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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL FROM ROUND KUMQUAT (*FORTUNELLA JAPONICA*) PEEL USING SUPERCRITICAL CO_2 EXTRACTION

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Abstract

In this study, the highest oil yield of kumquat peel essential oil extraction of 3.89±0.03% was achieved under the supercritical carbon dioxide (SC-CO_2) extraction condition including 250 bars, 50°C, fluid rate of 20 g/min and 120 min of extraction. Limonene comprising 39.25% was the main component of the oil. It showed 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical ($ABTS^{\bullet+}$) scavenging activity and ferric reducing antioxidant power (FRAP) value of 25.29±0.17mM Trolox equivalent (mM TE)/g dry weight and 6.25±0.15 mM TE/g dry weight, 4565 and 4623 folds lower than those of vitamin C, respectively. Inhibition zone diameter (IZD) of pure oil equaled to that of (i) 9.2 µg/mL Ampicillin solution against *Bacillus subtilis*, (ii) 23.4 µg/mL Ampicillin solution against *Staphylococcus aureus*, (iii) 7.2 µg/mL Streptomycin solution against *Klebsiella pneumoniae*, (iv) 35.1 µg/mL Amikacin solution against *Pseudomonas aeruginosa*, and (v) 37.7 µg/mL Amikacin solution against *Proteus mirabilis*. Meanwhile, minimal inhibitory concentration (MIC) value of the oil was 5 mg/mL for *B. subtilis* and 10 mg/mL for the rest bacteria.

Keywords: antioxidant activity, antimicrobial activity, essential oil, round kumquat peel, supercritical-CO_2 extraction

Introduction

In certain circumstances, enzymatic and non-enzymatic antioxidant defense system fails to protect human body against oxidative stress triggered by reactive radicals, resulting in numerous diseases such as cancer, atherosclerosis, diabetes, arthritis, coronary heart disease, and Alzheimer's disease (Wang *et al.*, 2015). The use of

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chemical synthesis antioxidants such as 2-3-tert-butyl-4-methoxyphenol, 2,6-ditertbutyl-4-methylphenol has been restricted in numerous countries due to their highly risk of liver damage and carcinogenesis (Shao *et al.*, 2014). Similarly, concerns about the safety of chemically synthetic antimicrobial agents used in the food industry to prevent growth of food spoilage microbes have increased (Yang *et al.*, 2015). One promising strategy to tackle these problems is fortification of food products with plant-derived antioxidant and antimicrobial compounds (Bouyahya *et al.*, 2017; Sodeifian and Sajadian, 2017). Plant essential oils, which are a safe complex of hydrocarbons, oxygenated compounds and nonvolatile residues, have been interpreted to possess various bioactivities including antioxidant and antimicrobial activity (Hsouna *et al.*, 2013).

Kumquat juice processing industry has discarded a huge amount of peel, a rich source of essential oil. However, this source has not been used effectively. Furthermore, publications on the antioxidant and antimicrobial capacity of essential oils originating from kumquat peels are limited.

SC-CO_2 extraction is known as a green, efficient and sustainable extraction method which is widely used to recover bioactive components from natural sources in recent years (Bimakr and Ganjloo, 2016). CO_2, an inexpensive generally recognized as safe solvent, reaches its supercritical state at 304.25 K and 7.39 MPa and returns to gas state under ambient conditions (Rovetto and Aieta, 2017). As a result, SC-CO_2 extraction expresses several advantages such as simple solvent recovery, solvent-free product, safe operation and favor for heat sensitive bioactive compounds (Bimakr and Ganjloo, 2016; Rovetto and Aieta, 2017).

This study aims to recover essential oil from round kumquat peel using SC-CO_2 extraction. Chemical composition and bioactivities (antioxidant and antimicrobial activity) of the essential oil are evaluated as well.

Materials and methods

Materials

After peeling the 4-5 months old round kumquat purchased from Ben Tre province, Vietnam, the peels were dried at 40-45°C until their moisture content was below 10%. Then, they were ground into small pieces with a diameter of approximately 0.3 mm before storing at room temperature in polyethylene bags.

Bacteria strains including *Pseudomonas aeruginosa* (ATCC 27853)(G-); *Proteus mirabilis* (ATCC 12453) (G-); *Klebsiella pneumoniae* (ATCC 700603)(G-); *Staphylococcus aureus* (ATCC 25923) (G+) and *Bacillus subtilis* (ATCC 6633) (G+) were provided by Cu Chi General Hospital, University of medicine and pharmacy Ho Chi Minh City and Research Institute for Aquaculture No.2.

Chemicals were purchased from Sigma-Aldrich and Merck. All reagents were of analytical grade. Double-distilled water was used in experiments.

SC- CO_2 extraction

The SC-CO_2 extraction was performed using the Thar SFC extraction system (USA). The CO_2 was cooled by the mixture of water and ethylene glycol with the ratio 1:1 (v/v) to ensure liquid state of CO_2 before reaching the high pressure pump which delivered liquid CO_2 through heat exchanger to bring it to supercritical state before entering into the extractor vessel. The vessel was also heated up to the desired temperature while pumping the supercritical solvent at required rate monitored by flow meter. The extraction pressure and temperature were maintained by an automatic pressure regulator and electrical jacket, respectively. The supercritical stream dissolved the target components from the peel and carried them from the extraction vessel to the collection vessel for a controlled depressurization process. After determined extraction time, the extract was obtained from the collection vessel and recorded its weight to calculate the oil yield.

$$\text{Oil yield}(\%) = \frac{\text{mass of extracted oil}}{\text{mass of dried peel}} \times 100 \qquad (1)$$

Effect of SC-CO_2 extraction condition on oil yield

Effect of pressure, temperature, SC-CO_2 fluid rate and extraction time on oil yield of round kumquat peel extract was examined using a single factor test method which was performed by one factor varied with different levels while other factors were fixed.

Gas chromatography-mass spectrometry (GC-MS) analysis

GS-Time of flight- MS (Leco, USA) equipped with a DB-5 capillary column (30m, 0.25mm, 0.25µm) was employed to qualify and quantify chemical components of the round kumquat peel essential oil. For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas serving as carrier gas was supplied at a constant flow rate of 1 mL/min. Temperature of the injector and mass transfer line were fixed at 250 and 280°C, respectively. The temperature program of the oven was set as follows: held at 50°C for 1 min, then heated to 270°C at a rate of 10°C/min, and held for 5min. Components of essential oil were identified based on their retention indices while their contents were expressed as percentage by peak area normalization.

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

The method of Dudonné *et al.* (2009) was applied to determined $ABTS^{\bullet+}$ radical scavenging activity of the essential oil. The $ABTS^{\bullet+}$ solution containing 7 mM of ABTS and 2.45 mM potassium persulfate was diluted in distilled water to an absorbance of 0.7±0.02 at 734 nm. After the addition of 200 µL of the oil to 5.7 mL of $ABTS^{\bullet+}$ solution, the absorbance reading was taken at room temperature after 1 min. The $ABTS^{\bullet+}$ scavenging activity of the essential oil was expressed as mM TE/g dry weight using standard curve which was determined with Trolox. Besides, the antioxidant activity was calculated as inhibition percentage as follows:

$$\text{Inhibition percentage}(\%)=\frac{A_b-A_s}{A_b}\times 100 \qquad (2)$$

where A_b is the absorbance of the blank (the essential oil was replaced by distilled water), and A_s is the absorbance of the sample.

FRAP assay

A modified method of Bordbar *et al.* (2013) was used to determine the ferric reducing capacity of the extract. 300 µL of the oil was mixed with 5.7 mL R6 solution including R2, R4 and R5 solution with R2:R4:R5 volumetric ratio of 10:1:1; where R2 was 300 mM acetate buffer, pH 3.6 solution; R4 was 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine in 40 mM HCl solution and R5 was 20 mM $FeCl_3$ solution. Then, the mixture was placed in the dark at room temperature for 30 min before recording its absorbance at 593nm. The FRAP value of the essential oil was expressed as mM TE/g dry weight using a standard curve which was determined with Trolox.

Determination of antimicrobial activity of the round kumquat peel essential oil

Kirby-Bauer disk diffusion method of Gerbig *et al.* (2013) with a slight modification was applied to access antimicrobial activity of the essential oil. Firstly, 5 various bacteria strains were individually grown in MacConkey agar at 37°C for 24h, then suspended in sterile saline solution to match the turbidity of a 0.5 McFarland Standard (standardized suspension). Subsequently, the culture was diluted to contain 10^6 CFU/mL using sterile saline before uniformly spreading onto Mueller-Hinton (MH) agar plates. Paper disks with a diameter of 6 mm were impregnated with 20 µL/disk of essential oils with various concentrations including the pure oil, the diluted oil with concentrations of 5.10^3 and 10^4 µg/mL in dimethyl sulfoxide (DMSO) or antibiotic solutions (streptomycin, ampicillin and amikacin) placed on inoculated MH agar. The plates were left at room temperature for 30 min to allow the oil or antibiotic compounds to diffuse into the agar, after which the plates were incubated at 37°C for 16-20h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone against the tested bacteria.

The MIC of essential oil was determined using the method of Yang *et al.* (2015) with a slight modification. The oil was dissolved in brain heart infusion (BHI) medium. Standardized suspension of each tested microorganism was diluted to contain 10^6 CFU/mL using BHI medium, and 2mL of the culture was transferred to separate tubes containing 2 mL of essential oil, which were then incubated at 37 °C in a shaking incubator for 24 h. The lowest concentration of the test samples, at which any visual growth of test organisms after macroscopic evaluation was not observed, was determined as MIC.

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.

Results and discussion

Effect of SC-CO_2 extraction condition on oil yield

Effect of pressure

As seen in Figure 1a, oil yield was directly proportional to pressure in the range from 150-250 bars and reached a peak of 3.28±0.22% at 250 bars. It was interpreted that at constant temperature, the increment in pressure boosted SC-CO_2 density and its solvent power, enhancing solubility of targeted compounds and oil yield (Bimakr and Ganjloo, 2016; Wang *et al.*, 2012a). Conversely, Figure 1a also indicated the decrease in oil yield as pressure was over 250 bars. Ozkal and Yener (2016) revealed that high pressure possibly resisted mass transfer, being difficult for diffusing solvent into solid matrix to dissolve targeted compounds. Similar observations could be found in the study of Chen *et al.* (2014), Wang *et al.* (2012a) and Bimakr and Ganjloo (2016). Hence, the pressure of 250 bars was set as working pressure for further research.

Effect of temperature

Temperature was reported to have two opposing impacts on the oil yield including (i) negative effect due to the diminution of SC-CO_2 density as a consequence of high temperature and (ii) positive effect owing to the augment of solute vapor pressure, leading to enhance supercritical-CO_2 solubility (Wang *et al.*, 2012a). Therefore, it was harsh to envisage the influence of temperature on oil yield. In this study, as temperature from 40 to 50°C, the essential oil yield was highest and then reduced at 60°C (Figure 1b). Wang *et al.* (2012a) reckoned that in the early stage of extraction, the positive effect of temperature probably outweighed its negative effect, resulting in high oil yield at high temperature. Besides, high temperature may breakdown cell structure, stimulating the diffusion of the targeted compounds into solvent, accelerating the extraction process (Bimakr and Ganjloo, 2016). However, as temperature gained a certain value under constant pressure, its effect was inverted (Wang *et al.*, 2012a). Therefore, the temperature for the next experiment was 50°C.

SC–CO_2 fluid rate

Flow rate of SC-CO_2 had a strong effect on oil yield since it tended to reduce the residence time and simultaneously improve film coefficient (the mass transfer coefficient across the stagnant fluid film surrounding the particles) (Wang *et al.*, 2012a). In this study, a positive relationship between SC-CO_2 fluid rate and oil yield was observed as presented in Figure 1c. According to Li *et al.* (2019), high solvent flow rate enhanced the mass transfer driving force and solvent ratio during extraction, boosting solubilizing rate of targeted compounds. Besides, weak parts of oil cells were additionally damaged and extra oils were released into the extract (Wang *et al.*, 2012a). Thus, SC-CO_2 fluid rate of 20 g/min was chosen for further experiments.

Extraction time

To enhance the oil yield, it was necessary to prolong the contact time between SC-CO_2 and the kumquat peel. Figure 1d showed that oil yield reached the peak of

3.89±0.03% at 120 min and kept significantly unchanged as prolonging extraction time up to 180 min. Similar oil yield-extraction time profiles were published by Sodeifian and Sajadian (2017) and Bimakr and Ganjloo (2016). Considering the oil yield and manufacturing cost, the extraction time of 120 min was chosen for further investigations.

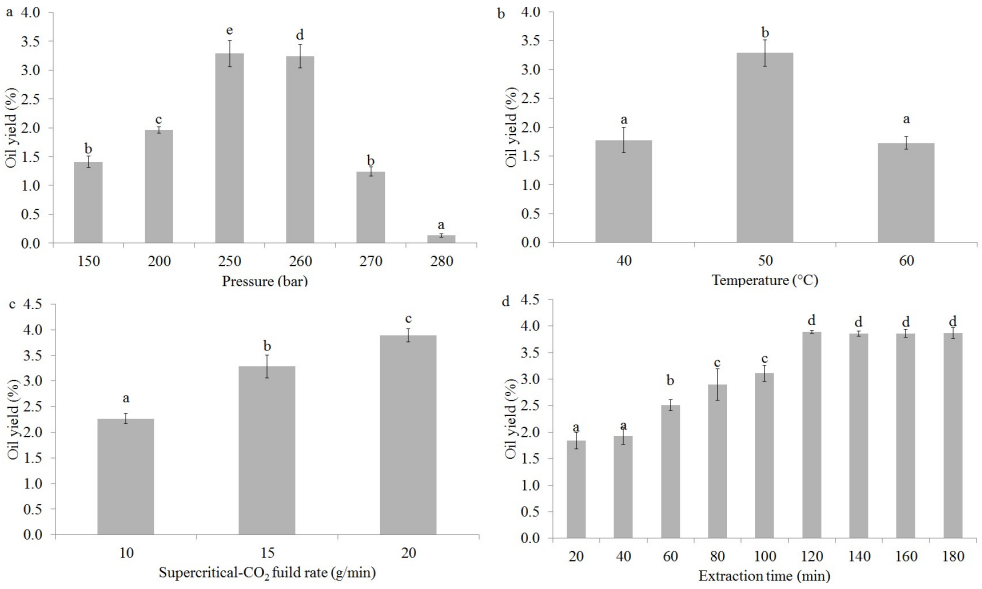

Figure 1. Effect of pressure (a), temperature (b), supercritical-CO_2 fluid rate (c) and extraction time (d) on oil yield of essential oil. Bars with different letters indicate significant differences ($p<0.05$).

Chemical composition of essential oil

The GC-MS results (Table 1) showed that the round kumquat peel essential oil contained 33 compounds which made up 99.06% of the oil. The main constituent of the oil was terpenoid compounds including two monoterpenoids (D-limonene (39.25%), β-Myrcene (0.46%)), five sesquiterpenoids (α-eudesmol (6.46%), Elemol (2.2%), Aristolene ((-)-Aristolene (1.53%), Aristolene (1.05%)) Germacrene (Bicyclogermacrene (1.37%), Germacrene D (1.23%)), and Elemene (γ-Elemene (0.76%), τ-Elemene (0.34%) and β-Elemene (0.25%)) and one oxygenated monoterpenes (Geranyl acetate (2.43%). All of these compounds displayed distinctive odors contributing to the character of the kumquat peel essential oil. The presence of sesquiterpenoids created the spicy and woody flavor of kumquat (Al-Saman *et al.*, 2019). Meanwhile, D-limonene possessed favorable lemon-like odor, which was widely used as flavor and fragrance agent in various food and cosmetic products (Vieira *et al.*, 2018). Besides, it was interpreted to exhibit different bioactivities including antioxidant and antimicrobial activity (Vieira *et al.*, 2018). The D-limonene content of the essential oil in our study was 1.42, 1.28 and 1.27 folds higher than that of essential oil from *Citrus sinensis* L.

Osbeck peel (Ghadiri *et al.*, 2020), mandarin peel (Šafranko *et al.*, 2021) and lemon peel (Romano *et al.*, 2022), respectively.

It is clear that the difference in kumquat variety, locality and extraction method results in the difference in composition of the essential oils. In the study of Yu *et al.* (2021), essential oils obtained from kumquat peel using ultrasound or microwave-assisted hydrodistillation extraction only encompassed three compounds, of which, D-limonene accounted for 96.58-97.02%. While six compounds from the kumquat peel essential oil were achieved when using 70% ethanol solution as extraction solvent (Al-Saman *et al.*, 2019). Besides, the essential oil from *Fortunella crassifolia* peel (Peng *et al.*, 2013) contained 97.06% of monoterpenes, and 1.59% of sesquiterpenes when employing hot water for extraction. However, the three essential oils from peels of *Fortunella crassifolia*, Iranian kumquat and Yangshuo kumquat were rich in monoterpenes with D-limonene proportion of 74.8%, 51.0% and 91.5%, respectively when using hydrodistillation for extraction (Lin *et al.*, 2021; Nouri and Shafaghatlonbar, 2016; Wang *et al.*, 2012b). Although the D-limonene of the essential oil from our study was lower than that of the essential oils mentioned above, the sesquiterpenoids was still higher and the essential oil comprised 33 compounds (Table 1).

Bioactivity of the kumquat peel essential oil

Antioxidant activity

FRAP assay was used for evaluating the electron donating capability of the essential oil. The antioxidative principle involves the reduction of ferric tripyridyl triazine complex to ferrous form (which has an intense blue color) by taking an electron from antioxidants at low pH, which can be monitored by measuring the change in absorbance at 593 nm (Bordbar *et al.*, 2013). The essential oil expressed the FRAP value of 6.25± 0.15 mM TE/g dry weight, 4623 folds lower than that of 5mg/ml vitamin C solution.

Meanwhile, $ABTS^{\bullet+}$ radical scavenging activity of the essential oil was exclusively measured by the ability of an antioxidant compound to be involved in a hydrogen atom transfer, which neutralizes generated $ABTS^{\bullet+}$(Olagunju *et al.*, 2018). $ABTS^{\bullet+}$ scavenging activity of the oil was 25.29 ± 0.17mM TE/g dry weight, 4565 times lower than that of 5mg/ml vitamin C solution. The essential oil expressed $ABTS^{\bullet+}$ radical inhibition percentage of 27.52 ± 0.47%, which was 3.11 folds lower than that of the vitamin C solution of 5 mg/ml.

Hsouna *et al.* (2017) revealed a general trend that great antioxidant potential of essential oils was attributed to the complex mixture of monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes hydrocarbons. Monoterpenes, particularly limonene, a main component of the kumquat peel essential oil, were reported to exhibit antioxidant activity both *in vitro* and *in vivo* models (Vieira *et al.*, 2018). However, the antioxidant potential of monoterpenes was lower than that of oxygenated monoterpenes because of the presence of strongly activated methylene groups (Hsouna *et al.*, 2013). On the other hand, sesquiterpenes hydrocarbons and their oxygenated derivatives have very low antioxidant activity

(Hsouna *et al.*, 2017). In general, synergism, antagonism and additivity were considered as predominant conceptions for antioxidant potential of the essential oil (Hsouna *et al.*, 2013).

Table 1. Chemical components of round kumquat peel essential oil.

Components	Retention time (min)	Content (%)
D – Limonene	11.543	39.25
n-Butyl ether	5.369	7.38
Diethyl phthalate	28.560	7.30
α-eudesmol	15.583	6.46
Propane,2,2-diethoxy	2.950	4.10
n-Hexadecanoic acid	18.566	3.07
Cyclohexanemethanol, 4-ethenyl-.alpha.,alpha.,4-trimethyl	27.741	2.45
Geranyl acetate	11.850	2.43
Elemol	14.183	2.20
Acetic acid	1.800	2.08
2,6 – Octadien-1-ol,3,7 – dimethyl-, acetate,(E)-	23.427	1.92
Phytol	19.900	1.88
Bicyclo[2.2.1]heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-	9.744	1.77
Bis(2-ethylhexyl)phthalate	28.560	1.62
(-)-Aristolene	29.766	1.53
Bicyclogermacrene	13.566	1.37
1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylen-1-(1-metyletyl)naphthalene	26.082	1.31
Germacrene D	13.400	1.23
1,2 – Benzenedicarboxylic acid, diisooctyl ester	46.501	1.17
β-Selinene	13.516	1.16
Aristolene	15.300	1.05
Guaiol	15.250	0.80
γ-Elemene	11.350	0.76
Propane,2-hydroxy-	3.350	0.75
Linalool	7.866	0.66
2-Naphthalenemethanol, decahydro-.alpha.,.alpha.,4a-trimethyl	30.236	0.55
Ethane,1,1-diethoxy	2.583	0.52
Nerylacetat	11.583	0.48
β-Myrcene	6.100	0.46
α-Terpineol	9.466	0.40
Decanal	9.483	0.36
τ-Elemene	14.400	0.34
β-Elemene	12.100	0.25

Antimicrobial activity

The IZD and MIC of the kumquat peel essential oil on 5 tested bacteriawith initial concentration of 10^6 CFU/mL were presented in table 2 and table 3, in order. In general, IZD of the essential oil was positively proportional to its concentration. The oil had no effect against five investigated bacteria as its concentration was below $5x10^3$ µg/mL. On the other hand, among five tested microorganisms, *B. subtilis* was the most susceptible strain to antimicrobial potential of essential oil with IZD of pure oil of 25.33 ± 0.58 mm and MIC of 5 mg/mL. Conversely, the essential oil displayed less effectiveness against three tested Gram negative bacteria including *K. pneumoniae*, *P. aeruginosa* and *Proteus mirabilis*. It could be due to the outer membrane surrounding the cell wall of Gram negative bacteria, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide (Hsouna *et al.*, 2017). In addition, it was reported that the gram-positive bacteria are more sensitive to essential oils than the gram-negative (Kupnik *et al.*, 2022; Ndayishimiye *et al.*, 2018). As comparing to antimicrobial activity of popular antibiotics, IZD of pure kumquat peel essential oil was comparable to that of (i) 9.2 µg/mL Ampicillin solution against *B. subtilis*¸ (ii) 23.4 µg/mL Ampicillin solution against *S. aureus*, (iii) 7.2 µg/mL Streptomycin solution against *K. pneumoniae*, (iv) 35.1 µg/mL Amikacin solution against *P. aeruginosa*, and (i) 37.7 µg/mL Amikacin solution against *Proteus mirabilis*.

Table 2. IZD of various concentration essential oils against five tested bacteria.

IZD (mm)	Concentration (µg/mL)		
	Pure	**104**	**5 x 10^3**
B. subtilis	$25.33^{i} \pm 0.58$	$23.33^{h} \pm 0.58$	$19.33^{g} \pm 0.58$
S. aureus	$15.67^{e} \pm 0.58$	$11.33^{c} \pm 0.58$	0
K. pneumoniae	$16.67^{f} \pm 0.58$	$9.33^{a} \pm 0.58$	0
P. aeruginosa	$13.67^{d} \pm 0.58$	$9.33^{a} \pm 0.58$	0
Proteus mirabilis	$16.33^{ef} \pm 0.58$	$10.33^{b} \pm 0.58$	0

Different letters indicate significant differences (P<0.05)

Table 3.MIC of essential oil against five tested bacteria.

Bacteria strain	**MIC (mg/mL)**
Staphylococcus aureus ATCC 25923	10
Bacillus subtilis	5
Klebsiella pneumoniae	10
Pseudomonas aeruginosa	10
Proteus mirabilis	10

It is clear that monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes hydrocarbons play important roles in the antimicrobial activity of essential oils. Their hydrophobic property allows them to partition the bacterial phospholipid bilayer, disrupting the cell membrane; as a result, proteins and other cell components are released and bacteria are deactivated (Ndayishimiye *et al.*,

2018). Limonene, the predominant compound in the kumquat peel essential oil, was reported to display strong antimicrobial activity by disrupting bacteria membrane integrity (Hsouna *et al.*, 2017). Besides, it is necessary to focus on some compounds in low content which may also contribute to the antimicrobial activity of the oil. For instance, α-terpineol, which merely comprised 0.46% in the oil, also exhibited great anti-microorganism potential by disrupting bacterial cell walls, inhibiting bacterial enzyme activity, and suppressing translation of certain regulatory gene products (Park *et al.*, 2012). Thus, the antimicrobial of the essential oil may be owing to the additive, synergistic, or antagonistic effects of its components (Hsouna *et al.*, 2013). The antimicrobial mechanism of the remainders possibly included the leakage of potassium, the disturbance in the electron respiratory chain and the loss of chemi-osmotic balance (Al-Saman *et al.*, 2019; Bouyahya *et al.*, 2017).

Conclusions

This study applied a green method of SC-CO_2 extraction to recover essential oil from the round kumquat peel which is usually discarded into the environment. This study also partially clarified the impact of extraction conditions on oil yield and the contribution of essential oil components to its bioactivities including antioxidant and antimicrobial activity. It could be suggested that the essential oil could be simultaneously served as a fragrance agent and a preservative agent which prevented oxidation and microbial-related spoilage in food and cosmetic products. However, sensory and clinical tests should be done.

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