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Rapid Communication

Polymethoxy flavones are responsible for the anti-inflammatory activity of citrus fruit peel

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ABSTRACT

In traditional Chinese medicine, dried citrus fruit peels are widely used as remedies to alleviate some respiratory inflammatory syndromes and are considered as potential sources of anti-inflammatory components. Seven citrus fruits were selected for this study. We determined the inhibitory ability of citrus peel extracts on the production of pro-inflammatory mediators, prostaglandin E_2 (PGE_2) and nitric oxide (NO), in lipopolysaccharide (LPS)-activated RAW 264.7 cells. Among the tested citrus peels, Ponkan (*Citrus reticulata* Blanco) and Tonkan (*Citrus tankan* Hayata) deserve special attention due to their outstanding inhibitory effect on PGE_2 and NO secretion. We have also examined the composition, flavanone glycosides and polymethoxy flavones. The polymethoxy flavone content, especially nobiletin, appears to correlate well with the anti-inflammatory activities of certain citrus peel extracts. Thus, our results suggest that polymethoxy flavones contribute crucially to the anti-inflammatory activity of citrus peels.

1. Introduction

At the site of inflammation in the airway, mitogen-activated cells, such as macrophages, neutrophils, eosinophils, and epithelial cells, synthesize and release various pro-inflammatory mediators (Rahman, 2003). Prostaglandin E_2 (PGE_2) and nitric oxide (NO) are two pivotal pro-inflammatory mediators. PGE_2 promotes inflammation by increasing the vascular permeability and vasodilation which give rise to redness, swelling, stiffness and pain (Vane et al., 1994). The synthesis of prostaglandins depends mainly on the activity of the cyclo-oxygenase (COX) enzymes. Cyclo-oxygenase 1 (COX-1) is constitutively expressed and catalyzes the synthesis of prostaglandins for normal physiological functions, whereas cyclo-oxygenase 2 (COX-2) is induced by cytokines and mitogenic factors in different cells associated with inflammation (Smith, Garavito, & Dewitt, 1996). The uncontrolled NO produced by the inducible NO synthase (iNOS) gives rise to reactive nitrogen species, which induce biomolecular and cellular damage. Both cytokines are implicated in inflammatory disorders and carcinogenesis (Mayer & Hemmens, 1997). Thus, inhibition of COX-2 and iNOS expression and PGE_2 and NO overproduction are targets of much research to develop the anti-inflammatory phytochemicals.

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 (Li, Lo, & Ho, 2006). Citrus peels, which comprise the dominant residue, exhibit potent anti-oxidant and anti-inflammatory activities (Lin, Hung, & Ho, 2008; Murakami et al., 2000), and are considered potential sources of functional components (Schieber, Stintzing, & Carle, 2001). Except for ascorbic acid, citrus peels contain more bioactive compounds, such as phenolic acids, flavonoids, limonoids, and fibre than do juices (Bocco, Cuvelier, Richard, & Berset, 1998; Gorinstein et al., 2001). In the traditional Chinese medicine, *chen-pi*, the dried mature fruit peels of *Citrus reticulata and Citrus sinensis* and their varieties, have been widely used for centuries as remedies to treat indigestion and to improve inflammatory syndromes of the respiratory tract, such as bronchitis and asthma (Ou, 1999).

Among the well-known citrus bioactive compounds, flavonoids, especially the citrus unique polymethoxy flavones and flavanone glycosides, attract considerable attention for their significant biological activities (Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). The most extensively studied actions proved are their anti-inflammatory and anti-cancer activities. It has been suggested that inhibition of the synthesis and biological activities of different inflammatory mediators, mainly arachidonic acid derivatives, prostaglandin E_2 and F_2 and thromboxane A_2, are responsible for the anti-inflammatory properties of citrus flavonoids (Benavente-Garcia & Castillo, 2008; Benavente-Garcia, Castillo, Marin, Ortuno, & Del Rio, 1997). In this study, we compared

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the anti-inflammatory activities of methanol extracts of seven citrus fruit peels by examining their ability to inhibit the lipopolysaccharide (LPS)-induced PGE_2 and NO production in RAW 264.7 macrophages. Moreover, the content of specific flavanone glycoside and polymethoxy flavone was analyzed, and relationships between specific flavonoids and anti-inflammatory ability were assessed. The purpose of this study was to elucidate the major components which contributed to the anti-inflammatory activities of citrus peels.

2. Materials and methods

2.1. Materials and chemicals

RAW264.7 is a mouse macrophage cell line and can be induced to secrete (considerably) a variety of pro-inflammatory mediators by LPS, and therefore is considered as a feasible cellular model to test inflammation-related reaction or mechanisms. In this study, RAW264.7 cells were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in Dulbecco modified eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Gibco BRL Life Technologies Inc., Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), sulphanilamide, naphthylethylenediamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), catechin, nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (NBT/BCIP), rutin and the reference authentic standards (i.e. hesperidin, naringin, neohesperidin, nobiletin and tangeretin), used in HPLC analyses, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other authentic reference standards, narirutin and sinensetin were purchased from ChromaDex Inc. (Irvine, CA, USA). All chemicals used in this study were of analytical grade.

2.2. Preparation of citrus peel extract

Seven citrus fruits, including Grapefruit (*Citrus paradis*), Hutoukan (*Citrus kotokan* Hayata), Lemon (*Citrus limon* (L.) Bur), Liucheng (*C. sinensis* (L.) Osbeck), Ponkan (*C. reticulata* Blanco), Murcott (*C. reticulata* × *C. sinensis*), and Tonkan (*Citrus tankan* Hayata), were purchased from a local market (Hsinchu, Taiwan) in the winter of 2006 and hand-peeled immediately after purchase. All of the peels were oven-dried at 45 °C for 48 h and then ground into powders. Aliquots (10 g) of the dried citrus peel powders were placed into glass flasks and extracted with a 30-fold volume of methanol for 20 h, under vigorous shaking. Afterwards, the samples were filtered through Whatman No. 1 filters; the filtrates were evaporated under a vacuum at <50 °C using a rotary evaporator to obtain the solid extract. The extraction yields are listed in Table 1.

For the experiments to follow, the solid extract was weighed and dissolved in DMSO to a concentration of 500 mg/ml. The stock solution was then diluted with optimal solvent or culture medium to the required concentration. Control experiments were carried out by adding an equivalent amount of DMSO without the peel extract.

2.3. Determination of total flavonoids

Total amount of flavonoids in the citrus peel extract was determined by a colorimetric method. Briefly, 0.25 ml of an optimally diluted sample was placed in a tube containing 1 ml of double-distilled 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 in sequence 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 measured using a spectrophotometer. The total amount of flavonoids in each extract sample was then calculated, using a standard curve prepared with rutin, and expressed in terms of milligrams of rutin equivalents (CE) per gram solid extract of citrus peels.

2.4. Analysis of flavonoids composition

A high performance liquid chromatography system (SCL-10Avp, Shimadzu, Kyoto, Japan) equipped with two pumps (LC-10ATvp), a UV–Vis detector (SPD-10Avp) and an autosampler (SIL-10ADvp) was used to determine the contents of the flavanone glycosides and polymethoxy flavones. Briefly, 10 μl of diluted and filtered sample were 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 nm and 332 nm. The mobile phase was composed of (A) 2% acetic acid and (B) 0.5% acetic acid-acetonitrile (50:50, v/v) and the gradient elution programme was performed as follows: 0 min, 95:5; 10 min, 90:10; 40 min, 60:40, 55 min, 45:55, 60 min, 20:80; 65 min, 0:100 and held for 10 min. Identification of the specific flavanone glycosides and polymethoxy flavones was based on the retention times of the sample peaks compared to those of the authentic reference standards. The amount of each constituent in the citrus peel extracts was estimated by the use of external standard calibration.

2.5. Determination of PGE_2 and NO secretion

The anti-inflammatory activity of citrus samples was evaluated, based on their inhibitory effect on the PGE_2 and NO secretion in LPS-activated RAW 264.7 macrophages. In brief, 6×10^5 RAW 264.7 cells were seeded into each well of a 24-well culture plate and incubated for 24 h. After they were washed three times with PBS, adherent cells were incubated with the peel extract (dissolved in DMEM), with or without LPS (1 μg/ml). After incubation for a further 20 h, the medium was collected for the PGE_2 and NO assay,

Table 1
Yield and total flavonoids content of methanolic extract of different citrus fruit peels.

Scientific name	Local name	Extraction yield[a] (%)	Total flavonoids[b,c]	
			(mg RE/g solid extract)	(mg RE/100 g dried peels)
Citrus paradis	Grapefruit	31.4	6.97 ± 0.40[c]	219 ± 12[d]
Citrus kotokan Hayata	Hutoukan	42.2	4.43 ± 0.21[e]	187 ± 9[e]
Citrus limon (L.) Bur	Lemon	20.5	11.9 ± 0.66[a]	254 ± 13[c]
Citrus sinensis (L.) Osbeck	Liucheng	31.0	4.89 ± 0.13[d,e]	151 ± 4[f]
Citrus reticulata × *Citrus sinensis*	Murcott	36.5	5.29 ± 0.22[d]	193 ± 8[e]
Citrus reticulate Blanco	Ponkan	43.2	11.1 ± 0.17[b]	478 ± 7[a]
Citrus tankan Hayata	Tonkan	31.5	10.5 ± 0.01[b]	331 ± 13[b]

[A] The extraction yield was expressed as weight percentage (%) of extract solid relative to dried peel.
[B] The values are expressed as means ± SD of triplicate tests. Means not sharing a common letter in the same column were significantly different ($p < 0.05$) when analyzed with ANOVA and Duncan's multiple range test.
[C] The total flavonoids content was expressed as milligrams of rutin equivalents (RE) per gram of extract solid and per one hundred grammes of dried peels, respectively.

while cell viability was evaluated using the MTT method. The PGE_2 concentration in the medium was measured by a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) and the NO concentration was measured by use of the Griess reagent.

2.6. Evaluation of COX-2 and iNOS protein levels

The RAW 264.7 cells were seeded at a density of 3×10^6 cells per 6 cm culture dish in a total volume of 5 ml and incubated for a period of 24 h. Following three washes with PBS, the adherent cells were incubated for 12 h in the presence or absence of 1 μg/ml of LPS and 1 mg/ml of citrus peel extracts. After incubation, the adherent cells were washed with PBS, collected, suspended in the lysis buffer (50 mM Tris, pH 7.6, 0.01% ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 1 μg/ml leupeptin) and centrifuged at 12,000 *g* for 20 min at 4 °C. COX-2 and iNOS protein levels in cell lysates were then determined by immunoblot analyses. Briefly, supernatants of cell lysates containing equal protein contents were loaded into and separated on a 10% sodium dodecylsulfate (SDS)–polyacrylamide gel, and then transferred to polyvinylidene fluoride filters. Following this, filters were blocked and probed with primary antibodies, including anti-COX-2, anti-iNOS (Cayman, Ann Arbor, MI, USA) and anti-β-actin antibodies (Biovision, Mountain View, CA, USA), respectively. The filters were then incubated with secondary antibody conjugated to alkaline phosphatase and detected using a NBT/BCIP solution. Finally, the band intensities of each protein were quantified using a software-supported photo-imager (ImageMaster VDS; Amersham Pharmacia Biotech Co., Piscataway, NJ, USA) and were normalized with β-actin.

2.7. Evaluation of COX-2 and iNOS mRNA levels

The cells were cultured, with or without the peel extract (1 mg/ml) and/or LPS (1 μg/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 mRNAs was determined by semi-quantitative 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′-GTCTGATGATGTATGCCACCATCTG-3′ (forward) and 5′-GCATCTGGACGAGGTTTTTC-3′ (backward) for mouse COX-2, 5′-CAGTTCTGCGCCTTTGCTCAT-3′ (forward) and 5′-GGTGGTGCGGC TGGACTTT-3′ (backward) for mouse iNOS, and 5′-AGGCCCA GAGCAAGAGAG-3′ (forward) and 5′-GGGTGTTGAAGGTCTCAAAC-3′ (backward) for mouse β-actin. Finally, aliquots (20 μl) 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 COX-2 and iNOS PCR products were quantified using a software-supported photo-imager and were normalized with β-actin.

2.8. Statistical analyses

All results are expressed as means ± SDs of at least three independent tests. The significance of the differences between the treatments was analyzed using analysis of variance (ANOVA), 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 using SPSS software (Statistical Package for the Social Sciences for Windows, ver. 10.0; SPSS Inc., Chicago, IL, USA), with the level of significance set at $p < 0.05$.

3. Results

3.1. Total flavonoid contents of different citrus peels

A summary of the total flavonoid content of different citrus peels is shown in Table 1. The flavonoid content of the citrus peel extracts varied greatly. Lemon peel extract contained 11.9 mg/g of rutin equivalents, which was the highest value among the tested citrus peels. In contrast, Hutoukan peel extract had the lowest level, containing only 4.43 mg/g rutin equivalents, which was less than half of the content for lemon peel. For convenience of comparison between findings found in this study and those reported in other studies, the flavonoid contents were also expressed on a weight basis of the dried peels. Ponkan had the greatest level of flavonoids, at 478 mg/100 g of dried peels. By contrast, the level of flavonoids in Liucheng (151 mg/100 g of dried peels) was only one-third of that found in Ponkan.

3.2. Flavonoid compositions of the different citrus peel extracts

By HPLC analysis, four flavanone glycosides, hesperidin, naringin, neohesperidin and narirutin, and three polymethoxy flavones, nobiletin, sinensetin and tangeretin, in the methanol extract of different citrus peels, have been identified. As shown in Table 2, the total amount of flavanone glycosides in the extracts of the tested citrus peels varied widely. Grapefruit and lemon peel extracts contained the flavanone glycosides at levels of 106 mg/g and 103 mg/g, respectively, which were the richest amongst the tested citrus peel extracts. In contrast, Murcotts peel extract had the lowest level of flavanone glycosides at 16.0 mg/g. Previously, Wang, Chuang, and Hsu (2008) have reported that hesperidin was the most dominant flavanone glycoside in most of the citrus peel extracts, except for those from the Grapefruit and Murcott. In this study, hesperidin accounted for ⩾92% of the total flavanone glycosides in the peel extracts of lemon, Liucheng, Ponkan and Tonkan. On the other hand, naringin was the most dominant flavanone glycoside (92% of the total flavanone glycosides) in Grapefruit, and narirutin was the main flavanone glycoside in Murcott.

Table 2
Compositions of flavanone glycosides in methanol extracts of different citrus fruit peels.[a,b].

Local name	Total	Hesperidin	Naringin	Neohesperidin	Narirutin
		(mg/g solid extract)			
Grapefruit	106 ± 9.1[a]	ND[f]	98.0 ± 8.12[a]	0.101 ± 0.089	8.28 ± 0.95[bc]
Hutoukan	30.9 ± 1.5[d]	22.0 ± 1.01[e]	ND[b]	ND	8.97 ± 0.46[b]
Lemon	103 ± 6.5[a]	94.0 ± 5.97[a]	0.58 ± 0.10[b]	0.129 ± 0.258	7.74 ± 0.60[c]
Liucheng	84.6 ± 4.4[b]	79.1 ± 4.02[b]	ND[b]	ND	5.44 ± 0.43[d]
Murcott	16.0 ± 1.2[e]	1.39 ± 0.07[f]	ND[b]	0.119 ± 0.033	14.5 ± 1.15[a]
Ponkan	68.4 ± 3.87[c]	65.5 ± 3.39[c]	0.23 ± 0.20[b]	ND	2.67 ± 0.27[e]
Tonkan	63.7 ± 5.0[c]	58.5 ± 4.45[d]	ND[b]	ND	5.22 ± 0.62[d]

[a] The values are expressed as means ± SDs of triplicate tests. Means not sharing a common letter in the same column were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.
[b] The total and specific flavanone glycoside content was expressed as mg/g of extract solid.

Table 3
Composition of polymethoxy flavones in the methanolic extract of different citrus fruit peels.[a,b]

Local name	Total	Nobiletin	Sinensetin	Tangeretin
	(mg/g solid extract)			
Grapefruit	0.10 ± 0.04[c]	0.10 ± 0.03[b]	ND[c]	ND[c]
Hutoukan	0.35 ± 0.27[c]	ND[b]	0.352 ± 0.268[a,b,c]	ND[c]
Lemon	ND[c]	ND[b]	ND[c]	ND[c]
Liucheng	1.36 ± 0.43[c]	1.12 ± 0.31[b]	0.242 ± 0.125[b,c]	ND[c]
Murcott	7.96 ± 1.59[b]	5.82 ± 1.09[a]	0.511 ± 0.113[a,b]	1.63 ± 0.39[b]
Ponkan	12.8 ± 1.77[a]	5.89 ± 0.58[a]	0.472 ± 0.339[a,b]	6.41 ± 1.03[a]
Tonkan	9.01 ± 1.99[b]	6.93 ± 1.46[a]	0.646 ± 0.173[a]	1.43 ± 0.36[b]

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

Table 3 shows the total quantities and distribution of polymethoxy flavones in the extract of different citrus peels. The total amounts of polymethoxy flavones in Grapefruit, Hutoukan, and Liucheng peel extracts were quite low, and were not detected (ND) in lemon. In contrast, Ponkan, Tonkan and Murcott were rich in polymethoxy flavones. In agreement with the finding reported by Nogata et al. (2006), our results show that nobiletin was the most abundant, while sinensetin was the least abundant of the polymethoxy flavones. Nobiletin comprised 73% and 77%, respectively, of the total polymethoxy flavones in Murcott and Tonkan peel extracts. In Ponkan, tangeretin comprised 50%, whereas nobiletin comprised 46% of the total polymethoxy flavones.

3.3. Anti-inflammatory activities of different citrus peel extracts

Prior to studying the effect of citrus peel extract upon secretion of PGE_2 and NO, cell viability was determined using the MTT method. Results show that cytotoxicity was evident only when the concentration of lemon peel extract was equal to or greater than 0.5 mg/ml, whereas no cytotoxicity (cell viability >95% of the control group) was observed in other citrus peel extracts at concentrations of $\leqslant$1 mg/ml. Lemon extract was thus excluded from the following experiments. As shown in Fig. 1, both PGE_2 and NO concentrations in the medium were markedly increased after treated with LPS (1 μg/ml) for 20 h, as compared to those of the un-stimulated control. Significant concentration-dependent inhibitions of PGE_2 and NO secretion were observed when the macrophages were co-treated with LPS and different concentrations (0.2, 0.5 and 1.0 mg/ml) of the citrus peel extract (Fig. 1). At a concentration of 1 mg/ml, the inhibitory abilities on PGE_2 secretion of different citrus peel extracts were ranked: Tonkan and Murcott and Ponkan > Liucheng > Hutoukan and Grapefruit. The inhibitory abilities on NO secretion were ranked: Tonkan > Murcott > Ponkan > Liucheng > Grapefruit > Hutoukan. Additionally, the levels of PGE_2 and NO secretion, in responding to different testing concentrations, were highly correlated (data not shown), suggesting that either PGE_2 or NO could be used as an indicator to assess the anti-inflammatory activity of citrus peels with very similar results.

3.4. Effect of different citrus peel extracts on the expression of COX-2 and iNOS

To explore the inhibitory effect of citrus peel extracts on the expression of COX-2 and iNOS, we determined the protein and mRNA levels by immunoblotting and RT-PCR analysis, respectively. Fig. 2 shows that RAW 264.7 macrophages did not express detectable levels of COX-2 and iNOS proteins when incubated in culture medium without LPS but, when incubated in the medium containing LPS (1 μg/ml), cells markedly increased both COX-2 and iNOS

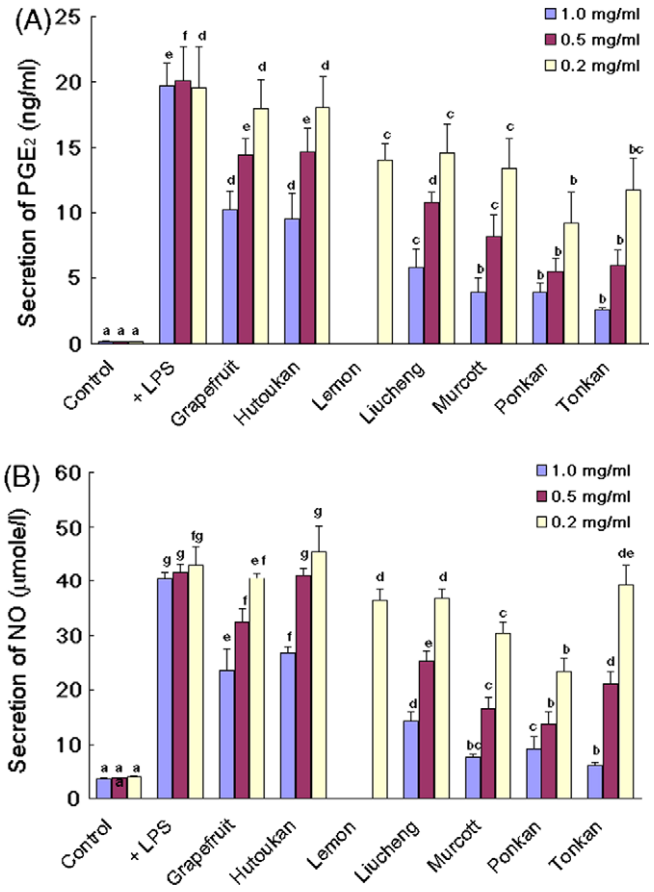

Fig. 1. Effect of different citrus peel extracts on PGE_2 (A) and NO (B) secretion in LPS-activated RAW 264.7 cells. The values are expressed as means ± SD of triplicate tests. Means not sharing a common letter at the same tested concentration were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

protein expression. At a concentration of 1 mg/ml, all tested peel extracts exerted a significant inhibitory effect on the LPS-induced COX-2 protein expression. Among different peel extracts, Ponkan suppressed 57.6% of COX-2 protein expression, and was the most potent inhibitor (Fig. 2). Although all citrus peel extracts at the test concentration of 1 mg/ml exerted a significant inhibitory effect upon NO secretion, as shown in Fig. 1, the expression of iNOS protein was only suppressed by extracts of Murcott, Ponkan and Tonkan. Interestingly, the peel extract of Grapefruit even had an enhancing effect on iNOS protein expression as compared to the LPS group. These results indicated that the extracts of citrus peels, at least, in Murcott, Ponkan and Tonkan, exerted an inhibitory effect on post-translational levels (enzyme catalytic function) of COX-2 and iNOS. Fig. 2 also shows that only peel extracts of Ponkan and Tonkan exerted inhibitory effect on the mRNA levels of COX-2 and iNOS, suggesting that the inhibitory effect of the Ponkan and Tonkan peel extract on COX-2 and iNOS mRNA expression could be at transcriptional level. Collectively, citrus peel extracts exerted their anti-inflammatory activity to inhibit PGE_2 and NO secretion through multiple mechanisms, including transcriptional, translational and post-translational suppression of COX-2 and iNOS.

3.5. Correlation between specific flavonoid and anti-inflammatory activity

To explore whether a possible relationship existed between the specific flavonoid and the anti-inflammatory activity of citrus peel extracts, a correlative analysis was performed. As shown in Table 4,

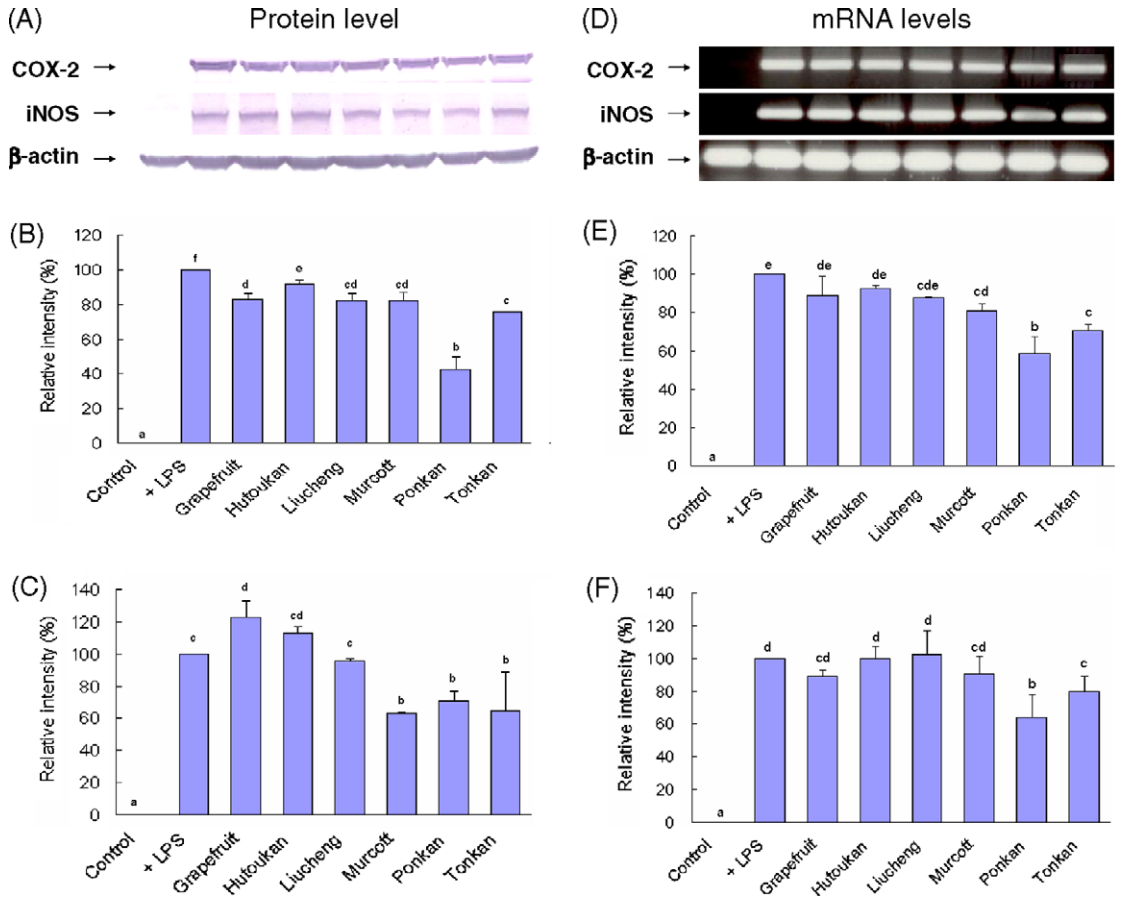

Fig. 2. Effect of different citrus peel extracts on the protein and mRNA levels of COX-2 and iNOS in LPS-activated RAW 264.7 cells. (A) Immunoblotting results of COX-2 and iNOS. (B) Quantitative results of COX-2 protein levels in cells treated with 1 mg/ml of different citrus peel extracts and 1 μg/ml of LPS. (C) Quantitative results of iNOS protein levels in cells treated with 1 mg/ml of different citrus peel extracts and 1 μg/ml of LPS. (D) RT-PCR results of COX-2 and iNOS. (E) Quantitative results of COX-2 mRNA levels. (F) Quantitative results of iNOS mRNA levels. The values are expressed as 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.

concentrations of PGE_2 and NO in the conditioned media treated with a particular citrus peel extract were not associated with either the total or the individual flavanone glycosides (naringin, hesperidin, narirutin and neohesperidin). However, they correlated significantly ($p = 0.034$) with the total amount of polymethoxy flavones, more especially, the nobiletin content ($p = 0.007$). Similarly, the levels of protein and mRNA of both COX-2 and iNOS also correlated significantly with the total polymethoxy flavones (data not shown). These results suggest that the polymethoxy flavones, especially nobiletin, were responsible for the anti-inflammatory activity of citrus peels.

Table 4
Correlation coefficient between the anti-inflammatory activity and total flavonoid as well as specific flavonoid.

	PGE_2 secretion	NO secretion
	Correlation coefficient (probality)	
Total flavonoids	−0.550 (0.258)	−0.559 (0.249)
Total flavanone glycosides	−0.283 (0.586)	−0.189 (0.720)
Naringin	−0.650 (0.162)	−0.500 (0.312)
Hesperidin	−0.503 (0.309)	−0.413 (0.416)
Narirutin	−0.184 (0.727)	−0.129 (0.808)
Neohesperidin	−0.196 (0.710)	−0.030 (0.955)
Total polymethoxy flavones	−0.846 (0.034)*	−0.846 (0.034)*
Nobiletin	−0.932 (0.007)**	−0.929 (0.007)**
Sinensetin	−0.680 (0.138)	−0.540 (0.269)
Tangeretin	−0.543 (0.266)	−0.537 (0.272)

* $p < 0.05$.
** $p<0.01$.

4. Discussion

Previously, the content of polymethoxy flavones, especially nobiletin in the peel extracts of 20 citrus fruits, has been shown to correlate well with their inhibitory activities on NO production in the LPS-activated RAW264.7 cells (Choi, Hwang, Ko, Park, & Kim, 2007; Choi et al., 2007). However, the relationship between the polymethoxy flavones and their impacts on the PGE_2 production is unclear. In this study, we demonstrated, for the first time, that polymethoxy flavones, especially nobiletin, were responsible for the inhibitory activity of citrus fruit peels on PGE_2 production. Although no study has examined the relative anti-inflammatory potentials of different citrus flavonoids, they could still be evaluated indirectly by comparing the lowest effective concentrations for suppressing the inflammatory mediator production reported in different studies. For example, at concentrations as low as 6 μM, nobiletin displayed a significant inhibition on NO and PGE_2 production in the LPS-activated RAW264.7 cells (Choi, Hwang, et al., 2007; Choi, Ko, et al., 2007). However, the lowest concentrations of flavanone glycosides, such as hesperidin and naringin, needed to suppress NO and PGE_2 production were 20 and 100 μM, respectively (Kanno et al., 2006; Sakata, Hirose, Qiao, Tanaka, & Mori, 2003). These results suggest that polymethoxy flavones exhibit a greater anti-inflammatory capacity than do the flavanone glycosides. Furthermore, these results support our finding that polymethoxy flavones were the key determinants of the anti-inflammatory activity of citrus peels.

Polymethoxy flavones, present exclusively in the peel tissues (Nogata, Ohta, Sumida, & Sekiya, 2003) of citrus fruits, and their distribution vary among the species. Citrus species of the *Acrumen* and *Aurantium* sections, namely, tangerines and oranges, have high concentrations of polymethoxy flavones (Nogata et al., 2006). Dancy tangerine (*Citrus tangerina*) and Ponkan, the most two widely used citrus species for the production of *chen-pi* in Chinese medicine, have the highest amounts of polymethoxy flavones among citrus species. Such a coincidence suggests that the beneficial function of *chen-pi* could be attributed to the polymethoxy flavones present in the peels.

In this study, we demonstrated that polymethoxy flavones in citrus peel extracts exerted their inhibitory activity upon PGE_2 and NO production primarily through transcriptional regulation of COX-2 and iNOS genes. Indeed, Jung et al. (2007) has recently reported that inhibition of activation of transcription factor nuclear factor-κB, as well as subsequent expression of the iNOS and COX-2 gene, underlies the anti-inflammatory mechanism of tangerine peels and nobiletin. Since the magnitudes of suppression of COX-2 and iNOS expression were smaller than those of PGE_2 and NO production, it is possible that there is an additive inhibitory effect of other citrus flavonoids on the COX-2 and iNOS catalytic function. This speculation is supported by the report that several citrus flavonoids exhibited potent inhibitory activity on the catalytic function of lipo-oxygenase and cyclo-oxygenase (Benavente-Garcia et al., 1997).

Previously, flavanone glycosides have been recognized as major contributors to the anti-inflammatory activity of citrus peels, because they are more abundant than polymethoxy flavones (Xu, Ye, Chen, & Liu, 2007). In fact, hesperidin, the most abundant flavanone glycoside, is still being used as a quality control indicator for production of the traditional citrus remedy, *chen-pi* (Lu, Zhang, Bucheli, & Wei, 2006). However, recently, the rule that flavanone glycosides are the major biologically active ingredients of *chen-pi* is being challenged (Choi, Hwang, et al., 2007; Choi, Ko, et al., 2007). In our earlier research, we have shown that polymethoxy flavones, but not flavanone glycosides, were the major contributors to the NO-suppressing activity of heat-treated *chen-pi* (Ho & Lin, 2008). Manthey and Guthrie (2002) have also shown that polymethoxy flavones exhibited a higher anti-proliferative activity than had flavanone glycosides and could serve as potent anti-cancer agents. Indeed, the fact that the hydrophobic nature of the polymethoxy flavones enables them to cross the intestinal membrane easily, and makes them absorbed more readily into the human body (Li, Wang, Sang, Huang, & Ho, 2006). These results indicate polymethoxy flavones to be promising therapeutic phytochemicals of citrus by-products (Li, Yu, & Ho, 2006).

In America, citrus juices and fruits are the major sources of dietary flavonoids (Chun, Chung, & Song, 2007). Since different manufacturing processes can modify the level of the biologically active polymethoxy flavones present in processed juices (Nogata et al., 2003), selection of the optimal processing method to increase the yield and supplementation (with peel extract into the processed juices) would be a practicable stratagem to increase polymethoxy flavone intake.

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References

Benavente-Garcia, O., & Castillo, J. (2008). Update on uses and properties of citrus flavonoids: New finding in anticancer, cardiovascular, and anti-inflammatory activity. *Journal of Agricultural and Food Chemistry, 56*, 6185–6205.

Benavente-Garcia, O., Castillo, J., Marin, F. R., Ortuno, A., & Del Rio, J. A. (1997). Uses and properties of citrus flavonoids. *Journal of Agricultural and Food Chemistry, 45*, 4505–4515.

Bocco, A., Cuvelier, M. E., Richard, H., & Berset, C. (1998). Antioxidant activity and phenolic composition of citrus peel and seed extract. *Journal of Agricultural and Food Chemistry, 46*, 2123–2129.

Choi, S.-Y., Hwang, J.-H., Ko, H.-C., Park, J.-G., & Kim, S.-J. (2007). Nobiletin form citrus fruit peel inhibits the DNA-binding activity of NF-kB and ROS production in LPS-activated RAW264.7 cells. *Journal of Ethnopharmacology, 113*, 149–155.

Choi, S.-Y., Ko, H.-C., Ko, S.-Y., Hwang, J.-H., Park, J.-G., Kang, S.-H., et al. (2007). Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits. *Biological and Pharmaceutical Bulletin, 30*(4), 772–778.

Chun, O. K., Chung, S. J., & Song, W. O. (2007). Estimated dietary flavonoid intakes and major food source of US adults. *Journal of Nutrition, 137*, 1244–1252.

Gorinstein, S., Martín-Belloso, O., Park, Y.-S., Haruenkit, R., Lojek, A., Ciz, M., et al. (2001). Comparison of some biochemical characteristics of different citrus fruits. *Food Chemistry, 74*, 309–315.

Ho, S.-C., & Lin, C.-C. (2008). Investigation of heat treating conditions for enhancing the anti-inflammatory activity of citrus fruit (*Citrus reticulata*) peels. *Journal of Agricultural and Food Chemistry, 56*, 7976–7982.

Jung, K. H., Ha, E., Kim, M. J., Won, H. J., Zheng, L. T., Kim, H. K., et al. (2007). Suppressive effects of nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression by *Citrus reticulata* extract in RAW 264.7 macrophage cells. *Food Chemistry and Chemical Toxicology, 45*, 1545–1550.

Kanno, S., Shouji, A., Tomizawa, A., Hiura, T., Osanai, Y., Ujibe, M., et al. (2006). Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. *Life Science, 78*(7), 673–681.

Li, S., Lo, C.-Y., & Ho, C.-T. (2006). Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. *Journal of Agricultural and Food Chemistry, 54*, 4176–4185.

Li, S., Wang, Z., Sang, S., Huang, M.-T., & Ho, C.-T. (2006). Identification of nobiletin metabolites in mouse urine. *Molecular Nutrition and Food Research, 50*, 291–299.

Li, S., Yu, H., & Ho, C.-T. (2006). Nobiletin: Efficient and large quantity isolation from orange peel extract. *Biomedical Chromatography, 20*, 133–138.

Lin, C.-C., Hung, P.-F., & Ho, S.-C. (2008). Heat treatment enhances the NO-suppressing and peroxynitrite-intercepting activities of kumquat's (*Fortunella margarita* Swingle) peel. *Food Chemistry, 109*, 95–103.

Lu, Y., Zhang, C., Bucheli, P., & Wei, D. (2006). Citrus flavonoids in fruit and traditional Chinese medical food ingredients in China. *Plant Food for Human Nutrition, 61*, 57–65.

Manthey, J. A., & Guthrie, N. (2002). Antiproliferative activities of citrus flavonoids against six human cancer cell lines. *Journal of Agricultural and Food Chemistry, 50*, 5837–5843.

Mayer, B., & Hemmens, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends in Biochemical Sciences, 22*, 477–481.

Murakami, A., Nakamura, Y., Ohto, Y., Yano, M., Koshiba, T., Koshimizu, K., et al. (2000). Suppressive effects of citrus fruits on free radical generation and nobiletin, an anti-inflammatory polymethoxyflavonoid. *Biofactors, 12*, 187–192.

Nogata, Y., Ohta, H., Sumida, T., & Sekiya, K. (2003). Effect of extraction method on the concentrations of selected bioactive compounds in mandarin juice. *Journal of Agricultural and Food Chemistry, 51*, 7346–7351.

Nogata, Y., Sakamoto, K., Shiratsuchi, H., Ishii, T., Yano, M., & Ohta, H. (2006). Flavonoid composition of fruit tissues of citrus species. *Bioscience, Biotechnology and Biochemistry, 70*(1), 178–192.

Ou, M. (1999). *Regular Chinese medicine handbook*. Taiwan: Warmth Publishing Ltd.

Rahman, I. (2003). Oxidative stress, chromatin remodeling and gene transcription in inflammation and chronic lung diseases. *Journal of Biochemistry and Molecular Biology, 36*, 95–109.

Sakata, K., Hirose, Y., Qiao, Z., Tanaka, T., & Mori, H. (2003). Inhibition of inducible isoforms of cyclooxygenase and nitric oxide synthase by flavonoid hesperidin in mouse macrophage cell line. *Cancer Letters, 199*(2), 139–145.

Schieber, A., Stintzing, F. C., & Carle, R. (2001). By-products of plant food processing as a source of functional compounds – recent developments. *Trends in Food Science and Technology, 12*, 401–413.

Smith, W. L., Garavito, R. M., & Dewitt, D. L. (1996). Prostaglandin endoperoxide H synthases (cycloxygenase)-1 and 2. *Journal of Biological Chemistry, 271*, 33157–33160.

Tripoli, E., Guardia, M. L., Giammanco, S., Majo, D. D., & Giammanco, M. (2007). Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chemistry, 104*, 466–479.

Vane, J. R., Mitchell, J. A., Appleton, I., Tomlinson, A., Biohop-Bailey, D., Croxtall, J., et al. (1994). Inducible isoforms of cycloxygenase and nitric oxide synthase in inflammation. *Proceedings of the National Academy of Sciences of the United States of America, 91*, 2046–2050.

Wang, Y.-C., Chuang, Y.-C., & Hsu, H.-W. (2008). The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. *Food Chemistry, 106*, 277–284.

Xu, G., Ye, X., Chen, J., & Liu, D. (2007). Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract. *Journal of Agricultural and Food Chemistry, 55*, 330–335.