Available at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Anti-inflammatory activity of mulberry leaf extract through inhibition of NF-κB

Eunkyo Park[1], *Seung-Min Lee*[1], *Jung eun Lee*, *Jung-Hyun Kim*[*]

Department of Home Economics Education, Chung-Ang University, Seoul 156-756, Republic of Korea

ARTICLE INFO

Article history:
Received 21 August 2012
Received in revised form
27 September 2012
Accepted 1 October 2012
Available online 3 November 2012

Keywords:
Inflammation
Mulberry leaf
Cytokines
NO
PGE_2

ABSTRACT

Mulberry leaf has been traditionally used to treat chronic diseases such as diabetes and cancer. The effect and mechanism of mulberry leaf extract (MLE) in LPS-induced activation of macrophage was investigated by the levels of production of proinflammatory mediators and cytokines, and their transcriptions. MLE effectively decreased proinflammatory mediators and cytokines production. Their expression levels were decreased at both transcript and protein levels by the pretreatment of MLE. Since all these genes are transcribed by NF-κB and MAPK, their activities were also examined. NF-κB activity was proved to be reduced by the preincubation with MLE through inhibition of IκBα phosphorylation. However, MLE-treatment had no effect on phosphorylation levels of MAPK. There results suggest that MLE can be used as an anti-inflammatory agent to inhibit NF-κB-mediated inflammatory response. In addition, our data support the idea that MLE can inhibit the activities of pro-inflammatory mediators and cytokines to ameliorate the disease conditions.

1. Introduction

Primarily inflammation is essential to protect the body against pathogens or dangerous cells. However, abnormal regulation of this process can result in destruction of cells or disturbed cellular metabolism, which often contributes to chronic diseases characterized by a low grade of inflammation. Upon stimuli by proinflammatory agents including pro-inflammatory cytokines and bacterial lipopolysaccharides (LPS), immune cells are activated through various receptors (Zhang & Ghosh, 2000). Then, leukocytes or monocytes are recruited to the site of infection by coordinated actions of adhesion molecules, chemokines and metabolites of arachidonic acid (Radi, Kehrli, & Ackermann, 2001). Inflammatory mediators including nitric oxide (NO) and prostaglandins (PGs) mediate this process by diluting vasculature. Once they are activated, they phagocytose targets and degrade them in the lysosomal compartment via oxidative burst. Nicotinamide adenine dinucleotide phosphate-oxidase and inducible nitric oxidase synthase (iNOS) are responsible for the generation of reactive oxygen species (ROSs) and reactive nitrogen species (RNS) (Guzik, Korbut, & Adamek-Guzik, 2003). Macrophages differentiated from monocytes activate inflammation-related genes via signaling pathways of

* *Corresponding author.* Address: Department of Home Economics Education, Chung-Ang University, Heuksuk-Dong, Dongjak-Gu, Seoul 156-756, Republic of Korea. Tel.: +82 2 820 5378, fax: +82 2 817 7304.
E-mail address: jjhkim@cau.ac.kr (J.-H. Kim).

[1] Equal contribution as the first author.

Abbreviations: LPS, lipopolysaccharides; NO, nitric oxide; PG, prostaglandins; iNOS, inducible nitric oxidase synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; NF-κB, nuclear factor-κB; AP1, activator protein 1; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-alpha; IL, interleukin; MLE, Mulberry leaf extracts; 1-DNJ, 1-deoxynojirimycin; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PVDF, polyvinylidene fluoride; AA, arachidonic acid; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; DMSO, Dimethyl sulfoxide

1756-4646/$ - see front matter
http://dx.doi.org/10.1016/j.jff.2012.10.002

nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK). The activated NF-κB translocates to the nucleus, where it binds to the promoter regions of target genes that include iNOS, cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and IL-6 (Baeuerle & Baltimore, 1996; Fujiwara & Kobayashi, 2005). Expression of these genes further elicits immune responses.

Normally inflammation lasts within days because of the short half lives of neutrophils and inflammatory mediators, and the delayed production of anti-inflammatory cytokines. This type of inflammation is referred to as acute inflammation. On the other hand, longer lasting inflammation is referred to as chronic inflammation, which can last weeks to years. Chronic inflammation is triggered by prolonged exposure to infections or by persistent inflammatory responses. The activation of macrophages plays a central role. Recent studies showed that sustained activation in signaling pathways relevant to inflammation are found in various pathological conditions and suggested to be a crucial underlying cause of many chronic diseases such as atherosclerosis, cardiovascular disease, autoimmune disease, rheumatoid arthritis, cancer, and diabetes (Aggarwal & Gehlot, 2009; McGeer & McGeer, 2004). Obesity is also considered a state of chronic inflammation (Dandona, Aljada, & Bandyopadhyay, 2004), which is related to insulin resistance or metabolic syndrome (Despres & Lemieux, 2006). The direct implication of inflammation in obesity is shown by the constitutive expression of a proinflammatory cytokine TNF-α in genetically obese mice (Hotamisligil, Shargill, & Spiegelman, 1993). Insulin-resistant lipolysis in adipocyte was detected when adipocytes were incubated with secretory factors from macrophages (Permana, Menge, & Reaven, 2006). Hence, macrophages and their immune reactions appear to contribute to insulin resistance and its related metabolic disorders. At the same time the fact that the inhibition of TNF-α activity decreased insulin resistance in mouse models (Hotamisligil et al., 1993) suggests that the modulation of inflammatory status can be targeted for therapeutic purposes to improve abnormal metabolic conditions.

Mulberry (*Morus alba* L.) has been traditionally used as oriental medicine either in a form of herbal tea in South East Asia including Korea. Its leaves contain many phenolic compounds including gallic acid, protocatechuic acid, catechin, gallocatechin gallate, caffeic acid, epicatechin, rutin, resveratrol and quercetin. Most of constituents are bioactive and have antioxidant properties (Chan et al., 2009). In addition, recent studies have reported that 1-deoxynojirimycin (1-DNJ) in mulberry leaf extract has anti-diabetic effects by reduction of postprandial blood glucose through inhibition of α-glucosidase and decrease of serum triglyceride (Kojima et al., 2010; Kwon, Chung, Kim, & Kwon, 2011). Biological effects of mulberry leaf extract are shown in rheumatic arthritis (Kim & Park, 2006), diabetes (Chen et al., 1995; Naowaboot et al., 2012), neurodegenerative diseases, atherosclerosis (Yang et al., 2011), and cancer. Although there have been reports regarding the mechanisms of action of polyphenols responsible for their anti-inflammatory action in other types of natural products, the knowledge about mulberry's immunomodulatory action is still limited.

Here we investigated if mulberry leaf organic extracts containing various bioactive substances exert anti-inflammatory effects in murine macrophages Raw 264.7 cells and studied the mechanisms of its action as naturally occurring anti-inflammatory agents.

2. Methods and materials

2.1. Preparation of mulberry leaf extraction

Mulberry leaves were collected from YangPyeong Agricultural Development & Technology Center (YangPyeong-Gun, South Korea). After harvest, mulberry leaf was cleaned, freeze-dried, and pulverized then mulberry leaf extracts (MLE) were prepared with 70% ethanol from dry powered leaves (1.0 kg). The mixture was filtered, evaporated in rotary evaporator, and lyophilized. The yield of extract was 20% of the starting dry weight of mulberry leaves. The obtained ethanol extracts of mulberry leaves were dissolved in dimethyl sulfoxide (DMSO) and kept at −20 °C until it was used.

2.2. Cell culture

The murine macrophage RAW 264.7 cells were purchased from Korea Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (v/v), 5% penicillin (100 U/ml), and streptomycin (100 μg/ml; Invitrogen, Carlsbad, USA) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. MLE (1, 10, and 50 μg/ml) was added on plated cells for 24 h, and then stimulated with 1 μg/ml of LPS (Sigma–Aldrich, St. Louis, MO, USA) for the indicated time.

2.3. Cell viability assay

Cells were treated at increasing doses of MLE for 24 h and then induced with 1 μg/ml of LPS for 18 h. Cell viability was determined by adding 500 μg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis, MO, USA) to each well and incubated for another 2 h at 37 °C. The cells were lysed with isopropanol after removal of the medium. The absorbance of the colored solution was quantified by measuring at 450 nm by a microplate reader (Molecular Devices, Spectra MAX 340, Sunnyvale, CA, USA).

2.4. Measurement of NO and PGE_2 production

Raw 264.7 cells were pretreated with various concentrations (1, 10, and 50 μg/ml) of MLE for 24 h then stimulated with LPS (1 μg/ml). After 18 h, the levels of nitrate and PGE_2 in the culture media were determined by Nitrate/Nitrite Colorimetric Assay kits and PGE_2 EIA kits (Cayman Chemical, Ann Arbor, MI, USA), respectively.

2.5. Measurement of pro-inflammatory cytokines production

Inhibitory effects of MLE on the production of cytokines for TNF-α, IL-1β, and IL-6 were measured by commercially enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA). The supernatants collected from the cells were analyzed according to the manufacturer's protocols.

2.6. RNA preparation and reverse transcriptase–polymerase chain reaction (RT-PCR)

RAW 264.7 cells were incubated with MLE for 24 h then induced by LPS (1 μg/ml) for 4 h. Total RNA was isolated by using RNeasy mini kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions and stored at −80 °C before use. Total RNA was reverse-transcribed using Superscript First Strand Synthesis Systems kits (Invitrogen, Carlsbad, CA, USA). All PCR analyses were subsequently carried out using a GenePro Thermal Cycler (Bioer Technology CO., LTD, Hangzhou, China). After an initial denaturation for 5 min at 95 °C, 25 ∼ 30 amplification cycles were conducted in the following order; denaturation at 95 °C for 30 s, annealing at varying temperature for 30 s, elongation at 72 °C for 1 min. Annealing temperatures and cycle numbers are as followed; iNOS (53 °C, 30 cycles) COX-2 (62 °C, 28 cycles), TNF-α (56 °C, 25 cycles), IL-1β and IL-6 (56 °C, 30 cycles), and GAPDH (62 °C, 30 cycles). Last extension step of PCR reaction was done at 72 °C for 10 min. The sequences of gene specific primers and the PCR product sizes are following; iNOS (175 bp) (forward) 5′-AGTGG TGTTCTTTGCTTC-3′ and (reverse) 5′-GCTTGCCTTATACTGG TC-3′, COX-2 (141 bp) (forward) 5′-GGTCTGGTGCCTGGTCTG-3′ and (reverse) 5′–CTCTCCTATGAGTATGAGTCTGC-3′, TNF-α (138 bp) (forward) 5′-ACGGCATGGATCTCAAAGAC-3′ and (reverse) 5′-AGATAGCAAATCGGCTGACG-3′, IL-1β (196 bp) (forward) 5′-AATCTATACCTGTCCTGTGTAATG-3′ and (reverse) 5′-GCTTGTGCTCTGCTTGTG-3′, IL-6 (165 bp) (forward) 5′-CTT CCATCCAGTTGCCTTCTT-3′ and (reverse) 5′-ACGATTTCCCA-GAGAACATGT-3′, GAPDH (149 bp) (forward) 5′-CCATCACCATC TTCCAGGAGCG-3′ and (reverse) 5′-AGAGATGATGACCCTTTT GGC-3′. After amplification, PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV light.

2.7. Western blot analysis

After MLE treatments, cells were stimulated with LPS and lysated in RIPA lysis buffer containing cocktail protease inhibitors and phosphatase inhibitors (Sigma–Aldrich, St. Louis, USA). Cell debris was removed by centrifugation for 30 min at 10,000*g* at 4 °C. Protein concentrations were measured using a Bradford Assay kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instruction. Lysates were boiled in sample buffer for 5 min. The protein equivalents of samples were then separated by 10% SDS–polyacrylamide gel (PAGE) and transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.05% tween 20 for 1 h at room temperature, and then probed with antibodies against iNOS, COX-2, p-IκB-α, ERK 1/2, p-ERK 1/2, p38, p-p38, JNK, p-JNK, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed and then exposed to HRP-conjugated secondary antibodies (1:4000 dilutions). Immunoreactive bands were detected by enhanced cheiluminescence (ECL) solution (Animal Genetics, Seoul, South Korea) and exposed to X-ray film.

2.8. Nuclear extraction and NF-κB transcriptional activity assay

Raw 264.7 cells were harvested after 24 h treatment of MLE and subsequent LPS stimulation for another 20 min. Nuclear proteins were extracted using a Nuclear Extraction kit (Cayman, Ann Arbor, MI, USA), and NF-κB transcriptional activity was measured using a NF-κB Transcription Factor Assay kit (Cayman, Ann Arbor, MI, USA). Two micrograms of nuclear extracts were incubated overnight at 4 °C and p65 primary antibody was incubated. After wash, secondary antibodies were incubated and developed with transcription factor developing solution. The absorbance of the colored solution was quantified by measuring at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.9. Statistical analysis

All data presented are representative of three or more independent experiments and expressed as the mean ± standard error (SE). Statistical comparisons were determined by analysis of variance (ANOVA), followed by Duncan's multiple range test. In all cases, results were considered significant if p value was less than 0.05. All statistical tests were performed using statistical analysis software (SPSS, Chicago, IL, USA)

3. Results

3.1. Non-cytotoxic level of MLE on Raw 264.7 cells

We first determined the bioactive compounds such as 1-DNJ and phenolic compounds in MLE. MLE we used contained 3.75% (wt/wt) of 1-DNJ and 0.015% of resveratrol. After that, we examined whether MLE has cytotoxicity on RAW 264.7 murine macrophages. The cells were treated with MLE at concentrations of 1, 10, 50 μg/ml for 24 h and then stimulated with LPS (1 μg/ml) for 18 h. Cell viability was measured using MTT assays. There were no significant changes in cell viability (Fig. 1), indicating that MLE is not cytotoxic at a dose of up to 50 μg/ml.

3.2. Effects of MLE on LPS-induced NO production and iNOS expressions in Raw 264.7 cells

To investigate the role of MLE on the production of NO in activated RAW 264.7 cells, we challenged the cells with 1 μg/ml of LPS for 18 h in the absence and presence of MLE with varying amounts (1, 10, 50 μg/ml). Then the cells were examined for the levels of nitrite that is a stable metabolite of NO and the expression levels of iNOS gene that synthesizes NO in response to LPS. LPS treatment increased the production of nitrite about 3-fold greater than the unstimulated control cells (Fig. 2A). The elevated nitrite production upon LPS stimulation was significantly inhibited by MLE at a concentration of 50 μg/ml (Fig. 2A). LPS increased mRNA and protein levels of iNOS gene (Fig. 2B and C). MLE at a concentration of greater than 1 μg/ml significantly reduced the expression of iNOS at both mRNA and protein levels in LPS-stimulated cells (Fig. 2B and

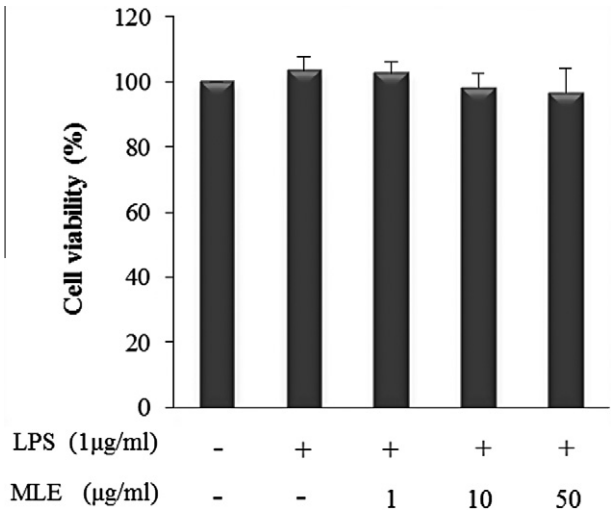

Fig. 1 – Effects of MLE on the viability of Raw 264.7 cells using MTT assay. The cells were treated with MLE for 24 h and then stimulated with LPS for 24 h. The values are expressed as the means ± SE of three individual experiments.

C). Especially the effects of MLE on the protein amounts of iNOS were shown to be dose-dependent (Fig. 2C). These results indicated that MLE could reduce the expression of iNOS gene at as low as 1 μg/ml dose but the noticeable reduction in NO production was elicited by at least 50 μg/ml of MLE.

3.3. *Effects of MLE on LPS-induced PGE2 production and COX-2 expressions Raw 264.7 cells*

The effects of MLE on the production of proinflammatory mediators were assessed by measuring the production of PGE_2 and the expression levels of COX-2 gene. COX-2 catalyzes the conversion of arachidonic acid (AA) to PGE_2. The cells were pretreated for 24 h with various concentrations of MLE and subsequently stimulated by LPS for 18 h to assess PGE_2 production or for 4 h to measure the transcript and protein abundances of COX-2 gene. MLE inhibited the production of LPS-induced PGE_2 in a dose-dependent manner (Fig. 3A). The lowest concentration of MLE to lessen PGE_2 production was 10 μg/ml. In addition, MLE was effective to decrease the expression of COX-2 gene at both mRNA and protein levels. 10 μg/ml of MLE was enough to reduce COX-2 expression. These results suggest that MLE is effective to lower the production of proinflammatory mediator PGE_2 and the expression of COX-2, an enzyme responsible for the synthesis of PGE_2.

3.4. *Effects of MLE on LPS-induced productions and expressions of TNF-α, IL-1β, and IL-6*

Then we investigated whether MLE can influence the production of pro-inflammatory cytokines which are TNF-α, IL-1β, and IL-6 as well as the expressions of these genes. 24 h treatment of MLE at a concentration of 1 μg/ml was sufficient to decrease the transcript and protein levels of TNF-α in RAW264.7 macrophage cells even if there was only 20% reduc-

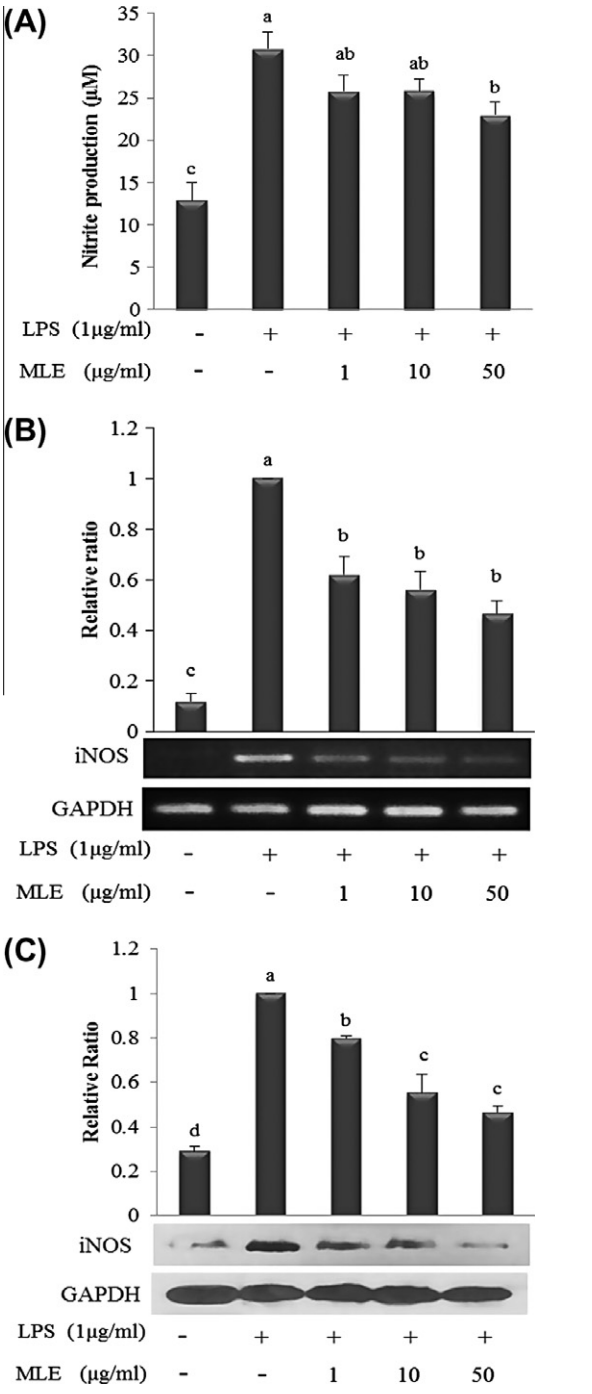

Fig. 2 – Effects of MLE on NO formation (A), and iNOS mRNA (B) and protein (C) expression in LPS-induced Raw 264.7 cells. The macrophage cells were pretreated with various concentrations of MLE for 24 h, and then stimulated with LPS (1 μg/ml). (A) NO formation. After 18 h incubation with LPS, the amount of NO in the culture supernatants was measured. (B) iNOS mRNA expression. After 4 h incubation with LPS, total RNA was subjected to semi-quantitative PCR. (C) iNOS protein expression. After the removal of supernatants, the cells were lysed to obtain total protein lysates, which were subjected to Western blot analysis. The values are expressed as the mean ± SE of three individual experiments. Values not sharing the same superscript were significantly ($P < 0.05$) different.

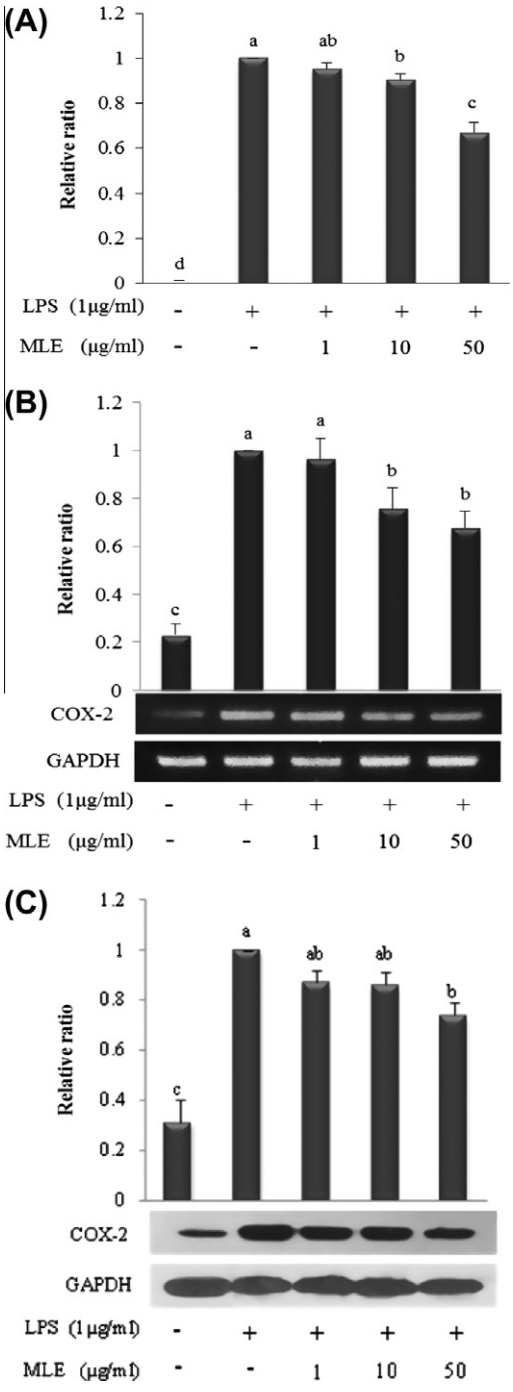

Fig. 3 – Effects of MLE on PGE_2 formation (A), and COX-2 mRNA (B) and protein (C) expression in LPS-induced Raw 264.7 cells. Cells were pretreated with various concentration of MLE for 24 h then stimulated with LPS (1 μg/ml). (A) PGE_2 formation. After 18 h incubation with LPS, the amount of PGE_2 in the culture supernatants was measured. (B) COX-2 mRNA expression. After 4 h incubation with LPS, total RNA was subjected to semi-quantitative PCR. (C) COX-2 protein expression. After LPS stimulation for 18 h, the cells were collected and lysed. Total protein lysates were subjected to Western blot analysis. The values are expressed as the mean ± SE of three individual experiments. Values not sharing the same superscript were significantly ($P < 0.05$) different.

tion in TNF-α level as compared to the LPS-induced control cells (Fig. 4A). MLE also noticeably inhibited the expression and production of two other cytokines that are IL-1β, and IL-6 (Fig. 4B and C). 10 μg/ml of MLE significantly decreased the mRNA levels of IL-1β and IL-6 and the secreted forms of IL-1β and IL-6 dose-dependently (Fig. 4B and C). In the case of IL-1β, 1 μg/ml of MLE was enough to reduce the secretion of IL-1β (Fig. 4 B). Taken together, MLE was able to markedly reduce the LPS-stimulated mRNA expressions of TNF-α, IL-1β, and IL-6 genes and the protein production and secretion of these genes. The MLE effects appear to be dose-dependent.

3.5. *Effects of MLE on LPS-induced NF-κB activity and MAPK phosphorylation*

Since MLE had effects on the transcript levels of inflammatory mediators such as iNOS and COX-2 and proinflammatory cytokines, we next investigated whether MLE modulates NF-κB and AP-1 activities, a transcription factor known to regulate the expression of many proinflammatory genes. Nuclear extracts from Raw 264.7 cells that were treated with MLE and then stimulated by LPS, were assayed to measure transcriptional activity of NF-κB. As shown in Fig. 5A, MLE reduced the LPS-induced DNA binding activity of NF-κB in a dose-dependent manner. Furthermore phosphorylation status of cytosolic IκB proteins was less detected in macrophage cells pre-treated with MLE for 24 h (Fig. 5B). Surprisingly 50 μg/ml of MLE lowered the amount of p-IκB to a degree of that found in unstimulated cells (Fig. 5B). However, phosphorylation levels of MAPK, ERK 1/2, p38, and JNK, had not been changed by MLE treatment (Fig. 5C).These results indicate that MLE decreased the transcriptional activity of NF-κB by reducing phosphorylation of IκB proteins.

4. Discussion

Inflammation is involved in many pathological diseases such as atherosclerosis (Libby, Ridker, & Maseri, 2002), rheumatoid arthritis (Manzi & Wasko, 2000), and pulmonary fibrosis (Coker & Laurent, 1998). Because constantly activated macrophages are closely linked to many chronic diseases including obesity-related disorders, the modulation of activation of macrophages has been considered one way to regulate the development of this type of diseases. Indeed previously inhibition of proinflammatory cytokines was shown to ameliorate disease conditions including chronic inflammatory rheumatical disorders (Agirbasli, Inanc, Baykan, & Direskeneli, 2006), LPS-induced cardiovascular failure (Hocherl, Dreher, Kurtz, & Bucher, 2002), and atherosclerosis (Schieffer et al., 2004). Here we provide an evidence to show that mulberry leaves often used in oriental medicine can be used as another anti-inflammatory agent to modulate inflammation.

We have investigated that the role of MLE in the control of LPS-induced activation of murine macrophage RAW264.7 cells and observed that MLE suppressed the expression of proinflammatory genes such as iNOS, COX-2, TNF-α, IL-1β, and IL-6. Macrophages do not produce detectable levels of inflammatory mediators such as NO and PGE_2 without proinflammatory stimuli (Epinat & Gilmore, 1999). The activation of

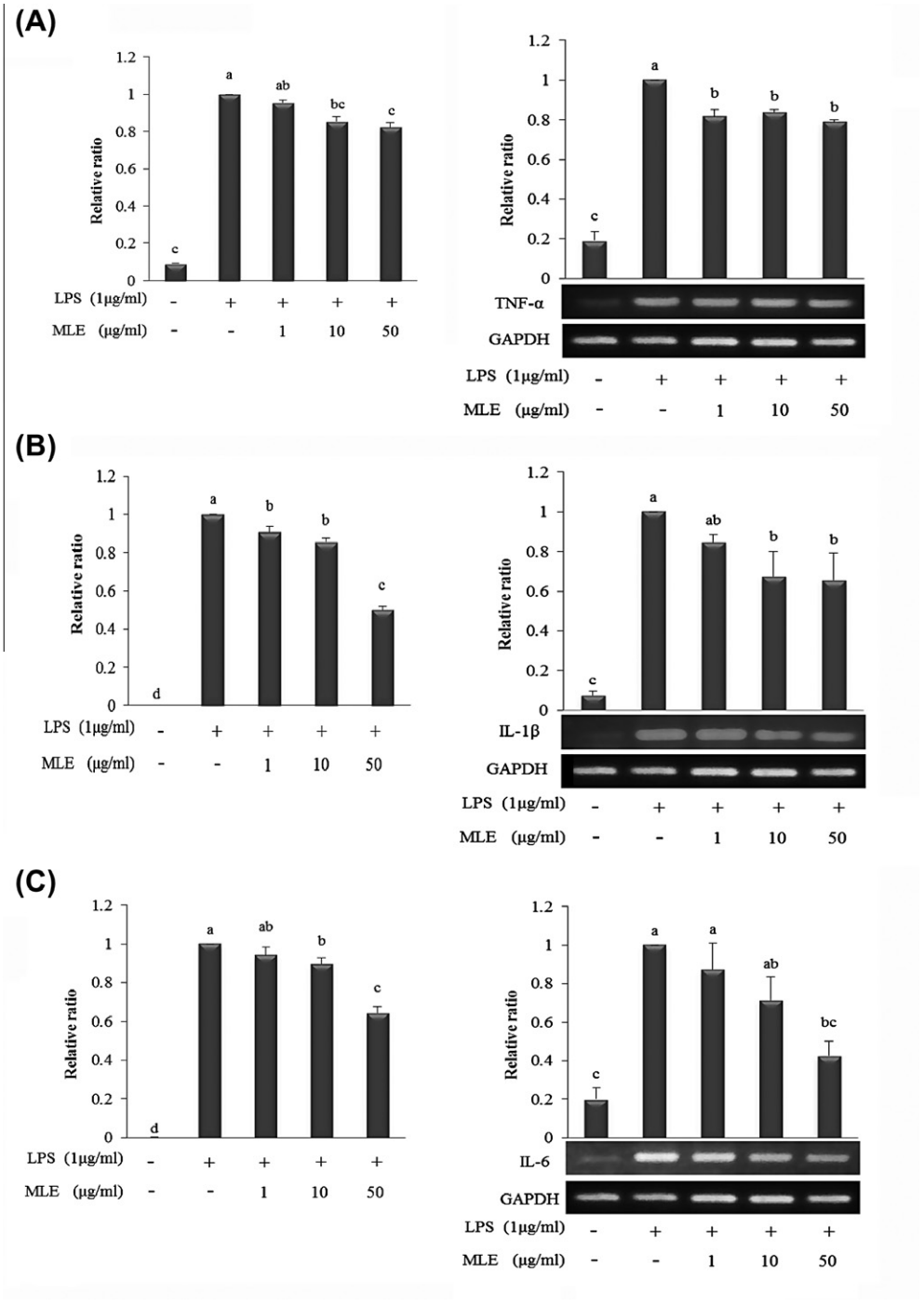

Fig. 4 – Effects of MLE on TNF-α (A), IL-1β (B), and IL-6 (C) mRNA and protein expression. The cells were pretreated with various doses of MLE for 24 h and then stimulated with LPS (1 μg/ml) for 4 or 18 h. Total RNA and protein lysates were subjected to semi-quantitative PCR or Western blot analysis. The values are expressed as the mean ± SE of three individual experiments. Values not sharing the same superscript were significantly ($P < 0.05$) different.

the NF-κB pathway results in the production of these inflammatory mediators as part of immune response, which is shown in our LPS-stimulated RAW 264.7 cells. The release of NO is mainly decided by the catalytic activity of iNOS, which is an inducible enzyme that catalyzes the production of NO from L-arginine. Thus, iNOS expression levels often indicate inflammation. On the other hand, PGE_2 is converted from arachidonic acid by COX-2. The elevated expression of COX-2 in LPS-treated macrophage cells also suggests inflammation. The marked increases in mRNA and protein levels of iNOS and COX-2 genes enabled to be diminished by the pretreatment of MLE dose-dependently. Subsequent decreases in the production of NO and PGE_2 were detected with the treatment of MLE. Furthermore, macrophages secrete proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 as part of an inflammatory response. IL-6 is primary mediator of the

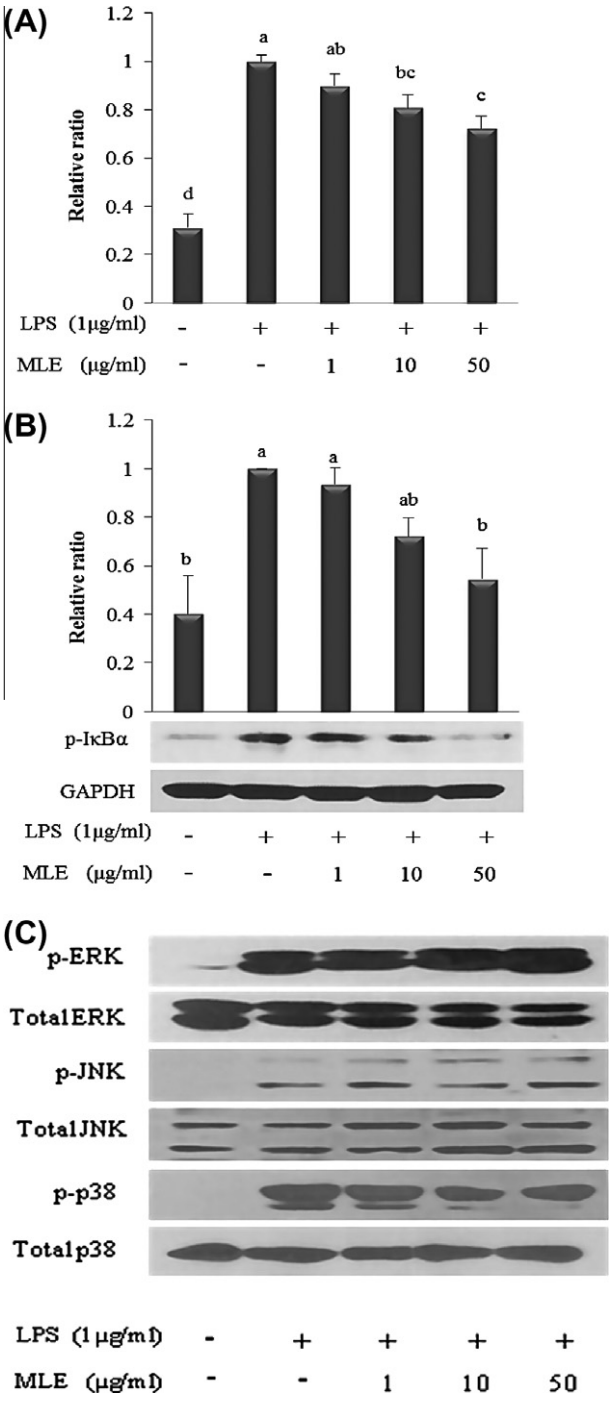

Fig. 5 – Effects of MLE on NF-κB activation (A), IκB phosphorylation (B), and MAPK phosphorylation (C). (A) NF-κB activation. The cells were pretreated with different doses of MLE for 24 h prior to stimulation with LPS (1 μg/ml) for 20 min. Nuclear extracts were prepared and used to measure NF-κB transcriptional activity. (B, C) IκB and MAP kinase phosphorylation. Cells were pretreated with various concentrations of MLE for 24 h and then induced with LPS (1 μg/ml) for 15 min. Cells were pretreated with various concentration. Total proteins lysates were subjected to western blot analysis. The values are expressed as the mean ± SE of three individual experiments. Values not sharing the same superscript were significantly ($P < 0.05$) different.

acute phase immune response. The promoter regions of the tested genes that are iNOS, COX-2, TNF-α, IL-1β, and IL-6 contain a consensus sequence for NF-κB transcription factor. All of these genes were down-regulated by the treatment of MLE.

Given the fact that MLE decreased the expression of NF-κB target genes, MLE appear to affect NF-κB signaling pathway to exert its biological effects. NF-κB plays an essential role during inflammation and immune responses as well as in other physiological functions such as cell growth, apoptosis, and developmental processes (Epinat & Gilmore, 1999). Under unstimulated conditions, NF-κB is present in an inactive form sequestered by a regulatory protein called I-κB in the cytoplasm. When cells are stimulated by the proinflammatory signals, phosphorylation of I-κB occurs, which releases the NF-κB and allow it to translocate to the nucleus (Yamamoto & Gaynor, 2004). Once in the nucleus, NF-κB binds to its IκB site in the promoter of target genes. The importance of NF-κB during inflammation was also confirmed in our LPS-activated macrophage by a great induction of the expression of NF-κB target genes including iNOS, COX-2, TNF-α, IL-1β, and IL-6. The LPS-induced activation of NF-κB was suppressed by the pretreatment of MLE as shown by the decreases in the transcriptional activities of NF-κB and in the expression levels of its target genes. Further investigation of the phosphorylation status of IκBα after MLE treatment indicates that the effects of MLE occur at earlier steps in the activation of NF-κB signaling pathway. MLE blocked nuclear translocation of NF-κB via maintaining unphosphorylated status of IκBα and thereby prevent NF-κB from being activated to transcribe its target genes. Therefore it is likely that MLE influenced the transcriptional activity of NF-κB by restricting its nuclear accessibility via sequestering it in the cytosol with IκB.

The anti-inflammatory effects of MLE via NF-κB can be derived from the presence of diverse polyphenols. Mulberry leaf extracts can be obtained in either in water or organic solvent. It was reported that the amount of total phenolic compounds was greater in organic extract than one in water extract about 2–3-fold (Naowaratwattana, De-Eknamkul, & De Mejia, 2010). Major components of organic extraction of Mulberry extract using methanol or butanol were rutin, isoquercetin, kaempferol, quercetin glycosides (Naowaratwattana et al., 2010). Also nonanthocyanin phenolics containing procatechuic acid, chlorogenic acid, 4-caffeolyquinic acid, taxifolin, rutin, and quercetin was shown to have high antioxidant activity (Zhang, Han, He, & Duan, 2008). Previously individual polyphenolic compounds were shown to exert immunomodulatory effects via the inhibition of NF-κB activity, which include piceatannol, curcumin, and epigallocatechin gallate (Jobin et al., 1999; Kim et al., 2012; Nomura, Ma, Chen, Bode, & Dong, 2000). In many cases the biological effects of polyphenols are closely linked to their role as antioxidants because of their ability to scavenge free radicals and metal ions (Rahman, Biswas, & Kirkham, 2006) but it can be achieved by modulating signaling processes apart from their antioxidant effects (Epinat & Gilmore, 1999). Considering that the activities of NF-κB can be modulated by oxidants and antioxidants (McGeer & McGeer, 2004; Pahl, 1999), polyphenols in MLE may play a major role in inhibition of inflammation as antioxidants roles.

In many other studies, mulberry extracts were also shown to have hypolipidemic effects by improving lipoprotein profiles and decreasing triglyceride and cholesterol levels (Aramwit, Petcharat, & Supasyndh, 2011; Peng et al., 2011). Also mulberry leaf decreased blood glucose, which effects was proven at the levels of intestinal absorption of glucose (Kwon et al., 2011) as well as at the postprandial levels to lower blood glucose (Kim, Kwon, & Jang, 2011).

Here we provide an evidence of that MLE plays a role to modulate the LPS-induced activation of macrophage cells by suppressing NF-κB activation. Also the effective concentrations of MLE to exert anti-inflammatory activities did not show cytotoxicity. Further in vivo studies are warranted to confirm the anti-inflammatory roles of MLE in people who are susceptible to develop the diseases with the low grade of inflammation. This study will further ensure that MLE can be used for a therapeutic purpose to prevent or inhibit the diseases characterized by a chronic inflammation with dys-regulated NF-κB activity.

Acknowledgment

This research was supported by Chung-Ang University Research Scholarship Grants in 2011.

REFERENCES

Aggarwal, B. B., & Gehlot, P. (2009). Inflammation and cancer: how friendly is the relationship for cancer patients? *Current Opinion in Pharmacology, 9*, 351–369.

Agirbasli, M., Inanc, N., Baykan, O. A., & Direskeneli, H. (2006). The effects of TNF alpha inhibition on plasma fibrinolytic balance in patients with chronic inflammatory rheumatical disorders. *Clinical and Experimental Rheumatology, 24*, 580–583.

Aramwit, P., Petcharat, K., & Supasyndh, O. (2011). Efficacy of mulberry leaf tablets in patients with mild dyslipidemia. *Phytotherapy Research: PTR, 25*, 365–369.

Baeuerle, P. A., & Baltimore, D. (1996). NF-kappa B: ten years after. *Cell, 87*, 13–20.

Chan, K. C., Ho, H. H., Huang, C. N., Lin, M. C., Chen, H. M., & Wang, C. J. (2009). Mulberry leaf extract inhibits vascular smooth muscle cell migration involving a block of small GTPase and Akt/NF-kappaB signals. *Journal of Agricultural and Food Chemistry, 57*, 9147–9153.

Chen, F., Nakashima, N., Kimura, I., Kimura, M., Asano, N., & Koya, S. (1995). Potentiating effects on pilocarpine-induced saliva secretion, by extracts and N-containing sugars derived from mulberry leaves, in streptozocin-diabetic mice. *Biological & Pharmaceutical Bulletin, 18*, 1676–1680.

Coker, R. K., & Laurent, G. J. (1998). Pulmonary fibrosis: cytokines in the balance. *The European Respiratory Journal: Official Journal of the European Society for Clinical Respiratory Physiology, 11*, 1218–1221.

Dandona, P., Aljada, A., & Bandyopadhyay, A. (2004). Inflammation: the link between insulin resistance, obesity and diabetes. *Trends in Immunology, 25*, 4–7.

Despres, J. P., & Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. *Nature, 444*, 881–887.

Epinat, J. C., & Gilmore, T. D. (1999). Diverse agents act at multiple levels to inhibit the Rel/NF-kappaB signal transduction pathway. *Oncogene, 18*, 6896–6909.

Fujiwara, N., & Kobayashi, K. (2005). Macrophages in inflammation. *Current Drug Targets. Inflammation and Allergy, 4*, 281–286.

Guzik, T. J., Korbut, R., & Adamek-Guzik, T. (2003). Nitric oxide and superoxide in inflammation and immune regulation. *Journal of Physiology and Pharmacology: An Official Journal of the Polish Physiological Society, 54*, 469–487.

Hocherl, K., Dreher, F., Kurtz, A., & Bucher, M. (2002). Cyclooxygenase-2 inhibition attenuates lipopolysaccharide-induced cardiovascular failure. *Hypertension, 40*, 947–953.

Hotamisligil, G. S., Shargill, N. S., & Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science, 259*, 87–91.

Jobin, C., Bradham, C. A., Russo, M. P., Juma, B., Narula, A. S., Brenner, D. A., & Sartor, R. B. (1999). Curcumin blocks cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity. *The Journal of Immunology, 163*, 3474.

Kim, A. J., & Park, S. (2006). Mulberry extract supplements ameliorate the inflammation-related hematological parameters in carrageenan-induced arthritic rats. *Journal of Medicinal Food, 9*, 431–435.

Kim, G. N., Kwon, Y. I., & Jang, H. D. (2011). Mulberry leaf extract reduces postprandial hyperglycemia with few side effects by inhibiting α-glucosidase in normal rats. *Journal of Medicinal Food, 14*, 712–717.

Kim, Y., Han, J., Sung, J., Sung, M., Choi, Y., Jeong, H. S., & Lee, J. (2012). Anti-inflammatory activity of *Chrysanthemum zawadskii* var. *latilobum* leaf extract through heme oxygenase-1 induction. *Journal of Functional Foods, 4*, 474–479.

Kojima, Y., Kimura, T., Nakagawa, K., Asai, A., Hasumi, K., Oikawa, S., & Miyazawa, T. (2010). Effect of mulberry leaf extract rich in 1-deoxynojirimycin on blood lipid profiles in humans. *Journal of Clinical Biochemistry and Nutrition, 47*, 155–161.

Kwon, H. J., Chung, J. Y., Kim, J. Y., & Kwon, O. (2011). Comparison of 1-deoxynojirimycin and aqueous mulberry leaf extract with emphasis on postprandial hypoglycemic effects: in vivo and in vitro studies. *Journal of Agricultural and Food Chemistry, 59*, 3014–3019.

Libby, P., Ridker, P. M., & Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation, 105*, 1135–1143.

Manzi, S., & Wasko, M. C. (2000). Inflammation-mediated rheumatic diseases and atherosclerosis. *Annals of the Rheumatic Diseases, 59*, 321–325.

McGeer, P. L., & McGeer, E. G. (2004). Inflammation and the degenerative diseases of aging. *Annals of the New York Academy of Sciences, 1035*, 104–116.

Naowaboot, J., Pannangpetch, P., Kukongviriyapan, V., Prawan, A., Kukongviriyapan, U., & Itharat, A. (2012). Mulberry leaf extract stimulates glucose uptake and GLUT4 translocation in rat adipocytes. *The American Journal of Chinese Medicine, 40*, 163–175.

Naowaratwattana, W., De-Eknamkul, W., & De Mejia, E. G. (2010). Phenolic-containing organic extracts of mulberry (*Morus alba* L.) leaves inhibit HepG2 hepatoma cells through G2/M phase arrest, induction of apoptosis, and inhibition of topoisomerase II alpha activity. *Journal of Medicinal Food, 13*, 1045–1056.

Nomura, M., Ma, W., Chen, N., Bode, A. M., & Dong, Z. (2000). Inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced NF-κB activation by tea polyphenols,(–)-epigallocatechin gallate and theaflavins. *Carcinogenesis, 21*, 1885–1890.

Pahl, H. L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene, 18*, 6853–6866.

Peng, C. H., Liu, L. K., Chuang, C. M., Chyau, C. C., Huang, C. N., & Wang, C. J. (2011). Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. *Journal of Agricultural and Food Chemistry, 59*, 2663–2671.

Permana, P. A., Menge, C., & Reaven, P. D. (2006). Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. *Biochemical and Biophysical Research Communications, 341*, 507–514.

Radi, Z. A., Kehrli, M. E., Jr., & Ackermann, M. R. (2001). Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. *Journal of Veterinary Internal Medicine/American College of Veterinary Internal Medicine, 15*, 516–529.

Rahman, I., Biswas, S. K., & Kirkham, P. A. (2006). Regulation of inflammation and redox signaling by dietary polyphenols. *Biochemical Pharmacology, 72*, 1439–1452.

Schieffer, B., Selle, T., Hilfiker, A., Hilfiker-Kleiner, D., Grote, K., Tietge, U. J., Trautwein, C., Luchtefeld, M., Schmittkamp, C., Heeneman, S., Daemen, M. J., & Drexler, H. (2004). Impact of interleukin-6 on plaque development and morphology in experimental atherosclerosis. *Circulation, 110*, 3493–3500.

Yamamoto, Y., & Gaynor, R. B. (2004). IkappaB kinases: key regulators of the NF-kappaB pathway. *Trends in Biochemical Sciences, 29*, 72–79.

Yang, M. Y., Huang, C. N., Chan, K. C., Yang, Y. S., Peng, C. H., & Wang, C. J. (2011). Mulberry leaf polyphenols possess antiatherogenesis effect via inhibiting LDL oxidation and foam cell formation. *Journal of Agricultural and Food Chemistry, 59*, 1985–1995.

Zhang, G., & Ghosh, S. (2000). Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. *Journal of Endotoxin Research, 6*, 453–457.

Zhang, W., Han, F., He, J., & Duan, C. (2008). HPLC–DAD–ESI-MS/MS analysis and antioxidant activities of nonanthocyanin phenolics in mulberry (*Morus alba* L.). *Journal of Food Science, 73*, C512–518.