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Cultivar variations in antioxidant and antihyperlipidemic properties of pomelo pulp (*Citrus grandis* [L.] Osbeck) in Thailand

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ABSTRACT

Pomelo (*Citrus grandis* L. Osbeck) is a native fruit of great economic importance in Southeast Asia. To provide experimental evidence for the antioxidant and antihyperlipidemic properties of pomelo, 6 cultivars, including Kao-Yai (KY), Thong-dee (TD), Kao-Tangkwa (KT), Kao-Numpueng (KN), Ta-Koi (TK), and Tubtim Siam (TS) were evaluated. KY had the highest phenolic content, and the strongest 1,1-diphenyl-2-pireyhydrazyl radical scavenging capacity and hydroxyl radical scavenging activity. From the high-performance liquid chromatography analysis, naringin and naringenin were the major flavonoids in the KT and TK cultivars. Six pomelo cultivars had antihyperlipidemic activities including the inhibition of pancreatic lipase and cholesterol esterase, as well as cholesterol micelle formation and bile acid binding. Hierarchical clustering analysis showed that the 6 cultivars were separated into 2 classifications. In addition, the total phenolics of the pomelo cultivars were significantly correlated with ferric reducing antioxidant power and Trolox equivalent antioxidant capacity. The results suggest that pomelo provides significant health benefits and may be used for developing functional foods.

1. Introduction

Consumption of fruits and vegetables plays a vital role in the prevention and treatment of various diseases. Fruits and vegetables are known to be rich sources of polyphenolic compounds, particularly flavonoids. Plant flavonoids have been attracting interest because of their significant bioactivities. The health benefits of flavonoids, including antioxidant, antithrombotic, antidiabetic, anticancer, and vasodilatory activities, have been reported (Duarte et al., 1993; Pandey & Rizvi, 2009; Ren, Qiao, Wang, Zhu, & Zhang, 2003; Vessal, Hemmati, & Vasei, 2003). Recent research has shown that the consumption of plant flavonoids may help protect against cardiovascular diseases (Knekt et al., 2002). The *Citrus* genus includes some of the most widely cultivated crops in the world because of their many nutritional and health benefits. Originating in the warm tropical climates of Southeast Asia, pomelo (*Citrus grandis* L. Osbeck), belongs to the family Rutaceae, and is one of the most widely cultivated crops under a variety of ecological conditions in Thailand. There are a rich variety of pomelo cultivars, including *C. grandis* 'Kao-Yai', *C. grandis* 'Thong-dee', *C. grandis* 'Kao-Tangkwa', *C. grandis* 'Kao-Numpueng', *C. grandis* 'Ta-Koi', and *C. grandis* 'Tubtim Siam'. Since ancient times, the pulp has been used as appetizer, antitoxic, cardiac stimulant, and stomach tonic (Arias & Ramón-Laca, 2005). The major flavanoids of pomelo are neohesperidin, hesperidin, naringenin, and naringin, which are high in pulp and fruit juice (Kanes, Tisserat, Berhow, & Vandercook, 1993; Kawaii, Tomono, Katase, Ogawa, & Yano, 1999; Xu et al., 2008). Recent reports have shown that phenolic-enriched extracts from pomelo inhibit α-amylase, α-glucosidase and angiotensin I-converting enzyme (ACE) enzyme activities (Oboh & Ademosun, 2011). Extensive studies of pomelo extract have revealed its favourable antioxidant properties using the ferric reducing antioxidant power (FRAP) assay *in vitro* (Guo et al., 2003). In addition, it has been shown to reduce reactive oxygen species in H_2O_2-treated HepG2 cells (Lim, Yoo, Moon, Jeon, & Cho, 2006). Interestingly, it has been reported that the phytochemical profile varies with the species and cultivars, which can exhibit different biological properties, especially antioxidant activity (Balasundram, Sundram, & Samman, 2006; Kim, Jeong, & Lee, 2003; Lee, Kim, Kim, Lee, & Lee, 2003). Furthermore, so far, there has been no report on the possible antihyperlipidemic activity of pomelo cultivars.

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Therefore, it was interesting to investigate the phenolic and flavonoid content and bioactivity of six pomelo cultivars related to antioxidants, including 1,1-diphenyl-2-pireyhydrazyl (DPPH) radical scavenging activity, Trolox equivalent antioxidant capacity assay (TEAC), FRAP assay, oxygen radical absorbance capacity (ORAC) assay, hydroxyl radical scavenging activity (HRSA), and superoxide radical scavenging activity (SRSA). In addition, antihyperlipidemic activity, including the inhibition of pancreatic lipase and pancreatic cholesterol esterase activities, as well as cholesterol micelle formation and bile acid binding, were also determined.

2. Materials and methods

2.1. Chemicals

Naringin, hesperidin, neohesperidin, naringenin, neohesperidin dihydrochalcone, hesperitin, *p*-nitrophenylbutylrate (*p*-NPB), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), oleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis3-ethylbenzothiazoline-6-sulphonic acid (Trolox), 2,4,6-tripyridyl-*S*-triazine (TPTZ), fluorescein, 2,2′-azobis-2-methyl-propanimidamide, dihydrochloride (APPH), xanthine, xanthine oxidase, phosphatidylcholine, glycodeoxycholic acid, taurodeoxycholic acid, taurocholic acid, deoxyribose, porcine cholesterol esterase, porcine pancreatic lipase, and 4-methylumbelliferone were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Cholesterol test kits were purchased from HUMAN GmbH Co. (Wiesbaden, Germany). A total bile acid kit was purchased from Bio-Quant Co. (San Diego, CA, USA). All other chemical reagents used in this study were of analytical grade.

2.2. Preparation of extract

As shown Fig. 1, the 6 pomelo cultivars (Kao-Yai, Thong-dee, Kao-Tangkwa, Kao-Numpueng, Ta-Koi, Tubtim Siam) were obtained from a local market and harvested at the mature stage. The pulp of pomelo was collected by manual peeling and then homogenized using a commercial blender (Moulinex, Thailand). The pulp was lyophilized and exhaustively extracted in a 2-step aqueous methanol process at 4 °C for 6 consecutive days. The supernatant was evaporated in a rotary evaporator at 60 °C and stored in the dark under vacuum desiccation, at room temperature. Thereafter, the dried extract was purified to remove sugars and organic acids using a Sep-Pak C_{18} Cartridge (Li, Smith, & Hossain, 2006). The purified extracts were evaporated in a rotary evaporator at 60 °C and stored at −20 °C.

2.3. Total phenolic content

The total phenolic content in the pomelo extract was determined using Folin–Ciocalteu reagent (Yoo, Hwang, Park, & Moon, 2009). A sample of purified extract (50 μL) was mixed with 1.5 mL of the reagent (previously diluted 10-fold with distilled water), followed by 50 μL of aqueous Na_2CO_3 (60 g/L). The absorbance was then measured at 725 nm after incubation for 90 min. The results were expressed as mg gallic acid equivalent/g dry weight of extract.

2.4. Quantification of flavonoid constituents

The method of analysis was slightly modified according to a previous report (Zhang, Duan, Zang, Huang, & Liu, 2011). The flavonoid content in the pomelo extract was determined by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) consisting of a binary pump (model LC-10A), auto-injector (model SIL-10A), and UV detector (model SPD-10A). Flavonoid separation was carried out using reversed-phase Vertic Sep™ UPS C-18 column (4.6 × 250 mm, 5 μm, Vertical Chromatography, Bangkok, Thailand). The mobile phase consisted of (A): water/acetic acid

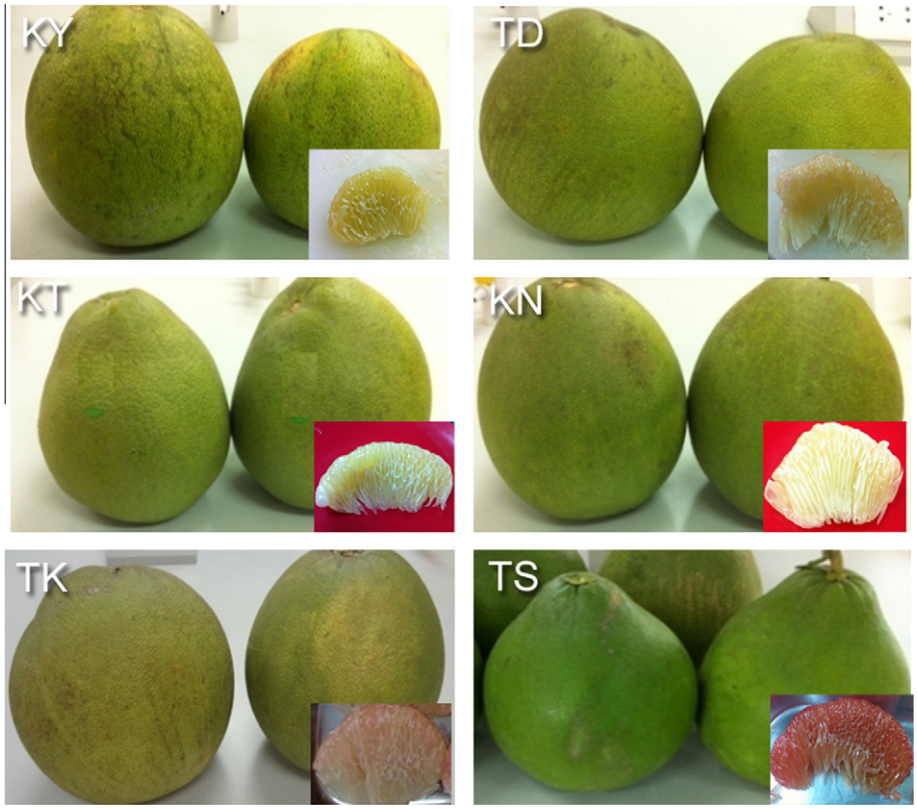

Fig. 1. Photos of the 6 cultivars of pomelo used in the present study.

(99:1, v/v) and (B): acetone nitrile/acetic acid (99:1, v/v). The gradient was as follows: 0 min, 5% B; 5 min, 8% B; 7 min, 12% B; 12 min, 18% B; 17 min, 22% B; 22 min, 25% B; 27 min, 35% B; 37 min, 53% B; 38 min, 53% B; 40 min, 55% B; 42 min, 60% B; 57 min, 80% B; 60 min, 85% B; 65 min, 85% B. The flow rate was 1.2 mL/min. The chromatograms were captured at 280 nm. Flavonoids were quantified using naringin, hesperidin, neohesperidin, neohesperidin dihydrochalcone, naringenin, and hesperitin as the standards. Chromatographic identification and confirmation of flavonoid compounds was based on comparing their retention times with those of authentic standards and spiking the samples with standard solutions. Eight-point calibration curves (0–250 μg/mL) were used for each standard. For naringin, hesperidin, neohesperidin, neohesperidin dihydrochalcone, naringenin, and hesperitin, the equations for the standard curve were $y = 12{,}472x + 22{,}775$ with R^2 value of 0.9979, $y = 1486.5x - 1710.2$ with R^2 value of 0.9950, $y = 5351.9x - 37{,}221$ with R^2 value of 0.9819, $y = 13{,}293x - 79{,}094$ with R^2 value of 0.9900, $y = 10{,}337x - 81{,}350$ with R^2 value of 0.9753, and $y = 15{,}854x - 83{,}875$ with R^2 value of 0.9839, respectively.

2.5. DPPH radical scavenging activity

Antioxidant capacity was measured using the DPPH assay as described previously (Tippani et al., 2010). Briefly, the sample (100 μL) was added to 100 μL DPPH solution (0.2 mM in ethanol) and incubated for 30 min at room temperature. The decrease in the solution absorbance was measured at 515 nm. The DPPH radical scavenging activity was calculated from a standard curve using ascorbic acid. The DPPH radical scavenging activity was expressed as an equivalent of ascorbic acid (mg ascorbic acid/g dried extract).

2.6. Trolox equivalent antioxidant capacity assay

The 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) free radical scavenging activity of each sample was determined according to a previously described method (Madhujith, Izydorczyk, & Shahidi, 2006). The $ABTS^{\cdot+}$ radical was generated by persulfate oxidation of ABTS by incubation at room temperature for at least 16 hours in the dark. An $ABTS^{\cdot+}$ solution was diluted with 0.1 M phosphate buffer solution to absorbance values of 0.70 ± 0.02 at 734 nm. For measuring antioxidant capacity, 25 μL of the sample was added to 75 μL of the $ABTS^{+}$ solution. The decrease in the absorbance was measured at 734 nm after 6 min. TEAC values were calculated from a standard curve using Trolox. TEAC values were expressed as nano moles of Trolox equivalents per gram of dried extract.

2.7. Ferric reducing antioxidant power assay

The reducing power was measured by a modified method of Benzie and Strains (Benzie & Strain, 1996). Briefly, a FRAP solution was mixed with 10 mL of 0.3 M sodium acetate buffer solution (pH 3.6), 1 mL of 10 mM 2,4,6-tripyridyl-*S*-triazine (TPTZ) in 40 mM HCl, and 1 mL of 20 mM $FeCl_3$. The sample (10 μL) was added to 190 μL of FRAP solution as an oxidizing reagent and incubated for 4 min at room temperature. The increase in the solution absorbance was measured at 593 nm. FRAP values were calculated from a standard curve prepared using ascorbic acid. FRAP values were expressed as micromole ascorbic acid/gram dried extract.

2.8. Oxygen radical absorbance capacity assay

The ORAC assay was modified according to a previously described method (Wang, Meckling, Marcone, Kakuda, & Tsao, 2011). Briefly, 25 μL of the extract and 150 μL of 48 nM fluorescein solution were mixed and placed in the wells of a microplate. The mixture was preincubated for 10 min at room temperature. A free radical generator solution (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride; APPH; 25 μL; 64 mM) was added. The fluorescence intensity was recorded every 2 min for 60 min with emission and excitation at 535 and 485 nm, respectively. A standard curve was generated with a Trolox concentration range from 0.024 to 3.125 μM. The ORAC values were calculated as the area under the curve (AUC) and expressed as micromoles of Trolox equivalent (TE) per gram of dry extract.

2.9. Hydroxyl radical scavenging activity

The HRSA measurement was done according to a previously described method. (Halliwell, Gutteridge, & Aruoma, 1987). The reaction mixture was generated by adding 30 μL of 2-deoxy-2-ribose (17 mM), 30 μL of the extract, 30 μL of 1.2 mM EDTA, 60 μL of 0.3 mM $FeCl_3$, 30 μL of 34 mM hydrogen peroxide (H_2O_2), and 60 μL of 0.6 mM ascorbic acid. The reaction was performed at 37 °C for 1 h. Thereafter, 150 μL of 1% (w/v) thiobarbituric acid (TBA) and 300 μL of 2.8% (w/v) trichloroacetic acid (TCA) were added to the mixture, which was then incubated at 100 °C for 15 min. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. HRSA values were calculated from a standard curve using Trolox. HRSA values were expressed as milligrams of Trolox equivalents per milligram of dried extract.

2.10. Superoxide radical scavenging activity

SRSA measurement was done according to a previously described method (Kweon, Hwang, & Sung, 2001). In brief, 7.5 μL of the extract, 150 μL of 0.30 mM xanthine, 50 μL of 0.15 mM nitrobluetetrazolium (NBT), 50 μL of 0.60 mM EDTA, and 7.5 μL of xanthine oxidase (0.05 U/mL) were mixed and placed in the wells of a microplate. After incubation for 40 min at 37 °C, the absorbance was measured at 560 nm against an appropriate blank to determine the quantity of formazan generated. SRSA values were calculated from a standard curve using Trolox. SRSA values were expressed as milligrams of Trolox equivalents per milligram of dried extract.

2.11. Pancreatic lipase inhibition

Pancreatic lipase activity was slightly modified according to a previously described method with minor modifications (Adisakwattana, Intrawangso, Hemrid, Chanathong, & Mäkynen, 2012). The extract (5 μL) dissolved in DMSO and 25 μL of the pancreatic lipase solution (50 U/mL) was mixed in a well of a microplate. Then, a 50-μL volume of 0.1 mM oleate ester of fluorescent 4-methylumbelliferone (4-MUO) solution (0.1 mM) and 20 μL of 13 mM Tris–HCl buffer containing 150 mM NaCl, and 1.3 mM $CaCl_2$, pH = 8.0, were added to the solution. After incubation at room temperature for 30 min, 100 μL of 0.1 M sodium citrate (pH = 4.2) was added to stop the reaction. The amount of 4-MUO released by the lipase was measured using a fluorescence microplate reader with excitation at 320 nm and emission at 450 nm.

2.12. Pancreatic cholesterol esterase inhibition

Pancreatic cholesterol esterase activity was measured according to a previously described method (Adisakwattana et al., 2012). The extracts (5 μL) were incubated with 12 mM taurocholic acid (50 μL), 20 mM *p*-NPB (5 μL), and 100 mM sodium phosphate-buffered saline (PBS; 30 μL), pH = 6.9. The reaction was initiated by adding 10 μL of porcine pancreatic cholesterol esterase (1 μg/

mL). After incubation for 5 min at room temperature, the absorbance of the mixtures was measured at 405 nm.

2.13. Cholesterol micellization

Artificial micelles were prepared according to a previously described method with minor modifications (Ngamukote, Mäkynen, Thilawech, & Adisakwattana, 2011). In short, the mixtures (2 mM cholesterol, 1 mM oleic acid, and 2.4 mM phosphatidylcholine) were dissolved in methanol and dried under nitrogen before adding 475 μL of 15 mM PBS containing 6.6 mM taurocholate salt, at pH = 7.4. The emulsion was sonicated twice for 30 min using a sonicator. The micelle solution was incubated overnight at 37 °C. The extract (25 μL) or equivalent PBS used as control were added to the mixed micelle solution and incubated for a further 2 h at 37 °C. The mixture was then centrifuged at 16,000 rpm for 20 min. The supernatant was collected for the determination of cholesterol using total cholesterol test kits.

2.14. Bile acid binding

The bile acid binding assay was slightly modified according to a previously described method (Adisakwattana et al., 2010). Taurocholic acid, glycodeoxycholic acid, and taurodeoxycholic acid were used as bile acids in this experiment. Briefly, 100 μL of the extract was incubated with 100 μL of 2 mM bile acid and 800 μL of 0.1 M PBS, pH = 7, at 37 °C for 90 min. The mixture was filtered through a 0.2 μm filter to separate the bound from the free bile acids. The bile acid concentration was analysed using a bile acid analysis kit.

2.15. Statistical analyses

The IC_{50} values were calculated from plots of log concentration of inhibitor vs. percentage inhibition. Values were expressed as mean ± standard error of the mean (SEM) for $N = 3$. Data were analysed using one-way analysis of variance (ANOVA) and Duncan's multiple-range tests with $p < 0.05$ were considered significant. Due to the relatively small sample size, hierarchical cluster analysis was used to group cultivars based on similarities in their antioxidant capacities and antihyperlipidemic activities. Between-groups linkage was performed by the cluster method with squared Euclidian distance measurement intervals. Hierarchical cluster analysis was done using average DPPH, TEAC, FRAP, ORAC, HRSA, SRSA, the percentage inhibition of pancreatic lipase, cholesterol esterase, and cholesterol micellization as well as the percentage of bile acid binding for each cultivar with standardization of all variables using z scores. The results of the analysis were visualized using a dendrogram plot. A 2-tailed Spearman's correlation was conducted to determine the correlations among means.

3. Results and discussion

Phenolic compounds are considered major contributors to the antioxidant and antihyperlipidemic activities of edible fruits. We examined the total phenolic content of 6 varieties of pomelo. As shown in Table 1, the total phenolic content of all pomelo cultivars tested in this study ranged from 101.32 to 113.73 mg gallic acid equivalent/g extract. We used HPLC to quantify the flavonoids in the pulp extracts of these 6 pomelo cultivars. The content of flavonoids in KY, TD, KT, KN, TK, and TS is shown in Table 1. For the 6 pomelo cultivars, naringenin determined by HPLC ranged from 7.39 to 29.52 μg/mg dried extract. The KN had the highest naringenin content in the pulp, followed by KT > TD > KY > TS ≅ TK. The naringin content ranged from 2.34–41.29 μg/mg dried extract. TK and KN had the highest and lowest concentration of naringin in all the pomelo varieties studied, respectively. Neohesperidin was found in 5 pomelo cultivars, and its concentration generally followed the order of TK, TS, TD, and KY. In the meantime, hesperidin was also detected in KY, TD, and KN, ranging from 10.08 to 22.78 μg/mg dried extract. In addition, hesperitin was found in both TD and KT, while neohesperidin dihydrochalcone was the only flavonoid found in KT. In a previous study, citrus flavonoids have also been identified in the pulp of pomelo such as naringin, narirutin, neohesperidin, and kaempferol (Abeysinghe et al., 2007). Our results indicate that the identified flavonoids in the present study are consistent with previous findings (Kim, Shin, & Jang, 2009; Zhang et al., 2011). In general, naringin has been used as a marker compound for the chemical evaluation or quality standardization of pomelo (Sudto, Pornpakakul, & Wanichwecharungruang, 2009). Our results have shown that naringin was the dominant flavonoid in 2 pomelo cultivars (KT and TK), consistent with previous studies (Xu et al., 2008; Zhang et al., 2011). The naringin content in pomelo juices has been shown in various studies. For example, the naringin content in the juice of 2 pomelo cultivars (Miyou and Sijiyou) was found to be 10.8% and 12.59%, respectively (Xu et al., 2008). In addition, the naringin content was 11.12% in Cheju pomelo juice (Kim et al., 2009), and 1.62% in the fruit pulp of Mauritian pomelo (Ramful, Tarnus, Aruoma, Bourdon, & Bahorun, 2011).The naringin content in the 6 pomelo cultivars in our study ranged from 0.234% to 4.129%; this differs from other studies. It has been reported that the extraction method affects the naringin content in citrus pomelo (Sudto et al., 2009). Prior to chemical identification using HPLC, we used methanol for the extraction of freeze-dried pomelo pulp powder followed by a Sep-Pak C_{18} Cartridge to remove sugars and organic acids that might interfere with the biological activity. Other reports have reported extraction using distilled water, 80% methanol, or fresh pomelo juice, followed by chemical identification.

The DPPH, TEAC, FRAP, ORAC, HRSA, and SRSA values of the pulp extract of the 6 pomelo cultivars are shown in Table 2. KY had the highest DPPH radical scavenging activity, followed by TD, TS, KT, and KN, while TK exhibited the lowest DPPH radical scavenging activity. In the TEAC assay, the antioxidant capacity ranged from 356.17–1,139.87 μmol Trolox/g dried extract. The TEAC value was in the following order, from highest to lowest: KY ≅ KN ≅ TK > TS > KT > TD. Furthermore, KN had the highest FRAP antioxidant capacity, while TD had the lowest FRAP antioxidant capacity among all the pomelo varieties studied. Among the 6 pomelo cultivars, TD and KT had the highest and lowest ORAC values, at 52.81 ± 1.85 and 3.23 ± 0.45 μmol Trolox/g dried extract, respectively. Consequently, the highest HRSA value was found in KY, whereas TK showed the lowest value, which was in agreement with the DPPH result. In the SRSA assay, KT demonstrated the highest antioxidant activity, whereas KY showed the lowest antioxidant activity among the 6 pomelo cultivars. Free radicals play a crucial role in the pathogenesis of several human diseases, such as rheumatoid arthritis, diabetes and its complications, cancer, and various neurodegenerative, and pulmonary diseases (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2008). Antioxidants from natural products protect against these radicals and are therefore important to the diet in obtaining and preserving good health. Many epidemiological studies show that phenolics and flavonoids have beneficial effects on human health because of their antioxidant activity (Fang, Yang, & Wu, 2002). Juices from 15 citrus varieties of China have been investigated for their antioxidant capacities. Sweet orange (*Citrus sinensis* Osbeck) was found to have the highest FRAP value of 899.31 ascorbic acid equivalent antioxidant capacity (AEAC), mg/L) among the 15 selected citrus varieties (Xu et al., 2008). Two cultivars of pomelo (Miyou and Sijiyou) had FRAP values of 510.16 and 442.22 AEAC, mg/L) which were lower than that of sweet orange. The literature has documented the

Table 1
Total phenolic content (TPC) and flavonoid constituents in the pulp extract of the 6 pomelo cultivars.

Pomelo cultivars	TPC	Naringin	Hesperidin	Neohesperidin	Neohesperidin dihydrochalcone	Naringenin	Hesperitin
KY	113.73 ± 0.67[a]	11.90 ± 0.21[a]	12.04 ± 0.12[a]	25.4 ± 0.12[a]	ND	9.20 ± 0.19[a]	ND
TD	101.32 ± 1.62[b]	8.13 ± 0.13[b]	10.08 ± 0.12[a]	10.76 ± 0.03[b]	ND	10.89 ± 0.15[a]	3.13 ± 0.01
KT	102.57 ± 0.51[b]	40.65 ± 0.39[c]	ND	ND	12.27 ± 0.66	12.23 ± 0.98[b]	4.79 ± 0.10
KN	115.02 ± 0.83[a]	2.34 ± 0.11[d]	22.78 ± 0.33[b]	14.76 ± 0.15[c]	ND	29.52 ± 0.40[c]	ND
TK	110.52 ± 1.00[a,c]	41.29 ± 0.43[e]	ND	36.79 ± 0.25[d]	ND	7.39 ± 0.15[d]	ND
TS	107.23 ± 0.62[c]	26.31 ± 0.44[f]	ND	29.92 ± 0.18[e]	ND	7.40 ± 0.04[d]	ND

ND, not detected. Data are expressed as mean ± SEM; $n = 3$. KY, Kao-Yai; TD, Thong-dee; KT, Kao-Tangkwa; KN, Kao-Numpueng; TK, Ta-Koi; TS, Tubtim Siam. Values in the same column sharing different letters are expressed as significantly different ($p < 0.05$). Total phenolic content of pomelo was expressed as mg gallic acid equivalent/g dry weight of extract. The flavonoid composition was determined by HPLC and expressed as μg/mg dry weight of extract.

Table 2
DPPH, TEAC, FRAP, ORAC, HRSA, and SRSA values of the pulp extract of the 6 pomelo cultivars.

Pomelo cultivars	DPPH	TEAC	FRAP	ORAC	HRSA	SRSA
KY	13.77 ± 0.66[a]	1,055.60 ± 3.99[a]	443.56 ± 1.47[a]	14.62 ± 2.63[a]	9.86 ± 0.40[a]	0.48 ± 0.06[a]
TD	10.97 ± 0.99[b]	356.17 ± 1.58[b]	345.78 ± 2.42[b]	52.81 ± 1.85[b]	6.81 ± 0.44[b]	0.72 ± 0.03[b]
KT	6.34 ± 0.63[c]	502.46 ± 4.20[c]	395.22 ± 0.56[c]	3.23 ± 0.45[c]	5.97 ± 0.03[b]	0.80 ± 0.14[b]
KN	1.45 ± 0.49[d]	1,129.05 ± 24.96[a]	616.89 ± 7.09[e]	6.30 ± 0.85[c]	6.19 ± 0.21[b]	0.62 ± 0.06[a,b]
TK	0.41 ± 0.27[d]	1,139.87 ± 139.86[a]	386.33 ± 6.01[c,d]	51.44 ± 1.88[b]	3.69 ± 0.14[c]	0.65 ± 0.09[a,b]
TS	8.64 ± 0.79[c,b]	634.83 ± 5.40[c]	377.44 ± 7.09[d]	29.24 ± 4.04[d]	3.74 ± 0.41[c]	0.61 ± 0.06[a,b]

Data are expressed as mean ± SEM; $n = 3$. KY, Kao-Yai; TD, Thong-dee; KT, Kao-Tangkwa; KN, Kao-Numpueng; TK, Ta-Koi; TS, Tubtim Siam. DPPH radical scavenging activity was expressed as milligram ascorbic acid/gram dried extract. TEAC was expressed as micromole Trolox/gram dried extract. FRAP was expressed as micromole ascorbic/gram dried extract. ORAC was expressed as micromole Trolox/gram dried extract. Hydroxyl radical scavenging activity (HRSA) was expressed as milligram Trolox/milligram dried extract. Superoxide radical scavenging activity (SRSA) was expressed as milligram Trolox/milligram dried extract. Values in the same column that have different superscripted letters are significantly different ($p < 0.05$).

antioxidant activity of pomelo through its ability to inhibit the formation of free radicals generated by the 1,1-diphenyl-2-picrylhydrazyl radical and to inhibit the oxidation of $ABTS^{+\cdot}$ (Jayaprakasha, Girennavar, & Patil, 2008; Lim et al., 2006). In addition, pomelo extract has also been shown to decrease peroxyl radicals through the oxygen radical absorbance capacity (ORAC) assay (Jayaprakasha et al., 2008). The results demonstrated that methanol extracts from the freeze-dried edible parts of pomelo showed the highest ORAC value among various types of solvent extractions. Moreover, juices from Thai pomelo could reduce Fe^{3+} to Fe^{2+}, which suggests its ability to suppress the formation of the Fenton reaction and hence impede the formation of a highly reactive hydroxyl radical (Pichaiyongvongdee & Haruenkit, 2009). This study reveals that the juice from TK and TD cultivars is valuable, with a higher total antioxidant capacity (DPPH and FRAP) than other pomelo cultivars. However, contrasting reports at present indicate that KY and KN have the highest DPPH and FRAP values among the 6 pomelo cultivars, respectively. It has been reported that the antioxidant capacities of cultivars can exhibit different biological properties because of growing in different environmental conditions during years (Dhuique-Mayer, Caris-Veyrat, Ollitrault, Curk, & Amiot, 2005; Yoo, Lee, Park, Lee, & Hwang, 2004). Further studies are needed to determine the antioxidant activities of different pomelo cultivars from various climatic regions and seasons in Thailand.

There has recently been a great deal of interest in polyphenolic compounds, particularly flavonoids, as antioxidants. The current study was the first comprehensive investigation to determine the antioxidant activity of 6 different cultivars of pomelo. In some studies, flavonoids in the pomelo extract have shown antioxidant activity that is directly related to their ability to inhibit free radical formation and lipid peroxidation (Zarina & Tan, 2013).

In addition, the main flavonoids in pomelo such as hesperidin and neohesperidin dihydrochalcone have shown to be effective in inhibiting superoxide formation and scavenging reactive oxygen species (Suarez, Herrera, & Marhuenda, 1998). These flavonoids can trap reactive oxygen species and inhibit free radical-induced oxidation by direct scavenging of free radicals. This is because of the phenolic hydroxyl groups in the structure, which allow the ready donation of electrons to unstable free radicals. Consequently, these radicals become more stable and less reactive (Nijveldt et al., 2001). These results suggest that the antioxidant activity of pomelo may be mediated by flavonoids.

Citrus fruits contain an array of potent antioxidants including flavonoids, vitamin C, and carotenoids. A limited amount of literature exists on antioxidant activity in humans associated with an assessment of risks markers as a result of citrus juice intake. The supplementation of blood with orange juice improves lymphocyte DNA resistance to oxidative stress in healthy humans (Riso et al., 2005). The consumption of mandarin juice (500 mL/day) significantly decreases plasma malondialdehyde (MDA) and protein carbonyl levels in hypercholesterolemic children (Codoñer-Franch, López-Jaén, Muñiz, Sentandreu, & Bellés, 2008). Moreover, orange juice (150 mL) improves antioxidant status and suppresses reactive oxygen species generation in healthy humans 30 min after consumption (Ko et al., 2005). Citrus fruit juices contain mainly naringin, naringenin, and hesperidin. The hesperidin ingested with citrus juices is metabolized by human intestinal bacterial microflora to aglycones hesperetin (Gardana, Guarnieri, Riso, Simonetti, & Porrini, 2007). Naringin and naringenin can be absorbed from the human gastrointestinal tract (Ameer, Weintraub, Johnson, Yost, & Rouseff, 1996; Kanaze, Bounartzi, Georgarakis, & Niopas, 2006). Although the effects of orange juice in healthy humans are well documented, studies regarding the consumption of pomelo juice in clinical trials have been not conducted. An increase in plasma antioxidant status in humans and the plasma pharmacokinetics of naringin, naringenin, and hesperetin after the intake of pomelo juice must be further investigated.

The results in Table 3 show the IC_{50} values of the 6 pomelo cultivars against pancreatic lipase. All extracts markedly inhibited pancreatic lipase activity in a dose-dependent manner with IC_{50} values ranging from 0.25 to 0.34 mg/mL. According to the results, there were no significant differences in the IC_{50} values between the 6 pomelo cultivars. As shown in Table 3, the IC_{50} values of the 6 pomelo extracts demonstrated a potent inhibitory activity

Table 3
IC_{50} values of the pulp extract of the 6 pomelo cultivars against pancreatic lipase and cholesterol esterase

Pomelo cultivars	IC_{50} values	
	Pancreatic lipase (mg/mL)	Pancreatic cholesterol esterase (mg/mL)
KY	0.28 ± 0.05[a]	2.10 ± 0.06[a]
TD	0.26 ± 0.04[a]	1.50 ± 0.30[a]
KT	0.32 ± 0.08[a]	2.97 ± 0.97[a]
KN	0.25 ± 0.07[a]	1.87 ± 0.12[a]
TK	0.36 ± 0.03[a]	2.89 ± 0.32[a]
TS	0.34 ± 0.02[a]	2.31 ± 0.27[a]

Data are expressed as mean ± SEM (n = 3). Values in the same column with different superscripted letters are significantly different ($p < 0.05$)

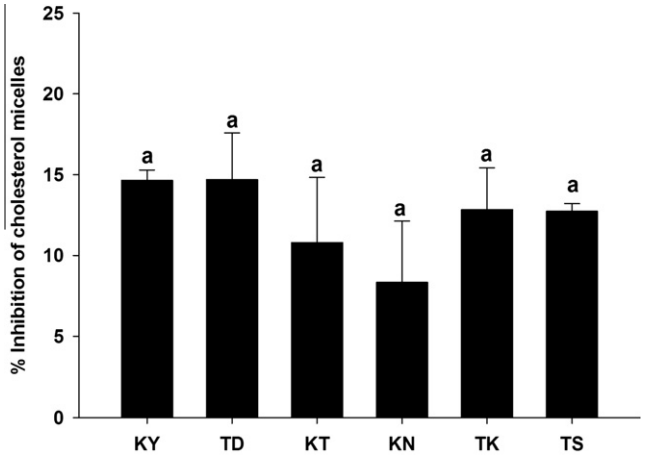

Fig. 2. The effect of pomelo extracts on inhibiting the formation of cholesterol micellization. Results were expressed as mean ± SEM; n = 3. KY, Kao-Yai; TD, Thong-dee; KT, Kao-Tangkwa; KN, Kao-Numpueng; TK, Ta-Koi; TS, Tubtim Siam. Values in the same column with different superscripted letters are significantly different ($p < 0.05$).

against pancreatic cholesterol esterase, ranging from 1.50 to 2.90 mg/mL. The results in Fig 2 show the percentage inhibition of cholesterol micellization by the 6 pomelo cultivars at a concentration of 5 mg/mL. In general, artificial micelles have been used as a model system for *in vitro* cholesterol solubilization; these principally contain uniform particles based on sodium taurocholate, egg lecithins, cholesterol, and oleic acid to reflect the natural mixed micelle. The 6 pomelo cultivars inhibited the formation of cholesterol micellization, ranging from 8.34 to 14.70%. However, we found no significant differences in the IC_{50} against pancreatic cholesterol esterase and the percentage inhibition of cholesterol micellization among the 6 pomelo cultivars. The percentage of bile acid binding by the 6 pomelo cultivars (2 mg/mL) is detailed in Fig 3. The results show that TD, KN, and TK had the highest binding capacity for taurocholic acid (primary bile acid), with values of 19.58 ± 3.71%, 19.67 ± 2.49%, and 19.63 ± 0.71%, respectively. Taurodeoxycholic acid (secondary bile acid) was markedly bound by all the pomelo cultivars with binding capacities around 14.65–20.50%, whereas all the pomelo cultivars were able to bind glycodeoxycholic acid, ranging from 5.60 to 16.93%.

Hyperlipidemia is a group of metabolic disorders characterized by elevated levels of triglycerides and cholesterol in the blood. One of the most important strategies in the prevention and treatment of hyperlipidemia includes delaying fat digestion and absorption through gastrointestinal mechanisms such as the inhibition of pancreatic lipase and pancreatic cholesterol esterase activities, as well as the inhibition of cholesterol micellization and bile acid binding.

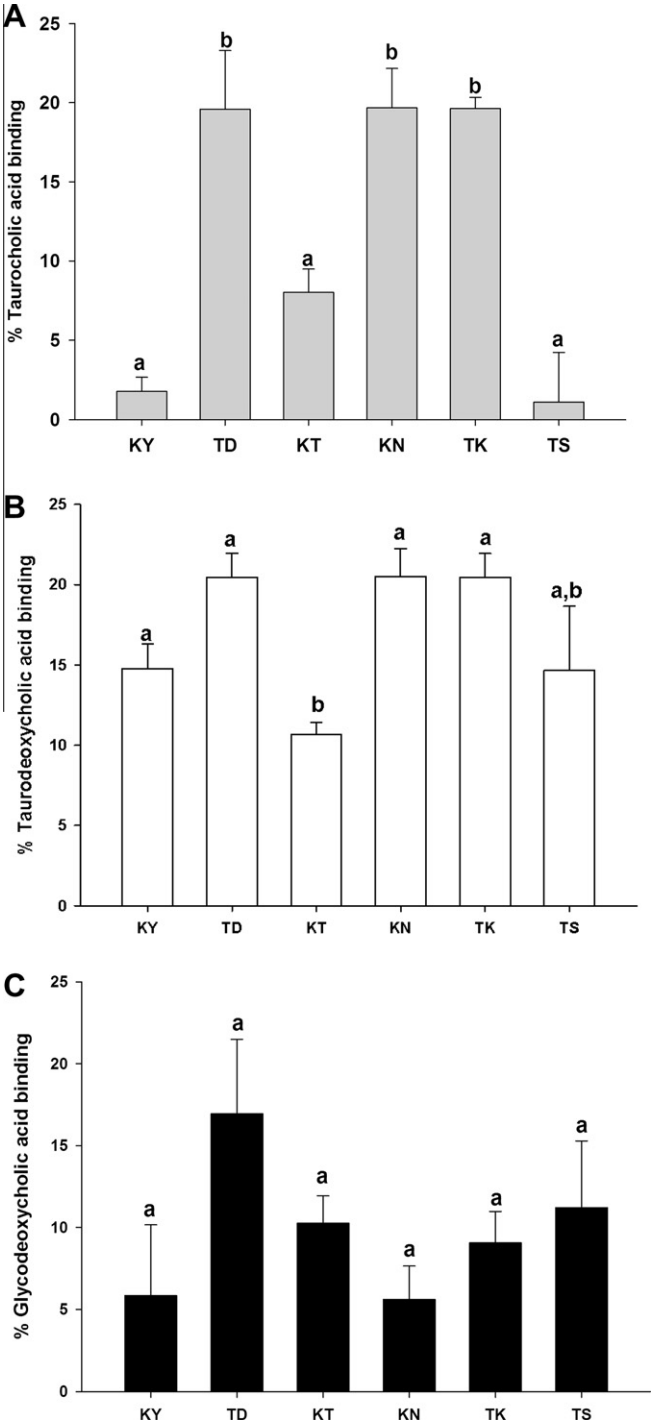

Fig. 3. The effect of pomelo extracts on (A) taurocholic acid, (B) taurodeoxycholic acid, and (C) glycodeoxycholic acid binding. Results were expressed as mean ± SEM; n = 3. KY, Kao-Yai; TD, Thong-dee; KT, Kao-Tangkwa; KN, Kao-Numpueng; TK, Ta-Koi; TS, Tubtim Siam. Values in the same column with different superscripted letters are significantly different ($p < 0.05$).

Pancreatic lipase inhibition is one of the most widely studied mechanisms of the antihyperlipidemic activity of natural products. The inhibition of this enzyme delays the digestion of triglyceride to absorbable free fatty acids, resulting in reduction of postprandial hypertriacylglycerolemia (Birari & Bhutani, 2007). The hydrolysis of dietary cholesterol esters in the small intestine is generally catalysed by pancreatic cholesterol esterase, which liberates free

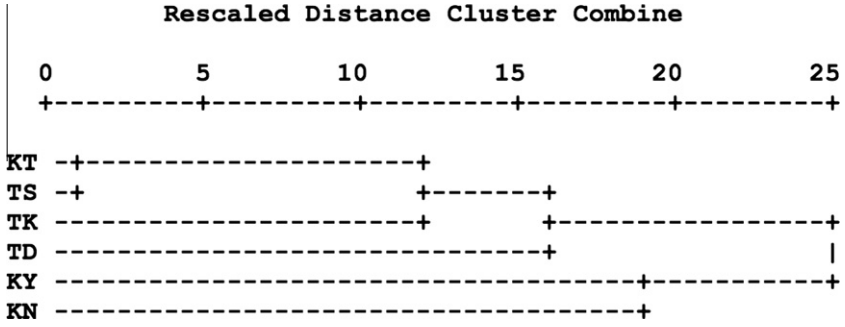

Fig. 4. A dendrogram plot visualizing the clustering of the 6 cultivars of pomelo used in this study based on their phenolic content, and antioxidant and antihyperlipidemic properties.

cholesterol (Brodt-Eppley, White, Jenkins, & Hui, 1995). In addition, the function of pancreatic cholesterol esterase is to enhance the incorporation of cholesterol into the mixed micelle, leading to the transport of free cholesterol to the enterocyte (Myers-Payne, Hui, Brockman, & Schroeder, 1995). Therefore, the ability to block cholesterol esterase may result in a delay in cholesterol digestion and absorption (Heidrich, Contos, Hunsaker, Deck, & Vander Jagt, 2004). Consequently, one of the principal mechanisms for dietary cholesterol absorption is micellar solubilization in the small intestine (Kirana, Rogers, Bennett, Abeywardena, & Patten, 2005). Recently, it has been reported that the reduction of cholesterol absorption by inhibiting cholesterol micellization in the intestinal lumen is a new target site of intervention for the treatment of hyperlipidemia (Kirana et al., 2005). Moreover, binding bile acids by forming insoluble complexes and increasing their faecal excretion have been demonstrated as one possible mechanism *of* lowering plasma cholesterol level (Insull, 2006). Our data clearly indicates that pomelo extract inhibits pancreatic lipase and cholesterol esterase. In particular, it also binds to primary bile acid and reduces the solubility of cholesterol in micelles. A previous study has shown the antihyperlipidemic effect of pomelo. For example, the administration of pomelo fruit juice significantly decreases blood cholesterol and triglyceride levels in alloxan-induced diabetic rats (Oyedepo, 2012). According to this result, we suggest that the pomelo extracts may help reduce blood cholesterol and triglycerides by inhibiting lipid digestion and absorption. Interestingly, it has been reported that a high level of secondary bile acid is associated with an increased risk of developing colorectal cancer (Peterlik, 2008). The results also show that pomelo extract binds to secondary bile acids. Therefore, the decrease in secondary bile acid concentration by pomelo extract may reduce the risk factor in developing colorectal cancer.

Hierarchical cluster analysis was used to compare antioxidant capacities and antihyperlipidemic activities of different pomelo cultivars and the results are presented in Fig 4. Hierarchical cluster analysis of 6 pomelo cultivars was performed on the average polyphenol, DPPH, TEAC, FRAP, ORAC, HRSA, SRSA, the percentage inhibition of pancreatic lipase (2 mg/mL), cholesterol esterase (0.5 mg/mL), and cholesterol micellization (5 mg/mL) as well as the percentage of bile acid binding (2 mg/mL). The 6 pomelo cultivars were divided into 2 main clusters, cluster 1 (TD, TS, KT, and TK) and cluster 2 (KY and KN). Cluster 1 included 2 very similar groups of pomelo, KT and TS, characterized by low antioxidant capacity, antihyperlipidemic activity, and polyphenol content. TK belongs to the second group with moderate activity, whereas TD belongs to the third group characterized by higher activity. Cluster 2 included 2 very similar pomelo cultivars, KY and KN, associated with the highest antioxidant capacity, antihyperlipidemic activity, and polyphenol content.

Published research has reported that polyphenolic compounds have been linked with the ability to inhibit pancreatic lipase activity (Nakai et al., 2005), the formation of cholesterol micelles (Vermeer, Mulder, & Molhuizen, 2008), and antioxidant capacity (Proteggente, Saija, De Pasquale, & Rice-Evans, 2003). Total phenolics were found to correlate well with the FRAP ($R^2 = 0.829$; $p < 0.05$) and TEAC ($R^2 = 0.829$; $p < 0.05$) assay results. These correlations are in agreement with many previous studies (Fu et al., 2010; Ikram et al., 2009; Lamien-Meda et al., 2008). However, no correlation existed among DPPH, ORAC, HRSA, SRSA, the IC_{50} values of pancreatic lipase, cholesterol esterase, and the percentage of cholesterol micellization as well as the percentage of bile acid binding. This lack of relationship between phenolic compounds in citrus species and DPPH activity is in agreement with other studies (Ghasemi, Ghasemi, & Ebrahimzadeh, 2009). Despite the considerable amount of data in the literature showing strong linear correlations, the antioxidant activity might not always correlate with phenolic content (Heinonen, Meyer, & Frankel, 1998; Kähkönen et al., 1999). The negative correlation is possibly owing to the presence of the following factors. For example, the antioxidant capacity observed was not solely from the phenolic content, but could possibly be due to the presence of some other phytochemical compounds in citrus juice such limonoids, which acts as an antioxidant (Andrew & Manners, 2006; Pichaiyongvongdee & Haruenkit, 2009). Consequently, the synergistic effects among phenolic compounds in pomelo extract may also contribute to the total antioxidant capacity. Moreover, the total phenolic content was determined according to Folin–Ciocalteu method, which may not be an absolute measurement of the amount of phenolic compounds. (Martins, Aguilar, Teixeira, & Mussatto, 2012).

4. Conclusion

Our results indicate that the phenolic composition, antioxidant properties, and antihyperlipidemic activity of pomelo vary in different cultivars. Statistically significant differences in total phenolic content, DPPH, FRAP, TEAC, ORAC, HRSA, and SRSA antioxidant capacities, and bile acid binding were seen in different cultivars. Hierarchical clustering analysis indicates that the cultivars in this study could be placed into 2 classifications: the first group, containing 4 cultivars (TD, TS, KT, and TK) and the second group, containing 2 cultivars (KY and KN). To our knowledge, these pomelo cultivars are the first to have been investigated for antihyperlipidemic activity. This activity appears to underlie the potential utility of pomelo as an edible fruit and offers remarkable prospects for the prevention of oxidative stress and hyperlipidemia, suggesting that it might be developed into functional foods in the future.

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