *p*-kumarna, cimetna in rožmarinska kislina, rutin, apigenin, in kvercetin so bile najbolj pogoste fitokemične komponente v tej raziskavi. Na osnovi multivariatne analize so bile sorte granatnega jabolka razdeljene v štiri glavne skupine. Sočen ovoj semen granatnega jabolka je najbolj pogost vir antioksidantov in drugih ugodnih sestavin. Sorti Sidakan Sweet in Shaqlawa sta se izkazali z značilno veliko vsebnostjo polifenolov.

**Ključne besede:** antioksidanti; galna kislina; klorogenska kislina; *Punica granatum*; fenolne spojine

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## 1 INTRODUCTION

The pomegranate (*Punica granatum* L.) which is originally described throughout Mediterranean region is a fruit crop that belongs to the Punicaceae family and has gained popularity in recent years due to its multi-functionality and high nutritional value when consumed as part of a healthy diet. In present it is grown worldwide in a variety of geographical regions, meeting the nutritional and medicinal requirements of people in a variety of countries. (Holland et al., 2009). Countries such as Iran, India, Egypt, China, Israel, Tunisia, Syria, Lebanon, Turkey; Greece; Cyprus; Italy; France; Spain; Chile; Portugal; the United States; Oman; and most recently, South Africa are growing in commercial pomegranate orchards a variety of pomegranate cultivars (Caleb et al., 2012; Holland et al., 2009). Among the many nutrients found in the edible part of the fruit are high concentrations of acids, sugars, polyphenolics, and essential minerals (Al-Maiman & Ahmad, 2002). The fruit is most commonly consumed as fresh arils or processed products, primarily juice. The color of the pomegranate fruit's outer peel does not indicate how ripe it is or whether if it is ready for consumption (Holland et al., 2009). Fruit quality assessment and classification are frequently based on factors such as the color of the aril, the total soluble solids content, and the presence of organic acids (Cristosto et al., 2000; Martinez et al., 2006). The extraordinarily high antioxidant capacity of pomegranates, directly correlated with the high amount and unique composition of phenolic compounds in the fruit, is credited with the fruit's favorable health properties. (Borochov-Neori et al., 2011; Fischer et al., 2011; Gil et al., 2000).

Pomegranate seeds, which are a byproduct of pomegranate juice manufacturing, include a variety of nutraceutical components, including sterols, alpha-tocopherol, punical acid, and hydroxybenzoic acids, among other substances (Kwan & Kowalski, 1978), suggesting that the extracts of pomegranate seed residue might be used as a nutraceutical resource, according to the research (Revilla & González-SanJosé, 2002) On the other hand, according to some researchers Pomegranate aril and juice contain a significant amount of polyphenolics, primarily composed of ellagitannins (punicalagin), gallic acid, ellagic acid, anthocyanins, catechins, caffeic acid, and quercetin derivatives. (Viuda-Martos et al., 2010). The amounts of these substances are dependent on the cultivars, the environment conditions, of the growing location where pomegranate orchards are established (Melgarejo et al., 2000). Many different pomegranate cultivars from Iran, Turkey, the United States, Italy, and South Africa have been investigated thus far for their polyphenolic content in their juice. However, prior to this study, no profile

comparison of polyphenol composition and antioxidant capacity in sweet and sour pomegranates from different regions of Iraq's Kurdistan region was conducted.

Kurdistan is a regional supplier of pomegranate, and it has more favorable growing circumstances than any other region where the fruit crop is grown.

Pomegranates grown in the neighborhood are diverse and have been adapted to the many natural states of Kurdistan's environment. As a result, the loss of genetic diversity in crop species as a result of commercialization has prompted the need to safeguard the genetic resources that are currently available. Unfortunately, there is currently no information available on the diversity in chemical composition of pomegranate fruit in Kurdistan, aside from a recent article by Mohammad et al. (2018) that looked at the physico-chemical properties of selected fruit cultivars at commercial harvest. In this study, the goal was to develop; using chemical analyses, as a classification model that would allow to classify Kurdistan pomegranates according to cultivars without regard to the effects of climate or geographical origin, and that would be based on the phenolic composition of the seed arils of pomegranates.

## 2 MATERIAL AND METHODS

### 2.1 POMEGRANATE SAMPLES

Fruits of selected cultivars of pomegranate (*Punica granatum* L.) (4 kg) were manually collected at commercial maturity from commercial Kurdistan region vineyards in the eight geographical regions (see Table 1). Samples were transported on ice and stored at -20 °C until required and destemmed while frozen.

### 2.2 POLYPHENOLICS

#### 2.2.1 Extraction and analysis of polyphenolics:

In order to extract polyphenolics, 2 g of the powdered sample was removed and after adding 4 ml of methanol solvent containing 1 % acetic acid, the extraction process was performed under ultrasonic waves for 20 minutes. The phenolic acids studied in this study were isolated, identified, and quantified applying HPLC (high-performance liquid chromatography) device model 1100 series (Agilent USA), prepared with an injection loop of 20 microliters, four solvent gradient pump, degassing system, Column oven (set at 25 °C), and diode array detector, set at 250, 272 and 310 nm, respectively. Isolation on the Ceylon octadecyl column (inner diameter 4.6 mm,

**Table 1:** Sampling locations of the different pomegranate cultivars studied

| Cultivar        | province     | Height (m) | Latitude | Longitude |
|-----------------|--------------|------------|----------|-----------|
| 'Sidakan Sweet' | Erbil        | 1502.0     | 36.5579  | 44.8283   |
| 'Sidakan Sour'  | Erbil        | 777.0      | 36.6085  | 44.5239   |
| 'Shaqlawā'      | Erbil        | 975.0      | 36.7990  | 44.6704   |
| 'Balakayati'    | Erbil        | 1145.0     | 36.4098  | 44.3201   |
| 'Halab Sweet'   | Sulaymaniyah | 928.0      | 36.2995  | 44.4136   |
| 'Halab Sour'    | Sulaymaniyah | 927.0      | 36.4098  | 44.3202   |
| 'Faqyan'        | Erbil        | 870.0      | 36.54    | 44.539    |
| 'Hiran'         | Erbil        | 650.0      | 36.283   | 44.496    |

length 25 cm, a particle size of 5 micrometers ZORBAX Eclipse XDB). In order to process the data, Chemstation software was applied.

### 2.3 TOTAL ANTIOXIDANT

Total antioxidant was measured according to the method of Brand Williams et al. (1995) (Brand-Williams et al., 1995) by DPPH standard.

### 2.4 TOTAL PHENOLIC CONTENT

Following the colorimetric oxidation/reduction reaction of phenolics, the total phenolics content was measured using the Folin Ciocalteu technique described by Singleton et al. (1999), with minor modifications. Polyphenolics were extracted by adding 10 ml 85 percent methanol to 1 g fine powder of samples and mixing thoroughly. After that, by the addition of 2.5 ml of Folin-Ciocalteu reagent and 2 ml of 7.5 % sodium carbonate. For 1.5 to 2 hours, the samples were shaken vigorously. A spectrophotometer was used to measure the absorbance of the samples at 765 nm (PG Instruments T80 UV, UK). The calibration curve was created using gallic acid. The results were presented in milligrams of GAE per 100 grams of fresh mass (FM).

### 2.5 STATISTICAL ANALYSIS

SAS 9.2 software was used to analyze all of the data (SAS, 2009). A one-way analysis of variance (ANOVA) was performed, and significant differences between groups were determined using Tukey's multiple range tests at a  $p \leq 0.05$ . In addition, distinct genotypes were classified based on the presence of phytochemical substances. In order to analyze the variables, principal com-

ponent analysis (PCA) and hierarchical cluster analysis (HCA) were carried out with the help of the Excel spreadsheet program XLSTAT (2018).

## 3 RESULT AND DISCUSSION

### 3.1 HPLC ANALYSIS OF THE SAMPLES

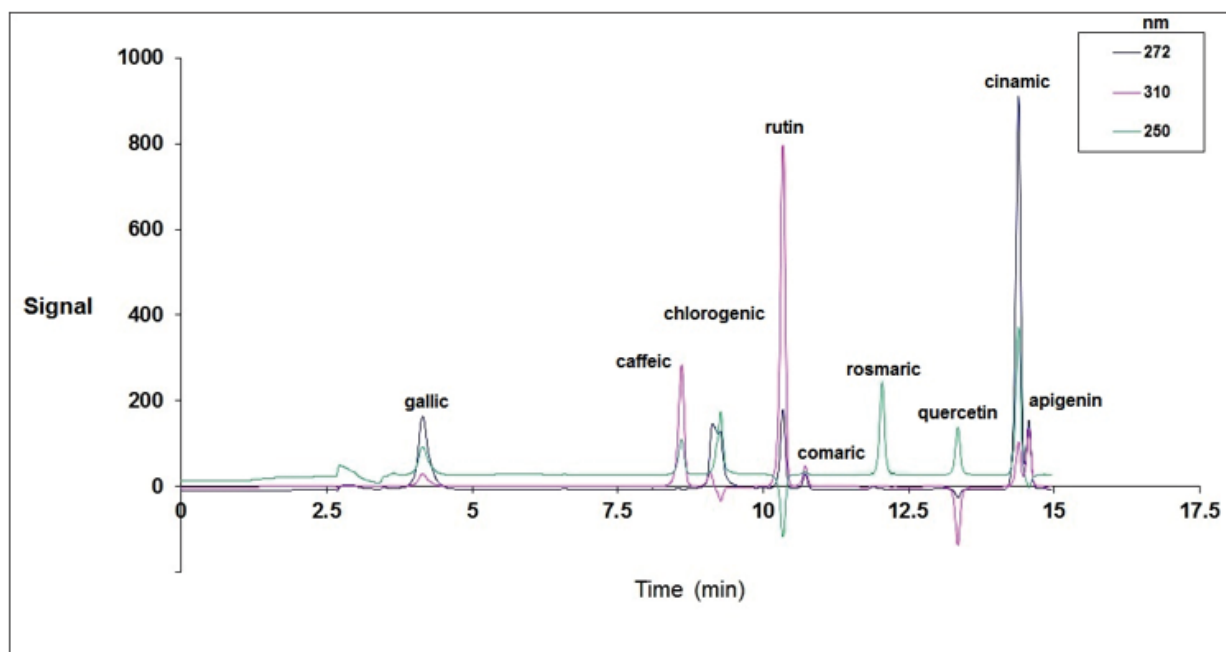
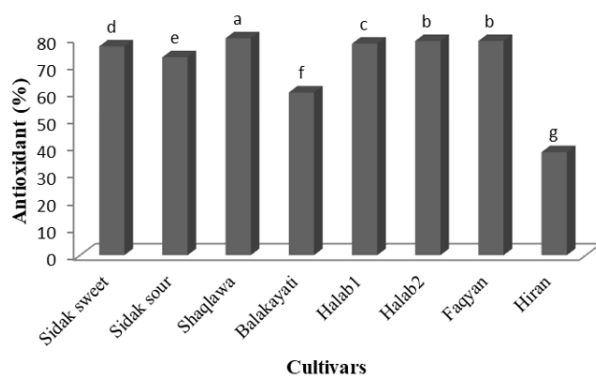
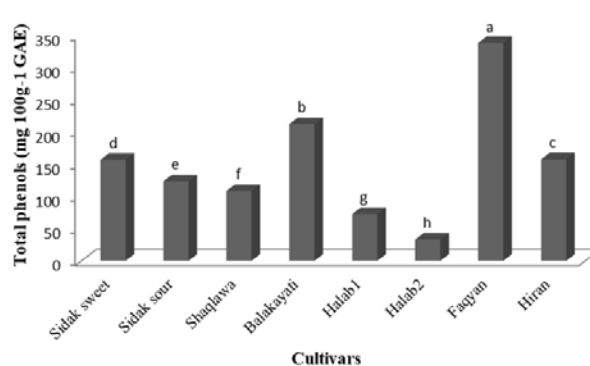
Figure 1 demonstrates the chromatogram of nine standards that were injected into an HPLC system. Between the eight cultivars studied in this study, the amounts of specific phenolic acid acids (gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, cinnamic acid, and rosmarinic acid), as well as flavonoids (rutin, apigenin, and quercetin), were found to be considerably different (Table 2). Gallic acid, chlorogenic acid, and coumaric acid were found as the most abundant phenolic compounds in the extracts of grape fruits. The high concentrations of gallic acid ( $68.6 \text{ mg kg}^{-1}$ ), caffeic acid ( $19.2 \text{ mg kg}^{-1}$ ), chlorogenic acid ( $202.2 \text{ mg kg}^{-1}$ ), *p*-coumaric acid ( $74.25 \text{ mg kg}^{-1}$ ), cinnamic acid ( $3.3 \text{ mg kg}^{-1}$ ), rutin ( $6.12 \text{ mg kg}^{-1}$ ), apigenin ( $11.26 \text{ mg kg}^{-1}$ ), rosmarinic acid ( $2.51 \text{ mg kg}^{-1}$ ) and quercetin ( $10.17 \text{ mg kg}^{-1}$ ) were obtained in 'Sidak Sweet', 'Hiran', 'Shaqlawā', and 'Balakayati' pomegranate seed fruit extracts, respectively. The highest concentrations of gallic acid, caffeic acid, rutin, and apigenin were observed in Sidak Sweet cultivar fruit extracts and *p*-coumaric acid, rosmarinic acid, and cinnamic acid concentration in 'Shaqlawā' fruit seed aril grown in Erbil province.

### 3.2 ANTIOXIDANT ACTIVITY

According to Figure 2, the antioxidant activity was influenced by both the cultivar and the samples' location. According to the results, the highest antioxidant activity was achieved from 'Shaqlawā' fruit extract (80 %), and

**Table 2:** Content of biochemical compounds in fruits of different pomegranate cultivars

| Cultivars   | Gallic acid (mg kg <sup>-1</sup> ) | Caffeic acid (mg kg <sup>-1</sup> ) | Chlorogenic acid (mg kg <sup>-1</sup> ) | Rutin (mg kg <sup>-1</sup> ) | p-Coumaric acid (mg kg <sup>-1</sup> ) | Rosmaric acid (mg kg <sup>-1</sup> ) | Quercetin (mg kg <sup>-1</sup> ) | Cinnamic acid (mg kg <sup>-1</sup> ) | Apigenin (mg kg <sup>-1</sup> ) |
|-------------|------------------------------------|-------------------------------------|-----------------------------------------|------------------------------|----------------------------------------|--------------------------------------|----------------------------------|--------------------------------------|---------------------------------|
| Sidak Sweet | 68.6a                              | 19.2a                               | 95.7c                                   | 6.12a                        | 28.08c                                 | 0.78f                                | 3.29e                            | 0.57d                                | 11.26a                          |
| Sidak Sour  | 39.45b                             | 10.77b                              | 56.88e                                  | 0.72d                        | 10.85e                                 | 0.36g                                | 1.77g                            | 0.31g                                | 2.9f                            |
| Shaqlawā    | 28.5c                              | 2.91e                               | 136.5b                                  | 0.6f                         | 74.25a                                 | 2.51a                                | 9.99b                            | 3.3a                                 | 7.83b                           |
| Balakayati  | 28.35d                             | 1.92f                               | 82.26d                                  | 3.24b                        | 28.15b                                 | 1.06d                                | 10.17a                           | 0.86c                                | 6.02d                           |
| Halab Sweet | 13.32e                             | 0.26h                               | 18.51f                                  | 0.1g                         | 6.71g                                  | 1.35b                                | 1.28h                            | 0.54e                                | 2.48g                           |
| Halab Sour  | 13.23f                             | 0.55g                               | 19.97g                                  | 0.06h                        | 4.47h                                  | 1.17c                                | 2.81f                            | 0.47f                                | 3.79e                           |
| Faqyan      | 11.75g                             | 4.66c                               | 11.96h                                  | 0.67e                        | 10.15f                                 | 0.3h                                 | 4.64c                            | 1.1b                                 | 6.89c                           |
| Hiran       | 11.31h                             | 3.73d                               | 202.25a                                 | 0.93c                        | 11.69d                                 | 1e                                   | 3.68d                            | 0.31g                                | 1.49h                           |

**Figure 1:** HPLC chromatograms of nine biochemical standards**Figure 2:** Antioxidant activities of different cultivars of pomegranate seeds by DPPH assay**Figure 3:** Total Phenolic content (TPC) of different cultivars of pomegranate seed

the lowest antioxidant activity was found in 'Hiran' fruit extract (38 %).

### 3.3 TOTAL PHENOLIC CONTENT (TPC)

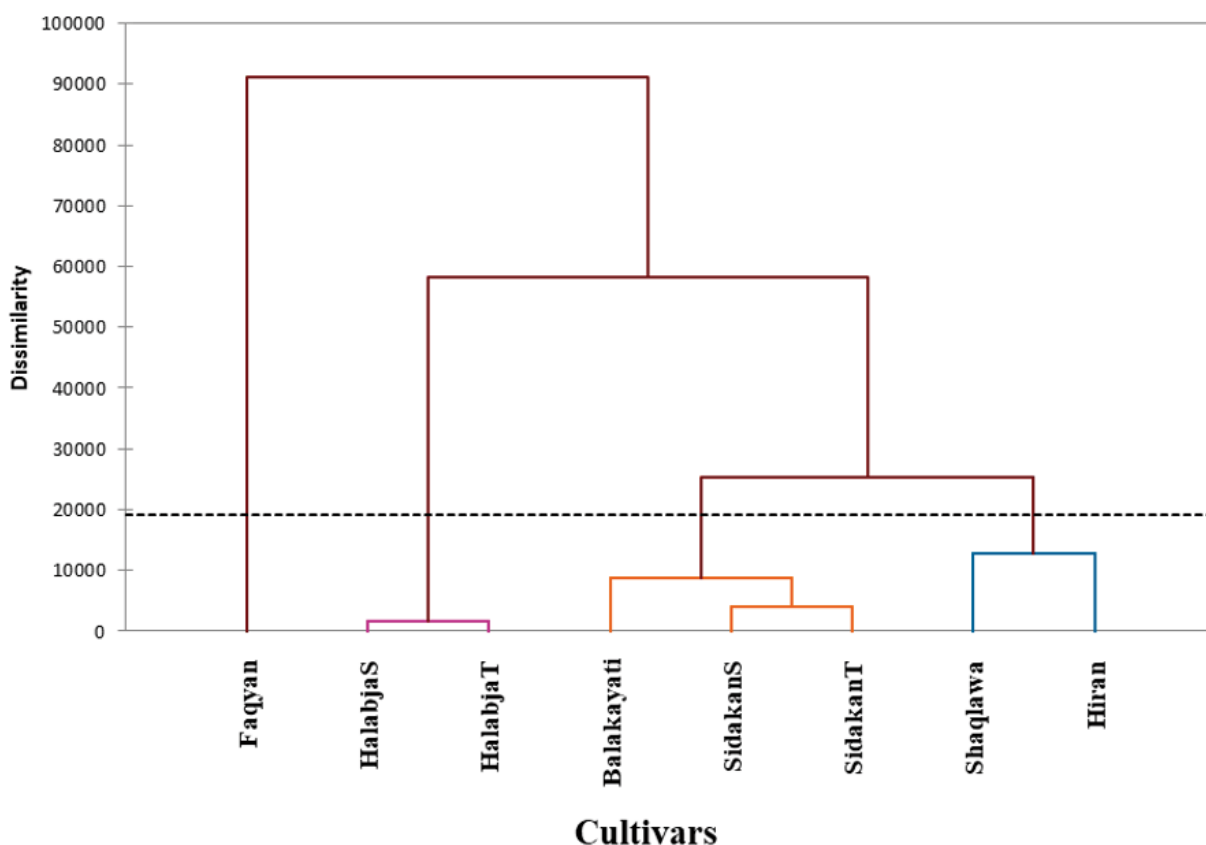
The TPC values of fruit extracts of pomegranate seed cultivars are presented in Figure 3. The amount of TPC in the fruits extracts obtained varied from 33 mg GAE 100 ml<sup>-1</sup> extract in Halabja sour cultivar to 338.2 mg GAE 100 ml<sup>-1</sup> extract in Faqyan cultivar. Results showed that TPC of extracts was influenced significantly by sampling location.

### 3.4 CLASSIFICATION OF POMEGRANATE CULTIVARS

HCA, PCA and were performed to classify the pomegranate cultivar regarding the 11 main traits (TPC, DPPH, gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, cinnamic acid, rosmarinic acid, rutin, apigenin, and quercetin). The Ward linkage method carried out

the cluster analysis (Figure 4). Based on this analysis, the pomegranate cultivars were classified into four main clusters group. In the first cluster, cultivars of 'Faqyan' were designated as the cultivars with the highest TPC. In the second cluster, a cultivar of 'Halabja' sweet and 'Halabja' sour were determined due to high amounts of antioxidant capacity. As a result of significant concentrations of gallic acid, chlorogenic acid, *p*-coumaric acid, and TPC, the 'Balakayati', 'Sidakan Sweet', and 'Sidakan Sour' were found in the third cluster. Finally but not least, the cultivar of Shaqlawa and Hiran was found to have a significant concentration of chlorogenic acid, coumaric acid, and TPC, which contributed to its classification as the fourth cluster.

PCA graph is shown in Figure 5. A PCA was performed, which reduced the multidimensional structure of the data and produced a two-dimensional map that could be used to explain the observed variation. 65.1 % of the total variation could be attributed to the first two components of the PCA (35.06 % for component 1 and 30.05 % for component 2). Correlations between the first component (PC1) and coumaric acid, quercetin, cinnamic acid, and apigenin are extremely strong posi-



**Figure 4:** Hierarchical cluster analysis (HCA) of pomegranate cultivars based on the 11 main traits

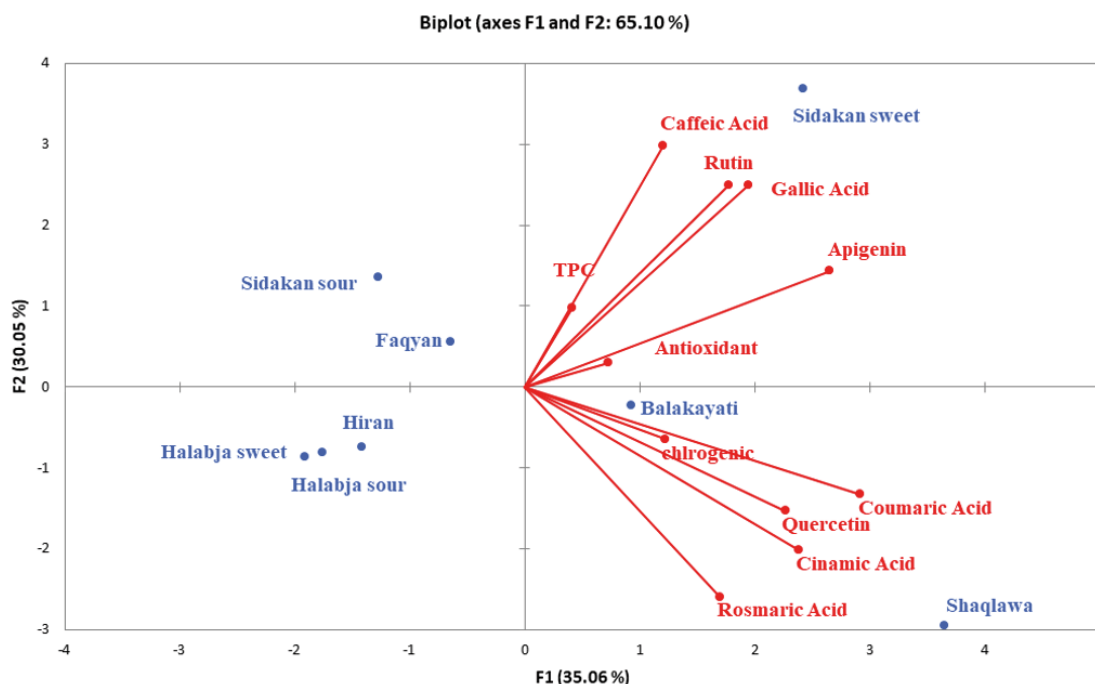


Figure 5: Principal component analysis (PCA) of pomegranate cultivars based on the 11 main traits

tive. Gallic acid, caffeic acid, and rosmaric acid are the primary constituents of the second principal component (PC2), separating the samples.

## 4 DISCUSSION

Results exhibited considerable variation in genotypes. According to previous research, the levels of polyphenolic compounds of gallic acid, chlorogenic acid, and caffeic acid in pomegranates grown in Chinese farms have been reported in 70-1400, 900-4800, and 1100-2440 mg kg<sup>-1</sup> DM, respectively (Li et al., 2015). The total phenolic content (TPC) of the juice samples ranged from 0.87 to 1.93 mg of gallic acid equivalents per mL (Russo et al., 2018). Other researchers have also discovered inconsistencies in the concentration of particular phenolic acids in different cultivars of the same plant (Fischer et al., 2011; Gundogdu & Yilmaz, 2012; Lansky & Newman, 2007). These differences were generated not only by variations in cultivar, growing region, and maturity level of the pomegranates under investigation but also by variations in the analytical procedures employed. It is possible to endogenously regulate the biosynthesis of the phenolic composition of fruits during various developmental variations in their life cycle (Gholizadeh-Moghadam et al., 2019) which exogenous agents can influence metabolism pathways. It is obvious that the antiradical activity against DPPH and ABTS radicals increases along with

the overall phenolic concentration regardless of the matrix. This is something that can be observed. This indicates that the phenolic compounds that possess antiradical characteristics are the ones that were discovered in the samples (Russo et al., 2018). Exogenous factors such as environmental conditions (temperature, biotic and abiotic stress, light intensity, humidity) and agricultural practices (soil fertility, irrigation) have an impact on the biosynthesis and accumulation of phenolic compounds in medicinal plants, which are fully addressed below (Alirezalu et al., 2018; Ghasemzadeh et al., 2012). The amino acid phenylalanine, which is synthesized via the shikimic acid pathway, is a precursor for several phenolic compounds. Phenylalanine ammonia-lyase is responsible for forming these compounds due to the de-amination of phenylalanine (PAL) (Shahidi & Chandrasekara, 2010). Environmental factors, remarkably light, are one of the most potent factors in phenolic metabolism, and they are significant. The light that impacts PAL increases the synthesis of phenolic compounds in the presence of oxygen (Macheix et al., 2018).

Wang et al. (2003) reported that the amount of phenolic compounds in a solution increases dramatically with increasing temperature and carbon dioxide (CO<sub>2</sub>) concentration. There is a possibility that the differences discovered in phenolic compounds between genotypes in this study are related to environmental factors such as geographic variations (altitude, latitude, and height), light intensity, and temperature.



According to the DPPH assay, pomegranate has a range of antioxidant activity ranging from 45 percent to 82 percent, consistent with our present findings. The examination of the antioxidant capacity of the fruit extract revealed that this cultivar possesses significant antioxidant potential due to the presence of simple phenolics, anthocyanins, phenolic acids, and flavonoids in the extract (Çam et al., 2009). Investigators have suggested that carotenes, vitamin C, butylated hydroxytoluene, and phenolic compounds are among the components in fruit extracts with significant radical scavenging capability and that these compounds are found in high concentrations in some fruits (Sayyah et al., 2010; Yildiz et al., 2014). The antioxidant activity of the pomegranate extract is highly correlated with the extract's phenolic contents, including rutin, caffeic acid, chlorogenic acid, and apigenin. This study found a substantial correlation between rutin and caffeic acid and antioxidant activity, which is consistent with earlier studies (Chen & Ho, 1997; Mariangel et al., 2013). Considering a study conducted by Castelluccio et al. (1995) on the antioxidant activity of chlorogenic acid and caffeic acid, they concluded that these compounds were more active than *p*-coumaric acid. Furthermore, Cos et al. (2002) noted that, caffeic acid had the highest scavenging activity in fruits.

According to earlier research, the TPC of pomegranate fruits has previously been observed to range from 53 to 200 (mg GAE ml<sup>-1</sup>) across pomegranate cultivars obtained from South Africa (Fawole & Opara, 2013). The relationship between latitude and total polyphenolic concentration, total reducing capacity, and DPPH radical scavenging capacity (Li et al., 2015) was positive, revealing that pomegranates grown in high latitude and low latitude longitude regions are more likely to accumulate more polyphenolics and have more significant antioxidant potential in their aril juice. As far as we know, the factors that regulate and control the production of fruit polyphenols have not been identified because the factors that influence polyphenol production among cultivars range from intrinsic genetic factors to various extrinsic environmental factors their interactions have varied over time and space (Hättenschwiler & Vitousek, 2000).

The PCA and cluster analysis were acceptable methodologies for determining cultivar classification among pomegranate varieties. The antioxidant capabilities of pomegranates have been demonstrated in several phytochemical investigations conducted on several pomegranate cultivars. In addition, it has been established by many research groups that the polyphenol content and flavonoids present in the fruits of pomegranate varieties have antioxidant properties.

## 5 CONCLUSION

There were significant variances in polyphenolic content amongst the different cultivars, which was noticed. This observation demonstrates that the relationship between genotype and environment is one of the most important elements influencing the accumulation and concentration of polyphenolics in pomegranate fruit. Furthermore, these findings demonstrated that pomegranate varieties are prospective sources of natural antioxidants and phenolic compounds employed in the food industry and that multivariate analysis was an appropriate method for classifying the pomegranate samples.

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