A DOCTORAL DISSERTATION

Anti-inflammatory Action and Mechanism of Nobiletin, a Citrus Polymethoxylated Flavone

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Anti-inflammatory Action and Mechanism of Nobiletin, a Citrus Polymethoxylated Flavone

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BACKGROUND

Plants of the genus *Citrus* are primarily valued for their edible fruit, but they also have traditional medicinal value. The peel of citrus fruits has been used in traditional Asian medicine for centuries. Two Chinese traditional medicines, which are used to treat indigestion, *zhi qiao* and *zhi shi*, are obtained from the mature and the immature fruit peels of *Citrus aurantium* L., respectively (Huang and Wang, 1993). In modern European herbal medicine, the fruit peel of *C. aurantium* L. is used to treat dyspepsia and related conditions. According to the package insert from the German Standard License, this fruit peel is used as a supportive medication in treating stomach complaints, such as insufficient formation of gastric juice, and in stimulating the appetite (Bisset and Wichtl, 1994). The German Commission E recognizes the medicinal value of the fruit peel of *C. aurantium* L. for loss of appetite and dyspeptic complaints (Blumenthal *et al*., 1998). The dried peel of *C. unshiu* Marc. has been used as a traditional medicine in China and Japan to improve bronchial and asthmatic conditions or cardiac and blood circulation and is known as 'Chinpi' (Tokyo Society of Pharmacognosy, 2006).

The peel of citrus fruits contains a wide range of flavonoid constituents, some of which, including hesperidin, naringin, and polymethoxylated flavones, are very rare in other plants while others, such as rutin and quercetin, are common in the plant kingdom (Manthey and Grohmann, 1998). Attempts have recently been made to determine the biological activities among citrus flavonoids (Bracke *et al*., 1994; Ishiwa *et al*., 2000; Jung *et al*., 2003; Kim *et al*., 2003; Tanaka *et al*., 1997). The focus of studies aimed at preventing chronic diseases through the daily intake of

citrus fruits and at increasing their added value. Two of the main areas of research into the biological actions of citrus flavonoids have been inflammation and cancer. Each citrus flavonoid and various possible mixtures exhibit a number of *in vitro* and/or *in vivo* anti-inflammatory and anticancer actions. Excessive nitric oxide (NO) production has been implicated in a variety of pathophysiological conditions, including inflammation and carcinogenesis (Kronche *et al*., 1998; Mordan *et al*., 1993; Ohshima and Bartsch, 1994). Although many reports describing the inhibitory activity against NO production by several *Citrus* plants or citrus-derived bioactive flavonoids, there are few evidences on the relationship between the content of each flavonoid and the NO production inhibitory efficacy in *Citrus* plants.

Jeju Island in Korea is a unique place because citrus plants are cultivated on a large scale in the island's subtropical climate. As part of the Korean fruit diet, the majority of citrus fruits are converted into juice. As citrus juice production generates large amounts of peel and mash, the isolation of functional compounds from citrus peel will be very beneficial to the food industry and will improve the quality and nutritional value of food.

The present study analyzed the content of the principal flavanones and flavones present in the fruit peel of various *Citrus* plants which are wildly grown in Jeju Island, for the purpose of the isolation of any pharmacologically active ingredients and its subsequent industrial use. This study also examined contribution of individual flavonoid components to the inhibitory activity of NO production by lipopolysaccharide (LPS)-activated RAW 264.7 cells. Inhibitory efficacy of the ethanol extracts of citrus fruit peels against NO production exhibited a highly positive correlation with the nobiletin content. Nobiletin is a polymethoxylated

flavone exclusively found in citrus fruit peel, and has been reported to be a promising anti-inflammatory agent. However, the precise mechanisms of the anti-inflammatory action of nobiletin have not been investigated. Thus, the present study investigated the action mechanism of nobiletin on the activation of nuclear factor-κB (NF-κB), which is a transcription factor that regulates the expression of many genes related to inflammatory and immune responses.

CONTENTS

LIST OF ABBREVIATIONS

cDNA	Complementary deoxyribonucleic acid
CM-H_2DCFDA	5- and-6 -Chloromethyl-2 7 –dichlorodihydrofluorescein diacetate acetyl ester
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's modified Eagle's minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FT-NMR	Fourier transform-nuclear magnetic resonance
HEPES	4-(2-Hydorxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IFN-γ	Interferon gamma
I-κB	Inhibitory kappa-B
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase

JNK	c-Jun NH_2-terminal kinase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tertazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NF-κB	Nuclear factor kappa-B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PGE_2	Prostaglandin E_2
PMSF	Phenylmethylsulphonylfluoride
PDTC	Pyrrolidine dithiocarbamate
RSD	Relative standard deviation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SNP	Sodium nitroprusside
TNF-α	Tumor necrosis factor alpha

PART 1

Comparative study of flavonoids contents and inhibitory activity on NO production by LPS-activated RAW 264.7 cells in the ethanol extracts from various citrus fruit peels: Correlation of nobiletin content to inhibition of NO production

1.1 ABSTRACT

The aims of the present study were to determine: 1) the contents of flavonoids in the fruit peel of 20 citrus species (including cultivars) within the *Citrus* genus grown on Jeju Island, 2) the inhibitory effect of citrus fruit peel on lipopolysaccharide (LPS)-induced NO production in RAW 264.7 cells, and 3) the relationship between the flavonoids contents of the citrus fruit peel and its inhibitory effect on NO production. Using 70% ethanol extracts prepared from immature and mature citrus fruit peels, the contents of seven flavonoids (naringin, naringenin, hesperidin, hesperetin, rutin, nobiletin, and tangeretin) were determined by HPLC analysis. Each citrus peel extract varied in flavonoid content, but the contents of nobiletin and tangeretin, which were contained in all 20 fruit peels, showed a positive and significant correlation with each other (r = 0.879, P < 0.0005 for immature fruit peels; r = 0.858, P < 0.0005 for mature fruit peels). The ethanol extracts from immature citrus fruit peels have not only higher flavonoids contents, but also a greater inhibitory effect on NO production by LPS-activated RAW 264.7 cells than those of mature citrus fruit peels. All citrus peel extracts dose-dependently inhibited LPS-induced NO production in RAW 264.7 cells. This inhibitory effect was significantly and positively correlated with the content of nobiletin and tangeretin. From the comparison of the inhibitory activity of each flavonoid, nobiletin , but not tangeretin, showed the most potent inhibitory effect on NO production among the seven tested flavonoids. Taken together, these results strongly suggest that the nobiletin content within citrus fruit peel is a key element in determining the inhibitory activity on NO production by LPS-activated RAW 264.7 cells inherent in

each citrus fruit peel. This result supports the promise that nobiletin-rich citrus may provide protection against diseases resulting from excessive NO production.

Key words: Citrus fruit peel, Flavonoid, NO production-inhibitory activity, Nobiletin

1.2 LIST OF TABLES

1.3 LIST OF FIGURES

1.4 INTRODUCTION

The peel of citrus fruits is a rich source of flavanones, as well as many polymethoxylated flavones, which are very rare in other plants (Nogata *et al*., 2006). The most prevalent flavanones are hesperetin and naringenin, both of which are found in the fruit peel largely as their glycosides, hesperidin and naringin, respectively. Tangeretin and nobiletin are two polymethoxylated flavones that are commonly found in citrus fruit peels. These compounds not only play important physiological and ecological roles but are also of commercial interest because they have a multitude of applications in the food and pharmaceutical industries. For example, naringin may act as an antioxidant (Chen *et al*., 1990), a lipid peroxidation protective agent (Guengerich and Kim, 1990), and an antimutagenic agent (Francis *et al*., 1989). Hesperidin influences vascular permeability, increases capillary resistance and exhibits analgesic and anti-inflammatory properties (Emim *et al*., 1994; Gabor, 1988; Kobayashi and Tanabe, 2006). It is also an effective antioxidant, since it is able to quench the oxygen free radicals which are involved in cancer growth (Berkarda *et al*., 1998). Polymethoxylated flavones are also of interest for their various pharmacological potentials, the most important of which are antitumor (Kandaswami et al., 1991; Kawaii *et al*., 1999; Murakami *et al*., 2000a, 2000b), antimutagenic (Miyazawa *et al*., 1999), anti-inflammatory (Ishiwa *et al*., 2000; Murakami *et al*., 2000b), and anti-allergic (Kobayashi and Tanabe, 2006) properties. Rutin, a flavone commonly found in citrus fruit peels, has been shown to have significant anti-inflammatory properties (Guardia *et al*., 2001). In general, the contents and distributions of flavonoids in different *Citrus* species are highly variable

and depend on genetic and environmental factors (Federica and Sergio, 2005; Nogata *et al*., 2006).

Two of the main areas of research about the biological actions of citrus flavonoids have been inflammation and cancer. Each citrus flavonoid and its mixtures can modulate the inflammation responses at a number of key regulatory points with a number of different mechanisms. Thus, it can be a potentially important source of anti-inflammatory flavonoids in the human diet (reviewed by Manthey *et al*., 2001). In addition to their anti-inflammatory properties, citrus flavonoids also have a variety of anti-cancer activities. Regulation of cancer may be accomplished by a variety of means, including suppressing, blocking, and transforming agents. Citrus flavonoids exert their anti-cancer effects through diverse mechanisms (reviewed by Manthey *et al*., 2001).

Nitric oxide (NO) has been implicated in a variety of pathophysiological conditions including inflammation, carcinogenesis, and atherosclerosis (Kronche *et al*., 1998; Mordan *et al*., 1993; Ohshima and Bartsch, 1994;). Since overproduction of NO is responsible for inflammation, the development of new substances to treat chronic inflammatory diseases has become a new research target (Hobbs *et al*., 1999). NO has also been considered to be an important mediator of carcinogenesis as it can react with reactive oxygen species (ROS) and then produce reactive nitrogen species which contribute to DNA damage and mutagenesis (Wiseman and Halliwell, 1996). Excessive NO causes the neoplastic transformation of mouse fibroblasts (Mordan *et al*., 1993) and has also been detected in several human cancerous tumors (Gallo *et al*., 1998; Rosbe *et al*., 1995; Thomsen *et al*., 1995). Although the inhibition of NO production by several *Citrus* plants or bioactive flavonoids has been recognized, little

is known about the relationship between the content of each flavonoid and its NO production inhibitory efficacy in *Citrus* plants. To examine such health-promoting properties of the fruit peel of the *Citrus* plants grown natively and cultivated in Jeju Island, the study measured the inhibitory efficacy of citrus fruit peel against NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage cells. Macrophages play a crucial role in the initiation and maintenance of inflammation. During endotoxemia and inflammation, macrophages are activated by LPS and pro-inflammatory cytokines.

The present study firstly analyzed the composition of common, citrus-derived flavonoids in the ethanol extracts from citrus fruit peels to provide information for the more effective utilization of flavonoid constituents. Thereafter, the study examined the inhibitory activity of the ethanol extracts from citrus fruit peels on NO production in LPS-activated RAW 264.7 cells. The present study also examined the correlation between flavonoids contents and inhibitory efficacy of NO production in the ethanol extracts from citrus fruit peels. A better understanding of the relationship between the flavonoid content and NO production inhibitory effect may be useful in developing a program for the effective utilization of citrus fruit peels and flavonoids in human health.

1.5 MATERIALS AND METHODS

1.5.1 Reagents

Nobiletin and tangeretin were obtained from Wako (Osaka, Japan). Other analytical-grade flavonoid standards and all other chemicals of analytical grade were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). LPS (*Escherichia coli* 026:B6), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and all other reagents, unless otherwise noted, were purchased from Sigma (St. Louis, MO, USA).

1.5.2 Plant materials

Twenty species (including cultivars) of *Citrus* grown on Jeju Island, Korea were selected for fruit peel testing (Table 1.1). The tested citrus fruits were grown and harvested at the Citrus Research Division of the National Institute of Subtropical Agriculture (Jeju, Korea). From the same tree, immature-green fruits were harvested from July through September 2005, and mature fruits were harvested from November 2005 through January 2006. The fruit peels were dissected, weighed, lyophilized and ground with a mortar mill. The powered peels were stored at -20°C prior to use.

1.5.3 Sample preparation

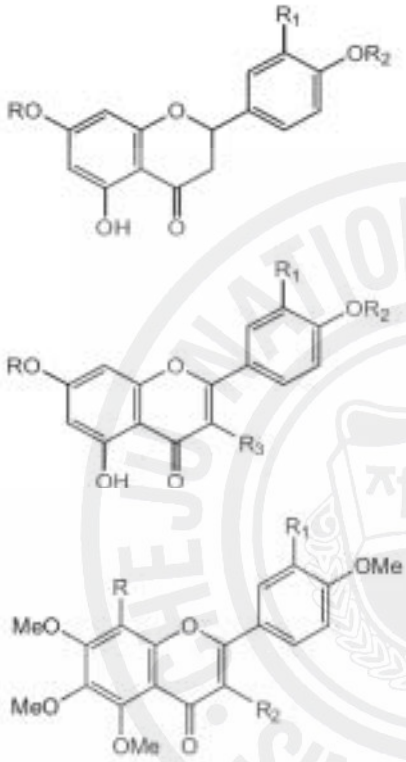
Table 1.1. *Citrus* plants investigated in this study

Common name	Tanaka name
Jikak	*Citrus aurantium* L.
Kamja	*C. benikoji* hort. ex Tan.
Dongjeongkyool	*C. erythrosa* hort. ex Tan.
Dangyooja	*C. grandis* (L.) Osb.
Cheongkyool	*C. nippokoreana* Tan.
Byungkyool	*C. platymamma* hort. ex Tan.
Sadookam	*C. pseudogulgul* hort. ex Tan
Jinkyool	*C. sunki* hort. ex Tan
Hongkyool	*C. tachibana* (Mark.) Tan.
Yooja	*C. junos* Sieb. ex Tan.
Binkyool	*C. leiocarpa* hort. ex Tan
Pyunkyool	*C. tangerina* hort. ex Tan
Hakyool	*C. natsudaidai* Hayata
Sambokam	*C. sulcata* hort. ex Takahashi
Shiikuwasha	*C. depressa* Hayata
Iyekam	*C. iyo* hort. ex Tan.
Cheungkyoun	*C. unshiu* Marc. × *C. sinensis* (L.) Osb.
Gungcheonjosaeng	*C. unshiu* Marc.
Palsak	*C. hassaku* hort. ex Tan.
Hallabong	*C. unshiu* × *C. sinensis* × *C. reticulatia*

Portions (10 g) of the powdered peels were extracted for 3 days with 200 ml of ethanol-water (7:3, v/v) at ambient temperature. The extract was decanted, and the remaining residue was extracted once more with 100 ml of the same solution. The combined extract was evaporated, lyophilized, and reconstituted with ethanol-water (1:1, v/v) to a final concentration of 100 mg/ml. This was termed the ethanol extract of citrus fruit peels.

1.5.4 Flavonoid analysis

The structures of seven flavonoids analyzed in this study are shown in Figure 1.1. Reconstituted samples were filtered through a membrane filter (0.45 μM). A 10-μl aliquot of filtered sample was injected into a high performance liquid chromatography (HPLC) System (Waters, Milford, MA, USA) equipped with a pump, UV-vis detector, column oven, and injector. A C_{18} RP column (Atlantis dc-18, 150×3.9 mm-i.d.; Waters, MA, USA) with a cartridge guard column was used for the HPLC system. The mobile phase for the HPLC system was acetonitrile (A) and water (B) with a flow rate of 1 ml/min. The mobile phase program consisted of four periods: (1) 0-10 min, 20% A, (2) 10-16 min, 45 % A, (3) 16-20 min, 75% A, and (4) 20-22 min, 20% A. The column was operated at 40°C and the eluent was monitored with a single-channel UV detector at a wavelength of 280 nm. The flavonoids were identified by comparing their retention times and UV spectra with those of authentic standards stored in a data processor. The content of each flavonoid was calculated from the integrated peak area of the sample and the corresponding standard.



Flavanone

Naringin: R=neohesperidose, R_1=R_2=H

Hesperidin: R=rutinose, R_1=OH, R_2=Me

Naringenin: R=R_1=R_2=H

Hesperetin: R=H, R_1=OH, R_2=Me

Flavone

Rutin: R=H, R_1=OH, R_2=H, R_3=O-rutinose

Polymethoxylated Flavone

Nobiletin: R=R_1=OMe, R_2=H

Tangeretin: R=OMe, R_1=R_2=H

Figure 1.1. Structures of the flavonoids

1.5.5 Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in DMEM medium, with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C.

1.5.6 Nitrite assay

To evaluate the inhibitory activity of the peel extracts on LPS-induced NO production, cells were plated in 96-well plates (5×10^4 cells/well) and incubated for 24 h. Cells were then treated with either LPS (100 ng/ml) or LPS (100 ng/ml) plus interferon-γ (IFN-γ, 100 U/ml) in the presence or absence of test materials. After an additional 24 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction (Green *et al*., 1982). The percent (%) inhibition was expressed as [1-(NO level of test samples/NO level of vehicle-treated control)]×100. The IC_{50} value, equivalent to the sample concentration that inhibits NO production by 50%, was determined using non-linear regression analysis (% inhibition versus concentration).

1.5.7 MTT assay

Cell viability was determined by MTT assay (Ferrari *et al*., 1990). After the cells were cultured as described above, MTT solution (final 0.5 mg/ml) was added to each

well and further incubated for 1 h at 37°C. Media were discarded, and dimethyl sulfoxide (DMSO) was added to each well to dissolve the generated formazan. The absorbance was measured at 570 nm, and the percentage survival was determined by comparison with the control group.

1.5.8 Statistical analysis

Data were presented as mean ± (relative) standard deviation, and all experiments were conducted in triplicate. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Correlations between variables were analyzed using Spearman's rank correlation coefficient. Wilcoxon's signed-rank test or paired *t*-test was used to determine the statistical significance. $P < 0.05$ was considered significant.

1.6 RESULTS

1.6.1 Distrubutions of the flavonoids among the 70% ethanol extracts from various citrus fruit peels

The flavonoid contents of the immature and mature fruit peel extracts derived from the 20 citrus species are presented in Tables 2 and 3, respectively. The flavonoids contents varied among the citrus plants investigated with hesperidin being widely distributed in large amounts in the ethanol extracts from both immature and mature citrus fruit, whereas naringin was present at high levels only in certain citrus fruit ethanol extracts (Figure 1.2). Especially, the Korea-native citrus Jikak (*C. aurantium* L.) contained the highest amount of naringin among ethanol extracts from both immature and mature citrus fruit peels. Naringenin and hesperetin, the aglycones of naringin and hesperidin, were distributed in lower concentrations in the citrus fruit peels. Nobiletin and tangeretin occurred ubiquitously in most citrus species with similar distribution patterns. The correlation analysis between flavonoid contents revealed that the nobiletin content was positively and significantly correlated with the tangeretin content in peel extracts of both the immature ($r = 0.879$, $P < 0.0005$) and mature ($r = 0.858$, $P < 0.0005$) citrus fruits (Figure 1.3). However, no significant correlation was detected among the contents of the other flavonoids (Table 1.4). The contents of naringin, hesperidin, nobiletin, and tangeretin in the peels of mature citrus fruits were significantly less than those of immature citrus fruits (Table 1.5, Figure 1.4).

Table 1.2. Flavonoids contents among the 70% ethanol extracts from immature citrus fruit peels

Citrus	Flavanone								Flavone		Polymethoxylated flavone			
	Naringin		Naringenin		Hesperidin		Hesperetin		Rutin		Nobiletin		Tangeretin	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
Jikak	**112.57**	0.36	-		23.80	2.18	0.60	4.81	7.02	0.16	0.92	0.63	0.42	0.00
Kamja	0.20	0.00	1.67	3.46	23.17	3.67	0.72	3.60	0.37	15.75	2.67	0.43	3.57	0.71
Dongjeongkyool	4.72	1.48	-		7.17	0.81	-		0.23	24.74	1.85	0.31	2.85	0.20
Dangyooja	55.41	0.39	-		27.59	0.70	1.97	3.85	1.96	1.77	2.11	0.81	1.46	0.47
Cheongkyool	57.07	0.10	-		28.83	0.20	0.45	1.59	1.74	1.00	1.90	0.53	1.27	0.79
Byungkyool	1.83	3.15	-		47.03	0.12	-		1.60	0.00	11.10	0.41	6.48	0.46
Sadookam	0.10	0.00	-		2.42	2.19	-		0.20	0.00	0.04	13.32	0.03	21.65
Jinkyool	4.60	0.00	-		5.87	0.98	3.11	0.10	0.20	0.00	**16.40**	0.16	**23.10**	0.41
Hongkyool	11.43	1.01	-		17.60	5.48	-		-		**15.19**	1.81	**10.72**	1.83
Yooja	6.37	2.40	-		23.20	1.14	1.51	0.38	-		-		-	
Binkyool	2.83	2.04	-		12.90	0.78	-		-		7.92	0.46	6.11	0.50
Pyunkyool	21.50	1.61	-		23.27	2.86	0.87	6.66	-		3.69	0.31	3.90	0.15
Hakyool	19.63	1.18	-		2.30	0.00	-		-		0.24	4.17	0.45	1.29
Sambokam	1.30	0.00	-		14.00	0.71	-		1.39	1.10	0.81	0.72	1.23	0.47
Shiikuwasha	-		-		11.93	0.48	-		-		**15.64**	0.29	**10.17**	0.15
Iyekam	3.73	4.09	-		15.83	0.96	-		1.80	0.64	2.53	0.23	1.21	0.48
Cheungkyoun	0.53	21.65	-		12.63	0.46	-		0.30	0.00	4.63	0.25	0.66	0.00
Gungcheonjosaeng	-	-	-		17.23	0.34	-		3.07	1.88	1.21	0.48	0.76	0.00
Palsak	62.27	0.33	-		5.80	0.00	-		-		0.25	2.28	0.30	3.33
Hallabong	9.55	1.64	-		26.60	2.29	-		-		8.26	1.65	1.27	2.52

Each mean calculated from values of three experiments (mg/g 70% ethanol extract). RSD; relative standard deviation (%), - ; not detected

Table 1.3. Flavonoids contents among the 70% ethanol extracts from mature citrus fruit peels

Citrus	Flavanone								Flavone		Polymethoxylated flavone			
	Naringin		Naringenin		Hesperidin		Hesperetin		Rutin		Nobiletin		Tangeretin	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
Jikak	**103.63**	0.06	-		20.33	16.06	2.60	0.00	7.43	0.78	0.80	0.00	0.51	1.14
Kamja	9.03	0.64	-		13.87	1.67	-		-		1.49	2.32	1.86	2.48
Dongjeongkyool	3.93	3.88	-		8.93	0.65	-		1.40	0.00	1.15	0.00	1.71	0.58
Dangyooja	31.03	0.37	-		14.27	0.81	-		3.10	0.00	1.19	0.48	0.91	0.64
Cheongkyool	6.00	0.00	-		7.80	0.00	-		1.87	3.09	**4.69**	0.00	**1.43**	0.00
Byungkyool	-		-		14.70	0.00	-		1.80	0.00	**5.72**	0.00	**3.66**	0.16
Sadookam	-		-		8.20	0.00	-		-		0.09	0.00	0.06	0.00
Jinkyool	3.83	1.51	-		7.13	0.81	-		-		**4.55**	0.00	**5.90**	0.20
Hongkyool	-		-		6.33	0.91	-		-		3.52	0.16	2.79	0.00
Yooja	-		-		5.60	6.19	-		3.20	0.00	-		-	
Binkyool	4.90	0.00	-		6.73	0.86	-		-		2.69	0.37	2.16	0.53
Pyunkyool	18.70	0.53	-		10.37	2.23	-		-		1.95	0.00	2.17	0.27
Hakyool	46.17	0.13	-		-		-		-		0.20	0.00	0.29	0.00
Sambokam	1.87	3.09	-		6.87	0.84	-		1.77	3.27	0.49	0.00	0.72	0.00
Shiikuwasha	-		-		6.67	0.87	-		-		**5.64**	0.18	**2.39**	0.00
Iyekam	-		-		6.17	0.94	-		-		1.27	0.00	0.74	0.78
Cheungkyoun	-		-		6.33	3.29	-		-		1.46	1.04	0.38	0.00
Gungcheonjosaeng	-		-		4.47	1.29	-		2.27	2.55	0.75	0.00	0.44	0.00
Palsak	58.93	0.52	-		20.50	0.00	-		-		0.24	0.00	0.22	2.59
Hallabong	-		-		21.07	0.27	-		-		4.06	0.00	0.78	0.00

Each mean calculated from values of three experiments (mg/g 70% ethanol extract). RSD; relative standard deviation (%), - ; not detected

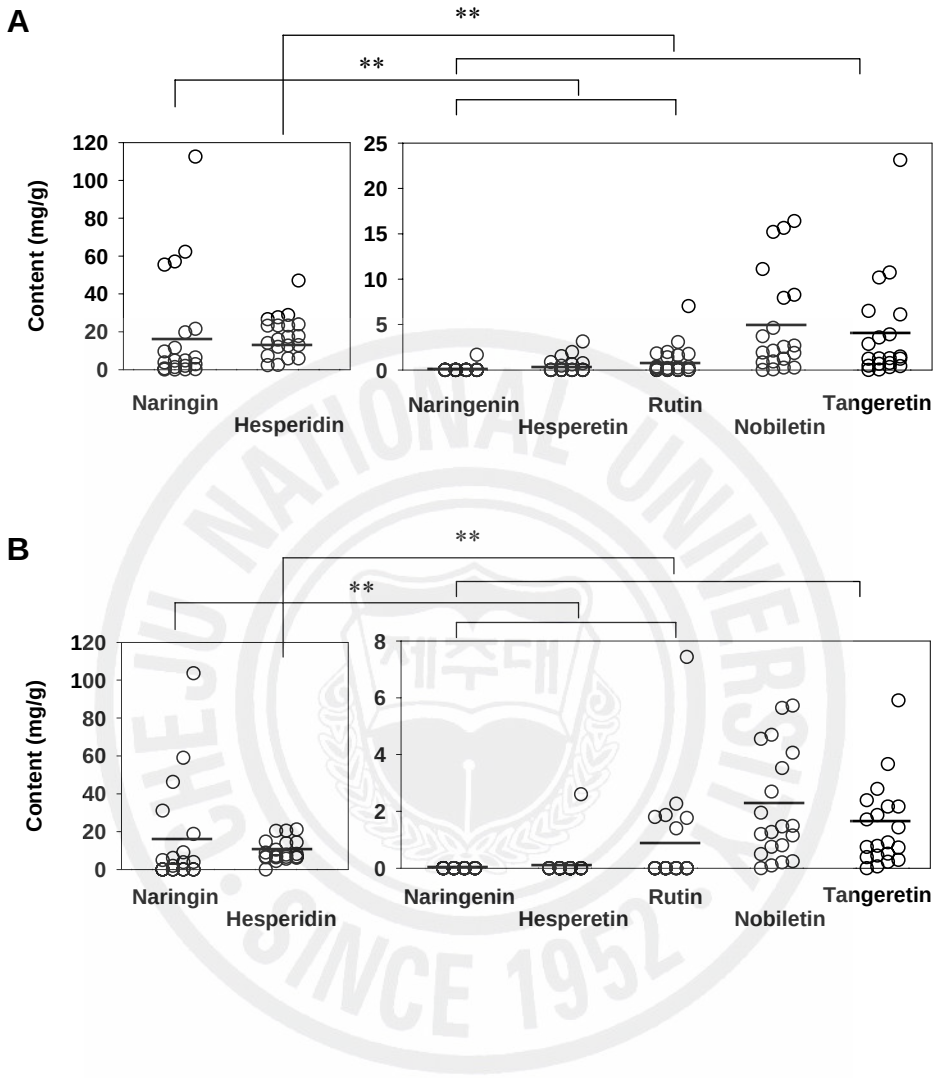


Figure 1.2. Flavonoids contents among the 70% ethanol extracts from immature (A) and mature (B) citrus fruit peels. The flavonoids contents were calculated from the integrated peak area of the sample and the corresponding standard. The open circle represents the flavonoid content in each peel extract. The horizontal bar represents the mean of 20 citrus fruit peel extracts. $^{**}P < 0.005$ by Wilcoxon signed rank test.

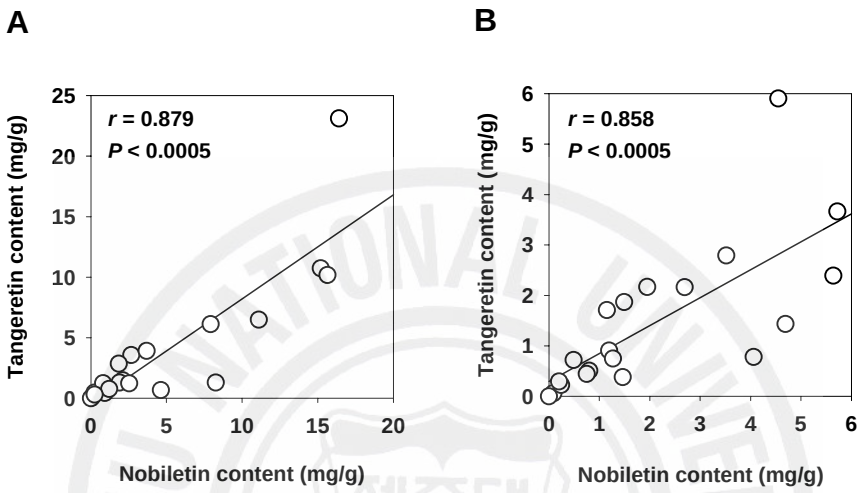


Figure 1.3. Correlation between nobiletin and tangeretin contents in the 70% ethanol extracts from immature (A) and mature (B) citrus fruit peels. The nobiletin and tangeretin content was calculated from the integrated peak area of the sample and the corresponding standard. The correlation between variables was obtained using Spearman's correlation. *r*: Spearman's rank correlation coefficient.

Table 1.4. Correlation between flavonoids contents in the 70% ethanol extracts from citrus fruit peels

Citrus		Spearman rank correlation coefficient						
		Naringin	Hesperidin	Naringenin	Hesperetin	Rutin	Nobiletin	Tangeretin
Immature	Naringin	1.000						
	Hesperidin	-0.259	1.000					
	Naringenin	0.285	0.119	1.000				
	Hesperetin	0.376	0.257	0.378	1.000			
	Rutin	-0.070	0.103	0.400	0.173	1.000		
	Nobiletin	-0.193	0.060	0.225	0.019	-0.147	1.000	
	Tangeretin	-0.138	0.139	0.213	0.125	-0.135	**0.879*****	1.000
Mature	Naringin	1.000						
	Hesperidin	n.c.	n.c.					
	Naringenin	0.387	n.c.	1.000				
	Hesperetin	0.396	n.c.	0.299	1.000			
	Rutin	0.096	n.c.	0.121	0.466	1.000		
	Nobiletin	-0.225	n.c.	0.195	-0.159	0.007	1.000	
	Tangeretin	-0.044	n.c.	0.197	-0.139	-0.015	**0.858*****	1.000

$^{***}P < 0.0005$, n.c.; not calculated

Table 1.5. Comparison of flavonoids contents between the 70% ethanol extracts from immature and mature citrus fruit peels

Flavonoid	Immature	Mature	*P* value[a]
Naringin	18.8 ± 29.9	14.4 ± 26.8	< 0.05
Naringenin	0.1 ± 0.4	0.0 ± 0.0	> 0.2
Hesperidin	17.5 ± 10.9	9.8 ± 5.8	< 0.005
Hesperetin	0.5 ± 0.8	0.1 ± 0.6	0.15
Rutin	1.0 ± 1.7	0.8 ± 1.8	> 0.2
Nobiletin	4.9 ± 5.6	2.1 ± 1.9	< 0.005
Tangeretin	3.8 ± 5.6	1.5 ± 1.5	< 0.005

Data are presented as Mean ± SD (mg/g 70% ethanol extract). [a] Wilcoxon signed rank test.

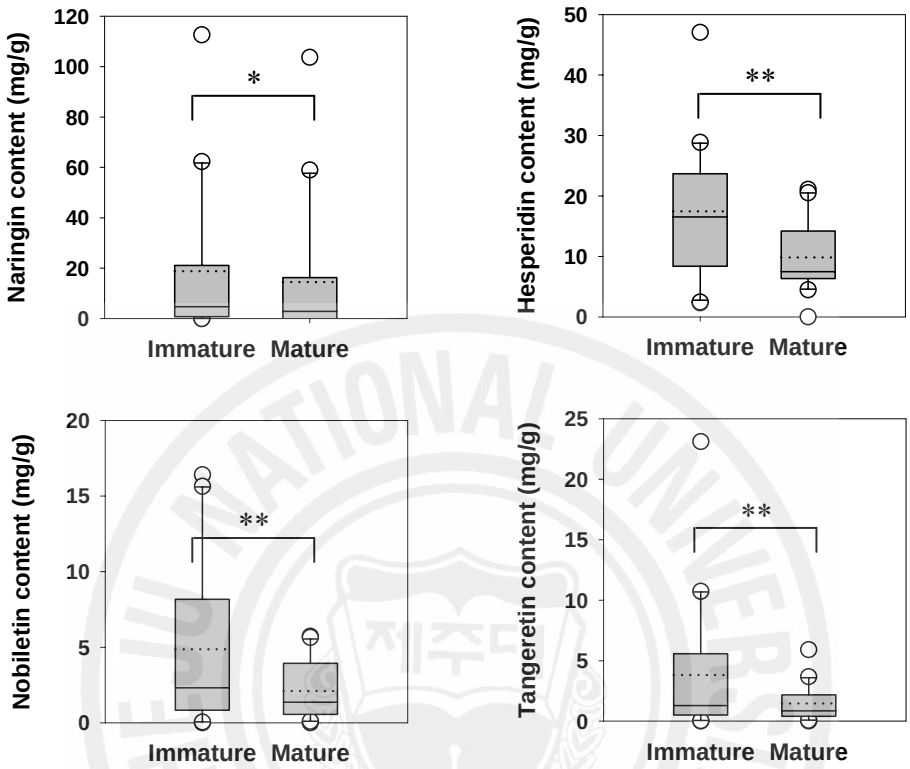


Figure 1.4. Box plots of naringin, hesperidin, nobiletin, and tangeretin contents in the 70% ethanol extracts from immature and mature citrus fruit peels. The flavonoids contents were calculated from the integrated peak area of the sample and the corresponding standard. The box represents the interquartile range, the line within the box the median, and the dotted line the arithmetic mean. The ends of the 'whiskers' show the maximum and minimum values. $^{*}P < 0.05$, $^{**}P < 0.005$ by Wilcoxon signed rank test.

1.6.2 NO production inhibitory effecs of the 70% ethanol extracts from various citrus fruit peels in LPS-activated RAW 264.7 cells

The potential anti-inflammatory effect of the ethanol extracts from immature and mature citrus fruit peels was evaluated based on their inhibitory activity on LPS-induced NO production in RAW 264.7 cells. A marked difference was observed in the suppressive potential for NO production (Table 1.6). Among the 20 citrus plants, the ethanol extracts from Jinkyool (*C. sunki* hort. ex Tan), and Shiikuwasha (*C. depressa* Hayata) were considered to be the most potent NO production inhibitory agents. The NO production inhibitory activities of immature fruit peels showed significantly higher inhibitory effects than those of the mature fruit peels at all tested concentrations ($P < 0.05$ for 250 μg/ml, $P < 0.005$ for 500 μg/ml, $P < 0.0005$ for 1000 μg/ml) (Figure 1.5). No detectable cytotoxicity was observed in any of the citrus samples except ethanol extracts from immature fruits of Kamja (*C. benikoju* hort. Ex Tan.), Jinkyool (*C. sunki* hort. ex Tan), Pyunkyool (*C. tangerina* hort. ex Tan), and Shiikuwasha (*C. depressa* Hayata) (Table 1.7).

1.6.3 Correlation between flavonoid content and NO production inhibitory activity in LPS-activated RAW 264.7 cells

The NO production inhibitory activity of citrus fruit peels was significantly and positively correlated with both nobiletin and tangeretin contents at all tested concentrations (Table 1.8, Figures 1.6 and 1.7). In accordance with these results, the IC_{50} values were always negatively correlated with both nobiletin and tangeretin contents (Figures 1.6 and 1.7). However, no significant correlation was found among

Table 1.6. Effects of the 70% ethanol extracts from citrus fruit peels on NO production in LPS-activated RAW 264.7 cells

Citrus	Peel of immature citrus fruits				Peel of mature citrus fruits			
	μg/ml 250	500	1000	IC_{50}(μg/ml)	250	500	1000	IC_{50}(μg/ml)
Jikak	-	13.2 ± 6.4	33.7 ± 5.5	*	6.3 ± 2.6	14.5 ± 1.7	13.7 ± 0.2	*
Kamja	25.2 ± 9.5	54.8 ± 2.5	92.6 ± 1.3	443.3 ± 49.0	14.7 ± 0.2	24.4 ± 1.2	41.8 ± 4.8	*
Dongjeongkyool	15.7 ± 1.5	34.1 ± 1.8	66.1 ± 0.8	716.6 ± 25.2	9.6 ± 0.2	22.0 ± 0.2	36.6 ± 1.1	*
Dangyooja	6.3 ± 4.1	21.6 ± 4.3	50.6 ± 2.7	996.5 ± 12.3	7.6 ± 0.7	18.3 ± 0.3	32.4 ± 1.1	*
Cheongkyool	-	27.4 ± 9.4	59.9 ± 1.9	745.6 ± 60.4	22.2 ± 1.0	23.0 ± 2.6	53.9 ± 0.2	930.7 ± 17.1
Byungkyool	41.6 ± 3.0	71.8 ± 3.7	92.8 ± 0.6	303.0 ± 27.6	23.4 ± 0.4	37.6 ± 4.1	72.1 ± 0.0	631.9 ± 66.4
Sadookam	-	-	19.6 ± 4.8	*	-	-	21.4 ± 0.3	*
Jinkyool	64.8 ± 1.2	88.9 ± 1.3	96.8 ± 0.8	188.4 ± 7.5	15.4 ± 2.2	42.3 ± 5.5	71.1 ± 2.3	580.9 ± 64.7
Hongkyool	51.7 ± 5.5	79.8 ± 2.1	93.9 ± 0.7	239.9 ± 14.4	11.1 ± 0.4	22.0 ± 2.2	52.2 ± 2.3	945.9 ± 54.1
Yooja	-	18.4 ± 4.0	34.1 ± 3.5	*	11.6 ± 1.4	21.6 ± 1.7	33.0 ± 2.8	*
Binkyool	22.5 ± 0.0	52.6 ± 2.5	81.5 ± 3.3	477.3 ± 20.3	19.6 ± 2.7	33.9 ± 0.4	56.7 ± 1.9	768.3 ± 47.7
Pyunkyool	24.9 ± 13.0	51.2 ± 8.2	93.4 ± 0.6	476.5 ± 91.5	12.3 ± 1.9	17.4 ± 4.8	37.3 ± 3.2	*
Hakyool	-	7.2 ± 7.1	29.1 ± 2.1	*	11.3 ± 1.0	13.9 ± 1.4	30.3 ± 1.2	*
Sambokam	-	9.0 ± 2.5	27.9 ± 0.0	*	17.0 ± 3.3	17.5 ± 2.0	23.7 ± 8.5	*
Shiikuwasha	45.9 ± 3.7	85.6 ± 0.1	95.4 ± 0.1	259.8 ± 13.7	15.2 ± 2.4	48.6 ± 1.1	70.2 ± 0.4	521.6 ± 14.7
Iyekam	6.0 ± 5.5	24.1 ± 0.7	45.5 ± 1.4	*	13.4 ± 2.2	26.2 ± 0.7	37.9 ± 2.2	*
Cheungkyoun	13.5 ± 0.1	43.5 ± 1.1	74.1 ± 1.3	565.7 ± 16.4	7.2 ± 3.2	30.2 ± 1.4	47.6 ± 0.2	*
Gungcheonjosaeng	-	26.5 ± 3.5	57.4 ± 0.7	795.7 ± 34.0	6.7 ± 1.9	12.9 ± 0.8	27.9 ± 4.7	*
Palsak	8.2 ± 7.3	18.2 ± 0.8	31.5 ± 1.7	*	14.7 ± 0.6	22.1 ± 2.5	26.9 ± 1.0	*
Hallabong	30.1 ± 1.4	63.5 ± 4.9	86.4 ± 0.3	353.7 ± 70.9	15.3 ± 0.6	31.9 ± 4.2	54.8 ± 2.7	815.9 ± 54.3

Data are presented as average of inhibitory activity (%)±SD (*n*=3), -; Inhibitory activity < 5%, *; IC_{50} > 1000 μg/ml.

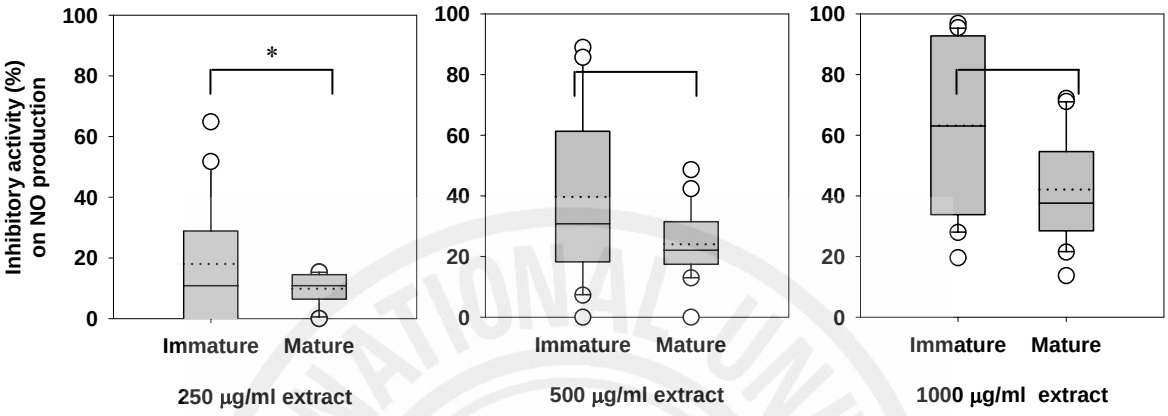


Figure 1.5. Box plots of inhibitory activity of the 70% ethanol extracts from immature and mature citrus fruit peels on NO production in LPS-activated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) in the presence or absence of citrus fruit ethanol extracts at the indicated concentrations for 24 h. The amount of nitrite in the culture medium was determined with Griess reagent. The box represents the interquartile range, the line within the box the median, and the dotted line the arithmetic mean. The ends of the 'whiskers' show the maximum and minimum values. $^{*}P < 0.05$, $^{**}P < 0.005$ by Wilcoxon signed rank test.

Table 1.7. Cytotoxicity of the 70% ethanol extracts from citrus fruit peels on RAW 264.7 cells

Citrus	Peel of immature citrus fruits				Peel of mature citrus fruits			
	μg/ml 250	500	1000	TC_{50}(μg/ml)	250	500	1000	TC_{50}(μg/ml)
Jikak	-	-	-	*	-	-	-	*
Kamja	-	-	79.6 ± 0.8	796.4 ± 43.3	-	-	-	*
Dongjeongkyool	-	-	-	*	-	-	-	*
Dangyooja	-	-	75.5 ± 1.4	*	-	-	-	*
Cheongkyool	-	-	19.6 ± 8.3	*	-	-	-	*
Byungkyool	-	-	35.2 ± 3.8	*	-	-	-	*
Sadookam	-	-	-	*	-	-	-	*
Jinkyool	-	17.0 ± 2.4	92.9 ± 1.7	668.8 ± 9.1	-	-	-	*
Hongkyool	-	-	35.1 ± 1.6	*	-	-	-	*
Yooja	-	-	8.3 ± 10.2	*	-	-	-	*
Binkyool	-	-	33.9 ± 8.0	*	-	-	-	*
Pyunkyool	-	-	71.9 ± 4.8	920.3 ± 2.7	-	-	-	*
Hakyool	-	-	-	*	-	-	-	*
Sambokam	-	-	-	*	-	-	-	*
Shiikuwasha	-	-	59.9 ± 1.1	946.5 ± 15.0	-	-	-	*
Iyekam	-	-	-	*	-	-	-	*
Cheungkyoun	-	-	6.7 ± 5.7	*	-	-	-	*
Gungcheonjosaeng	-	-	-	*	-	-	-	*
Palsak	-	-	-	*	-	-	-	*
Hallabong	-	-	16.2 ± 2.7	*	-	-	-	*

Data are expressed as average of inhibitory activity (%)±SD (*n*=3), -; Inhibitory activity < 5%, *; TC_{50} > 1000 μg/ml.

Table 1.8. Correlation between flavonoids contents and inhibitory activity on NO production by LPS-activated RAW 264.7 cells in the 70% ethanol extracts from citrus fruits

Citrus fruit	Concentration (μg/ml)	Spearman rank correlation coefficient						
		Naringin	Naringenin	Hesperidin	Hesperetin	Rutin	Nobiletin	Tangeretin
Immature	250	-0.135	0.181	0.070	0.067	-0.436	**0.869*****	**0.811*****
	500	-0.232	0.179	0.228	0.074	-0.245	**0.942*****	**0.870*****
	1000	-0.170	0.179	0.244	0.180	-0.251	**0.923*****	**0.881*****
	IC_{50}	0.195	-0.077	0.236	0.041	0.614	**-0.907*****	**-0.787****
Mature	250	-0.331	n.c.	0.168	-0.219	-0.226	**0.671****	**0.576***
	500	-0.282	n.c.	0.135	-0.259	-0.267	**0.762*****	**0.605****
	1000	-0.361	n.c.	-0.002	-0.378	-0.237	**0.901*****	**0.763*****
	IC_{50}	0.158	n.c.	-0.054	n.c.	0.223	**-0.500**	**-0.429**

$^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, n.c.; not calculated.

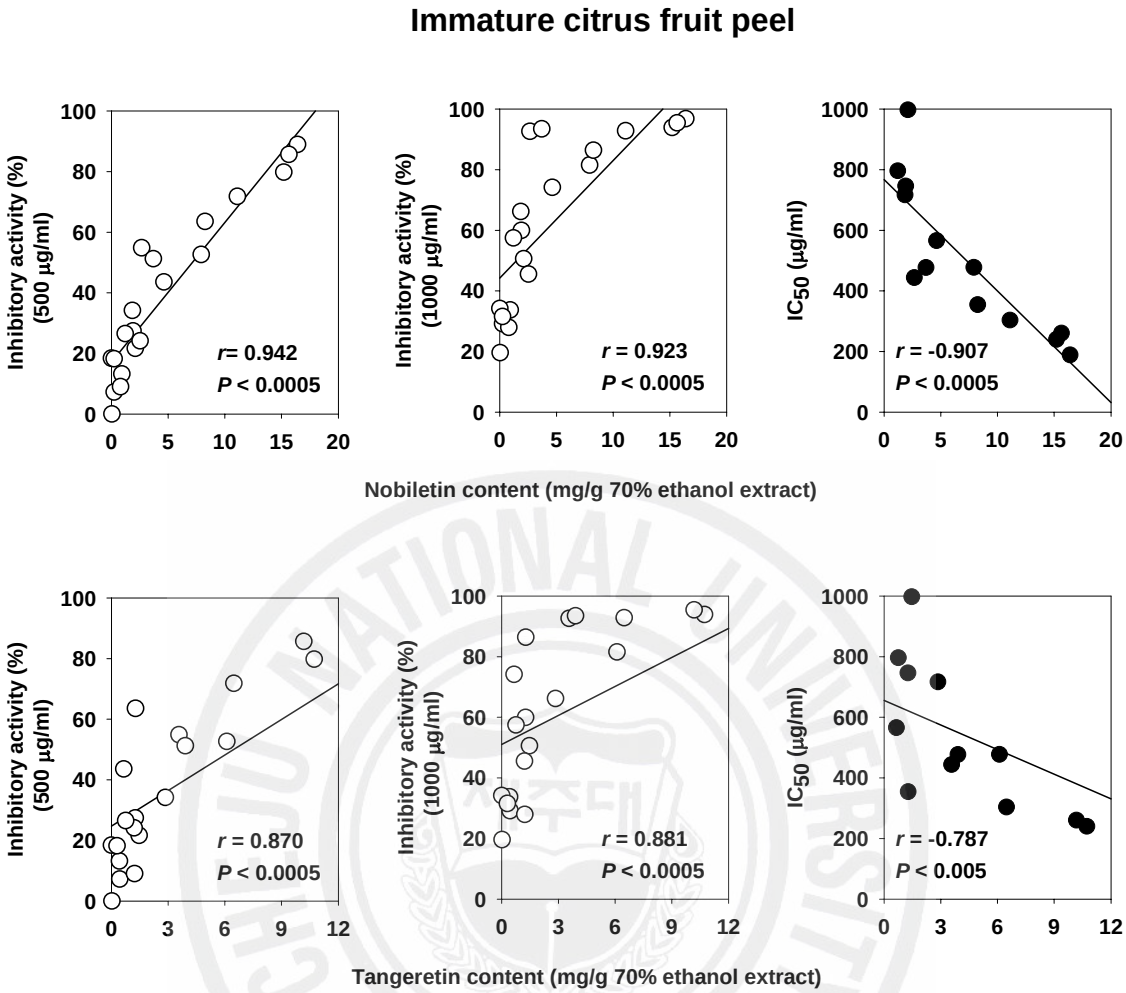


Figure 1.6. Correlation between the content of nobiletin or tangeretin and the inhibitory activity on NO production by LPS-activated RAW 264.7 cells in the 70% ethanol extracts from immature citrus fruit peels. Cells were treated with LPS (100 ng/ml) in the presence or absence of ethanol extracts from immature citrus fruit peels at the indicated concentrations for 24 h. The amount of nitrite in the culture medium was determined with Griess reagent. IC_{50} is the concentration of ethanol extracts from immature citrus fruit peels that inhibits LPS-induced NO production by 50%. The correlation between variables was obtained using Spearman's correlation. *r*: Spearman's rank correlation coefficient.

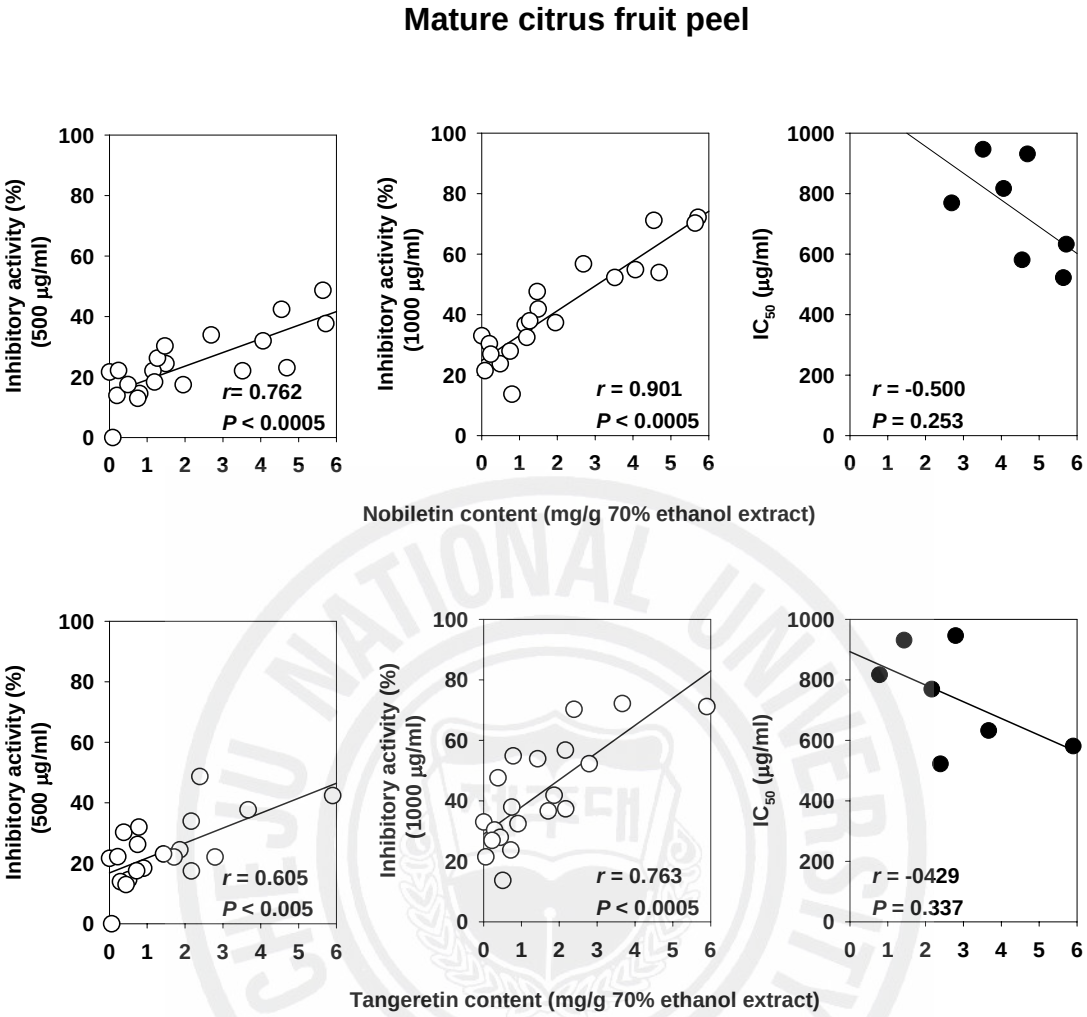


Figure 1.7. Correlation between the content of nobiletin or tangeretin and the inhibitory activity on NO production by LPS-activated RAW 264.7 cells in the 70% ethanol extracts from mature citrus fruit peels. Cells were treated with LPS (100 ng/ml) in the presence or absence of ethanol extracts from mature citrus fruit peels at the indicated concentrations for 24 h. The amount of nitrite in the culture medium was determined with Griess reagent. IC_{50} is the concentration of ethanol extracts from mature citrus fruit peels that inhibits LPS-induced NO production by 50%. The correlation between variables was obtained using Spearman's correlation. *r*: Spearman's rank correlation coefficient.

the other flavonoid contents and respective NO-production inhibitory activities (Table 1.8).

1.6.4 Effects of citrus flavonoids on NO production in LPS or LPS plus IFN-γ-activated RAW 264.7 cells

To define the extent of each flavonoid's contribution to the potential anti-inflammatory activity, the NO production inhibitory efficacy of each flavonoid (narignin, hesperidin, naringenin, hesperetin, rutin, nobiletin, and tangeretin) was compared to each other in LPS-activated RAW 264.7 cells. Moreover, this study evaluated effects of these flavonoids on NO production in LPS plus IFN-γ-activated RAW 264.7 cells. Among the tested flavonoids, nobiletin was found to be the most potent NO production inhibitor (IC_{50} = 26.5 μM for LPS, 49.1 μM for LPS plus IFN-γ) (Figures 1.8 and 1.9). In comparison, the IC_{50} values of tangeretin were found to be 136.6 μM for LPS and > 100 μM for LPS plus IFN-γ. This result indicates that the correlation between the tangeretin content and NO production inhibitory activity shown in Figure 1.3 could have been caused by the highly positive correlation between the nobiletin and tangeretin contents of the citrus fruit peels.

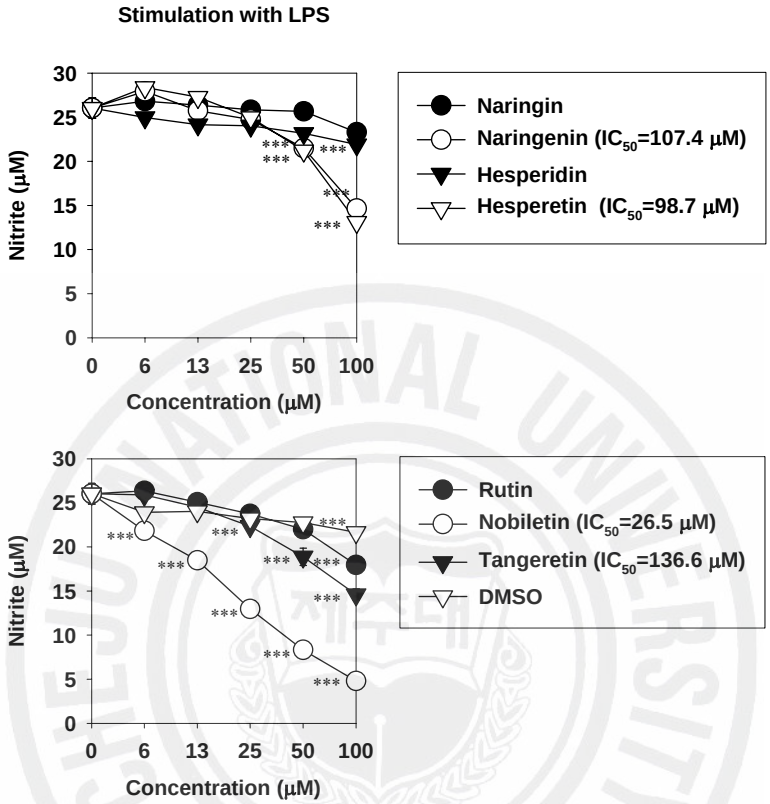


Figure 1.8. Inhibitory effect of citrus flavonoids on NO production in LPS-activated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) in the presence or absence of flavonoid at the indicated concentrations for 24 h. The amount of nitrite in the culture medium was determined with Griess reagent. IC_{50} is the concentration of flavonoid that inhibits LPS-induced NO production by 50%. Each data shows the mean ± SD of n=5. $^{***}P < 0.0005$ by paired t test.

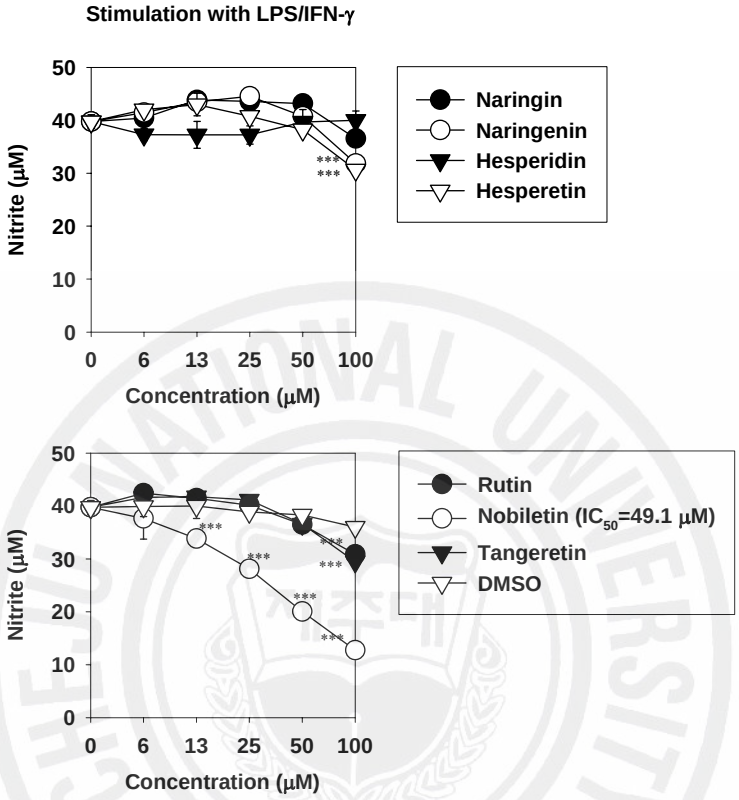


Figure 1.9. Inhibitory effect of citrus flavonoids on NO production in LPS plus IFN-γ-activated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) plus IFN-γ (100 U/ml) in the presence or absence of flavonoid at the indicated concentrations for 24 h. The amount of nitrite in the culture medium was determined with Griess reagent. IC_{50} is the concentration of flavonoid that inhibits LPS-induced NO production by 50%. Each data shows the mean ± SD of n=5. $^{***}P < 0.0005$ by paired t test.

1.7 DISCUSSION

Citrus fruit-derived flavonoids and their metabolites have been shown to have significant biological activities, including anti-inflammatory properties (Balestrieri *et al*., 2003; Kawaguchi *et al*., 1999; Manthey *et al*., 1999). A large body of evidence implicates flavonoids extracted from citrus fruit peels as being the most bioactive as supported by the high biological activities of peel components compared to fruit juice sac components (Murakami *et al*., 2000).

Among the seven flavonoids analyzed in this study, hesperidin was widely distributed with a varying amount in the majority of the tested citrus fruit peels, whereas naringin was present at high levels in only certain citrus fruits such as Jikak (*C. aurantium*). Both immature and mature fruit peels of *C. aurantium* contained the highest amounts of naringin and rutin. It is known that the immature and mature fruit peels of *C. aurantium* L. are used to treat indigestion in traditional Chinese medicine (Huang and Wang, 1993) and modern European herbal medicine (Blumenthal *et al*., 1998). Naringenin, a metabolite of naringin, has been shown in many studies to prevent gastric mucosal ulceration in several animal models, including restraint stress, pyloric occlusion, and ethanol-induced chronic ulceration (Martin *et al*., 1993; Motilva *et al*., 1992, 1993; Parmar, 1983). It has been reported that naringin also possesses antioxidant and superoxide anion scavenger properties that could contribute to its gastro-protective effect (Robak *et al*., 1988; Martin *et al*., 1994). This study suggests that the fruit peel of *C. aurantium* is an excellent source for the isolation of naringin to fulfill potential industrial and pharmacological applications. Hesperidin was ubiquitously contained to varying degrees in the peels of both

immature and mature fruits from all of the citrus varieties tested except mature Hakyool (*C. natsudaidai* Hayata). Both nobiletin and tangeretin also showed ubiquitous distributions among the peels of both immature and mature fruits from all the citrus varieties except Yooja (*C. junos* Sieb. Ex Tan). The study found that the nobiletin content was correlated significantly and positively with the tangeretin content. In general, the total amount of flavonoids within the mature citrus fruit peels was significantly lower than those of the immature fruit peels, confirming that the flavonoid contents in citrus fruit peels change dramatically during maturation.

The majority of the citrus fruit peels suppressed LPS-induced NO production in RAW 264.7 cells, although the inhibitory activities varied. The NO-production inhibitory activities of the mature fruit peels were significantly lower than those of the immature fruit peels, suggesting that the NO production inhibitory activity of a citrus fruit peel can be determined by the flavonoid composition, which was unique to each citrus plant. Notably, the NO production inhibitory activities of the citrus fruit peels were correlated with both the nobiletin and tangeretin contents.

By comparing the NO production inhibitory activities of the flavonoids, the study found that nobiletin was the most potent among the tested flavonoids. Unexpectedly, however, the NO production inhibitory activity of tangeretin was about five times lower than that of nobiletin, suggesting that the positive correlation observed between tangeretin content and NO production inhibitory activity may have been merely due to the high positive correlation between the nobiletin and tangeretin contents in citrus fruit peels.

In conclusion, the flavonoid contents and NO production inhibitory effects of the peel extracts investigated in this study were unique to each citrus plant. Among the seven flavonoids analyzed, the nobiletin content and NO production inhibitory effect

of each citrus fruit peel were highly correlated. This finding suggests various beneficial features of citrus fruit peels such as the prevention of diseases that involve excessive NO release.

PART 2

The anti-inflammatory mechanism of nobiletin in LPS-activated RAW 264.7 cells

2.1 ABSTRACT

Nobiletin (5,6,7,8,3',4'-hexamethoxy flavone), which is a polymethoxylated flavone found exclusively in the peels of citrus fruits, exhibits diverse biological potential. This study investigated the anti-inflammatory activity of nobiletin in terms of its regulation of nuclear factor κB (NF-κB), a transcription factor that is associated with the expression of many immune and inflammatory genes. Nobiletin suppressed the transcriptional activation of NF-κB in lipopolysaccharide (LPS)-activated RAW 264.7 cells. Nobiletin inhibited neither LPS-induced degradation of inhibitory κB-α nor nuclear translocation of NF-κB. However, it interrupted the DNA-binding activity of activated NF-κB. Nobiletin inhibited nitric oxide (NO) and prostaglandin E_2 production through the inhibition of inducible NO synthase (iNOS) and cyclooxygenase-2 gene expression in LPS-activated RAW 264.7 cells. Nobiletin also inhibited the production of three pro-inflammatory cytokines, tumor necrosis factor-α, interleukin-1β and interleukin-6, in gene expression levels. In addition, nobiletin inhibited the production of LPS-mediated reactive oxygen species (ROS) in RAW 264.7 cells. These results indicate that nobiletin inhibits LPS-induced NF-κB activation by interrupting DNA-binding activity without interfering with the nuclear translocation of NF-κB, and that nobiletin suppresses the accumulation of ROS, resulting in redox-based NF-κB activation in LPS-activated RAW 264.7 cells.

Key words: IκBα; lipopolysaccharide; NF-κB; nobiletin; reactive oxygen species

2.2 LIST OF TABLES

2.3 LIST OF FIGURES

2.4 INTRODUCTION

The production of pro-inflammatory mediators is primarily regulated at the level of gene transcription through the activities of several transcription factors. One of the most ubiquitous transcription factors is nuclear factor κB (NF-κB), which is activated by numerous inflammatory and pathologic stimuli, including cytokines, oxidative stress, ultraviolet light, and bacterial lipopolysaccharides (LPSs; Baeuerle & Henkel, 1994; Baldwin, 1996; Barnes & Karin, 1997). NF-κB typically exists in the form of a p65/p50 heterodimer, which is the most potent activator of pro-inflammatory gene expression (Baeuerle and Henkel, 1994; Kopp and Ghosh, 1995). In unstimulated cells, NF-κB is present constitutively in the cytosol and is linked to inhibitory κB (I-κB) proteins. The activation of NF-κB involves the phosphorylation by I-κB kinases followed by the translocation of NF-κB to the nucleus, its binding to the promoter κB site of the target gene, and finally, the proteolytic degradation of I-κBs in the proteasomes (Baldwin, 1996). Most anti-inflammatory drugs have been shown to suppress the expression of these genes by inhibiting the NF-κB activation pathway (Gilroy *et al*., 2004). Thus, an NF-κB inhibitor may be useful in the development of therapeutic drugs to control the inflammation associated with human diseases in a clinical environment. In addition, three well-defined, mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun NH_2-terminal kinase (JNK), have been implicated in the transcriptional regulation of pro-inflammatory mediators because the specific MAPK inhibitors suppress the expression of inflammatory-related genes (Kefaloyianni *et al*., 2006; Palsson-McDermott and O'Neill, 2004; Wong, 2005).

Macrophages play crucial roles in the initiation and maintenance of inflammation. During endotoxemia and inflammation, macrophages are activated by LPSs and cytokines. LPSs stimulate the production of reactive oxygen species (ROS) via the activation of NADPH-oxidase in macrophages (Uchikura *et al.*, 2004; Reis *et al*., 2006). ROS are mediators of cellular injury and are involved in the onset of cellular damage during endotoxemia (Pattanaik & Prasad, 1996; Spolarics, 1998). ROS are also thought to be involved in inflammatory gene expression through redox-based activation of the NF-κB signaling pathway (Kabe *et al*., 2005).

Nobiletin, which is a polymethoxylated flavone found exclusively in the peels of citrus fruits, is of commercial interest because of its multitude of applications in the food and pharmaceutical industries. For example, nobiletin has been shown to have an anti-inflammatory effect on phorbol ester-induced skin inflammation in mice (Murakami *et al*., 2000a), and higher protective activity than indomethacin in the TPA-induced edema formation test on mouse ears (Murakami *et al*., 2000b). Recently, it has been shown that nobiletin inhibits the eosinophilic airway inflammation of asthmatic rats (Wu *et al*., 2006). It has also been reported that nobiletin inhibits LPS-induced NF-κB transcriptional activation in mouse macrophages (Murakami *et al*., 2005), the production of pro-inflammatory mediators by LPS-activated and/or interferon-gamma-activated murine macrophages (Lin *et al*., 2003; Murakami *et al*., 2003; Murakami *et al*., 2005), UVB-irradiated human keratinocytes (Tanaka *et al*., 2004), and interleukin (IL)-1-stimulated human synovial fibroblasts (Ishiwa *et al*., 2000; Lin *et al*., 2003). However, the molecular mechanism by which nobiletin exerts its anti-inflammatory effect remains largely unknown. This study investigated the anti-inflammatory activity of nobiletin in terms of its regulation of NF-κB and ROS production in LPS-activated RAW 264.7 cells using

nobiletin purified from the fruit peel of Jinkyool (*C. sunki* hort. ex Tan), which is a polymethoxy flavone-rich citrus native to Korea.

2.5 MATERIALS AND METHODS

2.5.1 Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Antibody against inducible NOS (iNOS) was purchased from Calbiochem (San Diego, CA, USA), antibodies to p38, phospho-p38, JNK1/2, phospho-JNK1/2, ERK1/2 and phosphor-ERK1/2 from Cell Signaling Technology (Beverly, MA, USA), and antibodies to IκBα, and phospho-IκBα from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was obtained from Takara Shuzo Co (Otsu, Shiga, Japan). LPS (*Escherichia coli* 026:B6), sodium nitroprusside (SNP), pyrrolidine dithiocarbamate (PDTC) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were from Sigma (St. Louis, MO, USA). All other reagents were purchased from Sigma, unless otherwise indicated.

2.5.2 Isolation of nobiletin

Nobiletin was isolated from the mature fruit peel of Jinkyool (*C. sunki* hort. ex Tan), which is a citrus fruit that is native to Korea. The fruits were collected from the Seogwipo area of Jeju Island, Korea. The fruit peels (dry weight, 900 g) were extracted with 80% methanol (MeOH, 10 L) for 48 h twice at room temperature. The MeOH extract was eluted successively with *n*-hexane (hexane), ethyl acetate

(EtOAc), *n*-butanol (BuOH), and finally, with water (2 L each). The EtOAc eluted fraction (6.2 g) was subjected to chromatography over a silica gel, eluted with hexane-EtOAc (4:1, 3:2, 1:4), EtOAc-MeOH (4:1, 1:1, 1:4), and MeOH, and divided into five fractions (I–V). Fraction III (0.92 g) was purified by recycling preparative-HPLC with a JAIGEL-ODS-BP column (JAI, Japan), and eluted with acetonitrile-water (1:1). The isolated nobiletin was dried (312 mg). ^{1}H- and ^{13}C- nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a JNM-LA 400 Fourier transform (FT)-NMR spectrometer (JEOL, Akishima, Japan) operating at 270 MHz.

Nobiletin: Pale-yellow needles ($CHCl_3$–MeOH); ^{1}H NMR ($CDCl_3$): δ 7.68 (1H, *dd*, *J* = 8.4, 2.1Hz), 7.58 (1H, *d*, *J*=2.1 Hz), 7.14 (1H, *d*, *J*=8.4Hz), 6.62 (1H, *s*), 4.07 (3H, *s*); 13C NMR ($CDCl_3$) δ 176.6 (C=O), 161.5 (C), 153.2 (C), 152.2 (C), 150.5 (C), 149.0 (C), 148.5 (C), 145.0 (C), 139.1 (C), 124.6 (C), 120.2 (CH), 115.7 (C), 112.4 (CH), 109.7 (CH), 107.1 (CH), 62.3 (OMe), 62.2 (OMe), 61.9 (OMe), 61.8 (OMe), 56.1 (OMe×2). A stock solution of nobiletin (100 mM) was prepared in DMSO for the experiment; the final DMSO concentration was less than 0.2%.

2.5.3 Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in DMEM medium that contained 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C.

2.5.4 MTT and LDH release assays

Cell viability was determined by the MTT cell viability assay (Ferrari *et al.*, 1990). Cells were seeded at a density of 5×10^4 cells/well into 96-well, flat-bottom culture plates in the presence or absence of nobiletin. Mitochondrial enzyme activity, which is an indirect measure of the number of viable respiring cells, was determined using the MTT reagent after 40 h of nobiletin treatment. The MTT absorbance was read using the μQuant microplate reader (Bio-Tek Instrument, Winooski, VT, USA). The effect of nobiletin on cell viability was evaluated as the relative absorbance compared with that of control cultures. The cytotoxic effect of nobiletin was also estimated by measuring the LDH levels in the culture supernatants (He *et al.*, 2002). Leakage of LDH is a well-known marker of damage to the cell membrane (Hsieh, & Acosta, 1991). Cytotoxicity was expressed as the percentage of LDH released (LDH released into the medium / maximal LDH release × 100). Maximal LDH release was measured after lysis of the cells with 0.5% Triton X-100.

2.5.5 Measurement of NO, PGE_2, TNF-α, IL-1β and IL-6 production

The amount of nitrite, which is the end-product of NO generation by activated macrophages, was determined by a colorimetric assay (Green *et al*., 1982). Briefly, 100 μl of cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was read in a microplate reader. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve. Concentrations of prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), IL-1β and IL-6 in culture medium were quantified by ELISA according to the manufacturer's instructions.

2.5.6 NO scavenging activity and iNOS activity

SNP induces NO release under physiological condition by a light irradiation (Feelisch and Stamler, 1996). To estimate the possible direct NO-scavenging activity of nobiletin, SNP solution (1 mM) was incubated alone or in combination with a 6-100 μM concentration of nobiletin. SNP solution was made immediately before use in 0.1 M phosphate buffer (pH 7.4). The reaction mixtures were incubated in light, at room temperature, and nitrite levels were determined exactly after 3 h.

The iNOS activity in the cell lysate was measured as the L-arginine- and NADPH-dependent generation of nitrite as described previously (Wang *et al*., 2001). The assay was performed by incubating with 200 μg of the cellular extract from LPS-activated RAW 264.7 cells in the absence or presence of nobiletin for 180 min at room temperature in 100-μl reaction buffer containing 20 mM sodium phosphate buffer, 2 mM NADPH, 2 mM L-arginine, and 10 μm FAD at pH 6.7. The reaction was stopped by the addition of 10 U/ml LDH and 10 mM pyruvate. The reaction mixture was incubated with an equal volume of Griess reagent. The absorbance at 540 nm was read in a microplate reader. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

2.5.7 Western blot analysis

Cells were incubated with LPS (100 ng/ml) in the absence or presence of nobiletin for the indicated time. The cells were washed with ice-cold phosphate-buffered saline, collected, and centrifuged. The cell pellets were resuspended in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5%

deoxycholate, 0.1 mM EGTA, 1.0 mM EDTA, 0.1% SDS, and proteinase inhibitors] and incubated at 4°C for 20 min. Cell debris was removed by microcentrifugation, and the protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates were subjected to 7.5% or 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with a solution of 0.1% Tween 20/Tris-buffered saline that contained 5% nonfat dry milk for 1 h at room temperature. After incubation overnight at 4°C with the indicated primary antibody, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunodetection was carried out using enhanced chemiluminescence (Pierce, Rockford, IL, USA). The relative band densities were determined by densitometry using image acquisition and analysis software (LabWorks, UVP, UK).

2.5.8 RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Cells were incubated with LPS (100 ng/ml) in the absence or presence of nobiletin for 6 h. Total cellular RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). The purity of the RNA preparation was checked by measuring the absorbance at the 260/280 nm ratio. From each sample, 1 μg of RNA was reverse-transcribed using the ImProm-II™ Reverse Transcription System (Promega corp. Madison, WI, USA) with an oligo dT-15 primer, as recommended by the supplier. PCR assay was then performed on the aliquots of the cDNA preparations to detect the expression of mRNA for iNOS, PGE_2, TNF-α, IL-

1β, IL-6 and β-actin (as an internal standard). The reactions were carried out in a 25 μl volume containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5' and 3' primers. The cycle number was optimized to ensure product accumulation in the exponential range. PCR primers used in this study were purchased from Bioneer (Seoul, Korea) and are listed in Table 2.1. β-Actin was used as an internal control for normalizing RNA content of each sample. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation. The relative band density was determined by densitometry using image acquisition and analysis software (LabWorks, UVP, UK).

2.5.9 Cytoplasmic and nuclear protein preparations

Cells were incubated with LPS (100 ng/ml) in the absence or presence of nobiletin for the indicated time. Cytoplasmic and nuclear protein fractions were prepared by cell lysis followed by nuclear lysis. Briefly, cells were suspended in hypotonic buffer [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin]. After incubation for 20 min at 4°C, the cell lysates were centrifuged at 12000 × g for 6 min. The supernatant cytoplasmic fractions were removed and stored at -80°C until used. The nuclear pellet was suspended in high-salt buffer [20 mM Hepes (pH 7.9), 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate] for 40 min on ice, and then centrifuged at 10000 × g for 20 min. The supernatant nuclear protein extract was stored at -80°C until used. The protein concentration was determined using the Bio-Rad protein assay reagent.

Table 2.1. The sequences of primers used in RT-PCR analysis

Gene		Primer sequences
iNOS	Forward	5’-CCCTTCCGAAGTTTCTGGCAGCAGC-3’
	Reverse	5’-GGCTGTCAGAGCCTCGTGGCTTTGG-3’
COX-2	Forward	5’-CACTACATCCTGACCCACTT-3’
	Reverse	5’-ATGCTCCTGCTTGAGTATGT-3’
TNF-α	Forward	5’-TTGACCTCAGCGCTGAGTTG-3’
	Reverse	5’-CCTGTAGCCCACGTCGTAGC-3’
IL-1β	Forward	5’-CAGGATGAGGACATGAGCACC-3’
	Reverse	5’-CTCTGCAGACTCAAACTCCAC-3’
IL-6	Forward	5’-GTACTCCAGAAGACCAGAGG-3’
	Reverse	5’-TGCTGGTGACAACCACGGCC-3’
β-Actin	Forward	5’-AGGCTGTGCTGTCCCTGTATGC-3’
	Reverse	5’-ACCCAAGAAGGAAGGCTGGAAA-3

2.5.10 Transient transfection and luciferase assay

Cells were cotransfected with or without 10 ng of the NF-κB-promoted luciferase reporter gene plasmid pNF-κB-Luc (Promega, Madison, WI, USA) and 4 ng of the *Renilla* luciferase reporter plasmid pRL-TK (Stratagene, La Jolla, CA, USA), which served as the internal standard, using FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA). After 24 h, the cells were incubated with LPS (100 ng/ml) in the absence or presence of nobiletin for 24 h, at which point the luciferase activity in the cell lysate was determined using the Dual-Luciferase Reporter Assay Kit (Promega). The luciferase activity was normalized to the transfection efficiency, as monitored by the *Renilla* luciferase expression vector. The level of induced luciferase activity was determined as a ratio to the luciferase activity of unstimulated cells.

2.5.11 Electrophoretic mobility shift assay (EMSA)

NF-κB binding activity to DNA was measured by EMSA using a gel shift system (Promega) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides that contain the consensus NF-κB sequence 5'-AGTTGAGGGGACTTTCCCAGGC-3' were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ, USA) using T4 polynucleotide kinase, and were used as probes for EMSA. Nuclear extract proteins (2 μg) were preincubated with the gel shift binding buffer [4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.05 mg/ml poly(deoxyinosine-deoxycytosine)] for 10 min, and then incubated with the

labeled probe for 20 min at room temperature. Each sample was electrophoresed on a 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate EDTA buffer at 250 V for 20 min. The gel was dried and subjected to autoradiography.

The binding specificity was examined by competition experiments in which a 100-fold excess of unlabeled oligonucleotide with the same sequence was added to the reaction mixture prior to the addition of radioactive labeled oligonucleotide. For supershift assays, nuclear extracts prepared from LPS-treated cells were incubated with antibody to the p65 subunit of NF-κB for 30 min at room temperature before EMSA analysis.

2.5.12 Measurement of intracellular ROS levels

Intracellular ROS levels were measured by detecting the fluorescence intensity of the oxidant-sensitive probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA). The cells were incubated for 30 min with 50 μM CM-H_2DCFDA, and washed twice with phosphate-buffered saline. Then cells were incubated with nobiletin and LPS, and the fluorescence of DCF was measured at 485 nm (excitation) and 520 nm (emission) for 24 h. The experiments were performed with at least three independent replications, and the results from one representative experiment (n=6 wells) are presented.

2.5.13 Statistical analysis

All experiments were conducted in triplicate. However, the data from one representative trial are presented. Statistical analyses were carried out using the SAS

statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using the paired *t*-test; $P < 0.05$ was considered to be statistically significant.

2.6 RESULTS

2.6.1 Cytotoxicity of nobiletin on RAW 264.7 cells

The cytotoxicity of the extracted nobiletin was examined to determine the effective concentration for treatment. Exposure to 100 μM of nobiletin for 40 h did not affect the viability of the RAW 264.7 cells (Figure 2.1B). The viability of cells exposed to 200 μM nobiletin for 40 h was reduced to 74% of that of the control. Cells treated with nobiletin showed a similar dose-response for LDH release (Figure 2.1B). These data indicate that nobiletin does not affect the viability of RAW 264.7 cells at a concentration lower than 100 μM.

2.6.2 Nobiletin inhibits NO production and iNOS expression in LPS-activated RAW 264.7 cells

Treatment of cells with LPS (100 ng/ml) resulted in nitrite accumulation in the culture medium for the 24 h observation period, indicating NO production (Figure 2.2A). Simultaneous treatment of nobiletin with LPS significantly and dose-dependently decreased the production of NO. Nobiletin alone had no effect on the production of NO. To determine whether the altered synthesis of iNOS was responsible for the observed effect on NO production,the expression of iNOS mRNA and protein examined by RT-PCR and Western blot analyses, respectively. LPS increased the amounts of cellular iNOS protein and mRNA after 24- and 6-h treatments, respectively. Simultaneous treatment of nobiletin with LPS significantly

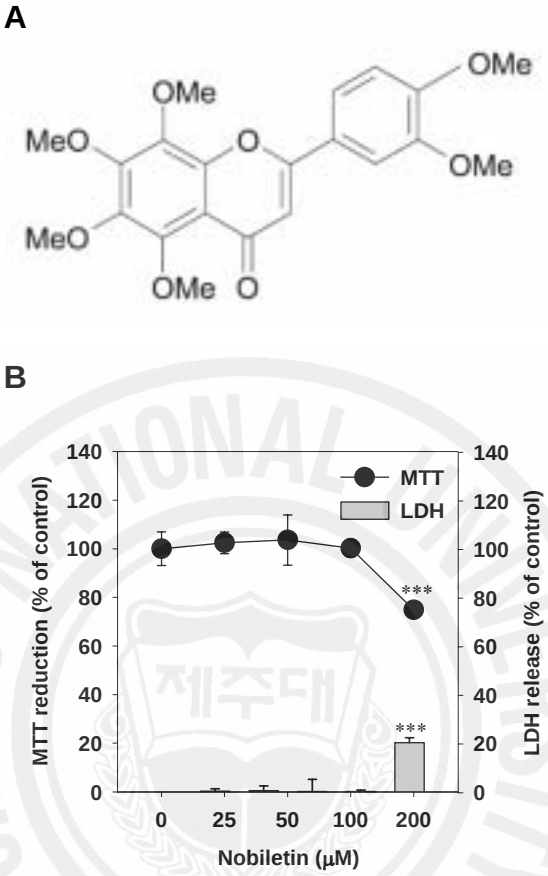


Figure 2.1. (A) Chemical structure of nobiletin. (B) Cytotoxicity of nobiletin on RAW 264.7 cells. Cells were treated with various concentrations of nobiletin for 40 h. Cell viability was represented by relative absorbance compared with controls. The LDH release was expressed as a percentage of total cellular LDH. Results from a representative experiment are expressed as mean ± SD (n = 4 in a single experiment). *** $P < 0.0005$ vs control without nobiletin.

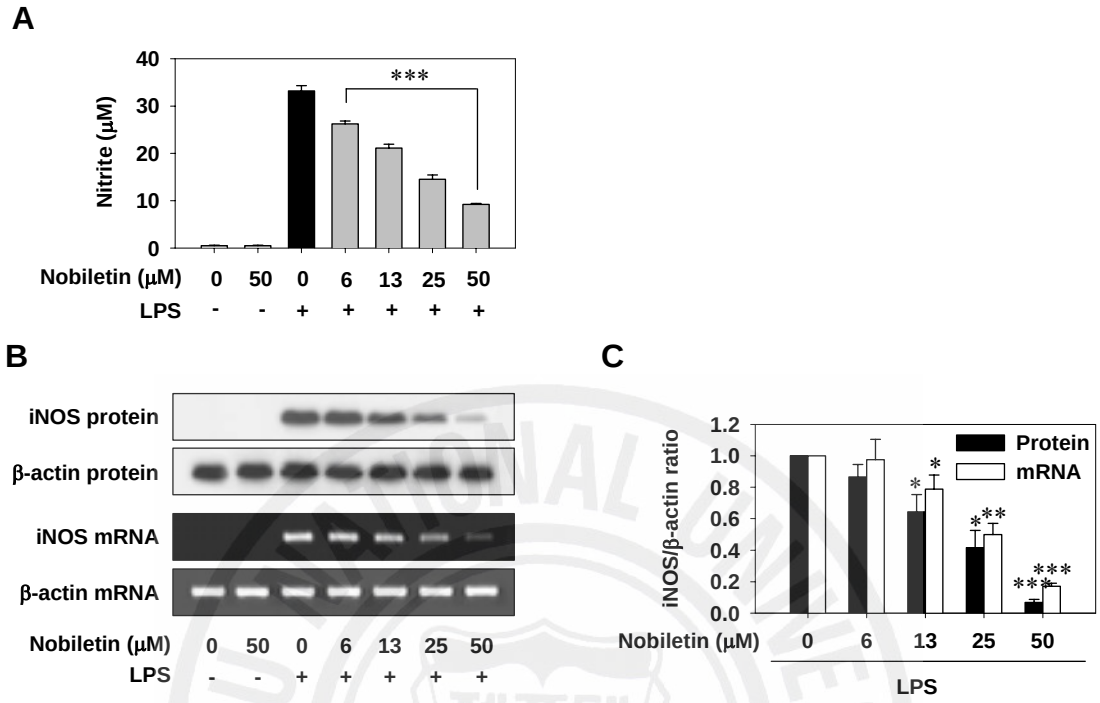


Figure 2.2. Effect of nobiletin on NO production and iNOS expression in LPS-activated RAW 264.7 cells. (A) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin at the indicated concentrations for 24 h. The amount of nitrite in the culture medium was determined by Griess reagent. (B) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin at the indicated concentrations for 24 h (for protein analysis) or 6 h (for mRNA analysis). Protein extracted from the cells was subjected to Western blot analysis and total RNA was subjected to RT-PCR. The figure shows a representative western blot or RT-PCR analysis from three independent experiments. (C) Graph representing the changes in the protein and mRNA levels of iNOS normalized by β-actin. The data represent the mean ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ vs LPS alone group.

attenuated both LPS-induced iNOS mRNA and protein in RAW 264.7 cells (Figure 2.2B). These results suggest that nobiletin inhibits the induction of NO production at the transcriptional level.

2.6.3 Nobiletin inhibits PGE_2 production and COX-2 expression in LPS-activated RAW 264.7 cells

PGE_2 was quantified in the culture medium of RAW 264.7 cells stimulated with LPS (100 ng/ml) for 24 h. Simultaneous treatment of nobiletin with LPS dose-dependently inhibited the production of PGE_2. Nobiletin in the absence of LPS did not alter the basal PGE_2 production (Figure 2.3A). Western blot and RT-PCR analyses showed that LPS stimulated expressions of COX-2 protein and mRNA. Simultaneous treatment of nobiletin with LPS significantly attenuated both LPS-induced COX-2 protein and mRNA expression (Figure 2.3B).

2.6.4 Nobiletin inhibits pro-inflammatory cytokines production and their mRNA expression in LPS-activated RAW 264.7 cells

The study examined whether nobiletin interfered with the production of pro-inflammatory cytokines in LPS-activated RAW 264.7 cells. When the cells were treated with LPS for 6 h, the production of TNF-α, IL-1β and IL-6 was augmented. Nobiletin dose-dependently and significantly diminished LPS-induced TNF-α, IL-1β and IL-6 production (Figure 2.4A). Nobiletin also significantly attenuated the mRNA levels of TNF-α, IL-1β and IL-6 (Figure 2. 4B).

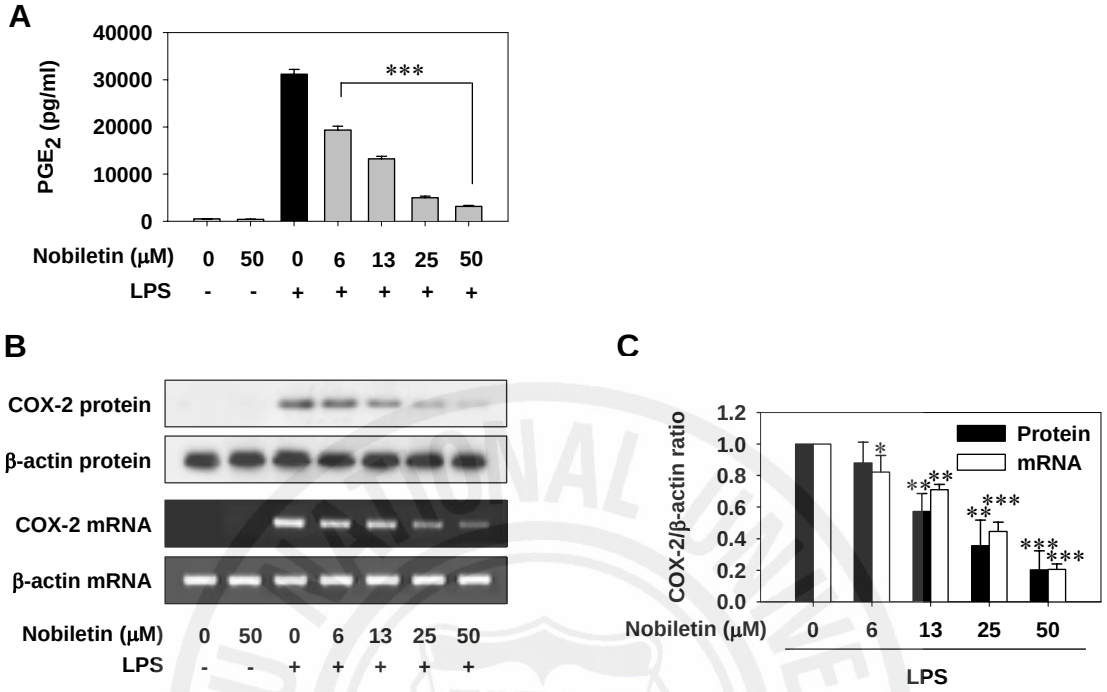


Figure 2.3. Effect of nobiletin on PGE_2 production and COX-2 expression in LPS-activated RAW 264.7 cells. (A) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin at the indicated concentrations for 24 h. The amount of PGE_2 in the culture medium was determined by enzyme-immunoassay. (B) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin at the indicated concentrations for 24 h (for protein analysis) or 6 h (for mRNA analysis). Protein extracted from the cells was subjected to Western blot analysis and total RNA was subjected to RT-PCR. The figure shows a representative western blot or RT-PCR analysis from three independent experiments. (C) Graph representing the changes in the protein and mRNA levels of COX-2 normalized by β-actin. The data represent the mean ± SD. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$ vs LPS alone group.

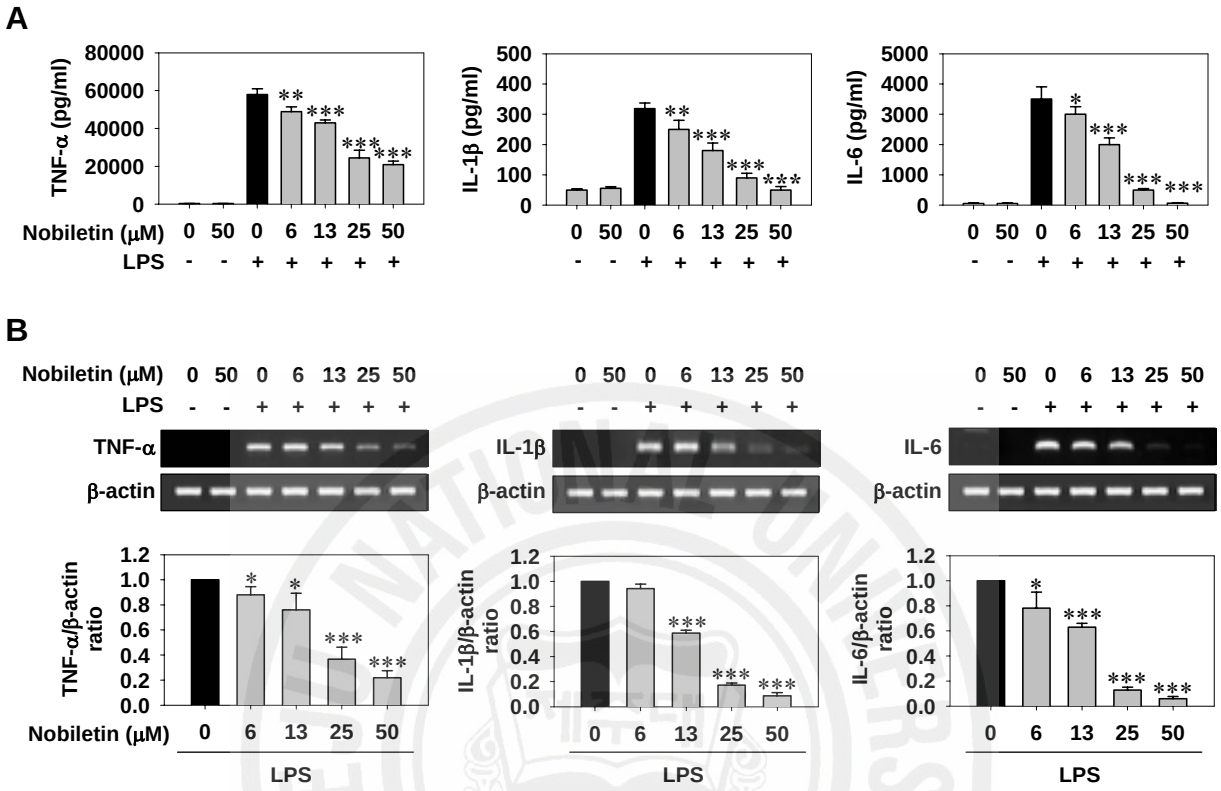


Figure 2.4. Effect of nobiletin on TNF-α, IL-1β and IL-6 production and their mRNA expression in LPS-activated RAW 264.7 cells. (A) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin at the indicated concentrations for 18 h. The amount of TNF-α, IL-1β and IL-6 released in the culture medium was determined by enzyme-immunoassay. (B) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin at the indicated concentrations for 6 h. Total RNA was subjected to RT-PCR. The figure shows a representative RT-PCR analysis from three independent experiments. The graph represents the changes in the mRNA levels of TNF-α, IL-1β and IL-6 normalized by β-actin. The data represent the mean ± SD. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005 vs LPS alone group.

2.6.5 Nobiletin inhibits the transactivation and DNA binding activity of NF-κB in LPS-activated RAW 264.7 cells

A transient transfection assay with an NF-κB-promoted luciferase reporter gene plasmid (pNF-κB-Luc) was carried out to investigate the effect of nobiletin on the NF-κB activation induced by LPS in RAW 264.7 cells. LPS treatment (100 ng/ml, 24 h) of the cells increased the luciferase activity by 8.5 ± 1.1-fold compared to the unstimulated controls. Nobiletin exposure suppressed the LPS induction of luciferase activity in a dose-dependent manner (Figure 2.5A). The inhibition of NF-κB activity may be the result of interference with various steps in the NF-κB activation pathway. The transactivating potential of transcription factors can be modulated by interacting coactivators or corepressors without affecting the DNA-binding potential of the transcription factor, the translocation of NF-κB from the cytoplasm to the nucleus, and/or the binding to DNA of the translocated transcription factor.

The effect of nobiletin on the DNA-binding activity of NF-κB was evaluated by EMSA. The nuclear extract from RAW 264.7 cells stimulated with LPS (100 ng/ml) caused a gel mobility shift of NF-κB-binding DNA oligomers at 30 min, 1 h and 4 h after LPS stimulation (Figure 2.5B, lanes 3, 5 and 7). However, the nuclear extract from cells treated simultaneously with nobiletin showed attenuation of the DNA-binding activity of NF-κB activated by LPS (Figure 2.5B, lanes 4, 6 and 8).

To confirm that the band associated with LPS-treated cells was indeed NF-κB protein, nuclear extracts were incubated with the antibody directed against the p65 subunits of NF-κB before conducting the EMSA. This supershift assay showed that the addition of the p65 antibody shifted the band to a higher molecular weight, which confirmed that the LPS-activated complex consisted of the p65 subunit (Figure 2.5C).

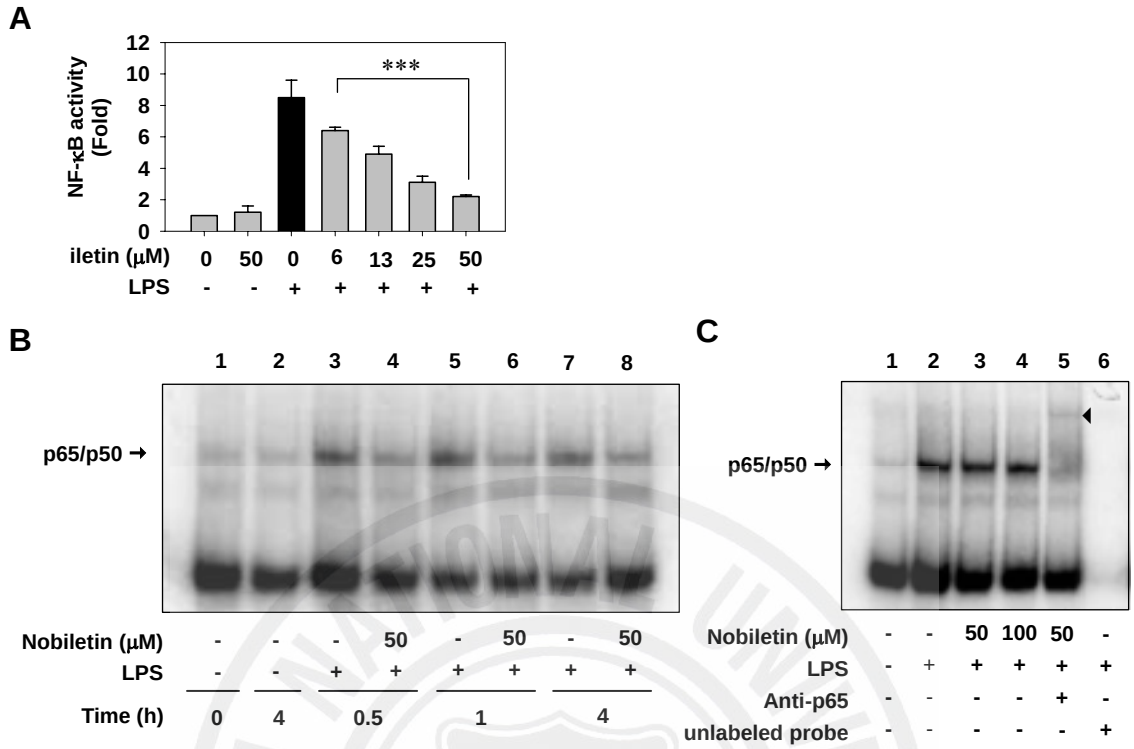


Figure 2.5. Effect of nobiletin on NF-κB activation in LPS-activated RAW 264.7 cells. (A) Cells were transiently cotransfected with NF-κB promoted luciferase reporter plasmid (pNF-κB-Luc) and Renilla luciferase reporter plasmid (pRL-TK) as internal control for 24 h, and then treated with LPS (100 ng/ml) in the presence or absence of nobiletin at the indicated concentrations for 24 h. The luciferase activity was measured and data were normalized by Renilla luciferase expression vector. The data represent the mean ± SD. ***$P < 0.0005$ vs LPS-activated group without nobiletin treatment. (B) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin (50 μM) for the indicated times. Then, nuclear extracts were prepared and assayed for nuclear NF-κB binding activity by EMSA: unstimulated (lanes 1 and 2); LPS-activated (lanes 3, 5, and 7); and LPS + nobiletin (lanes 4, 6, and 8). (C) Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin (50 or 100 μM) for 1 h. Nuclear extracts prepared from LPS-activated RAW 264.7 cells were incubated for 30 antibody against NF-κB subunit p65 (lane 5), or unlabeled NF-κB oligonucleotide as a competitor (lane 6).

Furthermore, this band disappeared when the unlabeled oligonucleotide was used, and the band was not affected by nobiletin addition (50 or 100 μM) prior to the reaction of the nuclear extracts (Figure 2.5C).

2.6.6 Nobiletin does not affect the phosphorylation and degradation of IκBα in LPS-activated RAW 264.7 cells

The effects of nobiletin on the phosphorylation and degradation of IκBα were investigated in LPS-activated RAW 264.7 cells. The amount of IκBα decreased markedly at 10 min, disappeared by 20 min, and reappeared approximately 45 min after LPS stimulation (Figure 2.6A). The time course and IκBα levels were not altered in the presence of nobiletin, indicating that nobiletin did not block IκBα degradation by LPS (Figure 2.6A). As a positive control, the study used MG-132, which is a peptide that blocks proteasome activity, thereby allowing phosphorylated IκBα to accumulate in the cells. Pre-incubation of cells in the presence of 10 μM MG-132 greatly enhanced the phospho-IκBα signal compared to MG-132-untreated cells after 30 min and 60 min of activation. However, nobiletin did not inhibit the accumulation of phospho-IκBα after 30 min and 60 min of activation (Figure 2.6B).

2.6.7 Nobiletin does not affect the nuclear translocation of NF-κB in LPS-activated RAW 264.7 cells

The effect of nobiletin on LPS-induced NF-κB nuclear translocation was analyzed by Western blot using the nuclear protein fractions. LPS induced the translocation from cytosol to the nucleus of the NF-κB p65 subunit in RAW 264.7

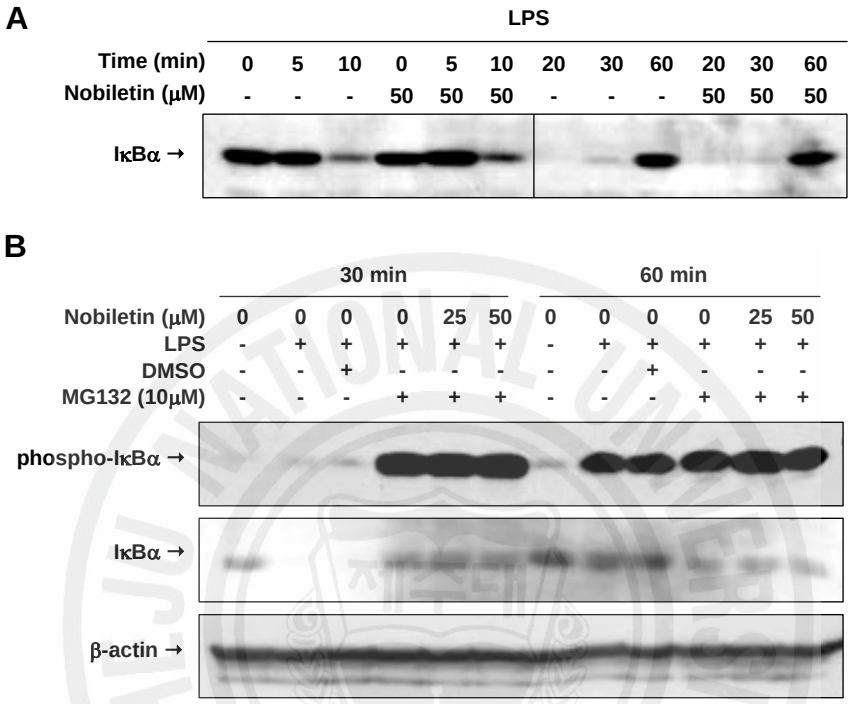


Figure 2.6. Effect of nobiletin on the phosphorylation and degradation of IκBα in LPS-activated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin for the indicated times. Whole-cell lysates were prepared and subjected to western blot analysis with (A) anti-IκBα antibody, and (B) anti-phospho-IκBα and anti-IκBα antibodies.

cells within 30 min and the translocation was sustained for up to 2 h (Figure 2.7 A). Nobiletin did not affect the LPS-induced nuclear translocation of the NF-κB p65 subunit. Because the maximal level of the NF-κB p65 subunit was observed at 30 min LPS-stimulation, this study examined the levels of the NF-κB p65 subunit in the nuclear extract at earlier times (5 and 10 min). LPS induced the nuclear translocation of the NF-κB p65 subunit in RAW 264.7 cells within 10 min while nobiletin did not affect the nuclear translocation of the NF-κB p65 subunit at these times (Figure 2.7 B).

2.6.8 Post-treatment with nobiletin inhibits NF-κB activation and iNOS and COX-2 expression in LPS-activated RAW 264.7 cells

It is well accepted that post-treatment with a therapeutic agent after the induction of a disorder more closely resembles a realistic clinical treatment regimen than pretreatment or cotreatment. In view of its pharmacological properties, this study investigated the effect of post-treatment of nobiletin on NF-κB activation and the production of inflammatory mediators in LPS-activated RAW 264.7 cells (Figure 2.8). Treatment with nobiletin 18 h after LPS activation significantly decreased LPS-induced NF-κB activation (Figure 2.8A), inhibited LPS-induced NO and PGE_2 production (Figure 2.8B and C), and decreased iNOS and COX-2 protein expression after 30 h of LPS activation (Figure 2.8D). The potent anti-inflammatory activities of nobiletin were comparable to those of PDTC (Figure 2.8).

To further confirm the evidence that nobiletin post-treatment attenuated NO production by inhibiting the prolonged expression of iNOS protein in LPS-activated RAW 264.7 cells, the study examined whether nobiletin has a direct NO scavenging

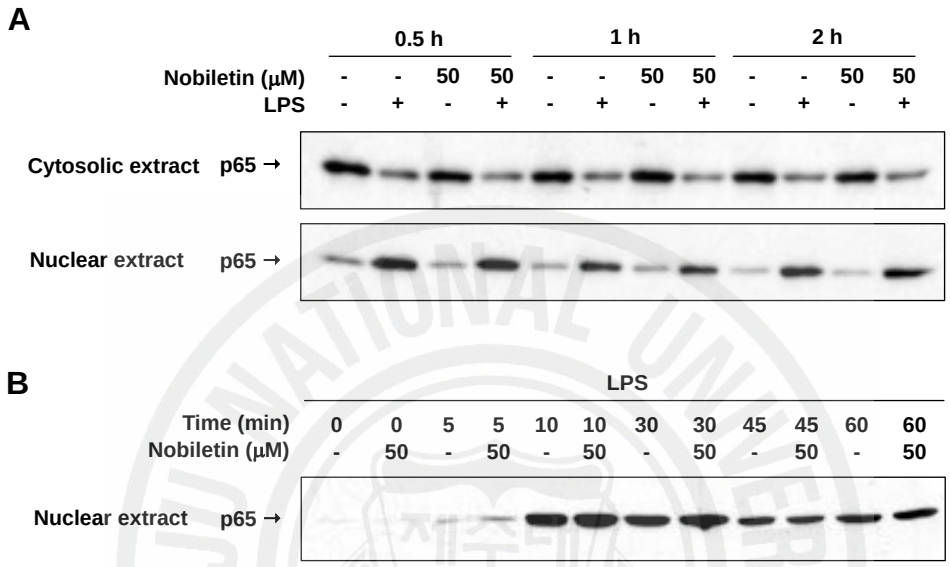


Figure 2.7. Effect of nobiletin on the nuclear translocation of NF-κB in LPS-activated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin for the indicated times. (A) Cytosolic and nuclear extracts were prepared and subjected to western blot analysis with anti-p65 antibody for NF-κB p65 subunit. (B) Nuclear extracts were prepared and subjected to western blot analysis with anti-p65 antibody for NF-κB p65 subunit.

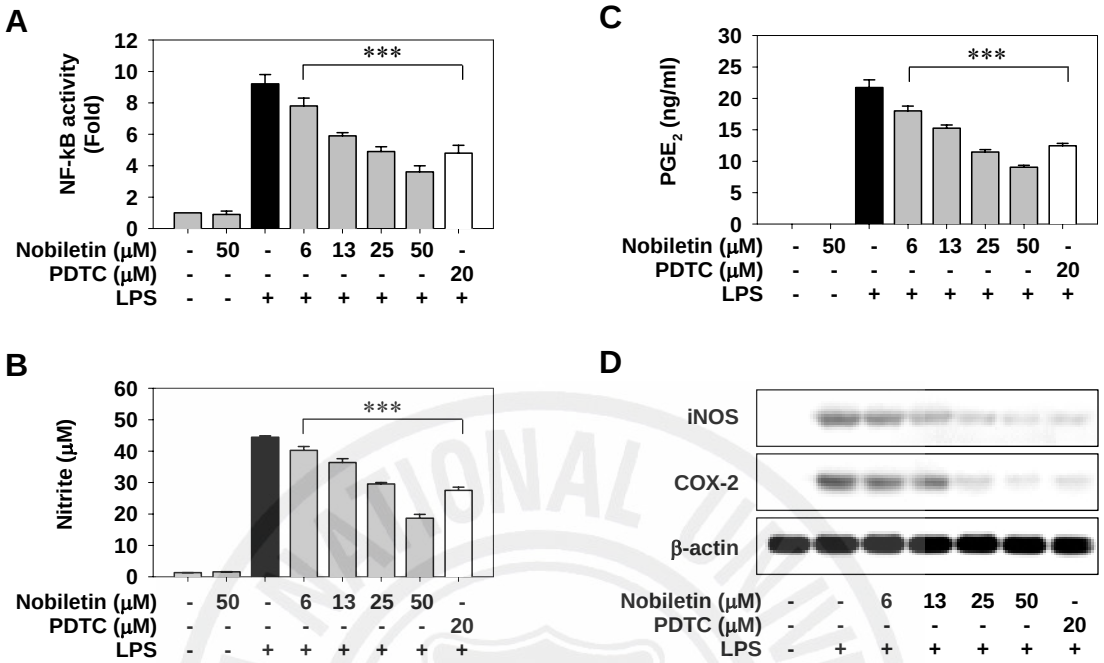


Figure 2.8. Effect of post-treatment with nobiletin in LPS-activated RAW 264.7 cells. (A) Cells were cotransfected with luciferase reporter gene plasmid and Renilla luciferase reporter plasmid as an internal control for 24 h. After stimulation by LPS for 12 h, cells were treated with nobiletin for 18 h. The cell extracts were assayed for luciferase activity and data were normalized by an internal Renilla luciferase vector. In other experiments, after stimulation by LPS for 12 h, cells were treated with nobiletin for 18 h. The levels of nitrite (B) and PGE_2 (C) released in the culture medium were assayed by Griess reagent and enzyme-immunoassay, respectively. (D) Protein extracted from the cells was subjected to Western blot analysis. The data represent the mean ± SD. ***$P < 0.0005$ vs LPS-activated group without nobiletin treatment.

activity or inhibitory effect on iNOS enzymatic activity. Nobiletin did not have either a direct scavenging effect on NO derived from SNP or an inhibitory effect on the enzymatic activity of iNOS. Astaxanthin and 1400W (a selective iNOS inhibitor), used as positive controls, significantly scavenged NO and inhibited iNOS activity, respectively (Figure 2.9).

2.6.9 Nobiletin inhibits ROS production in LPS-activated RAW 264.7 cells

It has been reported that LPS-triggered inflammatory responses induce ROS production and increase cellular oxidative stress (Pattanaik & Prasad, 1996). When RAW 264.7 cells were cultured with LPS, the accumulation of intracellular ROS was not detected until 4 h after treatment. However, the ROS levels increased in a time-dependent manner during prolonged LPS exposure for 12–40 h (Figure 2.10A), which is in accordance with the results of another study (Chandel *et al*., 2000). Nobiletin treatment blocked the elevation of intracellular ROS levels by LPS (Figure 2.10B). Thakur *et al*. (2006) have implicated NADPH-oxidase in ROS generation in macrophages. Thus, using the NADPH-oxidase inhibitor diphenyleneiodonium (DPI), this study tested whether NADPH-oxidase contributed to the LPS-induced ROS production. As expected, DPI attenuated the LPS-induced ROS elevation in RAW 264.7 cells (Figure 2.10B). PDTC also decreased LPS-induced ROS production, whereas nobiletin, PDTC or DPI alone had no effect on ROS production (Figure 2.10B). RAW 264.7 cells by using the NADPH oxidase inhibitor, diphenyleneiodonium (DPI). DPI attenuated the LPS-induced ROS elevation in RAW 264.7 cells. PDTC, NF-κB inhibitor, also decreased LPS-induced ROS production. Nobiletin, PDTC and DPI alone had no effect on ROS production (Figure 2.10B).

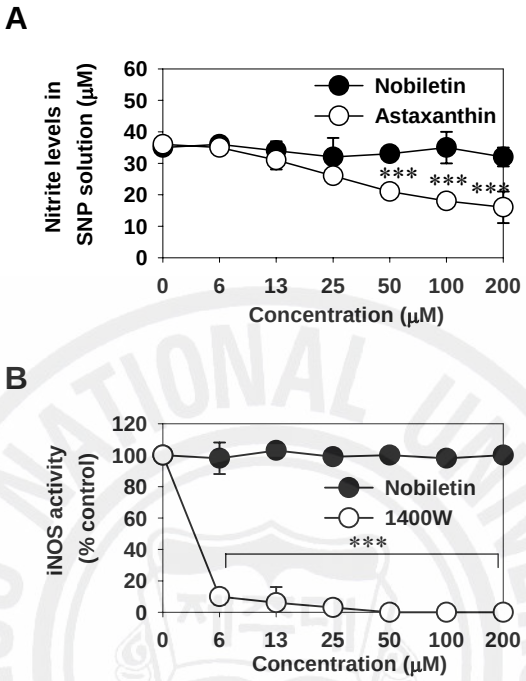


Figure 2.9. Effect of nobiletin on nitrite levels in SNP solution and iNOS activity. (A) SNP solution was incubated alone or in combination with different concentrations of nobiletin at room temperature and nitrite levels were estimated after 180 min using Greiss reagent. Astaxanthin was used for positive control. (B) Cell lysates were prepared at 20 h after treatment with LPS (100 ng/ml). Nobiletin was added to the crude extracts of LPS-activated RAW 264.7 cells 5 min prior to the iNOS activity assay. 1400W, a selective iNOS inhibitor was used for positive control. The data represent the mean ± SD (n=3). ***P < 0.0005 vs control or positive control.

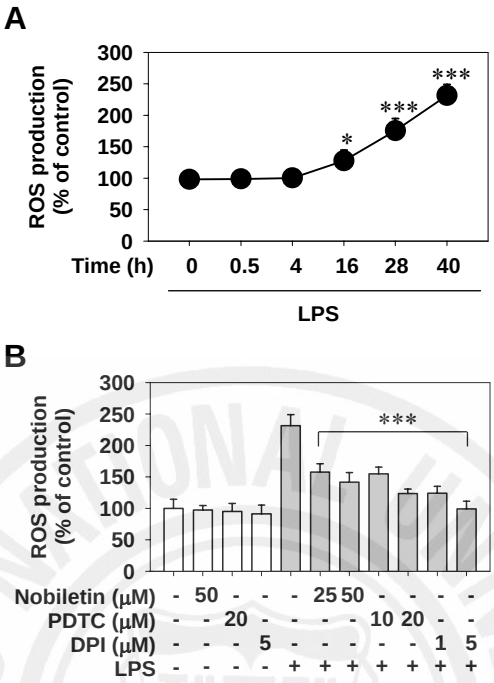


Figure 2.10. Effect of nobiletin on ROS accumulation in LPS-activated RAW 264.7 cells. (A) Cells were incubated for 30 min with 50 μM CM-H_2DCFDA, washed twice with phosphate-buffered saline, and then incubated with LPS. The fluorescence of DCF was measure at 485 nm (excitation) and 520 nm (emission) at the indicated times. (B) Cells were incubated for 30 min with 50 μM CM-H_2DCFDA, washed twice with phosphate-buffered saline, and then incubated with LPS in the presence or absence of nobiletin, PDTC, or DPI. The fluorescence of DCF was measure at 485 nm (excitation) and 520 nm (emission) for 40 h. The data represent the mean ± SD (n=3). *P < 0.05, ***P < 0.0005 vs control group without nobiletin treatment

2.6.10 Nobiletin does not affect the activation of ERK1/2, JNK1/2 and p38 MAPK in LPS-activated RAW 264.7 cells

LPS activates MAPK signal-transduction pathways in RAW 264.7 cells (Rao *et al*., 2002). At the molecular level, MAPK activation relies on the phosphorylation of both tyrosine and threonine residues. The study assayed the effect of nobiletin on MAPK activation by examining the phosphorylation of ERK1/2, JNK1/2, and p38 MAPK by Western blot analyses (Figure 2.11). Stimulation of cells with LPS increased the phosphorylation of all three MAPKs. However, this phosphorylation of ERK1/2, JNK1/2 and p38 was transient: reaching the maximum stimulation at 30 min and returning to the basal level at 1 h. Nobiletin did not affect LPS-induced phosphorylation of ERK1/2, JNK1/2, and p38 MAPK.

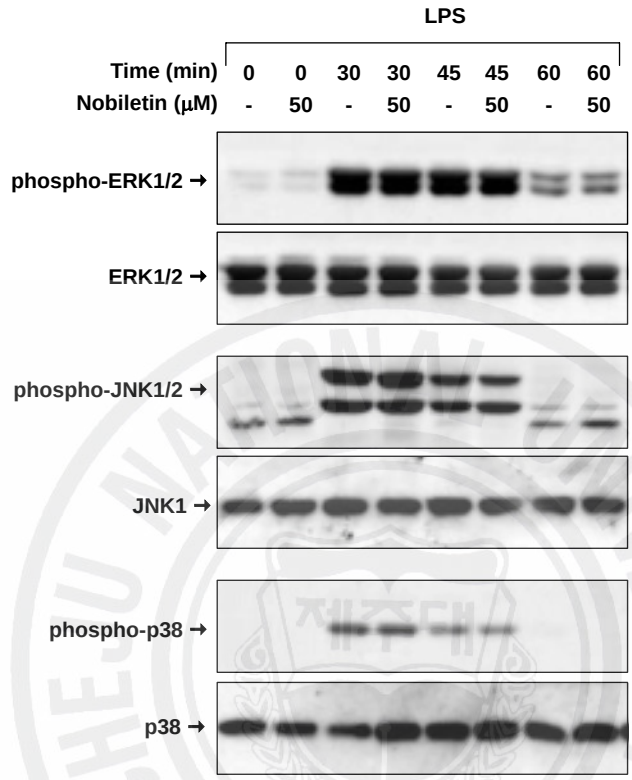


Figure 2.11. Effect of nobiletin on the activation of ERK1/2, JNK1/2 and p38 MAPK in LPS-activated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) in the presence or absence of nobiletin (50 μM) for the indicated times. Whole-cell lysates were prepared and subjected to Western blot analysis with antibodies against activated ERK1/2, JNK1/2 or p38 MAPK. Total ERK1/2, JNK1/2 and p38 MAPK were detected to ensure equal loading of proteins.

2.7 DISCUSSION

Nobiletin, a polymethoxylated flavone found exclusively in citrus fruit peel, is currently recognized as a promising anti-inflammatory and anti-tumor agent (Kandaswami *et al*., 1991; Kawaii *et al*., 1999; Wu *et al*., 2006). Despite various biological activities, the molecular mechanism of its action has not been sufficiently understood. Recently, it has been shown that nobiletin inhibits LPS-induced NF-κB transcriptional activation in mouse macrophages (Murakami *et al*., 2005). However, it remains to be elucidated how nobiletin inhibits NF-κB activation. The present study shows that nobiletin inhibits NF-κB activation by targeting the DNA-binding activity.

Our present study has shown that simultaneous treatment of nobiletin dose-dependently inhibits the production of three LPS-induced pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, as well as two pro-inflammatory enzymes, iNOS and COX-2 at the transcriptional levels. In addition, post-treatment with nobiletin also significantly reduced the expression of iNOS and COX-2 protein at 12 h after LPS stimulation. Nobiletin inhibited the transactivation of NF-κB and DNA binding activity of p50/p65, the heterodimer of NF-κB, following LPS stimulation. The suppression was observed at the concentration range required for the inhibition of pro-inflammatory mediator production. Decreased p50/p65 nuclear binding in nobiletin-treated cells is of particular interest because NF-κB with this subunit composition potently trans-activated the target genes (Baeuerle and Henkel, 1994; Kopp and Ghosh, 1995).

The release of cytosolic NF-κB from the NF-κB/IκB complex requires

phosphorylation of IκB by the IKK multiprotein complex, followed by ubiquitination and degradation by the 26S proteasome to generate transcriptionally active NF-κB that undergoes rapid translocation to the nucleus for the DNA binding (Baldwin, 1996). Although there are a number of IκB proteins, IκBα is the primary regulator of rapid signal-induced activation of NF-κB. After degradation, the cytoplasmic IκBα is rapidly replenished by an accelerated production of the protein which is, at least in part, transcriptionally regulated (Sun *et al*., 1993). To investigate the mechanism by which nobiletin inhibits NF-κB activation, the study examined the effect of nobiletin on LPS-induced nuclear translocation of NF-κB. The time/NF-κB level course was not altered in the presence of nobiletin. Nor nobiletin affected LPS-induced phosphorylation and degradation of IκBα protein, suggesting that the molecular target for nobiletin is not the dissociation of NF-κB from IκBα. These results indicate that the inhibitory effect of nobiletin on the production of pro-inflammatory mediators may involve transcriptional regulation through suppression of NF-κB DNA binding activity without interfering with nuclear translocation of NF-κB in LPS-activated RAW 264.7 cells.

Inhibition of NF-κB activation without interfering with nuclear translocation of NF-κB has been reported for PDTC (NF-κB inhibitor) and herbimycin A (a tyrosine kinase inhibitor). PDTC and herbimycin A have been shown to block DNA binding by a covalent modification on the NF-κB p50 subunit in EL4.NOB-1 cells (Brennan and O'Neill, 1996; Mahon and O'Neill, 1995). Nobiletin itself is unlikely to react directly with NF-κB, since adding nobiletin to nuclear extracts directly did not inhibit DNA binding. This suggests either that a metabolite of nobiletin generated in cells is responsible for the effect or that a nobiletin-induced product drives the inhibition of interaction between NF-κB and DNA. Brennan and O'Neill (1996) have

shown that PDTC increases oxidation of glutathione disulfide to glutathione disulfide leading to the formation of a mixed disulphide with NF-κB, thereby inhibiting DNA binding. How nobiletin suppresses NF-κB DNA binding activity in LPS-activated RAW 264.7 cells is unclear at present. Further investigations will be focused on elucidating the precise mechanism of nobiletin-dependent modulation of DNA binding of NF-κB protein.

Many signaling pathways, including the MAPK pathway, are proposed in response to LPS stimulation. LPS activates all three types of MAPKs (ERK1/2, p38, and JNK1/2) in mouse macrophages and human monocytes (Scherle *et al*., 1998; Suh *et al*., 2006). Although the mechanism by which MAPKs regulate LPS-induced inflammatory responses in macrophages is still unclear, it is known that the MAPK signaling pathway is involved in the production of pro-inflammatory mediators in LPS-activated macrophages. In the present results, LPS resulted in the phosphorylation of ERK1/2, JNK1/2 and p38 MAPK in RAW 264.7 cells, and this LPS-activated phosphorylation was not affected by nobiletin.

LPS-triggered inflammatory responses induce ROS production via the activation of NADPH oxidase in macrophages (DeLeo *et al*., 1998). ROS stimulates the redox-based activation of NF-κB and proinflammatory cytokine gene transcription (Kabe *et al*., 2005; Leeper-Woodford and Detmer, 1999). In our study, LPS did not acutely increase ROS production (within 120 min), but did induce ROS generation after 8–48 h. These results indicate that LPS induces NF-κB activation in a ROS-independent manner. Previous studies indicated that ROS were required for the hypoxic activation of NF-κB and TNF-α gene transcription, but not for the LPS-induced activation of NF-κB (Chandel *et al*., 2000). In the present study, nobiletin displayed the ability to alleviate oxidative stress by inhibiting the LPS-mediated ROS

accumulation in RAW 264.7 cells.

In conclusion, the present study has shown that nobiletin inhibits the expression of inflammation-associated genes by targeting the DNA-binding activity without interfering with the nuclear translocation of NF-κB (Figure 2.12), and that nobiletin suppresses ROS production, resulting in redox-based NF-κB activation in LPS-activated RAW 264.7 cells.

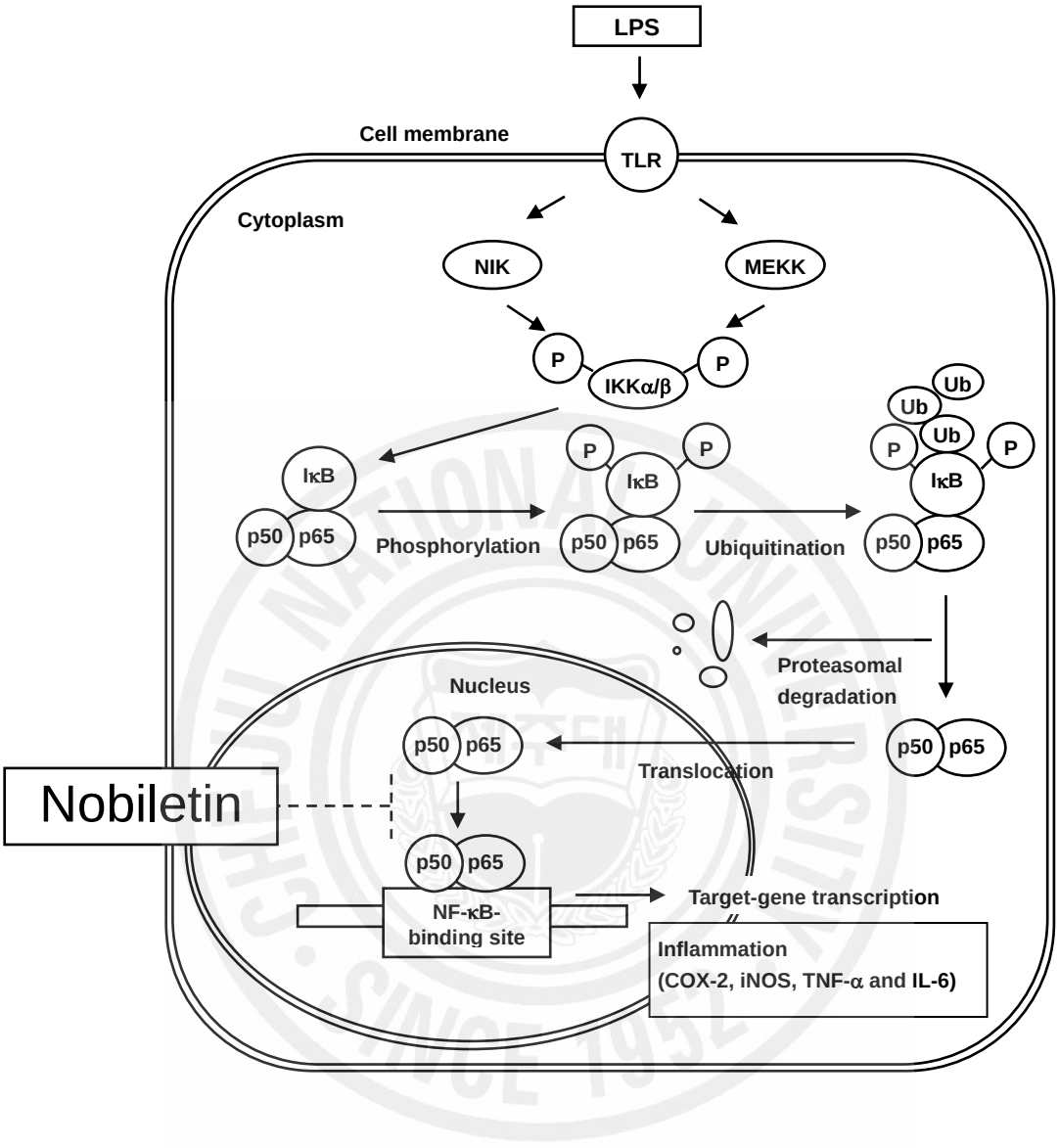


Figure 2.12. Illustration of the proposed action mechanisms by which nobiletin attenuates the expression of pro-inflammatory mediators in LPS-activated RAW 264.7 cells. Nobiletin may disturb the DNA binding process of NF-κB, because it suppressed NF-κB-DNA binding activity without interfering with the nuclear translocation of NF-κB.

REFERENCES

Baeuerle PA and Henkel T (1994) Function and activation of NF-κB in the immune system. Annu Rev Immunol 12:141-179.

Baldwin Jr AS (1996) The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 14:649-683.

Balestrieri ML, Castaldo D, Balestrieri C, Quagliuolo L, Giovane A and Servillo L (2003) Modulation by flavonoids of PAF and related phospholipids in endothelial cells during oxidative stress. J Lipid Res 44:380-387.

Barnes PJ and Karin M (1997) Nuclear factor-κB, a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066-1071.

Berkarda B, Koyuncu H, Soybir G and Baykut F (1998) Inhibitory effect of hesperidin on tumor initiation and promotion in mouse skin. Res Exp Med 198: 93-99.

Wichtl M and Bisset NG (1994) Herbal Drugs and Phytopharmaceuticals. 2nd Ed. Stuttgart: Medpharm Scientific Publishers.

Blumenthal M, Busse WR, Goldberg A, Gruenwald J, Hall T, Riggins CW and Rister RS (1998) The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines. Austin: American Botanical Council.

Bracke ME, Bruyneel EA, Vermeulenm SJ, Vennekens K, Marck VV, Mareel MM (1994) Citrus flavonoid effect on tumor invasion and metastasis. Food Techonol 48:121-124.

Brennan P and O'Neill LA (1996) 2-mercaptoethanol restores the ability of nuclear factor κB (NF κB) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrollidine dithiocarbamate (PDTC). Evidence for oxidation of glutathione in the mechanism of inhibition of NF-κB by PDTC. Biochem J 320:975-981.

Chandel NS, Trzyna WC, McClintock DS and Schumacker PT (2000) Role of oxidants in NF-κB activation and TNF-α gene transcription induced by hypoxia and endotoxin. J Immunol 165:1013-1021.

Chen YT, Zheng RL, Jia ZY and Ju Y (1990) Flavonoids as superoxide scavengers and antioxidants. Free Radic Biol Med 9:19-21.

Delaney B, Phillips K, Buswell D, Mowry B, Nickels D, Cox D, Wang HB and Manthey J (2001) Immunotoxicity of a standardized citrus polymethoxylated flavone extract. Food Chem Toxicol 39:1087-1094.

DeLeo FR, Renee J, McCormick S, Nakamura M, Apicella M, Weiss JP and Nauseef WM (1998) Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. J Clin Invest 101:455-463.

Emim JAS, Oliveira AB and Lapa AJ (1994) Pharmacological evaluation of the anti-inflammatory activity of a Citrus bioflavonoid, hesperidin, and the isoflavonoids, duartin, and claussequinone, in rats and mice. J Pharm Pharmacol 46: 118–122.

Federica DL and Sergio FDB (2005) Citrus flavonoids as bioactive compounds: role, bioavailability, socio-economic impact and biotechnological approach for their modification, Contributed paper, 9th ICABR International Conference on Agricultural Biotechnology: Agricultural Biotechnology: Ten Years After,

Ravello (Italy), July 9- 10.

Feelisch M and Stamler JS (1996) Donors of nitrogen oxides. In: Feelisch, M., Stamler, J.S. (Ed.), Methods in Nitric Oxide Research, John Wiley & Sons, Chichester, England, pp 2-115.

Ferrari M, Fornasiero MC and Isetta AM (1990) MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. J Immunol Methods 131: 165-172.

Francis AR, Shetty TK and Bhattacharya RK (1989) Modulating effect of plant flavonoids on the mutagenicity of N-methyl-N-nitrosoguanidine, Carcinogenesis 10: 1953-1955.

Gabor M (1988) Szent-Györgyi and the bioflavonoids: new results and perspectives of pharmacological research into benzo-pyrone derivatives. In: V. Cody, E. Middleton Jr., J.B. Harborne and A. Beretz, Editors, Plant flavonoids in biology and medicine II: biochemical, cellular and medicinal properties, Alan R Liss, Inc., New York, pp. 1-15.

Gallo O, Masini E, Morbidelli L, Franchi A, Fini-Storchi I, Vergari WA and Ziche M (1998) Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. J Natl Cancer Inst 90: 587-596.

Gilroy DW, Lawrence T, Perretti M and Rossi AG (2004) Inflammatory resolution: new opportunities for drug discovery. Nat Rev Drug Discov 3: 401-416.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal Biochem 126: 131-138.

Guardia T, Rotelli ae, Juarez AO and Pelzer LE (2001) Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Farmaco 56: 683-687.

Guengerich FP and Kim DH (1990) In vitro inhibition of dihydropyridine oxidation and aflatoxin B1 activation in human liver microsomes by naringenin and other flavonoids. Carcinogenesis 11: 2275–2279.

He Q, Riley RT and Sharma RP (2002) Pharmacological antagonism of fumonisin B1 cytotoxicity in porcine renal epithelial cells (LLC-PK1): a model for reducing fumonisin-induced nephrotoxicity in vivo. Pharmacol Toxicol 90: 268-277.

Hobbs AJ, Higgs A and Moncada S (1999) Inhibition of nitric oxide synthase as a potential therapeutic target. Annu Rev Pharmacol Toxicol 39: 191-220.

Hsieh GS and Acosta D (1991) Dithranol-induced cytotoxicity in primary cultures of rat epidermal keratinocytes. Toxicol Appl Pharmacol 107: 16-26.

Huang B and Y Wang (1993) Thousand Formulas and Thousand Herbs of Traditional Chinese Medicine, vol. 1, Heilongjiang Education Press, Harbin.

Ishiwa J, Sato T, Mimaki Y, Sashida Y, Yano M and Ito A (2000) A citrus flavonoid, nobiletin, suppresses production and gene expression of matrix metalloproteinase 9/gelatinase B in rabbit synovial fibroblasts. J Rheumatol 27: 20-25.

Jung UJ, Kim HJ, Lee JS, Lee MK, Kim HO, Park EJ, Kim HK, Jeong TS and Choi MS (2003) Naringin supplementation lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects. Clin Nutr 22: 561-568.

Kabe Y, Ando K, Hirao S, Yoshida M and Handa H (2005) Redox regulation of NF-κB activation: distinct redox regulation between the cytoplasm and the nucleus. Antioxid Redox Signal 7: 395-403.

Kandaswami C, Perkins E, Soloniuk DS, Drzewiecki G and Middleton E (1991) Antiproliferative effect of Citrus flavonoids on a human squamous cell carcinoma in vitro. Cancer Lett 56: 147–152.

Kawaguchi K, Kikuchi S, Hasegawa H, Maruyama H, Morita H and Kumuzawa Y (1999) Suppression of lipopolysaccharide-induced tumor necrosis factor-release and liver injury in mice by naringin. Eur J Pharmacol 368: 245-250.

Kawaii S, Tomono Y, Katase E, Ogawa K and Yano M (1999) HL-60 differentiating activity and flavonoid content of the readily extractable fraction prepared from citrus juices. J Agric Food Chem 47: 128-135.

Kefaloyianni E, Gaitanaki C and Beis I (2006) ERK1/2 and p38-MAPK signalling pathways, through MSK1, are involved in NF-κB transactivation during oxidative stress in skeletal myoblasts. Cell Signal 18: 2238-2251.

Kim HK, Jeong TS, Lee MK, Park YB and Choi MS (2003) Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats. Clin Chim Acta 327: 129-137.

Kobayashi S and Tanabe S (2006) Evaluation of the anti-allergic activity of Citrus unshiu using rat basophilic leukemia RBL-2H3 cells as well as basophils of patients with seasonal allergic rhinitis to pollen. Int J Mol Med 17: 511-515.

Kopp E and Ghosh S (1995) NF-κB and Rel proteins in innate immunity. Adv

Immunol 58: 1-27.

Kronche KD, Fehsel K and Kolb-Bachofen V (1998) Inducible nitric oxide synthase in human diseases. Clin Exp Immunol 113: 147-156.

Leeper-Woodford SK and Detmer K (1999) Acute hypoxia increases alveolar macrophage tumor necrosis factor activity and alters NF-κB expression. Am J Physiol 276: L909-L916.

Lin N, Sato T, Takayama Y, Mimaki Y, Sashida Y, Yano M and Ito A (2003) Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. Biochem Pharmacol 65: 2065-2071.

Mahon TM and O'Neill LA (1995) Studies into the effect of the tyrosine kinase inhibitor herbimycin A on NF-κB activation in T lymphocytes. Evidence for covalent modification of the p50 subunit. J Biol Chem 270: 28557-28564.

Manthey JA and Grohmann K (1998) In Flavonoids in the Living System; Manthey, J.A.; Buslig, B.S., Eds.; Plenum Press: New York, pp. 85.

Manthey JA, Grohmann K, Montanari A, Ash K and Manthey C (1999) Polymethoxylated flavones derived from citrus suppress tumor necrosis factor-α expression by human monocytes. J Nat Prod 62: 441-444.

Manthey JA, Guthrie N and Grohmann K (2001) Biological Properties of Citrus Flavonoids Pertaining to Cancer and Inflammation. Curr Med Chem 8: 135-153.

Martin MJ, Marhuenda E, Perz-Guerrero C and Franco JM (1994) Antiulcer effect of naringin on gastric lesions induced by ethanol in rats. Pharmacology 49: 144-150.

Martin MJ, Motilva V and Alarcon de la Lastra C (1993) Quercetin and naringenin:

Effects on ulcer formation and gastric secretion in rats. Phytother Res 7: 150-153.

Miyazawa M, Okuno Y, Fukuyama M, Nakamura S and Kosaka H (1999) Antimutagenic activity of polymethoxyflavonoids from Citrus aurantium. J Agric Food Chem 47: 5239-5244.

Mordan LJ, Burnett TS, Zhang LX, Tom J and Cooney RV (1993) Inhibitors of endogenous nitrogen oxide formation block the promotion of neoplastic transformation in C3H10T1/2 fibroblasts. Carcinogenesis 14: 1555-1559.

Motilva V, Alarcon De La Lastra C and Martin MJ (1992) Effects of naringenin and quercetin on experimental chronic gastric ulcer in rats: Studies on the histological findings. Phytother Res 6: 168-170.

Motilva V, Alarcon de la Lastra C and Martin MJ (1993) Ulcer protecting effects of naringenin on gastric lesions induced by ethanol in rat: Role of endogenous prostaglandins. J Pharm Pharmacol 46: 91-94.

Murakami A, Matsumoto K, Koshimizu K and Ohigashi H (2003) Effects of selected food factors with chemopreventive properties on combined lipopolysaccharide- and interferon-gamma-induced IκB degradation in RAW264.7 macrophages. Cancer Lett 195: 17-25.

Murakami A, Shigemori T, Ohigashi H (2005) Zingiberaceous and citrus constituents, 1'-acetoxychavicol acetate, zerumbone, auraptene, and nobiletin, suppress lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 murine macrophages through different modes of action. J Nutr 135: 2987S-2992S.

Murakami A., Nakamura Y, Torikai K, Tanaka T, Koshiba T, Koshimizu K,

Kuwahara S, Takahashi Y, Ogawa K, Yano M, Tokuda H, Nishino H, Mimaki Y, Sashida Y, Kitanaka S and Ohigashi H (2000a) Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. Cancer Res 60: 5059-5066.

Murakami A, Nakamura Y, Ohto Y, Yano M, Koshiba T, Koshimizu K, Tokuda H, Nishino H and Ohigashi H (2000b) Suppressive effects of citrus fruit peels on free radical generation and nobiletin, an anti-inflammatory polymethoxyflavonoid. Biofactors 12: 187-192.

Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, Yano M and Ohta H (2006) Flavonoid composition of fruit tissues of citrus species. Biosci Biotechnol Biochem 70: 178-192.

Ohshima H and Bartsch H (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat Res 305: 253-264.

Palsson-McDermott EM and O'Neill LA (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 113: 153-162.

Parmar NS (1983) The gastric antiulcer activity of naringenin, a specific histidine decarboxylase inhibitor. Int J Tiss Res 4: 415-420.

Pattanaik U and Prasad K (1996) Endotoxemia and oxidative stress. Ann N Y Acad Sci 793: 506-510.

Rao KM, Meighan T and Bowman L (2002) Role of mitogen-activated protein kinase activation in the production of inflammatory mediators: differences

between primary rat alveolar macrophages and macrophage cell lines. J Toxicol Environ Health A 65: 757-768.

Reis K, Halldin J, Fernaeus S, Pettersson C and Land T (2006) NADPH oxidase inhibitor diphenyliodonium abolishes lipopolysaccharide-induced down-regulation of transferrin receptor expression in N2a and BV-2 cells. J Neurosci Res 84: 1047-1052.

Robak J and Gryglewski R (1988) Flavonoids are scavengers of superoxide anions. Biochem Pharmacol 37: 837-841.

Rosbe KW, Prazma J, Petrusz P, Mims W, Ball SS and Weissler MC (1995) Immunohistochemical characterization of nitric oxide synthase activity in squamous cell carcinoma of the head and neck. Otolaryngol Head Neck Surg 113: 541-549.

Scherle PA, Jones EA, Favata MF, Daulerio AJ, Covington MB, Nurnberg SA, Magolda RL and Trzaskos JM (1998) Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes. J Immunol 161: 5681–5686.

Schreck R, Rieber P and Baeuerle PA (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 10: 2247-2258.

Spolarics Z (1998) Endotoxemia, pentose cycle, and the oxidant/antioxidant balance in the hepatic sinusoid. J Leukoc Biol 63: 534-541.

Suh SJ, Chung TW, Son MJ, Kim SH, Moon TC, Son KH, Kim HP, Chang HW and

Kim CH (2006) The naturally occurring biflavonoid, ochnaflavone, inhibits LPS-induced iNOS expression, which is mediated by ERK1/2 via NF-κB regulation, in RAW264.7 cells. Arch Biochem Biophys 447: 136-146.

Sun SC, Ganchi PA, Ballard DW and Greene WC (1993) NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science 259: 1912-1915.

Tanaka S, Sato T, Akimoto N, Yano M and Ito A (2004) Prevention of UVB-induced photoinflammation and photoaging by a polymethoxy flavonoid, nobiletin, in human keratinocytes in vivo and in vitro. Biochem Pharmacol 68: 433-439.

Tanaka T, Makita H, Kawabata K, Mori H, Kakumoto M, Satoh K, Hara A, Tanaka T and Ogwa H (1997) Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. Carcinogenesis 18: 957-965.

Thakur V, Pritchard MT, McMullen MR, Wang Q and Nagy LE (2006) Chronic ethanol feeding increases activation of NADPH oxidase by lipopolysaccharide in rat Kupffer cells: role of increased reactive oxygen in LPS-activated ERK1/2 activation and TNF-α production. J Leukoc Biol 79: 1348-56.

Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowledges RG and Moncada S (1995) Nitric oxide synthase activity in human breast cancer. Br J Cancer 72: 41-45.

Tokyo Society of Pharmacognosy, http://www.aa.alpha-net.ne.jp/rei03/chinpi.htm (October 31, 2006)

Uchikura K, Wada T, Hoshino S, Nagakawa Y, Aiko T, Bulkley GB, Klein AS and Sun Z (2004) Lipopolysaccharides induced increases in Fas ligand expression by Kupffer cells via mechanisms dependent on reactive oxygen species. Am J Physiol Gastrointest Liver Physiol 287: G620-626.

Wang MJ, Huang HM, Chen HL, Kuo JS and Jeng KC (2001) Dehydroepiandrosterone inhibits lipopolysaccharide-induced nitric oxide production in BV-2 microglia. J Neurochem 77: 830-838.

Wiseman H and Halliwell B (1996) Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J 313: 17-29.

Wong WS (2005) Inhibitors of the tyrosine kinase signaling cascade for asthma. Curr Opin Pharmacol 5: 264-271.

Wu YQ, Zhou CH, Tao J and Li SN (2006) Antagonistic effects of nobiletin, a polymethoxyflavonoid, on eosinophilic airway inflammation of asthmatic rats and relevant mechanisms. Life Sci 78: 2689-2696.

Zhang L, Ma Y, Zhang J, Cheng J and Du J (2005) A new cellular signaling mechanism for angiotensin II activation of NF-κB: An IκB-independent, RSK-mediated phosphorylation of p65. Arterioscler Thromb Vasc Biol 25: 1148-1153.

초 록

1. 제주 감귤 과피 추출물의 플라보노이드 함량 분석 및 LPS로 활성화된 대식세포주 RAW 264.7 세포에서 NO 생성에 미치는 영향:

감귤 과피 추추물의 NO 생성 억제 활성에 대한 노빌레틴 함량의 기여

감귤의 과피는 중국전통의학과 서양근대의학에서 염증을 포함한 다양한 질병의 치료를 위하여 사용되었다. 감귤 과피에는 다양한 생리활성을 나타내는 플라보노이드들이 다량 함유되어 있는 것으로 알려져 있으며 플라보노이드의 함량 및 분포는 유전적 요인 뿐 만 아니라 환경적 요인에 의해서도 변이가 매우 높은 것으로 보고되었다.

염증과 암은 감귤 유래 플라보노이드의 생물학적 활성 연구에 있어서 핵심이 되는 분야들로서 감귤 유래 플라보노이드의 각각 또는 혼합물이 *in vitro* 및 *in vivo*에서 항염 및 항암 작용을 나타내는 것으로 보고되었다. 세포의 염증반응에서 과다한 nitric oxide (NO)의 생성은 만성 염증 및 암화 과정을 포함한 다양한 병리생리학적 작용에 관여하는 것으로 알려져 있다.

이에 저자는 제주도에서 재배되고 있는 육성품종을 포함한 20종의 감귤류의 과피로부터 얻은 70% 에탄올추출물에서 1) 감귤 유래 플라보노이드 중 생리활성이 높은 것으로 보고된 나린진, 나린제닌, 헤스페리딘, 헤스페레틴, 루틴, 노빌레틴 및 탄저레틴의 함량을 HPLC를 사용하여 조사하고, 2) lipopolysaccharide (LPS)로 활성화된 대식세포주 RAW 264.7 세포에서 NO 생성에 대한 억제 활성을 관찰하였으며 3) 감귤 과피 추출물에서 플라보노이드 함량과 NO 생성 억제 효능과의 상관성을 살펴보았다.

1. 분석한 플로보노이드 함량 및 LPS로 활성화된 대식세포 RAW 264.7 세포에서 NO 생성 억제 활성은 감귤종에 따라 다르게 나타났다.

2. 폴리메톡시 플라본인 노빌레틴과 탄저레틴의 함량은 서로 높은 양의 상관성을 나타냈다.
3. 미성숙 과피 추출물이 성숙한 과피 추출물 보다 유의적으로 더 많은 플라보노이드를 함유하고 있었다.
4. 분석한 모든 감귤 과피 추출물은 농도의존적으로 NO 생성에 대한 억제 활성을 보였으며, 미성숙 과피 추출물이 성숙한 과피 추출물보다 유의적으로 높은 억제 활성을 나타냈다.
5. 플라보노이드 함량과 NO 생성 억제 활성과의 연관성 분석 결과는 감귤 과피 추출물의 NO 생성 억제 활성이 노빌레틴의 함량과 높은 양의 상관성을 가지는 것으로 나타났다.

이상의 결과에서, 감귤 과피 추출물의 플로보노이드 함량 및 LPS로 활성화된 NO 생성 억제 활성은 감귤종에 따라 다르게 나타나며, 노빌레틴의 함량이 감귤 과피 추출물의 NO 생성 억제 효능에 기여한다는 것을 알 수 있었다. 이는 노빌레틴을 다량 함유하고 있는 감귤종은 과다한 NO의 생성으로 초래되는 질병에 대해 보호 효과를 제공할 수 있음을 알게 해준다.

주요어: 감귤 과피, 플라보노이드, NO 생성 억제 활성, 노빌레틴, 대식세포, lipopolysaccharide

2. LPS에 의한 RAW 264.7세포의 염증작용에 대한 노빌레틴의 항염증 효과와 NF-κB 신호 전달 경로에 관한 연구

노빌레틴(nobiletin)은 감귤 과피에 다량으로 존재하는 것으로 알려진 폴리메톡시 플라본이며 *in vitro* 및 *in vivo*에서 뛰어난 항염 효과가 있는 것으로 보고되었다. 그러나, 노빌레틴의 항염 작용에 대한 자세한 분자적 기전은 아직까지 알려져 있지 않다. 이에 저자는 기존의 연구 결과들에 근거하여, 노빌레틴의 항염 작용의 분자적 기전을 이해하기 위해서 면역 및 염증과 관련된 유전자들의 발현 조절에 관여하는 nuclear factor-kappaB (NF-κB)의 활성화 경로에 대한 노빌레틴의 영향을 LPS로 활성화된 대식세포주 RAW 264.7 세포에서 관찰하였다. 이를 규명하기 위하여 electrophoretic mobility shift assay (EMSA)를 이용하여 NF-κB의 DNA 결합 활성, luciferase 분석법을 이용하여 NF-κB의 전사 활성, western blot 분석법을 이용하여 NF-κB의 핵으로의 이동 및 inhibitory-κB-alpha (IκBα)의 인산화 및 분해 활성에 미치는 노빌레틴의 영향을 살펴보았다.

1. 노빌레틴은 세포독성을 나타내지 않는 농도에서 염증매개인자들인, NO, prostaglandin E2 및 전염증성 사이토카인 tumor necrosis factor alpha, interleukin-1 beta 및 IL-6의 생성을 전사수준에서 억제하였다.
2. 노빌레틴은 핵내에서의 NF-κB의 전사 활성 및 DNA 결합 활성을 억제하였다.
3. 노빌레틴은 NF-κB의 핵내로의 이동 경로에 관여하는 IκBα의 인산화 및 분해 활성과 NF-κB의 핵내 이동에는 영향을 미치지 않았다.
4. 노빌레틴은 LPS로 매개되는 활성산소종(ROS)의 생성을 억제하였다.

이러한 결과는 노빌레틴이 NF-κB의 핵내 이동 경로에는 영향을 미치지 않고 NF-κB와 DNA 결합 부위간의 결합 활성을 저해하여 염증매개인자들의 유전자 발현을 억제 함으로써 염증 억제 효능을 가진다는 것을 시사한다.

주요어 : 노빌레틴, NF-κB, lipopolysaccharide, 대식세포, 염증, 유전자 발현 조절