Rhamnus crenata leaves exhibit anti-inflammatory activity via modulating the Nrf2/HO-1 and NF-κB/MAPK signaling pathways

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Article

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Abstract

Rhamnus crenata Siebold & Zucc. (RC), which belongs to the *Rhamnaceae* family, is distributed in China, Japan, and Korea. There have been no studies on mechanisms underlying the anti-inflammatory activity of RC. Here, we aimed to elucidate the potential anti-inflammatory mechanisms of RC using RAW264.7 cells. We evaluated the anti-inflammatory effects of a 70% ethanol extract of RC leaves (RC-L) and elucidated the potential signaling pathways in lipopolysaccharide (LPS)-induced RAW264.7 cells. RC-L exhibited anti-inflammatory effects, such as inhibition of nitric oxide (NO) production; it dose-dependently decreased NO production by suppressing the expression of inducible NO synthase, cyclooxygenase-2, interleukin 1β, and tumor necrosis factor alpha in LPS-induced RAW264.7 cells. Additionally, RC-L inhibited nuclear factor kappa B (NF-κB) activation and the degradation and phosphorylation of NF-κB inhibitor alpha and suppressed the phosphorylation of p38, extracellular signal-regulated kinase 1/2, and c-Jun N-terminal kinase. Furthermore, it increased the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1). These results suggest that RC-L exerts anti-inflammatory effects by inhibiting the NF-κB and mitogen-activated protein kinase signaling pathways and inducing Nrf2/HO-1 expression and could act as a natural anti-inflammatory drug.

Introduction

Inflammation is an innate immune response caused by pathogen infestation or tissue damage and is a complex process carried out by various immune cells [1, 2]. The prolonged production of inflammatory mediators by macrophages can damage the host cells [3]. Accordingly, a continuous inflammatory reaction can cause dysfunction, such as pain, fever, edema, and mucosal damage, and lead to the development of various diseases and cancers [4].

The treatment of inflammatory diseases primarily focuses on the suppression of inflammatory mediators, such as nitric oxide (NO), inducible NO synthase (iNOS), cyclooxygenase 2 (COX-2), nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and reactive oxygen species (ROS), as well as the inhibition of complex networks of signaling pathways [5]. Nuclear factor erythroid-2-related factor-2 (Nrf2) regulates antioxidant enzymes and detoxification-related enzymes in the body; after binding to antioxidant response elements, it increases the expression of these enzymes and cytoprotective genes [6]. Additionally, Nrf2 regulates the expression of heme oxygenase (HO-1) [7], which is involved in the production of carbon monoxide, biliverdin, bilirubin, and iron to reduce the generation of inflammation-inducing cytokines, and has antioxidant and anti-inflammatory effects.

Recently, various studies have been conducted to identify novel plant-based drugs with low toxicity and high anti-inflammatory effects, from various medicinal herbs [8]. *Rhamnus crenata* Siebold & Zucc, which belongs to Rhamnaceae, is a deciduous shrub or small tree plant that distributed in China, Japan, Taiwan and Korea. The leaves, branch and fruit of *Rhamnus crenata* have been reported to antioxidant and immunity properties [9, 10]. However, research on the action mechanism underlying the anti-inflammatory activity of *Rhamnus crenata* is insufficient. Therefore, in this study, the anti-inflammatory activity and

action mechanism of *Rhamnus crenata* extracts were investigated using RAW264.7 mouse macrophages induced by lipopolysaccharide (LPS).

Materials And Methods

Chemical reagents

1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture, 2.50 mM L-Glutamine, and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (DMEM/F-12) was used to culture mouse macrophages, which were purchased from Lonza (Walkersville, MD, USA). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA), and antibodies against IκB-α, phosphorylated (p)-ERK1/2, total-ERK1/2, p-p38, total-p38, and β-actin for western blotting were purchased from Cell Signaling Technology (Danvers, MA, USA).

Sample preparation

The *Rhamnus crenata* leaves (RC-L) used in this study was collected from Yeosu-si, Jeollanam-do, Republic of Korea, in June 2019 (voucher specimen: FMCBcYS-1906-1) and formally identified by Gyu Young Chung as a professor of Andong National University, Korea. The use of RC-L material in the present study complies with international, national and institutional guidelines. The RC-L was stored in Forest Medicinal Resources Research Center. After lyophilization, 400 mL of 70% ethanol was added to 20 g of the powdered leaves, stirred, and extracted at for 48 h at 25 ℃. After extraction, the sample was filtered through a filter paper (No. 2, Whatman Co., Maidstone, England), concentrated in a vacuum evaporator in a bath below 40 ℃, and lyophilized. The sample extract was then dissolved in dimethyl sulfoxide (DMSO) and used in subsequent experiments.

Cell culture

Mouse macrophage RAW264.7 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). After the addition of 10% fetal bovine serum, the cells were cultured in DMEM/F-12 medium, containing penicillin and 100 μg/mL streptomycin, at 5% CO_2. When the density of the cells was more than 80%, trypsin-ethylenediaminetetraacetic acid solution was used for passaging.

Measurement of NO production inhibition

NO production was measured using the method described by Namkoong et al. [27], with some modifications. Mouse macrophage RAW264.7 cells were dispensed into 12-well plates and cultured for 24 h at 37 ℃. Subsequently, the cells were treated with 0, 25, and 50 mg/mL RC-L and incubated for 2 h at 37 ℃. Afterward, they were treated with 1 μg/mL LPS and cultured for 18 h at 37 ℃. NO production was measured using the Griess reagent (Sigma-Aldrich Co., St. Louis, MO, USA). Absorbance was measured at 540 nm using a microplate reader (PerkinElmer, Waltham, MA, USA).

Measurement of cell viability

Cell viability was measured using the MTT assay. RAW264.7 cells were cultured at a concentration of 1×10^6 cells/well in 12-well culture plates and treated with RC-L for 12 h. Subsequently, the cells were incubated with 200 μL of MTT solution (1 mg/mL) for an additional 2 h. The resulting crystals were dissolved in DMSO. Formazan formation was determined by measuring the absorbance at a wavelength of 570 nm (Perkin Elmer, Waltham, MA, USA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cells were washed with 1× phosphate-buffered saline to extract proteins from the cells. Protease and phosphatase inhibitor cocktails (Sigma-Aldrich Co., St. Louis, MO, USA) were added to the radioimmunoprecipitation assay buffer (Boston Bio Products, Ashland, MA, USA) to obtain lysed proteins. After quantifying the proteins using the bicinchoninic acid protein assay (Pierce Biotechnology Inc., Waltham, MA, USA), proteins were loaded onto 10% SDS acrylamide; the resolved proteins were then transferred to a nitrocellulose membrane (GE Healthcare Life Science, Germany), which was blocked with 5% non-fat dry milk for 1 h at 25 ℃. After 1 h, the membrane was incubated with the primary antibody, dissolved in 5% non-fat milk, at 4°C overnight. The membrane was washed thrice for 5 min with Tris-buffered saline (1× TBS-T) containing 0.005% Tween 20. Then, the membrane was incubated with a secondary antibody, dissolved in 5% non-fat milk, for 1 h. After washing thrice for 5 min with TBS-T, the proteins were visualized using the enhanced chemiluminescence western blotting substrate (Amersham Biosciences Co., Little Chalfont, England).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using the RNeasy Mini kit (QIAGEN GmbH., Hilden, Germany). Then, 1 μg RNA was used for cDNA preparation using the Verso cDNA kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR was performed using a PCR master mix kit (Promega Co., Madison, WI, USA); the primers used are listed in Table 1. The quality of cDNA was determined through PCR; the amplified PCR products were analyzed through 1% agarose gel electrophoresis using stain Safe Shine Green stain(10,000×Biosesang) the gel was visualized in Chemidoc (Bio-rad, Chemi Doc MP Imaging system, Hercules, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (a housekeeping gene) was used as an internal control.

Table 1
Sequences of oligonucleotide primers used for RT-PCR

Gene name	Sequence
iNOS	Forward: 5'-aatggcaacatcaggtcggccatcact-3'
	Reverse: 5'-gctgtgtgtcacagaagtctcgaactc-3'
COX-2	Forward: 5'-ggagagactatcaagatagt-3'
	Reverse: 5'-atggtcagtagacttttaca-3'
IL-1β	Forward: 5'-acctgtcctgtgtaaatgaaagacg-3'
	Reverse: 5'-ttggtattgcttgggatcc-3'
TNF-α	Forward: 5'-tccaggcggtgcctatgt-3'
	Reverse: 5'-cgatcaccccgaagttcagt-3'
GAPDH	Forward: 5'-ggcatggccttccgtgt-3'
	Reverse: 5'-ggtttctccaggcggca-3'

RT-PCR, reverse transcription polymerase chain reaction; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

All data are presented as mean ± standard deviation (SD). The differences between treatments were determined with Student's *t*-test, and $p < 0.05$ was considered statistically significant.

Results

Inhibitory effects of RC-L on NO production and proinflammatory expression in LPS-stimulated RAW264.7 macrophages

The RC-L reduced the production of LPS-induced NO in RAW264.7 macrophages in a concentration-dependent manner (Fig. 1A). The 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay showed that RC-L did not exhibit toxicity (Fig. 1B). Therefore, LPS-stimulated RAW264.7 macrophages were used to determine the inhibitory activity of inflammatory mediator genes encoding iNOS, COX-2, interleukin-1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α), which are involved in the pathogenesis of inflammatory diseases. The RC-L decreased the expression of these inflammatory mediators in a concentration-dependent manner (Fig. 1C). These findings confirm that RC-L exhibits anti-inflammatory activity.

Effects of RC-L on NF-κB signaling activity in LPS-stimulated RAW264.7 macrophages

Inhibition of NF-κB signaling activity is considered an important target for anti-inflammatory activity; accordingly, we investigated whether RC-L affects the LPS-induced NF-κB signaling activation. RC-L blocked the LPS-induced degradation and phosphorylation of NF-κB inhibitor alpha (IκB-α) in a concentration-dependent manner (Fig. 2A and 2B). Additionally, it inhibited the nuclear translocation of p65 (Fig. 2C). These results suggest that RC-L exerts an anti-inflammatory effect by inhibiting NF-κB activity.

Effects of RC-L on MAPK signaling activity in LPS-stimulated RAW264.7 macrophages

Extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) exist in various conformations. To investigate the effects of RC-L on the inhibition of MAPK activity, different concentrations of RC-L were used to treat LPS-induced RAW264.7 macrophages. Phosphorylation of ERK1/2, p38, and JNK was found to be inhibited in a concentration-dependent manner (Fig. 3A–3C). These findings suggest that RC-L exerts anti-inflammatory effects by inhibiting MAPK activity.

Effects of RC-L on HO-1 expression in RAW264.7 macrophage cells

The RC-L dose-dependently increased HO-1 protein levels, and HO-1 expression was induced 9 h after RC-L treatment, with the highest expression being observed after 24 h (Fig. 4A and 4B), which indicates that HO-1 expression is influenced by RC-L activity.

HO-1 expression is induced by RC-L through p38, ERK1/2, JNK, and IκB kinase (IKK) activation in RAW264.7 cells

We evaluated whether RC-L affect HO-1 expression in RAW264.7 cells. To investigate the upstream kinases such as ERK1/2, p38, and JNK associated with HO-1 expression by RC-L, we pretreated RAW264.7 cells with PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), or BAY11-7082 (IKK inhibitor) and then co-treated them with RC-L. As shown in Fig. 5A-5D, the pretreatments of PD98059, SB203580, SP600125, and BAY11-7082 blocked HO-1 expression by RC-L in RAW264.7 cells. These results indicate that the HO-1 protein expression induced by RC-L is dependent on p38, ERK1/2, JNK, and IKK.

HO-1 expression induced by RC-L is dependent on Nrf2 activation in RAW264.7 cells

We investigated whether Nrf2 activation is involved in the RC-L-induced HO-1 expression. RC-L dose-dependently increased the nuclear levels of Nrf2, which indicated that RC-L may induce Nrf2 activation (Fig. 6A and 6B). AS HO-1 expression induced by RC-L was attenuated by the inhibition of p38, ROS, and glycogen synthase kinase 3 beta, we investigated whether ERK1/2 contributes to the Nrf2 activation induced by RC-L. These findings indicate that RC-L may upregulate HO-1 expression through Nrf2 activation via the ERK1/2 pathway.

Discussion

In the immune system, macrophages play an important role in regulating immune function and maintaining homeostasis by suppressing the inflammatory reactions caused by various stimuli, such as free radicals and stress [11]. In particular, macrophages are involved in the production of NO, a representative inflammatory mediator, which is a highly reactive biomolecule produced from L-arginine by NOS [12].

iNOS and COX-2 are representative pro-inflammatory cytokines. iNOS is primarily produced by stimulated macrophages, resulting in the inflammatory of NO [13, 14]. COX-2 is an enzyme that converts arachidonic acid into prostaglandins; although COX-1 is primarily found in normal cells, COX-2 is expressed at the site of inflammation and is associated with cancer [15]. Since cytokines such as IL-1β, IL-6, and TNF-α are inflammatory cell signaling proteins [16], inhibition of these inflammatory mediators is a promising target for the treatment of inflammatory diseases [17].

In this study, the effects of RC-L on NO production, cell viability, and expression of pro-inflammatory cytokines were investigated using the RAW264.7 cell line. NF-κB is a transcription factor that participates in various processes, such as general immunity, inflammation, and cell growth regulation. Usually, it exists in an inactive state, bound to the inhibitory protein IκB in the cytoplasm [18]. As p65 and p50, which are bound to the cell, move from the cytoplasm to the cell nucleus, NF-κB activation occurs, and inflammation is promoted [19]. When NF-κB signaling is activated, p65 (a component of the NF-κB complex) is transferred to the nucleus [20], and its phosphorylation plays a major role in NF-κB activity because it regulates migration from the cytoplasm to the nucleus. Inhibition of phosphorylation is associated with the inhibition of NF-κB activity [21]. RC-L inhibited LPS-induced NF-κB activity and intranuclear migration in RAW264.7 cells. Western blot analysis revealed that the quantity of protein transferred in the nucleus was significantly decreased in cells treated with 25 and 50 mg/mL of RC-L compared to that in the with LPS-treated control group and the LPS-only treatment group.

The MAPK family is a signal transduction mediator in cellular responses to various external stimuli and includes ERK, p38, and JNK [22]. In this study, western blotting results showed that RC-L inhibited the LPS-induced phosphorylation of ERK, p38, and JNK in RAW264.7 cells.

HO-1 induction acts against the mechanisms that protect cellular lipids and proteins from oxidative damage, thereby weakening the inflammatory response [23]. Nrf2 increases the expression of certain genes after binding to antioxidant response elements [24]. Under normal conditions, there are low levels

of Nrf2 in the cytoplasm because it forms a complex with Kelch-like ECH-associated protein 1 (Keap1), which degrades it. However, it can separate from Keap1 by external stimulation or oxidative stress and move to the nucleus [25]. Of note, Nrf2 and HO-1 are closely related, and HO-1 expression is increased by Nrf2.

When RAW264.7 macrophages were treated with RC-L, Nrf2 accumulated in the nucleus in a concentration-dependent manner, and the highest value was observed after 1 h of treatment. In addition, to determine whether HO-1 plays a major role in the anti-inflammatory effect of RC-L, inhibitors such as SB203580, PD98059, SP600125, BAY11-7082, and NAC were used. As expected, treatment with these inhibitors suppressed HO-1 expression. Accordingly, the HO-1 induction ability of RC-L decreased when HO-1 inhibitors were administered. These findings confirmed that the major anti-inflammatory mechanism of RC-L is HO-1 expression. Moreover, the translocation of the transcriptional regulatory factor of the HO-1 gene into the nucleus migration of Nrf2 was also significantly increased in a concentration-specific manner. Western blotting was performed to confirm the intranuclear migration of Nrf2; it was found that the amount of Nrf2 increased in the nucleus following RC-L treatment. These findings indicated that RC-L promoted the migration of Nrf2 into the nucleus and increased the expression of HO-1.

We also investigated RC-L-induced Nrf2 expression over time and found that this expression increased after 1 h of treatment. In the group treated with RC-L for 24 h, the expression of Nrf2 showed a similar pattern to that at 0 h without treatment, indicating that treatment with RC-L for 24 h did not affect the expression of Nrf2. However, owing to the activation of Nrf2, the expression of HO-1 was highest at 24 h. We expected that HO-1 expression would increase with the expression of Nrf2, but the expression of Nrf2 and HO-1 in the group treated with RC-L for 24 h showed a contrasting trend, consistent with the results of a previous study [26]. Similar to our results, previous studies have reported that the anti-inflammatory activity of biologically active substances derived from various natural substances is regulated by the Nrf2/HO-1 pathway [27]. In conclusion, our results demonstrated that RC-L reduces the expression of various inflammatory genes and can regulate inflammation through various pathways, such as the NF-κB, MAPK, and Nrf2/HO-1 pathways.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Availability of data and materials All data/material is available on request from the corresponding author.

Conflict of Interest. The authors declare no competing interests.

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Authors' contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Da Som Kim, Hyun Ji Eo, and Gwang Hun Park. The first draft of the manuscript was written by Da Som Kim and all authors commented on previous versions of the manuscript. All authors reviewed and approved the final manuscript.

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Figures

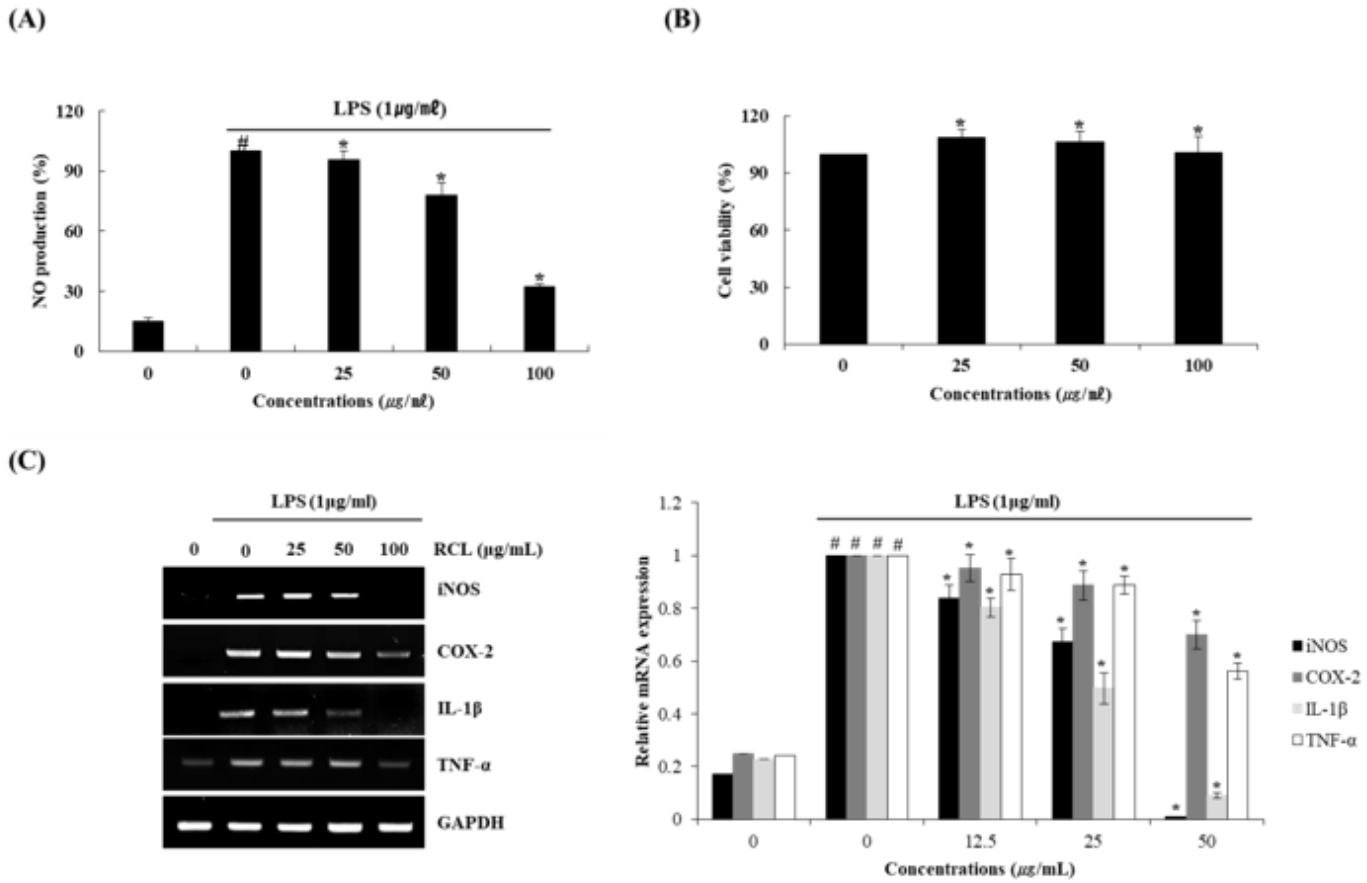

Figure 1

Effects of *Rhamnus crenata* leaves (RC-L) on the production of pro-inflammatory mediators, such as nitric oxide (NO), inducible NO synthase (iNOS), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α), in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. (A) RAW264.7 cells were pretreated with 25, 50, and 100 μg/mL of RC-L for 6 h and then further treated with 1 μg/mL of LPS for 18 h before the Greiss assay was performed. (B) RAW264.7 cells were treated with 25, 50, and 100 μg/mL of RC-L for 24 h, and then the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide assay was performed. (C) Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for RT-PCR. *$p < 0.05$ compared to the cells without RC-L.

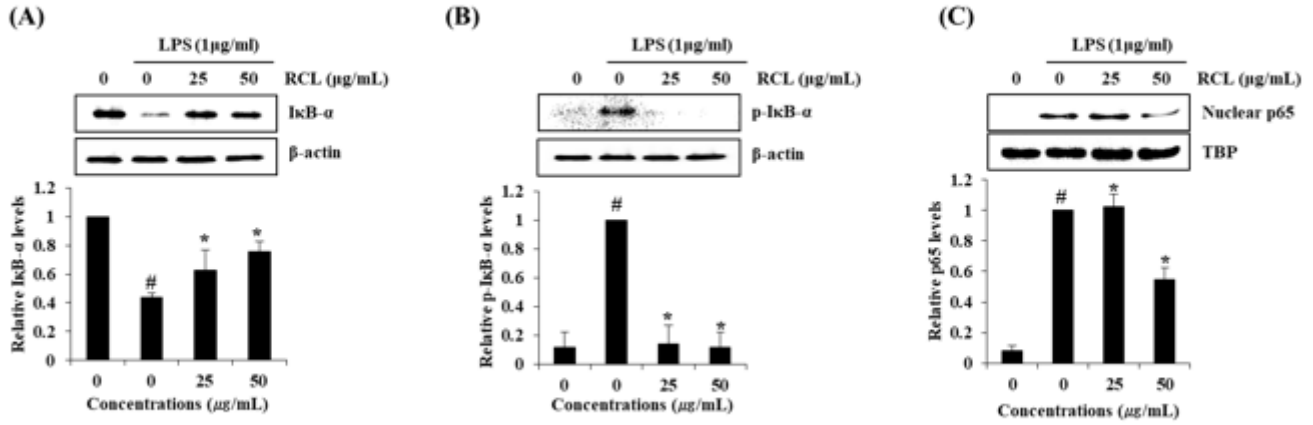

Figure 2

Effects of *Rhamnus crenata* leaves (RC-L) on nuclear factor kappa B (NF-κB) signaling activation in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. (A and B) RAW264.7 cells were pretreated with RC-L (0, 25, or 50 μg/mL) for 6 h, followed by treatment with LPS (1 μg/mL) for 40 min. (C) RAW264.7 cells were pre-treated with RC-L (0, 25, or 50 μg/mL) for 40 min. After the treatment, the cytosol and nucleus were isolated from the cells. The cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and western blotting was performed using antibodies against p65, NF-κB inhibitor alpha (IκB-α), and its phosphorylated form. β-actin was used as an internal control. #$p < 0.05$ compared to that for untreated cells, and *$p < 0.05$ compared to that for cells treated with LPS alone.

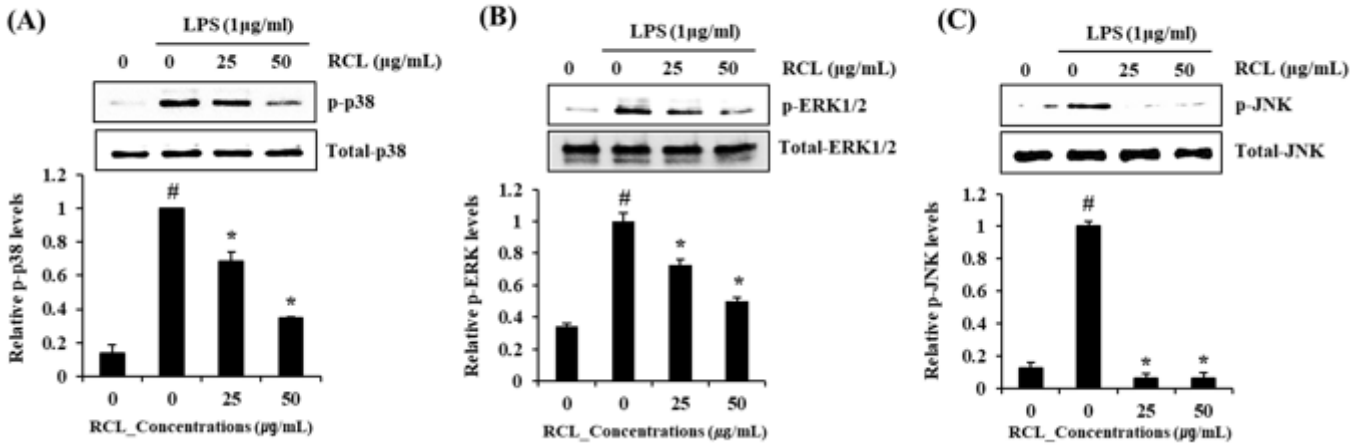

Figure 3

Effects of *Rhamnus crenata* leaves (RC-L) on mitogen-activated protein kinase (MAPK) signaling activation in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. (A, B and C). RAW264.7 cells were pretreated with RC-L (0, 25, or 50 μg/mL) for 6 h and then co-treated with LPS (1 μg/mL) for 40 min. The

cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and western blotting was performed using antibodies against phosphorylated p38, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK). #p < 0.05 compared to that for the untreated cells, and *p < 0.05 compared to that for cells treated with LPS alone.

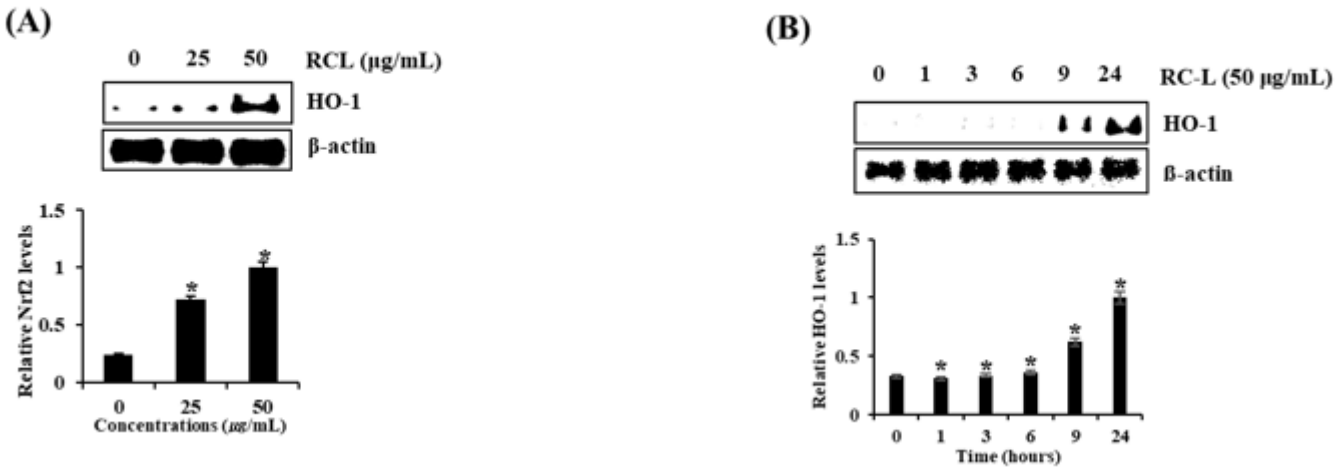

Figure 4

(A) Effects of *Rhamnus crenata* leaves (RC-L) on heme oxygenase-1 (HO-1) expression in RAW264.7 cells. (B) RAW264.7 cells were treated with 50 μg/mL RC-L for the indicated time periods and then western blot analysis was performed. RAW264.7 cells were treated with RC-L (50 μg/mL) for 24 h and then western blot analysis was performed using HO-1 antibodies. β-actin was used as an internal control for western blot analysis.

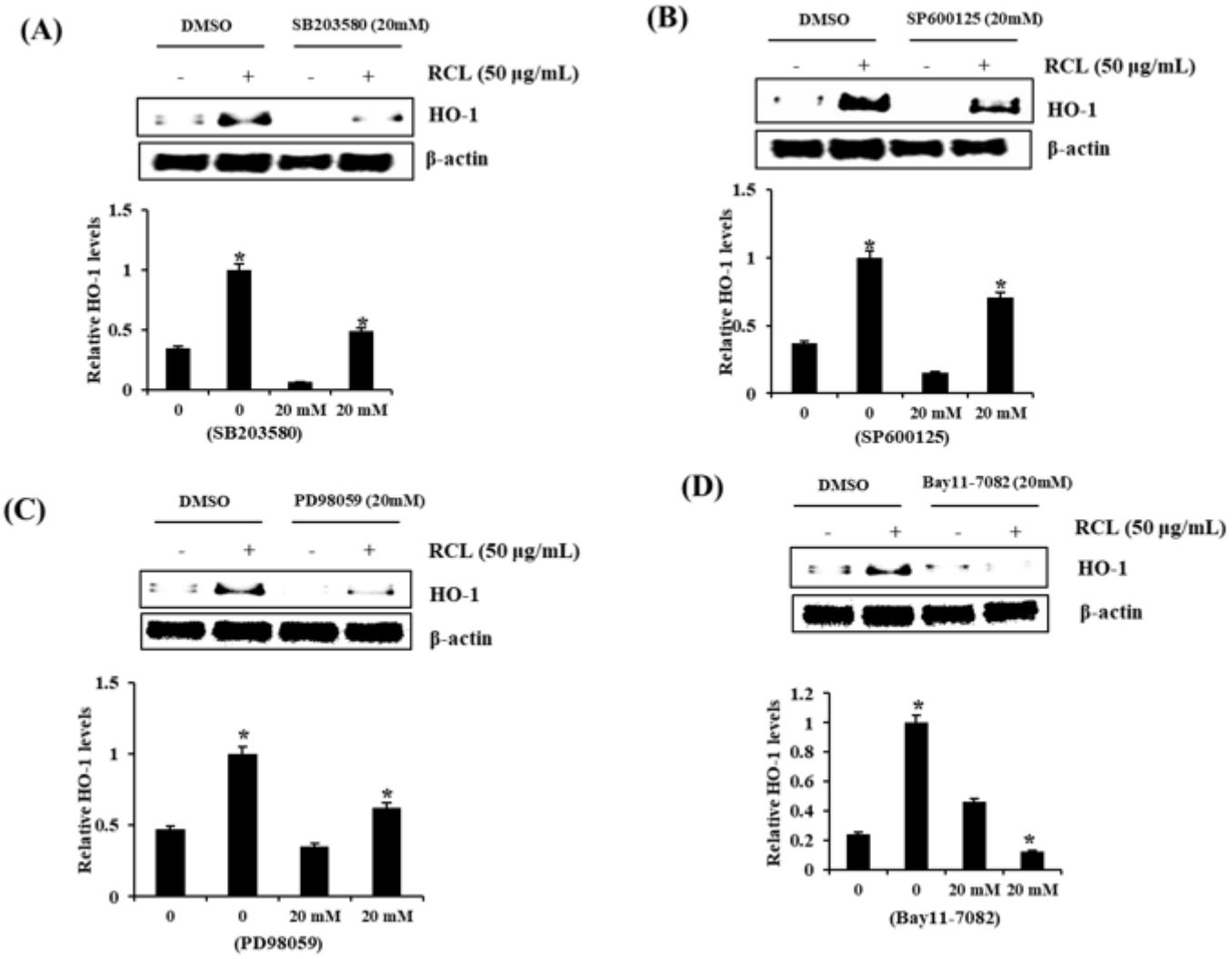

Figure 5

Effects of inhibitors on *Rhamnus crenata* leaf (RC-L)-mediated heme oxygenase-1 (HO-1) expression in RAW264.7 cells. RAW264.7 cells were pretreated with 20 μM each of (A) SB203580, (B) SP600125, (C) PD98059, and (D) BAY11-7082 for 2 h and then co-treated with 50 μg/mL of RC-L for 24 h. Western blot analysis was performed using anti-HO-1 antibodies. *P < 0.05 compared to the cells without RC-L.

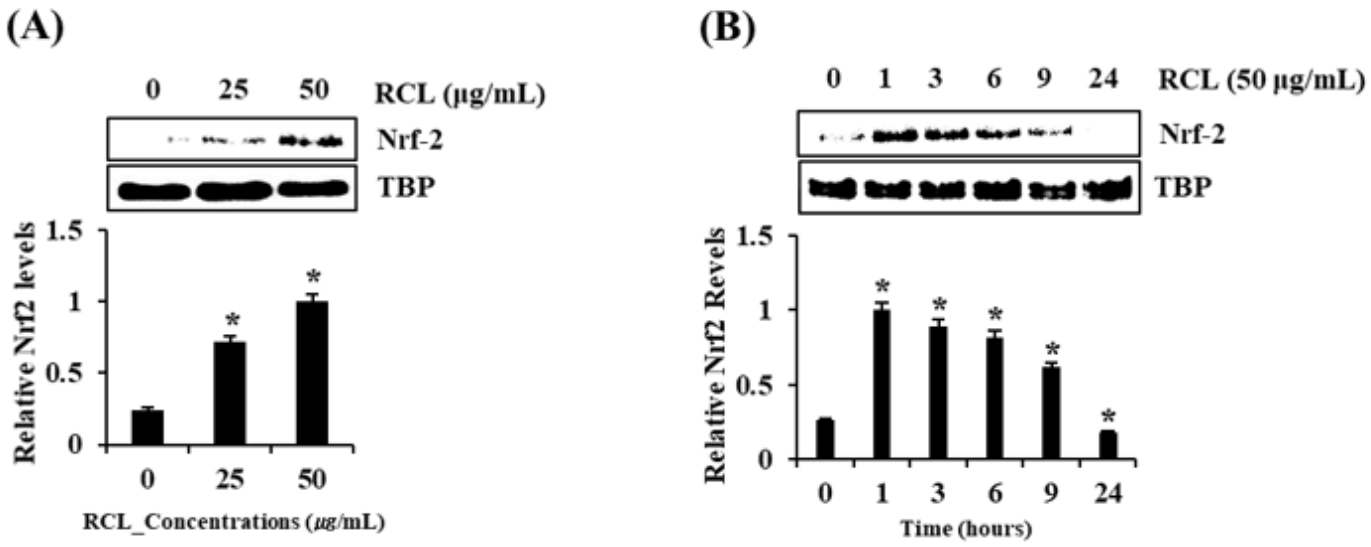

Figure 6

Effects of *Rhamnus crenata* leaves (RC-L) on nuclear factor erythroid 2-related factor 2 (Nrf2) activation in RAW264.7 cells. (A) RAW264.7 cells were treated with 0, 25, and 50 μg/mL of RC-L for 1 h; then, the nuclear proteins were subjected to western blot analysis using an anti-Nrf2 antibody. (B) RAW264.7 cells were treated with RC-L (50 μg/mL) for 24 h and then western blot analysis was performed using an anti-Nrf2 antibody. *$p < 0.05$ compared to that for cells without RC-L treatment.