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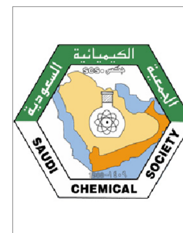

ORIGINAL ARTICLE

Hibiscus syriacus L. Extract by ultrasonic assistance displays anti-inflammatory and pro-apoptotic activity in LPS-stimulated Raw 264.7 cells

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Abstract Hibiscus syriacus L. extract (HSE) traditionally has been recognized as promising natural resources for the treatment of diseases related to hyper-inflammation. This study aimed to investigate the anti-inflammatory effect of HSE in LPS-stimulated Raw 264.7 cells. For this purpose, we determined the optimal extraction method and found that the ultrasonic extraction showed the most effective activity for reduction of free radical species by DPPH assay. Then, we performed PGE2 concentration analysis, COX-2 activity assay, proliferation assay, apoptosis detection, and western blotting analysis. HSE decreased PGE2 concentration and COX-2 activity and reduced the expression of COX-2 and IL6 in LPS stimulated Raw 264.7 cells. In addition, we found that HSE inhibited the proliferation and induced apoptosis with increase in the expression of pro-apoptotic proteins such as caspase-3, -7, -9, Bax, Bim, FOXO3, and p53 in LPS-stimulated Raw 264.7 cells. Taken together, we found that HSE effectively suppressed LPS-stimulated Raw 264.7 cells suggesting that HSE has the potential to treat inflammation via regulation of proinflammatory COX-2, IL6, and PGE2 and induction of apoptosis.

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1. Introduction

Hibiscus syriacus L. is a popular plant in South Korea and is also known as the Rose of Sharon or Mugunghwa (Yang et al., 2019). According to the book of traditional eastern medicine (Donguibogam 1613), Mugunghwa has the therapeutic effects on fever and insomnia (Yang et al., 2020). H. syriacus L. contains the various components such as fatty acids, naphthalene pentacyclic triterpene esters, and 9,9-O-Ferulolyl-(–)-secisolaricinresinol that are known to have anti-oxidant and anti-fungal effects. Recent studies have shown that H. syriacus L. extract (HSE) has the therapeutic effects on a few cancer cell lines in vitro (Cheng et al., 2008, Nguyen et al., 2019). Moreover, HSE upregulated the cell growth and migration of HaCaT cells, which suggests that HSE has the potential in the cosmetic industry as an alternative medicine (Yoon et al., 2017). In this study, we aimed to investigate the anti-inflammatory effect of HSE in Raw 264.7 cells.

Inflammation is a complex biological response triggered by harmful stimuli such as infections and injuries (Ambriz-Pérez et al., 2016). In modern society, the incidence of inflammatory diseases has been increasing due to westernized diet, pollution, and stress (Vlassara 2005). Recent research has shown that a westernized diet is an important environmental factor of inflammatory bowel disease (Chiba et al., 2019). Air pollution caused by traffic causes non-allergic eosinophilic airway inflammation in guinea-pigs (Fang et al., 2019). Furthermore, many chronic diseases such as cancer, diabetes, and auto-immune diseases are caused by the constant exposure to oxidative species that induce an inflammatory response (Ginwala et al., 2019). A recent study reported that inflammation is a major factor in cellular senescence, a process in which cell proliferation is stopped by the release of inflammatory molecules (Serino and Salazar 2019). Therefore, the effective therapeutic methods for suppressing inflammation need to be developed.

Apoptosis, also known as programmed cell death, is a mechanism that allows cells to commit suicide when appropriate triggers stimulate cells (Elmore 2007). Apoptosis is characterized by various morphological and physiological features including cell shrinkage, membrane blebbing, and DNA fragmentation (Lane et al., 2005). Apoptosis plays a crucial role in the regulation of cell population and multiple signaling pathways in cells (Strasser et al., 2000). When cells undergo apoptosis, the two major cell signaling pathways involved are the extrinsic pathway (induced by external factors) and intrinsic pathway (associated with mitochondria) (Jin and El-Deiry 2005). Caspase family including caspase-3, caspase-7, and caspase-9 are important mediators of the intrinsic apoptotic signaling pathway whereas caspase-8 is known to mediate the extrinsic pathway (Brentnall et al., 2013). The exact working mechanism of HSE on the anti-inflammatory activity has not been fully understood. Thus, in this study, we investigated whether the anti-inflammatory effect of HSE in LPS-stimulated Raw 264.7 cells was associated with apoptosis.

2. Materials and methods

2.1. Preparation of HSE

H. syriacus L. was collected from the various geographical regions in South Korea and was rinsed carefully with the fresh water and air dried. First, the dried H. syriacus L. was ground and sifted through a 30-mesh sieve (600 μm particle size). After then, 70% ethanol was added at a ratio of 1:50 (w/v) to extract them with ultra-sonification (20 KHz, 500 Watt ± 2, Sonic & Materials, CT, USA) for 1 h. Then, each supernatant extraction was filtered by a 110 nm filter paper (No. 2, Advantec, Tokyo, Japan) and the redundant ethanol was removed by rotary evaporation (Eyela, Tokyo, Japan). Finally, the sample was dried by a vacuum freeze dryer (Labogene, Lillerød, Denmark) for 72 h. The extract was dissolved in DMSO (100 mg/mL) and stored in −20 °C.

2.2. High-performance liquid chromatography (HPLC) analysis

Analytical HPLC was adopted for comparing the relative profile of the HSE by the various EtOH concentration using C18 column (WATERS, Seoul, Korea, 4.6 × 220 nm). The flow rate of the solvent was 1 ml/min and absorption peaks were identified at 254 nm.

2.3. Cell culture

Raw 264.7, murine macrophage cell line, was purchased from the Korean Cell Bank (Seoul, South Korea) and maintained in a humidified incubator (37 °C and 5% CO_2). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin (Gibco).

2.4. Free radical scavenging assay

Free radical scavenging activity of HSE was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma) scavenging photometric assay. DPPH powder was dissolved in methanol (0.2 mM). HSE were added to 96 well plates. Then, DPPH solution was added to each well (final concentration of HSE is 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 μg/mL and final concentration of DPPH is 0.1 mM). Plates were incubated for 30 min at room temperature in the dark. Then, the absorbance was read at 517 nm using a multi well microplate reader (Molecular Devices, Mountain View, CA, USA).

2.5. WST-1 assay

Raw 264.7 cells (1×10^3 cells per well of 96 well plate) were incubated in a humidified incubator (37 °C and 5% CO_2) for 24 h. After incubation, cells were treated with 10 μg/mL LPS (Sigma, 10 μg/mL) and HSE (0, 62.5, 125, and 250 μg/mL) for 24 h. Then, 10 μL of EZ-CYTOX (DoGenBio, Seoul, South Korea) was added to each well. After 1 h at 37 °C in the dark, absorbance was read at 570 nm using a multi-well microplate reader.

2.6. Annexin V/propidium iodide (PI) double staining assay

The population of live and apoptotic cells was determined by FITC Annexin V Apoptosis Detection Kit (Cat# 556547, BD Bioscience, Franklin Lakes, NJ, USA) and we followed the manufacturer's protocol. Raw 264.7 cells (1×10^6 cells in 60 mm cell culture dish) were incubated in a humidified incubator (37 °C and 5% CO_2) for 24 h. After incubation, cells were treated with 10 μg/mL LPS and HSE (0, 62.5, 125, and 250 μg/mL) for 24 h. Then, cells were collected through trypsinization and centrifuged. Cell pellet was re-suspended with binding buffer containing Annexin V labelling reagent and PI and incubated at room temperature in the dark for 15 min. After incubation, the population of live and apoptotic

cells was evaluated using flow cytometry (Beckman, Brea, CA, USA).

2.7. PGE2 concentration assay

PGE2 concentration assay was performed using prostaglandin E2 ELISA kit (Cat# ab133021, Abcam, Cambridge, UK) and we followed the manufacturer's protocol. Raw 264.7 cells (1×10^6 cells in 60 mm cell culture dish) were incubated in a humidified incubator (37 °C and 5% CO_2) for 24 h. After incubation, cells were treated with 10 μg/mL LPS and HSE (0, 62.5, 125, and 250 μg/mL) for 24 h. Then, cell culture media were added to appropriate wells followed by addition of assay buffer, PGE2 alkaline phosphatase conjugate, PGE2 antibody, and pNpp substrate, sequentially. After 1 h at 37 °C in the dark, absorbance was read at 420 nm using a multi-well microplate reader.

2.8. COX-1 and 2 activity assay

COX-1 and 2 activity assay was performed using COX activity assay kit (Cat# ab204699, Abcam) and we followed manufacturer's protocol. Raw 264.7 cells (1×10^6 cells in 60 mm cell culture dish) were incubated in a humidified incubator (37 °C and 5% CO2) for 24 h. After incubation, cells were treated with 10 μg/mL LPS and HSE (0, 62.5, 125, and 250 μg/mL) for 24 h. Then, cells were harvested and lysed in ice-cold lysis buffer. Cell lysates were added to appropriate wells followed by addition of assay buffer, arachidonic acid, and COX-1 or 2 inhibitors, sequentially. After 1 h at 37 °C in the dark, fluorescence was read at excitation 535 and emission 587 nm using a multi-well microplate reader.

2.9. Western blotting analysis

Cells were harvested and lysed in ice-cold RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing phenylmethylsulfonyl fluoride (PMSF; Sigma). Proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Burlington, MA, USA), and blocked with 5% bovine serum albumin (BSA, Sigma). Membranes were incubated with primary antibodies (1:1000 dilution) at 4 °C overnight. Primary antibodies against COX-2, caspase-3, caspase-6, caspase-7, caspase-9, cleaved (c)-caspase-3, c-caspase-6, c-caspase-7, c-caspase-9, Bim, Bax, p53, Bcl-2, FOXO3, JNK, phospho (p) JNK, ERK, and pERK were purchased from Cell Signaling Technology and primary antibodies against PARP, c-PARP, IL6, p38, and pp38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Then, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10000 dilution, Cell Signaling Technology) at room temperature for 1 h. Each protein was detected by the enhanced chemiluminescence (ECL; GE Healthcare, Little Chalfont, UK) and Chemi-doc system (Bio-Rad, Hercules, CA, USA).

2.10. Statistical analysis

All data were analyzed by Student's *t*-test and $p < 0.05$ was considered to indicate statistical significance. The results are expressed as mean ± SED (the standard deviation of the mean). The representative data were shown from experiments repeated at least three times.

3. Results

3.1. HSE shows the anti-oxidant activity

As shown in Fig. 1A, HSE was prepared with ultrasonification. As a result of HPLC analysis of the extract for each EtOH solvent, the chromatograph was analyzed for each polarity and it was confirmed that the most non-polar compounds were extracted from the 70% EtOH extract (Fig. 1B). Then, to determine the antioxidant activity of HSE, DPPH assay was conducted. Different concentrations of HSE were aliquoted into a 96 well plate, and DPPH solution was then added to each well. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm using a multi-well microplate reader. The results representing DPPH scavenging rates of HSE are shown in Fig. 1C. Our results showed that HSE decreased the absorbance in a dose dependent manner, which indicated that HSE decreased free radicals. The IC_{50} value (IC_{50} value is the concentration of chemical required to inhibit 50% of DPPH free-radical) of HSE was found to be about 300 μg/mL. We found that absorbance was minimized in the 700 μg/ml HSE-treated group.

3.2. HSE decreases PGE2 concentration and COX-2 enzyme activity and downregulates COX-2 and IL6 expression in LPS-stimulated Raw 264.7 cells

The COX-2/PGE2 pathway plays a crucial role in active inflammation. Thus, to investigate the anti-inflammatory effect of HSE, we measured the concentration of PGE2 in the media as well as the activity of COX-2 enzyme in LPS-stimulated Raw 264.7 cells. We found that PGE2 concentration was undetectable in unstimulated Raw 264.7 cells, whereas it increased markedly after treatment with 10 μg/mL LPS (Fig. 2A). PGE2 concentration decreased dramatically after treatment with HSE (62.5 and 125 μg/mL). COX-2 enzyme activity increased after treatment with 10 μg/mL LPS compared to unstimulated Raw 264.7 cells (Fig. 2B). The results indicated that COX-2 enzyme activity decreased markedly after treatment with 125 and 250 μg/mL HSE compared to the LPS-treated group whereas COX-1 enzyme activity was not much decreased. To investigate the effect of HSE on COX-2 and IL6 expression in LPS-stimulated Raw 264.7 cells, western blotting was conducted. Our results indicated that LPS treatment markedly upregulated the expression of COX-2 and IL6 compared to the control group, whereas the expression of COX-2 and IL6 was suppressed after treatment with HSE in LPS-stimulated Raw 264.7 compared to the LPS-treated group (Fig. 2C and D).

3.3. HSE decreases viability and induces apoptosis in LPS-stimulated Raw 264.7 cells

To clarify the effect of HSE in Raw 264.7 cells, we observed the morphological changes and measured cell viability using WST-1 assay. Raw 264.7 cells were stimulated by LPS, treated with

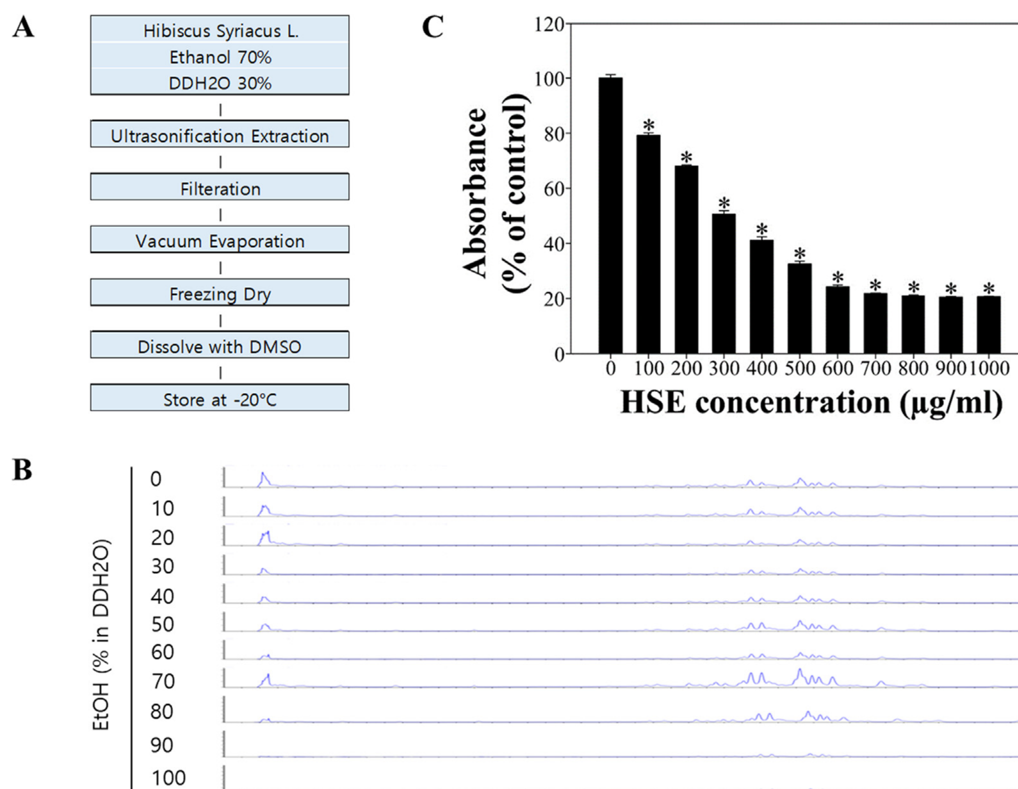

Fig. 1 Preparation and free-radical scavenging activity of HSE. (A) Schematic diagram for preparing HSE by the ultra-sonification. (B) HPLC chromatograph of the HSE by each EtOH solvent. (C) Free-radical scavenging activity of HSE by DPPH assay. Experiments were conducted three times independently and the results are shown as the means ± standard deviation (*p < 0.05 compared to control group). HSE: *Hibiscus syriacus* L. Extract.

the different concentrations of HSE (0, 62.5, 125, and 250 μg/mL) for 24 h, and observed by microscopy (Fig. 3A). We found that HSE treatment led to morphological changes in LPS-stimulated Raw 264.7 cells. Compared to the control and LPS-treated group, the number of the shrunken cells increased in HSE-treated group. Furthermore, the images showed that the density of cells decreased in the HSE-treated groups in a dose-dependent manner, which suggested that HSE suppressed the proliferation of Raw 264.7 cells. Additionally, WST-1 assay was performed to evaluate the anti-proliferative effect of HSE in Raw 264.7 cells (Fig. 3B). Raw 264.7 cells were treated with different concentrations of HSE (0, 62.5, 125, and 250 μg/mL) for 24 h and cell viability was measured. The results showed that HSE decreased the viability of Raw 264.7 cells in a dose-dependent manner. Furthermore, the cells were treated with 10 μg/mL LPS and different concentrations of HSE (0, 62.5, 125, and 250 μg/mL) for 24 h, and cell viability was measured by WST-1 assay (Fig. 3C). The results indicated that HSE decreased the viability of LPS-stimulated Raw 264.7 cells. To further evaluate the anti-proliferative effect of HSE in LPS-stimulated Raw 264.7 cells, we performed Annexin V/PI double staining assay. The proportion of apoptotic cells was assessed and is shown in Fig. 3D. We found that the proportion of viable cells (Annexin V negative/PI negative cells) decreased in the HSE and LPS-treated groups compared to the LPS-treated group. The proportion of total apoptotic cells (Annexin V positive/PI negative cells, and annexin V positive/PI positive cells) increased significantly in the HSE and LPS-treated groups compared to the LPS-treated group.

3.4. HSE activates apoptosis signaling pathways in LPS-stimulated Raw 264.7 cells

To demonstrate the apoptotic activity of HSE in LPS-stimulated Raw 264.7 cells, we performed western blotting of proteins related apoptosis signaling pathways. We measured the expression levels of the caspase family members (caspase-3, caspase-6, caspase-7, caspase-9, and cleaved (c)-caspase-3, c-caspase-6, c-caspase-7, and c-caspase-9). Our results showed that HSE increased c-caspase-3, c-caspase-6, c-caspase-7, and c-caspase-9 levels in LPS-stimulated Raw 264.7 cells (Fig. 4A). Furthermore, we found that HSE induced PARP cleavage and increased Bim, FOXO3, Bax, and p53 expression levels, but decreased Bcl-2 expression levels in LPS-stimulated Raw 264.7 cells (Fig. 4A). Additionally, we evaluated the expression levels of the mitogen-activated protein kinase (MAPK) family members (ERK, phosphorylated (p) ERK, JNK, pJNK, p38, and pp38). We found that HSE increased pJNK levels in LPS-stimulated Raw 264.7 cells (Fig. 4B). Furthermore, we observed that HSE increased p38 and pp38 expression levels in LPS-stimulated Raw 264.7 cells.

4. Discussion

Hibiscus syriacus L., called rose of Sharon or Moogoonghwa, belongs to family Malvaceae and is widely distributed in area with warm temperature such as South Korea (Kim et al., 2019). In Chinese herbal medicine, *Hibiscus syriacus* L. has

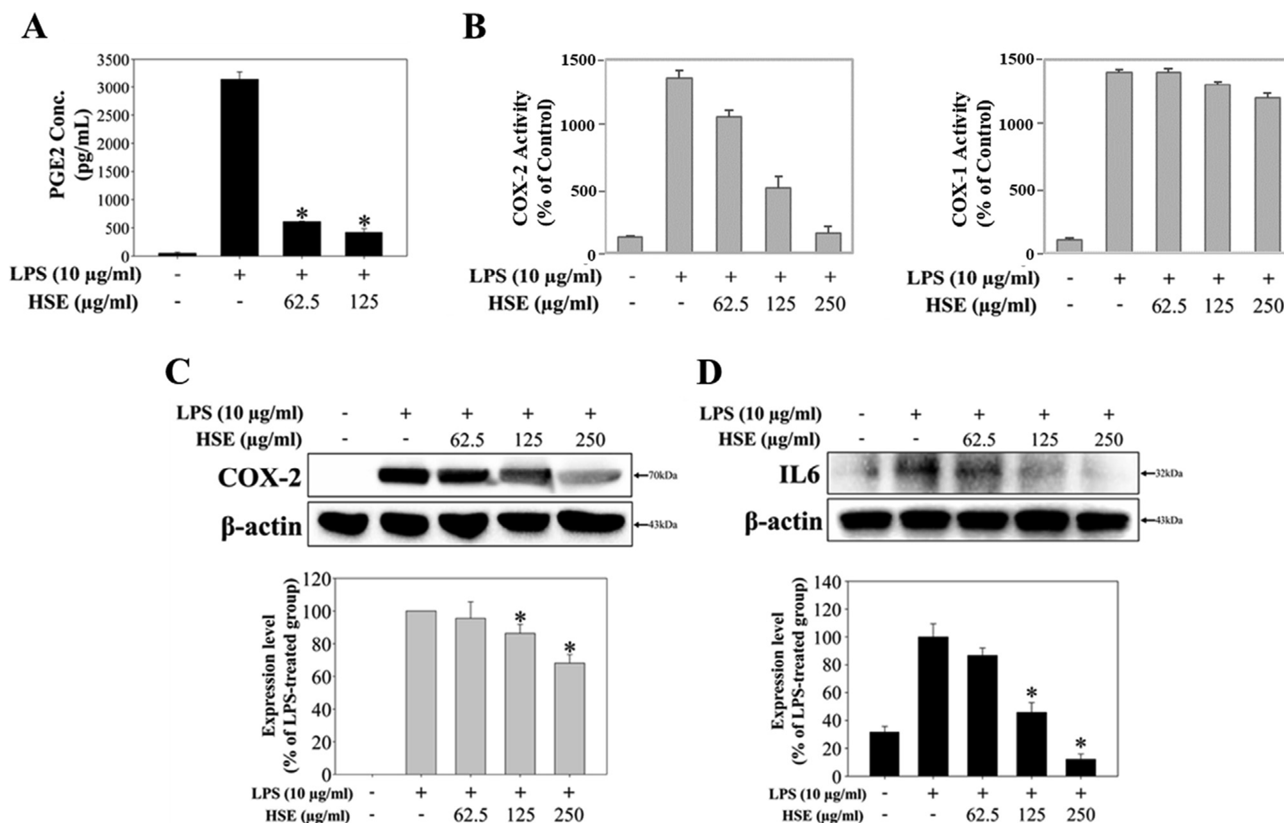

Fig. 2 Inhibition of PGE2 generation, COX-2 enzyme activity, COX-2 expression, and IL6 expression by HSE in LPS-stimulated Raw 264.7 cells. (A) PGE2 production in Raw 264.7 cells treated with HSE and LPS. (B) COX1-1 and COX-2 enzyme activity in Raw 264.7 cells treated with HSE and LPS for 24 h. (C) COX-2 expression analyzed by western blotting in Raw 264.7 cells treated with HSE and LPS for 24 h. (D) IL6 expression analyzed by western blotting in Raw 264.7 cells treated with HSE and LPS for 24 h. The bands are analyzed by densitometry. Experiments were conducted three times independently and the results are shown as the means ± standard deviation (*$p < 0.05$ compared to LPS-only treatment group). HSE: *Hibiscus syriacus* L. Extract, LPS: Lipopolysaccharide.

been prescribed to alleviate fever or treat athlete's foot (Oku and Ishiguro 2008). Furthermore, *Hibiscus syriacus* L. has used to soothe the skin and treat digestive disorders (Rakesh et al., 2014). Recently, *Hibiscus syriacus* L. has been recognized as a promising natural therapeutic compound and the nutritional and functional quality of its edible petals have been reported (Yang et al., 2018). Previous research has also shown that *Hibiscus syriacus* L. contains the high amounts of antioxidants and significantly improves blood circulation (Shin and Ha 2016). However, the anti-inflammatory effect of *Hibiscus syriacus* L. has not been well investigated. In this study, we aimed to study the anti-inflammatory effect of *Hibiscus syriacus* L. and elucidated its mechanism in LPS-stimulated Raw 264.7 cells, murine macrophage cell line. According to recent studies, researcher have identified various kinds of compounds from *Hibiscus syriacus* L. such as saponarin, coumaric acid, catechin, naringenin and apigetrin as the active compound candidates (Kim et al., 2022, Park et al., 2022). For the further study, we are planning to identify the active compounds from HSE which are involved in the anti-inflammatory activity and pro-apoptotic activity in LPS-stimulated Raw 264.7 cells.

Inflammation is a complex biological response to harmful stimuli such as infections and injuries. Inflammatory diseases caused by various factors such as westernized diet and air pollution have become a global health concern (Fernando et al., 2016). Therefore, effective therapeutic methods need to be developed to address this issue. Recently, natural compounds derived from various plants have been recognized as promising and effective substances for the treatment of many kinds of inflammatory diseases (Kishore et al., 2019). Several studies have shown the effectiveness of natural compounds on inflammation through in vitro and in vitro studies. P-coumaric acid derived from Oldenlandia diffusa was reported to have the anti-inflammatory effects by regulating TNF-α and IL-6 in a rat model (Zhu et al., 2018). Another study showed that notopterol from Notopterygium incisum, a Chinese medicinal herb, has a therapeutic effect on macrophage-induced inflammation and arthritis by targeting the JAK2/3 signaling pathway (Wang et al., 2019). A recent study revealed that myricetin, a flavonoid derived from Myrica ruba, has chemopreventive effect on chronic colonic inflammation in mice (Zhang et al., 2018). Thus, scientists have been trying to find more effective natural products to improve therapeutic options against inflammatory diseases.

COX, also called as prostaglandin-endoperoxide synthase, plays a crucial role in the conversion of arachidonic acid to prostaglandins. There are two isoforms of this enzyme, COX-1 and COX-2 (Belton and Fitzgerald 2003). COX-2 is considered an important target for anti-inflammatory treatment (Chen 2010). Celecoxib, a kind of nonsteroidal anti-

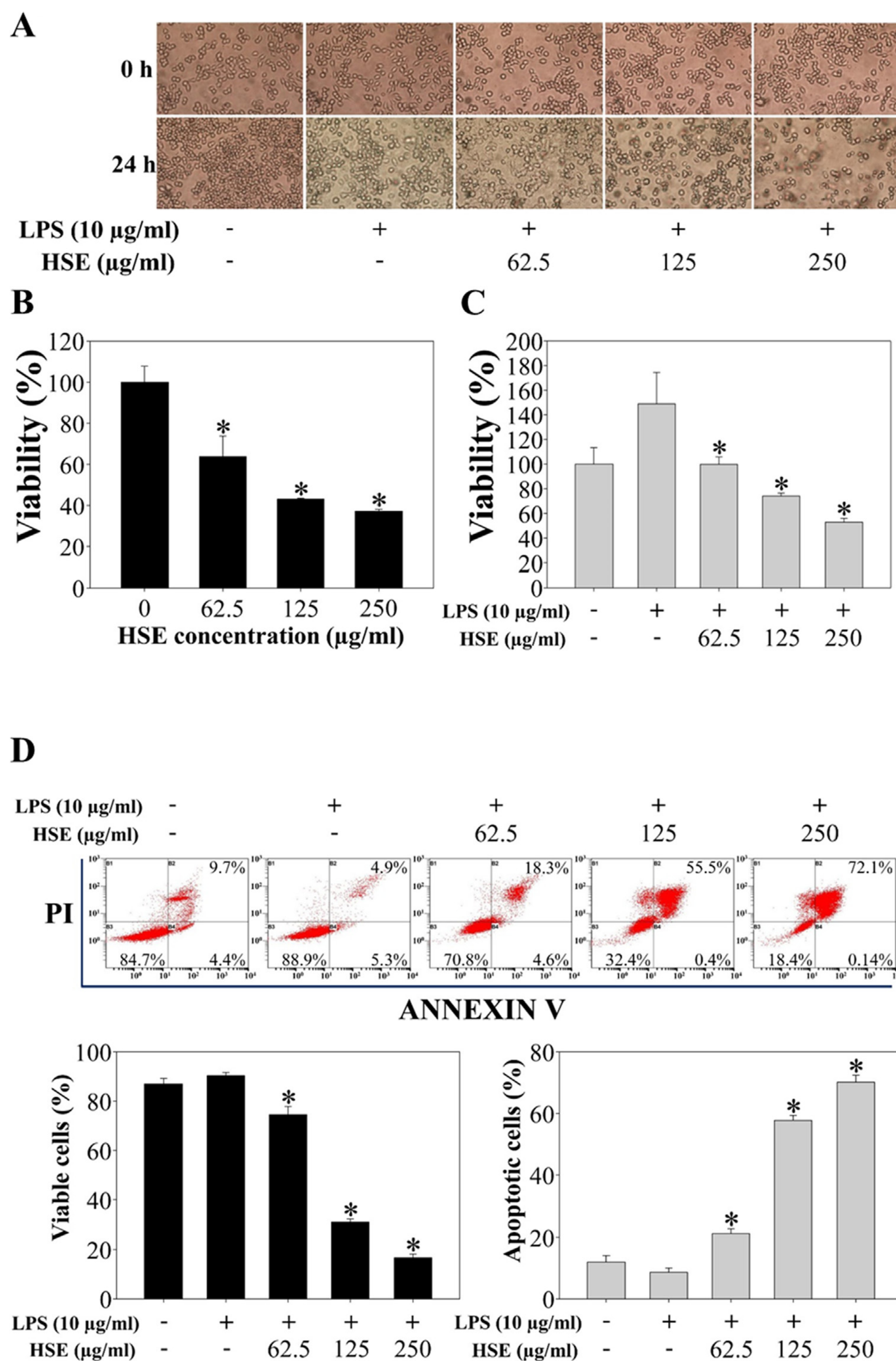

Fig. 3 Anti-proliferative activity of HSE and induction of apoptosis by HSE in LPS-stimulated Raw 264.7 cells. (A) Morphological changes in Raw 264.7 cells after treatment of HSE and LPS (400× magnification). (B) Viability of Raw 264.7 cells treated with HSE for 24 h was determined by WST-1 cell assay. (C) Viability of Raw 264.7 cells treated with HSE and LPS for 24 h was determined by WST-1 cell assay. (D) Apoptosis analysis using Annexin V/PI double staining in Raw 264.7 cells treated for 24 h with HSE and LPS. Experiments were conducted at least three times independently and the results are shown as the means ± standard deviation (*$p < 0.05$ compared to LPS-only treatment group). HSE: *Hibiscus syriacus* L. Extract, LPS: Lipopolysaccharide.

inflammatory drug, is a typical option for treating inflammation by targeting and inhibiting COX-2 (Davies et al., 2000). Interestingly, in this study, we found that HSE effectively decreased COX-2 activity and expression in LPS-stimulated Raw 264.7 cells, which suggests that HSE may provide promising phytocompounds for the development of the novel anti-

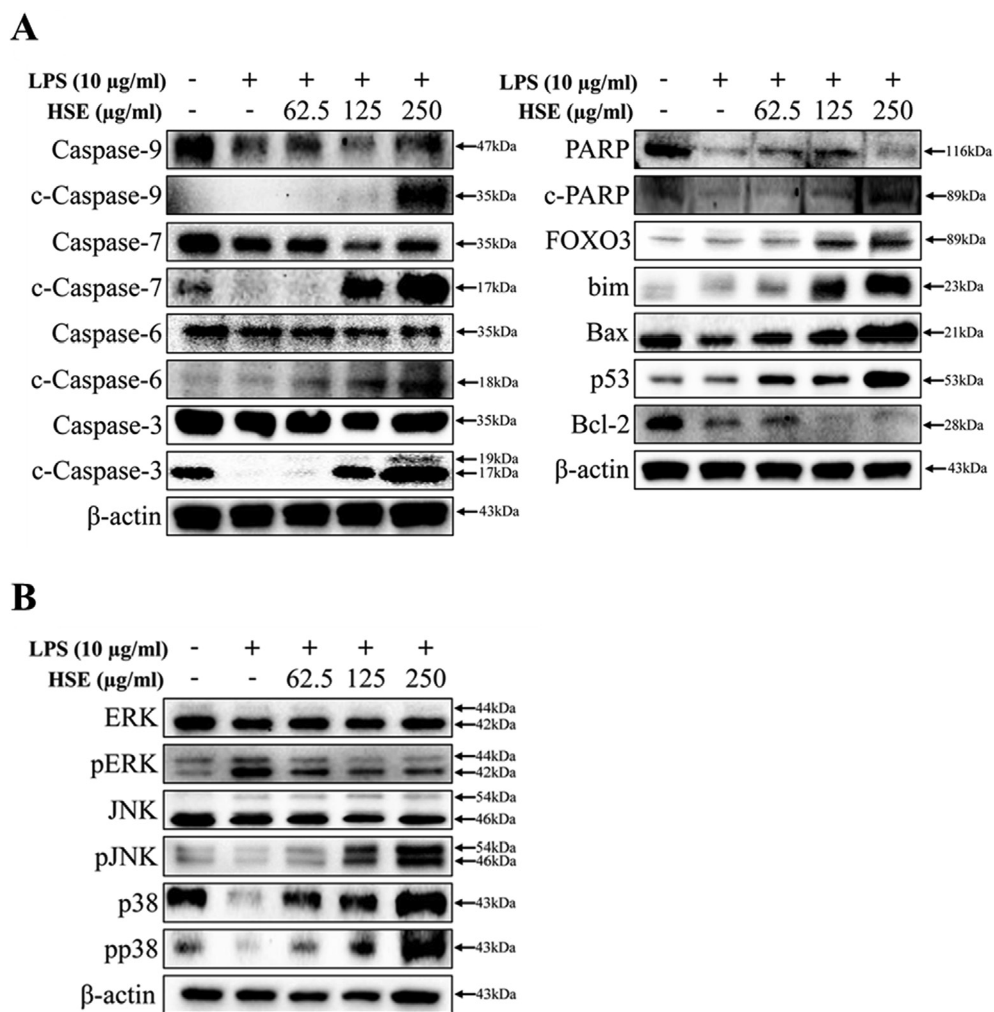

Fig. 4 Western blotting analysis of apoptosis related proteins and MAPK family in Raw 264.7 cells treated with HSE and LPS. (A) The expression levels of caspase family of proteins (caspase-3, caspase-6, caspase-7, and caspase-9, c-caspase-3, c-caspase-6, c-caspase-7, and c-caspase-9) and expression levels of apoptosis-related proteins (PARP, c-PARP, FOXO3, Bim, Bax, p53, and Bcl-2) were evaluated. β-actin was used as a loading control. (B) Expression levels of MAPK family of proteins (ERK, pERK, JNK, pJNK, p38, and pp38) were evaluated. β-actin was used as a loading control.

inflammatory agents. As shown in Fig. 2, our results showed that HSE decreased not only COX-2 activity and but also expression of COX-2.

PGE2 is an important downstream effector of COX-2 and has a wide variety of biological functions such as cell proliferation, apoptosis, and immune response (Piccolella and Pacifico 2015). PGE2 is a major inflammatory mediator in diseases and produced by COX-2 from arachidonic acid (Giuliano and Warner 2002). PGE2 is generated by a broad range of tissues and upregulated in many body fluids during inflammation (Loynes et al., 2018). PGE2 is an important mediator of inflammation because it regulates all processes that lead to signs of inflammation such as redness, swelling, and pain (Funk 2001). Thus, it is crucial to inhibit PGE2 expression to alleviate inflammation. Our results indicated that HSE significantly decreased PGE2 level in culture media of LPS-stimulated Raw 264.7 cells.

Because overexpression of COX-2 and PGE2 are major causes of inflammation, it is necessary to downregulate these factors (Ricciotti and FitzGerald 2011). Raw 264.7 cells are commonly used to study the anti-inflammatory effects because these cells constitute an appropriate macrophage model to study inflammation (Taciak et al., 2018). Recently, apoptosis is known to plays a crucial role in inflammatory responses through regulation of COX-2 and PGE2. C-phycocyanin induced apoptosis in LPS-stimulated Raw 264.7 cells through the downregulation of PGE2 as a result of selective COX-2 inhibition (Reddy et al., 2003). This study suggested that apoptosis and inhibition of COX-2 and PGE2 in Raw 264.7 cells are important anti-inflammatory mechanisms. Additionally, several studies have shown the anti-inflammatory effect in Raw 264.7 cells stimulated by LPS by evaluating the apoptotic process (Kim et al., 2006, Wang et al., 2017). Our results indicated that HSE downregulated COX-2 and PGE2 and induced apoptosis in LPS-stimulated Raw 264.7 cells.

FOXO3 is a major transcription factor involved in various cellular stimuli and plays a crucial role in tumor progression (Song et al., 2020). p53, also known as a tumor suppressor pro-

tein, is involved in various cellular processes such as the cell cycle, tumor suppression, and apoptosis (Harris 1996). Several studies have demonstrated that anti-inflammatory effects are mediated through FOXO3 and p53 associated apoptosis in Raw 264.7 cells. Methylsulfonylmethane has been reported to induce apoptosis in LPS/IFN-γ-induced Raw 264.7 cells by targeting apoptotic proteins, such as p53, Bax, and Bcl-2 (Karabay et al., 2014). FOXO proteins are known to modulate several inflammatory processes (Link and Fernandez-Marcos 2017). These studies suggested that the apoptosis signaling pathway may be a crucial mediator of the inflammatory response. Our results showed that both p53 and FOXO3 expression levels increased after treatment of HSE in LPS-stimulated Raw 264.7 cells, suggesting that the mechanism of apoptosis mediated by p53 and FOXO3 may be an important modulator for alleviating inflammation.

MAPK signaling pathways are involved in various cellular processes such as proliferation, apoptosis, cell stress response, and inflammation (Guo et al., 2020). Many studies have shown the anti-inflammatory effects are mediated via the regulation of MAPK signaling pathways (Jin et al., 2016, Lv et al., 2016, Zhan et al., 2018). Interestingly, our results showed that the expression levels of pJNK, p38, and pp38 were increased but the expression of pErk was slightly decreased after treatment HSE in LPS-stimulated Raw 264.7 cells. We found that the increase in phosphorylated JNK and p38 but the decrease in the phosphorylated ERK. Although all of MAPKs are known to be involved in activation of inflammation, there is a report describing that ERK is the sole regulator to control inflammation independent of JNK and p38 (Park et al., 2021). According to their study, 3,4,5 trihydroxycinnamic acid showed anti inflammatory activity in TNF α/IFN γ stimulated HaCaT cells without decrease in the phosphorylated JNK and p38. 3,4,5 trihydroxycinnamic acid inhibited only ERK phosphorylation in TNF α/IFN γ stimulated HaCaT cells. Furthermore, in this study, we are trying to show the anti-inflammatory and pro-apoptotic activity of HSE in LPS-stimulated Raw 264.7 cells. A recent report suggested that sorafenib treatment caused apoptosis through activation of JNK and p38 rather than ERK in EBV-transformed B cells (Park et al., 2014). Thus, we thought HSE has the dual physiological activity to cause the apoptosis via activation of JNK and p38 and show the anti-inflammation via inhibition of ERK in LPS-stimulated Raw 264.7 cells. These results imply that MAPK signaling pathway may be involved in HSE mediated anti-inflammatory response with different manner.

5. Conclusion

In conclusion, our results showed that HSE by the ultrasonic extraction from *Hibiscus syriacus* L. has the anti-inflammatory effects via regulation of COX-2, IL6, and PGE2 and the pro-apoptotic activity through FOXO3 and p53 in LPS-stimulated Raw 264.7, macrophage cell line. Our results suggest that HSE may provide promising phytochemicals for the treatment of inflammatory diseases. For additional study, we are currently designing to demonstrate the detailed anti-inflammatory and pro-apoptotic mechanisms of HSE by using proper animal model such as collagen-induced rheumatoid arthritis mouse model.

Author contribution

CML, MAK, JBK, KMP, HTK, and YHY: Performed experiments, analyzed the results, and co-wrote the manuscript. JSL: conceived the presented idea, co-wrote the manuscript, and analyzed the results. SHP: conceived the presented idea, performed experiments, and co-wrote the manuscript, analyzed the results, and supervised this study.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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