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Original Article

Accumulation of Naringin and Limonin and Genes Expression of their Corresponding Enzymes during Fruit Maturation of Three *Citrus* Genotypes

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ABSTRACT

Naringin and limonin are important antioxidant compounds in *Citrus* species. Due to the high biological effects of these compounds, in the present study, the variation of naringin and limonin content during fruit development was investigated at two fruit tissues of pulp and albedo in three *Citrus* genotypes (grapefruit, orange and mandarin). Also, the gene expression of involved enzymes in naringin and limonin metabolism was assayed. In all studied *Citrus* genotypes, the naringin content in albedo tissue was higher than the pulp and grapefruits showed the highest naringin content (940.26 mg per 100 g DW) followed by orange (791.17 mg per 100 g DW) and mandarin (602.10 mg per 100 g DW). The naringin content of studied genotypes was decreased during the fruit development and the highest decrement was observed at ripening stages. The decreasing of naringin content was attributed to the changes in genes expression of main involved enzymes in naringin biosynthesis pathways including chalcone-flavanone isomerase and naringenin glycoside biosynthesis (1.2 RhaT) during studied harvesting times. In both tissues of mandarin and orange fruits, the limonin content was increased up to 80 days after full bloom then decreased. In grapefruit, due to the late-ripening of the fruit, the peak of limonin increment was occurred later, which can be attributed to the long growth process of grapefruits compared to orange and mandarin. The reduction of limonin content with fruit ripening was in accordance with the increased gene expression of limonoid UDP-glucosyl transferase that converts limonin to tasteless compounds.

INTRODUCTION

The *Citrus* genus as flowering trees related to the Rutaceae family is grown in more than 64 countries of the world. Citrus fruit can be divided into four main groups including mandarins, sweet oranges, lemon and limes, pummelo and grapefruit species. The *Citrus* fruit in addition to pulp consists of epicarp or flavedo, mesocarp or albedo, which together constitute the fruit peel [1]. The *Citrus* flavor and taste are determined by the concentration of soluble solids and titrable acids [2]. *Citrus* are also rich sources of a vast array of metabolites such as flavonoids and limonoids which are abundant in fruit pulp and have beneficial health effects [3,4]. Flavonoids and limonoids determine the bitter flavor trait in *Citrus* fruits and their distributions greatly vary among species, varieties and tissues. Flavonoids as low molecular weight polyphenolic metabolites are synthesized in the phenylpropanoid pathway which consists of different groups including flavanones, flavones, flavonols, and anthocyanins [5]. A part of *Citrus* bitterness is attributed to flavanone O-glycosides especially naringin and neohesperidin [6] which a wide biological properties of these compounds were previously reported [7,8]. Limonoids are oxygenated triterpenes whose biosynthesis occurs in mevalonic acid and methylerythritol 4-phosphate pathways. In the *Citrus* genus, the higher concentrations of aglycones derivatives of

limonoids such as limonin and nomilin are associated with bitterness taste [9]. The accumulation of these compounds is affected by genotype, environmental conditions and fruit maturation stage [4,10,11]. During the fruit ripening process, limonoid UDP-glucosyl transferases catalyze the glycosylation of limonoid aglycones to tasteless compounds that cause the debittering of fruits [4,10]. Also, the concentration of total flavonoids decreases over the ripening process in different fruit tissues related to the down-regulation of chalcone synthase and chalcone isomerase gene expression [12,13]. The ratio between flavonoids and limonoids content characterizes the final taste of ripe *Citrus* fruits as sweet, bittersweet and bitter [6]. The endocarp or pulp as an edible part of *Citrus* fruits is an important source of metabolites with commercial value [14,15]. Despite to high level of flavonoids concentration in the albedo of several *Citrus* species [16], this issue has received less attention.

Various techniques have been used to evaluation of bitterness in *Citrus*. An efficient technique for the profiling of different secondary metabolites in *Citrus* fruit is liquid chromatography/electrospray ionization-quadrupole/time-of-flight-mass spectrometry (LC/ESI-QqTOF-MS) which has little sample processing (squeezing, centrifuging and filtering) compared with other techniques [17]. This study was conducted to the evaluation the effects of genotype, tissue and developmental stage on the accumulation of naringin and limonin contents. For this purpose, the most important *Citrus* fruit types with different tastes included mandarin (sweet), orange (bittersweet) and grapefruit (bitter) were selected focusing on the pulp and the albedo tissues. Also, the expression of three main involved enzymes in flavonoids and limonoids biosynthesis was assayed in pulp and albedo samples over the developmental process of fruits.

MATERIALS AND METHODS

Preparation of Plant Material

Samples of three *Citrus* genotypes included grapefruit (*Citrus paradisi* Macfad. cv Red blush), orange (*C. sinensis* L. cv Navelina) and mandarin (*C. clemantina* Hort. cv Clementine) were collected from adult trees at the germplasm bank (*Citrus* and Subtropical Fruits Research Center, Ramsar, Iran). Trees from studied genotypes were grafted onto bitter orange (*C. aurantium* L.) rootstock. The fruit was harvested at four stages including 20, 80, 110, and 200 days after full bloom. The evaluation of naringin and limonin content dependent on genotype, tissue and fruit developmental stage was investigated in separate experiments arranged in a randomized complete block design with three replicates. From each replicate tree (n = 3), four fruits in the different directions on the tree were harvested and pooled. In each sample, albedo and pulp tissues were accurately separated without cross-contamination and then immediately frozen in liquid nitrogen. Frozen samples were subsequently subjected to freeze-drying and then powered and kept at -20 °C until further analyses.

Frozen plant materials of pulp and albedo were weighed and extracted with 80% (v/v) aqueous methanol and biochanin A (1 mg/L concentration). The supernatants were used for assay of naringin and limonin contents with liquid chromatography/electrospray ionization-quadrupole/time-of-flight-mass spectrometry (LC/ESI-QqTOF-MS).

Chromatographic and QqTOF-MS Conditions

Sample extracts were separated by reversed-phase LC using LC/MS-degree acetonitrile (B) and water (A), both supplemented with formic acid to a concentration of 0.1% (v/v) as solvents. Separations were carried out on a C18 column (Luna Omega Polar C_{18}, 100 × 2.1 mm, 1.6 µm particle size, Phenomenex). The separation module, Waters Acquity SDS was operated in gradient mode for 25 min as follows: 0–2 min 95:5 (A:B) followed by an increase in B from 5 to 95 in the following 17 min (2.01–17.00 min), thereafter returning to initial conditions (17.01–20.00 min) that were maintained for 5 min for column reconditioning. During mass chromatographic data acquisition, the flow rate was maintained at 300 µL/min and column temperature at 40 °C. Column eluates were introduced into a QqTOF-MS through orthogonal electrospray ionization (ESI) source operated in positive and negative ionization modes. Before analyses, the QqTOF-MS was calibrated by infusing a mixture of NaOH and HCOOH at a flow rate of 25 µL/min. After calibration, the average mass error was less than 5 ppm. Samples were analyzed in both negative and positive ionization modes. Co-injection with

pure standards of naringin and limonin was carried out to identify these metabolites. Raw mass chromatograms were converted to net CDF prior to xcms processing. Extracted mass chromatographic data were corrected to the internal standard intensity and actual sample weight before any statistical analysis.

RNA Isolation and Quantitative Real-Time PCR

Quantitative real-time PCR was conducted to evaluate the genes expression level of chalcone-flavanone isomerase (EC:5.5.1.6), naringenin glycoside biosynthesis (1.6 RhaT) (AY048882) and limonoid UDP-glucosyl transferase (EC:2.4.1.210).

The frozen samples of pulp and albedo were ground into a fine powder using a mortar and pestle, and total RNA was isolated with the RNeasy Plant Mini Kit using RLC buffer for cell lysis due to the high presence of secondary metabolites in studied tissues. Genomic DNA contamination was removed with Turbo DNA-free treatment for 30 min at 37 °C. RNA was spectrophotometrically quantified and stored in RNase-free H_2O at 20 °C. The cDNA was synthesized by using a Reverse Transcription kit, and then qRT-PCR was carried out in triplicates using QuantiTect SYBR Green PCR kit. The conditions for PCR amplification were 95 °C for 10 min, 95 °C for 10 s, 60 °C for 10 s, 72 °C for 20 s (40 cycles), then at 60 °C for 10 s and 95 °C for 15 s. Melting curve analysis was applied to test the amplification specificity. Actin was used as a housekeeping gene, and the relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method [18,19]. For gene amplification, specific primers at least 20 nucleotides in length with similar annealing temperatures were designed for each studied gene.

Statistical analysis

Data were analyzed by SPSS software and means were compared according to Duncan's multiple ranges test at $P \leq 0.05$ (SPSS ver.16.0). The REST software was used to analyze the gene expression level of studied enzymes.

RESULTS AND DISCUSION

According to variance analysis, all studied traits were significantly influenced by the fruit development stage as well as fruit tissues and genotypes (Table 1). The highest limonin content was found in orange (66.51 mg per 100 g DW), mandarin (41.81 mg per 100 g DW) and grapefruit (36.50 mg per 100 g DW), respectively (Table 1). As shown in Table 1, in all studied *Citrus* genotypes, the naringin content in albedo tissue was higher than the pulp and grapefruits showed the highest naringin content (940.26 mg per 100 g DW) followed by orange (791.17 mg per 100 g DW) and mandarin (602.10 mg per 100 g DW). Naringin and limonin as important antioxidant compounds have been reported in various *Citrus* species [20,21]. Bilal *et al*. [22] and Huang *et al*. [23] reported the differences in the amount of limonin in different *Citrus* genotypes. According to reports of Raithore *et al*. [24], different varieties of *Citrus* species have also differed regards to limonin content. Huang *et al*. [23] reported the highest amount of limonin in orange varieties. Bermejo *et al*. [21] quantified the flavonoid compounds along with limonoid compounds in several cultivars related to Clementine, Satsume and Navel oranges. These authors reported the significant differences in naringin and limonin contents among the studied species. According to Chang and Azrina [16], the much bitterness taste of grapefruit is related to high flavonoids content as the results of the present study showed the highest concentration of naringin in both tissues of grapefruit (Table 1). The discrepancy in the results of the literature shows that besides genotype, the biosynthesis and accumulation of flavonoid and limonoid compounds are influenced by environmental conditions, harvest season, extraction methods, and storage conditions [6,20].

The naringin content of studied genotypes was decreased during the fruit development and the highest decrement was observed at ripening stages. So, the highest (449.96 mg/100 g FW) and lowest (352.61 mg/100 g FW) amount of naringin was obtained at 20 and 200 days after full bloom, respectively (Table 1).

The decreasing of naringin content was attributed to the changes in genes expression of main involved enzymes in naringin metabolism pathways such as chalcone-flavanone isomerase and naringenin glycoside biosynthesis (1.2 RhaT) during studied harvesting times. The gene of chalcone-flavanone isomerase is responsible to convert naringenin chalcone to naringenin [25] and as shown in Fig. 1 the expression level of this enzyme was decreased during fruit developmental stages.

Table 1 Effect of different harvesting times (days after full bloom) on the naringin and limonin content of pulp and albedo in three *Citrus* genotypes

Pulp						
Harvest time	Naringin (mg 100 g/ DW)			Limonin (mg 100 g/ DW)		
	Grapefruit	Orange	Mandarin	Grapefruit	Orange	Mandarin
20	449.96 ± 3.21 a	376.30 ± 2.22 a	325.46 ± 0.92 a	14.38 ± 2.06 c	28.61 ± 4.08 b	19.17 ± 0.08 b
80	441.90 ± 2.40 a	346.90 ± 42.71 a	326.60 ± 5.35 a	34.36 ± 0.79 a	62.83 ± 0.29 a	41.14 ± 1.67 a
110	407.29 ± 5.15 b	300.12 ± 0.46 b	278.63 ± 2.20 b	35.35 ± 1.21 a	30.06 ± 1.44 b	20.20 ± 1.10 b
200	352.61 ± 5.84 c	282.50 ± 1.59 b	239.26 ± 2.90 c	31.18 ± 0.69 b	24.06 ± 0.81 c	14.35 ± 1.05 c

		Naringin			Limonin		
Source of variation	df	Grapefruit	Orange	Mandarin	Grapefruit	Orange	Mandarin
Harvesting time	3	5880.740 **	5523.791**	5275.791**	287.521 **	951.784 **	578.535 **

Albedo						
Harvest time	Naringin (mg 100 g/ DW)			Limonin (mg 100 g/ DW)		
	Grapefruit	Orange	Mandarin	Grapefruit	Orange	Mandarin
20	934.36 ± 5.03 a	791.17 ± 9.85 a	600.42 ± 10.58 a	11.93 ± 0.08 c	28.44 ± 0.51 c	15.98 ± 0.60 b
80	940.26 ± 2.29 a	773.20 ± 4.45 b	602.10 ± 5.31 a	32.59 ± 1.77 b	66.18 ± 0.17 a	32.53 ± 2.32 a
110	923.01 ± 9.35 b	738.06 ± 5.24 c	530.77 ± 9.69 b	36.95 ± 0.09 a	36.36 ± 0.66 b	15.76 ± 0.21 b
200	900.45 ± 0.42 c	712.26 ± 12.28 d	449.73 ± 1.55 c	30.92 ± 2.50 b	8.84 ± 0.24 d	10.23 ± 0.13 c

		Naringin			Limonin		
Source of variation	df	Grapefruit	Orange	Mandarin	Grapefruit	Orange	Mandarin
Harvesting time	3	926.153 **	3745.829 **	788.348 **	367.815 **	1701.795 **	279.029 **

Mean square for harvesting time effect on naringin and limonin content in albedo of different citrus genotypes (** is significant at 1 percent probability level)

Values followed by the same letter within a column indicate they are not significantly different ($P < 0.01$).

The gene of naringenin glycoside biosynthesis is responsible to convert naringenin-7-O-glucoside to naringenin-7-O-neohesperidoside or naringin [25,26] the expression of this gene in both pulp and albedo tissues showed a descending trend with fruit ripening (Fig. 2). According to obtained results, the high content of naringin in grapefruit (Table 1) is due to the up-regulation of naringenin glycoside biosynthesis (1.2 RhaT) compared to orange and mandarin (Fig. 2). Decreasing of flavonoids content during fruit ripening of *Citrus* species was previously reported by Barreca *et al*. [12]; Raithore *et al*. [24]; Wang *et al*. [25]; Ahmed *et al*. [27]. According to the results of Wang *et al*. [25], the expression of the chalcone isomerase gene was positively correlated with flavonoid accumulation in the fruit of Guoqing No. 4 satsuma mandarin (*C. unshiu* Marcow). These authors reported that during fruit maturation, the expression of the chalcone isomerase gene declined gradually which results in the reduction of total flavonoids concentrations. Moriguchi *et al*. [28] reported that the flavonoid compounds in the juice sacs of *Citrus* fruit were decreased with fruit ripening and the chalcone isomerase gene signals in RNA blot analysis were rarely detectable in the later stage of fruit development indicating the expression of this gene in the mature fruit was in lower level.

During the orange fruit development, the highest content of limonin in the pulp (62.83 mg per 100 g DW) and albedo (66.18 mg per 100 g DW) was observed at 80 days after full bloom. The lowest content in both tissues was found in harvested fruits at 200 days after full bloom (Table 1). Similar to orange, in both tissues of mandarin fruit, the limonin content was increased up to 80 days after full bloom then decreased so the lowest content was obtained at 200 days after full bloom (Table 1).

In grapefruit, due to the late-ripening of the fruit, the peak of limonin increment was occurred later, which can be attributed to the long growth process of grapefruits compared to orange and mandarin. In grapefruit tissues, the limonin content was increased during the fruit developing stages until 110 days after full bloom then decreased (Table 1).

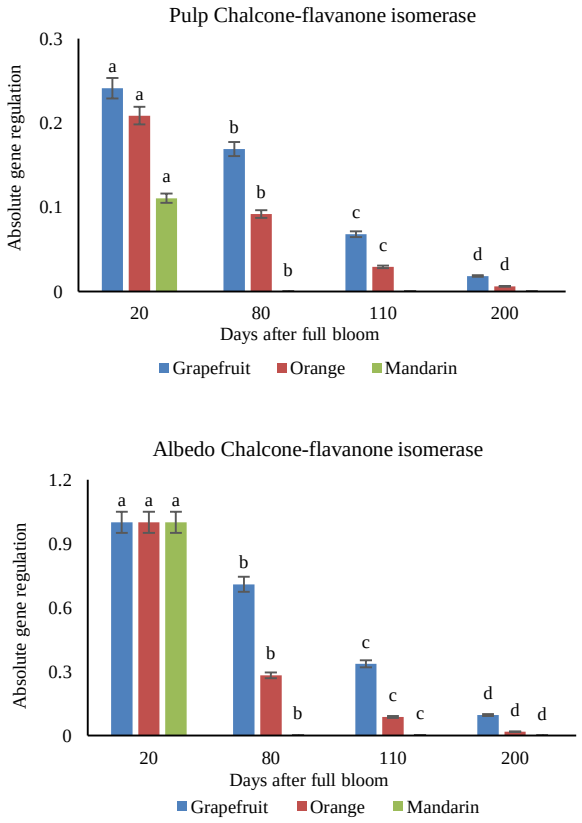

Fig. 1 Gene expression levels of chalcone-flavanone isomerase at different fruit developmental stages in pulp and albedo of three *Citrus* genotypes

The same trend of limonin content in pulp and albedo tissues in all three genotypes indicates that under stress conditions limonin may be transmitted between two tissues which can increase plant resistance to different biotic and abiotic stress conditions. Sun *et al*. [20]; Pichaiyongvongdee and Haruenkit [29] also reported the differences in the limonin content in different parts of the *Citrus* fruits. According to a report of Sun *et al*. [20] the amount of limonin was affected by genotype, and in some species, limonin content of pulp was higher than the albedo and in some others was versus.

Pichaiyongvongdee and Haruenkit [29] reported that the naringin and limonin accumulation in different tissues of *Citrus* fruit is influenced by genotype, environmental conditions, agricultural operations, soil nutritional conditions, and growing location. The observed trends of limonin changes at different fruit developing stages in studied genotypes are similar to the previous literatures [23,24,27]. Huang *et al*. [23] assayed the limonin content of different Citrus species during fruit development stages. The results of these authors have shown that the amount of limonin in most of the studied species first has an upward trend and then decreases. In most of the studied species, the amount of limonin was significantly decreased during fruit ripening and the highest limonin content in lemons (103.39 mg per 100 g DW) and orange of Ehime No. 38 (74.38 mg per 100 g DW) was observed in unripe fruits. Ahmed *et al*. [27] evaluated the variation of limonin content in grapefruit varieties at different fruit harvesting times. Their results indicate that limonin content increased with the development of fruits but with the ripening of the fruit gradually decreased. Daniel *et al*. [30] reported that the changes of flavonoids and limonoids content in hybrid varieties of *Citrus* follow the observed trends in their parents.

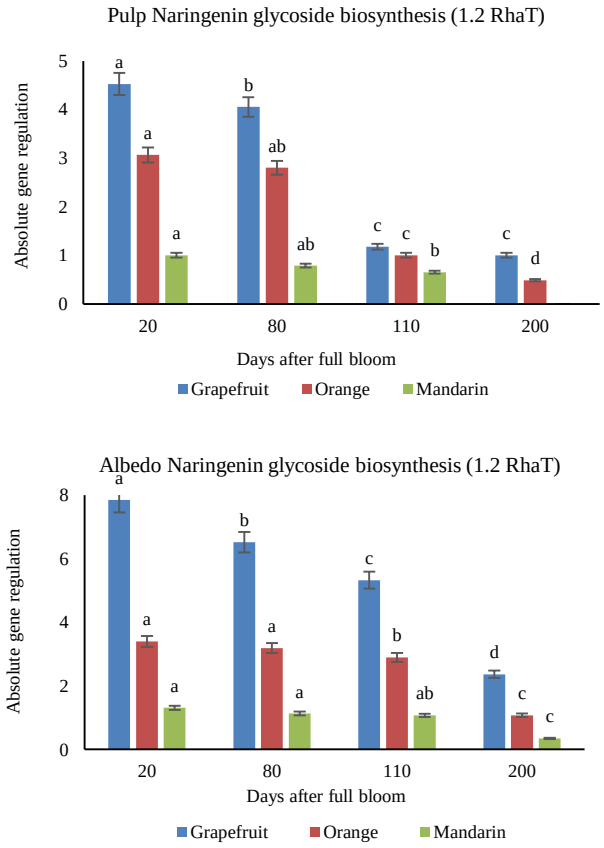

Fig. 2 Gene expression levels of naringenin glycoside biosynthesis (1.2 RhaT) at different fruit developmental stages in pulp and albedo of three *Citrus* genotypes

The reduction of limonin content with fruit ripening is probably due to the decreasing of limonic acid content (a precursor of limonin) and the activation of enzymes such as limonoid UDP-glucosyl transferase (Fig. 3) that convert limonin to tasteless compounds [31]. Limonoid UDP-glucosyl transferase is responsible to convert limonoate A ring lactone to limonin 17 beta D glucopyranoside [10]. Studies on different species of *Citrus* have shown that the activity of this enzyme decreases with fruit ripening and the reduction of limonin is simultaneous with increasing of soluble sugars [9,31]. Zaare-Nahandi *et al*. [10] reported the key role of limonoid UDP-glucosyl transferase in debittering *Citrus* fruits.

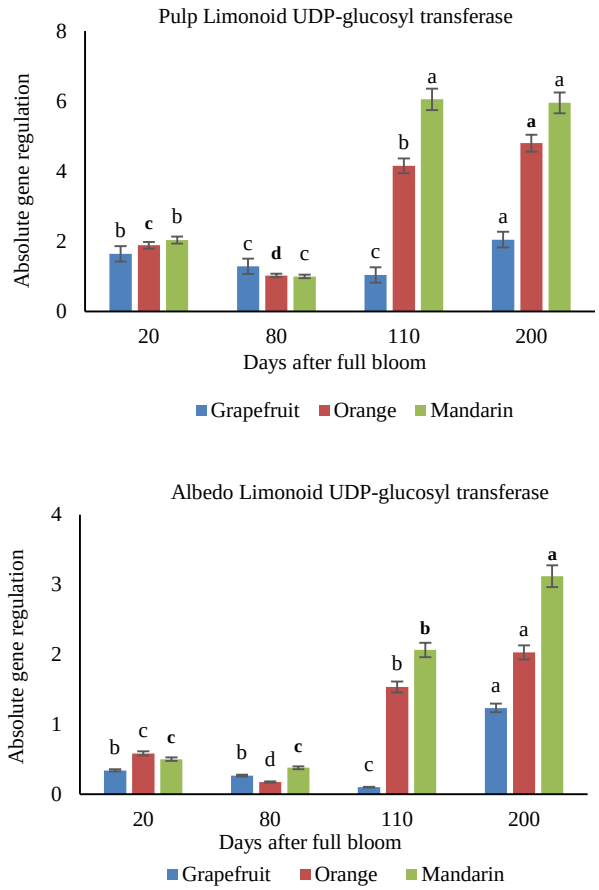

Fig. 3 Gene expression levels of limonoid UDP-glucosyl transferase at different fruit developmental stages in pulp and albedo of three *Citrus* genotypes

In general, the results of this study showed that the three factors of genotype, developmental stage and fruit tissue are effective on the accumulation of naringin and limonin in *Citrus*. It was also found that the content of these compounds is decreased with fruit ripening which is simultaneous with the changes of gene expression of involved enzymes in the biosynthesis pathway of naringin and limonin.

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REFERENCES

1. Iglesias I., Echeverría G. Differential effect of cultivar and harvest date on nectarine colour, quality and consumer acceptance. Sci Hortic. 2009;120:41-50.
2. Terol J., Soler G., Talon M., Cercos M. The aconitate hydratase family from Citrus. BMC Plant Biol. 2010;10:1-12.
3. Frydman A., Liberman R., Huhman D.V., Carmeli Weissberg M., Sapir Mir M., Ophir R., Sumner L., Eyal Y. The molecular and enzymatic basis of bitter/nonbitter flavor of citrus fruit: Evolution of branch forming rhamnosyltransferases under domestication. Plant J. 2013;73:166–178.
4. Wang F., Wang M., Liu X., Xu Y., Zhu S., Shen W., Zhao X. Identification of putative genes involved in limonoids biosynthesis in citrus by comparative transcriptomic analysis. Front Plant Sci. 2017;8:782.
5. Ververidis F., Trantas E., Douglas C., Vollmer G., Kretzschmar G., Panopoulos N. Biotechnology of flavonoids and other phenylpropanoid derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. Biotechnol J Healthc Nutr Technol. 2007;2:1214–1234.
6. Wang S., Tu H., Wan J., Chen W., Liu X., Luo J., Xu J., Zhang H. Spatio-temporal distribution and natural variation of metabolites in citrus fruits. Food Chem. 2016;199:8–17.
7. Bai J., Li S., Wu G., Ma L., Li C., Liu H., Zhang P. Naringin inhibits lipopolysaccharide-induced activation of microglia cells. Cell Mol Biol. 2019;65:38–42.
8. Farooqi A.A., Tahir F., Fakhar M., Butt G., Colombo Pimentel T., Wu N., Yulaevna I.M., Attar R. Antimetastatic effects of Citrus-derived bioactive ingredients: Mechanistic insights. Cell Mol Biol. 2021;67:178–186.
9. Dea S., Plotto A., Manthey J.A., Raithore S., Irey M., Baldwin E. Interactions and thresholds of limonin and nomilin in bitterness perception in orange juice and other matrices. J Sens Stud. 2013;28:311–323.
10. Zaare-Nahandi F., Hosseinkhani S., Zamani Z., Asadi-Abkenar A., Omidbaigi R. Delay expression of limonoid UDP-glucosyltransferase makes delayed bitterness in citrus. Biochem Biophys Res Commun. 2008;371:59–62.
11. Zandalinas S.I., Sales C., Beltrán J., Gómez-Cadenas A., Arbona V. Activation of secondary metabolism in citrus plants is associated to sensitivity to combined drought and high temperatures. Front Plant Sci. 2017;7:1954.
12. Barreca D., Bellocco E., Caristi C., Leuzzi U.G.O., Gattuso G. Flavonoid composition and antioxidant activity of juices from chinotto (*Citrus* × *myrtifolia* Raf.)

fruits at different ripening stages. J Agric Food Chem. 2010;58:3031–3036.

13. Chaudhary P.R., Bang H., Jayaprakasha G.K., Patil B.S. Variation in key flavonoid biosynthetic enzymes and phytochemicals in 'Rio Red'grapefruit (*Citrus paradisi* Macf.) during fruit development. J Agric Food Chem. 2016;64:9022–9032.
14. Asikin Y., Taira I., Inafuku S., Sumi H., Sawamura M., Takara K., Wada K. Volatile aroma components and antioxidant activities of the flavedo peel extract of unripe Shiikuwasha (*Citrus depressa* Hayata). J Food Sci. 2012;77:C469–C475.
15. Liu C., Yan F., Gao H., He M., Wang Z., Cheng Y., Deng X., Xu J. Features of citrus terpenoid production as revealed by carotenoid, limonoid and aroma profiles of two pummelos (*Citrus maxima*) with different flesh color. J Sci Food Agric. 2015;95:111–119.
16. Chang S.Q., Azrina A. Antioxidant content and activity in different parts of pomelo [*Citrus grandis* (L.) Osbeck] by-products. III International Conference on Agricultural and Food Engineering. 2016;27–34.
17. Arbona V., Iglesias D.J., Gómez-Cadenas A. Non-targeted metabolite profiling of citrus juices as a tool for variety discrimination and metabolite flow analysis. BMC Plant Biol. 2015;15:1–16.
18. Fallah F., Nokhasi F., Ghaheri M., Kahrizi D., Agha A.B., Ghorbani T., Kazemi E., Ansarypour Z. Effect of salinity on gene expression, morphological and biochemical characteristics of Stevia rebaudiana Bertoni under in vitro conditions. Cell Mol Biol. 2017;15:63:102-106.
19. Kahrizi D., Ghari S.M., Ghaheri M., Fallah F., Ghorbani T., Agha A.B., Kazemi E., Ansarypour Z. Effect of KH2PO4 on gene expression, morphological and biochemical characteristics of stevia rebaudiana Bertoni under in vitro conditions. Cellular and Molecular Biology. 2017;15:63:107-111.
20. Sun C., Chen K., Chen Y., Chen Q. Contents and antioxidant capacity of limonin and nomilin in different tissues of citrus fruit of four cultivars during fruit growth and maturation. Food Chem. 2005;93:599–605.
21. Bermejo A., Llosá M.J., Cano A. Analysis of bioactive compounds in seven citrus cultivars. Food Sci Technol Int Sage Publications Sage UK: London, England. 2011;17:55–62.
22. Bilal H., Akram W., Hassan S.A., Sahar S., Munir Iqbal M. Determination of limonin and nomilin contents in different citrus cultivars using high performance liquid chromatography. Pak J Sci Ind Res. 2013;56:36-40.
23. Huang S., Liu X., Xiong B., Qiu X., Sun G., Wang X., Zhang X., Dong Z., Wang Z. Variation in limonin and nomilin content in citrus fruits of eight varieties determined by modified HPLC. Food Sci Biotechnol. 2019;28:641–647.
24. Raithore S., Dea S., McCollum G., Manthey J.A., Bai J., Leclair C., Hijaz F., Narciso A.J., Baldwin E.A., Plotto A. Development of delayed bitterness and effect of harvest date in stored juice from two complex citrus hybrids. J Sci Food Agric. 2015;96:2.
25. Wang Y., Li J., Xia R. Expression of chalcone synthase and chalcone isomerase genes and accumulation of corresponding flavonoids during fruit maturation of Guoqing No. 4 satsuma mandarin (*Citrus unshiu* Marcow). Sci Hortic. 2010;125:110-116.
26. Lewinsohn E., Britsch L., Mazur Y., Gresse J. Flavanone glycoside biosynthesis in citrus. Plant Physiol. 1989;91:1323-1328.
27. Ahmed W., Ahmad S., Malik A.U., Ahmed R. Harvesting times modulates the human health promotoing substances in grapefruit. Pak J Agric Sci. 2015;52:393-401.
28. Moriguchi T., Kita M., Tomono Y., Endo-Inagaki T., Omura M. Gene expression in flavonoid biosynthesis: correlation with flavonoid accumulation in developing citrus fruit. Plant Physiol. 2001;111:66-74.
29. Pichaiyongvongdee S., Haruenkit R. Comparative studies of limonin and naringin distribution in different parts of Pummelo [Citrus grandis (L.) Osbeck] cultivars grown in Thailand. Nat Sci. 2009;43:28-36.
30. Daniel J.J., Owens D.K., McIntosh C.A. Secondary product glucosyltransferase and putative glucosyltransferase expression during Citrus paradisi (c.v. Duncan) growth and development. Phytochem. 2001;72:1732-1738.
31. Bai J., Baldwin E.A., McCollum G., Plotto A., Manthey J.A., Widmer W.W., Luzio G., Cameron R. Changes in volatile and non-volatile flavor chemicals of "Valencia" orange juice over the harvest seasons. Foods. 2016;5:4.