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Anti-inflammatory activity of hispidol A 25-methyl ether, a triterpenoid isolated from *Ponciri Immaturus Fructus*Eun Myoung Shin^a, Hong Yu Zhou^a, Guang Hua Xu^b, Seung Ho Lee^b, Irmgard Merfort^c, Yeong Shik Kim^{a,*}^a Natural Products Research Institute, College of Pharmacy, Seoul National University, 599 Gwanangno, Gwanak-gu, Seoul 151-742, Republic of Korea^b College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Republic of Korea^c Institute for Pharmaceutical Sciences, Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Stefan-Meier-Str. 19, Freiburg 79104, Germany

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ABSTRACT

The anti-inflammatory activity of hispidol A 25-methyl ether (hispidol A 25-Me ether), a triterpenoid isolated from *Ponciri Immaturus Fructus*, was studied in lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophages. It was revealed that hispidol A 25-Me ether dose-dependently inhibits nitric oxide (NO) production by down-regulating inducible nitric oxide synthase (iNOS). It also reduces prostaglandin E₂ (PGE₂) production by inhibiting cyclooxygenase-2 (COX-2) expression proven on both mRNA as well as on protein levels. In addition, hispidol A 25-Me ether inhibits mRNA expressions of major pro-inflammatory cytokines including the tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). Interestingly, hispidol A 25-Me ether probably exhibits a glucocorticoid-like activity, exerting functional inhibition of NF- κ B without inhibition of DNA binding as *de novo* synthesis of I κ B- α was induced and thereby NF- κ B activity was reduced. Furthermore, administrations of hispidol A 25-Me ether (1 and 10 mg/kg, *i.p.*, *v/w.*) were tested in two animal experiments involving acute inflammation, namely, the carrageenan-induced paw edema swelling test and the acetic acid-induced vascular permeability assay, and showed concentration-related inhibitory activities. The anti-inflammatory property of hispidol A 25-Me ether seems to resemble the effects of the class of naturally occurring anti-inflammatory agents, glucocorticoids, which inhibit transcriptions of important inflammatory mediators.

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1. Introduction

Inflammation is a crucial function of the innate immune system and it is necessary to protect the host against pathogens and initiate specific immunity. Upon bacterial invasion, mammalian monocytes/macrophages recognize lipopolysaccharides (LPS) of Gram-negative bacteria through the transmembrane signaling Toll-like receptor-4 (TLR-4), thus signaling the initiation of the innate host defense mechanism (Faure et al., 2000; Zhang and Ghosh, 2000). Instant cellular signal cascades cause monocytes to trigger the I κ B kinase (IKK)-NF- κ B pathway and the three mitogen-activated protein kinase (MAPK) pathways: extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) and p38 (Guha and Mackman, 2001; Zhang and Ghosh, 2000). These signaling pathways in turn activate a variety of transcription factors including NF- κ B (p50/p65) and AP-1 (c-Fos/c-Jun), which coordinate the induction of a range of inflammatory enzymes, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and IL-6. Acute inflammation is a rapid and securely self-terminating process that can, however, be harmful to the

host if subclinical inflammation survives and it is followed by the development of local chronic inflammation. Such inflammation provides a cellular micro-environment that favors malignant progressions such as tumor promotion (Balkwill et al., 2005).

In search for new treatments for inflammatory ailments, attention has turned back to natural products that have long been used clinically due to their proven safety over long periods of time. One such example is the herbal medicine Ji-Sil, made of dried immature fruits of *Poncirus trifoliata*. It has long been used in traditional medicine to treat human ailments such as gastritis, ulcers, and other inflammation-related diseases (Lee et al., 1996; Shin et al., 2006). In our continuing effort to evaluate the medicinal potentials of the singly isolated compounds of this herbal medicine, a novel triterpenoid, hispidol A 25-methyl ether (Xu et al., 2008), was studied that proved to inhibit LPS-induced inflammation in both the RAW264.7 macrophage cell line and in two animal experimental systems.

2. Materials and methods

2.1. Plant material

The premature fruits of *P. trifoliata* were purchased at Yangyeongsi, Daegu, South Korea and identified by Prof. Je-Hyun Lee,

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College of Oriental Medicine, Dongguk University, Gyeongju, South Korea. A voucher specimen was deposited at the College of Pharmacy, Yeungnam University, South Korea.

2.2. Isolation of hispidol A 25-methyl ether

A novel triterpenoid hispidol A 25-methyl ether (hispidol A 25-Me ether) was isolated and identified by comparison with spectral data (MS, ^1H and ^{13}C NMR), reported in the literature (Xu et al., 2008).

2.3. Animal, chemicals and reagents

Male ICR mice (Samtako, Osan, Korea) 4–5 weeks old and weighing 25–30 g were used in this study. All animal studies were carried out in a pathogen-free barrier zone of the Seoul National University Animal Laboratory, according to the procedures outlined in the Guide for the Care and Use of Laboratory Animals (Seoul National University, Korea). All animals were acclimated for at least one week, caged ten per group or fewer and fed a diet of animal chow and water *ad lib*. The animals were housed at 23 ± 0.5 °C and 10% humidity in a 12 h light–dark cycle. Unless otherwise indicated, all of the chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO). The antibodies against iNOS, COX-2, I- κ B α , and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4. Cell culture

RAW264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA). These cells were maintained at sub-confluence in a 95% air and 5% CO₂ humidified atmosphere at 37 °C. The medium used as the routine subculture was DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μ g/mL). The RAW264.7 cells harboring the pNF- κ B-secretory alkaline phosphatase (SEAP)-NPT reporter construct were cultured under the same conditions except that the media were supplemented with 500 μ g/ml geneticin (Invitrogen, Carlsbad, CA) (Moon et al., 2001).

2.5. Cell viability

The effect of the concentration of hispidol A 25-Me ether on cell viability was evaluated using a cell counting kit (CCK-8) purchased from Dojindo Laboratories (Tokyo, Japan), as previously described (Shin et al., 2008). Briefly, RAW264.7 cells were plated at a density of 1×10^4 per well in a 96-well plate and incubated at 37 °C for 24 h. The cells were treated with various concentrations of hispidol A 25-Me ether or a vehicle alone and were incubated at 37 °C for additional 24 h. After incubation, 10 μ l of CCK-8 solution was added to each well, and the plate was incubated under the same conditions for another 3 h. Water-soluble tetrazolium salt (WST8-[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disubstituted-phenyl)-2H-tetrazolium, monosodium salt]) in CCK-8 produces a water-soluble formazan dye upon reduction, and the resulting color was assayed at 450 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA).

2.6. Nitric oxide determination

Nitrite concentration in the medium was measured as an indicator of nitric oxide production according to the Griess method. In brief, RAW264.7 cells (1×10^5) were plated in 24-well plates, incubated for 24 h and pre-treated with the indicated concentrations of hispidol A 25-Me ether or a vehicle for another 2 h, then challenged with LPS (1 μ g/ml) for an additional 18 h. Equal volumes of culture medium and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) were mixed, and the absorbance was determined at 540 nm with a

microplate reader (Molecular Devices, Emax, Sunnyvale, CA). The absorption coefficient was calibrated using a sodium nitrite solution standard. For this experiment, 2-amino-5, 6-dihydro-6-methyl-4H-1,3-thiazine (AMT) was used as a positive control.

2.7. Semi-quantitative reverse transcriptase (RT-PCR)

To perform a semi-quantitative RT-PCR, 1 μ g of isolated total RNA (easy-BLUE™, iNtRON Biotech, Seongnam, Korea) was reverse-transcriptased to cDNA, employing an RT PreMix Kit (iNtRON Biotech) according to the manufacturer's instructions. The amount and purity of the RNA preparation were confirmed by measuring both the absorbance ratio at 260/280 nm and the 28S/18S intensity ratio in agarose gel electrophoresis. Two microliters of cDNA products was directly used in the PCR. The primers used for amplifications of the iNOS, COX-2, TNF- α , IL-1 β and GAPDH transcripts and the conditions for the amplifications were the same as previously described (Shin et al., 2008). *N-p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), an NF- κ B inhibitor was used as a positive control in this study. The amplified cDNA products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide. The gels were viewed using Doc-It® LS Image Analysis software (UVP Inc, Upland, CA) and quantified using UN-SCAN-IT™ software (Silk Scientific Corp, Orem, Utah). The PCR products were normalized to the amount of β -actin for each band.

2.8. Western immunoblot analysis

RAW264.7 cells were pre-treated with the indicated concentrations of hispidol A 25-Me ether or a vehicle for 2 h and stimulated with LPS (1 μ g/ml) for 18 h to detect β -actin, COX-2 and iNOS. Twenty micrograms of total protein extracts, except COX-2 (1 μ g), was separated on 10% SDS-PAGE, electro-transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany), blotted to each antibody (Santa Cruz, CA), and detected with WEST-SAVE Up™ luminol-based ECL reagent (ABFrontier, Seoul, Korea). The target bands were quantified using UN-SCAN-IT™ software (Silk Scientific Corp, Orem, Utah). TPCK, an inhibitor of NF- κ B was also used as a positive control.

2.9. Determination of PGE₂ production

The amount of PGE₂ produced from activated macrophages was quantified using an enzyme immunoassay (EIA) kit for PGE₂ (Cayman Chemical, Ann Arbor, MI). Briefly, 1×10^5 cells were seeded in 24-well plate and incubated for 24 h. After changing to new media, the cells were pre-treated with several concentrations of hispidol A 25-Me ether or a vehicle for 2 h and then activated by LPS (1 μ g/ml) to express COX-2 for an additional 18 h. These media were diluted 2 times with PBS and transferred to a PGE₂ antibody-coated 96-well culture plate in the EIA kit. Further treatment was according to the manufacturer's instructions. The produced PGE₂ in the specimen was quantified to determine COX-2 expression using a PGE₂ standard curve. Absorbance at 405 nm was recorded using a microplate reader (Molecular Devices, Emax, Sunnyvale, CA). For comparison, 50 μ M celecoxib (Celebrex®, Pfizer, NY) was used as a positive control.

2.10. Electrophoretic mobility shift assay (EMSA)

In order to check the inhibitory effect of hispidol A 25-Me ether on NF- κ B and AP-1 DNA binding, electrophoretic mobility shift assays (EMSA) were performed as described previously (Zabel et al., 1991). Briefly, nuclear extracts prepared from LPS-treated cells were incubated with the ³²P-end-labeled 22-mer double-stranded NF- κ B consensus oligonucleotide (Promega, Madison, WI) sequence: 5'-

AGT TGA GGG GAC TTT CCC AGG C-3' or the AP-1 consensus oligonucleotide (Promega) sequence: 5'-CGC TTG ATG AGT CAG CCG GAA-3' for 30 min at room temperature, and the DNA-protein complexes were separated from the free oligonucleotides on 6% native polyacrylamide gels. The signals obtained from the dried gel were quantified with an FLA-3000 apparatus (Fuji, Tokyo, Japan) using the BAS reader version 3.14 and Aida Version 3.22 software (Fuji-Raytest, Straubenhardt, Germany).

2.11. Carrageenan-induced paw edema test in mice

To test inhibitory effects of hispidol A 25-Me ether on acute inflammation in an animal model, paw edema was induced by subcutaneous injection of 0.05 ml of carrageenan (1%) into the right hind paw, as previously described (Shin et al., 2008). Control animals received identical treatment but with the vehicle, which, in this study, was 10% Tween 80 (10 ml/kg, *i.p.*) in saline. Thirty minutes prior to carrageenan administration, the animals received an *i.p.* injection of either of saline, dexamethasone (50 mg/kg) or hispidol A 25-Me ether (1 or 10 mg/kg), and the paw thickness was measured using a dial thickness gauge (No. 2046F, Mitutoyo, Kawasaki, Japan) before edema induction and every hour afterwards for 5 h. The percent increase of paw thickness was calculated based on the volume difference between the two paws with/without carrageenan injection.

2.12. Acetic acid-induced vascular permeability test in mice

A vascular permeability assay was conducted using a modified method originally described by Whittle (1964). Forty mice were divided into four groups. All samples were prepared in common vehicle, 10% Tween 80, 10 ml/kg. Thirty minutes after administration

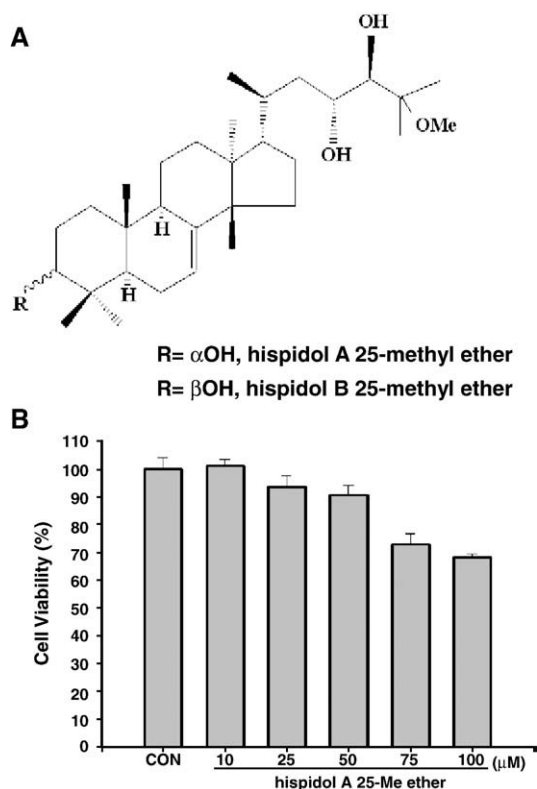


Fig. 1. Structure of hispidol A 25-Me ether (A). Effects of 25-methoxyhispidol A on cell viability (B) in RAW264.7 macrophages using a cell counting kit. The RAW264.7 cells were cultured with the indicated concentrations of hispidol A 25-Me ether or a vehicle at 37 °C in a 96-well plate for 24 h. Cell viability was evaluated as described in Materials and methods and is expressed as percent values compared to the vehicle control. Values are expressed as the mean \pm S.D. of three individual experiments. CON stands for vehicle control.

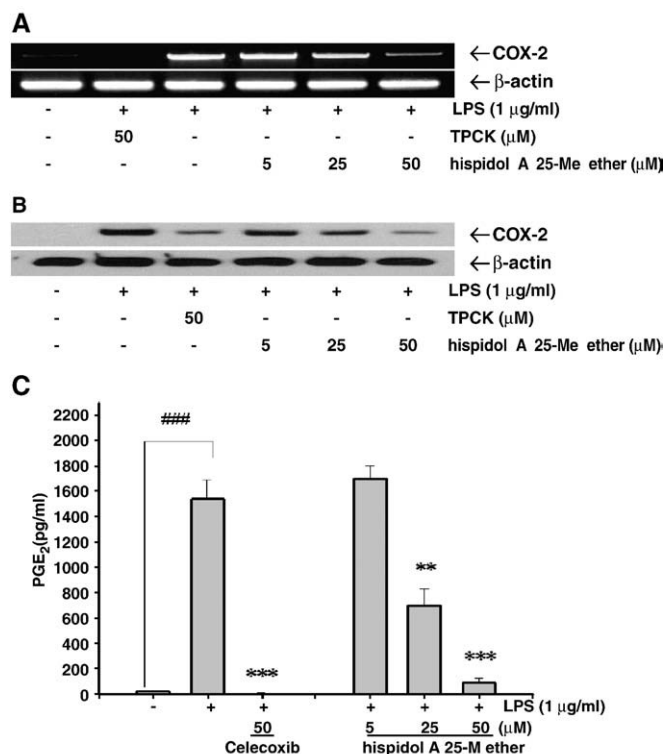


Fig. 2. Hispidol A 25-Me ether inhibits COX-2 in both mRNA and protein expression and in subsequent PGE₂ production in LPS-challenged RAW264.7 macrophages. Semi-quantitative RT-PCR (A), Western blot (B), and PGE₂ EIA (C) analysis, which were carried out as described in Materials and methods. Data were obtained from at least three independent experiments and are summarized expressed as mean \pm S.D. ** P < 0.01 and *** P < 0.001 indicate a significant difference from the LPS-challenged group. ### P < 0.001 stands for a significant difference from vehicle control. Con, Control; LPS, LPS-treated cells alone; TPCK, *N*-*p*-tosyl-L-phenylalanyl chloromethyl ketone at 50 μ M.

of *i.p.* injections (50 mg/kg dexamethasone or 1 mg/kg or 10 mg/kg hispidol A 25-Me ether), 10 ml/kg body weight of 1% Evans blue in normal saline was injected *i.v.* into the tail vein of each animal. After 60 min, 10 mg/kg of 0.6% acetic acid in normal saline was injected *i.p.* After 50 min, the mice were put down by cervical dislocation. Eight milliliters of normal saline was injected into the peritoneal cavity, and the washing solutions were collected in test tubes. To clear the turbidity caused by the protein, 80 μ l of 1 N NaOH solution was added to each tube, and the tubes were allowed to stand overnight at 4 °C. The solution was subjected to colorimetry using a UV/VIS JASCO V-550 spectrophotometer (Tokyo, Japan) at 610 nm. The vascular permeability effects were expressed according to the concentration of dye (μ g/ml) that had leaked into the peritoneal cavity.

2.13. Statistics

The results, unless otherwise stated, are summarized as mean \pm standard deviations (S.D.) from three independent experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test or Tukey's multiple comparison test was applied to assess the statistical significance of the differences among the study groups (SPSS version 10.0, Chicago, IL). A value of P < 0.05 was chosen as the criterion of statistical significance.

3. Results

3.1. Cell viability

The cytotoxic effects of hispidol A 25-Me ether are shown in Fig. 1B. No cytotoxic effect was observed up to a concentration of 50 μ M.

3.2. Hispidol A 25-Me ether inhibits COX-2 in both mRNA and protein expression and also inhibits subsequent PGE₂ production in LPS-stimulated RAW264.7 macrophages

Hispidol A 25-Me ether dose-dependently inhibited COX-2 in both mRNA and in subsequent protein expression levels. A significant decrease in PGE₂ expression was indicated when hispidol A 25-Me ether was used, which is in good agreement with the COX-2 inhibitory activity of hispidol A 25-Me ether in LPS-stimulated RAW264.7 macrophages. Celecoxib, a COX-2 inhibitor, at a concentration of 50 μM was also shown to have potent inhibitory activity on PGE₂ expression. TPCK at a concentration of 50 μM was used as a positive control to compare both the mRNA and protein expression (Fig. 2A and B). Hispidol A 25-Me ether was shown to down-regulate the production of PGE₂ to 54.9% at a concentration of 25 μM and to 94.2% at a concentration of 50 μM. Celecoxib, on the other hand, showed a 99.6% inhibition at 50 μM (Fig. 2C). The results indicated that hispidol A 25-Me ether can suppress COX-2 in LPS-stimulated RAW264.7 macrophages at the transcriptional and consequently on the translational level.

3.3. Hispidol A 25-Me ether suppresses iNOS in both mRNA and protein expression and in subsequent NO production in LPS-stimulated RAW264.7 macrophages

Hispidol A 25-Me ether showed a dose-dependent inhibitory activity when tested in the LPS-stimulated RAW264.7 macrophage system on iNOS both on mRNA and protein expression, in a concentration-

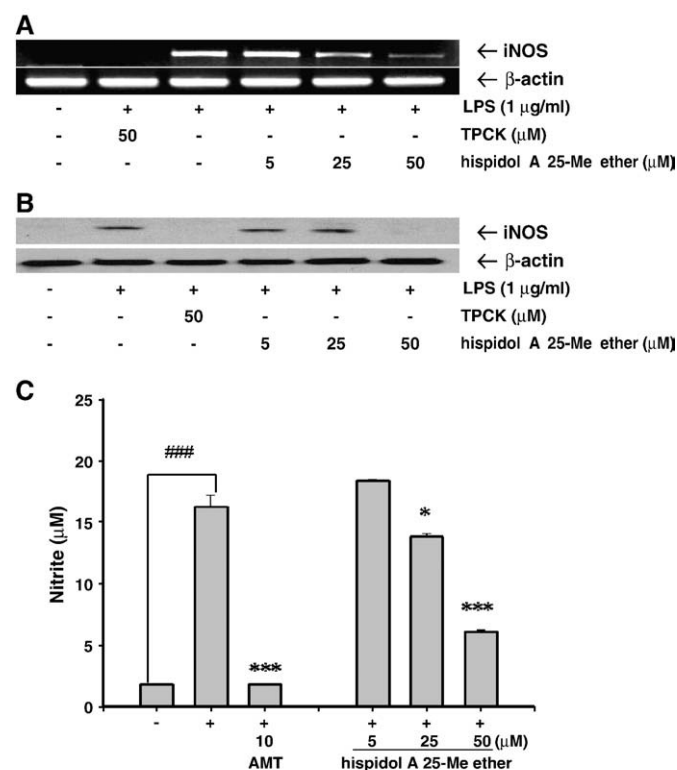


Fig. 3. Hispidol A 25-Me ether inhibits iNOS in both mRNA and protein expression and in subsequent NO in LPS-activated RAW264.7 macrophages. Semi-quantitative RT-PCR (A), Western blot (B), and Griess method (C) analyses were carried out as described in Materials and methods. Data were derived from three independent experiments and are summarized as mean ± S.D. **P* < 0.05 and ****P* < 0.001 indicate a significant difference from the LPS-challenged group. ###*P* < 0.001 means a significant difference from vehicle control group. Con, Control; LPS, LPS-treated alone; AMT, 2-amino-5, 6-dihydro-6-methyl-4H-1,3-thiazine; TPCK, *N*-*p*-tosyl-L-phenylalanyl chloromethyl ketone at 50 μM.

dependent manner, and these inhibitory activities were matched to the reduced NO production against the LPS challenge (Fig. 3). The NO inhibitory activity was 16.1% at 25 μM and 62.2% at 50 μM, but the NO inhibitor AMT showed a potent inhibition of 98.2% at 10 μM (Fig. 3C). From these results, it can be concluded that the decreased NO production originates from the suppressed iNOS mRNA and the later protein expression.

3.4. Hispidol A 25-Me ether inhibits pro-inflammatory cytokines IL-1β, IL-6 and TNF-α mRNA expressions in LPS-stimulated RAW264.7 macrophages

Pro-inflammatory cytokines enhance inflammation at locally inflamed tissues by maintaining the inflammatory process at various steps. Among the pro-inflammatory cytokines tested, hispidol A 25-Me ether most potently inhibited the expression of IL-1β, corresponding to 13.1% at 25 μM and 83.5% at 50 μM. For the positive control TPCK, a serine protease and NF-κB inhibitor, the inhibition was 98.1% for IL-6, 80% for TNF-α and 97.1% for IL-1β at the concentration of 50 μM (Fig. 4B).

3.5. Hispidol A 25-Me ether does not inhibit LPS-induced NF-κB and AP-1 nuclear translocation, but increases NF-κB and AP-1 DNA binding without LPS

To study whether the decrease in cytokine expression is due to inhibition of NF-κB and/or AP-1, EMSA was performed after preparation of nuclear extracts. Surprisingly, hispidol A 25-Me ether did not show any dose-dependent reduction of either NF-κB or AP-1 nuclear localization and consequently DNA binding (Fig. 5A and B). Parthenolide, a sesquiterpene lactone, was used as positive control and showed potent inhibition of both NF-κB and AP-1 DNA binding in the nucleus at 20 μM (Fig. 5A and B). In contrast, hispidol A 25-Me

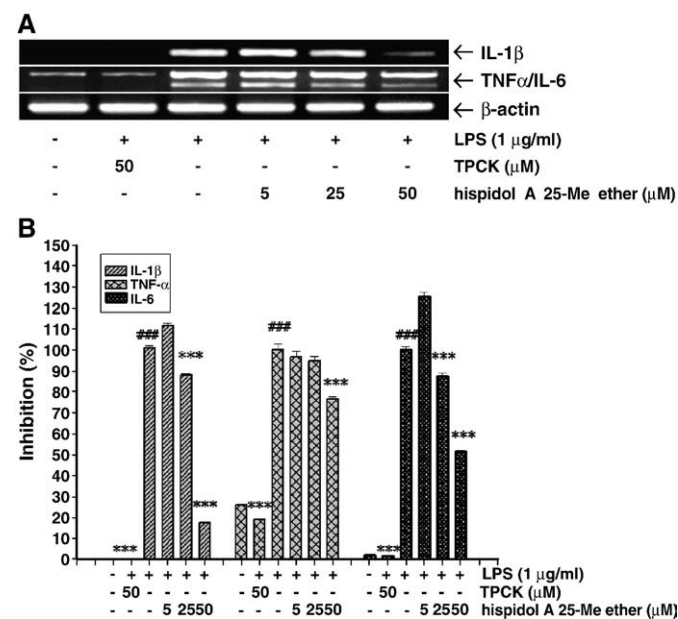


Fig. 4. Down-regulation of TNF-α, IL-1β, and IL-6 mRNA expression by hispidol A 25-Me ether in LPS-stimulated RAW264.7 macrophages. Two microliters of cDNA products was directly used in cDNA-based IL-1β, TNF-α, and IL-6 gene amplification (A), and the results are depicted as quantified bar graphs (B) as described in Materials and methods. Data are expressed as mean ± S.D. from three separate experiments. For quantification, the mRNA expression data were normalized to the β-actin signal. TPCK served as a positive control. ****P* < 0.001 indicates a significant difference from the LPS-treated group. ###*P* < 0.001 indicates a significant difference from the un-stimulated control group.

ether concentration dependently increased NF- κ B and AP-1 DNA binding in the absence of LPS (Fig. 5C).

3.6. Hispidol A 25-Me ether induces de novo I- κ B α synthesis in RAW264.7 macrophages

In order to figure out if there are any correlations between inhibition of I- κ B α phosphorylation and degradation (data not shown) and the missing inhibitory effect on NF- κ B DNA binding, hispidol A 25-Me ether was studied for its effect on de novo synthesis of I- κ B α in western immunoblot analysis. Glucocorticoids are known for its property. As shown in western blot (Fig. 5D) hispidol A 25-Me ether dose-dependently induced I- κ B α de novo synthesis and increased the I- κ B α expression level, and this was detected after 5 min of treatment with hispidol A 25-Me ether (Fig. 5D). This new synthesis may superimpose a simultaneous decrease of NF- κ B DNA binding.

3.7. Hispidol A 25-Me ether alleviates carrageenan-induced paw edema in mice

Carrageenan can induce local edema in mammals and be a sensitive method to test inhibitory activity of given compound in terms of acute inflammation in rodent animal systems. When tested, hispidol A 25-Me ether significantly inhibited the edema in a concentration-dependent manner. Interestingly, hispidol A 25-Me ether showed an inhibitory activity against carrageenan in early hours (Fig. 6). Furthermore, hispidol A 25-Me ether showed higher inhibitory activity in paw swelling than dexamethasone of 50 mg/kg ($P=0.022$, Tukey's). However, dexamethasone, having a half life of

36–54 h, showed continuous inhibitory activity even after 5 h of the carrageenan challenge. Hispidol A 25-Me ether seems to be metabolized more quickly 4 h after administration (Fig. 6).

3.8. Hispidol A 25-Me ether suppresses acetic acid-induced vascular permeability in mice

From the acetic acid-induced vascular permeability assay, we saw that two doses of hispidol A 25-Me ether (1 and 10 mg/kg) exert a dose-dependent inhibitory effect against acute inflammation induced by acetic acid. The inhibition rates of dye leakage were 37.7% at 1 mg/kg and 57.5% at 10 mg/kg (Fig. 7), and dexamethasone at a dosage of 50 mg/kg showed a 49.7% inhibitory activity.

4. Discussion

The immature fruits of *P. trifoliata* are widely used in traditional medicine as a remedy for allergic inflammation (Park et al., 2005; Shin et al., 2006), gastritis and dysentery (Lee et al., 2005). To date, more biological activities such as cancer inhibition and apoptosis induction are known effects of the extracts or their single components (Hong et al., 2008; Jayaprakasha et al., 2007; Pokharel et al., 2006a,b; Yi et al., 2004). In addition, anti-inflammatory (Kim et al., 2007; Shin et al., 2006; Tsai et al., 1999; Zhou et al., 2007), anti-*Helicobacter pylori* (Kim et al., 1999) and anti-anaphylactic activities (Lee et al., 1996) (Park et al., 2005; Pokharel et al., 2006a,b) have been reported up to now. The major effective principle of the species has been reported to be poncirin, a flavonoid, which has anti-inflammatory activity due to NF- κ B inhibition (Kim et al., 2007). More recently, the anti-inflammatory activities of 21 (α,β)-methylmelianodiols, forms of novel triterpenoids, were reported

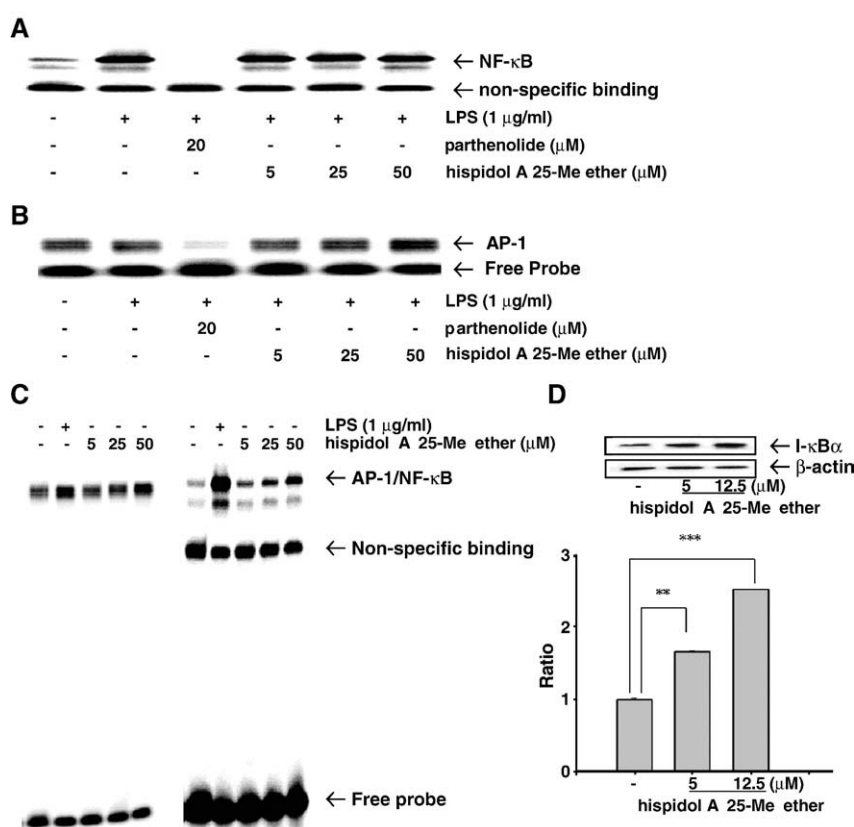


Fig. 5. Hispidol A 25-Me ether does not inhibit LPS-induced NF- κ B and AP-1 DNA binding but concentration dependently increases NF- κ B and AP-1 DNA binding and induces I- κ B α de novo synthesis in the absence of LPS. EMSA was conducted as described in Materials and methods. LPS-induced NF- κ B (A) and AP-1 (B) DNA binding and AP-1 and NF- κ B (C) DNA binding without LPS was checked using five micrograms of nuclear extracts incubated with [32 P]-labeled respective oligonucleotides. Parthenolide at a concentration of 20 μ M was used as a positive control (A and B). Western blotting was performed to determine I- κ B α de novo synthesis (D). I- κ B α normalized to β -actin signal of each treatment is expressed in relative ratio (%) as mean \pm S.D. of three individual experiments. ** $P<0.01$ and *** $P<0.001$ indicate significant differences from the control group.

under the conditions of LPS-stimulated RAW264.7 macrophages by our group (Zhou et al., 2007). To continue the search for effective anti-inflammatory compounds, other isomers, namely, hispidol A and B 25-Me ether were isolated from the fruits of *P. trifoliata* and investigated for their anti-inflammatory potencies against LPS challenge. From this result, it was revealed that hispidol A 25-Me ether potently decreased the iNOS and COX-2 protein expression, regardless of pre- or post-treatment (Supplementary Fig. 1). In addition, the down-regulated iNOS and COX-2 expressions in both mRNA and protein were followed by a decrease of NO and PGE₂ production. PGE₂ is known to increase vascular permeability and edema in locally inflamed tissue.

Other important inflammatory mediators produced by macrophages are pro-inflammatory cytokines such as IL-1, IL-6 and TNF- α . Pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α are effectively down-regulated by hispidol A 25-Me ether in LPS-stimulated RAW264.7 macrophages. In this study, hispidol A 25-Me ether showed a most pronounced inhibitory effect on IL-1 β mRNA expression, followed by the inhibitory effects on IL-6, whereas TNF- α was only slightly reduced in the LPS-stimulated RAW264.7 macrophages.

The anti-inflammatory effect of hispidol A 25-Me ether was further studied in two rodent acute inflammation models. Reportedly, carrageenan-induced paw edema is a highly sensitive tool to evaluate the efficacy of acute inflammation (Lallouette et al., 1970; Morris, 2003). Decreased carrageenan-induced paw swelling by reduced levels of IL-8, IL-1 β and TNF- α , NO via iNOS inhibition, and PGE₂ via COX-2 inhibition have all been well documented (Park et al., 2006, 2007). In the present study, hispidol A 25-Me ether showed a dose-dependent inhibitory activity in carrageenan-induced paw edema. The acetic acid-induced vascular permeability assay, as another acute inflammation animal model, was tested to further investigate the compound's inhibitory activity. Hispidol A 25-Me ether successfully decreased the acetic acid-induced Evans blue dye leakage in a dose-dependent manner.

In this study, the mode of action, especially the inhibitory activity of hispidol A 25-Me ether on NF- κ B action, was the primary focus. From the NF- κ B-SEAP reporter gene assay, hispidol A 25-Me ether showed a dose-dependent inhibitory activity on NF- κ B-SEAP transcription (data not shown). However, hispidol A 25-Me ether was revealed not to inhibit either NF- κ B or AP-1 probe binding to target DNA in the EMSA when studied with ³³P-labeled consensus NF- κ B and AP-1 probes. Moreover, hispidol A 25-Me ether induced dose-dependently NF- κ B and AP-1 probe binding to target genes (Fig. 5C). Hispidol A 25-Me ether also induced dose-dependently I- κ B α production at early times of treatment, such as 5 min. To explain such a behavior, a glucocorticoid-like mode of action can be assumed

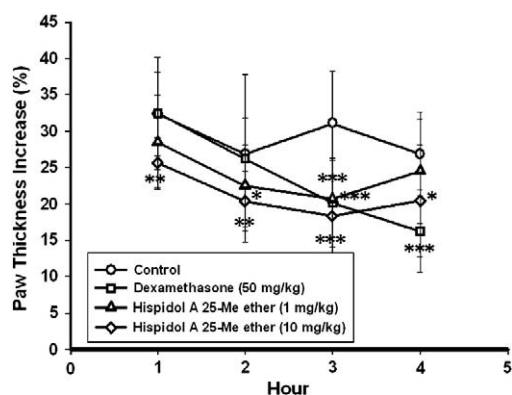


Fig. 6. Inhibitory effects of hispidol A 25-Me ether on carrageenan-induced paw edema in mice. Paw edema was induced as described in Materials and methods. Each point represents the percent increase of paw thickness, which was calculated on the thickness of the paw before carrageenan injection. The values are presented as mean \pm S.D. of 10 mice per group. * P < 0.05, ** P < 0.01, and *** P < 0.001 indicate significant differences from the vehicle control group.

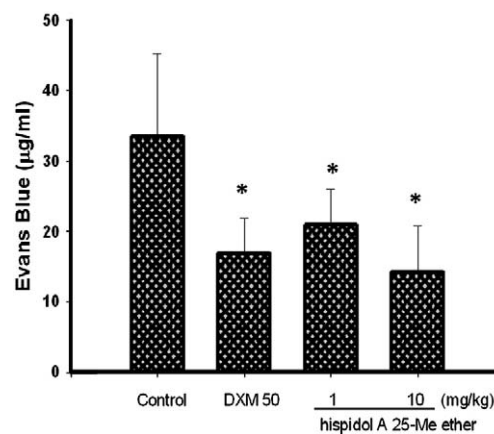


Fig. 7. Effect of hispidol A 25-Me ether on acetic acid-induced vascular permeability in mice. Vascular permeability assays were conducted as described in Materials and methods. The values of the relative ratios (%) are expressed as mean \pm S.D. (n = 10). * P < 0.05 indicates significant differences from the vehicle control group.

(Park et al., 2004; Yasuda et al., 2005). Glucocorticoids exert unique anti-inflammatory activity through two well-defined mechanisms: transactivation and transrepression. Transactivation refers to the direct induction property of anti-inflammatory genes for the benefit of the host, whereas, transrepression refers to the inhibitory activity on transcription factors, such as AP-1 and NF- κ B, by binding to GRE or GRE-like sequences in the promoter regions of the target genes (Chang et al., 1997; Sakurai et al., 1997; Sorrells and Sapolsky, 2007; van der Burg et al., 1997; Xu et al., 2001). Moreover, glucocorticoids induce I- κ B α expression, reduce phosphorylation at p65 and influence p300, by which the transactivating activity is reduced. In doing so, they still bind to the promoter regions of various genes. In summary, glucocorticoids target the NF- κ B pathway at different sites. Although further investigation is required to clarify the more detailed mechanism of hispidol A 25-Me ether activity, hispidol A 25-Me ether from *Ponciri Immaturus Fructus* can be regarded as a promising candidate for treating various inflammatory diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.10.036.

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