

LPS로 유도된 대식세포에서 카바뿌리로부터 분리한 Flavokavain C의 항염증 효과

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(2016년 8월 21일 접수, 2016년 11월 13일 수정, 2016년 11월 17일 채택)

Anti-inflammatory Effects of Flavokavain C from Kava (*Piper methysticum*) Root in the LPS-induced Macrophages

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(Received August 21, 2016; Revised November 13, 2016; Accepted November 17, 2016)

요약: 카바(*Piper methysticum*, *P. methysticum*)는 비뇨생식기 질환, 류머티즘, 위장 장애, 호흡기 자극 및 폐 통증에 대해 전통적으로 사용되는 것으로 알려져 있다. 본 연구에서는 카바에서 분리된 flavokavain C (FKC)가 염증성 유전자의 발현에 관여하는 전사인자인 핵요소- κ B (NF- κ B) 의존성 산화 질소(NO) 생산 및 산화 질소 합성 효소(iNOS)의 발현에 리포폴리사카라이드(LPS) 처리된 대식세포에서 항 염증 활성을 나타낸다는 것을 확인하였다. FKC는 과산화수소와 같은 반응성 산소 종(ROS)의 축적을 억제하고, LPS로 유도된 NO 생성 및 각종 염증 관련 유전자(iNOS, IL-1 β , IL-6)의 발현을 NF- κ B 및 MAPKs (ERK 및 JNK)의 억제를 통해 농도 의존적으로 줄일 수 있었다. 결론적으로, 이러한 결과는 FKC가 NF- κ B 경로와 MAPKs 포함한 염증 프로세스를 억제하는 능력을 가지고 있음을 나타내며, 이는 항 염증 및 항산화 효능 기반 기능성 화장품에 적용될 수 있음을 암시한다.

Abstract: Kava (*Piper methysticum*, *P. methysticum*) is used as traditional herbal medicine for urogenital diseases, rheumatism, gastrointestinal problems, respiratory irritations, and pulmonary pains. We identified a flavokavain C (FKC) from *P. methysticum*, which showed anti-inflammatory activity on nuclear factor κ B (NF- κ B)-dependent nitric oxide (NO) production and expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-induced RAW264.7 macrophages. FKC inhibited accumulation of reactive oxygen species (ROS), such as hydrogen peroxide, and was able to dose-dependently reduce the LPS-induced NO production and the expression of various inflammation-associated genes (iNOS, IL-1 β , IL-6) through inhibition of NF- κ B and MAPKs (ERK and JNK). In conclusion, these results indicate that FKC may have the potential to prevent inflammation process including NF- κ B and MAPKs pathways, and it could be applicable to functional cosmetics for anti-inflammation and antioxidant properties.

Keywords: Kava, flavokavain C, chalcone, NF- κ B, mitogen-activated protein kinases

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1. Introduction

Inflammation is a physiological response of many dermatologic disease processes, and plays a critical role in the pathogenesis of a large variety of inflammatory skin disorder[1]. During inflammatory process, activated macrophages secrete a large amount of cytokines, including interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), as well as nitric oxide (NO) via inducible NO synthase (iNOS)[2,3]. Especially, IL-1 β , IL-6, and TNF- α have a very important role that can be localized to the infected tissue in earliest phases, manifested systemically throughout the body, and caused vasodilatation as well as classic signs of inflammation such as heat, redness, swelling, and pain[4]. Regulation of the expression of iNOS and variety of cytokines has been known to be related with oxidative stress through specific reduction-oxidation (redox)-sensitive signaling pathways and nuclear factor κ B (NF- κ B) transcription factor and mitogen-activated protein kinases (MAPKs) pathways[5,6]. NF- κ B is a transcription factor which plays a major role in inflammation and cancer development. NF- κ B is situated at the crossroads of inflammation and oxidative stress, and also regulates target gene expression of adhesion molecules, cytokines, and other molecules. The activated NF- κ B translocates to the nucleus, binds to DNA and regulates the expression of many target genes including cytokines and chemokines, and leukocyte adhesion molecules[7]. Hence, NF- κ B signaling pathway provides a potential therapeutic target, more than 700 different inhibitors have been reported[8]. MAPKs plays pivotal roles in regulation of inflammatory and immune responses in mammalian cells, and their signaling pathways including extracellular signal-regulated kinase (ERK)-1/2, c-jun N-terminal kinase (JNK), and p38 are involved in LPS-induced COX-2 and iNOS expression in macrophages[9]. Therefore, pharmacological modulations of MAPKs activity has become important issues for preventing or ameliorating many diseases as described above[10].

Kava (*P. methysticum*, *Piperaceae*) is a perennial shrub distributed throughout the Pacific islands. The aqueous

extract from the root has been used for ceremonial and informal reasons and has a relaxing and mild euphoric effect[11]. The root is also used traditional herbal medicine for treating urogenital diseases, rheumatism, gastrointestinal problems, respiratory irritations, and pulmonary pains[12]. In Europe, kava extracts were utilized in the treatment of chronic inflammations of the urinary tract at the beginning of the 20th century[13]. Major constituents of kava include a class of lactone compounds referred to as kavalactones exemplified by kavain, methysticin, dihydrokavain, dihydromethysticin, yangoin, and demethoxy-yangonin. Kava also includes three chalcones, essential oil and traces of piperidine alkaloids[14]. It was previously reported that the chalcones flavokavain (FK) A, B, and C cause strong anti-proliferative and apoptotic effects in human bladder cancer cells. Among these compounds, FKA resulted in a decrease in formation of Bax and Bcl-xL heterodimer and conversion of Bax protein to its active form[15]. In addition to their effects on anti-proliferation and apoptosis, FKA and FKB were shown to inhibit TNF- α -induced NF- κ B-DNA binding in a concentration-dependent manner. Although kavachalcone derivatives have been known for beneficial effects on anti-proliferation and apoptosis, there was no report about the anti-inflammatory effects and molecular mechanisms of FKC among kavachalcone derivative in animal cells. In the present study, we evaluated the effects of FKC on LPS-mediated iNOS-dependent NO production and expression of a variety of cytokines through NF- κ B or MAPKs activation in RAW264.7 macrophages.

2. Materials and Methods

2.1. Compounds Isolation of *P. methysticum* Roots

Kava roots were collected and dried in Kosrae State, Federated States of Micronesia. The dried roots of kava (956.18 g) were extracted with methanol (3 L) and CH₂Cl₂ (3 L) at room temperature. The extract was filtered and concentrated under reduced pressure to afford 54.81 g of crude extract. A portion (19.46 g) of the crude extract was partitioned between H₂O and n-BuOH to yield

16.87 g of organic-soluble material. The n-BuOH layer was re-partitioned between 15% aqueous MeOH and n-hexane. The residue of aqueous MeOH layer (14.06 g) was subjected to C₁₈ reversed phase flash chromatography using gradient mixture of MeOH and H₂O. The fraction eluted with 20% aqueous MeOH was dried (5.06 g) and subjected to reversed phase HPLC (YMC ODS-AQ column) with a solvent system of 35% MeOH/H₂O to afford 14 fractions. Fraction 5 (98 mg) was subjected to successive normal phase HPLC (YMC SIL column) by eluting with 25% EtOAc in n-hexane to afford dihydrokavain (20 mg) and kavain (29.2 mg). Fraction 7 was subjected to reversed phase HPLC (YMC ODS-AQ column) by eluting with 35% aqueous MeOH to afford *cis*-yangonin (3.8 mg) and yangonin (2.3 mg). Fraction 9 was further purified by reversed phase HPLC (YMC ODS-A column) using 30% aqueous MeOH as the mobile phase to afford desmethoxyyangonin (17 mg). Finally, FKC (2.2 mg) was obtained from fraction 14 by reversed phase HPLC (YMC ODS-A column) using 25% aqueous MeOH as the mobile phase.

2.2. Determination of the Chemical Structures

NMR spectra were recorded in CDCl₃ on a Varian Unity 500 spectrometer. Proton and carbon NMR spectra were measured at 500 and 125 MHz, respectively. All solvents used were spectral grade or were distilled from glass prior to use. All spectral data were compared with those in previous literatures[16].

2.3. Reagents and Cell Culture

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Lonza Bioscience (Belgium). Lipopolysaccharide (LPS) was obtained from Sigma (USA). Antibody against iNOS was obtained from BD Transduction Laboratories (USA), antibodies against I κ B α , phospho-I κ B α , NF- κ B p65, and actin were obtained from Santa Cruz Biotechnology (USA), and antibodies against ERK, phospho-ERK, JNK, phospho-JNK, p38, and phospho-p38 were obtained from Cell Signaling (USA). 2',7'-dichlorofluorescein diacetate (DCFH₂-DA)

was purchased from Molecular Probe (USA). RAW264.7 cells (murine macrophage cell line) obtained from American Type Culture Collection (USA), was cultured in DMEM containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% FBS in a humidified incubator with 5% CO₂ / 95% air at 37 °C.

2.4. Measurement of NO Production and ROS Accumulation

RAW264.7 cells (1×10^4 cells/well) contained in wells of a 96-well plate were untreated or pretreated with compounds 1 ~ 6 for 2 h, followed by incubation with 1 μ g/mL of LPS for 18 h. To measure NO production, isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated for 10 min at room temperature. Nitrite production was determined by measuring the optical density at 540 nm using a model 680 microplate reader (BioRad, USA). Subsequently, after removal of the medium from each well, ROS was measured by incubation of the cells with 10 μ M DCFH₂-DA for 45 min. DCFH₂-DA is widely used to measure ROS generation in cells. Fluorescence was measured on a Wallac 1420 spectrofluorometer (Perkin-Elmer, Finland) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Fluorescence intensity of the cells was calibrated to 100%.

2.5. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

RAW264.7 cells were stimulated for 12 h with or without indicated concentrations of compounds 1 ~ 6, and 1 μ g/mL of LPS, were harvested and total RNA was isolated by RNA extraction kit (Ambion, USA), and cDNA was then synthesized using 2 μ g of RNA template in a 20 μ L reaction using the high-capacity cDNA reverse transcription kit (Ambion, USA) according to the manufacturer's instructions. PCR amplifications were quantified using the SYBRGreen PCR master mix with the 7500-real-time PCR system (Applied Biosystems, USA).

Table 1. Sequences of the Primers Used in qRT-PCR Analysis

Target genes	Accession number	Forward (5'-3')	Reverse (5'-3')
mCCL2	NM_011333	TCACCTGCTGCTACTCATTC	TACAGAAGTGCTTGAGGTGG
mCCL3	NM_011337	CACCCTCTGTACCTGCTCAA	TGGCGCTGAGAAGACTTGGT
mCCL4	nm_013652	CTAACCCCGAGCAACACCAT	AGCCATTGGTGCTGAGAAC
mTNF- α	nm_013693	CTCAGATCATCTTCTCAAAATTCGAGTGACA	CTTCACAGAGCAATGACTCCAAAAGT
mIL-1 β	nm_008361	ATGGCAACTGTTCTGAACTCAACT	ATATTCTGTCCATTGAGGTGGAGAGCT
miNOS	NM_010927	TTTGAGCAGAAGTGCAAAGTCTC	GATCAGGAGGGATTTCAAAGACCT
mIL-6	NM_031168	TGGAGTCACAGAAGGAGTGGCTAAG	CATCTGGCTAGGTAACAGAATATTTATATC

The primers were listed in Table 1. All primer sets produced amplicons of the expected size, and their identities were also verified by sequencing. The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 33 s. To detect and eliminate possible primer-dimer artifacts, a dissociation curve was generated by adding a cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Results were normalized by using the reference gene, GAPDH, and are represented as fold changes versus the reference gene.

2.6. Western Blot Analysis

Total cell extracts (for iNOS), cytoplasmic extracts (for phosphorylation and degradation of I κ B α ; 3 MAPKs pathways), and nuclear extracts (for NF- κ B translocation) were fractionated by electrophoresis on 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. To investigate proteolysis of I κ B α and activation of MAPK, each compounds-pretreated cell were stimulated for 0 ~ 1 h with LPS. Cytosolic extracts were prepared in lysis buffer (10 mM HEPES, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM PMSF). Each membrane was blocked overnight at 4 °C with blocking solution (10 mM Tris-HCl, pH 7.4; 125 mM NaCl; 0.1% Tween 20; 5% skim milk) and then incubated with specific antibodies against each targets or pathways at room temperature for 3 h. The blots were washed three times with washing buffer (20 mM Tris, 160 mM NaCl and 0.1% Tween 20), followed by a 1 h incubation with appropriate horseradish peroxidase-con-

jugated secondary antibody. The peroxidase activity was detected using the Immobilon western HRP detection reagent (Millipore, USA) using an image reader (LAS-3000 imaging system, Fuji Film, Japan).

2.7. Nuclear Extracts and Gel Electromobility Shift Assay (EMSA)

Nuclear lysis was performed using a hypertonic buffer (50 mM Hepes (pH 7.9), 400 mM KCl, 0.1 mM EDTA, 10% glycerol). Following lysis, the samples were centrifuged at 14,000 \times g for 15 min, and supernatant was retained for use in the DNA binding assay. EMSA (Promega, USA) was conducted with ³²P-labeled double-strand oligonucleotides having consensus recognition sequences for NF- κ B (5'-AGTTGAGGGGACTTTCCCAGGC-3') and Oct-1 (5'-TGTCGAATGCAAATCACTAGAA-3'). The DNA-protein complexes were then resolved on a 5% non-denaturing polyacrylamide gel in 0.5X TBE running buffer (44.5 mM Tris-HCl, pH 8.0, 44.5 mM boric acid, 1 mM EDTA). The gels were dried and exposed to X-ray film.

2.8. Statistical Analysis

All values are expressed as mean \pm S.D. The statistical significance of differences between groups was evaluated for two parallel experiments using the Student's *t*-test.

3. Results and Discussion

In the present study, we mainly described one chalcone flavonoid, namely, flavokavain C (FKC, 1) isolated from

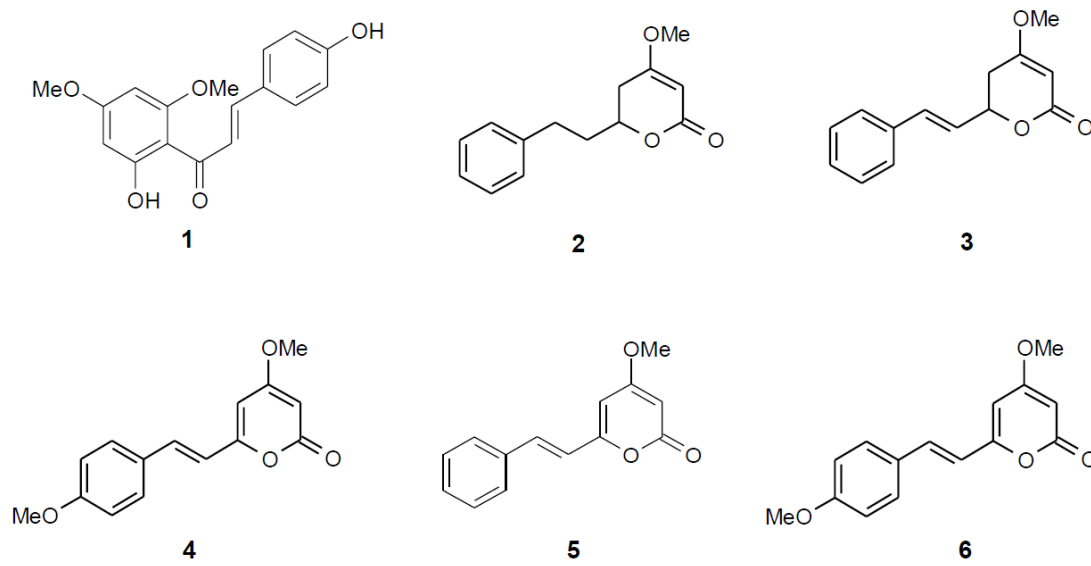


Figure 1. Chemical structures of one kava chalcone (1, flavokavain C) and five kavalactones (2, dihydrokavain; 3, kavain; 4, *cis*-yangonin; 5, desmethoxyyangonin; 6, yangonin).

P. methysticum. We also identified five kavalactones including dihydrokavain (2), kavain (KB, 3), *cis*-yangonin (4), desmethoxyyangonin (5), and yangonin (6) from *P. methysticum* (Figure 1). However, 5 kavalactones have not altered the inflammatory signals in LPS-stimulated macrophages. According to our results, we could speculate that chalcone derivatives have more protective role than kavalactone derivatives as a potent inhibitor on inflammatory processes. Although there were many studies on the beneficial effects of Kava [17] or its constituents [18,19] on inflammatory and oxidative mediators, there was no report on the anti-inflammatory activities of FKC. The objective of this study was to investigate the inhibitory effects of FKC from the Kava in both LPS-stimulated macrophages.

3.1. FKC Suppresses the LPS-induced NO Production and ROS Accumulation in RAW264.7 Cells

Anti-inflammatory activity of FKC has been attributed to its ability to inhibit LPS-induced iNOS-dependent NO production as well as ROS generation in macrophages. In general, NO and ROS productions are responsible for regulation on the NF- κ B transcriptional pathways. Low concentrations of NO play a major physiological role in the

immune system, whereas excess production of NO and cytotoxic NO metabolites are related to numerous pathological processes[2].

We initially tested anti-inflammatory activities of 5 kavalactones (compounds 2 ~ 6) by measuring NO production and ROS accumulation, but none of them showed meaningful activity (data not shown). We found that the only FKC (compound 1) have potent effect to inhibit the LPS-induced inflammatory signal.

To investigate whether FKC inhibits ROS generation, we measured ROS generation with DCF fluorescence intensity in LPS-stimulated RAW264.7 cells. DCFH₂-DA is widely used to measure ROS generation in cells. Treatment with only LPS resulted in about a 2.5-folds increase in DCF fluorescence intensity, but pretreatment with a concentration-dependent manner as FKC significantly reduced the intracellular levels of ROS in RAW264.7 cells (Figure 2A). Proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α as well as NO production are elevated in inflammatory disease and these cytokines play crucial role in immune and inflammatory response. Thus, we investigated the effects of FKC on LPS-induced NO production in RAW264.7 cells. When mouse macrophage cells were stimulated with LPS, a sig-

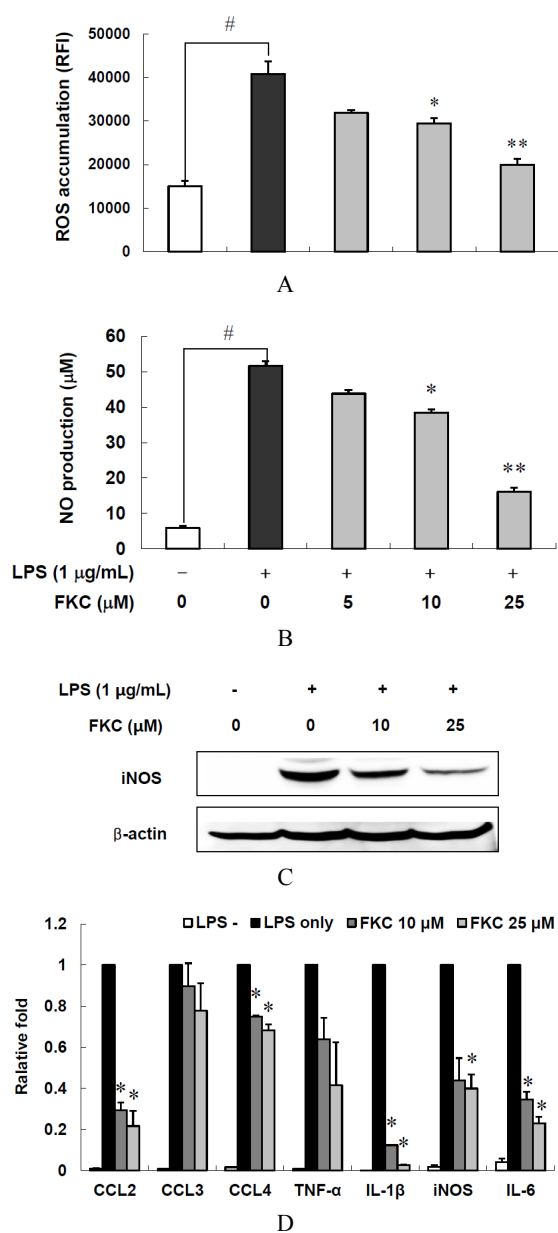


Figure 2. Effects of FKc on the LPS-induced inflammatory mediators in RAW264.7 cells. (A) Quantification of intracellular ROS levels. [#] $p < 0.01$ vs. media alone-treated group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. LPS only-treated group. (B) Quantification of nitrite levels. (C) The iNOS protein levels were analyzed by western blotting. Actin was used as an internal control. (D) The mRNA expression levels of CCL2, CCL3, CCL4, TNF- α , IL-1 β , iNOS, and IL-6 were determined using qRT-PCR (LPS only = 1). Data reported are mean \pm S.D. of two independent experiments. ^{*} $p < 0.01$ vs. LPS only-treated group.

nificant NO production was observed, which was then inhibited markedly by FKc pretreatment (Figure 2B). Production of NO in macrophage is mainly controlled by iNOS gene expression, which is in turn regulated by NF- κ B. FKc pretreatment inhibited LPS-induced iNOS protein induction in a concentration-dependent manner (Figure 2C). The potential toxicity of FKc to RAW264.7 cells was assessed by MTT assay after 48 h incubation. FKc displayed toxicity at high concentrations ($> 50 \mu\text{M}$) (data not shown).

Many inflammatory processes result in upregulation of iNOS in macrophages, leading to the excessive NO production and skin inflammation[20]. Thus, suppression of iNOS expression by drugs might be an attractive therapeutic target for the treatment of numerous pathological processes including UV-induced skin inflammation[21]. In our study, FKc showed inhibitory activity of LPS-induced NO production and ROS generation parallels the inhibition of iNOS protein and mRNA. Especially, we found that FKc (compound 1) is most potent inhibitor among identified compounds from kava, and is kavachalcone different with other 5 kavalactones (compounds 2 ~ 6).

3.2. FKc Inhibits LPS-induced mRNA Expression of Inflammation-related Genes

Several inflammatory cytokines or chemokines, particularly MCP-1 (CCL2), TNF- α , IL-1 β , and IL-6, are known to be a key mediator in the induction and prolongation of inflammation in macrophages[22]. To investigate the effect of FKc on the LPS-induced iNOS and inflammation-related gene expression, RAW264.7 cells were pretreated with 10 and 25 μM of FKc for 2 h, and subsequently stimulated with LPS for 12 h. As shown in Figure 2D, LPS stimulation caused a significant increase of the expression levels of CCL2 (MCP-1), CCL3, CCL4, TNF- α , IL-1 β , iNOS, and IL-6. However, pretreatment with concentration-dependent FKc markedly suppressed the induced mRNA expressions of these proinflammatory cytokines. One of the notable findings is treatment of the cells with 25 μM FKc completely abolished mRNA ex-

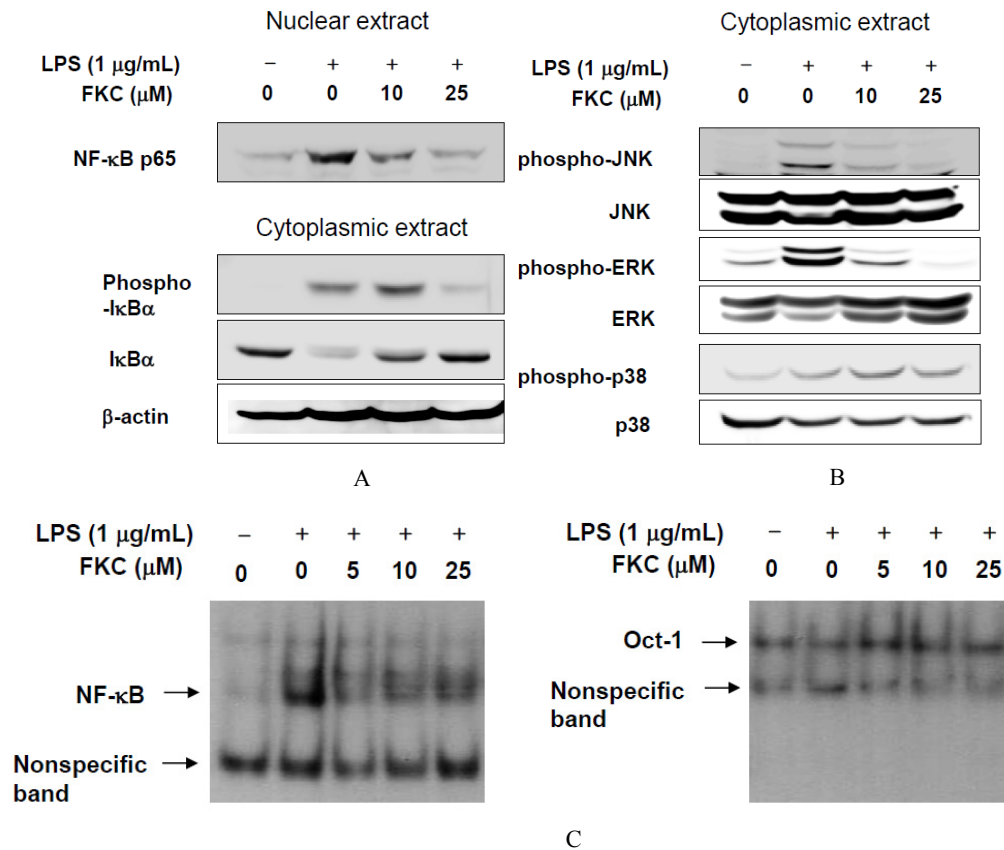


Figure 3. Effects of FKC on the activation of NF- κ B and MAPKs in LPS-stimulated RAW264.7 Cells. NF- κ B translocation and proteolysis of I κ B α (A) as well as MAPKs signals (B) were assessed by western blotting. (C) DNA-binding activity of NF- κ B complex. EMSA analysis of the nuclear extracts was conducted using a 32 P-labeled NF- κ B and Oct-1 oligonucleotide probe.

pressions of IL-1 β as well as an inhibitory ability of FKC to block LPS-induced NO production through suppressing iNOS expression at both mRNA and protein levels.

3.3. FK C Attenuates Activation of NF- κ B and MAPKs

NF- κ B is essential intermediary for the expression of variety of genes and is modulated by MAPK/ERK kinase kinase-1 (MEKK-1). It is a dimer of members of the Rel family, consists of RelA (p65), RelB, c-Rel, p50 (NF- κ B1), and p52[23]. In resting cells, NF- κ B is bound to cytoplasmic inhibitory protein, I κ B α . A variety of extracellular signals including viral antigens, LPS and physiological cytokines (TNF- α , IL-1 β , fractalkine) induce the phosphorylation cascade secondary to I κ B kinase (IKK) complex activation, I κ B α is then phosphorylated,

and its subsequent ubiquitination and degradation by the proteasome is followed[24].

To understand the molecular mechanism by which inhibits LPS-induced expression of proinflammatory cytokines, we next investigated the possible connection between increasing concentrations of FK C and NF- κ B/MAPKs activation (Figure 3). Hence, we performed western blotting to examine NF- κ B nuclear translocation and proteolysis of I κ B α . LPS significantly increased NF- κ B p65 translocation to the nucleus and phosphorylation and degradation of I κ B α within 5 ~ 10 min of stimulation. However, dose-dependent FK C dramatically attenuated NF- κ B p65 translocation as well as phosphorylation and degradation of I κ B α (Figure 3A). To further confirm the inhibitory effect of FK C on NF- κ B activity, we performed LPS-induced DNA binding of NF- κ B in

RAW264.7 cells using electrophoretic mobility shift assay. NF- κ B DNA binding was also induced by LPS and FKC remarkably inhibited DNA binding of NF- κ B in a dose-dependent manner (Figure 3C). Taken together, these results suggest that NF- κ B may be involved in the inhibitory activity of FKC on the gene expression of proinflammatory cytokines.

Additionally, we examined the effect of FKC on MAPK activation. As shown in Figure 3B, LPS caused a rapid and significant increase in the phosphorylation of ERK1/2, JNK, and p38 MAP kinases within 10 min. However, FKC treatment reduced phosphorylation of JNK and ERK MAP kinases in a concentration-dependent manner. MAPKs play a crucial role in cellular responses to cytokines and stress as well as modulation of NF- κ B activity[25]. It has been demonstrated that the inhibition of ERK directly suppresses LPS-induced NO synthesis, in mouse macrophages[26]. Our results demonstrated that FKC of kava markedly inhibited ERK and JNK as well as NF- κ B activation in a concentration-dependent manner. This is the first report demonstrating that FKC could inhibit inflammatory process by attenuating NF- κ B and MAPK activation in LPS-stimulated RAW264.7 macrophages.

The structure-activity relationships study on chalcone and flavone derivatives suggested that the inhibitory activity of the chalcone derivatives related to isoliquiritigenin is attributable to the 4-hydroxy group as well as the possible coplanarity between the phenyl ring and the adjacent conjugated ketone[27]. A similar chemical structure compound to isoliquiritigenin, FKC had inhibitory effect on the expression of inflammatory genes. It seems that the inhibition of inflammatory cytokines by FKC may arise from its similar structural components including 4-hydroxy group.

4. Conclusions

We identified one chalcone flavonoid, flavokavain C (FKC, 1) and five kavalactones including dihydrokavain (2), kavain (KB, 3), *cis*-yangonin (4), desmethoxy-

yangonin (5), and yangonin (6) from *P. methysticum*. 5 kavalactones have not altered the inflammatory signals in LPS-stimulated macrophages. However, FKC may have the potential to prevent inflammation through the suppression of NO production by inhibition of inflammation-related signal transduction (both NF- κ B and MAPKs pathways). Therefore, our results provide supporting data that FKC exerts anti-inflammatory effects, and this reveals feasibility of FKC becoming useful agents in treating inflammatory diseases including rheumatoid arthritis, and skin inflammation.

Acknowledgements

This research was supported by the Daejeon University fund (2013).

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