

Hispidulin-7-O-Neohesperidoside from *Cirsium japonicum* var. *ussuriense* Attenuates the Production of Inflammatory Mediators in LPS-Induced Raw 264.7 Cells and HT-29 Cells

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ABSTRACT

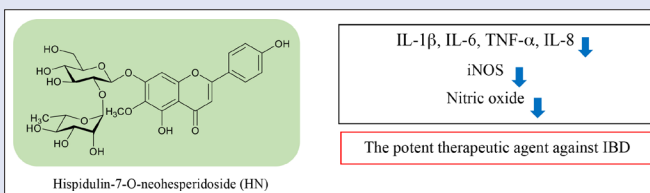
Background: Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract and involves secretion of inflammatory mediators. The flavone diglycoside hispidulin-7-O-neohesperidoside (HN) isolated from the methanolic extract of aerial parts of *Cirsium japonicum* var. *ussuriense*, but its pharmacologic activities, with the exception of alleviation of alcohol toxicity, have not been investigated to date. **Objective:** The aim of the present study was to investigate the anti-inflammatory activities of HN for the treatment of chronic inflammatory illnesses, including IBD. **Materials and Methods:** In lipopolysaccharide (LPS)-induced RAW264.7 cells and HT-29 cells, the effects of HN on cell viability and nitric oxide (NO) production were examined via MTT assay and the Griess reaction, respectively. The expression levels of interleukin (IL)-1 α , IL-8, and tumor necrosis factor (TNF)- α and inducible nitric oxide synthase (iNOS) protein levels were measured by enzyme-linked immunosorbent assay and Western blotting, respectively. **Results:** HN concentration-dependently inhibited NO production in LPS-induced RAW 264.7 cells. Treatment with HN considerably downregulated the levels of the pro-inflammatory cytokines, IL-1 β and TNF- α and the iNOS protein level in LPS-induced RAW 264.7 cells. Furthermore, HN inhibited the production of the chemotactic cytokine, IL-8, in LPS-induced HT-29 cells. **Conclusion:** HN has potential as an anti-inflammatory agent to prevent and/or treat IBD.

Key words: Hispidulin-7-O-neohesperidoside, *Cirsium japonicum* var. *ussuriense*, inflammation, inflammatory bowel disease

SUMMARY

- Hispidulin-7-O-neohesperidoside (HN) is flavone diglycoside isolated from the methanolic extract of aerial parts of *Cirsium japonicum* var. *ussuriense*.

- HN concentration-dependently inhibited NO production and considerably downregulated the levels of the proinflammatory cytokines, IL-1 β and TNF- α , and the iNOS protein level in LPS-induced RAW 264.7 cells.
- HN inhibited the production of the chemotactic cytokine, IL-8, in LPS-induced HT-29 cells.
- HN has potential as an anti-inflammatory agent to prevent and/or treat IBD.



Abbreviations used: IBD: Inflammatory bowel disease, HN: hispidulin-7-O-neohesperidoside, LPS: lipopolysaccharide, NO: nitric oxide, IL: interleukin, TNF: tumor necrosis factor, iNOS: inducible nitric oxide synthase, CD: Crohn's disease, UC: ulcerative colitis, RT: room temperature, DMEM: Dulbecco's modified Eagle's medium, FBS: fetal bovine serum, PBS: phosphate buffered saline, SDS: sodium dodecyl sulfate, PVDF: polyvinylidene difluoride, SD: standard deviation

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INTRODUCTION

Inflammation is a biologic response to stimuli such as pathogen infection and presents a major obstacle in maintaining a high quality of life.^[1] Chronic or recurrent inflammatory reactions within the colon possibly due to viruses or bacteria may initiate or promote colon cancer development or progression.^[2] Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract and is categorized as Crohn's disease (CD) and ulcerative colitis (UC).^[3] For development of new therapeutic strategies against these diseases, a better understanding of the processes that initiate, modulate, and perpetuate intestinal mucosal inflammation is required.^[4] Also, the chronic immune response in IBD may be regulated by increased secretion of pro-inflammatory cytokines due to an inappropriate response to initial stimulating events and/or impaired downregulation of cytokine secretion.^[4]

Nitric oxide (NO), an important mediator of inflammation, is synthesized by nitric oxide synthase (NOS), which exists as three isoforms, endothelial, neuronal, and inducible NOS. Among them, inducible nitric oxide

synthase (iNOS) plays a pivotal role in regulation of inflammation as well as ultimate repair of injury and carcinogenesis.^[5] Lipopolysaccharides (LPSs), the major outer membrane constituent of Gram-negative bacteria, stimulate production of pro-inflammatory cytokines such as iNOS, interleukin (IL)-1 β , IL-6, IL-8, and tumor necrosis factor-alpha (TNF- α), as well as immune mediators such as NO, in various cell types, including RAW264.7 macrophages and HT-29 cells.^[6-8]

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Cirsium japonicum DC var. *ussuriense* (Rege) Kitam (Compositae) is a perennial herb indigenous to Korea. The aerial parts of this plant are used in oriental medicine as a diuretic, tonic, neuralgia stomachic, antiphlogistic, and detoxicant.^[9] *C. japonicum* var. *ussuriense* is also known as “Korean thistle.” Pharmacologic studies on extract of *C. japonicum* var. *ussuriense* have reported hepatoprotective, anti-inflammatory, antitumor, antimutagenic, elimination, and antioxidant effects.^[9-12] Polyacetylene and flavonoid have been reported as major constituents of *C. japonicum* var. *ussuriense*.^[9,13] Hispidulin-7-O-neohesperidoside (HN) is a flavone diglycoside from *C. japonicum* var. *ussuriense* that alleviates alcohol toxicity by enhancing ethanol oxidation and inhibiting lipid peroxidation.^[9,14] However, the pharmacologic activities of HN have not been investigated to date.

In this study, to assess the anti-inflammatory activities of HN, we used LPS-stimulated RAW 264.7 macrophages and HT-29 colonic epithelial cells. HN isolated from *C. japonicum* var. *ussuriense* inhibited the production of NO and pro-inflammatory mediators. Our findings indicate that HN can modulate inflammation, suggesting it to have potential for treatment of IBD.

MATERIALS AND METHODS

Plant and phytochemical materials

The aerial parts of *C. japonicum* var. *ussuriense* were collected from Sanchung, Kyungnam, Korea, on July 20, 1997. A voucher specimen (NM018) was deposited at the herbarium of Sunchon National University, Suncheon, Korea. Dried and pulverized *C. japonicum* var. *ussuriense* aerial parts were extracted with methanol using an ultrasonic apparatus at room temperature (RT). Methanolic extract of *C. japonicum* var. *ussuriense* aerial parts was concentrated *in vacuo* to give a crude extract, which was suspended in H₂O and partitioned successively in CHCl₃, *n*-butanol, and H₂O. HN was isolated from the *n*-butanol fraction of *C. japonicum* var. *ussuriense* aerial parts.^[14]

Cell culture

HT-29 human colonic epithelial cells and RAW264.7 macrophages were obtained from the Korean Cell Line Bank (Seoul, Korea). These cell lines were separately maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), containing 100 IU/mL penicillin and 100 µg/mL streptomycin (Hyclone, Logan, UT, USA) at 37°C in a humidified atmosphere of 95% air–5% CO₂. Experiments were performed with RAW264.7 cells or HT-29 cells treated with HN at final concentrations of 25, 50, and 100 µM for 1 h and then 1 µg/mL lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) was applied for 24 h to induce inflammation.

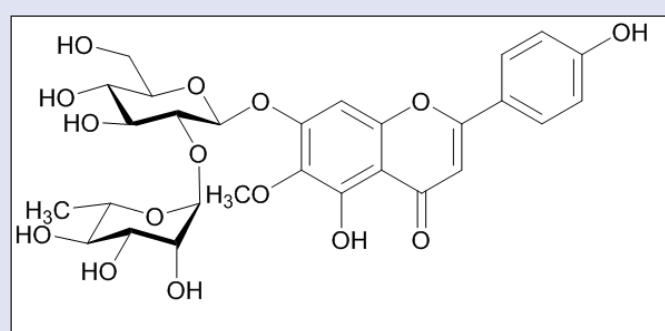


Figure 1: The chemical structure of hispidulin-7-O-neohesperidoside (HN)

Cell viability

Cells were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated for 24 h. HT-29 and RAW264.7 cells were treated with vehicle or HN for 24 h. Cell viability was assessed by MTT assay, in which MTT (final concentration 0.5 mg/mL) was directly added to cultures, followed by incubation at 37°C for 2 h. Subsequently, the supernatant was aspirated and 100 µL of DMSO was added to dissolve the formazan. Following dissolution of the insoluble crystals, absorbance at 570 nm was measured using a microplate reader. Data are expressed as percentages of viable cells relative to that of the control cultures.

Estimation of NO production

RAW264.7 cells were treated with HN for 1 h and then exposed to 1 µg/mL LPS. After incubation for 24 h, the nitrite level in culture medium was measured to evaluate NO production using Griess reagent. The supernatant was harvested and then 100 µL aliquots were mixed with an equal volume of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylene] in a 96-well plate and incubated at RT for 10 min. The absorbance at 550 nm was measured using a microplate reader (BioTek Instruments, Inc., Highland Park, Winooski, USA). Serum-free culture medium was used as the blank in all experiments. Nitrite was quantified by generating a standard curve using serial dilutions of NaNO₂. Relative NP (%) was calculated as (NP of sample treated – NP of control)/(NP of LPS-treated-NP of control) \times 100 (%).^[15]

Pro-inflammatory cytokine expression

RAW264.7 and HT-29 cells were plated overnight in 96-well plates at a density of 1×10^5 and 2×10^4 cells/well, respectively. The cells were treated with samples for 1 h before exposure to 1 µg/mL LPS. After incubation for 24 h, the supernatants were collected and stored at –70°C until cytokine assay. IL-1β, TNF-α, and IL-6 levels in RAW264.7 cells were determined using mouse ELISA kits (Cusabio, Wuhan, China) and IL-8 level in HT-29 human colonic epithelial cells was determined using ELISA kits (BD OptEIAM, CA, USA).

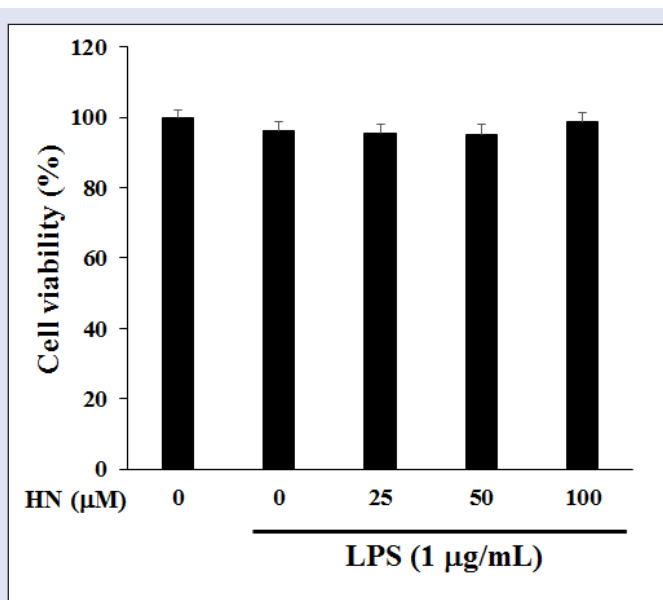


Figure 2: Effect of HN on the viability in LPS-induced RAW264.7 cells. Cytotoxicity on RAW264.7 cells treated with various concentrations of HN for 24 h. HN cytotoxicity was assessed by MTT assay. Data are mean \pm SD ($n = 3$) of three independent experiments

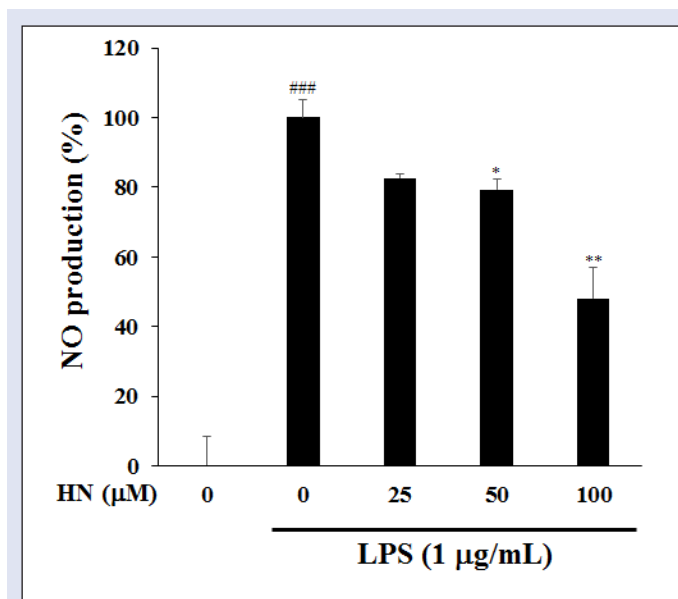


Figure 3: Inhibitory effect of HN on nitrite production in LPS-induced RAW264.7 cells. Nitrite in culture medium was quantified by Griess assay. Data are mean \pm SD ($n = 3$) of three independent experiments. ### $P < 0.001$, compared with the untreated control; * $P < 0.05$ and ** $P < 0.01$, compared with the LPS-treated control

Protein extraction and Western blot analysis

RAW264.7 cells were plated overnight in six-well plates at 1×10^5 cells/well. The medium was exchanged for fresh medium and cells were treated with samples for 1 h before exposure to 1 $\mu\text{g}/\text{mL}$ LPS. After incubation for 24 h, cells were washed twice with phosphate-buffered saline (PBS). Cell lysates were prepared using ice-cold lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSE, 25 $\mu\text{g}/\text{mL}$ leupeptin, and 20 $\mu\text{g}/\text{mL}$ pepstatin). Protein content was determined using Bio-Rad protein assay reagent according to the manufacturer's instructions. Equal amounts of protein (30 μg) were resolved in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), which were blocked with TBST (10 mM Tris [pH 7.4], 100 mM NaCl, and 0.5% Tween 20 containing 3% nonfat milk) for 1 h at RT. For immunodetection, membranes were incubated overnight with primary antibodies, including anti-iNOS (1:1000 dilution, Cell Signaling, BA, USA) in TBST containing 1% skim milk powder. After washing three times with TBST, immunoreactive bands were visualized using immunopure peroxidase-conjugated goat anti-mouse-IgG (1:1000, Santa Cruz, CA, USA). Finally, after rinsing in wash buffer, the membranes were visualized by enhanced chemiluminescence (ECL-kit, Thermo-Fisher Scientific, USA). The membranes were exposed to ECL detection reagents and quantified using a Bio imaging system (Micro Chemi 4.2 Chemilumineszenz-System, Israel).

Statistical analysis

Data are expressed as mean \pm standard deviation (SD) of at least three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by t -test and statistical significance was considered at P less than 0.05.

RESULT AND DISCUSSION

IBD, such as UC and CD, is a chronic and relapsing inflammatory condition of the gastrointestinal tract. Although the etiology of IBD is

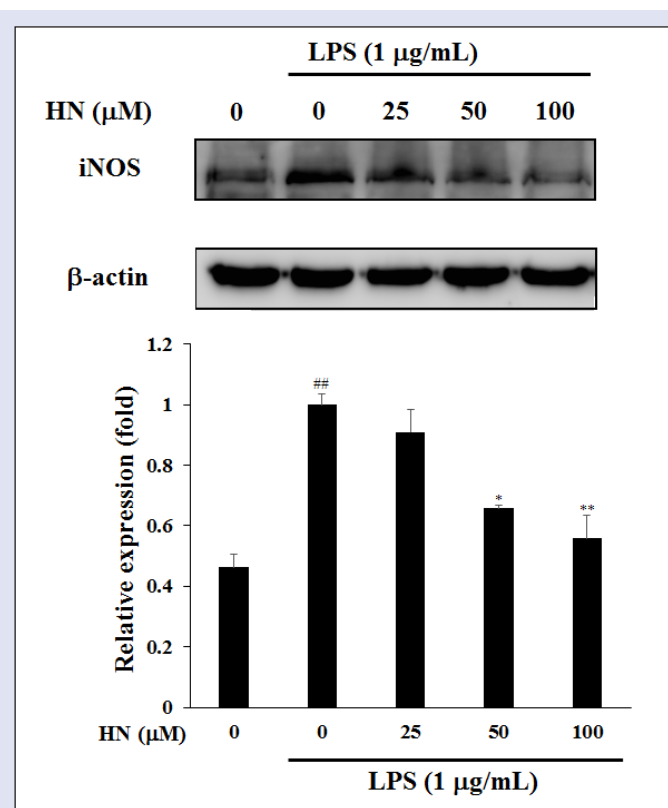


Figure 4: Inhibitory effect of HN on iNOS protein expression in LPS-induced RAW264.7 cells. iNOS and β -actin protein levels were determined by Western blotting. Data are mean \pm SD ($n = 3$) of three independent experiments. ## $P < 0.01$, compared with the untreated control; * $P < 0.05$ and ** $P < 0.01$, compared with the LPS-treated control

unknown, heredity, infection, environmental factors, and immunologic disorders have been suggested to be involved, and several models of experimental colitis have been developed to study the cellular and molecular mechanisms of inflammation and immunologic abnormality.^[16] To identify natural products with anti-inflammatory effects against IBD, we used LPS-stimulated RAW 264.7 cells and HT-29 colonic epithelial cells.

Cirsium japonicum DC var. *ussuriense* (Rege) Kitam (Compositae), also known as "Korean thistle," is a perennial herb indigenous to Korea. The aerial parts of this plant are used in oriental medicine as a diuretic, tonic, neuralgia stomachic, antiphlogistic, and detoxicant and have demonstrated hepatoprotective, anti-inflammatory, antitumor, antimutagenic, cytotoxic, and antioxidant effects in pharmacologic studies.^[9,10,12] Flavonoids are reported to be major constituents of *C. japonicum* var. *ussuriense*.^[9] These are plant-derived secondary metabolites distributed throughout the plant kingdom. In many studies, flavonoids such as quercetin and rutin have shown anti-inflammatory activities in cellular and rodent models.^[17] HN isolated from the methanolic extract of the aerial parts of *C. japonicum* var. *ussuriense* is a flavone diglycoside [Figure 1].^[14] Various pharmacologic activities of flavone glycosides have been reported in cellular and rodent models. HN has a neohesperidosyl [α -l-rhamnopyranosyl-(1 \rightarrow 2)- β -d-glucopyranosyl] moiety at the seventh position of hispidulin. Hispidulin is a natural bioactive flavone with various pharmacologic effects, for example, antioxidant, anticancer, antiepileptic, antihypnotic, anti-osteoclastogenesis, anti-inflammatory, anti-influenza, antidiabetic, antitrypanosomal, and hepatoprotective activities.^[18,19] However, the pharmacologic activities, including the anti-inflammatory effect, of HN

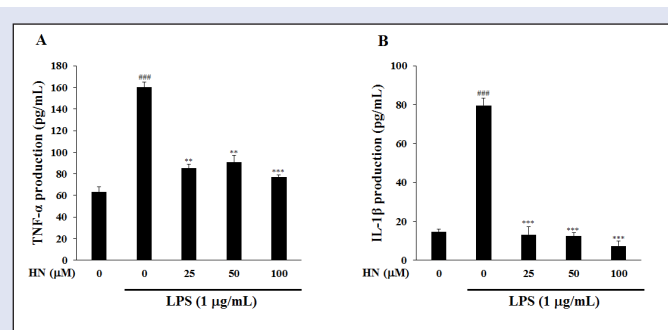


Figure 5: Inhibitory effect of HN on pro-inflammatory cytokine production in LPS-induced RAW264.7 cells. TNF-α was quantified by ELISA. (A) Cells were incubated with various concentrations of HN for 24 h. (B) Protein levels were determined by ELISA. Data are mean ± SD (n = 3) of three independent experiments. ###P < 0.001, compared with the untreated control; **P < 0.01 and ***P < 0.001, compared with the LPS-treated control

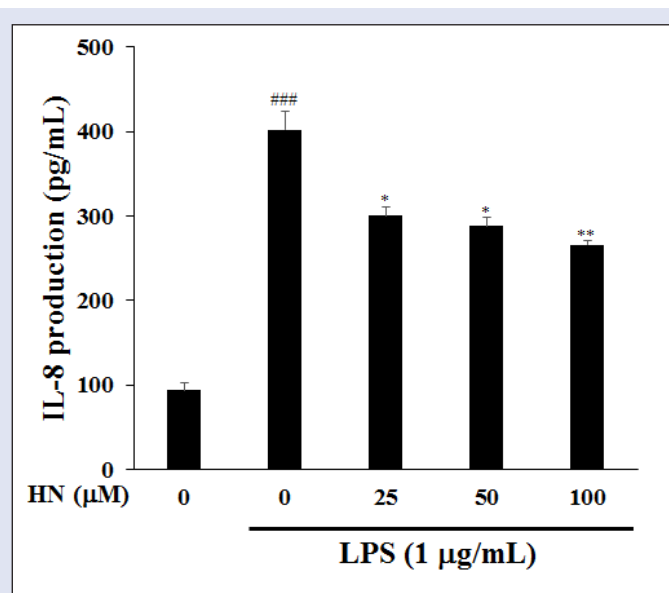


Figure 7: Inhibitory effect of HN on IL-8 expression in LPS-induced HT-29 colonic epithelial cells. IL-8 was quantified by ELISA. Values are means ± SD (n = 3) of three independent experiments. ###P < 0.001, compared with the untreated control; *P < 0.05 and **P < 0.01, compared with the LPS-treated control

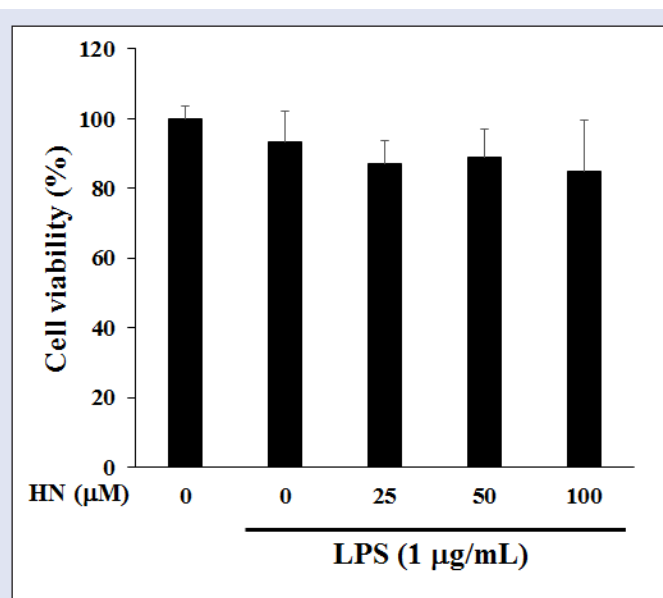


Figure 6: Effect of HN on the viability of LPS-induced HT-29 cells. Cells were pretreated with HN at various concentrations for 24 h, and cytotoxicity was analyzed by MTT assay. Data are mean ± SD (n = 3) of three independent experiments

have not been investigated to date, with the exception of alleviation of alcohol toxicity. In this study, we evaluated the effects on inflammatory mediators of HN treatment in LPS-treated RAW264.7 and HT-29 cells.

In LPS-induced RAW264.7 cells, the cytotoxicity of HN (25-100 μM) was measured by MTT assay. HN did not exert a cytotoxic effect at up to 100 μM compared with untreated control cells [Figure 2]. Thus, subsequent experiments used 25-100 μM HN. To elucidate its effects on inflammation, the inhibitory effect of HN on NO production was examined in LPS-induced RAW264.7 cells via the Griess reaction. HN showed potent inhibitory effects on NO production in LPS-induced RAW264.7 cells [Figure 3]. NO, which is involved in the development of intestinal inflammation, is generated by iNOS and contributes to inflammation. Therefore, we assessed the effect of HN on the iNOS protein level in LPS-induced RAW264.7 cells by Western blot analysis. The results confirmed that HN decreased the iNOS protein level in a concentration-dependent manner [Figure 4].

Moreover, HN at more than 25 μM suppressed the production of LPS-induced pro-inflammatory cytokines, IL-1β and TNF-α. In IBD patients, macrophages and intestinal immune cells secrete large amounts of IL-1β and TNF-α [Figure 5].^[17] IL-1β is produced by both inflammatory cells and mucosal epithelial cells during colonic inflammation. TNF-α is a potent cytokine with multiple immunologic and inflammatory effects related to IBD.^[19] Both IL-1β and TNF-α may regulate, amplify, and perpetuate mucosal inflammation by various mechanisms and increase the release of potent chemotactic cytokines such as IL-8, which is found in increased quantities in inflamed mucosa.^[4] The increased IL-8 production within the intestine of IBD patients contribute to neutrophil activation by interacting with IL-1β and TNF-α, and thus, may initiate or maintain IBD.^[20] Thus, inhibitors of IL-8 may be used to treat immune-associated diseases such as CD and UC. IL-8 is produced by various cell types, such as neutrophils, epithelial cells, and endothelial cells; in particular, LPS stimulates IL-8 secretion in HT-29 cells.^[21] To investigate the inhibitory effect of HN on IL-8 production, we evaluated its effect on IL-8 levels in LPS-induced HT-29 cells by ELISA. HT-29 cells were pretreated with HN or vehicle overnight, followed by a 1-h exposure to LPS. MTT assay indicated that HN at 50-200 μM was not toxic to LPS-induced HT-29 cells compared with the control group not treated with LPS [Figure 6]. HN treatment decreased IL-8 levels in a concentration-dependent manner [Figure 7].

HN, an active flavone diglycoside, was isolated from the methanolic extract of the aerial parts of *C. japonicum* var. *ussuriense* and significantly suppressed NO production in LPS-induced macrophages. HN downregulated the levels of the pro-inflammatory cytokines, IL-1β and TNF-α, as well as the iNOS protein level, in LPS-induced RAW 264.7 cells. In addition, HN inhibited the production of IL-8 in LPS-induced HT-29 colonic epithelial cells. Cytokines play a key role in the regulation of the intestinal immune system.^[22,23] Improvement in research of the immunology of IBD and in bioengineering have led to new therapeutic concepts that target aspect of the inflammatory process.^[24] Especially, the blockade of TNF is the currently most efficacious therapeutic target for IBD. Therefore, these results indicated

that HN inhibits the production of inflammatory mediators and could be a candidate therapeutic against IBD.

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Financial support and sponsorship

Nil

Conflicts of interest

There are no conflicts of interest.

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