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Anti-inflammatory effects of Juglanin H, a diarylheptanoid from *Juglans regia* in macrophages

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ABSTRACT

Anti-inflammatory effects of Juglanin H, a diarylheptanoid from *Juglans regia* in macrophages

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Inflammation is a complex biological response of body to endogenous and exogenous stimuli such as damaged cells, pathogens, or irradiation. Regulated inflammatory response plays an essential role in adjusting body functions and maintaining homeostasis. However, uncontrolled inflammatory response can be a principal factor of causing tissue damage or chronic inflammatory diseases, such as cardiovascular diseases, obesity, inflammatory bowel disease and cancer. Therefore, the regulation of uncontrolled inflammation responses is a strategy for prevention and therapeutic approach for inflammation associated diseases.

Macrophages are innate immune cells that initiate inflammation and immune responses. When exposed to various proinflammatory factors, monocytes are recruited to extravascular tissues and differentiate into macrophages under the pathogenesis of inflammation. Once activated, macrophages are able to produce proinflammation mediated cellular signaling molecules.

Diarylheptanoids are complex phenolic compounds with two phenyl rings at C-1 and C-7 positions. Juglanin H, a novel diarylheptanoid, was isolated from green walnut husk *Juglans regia* which is a traditional Chinese medicine used for the treatment of cancer and inflammation.

However, the bioactivity and underlying mechanisms remain to be elucidated. In the present study, the anti-inflammatory activities of Juglanin H were investigated. Juglanin H significantly suppressed the production of nitric oxide and the expression of pro-inflammatory mediators including inducible nitric oxide synthase (iNOS) and interleukin-1β (IL-1β) in both protein and mRNA levels in RAW 264.7 cells. Juglanin H also inhibited the translocation of NF-κB from the cytoplasm to the nucleus and modulated the activation of signaling molecules in MAPK and PI3K/Akt pathways. In addition, Juglanin H exhibited a significant suppression of interleukin-6 (IL-6) and Interferon-β (IFN-β) at mRNA level and inhibited NF-κB translocation into nucleus in THP-1 differentiated macrophages. Juglanin H was found to be able to decrease lipopolysaccharide (LPS)-stimulated reactive oxygen species (ROS) generation by activating Nrf2 leading to the initiation of hemeoxygenase-1 (HO-1), an anti-oxidant and cytoprotective enzyme. These findings suggest that Juglanin H is a potential

anti-inflammatory agent derived from natural products.

Keyword: Inflammation, ROS, NF-κB, MAPK, Nrf2, Juglanin H, macrophage

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I. Introduction

Inflammation is a complex biological response of body to endogenous and exogenous stimuli such as damaged cells, pathogens, or irradiation (Bai, Shi et al. 2015). Regulated inflammatory response plays an essential role in adjusting body functions and maintaining homeostasis. During acute inflammatory responses, mitigation process in which cellular and molecular events and interactions efficiently minimize impending injury or infection, contributes to restoration of tissue homeostasis and resolution of the acute inflammation (Chen, Deng et al. 2018). Therefore, inflammation is a defense mechanism that is vital to health. However, uncontrolled inflammatory response can be a principal factor of causing tissue damage or chronic inflammatory diseases, such as cardiovascular diseases, obesity, inflammatory bowel disease, and cancer (Chao, Kuo et al. 2009). On this line, the effective control of dysregulated inflammatory responses is a promising preventative and therapeutic strategy for many inflammation-associated diseases.

Macrophages are innate immune cells that initiate inflammation and immune responses. When exposed to various pro-inflammatory factors, monocytes are recruited to extravascular tissues and differentiate into macrophages under the pathogenesis of inflammation. Once activated, macrophages are the main sources of cellular signaling molecules and pro-inflammatory mediators (Fujiwara and Kobayashi 2005). RAW 264.7 cell, a murine macrophage cell line, is a mature model to study for

inflammation. THP-1 cell, which is monocyte derived from human leukemia cancer patient, can be differentiated into M0 macrophage and further polarized into pro-inflammatory macrophages which can be utilized as a human macrophage model (Luo, Shen et al. 2018). The THP-1 cells are widely used for investigation of human macrophage function as they acquire phenotypic and functional characteristics which closely resemble those of human macrophages (Lund, To et al. 2016). In response to environmental changes, macrophages can differentiate into two main functional phenotypes, classical activation phenotype (M1) and alternative activation phenotype (M2) (Mantovani, Sozzani et al. 2002). Based on their different phenotypes, macrophages can affect the outcomes of inflammation by producing either pro-inflammatory or anti-inflammatory factors.

Nitric oxide (NO) is a short-lived gaseous radical and is considered as a potent multifunctional reactive metabolite and a major effector molecule of immune cells against tumor cells and pathogens (Chi, Qui et al. 2003). NO produced from macrophages by nitric oxide synthase (NOS) plays an important role in implicating in the nonspecific immune defense against parasitic diseases and participating in the production of free radicals that are toxic to bacteria and parasites (Jimenez, Ros et al. 1999). However, excessive production of NO often exerts damage to endothelial tissues, especially of the vascular system (Rajendran, Rengarajan et al. 2013).

Reactive oxygen species (ROS), in particular mitochondrial-derived ROS, produced by cells that are involved in the host-defense response, has been implicated in chronic inflammation and the progression of many other inflammatory diseases (Mittal, Siddiqui et al. 2014). Mitochondrial-derived

ROS contributes to multiple functional outcomes such as activation of transcription factors (such as NF-κB) (Chandel, Trzyna et al. 2000), production of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α (Bulua, Simon et al. 2011) and activation of inflammasomes (Naik and Dixit 2011).The transcription factor NF-E2-related factor 2(Nrf2) appears to be a prominent regulator of overproduction of pro-inflammatory mediators including cytokines and ROS in activated macrophages (Sajadimajd and Khazaei 2018). Oxidative stress leads to dissociation of Nrf2 and Kelch-like ECH-associated protein 1 (KEAP1) and Nrf2 further translocates to the nucleus and binds to antioxidant response element (ARE) to initiate the transcription of antioxidant and cytoprotective gene expression including hemeoxygenase-1 (HO-1)(Liby, Yore et al. 2007). Therefore, the coordinated upregulation of Nrf2 is considered as a potent therapeutic strategy to protect against chronic inflammation.

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, can be transferred to cluster of differentiation14 (CD14) by LPS-binding protein(LBP) and recognized by Toll-like receptor4 (TLR4) on the cellular surface of macrophages (Aderem and Ulevitch 2000). The interaction causes overexpression and activation of nuclear factor(NF)-κB and mitogen-activated protein kinase (MAPK) through MyD-88 and TRIF intracellular signaling pathways, which are involved in inducing reactive oxygen species (ROS), nitric oxide and matrix metalloproteinases, upregulation of inflammatory gene transcription and release pro-inflammatory cytokines such as COX-2, IL-1β, IL-6 and TNF-α in macrophage cells (Yuk, Sanchez-Rodriguez et al. 2018).

Phenolic compounds have been well investigated for promoting health and preventative effects against diseases. Diarylheptanoids are complex phenolic compounds with a 7 carbon skeleton possessing two phenyl rings at the carbon 1- and 7- position as plant secondary metabolites (Jahng and Park 2018). Recently, a novel diarylheptanoid was isolated from green walnut husk *Juglans regia* L., which is a traditional Chinese medicine used for the treatment of cancer and inflammation and reported to inhibit the growth of HepG2 cells(Yang, Ma et al. 2019).The present study was designed to investigate the anti-inflammatory activity of Juglanin H for LPS-induced RAW 264.7 cells and THP-1 differentiated macrophages and to elucidate the precise underlying molecular mechanisms associated with the anti-inflammatory activity of Juglanin H.

II. Material and Methods

2.1 Reagents and material

Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotic/antimycotic solution, TRI reagent, Lipofectamine 2000 and trypsin-EDTA were purchased at Invitrogen (Grand Island, NY, USA). 12-O-Tetradecanoylphorbol -13-acetate (TPA), lipopolysaccharide (LPS), 3-(4,5-dimethylthisazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT), Dimethyl sulfoxide (DMSO), sulforhodamine B (SRB), trichloroacetic acid (TCA), bovine serum albumin (BSA), Iso-prophanoland other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against p-Akt (S473), Akt, p-p38, p38, KEAP1, SAPK/JNK, p-SAPK/JNK, p-IκB-α，HO-1，Histone H3, p-PI3K, PI3K, p-mTOR, mTOR, TLR4, MyD88 and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against IL-1β, p-ERK, ERK, NF-κB p65, NF-κB p50, Nrf2, NOS2, IκB-α, p-IKKα/β, IKKα/β, STAT3, p-STAT3 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The Reverse Transcription Kit was purchased from Toyobo (Osaka, Japan). The iQ SYBR Green Supermix was obtained from Bio-Rad Laboratories (Hercules, CA, USA). For real-time PCR, the gene-specific primers were designed by Roche (Basel, Switzerland) and synthesized by Bioneer (Dajeon, Korea).

2.2 Cell culture

RAW 264.7 murine macrophage cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HEK293 cells stably transfected with NRF2/ARE (NRF2/ARE HEK293) was purchased from Signosis (Santa Clara, CA, USA). THP-1 cells were obtained from Korean Cell Line Bank (Seoul, Korea). RAW 264.7 and NRF2/ARE HEK293 cells were maintained in DMEM and THP-1 in RPMI1640 media supplemented with 10% heated-inactivated fetal bovine serum (FBS), 100units/mL penicillin, 10μg/mL streptomycin and antibiotics-antimycotics (0.25μg/mL amphotericin B). The cells were incubated in a condition of 5% CO_2 in a humidified air at 37 ℃.

2.3 Nitricoxide production (NO) measurement

RAW 264.7 cellswere seeded at a density of 2.5 x 10^5cells per well in a 24-well culture plate and incubated for overnight. The cells were changed to fresh medium with 1% FBS and pre-treated with the compound for 1hr and then incubated with 1μg/mL LPS in the presence of absence of the compound for 20hr. The amount of nitric oxide released from the culture medium was measured by Griess reaction (Tracey, Nakane et al. 1995).

2.4 Measurement of Reactive Oxyge Species

THP-1 cells were seeded and treated with TPA (150nM/ml) immediately. After treated with or without Juglanin H in indicated concentrations for 1 hr, cells were added with 10μg/mL LPS for additional 1 hr. DCF-DA with a final concentration of 20μM was added directly in the media for 30min

following LPS treatment. Cells were collected with scraper and resuspended in 1mL of PBS. The cells were analyzed by flow cytometry (Becton Dickinson, FACS Calibur).

2.5 Cell viability assay (MTT)

After the Griess reaction, MTT solution with a concentration of 500μg/mL was added to each well and further incubated for 3.5 hr at 37℃ in the dark. The media were discarded, and 1mL DMSO was added to each well to dissolve the formazan. The absorbance was measured at the wavelength of 570nm.

2.6 Quantitative RT-PCR Analysis

The cells were seeded and treated by different concentration of Juglanin H, induced with LPS for indicated time and collected with TRI reagent(Invitrogen, Grand Island, NY, USA) for RNA isolation. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix(TOYOBO, Osaka, Japan). The real-time PCR was conducted with iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). β-Actin was utilized to normalized the relative expression. The following sequences were used for primers:

Mouse

iNOS F5'-ATGTCCGAAGCAAACATCAC-3'

iNOS R5'-TAATGTCCAGGAAGTAGGTG-3'

IL-1β F5'-AGTTGACGGACCCCAAAAG-3'

IL-1β R5'-AGCTGGATGCTCTCATCAGG-3'

β-actin F5'-TGTGATGGTGGGAATGGGTCAG-3'

β-actin R5'-TTTGATGTCACGCACGATTTCC-3'

Human

IL-6 F5'-GATGAGTACAAAAGTCCTGATCCA-3'

IL-6 R5'-CTGCAGCCACTGGTTCTGT-3'

IFN-β F5'-DNA-ACGCCGCATTGACCATCTAT-3'

IFN-β R5'-DNA-GTCTCATTCCAGCCAGTGCT-3'

Nrf2 F5'-TCAGCCAGCCCAGCACATCC-3'

Nrf2 R5'-TCTGCGCCAAAAGCTGCATGC-3'

HO-1 F5'-GTCCAACATCCAGCTCTTTGAGG-3'

HO-1 R5'-GACAAAGTTCATGGCCCTGGGA-3'

β-actin F5'-CCAACCGCGAGAAGATGA-3'

β-actin R5'-CCAGAGGCGTACAGGGATAG-3'

2.7 Westen Blotting Analysis

The cells were pre-treated with various concentrations of Juglanin H for 1hr, incubated with indicated time of LPSand lysed by lysis buffer(250nM Tris-HCL pH6.8, 4% SDS, 10% glycerol, 0.006% bromophenol, 2% β-mercaptoethanol, 50nM sodium fluoride, and 5nM sodium orthovanadate). The protein samples were subjected to SDS-PAGE gel and transferred onto PVDF membranes(Milipore, Bedford, MA, USA). The membranes were then blocked in 5%BSA in Tris-buffered saline containing 0.1% Tween-20(TBST) for 1hr at room temperature. According to different molecular weight of target protein, membranes were cut, put into respective primary antibodies in 2.5%BSA in TBST and shaked overnight at 4 ℃. The

membranes were washed three times with TBST for 10-15min with TBST and incubated with secondary antibodies diluted in TBST for 2hr at room temperature. After washing 2-3 times with TBST for 10min, the chemiluminescence signals of the membranes were captured with LAS4000 covered by enhanced chemiluminescence solution.

2.8 Reporter geneassay for secreted embryonic alkaline phosphatase (SEAP assay)

To examine the effect of Juglanin H on the NF-κB activation, a reporter gene assay was conducted. The cells were pre-treated with Juglanin H for 2 hr and then further induced with LPS for additional 16hr. Supernatants were heated at 65℃ to be inactivated and reacted with SEAP assay buffer[2M diethanolamine, 1mM $MgCl_2$, DDW] and 4-methylumbelliferyl phosphate(MU) for 1hr in the dark at room temperature. Fluorescence from the SEAP/MUP reaction was measured in relative fluorescence units with excitation at 360nm and emission at 449nm by a 96-well plate fluorometer and normalized by protein concentration.

2.9 Nrf2 reporter gene assay

NRF2/ARE HEK293 cells were seeded on 48-well plate and incubated until it reaches 80% confluency. Cells were treated with Juglanin H and further incubated for further 18 hr. Cells were lysed and were subjected to luciferase activity assay using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions.

2.10 THP-1 differentiation

THP-1 cells were differentiated into macrophages with TPA(150nM) treated for 48hr. To further change THP-1 differentiated M0 macrophages to proinflammatory macrophages, cells were changed to new media to eliminate the effect of TPA for 24hr and LPS (10μg/mL) was added for additional 24hr to induce pro-inflammatory macrophages.

2.11 ELISA kit assay

Human TNF-α ELISA Kit (Cat550610, BD Biosciences, San Diego, CA, USA) was performed according to manufacturer's instruction.

2.12 Molecular modeling

All docking studies were carried out using Sybyl-X 2.0 on a Windows workstation. The crystal structures of iNOS (PDB: 3e7g.pdb)were retrieved from the RCSB Protein Data Bank(Loza-Mejia and Salazar 2015). Before the docking process, the natural ligand (iNOS: AT2_1906) was extracted and water molecules were removed from the crystal structure. All docking studies were carried out as described previously.

2.13 Statistical analysis

Data are presented as the means ± standard deviation(SD) for at least three times repeated independently. The determination of statistical significance (***p < 0.001, **p< 0.01, *p< 0.05) was assessed by Student's t-test.p<0.05 was regarded as statistically significant.

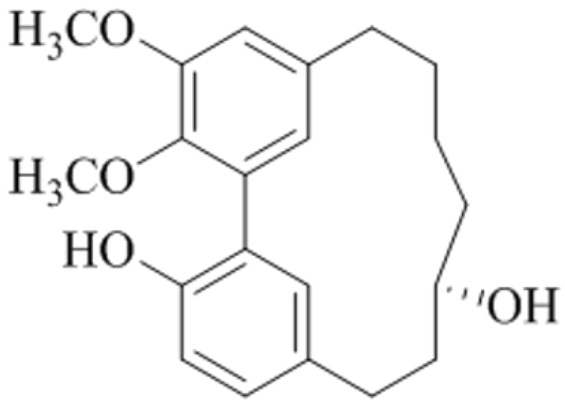

Figure 1. Structure of Juglanin

III. Results

3.1 Binding affinity of Juglanin H to iNOS protein

The docking study of Juglanin H (Figure 1) to iNOS protein was conducted using Sybyl-X 2.0 on a Windows workstation.The docking scores of Juglanin H and AT2_1906 (a natural ligand) binding to the binding pocket of iNOS were 6.6220 and 7.3860, respectively. These data indicated that Juglanin H and AT2_1906 show a comparable binding affinity to iNOS protein (Figure 2). In addition, the docking analysis exhibited the hydrogen bond interactions between Juglanin H and residues Tyr347 and in the binding pocket of iNOS. Moreover, it was suggested that Juglanin H is further stabilized in the binding pocket of iNOS by other residues including Glu263, Arg266 and Tyr373.

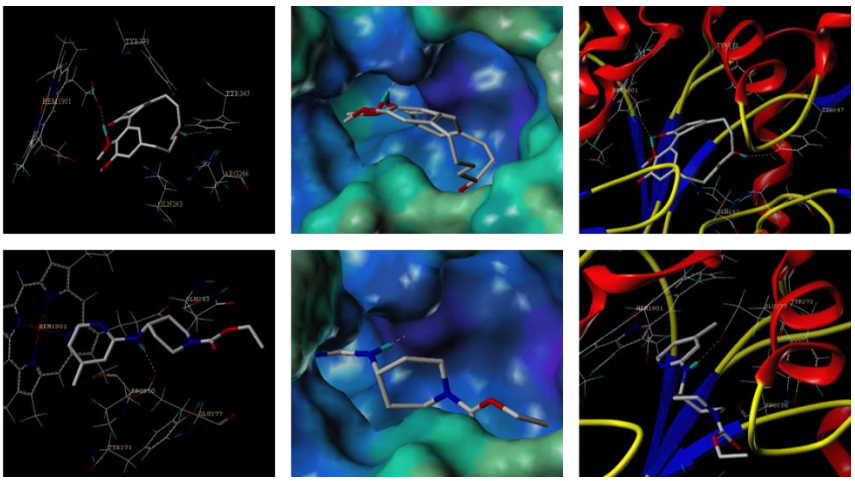

Figure 2. The binding interactions of Juglanin H (upper ones) and AT2_1906 (lower ones) with iNOS

The binding of Juglanin H to the corresponding amino acids of iNOS with hydrogen, carbon, nitrogen, and oxygen atoms(left). A surface representation(middle) and a ribbon diagram(right) of iNOS binding interaction with Juglanin H are shown. AT2_1906 was used as a natural ligand for iNOS.

3.2 Effects of Juglanin H on NO productionin LPS-stimulated RAW 264.7cells

To primarily evaluate the anti-inflammatory activity of Juglanin H, the effect of Juglanin H on the production of nitric oxide (NO) was determined in the LPS-stimulated RAW 264.7 cells. 2-Amino-5, 6-dihydro-6-methyl-4H-1,3-thiazine (AMT, 200nM)was used as a positive control in the same assay condition.The RAW 264.7 macrophage cells were pre-treated with various concentration of Juglanin H for 1hr and then further incubated in the presence of LPS (1 μg/ml) for 20 hr. Treatment with LPSsignificantly enhanced the production of NO in the cells, but Juglanin H effectively inhibited the LPS-stimulated NO production in a concentration-dependent manner (Figure 3). The cell viability of Juglanin H was also measured by MTT asssay. Juglanin H did not show any significant cytotoxicity to the cells up to 20 μM treatment (Figure 3), suggesting that the inhibitory effect of Juglanin H on the LPS-stimulated NO production was found without a cytotoxicity. Based on the findings, subsequent experiments were performed with the treatment of up to 20μM Juglanin H in the cells.

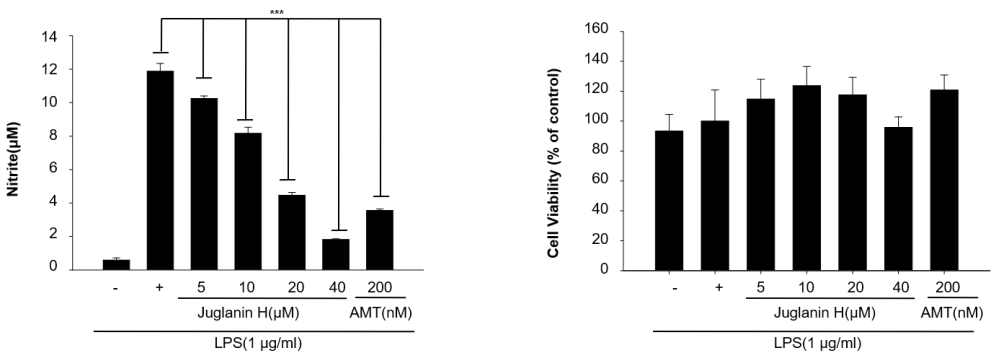

Figure 3. Effect of Juglanin H on No production

RAW 264.7 cells were treated with Juglanin H for 1hr and then stimulated in the presence of absence of LPS(1μg/ml) for 20hr. The NO production was measured by Griess reaction. Cell viability was monitored with MTT assay. Data were normalized with β-actin as an internal control. Data are expressed as mean ±SD (n=3) from three independent experiments, ** $p \leq 0.01$， *** $p \leq 0.001$ vs. the LPS-treated group.

3.3 Effects of Juglanin H on iNOS and IL-1β expressions in LPS-stimulated RAW 264.7 macrophages

Inflammatory mediators are important factors that regulate inflammation. Since JuglaninH was found to inhibit NO production, the effects of JuglaninH on the expressions of pro-inflammatory mediators in the protein and mRNA levels were evaluated by western blotting and real-time PCR analysis. The cells were pre-treated with indicated concentrations of Juglanin H for 1hr and then stimulated with LPS (1μg/ml) for 18 hr or 4hr. The treatment of LPS for 18 hr significantly enhanced the expressions of iNOS and IL-1β proteins, but the pre-treatment of Juglanin Heffectively suppressed the LPS-stimulated iNOS and IL-1β expressions in aconcentration-dependent manner (Figure 4). In addition, the treatment of LPS for 4 hr also significantly upregulated the expressions of iNOS and IL-1β mRNA levels. The levels of iNOS and IL-1β mRNA expressions in LPS-induced RAW 264.7 cells were effectively suppressed by the treatment of Juglanin H in a concentration-dependent manner(Figure 4)

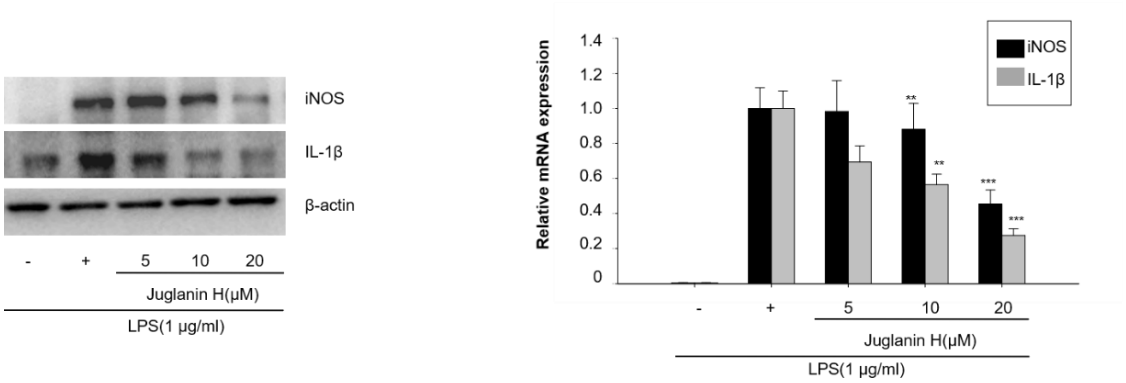

Figure 4. Effect of Juglanin H on pro-inflammatory mediators in RAW 264.7 cells

RAW 264.7 cells were pre-treated with Juglanin Hfor 1hrprior to LPS(1μg/ml) treatment for 4hr or 18hr, respectively. The mRNA and protein levels of iNOS and IL-1βwere determined by western blotting and real-time PCR. Data were normalized with β-actin as an internal control. Data are expressed as mean ±SD (n=3) from three independent experiments, ** $p \leq 0.01$， *** $p \leq 0.001$ vs. the LPS-treated group.

3.4 Effects of Juglanin H on NF-κB in LPS-stimulated RAW 264.7 macrophages

NF-κB is a critical transcription factor that regulates expression of pro-inflammatory cytokines. Since Juglanin H exhibitedthe significantly inhibition of pro-inflammatory mediator productions, the effect of Juglanin H on NF-κB activation was determined by SEAP reporter gene assay in LPS-induced RAW 264.7 macrophage cells. Juglanin H was able to alleviate the LPS-stimulated NF-κB activation in RAW 264.7 cells in a concentration-dependent manner (Figure 5). It is known that LPS treatment enhances the activation of NF-κB and thus translocates the NF-kB subunits into nucleus from cytosolic fractions. To further evaluate whether Juglanin H affects to the NF-κB translocation, the levels of expressions of NF-kB subunits were determined by western blotting in nuclear fractions. As a result, the enhanced translocation of NF-kB subunits (p65 and p50) into nuclear fractions by LPS treatment (1 μg/ml) for 1 hr was effectively suppressed by the treatment of Juglanin H (Figure 6). In addition, the cascade components of NF-kB activation including phosphorylation of IKKα/β and degradation of IκBα in cytoplasm were also suppressed byJuglanin H in LPS-stimulated RAW 264.7 cells (Figure 6).

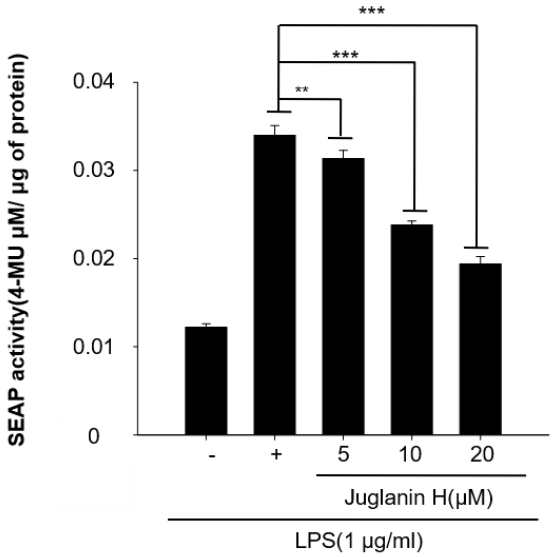

Figure 5. Effect of Juglanin H in SEAP assay in LPS-stimulated RAW 264.7 cells

The NF-κB transcriptional activity was assessed by a reporter gene assay. RAW 264.7 cells were stimulated with LPS(1μg/ml) for 16hr after pre-treatment with Juglanin H for 2hr. Relative fluorescence units (RFU) were measured by using a fluorometer for secreted alkaline phosphatase activity (SEAP). Data are representative of three separate experiments. ** $p \leq 0.01$， *** $p \leq 0.001$ vs. the LPS-treated group.

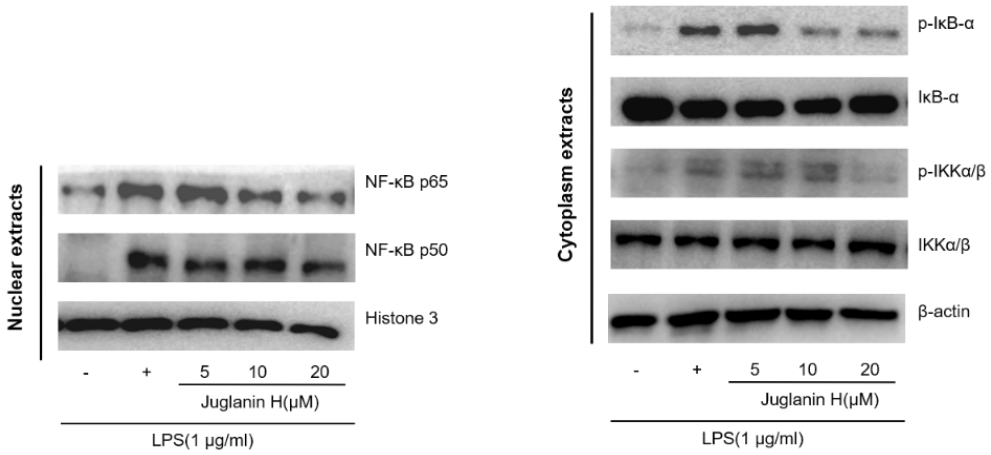

Figure 6. Effect of Juglanin H on NF-κB activation in LPS-stimulated RAW 264.7 cells

The nuclear cell lysates were analyzed for NF-κB（p65 and p50 subunits）expression by western blotting. The cytoplasmic protein expression levels of p-IκB-α, IκB-α, p-IKKαβ, and IKKαβ were detected by each specific antibody. Histone3 (nuclear fraction) and β-actin (cytoplasmic fraction) were used as the internal controls.

3.5 Effects of Juglanin H on MAPK and PI3K/Akt/mTOR signaling pathways in LPS-stimulated RAW 264.7 macrophages

MAPK signaling pathway has been implicated in the regulation of key cellular processes including gene induction, cell survival/apoptosis, proliferation and differentiation as well as cellular stress and inflammatory responses (Thalhamer, McGrath et al. 2008). Phosphoinositide-3 kinase (PI3K), Akt, and the mammalian target of the rapamycin (PI3K/Akt/mTOR) is also an upstream pathway for NF-κB-associated activation pathways. Therefore, the MAPK and PI3K/Akt/mTOR signaling pathways are considered as prominent therapeutic targets in inflammation processes. To further evaluate whether Juglanin H modulates the inflammatory signaling pathways, RAW 264.7 cells were treated with Juglanin H for 1hr and then further treated with LPS (1μg/ml) for an additional 4hr to analyze the protein expressions by western blotting. The MAPK signaling molecules such as p-ERK(1/2), p-SAPK, and p-p38 were upregulated by LPS treatment, but Juglanin H effectively suppressed the activation of MAPK signaling pathways in LPS-stimulated RAW 264.7 cells (Figure 7). The activation of PI3K/Akt/mTOR signaling pathways by LPS treatment was also alleviated by Juglanin H in a concentration-dependent manner in LPS-stimulated RAW 264.7 macrophage cells (Figure 8).

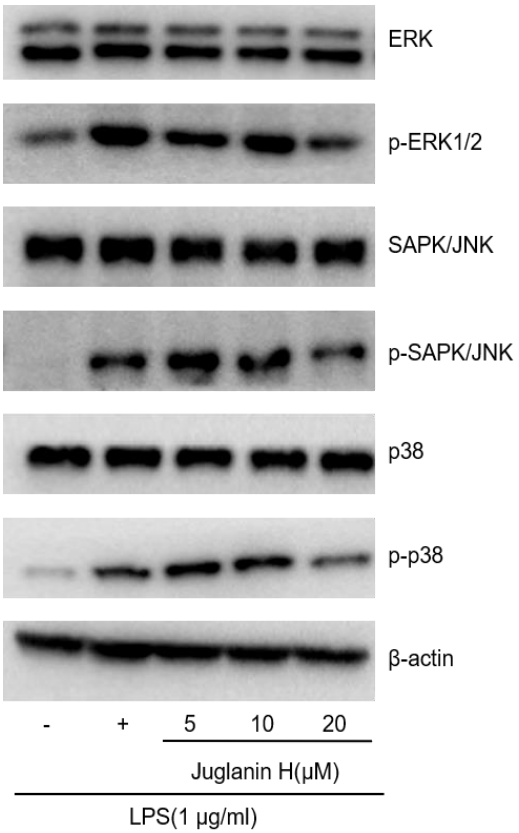

Figure 7. Effect of Juglanin H on protein expressions of MAPK signaling pathway in RAW 264.7 cells.

The cells were treated with indicated concentrations of Juglanin H for 1hr and then induced with LPS (1μg/ml) for 4 hr. MAPK signaling molecules ERK, SAPK/JNK, and p38 were analyzed using western blotting. β-actin was used as an internal control.

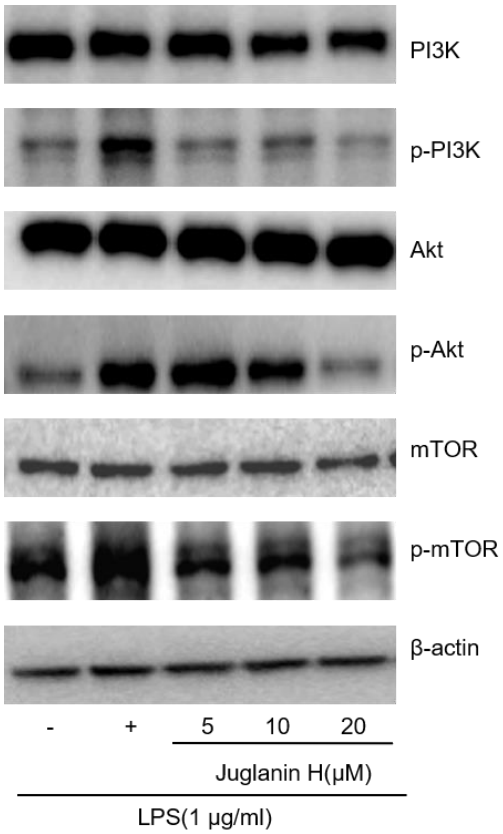

Figure 8. Effect of Juglanin H on protein expressions of PI3K/Akt/mTOR signaling pathway in RAW 264.7 cells

The cells were treated with indicated concentrations of Juglanin H for 1hr and then induced with LPS (1μg/ml) for 4 hr. PI3K/Akt/mTOR signaling molecules PI3K, Akt, and mTOR were analyzed using western blotting. β-actin was used as an internal control.

3.6 Effects of Juglanin H in THP-1 differentiated macrophage cells

To further elucidate the anti-inflammatory effects of Juglanin H, THP-1 differentiated macrophage cells which are mimic of human immune cell models were employed. The THP-1 cells were differentiated with the treatment of TPA (150nM) for 48hr. The cell medium without TPA was changed and incubated for an additional 24 hr. The cells were then treated with Juglanin H for 1hr, and LPS (10㎍/ml) was treated for 24 hr to polarize the cells into pro-inflammatory macrophages. In THP-1 differentiated macrophages, the expressions of NF-kB subunits (p50 and p65) were downregulated by the treatment of Juglanin H (Figure 9). These data are consistent with the findings of the suppressive effect of Juglanin H in LPS-stimulated RAW 264.7 macrophage cells. STAT3 plays a pivotal role in mediating inflammatory responses by regulating cytokine production(Schumann, Kirschning et al. 1996). Therefore, targeting of aberrantly activated STAT3 has been considered a promising approach for the treatment of inflammatory diseases (Goswami and Kaplan 2017). In THP-1 differentiated macrophages, LPS stimulation was found to enhance the activation of STAT3 (p-STAT3), but the treatment of Juglanin H effectively suppressed the protein expression of p-STAT3 (Figure 10). In addition, the over-expressions of IL-6 and IFN-β mRNA levels by LPS treatment in THP-1 differentiated macrophage cells were also effectively alleviated by Juglanin H in a concentration-dependent manner (Figure 11).

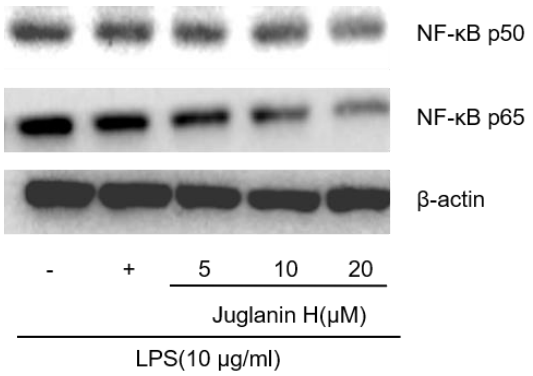

Figure 9. Effect of Juglanin H on NF-κB in LPS-stimulated THP-1 differentiated macrophage cells

THP-1 differentiated macrophages were treated with indicated concentrations of Juglanin H for 1hr and then incubated with or without LPS (10μg/ml) for 24hr. The effect of Juglanin H on activation of NF-κB (p50 and p65) were determined by western blotting. β-actin was used as the internal control.

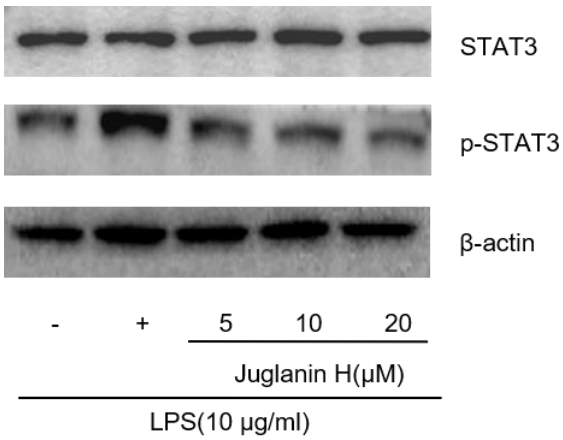

Figure 10. Effect of Juglanin H on STAT3 in LPS-stimulated THP-1 differentiated macrophage cells

THP-1 differentiated macrophages were treated with indicated concentrations of Juglanin H for 1 hr and then incubated with or without LPS (10 μg/ml) for 24 hr. The effect of Juglanin H on the activation of STAT3 was detected by western blotting. β-actin was used as the internal control.

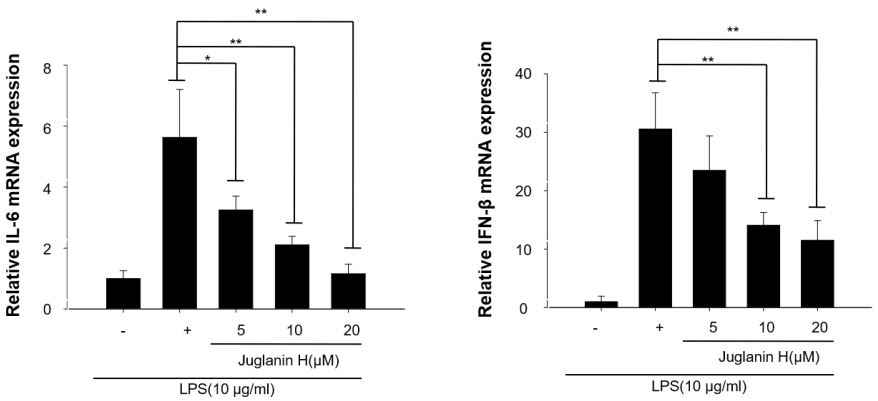

Figure 11. Effect of Juglanin H on IL-6 and IFN-β in LPS-stimulated THP-1 differentiated macrophage cells

THP-1 differentiated macrophages were treated with indicated concentrations of Juglanin H for 1 hr and then incubated with or without LPS (10 μg/ml) for 24 hr. The mRNA expressions of IL-6 and IFN-β were detected after treatment by Juglanin H with or without LPS induction. Data were normalized with β-actin as an internal control. Data are representative of three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$ vs. the LPS-treated group.

3.7 Effects of Juglanin H on ROS generation and expression of HO-1 and Nrf2 in LPS-stimulated THP-1 differentiated macrophages

To investigate whether Juglanin H attenuates the intracelluar accumulation of ROS, 2',7'-dichlorofluorescein diacetate (DCF-DA) assay was conductedin LPS-stimulated THP-1 differentiated macrophage cells. The flow cytometric analysis revealed that the ROS generation was increased by the treatment of LPS. However, the pre-treatment of Juglanin H effectively attenuated LPS-induced ROS generation (Figure 12). These data suggested that the anti-inflammatory effect of Juglanin H may be in part associated withthe antioxidant activity of Juglanin H in THP-1 differentiated macrophages. To further elucidate the detailed molecular mechanisms associated with the antioxidant activity of Juglanin H, the effect of Juglanin H on Nrf2 transcriptiona activity was perfomed with Nrf2 reporter gene assay in THP-1 differentiated macrophages. The Nrf2 luciferase activity by Juglanin H was found to be upregualted in a concentration-dependent manner without its cytotoxicity (Figure 13). In addition, Juglanin H also enhanced the expressions of Nrf2 protein and mRNA levels. Subsequently, the activation of Nfr2 led to the upregulation of the antioxidant enzyme HO-1 expression in the transcriptional and translational levels (Figure 14).

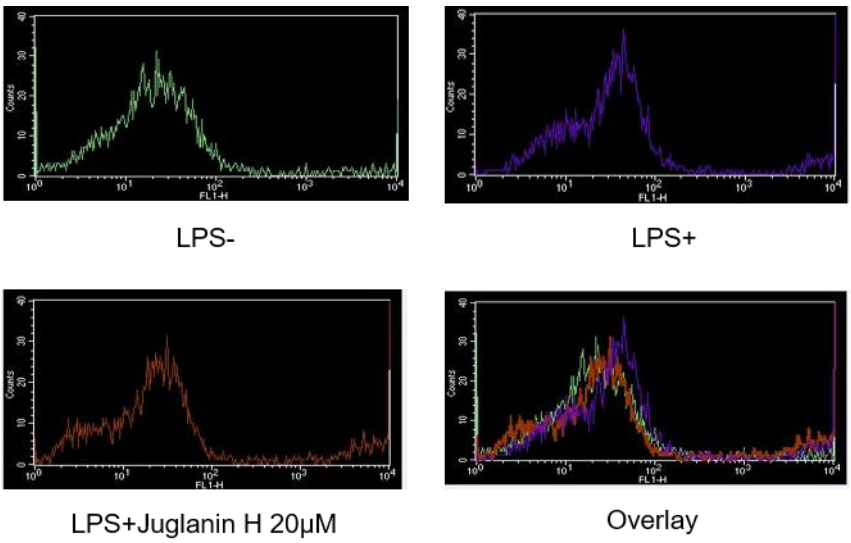

Figure 12. Effect of Juglanin H on ROS production

The THP-1 differentiated macrophages were prepared and incubated for 1hr after treatment with or without LPS (10μg/ml). DCF-DA was added to the cells and further incubated for 30 min. ROS production was analyzed by FACS.

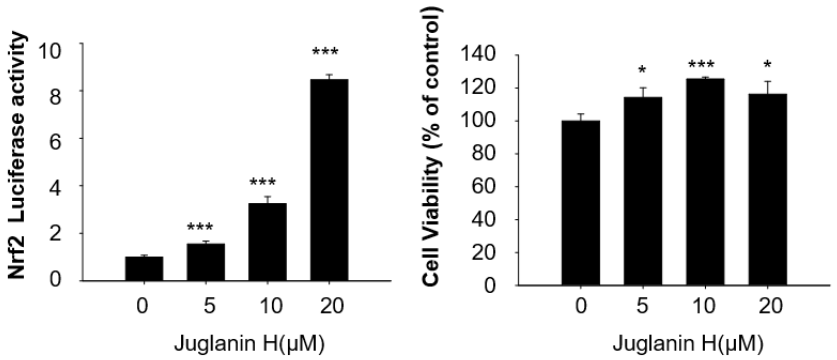

Figure 13. Effect of Juglanin on activation of Nrf2 luciferase activity

NRF2/ARE HEK293 cells were treated with indicated concentrations of Juglanin H and luciferase activity was measured as described in Materials and Methods. Cell viability was measured in NRF2/ARE HEK293 cells after 24hr with Juglanin H treatment. * $p \leq 0.05$, *** $p \leq 0.001$ vs. the LPS-treated group.

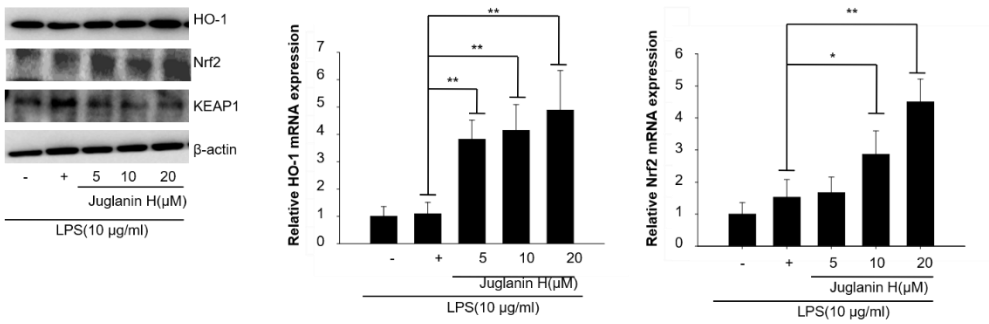

Figure 14. Effect of Juglanin H on activation of Nrf2 pathway

The protein expressions of HO-1, Nrf2, and KEAP1 were analyzed by western blotting. The mRNA expressions of HO-1 and Nrf2 were measured by real-time PCR. The results were normalized with β-actin as an internal control. Data are representative of three separate experiments. * $p \leq 0.05$, ** $p \leq 0.01$， *** $p \leq 0.001$ vs. the LPS-treated group.

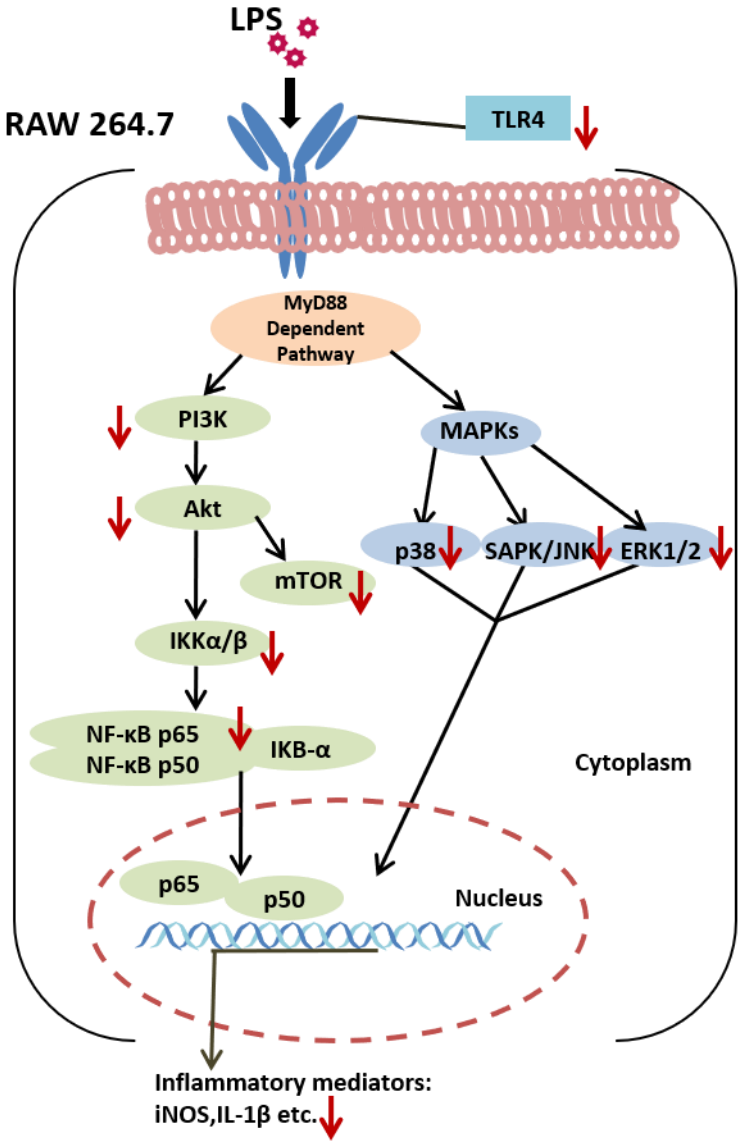

Figure 15. Graphical abstract in RAW 264.7 cells

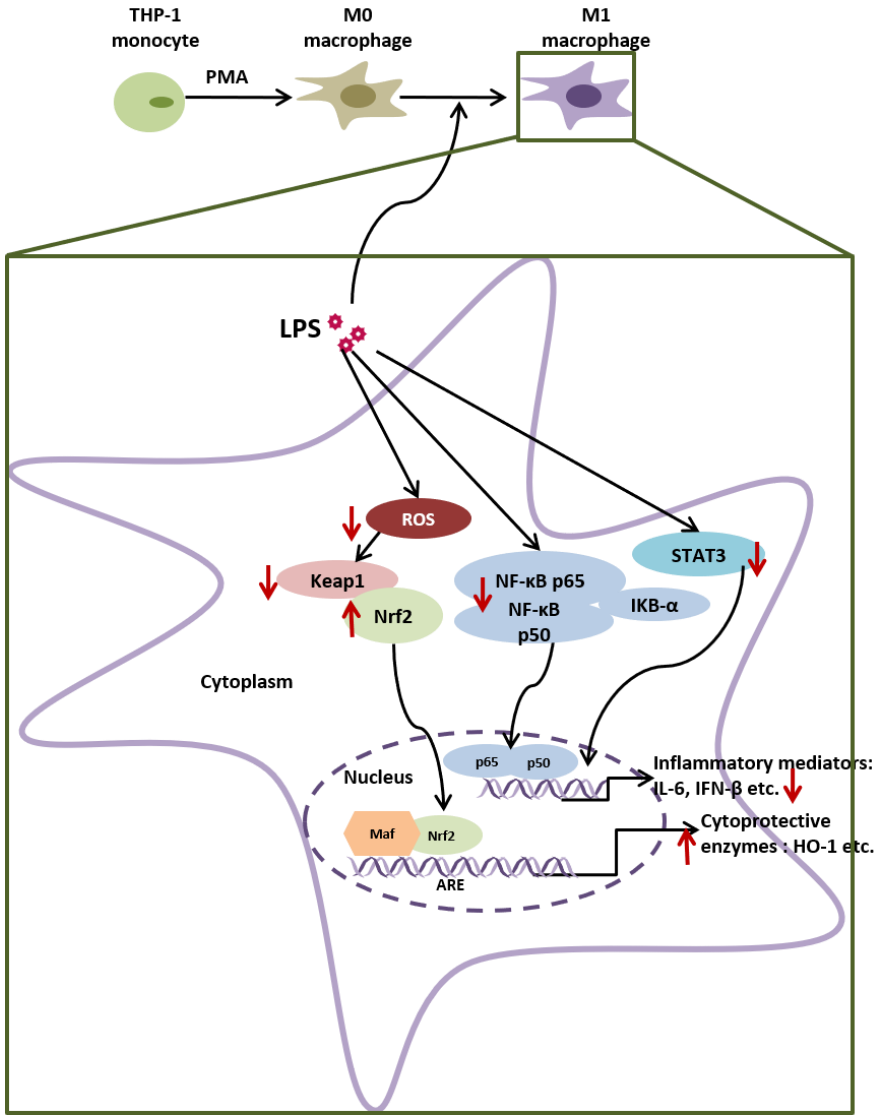

Figure 16. Graphical abstract in THP-1 differentiated macrophages

IV. Discussion

Inflammation is an essential immune response to maintain body homeostasis from various noxious conditions including microbial infection or tissue injury. Therefore, the function of inflammation is considered to eliminate the initial stimuli, necrotic cells and damaged tissues, and finally repair tissues(Nathan 2002, Hagemann, Haegele et al. 2013). However, since a transient decline in tissue fucntion occurs during inflammation, excessive inflammation may cause extensive damage to the host(Medzhitov 2010). Anti-inflammatory agents may aid to protect the tissue damages from inflammatory processes. A diverse class of natural products-derived compounds have been reported with potenial anti-inflammatory activities. In our continuous efforts to explore anti-inflammatory agents from natural sources, we found that Juglanin H, a novel diarylheptanoid isolated from green walnut husk *Juglans regia*, exhibits an anti-inflammatory activity.

Macrophages are essential in modulating acute and chronic inflammatory responses as they are the major source of cytokines, chemokines, and other inflammatory mediators which propagate or suppress the immune responses (Moldoveanu, Otmishi et al. 2009). Therefore, various cytokines and pro-inflammatory mediators which ae released by macrophages are important markers for detection of inflammation(Koh, Shin et al. 2017).In the present study, we found that Juglanin H effectively inhibits the production of nitric oxide (NO), a pro-inflammatory mediator, in LPS-induced murine macrophage cells, indicating the potential of anti-inflammatory activity of Juglanin H. In addition, the inhibition of NO

production by Juglanin H was found to be mediated by the suppression of a major target enzyme iNOS expressions. The anti-inflammatory activity of Jugalanin H was also associated with the suppressive activity of cytokine expressions such as IL-1β, TNF-α, IFN-βand IL-6 in protein and/or mRNA levels.

Furthermore, macrophages are professional phagocytes and antigen-presenting cells that remove dead cells and cellular debris, recruit other immune cells, modulate immune responses, and recover the damage cells and tissue in responses to environmental cues(Chawla, Nguyen et al. 2011, Wynn, Chawla et al. 2013, Chazaud 2014). In particular, M1 macrophage cells are mainly responsible for the inflammatory processes. Indeed, prolonged pro-inflammation is also highly correlated with many inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis(Straub and Schradin 2016). Based on the line, the anti-inflammatory activity of Juglanin H was also evaluated in THP-1 differentiated M1 macrophage cells regard.

NF-κB is a transcription factor that regulates expression of different pro-inflammatory cytokines-encoding genes in immune and inflammatory responses(Tak and Firestein 2001). Under the normal physiological status, NF-κB binds to its inhibitor κB (IκB) and locates in cytoplasm as a complex of two subunits. When inflammatory stimulation occurs IκB is phosphorylated by IκB kinases and subsequently degraded by proteasome which in turn results in release and translocation of NF-κB to nucleus to initiate transcription of pro-inflammatory-mediated genes(Li and Nabel 1997, Ghosh, Roy et al. 2007). The present study revealed that Juglanin H

is able to modulate the NF-kB activation and thus block the NF-kB-mediated pro-inflammatory responses in THP-1 differentiated macrophage cells.

Mitogen-activated protein kinases (MAPKs), serine/threonine protein kinases, mediate fundamental biological processes associated with a variety of cellular activities including cell proliferation, differentiation, survival, and transformation as well as inflammation(Dhillon, Hagan et al. 2007, Frazier, Xue et al. 2012). There are three major components of MAPK signaling pathways such as extracellular signal-regulated kinase (ERK1/2), p38 kinase, and c-Jun NH_2-terminal kinase (JNK)(Kim and Choi 2010). Activation of MAPKs enhances the expression of inflammatory mediators and also increases the production of pro-inflammatory cytokines. Therefore, the regulation of MAPK signaling pathways are also potential therapeutic targets for the anti-inflammatory agents (Kaminska 2005). The present findings suggested that Juglanin H also effectively regulates the activation of MAPK signaling pathways in LPS-stimulated macrophage cells, indicating that the anti-inflammatory activity of Juglanin H is in part associated with the suppression of MAPK signaling pathways.

Nrf2 is an essential transcription factor for encoding the ARE-mediated induction of phase II detoxifying and oxidative stress enzyme genes(Itoh, Wakabayashi et al. 1999). Under the basal condition, Keap1 binds to actin cytoskeleton and Nrf2 to retain Nrf2 as a cytoplasmic negative regulator (Zhang 2006). In response to oxidative stress, ROS liberates NRf2 from its cytoplasmic repressor Keap1, and provokes the accumulation of Nrf2 in the nucleus to initiate the transcription of antioxidant, cytoprotective, and

anti-inflammatory enzymes(Itoh, Wakabayashi et al. 2003). In the present study, we found that Juglanin H was able to inhibit ROS generation inmacrophage cells. Moreover, Juglanin H activated Nrf2, degraded KEAP1, and upregulated the expressions of antioxidant enzymes including hemeoxygenase-1 (HO-1).Althoughthe KEAP1 was initially defined as a negative regulator, some findings indicated that KEAP1 plays an active role in targeting Nrf2 for ubiquitination and proteasomal degradation(Cullinan, Gordan et al. 2004, Kobayashi, Kang et al. 2004). Therefore, the effect of Juglanin H on KEAP1 remains to be further elucidated.

The present study demonstrated that Juglanin H effectively inhibits the production of pro-inflammatory cytokines such as IL-1β and TNF-αwhich are induced by activation of NF-kB and MAPK signaling pathways in LPS-stimulated murine macrophage cells. In addition, Juglanin H also effectively attenuated the IL-6 and IFN-β production and enhanced HO-1 expression by activating Nrf2 in THP-1 differentiated macrophages. These findings suggest that a natural product-derived Juglanin H may be provided as a lead candidate for the treatment of chronic inflammation-associated disorders.

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