

Full Length Research Paper

Flavonoid, hesperidine, total phenolic contents and antioxidant activities from Citrus species

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Citrus has long been regarded as a food and also as a medicinal plant. Fruits of four species of citrus which are commonly available in Malaysia, namely *C. hystrix* (wild lime), *C. aurantifolia* (common lime), *C. microcarpa* (musk lime) and *C. sinensis* (orange), were chosen to investigate their total phenolic, flavonoid and hesperidine contents. Additionally, the antioxidant activities were also determined by ferric reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity. *C. hystrix* had the highest flavonoid and total phenolic contents while *C. aurantifolia* had the highest hesperidine content. The antioxidant activity of *C. hystrix* was highest determined by FRAP and DPPH assays compared to other citrus species. A strong positive correlation of $R^2 = 0.9090$ between total phenolic content and FRAP values was observed in this investigation. This study indicated that *C. hystrix* exhibited the highest antioxidant, flavonoid and phenolic content and can be used potentially as a readily accessible source of natural antioxidant.

Key words: Antioxidant activity, citrus juice, flavonoids, hesperidine, phenolics.

INTRODUCTION

Free radicals have been claimed to play an important role in affecting human health by causing several diseases including cancer, hypertension, heart attack and diabetes. These free radicals are generated during body metabolism. Exogenous intake of antioxidants can help the body scavenge free radicals effectively. Furthermore, many studies have shown that increased dietary intake of natural phenolics correlates with reduced coronary heart disease, cancer mortality with longer life expectancy (Halliwell, 2007). Moreover, these polyphenolic compounds have been found effective in many health-related properties, such as antioxidant, anticancer, antiviral and anti-inflammatory activities (Amin et al., 2006). On other hand, concern about the safety of the commonly used synthetic antioxidants such as butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) have led to increased interest on natural antioxidants which occur in plants as secondary metabolites.

The genus citrus belonging to the family *Rutaceae* comprises about 40 species which are distributed in India,

China, Malaysia, Srilanka and Australia. Citrus is one of the most important world fruit crops and is consumed mostly as fresh or as juice because of its nutritional value and special flavor. Consumption of citrus juice is found to be beneficial in preventing coronary diseases and chronic asthma (Dugo and Giacomo, 2002). Citrus fruit extracts are also found to have antioxidant, anti-inflammatory, anti-tumor, anti-fungal and blood clot inhibition activities (Garg et al., 2001; Kaur and Kapoor, 2001; Abeysinghe et al., 2007). The health benefits of citrus fruit have mainly been attributed to the presence of bioactive compounds, such as ferulic acid, hydrocinnamic acid, cyanidin glucoside, hesperidine, vitamin C, carotenoid and naringin content (Abeysinghe et al., 2007; Kelebek et al., 2008; Xu et al., 2008).

The consumption of citrus juices in Malaysia is increasing rapidly. Previous studies on biochemical activities from citrus were mainly focused on its essential oils which include antimicrobial properties (Chanthaphon et al., 2008), anti-aflatoxigenic activity (Razzaghi-Abyaneh et al., 2009). However, a comparison of antioxidant activity, phenolic, flavonoid and hesperidine content between several species of citrus which are commonly available in Malaysia is still unknown and hence, we investigated this study for the first time. This study can help the food

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Table 1. Physical characteristics of different species of citrus.

Citrus species	Color	Size	Shape	Taste	Texture
<i>C. hystrix</i>	Yellow green	5 - 6 cm	Pear	Sour	Smooth
<i>C. aurantifolia</i>	Greenish yellow	5 - 10 cm	Round	Sour	Fibrous
<i>C. microcarpa</i>	Greenish yellow	4 - 5 cm	Round	Sweet	Fibrous
<i>C. sinensis</i>	Orange	5 - 10 cm	Round	Sweet	Smooth

industry to use it as a natural compound for antioxidant activity, which might be used as an alternative to synthetic antioxidants since it is environmentally friendly and safe for consumption.

MATERIALS AND METHODS

Preparation of juice sample

Fresh fruits of *Citrus hystrix*, *C. aurantifolia*, *C. microcarpa* and *C. sinensis* at the commercial mature stage were harvested from a commercial orchard in the month of November - December, from Pasar Borong and Seri Kembangan, Selangor, Malaysia. Healthy fruits were selected randomly for uniformity of shape and color. Physical characteristics of each species of citrus fruit are described and given in Table 1. The fruits were washed thoroughly in potable water and then air-dried. The citrus fruit juice was extracted by cutting the fruit in half and careful hand-squeezing to obtain the juice. The juice was passed through a strainer to remove pulp and seeds. The freshly squeezed juice was centrifuged at 1610 g for 20 min and the supernatant was then collected and stored at -20°C until further analysis.

Chemicals and reagents

1,1-Diphenyl-2-picryl hydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), hesperidine, gallic acid and aluminium trichloride were from Sigma Chemical Co. (St Louis, MO, USA). Folin-Ciocalteu reagent (FC reagent), sodium carbonate and ferrous sulphate, were from Merck (Darmstadt, Germany). All other chemicals and solvents used in this study were of analytical grade.

Determination of total phenolic content

The total phenolic contents of the different species of citrus were determined with some modification as described by Velioğlu et al. (1998). Various species of citrus juice samples (100 μL) was mixed with 0.75 mL of Folin Ciocalteu reagent (10-fold dilution with distilled water) and allowed to stand at 22°C for 5 min. Then, 0.75 mL of sodium carbonate (60 g/L) solution was added to the mixture. Following 90 min incubation at 22°C , absorbance was measured at 725 nm using a UV-visible spectrophotometer (Prim, SECOMAM, France). The total phenolic content was determined using a standard curve of gallic acid at 0.02 - 0.1 mg/mL concentrations. Total phenolic content was calculated for each sample and expressed as milligrams of gallic acid equivalent per 100 mL of juice.

Determination of flavonoid content

The total flavonoid content was determined using the method of Quettier et al. (2000) with minor modifications. In brief, 1 mL of 2%

aluminium trichloride was mixed with the same volume of citrus juice. Absorbance readings at 430 nm were taken after 10 min against a blank sample consisting of 1 mL of sample solution and 1 mL of distilled water without aluminium trichloride. The total flavonoid content was determined using a standard curve of hesperidine at 0 - 50 mg/mL. The average of three readings was used and then expressed as milligrams of hesperidine equivalents per 100 mL of juice.

Identification of hesperidine by high performance liquid chromatography (HPLC)

High performance liquid chromatography coupled to diode array detector was used to analyze hesperidine content of citrus samples according to method of Ortuna et al. (1997) using separation module (Agilent, HP 1100, Delaware, USA) equipped with a C_{18} column (μ -Bondapak, Waters, USA, 250×4 mm, 5 μm particle size). The samples were eluted using isocratic system of water: methanol: acetonitrile: acetic acid (75:10:10:5, v/v/v/v) as the mobile phase at a flow rate of 1.0 mL/min. The temperature of the column was maintained at 35°C and the injection volume was 20 μL . The peak of standard hesperidine was monitored at 280 nm for quantification. Identification and quantification of hesperidine was accomplished by comparing the retention times of peaks in samples to those of standard. Calculation of hesperidine concentration (expressed as mg/100 mL of juice) was carried out by an external standard method using calibration curves of standard hesperidine.

DPPH radical scavenging activity

The scavenging activity of citrus species was estimated according to the procedure modified by Shimada et al. (1992). An aliquot of 0.5 ml of juice and ascorbic acid at different concentrations (25, 50, 75, 100 and 150 mg/mL) was mixed with 2.9 mL of 100 μM DPPH (dissolved in 80% ethanol). The mixture was vigorously shaken and left to stand at room temperature for 30 min in a dark room. The control contained only DPPH solution instead of sample while 80% ethanol was used as the blank. Absorbance was read at 515 nm by using UV-vis spectrophotometer. The scavenging effect was calculated using the following equation:

$$\text{Scavenging effect (\%)} = 1 - \left[\frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \right]$$

EC_{50} value was determined from the plotted graph of scavenging activity against the concentrations of the citrus samples, which is defined as total antioxidant necessary to decrease the initial DPPH radical by 50%. Triplicate measurements were carried out and EC_{50} was calculated based on the percentage of DPPH radicals scavenged.

Ferric reducing power (FRAP) assay

First, ferric reducing antioxidant power (FRAP) reagent was prepared

Table 2. Total phenolic, flavonoid and hisperidine contents of different species of citrus.

Citrus species	Common name	Malayan name	TPC	FC	HC
<i>C. hystrix</i>	Wild lime	Limau purut	490.74 ± 1.75 ^a	22.25 ± 0.20 ^a	7.01 ± 0.83 ^b
<i>C. aurantifolia</i>	Common lime	Limau nipis	211.70 ± 0.0 ^b	10.67 ± 0.27 ^b	16.67 ± 2.57 ^a
<i>C. microcarpa</i>	Musk lime	Limau kesturi	105.0 ± 3.0 ^d	8.70 ± 0.13 ^c	ND
<i>C. sinensis</i>	Orange	Limau manis	135.3 ± 0 ^c	2.99 ± 0.09 ^d	5.58 ± 0.66 ^c

TPC, Total phenolic content expressed as milligrams of gallic acid equivalent/100 mL of juice; FC, Flavonoid content expressed as milligrams of hesperidine equivalent/ 100 mL of juice; HC, Hesperidine content expressed as mg/100mL of juice; ND, Not detected. For each treatment, the means within the column followed by different letters were significantly different at $P < 0.05$.

as described by Benzie and Strain (1996). FRAP reagent (1.8 mL) was taken in a test tube and incubated at 30°C in water bath for 10 min. Then, absorbance was taken at 0 min (t_0). Immediately, 100 µL of sample or standard and 100 µL of distilled water were added to the test tube, mixed and incubated at 30°C for 4 min. Then, the absorbance was taken at 593 nm (t_4). Ferrous sulfate was used as standard (50 - 200 µM). The antioxidant potential of the sample extract from citrus was determined against a standard curve of ferrous sulphate and the FRAP value was expressed as µM Fe²⁺ equivalents per 100 mL of juice and calculated using the following equation.

$$\text{FRAP value} = \text{Absorbance}_{(\text{sample} + \text{FRAP reagent})} - \text{Absorbance}_{(\text{FRAP reagent})}$$

Statistical analysis

Data were expressed as means ± standard deviations (SD) of three replicate determinations and then analyzed by SPSS V.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). One way analysis of variance (ANOVA) and the Duncan's New Multiple-range test were used to determine the differences among the means. P values < 0.05 were regarded to be significant. The Pearson correlation analysis was performed between antioxidant activity and total phenolic content.

RESULTS AND DISCUSSION

Total phenolics, flavonoid and hisperidine content of citrus samples

Table 2 summarizes the contents of total phenolics, flavonoid and hisperidine contents of the different species of citrus. Phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activity. Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phospho-molybdic acids present in the Folin-Ciocalteu reagent (Prasad et al., 2005). However, it should be also noted that some chemical group of ascorbic acid, organic acids, sugars, aromatic amines can also react with FC reagent (Meda et al., 2005). *C. hystrix* had the highest total phenolic content (490.74 ± 1.75) significantly higher ($P < 0.05$) than other citrus species, while *C. microcarpa* had the lowest value (105.0 ± 3.0 mg GAE/100 mL of juice). Xu et al. (2008) have reported total phenolic content of 149.9 (GAE/100 mL of juice) from *C. sinensis*. This slight

difference from our results might be due to different extraction method adapted and also due to different environmental conditions.

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. The flavonoids in the citrus juice combine with aluminium to form a complex flavonoid-aluminium that could be measured at 430 nm (Quettier et al., 2000). Using the standard curve of hesperidine, the flavonoid content of citrus species varied from 22.25 ± 0.20 in *C. hystrix* to 2.99 ± 0.09 mg hesperidine equivalent/100 mL of juice in *C. sinensis*.

Hesperidin, a member of the flavanone group of flavonoids, can be commonly found in large amounts from citrus species and has been reported to have antioxidant, anticarcinogenic, antihypertensive and antimicrobial properties (Garg et al., 2001). Hesperidine was identified and then quantified by comparing their peak area with those of the standard. A great variation existed in the hesperidine composition among citrus species (Table 1). *C. aurantifolia* contained the highest hesperidine, while *C. sinensis* exhibited the lowest content; but in *C. microcarpa*, it was not detectable. Our results are in agreement with other authors who have reported similar results of hesperidin content in *C. sinensis* juice (Beljova and Suhaj, 2004).

DPPH radical scavenging activity

DPPH is a commercial oxidizing radical which can be reduced by antioxidants. In this assay, the violet colour of DPPH was reduced to a pale yellow color due to the abstraction of hydrogen atom from antioxidant compound. The more antioxidants occurred in the extract, the more the DPPH reduction will occur. High reduction of DPPH is related to the high scavenging activity performed by particular sample (Blois, 1958). EC₅₀ was calculated as amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50%. The lower the EC₅₀ value, the higher is the antioxidant activity.

C. hystrix had the highest scavenging activity against

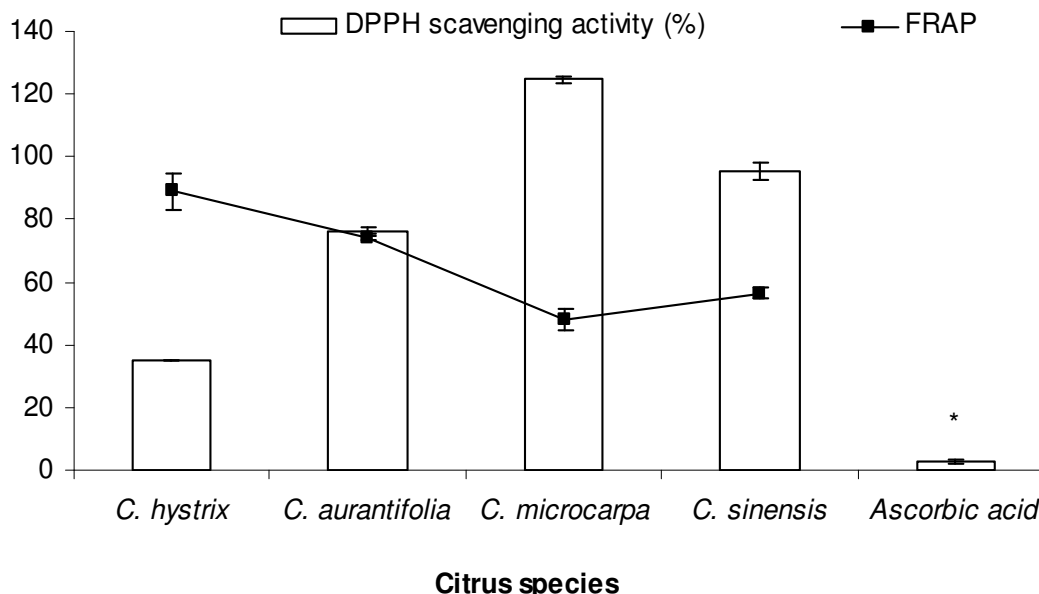


Figure 1. FRAP and DPPH radical scavenging activities of different species of citrus. FRAP, Ferric reducing antioxidant power expressed as $\mu\text{mol Fe}^{2+}$ equivalent/100 mL of juice; DPPH activity was expressed as EC_{50} values in mg/100 mL of juice concentration required to decrease the absorbance by 50%. Ascorbic acid used as positive control.

DPPH radicals compared to other species of citrus. *C. hystrix* at a concentration of 35 mg/100 mL of fresh juice was required to scavenge 50% of initial concentration of DPPH radical indicating its EC_{50} value (Figure 1). Our results are in agreement with Spada et al. (2008) who have reported high DPPH scavenging activity of lemon compared to orange. The high phenolic content of *C. hystrix* could be the main reason for its high antioxidant activity towards DPPH radicals. Jayaprakasha et al. (2008) have demonstrated the DPPH radical scavenging activities of citrus fractions is by their hydrogen donating ability.

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay was used to evaluate the antioxidant potential of different species of citrus. Principally, FRAP assay treats the antioxidants in the sample as reductant in a redox-linked colorimetric reaction (Guo et al., 2003). This assay is relatively simple and easy to conduct. FRAP assay measures the reducing potential of antioxidant to react on ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex and produce blue color of ferrous form (Benzie and Strain, 1996) which can be detected at absorbance 593 nm. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction. The higher the absorbance is, the higher is the antioxidant activity which is indicated by the high FRAP value.

The highest FRAP value of 89.0 ± 5.88 was obtained

from *C. hystrix* samples while lower value of 48.18 ± 3.34 was observed in *C. microcarpa* samples (Figure 1). The antioxidant potential of citrus species were in the increasing order of *C. hystrix* > *C. aurantifolia* > *C. sinensis* > *C. microcarpa*. In the present study, *C. hystrix* (wild variety) demonstrated the greatest reducing power significantly higher ($p < 0.05$) than other citrus species. This result is in agreement with those reported by Scalzo et al. (2005) who reported high antioxidant activity of wild strawberry than those of cultivated varieties. Jayaprakasha et al. (2008) demonstrated that the reducing power of citrus fractions were due to presence of phenolic compounds.

The antioxidant activities of citrus species are in accordance with their amount of phenolics. *C. hystrix* contained high phenolic content compared to other citrus species, which was responsible for its high antioxidant activity. Several reports showed a close relationship between total phenolic content and high antioxidant activity (Prasad et al., 2005; Amin et al., 2006; Li et al., 2009). The phenolic compounds exhibit extensive free radical scavenging activities through their reactivity as hydrogen or electron-donating agents and metal ion chelating properties (Rice-Evans and Bourdon, 1993).

A high linear correlations of $R^2 = 0.9090$ and 0.9470 between ferric reducing power, DPPH radical scavenging activity and total phenolic content were observed in this investigation. Li et al. (2009 and 2008) have reported the existence of similar linear relationships between reducing power and total phenol content. However, a negative correlation coefficients between total flavonoids and DPPH radical scavenging activity were determined to be $R^2 = -$

0.9580, much lower than those of total phenolics. Similar results of negative correlation were reported by Meda et al. (2005) between flavonoid content and antioxidant activity. The negative correlation between citrus species and flavonoid content are probably because of the presence of other phenolics in the citrus samples which might have contributed to antioxidant activity.

Conclusions

In the present study, the antioxidant activity from four species of citrus was evaluated. *C. hystrix* possessed high phenolic and flavonoid content and exhibited good antioxidant activity by DPPH and FRAP methods. The use of *C. hystrix* as a natural antioxidant source appears to be an alternative to synthetic antioxidants. This study was the first report about the antioxidant activities of different species of citrus which are commonly available in Malaysia. Further investigation to determine antioxidant activity by *in-vivo* methods of citrus species could be considered.

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