

Investigation of Limonoids, Flavanones, Total Polyphenol Content and Antioxidant Activity in Seven Thai Pummelo Cultivars

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ABSTRACT

Juices from seven pummelo cultivars: Kao Numpueng (KNP), Thong Dee (TD), Kao Pean (KP), Kao Yai (KY), Tha Khoi (TK), Kao Tanggkya (KTG) and Pattavee(PV) were selected for measurement of limonoids, flavanones, polyphenol content and antioxidant activity. Two limonoids were identified as limonin and nomilin. The limonin in pummelo juices ranged from 29.62 to 10.07mg/L, with the decreasing order of cultivars being TD, TK, KNP, KY, PV, KP and KTG. The nomilin ranged from 41.83 to 10.90 mg/L, with the decreasing order of cultivars being TK, KY, KNP, PV, KP, TD and KTG. The total limonoid ranged from 20.97 to 67.35 mg/L. Nine flavanones were identified: naringin, eriocitrin, neoeriocitrin, narirutin, neohesperidin, hesperidin, didymin, poncirin and quercetin. The last three were not found in all of the cultivars, whereas naringin and neoeriocitrin were found in all of them, with naringin being the major flavanone in all cultivars. The naringin content ranged from 386.45 to 242.63 mg/L, with the decreasing order of cultivars being PV, TK, KY, TD, KNP, KP and KTG. The total flavanones ranged from 245.63 to 393.96 mg/L. The antioxidant capacity was measured by DPPH and FRAPS assays, which gave good correlations with the total polyphenol content. The pink cultivars (TK and TD) had better antioxidant capacity than the white ones (KNP, KY, PV, KP and KTG). Therefore, these pink cultivars can be preferentially used for dietary prevention of cardiovascular diseases and are suitable for industrial processing.

Key words: limonoid, flavanones, antioxidant, pummelo

INTRODUCTION

Pummelo, *Citrus grandis* (L) Osbeck, is the largest citrus fruit and many cultivars are grown in Thailand. They can be divided into two groups according to juice color, being either white or pink. Tong Dee and Tha Khoi are in the pink group and the white group includes Kao Yai, Kao Paen, Kao Nampheung, Kao Tanggkya, Kao Hom, Kao Phuang and Pattavee. Citrus fruits have been recognised as a good source of vitamin C and

health-promoting compounds including carotenoids, flavonoids, linonoids and fiber (Yu *et al.*, 2005). Consumption of fruit through their anticarcinogenic antimutagenic activities has been proved beneficial to prevent diseases. Flavonoids have a wide range of biological effects including prevention and control of coronary heart disease and they have anti-inflammatory and antimicrobial activities (Harborne and Williams, 2000; Silberberg *et al.*, 2006). Flavonoids are divided into six classes: flavones, flavanones, flavonols,

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iso-flavones, anthocyanidins and flavanols (Horowitz and Gentili, 1977). The important flavanones found in fruits are: naringin, narirutin, neohesperidin, hesperidin, eriocitrin, neoeriocitrin, quercetin, poncirin and didymin (Albach *et al.*, 1969; Jourdan *et al.*, 1985; Julian *et al.*, 1992; Kawaii *et al.*, 1999). Seven flavanones (neohesperidin, hesperidin naringin, narirutin, poncirin and didymin) were identified in nine commercial grapefruit juices (Ross *et al.*, 2000). Naringin was the most predominant flavanone followed by narirutin and hesperidin or poncirin (Vanamala *et al.*, 2006; Wang *et al.*, 2007; Guihua *et al.*, 2008). Naringenin has been reported to reduce plasma and hepatic cholesterol, to inhibit HMG-CoA reductase and acyl Co-A cholesterol acyltransferase (ACAT) in rats and to reduce the apo-B secretion in Hep-G2 cells (Wilcox *et al.*, 2001). Silberberg *et al.* (2006) studied flavanone metabolism in healthy and tumor-bearing rats (TuB) and reported that total concentrations of naringenin metabolites reached $17.3 \pm 2.7 \mu\text{M}$ in plasma six hours after the beginning of the meal in healthy rats and only $10.6 \pm 1.3 \mu\text{M}$ in TuB rats. The lower concentration of flavanones in the TuB rats suggested that disease, and more particularly cancer, may affect the bioavailability of flavonoids.

Limonoids are a group of chemically related triterpene derivatives present in citrus fruit. The most prominent of this group are limonin and nomilin (Girard and Mazza, 1998). Limonoids can inhibit the development of cancer in laboratory animals and in human breast cancer cells as well as reducing cholesterol (Yu *et al.*, 2005). Guthrie *et al.* (2000) found that limonoids inhibited the proliferation of breast cancer cells grown in culture. The antioxidant activity of citrus fruit is due to the presence of many polyphenols and ascorbic acid. The characteristics of the polyphenols and the antioxidant potential of Thai pummelo have not been reported previously. The objectives of this study were to determine the

content of flavanones, limonoids and the antioxidant capacity of seven Thai pummelo cultivars. The aim is to use the results to promote the pummelo industry worldwide and to establish a database of health-promoting compounds in Thai pummelo fruits.

MATERIALS AND METHODS

Materials

Samples of seven pummelo cultivars, *Citrus grandis* (L.) Osbeck, were collected from orchards in five provinces. The most popular cultivars grown in each province were selected between November 2005 to February 2006 and fruit were harvested at the age of eight months. Five pummelo fruits of each cultivar were used for analysis. The cultivars were: Thong Dee (TD), Kao Paen (KP) and Kao Nampheung (KNP) (Nakhon Pathom province); Kao Yai (KY) (Samut Songkhram province); Tha Khoi (TK) (Phichit province); Kao Tanggkya (KTG) (Chainat province); and Pattavee (PV) (Nakhon Si Thammarat province).

Chemicals

Limonin, nomilin, hesperidin (hesperetin-7-rutinoside, HES), neohesperidin (hesperitin 7-neohesperidin-7-doside, NEH) and gallic acid monohydrate were purchased from Sigma-Aldrich (St Louis, USA). Narirutin (naringenin-7-rutinoside, NAT) was purchased from chromadex (ChromaDex, USA). Naringin (naringenin-7-rhamnosido-glucoside, NAR), eriocitrin (eriodictyol 7-O- β -rutinoside, DID), poncirin (isosakuranetin-7-neohesperidin-7-doside, PON), quercetin dihydrate (3,5,7,30,40-pentahydroxyflavone-dihydrate, QUE), DPPH (2,2-Diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine) were purchased from Fluka (Buchs, USA). Other common reagents were purchased from Merck (Darmstadt, Germany).

Extraction of limonoids from pummelo juices

To determine the limonoid content in the juice, 10 ml of each pummelo juice was prepared from fruit and centrifuged at 2500×g for 10 min. A millipore C18 Sep-pak cartridge was rinsed with 2 ml methanol and then with 5 ml deionized water, before 1 ml of juice supernatant was passed through the cartridge. The cartridge was rinsed with 5 ml of deionized water and limonoid was slowly eluted from the cartridge with 1 ml of methanol. The methanol effluent was filtered through a 0.22 µm nylon filter prior to injection for high performance liquid chromatography (HPLC) (Shaw and Wilson, 1984).

Extraction of flavanones from pummelo juices

The following process was used to determine the flavanone content in the juice. A sample of 1-2 ml of each pummelo juice was extracted with 4 ml of methanol by shaking for 1 min using a vortex mixer and then centrifuged at 2500×g for 10 min. The extract was passed through a 0.22 µm nylon filter prior to injection to HPLC (Rouseff, 1988).

Determination of limonoids and flavanones by HPLC

Limonoids and flavanones were determined by a reverse-phase HPLC method. The system consisted of the water HPLC (USA) system with two hydraulic pumps (model 515), an injection system (U6K), a Novapak C₁₈ Column (3.9×150 mm, pore size 4 µm), a C₁₈ guard column, a UV-VIS detector (model 2478) and a computerized recorder/integrator (model Millennium 32). For limonoid determination, the mobile phase consisted of acetonitrile: deionised water (35:65) with a flow rate of 1 ml/min. The injection volumes of the samples were 20 µl. The detection wavelength was 210 nm.

For flavanones, the mobile phase consisted of acetonitrile and water which was varied in ratio according to individual standards:

naringin (acetonitrile : DI water=25:75); eriocitrin, neoeriocitrin and narirutin (acetonitrile : DI water plus 1% acetic acid=15:85); hesperidin and neohesperidin (acetonitrile : DI water plus 1% acetic acid=17:83); poncirin and quercetin didymin (acetonitrile : DI water plus 1% acetic acid=20:80) with a flow rate of 1 ml/min and the injection volume of the samples was 20 µl. The detection wavelength was 280 nm.

Determination of total polyphenol content

The total polyphenol content in the pummelo juices was determined by the Folin-Ciocalteu method (Singleton *et al.*, 1999). The juice (0.5 ml) was added to 2 ml of 10% Na₂CO₃. After 5 min, 25% Folin-Ciocalteu reagent (0.5 ml) was added to the mixture and allowed to stand for 10 min before measurement. The absorbance was measured at 760 nm using a UV-VIS spectrophotometer (Shimadzu 1601, Japan). The total polyphenol content was expressed as mg gallic acid equivalent GAE/100mg FW.

Determination of antioxidant activity using a free radical scavenging assay (DPPH)

The free radical scavenging DPPH method was used according to Shyu and Hwang (2002), with 0.1 ml of pummelo juice added to 6 ml methanol followed by 0.6 ml of 0.8 mM solution of DPPH. The absorbance was read at 517 nm after 30 min of initial mixing. The same concentration of methanol (6 ml) was used as a control. The inhibitory percentage of DPPH was calculated using Equation 1:

$$\% \text{ inhibition} = [A_0 - A_1 / A_0] \times 100 \quad (1)$$

Where A₀ is the absorbance of the control, A₁ is the absorbance in the presence of the sample.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay method was used

according to Benzie and Strain (1999). The FRAP reagent was composed of: 0.1 M acetate buffer (pH 3.6); 40 mM TPTZ; and 20 mM ferric chloride at the ratio of 10:1:1 by volume. A sample of 0.1 ml of each pummelo juice was added to 3 ml reagent, the absorbance was read at 593 nm and the reaction was monitored for 8 min. The result was expressed as mg trolox equivalent TE/100 ml FW and mg ascorbic acid equivalent AAE/100 ml FW.

RESULTS AND DISCUSSION

Limonoid content in pummelo juices

The total limonoid content, consisting of limonin and nomilin, for the seven pummelo juice cultivars is presented in Table 1. Two major limonoids found in all cultivars were limonin (range 10.07-29.62 mg/L) and nomilin (range 10.90-41.83 mg/L). The amount of nomilin was higher than limonin in most cultivars except Thong Dee. The total limonoid range was from 20.97 to 67.35 mg/L and the mean was 50.49 mg/L. Ohta and Hasegawa (1995) reported that the total limonoid content in pummelo juice was 7 to 71 mg/L (the mean was 29 mg/L). In comparison to other fruits, the limonoid content in pummelo was lower than in grapefruit (190 mg/L), lemon (82 mg/L) and orange juice (320 mg/L) (Fong *et al.*, 1989). Therefore, differences in limonoid content

are partly due to the different kinds of citrus fruit. TK contained the highest limonoid content (67.35 mg/L), whereas the lowest was detected in KTG (20.97 mg/L).

The highest limonin content was in TD and the lowest was in KTG. The decreasing order of cultivars by limonin content was TD, TK, KNP, KY, PV, KP and KTG. Wattanasiritham *et al.* (2005) found that the limonin concentration in the juice of eight pummelo cultivars averaged 21.07 ppm. Ohta and Hasegawa (1995) reported the average limonin content in the pummelo juice of sixteen cultivars was 18 ppm.

Nomilin constituted the greater part of total limonoid in pummelo juice. TK contained the highest amount of nomilin, whereas KTG had the lowest. The decreasing order of cultivars by nomilin content was TK, KY, KNP, PV, KP, TD and KTG. Based on cultivar selection for the juice industry, the lowest limonin content cultivar is preferable due to the bitterness caused by limonin. Barmore *et al.* (1986) reported that the threshold point for sensory detection of limonin in distilled water was 1 mg/L. However, the study by Guadagni *et al.* (1973) showed that 75% of the taster panel could detect limonin in orange juice at a concentration of 5-6 mg/L. Kimball and Norman (1990) also reported that the lowest threshold point of limonin in orange juice was 6 mg/L. Thus, a reduction in limonin concentration

Table 1 Limonin and nomilin content (mg/L) in seven pummelo juices.

Cultivars	Limonin	Nomilin	Total limonoid
KNP	22.69±3.94	35.47±3.86	58.16
KP	18.27±3.70	31.01±4.21	49.28
KTG	10.07±1.64	10.90±3.04	20.97
KY	21.43±3.57	40.61±6.90	62.04
PV	20.02±1.68	31.36±4.38	51.38
TD	29.62±5.42	14.65±1.45	44.27
TK	25.52±3.54	41.83±7.03	67.35
Mean	21.09	29.40	50.49

KNP=Kao Nampheung, KP=Kao Paen, KTG=Kao Tanggkya, KY=Kao Yai, PV=Pattavee, TD=Thong Dee, TK=Tha Khoi.

Values are mean ±SD (N=5).

is necessary in the pummelo juice industry.

Naringin and neoriocitrin were detected in all cultivars. The cultivar order by decreasing naringin content was: PV (386.45 mg/L), TK (381.24 mg/L), KY (364.68 mg/L), TD (348.47 mg/L), KNP (323.00 mg/L), KP (315.71 mg/L) and KTG (242.63 mg/L). The cultivar order by decreasing eriocitrin content was: KY (21.55 mg/L), TD (15.07 mg/L), KNP (5.74 mg/L), TK (4.70 mg/L) and KP (4.20 mg/L). A small amount of hesperidin (2.15 mg/L) was found only in TK, while neohesperidin was found in KNP (0.59 mg/L)

L) and TK (2.18 mg/L). Narirutin was detected in most cultivars except TD and TK. Eriocitrin was not detected in KTG and PV. The total flavanone content ranged from 245.63 to 393.96 mg/L and the mean was 350.04 mg/L. The highest amount was found in TK (393.96 mg/L), whereas the lowest amount was in KTG (245.63 mg/L). Each cultivar contained different amounts of flavanones. Naringin was the most predominant flavanone in the seven cultivars and contributed about 96.40% of the flavanones in pummelo juice. Vanamala *et al.* (2006) found that naringin (304 mg/L) was the

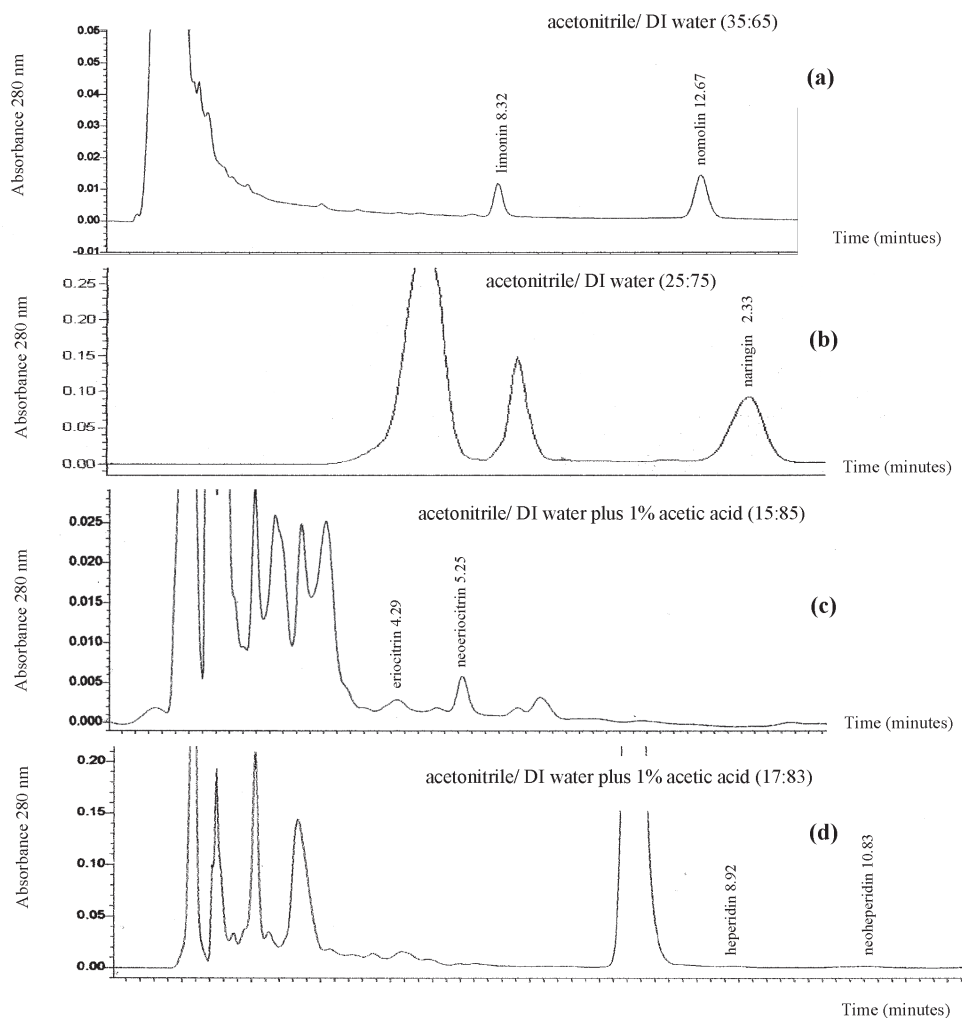


Figure 1 HPLC chromatograms of the pummelo juices in TK: (a) limonin and nomilin; (b) naringin, (c) eriocitrin, neoriocitrin and narirutin; (d) neohesperidin and hesperidin.

Table 2 Flavanone content (mg/L) in seven pummelo cultivars.

Cultivars	Naringin	Eriocitrin	Neo eriocitrin	Narirutin	Neo hesperidin	Hesperidin	Didymin	Poncirin	Quercetin	Total
KNP	323.00±43.62	5.74±0.84	2.70±0.30	0.30±0.03	0.59±0.04	ND	ND	ND	ND	332.33
KP	315.71±34.48	4.20±0.32	4.11±0.66	2.61±0.19	ND	ND	ND	ND	ND	326.63
KTG	242.63±33.64	ND	2.41±0.12	0.59±0.07	ND	ND	ND	ND	ND	245.63
KY	364.68±82.91	21.55±2.59	2.38±0.38	0.12±0.01	ND	ND	ND	ND	ND	388.73
PV	386.45±80.22	ND	1.01±0.09	0.07±0.01	ND	ND	ND	ND	ND	387.53
TD	348.47±54.93	15.07±2.34	11.93±1.93	ND	ND	ND	ND	ND	ND	375.47
TK	381.24±67.19	4.70±0.68	3.69±0.62	ND	2.18±0.32	2.15±0.11	ND	ND	ND	393.96
Mean	337.45	7.32	4.03	4.03	0.39	0.30	ND	ND	ND	350.04

KNP=Kao Nampheung, KP=Kao Paen, KTG=Kao Tanggkya, KY= Kao Yai, PV=Pattavee, TD =Thong Dee, TK =Tha Khoi. Values are mean ±SD (N=5).

ND = not detected

predominate flavanone in grapefruit juice followed by narirutin (101 mg/L) and pocerin (12.4 mg/L). Xu *et al.* (2008) also reported two pummelo cultivars, Miyou and Sijiyu, where naringin was the major flavanone followed by hesperidin. Narirutin was not detected in those two pummelo cultivars and neohesperidin was not detected in Sijiyu. Wattanasiritham *et al.* (2005) did not detect hesperidin and neohesperidin in pummelo juices.

The content of flavanones in each cultivar of pummelo juice could be used to identify pummelo cultivars.

Antioxidant capacity of pummel juices

A FRAP assay can evaluate antioxidant activities in a relatively short time compared with other methods. A DPPH assay measures the radical scavenging activity expressed as a percentage,

while FRAP measures the ability of antioxidants to reduce ferric tripyridyl triazining (F^{+3}) to a ferrous form (F^{+2}).

Polyphenols have reportedly been linked with the antioxidant capacity of fruits. Increasing the total polyphenol content also increased the antioxidant efficacy in fruits (Proteggente *et al.*, 2003). Total polyphenols were reported to be the major antioxidant of citrus fruits (Rapisarda *et al.*, 1999; Sun *et al.*, 2002). The total polyphenol content in seven pummelo cultivars is presented in Table 3. It was found that the polyphenol content ranged from 63.96 to 150.30 GAE mg/100ml FW. The polyphenol content in the pink-juice cultivars, TK and TD, was clearly higher than in the white-juice cultivars. Tsai *et al.* (2007) showed that pink pummelo juice had higher total polyphenol content and antioxidant ability than white pummelo juice due to pigments. The carotenoid content in pink

Table 3 Total polyphenol content and antioxidant activity in seven pummelo juices.

Cultivars	Total PPO (GAE mg /100ml FW)	FRAP		DPPH (%)
		mg AAE /100ml FW	mg TE /100ml FW	
KNP	58.64±4.22	19.28±1.41	28.44±2.21	22.21±0.71
KP	40.66±6.44	12.40±0.26	17.65±0.40	10.75±1.00
KTG	63.96±2.63	20.39±1.02	30.17±1.60	16.68±0.89
KY	65.82±2.60	21.37±0.63	31.71±0.99	16.99±0.74
PV	71.62±13.01	23.54±0.62	35.10±0.97	18.35±2.07
TD	137.04±7.16	30.32±0.88	43.95±1.38	25.03±2.89
TK	150.30±18.94	35.10±1.86	54.71±6.64	25.62±1.04
Mean	84.01	23.20	34.53	19.38

KNP=Kao Nampheung, KP=Kao Paen, KTG=Kao Tanggkya, KY= Kao Yai, PV=Pattavee, TD =Thong Dee, TK =Tha Khoi. Values are mean ±SD (N=5).

pummelo was also responsible for its characteristic color and was significantly higher than that found in white pummelo. Anthocyanins are a class of flavonoids and a large family of polyphenolic compounds synthesized by plants. It has been reported that anthocyanins and other flavonoids in fruits may be responsible for antioxidant capacity (Cao *et al.*, 1996; Prottengente *et al.*, 2003). Similar results were found in the current study, where TK and TD (pink cultivars) had a high content of flavanones, whereas the white cultivars, such as KTG, had very low flavanone content. Furthermore, the antioxidant capacity of TK and TD measured by FRAP and DPPH assay was higher than in other cultivars. The order of polyphenol content by cultivars was TK, TD, PV, KY, KTG, KNP and KP. This corresponded well to the antioxidant capacity measured by FRAP and DPPH assay. In the current study, TK and TD (pink pummelo juices) were valuable cultivars, with higher total polyphenol content and higher antioxidant capacity than KNP, KP, KY, KTG and PV (white pummelo juices). The variations in polyphenol content and antioxidant capacity were probably due to the pigments in the juices.

The correlation between total polyphenols and two scavenging assays provided a strong correlation between antioxidant capacity and total polyphenols (FRAP expressed as AAE($r=0.9639$); FRAP expressed as TE ($r=0.9531$); and DPPH ($r=0.8446$), respectively). Antioxidant capacity was not correlated with limonin, nomilin and naringin, which demonstrated that limonin, nomilin and naringin played a minimal role in the antioxidant capacity of pummelo juices. The antioxidant power of flavanones was also reported by Majo *et al.* (2005). They found that hesperidin had the highest antioxidant activity ($Ka/Kc=2.81$) followed by narirutin ($Ka/Kc=2.46$), naringin ($Ka/Kc=2.41$) and neohesperidin ($Ka/Kc=2.14$). In the current study, the highest antioxidant activity was found in the TK cultivar because only this one had

hesperidin. Majo *et al.* (2005) suggested that the antioxidant activity of flavanones *in vitro* depended on the kind of sugar in the 7th position (neohesperidoside or rutinose) and the arrangement of functional groups about the nuclear structure (position of methoxyl group; 3th or 4th position).

The results of this investigation have shown that the total polyphenol, limonoid and flavanone content and the antioxidant potential differed from values reported in other literature; these depended on the cultivars.

CONCLUSIONS

Seven Thai pummelo cultivars had high antioxidant capacity and total polyphenol content. The amount of bioactive compounds in pummelo juices was related to the cultivar. Two limonoids, limonin and nomilin, were identified in all cultivars. Nomilin was found in greater amounts than limonin. Nine flavanones were identified: naringin, neoriocitrin, eriocitrin, narirutin, neohesperidin, hesperidin, didymin, poncirin and quercetin. Naringin and neoriocitrin were found in all cultivars and no cultivars contained all nine flavanones. Didymin, poncirin and quercetin dihydrate were not found in all of the cultivars, whereas naringin was the predominant flavanone. Naringin was the predominant flavanone in all pummelo juices. The flavanones detected in each cultivar suggested they could be used for identification of pummelo juice cultivars.

The total polyphenol content and the antioxidant activity were higher in TK and TD (pink cultivars) than in PV, KY, KTG and KNP. The higher antioxidant activity of pink cultivars than white cultivars could make them more preferable for dietary prevention of cardiovascular diseases and suitable for industrial processing. Relationships between the total polyphenols and antioxidant potential (obtained from FRAP and DPPH) were high.

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