Master's Thesis

석사 학위논문

Flavonoids have modulatory effects on neuroinflammation in microglia

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A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain Science. The study was conducted in accordance with Code of Research Ethics[1)].

1. 5. 2015

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Flavonoids have modulatory effects on neuroinflammation in microglia

Hyojin Cho

Accepted in partial fulfillment of the requirements for the degree of Master of Science.

12. 3. 2014

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ABSTRACT

Microglia are the resident macrophages in the central nervous system. Under normal conditions, microglia cells contribute to brain development and maintenance of tissue homeostasis. However, chronic microglial activation can cause tissue damage and eventually lead to neurodegenerative diseases and neuronal death. In terms of phenotype, activated microglia can be divided into two different types: M1 state and M2 state. The first is classical activation, M1 state, which is typified by the production of inflammatory cytokines and reactive oxygen species (ROS). In contrast with pro-inflammatory M1 cells, alternative activation, M2 state has an anti-inflammatory phenotype promoting wound repair and debris clearance.

Recently, the effects of flavonoids on a variety of inflammatory processes and immune functions have been reported. Flavonoids, which are plant pigments, are anti-oxidants that possess anti-inflammatory properties and may modulate signaling pathways.

This study aims to investigate the anti-inflammatory effect of flavonoids and their role on the NF-kB pathway in BV2 microglial cells. Flavonoids treatment suppress production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), nitric oxide (NO) and interleukin-6 (IL-6) in lipopolysaccharide (LPS)-stimulated BV2 cells. Flavonoids treatment also attenuated activation of nuclear factor-kappaB (NF-κB) transcriptional activity. Furthermore, flavonoids suppress mRNA expressions related with pro-inflammatory M1 state. These observations suggest that flavonoids may contribute as an anti-inflammatory agent and provide evidence about its role in the prevention of neurodegenerative diseases associated with inflammation.

Key words: flavonoid, neuroinflammation, microglia, NF-κB

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I. Introduction

Microglia are glial cells that function as the prime effector cells in the immune defense in the central nervous system [1]. As sensitive immune cells of the brain, microglia act as the first responders to infection or tissue injury and initiate an inflammatory response [2]. However, prolonged microglial activation can cause chronic neuroinflammation and promote neuronal injury [3]. In terms of phenotype, activated microglia can be divided into two different states.: M1 state and M2 state. The first is classical activation, M1 state, which is typified by the production of inflammatory cytokines and reactive oxygen species. Microglia are able to recognize harmful stimuli and respond by producing inflammatory cytokines, such as TNFα, IL-6, IL-1β, interferon-γ (IFN-γ), and several chemokines. This cytokine production is essential for the polarization of microglia into M1 state, which is a classical activation.

The second is a state of alternative activation, M2 state, in which microglia take on an anti-inflammatory phenotype promoting wound repair and debris clearance [4, 5]. In contrast with proinflammatory M1 cells, alternatively activated macrophages express cytokines and receptors that are associated with protective functions [6]. In particular, cytokines associated with polarized type Ⅱ responses [Interleukin (IL)-4, IL-10, IL-13] induce an alternative activated M2 microglia. In addition, one of the best characterized markers in M2 alternative activation of microglia is the enzyme arginase 1 (Arg1), which catalyze arginine to polyamines, proline, and ornithines. Recently, it is known that macrophage-specific Arg1 functions as inhibitor of inflammation [7]. M2 microglia may also play important roles in

chronic inflammation and neurodegeneration [8]. It is therefore important to manipulate models of M1 and M2 polarized microglia to study their characteristics and phenotypes.

Naturally occurring polyphenols, such as flavonoids are the pigments for flower coloration and widely found in plants. They are widely found in seeds, citrus fruits, olive oil, red wine and tea [9]. Flavonoids have polyphenilic compound structures which are two benzene rings linked via a heterocyclic pyran ring. The various effects of flavonoids are determined by the types of substitutions of the pyran ring [10].

Recently, the effects of flavonoids on a variety of inflammatory processes and immune functions have been reported. Nitrite oxide synthesis is involved in inflammation and inducible isoforms of nitric oxide synthase (iNOS) are responsible for the production of a great amount of pro-inflammatory mediators [11]. It has been reported that flavonoids are able to inhibit nitric oxide production and iNOS expression [12]. The anti-inflammtory properties of flavonoids have been studied in order to establish and characterize their potential utility as therapeutic agents in the treatment of inflammatory disease [13].

Thus, it may be valuable to study the anti-inflammatory activity of flavonoids, not only to establish anti-inflammatory mechanisms, but also to develop a new class of safe anti-inflammatory agents, which may be useful in the treatment of neurodegenerative diseases.

Ⅱ. Materials and Methods

2.1 Chemicals and reagents

Hyclone DME/F-12 was purchased from Thermo scientific. Penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Lippopolysaccharide (LPS) is the component of gram-negative bacterial cell wall and elicit immune responses [14]. Lipopolysaccharide (LPS) was purchased from Calbiochem (La Jolla, CA). Synthetic flavonoids were provided by Prof. Hyoung-Su Kim (Ajou University). Detailed information of the synthetic flavonoids is not open yet for patent application.

2.2 Cell culture

BV2 microglial cells were maintained in DME/F-12 supplemented with 5% FBS and 1% penicillin-streptomycin. Cells were grown at 37^{o}C in an atmosphere containing 5% CO_2. For experiments, the cells were plated at a density of 1.0×10^5 cells per cm^2. For experiments, flavonoids were prepared in dimethyl sulfoxide (DMSO) and LPS was prepared in phosphate buffered saline (PBS), respectively.

2.3 Cell viability

Viability of BV2 cells was investigated using the CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI, USA). It provides a method for estimating the number of viable cells present in multiple plates. It uses the indicator dye resazurin is known to be metabolized

by viable cells to the fluorescent product, resorufin which can be measured. The 10 µl of reagent was directly added to the assay plate. After an incubation for 2h in 37ºC, data were recorded at 490nm with a Spectra-MAX 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4 Nitrite assay

The production of nitric oxide (NO) was determined by nitrite assay. This assay is to measure the accumulated level of the NO metabolite nitrite (NO_2^-) in the supernatant using a colorimetric reaction with Griess reagent (0.1% naphthylethylenediamine, 1% sulfanylamide and 2.5% H_3PO_4; Promega) 24 h after LPS treatment. Absorbance was measured at 540 nm with a microplate reader.

2.5 Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α and IL-6 were measured using a duo-set ELISA kit (R&D systems), respectively, according to the manufacturer's instructions.

2.6 NF-kB luciferase reporter assay

BV2 cells were co-transfected with pGL4.32 NF-κB luciferase reporter vector and pGL4.74 Renilla luciferase (Promega) using AMAXA SF-cell line transfection kit, according to the manufacturer's instructions. Renilla luciferase was used as an internal control reporter vector to normalize differences in transfection efficiency. Twenty four hours after transfection, the cells were treated with 100 ng/ml LPS for 3 h. The cells were lysed in dual luciferase lysis

buffer, and 10 µl aliquots of the cell lysate were assayed using a dual luciferase assay system (Promega) with a luminescence microplate reader. The transcriptional activity of each construct was nomalized with corresponding Renilla luciferase activity and is reported as fold of induction.

2.7 RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the QIAzol reagent (Qiagen, Valencia, CA, USA) and cDNA was prepared from total RNA using Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. cDNA generation and RT-PCR reaction were performed according to the previous report with slight modifications using TOPreal qPCR 2x PreMIX (Enzynomics) and the Rotor gene Q real-time amplification instrument (Qiagen). The PCR conditions and the sequences of the primers and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as reported. Each PCR reaction was performed in triplicates and for each sample, the levels of mRNA expression were normalized to GAPDH levels using the comparative C_T ($2^{-\Delta\Delta C_T}$) method and were expressed as fold induction.

2.8 Statistical methods

The difference between two groups was analyzed by the t-test. Multiple comparisons among groups were performed by one-way ANOVA. All statistical analyses were performed using GraphPad Prism (Graphpad software, Inc)

Table 1. Primer sequences used for qPCR analysis

cDNA	Primer sequences
TNF-α	Forward, 5'-ATG GCC TCC CTC TCA GTT C-3' Reverse, 5'-TTG GTG GTT TGC TAC GAC GTG-3'
iNOS	Forward, 5'-GCC ACC AAC AAT GGC AAC A-3' Reverse, 5'-CGT ACC GGA TGA GCT GTG AAT T-3'
IL-12	Forward, 5'-GCC CTC TCT CTC CTC TTG CT -3' Reverse, 5'-GTC TGC CTC TTT TGG TCA GG-3'
IL-10	Forward, 5'- TGT GAA AAT AAG AGC AAG GCA GTG-3' Reverse, 5'- CAT TCA TGG CCT TGT AGA CAC C -3'
Arg1	Forward, 5'-CGC CTT TCT CAA AAG GAC AG-3' Reverse, 5'-CCA GCT CTT CAT TGG CTT TC-3'
GAPDH	Forward, 5'- AAC TTT GGC ATT GTG GAA GG-3' Reverse, 5'- ACA CAT TGG GGG TAG GAA CA -3'

Ⅲ. Results

3.1 Flavonoids reduce NO production in LPS-stimulated BV2 microglia

There were ninety-six different types of synthetic flavonoid candidates. To investigate which ones are effective to inhibit microglia activation and neuroinflammation, flavonoids were treated 1 h prior to LPS treatment. With the results of nitrite oxide levels and cell viability, number 1, 53, 61 and 62 flavonoids were chosen among ninety-six flavonoid candidates.

BV2 cells are an immortalized murine microglia cell line [15]. Since LPS is the major component of the outer membrane of gram-negative bacteria and is commonly used as an inducer in immune cells [16], LPS was used to stimulate the increase of NO generation [17, 18]. Flavonoids were pretreated 1 h prior to LPS treatment in BV2 cell cultures. The levels of NO_2^- as a marker of BV2 cell activation were measured at 24 h after LPS treatment. The results showed that 100 ng/ml LPS treatments induced about five to seven fold increase in NO production, compared with untreated control. However, LPS-induced NO production was significantly attenuated by 1h pretreatment of 10 μM flavonoids (Fig. 1a-d). The levels of nitrite oxide with pretreatment of flavonoids were reduced half fold compared to the levels of nitrite following LPS treatment.

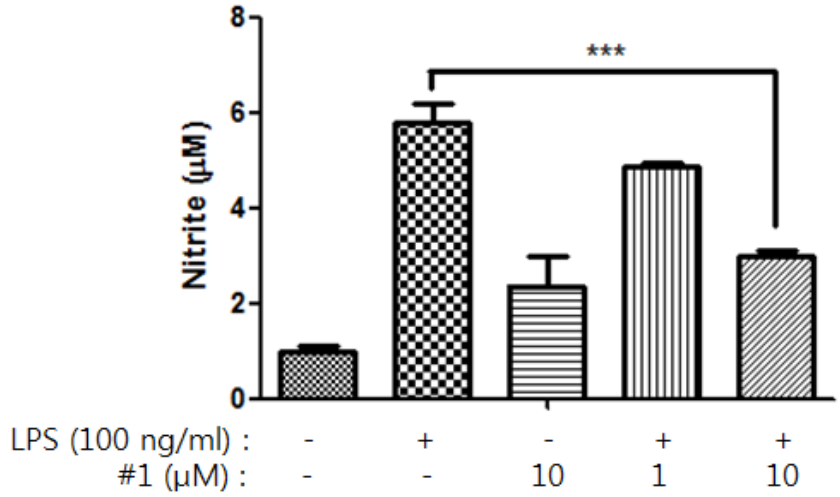
3.2 Number 1, 53, 61 flavonoids do not affect cell viability in LPS-stimulated BV2 microglia

To examine any neurotoxicity of flavonoids in BV2 cell, the cell viability was measured in 1 μM, 10 μM of flavonoid treatment in LPS-stimulated BV2 microglia. Number 1, 53, 61

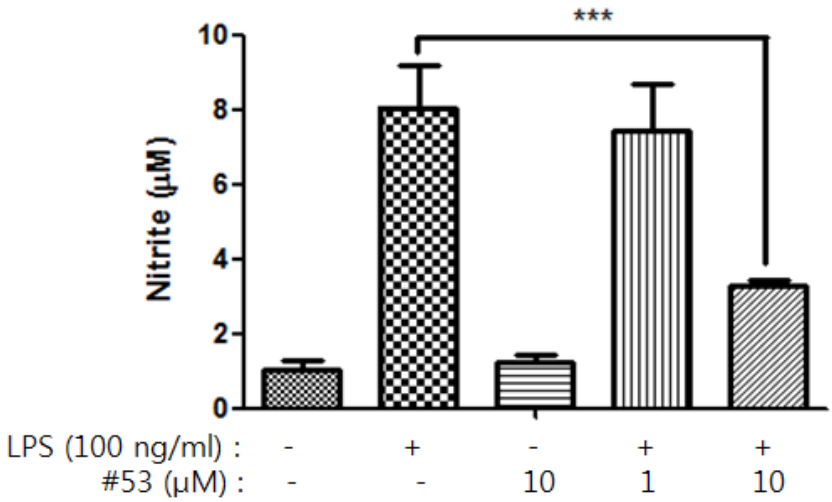
flavonoids used in this study showed no toxicity on BV2 cells, as confirmed by CellTiter Blue Assay (Fig.2a-c), while number 62 flavonoid showed a slight toxicity in 10μM dose (Fig. 2d).

Fig. 1.

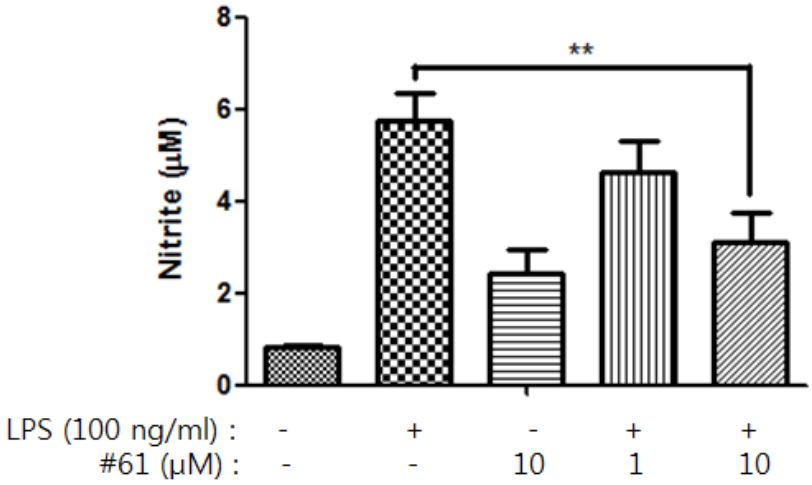
a


Nitrite (μM)
8
6
4
2
0
***
LPS (100 ng/ml) : - + - + +
#1 (μM) : - - 10 1 10

b


Nitrite (μM)
10
8
6
4
2
0
***
LPS (100 ng/ml) : - + - + +
#53 (μM) : - - 10 1 10

c



d

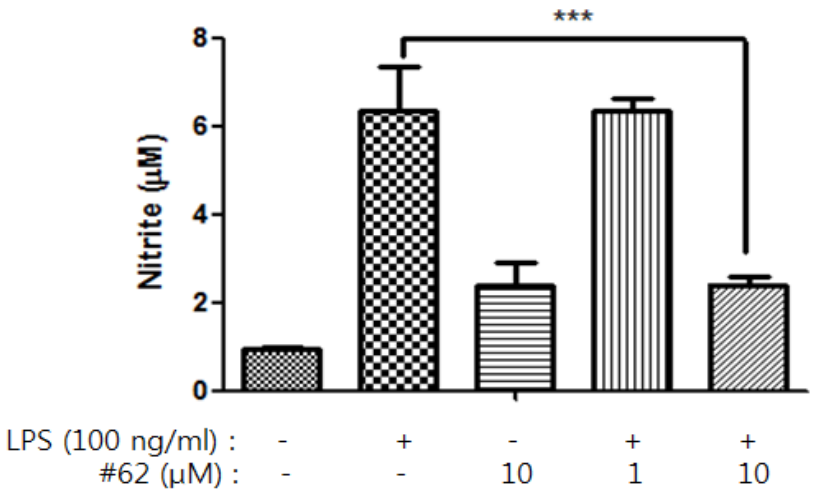
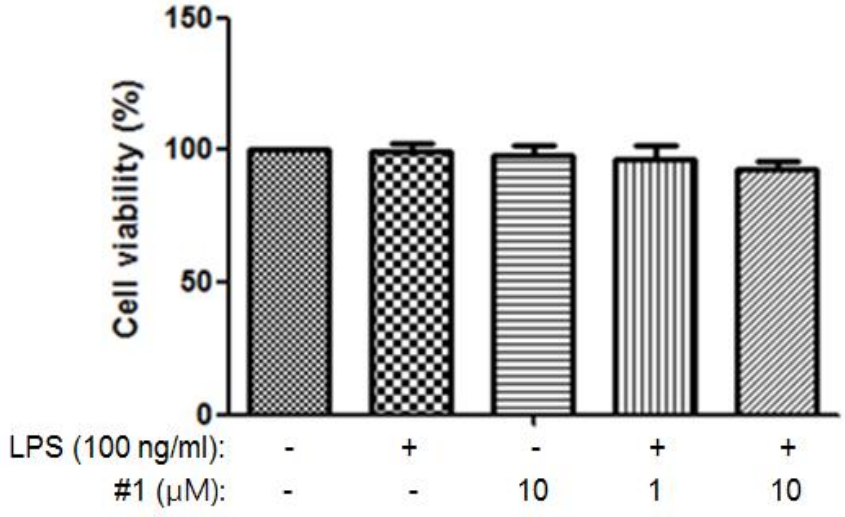


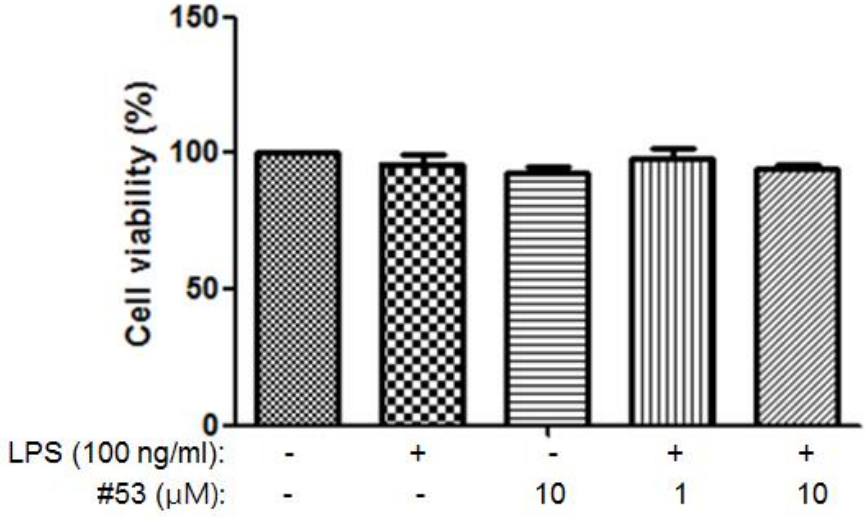
Fig. 1. The flavonoids were pretreated 1 hr prior to LPS (100 ng/ml) treatment in BV2 cell cultures and the levels of NO_2^- were measured as markers on BV2 cell activation. The NO_2^- levels were determined at 24 hr after LPS treatment. The data are presented as mean± SD (n=3), one-way ANOVA with Bonferroni correction: *p<0.05, **p<0.01, ***p<0.001.

Fig. 2.

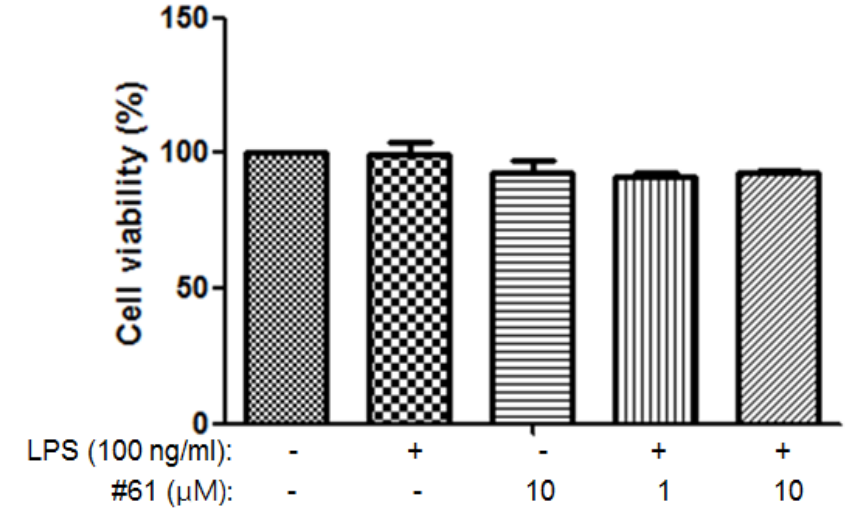
a


150
100
50
0
Cell viability (%)
LPS (100 ng/ml): - + - + +
#1 (μM): - - 10 1 10

b


150
100
50
0
Cell viability (%)
LPS (100 ng/ml): - + - + +
#53 (μM): - - 10 1 10

c



d

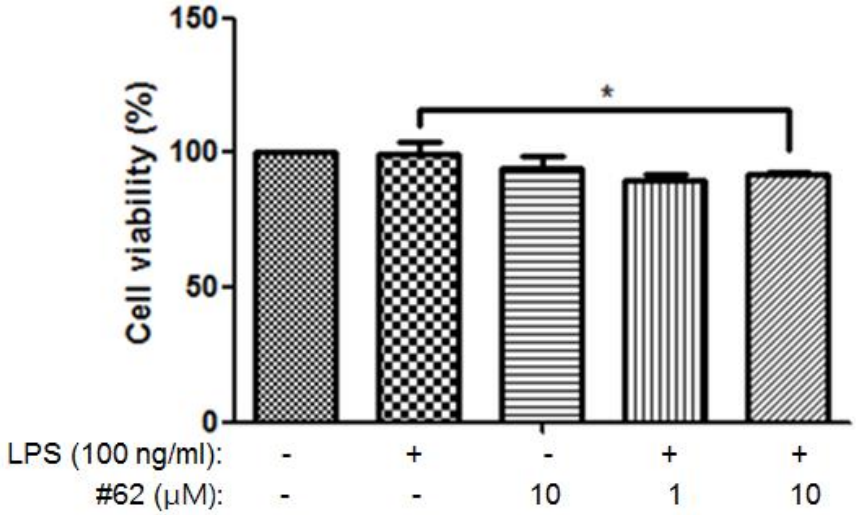


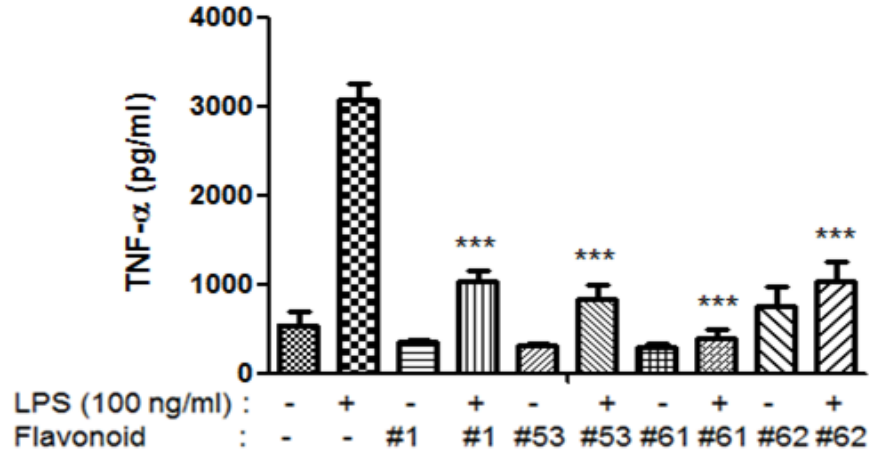
Fig. 2. The viability of BV2 cells following LPS (100 ng/ml) treatment in the presence or absence of the flavonoids. Results are the mean± S.D. (n=3), one-way ANOVA with Bonferroni correction: *p<0.05.

3.3 Flavonoids suppress production of inflammatory cytokines in LPS-stimulated BV2 microglia

Accumulating evidence points to activated microglia as a source of many neurotoxic factors, such as TNF-α, NO, IL-1β, and reactive oxygen species [19]. The flavonoids were pretreated 1 h prior to LPS treatment in BV2 cells and LPS-induced generation of cytokines by BV2 cells were measured after 24 h using ELISA assay. Tumor Necrosis Factor alpha (TNF-α) is produced by macrophages in response to immunological challenges such as bacteria (LPS). It is responsible for a diverse range of signaling events within cells [20]. Interleukin (IL)-6 is produced by innate immune cells. IL-6 is considered as a proinflammatory cytokine functioning the initiation and regulation of inflammatory responses. It is known that high concentrations of IL-6, which are thought to be a byproduct of inflammatory responses and a marker of inflammation, are associated with several inflammatory diseases [21]. The level of TNF-α following LPS treatment showed about five fold increase compare to the control group. However, number 1, 53, 61 and 62 flavonoid treatments significantly reduced the production of inflammatory factors in LPS-treated BV2 cells (Fig. 3a). The level of IL-6 following LPS treatment displayed over six fold increase compare to the control group, indicative of acute inflammation. Number 53 and 61 flavonoids substantially reduced the levels of IL-6, compared to these results, number 1 and 62 flavonoids more significantly attenuated the levels of IL-6 (Fig. 3b).

Fig. 3.

a



b

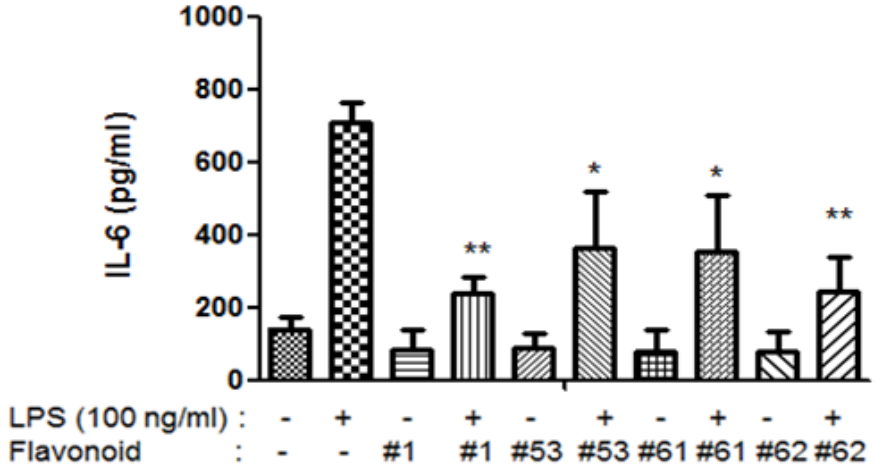


Fig. 3. ELISA (TNF-α, IL-6) assays 24 h after LPS treatment in BV2 cells treated with 10 μM of flavonoid. Results are the mean±S.D. (n=3), one-way ANOVA with Bonferroni correction: **p*<0.05, ***p*<0.01, ****p*<0.001.

3.4 Flavonoids blocks NF-κB activity in LPS-stimulated BV2 microglia

Nuclear factor kappa B (NF-κB) has been shown to play a key role in regulating the immune response to infection, including lipopolysaccharide (LPS) [22]. To examine whether the inhibitory effect of flavonoids on LPS-stimulated BV2 cells is associated with the suppression of the NF-κB signaling pathway, NF-κB transcriptional activity was measured using a luciferase reporter assay. As expected, LPS treatment induced about five fold increase in NF-κB transcriptional activity in BV2 cells, whereas LPS-induced NF-κB transcriptional activity was suppressed by pretreatment of flavonoids (Fig. 4). Although all number 1, 53, 61 and 62 flavonoid treatments significantly reduced NF- kB activity, number 53 and 61 flavonoid treatment more significantly reduced NF-kB activity, comparing the results of number 1 and 62. These results indicated that flavonoids attenuate LPS-induced inflammation, at least in part, by modulating NF-κB activation in BV2 cells.

Fig. 4.

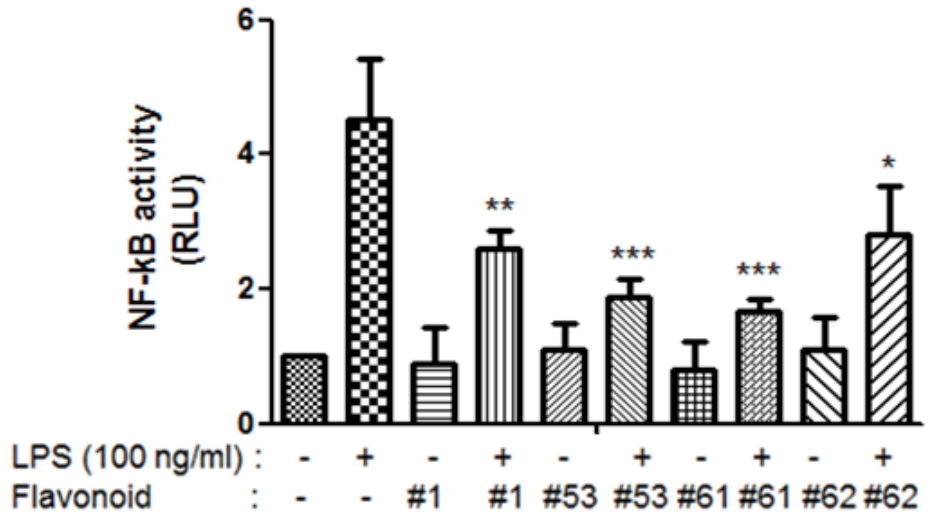


Fig. 4. Flavonoids were pretreated for 1 h prior to LPS treatment and luciferase activity was measured 3 h after LPS treatment (100 ng/ml). Results are the mean±S.D. (n=3), one-way ANOVA with Bonferroni correction: *p<0.05, **p<0.01, ***p<0.001.

3.5 Flavonoids significantly down-regulate expression of M1 state related genes

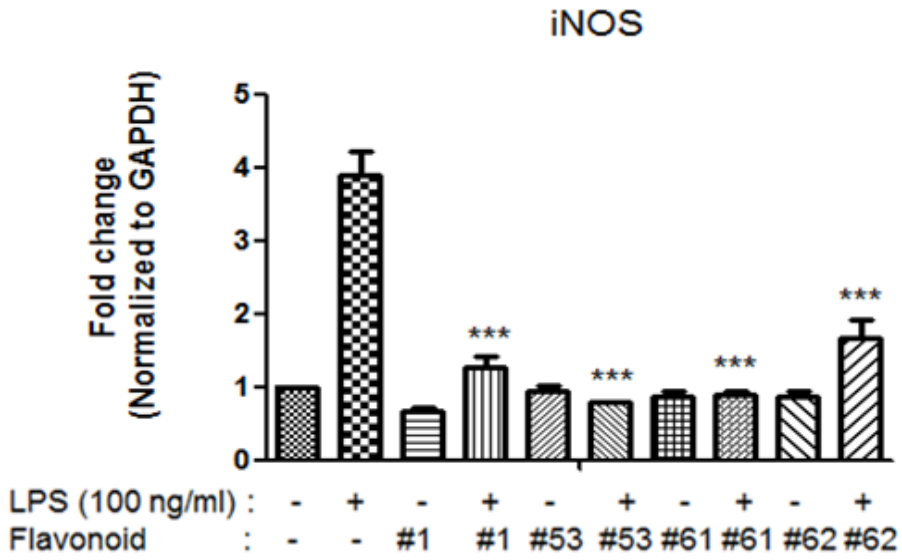
As introduced before, the ratio of particular cytokines can be used to identify the phenotype of activated microglia. A key factor to define M1 microglia is their ability to produce reactive oxygen species. An enzyme which is related to this process is inducible nitric oxide synthase (iNOS), which catalyze the production of nitrite. The flavonoids were pretreated 1 h prior to LPS treatment in BV2 cells and the mRNA level of iNOS was measured 1h after LPS treatment. Also, the mRNA levels of TNF-α and IL-12 were measured 3h after LPS treatment. As expected, LPS treatment increased mRNA levels of iNOS, TNF-α and IL-12 in BV2 cells, whereas LPS-induced mRNA levels were suppressed by pretreatment of flavonoids (Fig. 5a-c). These results indicated that flavonoids attenuate LPS-induced proinflammatory mediators from mRNA level in BV2 cells. However, number 53 flavonoid did not show significance in IL-12 mRNA level. IL-12 family is the only family of heterodimeric cytokines consisting of p35 and p40 subunits, which make them have several unique and distinctive features. It could be possible that the different effect of each subunit induced no substantial change in IL-12 mRNA level in flavonoid treatment except number 53 flavonoid [23, 24].

One of the best characterized markers in M2 alternative activation of microglia is the enzyme arginase 1 (Arg1), which catalyze arginine to polyamines, proline, and ornithines. Another cytokine to identify M2 state is IL-10. The mRNA expression levels of Arg1, IL-10 were measured 3h after LPS treatment. It was expected that mRNA level of Arg1 and IL-10 increase because M2 state can contribute to wound healing and matrix deposition [4]. However, mRNA levels Arg1 and IL-10 did not show a significant increase by pretreatment of flavonoids (Fig. 5d, e). The mRNA level of Arg1 in control group is similar to other LPS treatment and flavonoid treatment groups (Fig. 5d). The mRNA level of IL-10 showed over

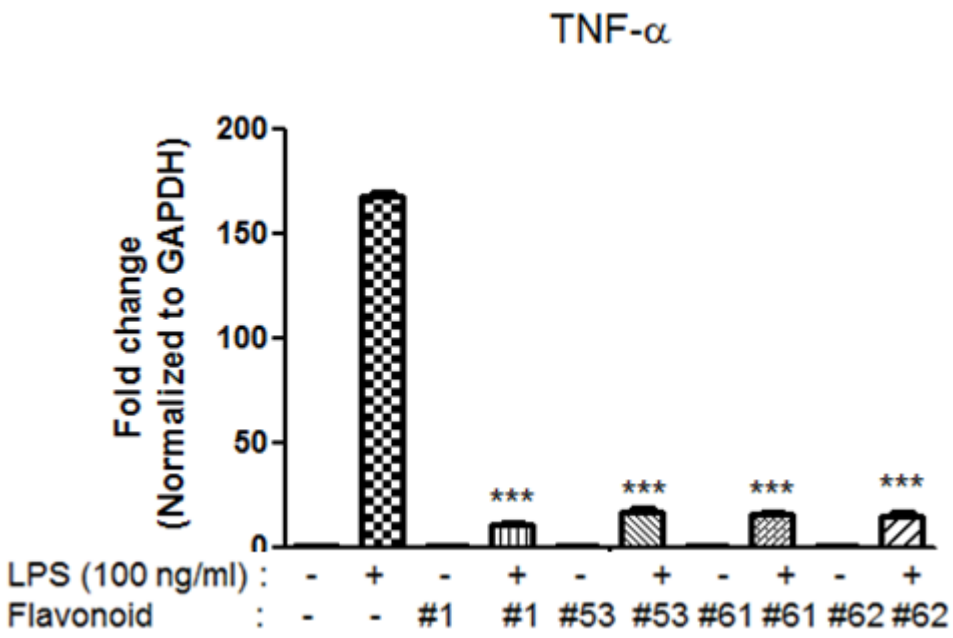
two hundred increase following LPS treatment and only flavonoid number 53 induced a substantial increase (Fig. 5e). With these results above, we concluded that flavonoids in this study could suppress proinflammatory cytokines from mRNA levels and inhibit NF-kB transcriptional activity. Meanwhile, they don’t have a significant effect on M2 state which is for the repairing and debris clearance.

Fig.5.

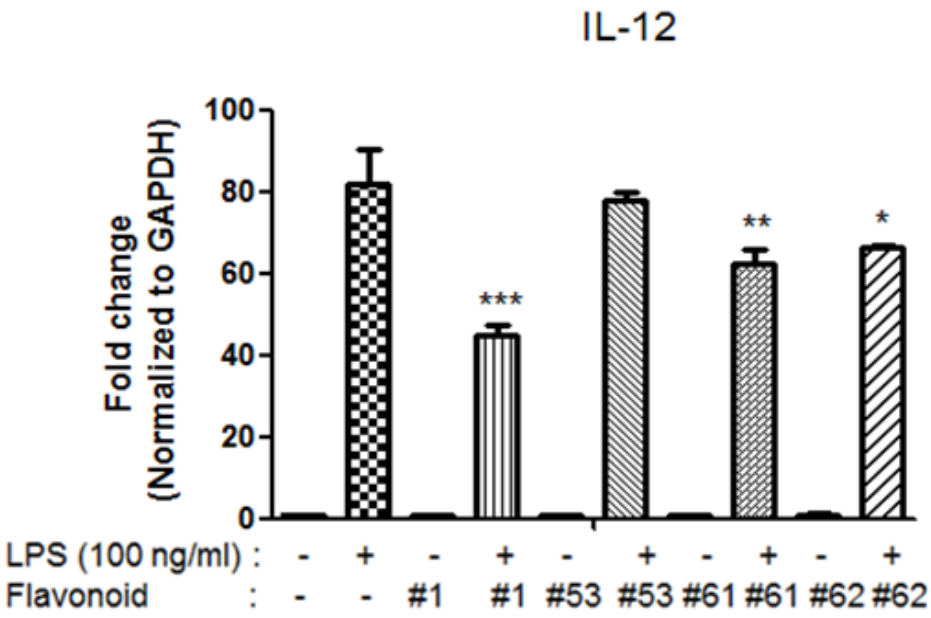
a


iNOS
Fold change
(Normalized to GAPDH)
5
4
3
2
1
0
***
***
***
***
LPS (100 ng/ml) : - + - + - + - + - +
Flavonoid : - - #1 #1 #53 #53 #61 #61 #62 #62

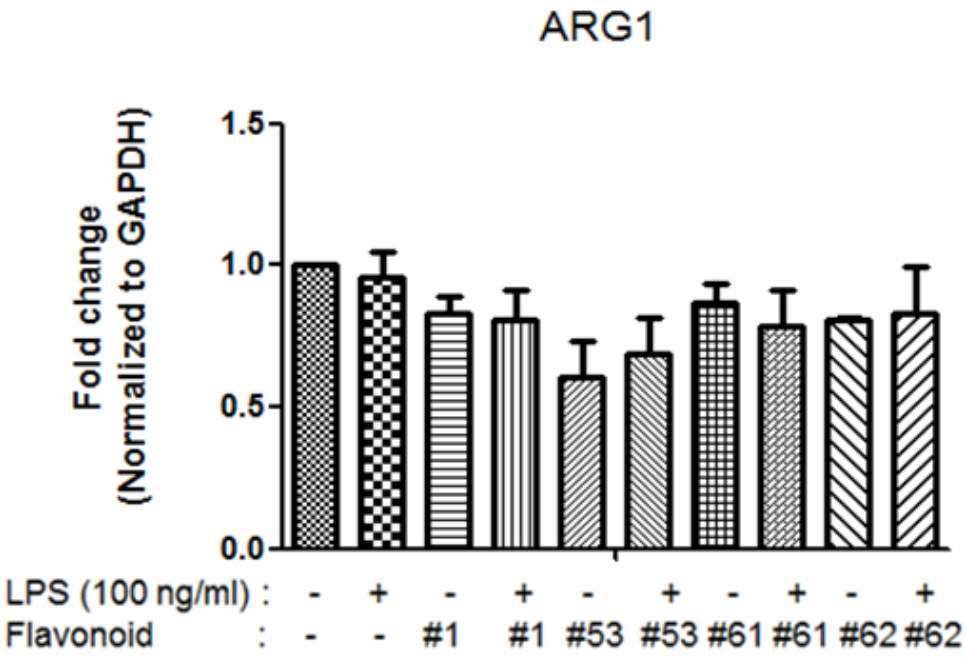
b


TNF-α
Fold change
(Normalized to GAPDH)
200
150
100
50
0
***
***
***
***
LPS (100 ng/ml) : - + - + - + - + - +
Flavonoid : - - #1 #1 #53 #53 #61 #61 #62 #62

c


IL-12
Fold change
(Normalized to GAPDH)
100
80
60
40
20
0
***
**
*
LPS (100 ng/ml) : - + - + - + - + - +
Flavonoid : - - #1 #1 #53 #53 #61 #61 #62 #62

d


ARG1
Fold change
(Normalized to GAPDH)
1.5
1.0
0.5
0.0
LPS (100 ng/ml) : - + - + - + - + - +
Flavonoid : - - #1 #1 #53 #53 #61 #61 #62 #62

e

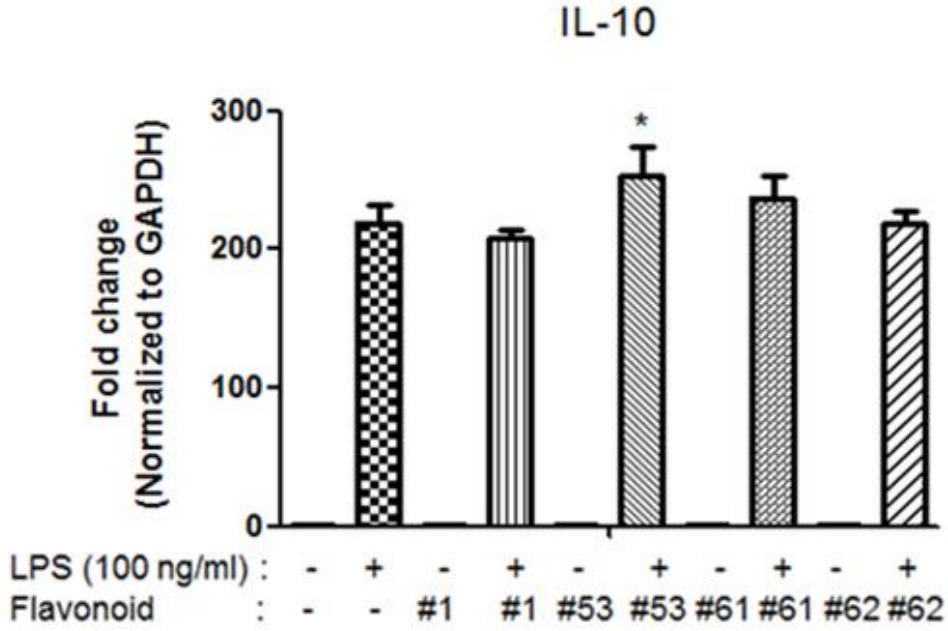


Fig. 5. mRNA expression level following LPS (100 ng/ml) treatment was measured by RT-PCR in BV2 cells. Results are the mean±S.D. (n=3), one-way ANOVA with Bonferroni correction: **p*<0.05, ***p*<0.01, ****p*<0.001.

Ⅳ. Discussion

Inflammation plays an important role in the pathology of neurodegenerative disorders in the brain [25]. In particular, neuroinflammation with prolonged activation of microglial cells leads to an increased production of proinflammatory mediators and cytokines. Chronic activation of microglia has been implicated in the pathology of neurodegenerative diseases [26, 27]. Therefore, inhibitors of these inflammatory molecules have been considered as candidate anti-inflammatory drugs for alleviation of the progression of neurodegenerative disease caused by activation of microglia [28]. Recently, several studies have demonstrated that the inhibition of pro-inflammatory mediators by flavonoids result in suppressing a number of key elements in cellular signal transduction pathways [11, 13]. In the present study, we demonstrated that flavonoids treatment of activated BV2 microglial cells resulted in significant inhibition of the production of the LPS-induced pro-inflammatory mediators (NO) and cytokines, (including TNF-α and IL-6). Therefore, inhibition of pro-inflammatory molecules by flavonoids, as shown in the present study, could be beneficial in the treatment of neurodegenerative diseases.

The cytokine production is essential for inducing the polarization of microglia into a classical activation, which is M1 state. In contrast with proinflammatory M1 cells, alternatively activated M2 macrophages express more anti-inflammatory cytokines and receptors to repair or protect the body from inflammation [4]. iNOS, TNF-α, the representative M1 markers, showed that LPS-induced mRNA levels were suppressed by pretreatment of flavonoids. However, number 53 flavonoid did not show significance in IL-12 mRNA level. It is known that IL-12 family is unique among a various cytokines because

they consist of the heterodimer of p35 and p40. Moreover, studies have demonstrated that homodimers and monomers of the p40 subunit also may act as antagonists of IL-12 function [23, 24]. So, there is a chance that p35 subunit had stronger effect while p40 subunit of IL-12 had weaker effect in flavonoid number 53 treatment. Meanwhile, mRNA levels of Arg1 and IL-10, which have M2 characteristics, did not show significant increases by pretreatment of flavonoids.

With these results, we could expect that the flavonoids used in this study have more protective effect on neuroinflammation. In this point, there is one important fact which must be considered as for the use of flavonoids as anti-inflammatory agents. Because microglial cells are located in the central nervous system, flavonoid must enter the Blood Brain Barrier (BBB) in order to have the neuroprotective effect. The relationship between flavonoid structure and permeability of the BBB is unveiled yet. Only low molecular weight and hydrophobic molecules are known to enter the brain through the BBB easily. The hydroxyl group of flavonoids may have a difficulty to enter the BBB [29]. Therefore, structural modifications of flavonoids should be considered for a pharmacological treat.

In this study, establishing a detailed mechanistic pathway for flavonoids' neuroprotective effect could ultimately result in broad therapeutic applications that benefit patients who suffer from neurodegenerative diseases .

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요약문

미세아교세포에서 flavonoid 의 신경염증 억제 효과

미세아교세포 (microglia)는 뇌와 척수를 포함하는 중추신경계(Central Nervous System; CNS)에서 주요하게 면역 기능을 수행하는 세포이다. 일반적인 상태에서 신경염증은 항상성을 유지하고 조직을 보호하는 기능을 한다. 하지만 오랜 시간 동안 외부에서 또는 내부의 자극이나 스트레스를 받게 되면 미세아교세포를 과도하게 활성화시켜 신경독성을 가진 물질을 배출하거나 조직에 손상을 주게 된다. 나아가 퇴행성 뇌질환을 유발하게 된다. 따라서 신경염증을 조절하는 메커니즘에 대한 연구는 퇴행성 뇌질환의 치료제 개발에 기여할 수 있다.

Flavonoid 는 식물의 색소로 알려져 있으며 최근 염증을 억제하는 기능을 가진다는 연구가 있다. 이 연구에서는 flavonoids 가 미세아교세포 BV2 세포에서 뇌신경염증을 억제하는 기능을 하는지 알아보고자 하였다. Flavonoid 를 lipopolysaccharide (LPS) 처리한 BV2 세포에 전처리 하였을 때 대표적인 신경염증 사이토카인 (cytokine)인 TNF-α 와 NO, IL-6 가 현저히 억제되는 효과를 관찰하였다. 또한, MAP kinase 단백질과 NF-κB 의 활성화 역시 억제되는 결과를 나타내었다. 정량 중합효소 연쇄반응 (Quantitative PCR) 분석 결과 염증 효과를 가지는 M1 연관 유전자의 발현이 감소되는 것을 관찰하였다.

이 연구에서는 Flavonoid 가 뇌신경염증을 억제하는 메커니즘을 밝힘으로서 안전한 항염증 치료제를 비롯, 더 나아가 퇴행성 뇌질환의 치료제제로의 발전 가능성을 제시하고 있다.

키워드: flavonoid, 신경염증, 미세아교세포, MAP kinases, NF-κB