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Investigation of Heat Treating Conditions for Enhancing the Anti-Inflammatory Activity of Citrus Fruit (*Citrus reticulata*) Peels

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In traditional Chinese medicine, dried citrus fruit peels are widely used as remedies to alleviate coughs and reduce phlegm in the respiratory tract. Induction of inducible nitric oxide synthase (iNOS) in inflammatory cells and increased airway production of nitric oxide (NO) are well recognized as key events in inflammation-related respiratory tract diseases. Despite the fact that the enhancing effect of heat treatment on the antioxidant activity of citrus fruit peels has been well documented, the impact of heat treatment on citrus peel beneficial activities regarding anti-inflammation is unclear. To address this issue, we determined the anti-inflammatory activities of heat-treated citrus peel extracts by measuring their inhibitory effect upon NO production by lipopolysaccharide-activated RAW 264.7 macrophages. Results showed that the anti-inflammatory activity of citrus peel was significantly elevated after 100 °C heat treatment in a time-dependent fashion during a period from 0 to 120 min. Inhibition of iNOS gene expression was the major NO-suppressing mechanism of the citrus peel extract. Additionally, the anti-inflammatory activity of citrus peel extract highly correlated with the content of nobiletin and tangeretin. Conclusively, proper and reasonable heat treatment helped to release nobiletin and tangeretin, which were responsible for the increased anti-inflammatory activity of heat-treated citrus peels.

KEYWORDS: Citrus fruit; heat treatment; nitric oxide; anti-inflammation; antioxidation

INTRODUCTION

Citrus fruits make up the largest sector of the world's fruit production, with more than 100 million tons produced each season. About 34% of citrus fruits are made into juices; therefore, large amounts of residues are formed every year (*1*). Citrus peels, the dominant residue, possess a large variety of bioactive compounds; they are considered as potential sources of functional components (*2*). Except for ascorbic acid, citrus peels contain more bioactive compounds such as phenolic acids, flavonoids, limonoids, and fibers than do the juices (*3, 4*).

In traditional Chinese medicine, *chen pi*, the dried fruit peels of *Citrus reticulata* has been widely used for centuries as a remedy to treat indigestion and to improve inflammatory syndromes of the respiratory tract such as bronchitis and asthma (*5*). Around inflammatory sites in the airway, mitogen-activated cells such as macrophages, neutrophils, eosinophils, and epithelial cells synthesize and release numerous reactive oxygen species and reactive nitrogen species (*6*). Most reactive nitrogen species are derived from nitric oxide (NO) in vivo. Inducible NO synthase (iNOS) produces NO in inflammatory cells, which can react with oxygen or superoxide anions ($O_2^{\bullet -}$) and generate reactive nitrogen species (*7*). Indeed, given the detection of tyrosine nitration, ample evidence suggests that reactive nitrogen species are involved in the pathogenesis of various diseases of the respiratory tract (*8, 9*). Undoubtedly, suppression of NO production by activated inflammatory cells appears to be a reasonable mechanism for adjunctive therapy of inflammatory respiratory disorders, and inducible nitric oxide synthase is recognized as a molecular target of anti-inflammatory agents (*10*).

Although heat treatment elevates the antioxidant activity of citrus peels, the high temperatures of heat treatment destroy the flavanone glycosides (*11, 12*). For this reason, Xu et al. suggested (*12*) that a heating temperature of ≤100 °C enhances the antioxidant activity of citrus peels, without a loss of anti-inflammatory flavanoid glucosides, which is optimal for the preparation of *chen pi*. Indeed, a 2-h heat treatment at 100 °C has been documented to elevate the antioxidant and anti-inflammatory activities of kumquat (*Fortunella margarita* Swingle) peels (*13*). Among the well-known citrus bioactive compounds, flavonoids attract considerable attention, not only for their abundance but also for their significant biological activities, including anti-inflammatory, anticancer, and anti-atherogenic activities (*14*). Although more than 60 flavonoids have been identified in citrus fruits, citrus flavonoids are

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Table 1. Total Phenolics Content and DPPH Radical Scavenging Capacity of Methanolic Extract of Citrus Peels Heated at 100 °C for Different Time Periods[a]

duration of heat treatment (min)	0	30	60	120	180
total phenolics content (mg GAE/g extract)[b]	40.8 ± 0.9 a	43.3 ± 1.3 ab	46.0 ± 3.2 bc	50.7 ± 4.4 cd	54.1 ± 1.8 d
DPPH scavenging capacity (μg extract/mL)[c]	371 ± 61 a	201 ± 11 b	186 ± 28 b	125 ± 24 c	117 ± 40 c

[a] The values are expressed as the means ± SD of triplicate tests. Means not sharing a common letter in the same row were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test. [b] The total phenolics content was expressed as milligrams of gallic-acid equivalents (GAE) per gram of solid extract. [c] The DPPH radical scavenging capacity was expressed as the IC_{50} value, which denoted the concentration of peel extracts that scavenged 50% of DPPH radicals.

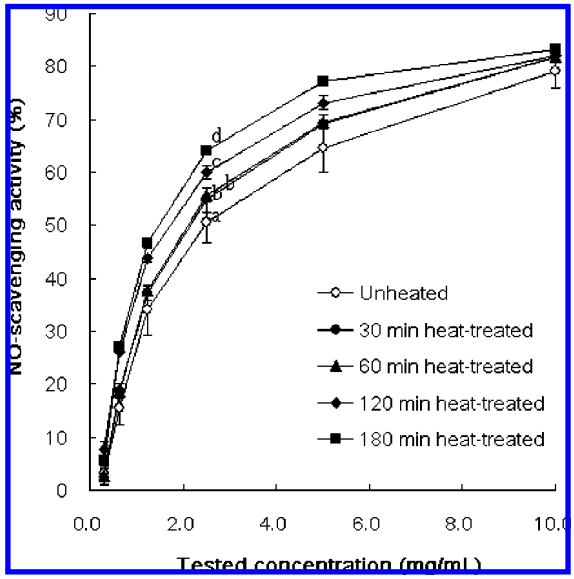

Figure 1. Effect of heating duration on the NO-scavenging activity of citrus peels. The values are expressed as the means ± SD of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

represented by two very peculiar classes of compounds: the polymethoxy flavones and the flavanone glycosides (*15*).

In spite of the long-time use of hesperidin, the dominant flavanone glycoside, as a quality control indicator for the production of *chen pi* (*16*), researchers have not agreed upon hesperidin contributing to the health function of *chen pi* (*Citrus reticulata* peels). Recently, a wide spectrum of biological activities of polymethoxy flavones has emerged (*17, 18*), and thus, the critical role of flavanone glycosides as biologically active ingredients of *chen pi* is being challenged (*19*).

In this study, 100 °C was adopted as the heating temperature to investigate the most optimal duration of heat treatment for enhancing the anti-inflammatory activity of citrus peels. Additionally, the key flavonoid involved in the enhanced anti-inflammatory activity of heated citrus peels would be identified further. We used lipopolysaccharide (LPS)-induced iNOS expression in murine RAW 264.7 macrophages as an inflammatory model. We determined the ability of *chen pi* samples to inhibit NO production as the method to assess anti-inflammatory activity. Moreover, the content of specific flavanone glycosides and polymethoxy flavones was analyzed, and the relationship between specific flavonoid and anti-inflammatory ability was assessed in order to elucidate the most possible contributor to the elevated anti-inflammatory activity of the heated citrus peels.

MATERIALS AND METHODS

Materials and Chemicals. We cultured RAW 264.7 cells obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies Inc., Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), sodium nitroprusside, LPS, sulfanilamide, naphthylethylenediamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Folin−Ciocalteu reagent, gallic acid, catechin, nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (NBT/BCIP), and the reference authentic standards used in HPLC analyses, hesperidin, naringin, neohesperidin, nobiletin, and tangeretin, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Heat Treatment and Preparation of Citrus Peel Extract. The ponkan mandarins (*Citrus reticulata*) used in this study were purchased from a local market (Shinchu, Taiwan) in the Winter of 2006 and hand-peeled immediately after purchase. All of the peels were oven-dried at 50 °C for 48 h and then ground into powders. Ten grams of the dried citrus peel powders were placed into 250 mL glass flasks and heated in an oven at 100 °C for periods of 30, 60, 120, and 180 min. After cooling to room temperature, the heated and unheated citrus peel powders were extracted twice with a 10-fold volume of methanol for 20 h, under vigorous shaking. Afterward, the samples were centrifuged at 10000 × *g* for 10 min, and the supernatants were evaporated under vacuum at <50 °C using a rotary evaporator to obtain the solid extract.

For the experiments to follow, the solid extract was weighed and dissolved in DMSO to a concentration of 500 mg/mL. It was then diluted with phosphate-buffered saline (PBS) or culture medium to the required concentration. Control experiments were carried out by adding an equivalent amount of DMSO without the peel extract. The highest final concentration of DMSO in the culture medium was 0.8%.

Determination of Total Phenolic Content. The quantity of total phenolic compounds in the extracted sample was determined by means of the Folin−Ciocalteu colorimetric method. Briefly, an optimally diluted sample was reacted with Folin−Ciocalteu phenol reagent in an alkaline solution. The absorbance at 765 nm was measured using a spectrophotometer. The total phenolic content for each citrus peel extract was then calculated by means of a standard curve prepared with gallic acid and expressed in terms of milligrams of gallic-acid equivalents (GAE) per gram of solid extract.

Determination of 2,2-Diphenyl-1-picryhydrazyl Radical-Scavenging Capacity. Twenty microliters of serially diluted sample extracts were pipetted into each well of a 96-well flat-bottomed plate. Then, 180 μL of 0.2 mM DPPH methanol solution was added into each well, and the plate was shaken on a plate shaker for 5 min. The absorbance at 540 nm was measured using an enzyme-linked immunosorbent assay reader (EL800, Bio-Tek Instruments Inc., Winooski, VT, USA). The scavenging capacity of the sample extracts to DPPH radicals was expressed as the IC_{50}, which denoted the concentration of peel extracts that scavenged 50% of DPPH radicals.

Determination of NO-Scavenging Activity. Sixty microliters of serial diluted sample extracts were pipetted into each well of a 96-well flat-bottomed plate. Following this, 60 μL of 10 mM sodium nitroprusside dissolved in PBS was added into each well, and the plate was then incubated under light at room temperature for 120 min. Finally, an equal volume of Greiss reagent (mixed solution of 1% of sulfanilamide and 0.1% of naphthylethylenediamine) was added into each well, and the absorbance at 540 nm was measured in order to measure the nitrite content.

The scavenging activity (%) of the sample extracts to NO was expressed as follows:

$$[1 - (A_{sample} - A_{sample\ blank})/(A_{control} - A_{control\ blank})] \times 100\% \quad (1)$$

where $A_{sample} - A_{sample\ blank}$ was the difference in the absorbances of a sample with 10 mM of sodium nitroprusside solution compared with PBS, and $A_{control} - A_{control\ blank}$ was the difference in the absorbances of the DMSO control with 10 mM sodium nitroprusside solution compared with PBS.

Evaluation of Anti-Inflammatory Activity. The anti-inflammatory activity was evaluated as the inhibitory effect of the citrus peel extracts on NO production by LPS-activated RAW 264.7 macrophages. Briefly, 1×10^5 RAW 264.7 cells were seeded into each well of a 96-well culture plate and incubated for 24 h. After they were washed three times with PBS, adherent cells were incubated in prepared DMEM containing peel extract with or without LPS (100 ng/mL). Following incubation for 20 h, the medium was collected for the nitrite assay, at which time cell viability was evaluated using the MTT method. Finally, the nitrite concentration of the conditioned medium was measured as an indicator of NO production by use of the Greiss reaction. The anti-inflammatory activity of the peel extracts was expressed as the IC_{50} value, which denoted the concentration of peel extracts that caused 50% inhibition of NO production by the LPS-activated RAW 264.7 cells. To calculate the percentage inhibition of LPS-stimulated NO production, NO production in the presence (+) and absence (−) of 100 ng/mL of LPS was taken as 0% and 100% inhibition, respectively. The percentage inhibition by a citrus extract at a specific concentration was calculated as percentage inhibition = $[1 - (NO_{citrus+LPS} - NO_{-LPS})/(NO_{+LPS} - NO_{-LPS})] \times 100\%$. The IC_{50} value, calculated from the curve of percentage inhibition versus concentration, is the concentration of sample that results in 50% inhibition.

Evaluation of iNOS Protein Levels. The RAW 264.7 cells were seeded at a density of 5×10^6 cells per 6-cm culture dish in a total volume of 5 mL and incubated for a period of 24 h. Following three washes of PBS, the adherent cells were incubated for 12 h in the presence or absence of LPS (100 ng/mL) and citrus peel extracts. Adherent cells were then washed with PBS, collected, suspended in lysis buffer (50 mM Tris, pH 7.6, 0.01% ethylenediaminetetra-acetic acid (EDTA), 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 1 μg/mL leupeptin), and centrifuged at 12,000 $\times$ g for 20 min at 4 °C. Additionally, iNOS protein levels were determined by immunoblot analysis. Briefly, supernatants of cell lysates containing equal protein content were loaded into and separated on a 10% sodium dodecylsulfate

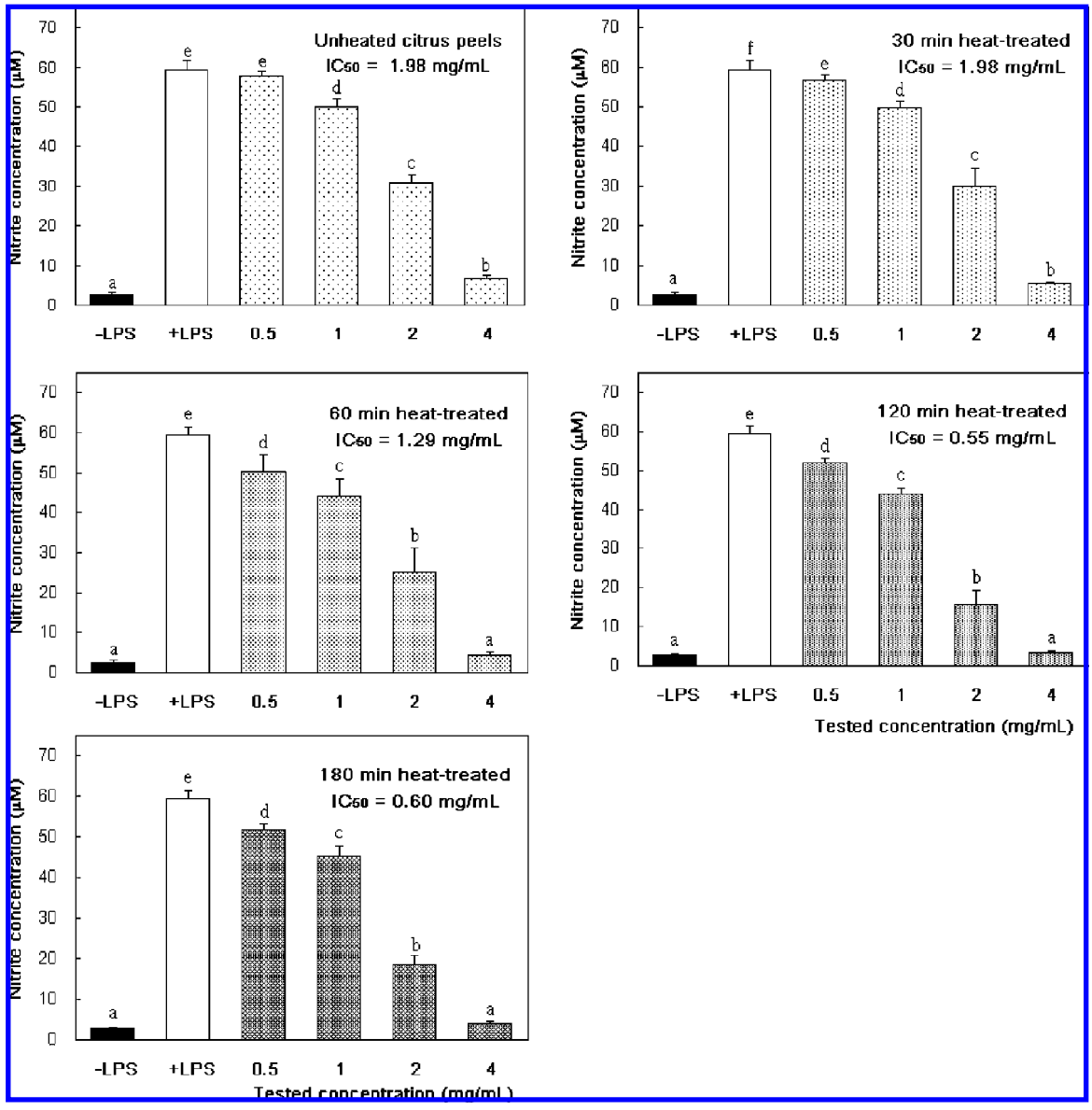

Figure 2. Effect of heating duration on the anti-inflammatory activity of citrus peels. The anti-inflammatory activity of the peel extract was expressed as the IC_{50}, which denoted the concentration of peel extracts that caused 50% inhibition of NO production by the LPS-activated RAW 264.7 cells. The values are expressed as the means ± SD of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

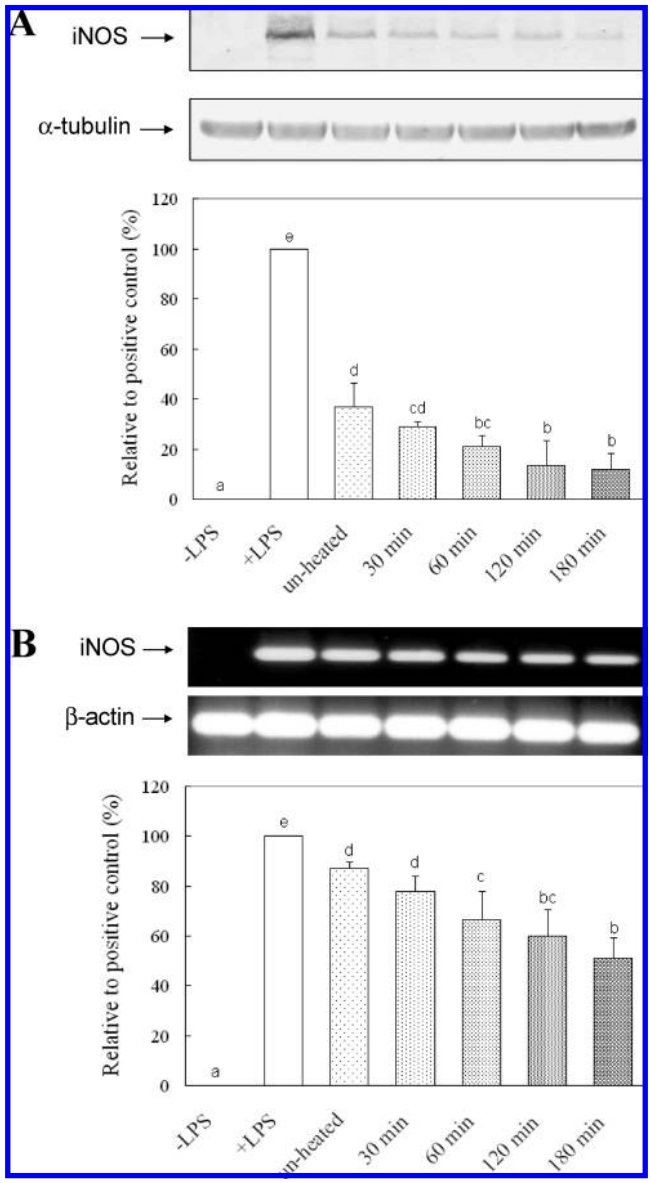

Figure 3. Inhibitory effect of citrus peel extracts on the iNOS protein (**A**) and mRNA (**B**) expression for LPS-activated RAW 264.7 macrophages. The protein and mRNA levels of iNOS were determined according to the description in Materials and Methods. The values are expressed as the means ± SD of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

(SDS)−polyacrylamide gel, and then transferred to polyvinylidene fluoride filters. Following this, filters were blocked and probed with anti-iNOS antibodies (BD Transduction Laboratories, San Jose, CA, USA) and anti-α-tubulin antibodies (Sigma, St. Louis, MO, USA). The filters were then incubated with secondary antibody conjugated to alkaline phosphatase and detected using NBT/BCIP solution. Finally, the band intensities of iNOS were quantified using a software-supported photoimager (ImageMaster VDS; Amersham Pharmacia Biotech Co., Piscataway, NJ, USA) and were normalized with α-tubulin.

Evaluation of iNOS mRNA Levels. The cells were cultured with or without sample extract and/or LPS (100 ng/mL) for 6 h. Adherent cells were then collected, and the total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The level of iNOS mRNA was determined by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). Briefly, the mRNA was transcribed to cDNA and amplified with PCR using a commercially available kit (Promega, Madison, WI, USA). The sequences of the PCR primers were as follows: 5′-CAGTTCTGCGCCTTTGCTCAT-3′ (forward) and 5′-GGTGGTGCGGCTGGACTTT-3′ (backward) for mouse iNOS and 5′-AGGCCCAGAGCAAGAGAG-3′ (forward) and 5′-GGGTGTTGAAG-GTCTCAAAC-3′ (backward) for mouse β-Actin. Finally, 20-μL aliquots of each PCR sample were analyzed by electrophoresis in 2% agarose gel in the presence of 5 ng/mL Gybr gold (Invitrogen, Carlsbad, CA, USA) and visualized under ultraviolet light. The band intensities of iNOS PCR products were quantified using a software-supported photoimager and were normalized with β-Actin.

Determination of Total Flavonoid Content. Total flavonoid content in the citrus peel extract was determined by a colorimetric method. Briefly, 0.25 mL of an optimally diluted sample was added into a tube containing 1 mL of double-distillated water. Then, 0.075 mL of 5% $NaNO_2$, 0.075 mL of 10% $AlCl_3$, and 0.5 mL of 1 M NaOH were added sequentially at 0, 5, and 6 min. Finally, the volume of reacting solution was adjusted to 2.5 mL with double-distilled water. The absorbance at 510 nm was detected using a spectrophotometer. The total flavonoid content in each extract sample was then calculated using a standard curve prepared with catechin and expressed in terms of milligrams of catechin equivalents (CE) per gram solid extract of citrus peels.

Analysis of Flavonoids Composition. The contents of specific flavanone glycosides and polymethoxy flavones were determined by high performance liquid chromatography (HPLC) according to Lu et al. (*16*). Briefly, 10 μL of sample was separated on a hypersil C18 column (250 × 4.6 mm i.d., Thermo Fisher Scientific, Waltham, MA, USA) at 35 °C, with a controlled flow rate of 1 mL/min and set wavelengths of 284 and 332 nm. The gradient elution program was as follows: initial 15-min run of 40% methanol (v/v), followed by a 5-min linear gradient to 100% methanol, which was held for 5 min; then, a linear gradient to 40% methanol in 1 min, which was held for 30 min. Identification of the specific flavanone glycosides and polymethoxylated flavones was based on the retention times of the sample peaks to those of the authentic reference standards. The amount of each constituent in the citrus peel extracts was estimated by the integrated datum.

Statistical Analysis. All results are expressed as the means ± SDs of at least three independent tests. The significance of the differences between the treatments was analyzed using analysis of variance followed by Duncan's multiple range test for multiple comparisons. The correlation between two variants was analyzed by application of the Pearson test. All of the statistical analyses were performed by means of SPSS software (SPSS for Windows, ver. 10.0; SPSS Inc. Chicago, IL, USA) with the level of significant difference between compared data sets being set at $p < 0.05$.

RESULTS

Effect of Heat Duration on Antioxidant Capacity. The antioxidant capacities of the methanol extracts of the citrus peels were evaluated by two assays, the total phenolic content, and the DPPH radical scavenging capacity assays. The total phenolic content of the citrus peel extracts increased significantly from 40.8 mg GAE/g of extract in the nonheated control to 54.1 mg GAE/g of extract when heated to 100 °C for 180 min (**Table 1**). The corresponding IC_{50} values for DPPH radical scavenging ability were calculated as corresponding with the different heating durations for making the citrus peel extracts. Similar to the results of total phenolic content, the DPPH-scavenging capacity of citrus peels increased as the heating time increased (**Table 1**).

Effect of Heat Duration on NO-scavenging Activity. All of the citrus peel extracts revealed NO-scavenging ability in a dose-dependent manner (**Figure 1**). Longer durations of heat treatment of the citrus peels increased their antioxidant activity as evidenced by their increasing ability to scavenge NO radicals.

Effect of Heat Treatment on Anti-Inflammatory Activity. The macrophages had a reduced survival rate of <95% when they were incubated with peel extracts at concentrations >5 mg/mL. Therefore, a concentration of <5 mg/mL was used to

Table 2. Total Flavonoids, Specific Flavanone Glycosides, and Polymethoxy Flavone Content in Methanolic Extract of Citrus Peels Heated at 100 °C for Different Time Periods[a]

duration of heat treatment (min)	0	30	60	120	180
total flavonoids (mg CE/g extract)[b]	7.62 ± 0.12 a	7.90 ± 0.10 b	8.45 ± 0.17 c	8.45 ± 0.11 c	9.07 ± 0.11 d
naringin (mg/g extract)[c]	5.87 ± 0.39 a	7.14 ± 0.23 b	7.28 ± 0.21 b	7.51 ± 0.16 bc	7.79 ± 0.16 c
hesperidin (mg/g extract)[c]	68.8 ± 2.9 a	88.7 ± 3.3 c	86.3 ± 1.6 bc	87.9 ± 1.0 bc	83.5 ± 2.6 b
neohesperidin (mg/g extract)[c]	ND[d]	ND	ND	ND	ND
nobiletin (mg/g extract)[c]	30.3 ± 1.0 a	32.3 ± 0.3 b	33.8 ± 0.3 c	35.4 ± 0.5 d	37.1 ± 0.9 e
tangeretin (mg/g extract)[c]	25.9 ± 0.2 a	26.6 ± 0.8 ab	27.2 ± 0.4 b	28.3 ± 0.1 c	29.4 ± 0.1 d

[a] The values are expressed as the means ± SD of triplicate tests. Means not sharing a common letter in the same row were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test. [b] The total flavonoid content was expressed as milligrams of catechin equivalents (CE) per gram of solid extract. [c] The specific flavonoid content was expressed as milligrams of per gram of solid extract. [d] ND, undetectable.

Table 3. Correlation coefficients between the specific flavonoid content of a particular citrus peel extract and its NO-scavenging and anti-inflammatory activity[a]

	correlation coefficient (probability)	
parameter	NO-scavenging[b]	anti-inflammatory[c]
total flavonoids	0.95 (0.015)*	−0.88 (0.049)*
naringin	0.89 (0.042)*	−0.77 (0.128)
hesperidin	0.54 (0.348)*	−0.43 (0.474)
nobiletin	0.99 (0.002)*	−0.93 (0.022)*
tangeretin	0.99 (0.002)*	−0.93 (0.022)*

[a] * represents significant correlation ($p < 0.05$) when analyzed by the Pearson test. [b] The values of NO scavenging activity of citrus peel extract at tested concentration of 2.5 mg/mL were used to analyze their correlation with total and specific flavonoids. [c] The IC_{50} values of citrus peel extracts on NO production for LPS-activated macrophages were used as indicators of anti-inflammatory activity to analyze their correlation with total and specific flavonoids.

treat the cells to prevent the citrus peel extracts from having a cytotoxic effect on NO production. When the cells were incubated with citrus peel extracts in the absence of LPS, the nitrite concentration of the medium was similar to the background level of the unstimulated control (data not shown). After treatment with LPS for 20 h, the nitrite concentration of the medium markedly increased, as compared to that of the unstimulated control (**Figure 2**). Significant concentration-dependent inhibition of NO production was observed when the macrophages were cotreated with LPS and various concentrations of the citrus peel extract (**Figure 2**). The IC_{50} values of unheated and 30, 60, 120, and 180 min heat-treated citrus peel extract on NO production were 1.98, 1.98, 1.29, 0.55, and 0.60 mg/mL, respectively. As the heating duration increased from 0 to 120 min, the anti-inflammatory activity of citrus peel also increased; 180 min of heat treatment did not incrementally boost the anti-inflammatory activity of the citrus peel extract. Therefore, we suggest that 120 min of heating was optimal for the maximum anti-inflammatory activity of the citrus peels. To elucidate the mechanism responsible for the NO-suppressing effect of citrus peel extracts, we determined the protein and mRNA levels of iNOS by immunoblotting and RT-PCR analysis, respectively. **Figure 3** shows that RAW 264.7 macrophages did not express a detectable level of iNOS protein or mRNA when incubated in culture medium without LPS, whereas incubation of the cells in the presence of LPS markedly increased both iNOS protein and mRNA content. All tested peel extracts at a concentration of 1 mg/mL revealed a significant inhibitory effect upon the level of iNOS protein and mRNA (**Figure 3**). Furthermore, the inhibitory effect of citrus peel on iNOS protein and mRNA expression was also boosted by heat treatment. These results indicated that unheated and heated citrus peels suppress LPS-stimulated NO production by the same mechanism, which is inhibition of iNOS gene expression.

Effect of Heat Duration on Flavonoid Composition. Two polymethoxylated flavones, nobiletin and tangeretin, and three flavanone glycosides, naringin, neohesperidin, and hesperidin, in the extract of citrus peels were determined by HPLC. The effect of heat treatment on flavonoid composition is shown in **Table 2**. The content of total flavonoids in citrus peel extract increased significantly upon heat treatment from 7.62 mg CE/g extract for the nonheated control to 9.07 mg CE/g extract by heating at 100 °C for 180 min. In accordance with previous studies, hesperidin was the most abundant flavanone glycoside present in mandarin peels, naringin was the second most abundant, which accounted for about 1/10 of hesperidin, while neohesperidin was almost undetectable (*20*). Although the content of naringin in the peel extract increased as the heating duration increased, interestingly, the content of the most abundant flavanone glycoside, hesperidin, did not increase as the heating time increased (**Table 2**). Heating for 30 min produced maximal release of hesperidin from the citrus peel. However, sustained heating destroyed them slightly. Both nobiletin and tangeretin are the two most abundant polymethoxylated flavones present in citrus peels, and their content in the peel extract increased as heating duration increased. The content of nobiletin and tangeretin in the extract of 180 min heated citrus peels were 22.4% and 13.8%, respectively, more than those in the unheated citrus peels.

To explore whether a possible relationship existed among the specific flavonoid contents, NO-scavenging, and anti-inflammatory activity of the citrus peel extracts, a correlative analysis was performed. The NO-scavenging activity correlated significantly ($p < 0.05$) to the contents of naringin, nobiletin, and tangeretin. However, the anti-inflammatory activity was only highly correlated with the content of the two polymethoxy flavones, nobiletin and tangeretin, but not with the content of the flavanone glycosides, naringin and hesperidin (**Table 3**).

DISCUSSION

The enhancing effect of simple heat treatment on the antioxidant capacity of citrus peels represented in this study was consistent with that of previous reports (*11*−*13*). Release of phenolic compounds from an unextractable form covalently bound to insoluble polymers to an extractable form was considered a reasonable mechanism to be responsible for the elevated antioxidant activity of heat-treated citrus peels (*11*). Additionally, heat treatment also affected the composition and distribution of phenolic compounds present in extracts of citrus peels (*11, 12*). In citrus peels, flavones and flavanone glycosides are the principle constituents of methanol extractable phenolic compounds, whereas polymer bound phenolic compounds mainly consist of phenolic acids and flavonols (*3*). Xu et al. (*12*) found that heat treatment cleaved the ester

and glycosidic bond, and thus, increased the amount of free form phenolic acids and decreased the amount of ester and glycoside forms of extractable phenolic acids. However, the increased amount of free form phenolic acid did not compensate for the lost amount of ester and glycoside forms by heat treatment and resulted in a decrease in the total amount of extractable phenolic acids. Therefore, they suggested that heat treatment affected the distribution rather than the total amount of extractable phenolic acids to elevate the antioxidant activity of citrus peels. At the same time, a warning regarding the impact of heat treatment on the methanol extractable flavanone glycosides, acknowledged antiinflammatory compounds abundantly present in citrus fruits, was published (*12*).

Heat treatment at 100 °C for 2 h significantly aided to increase the anti-inflammatory activity of citrus peels as evaluated by their ability to inhibit NO production in LPS-activated RAW264.7 macrophages. Moreover, both unheated and heat-treated citrus peels appeared to provide anti-inflammatory activity primarily through regulation of cellular iNOS expression. Indeed, inhibited activation of transcription factor nuclear factor-κB and subsequent expression of the iNOS gene underlie the NO-suppressing mechanism of citrus peels (*21*).

In agreement with the suggestion by Xu et al. (*12*), we found that a lower temperature (100 °C) heat treatment of the citrus peels reduced the destructive impact of high temperature (150 °C) on the most abundant flavanone glycoside, hesperidin. Additionally, we increased the duration of heat treatment from 30 to 180 min. Surprisingly, low temperature (100 °C) heat treatment released polymethoxy flavones in a time-dependent manner, at least during a 180 min heating duration. Most importantly, the content of polymethoxy flavones (nobiletin and tangeretin), but not flavanone glycosides (hesperidin and naringin), highly correlated with anti-inflammatory activity, suggesting that heat-released polymethoxy flavones were responsible for the increased anti-inflammatory activity of heat-treated citrus peels. Although flavanone glycosides are considered potential anti-inflammatory components, several other phytochemicals abundantly present in citrus fruits, including coumarins and polymethoxy flavones, reportedly exhibited potent anti-inflammatory activities as assessed by NO-suppressing abilities (*22*−*25*). Furthermore, polymethoxy flavones, especially nobiletin, were identified recently as major contributors to the NO-suppressing activity of citrus peels (*19*). Taking these results into consideration, we proposed that polymethoxy flavones were the key determinants of the anti-inflammatory activity of *chen pi*. Furthermore, the effect of heating time and temperature on the anti-inflammatory activity of citrus peels should be re-evaluated on the basis of the released polymethoxy flavones rather than flavanone glycosides in order to optimize the heating process.

The tissues of citrus peel, flavedo and albedo, account for 81%−82% and 16%−18%, respectively, of the total polymethoxy flavones in a whole fruit. Therefore, polymethoxy flavones present in processed juices come exclusively from the peel's tissues and are largely affected by the manufacturing process (*26*). Nonetheless, citrus peels rather than processed juices are the best source of polymethoxy flavones, and much effort has been spent to develop efficient and large quantity extraction methods (*27*). In traditional Chinese medicine, a stir-fry heating process is usually employed to decrease the warm and hot tastes of *chen pi*. Foods recognized as hot and warm tastes have been documented to stimulate, whereas in contrast, cold and cool foods attenuate pro-inflammatory PGE_2 production (*28, 29*). Therefore, we suggest that the change of tastes of *chen pi* after stir-frying was attributed to the release of polymethoxy flavones, well-known potent anti-inflammatory agents. This hypothesis requires further scientific research.

In conclusion, optimal heat treatment (for example, 100 °C for 2 h) boosted the release of some anti-inflammatory compounds, especially, polymethoxy flavones, from an unextractable form to an extractable form and thus elevates the antioxidant and anti-inflammatory activities of methanol extracts of citrus fruit peels.

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