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Heat treatment enhances the NO-suppressing and peroxynitrite-intercepting activities of kumquat (*Fortunella margarita* Swingle) peel

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Abstract

In Taiwan, folk remedies containing dried kumquats (*Fortunella margarita* Swingle) are used to cure inflammatory respiratory disorders. The induction of inducible nitric oxide (NO) synthase in inflammatory cells and increased airway production of NO and peroxynitrite, its derivative, are key events in such disorders. Although heat is known to affect the antioxidant activity of citrus peels, the effects of dehydration and heating on NO suppression and on the interception of peroxynitrite are unclear. We determined the NO-suppressing activities of freeze-dried, oven-dried, and heat-treated kumquat extracts by measuring their inhibition of NO production in lipopolysaccharide-activated RAW 264.7 macrophages. Furthermore, we evaluated the attenuation of peroxynitrite-mediated nitrotyrosine formation in albumin. Heating, but not oven drying, enhanced the ability of kumquat peels to suppress NO and intercept peroxynitrite, as compared with freeze drying. However, heat treatment and oven drying of kumquat flesh attenuated these activities; these effects were at least partially attributed to heat-susceptible ascorbate.
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Keywords: Citrus fruit; Heat treatment; Peroxynitrite; Nitric oxide; Antioxidant

1. Introduction

Oxidative stress, an imbalance between reactive oxygen species/reactive nitrogen species and antioxidants, is suggested to play an important role in the pathogenesis of several diseases of the respiratory tract, such as asthma (Rahman, Morrison, Donaldson, & MacNee, 1996). Both inflammation and environmental exposure to air pollution and cigarette smoke aggravate oxidative stress (Bowler & Crapo, 2002).

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 (Rahman, 2003). Most reactive nitrogen species are derived from nitric oxide (NO) *in vivo*. Inducible NO synthase (iNOS) produce NO in inflammatory cells, which can react with oxygen or superoxide anions (O_2^-) to generate reactive nitrogen species (Szabo, 2003). Among these reactive nitrogen species, peroxynitrite is formed during the diffusion-limited reaction between NO and O_2^-. Because peroxynitrite possesses a potent oxidizing and nitrating ability, it is regarded as the major contributor to the cytotoxicity of reactive nitrogen species (Arteel, Briviba, & Sies, 1999).

Indeed, given the detection of tyrosine nitration, ample evidence suggests that peroxynitrite and its related reactive nitrogen species are involved in the pathogenesis of various diseases of the respiratory tract (Dweik et al., 2001; MacPherson et al., 2001; van der Vliet, Eiserich, Shigenaga, & Cross, 1999). Hence, suppression of NO production by

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activated inflammatory cells and direct interception of peroxynitrite appear to be reasonable mechanisms for adjunctive therapy of inflammatory respiratory disorders (Bowler & Crapo, 2002).

Residues of the production of citrus juice, such as peels, membranes, and seeds, possess a large variety of bioactive components. Therefore, they have recently been considered potential sources of functional components (Li, Lo, & Ho, 2006; Cui et al., 2007). In traditional Chinese medicine, dried, whole immature and mature citrus fruits and peels, such as *zhi shi* (*Citrus anrantium* L.), *zhi quia* (*Citrus anrantium* L.), and *chen pi* (*Citrus reticulata* Blanco), are widely used as remedies to stimulate the appetite, aid digestion, improve menopausal syndromes, alleviate cough, and reduce phlegm in the respiratory tract (Ou, 1999). Another citrus fruit, the kumquat, is harvested from small trees in the genus *Fortunella* in the northeast region of Taiwan. Kumquats have a sweet outer coat and a tart, juicy center, and they can be dried and processed into preserves. Dried whole kumquats are often used as folk medicines to cure inflammatory syndromes of the respiratory tract, such as coughing, hoarseness, and sore throats (Chiu & Chang, 1998).

Sun drying is a common dehydration process used in the traditional food industry and in Chinese medicine to prepare citrus preserves and remedies. However, this uncontrolled drying process leads to considerable differences in the quality of citrus remedies. Therefore, standardization of this process for preparing citrus remedies, and thus, enhancement of their health benefits, is important. Another process, heat treatment elevates the antioxidant activity of citrus peels (Jeong et al., 2004; Xu, Ye, Chen, & Liu, 2007). Xu et al. suggested that a heating temperature of $\leqslant$100 °C enhances the antioxidant activity of citrus peels without a loss of anti-inflammatory flavanoid glucosides (Xu et al., 2007). However, the effect of different processing procedures, especially dehydration and heat treatment, on the beneficial activities of citrus fruits in suppressing NO and intercepting peroxynitrite are unclear.

The purpose of this study was to compare heat treatment before oven drying with simple oven drying and with freeze drying on the NO-suppressing and peroxynitrite-intercepting activities of kumquats. In addition, the phenolics and ascorbate contents and the antioxidant activity of each treatment were assessed to elucidate any relationship that might exist among them.

2. Materials and methods

2.1. Materials

We cultured RAW 264.7 cells obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), in Dulbecco modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies Inc., Grand Island, NY, USA). Dimethyl sulfoxide, lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), sulphanilamide, naphthylethylenediamine, bovine serum albumin (BSA), Folin-Ciocalteu reagent, gallic acid, ascorbic acid, nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (NBT/BCIP), and anti-3-nitrotyrosine antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Peroxynitrite was purchased from Upstate (Lake Placid, NY, USA). All other chemicals were of analytical-grade purity.

2.2. Preparation of the samples

The kumquats used in this study were cultivated in Yilan, Taiwan, and harvested in the winter of 2005. Fresh whole fruits were separated into peels and flesh by hand. Both the peels and flesh were divided into three parts. One part was heat treated; that is, it was heated at 100 °C for 2 h and then oven dried at 55 °C until a constant weight was obtained. Another part was oven dried at 55 °C, and one was freeze dried, until a constant weight was achieved.

All of the dried peels and flesh were milled into a powder by using a grinder (RT-04, Rong Tsung Iron Works, Taichung, Taiwan) and then extracted twice with a 10-fold volume of methanol for 30 min while it was vigorously shaken. After the samples were filtered with Whatman no.1 filter papers, the filtrates were evaporated under a vacuum at $<$50 °C by using a rotary evaporator (N-1000S, Eyela, Tokyo, Japan) to remove the methanol.

For the experiments to follow, the solid extract was weighed and dissolved in dimethyl sulfoxide to a concentration of 500 mg/ml. It was then diluted with phosphate-buffered saline or culture medium to the concentration required.

Control experiments were carried out by adding an equivalent amount of dimethyl sulfoxide without the kumquat extract.

2.3. Evaluation of NO-suppressing activity

NO-suppressing activity was evaluated as the inhibitory effect of the kumquat extracts on NO production by LPS-activated macrophages, as we have described (Lin, Lu, Chen, & Ho, 2006). In brief, 1×10^5 RAW 264.7 cells were seeded onto 96-well culture plates and incubated for 24 h. After they were washed three times with phosphate-buffered saline, adherent cells were incubated in prepared Dulbecco modified Eagle's medium containing kumquat extract with or without LPS (100 ng/ml). After incubation for 24 h, the medium was collected for nitrite assay, at which time cell viability was evaluated by using the MTT method. Finally, the nitrite concentration of the conditioned medium was measured as an indicator of NO production by use of the Griess reaction. The NO-suppressing activity of the kumquat extracts was expressed as the IC_{50}, which denoted the concentration of kumquat

extracts that caused 50% inhibition of the NO production by LPS-activated RAW 264.7 cells.

2.4. Evaluation of peroxynitrite-intercepting activity

Nitration of protein tyrosine residue is one mechanism by which peroxynitrite contributes to the pathogenesis of human disease (Radi, 2004). Therefore, we evaluated the peroxynitrite-intercepting activity of the extracts with regard to their inhibitory effect on peroxynitrite-mediated nitrotyrosine formation in BSA. The nitration of tyrosyl residues in BSA was performed according to the method of Ippoushi, Azuma, Ito, Horie, and Higashio (2003), with minor modification. In brief, we dissolved 200 μl of 500 μg/ml kumquat extracts in phosphate-buffered saline and mixed it with 100 μl of 500 μg/ml BSA. We then added peroxynitrite to a final concentration of 100 μM. The reaction solution was incubated for 30 min at 37 °C.

In addition, the 3-nitrotyrosine content was determined by means of immunoblot analysis. In brief, 10 μl of the reaction solution was loaded into and separated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride filters. The filters were blocked and probed with anti-3-nitrotyrosine antibodies. The filters were then incubated with secondary antibody conjugated to alkaline phosphatase and detected by using an NBT/BCIP solution. Finally, the band intensities were quantified with a software-supported photoimager (ImageMaster VDS; Amersham Pharmacia Biotech Co., Piscataway, NJ, USA). The peroxynitrite-intercepting activity of the kumquat extracts was expressed as percentage of inhibition on peroxynitrite-mediated nitrotyrosine formation.

2.5. Determination of 2,2-diphenyl-1-picryhydrazyl radical-scavenging activity

Many different methods have been established for evaluating the antioxidant capacity of certain biological samples, with such methods being classified, roughly, into one of two categories based upon the nature of the reaction that the method involved. The methods involving an electron-transfer reaction include the 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging assay, the ferric ion reducing antioxidant power (FRAP) and the Trolox-equivalent antioxidant capacity (TEAC) (Huang, Ou, & Prior, 2005). These three methods are the most commonly accepted assays for determining the antioxidant activity of food and therefore, were adopted in this study.

The DPPH radical-scavenging capacity of the kumquat extracts was determined, as previously described (Tsai, Tsai, Yu, & Ho, 2007). In brief, 20 μl of a serially diluted sample of the extract was pipetted onto a 96-well flat-bottomed plate. Then, 200 μl of 0.2 mM DPPH methanolic solution was added into each well, and the plate was shaken with a plate shaker for 5 min. The change in absorption at 540 nm after to the addition of DPPH was measured by using an enzyme-linked immunosorbent assay reader (EL800, Bio-Tek Instruments Inc., Winooski, VT, USA).

The scavenging activity (%) of the kumquat extracts to DPPH was expressed as $[1 - (A_{\text{sample}} - A_{\text{sample blank}})/(A_{\text{control}} - A_{\text{control blank}})] \times 100\%$, where $A_{\text{sample}} - A_{\text{sample blank}}$ was the difference in the absorbances of a sample with 200 μl of DPPH methanolic solution compared with 200 μl of methanol, and $A_{\text{control}} - A_{\text{control blank}}$ was the difference in the absorbances of the dimethyl sulfoxide control with 200 μl of DPPH methanolic solution compared with 200 μl of methanol.

2.6. Determination of reducing power

The reducing power of the kumquat extracts was determined according to the method Oyaizu reported (1986). First, 50 μl of serially diluted sample extracts was mixed with 500 μl of phosphate buffer (0.05 M, pH 6.6) and 500 μl of 1% potassium ferricyanide and incubated at 50 °C for 20 min. Then, 500 μl of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. Finally, 100 μl of the supernatant was mixed with 100 μl of methanol and 20 μl of 1% ferric chloride at room temperature for 10 min. The absorption at 700 nm was then measured by using a spectrophotometer (Ultrospec 2100 Pro, Amersham Biosciences Inc., UK). Results were represented as absorbance, which is positive correlated with the reducing power.

2.7. Determination of Trolox-equivalent antioxidant capacity (TEAC)

The TEAC assay is better than the DPPH assay for evaluating the total antioxidant capacity of foods (Lee, Kim, Lee, & Lee, 2003). The TEAC of the extracts was measured by using a commercially available kit (Randox Laboratories, Crumlin, UK). Assays were performed according to the manufacturer's instructions. The absorbance at 600 nm was measured by using a spectrophotometer. Results were expressed in micromoles of Trolox equivalents per gram of kumquat extract.

2.8. Determination of total phenolics

To exclude the interference of ascorbate and reducing sugar, sample extracts were passed through a solid-phase extraction cartridge (Oasis HLB, Waters Milford, Ireland) before the total phenolic content was determined (George, Brat, Alter, & Amiot, 2005). In brief, 2 ml of crude extract was passed through a cartridge previously conditioned with 3 ml of methanol and 3 ml of distilled water. The cartridge was then washed with 4 ml of distilled water to elute any reducing sugars and ascorbates.

Both the original crude extract and collected water-elute were reacted with Folin-Ciocalteu phenol reagent in alkaline solution. The absorbance at 760 nm was measured by using a spectrophotometer. Finally, the phenolics content

of the kumquat extracts was calculated by subtracting the phenolic content of collected water-elutes from those of the original crude extracts and expressed in terms of milligrams of gallic-acid equivalents (GAE) per gram of kumquat extract.

2.9. Determination of ascorbic acid

To avoid possible interference in determining the ascorbic acid content, the crude extracts were partially purified before high-performance liquid chromatography (HPLC) by using a solid-phase extraction cartridge, as described before. The ascorbic acid content of the collected water-elute was measured by means of HPLC according to the conditions established by Asami, Hong, Barrett, and Mitchell (2003).

The HPLC system consisted of a liquid chromatography system (Shimadzu) equipped with LC-10AD pumps, a detector (model SPD-10Avp UV−VIS), an auto injector (model SIL-10ADvp), and an auto sampler. Reverse-phase separation was attained by using a column (Thermo ODS Hypersil; 250 × 4.6 mm, particle size 5 μm). The mobile phase was deionized water brought to pH 2.2 with sulfuric acid. The flow rate was 0.5 ml/min, and the detection wavelength was 245 nm. Sample aliquots were filtered through a 0.22 μm polyvinylidene fluoride filter (Millipore) before injection. The linearity range was determined from 0.001 to 1.0 mg/ml with a 20 μl injection volume ($R^2 = 0.9984$), which yielded an absorbance range of 0.06–3.0 V.

The ascorbic acid content of each kumquat extract was expressed in terms of milligrams per gram of kumquat extract.

2.10. Statistical analysis

All results are expressed as the mean standard error of the mean for at least three independent tests. The significance of the differences between different dried procedures was analyzed by means of One-way ANOVA, followed by a Duncan multiple range test for multiple comparisons. All of the statistical analyses were performed by using software (SPSS for Windows, version 10.0, SPSS Inc. Chicago, IL, USA). The level of significant difference was set at $P < 0.05$.

3. Results

3.1. Effect of drying processes on NO-suppressing activity of kumquat extracts

The cells had an obviously reduced survival rate of <90% when they were incubated with kumquat extracts at a concentration of >5 mg/ml. Therefore, a concentration of <5 mg/ml was used to treat the cells to prevent the kumquat extracts from having a cytotoxic effect on NO production.

When the cells were incubated with kumquat extracts in the absence of LPS, the nitrite concentration of the medium was maintained at a background level similar to that of the unstimulated control (data not shown). After treatment with LPS for 24 h, the nitrite concentration of the medium markedly increased, as compared with that of the unstimulated control (Fig. 1). A significant concentration-dependent inhibition of NO production was observed when cells were co-treated with LPS and various concentrations of the kumquat extracts (Fig. 1).

The IC_{50} value was lower in freeze-dried peels than in freeze-dried flesh (0.48 vs 2.35 mg/ml); this finding indicated that the peels possessed more NO-suppressing activity than did the flesh. Oven drying increased the IC_{50} of the peels to 0.74 mg/ml, implying that this process attenuated their NO-suppressing activity. In contrast, heat treatment at 100 °C for 2 h before oven drying significantly enhanced the NO-suppressing activity of the kumquat peels, as their IC_{50} decreased to 0.27 mg/ml.

The IC_{50} values of freeze-dried, oven-dried, and heat-treated kumquat flesh were 2.35, 2.70, and 3.18 mg/ml, respectively. As a consequence, oven drying affected the NO-suppressing activity of kumquat flesh, and heat treatment further attenuated it.

3.2. Effect of drying processes on the peroxynitrite-intercepting activity of kumquat extracts

When BSA was exposed to peroxynitrite, a significant level of nitrotyrosine was formed (Fig. 2). Furthermore, the level of peroxynitrite-induced nitrotyrosine formation under such conditions was attenuated by the addition of kumquat extracts. At a tested concentration of 500 μg/ml, extracts from freeze-dried, oven-dried, and heat-treated kumquat peels respectively inhibited 57%, 47% and 69%, of the peroxynitrite-induced formation of nitrotyrosine. The heat-treated peel conveyed more protection against peroxynitrite-induced nitrotyrosine formation than did the freeze-dried peel. By comparison, freeze-dried, oven-dried, and heat-treated kumquat flesh attenuated peroxynitrite-induced nitrotyrosine formation by 63%, 29% and 24%, respectively. As a consequence, oven drying and heat treatment affected the peroxynitrite-intercepting activity of kumquat flesh.

3.3. Effect of the drying processes on the antioxidant capacity of kumquat extracts

All of the tested kumquat extracts demonstrated a dose-dependent scavenging effect on DPPH radicals (Fig. 3). At a tested concentration of 125 mg/ml, freeze-dried, oven-dried, and heat-treated peel extracts scavenged 66%, 66% and 85% of the presented DPPH radicals, respectively. Oven drying had not obvious effect, but heat treatment enhanced the DPPH radical-scavenging activity of the

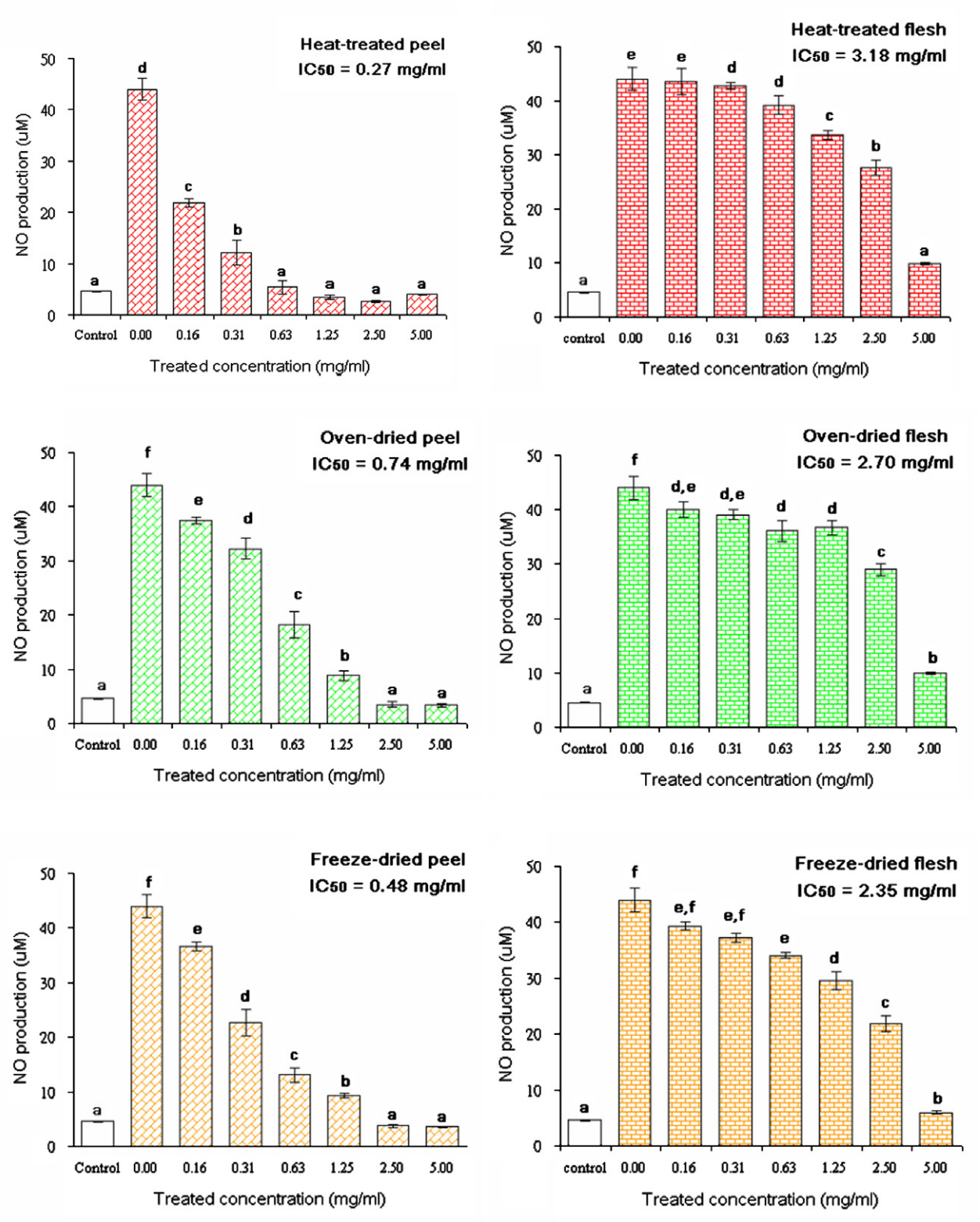

Fig. 1. Effect of drying processes on the NO-suppressing activity of kumquat extracts. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test. IC_{50} denotes the concentration of kumquat extracts causing 50% inhibition of NO production by LPS-activated RAW 264.7 cells.

peels. At the same tested concentration of 125 mg/ml, freeze-dried, oven-dried, and heat-treated flesh extracts had 65%, 47% and 47% DPPH radical-scavenging activity, respectively. These results indicated that even mild heating, as during oven drying, affected the DPPH radical-scavenging activity of the flesh.

Fig. 4 shows the predicted reducing powers of the kumquat extract. As observed with scavenging, heat treatment

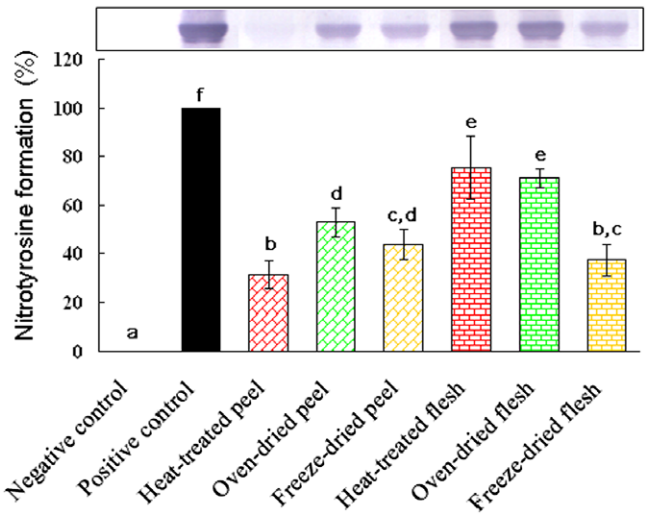

Fig. 2. Effect of drying processes on the peroxynitrite intercepting activity of kumquat extracts. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

but not oven drying enhanced the reducing power of the peels. In contract, both oven drying and heat treatment diminished the reducing power of the flesh.

Table 1 presents the TEAC results for the kumquat extracts. Extracts from freeze-dried, oven-dried, and heat-treated peels were had TEAC values of 18.6, 20.7 and 20.9 mmol Trolox equivalents per gram of extract solid, respectively. However, the TEAC results for oven-dried and heat-treated flesh decreased to 74% and 66%, respectively, of that noted with freeze-dried flesh.

3.4. Effect of drying processes on the total phenolics and ascorbic acid content of kumquat extracts

Table 1 lists the total phenolics and ascorbic acid contents of different kumquat extracts. Kumquat peels appeared to contain more phenolics than did the flesh. In addition, oven drying and heat treatment increased the phenolics content of both the peels and the flesh. Oven drying and heating increased the amount of phenolics of peels by 22% and 45%, respectively, compared with the level measured in the freeze-dried control. Likewise, oven drying and heat treatment increased the phenolics content of the flesh by 44% and 43%, respectively, compared with the level in the freeze-dried control.

Of all the extracts tested, only the sample of freeze-dried flesh contained ascorbic acid (12.1 mg/g). This finding suggested that the ascorbic acid in the flesh was susceptible to mild oven drying and that it was destroyed completely.

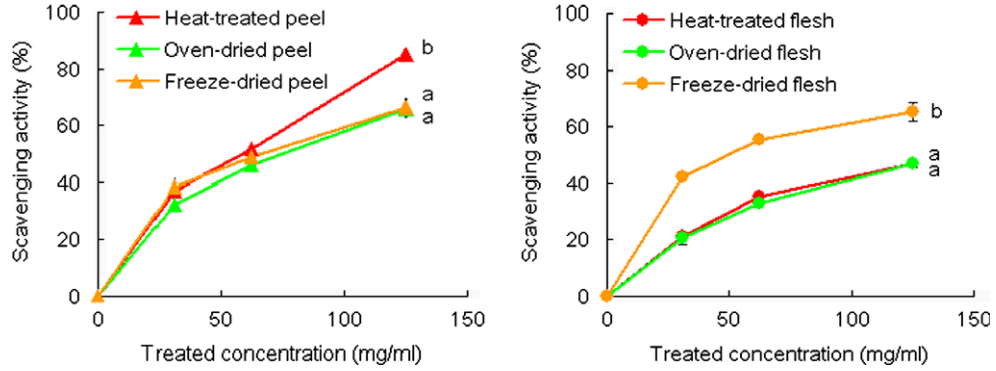

Fig. 3. Effect of drying processes on the DPPH radical scavenging activity of kumquat extracts. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

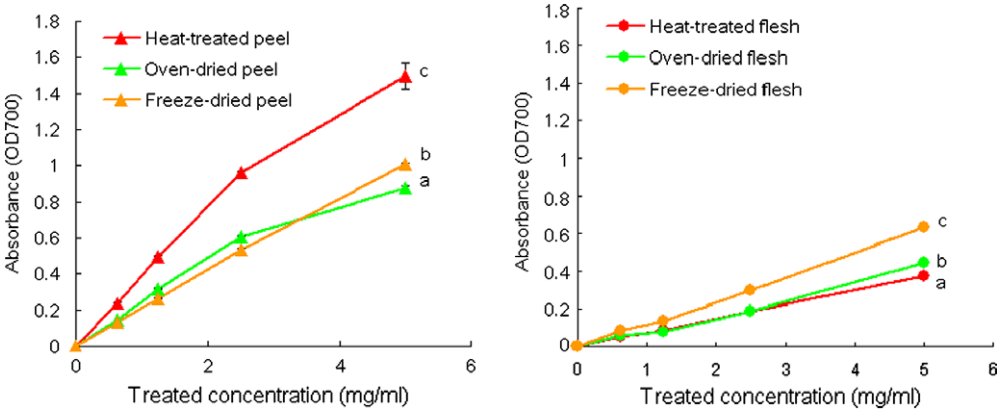

Fig. 4. Effect of drying processes on the reducing power of kumquat extracts. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

Table 1
Effect of drying processes on the total phenolics, ascorbate content and Trolox equivalent antioxidant capacity (TEAC) of kumquat extracts[A]

Treatment	Total phenolics (mg GAE/g)	Ascorbate (mg/g)	TEAC (μmol Trolox/g)
Heat-treated peel	3.99 ± 0.12^{d}	N.D.[B]	20.9 ± 1.2^{d}
Oven-dried peel	$3.35 \pm 0.33^{c,d}$	N.D.	$20.7 \pm 1.8^{c,d}$
Freeze-dried peel	$2.75 \pm 0.13^{b,c}$	N.D.	$18.6 \pm 1.9^{b,c}$
Heat-treated flesh	$1.94 \pm 0.21^{a,b}$	N.D.	12.6 ± 1.7^{a}
Oven-dried flesh	$1.96 \pm 0.04^{a,b}$	N.D.	$14.2 \pm 1.2^{a,b}$
Freeze-dried flesh	1.36 ± 0.15^{a}	12.11 ± 0.03	19.1 ± 2.4^{c}

[A] The values are expressed as means SEM of triplicate tests. Means in the same column not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.
[B] N.D.: undetectable.

4. Discussion

Because epidemiological studies have consistently demonstrated that diets rich in plant foods reduce the risk of chronic disease, researchers have increased their efforts to investigate and identify the bioactive components of plant foods (Stanner, Hughes, Kelly, & Buttriss, 2004). However, the possible effect of processing, such as extraction, heat treatment, dehydration, and other methods, on the healthy benefits of processed foods are usually ignored. For example, in our previous study, we found that heavy fermentation attenuated the antioxidant and NO-suppressing activities of tea (Lin et al., 2006). Therefore, to enable the preparation of highly valuable and functional food, an important issue to investigate is the effect of processing procedures on the health benefits of processed foods.

In accordance with previous investigators (Murakami et al., 2000), we found that kumquat peels appeared to exhibit more NO-suppressing and peroxynitrite-intercepting activities than did the flesh. The residues of citrus-juice production are undoubtedly abundant resources from which healthy foods can be developed (Schieber, Stintzing, & Carle, 2001).

Most importantly, we found that oven drying attenuated, whereas 100 °C heat treatment for 2 h before oven drying significantly enhanced, the NO-suppressing and peroxynitrite-intercepting activities of kumquat peels. In contrast, both oven drying and heat treatment affected the NO-suppressing and peroxynitrite-intercepting activities of kumquat flesh. Therefore, given our results, we suggest that proper heat treatment be added before oven drying in process of making kumquat-peel preserves to maintain its health benefits. For kumquat flesh, we suggested the avoidance of heat treatment and oven drying.

Inhibited activation of transcription factor nuclear factor- B and subsequent expression of the iNOS gene underlie the NO-suppressing mechanism of citrus peels (Jung et al., 2007). Several phytochemicals abundantly present in citrus fruits, including coumarins, flavanone glycosides, and polymethoxy flavones, reportedly exhibit potent NO-suppressing activities (Miyake et al., 1999; Murakami et al., 1999; Murakami et al., 2000; Sakata, Hirose, Qiao, Tanaka, & Mori, 2003). Polymethoxy flavones, especially nobiletin were recently identified as major contributors to the NO-suppressing activity of citrus peels (Choi et al., 2007). Indeed, our finding that the NO-suppressing activity was not correlated with the total phenolic content implied that the amount of specific phenolics – rather than total phenolics – was the key determinant of NO suppression by citrus fruits.

Like previous investigations, our study showed that heat treatment helps to increase the antioxidant activity of citrus peels (Jeong et al., 2004; Xu et al., 2007). Heat treatment affects the composition and distribution of phenolic compounds present in extracts of citrus peels. Jeong et al. (2004) proposed that heat treatment may liberate phenolic compounds from an un-extractable form covalently bound to insoluble polymers to an extractable, free form. Flavones and glycosylated flavanones are the principle constituents of methanol-extractable phenolic compounds, whereas polymer-bound phenolic compounds mainly consist of phenolic acids and flavonols (Bocco, Cuvelier, Richard, & Berset, 1998). Furthermore, increased amounts of free phenolic acids but not glycoside and ester-bound fractions are suggested to be responsible for the elevated antioxidant activity of heat-treated citrus peels (Xu et al., 2007). However, whether the liberated, free phenolic acids are responsible for elevated NO suppression of heat-treated kumquat peels is an issue in need of further study.

Our results indicated that heat treatment and oven drying attenuated the antioxidant activity of kumquat flesh. This effect was at least partly due to the destruction of ascorbic acid. Although phenolic compounds are considered the major contributors to the antioxidant capacity of most fruits, the relative contribution of ascorbic acid to the antioxidant potential of citrus juice is controversial (Gardner, White, McPhail, & Duthie, 2000; Hassimotto, Genovese, & Lajoli, 2005; Pretel, Botella, Zapata, Amoros, & Serrano, 2004; Rababah, Ereifej, & Hoeard, 2005; Rapisarda et al., 1999). Rapisarda et al. (1999) reported that the antioxidant activity of citrus juice was notably or at least partly attributed to their phenolic content, whereas ascorbic acid seemed to play a minor role. However, several researches have indicated that ascorbic acid contributed >50% of the antioxidant potential of citrus juices (Gardner et al., 2000; Pretel et al., 2004). In this study, the loss of TEAC of kumquat flesh during oven drying was totally attributed to the destruction of ascorbic acid; therefore,

ascorbic acid was estimated to contribute 26% of the antioxidant activity of fresh kumquat flesh.

Direct evidence that ascorbic acid can inhibit NO production by means of activated inflammatory cells is lacking. However, dehydroascorbate, (oxidised ascorbate) could inhibit IκB kinase and thus attenuate the activation of nuclear factor-κB. (Cárcamo et al., 2004). Therefore, we attribute the oven drying – and heating-induced decrease in NO suppression by kumquat flesh to the destruction of ascorbic acids, at least partially. We propose that an indirect pathway, such as an influence on the cellular prooxidant–antioxidant balance, is involved in this process.

Although we did not identify the bioactive components of heated peels in this study, we observed that proper heat treatment enhanced the antioxidant and anti-inflammatory activities of kumquat peels, as reflected in their suppression of NO and interception of peroxynitrite. The best conditions for heating and the anti-inflammatory compounds thus liberated are under investigation in our laboratory.

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