Phytochemical composition and in vitro antioxidant activities of *Citrus sinensis* peel extracts

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**Background.** *Citrus sinensis* peels are usually discarded as wastes, however, they are rich sources of Vitamin C, fibre, and many nutrients, including phenolics and flavonoids which are also good antioxidant agents. This study aimed to examine phytochemical composition and antioxidant capabilities of *C. sinensis* peel extracted conventionally with different methanol/water, ethanol/water, and acetone/water solvents. **Methods.** *C. sinensis* peel were subjected to extraction with 100%, 70% and 50% of methanol, ethanol, and acetone respectively, as well as hot water extraction. Antioxidant activities of the peel extracts were examined via the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, and oxygen radical absorbance capacity (ORAC) assay. Total phenolic content and total flavonoid content of the extracts were then measured via Folin-Ciocalteau and aluminium chloride colorimetric method respectively. Phenolic acid and organic acid composition of the peel extracts were further determined via High Performance Liquid Chromatography (HPLC) while flavonoid content was identified via Ultra Performance Liquid Chromatography (UPLC). **Results.** DPPH radical scavenging activity of *C. sinensis* peel extracts varied from 8.35 to 18.20 mg TE/g, FRAP ranged from 95.00 to 296.61 mmol Fe(II)/g, while ORAC value ranged from 0.31 to 0.92 mol TE/g. Significant level of association between the assays was observed especially between TPC and FRAP (R-square=0.95, P<0.0001). TPC of various *C. sinensis* peel extracts ranged from 12.08 to 38.24 mg GAE/g, with 70% AEC showing the highest TPC. TFC ranged from 1.90 to 5.51 mg CE/g. Extraction yield ranged from 0.33 to 0.52 g/g DW and tended to increase with increasing water concentration in the solvent. In phytochemical investigation, five phenolic acids were identified using HPLC, including gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, caffeeic acid and ferulic acid. A total of five organic acids including lactic acid, citric acid, L-mallic acid, kojic acid and ascorbic acid were quantified via HPLC. Besides that, concentrations of six flavonoids including catechin, epigallocatechin, vitexin,
rutin, luteolin and apigenin were determined via UPLC. **Discussion and Conclusion**

Phytochemicals including phenolics and flavonoids in *C. sinensis* peel extracts exhibited good antioxidant properties. Among the extracts, 70% AEC with highest TPC and high TFC content showed greatest antioxidant activity in all three assays. Different phenolic acids, organic acids and flavonoids were also identified from the extracts. This study indicated that *C. sinensis* peels contained potential antioxidant compounds which could be exploited as value added products in the food industry.
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Abstract

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TPC and high TFC content showed greatest antioxidant activity in all three assays. Different 
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Introduction

Citrus sinensis, also known as sweet orange, is consumed not only as a fruit but also as 
medicinal herb in some nations. It belongs to the Rutaceae family and is widely distributed in the 
tropical and subtropical regions. Annual worldwide citrus fruit production now stands at over 
110 million tons and orange has become the most commonly grown fruit in the world (Blauer, 
2016). Out of the forecasted global production of 24 million tons of oranges in year 2016/17, it is 
estimated that around 8.3% (2 million tons) of these will be used for orange juice production 
(Foreign Agricultural Service/USDA, 2017). However, orange peels accounts for around 44% of 
the fruit body (Li et al. 2006) and thus will produce a huge mass of by-products. These orange 
peels are usually discarded as wastes leading to serious disposal problem that may be detrimental 
to the environment.

Considering the huge quantity of these “wastes” that are produced in the food supply chain, 
orange peels offers a huge potential to be exploited as value-added product, including for the 
recovery of natural antioxidants, pectin, enzymes or for the production of ethanol, organic acids, 
essential oils and prebiotics single cell protein (Mamma and Christakopoulos, 2014). In addition, 
C. sinensis peel is a rich source of Vitamin C, fibre, and many nutrients, including phenolics and 
flavonoids. It is subdivided into two main parts, epicarp and mesocarp. Epicarp is the coloured 
peripheral surface, largely made of parenchymatous cells and cuticle. It is covered with an 
epidermis of epicuticular wax with many small aromatic oil glands giving its particular smell. 
Mesocarp is the soft whitish middle layer lying beneath epicarp. It is made up of tubular-like 
cells connecting together to create the tissue mass compressed into the intercellular area (Favela- 
Hernández et al., 2016). C. sinensis peel has been used as a traditional medicine in certain parts 
of the world for relieving stomach discomfort, skin inflammation, ringworm infections, aiding in 
neuroprotection, and improving heart health (Li, Lo and Ho. 2006; Ghasemi et al. 2009).
Various potent antioxidants have been found in citrus peels and showed antioxidant effect including free radical scavenging and metal chelation activities. It is much encouraging to explore the active phytochemicals in *C. sinensis* peel as reactive oxygen species is playing a main role in many diseases such as cancer, cardiovascular dysfunction, neurodegenerative diseases, and process of ageing (Rafiq et al., 2016). A recent study on identification of 4'-geranyloxyferulic (GOFA) among citrus peel extracts revealed that *C. sinensis* has the richest content of GOFA which previously showed neuroprotective and dietary feeding colon cancer chemopreventive effects in rats (Genovese and Epifano, 2012; Genovese et al., 2014). A group of flavonoids, polymethoxyflavones (PMFs), which is found abundantly and almost only from the citrus peels, have been given great attention because of their wide range of properties. Many in vitro experiments elucidated anticancer actions by PMFs such as antiproliferation, enzyme inhibition and cancer cell growth inhibition (Qiu *et al.*, 2011; Onda *et al.*, 2013; Rawson, Ho and Li, 2014).

Extraction is the key step for analysis and exploitation of plant bioactive compounds. An ideal extraction method should be quantitative, non-destructive, and time effective. Due to lower toxicity and ease of access to water, traditional method of using medicinal plants was boiled in water and consumed the extracts as additives in food or consumed directly as functional foods but the effectiveness of consuming boiled water extract was in doubt (Wong *et al.*, 2006). Conventional solvent extraction (CSE) is used widely for the recovery of bioactive compounds due to its simplicity. Despite its simplicity, disadvantages such as long extraction time, large consumption of solvents, exposure to flammable and hazardous liquid organic solvents, conventional method of extraction encourages use of more advanced technique. Some of the non-conventional extraction methods were suggested, however CSE was quite comparable to other extraction method in the TPC and antioxidant activity of extracts as shown in study by Nayak *et al.*, (2015). Phenolics or antioxidant content is greatly affected by properties of the extracting solvents. Some common solvent used in extraction of phenolics are methanol, ethanol, propanol, acetone and ethyl acetate (Spigno, Tramelli and De Faveri, 2007). Phenolic compounds dissolve better in solvent with higher polarity such as methanol. It is important to note that some organic solvents are identified as toxic such as methanol, therefore, ethanol as a
food-grade solvent is preferred to be used for the extraction of phenolic compounds from various citrus peels (Li, Smith and Hossain, 2006).

Due to their low cost and high availability in the world, *C. sinensis* peels and its phytochemical compounds could serve as a cheap and yet nutritional dietary supplement or even a potential therapeutic agent. However, its true properties should be proven with more reliable and systematic study to understand the role of *C. sinensis* in performing these beneficial health actions. It was hypothesized that conventional extraction with the solvents including methanol, ethanol, and acetone can isolate the useful bioactive compounds which will exert high antioxidant activities. Therefore, this study aims to examine antioxidant capabilities of *C. sinensis* peel extracts and the correlation to their phytochemical content.

**Materials & Methods**

**Extraction and preparation of samples**

*C. sinensis* peels are by-products collected from an orange juice manufacturer. The peels composed of flavedo and albedo were then washed and fully dried in an oven at 60°C for 72 hours. The dried peels were ground to powder with particle size ranging of 0.5 mm to 0.1 mm using mortar and pestle and were extracted using 100/0, 70/30, 50/50% (v/v) of methanol/water, ethanol/water, and acetone/water solvents respectively for 72 hours with a mass to volume ratio of 1:25 (g/ml). The extracts were then filtered through Whatman No.1 filter paper and collected into glass bottles. The whole process of extraction and filtration was repeated twice followed by evaporation of the collected extracts to dryness using a rotary evaporator at 37°C. The extracts were re-dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100% mg/mL and kept at 4°C until use. Extraction yield was expressed in g of extract per g of dry weight (g/g DW). For water extract, 4g of dried *C. sinensis* peel powder was boiled with distilled water for 1 hour before filtering it through Whatman No.1 filter paper. The whole process of extraction and collection of extract was repeated again followed by evaporating the collected extract to 40 mL left (Concentration: 4g/40mL=100 mg/mL) using a boiling water bath. Water extract stock was stored in -20°C and thawed before use. Extracts of *C. sinensis* peel by using 100, 70, 50 wt.% methanol/water and ethanol/water solvents were expressed as 100% MEC, 70% MEC, 50%
MEC, 100% EEC, 70% EEC, and 50% EEC, respectively. Extracts of *C. sinensis* peel by using 70 and 50 wt.% acetone/water solvents were expressed as 70% AEC, and 50% AEC respectively. Water extract of *C. sinensis* peel were expressed as WEC.

**Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity**

The scavenging activity of the extract against DPPH• radical was measured according to Brand-Williams et al. method (Brand-Williams, Cuvelier and Berset, 1995). 50 μL of 2.5 mg/mL extract was added to 150 μL of mixture (0.2 mM DPPH• and 90 mM Tris-Cl). The total volume was made up to 200 μL with methanol. The mixture was incubated in dark for 40 minutes at 25°C before taking the absorbance readings at 517 nm. DPPH• scavenging radical ability of each sample was expressed as mg of trolox equivalents per g of sample (mg TE/g).

**Determination of ferric reducing antioxidant power (FRAP)**

FRAP assay was performed with slight modification (Benzie and Strain, 1996). The FRAP reagent was prepared by mixing TPTZ (1 mM), FeCl₃•6H₂O (2 mM), and 300 mM acetate buffer in a ratio of 10:1:1 at 37°C. 25 μL of 1mg/mL extract was mixed with 175 μL FRAP reagent. A final volume of 200 μL reaction mixture was incubated in dark for 10 minutes at 25°C before taking the absorbance readings at 590 nm. FeSO₄•7H₂O with different concentrations (100-1000 μM) was used as standard for construction of calibration curve. FRAP value of each sample was expressed as mmol of Fe(II) per g of sample (mmol Fe(II)/g).

**Determination of oxygen radical absorbance capacity (ORAC)**

ORAC assay was carried out according to method of Huang et al. with modifications (Huang et al., 2002). First, 50 μL of 100 μg/mL extract was added to 800 nM fluorescein in 75 mM phosphate buffer pH 7.4. The reaction mixture was incubated at 37°C for 15 minutes following by addition of 200 mM AAPH (2,2’-azobis-2- methyl-propanimidamide, dihydrochloride) solution to a final volume of 200 μL. The fluorescence signal was measured using Hitachi F-7000 Fluorescence Spectrophotometer in 5 minutes interval over 90 minutes by excitation at 485 nm, emission at 520 nm. Inhibition of loss of fluorescein fluorescence is proportional to the antioxidant effect of the sample. Trolox was used as standard for construction of calibration curve.
The area under the curve (AUC) of each sample was calculated by integrating the relative fluorescence curve. Next, net AUC of the sample was calculated by subtracting the AUC of the blank from the AUC of the sample.

\[ \text{AUC} = 1 + \frac{\text{RFU1}}{\text{RFU0}} + \frac{\text{RFU2}}{\text{RFU0}} + \ldots + \frac{\text{RFU18}}{\text{RFU0}} \]

where RFU0 = relative fluorescence value of time point zero, RFUx = relative fluorescence value for the number of reading (eg. RFU5 is relative fluorescence value of fifth reading, which is at minute 25)

\[ \text{Net AUC} = \text{AUC (sample)} - \text{AUC (blank)} \]

The regression equation between net AUC and Trolox concentrations was determined and the ORAC value of extracts was expressed as mol trolox equivalents per gram of sample (mol TE/g).

**Determination of total phenolic content**

TPC of the extracts was determined according to the Folin–Ciocalteu method (Singleton and Rossi, Joseph A., 1965). 250 \( \mu \text{L} \) of 2 N Folin–Ciocalteu reagent was mixed with 50 \( \mu \text{L} \) of 10 mg/mL extract, following by addition of 750 \( \mu \text{L} \) of 7% w/v Na2CO3 after 5 minutes. The total volume was made up to 5 mL with distilled water. The mixture was incubated in dark for 2 hours at 25°C before absorbance was measured using a spectrophotometer (The VersaMax™ Microplate Reader, USA) at 765 nm. TPC results were expressed as mg gallic acid equivalents per g of dry weight (mg GAE/g DW).

**Determination of total flavonoid content**

TFC of extracts was determined using the aluminium chloride colorimetric assay with slight modification (Zhishen, Mengcheng and Jianming, 1999). 25 \( \mu \text{L} \) of 10 mg/mL extract was added with 7.5 \( \mu \text{L} \) of NaNO2 (5% w/v), and 7.5 \( \mu \text{L} \) of AlCl3 (10% w/v). The mixture was then allowed to stand for 10 minutes at 25°C. 50 \( \mu \text{L} \) of NaOH (1 M) was added subsequently and the total volume was made up to 250 \( \mu \text{L} \) with distilled water. The absorbance was measured at 510 nm using a spectrophotometer. TFC results were expressed as mg of catechin equivalents per g of dry weight (mg CE/g DW).

**Quantification of phenolic acids content**
Identification and quantification of phenolic acids in the orange peel extracts was performed using a High Performance Liquid Chromatography (HPLC) method using 2956 LC system (Waters, USA). Samples were filtered through 0.22-μm pore size membrane filters before injection. Presence of phenolic acids was then determined using a reversed phase XBridge C18 column (4.6 x 100mm, 3.5 μm particle size) and the detector was set at λ=270nm, and λ=306nm. The separation of phenolic acids was made in gradient condition at 30°C, using a mobile phase A made of acid water (0.1% formic acid) and mobile phase B, methanol (100%) with the flow rate of 0.7mL/min. The gradient elution was performed as follows: 0-10 min, from 95% to 85% A; 10 -20 min, - from 85% to 80% A; 20-52 min, from 80 to 70% A; 52-55 min, maintained at 70% A; 55 -58 min, from 70 to 50% A; 58-63 min, from 50 to 20% A; 63-70 min, from 20 to 95% A; 70-75 min, maintained at 95% A. Peak identification was made by comparing retention time of known phenolic acids and quantification was performed using calibration curves obtained by injecting known amounts of the pure phenolic acids (Gallic acid, vanillic acid, protocatechuic acid, syringic acid, 4-hydroxybenzoic acid, caffeic acid, o-coumaric acid, ferulic acid, sinapic acid and p-coumaric acid) as the external standards.

**Determination of organic acids content**

HPLC analyses of organic acid content was carried out using 2695 Alliance Separation Module (Waters, USA) equipped with a 2996 diode array detector (Waters, USA). A 10 μL aliquot of filtered sample was separated using Synergi Hydro-RP80A column (250 x 4.6 mm, 4 μm particle size) (Phenomenex, USA) with temperature controlled at 30°C. The mobile phase consisted of mobile phase A (20 mM KH2PO4 with adjusted pH 2.9) and mobile phase B (water) with a flow rate of 0.6 mL/min. Gradient elution was performed as follows: 0-30 min, maintained at 100% A; 30-31 min, from 100% to 0% A; 31-45 min, maintained at 0% A; 45-46 min, from 0 to 100% A; 46-55 min, maintained at 100%. Peak identification was made by comparing retention times and UV spectra at 190, 210 and 254 nm with authentic organic acids compounds. Quantification was performed using calibration curves obtained by injecting known amounts of pure organic acids (tartaric acid, lactic acid, acetic acid, citric acid, succinic acid, oxalic acid, L-mallic acid, kojic acid and ascorbic acid) as external standards.

**Determination of flavonoids content using UPLC**
The flavonoid content of the filtered extracts were separated using Acquity™ Ultra Performance Liquid Chromatography (UPLC) system (Waters, USA) with Kinetex C18 100A column (100mmx 2.1mm; 1.7µm particle size), at a flow rate of 0.4 ml/min with the temperature controlled at 40°C under the UV spectrum of 280, 330, 360 nm. The gradient elution consists of mobile phase A (water:acetic acid, 97:3) and mobile phase B (methanol). The gradient elution was conducted as follows; 0-1 min, maintained at 100% A; 1-10 min, from 100 to 40% A; 0-12 min, from 40 to 100% A and then maintained at 100% A for another 2 min. Quantification was performed using calibration curves obtained by injecting known amounts of flavonoids (Epigallocatechin, vitexin, rutin, quercetin, luteolin, apigenin, tannic acid and ellagic acid) as external standards with known retention time.

**Statistical analysis**

All experiments were performed in triplicate unless stated otherwise. Statistical analyses of the experimental data were performed with GraphPad prism 6 statistical software (GrapPad Software, USA). Results of the replicates were expressed as mean±standard error (SEM). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to evaluate differences between means in each experiment. Experimental results were further analyzed for Pearson correlation coefficient (R-square) between TPC, TFC and different antioxidant assays. P value of ≤0.05 was taken as statistically significant.

**Results**

**Antioxidant activity**

Antioxidant activities of the extracts were evaluated via 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC). DPPH radical scavenging activity of C. sinensis peel extracts varied from 8.35 to 18.20 mg TE/g, FRAP ranged from 95.00 to 296.61 mmol Fe(II)/g, while ORAC ranged from 0.31 to 0.92 mol TE/g. In all three assays, 70% AEC showed higher antioxidant activity and WEC showed a much lower antioxidant activity among the extracts.
However, DPPH, FRAP and ORAC values of extracts were much lower than the tested positive controls, ascorbic acid and gallic acid. Pearson correlation coefficients (R-square) between TPC, TFC and different antioxidant assays were tabulated in Table 2. Significant level of association between the assays was observed especially between FRAP values and TPC (R-square=0.95, P<0.0001) as well as TFC (R-square=0.93, P<0.0001) across all the extracts.

Extraction yield, total phenolic and total flavonoid content

In general, extraction yield of all samples ranged from 0.33 to 0.52 g/g DW and appeared to increase with increasing water concentration in the solvent (Table 3). 100% AEC was excluded from further study due to its low yield (<0.005g/g DW) and low solubility (Table 3). TPC of various C. sinensis peel extracts ranged from 12.08 to 38.24 mg GAE/g, with 70% AEC showing the highest TPC. The other extracts exhibited relatively high TPC too except for 100% EEC and WEC which showed significantly lower TPC than the other extracts. On the other hand, TFC ranged from 1.90 to 5.51 mg CE/g. 50% AEC showed the highest TFC, followed by 70% AEC. Generally, aqueous acetone extracts contained higher TPC and TFC than the other extracts while 100% EEC and WEC contained the lowest TPC and TFC among all.

Phytochemical analysis

Phenolic acids can be divided into derivatives of benzoic acid and derivatives of cinnamic acid and both derivatives were found in our peel extracts. Cinnamic acid derivatives namely ferulic acid and caffeic acid are found in highest abundance while the derivatives of benzoic acid such as gallic acid, protocatechuic acid and 4-hydroxybenzoic acid are present on lower abundance as compared to the former group (Table 4).

Flavonoids are classified into six groups including flavanone, flavonol, flavone, isoflavone, flavan-3-ols, and anthocyanin. The major class of flavonoids in the extracts appear to be the flavan-3-ols (catechin and epigallocatechin), followed by flavanone (luteolin, apigenin and vitexin) while flavonol (rutin) is present at low abundance in the extracts (Table 4).
On the other hand, a few organic acids were identified in the extracts via HPLC (Table 4). Interestingly, 100% MEC, 100% EEC and 70% EEC were shown to contain only lactic acid. Citric acid is the major organic acid in the remaining extracts, followed by lactic acid and L-malic acid. Kojic acid and ascorbic acid appear to be present in much lower abundance in the extracts.

Discussion

In this study, antioxidant activities of C. sinensis peel extracts were evaluated and correlated with the important phytochemical content including phenolic acid, flavonoid and organic acid. Antioxidants can deactivate radicals via two main mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET). In HAT, antioxidant donate hydrogen atoms to stabilise free-radical species to quench them from progressing further in radical reactions while in SET, free radicals are reduced through the donation of an electron from antioxidant compounds (Craft et.al,. 2012). Depending on the structure and properties of the antioxidants present, either HAT or SET may dominate in a given system (Prior, Wu and Schaich, 2005). Therefore, antioxidant capacities of plant extracts greatly depends on extract composition as well as conditions and mechanism of the test used. In order to evaluate antioxidant activity of components in the extract, three antioxidant assays operated on different mechanism were used in our study; FRAP and ORAC assay measures via HAT and SET respectively. DPPH assay determines antioxidant activity via both mechanism (Prior, Wu and Schaich, 2005). In both ethanol and acetone extracts, greatest antioxidant activity was observed in 70% followed by 50% and 100% of both extracts in all three assays (Table 1). This suggests that the water content in the extracts may not correlate proportionally to the antioxidant level but addition of water to the extraction could improve the antioxidant level of the ethanol and acetone extracts. In contrast, antioxidant level of the methanol extracts did not correlate well to water content in the extracts. 100% MEC showed antioxidant activity that was superior to 70% and 50% MEC in both FRAP and ORAC assays but the reverse in DPPH assay.
Antioxidant capacity of phenolics and flavonoids in plants is the main contributor to the specific biological actions in diseases prevention and treatment (Dai and Mumper, 2010). Therefore, bioactive phytochemical components may define the medicinal value of a plant source. From the results, we observed a high correlation between FRAP values and TPC and TFC across all the extracts (Table 2). The higher correlation of TPC and TFC to FRAP values suggest to us that the antioxidant secondary metabolites, in particular phenolics and flavonoids in the peel extracts, may react with free radicals mainly via SET mechanism. Overall, statistically significant correlation between TPC, TFC and the antioxidant assays suggests that the phenolic and flavonoid content contribute to antioxidant activities of the *C. sinensis* extracts.

Phenolics or antioxidant content is greatly affected by properties of the extracting solvents (Spigno et al. 2007). Phenolic compounds are generally known to dissolve better in solvents with higher polarity. Polar alcohol type solvent would produce higher yield as compared to other type of solvents. Extraction yield may be increased with addition of water to ethanol, but water content in the solvent would increase concomitant extraction of other compounds, yielding lower concentration of phenols in the extracts (Naczk & Shahidi 2006). In agreement to the former, we found that the yield of extract increased with increasing water percentage within each solvent extraction group. Although there isn’t a specific pattern observed with regards to the effect of addition of water to TPC or TFC content, 30% of water content in each of the solvent extraction group generally exhibited higher TPC and TFC content than those with 50% of water content (Table 3). The optimum extraction method, water at boiling temperature was one of the effective solvents for antioxidants extraction giving higher total phenol content (Sousa *et al.*, 2008). In our study, WEC had exerted antioxidant activities and tested to have phenolic and flavonoid content although being the least among the extracts (Table 1 & 3). It was understood that conventional solvent extraction (CSE) is generally being used to extract bioactive components from the plant materials at a small scale level. Our study which used CSE served as a control method to understand the bioactivities of *C. sinensis* peels and a reference method for small scale production or homemade level. However, the challenges to scale up the extraction with this convention method would be long extraction time and large consumption of solvent. Therefore, new and promising non-conventional extraction techniques were introduced for industrial application such as ultrasound-assisted extraction (UAE), molecular distillation, microwave-assisted extraction (MAE), pulsed electric field extraction, and supercritical fluid extraction.
(Selvamuthukumaran and Shi, 2017). A recent study comparing CSE, UAE, MAE and supercritical CO$_2$ extraction of Maltease citrus peel showed that MAE was a more effective method in phenols and flavonoids extraction while CSE gave an extract with more antioxidant activity (Boudhrioua, 2016). In another study by Ko, Kwon and Chung (2016), a pilot-scale subcritical water extraction plant was conducted to extract antioxidant flavonoids from dried satsuma mandarin peel (*Citrus unshiu* Markovich) and the proportion of flavonoids recovered with this extraction pilot plant was 96.3%.

Besides important role of extraction solvent, pre-extraction and extraction conditions and methods are equally important in extracting compounds from plant materials. A study by Hegde et al. (2015) recommended peel drying since loss of water content from the peel decreases the bulk of the material for easier handling and storage, lower risk of bacterial growth, as well as more efficient extraction. The study concluded that extraction of sundried peel with acidified aqueous methanol at 90°C for 5 hours yielded the highest polyphenol content. However, it is understood that conditions of sun drying is not controlled throughout the process and oven drying is the alternative option. Oven drying uses thermal energy to remove moisture from the samples rapidly and at the same time, preserves the phytochemicals. Grinding of samples into smaller particle size increases surface contact between samples and extraction solvents (Azwanida NN, 2015; Hegde et al. 2015). These findings supported the use of oven drying at 60°C following by grinding for pre-extraction method with *C. sinensis* peels in this study.In this study, we further identified the different phenolic acids, flavonoids and organic acids content in the various extracts. Phenolic compounds are secondary metabolites synthesized by plants for protection against excessive ultraviolet radiation or pathogenic aggression (Beckman, 2000). Their biological benefits especially antioxidant properties have been extensively studied and described in the literature. Five phenolic acids were identified from *C. sinensis* peel extracts including gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid and ferulic acid. Ferulic acid is the most abundant phenolic acid of *C. sinensis* peel in our study, in agreement with the previous study by M’hiri et al. (2017). Ferulic acid has been reported with various bioactivities including antioxidant, anti-diabetic, anti-tumor and cardio-protection (Kumar & Pruthi, 2014). Similar to ferulic acid, caffeic acid which was present as the second most abundant phenolic acid in all the extracts, are hydroxycinnamic acid derivatives which were shown to show concentration-dependent antioxidant effects. These compounds exhibited inhibition against
induced lipid peroxidation in mouse liver microsomes and scavenging activity against a range of radicals including nitric oxide, superoxide and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid radical (ABTS+) (Maurya & Devasagayam, 2010). In addition, both ferulic acid and caffeic acid are widely added as active ingredients in cosmetic product due to its anti-aging, anti-hyaluronidase and UV absorption capacities (Kumar & Pruthi, 2014; Taofiq et al., 2017).

Besides, our results show that C. sinensis peel extracts contain flavonoids including catechin, epigallocatechin, vitexin, rutin, luteolin and apigenin. Addition of water to the methanol and ethanol extraction appeared to enhance the concentration of most flavonoids such as catechin, epilocatechin, vitexin and luteolin (Table 4). All extracts with exception of 100% MEC and 100% EEC contained catechin as the most abundant flavonoid. Catechin is found abundantly in tea extracts and is well known for its multiple health benefits including anti-aging, anti-diabetic and anti-cancer effects (Pandey & Rizvi, 2009). Epigallocatechin and apigenin which were also present in high abundance in the extracts were suggested to be able to reverse epigenetic changes in disease prevention and regulate a number of biological processes (Li et al., 2016; Shankar et al., 2016; Zhou, Yang and Kong, 2017). On the other hand, rutin was claimed to demonstrate beneficial biological properties including antioxidant, anti-inflammatory and anticarcinogenic properties (Rawson, Ho and Li, 2014).

Apart from the polyphenols, the concentration of organic acids were also determined via HPLC method (Table 4). In comparison to the other phytochemicals, lactic acid, citric acid and L-malic acid were present in much higher concentration in all the extracts apart from 100% MEC, 100% EEC and 70% EEC. These organic acids are commonly found in citrus food and carry major economy value as they have been widely used as acidulant, preservative, emulsifier, flavorant and buffering agents across many industries particularly in food, beverage, pharmaceutical, nutraceutical and cosmetic manufacturing (Cirimina et al., 2017). For example, ascorbic acid and citric acid are normally added to fruit beverages, as acidulant, to enrich the nutrient content and palatability of juices from orange, grapefruit and lemon (Scherer et al., 2012). In addition, the presence of ascorbic acid, limonoids citric acid and flavonoids content of C. sinensis had been claimed to be useful for fermentation and for treating kidney stones in clinical application (Alok et al., 2014). As global supply of organic acid particularly citric acid has rose from less than 0.5 to more than 2 million tonnes for the last two decades, extracting organic acid from citrus...
industrial waste can serve as alternative source that supplying the market needs of natural organic acid (Cirimina et al., 2017).

Conclusions

The antioxidant activity, total phenolic and flavonoid content of the C. sinensis peels were evaluated. The C. sinensis peels showed high antioxidant activities, total phenolic and flavonoid content. Conventional solvent extraction in our study has produced extracts with high antioxidant activities and high phytochemicals content. In particular, extraction of C. sinensis peels with 70 wt.% acetone/water solvent was found to be most effective in extracting organic acid (citric acid and lactic acid) and phenolic acid (ferrulic acid and caffeic acid) of the C. sinensis peel extracts. However, for scale up industrial production, more effective technology can be considered such as MAE and supercritical CO\textsubscript{2} extraction.

The rich phytochemical constituents including phenolic and flavonoid content appeared to contribute to the antioxidant potential to the C. sinensis peel extracts. The bioactive phytochemicals could therefore be exploited for various applications such as for extraction of natural antioxidants, food additive and colourants in the food industry. Despite being agricultural wastes produced in the food supply chain, the enormous availability of C. sinensis peels could be benefited as value added products in line with green technology.

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Table 1 (on next page)

Antioxidant activities of *C. sinensis* peel extracts

Mean±SEM followed by different alphabets in the same column were significantly different between the *C. sinensis* peel extracts at P < 0.05 by one-way ANOVA. TE, trolox equivalents; Fe(II), amount of Fe$^{2+}$ reduced from Fe$^{3+}$. Extracts of *C. sinensis* peel by using 100, 70, 50 wt.% methanol/water and ethanol/water were expressed as 100% MEC, 70% MEC, 50% MEC, 100% EEC, 70% EEC, and 50% EEC, respectively. Extracts of *C. sinensis* peel by using 70 and 50 wt.% acetone/water solvents were expressed as 70% AEC, and 50% AEC respectively. Water extract of *C. sinensis* peel were expressed as WEC.
### Table 1. Antioxidant activities of *C. sinensis* peel extracts.

| Sample       | DPPH (mg TE/g) | FRAP (mmol Fe(II)/g) | ORAC (mol TE/g) |
|--------------|----------------|-----------------------|-----------------|
| 100% MEC     | 13.96±1.08abc  | 275.62±1.85ab         | 0.73±0.05a      |
| 70% MEC      | 16.69±1.20ab   | 240.94±4.95c          | 0.70±0.04ab     |
| 50% MEC      | 15.98±1.33ab   | 214.64±3.49d          | 0.56±0.03bcde   |
| 100% EEC     | 11.61±0.82ac   | 139.94±3.89e          | 0.46±0.03cef    |
| 70% EEC      | 16.52±1.29ab   | 219.02±3.87cd         | 0.68±0.02ad     |
| 50% EEC      | 15.96±1.32ab   | 194.73±5.81d          | 0.50±0.02g      |
| 70% AEC      | 18.20±1.62b    | 296.61±7.97a          | 0.92±0.03h      |
| 50% AEC      | 16.87±1.30ab   | 269.71±7.33b          | 0.60±0.02feg    |
| WEC          | 8.35±1.14c     | 95.00±2.11f           | 0.31±0.03f      |
| Ascorbic acid| 1883.97±22.09  | 14672.83±218.86       | 5.33±0.69       |
| Gallic acid  | 4133.73±360.07 | 26059.73±4427.54      | 7.88±0.60       |

Mean±SEM followed by different alphabets in the same column were significantly different between the *C. sinensis* peel extracts at P < 0.05 by one-way ANOVA. TE, trolox equivalents; Fe(II), amount of Fe^{2+} reduced from Fe^{3+}.

Extracts of *C. sinensis* peel by using 100, 70, 50 wt.% methanol/water and ethanol/water were expressed as 100% MEC, 70% MEC, 50% MEC, 100% EEC, 70% EEC, and 50% EEC, respectively. Extracts of *C. sinensis* peel by using 70 and 50 wt.% acetone/water solvents were expressed as 70% AEC, and 50% AEC respectively. Water extract of *C. sinensis* peel were expressed as WEC.
**Table 2** (on next page)

Correlation between antioxidant activities and phytoconstituents of *C. sinensis* peel extracts

* Correlation of the experimental values between the tests were statistical significant at P < 0.05.
Table 2. Correlation between antioxidant activities and phytoconstituents of *C. sinensis* peel extracts.

| Correlation R-square | DPPH | FRAP | ORAC | TFC |
|----------------------|------|------|------|-----|
| TPC                  | 0.83<sup>a</sup> | 0.95<sup>a</sup> | 0.80<sup>a</sup> | 0.91<sup>a</sup> |
| TFC                  | 0.76<sup>a</sup> | 0.93<sup>a</sup> | 0.66<sup>a</sup> |     |
| ORAC                 | 0.61<sup>a</sup> | 0.82<sup>a</sup> |     |     |
| FRAP                 | 0.72<sup>a</sup> |     |     |     |

<sup>a</sup> Correlation of the experimental values between the tests were statistical significant at P < 0.05.
Table 3 (on next page)

Extraction yield, total phenolic and flavonoid content of *C. sinensis* peel extracts

| Treatment   | Extraction Yield | Total Phenolic Content | Total Flavonoid Content |
|-------------|------------------|------------------------|-------------------------|
| 100% MEC    | 8.5 ± 0.2        | 12.3 ± 1.0             | 9.8 ± 0.5               |
| 70% MEC     | 7.2 ± 0.3        | 11.5 ± 0.8             | 8.9 ± 0.4               |
| 50% MEC     | 6.1 ± 0.1        | 10.7 ± 0.7             | 8.1 ± 0.3               |
| 100% EEC    | 9.1 ± 0.4        | 13.6 ± 0.9             | 10.2 ± 0.6              |
| 70% EEC     | 7.8 ± 0.2        | 12.3 ± 0.8             | 9.4 ± 0.5               |
| 50% EEC     | 6.5 ± 0.1        | 11.5 ± 0.7             | 8.5 ± 0.4               |
| 70% AEC     | 8.0 ± 0.3        | 12.1 ± 0.9             | 9.2 ± 0.5               |
| 50% AEC     | 6.7 ± 0.2        | 11.4 ± 0.8             | 8.6 ± 0.4               |
| Water Extract | 7.0 ± 0.2      | 11.0 ± 0.7             | 8.8 ± 0.5               |

Mean ± SEM followed by different alphabets in the same column were significantly different at P < 0.05 by one-way ANOVA. DW, dry weight; GAE, gallic acid equivalents; CE, catechin equivalents. Extracts of *C. sinensis* peel by using 100, 70, 50 wt.% methanol/water and ethanol/water were expressed as 100% MEC, 70% MEC, 50% MEC, 100% EEC, 70% EEC, and 50% EEC, respectively. Extracts of *C. sinensis* peel by using 70 and 50 wt.% acetone/water solvents were expressed as 70% AEC, and 50% AEC respectively. Water extract of *C. sinensis* peel were expressed as WEC.
Table 3. Extraction yield, total phenolic and flavonoid content of *C. sinensis* peel extracts.

| Sample     | Extraction yield (g/g DW) | TPC (mg GAE/g) | TFC (mg CE/g) |
|------------|---------------------------|----------------|---------------|
| 100% MEC   | 0.41                      | 36.09±2.87<sup>a</sup> | 4.61±0.08<sup>abc</sup> |
| 70% MEC    | 0.47                      | 34.55±2.09<sup>a</sup> | 4.32±0.19<sup>ab</sup> |
| 50% MEC    | 0.51                      | 29.48±2.49<sup>ab</sup> | 3.81±0.13<sup>a</sup> |
| 100% EEC   | 0.33                      | 21.38±0.93<sup>bc</sup> | 2.59±0.09<sup>e</sup> |
| 70% EEC    | 0.48                      | 33.07±2.66<sup>ab</sup> | 4.35±0.20<sup>af</sup> |
| 50% EEC    | 0.52                      | 29.65±2.25<sup>ab</sup> | 3.73±0.22<sup>a</sup> |
| 100% AEC   | 0.00                      | NA             | NA            |
| 70% AEC    | 0.52                      | 38.24±3.44<sup>a</sup> | 5.03±0.27<sup>bdf</sup> |
| 50% AEC    | 0.54                      | 35.58±2.81<sup>a</sup> | 5.51±0.43<sup>cd</sup> |
| WEC        | -                         | 12.08±0.96<sup>c</sup> | 1.90±0.09<sup>e</sup> |

<sup>abcdef</sup> Mean±SEM followed by different alphabets in the same column were significantly different at P < 0.05 by one-way ANOVA. DW, dry weight; GAE, gallic acid equivalents; CE, catechin equivalents. Extracts of *C. sinensis* peel by using 100, 70, 50 wt.% methanol/water and ethanol/water were expressed as 100% MEC, 70% MEC, 50% MEC, 100% EEC, 70% EEC, and 50% EEC, respectively. Extracts of *C. sinensis* peel using 100, 70 and 50 wt.% acetone/water solvents were expressed as 100% AEC, 70% AEC, and 50% AEC respectively. Water extract of *C. sinensis* peel were expressed as WEC. NA: Not available (The yield of 100% acetone was lower than 0.005g/g DW and was not soluble, thus not able to be used for subsequent analysis.)
Table 4 (on next page)

Phytochemical content of *C. sinensis* peel extracts.

Each data point indicates the average results of phytochemicals phenolic acid, organic acid and flavonoids in ug/g extract of C sinensis peel extracts.
Table 4. Phytochemical content of *C. sinensis* peel extracts.

| Phytochemicals (ug/g extract) | 100% MEC | 70% MEC | 50% MEC | 100% EEC | 70% EEC | 50% EEC | 70% AEC | 50% AEC | WEC |
|-------------------------------|-----------|---------|---------|----------|---------|---------|---------|---------|-----|
| **Phenolic acid**             |           |         |         |          |         |         |         |         |     |
| Gallic acid                   | 43.43     | 18.80   | 32.69   | 31.30    | 42.67   | 38.88   | 33.55   | 40.14   | 20.14|
| Protocatechuic acid           | 59.87     | 140.25  | 108.95  | 70.33    | 121.25  | 133.26  | 112.08  | 130.79  | 24.40|
| 4-hydroxybenzoic acid         | 65.22     | 53.93   | 69.79   | 63.48    | 73.05   | 81.74   | 50.51   | 54.25   | 24.07|
| Caffeic acid                  | 224.65    | 247.96  | 411.75  | 164.99   | 243.01  | 362.70  | 266.43  | 264.84  | 69.58|
| Ferulic acid                  | 377.61    | 821.87  | 769.19  | 579.33   | 404.34  | 742.22  | 917.88  | 683.44  | 108.79|
| **Organic acid**              |           |         |         |          |         |         |         |         |     |
| Lactic acid                   | 18660.69  | 20981.33| 16387.22| 20190.94 | 16316.47| 12708.52| 12444.50| 10117.39| 20929.25|
| Citric acid                   | 0         | 40900.07| 38405.91| 0        | 0       | 42559.18| 53673.85| 45076.92| 37364.21|
| L-mallic acid                 | 0         | 9207.74 | 9200.43 | 0        | 0       | 8701.07 | 5876.59 | 9378.31 | 1308.75|
| Kojic acid                    | 0         | 247.64  | 218.13  | 0        | 0       | 222.96  | 221.39  | 229.17  | 120.68|
| Ascorbic acid                 | 0         | 12.55   | 2.19    | 0        | 0       | 4.37    | 9.71    | 13.55   | 6.21 |
| **Flavonoids**                |           |         |         |          |         |         |         |         |     |
| Catechin                      | 248.76    | 514.63  | 627.22  | 123.99   | 573.81  | 669.62  | 572.26  | 679.32  | 352.72|
| Epigallocatechin              | 373.41    | 472.66  | 621.84  | 255.24   | 436.16  | 593.99  | 446.57  | 520.82  | 178.07|
| Vitexin                       | 103.92    | 190.17  | 196.80  | 93.12    | 107.60  | 202.40  | 225.52  | 195.38  | 69.61 |
| Rutin                         | 23.48     | 20.34   | 20.38   | 30.63    | 29.48   | 22.36   | 21.63   | 26.63   | 15.08|
| Luteolin | 227.97 | 496.83 | 539.49 | 334.51 | 300.51 | 458.37 | 516.27 | 478.70 | 324.74 |
|----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Apigenin | 141.24 | 285.77 | 313.08 | 203.65 | 174.91 | 270.45 | 311.19 | 279.59 | 194.38 |

Each data point indicates the average results of phytochemicals phenolic acid, organic acid and flavonoids in ug/g extract of C sinensis peel extracts.