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Anti-psoriatic and anti-inflammatory effects of *Kaempferia parviflora* in keratinocytes and macrophage cells

Mingkwan Na Takuathung [a,d], Saranyapin Potikanond [a,d], Siriwoot Sookkhee [b], Pitchaya Mungkornasawakul [c,e], Thanathorn Jearanaikulvanich [a], Kittinan Chinda [a], Nitwara Wikan [f], Wutigri Nimlamool [a,d,*]

[a] Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
[b] Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
[c] Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
[d] Research Center of Pharmaceutical Nanotechnology, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand
[e] Environmental Science Program, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
[f] Institute of Molecular Biosciences, Mahidol University, Salaya, Nakorn Pathom, Thailand

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ABSTRACT

Kaempferia parviflora (KP) has been used as folk medicine for curing various conditions, including anti-inflammatory diseases. However, anti-psoriatic effects in an aspect of suppression of NF-κB activation have not been explored. Therefore, our current study aimed to elucidate the anti-inflammation of KP in lipopolysaccharide (LPS)-induced RAW264.7 cells and anti-psoriatic effects of KP in cytokine-induced human keratinocytes, HaCaT cells. We discovered that KP extract significantly suppressed LPS-induced inflammation at both gene expression and protein production. Specifically, dramatic reduction of nitric oxide (NO) was explored by using Griess method. Consistently, data from RT-qPCR, ELISA, and western blot analysis confirmed that crucial inflammatory and psoriatic markers including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-17, IL-22, and IL-23 were significantly decreased by the action of KP. These events were associated with the results from immunofluorescence study and western blot analysis where the activation of NF-κB upon LPS stimulation was clearly inhibited by KP through its ability to suppress IκB-α degradation resulting in inhibition of NF-κB nuclear translocation. Furthermore, KP extract significantly inhibited LPS-stimulated phosphorylation of ERK1/2, JNK, and p38 in a dose-dependent manner, along with inhibition of ERK1/2 activation in both TNF-α- and EGF-induced HaCaT cells. Interestingly, HaCaT cells exposed to 15 μg/mL of KP also exhibited significant decrease of cell migration and proliferation. Our results revealed that KP extract has a potential to be developed as a promising agent for treating inflammation and psoriasis, in part through targeting the proliferation and the NF-κB pathways.

1. Introduction

Psoriasis is one of the most common dermatologic disease, affecting up to 2% of the world's population [1]. It is an immune-mediated disease clinically characterized by erythematous, sharply demarcated papules and rounded plaques covered by silvery micaceous scale [2]. The clinical presentation shows thick epidermis with infiltrated dermal inflammation and elongated rete ridges [3]. The pathogenesis of psoriasis is involved with interleukin (IL)-23 that is produced by dendritic cells and keratinocytes, and this cytokine stimulates T helper 17 cells within the dermis to produce IL-17A and IL-22 [4]. Inflammation is a complex physiologic response to harmful stimuli, which is characterized as pain, heat, redness, and swelling [5]. Macrophages play an important role in local host defense and inflammatory response which is orchestrated by proinflammatory cytokines such as nitric oxide (NO), tumor necrosis factor (TNF), IL-1, and IL-6 [6]. NO, a gas generated by mammalian cells, is synthesized from the amino acid L-arginine by enzymes called inducible nitric oxide synthases (iNOS) [7]. NO is responsible for several biological processes, including being a neurotransmitter, regulation of blood pressure, and control of gastrointestinal,

* Corresponding author at: Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.
E-mail address: wutigri.nimlamool@cmu.ac.th (W. Nimlamool).

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respiratory, and genitourinary tract function [8]. In addition, NO is produced excessively during host defense and immunologic reactions by activated macrophages, which is stimulated by lipopolysaccharide (LPS) [7]. LPS is associated with activation of the toll-like receptor (TLR) 4 [9], resulting in the phosphorylation and transcriptional activation of nuclear factor-κB (NF-κB) and mitogen-activated protein (MAP) kinases, including ERK, JNK and p38, which are important signals for iNOS [10]. Moreover, MAPKs can also mediate cellular responses to extracellular signals and proinflammatory cytokines, particularly TNF-α, IL-1, and IL-6, whereas prostaglandin E2 (PGE2) is synthesized by cyclooxygenase (COX)-2 in various models of inflammation [11]. Aberrant activation of this system leads to immunodeficiency, septic shock, or induction of autoimmunity [5]. Thus, the combined regulation of proinflammatory cytokine production, NO reduction, and NF-κB and MAPK pathway inhibition are considered therapeutic strategies to treat inflammatory diseases [12,13].

Effective treatment of autoimmune diseases and inflammatory, including psoriasis, is one of the greatest unmet medical needs. The use of conventional steroidal and immunosuppressant medicines for psoriatic inflammation is still the mainstay in a typical practice [14,15]. However, persistent long-term use safety concerns must be taken into consideration [16]. Thus, there is a greater interest in natural compounds which have been used for centuries to reduce psoriatic inflammation [17]. Many plants and their compounds have been demonstrated to exhibit strong anti-inflammatory activities [18–20]. *Kaempferia parviflora* (KP) or Krachaidam, also known as black ginger, which belongs to the Zingiberaceae family is native to North and Northeast of Thailand [21]. Its rhizomes are traditionally used as folk medicine for curing various diseases, including allergy, asthma, impotence, gout, diarrhea, dysentery, peptic ulcer, and diabetes [22,23]. Moreover, recent evidence demonstrates that KP has anticancer activities [24–26], cellular metabolism-regulating activities [27], vascular relaxant and cardioprotective activities [28,29], neuroprotective activities [30,31], and antioxidant and anti-inflammatory activities [21]. Specifically, for an anti-inflammatory effect of KP, previous studies showed that hexane and ethanol extracts of KP inhibited IgE antigen- or a calcium ionophore-induced degranulation in rat basophilic leukemia (RBL-2H3) cells [32] and inhibited degranulation associated with the suppression of calcium influx to the cells [33]. Interestingly, previous evidence also reported that ethanolic extract of KP inhibited prostaglandin E2 (PGE2) expression in LPS-induced RAW264.7 cells, whereas hexane and chloroform decreased paw edema in rats [34]. Consistently, the compounds isolated from KP showed inhibitory effects on nitric oxide (NO), inducible nitric oxide synthase (iNOS), PGE2, and TNF-α release in LPS-induced RAW264.7 cells through activation of spleen tyrosine kinase pathway but not ERK and JNK pathways [34–36]. Furthermore, KP extract and its compounds have been reported to suppress NO production in LPS-stimulated RAW264.7 cells, inhibit human monocytic leukemia, THP-1, cell adhesion to human umbilical vein endothelial cells (HUVECs), decrease the production of angiotensin-converting enzyme (ACE)-mediated Ang-II, and inhibit the production of cellular reactive oxygen species (ROS) in pyocyanin-stimulated HUVECs [37]. However, the anti-inflammatory effects of KP on LPS-induced RAW264.7 cells in other aspects including the suppression of NF-κB activation have not yet been investigated.

This present study aimed to elucidate the anti-inflammatory and anti-psoriatic effects and mechanisms of KP on inhibition of LPS-induced RAW264.7 cells and cytokines-induced human keratinocytes, HaCaT cells. We particularly investigated effects of KP on suppressing gene expression and protein production of nitric oxide, IL-1β, IL-6, TNF-α, iNOS, and COX-2. In addition, the inhibitory properties of KP on major molecular signal transduction pathways related to NF-κB and MAPKs were also determined. The study showed that KP possesses anti-inflammatory effects at least in part through its ability to suppress NF-κB activation and the proliferative signal transduction pathway, and this plant may be a good candidate as an alternative therapeutic agent for psoriatic inflammatory diseases.

2. Materials and methods

2.1. Plant material and extraction of Kaempferia parviflora Rhizomes

The rhizomes of KP were planted, harvested, authenticated, and extracted as previously reported [24]. Specifically, we obtained the fresh KP rhizomes from the cultivating area called "CMU-RSPG Kaempferia housing". This housing is located in Chiang Dao, Chiang Mai Province, Thailand. The plant was authenticated and the voucher specimen number (R-CMUKP002) was designated. The plant specimens were kept in the Herbarium at Faculty of Sciences, Chiang Mai University. For KP extract preparation, the harvested KP rhizomes were thoroughly cleaned, air-dried at room temperature, weighed, and grounded to small pieces. The materials were sonicated with 95% ethanol at room temperature for 72 h. Then, the solution was filtered, and the solvent was eliminated using a rotary evaporator. The percentage yield extracts from the plant (calculated on dry weight basis) was 9.9%. After extraction, 1 mL of 100% DMSO was added to 1 g of ethanolic KP crude extract to make a 1 g/mL KP stock solution. Prior to each experiment, KP stock solution was diluted in medium and the final concentration of DMSO was maintained below 0.02% v/v throughout the experiment. At least 3 independent trials were performed for each experiment.

The quality control of KP extract was evaluated by high performance liquid chromatograph (HPLC) analysis by detecting the presence of 9 methoxyflavones in KP extract (KP1 = 3,5,7,3′,4′-pentamethoxyflavone, KP2 = 5,7,4′-trimethoxyflavone, KP3 = 3,5,7-trimethoxyflavone, KP4 = 3,5,7,4′-tetramethoxyflavone, KP5 = 5-hydroxy-3,7,3′,4′-tetramethoxyflavone, KP6 = 5-hydroxy-7-methoxyflavone, KP7 = 5-hydroxy-7,4′-dimethoxyflavone, KP8 = 5-hydroxy-3,7-dimethoxyflavone, and KP9 = 5-hydroxy-3,7,4′-trimethoxyflavone). The assay was performed in comparison to nine standard methoxyflavones compounds [(1) = 3,5,7,3′,4′-pentamethoxyflavone, (2) = 5,7,4′-trimethoxyflavone, (3) = 3,5,7-trimethoxyflavone, (4) = 3,5,7,4′-tetramethoxyflavone, (5) = 5-hydroxy-3,7,3′,4′-tetramethoxyflavone, (6) = 5-hydroxy-7-methoxyflavone, (7) = 5-hydroxy-7,4′-dimethoxyflavone, (8) =5-hydroxy-3,7-dimethoxyflavone, (9) = 5-hydroxy-3,7,4′-trimethoxyflavone] which were obtained from the Eco-friendly Product Research laboratory (EfPRL), Department of Chemistry, Faculty of Science, Chiang Mai University. For HPLC analysis, the compound determination was performed on an Agilent 1100 series (Agilent Technologies, USA) equipped with UV-Vis detector (with detection wavelength at 330 nm). A C18 column (250 mm × 4.6 mm, internal diameter 5 μm, Hypersil™, Sigma-Aldrich, USA) was used for differential separation. The mobile phase was methanol – 0.5% acetic acid (65:35, v/v), and the operation was done at a flow rate of 1.0 mL/min. The injection volume was 20 μL.

2.2. Cell culture

RAW 264.7 macrophage cells were purchased from ATCC (ATCC, Manassas, VA, United States) and HaCaT cells were obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany). The cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, United States). Cells were grown at 37 °C in DMEM medium supplemented with 10% fetal bovine serum (Merck KGaA, Germany), and penicillin (100 U/mL) and streptomycin (100 μg/mL) (Gibco, United States) in a humidified atmosphere of 5% CO_2.

2.3. Cell viability assay

The potential cytotoxicity of KP to RAW 264.7 macrophage and HaCaT cells was evaluated by using 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [38]. Briefly, the cells (2×10^5 cells/mL) were seeded in 96-well plates

for 24 h in complete medium and treated with different concentrations of KP (0–1000 μg/mL) or with vehicle (DMSO 0.001–0.1%) for 24 h. Then the MTT solution (0.5 mg/mL in PBS) was added to each well, then the plates were incubated at 37 °C, 5% CO_2 for 2 h. After aspirating the culture supernatants, 200 μL of DMSO was added to each well, and the plates were incubated in the dark for 10 min. The absorbance at 590 nm was measured using a microplate reader (BioTek Instruments, United States). Cell viability assay was performed three times (triplicate for each individual trial).

2.4. Nitric oxide assay

Nitric oxide assay was performed by using commercial Griess reagent (Sigma, Unites States) for the determination of total nitrate and nitrite concentration as an index of nitric oxide production as described previously [39]. Briefly, RAW 264.7 cells (1.5×10^6 cells/mL) were seeded and cultured for 24 h. Prior to stimulation with LPS (Sigma, United States), cells were treated with KP for 4 h. After 24 h of LPS incubation, the culture supernatant (100 μL) of each well was collected and mixed with equal volume of Griess reagent in a new 96-well plate. After incubation at room temperature (RT) for 15 min, the absorbance was measured at 540 nm using a microplate reader (BioTek Instruments, United States. The nitrite concentration was assessed by a sodium nitrite standard curve.

2.5. Real-time reverse transcription polymerase chain reaction (RT-qPCR) assay

RAW 264.7 cells (1.5×10^6 cells/mL) were seeded and cultured for 24 h. After pre-treatment with KP extract at different concentrations (3.75, 7.5, and 15 μg/mL) for 4 h, cells were stimulated with LPS (1 μg/mL) for 24 h and harvested for detection of mRNA expression. Total RNA was extracted by using FavorPrep™ Total RNA Purification Mini Kit (Favorgen Biotech Corp., Taiwan). The concentration of total RNA was estimated using Nano-Drop spectrophotometer (Thermo Fisher Scientific, USA). Then, RNA was reverse-transcribed using ReverTra ACE® qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) as described in the manufacturer's protocol. The reverse transcription was performed at 37 °C for 15 min, 50 °C for 5 min, and 98 °C for 5 min. The consequent complementary (c)DNA was used for quantitative PCR using the PCRmax Eco 48 real-time PCR system (PCRmax Limited, UK) with PerfeCTa™ SYBR® Green FastMix™, Low Rox™ (Quanta Biosciences™, USA), following the manufacturer's instruction. The reaction mixture consisted of 2 μL cDNA, 1 μL nuclease-free water, 5 μL PerfeCTa™ SYBR® Green FastMix™, Low Rox™, and 1 μL (100 nM) of each specific primer (Table 1). PCR was performed under the following conditions: 95 °C for 3 min, 45 cycles of 95 °C for 15 s, 58 °C for 15 s, 72 °C for 30 s

Table 1
Amount (%) of methoxyflavones (KP1-KP9) in KP extract determined by HPLC analysis. RT: Retention time (min).

Peak	RT (min)	Component	Amount (%)
1	7.809	Unknown	0.67
2	10.459	Unknown	2.90
3	11.581	KP1:3,5,7,3`,4`-pentamethoxyflavone	18.97
4	15.844	Unknown	11.68
5	17.793	KP2:5,7,4`-trimethoxyflavone	25.62
6	19.370	KP3:3,5,7-trimethoxyflavone	3.80
7	20.897	KP4:3,5,7,4`-tetramethocuflavone	13.27
8	31.753	KP5:5-hydroxy-3,7,3`,4`-tetramethoyflavone	1.94
9	41.107	KP6:5-hydroxy-7-methoxyflavone	3.71
10	48.435	KP7:5-hydroxy-7,4`-dimethoxyflavone	6.02
11	51.676	KP8:5-hydroxy-3,7-dimethoxyflavone	4.70
12	58.269	KP9:5-hydroxy-3,7,4`-trimethoxyflavone	6.72

2.6. Enzyme-Linked Immunosorbent (ELISA) Assay

RAW 264.7 and HaCaT cells (1.5×10^6 cells/mL) were seeded in 3-cm dishes and cultured for 24 h. After treatment with KP extract at different concentrations (3.75, 7.5, and 15 μg/mL) for 4 h, the cells were stimulated with LPS (1 μg/mL) for 6 h and 24 h. Then, the culture supernatants were collected. The concentration of IL-6 and TNF-α in the supernatants of RAW 264.7 cells was measured using mouse IL-6 and TNF-α ELISA MAX™ Deluxe (BioLegend, USA), whereas the concentration of IL-6, IL-17, IL-22, and IL-23 in the culture supernatants of HaCaT cells was measured using human IL-6, IL-17, IL-22, and IL-23-α ELISA MAX™ Deluxe (BioLegend, USA). ELISA was performed in accordance with the manufacturer's protocol. Briefly, the ELISA 96-well plate was coated with 100 μL of 1x capture antibody in coating buffer and incubated overnight at 4 °C. The plate was washed with wash buffer (0.05% Tween-20 in PBS) four times and then blocked with 200 μL of blocking buffer for 1 h at RT. After washing the plate with wash buffer for four times, mouse IL-6 and TNF-α standards were diluted to create the concentration range of 0–1000 pg/mL. The supernatant samples or standards (100 μL) were added to each well in duplicate for 2 h at RT. The plate was washed with wash buffer for four times, then the specific detection antibody (100 μL) was added to each well for 1 h at RT and washed for four times. One hundred microliters of HRP-conjugated avidin (1:1000) was added, and the plate was incubated for 30 min, followed by washing for five times. Lastly, the freshly mixed TMB substrate solution (100 μL) was added to each well and incubated in the dark for 30 min. The stop solution (2 N of H_2SO_4) (100 μL) was added to each well and the absorbance at 450 nm and 570 nm was measured using a microplate reader (BioTek Instruments, United States) within 15 min

2.7. Immunofluorescence study

RAW 264.7 cells were grown on glass cover slips for 24 h. Then the cells were pre-treated with KP extract at 15 μg/mL for 4 h and were stimulated with LPS for 1 h. The cells were fixed with 4% paraformaldehyde dissolved in PBS for 15 min. After washing three times with PBS, cells were permeabilized using 0.3% TritonX-100 in PBS for 5 min. Then cells were incubated with 1% BSA in PBS solution for 1 h, and with 1:350 of NF-κB p65 (D14E12) XP Rabbit monoclonal antibody (Cell Signaling Technology, United States) at 4 °C overnight. Cells were washed three times with PBS for 5 min each time, and incubated with a 1:500 dilution of Alexa 594-conjugated goat anti-rabbit Ig (Life Technologies, USA) and 5 μg/mL of Hoechst 33342 (Thermo Fisher Scientific, USA) for 2 h, in the dark, at RT. After washing three times with PBS and one time with distilled water, cells were mounted using Fluoromount-G (0100-01: SouthernBiotech, USA). The sample coverslips were sealed with nail polish to prevent dryness. Observations were performed on a fluorescent microscope, AX70 Olympus®, Japan, with 40x magnification, and micrographs were captured with the DP-BSW Basic Software for the DP71 microscope digital camera.

2.8. Nuclear and cytosolic extraction

After treating cells with KP extract, nuclear and cytosolic extracts were prepared based on the previously described methods with some modifications [40,41]. Briefly, cells were harvested by centrifugation for 1 min at 2000×*g*, rinsed with cold PBS, and resuspended in buffer A (10 mM Hepes, pH 10, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 300 mM sucrose, 0.1% Nonidet P-40). Then, cells were centrifuged (15,000×*g*) for 10 min at 4 °C. The nuclear pellet was washed with 1 mL of cold PBS, and centrifuged (15,000×*g*) for 5 min at 4 °C. Three hundred microliters of cytosolic extracts were resuspended in 80 μL of 5x reducing Laemmli buffer, and the nuclear extracts were resuspended in 100 μL of 1x reducing Laemmli buffer before subjected to western blot analysis.

2.9. Western blotting

For COX-2, total ERK1/2, pERK1/2, NF-κB, IκB, pAKT, and pJNK detection, RAW 264.7 cells and HaCaT were pre-treated with 3.75, 7.5, and 15 μg/mL of KP extract for 4 h, then stimulated with 1 μg/mL of LPS for either 6 h or 24 h for the collection of whole cell lysate, and 1 h for the collection of nuclear and cytoplasmic extraction. RAW 264.7 and HaCaT cells were prepared by adding 1x reducing Laemmli buffer into the sample dishes. Samples were collected, heated at 95 °C for 5 min, separated by SDS-PAGE, and electroblotted onto PVDF membranes (GE Healthcare Life Sciences, Germany). Membranes were blocked with 5% BSA in TBS-T (0.02 M Tris-HCl, pH 7.6, 0.0137 M NaCl, and 0.1% Tween 20) at RT for 1 h. Membranes were then incubated with primary antibodies (Cell signaling Technology, USA) at 4 °C overnight. Primary antibodies used included COX-2, total ERK1/2, pERK1/2, NF-κB, IκB, pAKT, and pJNK (1: 1000 for each antibody), a 1:1000 dilution of lamin antibody, and a 1:10,000 dilution of an anti-β-actin antibody. After three washes with TBS-T, membranes were incubated with 1:5000 dilution of appropriate secondary antibodies [IRDye® 800cw anti-mouse or IRDye® 680RD anti-rabbit IgG (LI-COR Biosciences, USA)]. The immune complexes were detected using LI-COR odyssey CLx western blotting detector (LI-COR Biosciences, USA). The intensity of immunoreactive bands was analyzed and quantified using the ImageJ software.

2.10. Migration assay

HaCaT cells were grown in 12-well tissue culture plates with designated wells for untreated, 15 μg/mL of KP- and DMSO-treated groups. Once the cells were 95–100% confluent, a 200-μL pipette tip was used to scrape the cell monolayer in a vertical and horizontal cross-line to create a "scratch" to each well. The center of the cross, where the 2 scratch lines meet, was used to position the center of the wound gap. The wells were washed once with growth medium to clear any detached cells and then refilled with the treatment media. Cell migration (gap filling) was examined using a phase-contrast microscope with EOS Utility Software at 0 h (immediately after scratch) (Day 0), 24 h (Day 1), and 48 h (Day 2) after wounding. Gap areas were measured, and cell migration was quantified. Each treatment was done in triplicate, and each experiment was repeated at least twice.

2.11. Cell counting assay

HaCaT cells were grown in 12-well tissue culture plates at a density of 5×10^4 cells/well and incubated until cells were 90% confluent. Cells were treated with KP extract at 3.75, 7.5, and 15 μg/mL before the total number of cells were counted at different time point (0, 24, 48, and 72 h) by using hemocytometer. The doubling time of the cells was calculated regarding to the following equation: duration*log(2)/ [log(Final Concentration)-log(Initial Concentration)].

2.12. Statistical analysis

For comparison between treatment groups, Student's *t*-test was used for two groups and one-way ANOVA was used for multiple groups. Data were analyzed by using Statistical Package for the Social Sciences (SPSS) software. Data are presented as mean ± SEM. In all analyses, a *p*-value ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Chemical compositions of KP extract

After obtaining KP extract and performing HPLC analysis, the results, shown in Fig. 1, clearly demonstrated that KP ethanolic extract contains methoxyflavones as main compounds, which are 3,5,7,3′,4′-pentamethoxyflavone (KP1), 5,7,4′-trimethoxyflavone (KP2), 3,5,7-trimethoxyflavone (KP3), 3,5,7,4′-tetramethoxyflavone (KP4), 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (KP5), 5-hydroxy-7-methoxyflavone (KP6), 5-hydroxy-7,4′-dimethoxyflavone (KP7), 5-hydroxy-3,7-dimethoxyflavone (KP8), and 5-hydroxy-3,7,4′-trimethoxyflavone (KP9). The peak number, retention time, and percentage of each compound were shown in Table 1. The compound present in the highest quantity was KP2 (25.62%), followed by KP1(18.97%), KP4 (13.27%), KP9 (6.72%), KP7 (6.02%), KP8 (4.70%), KP3 (3.80%), KP6 (3.71%), and KP5 (1.94%).

3.2. The effects of KP on RAW 264.7 and HaCaT cell viability

Cell viability of RAW 264.7 and HaCaT cells treated with various concentrations of KP extract was measured using MTT assay. The percentage of viable cells was monitored for 24 h. The effect of cell survival suppression by KP extract, compared to DMSO vehicle control. Results showed that the viability of both RAW 264.7 and HaCaT cells was decreased when the concentration of KP extract was increased. The IC_{50} of KP extract in RAW 264.7 and KP extract was 43.83 ± 3.172 μg/mL and 48.86 ± 1.689 μg/mL, respectively (Fig. 2). KP extract at the concentrations less than 31.25 μg/mL was observed to be non-toxic to both

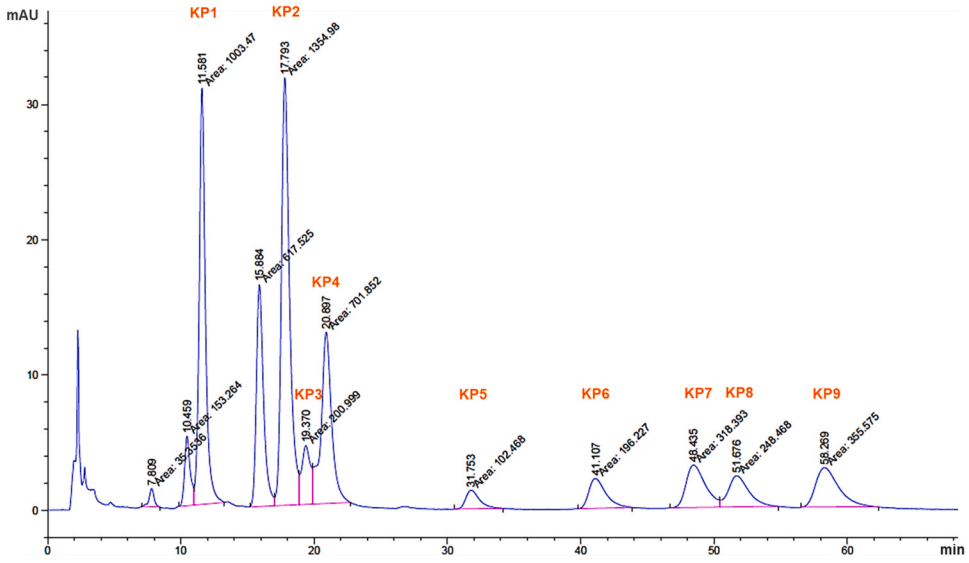

Fig. 1. HPLC chromatogram for ethanolic extract of *K. parviflora*. The abbreviation "KP" with peak numbers (at certain retention times and areas) indicates to the following compounds: KP1 = 3,5,7,3′,4′-pentamethoxyflavone, KP2 = 5,7,4′-trimethoxyflavone, KP3 = 3,5,7-trimethoxyflavone, KP4 = 3,5,7,4′-tetramethoxyflavone, KP5 = 5-hydroxy-3,7,3′,4′-tetramethoxyflavone, KP6 = 5-hydroxy-7-methoxyflavone, KP7 = 5-hydroxy-7,4′-dimethoxyflavone, KP8 = 5-hydroxy-3,7-dimethoxyflavone, and KP9 = 5-hydroxy-3,7,4′-trimethoxyflavone.

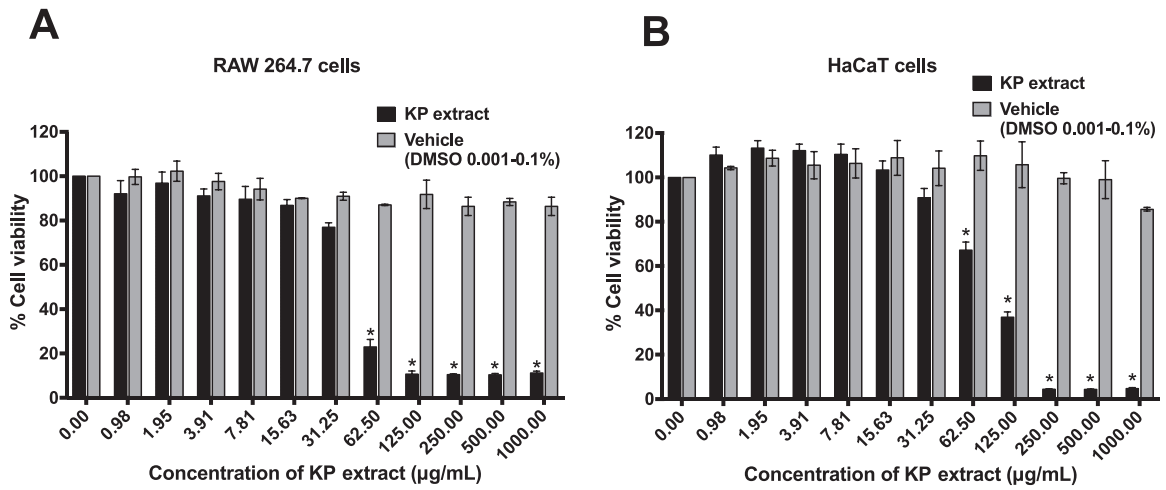

Fig. 2. The effects of KP on RAW264.7 cell viability measured by the MTT assay. The bars indicate percent cell viability of RAW264.7 cells treated with different concentrations of KP extract (0–1000 μg/mL) for 24 h. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the untreated cells (0 μg/mL).

cell lines. Therefore, KP extract at the range of non-toxic concentrations (3.75, 7.5, and 15 μg/mL) was selected for subsequent experiments in this study.

3.3. The effects of KP on inhibiting nitric oxide production

One major characteristic of RAW264.7 cells in response to inflammatory stimuli is the production of nitric oxide. As a step toward the determination whether KP has the ability to suppress inflammation, we detected the presence of nitric oxide in the culture supernatants of the untreated and KP-treated cells by using Griess reagent. We found that in comparison to the untreated cells, treatment of cells with LPS significantly increased the production of nitric oxide to approximately 50 μM (Fig. 3). Interestingly, cells treated with LPS with the presence of KP extract exhibited a dramatic decrease in nitric oxide production in a concentration-dependent manner. Specifically, KP extract at 7.5 and 15 μg/mL significantly reduced the level of nitric oxide to approximately 45 and 30 μM, respectively; however, DMSO vehicle control was proved to have no effect on suppressing LPS-induced nitric oxide production in RAW264.7 cells (Fig. 3).

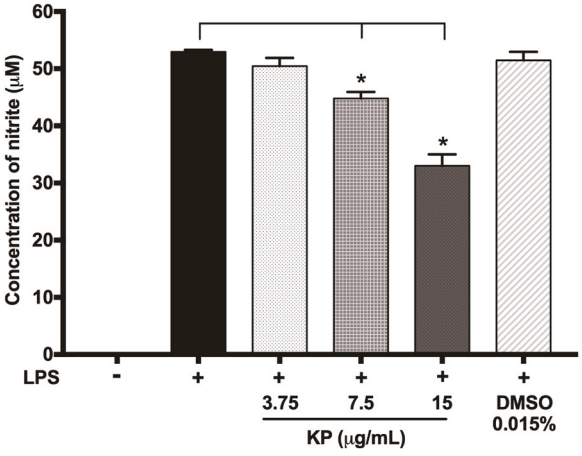

Fig. 3. Inhibitory effects of KP on LPS-induced production of nitric oxide. Cells (1.5 × 106 cells/mL) were pre-treated with various concentrations (3.75, 7.5, and 15 μg/mL) of KP extract for 4 h, and then incubated with LPS (1 μg/mL) for 24 h. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the LPS-treated group.

3.4. The effects of KP on inhibiting gene expression of inflammatory cytokines

Based on the observation that KP could reduce LPS-induced nitric oxide production, we further hypothesized that KP may have a potential to reduce the production of inflammatory cytokines. To test our hypothesis, we determined the effects of KP on mRNA expression levels of IL-1β, IL-6, iNOS, COX-2, and TNF-α by qRT-PCR. As shown in Fig. 4, LPS treatment markedly augmented transcription of all cytokine genes, whereas cells pre-treated with KP extract significantly attenuated LPS-induced IL-1β, IL-6, iNOS, COX-2, and TNF-α gene levels in a concentration-dependent manner. Specifically, the inhibitory activities of 3.75, 7.5, and 15 μg/mL KP extract on IL-1β expression in RAW264.7 cells were approximately 6-fold, 12-fold, and 24-fold, respectively, compared to IL-1β expression of cells stimulated with LPS alone (Fig. 4A). Furthermore, in comparison to RAW264.7 cells with LPS stimulation alone, the groups treated with KP extract at 15 μg/mL exhibited approximately a 10-fold decrease in IL-6 expression (Fig. 4B) and an 18-fold decrease in iNOS (Fig. 4C) and COX-2 expression (Fig. 4D). Moreover, the presence of 3.75 and 7.5 μg/mL of KP extract was able to suppress the expression of IL-6 and iNOS for 5-fold and 6-fold, respectively, as compared with RAW264.7 cells stimulated with LPS alone (Fig. 4B and C). Additionally, KP extract at concentrations of 3.75, 7.5, and 15 μg/mL inhibited the expression of TNF-α for 2-fold as compared with RAW264.7 cells stimulated with LPS alone (Fig. 4E).

3.5. The effects of KP on inhibiting inflammatory cytokine secretion

Since we observed strong inhibition of cytokine expression at the gene level, we would like to further investigate whether KP inhibits production and secretion of IL-1β, IL-6, and TNF-α in LPS-stimulated RAW264.7 cells by ELISA. Results revealed that compared with the untreated controls, LPS significantly increased the production of IL-1β, IL-6, and TNF-α in the culture supernatants of RAW264.7 cells (Fig. 5). However, treatment with KP extract significantly inhibited the production of IL-1β, IL-6, and TNF-α in a concentration-dependent manner (Fig. 5). Although the production levels of IL-1β in the LPS-stimulated cells at 6 h were not statistically different from the cells treated with 3.75, 7.5 and 15 μg/mL KP extract, treatment of cells with KP extract at all concentrations significantly reduced LPS-stimulated IL-1β production after 24 h of incubation for 1-fold ($p < 0.05$) (Fig. 5A and B). In addition, KP extract at 7.5 and 15 μg/mL significantly decreased the level of IL-6 in the culture supernatants of RAW264.7 cells to approximately 2-fold and 10-fold, respectively, compared with the LPS-stimulated cells

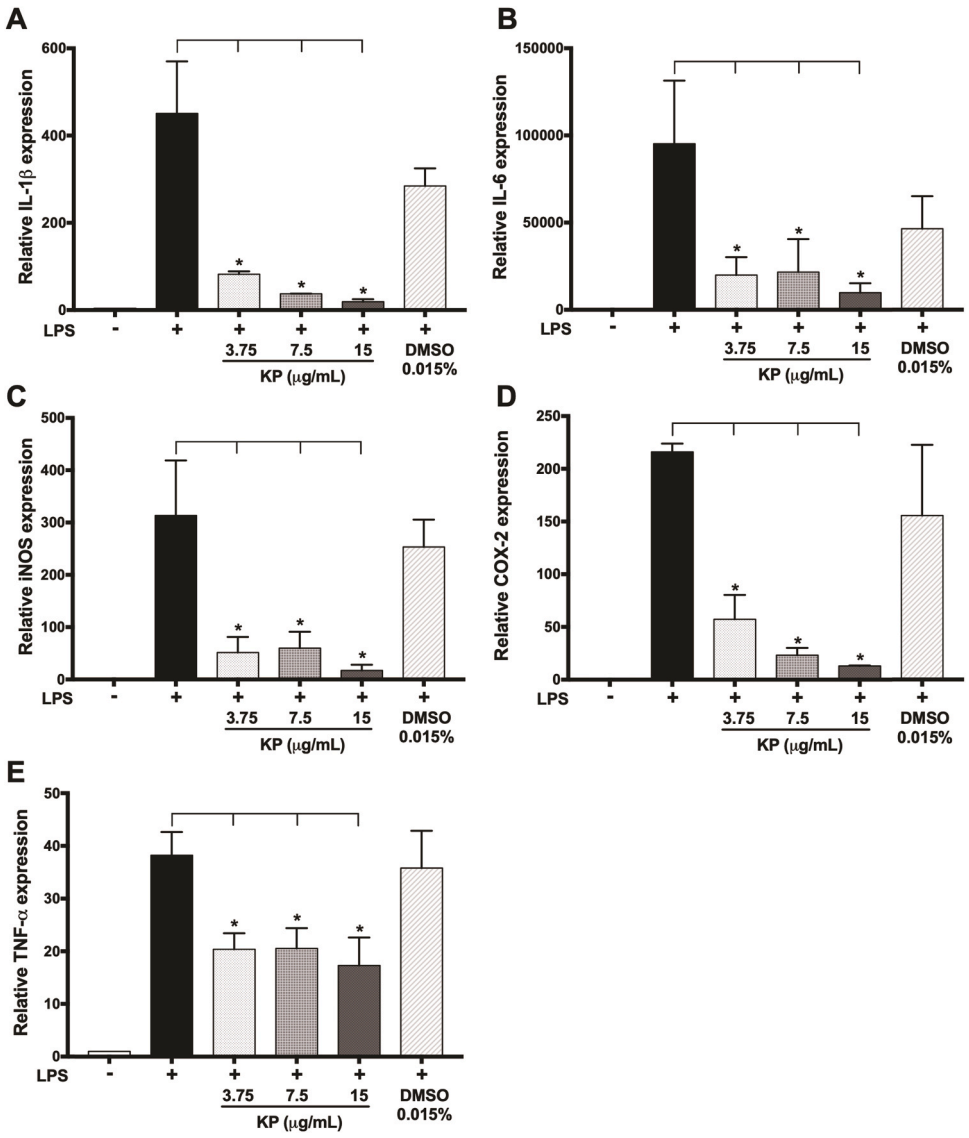

Fig. 4. Inhibitory effects of KP on LPS-induced mRNA expression of IL-1β, IL-6, iNOS, COX-2, and TNF-α. Cells (1.5 × 106 cells/mL) were pre-treated with various concentrations (3.75, 7.5, and 15 μg/mL) of KP extract for 4 h, and then incubated with LPS (1 μg/mL) for 24 h. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the LPS-treated group.

(Fig. 5C and D). Moreover, the presence of KP extract at 15 μg/mL significantly reduced the LPS-induced TNF-α production for 3-fold ($p < 0.05$) (Fig. 5E and F).

3.6. The effects of KP on suppressing LPS-induced NF-κB activation in RAW264.7 cells

This study investigated whether KP extract has ability to block the activation of NF-κB pathway because the regulation of inflammatory mediators in LPS-stimulated macrophages is transcriptionally implicated with NF-κB [42]. The analysis of NF-κB localization by immunostaining and fluorescence microscopy showed that KP extract retained NF-κB in the cytoplasm of cells (Fig. 6A). Free dimer activated subunits of NF-κB (p50/p65) can be translocated from the cytosol to the nucleus upon dissociation of IκB-α from NF-κB. Therefore, in order to clearly verify that KP can suppress the nuclear translocation of NF-κB, western immunoblotting for NF-κB was conducted. Exposure of LPS alone significantly increased the amount of NF-κB in the nucleus to 4.8-fold. However, KP extract at 15 μg/mL noticeably inhibited LPS-induced nuclear translocation of NF-κB (Fig. 6B and C). On the basis that the phosphorylation of inhibitory kappa B (IκB) and its subsequent degradation by various stimuli are critical steps in NF-κB activation [43], we further investigated the effects of KP extract on inhibiting LPS-induced degradation of IκB-α protein by immunoblotting analysis. We found that KP extract at concentration of 15 μg/mL markedly inhibited LPS-induced degradation of IκB-α (Fig. 6D and E) to 0.97-fold, compared to the LPS-treated group (0.7-fold).

3.7. Effects of KP on attenuating of MAPK phosphorylation and COX-2 production in LPS-stimulated RAW264.7 cells

To confirm whether inhibition of NF-κB activation is mediated through MAPK pathways, we examined the effect of KP extract on LPS-stimulated phosphorylation of ERK1/2, JNK, and p38 MAPK in RAW264.7 cells. As depicted in Fig. 7A, LPS markedly induced phosphorylation of ERK1/2, JNK, and p38. Nevertheless, pre-treatment with KP extract significantly inhibited LPS-stimulated phosphorylation of ERK1/2, JNK, and p38 in a dose-dependent manner. Specifically, 15 μg/mL of KP extract reduced phosphorylation of ERK1/2, JNK, and p38 to 1.8-fold, 2.2-fold, and 1.3-fold, respectively (Fig. 7A and B).

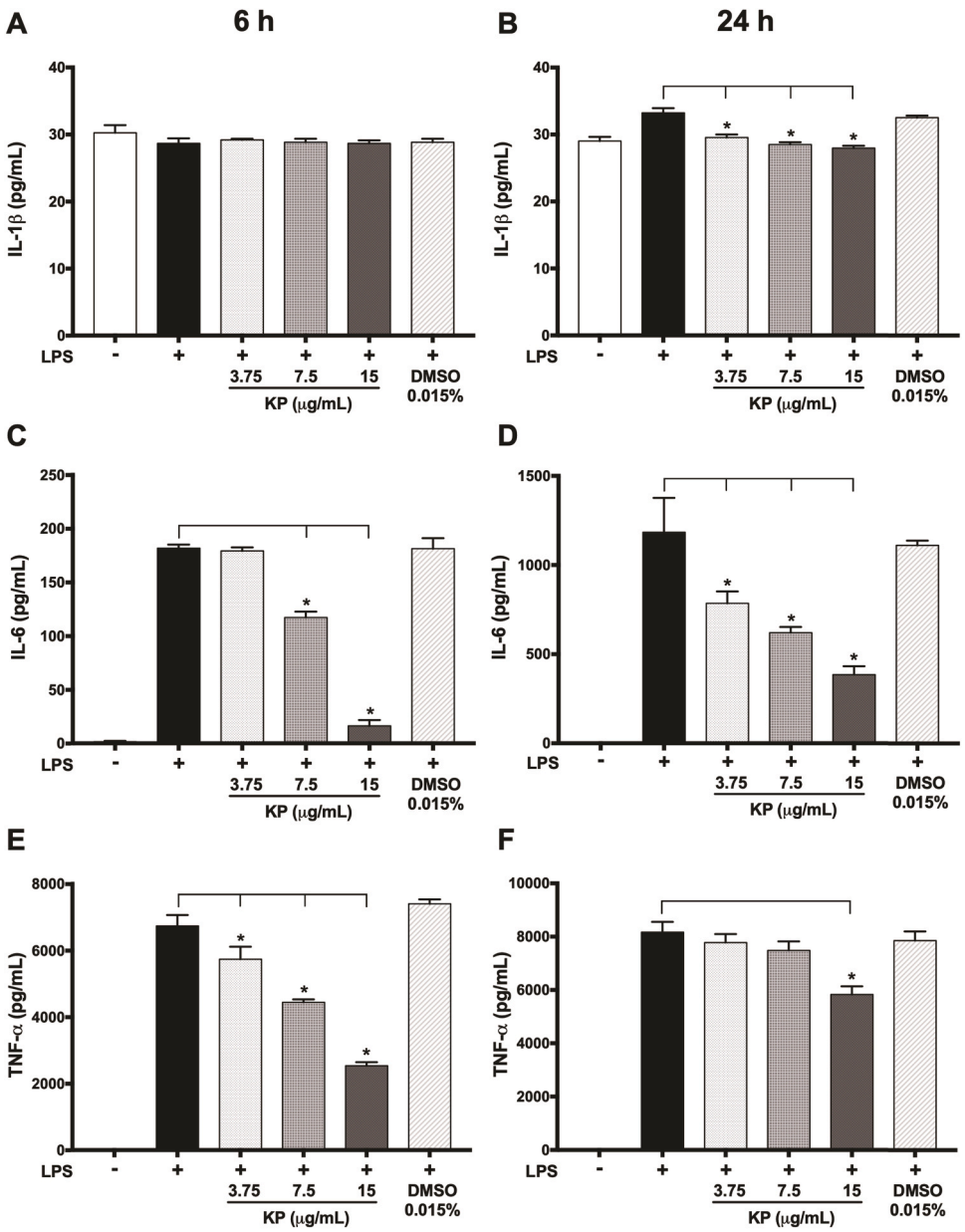

Fig. 5. Inhibitory effects of KP on LPS-induced production of IL-1β, IL-6, and TNF-α in RAW264.7 cells at 6 h and 24 h. Cells (1.5×10^6 cells/mL) were pre-treated with various concentrations (3.75, 7.5, and 15 μg/mL) of KP extract for 4 h and then incubated with LPS (1 μg/mL) for either 6 h or 24 h. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the LPS-treated group.

Furthermore, KP extract at 15 μg/mL could significantly decrease the production of COX-2 (approximately 30% reduction), compared to the LPS-treated cells (Fig. 7A and C).

3.8. The effects of KP on inhibiting keratinocyte migration

To explore the migratory ability, a scratch-wound assay was undertaken on HaCaT cells exposed to 15 μg/mL of KP treatment for 24 h and 48 h. To eliminate any confounding and variation in closure rate due to unequal of distance between wounded edges, wounding was repeated in many replicates and thoroughly chosen to ensure that all of them had similar initial gap width of approximately 500 μm. Our results found that HaCaT cells exposed to 15 μg/mL of KP exhibited significantly decelerated gap filling by 40% and 51% compared with untreated and DMSO-treated cells at 24 h and 48 h, respectively (Fig. 8).

3.9. The effects of KP on suppressing cell proliferation

The doubling time point was calculated using the number of HaCaT cells treated with KP at different time points. The time required for HaCaT cells to divide from one to two cells was approximately 24 h. Interestingly, the treatment of KP at 3.75, 7.5, and 15 μg/mL in HaCaT cells significantly increased cell doubling time to 51.62, 59.92, and 136.1 h, respectively (Fig. 9).

3.10. The effects of KP on anti-psoriatic inflammatory effects

Next, we sought to determine anti-psoriatic effects of KP on human keratinocytes in order to investigate the function of KP in T-helper 17 immune regulation. The production of key inflammatory cytokines, including IL-6, IL-17, IL-22, and IL-23 related to pathophysiology of psoriasis, were evaluated by ELISA as reported in Fig. 10. Overproduction of IL-6, IL-17, IL-22, and IL-23 has been detected in TNF-

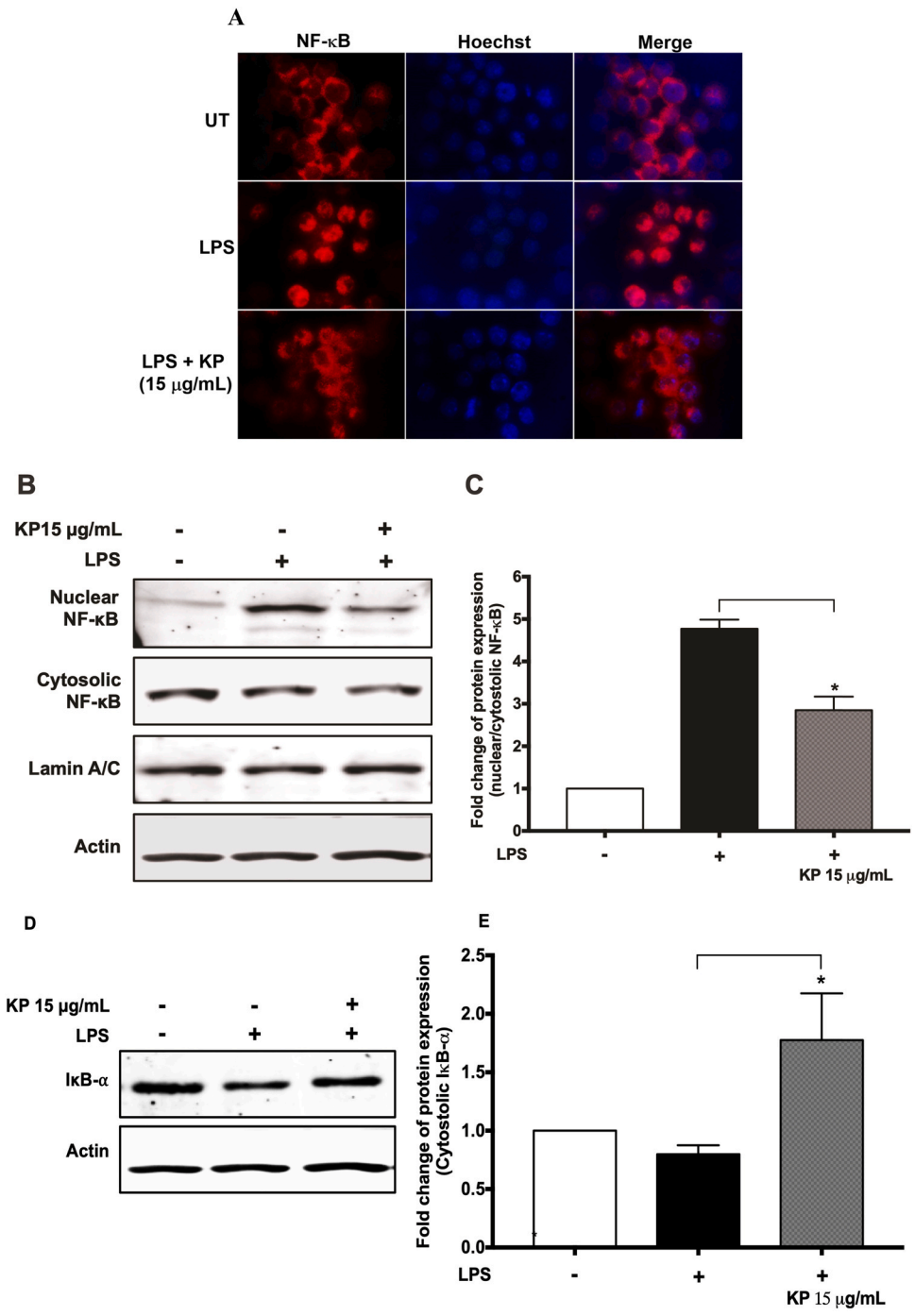

Fig. 6. Effects of KP on suppressing the upstream signaling for NF-κB activation in LPS-induced RAW264.7 cells. Cells were pretreated with 15 μg/mL of KP extract for 4 h, and then incubated with LPS (1 μg/mL) for 1 h. (A) Localization of NF-κB was visualized by a fluorescent microscope after staining for NF-κB (red). The nuclei of cells were stained with Hoechst 33342 (blue). (B, D) NF-κB and IκB-α in LPS-stimulated RAW264.7 cells by western blotting. Micrographs were captured with 40X magnification. (C, E) The quantification of relative band intensities from three independent experimental results was determined by densitometry. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the LPS-treated group.

α-stimulated HaCaT cells, however treatment with KP extract led to a marked reduction of these cytokines. Our results revealed that KP extract at 3.75, 7.5, and 15 μg/mL significantly suppressed production of IL-23 in the culture supernatants of TNF-α-induced HaCaT cells to approximately 2-fold, 2.5-fold, and 3-fold, respectively, compared with the TNF-α-induced HaCaT cells (Fig. 10D). Although 15 μg/mL of KP extract significantly inhibited TNF-α-induced IL-6, IL-17 and IL-22 production for approximately 2.5-fold, 1.5-fold, and 1.5-fold, respectively, KP extract at concentration of 3.75 and 7.5 μg/mL showed a decreasing trend, compared with the TNF-α-stimulated cells (Fig. 10A, B, and C).

Furthermore, we investigated the phosphorylation status of ERK1/2 kinase in KP-treated HaCaT cells by western blot. The data demonstrated that KP extract at 15 μg/mL significantly suppressed the activation of ERK1/2 signaling, as evidenced by approximately 1.5-fold reduction of the levels of pERK1/2 in both TNF-α- and EGF-induced HaCaT cells (Fig. 11). Therefore, the regulation of proliferative activity of KP extract is likely to be associated with the inhibition of the ERK1/2 signaling pathway.

4. Discussion

Psoriasis is a complicated inflammation with immune-related skin disease which is associated with a high degree of morbidity [2]. Most psoriatic patients have a reduced health-related quality of life and have profound psychosocial impact [44]. Inflammation is a biological response of immune system that has long been a well-known symptom leading to tissue damage and many common diseases, including

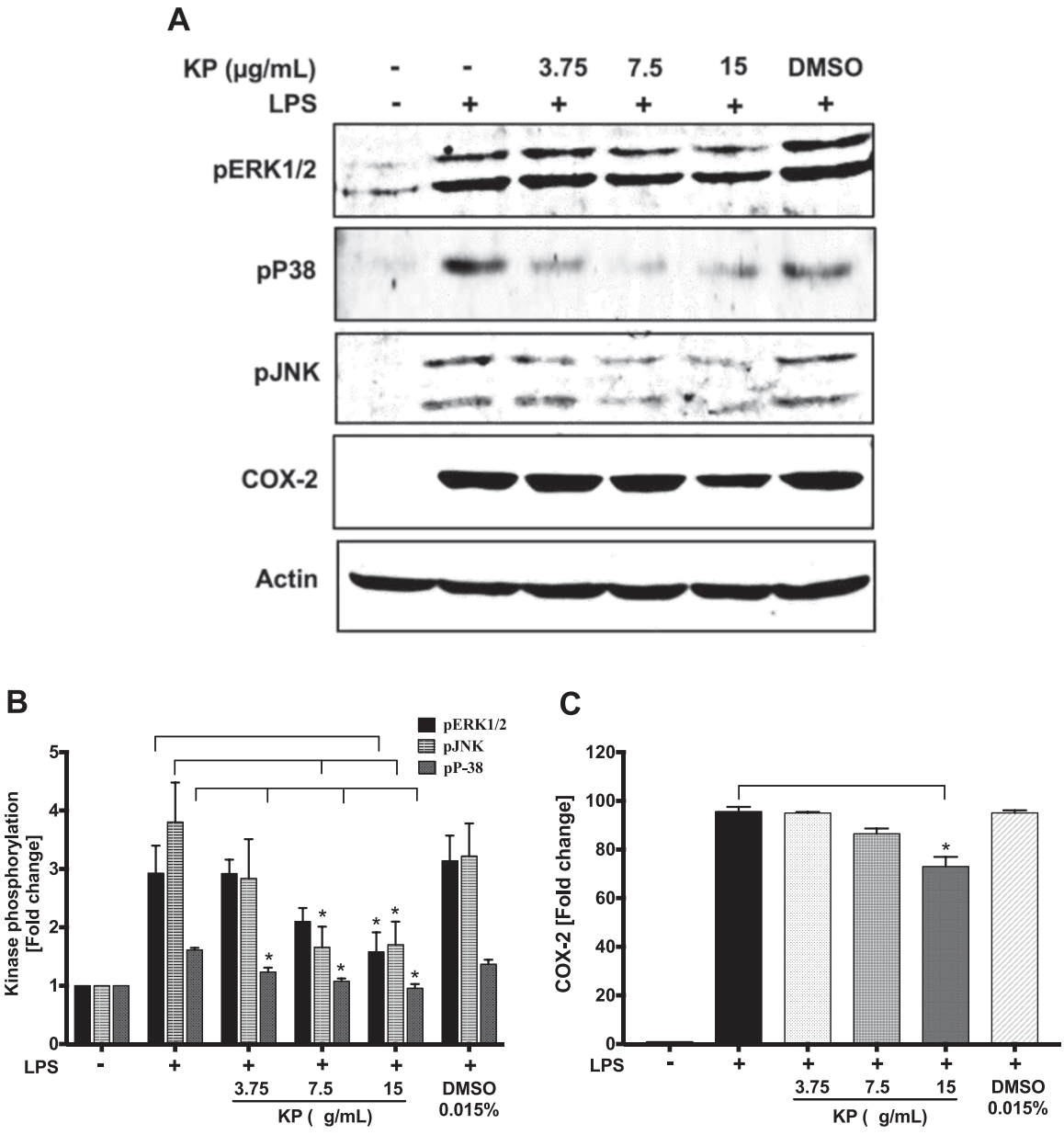

Fig. 7. Effects of KP on MAPK phosphorylation and COX-2 production in LPS-stimulated RAW264.7 cells. (A) The cellular proteins from the cells were used for the detection of phosphorylated or total forms of ERK1/2, p38, and JNK MAPKs. (B and C) The quantification of relative band intensities from three independent experimental results was determined by densitometry. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the LPS-treated group.

psoriasis [45]. Thus, conventional steroidal and immunosuppressant drugs have been used to slow down the inflammatory process with the subsequent potential risk of adverse reactions [46]. This opens new possibilities for an alternative therapy by using folklore medicine from plants and herbs to block the inflammatory processes with minimum or null side effects [47]. Several different medicinal plants and their active compounds have been claimed to have potent anti-inflammatory effects with potential mechanisms of actions identified [18,20]. Some plants exhibited cell proliferation promoting activities [48,49] while many of them demonstrated anti-proliferative effects [24,26,50,51]. *Kaempferia parviflora* extract is one of alternative medicines that has been reported to possess anti-inflammatory activities [21]. However, the anti-psoriatic effects of KP on TNF-α-induced HaCaT cells and anti-inflammatory effects of KP on LPS-induced RAW264.7 cells in other aspects, in particular the suppression of NF-κB activation, are not available.

In this study, we investigated the anti-inflammatory and anti-psoriatic effects of KP on macrophage cell line, RAW264.7, and human keratinocyte cell line, HaCaT. The results showed that KP extract decreased the cell viability of macrophages and keratinocytes in a concentration-dependent manner. The clinical presentation of psoriatic patients manifested as acanthosis, erythematous oval plaques with adherent silvery scales resulting from the hyperproliferation of keratinocytes [52]. Furthermore, evidence shows that the migratory effect of epidermal keratinocytes could stimulate wound re-epithelialization and recapitulate psoriatic plaques [53]. Therefore, it could be advantageous for psoriasis therapy, if keratinocyte proliferation and migration are inhibited [54]. We demonstrated that KP extract inhibited keratinocyte proliferation and migration. Our results are consistent with previous studies showing that methoxyflavonoids isolated from KP reduced cell viability in LPS-activated RAW264.7 cells [36]. Moreover, it has been reported that KP extract inhibited proliferation of JB6 P^{+} and HaCaT cells [55].

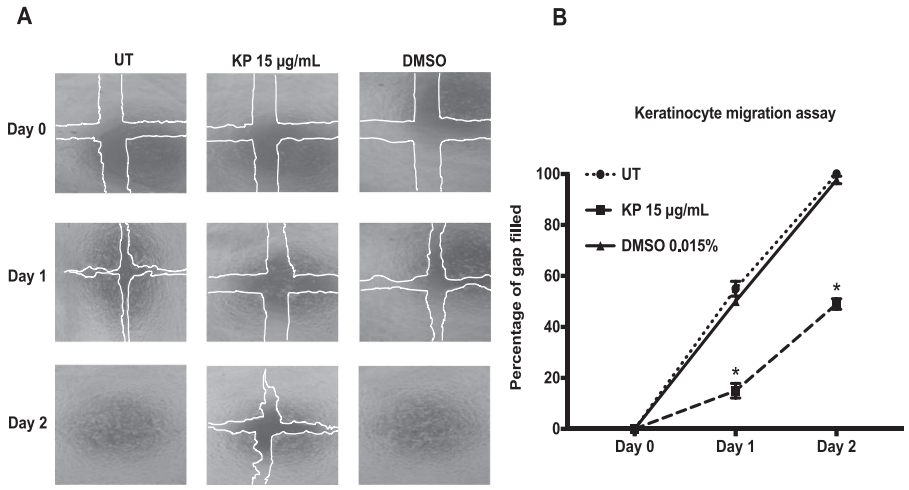

Fig. 8. (A) Microscopic pictures of HaCaT keratinocyte migration assay; and (B) graphical analysis of the effect of KP on keratinocyte migration (the percentage of gap filled). The cells treated with 15 µg/mL of KP were compared with the untreated cells and DMSO-treated-cells. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the untreated group.

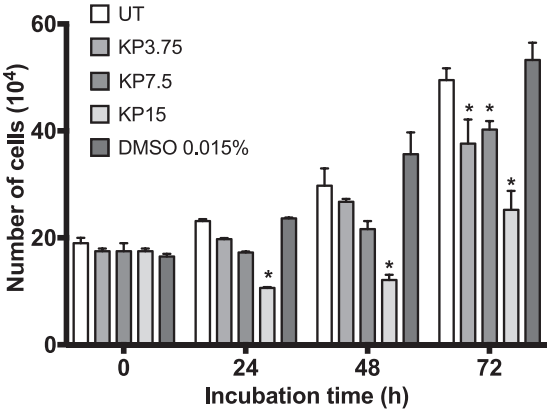

Fig. 9. The number of HaCaT keratinocyte cells treated with non-toxic concentrations of KP (3.75, 7.5, and 15 µg/mL) compared with the untreated cells and DMSO-treated-cells at 0, 24, 48, and 72 h. Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the LPS-treated group.

The pathophysiology of psoriasis involves key cytokines, including IL-23 that acts through signal transduction pathways and transcriptional regulation in the control of T-helper 17 differentiation, thereby producing IL-17, IL-22, TNF-α [2,4]. During the inflammation process, the major functions of macrophages are antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors [56], including NO, TNF-α, IL-1, and IL-6 [57]. NO, which is regulated by iNOS, is a potent reactive factor in inflammatory responses found in the stimulated macrophages and in the sites of inflammation [12]. Additionally, COX-2 is an inflammation-induced enzyme that is highly expressed at the sites of inflammation [58]. It was suggested that iNOS and COX-2 play an important role in inflammation. All of these molecular players, in concert, may induce recruitment of further excessive inflammatory cells, resulting in triggering of acute generalized inflammatory response characteristic of septic shock and multi-organ failure [59]. For this reason, therapeutic interventions targeted macrophages and their products could open new avenues for anti-inflammatory and anti-psoriatic treatment. In the present study, we showed that KP extract inhibited the gene expression of iNOS, IL-1β, IL-6, TNF-α, and COX-2 in LPS-activated macrophages. These data suggest that KP acts principally by suppressing iNOS, IL-1β, IL-6, TNF-α, and COX-2 in a concentration-dependent manner through regulation of gene transcription in activated macrophages. Our observations are similar to those reported in 2009 where the crude ethanol extract of KP and its compound (5,5-hydroxy-3,7,3′,4′-tetramethoxyflavone) inhibited PGE2 release and suppressed mRNA expression of iNOS, whereas COX-2 mRNA expression was partly affected [34].

It is well known that NF-κB plays an important role in the control of the gene encoding pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α as well as inducible enzymes, including iNOS and COX-2 [60]. In addition, activation of NF-κB stimulates phosphorylation, ubiquitination, and subsequent degradation of IκB-α by specific IκB kinases. This allows the free NF-κB p65-p50 heterodimer to translocate into the nucleus and upregulate the expression of pro-inflammatory genes [61]. Since we observed that KP decreased the expression of NF-κB downstream pro-inflammatory genes, it is reasonable to hypothesize that KP may interfere with the activation of NF-κB signaling. Therefore, we examined the effect of KP on inhibiting LPS-induced degradation of IκB-α protein and inhibiting LPS-induced translocation of NF-κB into the nucleus. Our results showed that LPS treatment caused a drastic decrease of NF-κB in the cytoplasm and an increase in the nucleus, but this effect could be reversed in a concentration-dependent manner by pre-treatment with KP extract. The present study suggests that KP inhibits LPS-induced NF-κB activation, in part through the suppression of degradation of IκB-α protein, that leads to the retention of NF-κB in the cytoplasm of RAW264.7 macrophages. Our results are in line with a recent study showing that 5,7-dimethoxyflavone regulates iNOS and NF-κB expression in *P.acnes*-stimulated human keratinocyte (HaCaT) cells as well as down-regulates phosphorylated IκB-α and nuclear translocation of NF-κB p65 in LPS-stimulated RAW264.7 cells [62]. In addition, it has been reported that the expression of NF-κB and COX-2 decreased by the treatment with KP extract in H_2O_2-induced senescent human dermal fibroblasts (Hs68) cells [63], whereas Lee et al. found that KP extract suppressed NF-κB transcriptional activity in 23 kJ/cm^2 of solar wavelengths (sUV)-induced JB6 P^+ cells [55]. These findings indicate the potential role of KP in inhibiting NF-κB activation and suppressing the production of nitric oxide and pro-inflammatory cytokines in activated macrophages. We further elucidated specific inflammatory signal transduction pathways for which KP may be able to interfere.

It is well known that MAPKs, which are highly conserved protein serine/threonine kinases, are responsible for the regulation of inflammatory mediator production. Upon LPS stimulation, the phosphorylation of JNK, ERK, and p38 MAPKs is activated, and these phosphorylated kinases play crucial roles in controlling the expression of cell survival genes and proinflammatory cytokines, including iNOS, IL-1β, IL-6, TNF-

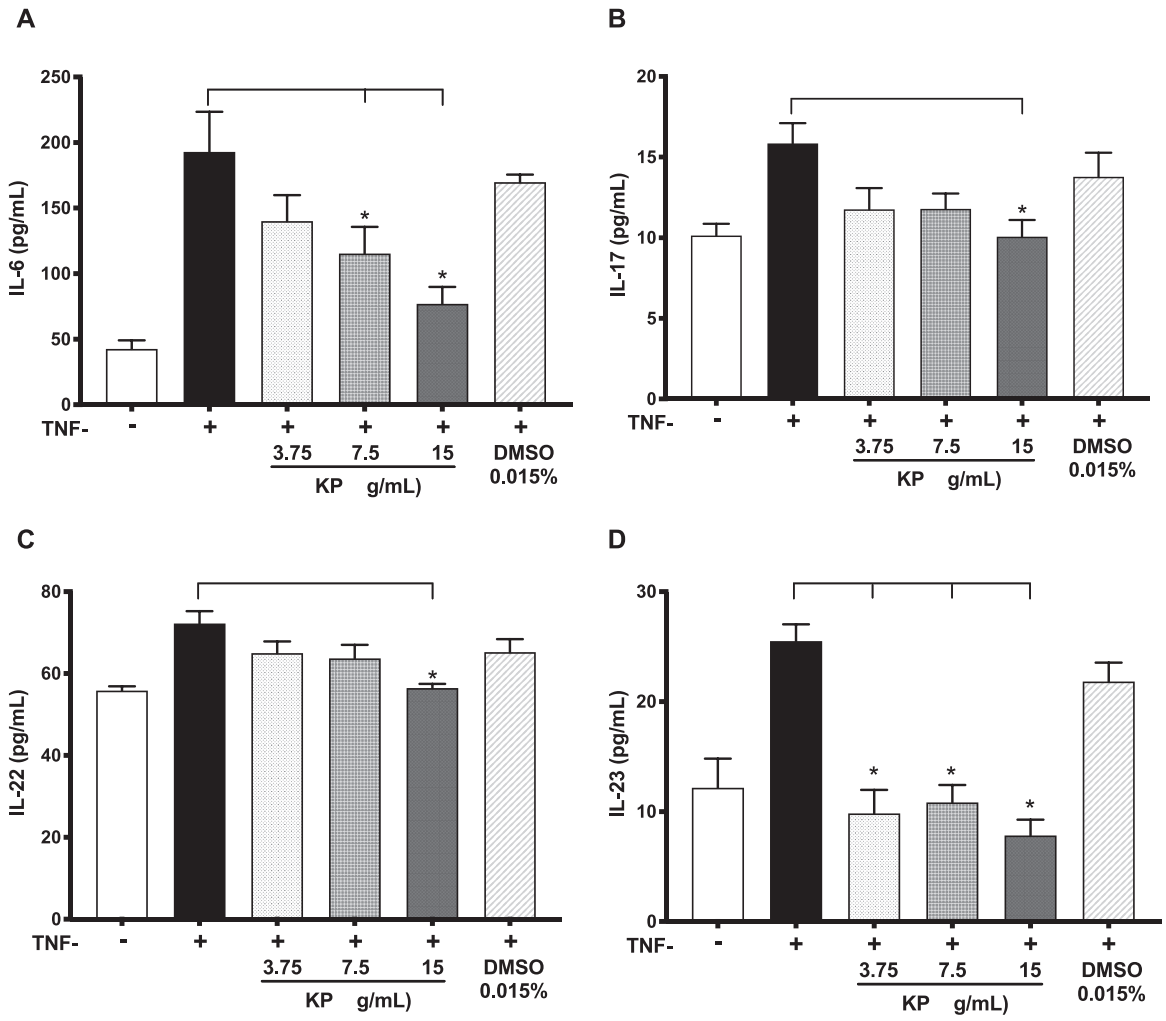

Fig. 10. Inhibitory effects of KP on TNF-α-induced production of IL-6, IL-17, IL-22 and IL-23 in HaCaT cells at 24 h. Cells (1.5 × 106 cells/mL) were pre-treated with various concentrations (3.75, 7.5, and 15 μg/mL) of KP extract for 4 h and then incubated with TNF-α (20 ng/mL) for 24 h. Data are presented as mean ± SEM of three independent experiments. $^{*}p < 0.05$ significant compared with the TNF-α-treated group.

α, and COX-2 [64]. Therefore, we investigated the effects of KP on MAPK phosphorylation. Our data showed that pre-treatment with KP extract significantly inhibited LPS-stimulated phosphorylation of ERK1/2, JNK, and p38 in a dose-dependent manner. Similarly, KP extract also had significant inhibition of TNF-α-induced phosphorylation of ERK1/2 pathway in human keratinocytes. These results clearly provide accumulated evidence that phosphorylation of ERK1/2, JNK, and p38 was potently inhibited by KP. However, the degree of inhibition was different for each kinase, with the maximum inhibitory effect exerted on JNK. These results indicate that signal transduction pathways mediated by ERK1/2, JNK, and p38 are effectively blocked by KP in activated macrophages. Recently, a report showed that inhibition of JNK phosphorylation, but not ERK, reduced LPS-stimulated NO production [65]. On the other hand, previous study by Hwang et. (2010) demonstrated that the inhibition of ERK and p-38 phosphorylation, but not JNK, decreased LPS-stimulated NO production [66]. Moreover, recent studies revealed that the compounds isolated from KP (5,7-dimethoxyflavone, trimethylapigenin, and tetramethylluteolin) showed inhibitory effects on nitric oxide (NO), inducible nitric oxide synthase (iNOS), PGE2, and TNF-α release in LPS-induced RAW264.7 cells through activation of spleen tyrosine kinase pathway but not ERK and JNK pathways [34–36]. Nevertheless, how KP regulates the kinase-substrate events upstream the MAPK and NF-κB signal transductions is not known. To define the accurate molecular mechanisms of action of KP, it requires structural manipulations that synthesize the orthogonal kinase-substrate pair as an effective tool to probe the functional consequences of specific phosphorylation events in living cells [67]. Based on our finding that KP could suppress the activation of both NF-κB and MAPK signaling pathways as well as the downstream pro-inflammatory mediators at the gene and protein levels, we strongly believe that KP possesses its authentic anti-inflammation and anti-psoriasis.

5. Conclusion

This study provided accumulated evidence that *Kaempferia parviflora* extract possesses anti-inflammatory and anti-psoriatic activities including the inhibition of pro-inflammatory cytokine production of IL-6, IL-17, IL-22, and IL-23 in TNF-α-induced keratinocytes, and inhibition of gene expression of NO, iNOS, IL-1β, IL-6, TNF-α, and COX-2 in LPS-stimulated macrophages. Moreover, KP exhibited inhibitory property in the proliferation and migration of HaCaT cells. This inhibitory process was proved to be regulated through the signal transduction pathways of NF-κB and MAPKs including ERK1/2, p38, and JNK in LPS-induced RAW264.7 cell and ERK1/2 in TNF-α-induced keratinocytes. Although further investigation is required to validate the detailed mechanism involved, our report strongly supports the traditional use of *Kaempferia parviflora* and suggests that this plant is a promising candidate for the development of novel anti-inflammatory and anti-psoriatic agents.

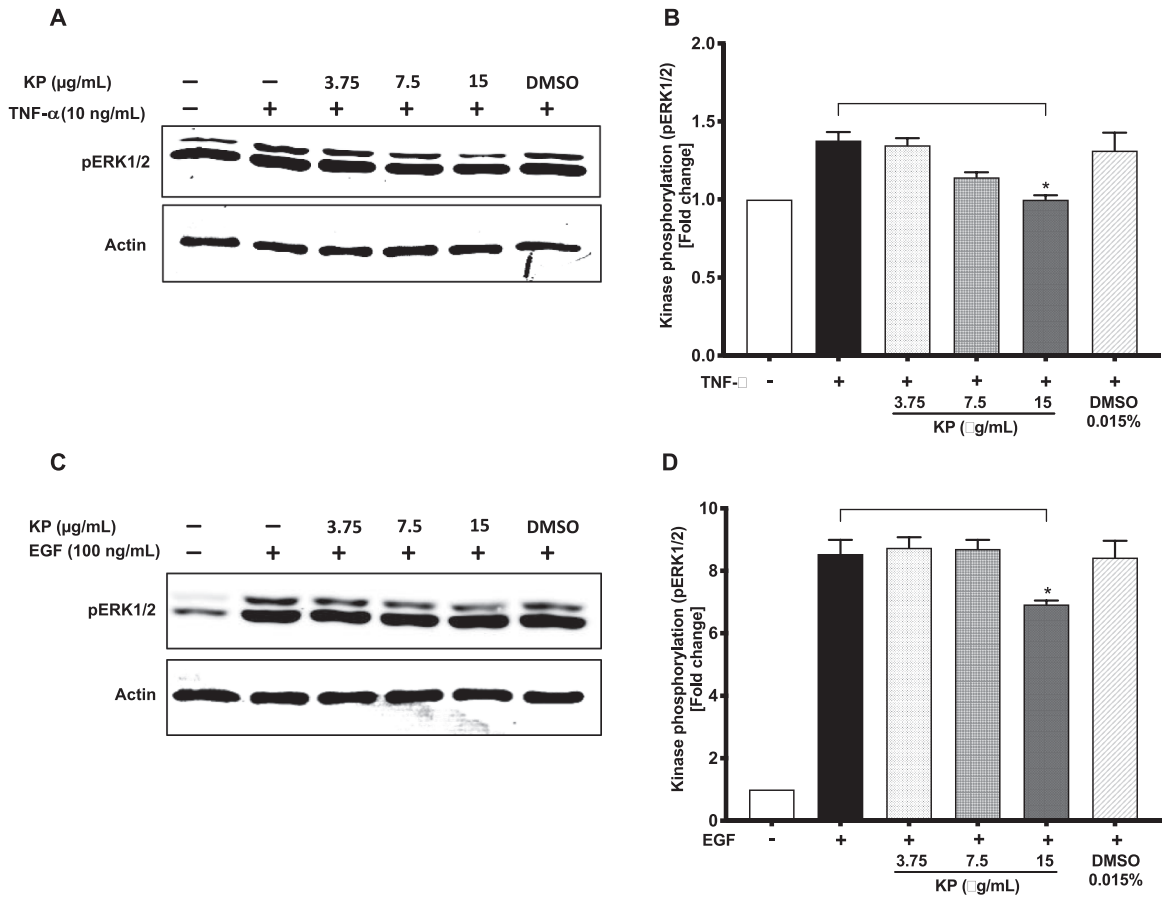

Fig. 11. Effects of KP on MAPK phosphorylation in TNF-α- and EGF-stimulated HaCaT cells. (A) The cellular proteins from the cells were used for the detection of phosphorylated or total forms of ERK1/2 (A and C). The quantification of relative band intensities from three independent experimental results was determined by densitometry (B and D). Data are presented as mean ± SEM of three independent experiments. *$p < 0.05$ significant compared with the either TNF-α- or EGF-stimulated group.

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Author Contributions

The experiments, data analysis, and manuscript writing were conducted by MNT and WN. SP, SS, TJ, PM, and KC performed experiments. NW performed data analysis, provided technical supports, and revised the manuscript. WN founded the research project, designed the experiments, and contributed to the funding of the project.

CRediT authorship contribution statement

Wutigri Nimalmool: Conceptualization, Methodology, Investigation, Writing – review & editing, Project administration, Funding acquisition. **Mingkwan Na. Takuathung:** Investigation, Writing – original draft. **Saranyapin Potikanond:** Investigation. **Siriwoot Sookkhee:** Resources. **Pitchaya Mungkornasawakul:** Resources. **Thanathorn Jearanaikulvanich:** Investigation. **Kittinan Chinda:** Investigation. **Nitwara Wikan:** Resources, Writing – review & editing.

Supplementary Material

Supplementary materials are available upon request.

Conflict of interest statement

The authors declare that they have no competing interests.

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