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Original Article

The Effect of Rootstocks on the Peel Phenolic Compounds of Clementine Mandarin (*Citrus clementina*)

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Abstract

Studies have shown that phenolic compounds are important in human health. The purpose of this research was to examine the influence of rootstocks on phenolic compounds. The content of individual phenolic compounds in fruits was determined by HPLC. Total flavonoids content was measured using colorimetric method. Free radical scavenging activity on stable DPPH radicals was also evaluated. HPLC analysis of the peel compounds extracted from Clementine mandarin allowed identification of 7 phenolic components. Hesperidin was the main component for all rootstocks. Among the six rootstocks examined, Flying dragon showed the highest content of phenolic compounds. As a result of our study, we can conclude that the rootstock can influence the quantity of phenolic compounds present in fruit.

Keywords: Citrus rootstocks, Clementine mandarin, Phenolic compounds
Abbreviations: HPLC, High performance liquid chromatography; DPPH, 2, 2-diphenyl-1-picryhydrazyl radical.

Introduction

Mandarin is one of the most economically important crops in Iran. In the period 2011-2012, the total mandarin production of Iran was estimated at around 825000 tones [1]. Clementine (*Citrus clementina*) is one of the most important mandarins are widely cultivated in Iran. Although it is an important crop, little research has been done on phenolic compounds of Clementine mandarin.

Phenolic compounds have been classified into two major categories: phenolic acids and flavonoids. All flavonoids can be classified into flavanones, flavones, flavonols [2]. Flavanones are identified as the major flavonoid in citrus fruit and are the most abundant. Hesperetin and naringenin are the most important flavanones in citrus fruit [3].

Flavonoids play an important role in the prevention of cardiovascular diseases, cancers, and other degenerative diseases [4]. In addition, recent studies have identified antimicrobial and antifungal properties for flavonoids [5]. Flavonoids are important compounds extensively used in food and pharmaceutical industry [6].

Citrus peel is an excellent source of flavonoids. The quantity of flavonoids present in the Citrus fruit, is variable and depends upon a number of factors, including: rootstock [7] cultivar [8], and etc.

Several studies have shown that the rootstocks used can influence the flavonoid compounds in Citrus [9, 10].

In this paper, we compared the Citrus rootstocks with the aim of determine whether the phenolic compounds were impressed by the rootstocks.

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Material and Methods

Chemicals and Standards

Hesperidin, naringenin, narirutin, diosmin, caffeic acid, *p*-coumaric acid, Chlorogenic acid, gallic acid standards, 1,1-diphenyl-2-picrylhydrazyl (DPPH), acetonitrile, methanol and Folin–Ciocaltaeu's reagent were purchased from Sigma Chemical Co. (St. Louis, MO) . Rutin and Na2CO3 were purchased from Merck (Darmstadt, Germany).

Rootstocks

In 2001, rootstocks were planted at 8×4 m with three replication at Ramsar research station [Latitude 36° 54' N, longitude 50° 40' E; Caspian Sea climate, average rainfall and temperature were 970 mm and 16.25°C per year respectively; soil was classified as loam-clay, pH ranged from 6.9 to 7]. Sour orange, Swingle citrumelo, Trifoliate orange, Flying dragon, Orlando tangelo and Murcott were used as rootstocks in this experiment (Table 1).

Preparation of peel sample

Fruits were collected from different parts of the same trees in January 2016, early in the morning (6 to 8 am) and only during dry weather. The selection method was on the basis of completely randomized design.

Peel Extraction Technique

The peel was extracted according to the method of Chen *et al.* [11] with slight modifications. In order to obtain the phenolic compounds from the Peel, 0.2 g of dried peel (powder) were placed in a 200 ml spherical flask, along with 20 mL of methanol. The flask was covered and then placed in an ultrasonic water bath for 15 min. Extraction were performed with an ultrasound cleaning bath-Fisatom Scientific-FS14H (Frequency of 40 KHz, nominal power 90 W and 24×14×10 cm internal dimensions water bath). The temperature of the ultrasonic bath was held constant at 40 °C. The extract was subsequently filtered through 0.45 mm filter paper. The concentration of the extract was finally reduced to 40 ml using methanol and placed in a vial. Vial sealed and was kept in the refrigerator at 4 °C until the HPLC analysis.

Analysis of Phenolic Compounds by HPLC

HPLC analysis was performed with a PLATIN blue system (Knauer, Berlin, Germany) equipped with binary pump and a photodiode array (PDA) detector. The separation was carried out on a ODS-2 C-18 reversed phase column (250 mm × 4.6 mm, i.d.) 5 μm. Column temperature was maintained at 25 °C, and the injection volume for all samples was 10 μL. Elution was performed isocratically with the mobile phase consisting of 0.05% (v/v) aqueous phosphoric acid (eluent A) and acetonitrile (eluent B) at a flow rate of 0.6 mL/ min. The column was washed with 100% methanol and equilibrated to initial conditions for 15 min before each injection. UV–visible spectral measurements were made over the range of 210–400 nm. Chromatograms were recorded at 329 nm for caffeic acid, p-coumaric acid, chlorogenic acid. Chromatograms were also recorded at 283 nm for narirutin, naringin and hesperidin. Identification of phenolic acids and flavanone glycosides was based on retention times and UV–visible spectra of unknown peaks in comparison with standards. The concentration of the phenolic acids and flavanone glycosides was calculated from peak area according to calibration curves.

Standard solutions of phenolic compounds were prepared by dissolving hesperidin, narirutin, narirutin, diosmin, caffeic acid, *p*-coumaric acid in HPLC grade methanol and stored at -20 °C between analyses.

Table 1 Common and botanical names for citrus taxa used as rootstocks and scion.

Common name	Botanical name	Parents	Category
Clementine (scion)	*Citrus clementina cv. Cadox*	Unknown	Mandarin
Sour orange (Rootstock)	*Citrus×auranthium L.*	Mandarin×Pomelo	Sour orange
Swingle citrumelo (Rootstock)	*Swingle citrumelo*	*C.paradisi cv. Duncan*× *C. trifoliate L.*	Poncirus hybrids
Trifoliate orange (Rootstock)	*Citrus trifoliata*	Unknown	Poncirus
Flying dragon (Rootstock)	*Citrus trifoliata*	Unknown	Poncirus
Orlando tangelo (Rootstock)	*Citrus sp. cv. Orlando*	*Citrus reticulata cv. Dancy* × *Citrus paradisi cv. Duncan*	Tangelo
Murcott(Rootstock)	*Citrus sp. cv. Murcot*	*C.reticulata*× *C.sinensis*	Tangor

Calibration was performed by injecting the standard three times at five different concentrations. Standard solution of hesperidin that diluted in ethanol at concentrations of 0, 7.33, 14.67, 22 and 30.69 ug/mL, used to obtain a standard curve.

Standard solutions of naringenin at concentrations of 0, 5, 10, 15 and 20 ug/mL, used to obtain a standard curve. Standard solutions of narirutin at concentrations of 0, 14.5, 29, 43.5 and 58 ug/mL, used to obtain a standard curve. Standard solutions of diosmin at concentrations of 0, 6.5, 13, 19.5 and 25 ug/mL, used to obtain a standard curve. Standard solutions of *p*-coumaric acid at concentrations of 0, 5.5, 11, 16.5 and 22 ug/mL, used to obtain a standard curve. Standard solutions of caffeic acid at concentrations of 0, 7, 14 and 28 ug/mL, used to obtain a standard curve. Standard solutions of Chlorogenic acid at concentrations of 0, 5.5, 11 and 22 ug/mL, used to obtain a standard curve. (Fig. 1 to 7)

The amount of each phenolic acid and flavanone glycosides was expressed as milligrams of compound per gram of dry weight (mg/g DW).

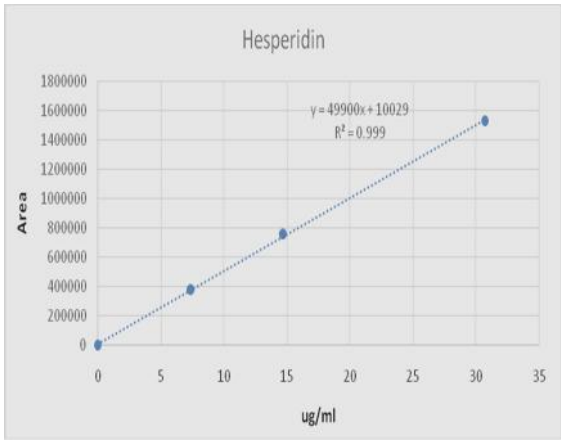

Fig. 1 The standard curve of hesperidin

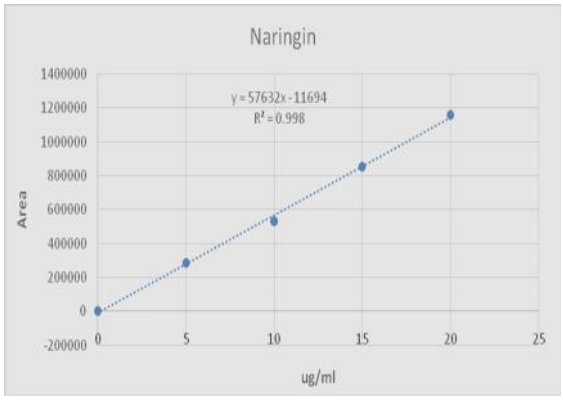

Fig. 2 The standard curve of naringin

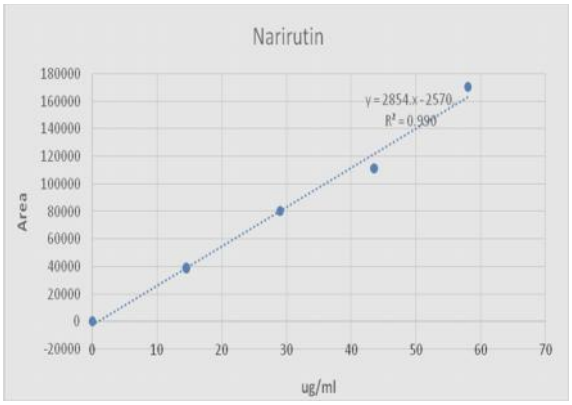

Fig. 3 The standard curve of narirutin

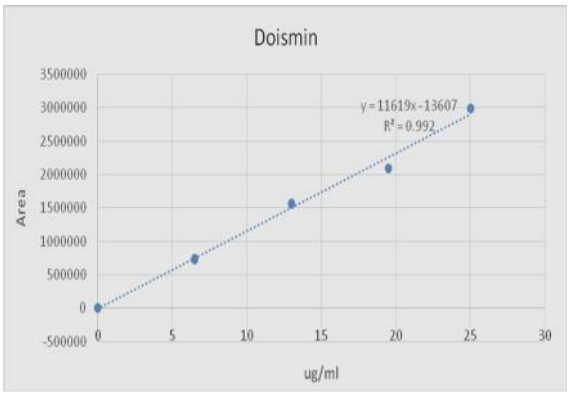

Fig. 4 The standard curve of doismin

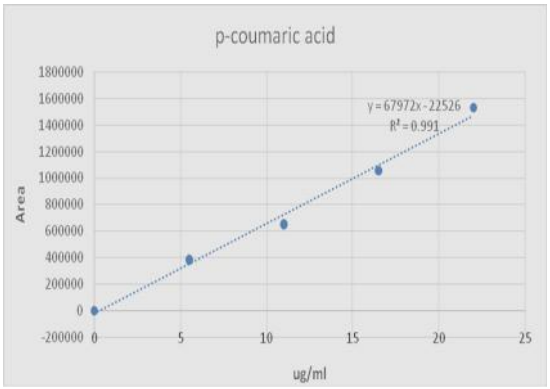

Fig. 5 The standard curve of p-coumaric acid

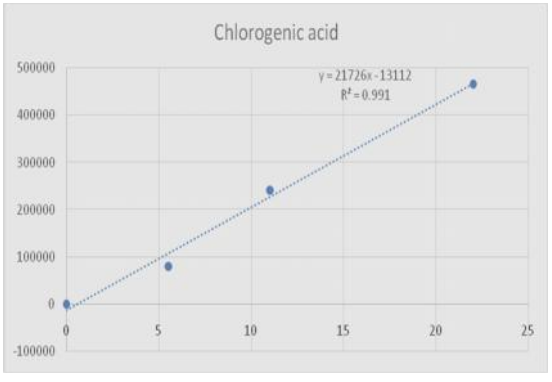

Fig. 6 The standard curve of chlorogenic acid

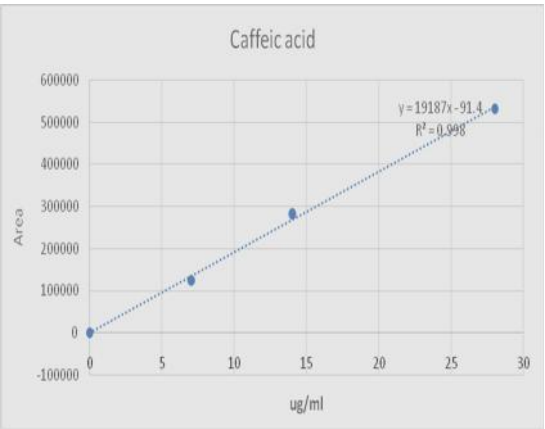

Fig. 7 The standard curve of caffeic acid

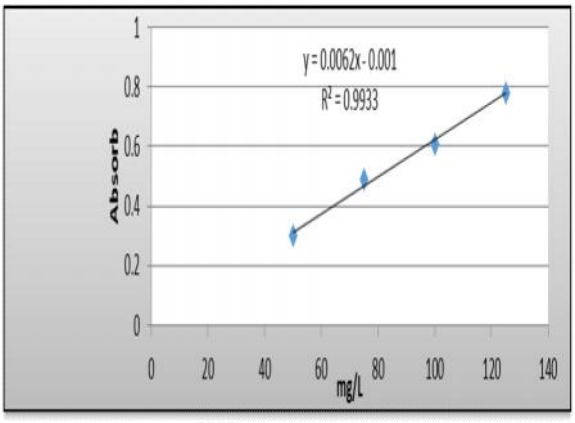

Fig. 8 The standard curve of rutin

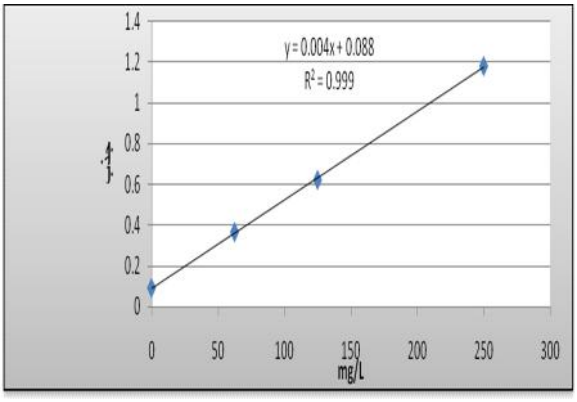

Fig. 9 The standard curve of gallic acid

Identification of Flavonoid Components

phenolic acids and flavonoids were identified by comparing the retention times, absorption spectra (210–400 nm) and mass spectra of unknown peaks with those of reference compounds.

Determination of Total Flavonoid Content

The flavonoid content was determined by the aluminum chloride colorimetric method. Standard solutions of rutin were prepared by dissolving 16.2 mg rutin with 70% ethanol into 100 ml after shaking evenly. Standard solutions of rutin at concentrations of 50, 75, 100 and 125 mg/L used to obtain a standard curve. Standard solutions of rutin were pipetted into four flasks of 10 ml, respectively, and diluted to 5 ml with 70% ethanol solution. Sodium nitrite solution (5%, 0.5 ml) was added to the standards and maintained for 5 min. Then, 0.5 ml of aluminium chloride (10%) was added. It remained at room temperature for 6 min. Finally, 5 ml of sodium hydroxide (1 M) was added. The mixture was diluted to 10 ml with distilled water.

The absorbance of all the samples was measured using a spectrophotometer (UV 1600 PC,Shimadzu, Tokyo, Japan) at 415 nm. The regression equation of rutin density and absorption value was obtained using rutin density (X) as the abscissa axis and absorption value (Y) as the vertical axis (Fig. 8). The total flavonoid content was calculated from calibration curve and the result was expressed as mg rutin equivalent per g dry weight [11]

Determination of Total Phenol Content

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Determination of Total Phenol Content

The total phenol content was determined by Folin-Ciocalteu's reagent. Standard compound of gallic acid (6.2 mg) was weighed accurately and dissolved with distilled water (25 ml). Standard solutions of gallic acid at concentrations of 0, 62.5, 125 and 150 mg/L used to obtain a standard curve. Standard solutions of gallic acid were pipetted and diluted to 5 ml with distilled water. Then Folin- Ciocalteau reagent (0.5 ml) was added. It remained at room temperature for 2 min. Finally, sodium carbonate (5%, 0.5 ml) was added. It remained at room temperature for 3 h.

Absorbance was measured using a spectrophotometer (UV 1600 PC,Shimadzu, Tokyo, Japan) at 760 nm. The regression equation of gallic acid (X) and absorption value (Y) was obtained by using gallic acid density (X) as the abscissa axis and absorption value (Y) as the vertical axis (Fig. 9.).The total phenol content was calculated from the calibration curve and the results were expressed as mg of gallic acid equivalent per g dry weight [11].

DPPH Free Radical Scavenging Activity

The free radical scavenging activity was measured according to the method of Umamaheswari and Asokkumar [12] with slight modification. Briefly, 0.2 ml of extract was mixed with 2 ml DPPH (2, 2-diphenyl-1-picryl-hydrazyl). It remained at room temperature for 30 min. Absorbance was measured at 517 nm. DPPH expressed as (%).

Data Analysis

SPSS 18 was used for analysis of the data obtained from the experiments. Analysis of variations was based on the measurements of 7 phenolic compounds. Comparisons were made using one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Differences were considered to be significant at $P < 0.01$. The correlation between pairs of characters was evaluated using Pearson's correlation coefficient.

Results

Peel Compounds of the Clementine Mandarin

HPLC analysis of the peel compounds extracted from Clementine mandarin allowed identification of 7 phenolic components (Table 2, Fig. 10): 3 flavanones, 1 flavones and 3 phenolic acid.

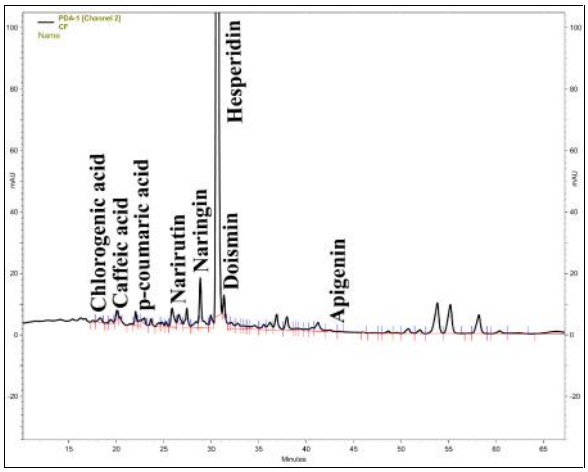

Fig. 10 HPLC chromatogram of phenolic components of Clementine mandarin

Flavanones

Three flavanones that identified in this analysis were narirutin, naringin, and hesperidin. In addition they were quantified from 12.72 to 20.26 mg/g DW. The concentration of hesperidin was higher in our samples. Among six rootstocks examined, Flying dragon showed the highest content of flavanones (Table 2).

Flavones

One compound identified in this analysis was doismin. The total amount of flavones ranged from 0.08 to 0.10 mg/g DW. Among six rootstocks examined, Flying dragon, Sour orange and Orlando tangelo showed the highest content of flavones (Table 2).

Phenolic Acids

Three phenolic acids identified in this analysis were chlorogenic acid, caffeic acid and p-coumaric acid. The total amount of phenolic acids ranged from 0.20 to 0.55 mg/g DW. Chlorogenic acid was identified as the major component in this study and was the most abundant. Among six rootstocks examined, Orlando tangelo showed the highest content of phenolic acids (Table 2).

Results of Total Flavonoid Content

The amount of total flavonoid ranged from 6.99 to 11.25 mg/g DW. Among six rootstocks examined, Flying dragon showed the highest content of flavonoid (Table 2).

Results of Total Phenol Content

The amount of total phenol ranged from 2.99 to 6.39 mg/g DW. Among six rootstocks examined, Flying dragon showed the highest content of phenol (Table 2).

Results of DPPH Free Radical

The amount of total DPPH ranged from 64.68 to 84.37%. Among six rootstocks examined, Flying dragon showed the highest content of DPPH free radical (Table 2).

Results of Statistical Analyses

Differences were considered to be significant at $P < 0.01$. These differences on the 1% level occurred in narirutin, naringin, hesperidin, doismin, chlorogenic acid, caffeic acid, total flavonoid, total phenol and DPPH free radical. This difference on the 5% level occurred in p-coumaric acid. (Table 2).

Results of Correlation

Simple intercorrellations between 7 components were presented in a correlation matrix (Table 3). The highest positive values or r (correlation coefficient) were observed between caffeic acid and chlorogenic acid. There was also a high positive correlation between P-coumaric acid and doismin.

Table 2 Statistical analysis of variation in peel phenolic compounds of Clementine mandarin on six different rootstocks.

Compounds (mg/g DW)	Sour orange		Swingle citrumelo		Trifoliate orange		Flying Dragon		Orlando tangelo		Murcott		
	Mean	St.err	Mean	St.err	Mean	St.err	Mean	St.err	Mean	St.err	Mean	St.err	F value
a) Flavanones													
1) Narirutin	1.86	0.10	3.37	0.13	1.36	0.10	1.98	0.12	2.59	0.17	1.26	0.10	F**
2) Naringin	0.09	0.01	0.22	0.02	0.19	0.01	0.35	0.03	0.26	0.03	0.16	0.02	F**
3) Hesperidin	16.41	0.44	14.45	0.45	12.63	0.26	17.94	0.11	14.64	0.19	11.31	0.22	F**
total	18.36	0.55	18.04	0.60	14.18	0.37	20.27	0.26	17.49	0.39	12.73	0.34	
b) Flavones													
1) Diosmin	0.10	0.01	0.09	0.01	0.08	0.00	0.12	0.01	0.10	0.01	0.08	0.00	F**
c) Phenolic acids													
1)Chlorogenic acid	0.12	0.02	0.16	0.02	0.24	0.03	0.22	0.02	0.25	0.03	0.22	0.01	
2) Caffeic acid	0.005	0.001	0.008	0.001	0.22	0.02	0.04	0.01	0.23	0.02	0.19	0.02	F**
3)p-coumaric acid	0.08	0.01	0.08	0.01	0.07	0.00	0.08	0.01	0.08	0.01	0.05	0.00	F*
total	0.21	0.03	0.25	0.03	0.53	0.06	0.34	0.04	0.56	0.06	0.46	0.05	
total flavonoid	10.32	0.27	9.01	0.53	7.66	0.22	11.25	0.20	9.26	0.26	6.99	0.22	F**
total phenol	5.52	0.27	3.49	0.20	4.55	0.32	6.39	0.20	4.60	0.15	2.99	0.22	F**
DPPH free%	83.43	0.31	70.62	0.18	69/06	0.18	84.37	0.20	73.43	0.32	64.68	0.19	F**

Mean is average composition (mg/g DW) in six different rootstocks used with three replicates. St. err = standard error. F value is accompanied by its significance, indicated by: NS = not significant, * = significant at P = 0.05, ** = significant at P = 0.01.

Table 3 Correlation matrix (numbers in this table correspond with components mentioned in Table 2).

	narirutin	naringin	hesperidin	doismin	chlorogenic acid	caffeic acid
naringin	0.30	-	-	-	-	-
hesperidin	0.33	0.42	-	-	-	-
doismin	0.37	-0.16	0.23	-	-	-
chlorogenic acid	-0.26	0.56*	-0.34	-0.19	-	-
caffeic acid	-0.43	0.03	-0.66**	-0.09	0.83**	-
p-coumaric acid	0.53*	0.32	0.64**	0.73**	-0.13	-0.36

*=significant at 0.05, **=significant at 0.01

Discussion

Our observation that rootstocks had effect on the flavonoids was in accordance with previous findings **[10]**. The compositions of the flavonoid obtained from six rootstocks of Clementine were very similar. However, the relative concentration of compounds was different according to the type of rootstock. Comparison of our data with those in the literatures revealed that some were not consistent with previous studies [13]. It might be related to rootstock and environmental factors that could influence the compositions. However, it should be noted that the extraction method might also affected the results. Studies showed that Fertilizer and irrigation were affected the content of flavonoids present in plant [14]. Fertilization, irrigation and other operations were carried out uniform in this study so we did not believe that these variations might be due to the variation in environmental conditions

The discovery of naringenin chalcone, as an intermediate between Malonyl CoA and flavonoids, led to a rapid description of the biosynthetic pathway of flavonoid compounds. The biosynthetic pathway of flavonoid compounds in higher plants is as follows:

Phenylalanine→ Malonyl CoA (+4-comaryol CoA) → Naringenin Chalcone → Naringenin → flavonoids

Reaction pathway catalyzed by chalcone synthase and chalcone isomerase respectively [15]. An increase in the amount of flavonoids, when Flying dragon used as the rootstock, showed that either the synthesis of naringenin chalcone was enhanced or activities of both enzymes increased.

Cytokinins were known to stimulate the synthesis of several types of phenolic compounds [16]. It is generally accepted that Cytokinins in higher plants are synthesized mainly in the root system and transported to the shoots through the xylem. In addition, cytokinin level in the xylem sap also can vary by rootstock and exhibit an extremely competitive source/ sink relationship for mineral elements and metabolites [17].

High positive correlations between pairs of phenolic compounds indicated a genetic control [18] and such dependence between pairs of phenolic components was due to derivation of one from another that was not known. Similarly, high negative correlations between pairs of phenolic components indicated that one of the two compounds was synthesized at the expense of the other or its precursor. Non-significant negative and positive correlation indicated genetic independence. However, without extended information into the biosynthetic pathway of each flavonoid compound, the true significance of the observed correlations is not clear.

Considering that naringenin chalcone is necessary for the synthesis of flavonoids, it can be assumed that there is a specialized function for this molecule and it may be better served by Flying dragon.

In the present study we found that the amount of flavonoids was significantly impressed by rootstocks and there was a great variation in most of the measured characters among six rootstocks. The present study demonstrated that the relative concentration of flavonoids was different according to the type of rootstock. Among six rootstocks examined, Flying dragon showed the highest content of flavonoids. The lowest of flavonoids content were produced by Murcott. Further research on the relationship between rootstocks and flavonoids is necessary.

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