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Effects of heat treatment of mandarin peel on flavonoid profiles and lipid accumulation in 3T3-L1 adipocytes

Jeehye Sung [1], *Joon Hyuk Suh* [1], *Yu Wang* [*]

Department of Food Science and Human Nutrition, Citrus Research and Education Center, University of Florida, 700 Experiment Station Rd, Lake Alfred, FL, 33850, USA

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ABSTRACT

Citrus peel, a primary byproduct of citrus fruits, contains a variety of flavonoids. Heat treatment is a favorable food processing for solid peel to release bioactive compounds from tissues and intensify nutritional effects. In this study, we explored alterations of flavonoids by thermal treatment of mandarin peel and their effects on lipid accumulation and intracellular levels during differentiation of 3T3-L1 cells to adipocytes. The heat-treated sample showed stronger inhibition on the formation of lipid droplets than the non-treated sample, along with enhanced intracellular levels of flavonoids. Overall flavonoids, especially flavonoid aglycones showing better efficacy, were found to increase in the peel after heat-treatment. Our findings indicate thermal processing could help release flavonoids from citrus peel and convert them into aglycone forms, leading to efficient cellular uptake and suppression of lipid accumulation in 3T3-L1 cells. This study provides useful information of heat-treated citrus peel as potential dietary supplements with anti-obesity-related effects.

1. Introduction

Citrus is one of the most popular fruit crops in the world. It is widely consumed as processed citrus products, juice or fresh fruit to increase the beneficial intake of antioxidant flavonoids [1,2]. Citrus flavonoid has received considerable attention as a valuable resource due to their potential health benefits such as antioxidant, and anti-inflammatory activities [3,4]. The major categories of citrus flavonoids are flavanone, flavone, and flavonols, which are found as aglycones, glycosides and methylated derivatives [1]. The most important citrus flavanones are hesperidin and naringin, and their corresponding aglycones, such as hesperetin and naringenin, respectively [1,5]. Flavone and flavonols also exist in both aglycone and glucoside forms in citrus. Moreover, numerous studies have demonstrated that citrus peels contain large amounts of the polymethoxylated flavones (PMFs), such as nobiletin and

* *Corresponding author.*
E-mail address: yu.wang@ufl.edu (Y. Wang).
[1] These authors contributed equally to this work.
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tangeretin, providing a broad spectrum of biological activities including anti-cancer, anti-inflammatory, anti-diabetic and hypolipidemic properties [6–8]. These compounds exist almost exclusively in the citrus genus, particularly in the peels of mandarin (*Citrus reticulata*) [2]. Due to intensive existence of functional molecules such as flavonoids, citrus peel previously regarded as byproduct and waste has received attention, and started to be developed into dietary supplements as granule, capsule and slice forms.

Heat treatment is a simple and cost-effective process to ensure microbial safety and enzyme deactivation, but also has beneficial effects on bioactive phytochemicals such as improving their bioavailability or modifying chemical structure to enhance absorption [9–11]. For the study on health benefits of bioactive citrus flavonoids, heat treatment has been used as an effective method to release bound phenolic and flavonoid compounds from citrus peels [12,13]. It has been reported that simple heating process at high temperature (i.e. $\geq$ 90 °C) promoted the major losses of cell wall polymers, which could contribute to efficient separation of phenolic and flavonoid compounds extracted from citrus peels [14]. Several studies also found that heat treatment of citrus peels released a variety of polyphenolic compounds and flavonoids, along with showing antioxidant and anti-inflammatory activities [15,16]. However, the anti-obesity-related effects of heat-treated citrus peel, as well as the intracellular changes of flavonoids in adipocytes have not been studied yet.

Considering the growing evidence and potentials, heat treatment might be a good strategy to improve health benefits of citrus peel, and will be helpful for development of dietary supplements. In this respect, it is important to understand alterations in chemical composition of functional molecules during thermal processing, and how these changes affect intracellular bioavailability and bioactivity. Therefore, in this study, we have investigated the changes of flavonoid profiles in mandarin peel via heat treatment, corresponding intracellular levels, and the effect on lipid accumulation in 3T3-L1 adipocytes.

2. Materials and methods

2.1. Standard solution and sample preparation

All flavonoid (Indofine Chemical Company, Inc., Hillsborough, NJ, USA) stock solutions were prepared in methanol at a concentration of 1000 μg/mL. Working standard and internal standard (salicylic acid-d_6) solutions were prepared by diluting and mixing each stock solution with methanol. Mandarin (*Citrus reticulata*) were obtained from the Citrus Research and Education Center at the University of Florida. The fruit peels were freeze-dried and ground to a fine powder. For heat-treated samples (n = 3), the peels were prepared by heating the powder in an oven at 150 °C for 50 min, and cooling down to room temperature. No heat was apply to non-treated samples (n = 3). Heat-treated and non-treated samples (10 g) were extracted with 500 mL of methanol/water (70:30, v/v) using sonication (40 kHz) for 1 h followed by agitation for 18 h. The extracts were centrifuged at 5000 rpm for 15 min and filtered through a filter paper. Methanol was evaporated under vacuum in rotary evaporator and the aqueous phase was freeze-dried. The samples were stored at −20 °C until analysis.

2.2. Cell culture and cell adipocyte differentiation

3T3-L1 mouse preadipocytes (American Type Culture Collection; ATCC CL-193, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), 100 units/mL penicillin, and 50 μg/mL streptomycin. The cells were incubated at 37 °C in 5% CO_2-humidified air. Adipocyte differentiation was induced as described previously [17]. Briefly, the preadipocytes were maintained at 2 day postconfluent stage (designed as day 0) in 10% fetal bovine serum (FBS)-DMEM including 1 μM dexamethasone (Dex), 5 μg/mL insulin, and 0.5 mM 3-isobutyl-1-methylxantine (IBMX). After 48 h, the medium was changed to 10% FBS-DMEM containing insulin and 10% FBS-DMEM, on Day 2 and Day 4, respectively. 3T3-L1 cells were simultaneously treated with or without non-treated or heat-treated citrus peel extracts from Day 0 to Day 4 during the differentiation. Samples were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the medium was adjusted to < 0.1%.

2.3. Oil Red O staining

Oil Red O staining was performed on Day 6. Briefly, the cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde (v/v) in PBS for 30 min at room temperature. The fixed cells were washed with distilled water twice and stained with filtered 0.35% Oil Red O solution (w/v) in 60% isopropyl alcohol for 15 min. Image of stained lipid droplets in 3T3-L1 adipocytes were photographed using an Olympus IX73 microscope (Olympus Corporation, Tokyo, Japan) at 200× magnification. Intracellular lipid content was extracted with 100% isopropanol and quantified at 500 nm using a FlexStation 3™ (Molecular Devices, Sunnyvale, CA, USA).

2.4. Cellular uptake

3T3-L1 cells were grown up to 2 day postconfluent stage and treated with samples (non-treated and heat-treated citrus peel extracts) and standards (narirutin, naringenin, hesperidin, and hesperetin) with 10% FBS-DMEM including 1 μM Dex, 5 μg/mL insulin, and 0.5 mM IBMX. After treatment, the cells were washed twice with PBS, detached with trypsin/EDTA solution and re-suspended in PBS. The number of viable cells was estimated by a trypan blue assay. The cell suspension was centrifuged by a Sorvall ST-8R centrifuge (10,000 rpm for 15 min at 4 °C).

2.5. LC–MS/MS assay

Ten to one hundred milligrams of peel extracts (heat-treated and non-treated), and cell samples (control and extract-treated) were added to 10 mL of methanol containing internal standard (salicylic acid-d_6), agitated, and after

centrifugation, the supernatants (5 μL) were injected into the LC-MS/MS (TSQ Quantiva, Thermo Fisher Scientific, San Jose, CA, USA). The assay was repeated in triplicate for each sample. Analytes were chromatographed on an Acclaim C30 column (150 mm × 2.1 mm, 3.0 μm particle size, Thermo Fisher Scientific, San Jose, CA, USA) at a column temperature of 25 °C using (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile as mobile phases. A gradient elution was carried out as follows: 0–30 min 20–70% B, 30–31 min 70–95% and 31–35 min 95%. The column was re-equilibrated with the initial mobile phase before next run. The flow rate was 0.2 mL/min. The mass spectrometer was operated in positive and negative electrospray ionization (ESI+ and ESI−) modes depending on analytes with selected reaction monitoring (SRM). The parameters of ESI source were as follows: spray voltage, 3500 V (ESI+) and 2500 V (ESI−); ion transfer tube temperature, 325 °C; vaporizer temperature, 275 °C; sheath gas, 35 Arb; aux gas, 10 Arb; and sweep gas, 0 Arb. Collision-induced dissociation (CID) gas was 2 mTorr, and dwell time was 100 msec. The parameters of MS/MS (SRM transition, collision energy and RF lens) are presented in Supplementary Table 1.

2.6. Procedures for method validation

The LC–MS/MS method was validated according to the International Conference on Harmonization (ICH) guideline in terms of specificity, limit of quantification (LOQ), linearity, accuracy, precision and recovery [18]. The specificity was evaluated by analyte chromatograms from standard solution and extracted samples to confirm the lack of interference at their retention time. The LOQ was determined as the analyte concentration at which the signal to noise ratio (S/N ratio) was over ten. The linearity was obtained by calibration curves constructed with peak area ratios of analyte to internal standard against nominal analyte concentration using six analyte concentrations in the range of 0.5, 2.5, 10, 50, 100 and 200 ng/mL for group A (taxifolin, naringin, diosmin, neodiosmin, neohesperidin, scutellarein, eriodictyol, poncirin, quercetin, naringenin, hesperetin, apigenin, diosmetin, 5,6,7,3′,4′,5′-hexamethoxyflavone, and isosakuranetin), and 5, 25, 100, 500, 1000 and 2000 ng/mL for group B (eriocitrin, neoeriocitrin, rutin, narirutin, rhoifolin, hesperidin, didymin, sinensetin, nobiletin, and tangeretin). The accuracy and precision were estimated by analyzing three levels (0.5, 50 and 200 ng/mL for group A, and 5, 500 and 2000 ng/mL for group B) of quality control (QC) samples within calibration ranges. Three replicate analyses were performed on the same day (intra-day assay) and three consecutive days (inter-day assay). The accuracy was calculated by comparing observed concentration and the corresponding concentration, and the precision was assessed as the relative standard deviation (RSD, %). Lastly, the recovery was tested by standard addition of analytes to sample matrix (mixture of heat-treated and non-treated extracts) at low and high concentrations (10 and 50 ng/mL for group A, and 100 and 500 ng/mL for group B). The recovery was performed in triplicate and was calculated by comparing peak areas of sample extract fortified with standard, peak areas of sample extract alone, and peak areas of standard added.

2.7. Statistical analysis

The quantification data of lipid accumulation on 3T3-L1 adipocytes represents mean ± standard error of the mean (SEM) and significant differences were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA), followed by Tukey's multiple comparison test. The data of flavonoid profiling represents mean ± standard deviation (SD) and significance comparisons were determined by unpaired t-test at $p < 0.05$ with GraphPad Prism version 5.0.

3. Results and discussion

3.1. Effects of heat-treated and non-treated mandarin peels on lipid accumulation in 3T3-L1 adipocytes

We prepared heat-treated and non-treated mandarin peel extracts and investigated their potentials to reduce lipid accumulation on differentiation of 3T3-L1 cells to adipocytes. Treatment with heat-treated and non-treated mandarin peel

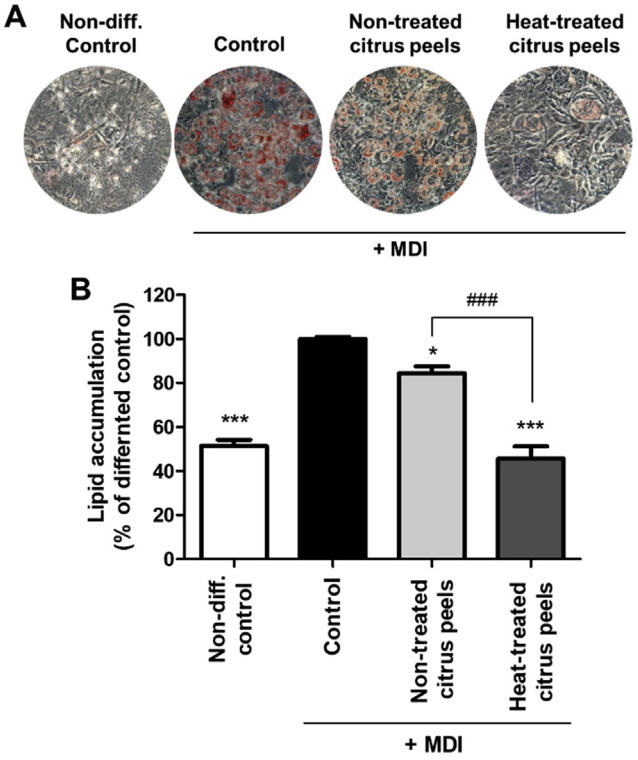

Fig. 1 – Effect of extracts from heat-treated and non-treated mandarin peel on intracellular lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 cells were differentiated for 6 days in the presence or absence of 1 mg/mL heat-treated or non-treated mandarin peel extracts. (A) Images of lipid droplets stained with Oil red O dye were photographed. (B) Intracellular lipid contents were extracted and quantified. Data are expressed as a mean ± SEM (n = 3).*, $^{***}P < 0.05$ and 0.001, respectively, significant difference compared to differentiated control. $^{###}P < 0.001$, significant difference between two groups.

extracts up to a concentration of 1 mg/mL did not induce significant cytotoxicity on 3T3-L1 cells (Supplementary Fig. 1A). During the differentiation of cells, cells were treated with each extract (heat-treated and non-treated peels) and intracellular lipid droplets were measured by Oil Red O staining. The extracts of heat-treated and non-treated peels significantly diminished the lipid accumulation in 3T3-L1 cells at 0.5 and 1 mg/mL (Supplementary Fig. 1B). As

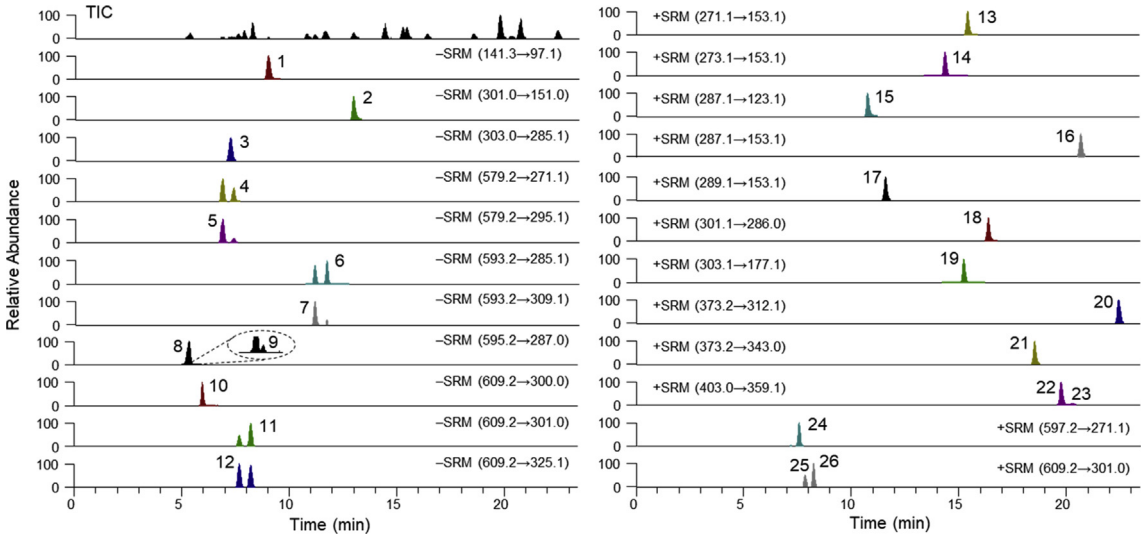

Fig. 2 – LC–MS/MS chromatograms of target flavonoids and internal standard: salicylic acid-d_6 (internal standard) (1), quercetin (2), taxifolin (3), naringin (4), narirutin (5), poncirin (6), didymin (7), eriocitrin (8), neoeriocitrin (9) rutin (10), neohesperidin (11), hesperidin (12), apigenin (13), naringenin (14), scutellarein (15), isosakuranetin (16), eriodictyol (17), diosmetin (18), hesperetin (19), tangeretin (20), sinensetin (21), 5,6,7,3′,4′,5′-hexamethoxyflavone (22), nobiletin (23), rhoifolin (24), diosmin (25), and neodiosmin (26).

Table 1 – Validation summary of LC–MS/MS method for flavonoids.

Compound	LOQ (μg/g peel)	Calibration Range (ng/mL)	r^2	Precision[a] ($n = 3$)		Accuracy[a] ($n = 3$)		Recovery[b] ($n = 3$)	
				Intra-day (RSD %)	Inter-day (RSD %)	Intra-day (%)	Inter-day (%)	Mean (%)	RSD (%)
Eriocitrin	0.1	5–2000	0.9979	4.8–6.4	1.4–12.1	95.9–112.7	99.3–104.4	102.5–105.6	2.1–11.4
Neoeriocitrin	5.0	5–2000	0.9990	7.3–9.9	4.1–11.2	103.0–108.8	102.8–107.2	97.3–101.7	4.5–5.4
Rutin	2.0	5–2000	0.9986	9.2–12.3	10.5–13.5	92.6–101.9	94.9–105.5	98.5–102.8	3.0–3.9
Narirutin	0.1	5–2000	0.9970	1.9–7.6	1.4–7.7	87.9–97.2	93.3–104.9	97.2–104.2	5.0–8.1
Taxifolin	0.05	0.5–200	0.9991	3.3–6.3	3.4–11.7	92.4–109.4	96.1–106.2	94.5–98.6	5.4–10.2
Naringin	0.5	0.5–200	0.9996	4.5–7.4	2.2–6.8	94.0–105.2	101.2–102.0	98.1–99.6	3.1–6.7
Rhoifolin	0.2	5–2000	0.9992	6.8–12.0	2.9–6.1	95.4–110.29	93.4–114.7	97.9–101.6	5.3–7.6
Hesperidin	0.1	5–2000	0.9975	2.7–14.6	5.0–10.6	93.2–99.6	96.6–97.3	103.5–105.3	3.5–6.8
Diosmin	0.1	0.5–200	0.9997	6.3–13.8	6.8–14.9	102.1–114.3	104.6–108.5	93.4–98.4	5.7–9.2
Neodiosmin	0.01	0.5–200	0.9994	5.2–6.1	2.2–7.3	96.0–109.9	99.1–107.8	95.2–96.4	2.5–5.7
Neohesperidin	0.5	0.5–200	0.9983	9.6–12.4	2.1–9.6	101.9–108.3	101.7–108.3	92.2–95.1	3.7–8.3
Scutellarein	0.05	0.5–200	0.9940	5.1–8.4	7.0–8.9	95.1–103.7	101.5–104.6	102.8–108.7	4.8–6.5
Didymin	0.1	5–2000	0.9972	3.1–11.6	3.5–6.9	98.8–107.3	97.7–100.7	91.1–98.4	2.7–12.3
Eriodictyol	0.05	0.5–200	0.9959	2.1–3.7	1.2–9.2	91.3–107.7	101.6–107.9	87.3–93.8	3.1–4.6
Poncirin	0.02	0.5–200	0.9954	4.5–9.5	2.6–5.0	97.7–114.1	96.1–104.5	95.7–96.5	2.4–5.8
Quercetin	0.05	0.5–200	0.9952	9.7–14.6	0.3–12.9	93.8–104.5	98.0–106.9	102.2–111.5	2.3–5.7
Naringenin	0.02	0.5–200	0.9997	5.8–11.0	3.1–8.7	104.1–107.7	98.9–108.8	100.5–108.3	1.1–9.0
Hesperetin	0.5	0.5–200	1.0000	5.7–7.9	5.0–7.6	99.1–102.0	96.2–113.3	95.3–96.5	6.8–7.8
Apigenin	0.01	0.5–200	0.9994	5.8–8.6	1.8–6.9	99.6–106.3	98.0–102.0	106.7–110.4	0.9–8.5
Diosmetin	0.02	0.5–200	0.9976	5.4–11.0	1.1–13.7	99.7–106.2	97.0–101.4	95.0–108.0	1.4–8.8
Sinensetin	0.5	5–2000	1.0000	1.7–7.2	1.5–8.6	96.2–107.2	93.2–104.9	96.4–100.2	6.2–8.5
5,6,7,3′,4′,5′-Hexamethoxyflavone	0.1	0.5–200	0.9976	4.8–7.7	1.7–13.7	97.2–106.3	99.1–107.7	92.8–103.1	3.0–12.9
Nobiletin	0.5	5–2000	0.9979	3.9–6.7	3.5–6.1	100.4–111.5	101.9–107.4	100.5–106.3	9.9–10.1
Isosakuranetin	0.02	0.5–200	0.9978	7.2–13.0	0.8–12.8	92.1–104.5	91.8–101.7	94.6–98.6	3.2–5.1
Tangeretin	0.1	5–2000	0.9996	4.8–9.0	2.3–7.3	99.1–102.8	98.8–104.5	91.3–91.5	7.6–9.0

[a] Three different concentration levels.
[b] Two different concentration (low and high) levels.

shown in Fig. 1A, the lipid droplets in differentiation media-treated cells became lager with a deeper red color; however, these phenomena were decreased by the treatment of 1 mg/mL of both treatments (heat-treated and non-treated peels) without cytotoxicity. Both extracts also reduced the elevated intracellular lipid contents, but the heat-treated peel obviously showed a better inhibitory effect on lipid accumulation in 3T3-L1 adipocytes than the non-treated peel (Fig. 1B). It has been reported that mandarin peel contains a plenty of flavanones and PMFs, contributing to various health benefits [7,15]. In addition, several studies demonstrated that heat treatment of the citrus peels increased their total polyphenol contents, followed by stronger antioxidant and anti-inflammatory activities [12,16]. Therefore, similarly, heat treatment of mandarin peel might contribute to enhancing inhibition of lipid accumulation on differentiation of 3T3-L1 cells to adipocytes, which appear to be due to changes in flavonoids. Further studies were performed to confirm the influence of the heat treatment on the flavonoid profile of mandarin peels.

3.2. Optimization of analytical conditions

Because many flavonoids have similar structures, and some are isomers producing same *m/z* trace from mass spectrometer (e.g. diosmin and neodiosmin), two columns were compared to obtain good separation efficiency: a Gemini C18 column (150 mm × 3.0 mm, 3.0 μm particle size) and an Acclaim C30 column (150 mm × 2.1 mm, 3.0 μm particle size). The C30 column exhibited better isomer separation, and was selected as the analytical column (data not shown). For mobile phase additives, formic acid solution resulted in favorable peak shape and suitable retention time of analytes. Thus, formic acid (0.1% as final concentration) was added to both water (mobile phase A) and acetonitrile (mobile phase B). For MS/MS detection, ESI + or ESI− was selected depending on ionization characteristics of analytes, and parameters including SRM transition, collision energy and RF lens were optimized using flow injection of individual standards. Precursor and product ions showing the highest signal response were chosen as optimum *m/z* values (Supplementary Table 1). Under developed LC–MS/MS conditions, all analytes were successfully separated and detected within 23 min (Fig. 2).

3.3. Method validation

The LC–MS/MS method was validated in terms of specificity, LOQ, linearity, accuracy, precision and recovery. No interference peak was observed at the analyte retention times. The LOQ ranged between 0.01 and 5 μg/g peel, demonstrating the method was sensitive to determine nanogram levels of trace analytes. Calibration curves showed good linearity with the coefficient of determination (r^2) values higher than 0.994 across the concentration ranges of analytes. The intra- and inter-day accuracy and precision were 87.9–114.7% and 0.3–14.9%, respectively, where all values were within an acceptable range. The mean recovery was in the range of 87.3–111.5%, with RSD values less than 15%. The summary of validation results are shown in Table 1.

3.4. Flavonoid composition of heat-treated and non-treated mandarin peels

The validated LC–MS/MS method was applied to quantification of flavonoids in heat-treated and non-treated mandarin peel extracts. We found 20 flavonoid compounds in heat-treated and non-treated peels (Table 2). According to the chemical structure, they were divided into: (1) flavanone glycosides (eriocitrin, neoeriocitrin, didymin, naringin, narirutin and hesperidin) and their aglycone compounds (eriodictyol, isosakuranetin, naringenin, and hesperetin); (2) a methoxylated flavone (diosmetin) and its aglycone compounds (diosmin and neodiosmin); (3) a flavonol glycoside (rutin) and its aglycone compound (quercetin); (4) various hydroxylation and methoxylation of flavones (apigenin, sinensetin, nobiletin and tangeretin); and (5) a flavanol aglycone (taxifolin). The major flavonoid was hesperidin, which represented 8361.19 ± 363.82 μg/g and 15,578.40 ± 324.05 μg/g in non-treated samples and heat-treated samples, respectively. Some PMFs were also found in high quantity in both samples such as nobiletin (4770.87 ± 253.62 and 5420.29 ± 300.32 μg/g, respectively), narirutin (1764.86 ± 80.71 and 2336.02 ± 126.40 μg/g, respectively), tangeretin (1553.17 ± 73.06 and 1854.40 ± 65.30 μg/g, respectively), and sinensetin (527.26 ± 22.55 and 595.69 ± 47.49 μg/g, respectively). The total flavonoid content of peels was significantly enhanced upon heat treatment. It was in accordance with previous reports

Table 2 – Mean concentrations of flavonoids in mandarin peels (n = 3).

Compound	Mean ± SD (μg/g)	
	Non-treated	Heat-treated
Eriocitrin	56.07 ± 1.22	85.80 ± 3.46***[a]
Neoeriocitrin	48.59 ± 0.83	125.09 ± 5.38***
Rutin	135.90 ± 4.97	128.98 ± 7.40
Narirutin	1764.86 ± 80.71	2336.02 ± 126.40**
Taxifolin	0.07 ± 0.01	0.16 ± 0.01***
Naringin	37.85 ± 5.48	56.73 ± 4.64*
Rhoifolin	–	–
Hesperidin	8361.19 ± 363.82	15,578.40 ± 324.05***
Diosmin	5.34 ± 0.29	36.31 ± 1.63***
Neodiosmin	–	–
Neohesperidin	–	–
Scutellarein	2.78 ± 0.04	3.76 ± 0.05***
Didymin	402.11 ± 12.31	656.54 ± 22.76***
Eriodictyol	0.14 ± 0.00	0.63 ± 0.01***
Poncirin	–	–
Quercetin	0.84 ± 0.03	4.17 ± 0.15***
Naringenin	0.24 ± 0.01	10.27 ± 0.64***
Hesperetin	0.85 ± 0.03	134.51 ± 29.68**
Apigenin	0.14 ± 0.01	2.53 ± 0.19***
Diosmetin	0.06 ± 0.01	1.15 ± 0.06***
Sinensetin	527.26 ± 22.55	595.69 ± 47.49
5,6,7,3′,4′,5′-Hexamethoxyflavone	–	–
Nobiletin	4770.87 ± 253.62	5420.29 ± 300.32*
Isosakuranetin	0.05 ± 0.00	2.01 ± 0.08***
Tangeretin	1553.17 ± 73.06	1854.40 ± 65.30**

The values are expressed as a mean ± SD (n = 3).
[a] Symbols in the same row are significantly different (*, **, ***P < 0.05, 0.01 and 0.001, respectively).

showing elevated phenolic and flavonoid compounds after heat treatment [19,20]. It seemed that heat treatment probably transferred thermal energy to liberate flavonoids through destruction of structural network within cell walls, leading to more release of them. Flavonoid aglycones were also found to increase after heat treatment ($P < 0.01$). Quercetin, naringenin, hesperetin, apigenin, and diosmetin were markedly raised by 5-, 43-, 158-, 18-, and 19-fold, respectively. Noteworthy, these aglycones showed significantly higher inhibitory effects on lipid accumulation in 3T3-L1 adipocytes than their glycosides (Supplementary Fig. 2). This could partially explain why heat-treated peels had the enhanced ability of suppression of lipid accumulation in 3T3-L1 cells (Fig. 1B). To confirm whether the aglycones were generated by heat treatment, the degradation of rutin, a representative flavonoid glycoside, was tested under the same thermal condition (150 °C, 50 min) with the peels. The results indicated a considerable amount of rutin (glycoside form) was found to convert into its aglycone form, quercetin, after heat treatment (Supplementary Fig. 3). This can be explained by deglycosylation (hydrolysis of sugar moieties) of flavonoid glycosides triggered by thermal energy, and several previous studies support this phenomenon [21]. Collectively, these findings suggest that heat treatment has an impact on the enhanced release of flavonoids from mandarin peel, accelerating degradation of flavonoid glucosides to aglycone forms, leading to the high efficiency on inhibition of lipid accumulation in 3T3-L1 cells. However, information on intracellular levels of flavonoids was still required to elucidate their bioactivities more clearly.

3.5. *Intracellular flavonoid change in 3T3-L1 cells treated with heat-treated and non-treated mandarin peel extracts*

To see intracellular change of flavonoids by heat treatment, flavonoids in 3T3-L1 cells were determine using LC–MS/MS after treating heat-treated and non-treated peel samples in cells for different times (0–24 h). The major intracellular flavonoids were hesperidin and nobiletin (Fig. 3), which were the most abundant compounds of flavonoids detected in the peel (Table 2). Treatment with the heat-treated samples resulted in significantly increased intracellular narirutin, hesperidin, diosmin, naringenin, hesperetin, didymin, nobiletin, sinensetin, and tangeretin, compared with those cells treated with the non-treated samples, supporting the better inhibitory efficacy of heat-treated samples on lipid accumulation in 3T3-L1 cells (Fig. 3). Apart from this, we additionally tested cellular uptake of individual flavonoid glycosides (narirutin and hesperidin) and their aglycones (naringenin, and hesperetin) to confirm which form shows favorable cellular uptake (Supplementary Fig. 3). Remarkably, the aglycones showed much higher cellular uptake than the

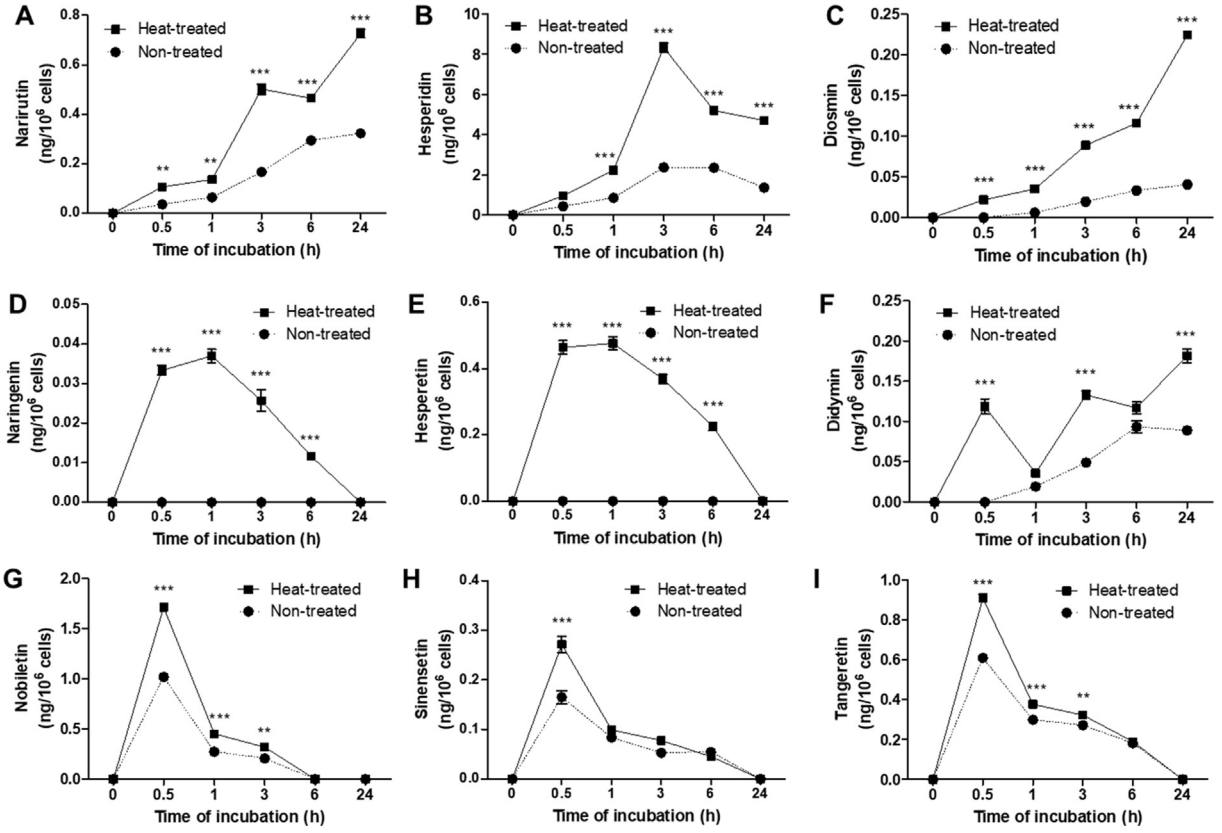

Fig. 3 – The contents of intracellular narirutin (A), hesperidin (B), diosmin (C), naringenin (D), hesperetin (E), didymin (F), nobiletin (G), sinensetin (H), and tangeretin (I) in 3T3-L1 cells treated with heat-treated and non-treated mandarin peel. The cells were incubated for indicated time with heat-treated or non-treated mandarin peel extracts (1 mg/mL). Intracellular flavonoid contents were quantified with the LC–MS/MS method. Data are expressed as a mean ± SEM (n = 3). *, **, ***P < 0.05, 0.01 and 0.001, respectively, significant difference between groups at the same time point.

glycosides when the same concentration was treated on 3T3-L1 cells, strengthening the biological effects of heat-treated samples. It has also been reported that isoflavone aglycones could be absorbed faster than their glucosides in humans [22]. Taken together, heat treatment of mandarin peel has high potential to alleviate lipid-related diseases such as obesity based on several biochemical evidences: (1) increase of releasing flavonoids from peel tissues; (2) conversion of glycosides to effective aglycones; (3) enhanced cellular uptake; and (4) high inhibitory efficacy of lipid accumulation on adipocytes. With increasing attention to the use of citrus peel, our results will be useful for future work of evaluation of citrus peel as applied to dietary supplements.

4. Conclusion

In the present study, the effect of heat treatment of mandarin peel on lipid accumulation in 3T3-L1 cells, and the alterations of flavonoid profiles in peels and the cells were investigated. With increased levels of flavonoids, especially flavonoid aglycones, heat-treated peel extracts showed a stronger inhibitory effect on differentiation of 3T3-L1 cells to adipocytes as well as more effective intracellular absorption of flavonoids than non-treated peel extracts. This work will expand our understanding of alterations in flavonoid profiles by heat treatment, and provide a new perspective regarding health benefits of heat-treated citrus peel as a potential dietary supplement.

Conflicts of interest

The author declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2019.05.002.

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