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Antioxidant activity of enzyme-assisted extract derived from round kumquat peel (*Fortunella japonica*)

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ABSTRACT

This study intends to apply enzyme-assisted extraction method to determine the antioxidant activity of round kumquat peel extract. Firstly, chemical composition of the kumquat peel was determined. Then, single factor test was employed to evaluate the effects of enzyme treatment conditions (enzyme amount and extraction time, ethanol concentration, material:ethanol ratio, extraction time and extraction temperature) on total phenolic content (TPC) and antioxidant activity of the kumquat peel extract. The result showed that the content of phenolic compounds in the kumquat peel achieved 1.3%. The peel extract exhibited the maximum TPC of 335.96±16.79 milligrams of gallic acid equivalents (mg GAE)/g dry matter, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) scavenging activity of 1993.26±99.66 µmol Trolox Equivalent (TE)/g dry matter and ferric reducing antioxidant power (FRAP) value of 3202.86±160.14 µmol TE/g dry matter. This extract was obtained under enzyme treatment condition including enzyme content of 9 U/g dry matter, enzyme-treating time of 90 min and the extraction condition comprising of kumquat peel: 96% ethanol ratio of 1:40 (w/v), extraction time of 150 min and temperature of 50°C. The results revealed that the kumquat peel extract could be used as a potential natural antioxidant in food and/or pharmaceutical products.

1. INTRODUCTION

In human body, damages of reactive radicals results in numerous diseases such as cancer, atherosclerosis, diabetes, arthritis, coronary heart disease, and Alzheimer's disease because of biological antioxidant defense systems including enzymatic (superoxide dismutase and glutathione peroxidase) and non-enzymatic antioxidants (vitamins, trace elements, coenzymes, and cofactors) inability to protect the body (Wang et al., 2015). In terms of food, undesirable secondary lipid peroxidation products might be released from lipid oxidation due to free radicals, decreasing the shelf life, quality and safety of food product (Chalamaiah et al., 2015). Oxidation of a substance could be delayed or hindered utilizing antioxidants, preventing these negative effects. Despite their cost-effective and high antioxidant potential, synthetic antioxidant substances including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ) and propyl gallate displayed several toxic and hazardous effects; hence, restriction on their use was made in some countries (Zhang et al., 2012).

Round kumquat peel has been proven to comprise of a source of phenolic compounds, which has been discarded in a huge amount by fruit juice industry (Sadek et al., 2009). These substances have been proven to have capacity to scavenge free radicals and form complex with pro-oxidant transition metal ions, inhibiting oxidation process (Sadek et al., 2009). Moreover, antioxidant activity of the round kumquat peel extracts using enzyme-assisted extraction has not been widely known.

There are many merits to enzyme-assisted extraction such as quick extraction rate, high recovery yield, low amount of solvent usage and low energy consumption (Puri et al., 2012). Furthermore, enzyme-assisted extraction is a well-known potential extraction in pharmaceutical and food applications for phenolic compounds with great durability and antioxidant activity (Gómez-García et al., 2012). In plant, it has been reported that phenolic compounds could bind to cell wall polysaccharides through hydrophobic and hydrogen bonds (Gómez-García et al., 2012). More bioactive compounds could be released into the extracts thanks to enzymes with the ability to degrade or disrupt cell walls and membranes as well as weaken or break down the phenol-polysaccharide links (Puri et al., 2012). Pectinex® Ultra SP-L, a commercial pectinolytic enzyme preparation including polygalacturonase, derived from *Aspergillus aculeatus*, which was used to improve the total phenolic content (TPC) in the extraction of some fruit juices including red dragon (Kunnika & Pranee, 2011) and mulberry juice (Nguyen & Nguyen, 2018), was used in this study.

This study aims to (i) investigate the chemical composition of the round kumquat peel and (ii) analyze the influences of enzyme treatment condition and extraction condition on TPC and antioxidant activity via 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) scavenging activity and ferric reducing antioxidant power (FRAP) assay.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Round kumquat peel

Round kumquat was purchased in local market at district 8, Ho Chi Minh City. Then, the peel was collected, ground and packed in polyethylene bags, labeled and stored at –20°C until used.

2.1.2. Chemicals and enzyme preparation

Chemicals and Pectinex preparation with the activity of 4560 polygalacturonase unit/ml, were purchased from Sigma-Aldrich and Merck companies. Analytic quality of all reagents was required. Tests were performed with double-distilled water.

2.2. Methods

2.2.1. Chemical composition analysis

The methods of Association of Official Agricultural Chemists (AOAC) (2000) were applied to determine the contents of moisture, ash, pectin, cellulose, total sugar, phenolic compounds, and vitamin C of round kumquat peel.

2.2.2. Effect of enzyme treatment condition and extraction condition on TPC and antioxidant activity of round kumquat peel extract

The effects of two parameters in enzyme treatment and four factors in extraction on the TPC and antioxidant activity of the kumquat peel extract were analyzed using a single factor test approach where one factor was modified on various levels while keeping others constant was conducted. Table 1 described the levels of these parameters.

Prior to the addition of enzyme Pectinex Ultra SP-L with the required content, the mixture of ground kumquat peel and water with the peel:water ratio of 1:20 (w/v) was adjusted to pH 4.5. Then, the enzyme was deactivated after desired hydrolysis time at 50°C in a laboratory water bath by heating the mixture to 95°C. The enzyme treated peel was then extracted using hot water at 50°C for 30 min. Subsequently, the extract was recovered using vacuum filter system and vacuum evaporation at 40°C.

After deactivating Pectinex Ultra SP-L, the mixture was continuously ethanolic extracted with the required ethanol concentration, kumquat peel:ethanol ratio, extraction time and temperature. The extract was recovered using vacuum filter system and vacuum evaporation at 40°C.

Table 1. Levels of enzyme treatment and extraction parameters

Parameters	Value of parameters	Fixed parameters	Target function
Enzyme amount	0; 4.5; 9; 13.5 and 18 (U/g dry matter)	Peel:water ratio = 1:20 (w/v) pH: 4.5 Temperature: 50°C Hydrolysis time: 30 min Hot water extraction condition: Enzyme-treated Peel:hot water ratio = 1:20 (w/v) Temperature: 50°C Extraction time: 30min	TPC, $ABTS^{\bullet+}$ scavenging activity and FRAP value
Enzyme treating time	0; 30; 60; 90; 120 and 150 (min)	Peel:water ratio = 1:20 (w/v) pH: 4.5 Temperature: 50°C Enzyme content in previous experiment Hot water extraction condition: Enzyme-treated Peel:hot water ratio = 1:20 (w/v) Temperature: 50°C Extraction time: 30min	
Ethanol concentration	70; 80; 90; 96 and 99 (%)	Enzyme treated peel:ethanol = 1:20 (w/v) Temperature: 25°C Extraction time: 30 min	
Enzyme treated peel:ethanol ratio	1:20; 1:30; 1:40; 1:50 and 1:60 (w/v)	Ethanol concentration in previous experiment Temperature: 25°C Extraction time: 30 min	
Extraction time	0; 30; 60; 90; 120; 150 and 180 (min)	Ethanol concentration and Enzyme treated peel:ethanol ratio in previous experiment Temperature: 25°C	
Extraction temperature	4; 25 and 50 (°C)	Ethanol concentration, Enzyme treated peel:ethanol ratio and extraction time in previous experiment	

2.2.3. Determination of TPC

The method of Lou et al. (2016) was modified to assess TPC of kumquat peel extract. The mixture of 400 µL of the extract and 3 mL distilled water was added with 200 µL of Folin-Ciocalteu's phenol reagent, then left for 5 min at room temperature. After that, the addition of 400 µL of 20% Na_2CO_3 solution followed by incubating at 40°C for 30 min. The absorbance was obtained at 760 nm. Gallic acid was employed to determine the standard curve, which was used to express the TPC of the extract as mg gallic acid equivalents (GAE)/g dry matter.

2.2.4. $ABTS^{\bullet+}$ scavenging activity assay

$ABTS^{\bullet+}$ radical scavenging activity of the extract was obtained by using the method presented in the study of Dudonné et al. (2009). Distilled water was use to distill the $ABTS^{\bullet+}$ solution of 7 mM of ABTS and 2.45 mM potassium persulfate to an absorbance of 0.7±0.02 at 734 nm. Then, 400 µL of extract (1mg/ml) was added to 5 mL of $ABTS^{\bullet+}$ solution, which was left at room temperature for 4 min before reading the absorbance. Trolox was used to obtain the standard curve, which was a tool to assess the $ABTS^{\bullet+}$ scavenging activity of the extract expressed as mg Trolox Equivalent (mg TE)/g dry matter.

2.2.5. FRAP assay

The ferric reducing capacity of extracts was determined by modifying the method of Bordbar et al. (2013). According to this method, at low pH, the reduction of a colorless ferric-2,4,6-Tris(2-pyridyl)-s-triazine complex (Fe^{3+}-TPTZ) to a blue-colored ferrous complex (Fe^{2+}-TPTZ) is carried out by electron-donating antioxidants. The reduction is regulated by the change of absorbance at 593 nm. Standard curve was determined with Trolox, and the FRAP value of the extract was expressed as mg TE/g dry matter using a standard curve.

2.2.6. Statistical analysis

Data were presented as means ± standard deviations of triplicate determinations. Analysis of variance (one-way ANOVA) was performed on the data, and the significance was determined using Tukey method ($P<0.05$). These analyses were performed using the Statgraphics Centurion 18 software.

3. RESULTS AND DISCUSSIONS

3.1. Chemical composition of round kumquat peel

The chemical composition of round kumquat peel comprised of 77.5% of moisture, 4.1% of ash, 3.4% of pectin, 3% of cellulose, 2.9% of total sugar, 1.3% of phenolic compounds, 0.012% of vitamin C (on wet weight basis). M'hiri et al. (2017) reported that the phenolic compounds content of kumquat peel was 1.9 and 1.7 times higher than that of orange and mandarin peels, respectively. Aromatic ring with one or more hydroxyl groups in phenolic compounds can act as antioxidants thanks to their electron donating, hydrogen donating and transition metal-chelating activity (Galanakis, 2019). Therefore, a high content of phenolic compounds in kumquat peel is suggested to potentially be a source of antioxidants. Moreover, antioxidant activity of the peel or its extract was also affected by the presence of vitamin C in the peel. However, the extraction of phenolic compounds may be inhibited because of the linkage between the pectin and cellulose in the peel. Therefore, in this study, antioxidant extract from the kumquat peel was produced by enzyme-assisted extraction.

3.2. Effect of enzyme treatment condition on TPC and antioxidant activity of kumquat peel extract

3.2.1. Effect of enzyme content

TPC, $ABTS^{\bullet+}$ scavenging activity and FRAP value of kumquat peel extract were the highest at the enzyme content of 9 U/g dry matter with 304.01 ± 10.27 mg GAE/g dry matter, 559.01 ± 27.95 and 360.09 ± 18.00 µM TE/g dry matter, respectively. The increase of the enzyme content enhanced the ability of enzyme to break down cell wall structure and liberate phenolic compounds from complex forms (Boulila et al., 2015). As a result, the TPC and antioxidant activity increased correspondingly with the higher contents of antioxidants such as phenolic compounds and vitamin C from the peel extracted into the extract. Nevertheless, the decrease of TPC and antioxidant activity of the peel extracts was observed with the higher enzyme content (more than 9 U/g dry matter). Pectinex Ultra SP-L includes various enzymes such as pectin transeliminase, polygalacturonase, pectinesterase, hemicellulases and cellulases. Therefore, the contact between substrate and enzyme active site was hindered sterically by the competition of excess enzyme to adsorb to the cell wall polysaccharides, reducing enzyme catalytic activity (Boulila et al., 2015). Hence, the enzyme content of 9 U/g dry matter was chosen for further investigations.

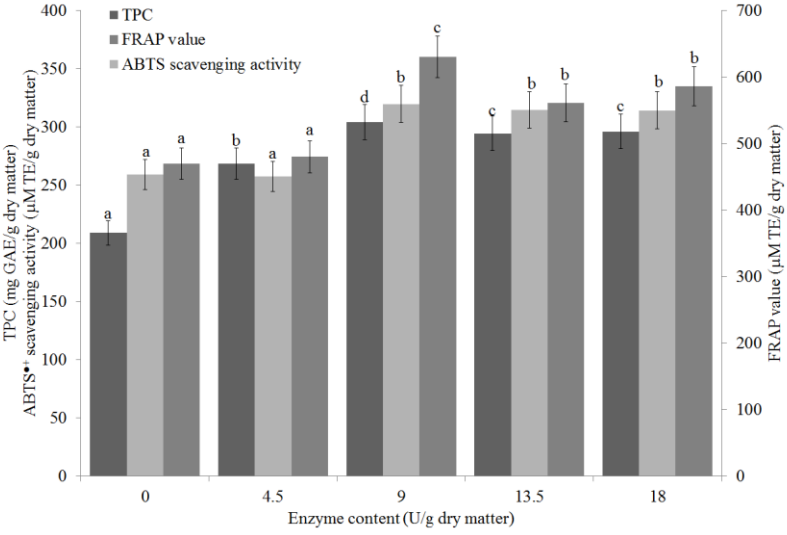

Fig. 1. Effects of enzyme content on TPC and antioxidant activity of kumquat peel extract

Same color bars with different letters indicate significant differences ($p<0.05$).

3.2.2. Effect of enzyme treatment time

Fig. 2 described that TPC and antioxidant activity of the kumquat peel extract increased with the time of enzymatic treatment. The extract reached the peak of TPC of 367.10 ± 7.19 mg GAE/g dry matter, $ABTS^{\bullet+}$ scavenging activity of 1739.34 ± 86.97 µM TE/g dry matter and FRAP value of 2804.20 ± 140.21 µM TE/g dry matter at enzyme treatment time of 90 min. However, beyond this point longer incubation time would result in lower TPC and antioxidant activity of the peel extract possibly because of the oxidation of phenolic compounds from the peel cell under the external environment condition (Ranveer et al., 2013). Ranveer et al. (2013) also reported similar result on enzyme-assisted extraction of lycopene from tomato peel. Hence, 90 min was set as enzymatic treatment time for further studies.

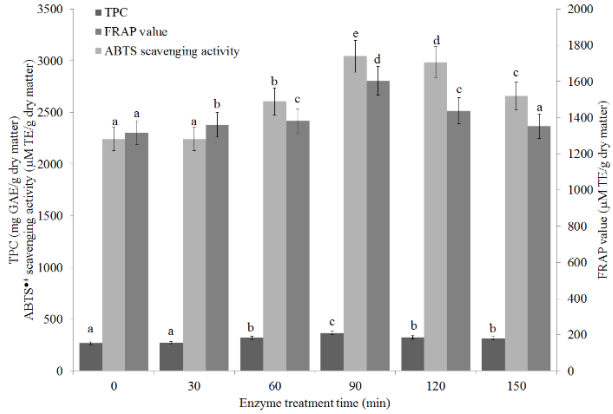

Fig. 2. Effects of enzyme treatment time on TPC and antioxidant activity of kumquat peel extract

Same color bars with different letters indicate significant differences (p<0.05).

3.3. Effect of extraction condition on TPC and antioxidant activity of kumquat peel extract

3.3.1. Effect of solvent concentration

The solubility of phenolic compounds is impacted by the ethanol concentration via changes on the polarity of solvent, which is according to a general principle in solvent extraction, "like dissolves like" - solvents only extracts the compounds with polarities similar to that of solvent (Chew et al., 2011). In this study, 96% ethanol solution was the most effective to produce the kumquat peel extract possessing the highest TPC, $ABTS^{\bullet+}$ scavenging activity and FRAP value of 347.70 ± 20.31 mg GAE/g dry matter, 1738.24 ± 12.82 and 2185.49 ± 19.81 µM TE/g dry matter, respectively (Fig. 3). Therefore, 96% ethanol solution was employed as solvent for further study.

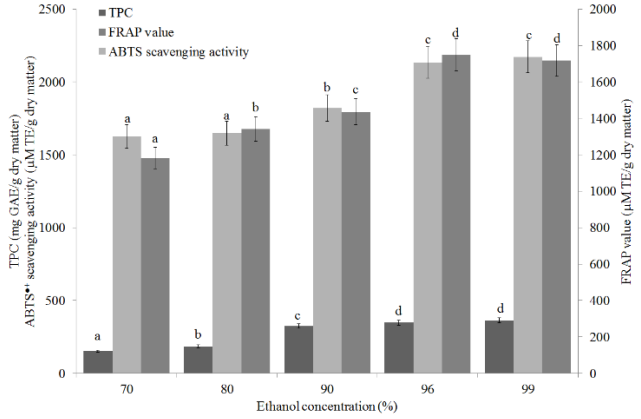

Fig. 3. Effects of ethanol concentration on TPC and antioxidant activity of kumquat peel extract

Same color bars with different letters indicate significant differences (p<0.05).

3.3.2. Effect of material:solvent ratio

Fig. 4 illustrated the effect of material:solvent ratio on TPC and antioxidant activity of the kumquat peel extract. It could be deducted that the higher the material:solvent ratio correlated with the greater antioxidant activity and TPC of the extract. It was published by Spigno et al. (2007) that in extraction process, according to mass transfer principles, the material:solvent ratio could increase the amount of solutes released from solid matrix to the extract. However, maximum $ABTS^{\bullet+}$ scavenging activity and FRAP value of the extract were obtained at the ratio 1:40 (w/v). Similar observation could be found in the study of Kankara et al. (2014). It could be due to the fact that the interaction between bioactive compounds and extracting solvent expands as a consequence of high extraction solvent ratio, increasing the leaching out of phenolic components (Kankara et al., 2014). Therefore, for further studies, the material:solvent ratio of 1:40 (w/v) was selected.

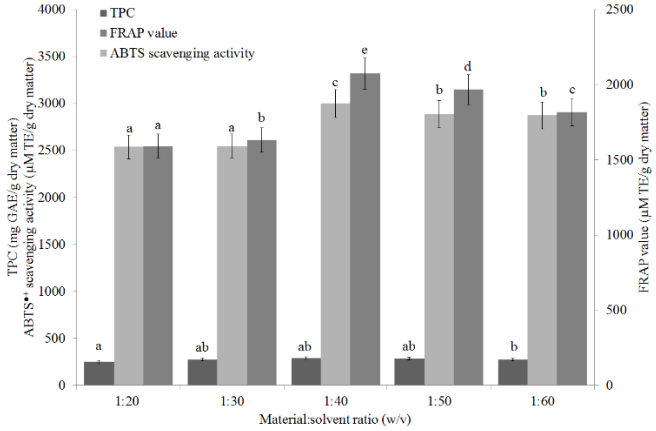

Fig. 4. Effects of material:solvent ratio on TPC and antioxidant activity of kumquat peel extract

Same color bars with different letters indicate significant differences ($p<0.05$).

3.3.3. Effect of extraction time

As described in Fig. 5, TPC and antioxidant activity of the kumquat peel extract directly correlated with the extraction time from 0 to 150 min and remained constant with the prolonging extraction time to 180 min. The research of Chew et al. (2011) also published similar results in TPC and antioxidant activity of *Centella asiatica* extracts. This phenomenon was in accordance with Fick's second law of diffusion that predicts a final equilibrium between the solute concentrations in the solid matrix and in the bulk solution after a certain time (Silva et al., 2007). 150 min was chosen for further researches after taking experimental and economic aspects into accounts.

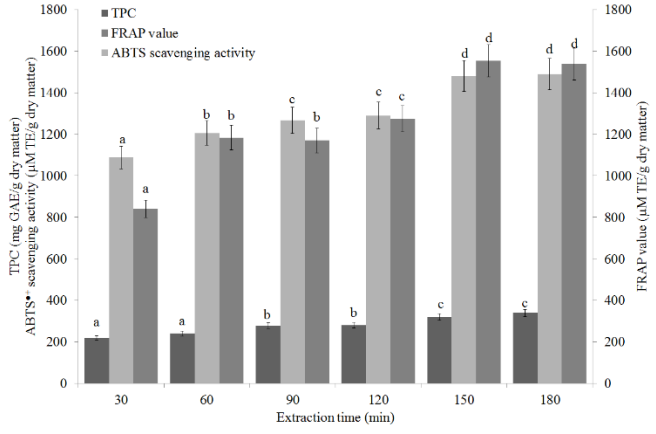

Fig. 5. Effects of extraction time on TPC and antioxidant activity of kumquat peel extract

Same color bars with different letters indicate significant differences ($p<0.05$).

3.3.4. Effect of extraction temperature

Temperature plays an important role in phenolic extraction process, due to its effect on diffusion coefficient, solubility and stability of phenolic compounds (Spigno et al., 2007). The kumquat peel extract had the highest TPC of 335.96 ± 16.79 mg GAE/g dry matter, $ABTS^{\bullet+}$ scavenging activity of 1993.26 ± 99.66 and FRAP value of 3202.86 ± 160.14 µM TE/g dry matter, respectively, at the temperature of 50°C (Fig. 6). The degradation of phenolic compounds antioxidant activity could be initiated by high temperature via cleaving their double bonds and/or adjusting their structures. However, in certain circumstances, phenolic compounds could be converted by high temperature into their intermediate oxidation state, which has been reported to achieve higher antioxidant activity than their normal forms (M'hiri et al., 2017). Therefore, extraction temperature of 50°C was chosen for further experiment.

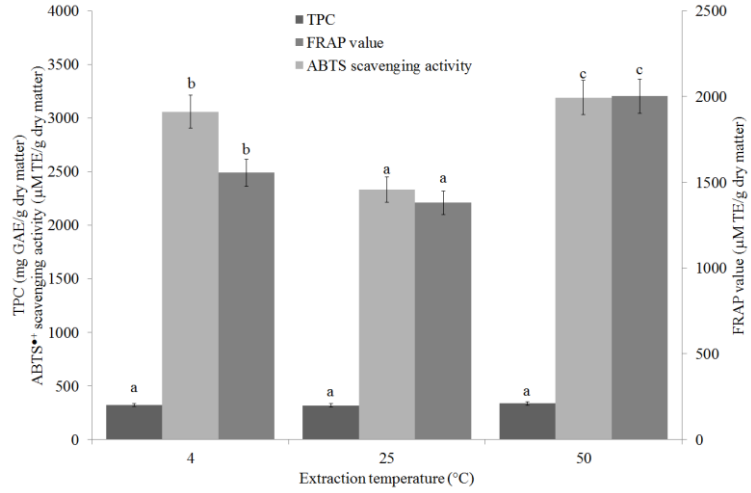

Fig. 6. Effects of extraction temperature on TPC and antioxidant activity of kumquat peel extract

Same color bars with different letters indicate significant differences ($p<0.05$).

4. CONCLUSIONS

This study partially contributes to interpret the effect of enzyme treatment and ethanolic extraction condition on TPC and antioxidant activity of the extracts. Besides, this research introduces a new application of round kumquat peel as an antioxidant extract by employing enzyme-assisted extraction. It could add the value of the peel as well as reduce its environmental pollution risk. With further researches on absorption ability, bioavailability and *in vivo*, antioxidant activity of the extract could be widely applied in food and pharmaceutical products.

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