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Screening of phenolic content and antioxidant capacity of Okitsu mandarin (*Citrus unshui* Marc.) juice extracted with various solvents

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Abstract

This study aims to compare the effect of using various solvents to obtain phenolic extract from 'Satsuma Okitsu' mandarin (*Citrus unshui* Marc.) juice and characterize their phenolic profiles and determine their antioxidant capacity. Three different solvents were used to extract phenolic content of mandarin juice as water, methanol and ethanol. A total of 17 phenolic compounds were identified and quantified in all samples by LC-DAD-ESI-MS/MS. According to results, among all phenolics, narirutin (naringenin-7-O-rutinoside) was the most abundant compound with the concentrations of 164.3, 201.2 and 203.7 mg/L for the aqueous, methanolic and ethanolic extracts respectively. In the aqueous extract, narirutin was followed by feruloyl-galactaric acid and luteolin-6,8-di-C-glucoside with 66.4 and 61.5 mg/L. However, hesperidin (hesperetin-7-O-rutinoside) was the second most abundant compound in methanolic and ethanolic extracts with the concentrations of 83.9 and 125.0 mg/L. In addition, beyond the phenolic content ranged from 532.1, 691.3 and 755.6 mg/L in the mandarin juice for the aqueous, methanolic and ethanolic extracts respectively. Beyond the phenolic profiles, the antioxidant capacity of three extracts was investigated using DPPH and ABTS assays. According to revealed results, the ethanolic extracts possessed the highest antioxidant activity and followed by methanolic extracts.

Keywords: *Okitsu, Citrus unshui mandarin, LC-MS, phenolic, antioxidant*

1. INTRODUCTION

In recent years, growing demand in healthy, nutritious, and preventive foods has enlarged consumers' perspective for the functional foods, including fruits, vegetables, and plant-based products such as fruit juices. Therefore, secondary metabolites, mainly known as plant polyphenols, gained considerable importance due to their remarkable properties in the face of cardiovascular diseases, various cancers, and many other health problems. In the extant literature, it was mentioned many times that the higher intake of fruits and vegetables was related to lower incidents of these kinds of degenerative diseases. Moreover, their existence in a food product highly affects taste and organoleptic characteristics. Among many beneficial fruits, citrus is a prominent and popular crop actively used in the food sector for its unique aroma, taste, and ample bioactive compounds. Many studies published about the phenolic composition of several citrus fruits, such as orange (Kelebek et al., 2009), lemon (Xi et al., 2017), lime (Singanusong et al., 2013), grapefruit (Xi et al., 2015), kumquat and mandarin (Nipornram et al., 2018), in extant literature. Mandarin is one of the most consumed citrus fruit as fresh, juice, flavouring agent. Its juice, peels, and peel oils possess high amount of phenolic compounds. The peel of the mandarin accounts for approximately half the total dry weight of the fruit. Bocco et al. (1998) cited that the methanolic extracts of citrus peels are especially rich in flavones and glycosylated flavanones, while aqueous extracts possessed high amount of flavonols and phenolic acids. Apart from the peels, the fresh juice of mandarin contains mostly flavanones and phenolic acids (Rapisarda et al., 1999; Nipornram et al., 2018). In another earlier study, Bilbao et al. (2007), performed research on citrus fruits such as

grapefruit *(C. paradisi)*, bergamot *(C. aurantium)*, sweet orange *(C. sinensis)* and tangerine *(C. reticulate)* to observe their phenolic profiles using LC-DAD-MS, and they found that the flavonoids were the major phenolics as rutin (326.59 mg/100 g), naringin (338.36 mg/100 g), quercetin (96.35 mg/100 g) and naringenin (2.35 mg/100 g). As the flavonoids are the important contributors for human health, especially their anti-cancer, anti-inflammatory, cytotoxic and anti-viral effects in the human body should be enlightened with multidisciplinary research activities. Numerous methods constituted for the extraction of phenolic compounds from plant materials so far. Each method used for this purpose showed that their several advantages. Generally, these methods vary in solvents and conditions used during the extraction procedure (Spigno et al., 2007; Mokrani & Madani, 2016). The extraction procedure is essential for the precise identification and quantification of phenolic compounds and to obtain antioxidant capacity accurately (Azwanida, 2015). The structure of phenols is quite fragile in case of extreme conditions such as high temperatures. Therefore, as Naczk and Shahidi (2006) mentioned before, several factors, especially the extraction methodology, influence the quantification of phenolics in plant materials.

In light of this information, the present study was aimed to observe the effect of using different solvents to mandarin juice phenolics and antioxidant capacity using LC-DAD-ESI-MS/MS and ABTS-DPPH, respectively.

2. MATERIALS AND METHODS

2.1. Mandarin juice samples

The mature mandarin samples of Okitsu (*Citrus unshui* Marc.) (10 kg) were harvested in October 2019 from three different orchards in the Adana province of Turkey and transported to the Food Engineering Department, Faculty of Engineering, Adana Alparslan Türkeş Science and Technology University.

2.2. Chemicals

In the present study, the deionized water (18.2 MΩ) was used in all analysis obtained by Millipore Q (Millipore Corp., Saint-Quentin, France) water purification instrument. Apart from water, all chemical solvents and phenolic standards (Feruloyl-galactaric acid, luteolin-6,8-di-C-glucoside, isorhamnetin-3-O-rutinoside-7-O-glucoside, apigenin-6,8-di-C-glucoside, flavanone-O-rutinoside 1, narirutin-4′-O-glucoside, apigenin-8-C-glucoside-2″-O-xyloside, eriodictyol-7-O-rutinoside, genistein-7-O-xylosylglucoside malonylated, phloretin-3′5′-di-C-glucoside, hesperetin derivative, flavanone-O-rutinoside 2, narirutin (naringenin-7-O-rutinoside), naringenin-7-O-xylosylglucoside malonylated, hesperidin (hesperetin-7-O-rutinoside), 1,2-diferuloylgentiobiose, didymin) were used including ethanol, methanol, formic acid, acetic acid, acetone, acetonitrile were supplied from Sigma Aldrich (Sigma-Aldrich, Steinheim, Germany) and Merck (Darmstadt, Germany).

2.3. Extraction and analysis of phenolic compounds

Prior to LC-MS and antioxidant capacity analysis, cleansed mandarin samples were freshly squeezed by a juice extractor (Indelicato Super Automatic, Model A2 104) and obtained juice Afterwards, the juice was divided into three equal lots. Three samples of the study were extracted from these lots using; *(i)* distilled water, *(ii)* ethanol (70:30, ethanol / distilled water), *(iii)* methanol (70:30, methanol / distilled water) with a 1:1 ratio. The extraction process carried out for 30 minutes at 25°C . Subsequently, each lot was subjected to a centrifugation at 4500 rpm for 15 minutes in a laboratory scale centrifuge (Eppendorf 3810 R, Hamburg, Germany). The supernatant of the liquid were taken and filtered through 0.45-µm membrane filters and were stored in a refrigerator at temperature of -18 °C until following analysis.

2.4. Analysis of phenolic compounds LC-DAD-ESI-MS/MS

An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a

Windows NT-based ChemStation software and a diode array detector (DAD) was used to identify the compounds. The system were composed of a binary pump, degasser, and auto sampler. The column used for the analysis was a Phenomenex

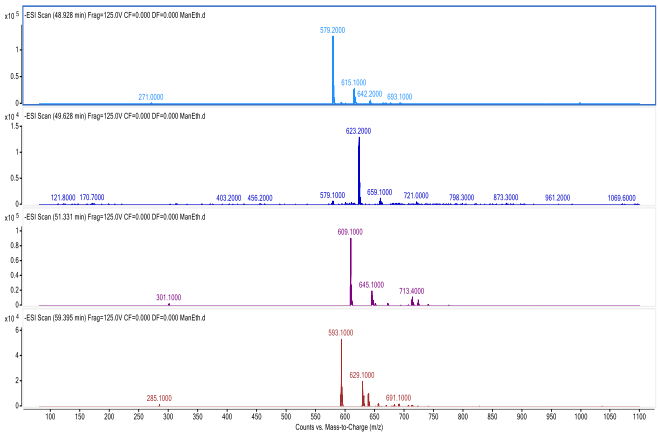

Figure 1. Fragmentations of some phenolic compounds of the sample

reversed-phase C-18 column (4.6 mm × 250 mm, 5 µm) (Torrance, CA, USA). Two different solvents were used as mobile phase: Solvent A, water/formic acid (99:1; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). The comprehensive identification and assignation of each phenolic compound was carried out by comparing retention times of compounds and UV spectra to authentic standards and these values confirmed by an Agilent 6430 LC-MS/MS spectrometer equipped with an electrospray ionization source. The ion source in negative mode was set up with the following conditions; capillary temperature 400°C, N2 12 L/min; nebulizer pressure, 45 psi). Data collection was performed by using the multiple reactions monitoring (MRM) method. Afterwards, the curves were gained using the commercial standards of the concentrations normally present in the mandarin juice sample. The concentration curves were possessed regression coefficients (R2) above 0.995 in all cases (Kelebek & Selli., 2011).

2.4. Measurement of antioxidant activity

In the present study, the DPPH and ABTS assays were used to investigate antioxidant capacity of mandarin juices obtained with three different solvents (water, 70 % ethanol, and 70 % methanol /). The extraction procedures of ABTS and DPPH were carried out similarly to the method used in the study of Kelebek & Selli (2014). In the DPPH assay, the dilutions of each extract (1, 20 and 50 mg/mL) were taken from the liquid obtained in phenolic extraction procedure by using water, ethanol and methanol respectively (v/v). An aliquot of 0.1 mL of diluted extracts was added to 3.9 mL of DPPH solution in related solvent (6×10−5 M). Afterwards, extracts were shaken vigorously and left standing at room temperature for half an hour. The absorbance of the final solution was then measured at 515 nm by a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA).

The ABTS radical cation was prepared by the reaction of 7 mM ABTS with 2.54 mM potassium persulfate, incubated extracts were stored at room temperature for 12–16 hours. Prior to the assay, the ABTS solution was diluted with water, ethanol and methanol to an absorbance of 0.70 ± 0.02 at 734 nm. A total of 3.9 mL of the diluted ABTS solution was added to 0.1 mL of each sample. The final mixture was allowed to stand at room temperature for 30 minutes and the absorbance value was recorded at 734 nm.

3. RESULTS AND DISCUSSION

3.1. Antioxidant capacity of Mandarin juice samples

The antioxidant capacities of samples were measured by means of DPPH and ABTS assays and the results were given in Table 1. DPPH values of aqueous, methanolic and ethanolic extracts were measured as 5.54, 7.20, 7.87 mM Trolox/L, respectively. Besides, ABTS values of aqueous, methanolic and ethanolic extracts were measured as 7.65, 9.94, 10.86 mM Trolox/L. According to the revealed results, the antioxidant activity of phenolic compounds were higher in ethanolic extracts for both assays. In the earlier studies, it has already been proved that the efficiency of ethanol is high for the extraction of phenolic compounds from citrus fruits (Li et al., 2006). Li et al. (2006), investigated the solvent effect on the extraction yield of phenolic compounds and the researchers observed that the increase in ethanol concentration provides an increase in the concentration of extracted phenolics up to a certain level.

3.2. Phenolic composition of Mandarin juice samples

The phenolic compounds identified and quantified in the mandarin juice extracts by using three different solvents. The phenolic composition of mandarin juice extracts were given in Table 2 and fragmentations of compounds and the total ion chromatogram were given in Figure 1, 2 and 3. A total of 17 phenolic compounds were found including, feruloyl-galactaric acid, luteolin-6,8-di-C-glucoside, isorhamnetin-3-O-rutinoside-7-o-glucoside, apigenin-6,8-di-C-glucoside, flavanone-O-rutinoside 1, narirutin-4′-O-glucoside, apigenin-8-c-glucoside-2″-O-xyloside, eriodictyol-7-O-rutinoside, genistein-7-O-xylosylglucoside malonylated, phloretin-3′5’-di-c-glucoside, hesperetin derivative, flavanone-O-rutinoside 2, narirutin (naringenin-7-O-rutinoside), naringenin-7-O-xylosylglucoside malonylated, hesperidin (hesperetin-7-O-rutinoside), 1,2-diferuloylgentiobiose and didymin (isosakuranetin-7-O-rutinoside). Total concentrations of phenolic compounds were quantified as 532.1, 691.3, and 755.6 mg/L for aqueous, methanolic and ethanolic extracts. Among three solvents used for the extraction, ethanol possessed the higher value of phenolic content, as same as the antioxidant capacity results shown in Table 1. Although, the distribution of phenolic compounds was similar in all solvents, their dissolution in different solvents changed. In other words, all 17 phenolic compounds existed in all three samples; however, their concentrations were altered related to the solvent used during the extraction procedure. Among phenolic compounds, narirutin were the most abundant compound in all three samples with the concentration of 164.3, 201.2 and 203.7 mg/L. As an important result of the present study, the concentration of hesperidin altered dramatically. In the ethanolic extract, hesperidin was the second most abundant compound with the 125 mg/L of concentration, while its concentration recorded only 24.6 and 83.9 mg/L in aqueous and methanolic extracts. Apart from those two well-known mandarin phenolics, feruloyl-galactaric acid, luteolin-6,8-di-C-glucoside, apigenin-6,8-di-C-glucoside, flavanone-O-rutinoside 1 and isorhamnetin-3-O-rutinoside-7-O-glucoside has already been mentioned in the extant literature that mandarin juice and other mandarin derived products, such as mandarin wine and mandarin peel oil, are high in phenolic compounds, particularly flavanone glycosides such as narirutin, naringin and hesperidin (Levaj et al., 2009; Kelebek and Selli, 2014). The extraction performance of phenolic compounds from

Table 1. Antioxidant capacity of mandarin juice samples

mM Trolox/L	Water	MeOH	EtOH
DPPH	5.54	7.20	7.87
ABTS	7.65	9.94	10.86

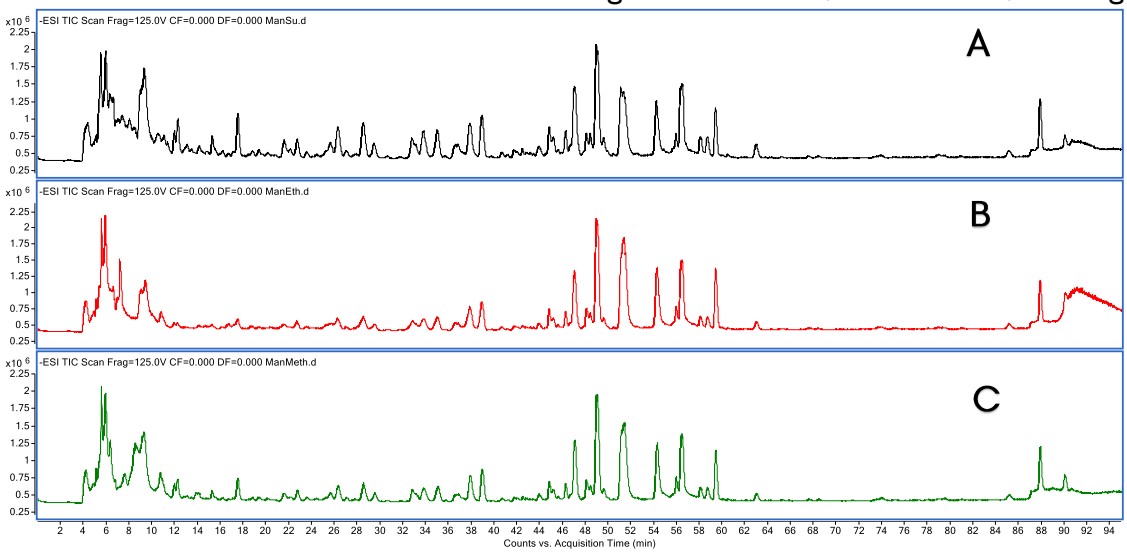

Figure 2. Total ion chromatograms of A water; B ethanolic; C methanolic extracts

Table 2. Retention Times (Rt), family, wavelength for detection (DAD), and mass spectral data for analyses of phenolic compounds in mandarin juice using LC-DAD-ESI-MSn detection

Peak	RT	Compound name	[M-H]⁻	Fragment ions (m/z)	Water	MeOH (70:30)	EtOH (70:30)
1	28.5	Feruloyl-galactaric acid	385	209, 191	66.4±0,72^{c}	70.1±0,76^{b}	73.2±0,79
2	45.5	Luteolin-6,8-di-*C*-glucoside	609	519, 489, 399, 369, 293	61.5±0,66^{c}	76.6±0,83^{b}	80.5±0,87
3	36.8	Isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside	785	623, 315	33.9±0,37^{a}	34.6±0,37^{a}	26.8±0,29
4	36.5	Apigenin-6,8-di-*C*-glucoside	593	503, 473, 383, 353, 325	48.2±0,52^{c}	55.2±0,6^{a}	53.0±0,57
5	37.8	Flavanone-*O*-rutinoside 1	625	317	36.9±0,4^{b}	34.8±0,38^{c}	40.7±0,44
6	38.9	Narirutin-4′-*O*-glucoside	741	433, 271	20.5±0,22^{a}	17.4±0,19^{c}	18.1±0,2
7	58.8	Apigenin-8-C-glucoside-2′′-*O*-xyloside	563	473, 413, 293	0.4±0,01^{c}	0.8±0,01^{b}	1.0±0,01
8	32.8	Eriodictyol-7-*O*-rutinoside	595	475, 287	3.8±0,04^{c}	11.3±0,12^{b}	12.3±0,13
9	47.0	Genistein-7-*O*-xylosylglucoside malonylated	649	605, 433, 209	0.3±0,01^{b}	0.9±0,01^{a}	0.9±0,01
10	46.7	Phloretin-3′5′-di-*C*-glucoside	597	577, 561, 489, 477, 387, 357	11.9±0,13^{c}	14.7±0,16^{b}	16.3±0,18
11	46.8	Hesperetin derivative	915	843, 771, 669, 505, 463	1.0±0,01^{b}	1.1±0,01^{a}	1.1±0,01
12	49.6	Flavanone-*O*-rutinoside 2	623	301	49.4±0,53^{c}	66.8±0,72^{b}	74.5±0,81
13	48.9	Narirutin (naringenin-7-*O*-rutinoside)	579	315	164.3±1,78^{b}	201.2±2,18^{a}	203.7±2,2
14	51.5	Naringenin-7-*O*-xylosylglucoside malonylated	651	271	1.2±0,01^{c}	4.2±0,05^{b}	6.3±0,07
15	51.2	Hesperidin (hesperetin-7-*O*-rutinoside)	609	565, 403, 271	24.6±0,27^{c}	83.9±0,91^{b}	125.0±1,35
16	56.3	1,2-diferuloylgentiobiose	693	301	0.5±0,01^{c}	2.6±0,03^{b}	3.2±0,03
17	59.3	Didymin (isosakuranetin-7-*O*-rutinoside)	595	285	7.5±0,08^{c}	15.1±0,16^{b}	19.1±0,21
				Total	**532.1**	**691.3**	**755.6**

plant based products is highly dependent on the solvent. Several studies have been carried out aiming to describe the differences between various solvents used during the extraction of phenolic compounds (Jakopič et al., 2009). In our study, ethanol was the most effective solvent to elute phenolic compounds from mandarin juice, as previously reported by others (Li et al., 2006; Vatai et al., 2009). The work of Vatai et al. (2009), resulted that for all plant materials the phenolic extraction by using 50% ethanol- and acetone–water solutions showed the highest yields. Similarly, Alothman et al. (2009) performed the phenolic extraction using three different solvents

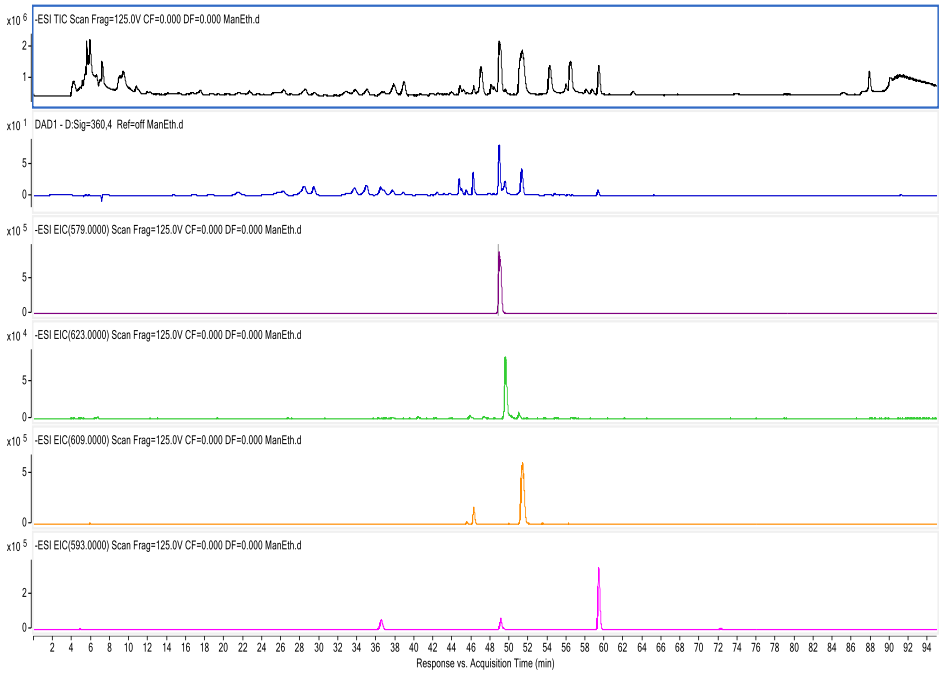

Figure 3. Total ion chromatograms of mandarin juices

(ethanol, methanol and acetone) in different rates (50, 70 and 90%) and distilled water (100%). Researchers reported that the ethanolic and acetone extracts of guava fruits possessed higher total phenolics and total flavonoids than methanolic and aqueous extracts. Hence, the extraction efficiency of phenolics is not stable under different experimental conditions and it is hard to configure a suitable standard procedure for the extraction of these bioactive compounds. However, least polar solvents are known to be appropriate for the extraction of lipophilic phenols unless a high pressure is applied (Alothman et al., 2009).

4. CONCLUSIONS

Phenolic profile and antioxidant activity of the Okitsu mandarin (*Citrus unshui* Marc.) have been examined by comparing the effect of solvent used during phenolic extraction. The revealed data indicate that there are significant differences in the concentrations of phenolic compounds and antioxidant activity among different solvents. The highest values of phenolic compounds and antioxidant activity were observed in ethanolic extracts, followed by methanolic and aqueous extracts of Okitsu mandarin juice. Narirutin and hesperidin possessed the highest concentrations. Although the concentration of hesperidin were not so high, its concentration increased dramatically with the use of ethanol as the solvent. So, the more rate of ethanol presence in the solvent mixture increased the total concentrations of phenolic compounds. This finding was in accordance with the earlier papers.

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