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Identification of Phenolic Contents in Malaysian Variety of Pummelo (*Citrus Grandis* L. Osbeck) Fruit Juice Using HPLC-DAD

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***Abstract*—**Pummelo is known to be the largest of all citrus fruits, with expected ratio of 2:1 (w/v) of producing juice, is an attractive opportunity for Malaysia to expand pummelo's influence and marketability over the international market of juices. The purpose of this study is to identify and quantify the phenolic compounds in two Malaysian varieties of pummelo fruit juice: Ledang (PO55) and Tambun (PO52). Identifications of polyphenols composition were done using High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD). The phenolic compounds that were found in both varieties were hydroxycinnamic acids and flavonones. This study proved that Tambun variety has the highest antioxidant and phenolic compounds in comparison to Ledang variety. However, considerations have to be made to suit consumer's taste bud and the amount of enzyme needed to clarify the juice for its marketability.

***Keywords*—**Antioxidant, HPLC, phenolic contents and pummelo fruit juice.

I. Introduction

CITRUS fruits are considered as the most important horticultural crops because of their nutritional value and health benefits. Citrus juice has long been believed to have a correlation with good health, due to their antioxidant potency and plasma lipid metabolism [1]. Citrus fruit extracts have also been found to demonstrate anti-cancer, anti-inflammatory, anti-tumor and blood clot inhibition activities [2]. The health benefits of citrus juice have mainly been attributed to the presence of bioactive compounds, such as phenolics, ascorbic acid and carotenoids. Citrus flavonoids have been said to be responsible for the beneficial effects and such compounds are identified as methoxylated flavones, flavonones, and flavonone glucosides [3].

Pummelo (*Citrus grandis* L. Osbeck), of *Rutaceae* family, is a citrus fruit native to Southeast Asia and the Indo-China regions. It is also known as pomelo, pommelo, shaddock, limau bali and Chinese grapefruit. The fruit is commonly eaten fresh and the taste varies from mildly sweet and bland to sub acid with a faint touch of bitterness [4]. Popular variations of the fruits are; PO51 (Sha Thing), PO52 (Tambun) and KK2 (Melo Mas). Recently, Department of Agriculture Malaysia has introduced a new hybrid known as Ledang variety (PO55), where the fruit is sweeter and has less bitter aftertaste pummelo usually known for. In Malaysia, about 1895 hectare of pummelo grown commercially and in 2009, production is estimated at 8830 metric tonnes. The largest growing state is Johor with 380 hectare and Perak with current 320 hectare of commercially grown trees and the harvest season is usually in January and September [5]. Singapore, Indonesia and Thailand are the biggest importer of pummelo with the amount reaching up to RM5.4 million per year.

Naringin, neohesperidin, and hesperidin are the major flavonoids formed in *Citrus grandis, Citrus paradise, Citrus aurantium,* and *Citrus limon* [6]. However, until the moment of writing, identification and/or quantification of flavonoids has been mainly carried out on lemon, orange, and grapefruit species [2], [6]-[9] and very little literature was done on pummelo. Pummelo have been shown to contain coumarins, furocoumarins, flavanones, flavones and flavonols in both free and glycosidic form [7], [8]. The flavonoid profile too, has been shown to vary with the species and cultivars, and therefore can be used to distinguish between the different varieties. In this study, two cultivars of pummelo fruit juice were analyzed using high-performance liquid chromatography diode array detection (HPLC-DAD) for the identification and quantification of flavonoids.

II. Material and Methods

A. Standards and Chemicals

Milli-Q water (Millipore, Bedford, MA, USA) was used in all work. HPLC-grade methanol and formic acid (Merck, Darmstadt, Germany) were used after filtration through a 0.45μm pore size membrane filter and sonication for 30 minutes. Phenolic acids (gallic, caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic acids) and flavanones (naringin, naringenin, narirutin, and hesperidin) were purchased from Sigma-Aldrich (Stenheim, Germany).

B. Preparation of Juice Sample

Fresh harvests of pummelo fruits, *Citrus grandis* L. Osbeck, of Ledang variety (PO55) were obtained from Jabatan Pertanian Daerah Segamat, Johor Darul Takzim, Malaysia and Tambun variety (PO52) were obtained from Jabatan Pertanian Daerah Kinta, Ipoh, Perak Darul Ridzuan, Malaysia. Pummelo

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fruits were kept in a refrigerator at 10°C until they were used for experimental works. Prior to peeling, the pummelos were washed with tap water to eliminate any microbial contaminations to the fruits. The thick fruit skin (flavedo and albedo) was peeled manually after a 1cm-deep horizontal incision was made using a knife to reveal the juicy segments. Pummelo was then peeled into segments and the inner skin of each segment was peeled and discarded. The white membrane surrounding the juicy segments, including seeds, were removed completely. The juice was extracted using a screw type extractor and nylon-filtered to remove the pulps. This process was repeated three times to optimize the juice extraction. The juice is then kept in a HDPE bottle in a freezer at -5°C until further experimental works.

C. Liquid Chromatographic Analysis of Phenolic Compounds

Samples were filtered through a 0.45m pore size membrane before injection. An Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) operated by Windows NT based ChemStation software was used. The HPLC equipment was used with a diode array detector (DAD). The system consisted of a binary pump, degasser and auto sampler. The column used was a Thermo Scientific C18 column (Waltham, MA, USA): 250 x 4.6mm x 5µm. The injection volume of the fruit juice was 20µL per sample. The mobile phase consisted of two solvents: Solvent A, 0.1% water in formic acid and Solvent B, 100% methanol. Phenolic compounds were eluted under the following conditions [9] with modifications: gradient conditions 0 to 20% solvent B (0mins), 20 to 30% solvent B (0 to 20mins), 30 to 50% solvent B (20 to 30mins), 50 to 90% solvent B (30 to 35mins), 90 to 20% solvent B (35 to 40mins), followed by washing and reconditioning of the column. The separations were performed with a flow rate of 1mL/min, which was directly injected in the ESI source, without any splitting. The column temperature was maintained at 25°C. The analysis time was of 40 minutes. The HPLC method was tested on 10 phenolic compounds (coumaric acid, sinapic acid, chlorogenic acid, gallic acid, ferulic acid, caffeic acid, narirutin, hesperidin, naringenin, and naringin). The polyphenols standard solutions (10µg/mL) were prepared in methanol. The ultra-violet-visible-spectra (scanning from 200 to 600nm) were recorded for all peaks. Triplicate analyses were performed for each sample. The identification of phenolic compounds was obtained by using authentic standards while quantification was performed by external calibration with standards.

D. Statistical Analysis

The data obtained in the study were analyzed using Minitab Release 14 (Minitab Inc., PA, USA). Analysis of variance was performed by ANOVA procedure and significant differences ($p<0.05$) between means were determined using Tukey's multiple range test. All analyses were done in triplicate.

III. Results and Discussion

A total of six phenolic compounds were identified and quantified in Ledang and Tambun pummelo fruit juice (Table I), including hydroxycinnamic acids and flavanones compounds. The total amount of phenolic compounds found though liquid chromatograph analysis was 19.66 and 39.34mg/100mL for Ledang and Tambun variety respectively, with naringin being the main contributor of the total phenolic compounds. A great difference ($p<0.05$) of total phenolic contents for the control juice could be affected by the species, growing season, ripening, and environmental factors such as light, temperature and as well as, processing treatment.

TABLE I
Phenolics Content (mg/100mL ± Standard Deviation) of Ledang and Tambun Pummelo Fruit Juice

Compounds	Peak no.	Retention Time (min)	Ledang (mg/100mL)	Tambun (mg/100mL)
Hydroxycinnamic acids				
Chlorogenic acid	1	7.85	1.60 ± 0.07	2.85 ± 0.61
Caffeic acid	2	10.84	0.09 ± 0.01	0.11 ± 0.01
Coumaric acid	3	16.80	1.44 ± 0.08	1.33 ± 0.20
Total	-	-	3.13	4.29
Flavanones				
Naringin	4	22.75	13.09 ± 0.10	26.76 ± 4.28
Hesperidin	5	8.36	3.57 ± 0.03	5.39 ± 1.10
Narirutin	6	28.26	3.00 ± 0.14	7.19 ± 1.18
Total	-	-	19.66[a]	39.34[b]

[a]For each treatment, the means within the row followed by different letters were significantly different at $p < 0.05$.

Through liquid chromatograph analysis, it was identified that three hydroxycinnamic acids and three flavanones found in both varieties at 280nm and 320nm. The summary of both phenolic compounds were shown in Table I. Two hydroxybenzoic acids that were tested for identification; gallic and protocatechuic acid were not detected in both varieties. This could be explained by the standard reference that was obsolete at the time of processing. Gallic acid, the major contributor to hydroxybenzoic acid, is a naturally abundant plant phenolic compound has been attracting considerable interests for its antioxidant properties [10].

Three hydroxycinnamic acids identified in the analysis were chlorogenic acid, caffeic acid and coumaric acid. Sinapic and ferulic acid were not in found in either 280nm or 320nm chromatogram, with the reason being that the amount of acids were too nominal in comparisons to other acids in the fruit juice for the chromatograph to detect it at both wavelengths. Chlorogenic acid was the most dominant hydroxycinnamic acid in both varieties, as it accounted for the largest proportion of the total hydroxycinnamic acids contents (Table I). In both Ledang and Tambun, chlorogenic acid was found with 1.60-2.85mg/100mL, followed by coumaric acid (1.44–1.33mg/100mL) and caffeic acid (0.09–0.11mg/100mL). Chlorogenic and coumaric has long been known as the antioxidant that reduced the risk of stomach cancer by reducing the formation of carcinogenic nitrosamines [11].

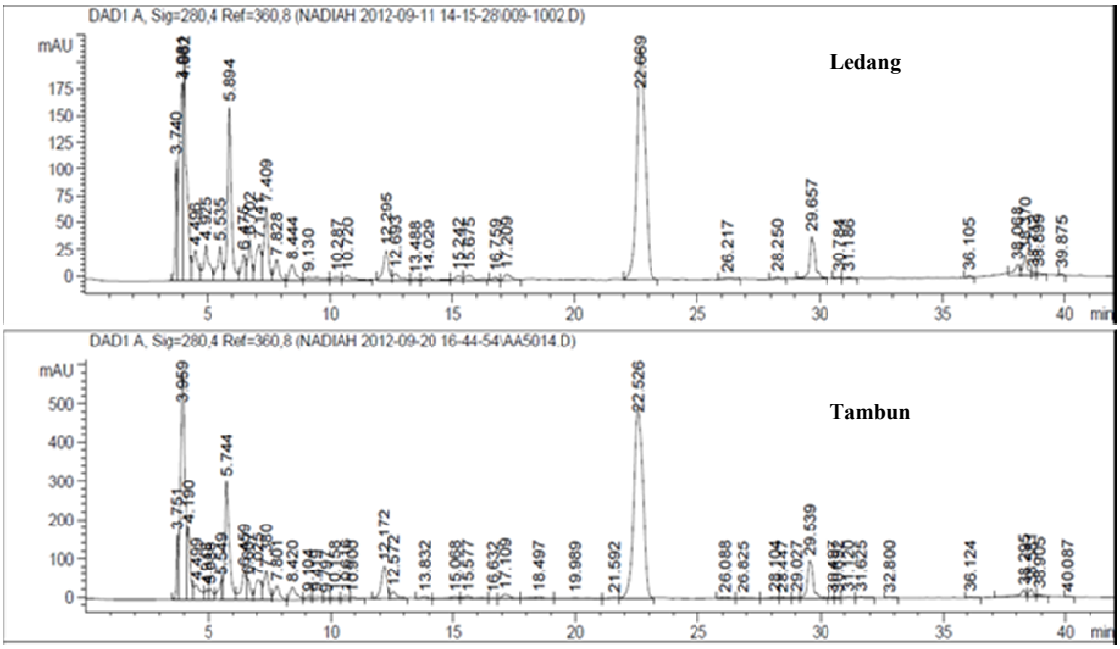

Fig. 1 HPLC-DAD chromatogram at 280nm of Ledang and Tambun variety

Flavanone is the major flavonoids found in citrus fruits, especially in pummelo. Three flavanones; naringin, hesperidin and narirutin were identified in both varieties. Table I and Fig. 1 showed that, the dominant neohesperidosyl flavanones, naringin was the most abundant phenols in all samples. Naringin may be instrumental in inhibiting cancer-causing compounds and thus may have potential chemotherapeutic value. Studies have also shown that naringin interferes with enzymatic activity in the intestines and, thus, with the breakdown of certain drugs, resulting in higher blood levels of the drug [12]. Naringin was found with 13.09 and 26.76mg/100mL respectively in Ledang and Tambun variety. However beneficial naringin as an antioxidant property, naringin is also known to give a bitter aftertaste to the juice, which may affect the marketability of the juice.

IV. Conclusion

In the present study, the antioxidant contents from two Malaysian varieties pummelo fruit juice were identified and quantified. Tambun variety was proven to possess high phenolic and flavonoid content, thus higher antioxidant capacity, due to the amount of naringin found in fresh squeezed juice. Naringin, the most dominant flavanone and main contributor of antioxidant property however, was found significantly less in Ledang variety. Theoretically Tambun would have made a better choice for juice development for the total phenolic content (naringin) it possessed, however, considerations have to be made to suit consumer's taste bud and the amount of enzyme needed to clarify the juice for its marketability.

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References

[1] Amin, I., Norazaidah, Y., Haninida, K.I.E., "Antioxidant activity and phenolic content of raw and blanched *Amaranthus* species" Food Chem vol 94, pp. 47–52, 2006.
[2] Garg, A., Garg, S., Zeneveld, L.J. & Singla, A.K., "Chemistry and pharmacology of the citrus bioflavonoid hesperidin" Photother Res vol 15, pp. 655–669, 2001.
[3] Jayaprakasha, G.K. & Bhimanagouda, S.P., "In vitro evaluation of the antioxidant activities in fruit extracts from citron and blood orange" Food Chem vol 101, pp. 410–418, 2007.
[4] Morton, J.F., "Pummelo" in *Fruits of Warm Climates*. Miami: Florida Flair Books, 1987, pp. 147–151.
[5] Department of Agriculture Malaysia, "Pomelo: Varieties/clones" unpublished.
[6] Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. & Yano, M., "Quantification of flavonoid constituents in citrus fruits" J Agric. Food vol 47, pp. 3565–3571, 1999.
[7] Bocco, A., Cuvelier, M.E., Richard, H. & Berset, C., "Antioxidant activity and phenolic composition of peel and seed extracts" J Agric. Food vol 41, pp. 2123–2129, 1998.
[8] Kanes, K., Tisserat, B., Berhow, M. & Vandercook, C., "Phenolic composition of various tissues of Rutaceae species" Phytochem vol 32, pp. 961–974, 1993.
[9] Shui, G. & Leong, L.P., " Analysis of polyphenolic ants in star fruit using liquid chromatography and mass spectrometry J Chromato A vol 1022, pp. 67 – 75, 2004.
[10] Strlic, M., Radovic, T., Kolar, J. & Pihlar, B., "Anti and pro-oxidative properties of gallic acid in Fenton-like systems" J Agric. Food vol 50, pp. 6313–6317, 2004.
[11] Nicoli, M.C., Anese, M., Parpinel, M.T., Franceschi, S. & Lerici, C.R., "Study on loss and/or formation of antioxidants during food processing and storage" Cancer Lett. vol 114, pp. 71-74, 1997.