Anti-inflammatory effects of methanol extract from *Peperomia dindygulensis* Miq. mediated by HO-1 in LPS-induced RAW 264.7 cells

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Abstract. Inflammation serves as a multifaceted defense mechanism activated by pathogens, cellular damage and irritants, aiming to eliminate primary causes of injury and promote tissue repair. *Peperomia dindygulensis* Miq. (*P. dindygulensis*), prevalent in Vietnam and southern China, has a history of traditional use for treating cough, fever and asthma. Previous studies on its phytochemicals have shown their potential as anti-inflammatory agents, yet underlying mechanisms remain to be elucidated. The present study investigated the regulatory effects of *P. dindygulensis* on the anti-inflammatory pathways. The methanol extracts of *P. dindygulensis* (PDME) were found to inhibit nitric oxide (NO) production and induce heme oxygenase-1 (HO-1) expression in murine macrophages. While MAPKs inhibitors, such as SP600125, SB203580 and U0126 did not regulate HO-1 expression, the treatment of cycloheximide, a translation inhibitor, reduced HO-1. Furthermore, PDME inhibited lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and TNF-α expression at both the mRNA and protein levels. The activity of NOS and the expression of TNF-α, iNOS and COX-2 decreased in LPS-stimulated Raw 264.7 cells treated with PDME and this effect was regulated by inhibition of HO-1 activity. These findings suggested that PDME functions as an HO-1 inducer and serves as an effective natural anti-inflammatory agent in LPS-induced inflammation.

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Introduction

Inflammation is a complex biological defense system activated by a pathogen, damaged cells and irritants (1,2). The inflammatory response eliminates the initial cause of cell damage, necrosis of cells and tissues damaged by the inflammatory process and initiates tissue repair (3). The inflammatory process is regulated by various cell types, including macrophages, neutrophils, eosinophils and mononuclear phagocytes (4,5). Macrophages are associated with the initiation and maintenance of inflammation, the presentation of antigens and the production of cytokines and growth factors (6). Therefore, the regulation of macrophages is essential for controlling the overall immune response.

Heme oxygenase 1 (HO-1) is an enzyme responsible for degrading heme into carbon monoxide, biliverdin and iron, playing crucial roles as a tissue homeostatic regulator, immune function regulator and inflammatory attenuator (7-9). Multiple isozymes of HO-1 and HO-2 have been identified, each encoded by different gene (10). While HO-2 is predominantly expressed in the brain and testicles and remains unstimulated by receptors or metabolism (10-12), HO-1, characterized by low basal expression levels in most cells and tissues, undergoes marked elevation in response to heme substrate and various stress such as UV light, lipopolysaccharides (LPS) or hydrogen peroxide (13,14). Carbon monoxide produced by HO-1 plays an anti-inflammatory role by inhibiting the secretion of LPS-induced inflammatory cytokines including TNF-α, interleukin (IL)-1β and IL-6. Additionally, it induces IL-10, which is known for its anti-inflammatory properties, in macrophages (15-18). When

exposed to damage and injury, tissues activate macrophages and produce prostaglandin E2 (PGE2) via cyclooxygenase-2 (COX-2), and nitric oxide (NO) via inducible nitric oxide synthase (iNOS) (19). Increased HO-1 expression inhibits LPS-mediated expression of COX-2 and iNOS, thereby inhibiting PGE2 and NO production (20,21). Although it has been reported that HO-1 induces NO during cellular senescence, the induction of HO-1 inhibits iNOS to reduce NO by oxidative injury (22). CO and biliverdin, the products of HO-1, are also known to inhibit iNOS (23-25). Therefore, controlling HO-1 expression is pivotal in modulating anti-inflammatory responses.

Peperomia dindygulensis Miq. (*P. dindygulensis*), a commonly found herb in southern China, has been used in folk medicine to address various ailments, including cough, asthma, phthisis and a range of cancers, such as stomach, lung, breast and liver cancer (26). The ethanol extract of *P. dindygulensis* repressed the growth of the lung cancer cell lines A549 and Lovo (5). Some compounds found in the extract demonstrate activity that hampers the growth of liver cancer cells (27). Moreover, *P. dindygulensis* ethanol extracts have demonstrated the capacity to inhibit cell growth and impede angiogenesis at specific concentrations in human umbilical vein endothelial cells (HUVECs) (28,29). No studies, to the best of the authors' knowledge, have explored the regulatory effects of *P. dindygulensis* methanol extract (PDME) on inflammatory responses in raw 264.7 cells.

The present study demonstrated that PDME acts as an anti-inflammatory effector in macrophages. This extract diminished NOS activity and inflammatory factor expression, such as iNOS, Cox-2 and TNF-α, in LPS-induced macrophages. The anti-inflammatory effects of PDME are contingent upon the HO-1 translation level. Based on these results, a new role was identified for PDME in inducing anti-inflammatory reactions through the regulation of the expression of HO-1.

Materials and methods

Cell culture. RAW 264.7 and U937 cells were purchased from the American Type Culture Collection (cat. nos. TIB-71 and CRL-1593.2). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Welgene, Inc.; cat. no. LM 001-05) containing 10% fetal bovine serum (GenDEPOT, LLC; cat. no. F0900-050) and 1% penicillin/streptomycin (Lonza Group, Ltd.; cat. no. 17-602E). U937 cells (30,31) were maintained in Dulbecco's modified Eagle's medium (Welgene, Inc.; cat. no. LM 001-05) containing 10% fetal bovine serum (GenDEPOT, LLC; cat. no. F0900-050), 1% penicillin/streptomycin (Lonza Group, Ltd.; cat. no. 17-602E) and 1% beta-mercaptoethanol (cat. no. MER002; BioShop Canada, Inc.). After exposure to 10 ng/ml PMA (MilliporeSigma; cat. no. p8139) for 24 h and the U937 cells underwent differentiation and were washed to eliminate remaining PMA. HUVECs were purchased from PromoCell GmbH. HUVECs were maintained in medium M199 (MilliporeSigma; cat. no. M4530) containing 20% fetal bovine serum, 30 μg/ml ECGS (Corning, Inc.; cat. no. 306006) and 100 μg/ml heparin (MilliporeSigma; cat. no. H3149). Cells were incubated in a humidified 5% CO_2 atmosphere at 37˚C incubator.

Plant material. *P. dindygulensis* was obtained from the Thang Loi community, located in the Ha Lang district of Cao Bang Province, Vietnam, in July 2015. It was identified by Dr Tran The Bach, from the Institute of Ecology and Biological Resources in Hanoi, Vietnam. Voucher specimens labelled as VK 6535 were archived in the herbarium of the Korea Research Institute of Bioscience and Biotechnology. The dried whole plant (50 g) was pulverized and extracted with methanol (500 ml; HPLC grade) using an ultrasonic extractor (SDN-900H; Sungdong Ultrasonic Co., Ltd.). The ultrasonic extraction procedure was systematically conducted over 30 cycles, with each cycle comprising a 15 min extraction phase followed by a 120 min standby period to optimize extraction efficiency and prevent temperature elevation effects. After the *P. dindygulensis* methanol extract was filtered, it was then concentrated under reduced pressure, yielding 2.8 g of extract, which corresponds to a 5.6% yield. The methanol extract of *P. dindygulensis* was supplied by the International Biological Material Research Center (cat. no. FBM259-078).

Reagents. SB203580 (cat. no. 559389), SP600125 (cat. no. 420119), U0126 (cat. no. U120), Cycloheximide (cat. no. 01810) and actinomycin D (cat. no. A1410) were purchased from MilliporeSigma. IX (ZnPP; cat. no. sc-200329) was purchased from Santa Cruz Biotechnology, Inc.

NOS assay. RAW 264.7 cells were seeded at a density of $1x10^5$/well in a 96-well plate and incubated for 24 h at 37˚C. Cells were treated with PDME (10 μg/ml) or ZnPP (10 μM) at the indicated concentration and time, followed by treatment with LPS (1 μg/ml). After a 24 h-incubation at 37˚C, 70 μl of the medium from each well was transferred to a new 96-well plate, mixed with 50 μl of Griess reagent (40 μg/μl; MilliporeSigma; cat. no. G4410) and incubated in a foil-wrapped plate for 15 min at room temperature. Absorbance was measured at 540 nm using Spark (Tecan Group, Ltd.). The amount of NO produced was calculated by using a standard curve.

MTT assay. RAW 264.7 cells were seeded at a density of $1x10^5$/well in a 96-well plate. HUVECs were seeded at a density of $0.5x10^4$/well in a 96-well plate. The cells were incubated for 24 h at 37˚C. PDME treatment was administered at the indicated concentrations and times. Then, 20 μl of MTT reagents (Merck KGaA; cat. no. 475989) was added per 96-well plate, with reagents added to designated wells as a blank. After incubation for 3.5 h at 37˚C, 100 μl DMSO solvent was added to each well. The foil-wrapped plate was incubated for 15 min at room temperature on the shaker, and then the absorbance was measured at 590 nm using a SPARK microplate reader (Tecan Group, Ltd.).

Western blot analysis and antibodies. For western blotting, proteins were extracted from cells lysed in lysis buffer [50 mM Tris (pH 7.4), 150 nM NaCl, 1% NP-40, 1 mM EDTA, protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Inc.; cat. no. 5872S)] for 30 min on ice and centrifuged at 21,000 x g for 15 min at 4˚C. Protein quantification was performed using the Bradford reagent (Bio-Rad Laboratories, Inc.; cat. no. 5000006) using a microplate reader (Bio-Rad Laboratories, Inc.; model 680) and calculated using standard

curves. The cell lysate (25 μg/lane) was loaded onto an 8-12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.; cat. no. 1620177). The membrane was blocked with a 5% blocking reagent (GenomicBase; cat. no. SKI400) for 1 h at room temperature and then incubated with the specific primary antibody overnight at 4˚C. The next day, the membrane was washed with PBS-T (0.2% Tween 20) and incubated with the secondary antibody for 3 h at room temperature. After incubation, the blot was washed with PBS-T and developed with the Clarity ECL Substrate Kit (Bio-Rad Laboratories, Inc.; cat. no. 1705061) using KwikQuant Pro Imager (Kindle Biosciences, LLC; cat. no. D1010). To western blot using other antibody on the same membrane, the membrane was incubated with stripping buffer [2% SDS; 50 mM Tris (pH 6.8), 0.7% β-mercaptoethanol] for 10 min at 55˚C. After incubation, the membrane was washed with PBS-T and every step repeated from blocking to detection. Phosphorylated (p-)JNK, p-p38 and p-ERK1/2 levels were semi-quantified using ImageJ software (version 1.53n; National Institutes of Health) and normalized to the intensity of JNK, p38 and ERK1/2.

The antibodies used were: HO-1 (1:500; cat. no. sc-390991), β-actin (1:2,000; cat. no. sc-47778), p-JNK (1:1,000; cat. no. sc-6254), JNK (1:1,000; cat. no. sc-7345), p-p38 (1:1,000; cat. no. sc-166182), p38 (1:1,000; cat. no. sc-7972), p-ERK1/2 (1:1,000; cat. no. sc-7383), ERK1/2 (1:1,000; cat. no. sc-514302), α-tubulin (1:1,000; cat. no. sc-58666), iNOS (1:1,000; cat. no. sc-7271) and COX-2 (1:1,000; cat. no. sc-514489). All antibodies were anti-mouse IgG and purchased from Santa Cruz Biotechnology, Inc. The mouse secondary antibody (HRP-linked antibody) was purchased from Cell Signaling Technology, Inc. (1:2,000; cat. no. 7076S).

Reverse transcription-quantitative (RT-q) PCR. The cells were seeded at a density of $1x10^4$ in a 6-cm plate and incubated for 24 h at 37˚C. Cells were treated with PDME (10 μg/ml) or ZnPP (10 μM) for 1 h at 37˚C, followed by treatment with LPS (1 μg/ml) for 24 h at 37˚C. Total RNA for analysis was obtained using NucleoSpin RNA (Macherey-Nagel; cat. no. 740955.250) according to the manufacturer's instructions. RNA (1 μg) was used for complementary DNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.; cat. no. BR-170-8891). The reverse transcription reaction was performed at 37˚C for 1 h and then terminated at 95˚C for 5 min. RT-PCR analysis was performed with cDNA and specific gene primers using IQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.; cat. no. BR1708882). Primers sequence for RT-qPCR were; HO-1, 5'-TGAACACTCTGGAGATGACA-3' (sense) and 5'-AACAGGAAGCTGAGAGTGAG-3' (antisense); TNF-a, 5'-CAGGAGGGAGAACAGAAACTCCA-3' (sense) and 5'-CCTGGTTGGCTGCTTGCTT-3' (antisense); iNOS, 5'-TGCATGGACCAGTATAAGGCAAGC-3' (sense) and 5'-CTCCTGCCCACTGAGTTCGTC-3' (antisense); COX-2, 5'-CCACTTCAAGGGAGTCTGGA-3' (sense) and 5'-AGTCATCTGCTACGGGAGGA-3' (antisense); GAPDH, 5'-CATCACTGCCACCCAGAAGACTG-3' (sense) and 5'-ATGCCAGTGAGCTTCCCGTTCAG-3' (antisense). Pre-denaturation step was conducted at 95˚C for 3 min, followed by denaturation and extension steps at 95˚C for 15 sec and 60˚C for 1 min, respectively, repeated for 40 cycles in a Rotor-Gene Q (Qiagen GmbH). RT-qPCR experiments were repeated three times independently.The RNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (32).

TNF-α ELISA. To measure the secretion level of TNF-α, Mouse TNF-α Quantikine ELISA Kit was used (R&D Systems, Inc.; cat. no. MTA00B) according to the manufacturer's protocol. First, the standard reagent was prepared and the cultured medium obtained from control, LPS-treatment and LPS with PDME treatment cells. Diluent RD1-63 (50 μl) was added to each well and then 50 μl of standard or sample medium added per well. The plate was gently tapped to mix and incubated at room temperature for 2 h with adhesive strips covering. Each well was washed four times with 400 μl wash buffer. The wash buffer was completely removed and 100 μl Mouse TNF-α conjugate added to each well. The wells were incubated at room temperature for 2 h while covered with adhesive strips. After incubation, each well was washed four times with 400 μl wash buffer. Substrate solution (100 μl) was added to each well and the foil-wrapped plates were incubated for 30 min at room temperature. Stop solution (100 μl) was added to each well and gently tapped to mix thoroughly. Absorbance was measured at 450 nm using Spark (Tecan Group, Ltd.).

Statistical analysis. Statistical analyses were conducted using GraphPad Prism version 7.04 (Dotmatics). For multiple comparisons, data were subjected to one-way ANOVA followed by the Bonferroni post hoc test to generate adjusted P-values. For comparisons between two groups, data were analyzed using an unpaired two-tailed Student's t-test. Error bars in all graphs represent the means of SEM. P-value was denoted with symbols (*P<0.05, **P<0.005, ***P<0.0005 and 'n.s' indicating no significance). All statistical analyses were performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibition of NOS activity by PDME in murine macrophages. To evaluate the anti-inflammatory properties of PDME, the present study conducted a NOS activity assay in Raw264.7 cells. LPS stimulation increased NOS activity. However, the combination of PDME and LPS resulted in a dose-dependent reduction in NOS activity (Fig. 1A). Cell viability decreased only in cells treated with 20 μg/ml PDME, with no changes observed at other concentrations (Fig. 1B). Cell cytotoxicity was measured in HUVECs following treatment with the PDME, as there have been reports indicating its impact on endothelial cell viability (29). However, it was confirmed that there was no cellular toxicity when treated with a concentration of 10 μg/ml for 24 h, indicating no impairment in endothelial cell function at the concentration demonstrating anti-inflammatory activity (Fig. S1). Collectively, these findings suggested that PDME exerted anti-inflammatory effects on LPS-stimulated Raw264.7 cells.

PDME induces HO-1 expression. HO-1 induces anti-inflammatory reactions in macrophages (21). Therefore, the present study tested whether PDME induced HO-1 expression in Raw264.7 cells. The expression of HO-1 gradually increased

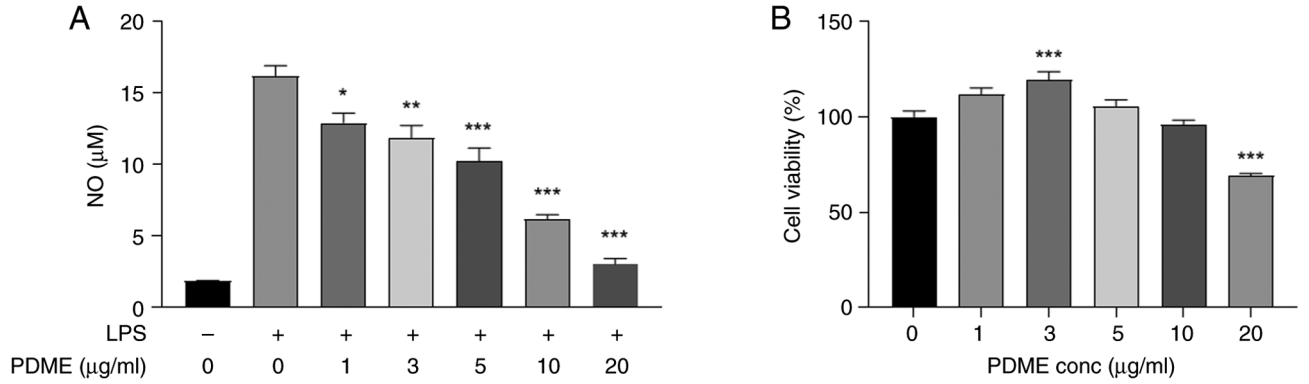

Figure 1. PDME reduce NOS activity in Raw246.7 cells. (A) NOS activity was measured in PDME-treated 264.7 cells at concentrations from 0 to 20 μg/ml for 1 h and then LPS (1 μg/ml) treatment for 24 h. For all controls, the solution used for extraction and dilution was treated in equal amounts. Data are presented with the mean ± SEM and statistically analyzed using one-way ANOVA; *P<0.05, **P<0.005, ***P<0.0005, vs. LPS only treated samples. (B) Cell viability was evaluated in PDME-treated cells at concentrations from 0-10 μg/ml for 24 h. Data are presented as the mean ± SEM and statistically analyzed using one-way ANOVA; ***P<0.0005, vs. PDME 0 μg/ml. PDME, *P. dindygulensis* methanol extracts; NOS, nitric oxide synthase; LPS, lipopolysaccharides.

with increasing treatment doses of PDME from 1-10 μg/ml over 24 h (Fig. 2A). Time course experiments conducted with 10 μg/ml PDME showed an increasing HO-1 protein level 1 h post-treatment, followed by a steady increase in expression up to 24 h (Fig. 2B). Furthermore, when human monocytic leukemia cells U937 were differentiated and treated with 10 μg/ml of PDME at various time points or with PDME ranging from 1-10 μg/ml for 24 h, the expression of HO-1 increased similarly to the previous results (Fig. S2A and B). These results indicated that PDME modulated HO-1 expression in murine macrophages and human monocytic leukemia cells.

Regulation of HO-1 expression by PDME at the translation level. The expression of HO-1 is primarily controlled at the transcriptional level through the MAPK signaling pathways, including JNK, ERK and p38 kinase (33). Therefore, the present study investigated the mRNA levels of HO-1 in a time- and dose-dependent manner upon PDME treatment in Raw264.7 cells. Notably, PDME treatment did not alter HO-1 mRNA expression (Fig. 3A and B). To determine whether the inhibition of MAPKs regulates the PDME-induced increase in HO-1 expression, Raw264.7 cells were treated with specific inhibitors: JNK inhibitor SP600125, p38 inhibitor SB203580, or MEK inhibitor U0126 (Fig. 3C). Treatment with these inhibitors and PDME partly affected HO-1 expression. In addition, PDME treatment did not affect the expression or activity of JNK, p38 and ERK1/2 (Fig. 3D and E). Furthermore, the present study examined whether PDME-induced changes in HO-1 expression occurred at the transcriptional or translational level by co-treating cells with actinomycin D (ActD) and cycloheximide (CHX) (Fig. 3F). While the expression of HO-1 remained unaffected when the transcription inhibitor ActD was administered, PDME-induced HO-1 expression was diminished when the translation inhibitor CHX was used. Collectively, these findings indicated that PDME specifically regulates HO-1 expression at the translational level.

PDME suppresses the expression of iNOS, COX-2 and TNF-α induced by LPS. The present study investigated the effect of PDME treatment on the expression levels of iNOS, COX-2 and TNF-α stimulated by LPS. The combination of PDME

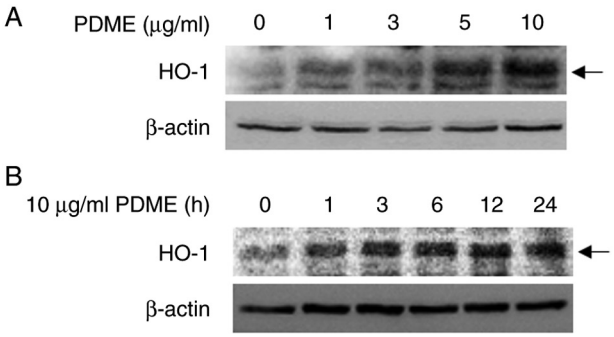

Figure 2. PDME induces HO-1 protein expression in dose- and time-dependent manner. (A) RAW264.7 cells were treated with PDME at concentrations ranging from 0-10 μg/ml for 24 h, followed by determination of HO-1 protein expression using western blotting. (B) The cells were treated with PDME (10 μg/ml) for time from 0-24 h. Next, HO-1 protein expression determined using western blotting. For all controls, the solution used for extraction and dilution was treated in equal amounts. PDME, *P. dindygulensis* methanol extracts; HO-1, heme oxygenase 1.

with LPS for 24 h resulted in a notable decrease in the mRNA expression of iNOS, COX-2 and TNF-α in Raw 264.7 cells (Fig. 4A). Furthermore, LPS-induced protein expression of iNOS and COX-2 was attenuated following PDME treatment for either 24 h (Fig. 4B) or 6 h (Fig. S3). In addition, even in U937 cells, the expression of iNOS and COX-2 stimulated by LPS was reduced with PDME treatment (Fig. S2C). The secretion of TNF-α, which was increased by LPS, is also decreased by treatment with 10 μg/ml PDME for 24 h (Fig. 4C). These findings demonstrated that PDME effectively reduced both mRNA and protein expression levels of iNOS, COX-2 and TNF-α induced by LPS treatment.

Inhibition of HO-1 activity suppresses the anti-inflammatory effect stimulated by PDME. In RAW 264.7, PDME demonstrated its anti-inflammatory effect by upregulating HO-1 expression. To establish whether the anti-inflammatory function induced by PDME was mediated by HO-1, the inflammatory response following treatment with ZnPP, a specific inhibitor of HO-1 was assessed. The increase in NO levels induced by LPS decreased when PDME was administered concurrently,

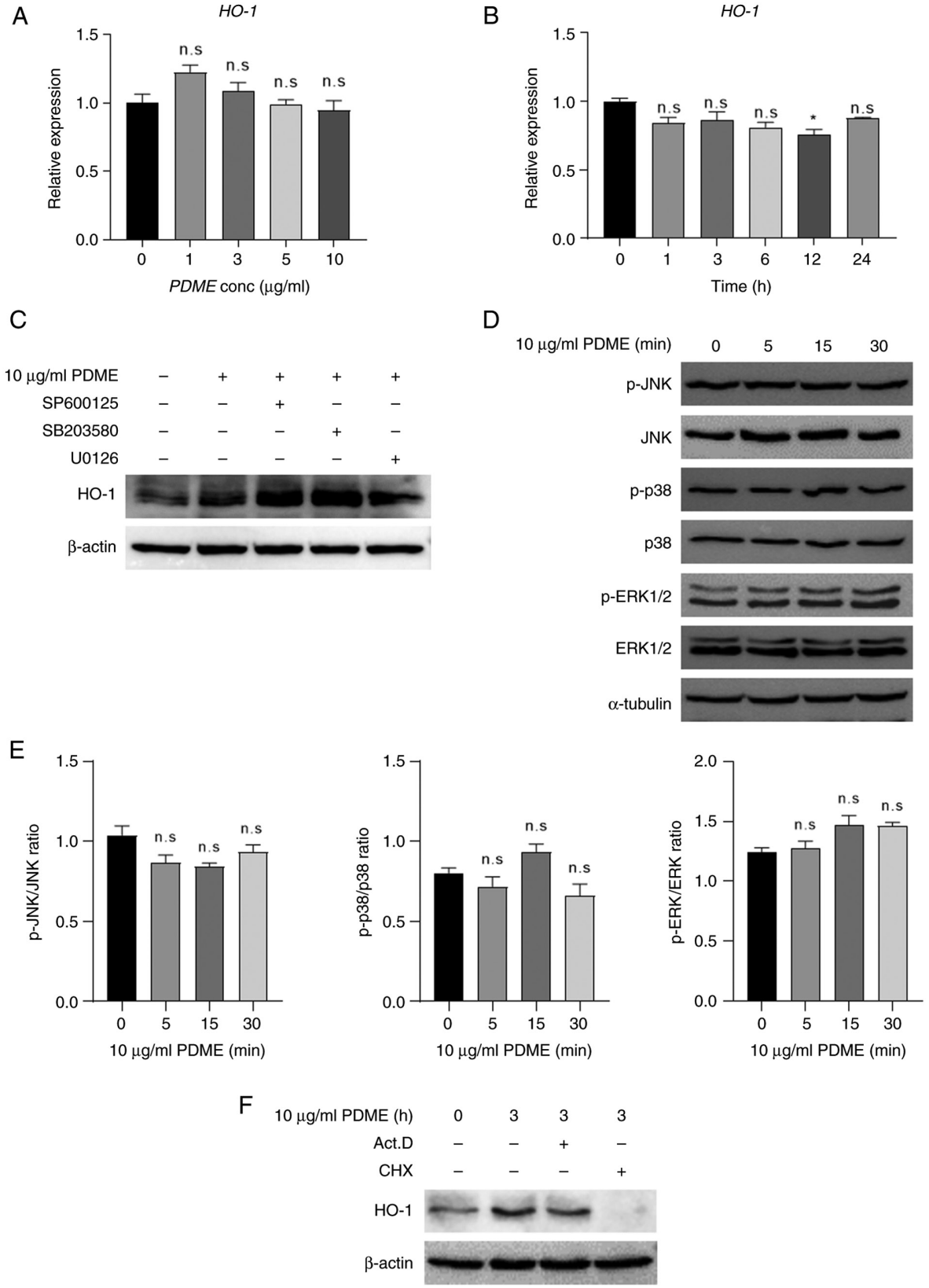

Figure 3. PDME regulate HO-1 expression at the translation level. (A) The RNA expression of HO-1 was evaluated in PDME-treated cells with concentrations from 0-10 μg/ml for 24 h and (B) PDME (10 μg/ml)-treated cells with time from 0-24 h. For all controls, the solution used for extraction and dilution was treated in equal amounts. Data are presented as the mean ± SEM and statistically analyzed using one-way ANOVA; n.s, not significant and *P<0.05 vs. control. (C) RAW246.7 cells were pre-treated with SP600125 (JNK inhibitor, 10 μM), SB203580 (p38 inhibitor, 10 μM), or U0126 (MEK inhibitor, 10 μM) for 30 min and treated with PBVN14063 (10 μg/ml) for 24 h. Subsequently, HO-1 protein expression was determined by western blotting using total lysate. (D) The cells were treated with PDME (10 μg/ml) for the indicated time (0-30 min), and the expression and phosphorylation levels of JNK, p38 and ERK1/2 were detected using western blotting. (E) Ratio of phospho-kinase/kinase semi-quantified using ImageJ. All data are presented as the mean ± SEM and were statistically analyzed using one-way ANOVA with the Bonferroni post hoc test. (F) The cells were pre-treated with Act. D (50 ng/ml) or CHX (10 μg/ml) for 30 min and treated with PDME (10 μg/ml) for 3 h. Subsequently, HO-1 protein expression was determined by western blotting using total lysate. PDME, *P. dindygulensis* methanol extracts; HO-1, heme oxygenase 1; Act. D, actinomycin D; CHX, cycloheximide; n.s, not significant; p-, phosphorylated.

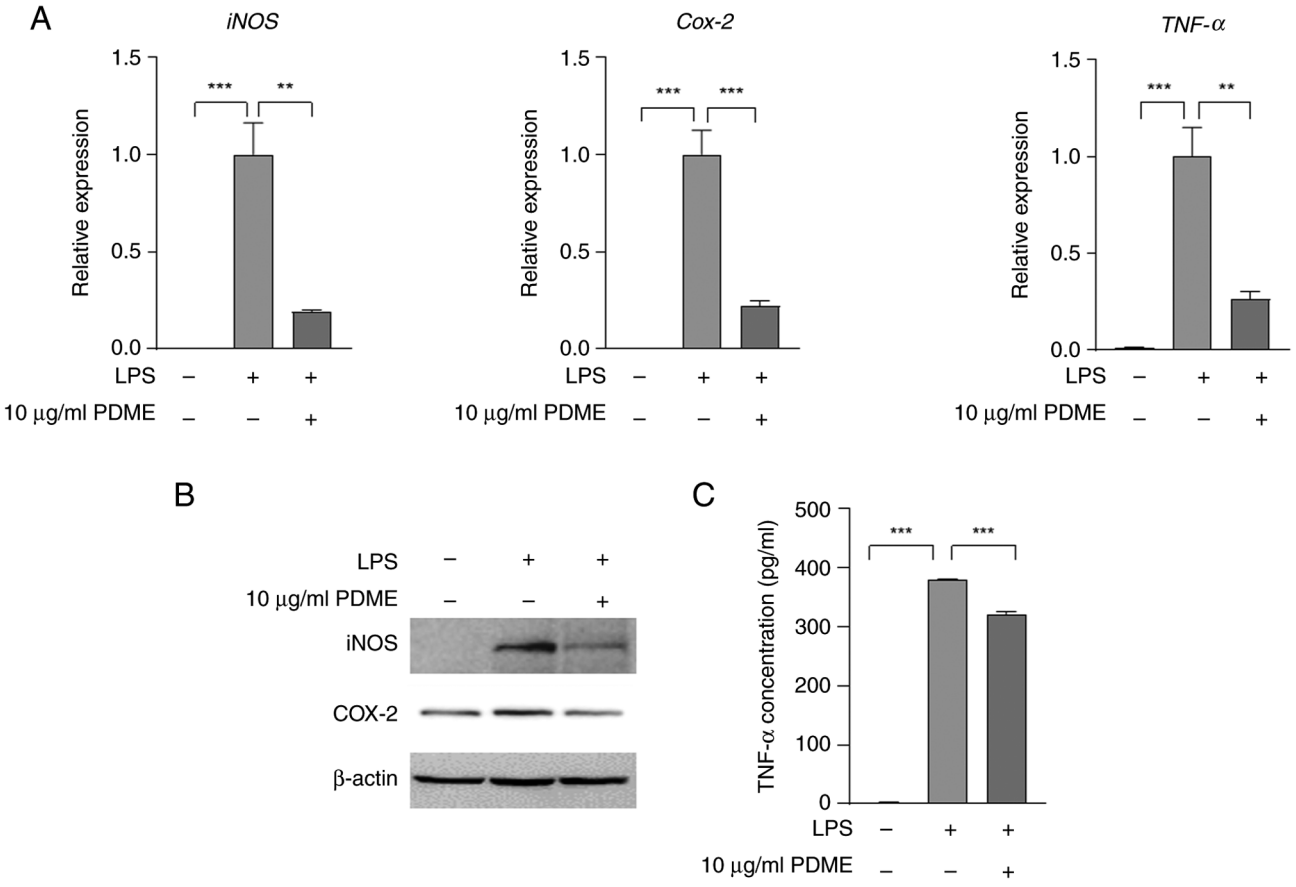

Figure 4. PDME reduces LPS-mediated iNOS, COX-2 and TNF-α expression. (A) RAW246.7 cells were pre-treated with PDME (10 μg/ml) for 30 min and treated with LPS (1 μg/ml) for 24 h. For all controls, the solution used for extraction and dilution was treated in equal amounts. Next, the mRNA expression of iNOS, COX-2 and TNF-α was determined using RT-qPCR. Columns are presented with the mean ± SEM and statistical analysis using one-way ANOVA; **P<0.005, ***P<0.0005, Statistical analyses was compared among the columns shown using triplicate results. (B) RAW246.7 cells were pre-treated with PDME (10 μg/ml) for 30 min treated with LPS (1 μg/ml) for 24 h. Next, the protein expression of iNOS and COX-2 was determined using a western blotting. (C) The cells were pre-treated with PDME (10 μg/ml) for 30 min and treated with LPS for 24 h. Next, the incubation medium was obtained from cells and TNF-α secretion was detected using ELISA. Columns are presented with the mean ± SEM and statistical analysis using one-way ANOVA. ***P<0.0005. PDME, *P. dindygulensis* methanol extracts; LPS, lipopolysaccharides; iNOS. inducible nitric oxide synthase; COX-2, cyclooxygenins-2; RT-qPCR, reverse transcription-quantitative PCR.

whereas NO levels increased when ZnPP was administered as a pretreatment (Fig. 5A). Additionally, the mRNA expression of iNOS, COX-2 and TNF-α was analyzed under identical conditions and it was verified that the inflammatory factors induced by PDME were reduced by the addition of ZnPP (Fig. 5B). These findings strongly indicated that the anti-inflammatory response triggered by PDME relies on HO-1 in macrophages.

Discussion

P. dindygulensis, a traditional medicinal herb from southern China, has historically been employed to alleviate conditions such as cough, asthma and pharyngitis (26). Previous reports have highlighted the anticancer properties of compounds extracted from *P. dindygulensis*, particularly against lung and liver cancers (5,27-29). Despite the close relationship between its anticancer properties and its anti-inflammatory function, there has been limited investigation into the anti-inflammatory effects of natural products derived from *P. dindygulensis*. Traditionally, *P. dindygulensis* has been recognized for its pharmacological effectiveness in diseases associated with inflammation. Studies have indicated that treatment with an ethanol extract of this plant alleviate atherosclerosis by inhibiting the formation of the NOD-like receptor pyrin 3 inflammasome (26,34,35). Furthermore, Lin *et al* (28) demonstrated the potential for the structural constituents of ethanol extracts to act as regulators of the IFN-γ/STAT1 and IL-6/STAT3 pathway. Treatment of endothelial cells with the ethanol extract reduced angiogenic ability, such as tube formation (29). However, the efficacy of the methanol extract is unknown and changes in representative factors of the inflammatory response, such as HO-1 and NO, have not yet been studied. Hence, the objective of the present study was to investigate the anti-inflammatory effects and underlying mechanism of action of a methanol extract derived from *P. dindygulensis* against LPS-induced inflammation. While the anticancer effects are closely linked to its anti-inflammatory properties, the extent of the anti-inflammatory effects of PDME remain relatively unexplored.

LPS binds to Toll-like receptor 4, triggering inflammatory signals and inducing the expression and secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (36,37). Upon stimulation by LPS, macrophages activate inflammatory pathways including NF-κB, MAPKs and AKT

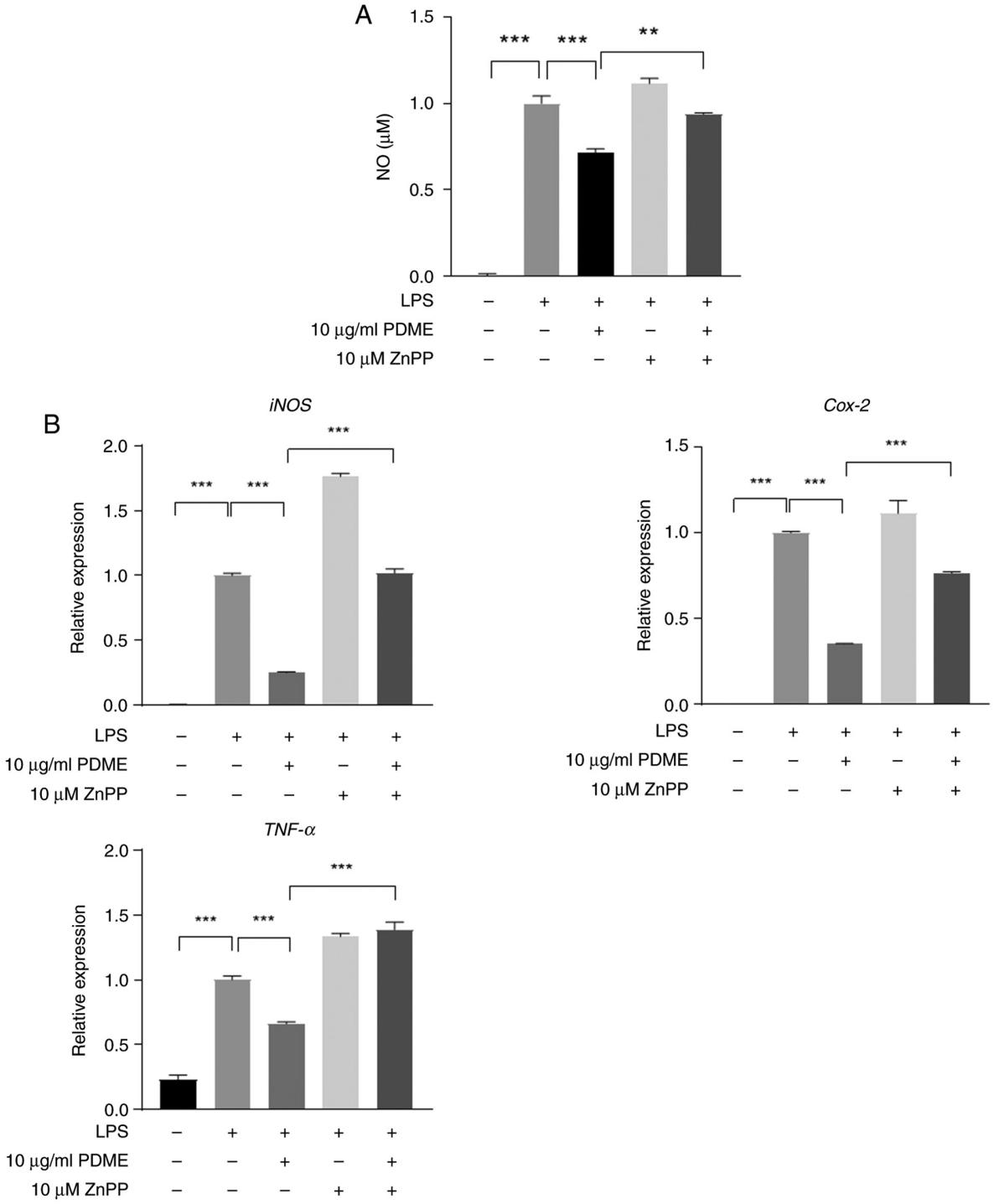

Figure 5. Inhibition of HO-1 activity decreases PDME-mediated anti-inflammatory effects in RAW264.7 cells. (A) The RAW264.7 cells were pre-treated with PDME (10 μg/ml) and ZnPP (10 μM) for 1 h. For all controls, the solution used for extraction and dilution was treated in equal amounts. NOS activity was measured in LPS (1 μg/ml) -stimulated cells for 24 h. (B) The cells were pre-treated with PDME (10 μg/ml) and ZnPP (10 μM) for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Next, the mRNA expression of iNOS, COX-2 and TNF-α were measured using RT-PCR. Data are presented as the mean ± SEM and statistically analyzed using one-way ANOVA; **P<0.005, ***P<0.0005. Statistical analyses were compared among the bars using triplicate results. HO-1, heme oxygenase 1; PDME, *P. dindygulensis* methanol extracts; NOS, nitric oxide synthase; LPS, lipopolysaccharides; iNOS. inducible nitric oxide synthase; COX-2, cyclooxygenins-2; RT-qPCR, reverse transcription-quantitative PCR.

through Toll-like receptor 4, leading to the secretion of various pro-inflammatory mediators such as NO, PGE2, iNOS and COX-2 (38,39). HO-1, an enzyme that catalyzes heme degradation, is a potent protective enzyme upregulated in response to various cellular stress conditions (4). HO-1 and its by-product, carbon monoxide, both possess anti-inflammatory activity by inhibiting LPS-induced expression of NO, PGE2, iNOS and COX-2 (20,21). Natural extracts exert their anti-inflammatory effects by upregulating HO-1 (40).

The present study observed that the treatment of macrophages with PDME reduced LPS-induced NOS activity and increased HO-1 production in RAW 264.7 and U937 cells.

HO-1 expression is subjected to regulation at the mRNA level by various transcription factors, including activator protein-1, NF-κB, hypoxia-inducible factor and notably, nuclear factor erythroid 2-related factor 2. Additionally, its regulation extends to the protein level (41,42). No significant changes were observed in HO-1 regulation by PDME at the mRNA level. The upregulation of HO-1 expression is typically associated with the activities of JNK, ERK and p38, which are the three representative kinases of the MAPK pathway. For instance, in mouse hepatocytes, sodium arsenite activates the JNK pathway to modulate HO-1 expression (43). Similarly, HO-1 is upregulated in rat hepatocytes through both the JNK and p38 pathways (43,44). In chicken hepatoma cells, arsenite triggers the ERK and p38 pathways, leading to increased HO-1 expression (45). However, in the present study, no significant changes in HO-1 expression were observed when MAPK inhibitors (JNK, p38 and MEK) were administered. Li *et al* (46) demonstrated that Fucoxanthin, a marine seaweed extract, exhibits anti-inflammatory effects by modulating pro-inflammatory factors and regulating TLR4/MyD88 signaling in RAW264.7 cells. Exposure to NO inhibits the activity of iron regulatory protein 1 and increases the expression of HO-1 in mouse lymphoma cells (47). The increase in the mRNA levels of HO-1 upon complete inhibition of inducible HO-1 expression with NO scavenger treatment demonstrate the presence of a direct regulatory system for HO-1 against NO exposure (48). The present study showed that resistance to LPS-induced inflammatory responses was not influenced by MAPK. These results demonstrated that PDME may trigger anti-inflammatory responses through TLR4 signaling or direct modulation of HO-1 and NO. The translation of HO-1 can be regulated through alternative mechanisms in the 5'-untranslated region and HO-1 protein levels may be modulated via proteasomal degradation (49,50). The expression of HO-1 decreased with CHX treatment, a translation inhibitor, while it remained unaffected by ActD, a transcription inhibitor. These results suggested that PDME regulates HO-1 protein expression in macrophages.

Furthermore, PDME treatment exhibited its capacity to inhibit the LPS-induced increase in NO, COX-2 and TNF-α. Notably, the effect of PDME was negated by the administration of ZnPP, an HO-1 inhibitor. Collectively, these results indicated that PDME induces anti-inflammatory effects in macrophages by upregulating HO-1 expression. Additional investigation concentrating on the specific compounds present in PDME is necessary. Nevertheless, the present study highlighted the anti-inflammatory capacity of PDME and could aid in uncovering novel natural therapeutics.

The present study confirmed the anti-inflammatory effects of whole methanol extracts obtained from *P. dindygulensis*. Although this marks the first validation of the anti-inflammatory effect of PDME, to the best of the authors' knowledge, this extract comprises various compounds. Therefore, it is necessary to analyze the distribution of individual compounds using experimental methods such as LC/MS and isolate them to ascertain their singular anti-inflammatory effect. The present study verified the anti-inflammatory effect of PDME against LPS-stimulated inflammation in human monocyte leukemia cells U937. PDME increased HO-1 expression and suppressed the upregulation of COX-2 and iNOS induced by LPS-mediated inflammation in U937 cells. However, further studies such as changes in the expression and release of TNF-α by PDME in U937 cells and effects by ZnPP are needed to identify clear efficacy in human cells. In addition, the effectiveness of PDME has not been confirmed in animal models. Verifying the anti-inflammatory effects of natural products in animal models will be an important step in confirming their effectiveness as well as their stability in organisms. These further investigations serve as a foundation for safer and more efficacious utilization of the anti-inflammatory properties of these extracts.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WHM and CYK conducted the experiments. WHM, CYK and HJK validated and curated the data. HJJ, HJK and CH conceived and designed the study. HK, JHL, HJK and CH conceptualized and supervised the study. HJJ, JHL, HJK and CH wrote the original manuscript. TTB, LNH, HKK and HJK participated in the manuscript modification of the important points and analyzed the data. HJJ, TTB, LNH, HKK, HJK and CH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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