Antioxidant Properties of Fresh and Frozen Peels of *Citrus* Species

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Abstract
Citrus peel is a functional food. It is rich in antioxidants. This study aims to investigate the antioxidant properties of selected fresh and frozen peels of *Citrus* species. Frozen and fresh peels of lemon (*Citrus limon*), key lime (*C. aurantifolia*) and musk lime (*C. microcarpa*) were screened for their antioxidant properties such as total phenolic content and total flavonoid content. DPPH radical scavenging activity and ferric ion reducing antioxidant power (FRAP) assays were also determined. Among the three *citrus* peels, musk lime peel had the significantly highest total phenolic content and total flavonoid content. Frozen *citrus* peels showed significantly higher antioxidant content than the fresh peels. The frozen peels also showed promising antioxidant activity as indicated by their significantly higher FRAP value compared with fresh citrus peels. Moreover, frozen citrus peel possessed higher antioxidant activity as indicated by its lower EC₅₀ values which ranged between 0.823 ± 0.1 and 3.16 ± 0.92 mg mL⁻¹. A moderately high correlation was determined between FRAP value and total phenolic content (r=0.783), and between FRAP value and total flavonoid content. This study shows that frozen peels of citrus are functional foods and sources of potent antioxidants.

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Introduction
Free radicals or reactive oxygen species (ROS) are the outcomes of normal essential metabolic processes. Exposure to air pollutants, cigarette smoking, industrial chemicals, ozone and X-rays also contribute to the production of free radicals. The implication of free radicals and ROS exposure employs a multitude of biological effects covering many chronic diseases. Therefore, food with a high content of antioxidant compounds may lower the chances of chronic disease development because natural antioxidant from plant owns a protective effect against the progress of chronic diseases. Thus, the antioxidant is essential in protecting the human body from harmful effects of oxidation. The Citrus genus belongs to the family of Rutaceae which includes around 40 species of citrus; the citrus trees are widely planted in China, India, Malaysia, Australia and Sri Lanka.

Citrus fruit has been largely cultivated in the tropical and subtropical countries with a total annual production of approximately 102 million tonnes. In 2014, the production of citrus in Malaysia alone was around 42,212 tonnes. Citrus is planted for domestic and export purposes on 6,208 hectares of land around the country. Fruit from Rutaceae plants are typically rich in phytochemicals, and these plants are the good sources of bioactive compounds that responsible for antioxidation and many other biological activities. Citrus fruit prevents free radical generation and reduces homocysteine level; it also possesses anti-carcinogenic, anti-diabetic, anti-inflammatory and anti-arthritic properties.

It is important to incorporate citrus fruit into our daily diets as it is rich in antioxidants. It supplies energy and nutrient to our body for combating diseases. Among the different parts of citrus fruit, citrus peel contains the highest proportion of natural antioxidants such as natural flavonoid, phenolic, ascorbic acid and carotenoids, as well as reducing sugar. Literature shows that citrus peel extracts had the highest antioxidant activity compared with pulp and seeds. The citrus peel that represents around 50-65% of the fruit is commonly treated as a primary citrus by-product and discarded as the massive load into the environment.

Antioxidants in a plant are susceptible to degradation, mostly due to the handling practices such as chilling injury, fungal decay, irradiation, incorrect holding temperature, relative humidity and other factors of stress that can contribute to the reduction of antioxidant content and nutritional value. There is an established investigation of postharvest exposure at a temperature above the freezing point that can contribute to a negative effect on nutrient quality and antioxidant. However, there are inconsistencies in the finding on the effect of freezing toward various plant samples, where some studies reported a positive effect of frozen samples and some were contradicted. The current study aims to determine antioxidant content and antioxidant activity of fresh and frozen selected citrus peels. This study used fresh and frozen peel samples of lemon, key lime and musk lime.

Material and Methods
Compound Name
In this study, the chemicals used were 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri-(2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium bicarbonate (Na\textsubscript{2}CO\textsubscript{3}), sodium nitrate (NaNO\textsubscript{3}), sodium acetate trihydrate (C\textsubscript{2}H\textsubscript{3}NaO\textsubscript{2}∙3H\textsubscript{2}O), ferric chloride hexahydrate (FeCl\textsubscript{3}∙6H\textsubscript{2}O), ferrous sulphate heptahydrate (FeSO\textsubscript{4}∙7H\textsubscript{2}O), aluminium chloride (AlCl\textsubscript{3}), glacial acetic acid, Folin-Ciocateu reagent, gallic acid and quercetin. These chemicals and reagents were supplied by Sigma-Aldrich, Co. (USA).

Sample Preparation
Selected citrus species were Citrus limon (lemon) C. aurantifolia (key lime) and C. microcarpa (musk line). All samples were purchased at the Selangor wholesale market, Malaysia. The fruit peel was removed from the pulp, and the peel was divided into two parts. Half of each fresh peel was extracted for antioxidants right after sample preparation; while the other half was the frozen sample. All samples were kept frozen at −80°C for 24 h prior to freeze-drying.

Extraction Method
Antioxidants from both fresh and frozen peels of citrus fruit were extracted according to Ferreira et al., with slight modification. The fresh citrus peels
(10.0 g) and freeze-dried (frozen) citrus peel powders (25.0 g) were added to 100 mL of 70% ethanol solution in a conical flask. The extracts were then sonicated for 60 min and filtered using a Whatman No. 1 filter paper. The ethanol was removed using a vacuum rotary evaporator (Buchi Rotavapor R-200, Switzerland) at 50°C. The extract was kept at −20°C before further analysis. The extraction yield was obtained by weighing the extract after the solvent removal; the percentage of the extraction yield was calculated based on the equation as follows:

\[
\text{Extraction yield (\%) } = \frac{\text{Weight of yield obtained}}{\text{Weight of fresh sample}} \times 100 \%
\]

**Estimation of Total Phenolic Content**

Folin-Ciocalteu (FC) reagent assay was used to determine total phenolic content (TPC) of citrus samples. The assay was performed according to the procedure described by Meda et al.,\textsuperscript{19} with slight modification. FC solution (0.2 N) was prepared by adding 0.15 mL of FC reagent with 15.0 mL of methanol, whereas Na\textsubscript{2}CO\textsubscript{3} solution was prepared by mixing 0.6 g Na\textsubscript{2}CO\textsubscript{3} with 10.0 mL of distilled water. Briefly, 1.0 mL of sample was added with 1.0 mL of FC reagent and incubated for 5 min in the dark before the addition of 2.0 mL of Na\textsubscript{2}CO\textsubscript{3} solution. The absorbance was measured spectrophotometrically at 725 nm. Gallic acid (1-1000 µg mL\textsuperscript{-1}) was used for standard calibration. The results were expressed as gallic acid equivalent (GAE) per gramme fresh sample.

**Estimation of Total Flavonoid Content**

Total flavonoid content (TFC) of citrus samples was estimated based on the aluminium chloride colourimetric method as described by Ghasemzadeh et al.,\textsuperscript{20} Briefly, 2.0 mL of the diluted sample was mixed with 0.2 mL of 5% NaNO\textsubscript{3} and incubated at room temperature for 5 min before the addition of 0.2 mL of 10% AlCl\textsubscript{3}. After 6 min of incubation, the mixture was added with 2.0 mL of 1 M NaOH. The volume was then made up to 5.0 mL by adding 70% ethanol and incubated for another 10 min. Absorbance of the reacting mixture was read at 430 nm. Quercetin was used as a standard at concentrations ranged between 1.0 µg mL\textsuperscript{-1} and 1000.0 µg mL\textsuperscript{-1}. The results were presented as milligramme quercetin equivalent per gramme (mg QE g\textsuperscript{-1}) fresh sample.

**DPPH Radical Scavenging Assay**

DPPH radical scavenging activity of citrus samples was determined according to the method described by Thaipong et al.,\textsuperscript{21} with slight modification. DPPH stock solution was prepared by adding 24 mg DPPH with 100 mL of methanol, whereas DPPH working solution was prepared by mixing 10 mL of the DPPH stock solution with 45 mL of methanol. Briefly, 500 µL of the sample was added with 500 µL of DPPH working solution. The mixture was incubated for 2 h at room temperature in the dark. Absorbance of the mixture was determined at 515 nm against a blank. The results were expressed as EC\textsubscript{50} value of DPPH assay in mg mL\textsuperscript{-1}.

**Ferric Reducing Antioxidant Power**

Ferric reducing antioxidant power (FRAP) of citrus samples was determined based on the method described by Ghasemzadeh et al.,\textsuperscript{20} FRAP reagent was prepared by adding 2.5 mL of TPTZ (10 mM in 40 mM HCl) with 2.5 mL of FeCl\textsubscript{3} solution (20 mM in distilled water) and 25 mL of acetate buffer (300 mM, pH 3.6). In brief, 3.0 mL of the freshly prepared FRAP reagent was added with 50 µL of the sample, and stand at 37°C for 30 min in the dark. Absorbance was determined at 593 nm after 30 min of incubation. Antioxidant activity was expressed as millimoles ferrous ion per gramme fresh weight (mM Fe\textsuperscript{2+} g\textsuperscript{-1} FW).

**Statistical Analysis**

The data were presented as mean ± standard deviation of three replicates (n = 3). The mean differences were statistically analysed based on one-way analysis of variance (ANOVA) with posthoc test (Tukey HSD). Pearson correlation coefficient was determined between the content of antioxidant compounds and antioxidant activity of the fresh and frozen citrus peels. The p-value of less than 0.05 was considered significant differences between different citrus samples. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 23.

**Results and Discussion**

**Extraction Yield**

Solvent extraction is the most commonly used technique in isolation of antioxidant compound because it is a widely applicable technique and efficient in extracting bioactive compounds. Ethanol
is one of the popular solvent used in extraction as it offers higher antioxidant properties compared with water extraction.\textsuperscript{22,23} The extraction yields obtained are presented in Table 1. Aqueous ethanol (70\%) was chosen as the extraction solvent due to the reason that it is an ideal solvent for extracting polyphenolic compounds; it is also not toxic to the environment and safe for human consumption.\textsuperscript{3,24} The results of this study showed that the extraction yields of all frozen citrus peels were significantly higher than the fresh citrus peels (p < 0.05).

The results showed that the extraction yield for fresh lemon peel (11.0\%) was significantly lower than frozen lemon peel (33.9\%). Similarly, extraction yields for fresh key lime peel (6.7\%) and fresh musk lime (7.9\%) were also significantly lower than frozen key lime peel (31.7\%) and frozen musk lime peel (33.0\%), respectively. Based on the result obtained, frozen lemon peel had 207.3\% total extractable yield higher than fresh lemon peel. Similarly, frozen key lime peel and frozen musk lime peel had 373.6\% and 315.9\% total extraction yield higher than fresh key lime peel and fresh musk lime peel, respectively. Freezing technique serves as a better method to preserve the antioxidant activity of polysaccharide from plant samples as the bioactive compound in the sample can be maximally protected under the freezing condition.\textsuperscript{25} Literature also showed that frozen carrot samples had higher extraction yield of carotenoids than the non-frozen samples.\textsuperscript{26}

**Total Phenolic Content**

In this study, all frozen citrus samples had significantly higher TPC than the fresh citrus samples (Table 1). The results showed that TPC of fresh lemon peel (72.0 ± 0.67 mg GAE g\(^{-1}\) FW) was significantly lowered than frozen lemon peel (100.0 ± 0.96 mg GAE g\(^{-1}\) FW). The TPC was also significantly different between fresh musk lime peel (108.6 ± 1.34 mg GAE g\(^{-1}\) FW) and frozen musk lime peel (136.5 ± 0.58 mg GAE g\(^{-1}\) FW). Moreover, TPC of fresh key lime peel (75.7 ± 1.34 mg GAE g\(^{-1}\) FW) and frozen key lime peel (118.6 ± 0.77 mg GAE g\(^{-1}\) FW) had the same trend, where the frozen samples possess significantly higher TPC than the fresh sample. Furthermore, the finding of this study shows that frozen lemon peel had 38.9\% increment in TPC compared with the fresh lemon peel. Similarly, the frozen key lime peel and frozen musk lime peel showed 56.7\% and 25.7\% increment in TPC, respectively.

Fruit possesses a high concentration of phenolic in the outer compartment. The phenolic compounds are known to be accumulated in the vacuoles. Food processing such as freezing helps to accelerate a more release of the bound phenolic compound from the breakdown of the cellular part.\textsuperscript{27} Besides, the enzymes that can destroy antioxidant such as oxidative and hydrolytic enzymes are deactivated during freezing.\textsuperscript{28,29} Our finding is supported by a previous report that the TPC of frozen Ajwain leaf sample was significantly higher than the fresh sample.\textsuperscript{30} On the contrary, freezing the freshly cut peach at −20 °C for 24 h did not significantly affect TPC of the peach compared with the freshly cut peach\textsuperscript{31}. The content of total phenolics of frozen raspberry and blackberry of different cultivars was also significantly lower than the fresh samples.\textsuperscript{32}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction yield (%)</th>
<th>TPC (mg GAE g(^{-1}) FW)</th>
<th>TFC (mg QE g(^{-1}) FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh lemon</td>
<td>11.03 ± 0.67(^c)</td>
<td>72.01 ± 0.67(^f)</td>
<td>50.51 ± 1.36(^d)</td>
</tr>
<tr>
<td>Frozen lemon</td>
<td>33.9 ± 0.74(^a)</td>
<td>100.00 ± 0.96(^d)</td>
<td>78.15 ± 0.61(^c)</td>
</tr>
<tr>
<td>Fresh key lime</td>
<td>6.7 ± 0.95(^f)</td>
<td>75.68 ± 1.34(^a)</td>
<td>50.75 ± 0.24(^d)</td>
</tr>
<tr>
<td>Frozen key lime</td>
<td>31.73 ± 0.91(^b)</td>
<td>118.61 ± 0.77(^b)</td>
<td>82.22 ± 1.29(^c)</td>
</tr>
<tr>
<td>Fresh musk lime</td>
<td>7.94 ± 0.75(^d)</td>
<td>108.57 ± 1.34(^c)</td>
<td>120.92 ± 2.56(^b)</td>
</tr>
<tr>
<td>Frozen musk lime</td>
<td>33.02 ± 0.23(^a)</td>
<td>136.48 ± 0.58(^a)</td>
<td>178.32 ± 2.13(^a)</td>
</tr>
</tbody>
</table>

The values are presented as mean ± standard deviation (n = 3). Different superscript lowercase letters denote a significant difference at p < 0.05, Tukey posthoc test. GAE - gallic acid equivalent; QE - quercetin equivalent; FW - fresh weight.
freshness of fruit samples reported in these studies could be varied. Postharvest storage of different fresh fruit samples should be further investigated. As the studied frozen fruit samples showed a better TPC than the non-frozen fresh samples; therefore, cold storing or freezing of freshly harvested fruit retains the bioactive antioxidants of the fruit.

**Total Flavonoid Content**

Fruit contains naturally occurring compounds. These compounds are hydroxybenzoic acid, anthocyanins, hydroxycinnamic acids, proanthocyanidins, flavonoids, lignans and stilbenes.\(^3^3\) As shown in Table 1, TFC of the frozen citrus peels was significantly higher than the fresh citrus peels. Based on the results obtained, TFC of fresh lemon peel (50.51 mg QE g\(^{-1}\) FW) and frozen lemon peel (78.15 mg QE g\(^{-1}\) FW) was significantly different. TFC of the fresh key lime peel (50.75 mg QE g\(^{-1}\) FW) was also significantly lower than the frozen key lime peel (82.22 mg QE g\(^{-1}\) FW). Similar with frozen lemon and key lime peels, TFC of frozen musk lime peel (178.32 mg QE g\(^{-1}\) FW) was significantly higher than the TFC of fresh musk lime peel (120.92 QE g\(^{-1}\) FW). The finding also showed that frozen lemon peel had 54.7% increment in TFC compared with the fresh lemon peel. Similarly, frozen key lime peel and frozen musk lime peel had 62.0% and 47.5% increment of TFC, respectively. Based on a previous study, frozen red raspberries had higher TFC than the fresh and refrigerated red raspberries.\(^3^4\) The reason might be after due to deactivation of oxidative and hydrolytic enzymes during the freezing process.\(^2^8\)

**DPPH Radical Scavenging Activity**

Antioxidants have the capability to slow down or prevent the oxidation process of other molecules.\(^3^5\) DPPH radical scavenging assay is a procedure for estimation of antioxidant capacity by measuring the decrease in absorbance after oxidation processes. The scavenging activity was determined based on the capability of the antioxidants to retard colour loss. The results of this study showed that no significant difference was found between the frozen and fresh citrus samples (Table 2). Among the samples, frozen lemon peel had the lowest EC\(_{50}\) value (0.82 mg mL\(^{-1}\)), followed by fresh lemon peel (1.30 mg mL\(^{-1}\)), fresh key lime peel (1.57 mg mL\(^{-1}\)), fresh key lime peel (1.83 mg mL\(^{-1}\)), frozen musk lime peel (2.70 mg mL\(^{-1}\)) and fresh musk lime peel (3.16 mg mL\(^{-1}\)). The lowest EC\(_{50}\) value represents the highest scavenging activity of a sample. Although no statistically significant difference was found between EC\(_{50}\) values of the frozen and fresh citrus peels, the frozen peels had better efficiency in scavenging DPPH radicals than the fresh citrus peels.

**Ferric Reducing Antioxidant Power**

Ferric reducing antioxidant power (FRAP) assay is another method for determination of the antioxidant activity of a plant extract. The assay signifies the power of antioxidants as reductants in the redox-linked colourimetric reaction. This assay is also used to quantify the capability of antioxidants in a sample to reduce ferric (III) ions to ferrous (II) ions at a low pH.\(^3^5\) As shown in Table 2, FRAP values of the fresh and frozen citrus peels ranged from 0.38 mM Fe\(^{2+}\) g\(^{-1}\) FW to 0.52 mM Fe\(^{2+}\) g\(^{-1}\) FW. FRAP value of frozen musk lime peel (0.53 mM Fe\(^{2+}\) g\(^{-1}\) FW) was significantly higher than the value of fresh musk lime peel (0.46 mM Fe\(^{2+}\) g\(^{-1}\) FW). Similarly, frozen peels of lemon and key lime had significantly higher FRAP values than the fresh peels (Table 2).

There are limited published data on FRAP values of fresh and frozen peels of lemon, key lime and musk lime. Only data on FRAP values of fresh and frozen guava samples are available for reference. Although no significant difference between the FRAP values of fresh and frozen samples was observed, the FRAP value of the frozen guava sample was slightly higher than the FRAP value of fresh guava sample.\(^3^6\) It could be due to the low storage temperature destructed the cell walls of guava, and also the release slightly higher bioactive compounds such as polyphenol and carotenoids from the frozen guava tissue. The higher free phenolics extracted from the frozen fruit sample contributed to the significant increase in antioxidant activity.\(^3^0\)

**Correlation between Antioxidant Content and Antioxidant Activity**

As shown in Table 3, EC\(_{50}\) values of DPPH radical scavenging assay were not significantly correlated with both TPC or TFC. Therefore, an increase in the TPC does not increase the DPPH scavenging activity. It might be due to the fact that the increased value of TPC is attributed to ascorbic acid content.
Table 2: Antioxidant activities of frozen and fresh citrus peels

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH EC&lt;sub&gt;50&lt;/sub&gt; (mg mL⁻¹)</th>
<th>FRAP (mM Fe&lt;sup&gt;2+&lt;/sup&gt; g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh lemon</td>
<td>1.30 ± 0.42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.38 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen lemon</td>
<td>0.82 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.51 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh key lime</td>
<td>1.83 ± 0.25&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.44 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen key lime</td>
<td>1.57 ±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fresh musk lime</td>
<td>3.16 ± 0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen musk lime</td>
<td>2.70 ± 0.42&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.53 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are presented as mean ± standard deviation
Different superscript lowercase letters denote a significant difference at p < 0.05, Turkey posthoc test.
FW - fresh weight.

Table 3: Correlation coefficient of total antioxidant content and antioxidant activity

<table>
<thead>
<tr>
<th>Antioxidant properties</th>
<th>TPC</th>
<th>TFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>FRAP</td>
<td>r = 0.783</td>
<td>r = 0.681</td>
</tr>
</tbody>
</table>

NC - no correlation; r - Pearson coefficient
**significant at p < 0.05.

A previous study revealed that ascorbic acid is a strong free radical scavenger. Literature also shows that citrus peels exhibited a high vitamin C content. As citrus peel contains a high amount of ascorbic acid, the compound might affect the DPPH radical scavenging activity of the peel. The finding of this study is supported by a study which determined vitamin C and antioxidant capacities of fresh and frozen guava samples. Based on the study, no correlation was determined between the phenolic compound and antioxidant activity of the guava samples. However, there was a high correlation between vitamin C content and antioxidant capacities. Thaipong et al., also showed that there was a strong correlation between ascorbic acid content and antioxidant capacity of the citrus and guava samples. On the contrary, Sir Elkhatim et al., reported a low correlation between vitamin C content and DPPH radical scavenging assay. Another study also revealed that no significant correlation was found between the antioxidant content and scavenging activity of the peels of 13 citrus species.

FRAP values of the fresh and frozen citrus peels reported in this study (Table 3) were positively correlated with TPC (r= 0.783) and TFC (r= 0.681). The higher the FRAP values of the frozen and fresh citrus peels, the higher the total antioxidants. Therefore, total phenolics and flavonoids were highly correlated with FRAP values. The finding suggests that phenolic compounds in the citrus samples highly contributed to the reduction of ferric ions to ferrous ions. A preceding study also supports our finding that the peels of C. sinensis and C. aurantium had a strong correlation between phenolic content and reducing power.

Conclusion
In brief, frozen citrus peels had higher antioxidant properties than the fresh peels. The frozen peels also possessed higher phenolics content and antioxidant activity than the fresh peels. Based on the results obtained, frozen citrus peels have high dietary antioxidants. Due to the high antioxidant capacity, frozen citrus peels can be used as raw ingredients for production of dietary supplements and in value-added products. Food industries are encouraged to fully utilise the fruit peels resulted from juice processing since these by-products contain many potentials in terms of their health benefits.
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