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THE EFFECT OF EXTRACTION PARAMETERS ON ANTIOXIDANT ACTIVITY OF SUBCRITICAL WATER EXTRACTS OBTAINED FROM MANDARIN PEEL

Evrim Özkaynak Kanmaz*[1], Özlem Saral[2]

[1] Artvin Çoruh University, Faculty of Health Sciences, Nutrition and Dietetics Department, Turkey
[2] Recep Tayyip Erdoğan University, Health College, Nutrition and Dietetics Department, Turkey

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Abstract

Mandarin peel, which discarded as by-product at large amounts during fruit juice production, is a valuable source of antioxidant compounds in the food industry. The aim of this study was to investigate the effect of extraction temperature and static extraction time on antioxidant activity and phenolic compounds during subcritical water extraction. The antioxidant activity of subcritical water extracts were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as IC_{50}, ferric ion reducing antioxidant power (FRAP) and total antioxidant potential assay using Cu(II) complex as an oxidant (CUPRAC). As the IC_{50} value of the subcritical water extracts obtained from mandarin peel decreased 40.8 times, FRAP and CUPRAC values increased 66.9 and 34.2 times with increasing extraction temperature from 50 to 180°C for 5 min, respectively. Besides, total phenolic content (TPC) of subcritical water extracts increased 4.9 and 5.0 times from 50 to 180°C for 5 and 15 min whereas, 9.6 and 9.9 times increase was obtained for total flavonoid content (TFC) in the subcritical water extracts, respectively.

Keywords: Subcritical water extraction, antioxidant activity, mandarin peel, phenolics, flavonoids.

MANDALİNA KABUĞUNDAN ELDE EDİLEN KRİTİK ALTI SU EKSTRAKTLARININ ANTİOKSİDAN AKTİVİTE DÜZEYİNE EKSTRAKSİYON PARAMETRELERİNİN ETKİSİ

Öz

Meyve suyu üretimi sırasında fazla miktarda yan ürün olarak açığa çıkan mandalina kabuğu gıda endüstrisinde içerdiği antioksidan bileşikler açısından önemli bir kaynaktır. Bu çalışmanın amacı, mandalina kabuğundan kritik altı su ekstraksiyonu tekniği ile elde edilen su ekstraktlarının antioksidan aktivite ve fenolik bileşik içeriğine ekstraksiyon sıcaklığının ve statik ekstraksiyon süresinin etkisini araştırmaktır. Kritik altı su ekstraktlarının antioksidan aktivitesi 1,1-difenil-2-pikrilhidrazil (DPPH)-IC_{50} yöntemi, demir iyonu indirgeyici antioksidan güç (FRAP) yöntemi ve oksidan olarak bakır (II) kullanan toplam antioksidan (CUPRAC) yöntemi ile belirlenmiştir. 5 dakika statik ekstraksiyon süresi için ekstraksiyon sıcaklığının 50°C'den 180°C'ye yükselmesi ile mandalina kabuğundan elde edilen kritik altı su esktraktlarının IC_{50} değerleri 40.8 kat düşmüş olup FRAP ve CUPRAC değerleri ise sırası ile 66.9 and 34.2 kat artmıştır. Ayrıca, kritik altı su esktraktlarının toplam fenolik madde (TPC) içeriği ekstraksiyon sıcaklığının 50°C'den 180°C'ye yükselmesi ile 5 ve 15 dakika statik ekstraksiyon sürelerinde sırası ile 4.9 ve 5.0 kat artmıştır. Buna karşın, ekstraksiyon sıcaklığının 50°C'den 180°C'ye yükselmesi ile 5 ve 15 dakika statik ekstraksiyon sürelerinde kritik altı su esktraktlarının toplam flavonoit içeriklerinde (TFC) sırası ile 9.6 ve 9.9 kat artış elde edilmiştir.

Anahtar kelimeler: Kritik altı su ekstraksiyonu, antioksidan aktivite, mandalina kabuğu, fenolikler, flavonoitler.

* Yazışmalardan sorumlu yazar / *Corresponding author;*
evrimka2000@yahoo.com, (+90) 466 215 1063 / 2127, (+90) 466 215 1064

INTRODUCTION

In the last years, novel sustainable extraction techniques such as Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Supercritical CO2 Extraction, Supercritical Fluid Extraction (SFE), Accelerated Solvent Extraction (ASE) and Pressurized Hot Water Extraction (PHWE) or Subcritical Water Extraction (SWE) which allow to reduce extraction time and solvent consumption, increase nutraceutical yield and improve plant extract quality (1-10). Subcritical water extraction is called green technology because it is a rapid and efficient recovery method as compared with conventional extraction methods. During the subcritical water extraction, water was kept in the liquid form using high pressure and high extraction temperature below its critical point, 273°C. Because of that, the solubility of less polar compounds increase at high temperatures during the subcritical water extraction. In the literature, subcritical water extraction is reported to have high extraction yields and short extraction times as compared conventional extraction methods. In the literature, antioxidant compounds from different food materials were successfully extracted with subcritical water, which is nontoxic extraction solvent (11-14).

Mandarin peel is discarded as by product during mandarin juice or mixed fruit juice production in the food industry and a rich source of especially flavonoids and phenolic acids (11, 15, 16). Nowadays, extraction studies of natural antioxidant compounds from various food waste products and also citrus peels have been increased. However, organic toxic solvents were used in the most of these studies. On the other hand, especially for human consumption as dietary supplements or food additives, several new techniques have been used and one of them is subcritical water extraction.

In the literature, there is lack of publication regarding the effect of extraction parameters on antioxidant activities of subcritical water extracts obtained from mandarin peel. Therefore, the objective of this study was to investigate the effect of extraction temperature and static extraction time on antioxidant activity and phenolic compounds during subcritical water extraction using accelerated solvent extractor, ASE 350 and also subcritical water extracts and solvent extracts from mandarin peel were compared for extraction efficiency. Besides, the correlation coefficients between phenolic compounds and antioxidant activities of subcritical water extracts obtained from mandarin peel were investigated in this study.

MATERIAL AND METHOD

Material

Domestic mandarin (*Citrus reticulata*) peels were used in this study. Mandarins were cultivated with natural farming using only goat fertilizer in the red and stony soil. Mandarins were harvested in November 2014 from a natural orchard by the Kızan mountainfoot in Köyceğiz, Muğla. The orange coloured mandarin peels were not covered with wax and they were peeled with a stainless steel knife and then dried in the vacuum air oven at 50°C until constant weight. Dried mandarin peels were vacuum packed for prevention of oxidation and then stored at -20°C until the extraction process. Samples were ground with a coffee grinder (Bosch, KM 13) between 600 and 1500 µm just before extraction.

The chemicals and reagents used in this study were Folin-ciocalteau phenol reagent, luteolin, aluminium chloride, sodium hydroxide, methanol, ethanol, acetone, DPPH, acetate buffer, hydrochloric acid, 2,4,6-tripyridyl-s-triazine (tptz), iron (III) chloride hexahydrate, iron(II) sulfate heptahydrate, copper(II) chloride, neocuproine, ammonium acetate, trolox (Sigma); sodium carbonate, sodium nitrite (Merck); ferrulic acid (Fluka). All the chemicals and solvents used were of analytical or HPLC grade.

Method

Solvent extraction by magnetic stirrer

Solvent extraction of mandarin peel was carried out using magnetic stirrer and applied according to the method of Çam and Hışıl (17). 1 gram of mandarin peel was mixed with 10 mL of organic solvent as methanol, ethanol and acetone at 40°C for 1 h using three magnetic stirrer and the supernatants were filtered and these processes were applied two times and the collected supernatants were adjusted to 25 mL in this study. Then, the solvent extracts were stored at -20°C. The extraction procedure was carried out two times.

Subcritical water extraction

In this study, six extraction temperatures (50, 100, 120, 140, 160 and 180°C) and two static extraction times (5 and 15 min) were studied to determine the effect of extraction temperature and static extraction time on phenolics, flavonoids and antioxidant activities of subcritical water extracts. The subcritical water extracts were obtained from mandarin peel using accelerated solvent extractor (ASE 350, Dionex Corporation). The subcritical water extraction was carried out in 25 ml metal extraction cell, at constant pressure of 1500 psi, fresh water of 5% and sample amount of 1.5 g. The all subcritical water extracts were obtained at one cycle by accelerated solvent extractor. The extraction procedure was carried out two times.

Determination of antioxidant activities

The Ferric Reducing Antioxidant Assay (FRAP) was carried according to the method of Benzie and Strain (18). FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6) with 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (tptz) solution in 0.5 mL of HCl (40 mM) and 2.5 mL of 20 mM iron (III) chloride hexahydrate solution. 3 mL FRAP reagent was mixed with 100 µL of sample extract and incubated at 37°C for 4 min. Absorbance of the solutions was measured by a Shimadzu UV-VIS 1800 spectrophotometer at 595 nm against a reagent blank containing distilled water. Trolox was used a positive control to construct a reference curve (62.5-1000 µM). FRAP values were expressed as µmol iron (II) sulfate heptahydrate equivalent of g.

The scavenging activity of DPPH radical was determined using the method of Molyneux (19). 1.5 mL of the sample extract was mixed with 1.5 mL of DPPH (0.1 mM in methanol), vortexed and incubated at room temperature in the dark for 50 min. Absorbance of the solutions was measured by spectrophotometer at 517 nm. Besides, the control solution without sample extract was used. The results were expressed as IC_{50} (mg/mL), which was calculated from the curves by plotting absorbance values. IC_{50} values represent the concentration of the extract (mg/mL) required to inhibit 50% of the radicals.

The cupric reducing antioxidant capacity (CUPRAC) was determined according to the method of Apak et al. (20). 1 mL of Copper(II) chloride solution ($1.0x10^{-2}$ M), 1 mL of ethanolic neocuproine solution ($7.5x10^{-3}$ M) and 1 mL of ammonium acetate (1M, pH 7.0) were mixed in a test tube. The sample extract with different concentrations was added to the initial mixture. The tubes were stoppered and the absorbance of the solutions was measured by spectrophotometer at 450 nm against a reagent blank after 30 min. The result was calculated using the molar absorption coefficient (ε; 1.7x104 L. $mol^{-1}.cm^{-1}$) against trolox, which was the standard reference compound. The result was expressed as mM Trolox/100 g.

Determination of total phenolic and total flavonoid content

Total phenolic content of subcritical water and solvent extracts were assayed as described by Skerget et al. (21). 0.5 mL of extract was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate and then the solution was incubated at 50°C for 5 min in the water bath (Memmbert, WNB 14) and cooled immediately. The absorbance was measured by spectrophotometer at 760 nm. The calibration curve was prepared with ferrulic acid solutions at five concentrations of aqueous methanol (80%). The results were expressed as ferrulic acid equivalent (mg of ferrulic acid per L of extract).

Total flavonoid content of subcritical water and solvent extracts were analysed by a spectrophotometric method by Chang et al. (22). 0.5 mL of extract was mixed with 2.5 mL of distilled water and 150 µL of 5% sodium nitrite solution. The vortexed solution was allowed to stand for 5 min and then 300 µL of 10% aluminium chloride solution was added to the mixure and allowed to stand for 5 min. Lastly, 1 mL of 1 M sodium hydroxide was added and 450 µL distilled water was added and final solution was vortexed at medium speed. The absorbance was measured by spectrophotometer at 510 nm. The calibration curve was prepared with luteolin solutions at five concentrations aqueous methanol (80%). The results were expressed as luteolin equivalent (mg of luteolin per L of extract).

Statistical analysis

All the analyses were performed in triplicate. Results were expressed as means±standard deviation. One-way analysis of variance, least significant difference (LSD) for extraction temperatures and T-test for extraction time and also univariate analysis of variance for the temperature effect, time effect and temperature-time interaction was applied at significance level 0.05 using SPSS statistical package whereas, Pearson's correlation coefficients were at significance level 0.01.

RESULTS AND DISCUSSION

Antioxidant activity of subcritical water extracts from mandarin peel

As shown from Table 1, the effect of extraction temperature and static extraction time on FRAP, CUPRAC and IC_{50} values of subcritical water extracts obtained from mandarin peel were statistically significant (P <0.05). Subcritical water extracts from mandarin peel showed higher scavenging activity at higher extraction temperatures. Besides, 15 min as static extraction time was more effective to extract of antioxidant compounds with subcritical water than 5 min (Table 1). The lowest IC_{50} value and the highest FRAP and CUPRAC value of subcritical water extracts from mandarin peel were determined at 180°C for 15 min. Subcritical water extracts from mandarin peel showed scavenging activity against DPPH radical between 37.12 and 0.11 g/L. As extraction temperature increased from 50 to 180°C, IC_{50} value of subcritical water extracts from mandarin peel decreased 40.8 times (from 37.12 to 0.91 g/L) and 239.4 times (from 26.33 to 0.11 g/L) for 5 and 15 min, respectively. Furtherly, subcritical water extracts at 180°C and 15 min had a high antioxidant activity with the lowest IC_{50} value (0.11 g/L) as compared the IC_{50} value of trolox (0.06 g/L).

Subcritical water extracts from mandarin peel had the significant (P <0.05) highest FRAP and CUPRAC value at 180°C for 15 min, followed by 180°C and 5 min. Besides, FRAP and CUPRAC values of subcritical water extracts from mandarin peel increased 66.9 times (from 1.40 to 93.63 µmol $FeSO_4.7H_2O$/g) and 34.2 times (from 2.27 to 77.70 mM troloks/g) with increasing extraction temperature from 50 to 180°C for 5 min, respectively. Also, for 15 min, FRAP and CUPRAC values of subcritical water extracts increased 39.9 times (from 3.37 to 134.41 µmol $FeSO_4.7H_2O$/g) and 24.4 times (from 4.10 to 100.11 mM troloks/g) with increasing extraction temperature from 50 to 180°C.

Also, static extraction time had a significant (P <0.05) effect on antioxidant activity of subcritical water extracts from mandarin peel. As static extraction time increased from 5 to 15 min, IC_{50} value of subcritical water extracts from mandarin peel decreased 6.6 times (from 3.42 to 0.52 g/L) and 8.3 times (from 0.91 to 0.11 g/L) at 160 and

Table 1. Antioxidant activity values of subcritical water extracts from mandarin peel.

Extraction temperature (°C)	Extraction time (min)	FRAP (µmol $FeSO_4.7H_2O$/g)	CUPRAC (mM troloks/g)	IC_{50} (g/L)
50	5	1.40±0.10^{e}	2.27±0.09^{e}	37.12 ±1.20^{a}
100		2.53±0.18^{e}	3.03±0.10^{d}	32.03±0.87^{b}
120		4.01±0.09^{d}	3.16± 0.20^{d}	12.74±2.42^{c}
140		11.34±0.11^{c}	9.51±0.21^{c}	9.59±1.11^{d}
160		25.85±0.50^{b}	22.94±0.14^{b}	3.42±0.72^{e}
180		93.63± 1.86^{a}	77.70±0.27^{a}	0.91±0.03^{f}
50	15	3.37±0.35^{e}	4.10±0.53^{e}	26.33±0.57^{a}
100		4.63±0.06^{e}	4.61±0.16^{e}	18.23±0.51^{b}
120		12.15±0.19^{d}	9.98±0.19^{d}	10.14±0.09^{c}
140		14.85±0.20^{c}	12.27±0.34^{c}	7.48±0.49^{d}
160		90.37±2.35^{b}	73.96±0.44^{b}	0.52±0.04^{e}
180		134.41±2.23^{a}	100.11±1.81^{a}	0.11±0.00^{e}
Trolox			0.06±0.00	

Values are means±standard deviations of three (n=3) measurements

$^{a-f}$ Values with different superscript letters within a column are significantly different at P <0.05

Table 2. The Pearson's correlation coefficients* between antioxidant activities and phenolics in the subcritical water extracts from mandarin peel.

Trait	FRAP	CUPRAC	IC_{50}	TFC
IC_{50}	-0.68*	-0.69*		
TPC	0.99*	0.99*	-0.72*	0.99*
TFC	0.99*	0.99*	-0.67*	
CUPRAC	0.99*			

* Correlation is significant at the P <0.01 level

180°C, respectively. However, FRAP value of subcritical water extracts increased 3.0 times (from 4.01 to 12.15 µmol $FeSO_4.7H_2O$/g) and 3.5 times (from 25.85 to 90.37 µmol $FeSO_4.7H_2O$/g) with increasing static extraction time from 5 to 15 min at 120 and 160°C, respectively. Besides, CUPRAC value of subcritical water extracts from mandarin peel increased 3.2 times (from 3.16 to 9.98 mM troloks/g) and 3.2 times (from 22.94 to 73.96 mM troloks/g) while static extraction time increased from 5 to 15 min at 120 and 160°C, respectively.

These results suggested that the compounds capable of reducing DPPH radical in subcritical water extracts from mandarin peel could be extracted with subcritical water at higher extraction temperatures. Rodr guez-Meizoso et al. (23) also reported that the highest antioxidant activity (EC_{50} equal to 10 mg/L) in subcritical water extracts from oregano leave was observed at the highest temperature, 200°C and 30 min. Also, Wiboonsirikul et al. (24) determined the highest EC_{50} value (25 and 22 mg/L, respectively) in subcritical water extracts from oregano leave at 150 and 200°C for 15 min and it was also reported that the radical scavenging activity would resulted from several substances depending upon their water solubility and heat stability.

On the other hand, the correlations between total phenolic contents and antioxidant activities and the correlations between total flavonoid contents and antioxidant activities and also between antioxidant activities of subcritical water extracts from mandarin peel were shown in Table 2. Both FRAP and also CUPRAC values of subcritical water extracts from mandarin peel were significantly (P <0.01) correlated as r^2=0.99 with total phenolic contents in this study. Also, Tezcan et al. (25) reported that the antioxidant activity, FRAP assay and total phenolic content levels were positively and significantly correlated as r^2>0.98 in commercial pomegranate juices. Also, Ozgen et al. (26) reported that levels of FRAP and total phenolic content of pomegranate juices were strongly correlated as r^2=0.93. In this study, total flavonoid content of subcritical water extracts from mandarin peel were also significantly (P <0.01) correlated as r^2=0.99 with both FRAP and also CUPRAC values.

Besides, in this study, significantly (P <0.01) high correlations between IC_{50} values with total phenolic and total flavonoid contents were found as r^2=-0.72 and -0.67 in the subcritical water extracts from mandarin peel, respectively (Table 2). IC_{50} values showed lower correlation with phenolic compounds than FRAP and CUPRAC values. It can be explained with β-caroten, essential oils and melanoidins could have higher correlation with DPPH. As seen from Figure 1, melanoidins increased at higher extraction temperatures and also with longer extraction times. Delgado Andrade et al. (27) also reported that coffee melanoidins showed the highest correlation with DPPH.

Subcritical water extraction of phenolic compounds from mandarin peel

The effect of extraction temperature and static extraction time on total phenolic and total flavonoid content of subcritical water extracts obtained from mandarin peel using accelerated solvent extractor was statistically significant (P <0.05). Total phenolic and total flavonoid content of subcritical water extracts from mandarin peel increased 3.8 times (from 3.52 to 13.40 mg/L) and 3.9 times (from 0.71 to 2.73 mg/L) with increasing extraction temperature from 140 to 160°C for 15 min, respectively. However, total phenolic and total flavonoid content of subcritical water extracts increased 2.4 times (from 5.50 to 13.40 mg/L) and 2.7 times (from 1.01 to 2.73 mg/L) with increasing static extraction time from

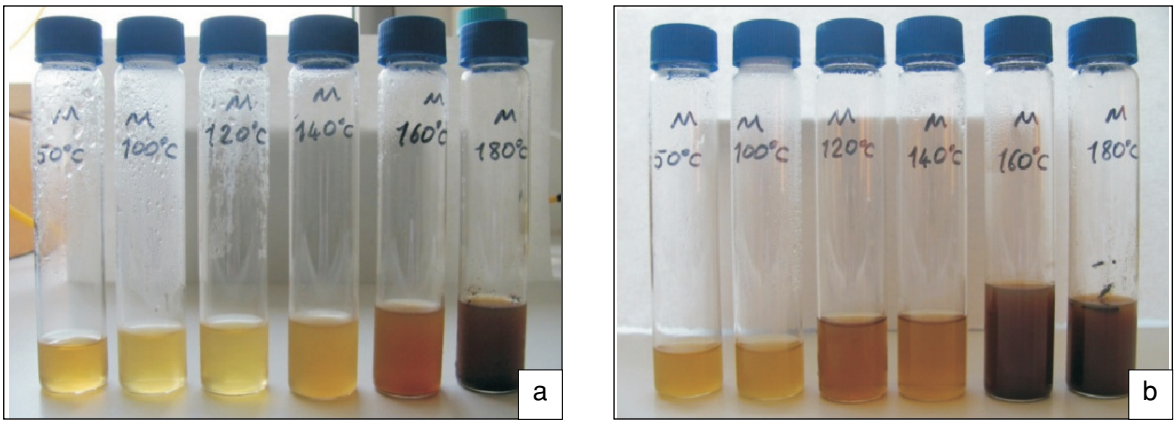

Figure 1. Subcritical water extracts from mandarin peel at 50-180°C for 5 (a) and 15 (b) min using accelerated solvent extractor, respectively.

5 to 15 min at 160°C. Besides, total phenolic and total flavonoid content of subcritical water extracts from mandarin peel increased 2.2 times (from 5.50 to 12.13) and 2.5 times (from 1.01 to 2.47) as extraction temperature increased from 160 to 180°C for 5 min, respectively (Table 3).

Total phenolic content of subcritical water extracts from mandarin peel increased 9.6 times (from 1.26 to 12.13mg/L) and 9.9 times (from 1.61 to 15.91 mg/L) with increasing extraction temperature from 50 to 180°C for 5 and 15 min, respectively whereas, total flavonoid content of subcritical water extracts increased 4.8 times (from 0.51 to 2.43) and 5.0 times (from 0.61 to 3.03) for 5 and 15 min, respectively (Table 3). The significantly (P <0.05) highest total phenolic and total flavonoid content of subcritical water extracts from mandarin peel were determined at 180°C and 15 min and it was followed by 160°C and 5 min in this study. These results suggested that phenolic acids and flavonoids of mandarin peel could be extracted with subcritical water between 160 and 180°C and also high amounts of phenolics especially at 180°C might be explained with formation of new phenolic compounds during decomposition of lignans and dietary fibers.

Özkaynak Kanmaz and Ova (12) reported that SDG lignan in subcritical water extracts from flaxseed meal increased 15.6 times from 120 to 180°C for 15 min whereas, there was a significantly (P <0.05) decrease at 180°C for 30 min. On the other hand, Özkaynak Kanmaz (13)

Table 3. Total phenolic and total flavonoid content of subcritical water extracts from mandarin peel.

Extraction temperature (°C)	Extraction time (min)	Total phenolic content (g/L)	Total flavonoid content (g/L)
50	5	1.26±0.06^{f}	0.51±0.04^{e}
100		1.50±0.04^{e}	0.55±0.04^{d}
120		1.57±0.07^{d}	0.53±0.03^{d}
140		2.96±0.13^{c}	0.72±0.01^{c}
160		5.50±0.13^{b}	1.01±0.02^{b}
180		12.13±0.28^{a}	2.47±0.09^{a}
50	15	1.61±0.03^{f}	0.61±0.03^{d}
100		1.90±0.04^{e}	0.51±0.04^{e}
120		2.72±0.03^{d}	0.63±0.04^{d}
140		3.52±0.24^{c}	0.71±0.04^{c}
160		13.40±1.09^{b}	2.73±0.22^{b}
180		15.91±1.08^{a}	3.03±0.07^{a}
Organic solvents*	Methanol	8.35±0.70	0.89±0.02
	Ethanol	4.17±0.32	0.65±0.01
	Acetone	1.94±0.09	0.54±0.01

Values are means±standard deviations of three (n=3) measurements
$^{a-f}$ Values with different superscript letters within a column are significantly different at P <0.05
* 40°C, 2h, magnetic stirrer

reported that the significantly (P <0.05) highest total phenolic and total flavonoid content of subcritical water extracts from flaxseed meal were determined at 180°C for 30 min. Besides, Ko et al. (14) studied with subcritical water extraction (110-210°C) of flavonoids from different materials using accelerated solvent extractor and the highest extracts were obtained for quercetin at 170°C/10 min (from onion skins), kaempferol and luteolin at 190°C/15 min (from carrots), naringin at 170°C/10 min and naringenin at 170-190°C/15 min (from grapefruit peels), hesperidin at 170°C/10 min (from orange peels) and also hesperetin at 190°C/10 min (from lemon peels).

On the other hand, in this study, subcritical water extracts from mandarin peel obtained at 180°C and 15 min had 3.4, 4.7 and 5.6 times higher total phenolic content than methanol, ethanol and acetone extracts, respectively. Besides, total flavonoid content of subcritical water extracts from mandarin peel at 180°C and 15 min was 1.9, 3.8 and 8.2 times higher than methanol, ethanol and acetone extracts, respectively (Table 3). These results showed that subcritical water was much more effective than methanol and also methanol was more effective to extract especially flavonoids from mandarin peel than ethanol and acetone solvents. In the literature, Min et al. (28) studied with subcritical water extract from *Citrus unshiu* peel only at 160°C and 15 min and found that total phenolic and total flavonoid content of subcritical water extract were higher than ethanol and hot water extracts. Cheigh et al. (11) also reported that hesperidin of subcritical water extracts from *Citrus unshiu* peel were 1.9 and 3.2 times higher than ethanol and methanol extracts, respectively.

CONCLUSION

Antioxidant activity, total phenolic content and also total flavonoid content of subcritical water extracts from mandarin peel increased with the increase in extraction temperature and static extraction time. The results in this study showed that subcritical water extracts obtained from mandarin peel at 180°C and 15 min are good source for functional foods with high antioxidant activity and phenolic compounds and also subcritical water extracts from mandarin peel at 180°C and 15 min are noticeably important as natural antioxidant.

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