

Full Length Research Paper

Functional antioxidant and tyrosinase inhibitory properties of extracts of Taiwanese pummelo (*Citrus grandis* Osbeck)

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In recent years, the overproduction of citrus fruits has resulted in an unnecessary increase in agricultural wastes in Taiwan. In an attempt to find an application for these potentially valuable wastes, we evaluated the antioxidant and whitening properties of six Taiwanese pummelo varieties (Miyu Shihtouyu Taipeiyu Touyu Wentan and Hsishihyu). The methanolic extract of *Citrus grandis* Osbeck Miyu (Miyu) had the highest phenolic content (9.99 mg of gallic acid equivalent/g). *C. grandis* Osbeck Shihtouyu (Shihtouyu) displayed the highest 2, 2-azino-bis-(3- ethylbenzthiazoline-6-sulfonic acid) content (9.3 mg trolox equivalent antioxidant content/g), indicating its good free radical-scavenging activity. *C. grandis* Osbeck Taipeiyu (Taipeiyu) showed the highest 1,1-diphenyl-2-picrylhydrazyl content and this compound too possesses good radical-scavenging activity. The ferrous-ion chelating effect of *C. grandis* Osbeck Touyu (Touyu) and *C. grandis* Osbeck Wentan (Wentan) was found to be 0.78 and 0.92 mg/ml, respectively. Taipeiyu showed the highest limonin content (1251.86 µg/ml). Touyu inhibited tyrosinase up to 90.8% (10 mg/ml), which was almost similar to the 95% inhibition shown by kojic acid (10 mg/ml). Thus, the components of pummelo have high potential for use as ingredients in products that prevent skin pigmentation. These results indicate that the methanolic extracts and the phytochemicals derived from pummelo are potential natural antioxidant agents.

Key words: Antioxidant, free radical chelating, limonin, pummelo, tyrosinase.

INTRODUCTION

Citrus grandis Osbeck, known as pummelo, is a popular seasonal fruit in Taiwan. It is regularly consumed in Asian countries as a whole fruit, juice or even in the form of preserved snacks (Tsai et al., 2007). Taiwan produces 70,000 metric tons of pummelo every year (Council of Agriculture Executive Yuan R.O.C., 2009). In China, pummelo is an important seasonal and festival fruit consumed for its unique flavour. However, considerably the festival, thereby, creating a large amount of large quantities of pummelo become unmarketable after

agricultural waste.

Citrus fruits contain several types of biologically active compounds such as limonoids, alkaloids, vitamin C and flavonoids that have potential health-promoting properties (Itoh et al., 2009; Dante et al., 1973; Rapisarda et al., 2009) and have been widely used in traditional Chinese medicine. The neutral extract of this fruit possesses strong antioxidant and antibacterial activities (Matook and Toshihiko, 2006). Limonoids, which are one of the major constituents of citrus fruits, have attracted attention because of their anticarcinogenic and antitumourigenic activities in insects (Jayaprakasha et al., 1997). Naringin and limonin protect against azoxymethane-induced aberrant crypt foci formation by suppressing proliferation and increasing apoptosis because of their anti-inflammatory

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Table 1. Location of samples and TSS content (Brix, 20°C), titratable acidity (TA, g citric acid/100 ml) and limonin equivalence of pummelo juice.

Species	Sample ID	Character	Location of sample	TA (%) [*]	TSS (Brix)	Limonin equivalence (µg/ml) ^{**}
<i>C. grandis</i> (L.) Osbeck Hsishihyu	CPH	White pummelo	Hsinchu/ Guansi	0.732	11.0	433.28±2.754 ^e
<i>C. grandis</i> (L.) Osbeck Miyu	CPM	White pummelo	Hsinchu/ Guansi	0.564	8.9	981.14±2.979 ^c
<i>C. grandis</i> (L.) Osbeck Shihtouyu	CPS	Red pummelo	Hsinchu/ Guansi	0.502	8.0	982.57±3.849 ^c
<i>C. grandis</i> (L.) Osbeck Touyu	CPTo	Red pummelo	Hsinchu/ Guansi	0.439	8.0	1059.71±4.049 ^b
<i>C. grandis</i> (L.) Osbeck Wentan	CPW	White pummelo	Hsinchu/ Guansi	0.282	10.2	753.29±5.010 ^d
<i>C. grandis</i> (L.) Osbeck Taipeiyu	CPTa	White pummelo	Taipei/ Hsin-Tien	0.575	9.9	1251.86±1.930 ^a

^{*}Data bearing different superscript letters in the same column are significantly different ($p < 0.05$); ^{**}Means $n = 3$.

Activities (Jairam et al., 2006). Tyrosinase, which is another major constituent of citrus fruits, is a copper-containing monooxygenase. It catalyses melanin biosynthesis in the human skin and causes various dermatological disorders such as melasma and freckles (Seo et al., 2003). The citrus peel extract containing nobiletin is an effective tyrosinase inhibitor and therefore, it effectively inhibits melanin production (Sasaki et al., 2002). Nobiletin and hesperidin contained in citrus peel crude extract also have inhibitory effects on tyrosinase diphenolase activity (Zhang et al., 2007)

In a functional study, the hexane fraction of immature *C. grandis* Osbeck was shown to be effective in inducing apoptosis in U937 human leukaemia cells (Lim et al., 2009). A Korean citrus species (*C. grandis* Osbeck, Danguyuja) has high flavonoid content and is effective in inducing apoptosis in human cervical carcinoma HeLa cells (Kim et al., 2010). Taiwanese Wentan (Buntan) pummelo (*C. grandis* Osbeck Wentan) has a high phenolic content and displays high free radical-scavenging activity and ferric-reducing ability (Jang et al., 2010)

Thus far, the antioxidant and tyrosinase inhibition properties of the Taiwanese pummelo fruit have not been reported. The evaluation of the total antioxidant capacity of certain foods has received much attention in recent years because of the potential synergistic effects of these food items. This study reveals the antioxidant and whitening property of the methanolic extract of pummelo. We investigated the free radical-scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ferrous ion-chelating ability, total phenolic content (TPC), 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging ability, limonin content and tyrosinase inhibitory activity. The findings thus obtained, have potential to promote the novel utility of agricultural waste and enhance the market value of pummelo fruits. Our study evaluated the functionality of pummelo extract as an antioxidant and whitening agent.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ferrozine, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$,

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu's phenol reagent and 4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma, St. Louis, USA and 4-(dimethyl) aminobenzaldehyde (DMAB) indicator reagent was purchased from Riedel-de-Haen.

Fruit samples

All commercially mature fruits were harvested from trees located in the experimental field of the Kuan-His country (Hsin-chu, Taiwan) (Table 1). The fruits were collected, freeze-dried, powdered and the albedo was separated from the fruit by hands. The samples were stored in a zipper bag in a dry cabinet (40% RH) until analysis. To obtain the extracts, dried pummelo samples (50 g) were stirred with 100 ml of methanol at 25°C and 1500 rpm for 6 h, followed by filtration through Whatman No. 1 filter paper. The solution was evaporated to dryness at 40°C, re-dissolved in methanol at a concentration of 10 mg/ml and stored at 4°C for further use. The analyses were carried out in triplicate.

DPPH radical-scavenging activity

Methanolic extracts of pummelo in various concentrations were added to 4 ml of 0.5 mM DPPH in methanol according to the method described previously (Shimada et al., 1992; Tung et al., 2009). The solution was kept in the dark for 30 min. The absorbance of reaction mixture was measured at 517 nm with a spectrophotometer (Beckman Coulter, DU[®]640, Minnesota, USA). The scavenging ability was calculated as follows:

$$\text{Scavenging effect (\%)} = [1 - (A_{517 \text{ nm sample}}/A_{517 \text{ nm control}})] \times 100.$$

Ferrous ion-chelating capacity assay

The ferrous ion-chelating capacity was measured using the modified method (Su et al., 2008; Mariken et al., 2003). Briefly, 1 ml of the methanolic extract was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$. The reaction was initiated by adding 0.2 ml of 5 mM ferrozine after 30 s. The absorbance was measured at 562 nm after the mixture was placed at room temperature for 10 min. Chelating effect (%) = $[(A_{562 \text{ nm control}} - A_{562 \text{ nm sample}}) / (A_{562 \text{ nm control}})] \times 100$

Total antioxidant capacity determined by trolox equivalent antioxidant capacity (TEAC)

The trolox equivalent antioxidant capacity (TEAC) was determined

by the procedure described by Mariken et al. (2003) with slight modifications, using trolox as a standard. The 2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) stock solution (2.45 mM potassium persulfate and 7 mM ABTS) was prepared as follows: the mixture was kept in the dark for 12 to 16 h until the reaction was complete and the absorbance was stable. The ABTS solution was diluted with water to obtain an absorbance value of 0.7 ± 0.02 at 734 nm. The antioxidant capacity was calculated based on the following formula:

$$\text{Total antioxidant activity (\%)} = [1 - (A_{734 \text{ nm sample}}/A_{734 \text{ nm control}})] \times 100.$$

Determination of total phenolic content

The total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu assay (Zhou et al., 2009). 1 ml of the extracted liquor was mixed with 0.5 ml of 1 M Folin-Ciocalteu's phenol reagent and 2.5 ml of 20% sodium carbonate solution after 1 min. The tubes were kept for 20 min at room temperature before the absorbance was measured at 765 nm with a spectrophotometer. The TPC was expressed as a gallic acid equivalent (GAE) in mg/g of sample. The standard curve was drawn using 10 to 100 mg GAE/g DW.

Limonin assay

Limonin assay was carried out as described previously (Breksa and Jr., 2007; Abbasis et al., 2005). Limonin extraction was done by mixing the extracts and CHCl_3 in the ratio of 1:2 (v/v). The mixture was vortexed for 2 min and centrifuged (9,500 rpm, 10 min and 4 °C) to accelerate the separation of the phases. The chloroform phase (the upper phase) (1 ml) was collected and evaporated until dried. The samples were reconstituted with acetonitrile (0.5 ml). A 96-well assay plate with the samples and standards was organized as follows: the standard (110 μl) was placed in a single column, the samples (110 μl) were placed in each well and a well was reserved for the blank, the DMAB indicator reagent (165 μl) was added to each well (including, blank, standard and samples) with a multichannel pipette, the plate was incubated at room temperature for 30 min and the absorbance was measured at 470 nm using a VersaMax™ Microplate spectrophotometer (Sunnyvale, CA, USA).

$$\text{Limonin equivalence (LG) (\mu g/ml)} = [(E_{\text{DMAN}} - E_{\text{Blank}})] \times \text{DF} \times N,$$

Where, DF is the dilution factor and *N* is the LG equivalence of the single point calibrator.

Tyrosinase *in vitro* assay

Tyrosinase inhibition activity was determined by the dopachrome method with L-3, 4-dihydroxyphenylalanine (L-DOPA) as a substrate (Chan et al., 2008; Kittisak et al., 2001). The amount of dopachrome produced in the reaction mixture was determined at 475 nm using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). The percent inhibition of tyrosinase activity was calculated as follows:

$$\% \text{ inhibition} = 100 \times [(A - B) - (C - D)] / (A - B)$$

Where, A is the absorbance at 475 nm without the test sample; B is the absorbance at 475 nm without the test sample and enzyme; C is the absorbance at 475 nm with the test sample; and D is the absorbance at 475 nm with the test sample, but without enzyme. Kojic acid (Sigma, St. Louis, USA) was used as a positive standard.

Statistical analysis

All analyses were performed in triplicate. The data were recorded as the mean \pm S.D. (standard deviation) and analyzed by the statistical analysis system software (SAS Inc., NC, USA). A one-way analysis of variance (ANOVA) was performed and differences between means at $P < 0.05$ level were considered significant.

RESULTS AND DISCUSSION

C. grandis Osbeck Touyu (Touyu) and *C. grandis* Osbeck Shihtouyu (Shihtouyu) varieties of pummelo are characterised by the red colour of their fruit flesh; the colour is possibly attributable to the presence of anthocyanins or flavonoids. TSS content (Brix, 20 °C), titratable acidity (TA, g citric acid/100 ml) and limonin equivalence of pummelo juice are shown in Table 1. The titratable acid of the six pummelo varieties ranged from 0.28 to 0.73% and the *C. grandis* Osbeck Hsishihyu (Hsishihyu) and Wentan varieties showed the highest and lowest values, respectively. Hsishihyu also displayed the highest total soluble solid content, which was 11 °Bx, but a low limonin content of merely 433 $\mu\text{g/ml}$. The *C. grandis* Osbeck Taipeiyu (Taipeiyu) variety showed the highest limonin content of 1250 $\mu\text{g/ml}$ (Table 1). Table 2 summarises the antioxidant activities of the six species. *C. grandis* Osbeck Miyu (Miyu) had the highest TPC (9.99 mg gallic acid equivalent (GAE)/g dry weight (DW)) followed by Taipeiyu (8.94 mg GAE/g DW) and Touyu (8.29 mg GAE/g DW), whereas Shihtouyu had the lowest value (4.5 mg GAE/g DW). When compared with a previous study, our study showed lower TPC values for the two different extract fractions of pummelo juice and the freeze-dried products (Lim et al., 2009). Herbal citrus products (*Aurantii immaturus* Fructus) had a lower TPC than Miyu, but other herbal products had significantly higher TPC than Miyu (Su et al., 2008). There were significant differences in the results of the ABTS radical scavenging assay among all samples. Shihtouyu had the highest trolox equivalent antioxidant content (TEAC). We compared the ABTS radical scavenging assay results and found that the half-maximal effective concentration (EC_{50}) for the white button mushroom ranged from 3.33 to 4.97 mg/ml. Because of its low EC_{50} value, the antioxidant power of Hsishihyu was higher than that of the mushroom samples (Savoie et al., 2008). Bayberry has a higher ferrous-reducing ability (71.4 mg of TEAC/g) and TPC value (GAE, >40 mg/g) than the six pummelo varieties evaluated in this study (Rapisarda et al., 2009). The effectiveness of *in vitro* antioxidants test model does not necessarily reflect their *in vivo* activity (Li et al., 2008). The correlation between phenolic and antioxidant properties of the various pummelo varieties is presented in Table 3. There was a significant correlation between TPC and free radical-scavenging and tyrosinase-inhibiting activities.

In previous studies, the methanol extract of the albedo tissues of the fruit exhibited significant antioxidant activities as determined by the free radical assay (Matook

Table 2. DPPH scavenging, ferrous ion-chelating, and ABTS assays of EC₅₀ values in antioxidant properties, total phenolic content and tyrosinase inhibition activity of methanolic extracts of albedo tissues of different species of pummelo.

Extract from species	DPPH scavenging (mg/ml)	Ferrous ion-chelating (mg/ml)	ABTS (mg of TEAC/g)	Total phenolic (mg of GAE/g)	Tyrosinase Inhibition activity (mg/g)
<i>C. grandis</i> (L.) Osbeck Hsishihyu	0.83±0.015 ^b	1.0±0.265 ^d	4.93±0.065 ^d	8.02±0.04 ^d	2.277±0.081 ^b
<i>C. grandis</i> (L.) Osbeck Miyu	0.71±0.006 ^c	1.56±0.016 ^c	1.59±0.044 ^e	9.99±0.035 ^a	2.087±0.208 ^c
<i>C. grandis</i> (L.) Osbeck Shihtouyu	0.46±0.015 ^e	3.95±0.040 ^a	9.30±0.132 ^a	4.50±0.010 ^f	2.183±0.055 ^e
<i>C. grandis</i> (L.) Osbeck Touyu	0.39±0.015 ^f	0.78±0.040 ^e	6.03±0.181 ^b	8.29±0.070 ^c	2.130±0.056 ^f
<i>C. grandis</i> (L.) Osbeck Wentan	0.52±0.010 ^d	0.92±0.035 ^e	5.38±0.140 ^c	5.65±0.093 ^e	2.522±0.200 ^d
<i>C. grandis</i> (L.) Osbeck Taipeiyu	0.95±0.015 ^a	2.37±0.165 ^b	5.38±0.036 ^c	8.94±0.032 ^b	2.173±0.010 ^a
BHT	0.07±0.006 ^g	-	-	-	-
EDTA	-	0.55±0.025 ^f	-	-	-
Kojic acid	-	--	-	-	0.076±0.030 ^d

Data bearing different superscript letters in the same column are significantly different ($p < 0.05$).

Table 3. Correlation of total phenolics of various pummelos versus DPPH scavenging, ferrous chelating and tyrosinase inhibition activity.

Pummelo	Correlation of DPPH scavenging activity	Correlation of ferrous-chelating activity	Correlation of tyrosinase inhibition activity
<i>C. grandis</i> (L.) Osbeck Hsishihyu	0.8209	0.9913	0.9019
<i>C. grandis</i> (L.) Osbeck Miyu	0.9663	0.7841	0.9908
<i>C. grandis</i> (L.) Osbeck Shihtouyu	0.9039	0.9274	0.891
<i>C. grandis</i> (L.) Osbeck Touyu	0.7553	0.973	0.8891
<i>C. grandis</i> (L.) Osbeck Wentan	0.9233	0.834	0.9748
<i>C. grandis</i> (L.) Osbeck Taipeiyu	0.3015	0.7852	0.9199

and Toshihiko, 2006). Pummelo extracts showed notable antioxidant activities in the DPPH scavenging assays, as depicted in Figure 1. For each extract, different concentrations (0.2 to 1 mg/ml) were prepared. The activities of the extracts ranged between 10 and 72% at 0.2 mg/ml and between 17 and 85% at 0.8 mg/ml. The DPPH free radical-scavenging activity of Touyu was similar to that of butylated hydroxytoluene, with an EC₅₀ value of 0.39 mg/ml (Table 2). On comparing our data with previous data, we found that the radical-scavenging ability of Touyu was higher, whereas that of herbal citrus products was lower (EC₅₀, 0.46 to 1.59 mg/ml), except in the case of *A. immaturus* Fructus. The methanol extracts previously obtained from red pummelo (Touyu and Hsishihyu) exhibited efficient radical-scavenging ability. The result obtained in this study was similar to that obtained by Tsai et al. (2010).

Figure 2 compares the ferrous ion-chelating activities of the pummelo extracts at various concentrations of ethylenediamine tetraacetic acid. The EC₅₀ values for the ferrous ion-chelating ability of Touyu, Wentan, Hsishihyu, Miyu, Taipeiyu and Shihtouyu were 0.78, 0.92, 1.0, 1.56, 2.37 and 3.95 (mg/ml), respectively. These results indicate that Touyu and Wentan were effective ferrous ion-chelating agents. In a previous study, the EC₅₀ values

for the ferrous ion-chelating ability of the ethanol extract of *Acacia confusa* bark and its fractions were 0.253 to 2.185 mg/ml (Tung et al., 2009). The results obtained by this method vary considerably, but the chelating ability of the tested samples notably increased as a function of their concentration.

Jang et al. (2010) investigated the antioxidant potentials and fermentation of Wentan (Buntan). The DPPH free radical-scavenging activity of fermented Wentan products was 15.5%. Our results revealed that the 0.52 mg/ml methanol extract of Wentan may achieve a half-maximal inhibitory concentration (IC₅₀) for DPPH scavenging. Although the antioxidant activity levels differed among the varieties, Wentan clearly showed the existence of a strong EC₅₀ for DPPH scavenging ranged from 0.1 to 0.7 mg/ml, which was similar to the result of our study. Their results antioxidant compound. Su et al. (2008) analysed the phenolic compounds present in the methanol extract of various citrus herbal ingredients. They reported that the be attributed to the presence of high levels of antioxidant substances present in the processed dried herbal ingredients. Touyu showed the highest antioxidant activity against DPPH, whereas Taipeiyu displayed the lowest. At the concentration of about 1 mg/ml, there was a steep rise in the scavenging activity, with an average of about 95%

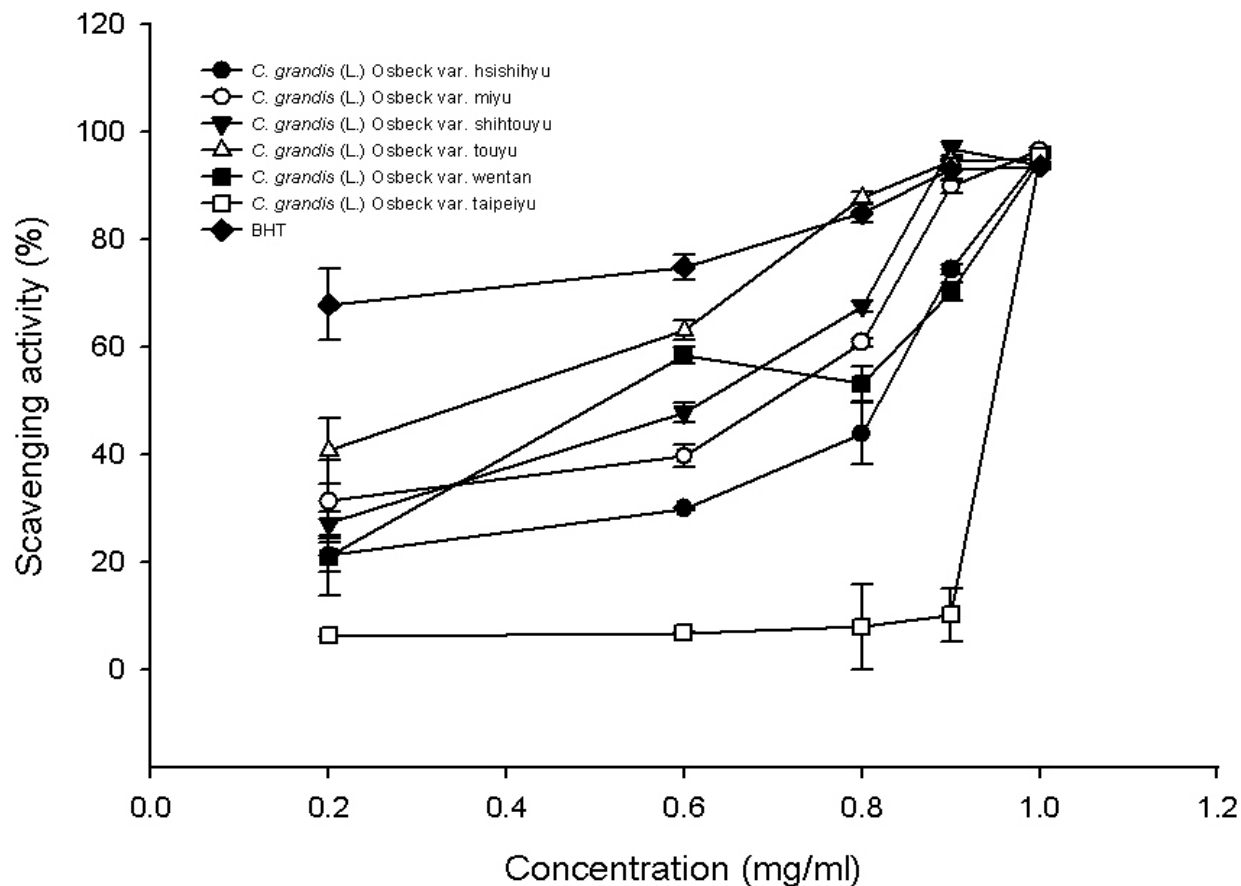


Figure 1. Antioxidant activity of *C. grandis* (L.) Osbeck species methanolic extracts at different concentrations using DPPH method.

(Figure 1). The results for ferrous-chelating activity were similar to those of DPPH scavenging activity: Touyu showed the highest activity, whereas the extracts of Taipeiyu, Miyu and Shihtouyu showed a chelating activity of less than 40% at a concentration of 1 mg/ml (Figure 2)

Limonoids are highly oxygenated triterpenes found in the plant family Rutaceae and few other plants. Limonin is predominantly found in the albedo tissues and pummelo peel. Because of physical damage or freeze injury, the juice obtained from citrus fruits is usually bitter. Limonin, when present at concentrations higher than 6 ppm, causes an unacceptable level of bitterness in citrus fruit juice and processed citrus products (Dan and Andrew, 1986). The presence of limonin in pummelo is responsible for the reduction in the risk of certain chronic diseases. Recent findings have shown that naringin and limonin exert protective activities by enhancing apoptosis through their anti-inflammatory activities. Future applications of purified limonin compounds extracted from pummelo waste may enhance the value of this citrus species in Taiwan.

The methanolic extracts of the albedo tissues from the six pummelo varieties were analysed for their tyrosinase inhibition activity by using the modified dopachrome

method with L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate. The results indicate that Miyu effectively inhibited tyrosinase activity and its IC_{50} value was 2.088 mg/ml (Table 2). A wide variation in the results of the ABTS assay was observed between Miyu and Shihtouyu, showing a nearly 6-fold difference in antioxidant capacity. However, the range of the variation is reasonable considering the diverse source of medicinal plants and it may even vary up to 400-fold (Li et al., 2008). In citrus herbal products, the phenolic content varied up to 15-fold (Su et al., 2008). Hsishihyu, Shihtouyu, Touyu and activity. The results of our study are similar to those of Sasaki et al. (2002). The methanol extract obtained from the citrus peels had an IC_{50} value of 2.427 mg/ml for Taipeiyu had similar anti-tyrosinase activity, but their values were slightly higher than that of Wentan. Pummelo extracts showed a significant inhibition of tyrosinase tyrosinase activity, whereas an ethyl acetate extract with an IC_{50} value of 0.097 mg/ml strongly inhibited tyrosinase activity. Touyu inhibited tyrosinase up to 90.8% (10 mg/ml), which was similar to kojic acid that showed 95% inhibition (10 mg/ml). Future studies should investigate the tyrosinase inhibition efficiency of extracts obtained by different solvent extraction methods. Thus, components of

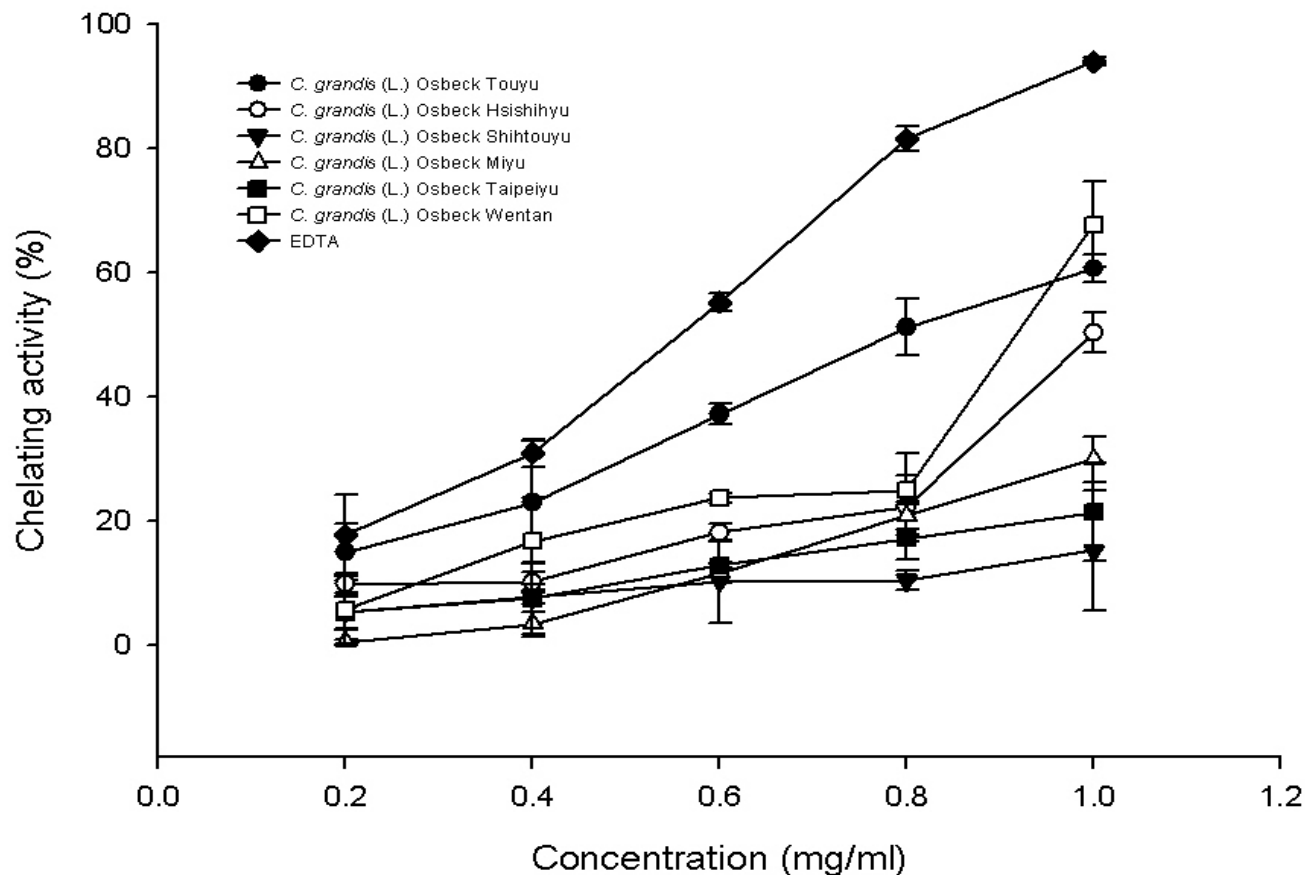


Figure 2. Antioxidant activity of *C. grandis* (L.) Osbeck species methanolic extracts at different concentrations using ferrous ion-chelating

pummelo extracts are expected to be valuable ingredients of products for the prevention of skin pigmentation. Some studies have indicated that TEAC not only reflects the antioxidant effect of the parent compound, but also reflects the potential antioxidant capacity of the reaction products. This concern has been raised, but a recent review by Niki (2010) elucidated that trolox assay is used as a standard method to determine oxygen radical absorbance capacity and that TEAC has been widely adapted to measure the hydrophilic as well as lipophilic antioxidant potential, especially from unknown or untested samples.

Conclusion

This study examined the antioxidant activities and phenolic content of methanolic extracts of pummelo. The results indicate that the methanol extract of Miyu showed the highest phenolic content (9.99 mg of GAE/g). Miyu was also found to be an effective tyrosinase inhibitor, with an IC_{50} value of 2.088 mg/ml. This study indicates that the methanolic extracts of pummelo or its derived phytochemicals have good antioxidant properties and they could be potentially used in the prevention of

diseases caused by free radicals and in the prevention of skin pigmentation.

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