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Research article

Chlorogenic acid rich in coffee pulp extract suppresses inflammatory status by inhibiting the p38, MAPK, and NF-κB pathways

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ABSTRACT

Coffee pulp (CP) is a coffee byproduct that contains various active ingredients, namely, chlorogenic acid (CGA) and caffeine. These active compounds show several benefits, including anti-hyperlipidemia, antioxidants, and anti-inflammation. However, the anti-inflammatory properties of Coffea pulp extract (CPE) are unknown. This work determined the impact of CPE on lipopolysaccharide (LPS)-activated murine macrophage cells and the molecular mechanism behind this action. RAW 264.7 cells were exposed to varying doses of CPE with or without LPS. Inflammatory markers and their mechanism were studied. CPE therapy has been shown to suppress the synthesis of inflammatory cytokines and mediators, namely, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and nitric oxide (NO), as well as prostaglandin E2 (PGE_2). Finally, CPE inactivated the nuclear factor-kappa B (NF-κB) and MAPK signaling pathways. Consequently, CPE might be used as a nutraceutical to treat inflammation and its related disorders.

1. Introduction

Pathogens, trauma, and irritants cause inflammation, which the body uses as a defensive mechanism. The response is defined by the production of proinflammatory cytokines and other inflammatory markers [1]. Macrophages mainly secrete cytokines. As the macrophage cell membrane is activated, the Toll-like receptor (TLR) complex attracts signaling proteins, such as IFN-γ receptor-associated kinase (IRAK), into the cytoplasm. Phosphorylated IRAK degrades the nuclear factor-kappa B (NF-κB) inhibitor (IκB). NF-κB then translocates into the nucleus and generates proinflammatory markers, including cytokines and markers, interleukin-6 (IL-6), IL-1β, tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE_2), and nitric oxide (NO) [2,3]. Currently, nonsteroidal anti-inflammatory drugs have been used as anti-inflammatory drugs. However, these drugs have side effects on several organ systems, such as the gastrointestinal, renal, and cardiovascular systems [4,5]. Thus, this research aims to

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find more effective methods for preventing and treating inflammation and its complications.

Coffee pulp (CP) showed high antioxidant, antimicrobial, and lipid-lowering effects *in vitro* and *in vivo* [6–9]. Moreover, CP has four significant constituents: chlorogenic acid, caffeine, epicatechin, and catechin [7]. Many medical benefits are associated with chlorogenic acid (CGA), including antioxidation, inflammation prevention, weight loss, diabetes prevention, and cancer prevention [10,11]. In addition, CGA scavenged superoxide anions in an *in vitro* model and drastically decreased iron-induced lipid peroxidation in bovine liver microsomes and temporary middle cerebral arterial occlusion (MCAo) [12,13]. CGA also protects against oxidative stress-related diseases induced by heavy metal exposure [14,15]. Furthermore, caffeine significantly lowers plasma lipid peroxidation and glucose levels in streptozotocin-treated rats; it also increases glutathione levels in cardiac, renal, and hepatic tissues, increasing the enzyme activities of glutathione peroxidase, superoxide dismutase, and catalase [16]. The underlying mechanisms and anti-inflammatory activities of CP extract (CPE) remain unknown. This work aimed to determine whether CPE had any anti-inflammatory actions on LPS-induced inflammatory RAW264.7 cells. Furthermore, the molecular mechanisms that cause inflammation were studied.

2. Materials and methods

2.1. Chemicals

Merck provided lipopolysaccharides, nicotinamide adenine dinucleotide phosphate, and lipopolysaccharide (Darmstadt, Germany). Thermo Fisher Scientific provided the PrestoBlue cell viability reagent (Waltham, MA, USA), and BioLegend provided the ELISA test kits (San Diego, CA, USA). Commercial sources were used to obtain all extra chemicals with a high purity level.

2.2. CPE preparation

Hillkoff (Chiang Mai, Thailand) kindly provided dried CP, and a voucher herbarium specimen (no. NU003806) was deposited at Naresuan University, Phitsanulok, Thailand. We weighed and thoroughly mixed 100 g of dry CP, followed by a 10-min infusion in 100 °C water and three times filtering through filter paper. The solution was lyophilized using a lyophilizer (Labconco, MO, USA).

2.3. CPE chemical characterization

The chemical constituents of CPE, CGA, and caffeine were used as a reference for validation and quantitation by HPLC with diode array detection on an Agilent 1200 series (LabX, Midland, Canada) at the Science and Technology Service Center, Faculty of Science, Chiang Mai University (STSC-CMU), according to the ISO/IEC 17025 method; International Organization for Standardization, 2005. The flow rate was 0.6 mL/min. The mobile phase was a binary solvent system consisting of 2% acetic acid dissolved in HPLC water (solvent A) and absolute methanol (solvent B). The column used was a Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 μm), and the absorption wavelength was selected at 327 nm for chlorogenic acid and 280 nm for caffeine. The HPLC chromatogram, calibration curve parameters, retention time, regression equation, and correlation coefficient (r^2) were recorded. Active compounds found in CPE were detected relative to the corresponding peaks of references, and the concentration of each component was subsequently quantified by peak area under the curve relative to the reference compound.

2.4. Cell culture

The American Type Culture Collection provided the RAW264.7 cell line (ATCC, Rockefeller, MD, USA). Cells at passages $^{2–22}$ were subcultured every 4–5 days in DMEM with 3.7 g/L NaHCO3 supplemented with 1*X* antibiotic-antimycotic at 37 °C and 5% CO_2. Cells were plated at 1 × 10^5 cells/mL and cultivated for two days before use in the following studies.

2.5. Cell viability determination

For 24 h, RAW264.7 cells were cultured in serum-free medium with or without CPE at concentrations ranging from 0.1 to 1000 μg/mL or its key ingredients at the molar ratios found in 1000 mg/mL CPE: CGA (13.38 μg/mL) and caffeine (3.82 μg/mL), polymyxin B sulfate; a TLR4 antagonist (10 μg/mL) as well as a nonsteroidal anti-inflammatory drug, celecoxib (CX) at 3.8 μg/mL. On the day of the experiment, the test compounds were removed, and a PrestoBlue solution (10% in free medium without phenol red) was added to each well before incubating the plates at 37 °C for 4 h. The viability was determined using a SynergyTM HT (Biotek, VT, USA) equipped with 560/590 nm excitation/emission wavelengths.

2.6. Determination of inflammatory cytokine and mediator levels

ELISA was used to determine the impact of CPE on the production of proinflammatory cytokines. For 24 h at 37 °C and 5% CO_2, cells were supplemented with CPE ranging from 0.1 to 1000 μg/mL, CGA (13.38 μg/mL) and caffeine (3.82 μg/mL), polymyxin B sulfate (10 μg/mL), and CX (3.8 μg/mL) with or without LPS at 1 μg/mL. RAW264.7 cell supernatants were centrifuged at 2000×*g* for 10 min at 4 °C. IL-6, IL-1β, TNF-α, and PGE_2 were analyzed using ELISA kits (BioLegend, CA, USA).

Table 1
Primer sequences and expected amplicon sizes for gene amplification.

cDNA	Genbank Acc. No.	Forward primer	Reverse primer	Amplicon size (bp)
TNF-α	NM013693.3	5′-ACCTGGCCTCTCTACCTTGT-3′	5′-CCCGTAGGGCGATTACAGTC-3′	161
IL-1β	NM008361.4	5′-GCCACCTTTTGACAGTGATGAG-3′	5′-AGTGATACTGCCTGCCTGAAG-3′	165
IL-6	NM031168.2	5′-CAACGATGATGCACTTGCAGA-3′	5′-TCTCTCTGAAGGACTCTGGCT-3′	201
COX-2	NM011198.4	5′-CCACTTCAAGGGAGTCTGGA-3′	5′-AGTCATCTGCTACGGGAGGA-3′	197
iNOS	NM010927.4	5′-GGAGCCTTTAGACCTCAACAGA-3′	5′-TGAACGAGGAGGGTGGTG-3′	123
GAPDH	NM001289726.1	5′- TGTGTCCGTCGTGGATCTGA-3′	5′- TTGCTGTTGAAGTCGCAGGAG-3′	150

TNF-α, *Tumor necrosis factor alpha*; IL-1β, Interleukin 1 beta; IL-6, *Interleukin-6*; COX-2, C*yclooxygenase-2*; iNOS, *Inducible nitric oxide synthase;* GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

2.7. Nitric oxide level evaluation

The nitric oxide (NO) concentration was evaluated using the Griess test kit (Cayman Chemical Company, MI, USA). RAW264.7 cells were treated with 1000 μg/mL CPE, CGA (13.38 μg/mL) and caffeine (3.82 μg/mL), polymyxin B sulfate (10 μg/mL), and CX (3.8 μg/mL) with or without 1 μg/mL LPS for 24 h. The cell supernatants were centrifuged at 10,000 g at 4 °C for 20 min. The supernatant was used to determine the NO level at 540 nm wavelength using a SynergyTM HT (Biotek, VT, USA).

2.8. Determination of the expression of proinflammatory genes

RAW264.7 cells were extracted, and total RNA was purified using TRIzol® reagent, and cDNA was reverse transcribed using the SensiFASTTM reverse transcription kit (Bioline, TN, USA). After that, qPCR was performed using QIAquant equipment (Hilden, Germany) and SYBR qPCR Master Mix (Bioline, Tennessee, USA). Macrogen (South Korea) provided the qPCR primers, which were utilized at 0.4 M. The primer sequences for IL-6, IL-1β, TNF-α, COX2, iNOS, and GAPDH in mice are listed in Table 1 [17–19]. Normalized to GAPDH, gene expression was represented as fold changes relative to GAPDH.

2.9. Detection of NF-κB activation

The activation of NF-κB was determined using immunofluorescence labeling. For 24 h, RAW264.7 cells were treated with 1000 μg/mL CPE, CGA (13.38 μg/mL), caffeine (3.82 μg/mL), polymyxin B sulfate (PMB) (10 μg/mL), and CX (3.8 μg/mL). Cells were then stimulated with 1 μg/mL LPS for 30 min [20]. The treated cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 1% BSA. RAW264.7 cells were incubated with the p65-NF-κB antibody at 4 °C overnight and then incubated for 1 h with goat anti-rabbit IgG (Alexa Fluor® 488) (Invitrogen, MA, USA). After staining the cells for 5 min with DAPI, images were captured using a Nikon Eclipse Ni–U fluorescence microscope (original magnification 40) (Nikon Corporation, Japan).

2.10. Expression of MAPK pathway and p65-NF-κB proteins

For 24 h, RAW264.7 cells were treated with 1000 μg/mL CPE, CGA (13.38 μg/mL), caffeine (3.82 μg/mL), polymyxin B sulfate (PMB) (10 μg/mL), and CX (3.8 μg/mL). Cells were then stimulated with 1 μg/mL LPS for 15 min [21]. RAW264.7 cells were homogenized in cell lysis solution with a protease inhibitor and centrifuged for 10 min at 5000 g at 4 °C. The pellet was then resuspended in cell lysis solution and centrifuged for 10 min at 10,000 g at 4 °C to be designated the nuclear fraction. The Bradford test was used to assess the protein level of each sample (Bio-Rad Laboratories, Inc.). Western blotting was electrophoresed and transferred to PVDF membranes (Merck KGaA), followed by overnight incubation with polyclonal anti-rabbit JNK, phosphorylated JNK, p38, phosphorylated p38, ERK, phosphorylated ERK, p65-NF-κB or anti-mouse β-actin antibodies. Following incubation for 1 h with goat anti-rabbit or anti-mouse IgG (Merck KGaA). The Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.) was used to identify proteins. The ImageJ 1.44p application from the National Institutes of Health's Research Services Branch was used to quantify them.

2.11. Statistical analysis

The data are shown using the mean ± SEM. The statistical analyses were completed using SPSS version 23 (IBM Corp.). One-way ANOVA was used to assess differences between different groups, followed by Dunnett's test. A difference of $P < 0.05$ was deemed statistically significant.

3. Results

3.1. Active components in CPE

The HPLC chromatogram of CPE reliably detected chlorogenic acid and caffeine with retention times of 9.913 and 11.701, respectively (Fig. 1A). The amount of each significant constituent found in CPE is shown in Table 2. A high content of CGA was obtained (13.38 ± 1.42 mg/g) compared to other phenolic compounds (Fig. 1B). In addition, caffeine, a significant component in the

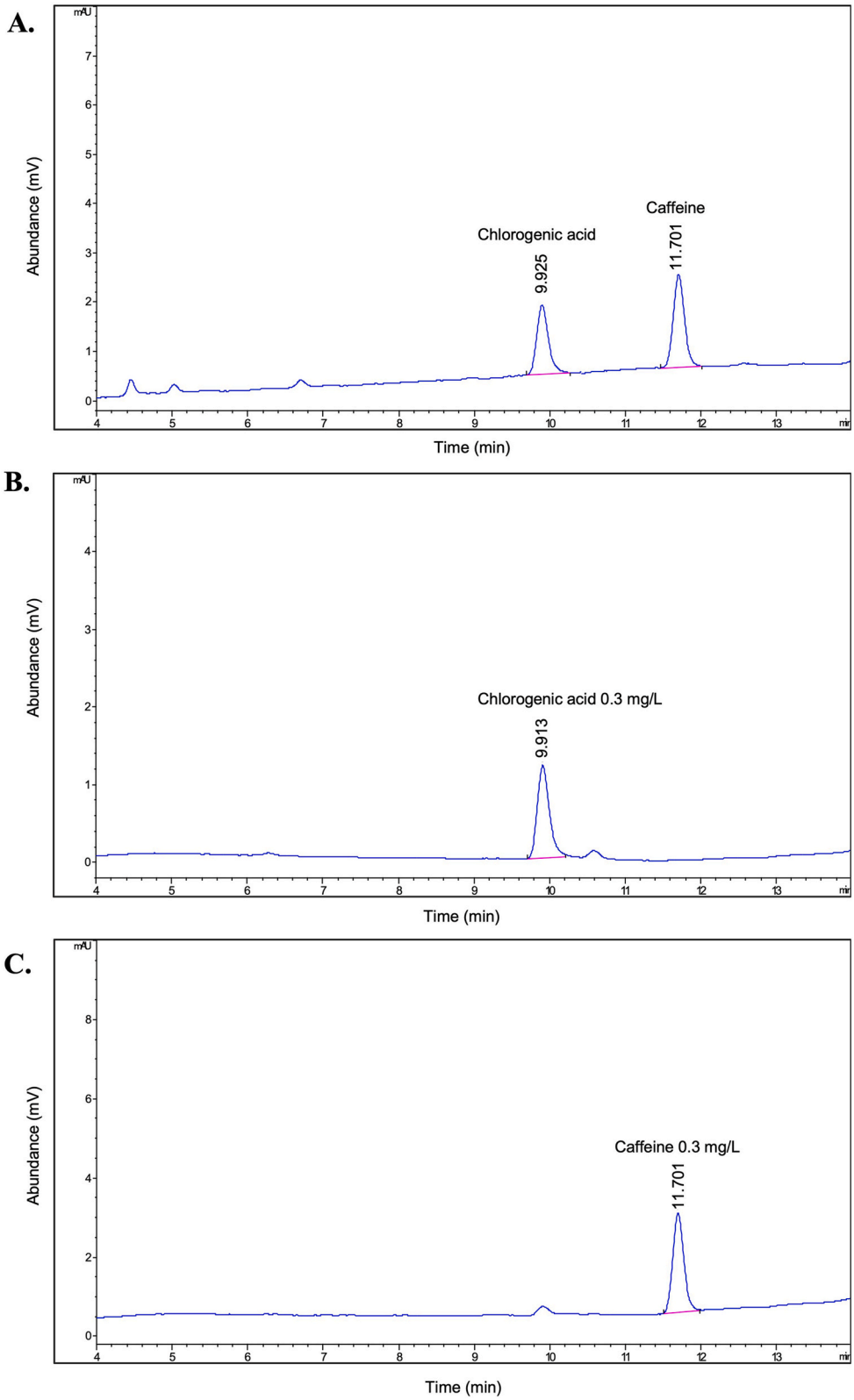

Fig. 1. A representative HPLC chromatogram of (A) Coffea pulp aqueous extract (CPE), (B) chlorogenic acid standard and (C) caffeine standard at an absorption wavelength of 280 nm.

Table 2
The amount of each significant constituent found in CPE.

Phenolic compound	Amount (mg/g of CPE)
Chlorogenic acid (CGA)	13.38 ± 1.42
Caffeine	3.82 ± 0.19

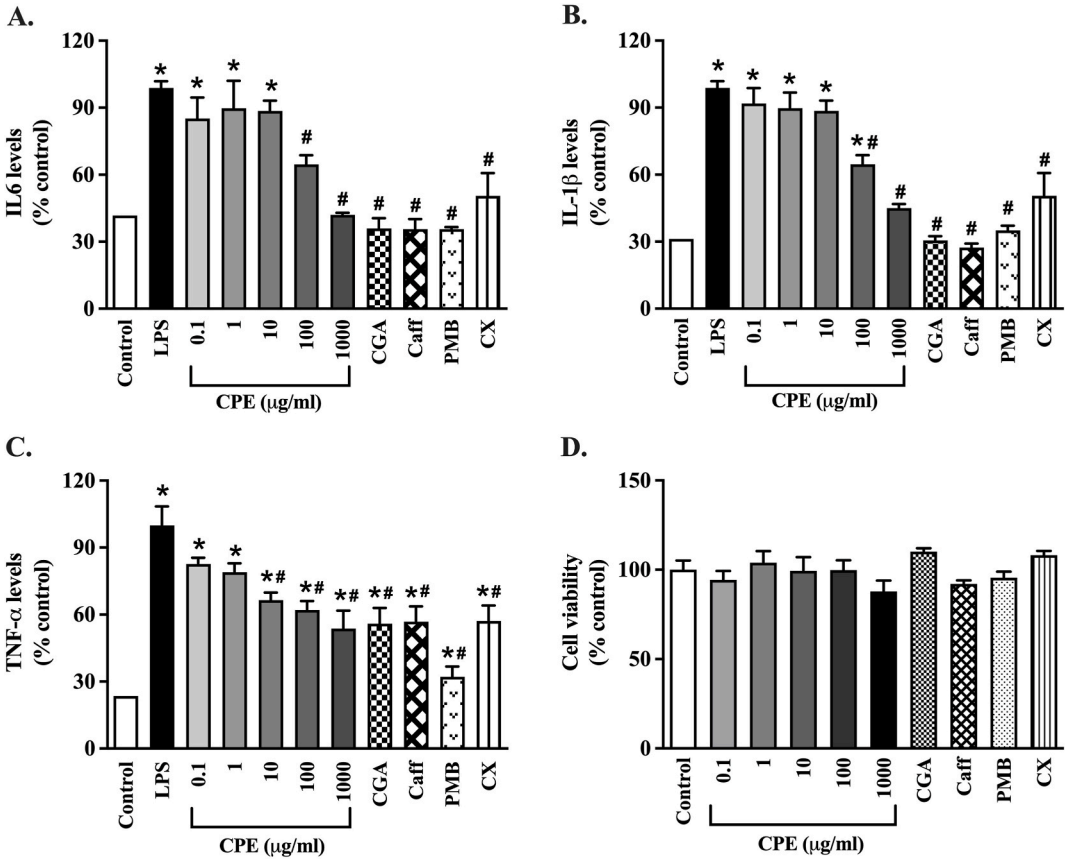

Fig. 2. The effects of CPE on inflammatory cytokines in LPS-stimulated RAW 264.7 cells. Cells were pretreated with 0.1–1000 μg/mL CPE or its major constituents at the molar ratios present in 1000 μg/mL CPE: 13.38 μg/mL CGA and 3.82 μg/mL caffeine, 10 μg/mL PMB, and 3.8 μg/mL CX for 24 h. The production of (A) IL-6, (B) IL-1β, and (C) *TNF-α* was measured via ELISA. (D) Viability of RAW 264.7 cells following exposure to 0.1–1000 μg/mL CPE for 24 h. Data are presented as the mean ± standard error of the mean (n = 5), *$p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. LPS.

coffee pulp, was found at 3.82 ± 0.19 mg/g CPE (Fig. 1C).

3.2. CPE suppresses proinflammatory cytokine production in RAW264.7 cells

The effect of CPE and its critical components on inflammatory cytokine production in LPS-activated macrophage cells was evaluated using ELISA. The results demonstrated that whereas LPS at a dose of 1 μg/mL significantly increased proinflammatory cytokine levels in RAW264.7 cells, CPE at 100 and 1000 μg/mL concentrations significantly decreased IL-6 and IL-1β production when compared to LPS alone (Fig. 2A and B). Additionally, CPE at doses of 10–1000 μg/mL reduced the generation of TNF-α in inflammatory cells activated by LPS (Fig. 2C). Similarly, CPE, CGA, and caffeine components significantly decreased IL-6, IL-1β, and TNF-α levels. Moreover, PMB, a TLR4 antagonist, decreased the levels of these three cytokines. Again, CX at 3.8 μg/mL significantly reduced IL-6, IL-1β, and TNF-α production. However, compared to control cells, CPE (0.1–1000 μg/mL), CGA, caffeine, PMB, and CX had no significant impact on RAW 264.7 cell viability (Fig. 2D). These results suggested that CPE and its effective ingredients suppressed proinflammatory cytokine production without cytotoxicity.

3.3. CPE suppresses proinflammatory cytokine mRNA expression in RAW 264.7 cells

This study performed real-time PCR to measure proinflammatory cytokine (IL-6, IL-1β, and TNF-α) gene expression in LPS-

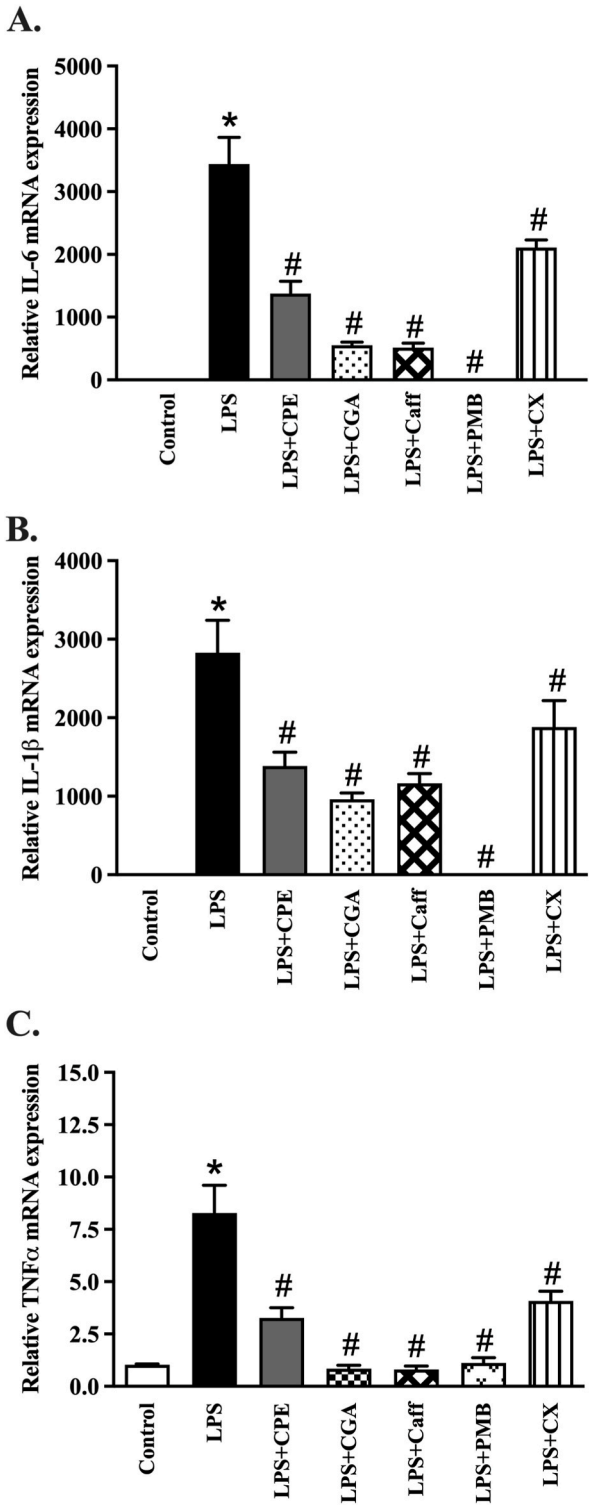

Fig. 3. The effects of CPE on inflammatory cytokine gene expression in LPS-stimulated RAW 264.7 cells. Cells were pretreated with 1000 μg/mL CPE or its major constituents at the molar ratios present in 1000 μg/mL CPE: 13.38 μg/mL CGA and 3.82 μg/mL caffeine, 10 μg/mL PMB, and 3.8 μg/mL CX with or without LPS for 24 h. The expression levels of (A) IL-6, (B) IL-1β, and (C) *TNF-α mRNA* were detected via quantitative PCR analysis. Data are presented as the mean ± standard error of the mean (n = 5). *$p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. LPS.

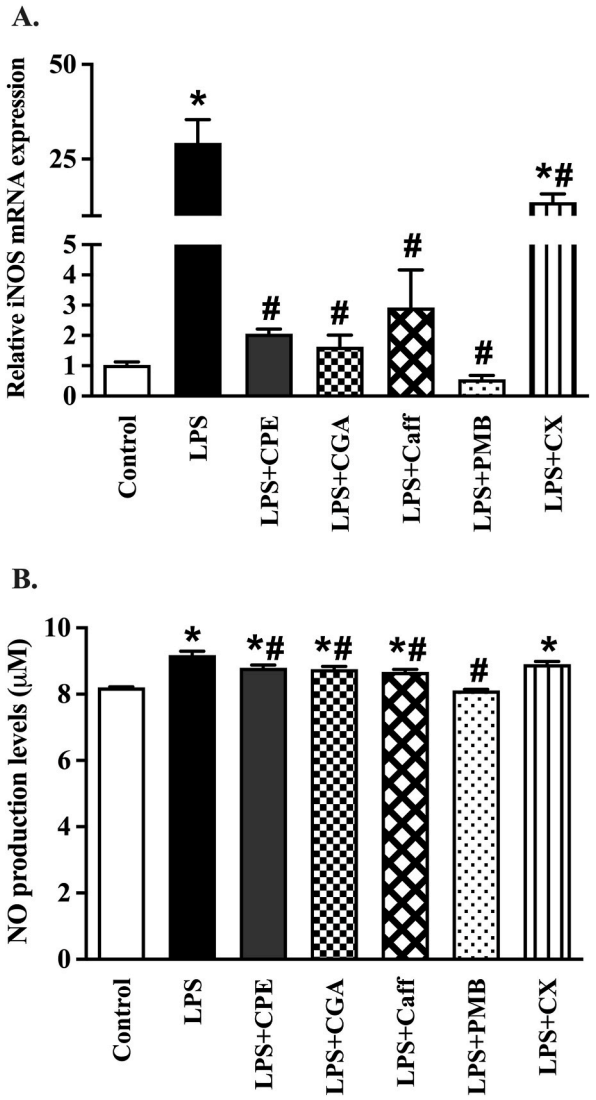

Fig. 4. The effects of CPE on iNOS mRNA expression and NO production in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were supplemented with 1000 μg/mL CPE or its major constituents at the molar ratios present in 1000 μg/mL CPE: 13.38 μg/mL CGA and 3.82 μg/mL caffeine, 10 μg/mL PMB, and 3.8 μg/mL CX with or without LPS for 24 h. (A) iNOS mRNA expression and (B) the production of NO were measured. Data are presented as the mean ± standard error of the mean (n = 5). *$p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. LPS.

stimulated inflammatory cells to confirm the effect of CPE and its critical constituents on inflammatory conditions. CPE was administered in a single dose (1000 μg/mL) since it dramatically decreased all inflammatory cytokines while remaining equivalent to control cells (Fig. 2). As shown in Fig. 3, the mRNA expression of IL-6, IL-1β, and TNF-α was significantly decreased in LPS-induced inflammatory cells. Additionally, treatment with CGA, caffeine, PMB, or CX resulted in a marked reduction in proinflammatory cytokine mRNA expression. Consequently, CPE inhibited proinflammatory cytokines at both the transcriptional and translational levels in activated macrophages.

3.4. CPE suppresses iNOS mRNA expression and nitric oxide production

As shown in Fig. 4A, the inducible NOS (iNOS) mRNA expression levels were upregulated under LPS induction. However, LPS-stimulated iNOS mRNA expression with CPE, CGA, caffeine, PMB, and CX was significantly lower than that without LPS treatment.

In addition, the Griess assay kit was used to examine the impact of CPE and its major components on the NO produced by LPS. As shown in Fig. 4B, inflammatory cells generated by LPS exhibited considerably greater intracellular NO levels than control cells. On the other hand, CPE significantly reduced NO production compared to the LPS group. Similarly, CGA, caffeine's CP components, and PMB inhibited NO production in LPS-induced inflammatory cells but did not affect CX cells.

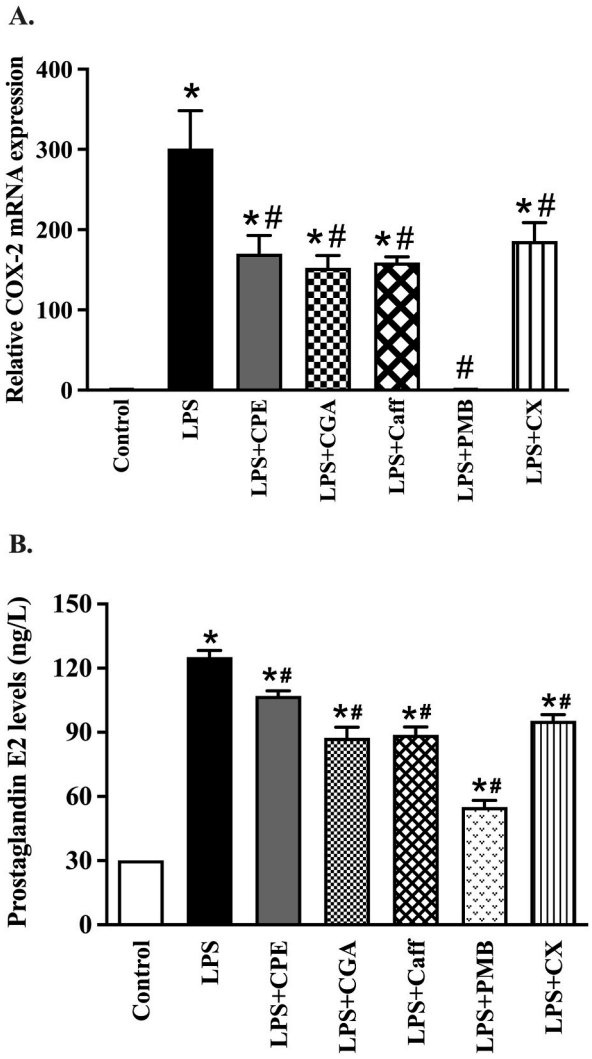

Fig. 5. The effects of CPE on inflammatory mediators in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were supplemented with 1000 μg/mL CPE or its major constituents at the molar ratios present in 1000 μg/mL CPE: 13.38 μg/mL CGA and 3.82 μg/mL caffeine, 10 μg/mL PMB, and 3.8 μg/mL CX with or without LPS for 24 h. (A) COX-2 mRNA expression and (B) PGE2 production were measured. Data are presented as the mean ± standard error of the mean (n = 5). $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. LPS.

3.5. CPE inhibits the inflammatory signaling cascade

We looked at molecules involved in inflammatory pathways to study CPE further and its key constituents' inhibitory impact on inflammatory conditions. As shown in Fig. 5A, LPS increased COX-2 mRNA expression considerably compared to the control group. However, compared to LPS-stimulated cells, CPE-, CGA-, caffeine-, PMB-, and CX-treated cells exhibited dramatically decreased COX-2 gene expression. PGE_2 production was considerably elevated in LPS-induced inflammatory cells compared to the control group, as shown in Fig. 5B. Furthermore, PGE_2 levels were much lower in CPE-supplemented cells than in LPS-treated cells. Similarly, CGA, caffeine, and CX reduced PGE_2 production (Fig. 5B). The PMB-treated group had the maximum inhibitory effect on COX-2 mRNA expression and PGE2 levels among the groups. These data suggest that CPE is anti-inflammatory and inhibits inflammatory signaling pathways.

3.6. CPE inhibits p65NF-κB activation

The effect of CPE on p65NF-κB activation was investigated using fluorescence microscopy. As shown in Fig. 6A, p65NF-κB was primarily detected in the cytoplasm of stimulated RAW264.7 cells (green color). However, p65NF-κB translocated into the nucleus of LPS-induced inflammatory cells but not into the nucleus of the CPE-treated group. Similarly, when cells were supplemented with CGA, caffeine, PMB, and CX, p65NF-κB activation was reduced. The effect of CPE on p65NF-κB protein expression was further investigated.

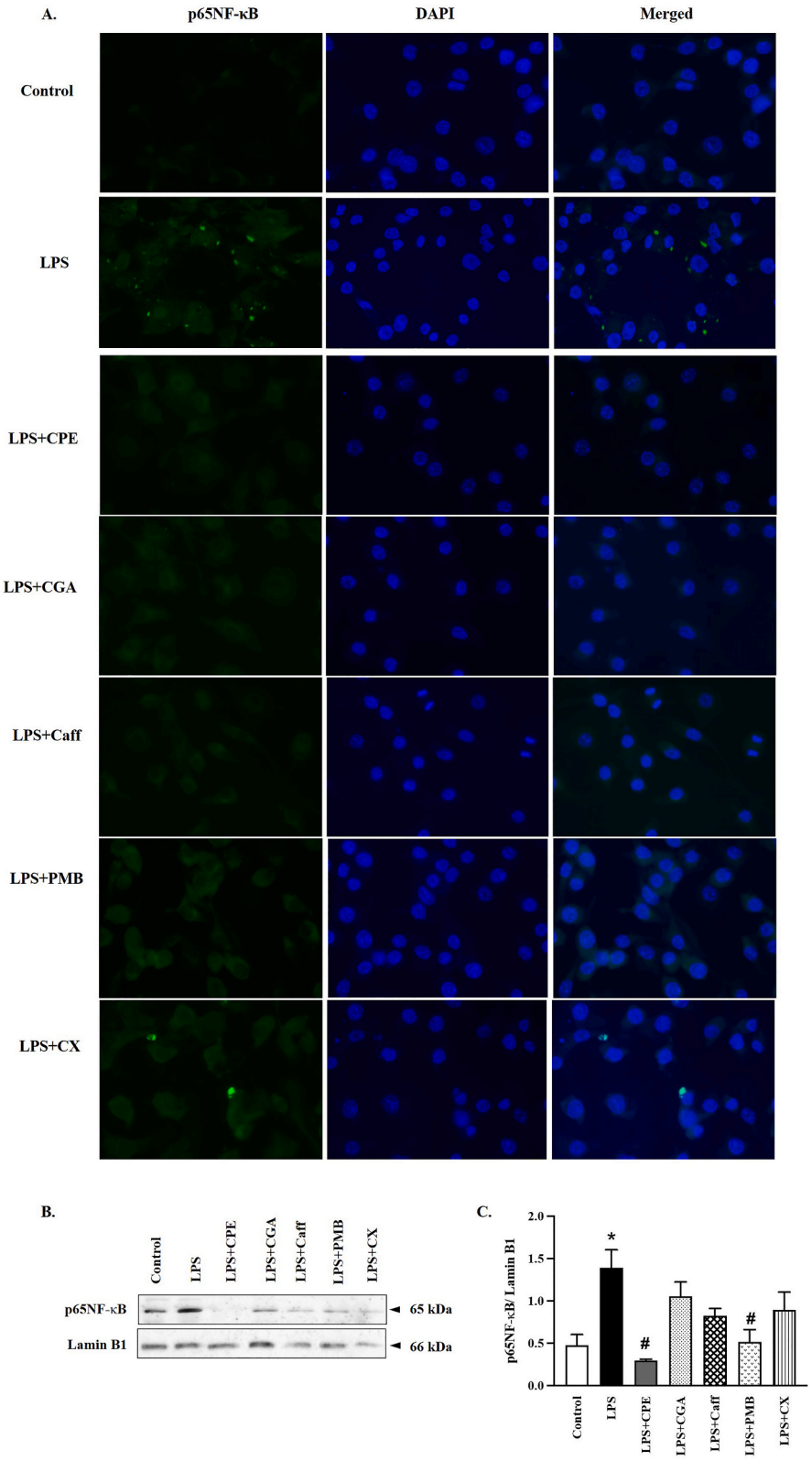

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Fig. 6. The effects of CPE on p65NF-κB nuclear translocation in LPS-stimulated RAW 264.7 cells. (A) Cells were pretreated with 0.1–1000 μg/mL CPE or its major constituents at the molar ratios present in 1000 μg/mL CPE: 13.38 μg/mL CGA and 3.82 μg/mL caffeine, 10 μg/mL PMB, and 3.8 μg/mL CX for 24 h and then stimulated with LPS for 30 min. The cells were stained with NF-κB p65 antibodies (green) and DAPI (blue). The nuclear translocation of NF-κB p65 was assessed using a fluorescence microscope. (B) Representative blot of NF-κB p65 and lamin B1. (C) Quantification of the relative NF-κB p65/lamin B1 protein expression. Data are presented as the mean ± standard error of the mean (n = 3). *$p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. LPS. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

As shown in Fig. 6B and C, LPS increased nuclear p65NF-κB protein expression compared to the control. However, CPE and the TLR4 antagonist PMB significantly decreased nuclear p65NF-κB protein expression. These data suggest that CPE's anti-inflammatory action is mediated by inhibiting the NF-κB pathway.

3.7. CPE acts as a MAPK signaling pathway inhibitor

The effect of CPE on the mitogen-activated protein kinase (MAPK) signaling pathway was further investigated by examining the phosphorylation of MAPK pathway proteins (c-Jun N-terminal kinase (JNK), p38 kinase (p38), and extracellular signal-regulated kinase (ERK)). As shown in Fig. 7, LPS phosphorylated JNK (Fig. 7A and B) and p38 (Fig. 7C and D) in RAW 264.7 cells. CPE, similar to PMB, dephosphorylated JNK proteins considerably. CPE, CGA, caffeine, PMB, and CX also reduced p38 phosphorylation. However, there was no effect on ERK phosphorylation (Fig. 7E and F). These results reveal that CPE inhibits NF-κB activation in LPS-activated cells by regulating MAPK signaling pathway phosphorylation.

4. Discussion

Inflammation is the body's immune system protecting itself from injury or disease. Toll-like receptor-4 (TLR4) plays a key role in inflammatory conditions [22]. Binding of LPS to TLR4 triggers signaling cascades such as the NF-κB and MAPK pathways, resulting in proinflammatory cytokine production [23]. Moreover, macrophages are activated by LPS through TLR4 binding and participate in the production of proinflammatory cytokines, including IL-6, IL-1β, and TNF-α. Irregular inflammation is linked to many health problems, including autoimmune disease and diabetes, cardiovascular disease, Alzheimer's disease, and cancer [24,25]. On the other hand, clinical anti-inflammatory drugs have several side effects on many organ systems, necessitating the urgent development of natural or traditional treatments for inflammation prevention and treatment. Thus, the modulation of the TLR4 receptor and its signaling molecules by a reagent or bioactive compound could be a therapeutic concept to reduce exogenous pathogen-mediated inflammation. In LPS-activated RAW264.7 cells, CPE and its principal components suppressed IL-6, IL-1β, and TNF-α gene expression and production, similar to NSAIDs and polymyxin B (PMB). PMB inhibits LPS-induced Toll-like receptor 4 (TLR4) activation [26]. These data suggested that CPE and its effective ingredients might suppress proinflammatory cytokine production through TLR4 inhibition.

There are several essential components in CPE, including CGA and caffeine. Myocardial infarction animal models show that CPE's primary component, CGA, effectively reduces proinflammatory factors and enhances antioxidant enzyme levels [27]. In rats with acetaminophen-induced hepatotoxicity, CGA also decreases the levels of the proinflammatory cytokines TNF-α, IL-6, and IL-1β [9]. Rats treated with carbon tetrachloride had lower IL-6, IL-1β, and TNF-α in their blood and lower expression of CGA mRNA in their livers [28]. In addition, caffeine-infused rats show a decrease in IL-6, TNF-α, and cell death compared to placebo-treated rats [29]. A reduction in cytokine expression and production might explain CPE's anti-inflammatory effects.

The activation of macrophages by LPS leads to the synthesis of inflammatory mediators, such as NO and PGE_2 [30]. When activated, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) produce PGE_2 and NO, respectively [31]. Many disorders, such as cancer, Parkinson's disease, and Alzheimer's disease, have been linked to COX-2 and iNOS. The generation of NO or PGE_2 is thus an important therapeutic target in treating inflammatory disorders. According to the present study, CPE and its components have been shown to reduce iNOS mRNA expression and NO production in LPS-stimulated RAW264.7 cells. CPE also inhibited LPS-induced COX-2 mRNA expression and PGE_2 production, although the extent of inhibition was much greater than that of NO and iNOS suppression. There was a possibility that the PGE_2 production pathway was the target of CPE. However, there are differences between iNOS mRNA and NO production data. The lack of correlation between mRNA and protein fluctuations might be due to the increase in mRNA molecules present in the cell faster than protein production [32].

PGE_2 release and NO expression may be inhibited by natural products such as Angelica sinensis, Shorea robusta, and Cryptostegia grandiflora leaf extracts [33–35]. In IL-1β-induced chondrocytes, CGA inhibits NO and PGE_2 generation and decreases the expression of iNOS and COX-2 [36]. Moreover, the plasma levels of LPS and IL-6 were also reduced in human subjects who took polyphenol extracts [37]. Similarly, the polyphenol activity of CPE inhibits the expression and synthesis of proinflammatory cytokines and mediators, and its inhibitory impact may protect against inflammation.

MAPK pathways involved in cell proliferation, death, immune defense, and stress responses are influenced by MAPKs [38,39]. MAPK pathways include the p38 kinase, JNK, and ERK pathways. Proinflammatory stimuli, such as oxidative stress, can trigger the production of these proteins [40]. The production of proinflammatory mediators NO, PGE_2, iNOS, COX-2, and NF-κB activation increases when these MAPK signaling pathways are activated [41,42]. Treating or alleviating inflammation may be improved by pharmacological inhibition of p38 MAPK and JNK [43,44]. CPE treatment simultaneously reduced the phosphorylation of JNK and p38.

NF-κB is a critical component of intracellular signaling pathways involved in inflammatory processes. The inflammatory mediators

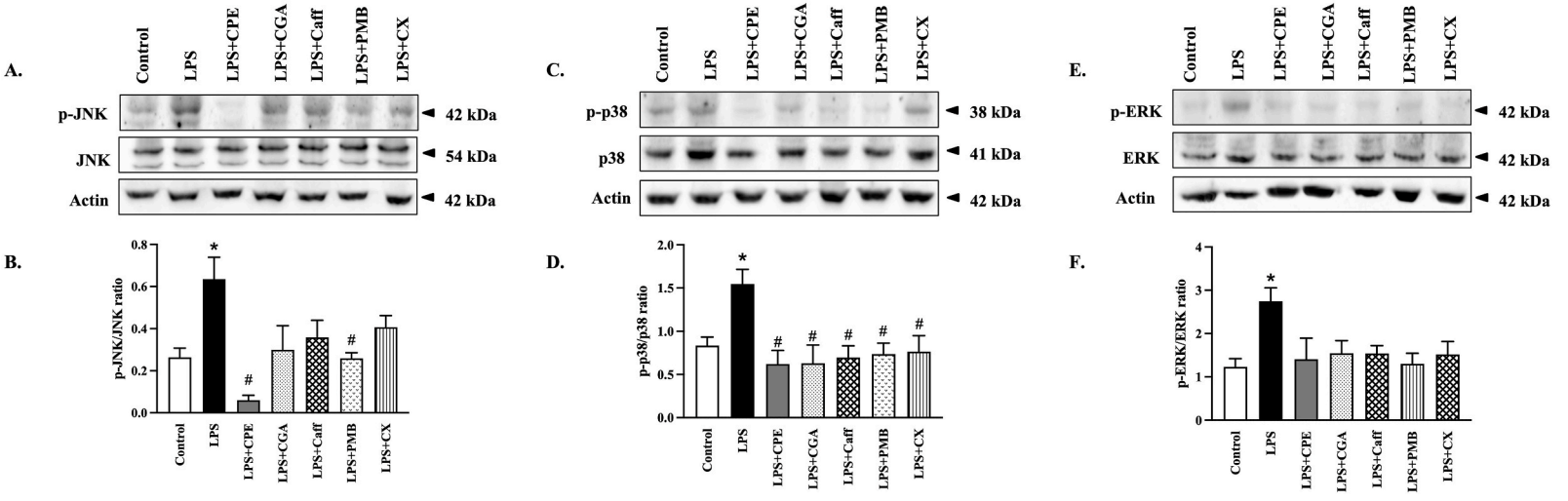

Fig. 7. The effects of CPE on JNK, p38 and ERK with LPS-induced phosphorylation in RAW 264.7 cells. (A) Representative blot of *p*-JNK, JNK, and actin. (B) Quantification of the relative *p*-JNK/JNK ratio. (C) Representative blot of p-p38, p38, and actin. (D) Quantification of the relative p-p38/p38 ratio. (E) Representative blot of *p*-ERK, ERK, and actin. (F) Quantification of the relative *p*-ERK/ERK ratio. Data are presented as the mean ± standard error of the mean (n = 3). *p < 0.05 vs. control; #p < 0.05 vs. LPS.

COX-2 and iNOS are synthesized in response to LPS activation of NF-κB [45]. LPS also dephosphorylates the NF-κB inhibitory protein IκB p50, activating NF-κB. p65NF-κB translocates into the nucleus and interacts with proinflammatory genes after activating it in macrophages by LPS [46]. This study found that CPE and its significant constituents prevented the activation of p65NF-κB. Populus inhibits NF-κB signaling in deltoid leaf extracts, simultaneously inhibiting IκB dephosphorylation and p65 NF-κB nuclear translocation [43]. It has been shown that Withania somnifera extract inhibits NF-κB translocation in stimulated human peripheral blood and synovial fluid mononuclear cells in the same way [47]. A similar effect is seen in rats with carbon tetrachloride-induced fibrosis of their livers when CGA is used as a treatment [28]. Because CPE's major constituents dephosphorylate MAPK pathways and inhibit the NF-κB pathway, it has anti-inflammatory action.

5. Conclusions

The anti-inflammatory effects of CPE were shown for the first time in this work. CPE inhibited IL-6, IL-1β, and TNF-α production and the generation of the inflammatory mediators iNOS, COX2, PGE_2, and NO, reduced p65NF-κB translocation and dephosphorylated MAPK signaling pathways. Caffeine and CGA may also have a role in the anti-inflammatory properties of CPE. CPE has this capability as a natural anti-inflammatory product. However, this limitation should be noted. The anti-inflammatory effect of CPE and its mechanism need further study in animal models and clinical trials.

Author contribution statement

Atcharaporn Ontawong: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper; Contributed reagents, materials, analysis tools or data.

Chutima S. Vaddhanaphuti: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Acharaporn Duangjai: Performed the experiments.

Sirinat Pengnet: Performed the experiments; Analyzed and interpreted the data.

Natakorn Kamkaew: Analyzed and interpreted the data.

Doungporn Amornlerdpison; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13917.

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