Rhamnus crenata leaf extracts exhibit anti−inflammatory activity *via* modulating the Nrf2/HO−1 and NF−κB/MAPK signaling pathways

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ABSTRACT

Objective: To elucidate the potential anti-inflammatory mechanisms of *Rhamnus crenata* leaf extracts using RAW264.7 cells.

Methods: We used 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay to measure cell viability. Nitric oxide (NO) production was measured using Griess reagent. Western blotting and RT-PCR assays were carried out for analyzing the protein and gene expressions of pro-inflammatory mediators, respectively. Moreover, PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and BAY11-7082 (NF-κB inhibitor) were used to evaluate the anti-inflammatory mechanism of *Rhamnus crenata* leaf extract.

Results: *Rhamnus crenata* leaf extracts significantly inhibited the production of the pro-inflammatory mediators such as NO, iNOS, COX-2, IL-1β, and TNF-α in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. *Rhamnus crenata* leaf extracts also suppressed LPS-induced degradation of IκB-α and nuclear accumulation of p65, which resulted in the inhibition of NF-κB activation in RAW264.7 cells. Additionally, the extracts attenuated the phosphorylation of p38, ERK1/2, and JNK in LPS-stimulated RAW264.7 cells. Moreover, HO-1 expression induced by *Rhamnus crenata* leaf extracts was significantly downregulated by SB230580, PD98059, SP600125 and BAY11-7082.

Conclusions: *Rhamnus crenata* leaf extract may upregulate HO-1 expression through inhibition of p38, ERK1/2, and NF-κB activation, which may contribute to the anti-inflammatory activity of the extracts. *Rhamnus crenata* leaf extracts may have great potential for the development of anti-inflammatory drugs to treat acute and chronic inflammatory diseases.

KEYWORDS: Anti-inflammatory activity; Heme oxygenase-1; Nrf2; Mitogen-activated protein kinase; Nuclear factor kappa B; *Rhamnus crenata*

1. 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,

Significance

Rhamnus crenata is widely distributed in China, Japan, and Korea. There is scarce information on mechanisms underlying the anti-inflammatory activity of this plant. In this study, *Rhamnus crenata* leaf extract significantly inhibited the production of the pro-inflammatory mediators and suppressed NF-κB signaling activation through blocking IκB-α degradation and p65 nuclear accumulation in lipopolysaccharide-stimulated RAW264.7. *Rhamnus crenata* leaf extract also inhibited MAPK signaling activation by attenuating the phosphorylation of ERK1/2, p38 and JNK and induced Nrf2/HO-1 expression. Therefore, this plant may have great potential for the development of anti-inflammatory drugs to treat acute and chronic inflammatory drugs.

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* (*R. crenata*) Siebold & Zucc, which belongs to Rhamnaceae, is a deciduous shrub or small tree plant that is distributed in China, Japan, Taiwan, and Korea. The leaves, branches, and fruit of *R. crenata* have been reported to have antioxidant and immune properties[9]. However, research on the action mechanism underlying the anti-inflammatory activity of *R. crenata* is insufficient. Therefore, in this study, the anti-inflammatory activity and action mechanism of *R. crenata* extracts were investigated using lipopolysaccharide (LPS)-induced RAW264.7 mouse macrophages.

2. Materials and methods

2.1. Chemical reagents

In this study, a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture, 2.50 mM *L*-glutamine, and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (DMEM/F-12) were used to culture mouse macrophages, which were purchased from Lonza (Walkersville, MD, USA). LPS and inhibitors (PD98059, SB203580, SP600125, and BAY11-7082) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and antibodies against IκB-α (#4148), phospho-IκB-α (#2859), phospho-ERK1/2 (#4377), ERK1/2 (#9102), phospho-p38 (#4511), p38 (#9212), phospho-JNK(#9251), JNK (#9252), NF-κB p65 (#8242), HO-1 (#70021), Nrf2 (#12721), TATA-binding protein (TBP, #8515), and β-actin (#5125) for Western blotting assays were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Sample preparation

The *R. crenata* leaves used in this study were collected from Yeosu-si, Jeollanam-do, Republic of Korea, in June 2019 (voucher specimen: FMCBcYS-1906-1) and formally identified by Gyu Young Chung, a professor of Andong National University, Korea. The use of *R. crenata* leaves material in the present study complied with international, national, and institutional guidelines. The *R. crenata* leaves were stored in Forest Medicinal Resources Research Center (voucher number FMRC-20190902A1). After lyophilization, 400 mL of 70% ethanol was added to 20 g of the powdered leaves, stirred, and extracted for 48 h at 25℃. After extraction, the sample was filtered through 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.

2.3. Cell culture

Mouse 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 a 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 passage.

2.4. Measurement of cell viability

Cell viability was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. RAW264.7 cells were cultured at a concentration of 1×10^6 cells/well in 12-well culture plates and treated with *R. crenata* leaf extracts 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).

2.5. Measurement of NO production

NO production was measured using the method described by Namkoong *et al.*[10], 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, 50, and 100 μg/mL *R. crenata* leaf extracts 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 Griess reagent (Sigma-Aldrich Co., St. Louis, MO, USA). Absorbance was measured at 540 nm using a microplate reader (PerkinElmer, Waltham, MA, USA).

2.6. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting assay

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 (1:1000), dissolved in 5% non-fat milk, at 4℃ 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 (1:500), 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).

2.7. Reverse transcription−polymerase chain reaction (RT−PCR) assay

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.

2.8. Statistical analysis

All data are presented as mean±standard deviation (SD). The differences between treatments were determined by one-way analysis of variance (ANOVA) and $P<0.05$ was considered statistically significant.

Table 1. Sequences of primers used for RT-PCR.

Gene	Sequences
iNOS	Forward: 5′-aatggcaacatcaggtcggccatcact-3′
	Reverse: 5′-gctgtgtgtcacagaagtctcgaactc-3′
COX−2	Forward: 5′-ggagagactatcaagatagt-3′
	Reverse: 5′-atggtcagtagactttaca-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′

iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin-1β; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

3. Results

3.1. Inhibitory effects of R. crenata leaf extracts on NO production and proinflammatory expression in LPS−stimulated RAW264.7 macrophages

The MTT assay showed that *R. crenata* leaf extracts did not exhibit toxicity (Figure 1A). *R. crenata* leaf extracts also reduced the production of LPS-induced NO in RAW264.7 macrophages in a concentration-dependent manner (Figure 1B). In addition, LPS-stimulated RAW264.7 macrophages were used to determine the inhibitory activity of inflammatory mediator genes including *iNOS*, *COX−2*, *IL−1β*, and *TNF−α*, which are involved in the pathogenesis of inflammatory diseases. The extracts decreased the expression of these inflammatory mediators in a concentration-dependent manner (Figure 1C). These findings confirm that *R. crenata* leaf extracts exhibit anti-inflammatory activity.

3.2. Inhibitory effects of R. crenata leaf extracts on NF−κB signaling activity in LPS−stimulated RAW264.7 cells

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

3.3. Inhibitory effects of R. crenata leaf extracts on MAPK signaling activity in LPS−stimulated RAW264.7 cells

MAPK signaling, such as ERK1/2, p38, and JNK, exists in various conformations. To investigate the effects of *R. crenata* leaf extracts on the inhibition of MAPK activity, different concentrations of *R. crenata* leaf extracts were used to treat LPS-induced RAW264.7 cells. Phosphorylation of ERK1/2, p38, and JNK was significantly inhibited in a concentration-dependent manner (Figures 3A-3C). These findings suggest that the extract exerts anti-inflammatory effects by inhibiting MAPK signaling pathway.

3.4. R. crenata leaf extracts activate HO−1 expression in RAW264.7 cells

R. crenata leaf extracts dose-dependently increased HO-1 protein levels (Figure 4A), and HO-1 expression was induced 9 h after treatment with *R. crenata* leaf extracts, with the highest expression being observed after 24 h (Figure 4B), which indicates that HO-1 expression is influenced by *R. crenata* leaf extracts. To investigate the upstream kinases such as ERK1/2, p38, JNK, and NF-κB associated with HO-1 expression, we pretreated RAW264.7 cells with PD98059

(ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), or BAY11-7082 (NF-κB inhibitor) and then co-treated them with *R. crenata* leaf extracts. As shown in Figures 5A-5D, the pretreatments of PD98059, SB203580, SP600125, and BAY11-7082 blocked HO-1 expression induced by *R. crenata* leaf extracts in RAW264.7 cells. These results indicate that the HO-1 protein expression induced by *R. crenata* leaf extracts is dependent on ERK1/2, p38, JNK, and NF-κB.

3.5. HO–1 expression induced by R. crenata leaf extracts is dependent on Nrf2 activation in RAW264.7 cells

We investigated whether Nrf2 activation is involved in the *R. crenata* leaf extracts-induced HO-1 expression. When RAW264.7 cells were treated with *R. crenata* leaf extracts, Nrf2 accumulated in the nucleus in a concentration-dependent manner (Figure 6A), and the highest value was observed after 1 h of treatment (Figure 6B).
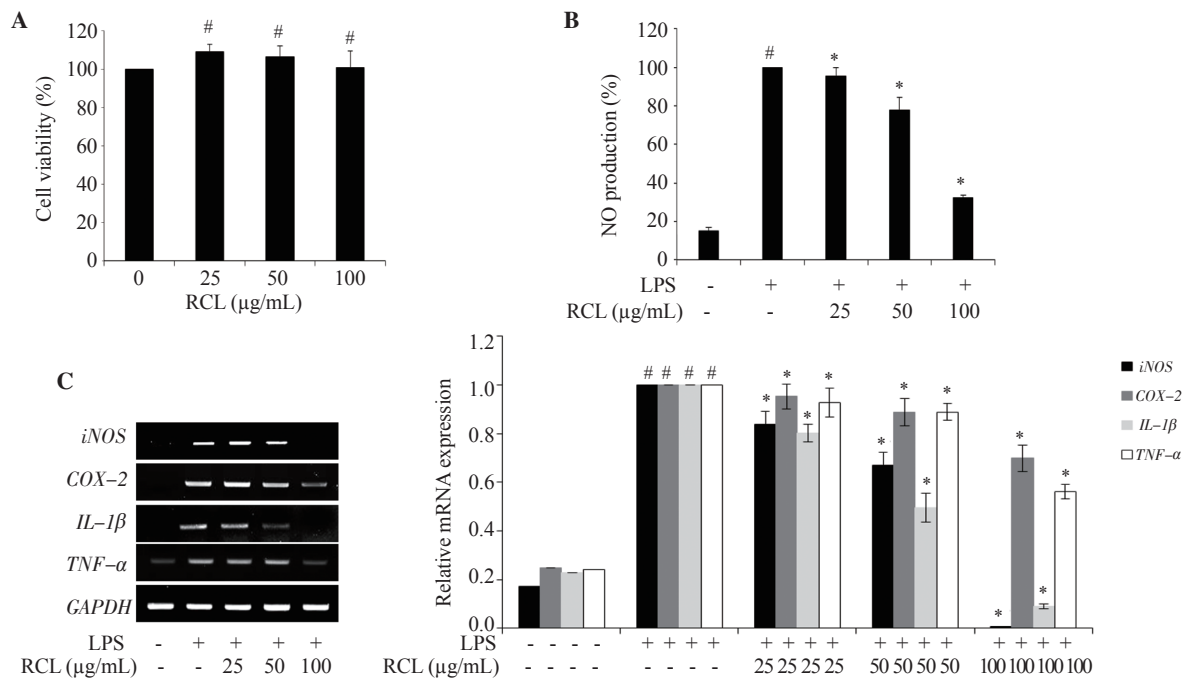

Figure 1. Effects of *Rhamnus crenata* leaf extracts (RCL) on cell viability and the production of pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. (A) Cell viability was determined by MTT assay. (B) RAW264.7 cells were pretreated with 0, 25, 50, and 100 μg/mL of RCL for 6 h and then co-treated with LPS (1 μg/mL) for 18 h. The nitric oxide (NO) production was measured by Griess assay. (C) The gene expressions of pro-inflammatory mediators were measured by RT-PCR assay. *GAPDH* was used as an internal control. The density of mRNA bands was calculated using the software Chemidoc. The data are expressed as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). [#]$P<0.05$ compared to the cells without the treatment of RCL and LPS, and [*]$P<0.05$ compared to the cells treated with LPS alone.
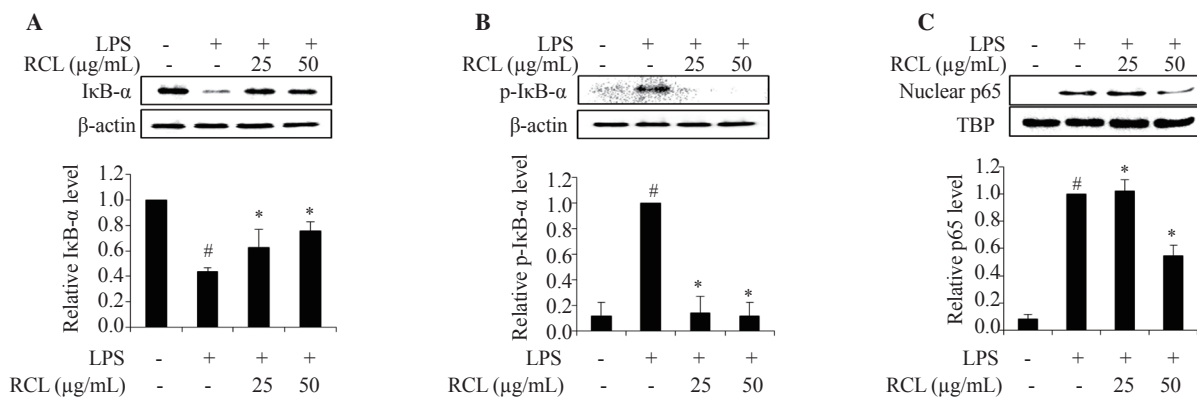

Figure 2. Inhibitory effect of RCL on the activation of NF-κB signaling pathway in RAW264.7 cells. (A and B) RAW264.7 cells were pretreated with RCL (0, 25, and 50 μg/mL) for 6 h, followed by treatment with LPS (1 μg/mL) for 40 min. (C) RAW264.7 cells were pretreated with RCL (0, 25, and 50 μg/mL) for 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 assay was performed using antibodies against p65, IκB-α, and phospho-IκB-α. β-actin and TBP were used as internal controls. The data are expressed as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). [#]$P<0.05$ compared to the cells without the treatment of RCL and LPS, and [*]$P<0.05$ compared to the cells treated with LPS alone. TBP: TATA-binding protein.
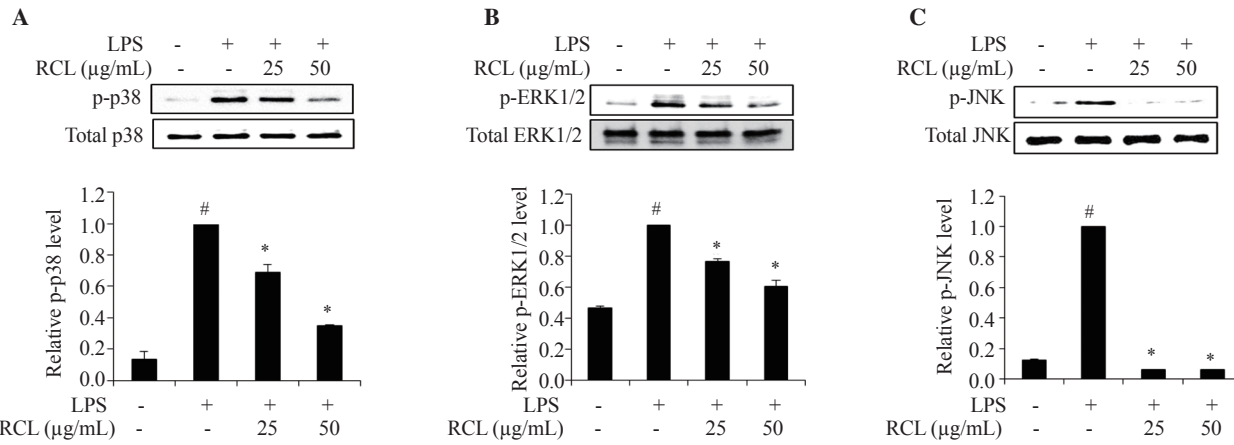
Figure 3. Inhibitory effects of RCL on the activation of MAPK signaling pathway in RAW264.7 cells. RAW264.7 cells were pretreated with RCL (0, 25, and 50 μg/mL) for 6 h and then co-treated with LPS (1 μg/mL) for 40 min. The cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting assay was performed using antibodies against phosphorylated p38, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK). The data are expressed as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). [#]$P<0.05$ compared to the cells without the treatment of RCL and LPS, and [*]$P<0.05$ compared to the cells treated with LPS alone.
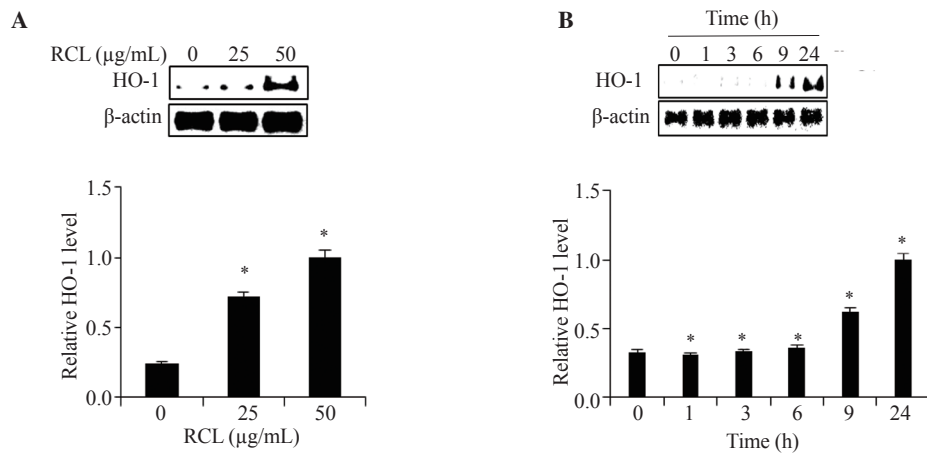

Figure 4. Effects of RCL on heme oxygenase 1 (HO-1) expression in RAW264.7 cells. RAW264.7 cells were treated with 50 μg/mL RCL for the indicated periods and then Western blotting analysis was performed. β-actin was used as an internal control. The data are expressed as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). [*]$P<0.05$ compared to the cells without the treatment of RCL.
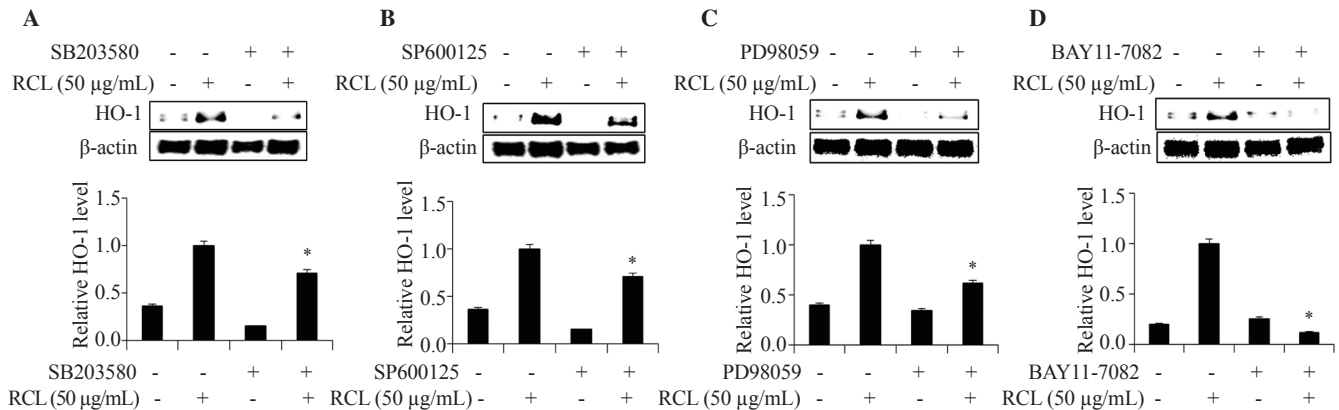
Figure 5. Effects of inhibitors on RCL-mediated HO-1 expression in RAW264.7 cells. RAW264.7 cells were pretreated with 20 μM each of (A) SB203580, (B) SP600125, (C) PD98059, and (D) BAY11-7082 for 2 h and then co-treated with 50 μg/mL of RCL for 24 h. Western blotting analysis was performed using anti-HO-1 antibodies. The data are expressed as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). [*]$P<0.05$ compared with the cells treated with RCL only.
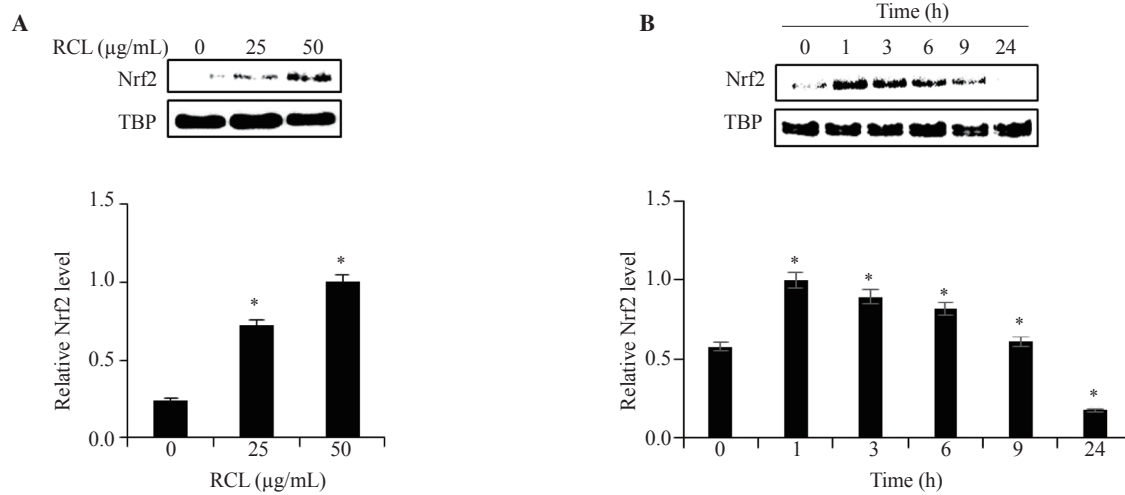
Figure 6. Effects of RCL on Nrf2 activation in RAW264.7 cells. (A) RAW264.7 cells were treated with 0, 25, and 50 μg/mL of RCL for 1 h; then, the nuclear proteins were subjected to Western blotting analysis using an anti-Nrf2 antibody. (B) RAW264.7 cells were treated with RCL (50 μg/mL) for 24 h and then Western blotting analysis was performed using an anti-Nrf2 antibody. TBP was used as an internal control. The data are expressed as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). $^*P<0.05$ compared to the untreated control cells.

These findings indicate that *R. crenata* leaf extracts may upregulate HO-1 expression through Nrf2 activation.

4. 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 production 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 *R. crenata* leaf extracts on NO production, cell viability, and expression of pro-inflammatory cytokines were investigated using RAW264.7 cells.

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, which is 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]. *R. crenata* leaf

extracts inhibited LPS-induced NF-κB activity and intranuclear migration in RAW264.7 cells. Western blotting analysis revealed that the quantity of protein transferred in the nucleus was significantly decreased in cells treated with 25 and 50 μg/mL of *R. crenata* leaf extracts compared to the LPS-treated control 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 *R. crenata* leaf extracts 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[26,27]. In this study, Western blotting was performed to confirm the intranuclear migration of Nrf2; it was found that the amount of Nrf2 increased in the nucleus following *R. crenata* leaf extract treatment. These findings indicated that *R. crenata* leaf extracts promoted the migration of Nrf2 into the nucleus and increased the expression of HO-1. In conclusion, our results demonstrated that *R. crenata* leaf extracts reduce the expression of various inflammatory genes and can regulate inflammation through various pathways, such as the NF-κB, MAPK, and Nrf2/HO-1 pathways.

Conflict of interest statement

The authors declare no conflict of interests.

Funding

Authors' contributions

All authors contributed to the conception and design. HJE mainly engaged in experimental design and operation, data collation, article writing, and commissioning. Material preparation, data collection, and analysis were performed by DSK. GHP helped in experimental design, paper writing and modification, and submission. All authors reviewed and approved the final manuscript.

References

[1] Zedler S, Faist E. The impact of endogenous triggers on trauma associated inflammation. *Curr Opin Crit Care* 2006; **12**(6): 595-601.

[2] Andersen CJ. Lipid metabolism in inflammation and immune function. *Nutrients* 2022; **14**(7): 1414.

[3] Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. *Cell* 2015; **160**(5): 816-827.

[4] Deretic V. Autophagy in inflammation, infection, and immunometabolism. *Immunity* 2021; **54**(3): 437-453.

[5] Joung EJ, Lee B, Gwon WG, Shin T, Jung BM, Yoon NY, et al. Sargaquinoic acid attenuates inflammatory responses by regulating NF-κB and Nrf2 pathways in lipopolysaccharide-stimulated RAW 264.7 cells. *Immunopharmacol Int* 2015; **29**(2): 693-700.

[6] Giudice A, Arra C, Turco MC. Review of molecular mechanisms involved in the activation of the Nrf2-ARE signaling pathway by chemopreventive agents. *Methods Mol Biol* 2010; **647**: 37-74.

[7] Chen H, Song L, Xu X, Han Z, Peng F, Zhang Q, et al. The effect of icariin on autoimmune premature ovarian insufficiency *via* modulation of Nrf2/HO-1/Sirt1 pathway in mice. *Reprod Biol* 2022; **22**(2): 100638.

[8] Luvián-Morales J, Varela-Castillo FO, Flores-Cisneros L, Cetina-Pérez L, Castro-Eguiluz D. Functional foods modulating inflammation and metabolism in chronic diseases: A systematic review. *Crit Rev Food Sci Nutr* 2022; **62**(16): 4371-4392.

[9] Eo HJ, Kim DS, Kang Y, Kim K, Park Y, Park GH. Antioxidant and immunoregulatory effects of Korea Rhamnaceae. *J Plant Biotechnol* 2020; **47**(3): 254-259.

[10] Namkoong S, Jang SA, Sohn EH, Bak JP, Sohn E, Koo HJ, et al. Comparative study of *Litsea japonica* leaf and fruit extract on the anti-inflammatory effects. *Korean J Plant Res* 2015; **28**(2): 145-152.

[11] Higuchi M, Higashi M, Taki H, Osawa T. Cytolytic mechanisms of activated macrophages. Tumor necrosis factor and *L*-argininedependent mechanisms acts synergistically as the major cytolytic mechanisms of activated macrophages. *J Immunol* 1990; **144**(4): 1434-1441.

[12] Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. *J Biol Chem* 1994; **269**(19): 13725-13728.

[13] Kim KW, Ha K, Park CS, Jin UH, Chang HW, Lee IS, et al. *Polygonum cuspidatum*, compared with baicalin and berberine, inhibits inducible nitric oxide synthase and cyclooxygenase-2 gene expressions in RAW264.7 macrophages. *Vascul Pharmacol* 2007; **47**(2-3): 99-107.

[14] Förstermann U, Kleinert H. Nitric oxide synthase: Expression and expressional control of the three isoforms. *Naunyn Schmiedebergs Arch Pharmacol* 1995; **352**(4): 351-364.

[15] Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, et al. Selective inhibition of inducible cyclooxygenase-2 *in vivo* is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci U S A* 1994; **91**(8): 3228-3232.

[16] Yang YZ, Tang YZ, Liu YH. Wogonoside displays anti-inflammatory effects through modulating inflammatory mediator expression using RAW264.7 cells. *J Ethnopharmacol* 2013; **148**(1): 271-276.

[17] Stokes KY, Cooper D, Tailor A, Granger DN. Hypercholesterolemia promotes inflammation and microvascular dysfunction: Role of nitric oxide and superoxide. *Free Radic Biol Med* 2002; **33**(8): 1026-1036.

[18] Jiang A, Zhang Y, Zhang X, Wu D, Liu Z, Li S, et al. Morin alleviates LPS-induced mastitis by inhibiting the PI3K/AKT, MAPK, NF-κB and NLRP3 signaling pathway and protecting the integrity of blood-milk barrier. *Int Immunopharmacol* 2020; **78**: 105972.

[19] Celec P. Nuclear factor kappa B-molecular biomedicine: The next generation. *Biomed Pharmacother* 2004; **58**(6-7): 365-371.

[20] Choi DJ, Kim GS, Choi B, Lee Y, Han KS, Lee D, et al. Anti-inflammatory effect of a mixture of *Astragalus membranaceus* and *Lithospermum erythrorhizon* extracts by inhibition of MAPK and NF-κB signaling pathways in RAW264.7 cells. *J Appl Biol Chem* 2020; **63**(4): 421-428.

[21] Hsieh CY, Hsu MJ, Hsiao G, Wang YH, Huang CW, Chen SW, et al. Andrographolide enhances nuclear factor-κB subunit p65 Ser536 dephosphorylation through activation of protein phosphatase 2A in vascular smooth muscle cells. *J Biol Chem* 2011; **286**(8): 5942-5955.

[22] An F, Xuan X, Liu Z, Bian M, Shen Q, Quan Z, et al. Anti-inflammatory activity of 4-(4-(Heptyloxy)phenyl)-2,4-dihydro-3H-1,2,4-triazol-3-one *via* repression of MAPK/NF-κB signaling pathways in β-amyloid-induced Alzheimer's disease models. *Molecules* 2022; **27**(15): 5035.

[23] Takahashi T, Moriga K, Akagi R, Sassa S. Heme oxygenase-1: A novel therapeutic target in oxidative tissue injuries. *Curr Med Chem* 2004; **11**(12): 1545-1561.

[24] Ahmed O, Xu M, Zhou F, Wein AN, Upadhya GA, Ye L, et al. NRF2 assessment in discarded liver allografts: A role in allograft function and salvage. *Am J Transplant* 2021; **22**(1): 58-70.

[25] Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (keap1) signaling in oxidative stress. *Free Radic Biol Med* 2009; **47**(9): 1304-1309.

[26] Gu L, Lin J, Wang Q, Li C, Peng X, Fan Y, et al. Dimethyl itaconate protects against fungal keratitis by activating the Nrf2/HO-1 signaling pathway. *Immunol Cell Biol* 2020; **98**(3): 229-241.

[27] Chulrik W, Jansakun C, Chaichompoo W, Tedasen A, Yotmanee P, Sattayakhom A, et al. Oxocrebanine from *Stephania pierrei* exerts macrophage anti-inflammatory effects by downregulating the NF-κB, MAPK, and PI3K/Akt signalling pathways. *Inflammopharmacology* 2022; **30**(4): 1369-1382.

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