Food and Chemical Toxicology 126 (2019) 332–342

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Anti-inflammatory effects of dicaffeoylquinic acids from *Ilex kudingcha* on lipopolysaccharide-treated RAW264.7 macrophages and potential mechanisms

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ARTICLE INFO

Keywords:
Ilex kudingcha
Dicaffeoylquinic acid
RAW264.7 macrophage cells
Anti-inflammatory
NF-κB
MAPKs

ABSTRACT

Increasing evidence has shown that dicaffeoylquinic acids (DiCQAs) have anti-inflammatory activity. However, the underlying molecular mechanisms of the anti-inflammatory effects of DiCQAs are still unclear. In the present study, the anti-inflammatory effects of DiCQAs from the leaves of *Ilex kudingcha* and the potential molecular mechanisms on LPS-induced inflammatory responses in RAW264.7 macrophage cells were investigated. The results showed that pretreatment with DiCQAs could suppress the production of NO, PGE_2 and also pro-inflammatory cytokines TNF-α, IL-1β and IL-6, and the mRNA expression of two major inflammatory mediators of COX-2 and iNOS. The phosphorylated IκBα, ERK, JNK and p38 proteins in LPS-treated cells were significantly increased, which could be reversed by pretreatment with DiCQAs in a concentration-dependent manner. Taken together, the results suggest that DiCQAs from *I. kudingcha* have potent anti-inflammatory effects on LPS-induced inflammatory responses by inhibiting the NF-κB and MAPKs pathways and may be a prophylactic for inflammation.

1. Introduction

Inflammation is a beneficial self-protective physiologic process in response to pathogen invasion, chemical irritation, tissue damage or exposure to endotoxin like lipopolysaccharide (LPS) (Nathan, 2002). Normal inflammatory response maintains a balance between pro-inflammatory and anti-inflammatory cytokines. But epidemiological studies have shown that the excessive production of pro-inflammatory cytokines may result in the development of chronic inflammatory diseases, such as cancers (Mantovani et al., 2008), inflammatory bowel diseases (Neuman, 2007) and metabolic syndromes (Hotamisligil, 2006). Therefore, novel effective therapy is in great request for chronic inflammation.

Macrophages are major inflammatory and immune cells that play an important role in the innate and adaptive immune responses. The murine macrophage cell line RAW264.7 is generally considered as a suitable anti-inflammatory macrophage model for research *in vitro* (Chu et al., 2016). LPS, a major component of the cell wall of Gram-negative bacteria, is widely used to establish inflammatory models of macrophages *in vitro* (Jiang et al., 2017). When RAW264.7 macrophages are stimulated by LPS, the toll-like receptor-4 (TLR-4) recognizes and binds to LPS, leading to the coordinated activation of various intracellular signaling pathways, which involves in the activation of transcription factor nuclear factor-kappa B (NF-κB) and the phosphorylation of mitogen-activated protein kinases (MAPKs) (Byun et al., 2013). Subsequently, the production of pro-inflammatory mediators such as cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, pro-inflammatory proteins including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and small molecules including nitric oxide (NO) and prostaglandin E_2 (PGE_2) are increased significantly, and they trigger the inflammatory cascade response (Zhou et al., 2015). Therefore, inhibition of pro-inflammatory cytokine and mediator production is thought to serve as a key mechanism in the prevention of inflammation.

NF-κB is a transcription factor that plays pivotal roles due to its ability to induce the expression of genes in immune and inflammatory responses as well as in cell survival (Tak and Firestein, 2001). In the unstimulated cells, NF-κB dimers are bound to inhibitor κBα (IκBα) and IκBβ as an inactive complex in the cytoplasm, which prevents it translocating to the nucleus. The activated form of NF-κB is a heterodimer, which usually consists of two proteins, a p65 subunit and a p50 subunit, regulating the transcription of multiple genes such as various

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https://doi.org/10.1016/j.fct.2019.01.011
Received 27 September 2018; Received in revised form 8 January 2019; Accepted 11 January 2019
Available online 14 January 2019
0278-6915/

inducible enzymes (COX-2 and iNOS) and pro-inflammatory cytokines (Shih et al., 2015). When the cells are stimulated, the cytoplasmic IκB kinase (IKK) complex induces phosphorylation of IκB, leading to ubiquitination and degradation of IκB and freeing NF-κB complex. Thereby, NF-κB is enabled to translocate to the nucleus, where it regulates the transcription of its target genes, including TNF-α, IL-1β, IL-6, iNOS and COX-2. In addition, recent literature demonstrate that the activation of NF-κB is regulated by MAPKs signaling pathways (Kim et al., 2008; Komatsu et al., 2017). MAPKs are important signaling pathways in the immune system and main signaling molecules including p38, extracellular signal-regulated protein kinase 1/2 (ERK1/2) and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) are involved in the signal transduction pathways that lead to upregulate pro-inflammatory cytokines genes expression (Lee et al., 2012). Therefore, any substances that inhibit the activation of NF-κB pathway and signaling cascades mediated by MAPKs can be used as therapeutic agents against inflammatory diseases.

Kudingcha, a particularly bitter-tasting tea that mainly made from the leaves of *Ilex* genus plants including *I. kudingcha* C.J. Tseng and *I. latifolia* Thunb, is widely used as a kind of herbal tea in China (Fan et al., 2014; Li et al., 2013). It has been demonstrated that kudingcha exhibited diverse biological activities such as antioxidant, anti-inflammatory, anticancer and prevention on neuronal damage and metabolic disorders (Fan et al., 2012; Kim et al., 2011; Liu et al., 2009; Song et al., 2013; Zhao et al., 2013; Zhu et al., 2014). In our previous study (Liu et al., 2009; Xu et al., 2015), it has been shown that *I. kudingcha* contained large amounts of caffeoylquinic acid (CQA) derivatives, including 3-CQA, 4-CQA, 5-CQA, 3,4-dicaffeoylquinic acids (3,4-diCQA), 3,5-diCQA and 4,5-diCQA. To date, Puangpraphant et al. (2011) investigated the anti-inflammatory and anti-cancer capabilities of dicaffeoylquinic acids (DiCQAs, Fig. S1) from Yerba mate tea leaves *in vitro* and explored their mechanism of action. Song et al. (2013) explored the anti-inflammatory effects of methanol extract from *I. kudingcha* C.J. Tseng in dextran sulfate sodium-induced ulcerative colitis in mice. Nevertheless, less is known regarding the anti-inflammatory effects of DiCQAs from *I. kudingcha* and the underlying mechanisms have not been systematically studied. In the current study, the anti-inflammatory activity of DiCQAs from *I. kudingcha* was investigated by using LPS-stimulated RAW264.7 macrophage cells as model of inflammation. The concentrations of pro-inflammatory mediators in the culture medium of DiCQAs pre-treated inflammatory cells were measured. The mRNA expression levels of iNOS, COX-2 and major cytokines in cells were evaluated by quantitative real time-polymerase chain reaction (qRT-PCR). In addition, the regulation of MAPKs and NF-κB by DiCQAs was examined to explore the underlying molecular mechanisms on inhibiting LPS-induced inflammatory responses.

2. Materials and methods

2.1. Materials and chemicals

Kudingcha, made from the leaves of *I. kudingcha* C.J. Tseng, was obtained from Hainan Yexian Bio-Science Co. (Haikou, China). The murine macrophage RAW264.7 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin stock solution were purchased from Gibco (Carlsbad, CA, USA). *Escherichia coli* O111:B4 LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). ELISA kits for determinations of PGE_2, IL-6, IL-1β and TNF-α were purchased from Neobioscience Biological Technology Co., Ltd. (Shenzhen, China). Primary antibodies against iNOS, COX-2, NF-κB p65, IκBα, phospho-IκBα, SAPK/JNK, phospho-SAPK/JNK, ERK1/2, phospho-ERK1/2, p38, phospho-p38, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). All other chemical reagents used were of analytical grade.

2.2. Preparation of DiCQAs from kudingcha

The preparation of DiCQAs was carried out according to our previously reported procedures (Xie et al., 2016) with some modifications. Briefly, the powder of kudingcha was extracted two times with hot water in a solid-liquid ratio of 1:10 for 30 min at 95 °C. After extraction, the extracts were filtered and centrifuged at 5000 *g* for 10 min, and the supernatants were loaded onto a column (5 × 50 cm) of macroporous HP-20 resin (Mitsubishi Chemical Corp., Tokyo, Japan). The column was washed with 4 bed volumes of distilled water, and then eluted with 70% (v/v) aqueous ethanol solution for targeted DiCQAs. The eluted fractions were concentrated by a rotary evaporator and lyophilized by freeze dryer, affording DiCQAs for use.

2.3. Cell culture and assay of cell viability

The RAW264.7 cells were transferred to 25 cm^2 plastic flasks and cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in a humidified incubator with 5% CO_2 at 37 °C. In all experiments, the cells were left to acclimate for 24 h before any treatments.

For analysis of the cytotoxicity of DiCQAs, cell viability was measured by MTT assay. The RAW264.7 cells at a density of 5 × 10^4 cells per well were cultured in 96-well plates for 24 h, treated with different concentrations of DiCQAs (1, 10, 50, 100 and 150 μg/mL) for 2 h, and then co-cultured with LPS (final concentration of 1 μg/mL) for another 24 h. The culture media with LPS alone and without LPS were used as positive control and blank control, respectively. After the incubation, the medium was discarded, 50 μL of MTT solution (2 mg/mL in phosphate-buffered saline (PBS), pH 7.4) was added to each well and incubated at 37 °C for 4 h. Subsequently, the supernatants were discarded and 150 μL DMSO was added to each well to dissolve the MTT formazan crystals. The absorbance (Abs) at a wave-length of 570 nm was determined using an ELISA microplate reader (BioTeK Instruments, Inc., Winooski, VT, USA). The cell viability rate was calculated according to the formula below:

Cell viability rate (%) = (Abs_{570} of treated cells/Abs_{570} of untreated cells) × 100.

2.4. Assay of NO

The nitrite concentration in the culture medium as an indicator of NO production was measured by the Griess assay. Briefly, the RAW264.7 cells (5 × 10^4/well) were incubated in a 96-well plate for 24 h and pretreated with 1–100 μg/mL of DiCQAs for 2 h prior to treatment with 1 μg/mL LPS. After co-cultured for 24 h in a 5% CO_2 incubator at 37 °C, 50 μL of cell culture medium was collected and mixed with 50 μL of Griess reagent I and II, and the mixture was incubated at room temperature for 10 min with horizontal shaking. The Abs of solution was measured at 540 nm using an ELISA microplate reader and the nitrite concentration was then calculated by using sodium nitrite ($NaNO_2$) as a standard.

2.5. Determinations of PGE_2, TNF-α, IL-1β and IL-6

The levels of PGE_2 and the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in cell culture medium were measured by using commercial mouse immunoassay ELISA kits according to the manufacturer's instructions. Briefly, the RAW264.7 cells (5 × 10^5/well) plated on 6-well plates were pretreated with 1–100 μg/mL DiCQAs for 2 h prior to treatment with 1 μg/mL LPS. After 24 h incubation, the culture medium

from each well was centrifuged at 2000 *g* for 10 min at 4 °C, and the supernatants were harvested for the determinations of PGE_2, TNF-α, IL-1β and IL-6. The concentrations of cytokines in the samples were calculated from standard curves. All of the experiments were performed in triplicate.

2.6. RNA extraction and qRT-PCR analysis

The mRNA expression levels of TNF-α, IL-1β, IL-6, COX-2 and iNOS in macrophages were measured by qRT-PCR according to our previously reported method (Chen et al., 2018). The RAW264.7 cells (5×10^5/well) were seeded in 6-well plates, cultured overnight, pretreated with various dose (1, 10, 50 and 100 μg/mL) of DiCQAs for 2 h and then stimulated with 1 μg/mL LPS for 6 h. The total RNA was extracted using MiniBEST Universal RNA Extraction Kit (TaKaRa Co., Ltd., Beijing, China) from the cells according to the manufacturer's instructions. The resulting RNA samples were DNase-treated by incubation at 37 °C for 15 min with the reaction buffer and recombinant RNase-free DNase I. The quantity and purity of the RNA were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and the ratio of Abs at 260/280 nm of all the samples was in the range of 1.8–2.0. Then, 1 μg total RNA in each group was reverse transcribed into single-stranded cDNA using PrimeScript™ Reverse Transcription Master Kit (TaKaRa Co., Ltd.). qRT-PCR was performed with the Quant Studio 6 Flex Real-Time PCR System (ABI, Carlsbad, CA, USA) using PowerUp™ SYBR® Green Master Mix (ABI). The mRNA expression levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, COX-2 and iNOS were quantified by Quant Studio™ 6 Flex (ABI). The PCR mix was subjected to the following thermal profile: 95 °C for 30 s followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The relative gene expression was calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method and GAPDH was used as the reference housekeeping gene for normalizing mRNA level. The oligonucleotide sequences of primers are shown in Table 1.

2.7. Preparation of total, cytoplasmic and nuclear proteins of cells

The RAW264.7 cells (1×10^6/well) plated on 6-well plates were pretreated with different concentrations (0, 1, 10, 50 and 100 μg/mL) of DiCQAs for 2 h and then stimulated with 1 μg/mL LPS for 30 min (for MAPKs, IκBα, p-IκBα and NF-κB p65) or 24 h (for iNOS and COX-2) at 37 °C. The cells were rinsed twice with ice-cold PBS, and lysed using 200 μL RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with 1 mM PMSF for 15 s. The cell suspension was centrifuged at 14000 *g* for 10 min at 4 °C, and the supernatant was collected as the total proteins. For cytoplasmic and nuclear fractions, the harvested cells were extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's instructions. All the resulting samples were stored at −80 °C until western blot analysis and their proteins concentrations were determined by using a BCA protein assay kit (Beyotime).

Table 1
Sequences of PCR primers used for qRT-PCR.

Gene	Primers	Sequence
iNOS	Forward	5′-GAAGCTGAGGCCCAGGAGGA-3′
	Reverse	5′-GAACAAGGTGGCCAGGTCCC-3′
COX-2	Forward	5′-CATCCCCCTCCTGCGAAGTT-3′
	Reverse	5′-TCTGCTACGGGAGGAAGGGC-3′
TNF-α	Forward	5′-GATCGGTCCCCAAAGGGATG-3′
	Reverse	5′-TCAGCCACTCCAGCTGCTCC-3′
IL-1β	Forward	5′-GGG CTG CTT CCA AAC CTT TG-3′
	Reverse	5′-GCT TGG GAT CCA CAC TCT CC-3′
IL-6	Forward	5′-TAGTCCTTCCTACCCCAATTTCC-3′
	Reverse	5′-TTGGTCCTTAGCCACTCCTTC-3′
GAPDH	Forward	5′-AAGGCTGTGGGCAAGGTCAT-3′
	Reverse	5′-CGTCAGATCCACGACGGACA-3′

2.8. Western blot analysis

Western blot analysis was carried out according to our previously reported method (Gan et al., 2015) with some modifications. Briefly, the supernatant of each sample with equal protein content was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) except 8% for iNOS. After electrophoresis, the protein was transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skim milk (BD, New Jersey, USA) in tris-buffered saline Tween 20 (TBST) for 2 h, and they were then incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: anti-iNOS antibody (1:2000), anti-COX-2 antibody (1:2000), anti-IκBα antibody (1:2000), anti-phospho-IκBα antibody (1:2000), anti-NF-κB p65 antibody (1:1000), anti-SAPK/JNK antibody (1:1000), anti-phospho-SAPK/JNK antibody (1:1000), anti-Erk1/2 antibody (1:1000), anti-phospho-Erk1/2 antibody (1:2000), anti-p38 antibody (1:1000), anti-phospho-p38 antibody (1:1000) and anti-GAPDH antibody (1:2000). The next day, the membranes were washed five times with TBST and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:50000) at room temperature for 2 h. After thorough washing with TBST, each band of protein was visualized by enhanced chemiluminescence (Pierce™, Thermo Fisher Scientific) and photographed using the Tanon 6000 imaging system (Tanon Science & Technology, Shanghai, China). Each band was quantitatively determined using ImageJ 1.50 software (http://rsb.info.nih.gov). The density ratio represented the relative intensity of each band against that of GAPDH as a loading control in each experiment.

2.9. Statistical analysis

The experimental data are presented as the means ± standard deviation (SD) at least three independent experiments for each condition. Duncan's multiple-range test and one-way analysis of variance (ANOVA) were used for multiple comparisons using SPSS 22 software (SPSS Inc., Chicago, IL, USA). P value < 0.05 was considered to be of statistically significant difference.

3. Results

CQA derivatives are the main phenolics in *I. kudingcha*, and 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA account about 90% of total kudingcha polyphenols. During preparation, DiCQAs were absorbed to HP-20 macroporous resin, while mono-CQAs were eluted out by pure water with other components such as carbohydrates and proteins. After complete washing with deionized water, DiCQAs were eluted by 70% aqueous ethanol solution. The resulting DiCQAs were composed of 3,4-diCQA (26.9 ± 1.7%), 3,5-diCQA (42.3 ± 2.0%) and 4,5-diCQA (30.8 ± 3.4%) by HPLC analysis.

3.1. Effect of DiCQAs on cell viability

MTT assay was carried out to evaluate the cytotoxic effects of DiCQAs on RAW264.7 macrophages. The cells were pretreated with various concentrations (1, 10, 50, 100 and 150 μg/mL) of DiCQAs for 2 h and then treated with 1 μg/mL LPS for 24 h. As results, DiCQAs at a concentration of 150 μg/mL significantly decreased cell viability to 84.27 ± 3.50% ($p < 0.05$) compared with the blank control (Fig. 1A). However, DiCQAs in the concentration of 1–100 μg/mL did not exhibit significant effect on the relative viability of cells. Therefore, the concentrations of 1, 10, 50 and 100 μg/mL of DiCQAs (no cytotoxicity on RAW264.7 cells) were selected for the subsequent experiments in the present study.

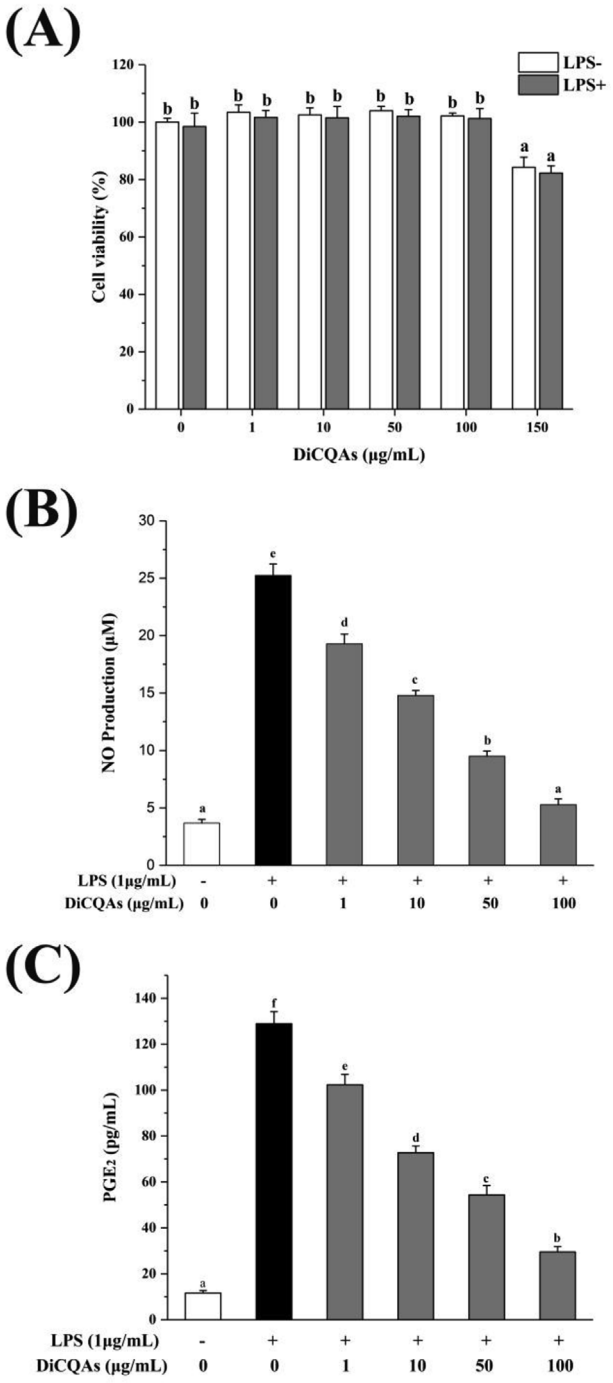

Fig. 1. Cytotoxic effects of DiCQAs on RAW264.7 cells (A). Cells were pretreated with different concentrations of DiCQAs for 2 h and then treated with 1 μg/mL LPS for 24 h. Effects of DiCQAs on production of NO and PGE_2 in RAW264.7 macrophage cells. RAW264.7 cells were pretreated with DiCQAs for 2 h and then incubated with LPS for 24 h. (B), The NO levels in the cell cultures were measured by Griess assays; (C), The PGE_2 levels were measured by ELISA. Each value is expressed as mean ± S.D. (n = 3). Data bearing different letters are significantly different ($P < 0.05$).

3.2. Effects of DiCQAs on production of NO and PGE_2

To evaluate the effects of DiCQAs on production of LPS-induced NO and PGE_2 in RAW264.7 cells, the concentrations of NO and PGE_2 in the culture medium were measured by Griess assays and ELISA, respectively. As shown in Fig. 1B and C, treatment with 1.0 μg/mL LPS caused a significant increase in NO and PGE_2 production of the macrophages ($P < 0.05$). The concentrations of NO and PGE_2 were significantly increased, approximately 7- and 11-fold compared to the blank control cells, respectively, by LPS stimulation. However, pretreatment with DiCQAs significantly suppressed LPS-induced NO production in a dose-dependent manner (Fig. 1B). DiCQAs at 1, 10, 50 and 100 μg/mL attenuated the concentration of NO to 19.28 ± 0.85, 14.79 ± 0.43, 9.5 ± 0.45 and 5.28 ± 0.51 μM, respectively, with an IC_{50} value of 33.81 ± 2.51 μg/mL. In addition, DiCQAs induced a dramatically decrease in PGE_2 production in a concentration-dependent manner with an IC_{50} value of 39.92 ± 2.97 μg/mL (Fig. 1C). The concentration of PGE_2 in the medium decreased to 102.32 ± 5.24, 72.73 ± 2.97, 54.35 ± 4.08 and 29.54 ± 2.37 pg/mL at dosage of 1, 10, 50 and 100 μg/mL, respectively. These results demonstrated that the pretreatment with DiCQAs could simultaneously inhibit LPS-induced NO and PGE_2 production. Moreover, DiCQAs exerted their inhibitory effects in a dose-dependent manner.

3.3. Effects of DiCQAs on expression levels of iNOS and COX-2 mRNA and protein

To further explore the molecular mechanisms of DiCQAs in inhibiting pro-inflammatory mediators NO and PGE_2, the effects of DiCQAs on iNOS and COX-2 mRNA and protein expression, which mediate the synthesis of NO and PGE_2 in LPS-stimulated RAW264.7 cells, were investigated by qRT-PCR and western blot. As shown in Fig. 2A and B, the results of qRT-PCR indicated that the expression levels of iNOS and COX-2 mRNA were strikingly increased by LPS stimulation compared to the blank control cells ($P < 0.05$). By contrast, pretreatment with DiCQAs at 10, 50 and 100 μg/mL markedly attenuated transcriptions of iNOS and COX-2 mRNA expression (to 82.03 ± 3.76%, 68.10 ± 2.37%, 15.76 ± 1.32% for iNOS, to 70.25 ± 6.74%, 38.10 ± 2.94%, 17.76 ± 1.35% for COX-2, respectively, compared to the cells treated with LPS only) after LPS-stimulation for 6 h in RAW264.7 cells. Western blot analyses (Fig. 2C and D, Fig. S2A) showed that the protein expression levels of iNOS and COX-2 were increased markedly after treatment with LPS for 24 h compared with those of blank control cells. As expected, a dose-dependent reduction in the protein levels of iNOS and COX-2 was observed in DiCQAs pretreated cells. When the cells pretreated with DiCQAs at a dose of 1, 10, 50 and 100 μg/mL, the protein levels of iNOS decreased to 74.20, 72.39, 53.27 and 0.83% relatively to the cells treated with LPS only, respectively. The protein levels of COX-2 decreased to 32.16, 29.50, 16.10 and 7.94% for pretreatment with DiCQAs at concentrations of 1, 10, 50 and 100 μg/mL, respectively. All these results clearly demonstrated that DiCQAs suppressed the production of NO and PGE_2 in the LPS-stimulated RAW264.7 cells by down-regulating iNOS and COX-2 mRNA and protein expression at the transcriptional and translation levels.

3.4. Effects of DiCQAs on LPS-induced pro-inflammatory cytokines production and mRNA expression

The effects of DiCQAs on LPS-induced IL-1β, IL-6 and TNF-α production and transcription levels were investigated by ELISA and qRT-PCR, respectively. It was found that the levels of IL-1β, IL-6 and TNF-α were noteworthy elevated from 5.34 ± 0.33 pg/mL to 34.56 ± 1.67 pg/mL (Fig. 3A), 41.63 ± 3.62 to 1933.12 ± 76.24 pg/mL (Figs. 3B) and 0.13 ± 0.01 to 19.52 ± 0.61 pg/mL (Fig. 3C) after a 24 h treatment with LPS alone compared with those of the blank control, respectively. Moreover, concentration-dependent suppressed levels of IL-1β, IL-6 and TNF-α were observed in DiCQAs pretreated groups. The IC_{50} values of IL-1β, IL-6 and TNF-α were estimated to be 64.37 ± 3.18, 66.92 ± 2.35 and 79.84 ± 2.61 μg/mL, respectively. Likewise, the expression levels of IL-1β, IL-6 and TNF-α mRNA increased significantly after LPS-stimulation for 6 h in RAW264.7 macrophage cells ($P < 0.05$) and decreased by pretreatment with

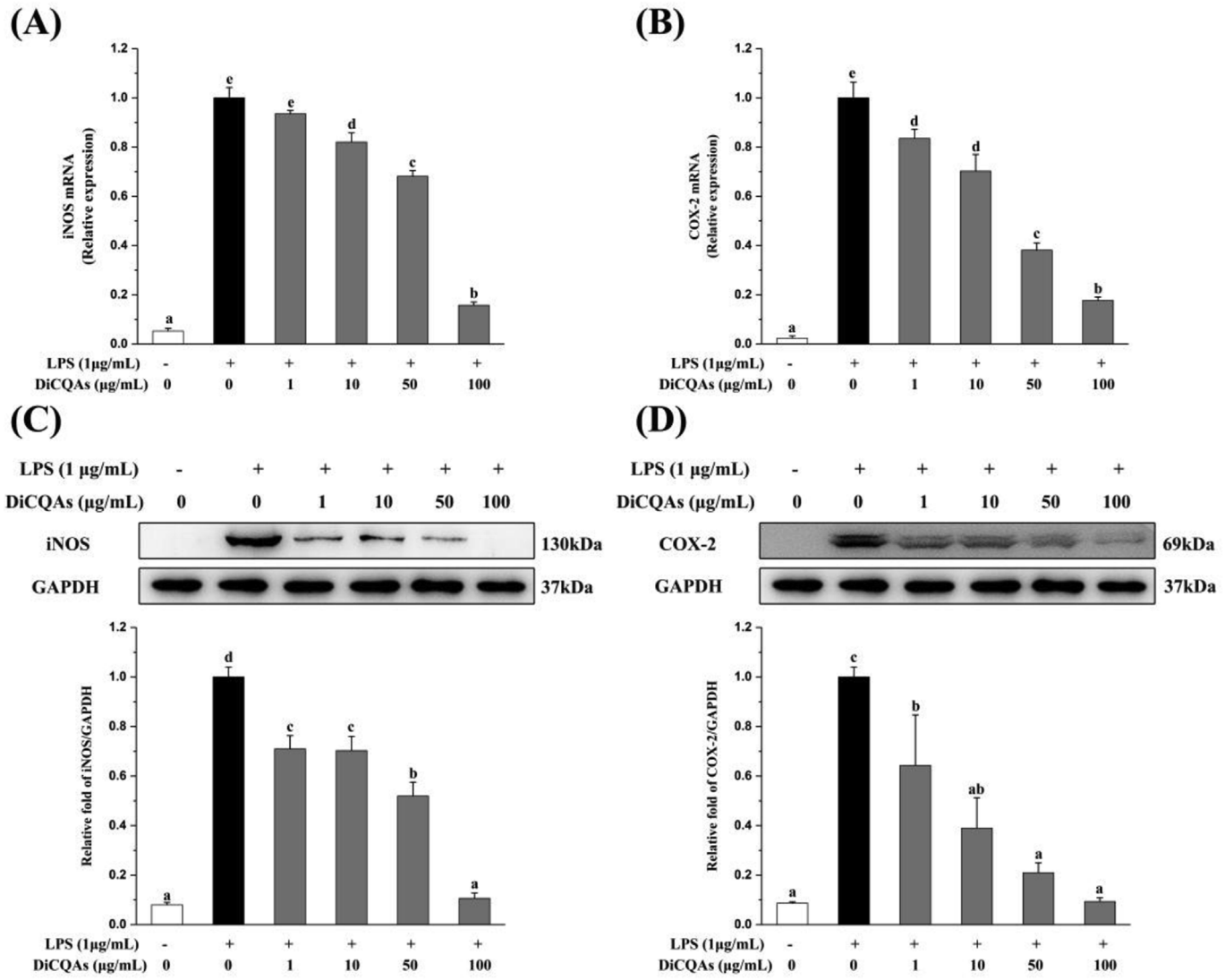

Fig. 2. Effects of DiCQAs on expression of LPS-induced iNOS and COX-2 mRNA and protein in RAW264.7 macrophage cells. RAW264.7 cells were pretreated with DiCQAs for 2 h and then incubated with LPS for 6 h. The total mRNA was extracted, and the mRNA levels of COX-2 (A) and iNOS (B) were ascertained by qRT-PCR. Graphs represent relative expression values, normalized against the reference gene. Each value is expressed as mean ± S.D. (n = 3). Data bearing different letters are significantly different ($P < 0.05$). RAW264.7 cells were pretreated with DiCQAs for 2 h and then incubated with LPS for 24 h. The cells were lysed, and the lysates were examined by Western blot for iNOS and COX-2 (C and D).

1–100 μg/mL DiCQAs (Fig. 3D and F). DiCQAs at 1, 10, 50 and 100 μg/mL reduced the mRNA expression levels of IL-1β to 85.54 ± 3.16, 66.18 ± 1.24, 37.64 ± 3.23 and 31.38 ± 2.51%, IL-6 to 94.45 ± 3.29, 83.76 ± 5.27, 49.24 ± 3.26 and 36.10 ± 3.24%, and TNF-α to 92.08 ± 3.73, 76.05 ± 2.74, 61.54 ± 5.36 and 54.14 ± 2.74%, respectively, compared to those of the cells stimulated with LPS alone. These results implied that the concentration-dependent decrease of the production of IL-1β, IL-6 and TNF-α by DiCQAs might be associated with the transcriptional inhibition of the genes of IL-1β, IL-6 and TNF-α.

3.5. Effects of DiCQAs on activation and translocation of NF-κB and phosphorylation of MAPKs

In order to determine the potential mechanisms working in DiCQAs function, the expression of proteins in NF-κB and MAPKs pathways was analyzed by western blot analysis. For NF-κB signaling pathway, the several NF-κB proteins such as p65, IκBα and p-IκBα were investigated. As shown in Fig. 4A and B and Fig. S2B, slight amount of p65 translocated into nucleus in the un-stimulated RAW264.7 cells, but LPS stimulation induced an increment by nearly 83.26% of nuclear translocation of p65 compared with the blank control. Nevertheless, the nuclear translocation of p65 was notably restrained in a dose-dependent manner with the pretreatment of DiCQAs. Pretreatment with DiCQAs at 10, 50 and 100 μg/mL significantly inhibited the nuclear translocation of p65 by 60.29, 76.98 and 79.04%, respectively, compared to the cells treated with LPS only.

Since IκBα ubiquitination and degradation are the key steps in NF-κB activation, the effect of DiCQAs on IκBα degradation was investigated. As shown in Fig. 4C and D and Fig. S2B, LPS treatment resulted in degradation of IκBα and increase of p-IκBα as compared to blank control group. However, the LPS-induced effect on IκBα was suppressed by DiCQAs pretreatment in a dose-dependent manner. Pretreatments with DiCQAs at 10, 50 and 100 μg/mL notably decreased the phosphorylation of IκBα to 39.86, 11.93 and 3.92%, respectively, compared to the cells treated with LPS only. All these results indicated that the DiCQAs inhibited effects on levels of NO, PGE_2 and pro-inflammatory cytokines and mRNA expression were via inhibiting IκBα degradation and phosphorylation to restrain nuclear translocation of p65, resulting in the blockage of the NF-κB pathway in LPS stimulated

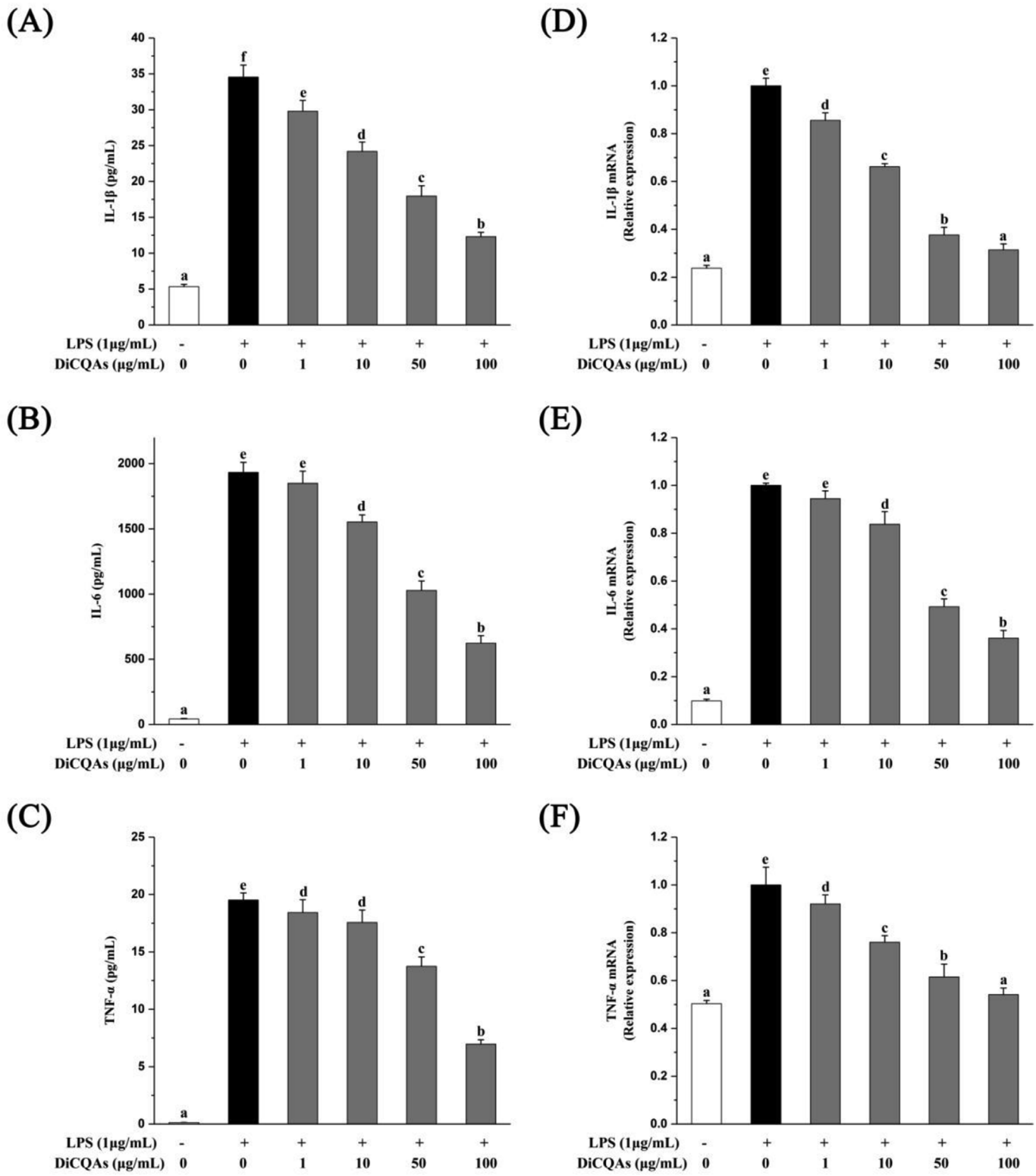

Fig. 3. Effects of DiCQAs on LPS-induced pro-inflammatory cytokine production and mRNA expression. RAW264.7 cells were pretreated with DiCQAs for 2 h and then incubated with LPS for 24 h. The culture medium was collected, and the levels of IL-1β (A), IL-6 (B) and TNF-α (C) were ascertained by ELISA. RAW264.7 cells were pretreated with DiCQAs for 2 h and then incubated with LPS for 6 h. The total mRNA was extracted, and the mRNA levels of IL-1β (D), IL-6 (E) and TNF-α (F) were ascertained by qRT-PCR. Each value is expressed as mean ± S.D. (n = 3). Data bearing different letters are significantly different ($P < 0.05$).

RAW264.7 macrophages.

To further investigate whether DiCQAs regulate signaling proteins of MAPKs signaling pathway, the several MAPKs proteins such as ERK, p-ERK, JNK, p-JNK, p38 and p-p38 were analyzed by western blot analysis. As shown in Fig. 4E and G and Fig. S2B, LPS notably increased the phosphorylation of ERK, JNK and p38 by approximately 3.0-, 3.4- and 4.4-fold, respectively, compared to the blank control cells. However, pretreatment of DiCQAs significantly reduced the phosphorylation

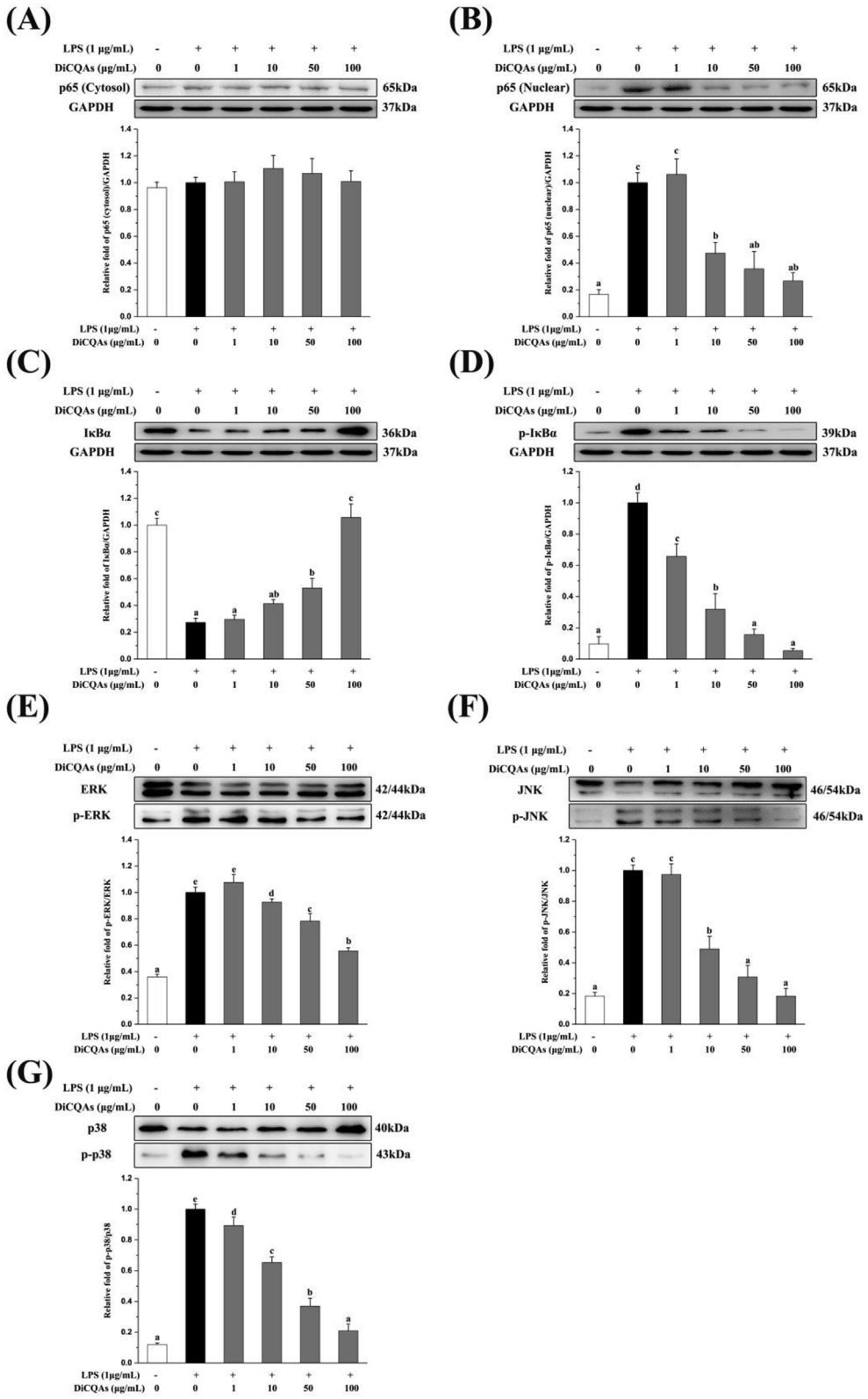

Fig. 4. DiCQAs inhibited related proteins expression of NF-κB (A–D) and MAPKs (E–G) signaling pathways in LPS-stimulated RAW264.7 macrophage cells. Cells were pretreated with the DiCQAs (1, 10, 50 and 100 μg/mL) for 2 h and LPS (1 μg/mL) for 30 min. The levels of these related proteins were measured by Western blotting. Protein samples from 3 independent experiments were quantified by Image J software. Each value is expressed as mean ± S.D. (n = 3). Data bearing different letters are significantly different ($P < 0.05$).

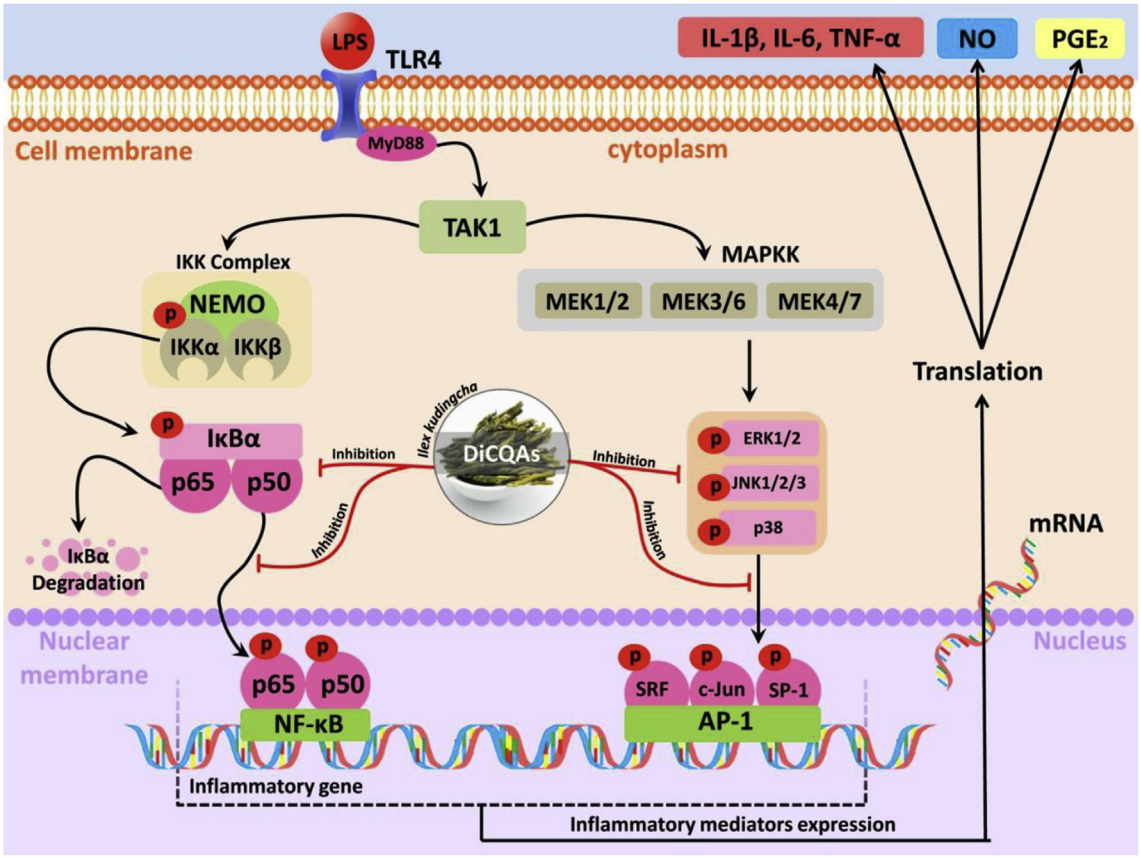

Fig. 5. Schematic diagram for potential mechanisms of anti-inflammtory activity of DiCQAs in LPS-induced RAW264.7 cells through inhibiting the NF-κB and MAPKs signal pathways.

of ERK, JNK and p38 in LPS-induced RAW264.7 macrophage cells. DiCQAs at 10, 50 and 100 μg/mL markedly decreased p-ERK to 86.69, 61.94 and 45.27%, p-JNK to 76.26, 52.54 and 31.93%, and p-p38 to 36.87, 16.47 and 9.22%, respectively, compared to the cells treated with LPS only. These results collectively suggested that the anti-inflammatory properties of DiCQAs were also associated with the blockage of the MAPKs signaling pathway and suppressing the production of various inflammatory mediators via the inhibition of ERK, JNK and p38 phosphorylations in RAW264.7 macrophage cells. As summarized in Fig. 5, the present results demonstrated that DiCQAs could suppress inflammation by regulating the signaling pathways including NF-κB and MAPKs.

4. Discussion

Kudingcha is a particularly bitter-tasting herbal tea, including species *I. kudingcha* C.J. Tseng and *I. latifolia* Thunb. The pharmacological active constituents of kudingcha have been extensively studied because of their various biological activities and low side effects or toxicity. Compounds isolated from kudingcha include phenolic acids, triterpenoids, flavonoids, and essential oils (Li et al., 2013). For *I. kudingcha* C.J. Tseng, it has been confirmed to have anti-inflammatory activity, which is likely related to CQA derivatives. In the present study, inflammation model using RAW264.7 cells was established to evaluate the anti-inflammatory properties and potential molecular mechanisms of DiCQAs from *I. kudingcha* C.J. Tseng. As shown in Fig. 5, DiCQAs could pronouncedly inhibit LPS-induced overproduction of NO and PGE_2 by down-regulating the expression of iNOS and COX-2 genes in RAW264.7 macrophage cells. Besides, DiCQAs exerted anti-inflammatory effect by blocking MAPKs and NF-κB pathways and inhibiting the transcription of IL-1β, IL-6 and TNF-α. To the best of our knowledge, this is the first systematic report about anti-inflammatory effects and potential mechanisms of DiCQAs from *I. kudingcha*.

Structure-activity relationship analysis indicated that the double bond and hydroxyl groups in flavonoids might be important for inhibition of NO, TNF-α and IL-6 in LPS-stimulated RAW264.7 macrophages, and hydroxyl groups on the benzene ring might enhance the anti-inflammatory activity (Hou et al., 2018; Wu et al., 2016). Coincidentally, each of DiCQAs from *I. kudingcha* including 3,4-diCQA, 3,5-diCQA and 4,5-diCQA has 6 hydroxyl groups and 2 double bonds, and the present result demonstrated that DiCQAs from *I. kudingcha* had potent anti-inflammatory activities, which could logically explain the *in vitro* experiment.

It also noted that the present results are basically consistent with other reports that CQA derivatives could decrease the production of NO, COX-2 and pro-inflammatory cytokines via inhibition of MAPKs (p38, ERK and JNK) and NF-κB nucleus translocation in LPS-stimulated macrophage RAW264.7 cells (Chen et al., 2015; Hong et al., 2015; Puangpraphant et al., 2011; Zhang et al., 2018). However, there is significant difference in the effective concentration of anti-inflammatory of DiCQAs, which may have an important relationship with the status of cells. The significant inhibition effect on the expression of iNOS, COX-2 and TNF-α genes was observed by Hong et al. (2015) at the toxic concentration (250 mg/mL) of 3,5-CQA, while the present results were obtained at the nontoxic concentration (100 μg/mL) of DiCQAs (Fig. 1A). The crude polyphenols extracted from *I. latifolia* containing tens of polyphenols, including quinic acid, shikimic acid, rutin, hyperoside, 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, showed strong inhibition on LPS-induced NO, COX-2 and pro-inflammatory cytokines in RAW264.7 cells, which might be related to their synergy activities of the polyphenols from *I. latifolia* (Zhang et al., 2018).

NO is a short-lived free radical and plays a central role in diverse biological processes, such as neurotransmission, gastric motility, wound healing, mitochondrial respiration, apoptosis and inflammation (Eiserich et al., 1998). NO is enzymatically produced by the NADPH dependent oxidation of L-arginine to L-citrulline, catalyzed by iNOS in a wide variety of cells. iNOS is expressed in response to inflammatory stimuli such as bacterial, cytokines and lipid mediators (Faro et al., 2014). In the present study, pretreatment with DiCQAs significantly suppressed LPS-induced NO production and expression of iNOS mRNA and protein in a dose-dependent manner (Figs. 1B and 2). The reduction in NO production might be directly related to the blockade of iNOS gene transcription and protein expression by DiCQAs. PGE_2, a bioactive lipid, is a metabolite of arachidonic acid produced from the reaction catalyzed by COX-2 (Jiang et al., 2017). Over-expression of COX-2 is beneficial for the synthesis of PGE_2, which regulates downstream target molecules to trigger inflammation through multiple signaling pathways (Gandhi et al., 2015). COX-2 can be induced by several intracellular and extracellular factors including LPS, epidermal growth factor (EGF) and TNF (Fontnieves et al., 2012). Similar to NO inhibition, pretreatment with DiCQAs remarkably reduced LPS-induced PGE_2 production in a dose-dependent manner (Fig. 1C). Moreover, DiCQAs significantly inhibited the expression of COX-2 at mRNA and protein levels (Fig. 2), suggesting that DiCQAs might inhibit PGE_2 production by inhibiting COX-2 expression.

IL-1β, a member of IL-1 family of cytokines, is an important mediator of the inflammatory response, and it is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis (Samad et al., 2001). IL-6, one of the most important inflammatory cytokines, is synthesized and secreted by many cell types including T-cells and macrophages to stimulate immune response (Schaper and Rosejohn, 2015). TNF-α is one of the cell signaling protein (cytokine) involved in systemic inflammation and plays critical roles in the regulation of immune cells and amplification of inflammatory response (Yang et al., 2013). In this study, DiCQAs concentration-dependently suppressed the LPS-induced production of IL-1β, IL-6 and TNF-α (Fig. 3A and C). As well, the mRNA expression levels of IL-1β, IL-6 and TNF-α were remarkably decreased by pretreatment with DiCQAs (Fig. 3D and F). DiCQAs decreased the production of IL-1β, IL-6 and TNF-α in concentration-dependent manner, which might be associated with the transcriptional inhibition of IL-1β, IL-6 and TNF-α genes.

NF-κB is a key nuclear transcription factor involved in the transcriptional regulation of inflammation-induced enzymes and cytokines such as iNOS, COX-2, IL-1β, IL-6 and TNF-α (Tak and Firestein, 2001). In un-stimulated cells, the NF-κB dimers are bound with an inhibitory subunit as IκB. Upon stimulation (such as LPS), IκBα is phosphorylated, which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteasome. The activated NF-κB is then translocated to the nucleus to bind target DNAs and induce gene expression (Perkins, 2007; Shih et al., 2015). Therefore, the suitable regulation of NF-κB may be beneficial for the therapy of many inflammatory diseases. The present results showed that pretreatment with DiCQAs at concentration of 10, 50 and 100 μg/mL significantly inhibited the LPS-induced activation and nuclear translocation of NF-κB p65 (Fig. 4A and B, Fig. S2B). However, the results showed that the total expression levels of p65 in the cytosol were not affected in the LPS or DiCQAs-treated groups compared with those of the blank control group. The present result is similar to that of some previous studies (Dong et al., 2017). In response to LPS, degradation of IκBα and nuclear translocation of NF-κB p65 are not sufficient to promote a maximal NF-κB transcriptional response. Rather, the NF-κB complex must undergo additional post-translational modifications (Chen and Greene, 2004; Perkins, 2006). IκB is a transcriptional target for NF-κB, creating a negative feedback loop. IκB contains both nuclear localization and export sequences, enabling its nuclear cytoplasmic shuttling. Newly synthesized free IκB can bind to nuclear p65, leading to the complex return to the cytoplasm. This complex is the target for IκB phosphorylation by IKK for maximum NF-κB transcriptional response (Nelson et al., 2002). Moreover, Nelson et al. (2004) found that following activation by LPS, p65 can dynamically oscillate between the nucleus and cytoplasm. In this study, the level of p65 in the cytosol had not significant difference between groups, which might associate with the dynamically oscillation of p65. Moreover, the phosphorylation of IκBα was suppressed in a dose-dependent manner in RAW264.7 macrophages pretreated with DiCQAs (Fig. 4C and D, Fig. S2B). The above results indicated that DiCQAs suppressed NF-κB activation and nuclear translocation in LPS-stimulated RAW264.7 macrophages by blocking the phosphorylation of IκBα.

MAPKs are important intracellular signaling pathways in the immune system and mainly signaling molecules including p38, ERK and JNK which are involved in the signal transduction pathways for up-regulation of the expression of pro-inflammatory cytokines genes (Lee et al., 2012). MAPKs regulate cell functions including cellular proliferation, gene expression, differentiation, apoptosis and inflammatory (Dong et al., 2017). LPS via TLR-4 stimulates MAPKs cascades. The MEK1/2 (MAPKs activated protein kinases 1/2), MEK3/6 and MEK4/7 kinases are highly specific upstream activators for ERK, p38 and JNK, respectively (Cargnello and Roux, 2011). The main biological response of MAPKs (in particular p38) involves the activation and production of inflammatory mediators at the transcription and translation levels. MAPKs positively regulate expression of many genes involved in inflammation, such as those coding for TNF-α, IL-1β, IL-6, iNOS and COX-2, make them potential targets for anti-inflammatory therapeutics (Kaminska, 2005). Using the DSS-induced experimental colitis model in mice, mimicking human inflammatory bowel disease, Hollenbach et al. (2004) found that inhibitor of p38 MAPK improved the clinical score, ameliorated the histological alterations, and reduced mRNA levels of proinflammatory cytokines. Another potential anti-inflammatory drug, a synthetic guanylhydrazone, which inhibits the phosphorylation of both p38 MAP kinase and JNK, can suppress macrophage activation and the production of several proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and macrophage inflammatory (Hommes et al., 2002). The inhibitors of p38, ERK and JNK signalling pathways are noticeably attractive because they are capable of reducing both the synthesis of pro-inflammatory cytokines and their intracellular signaling (Kumar et al., 2003). Therefore, any substances that inhibit the activation of NF-κB pathway and signaling cascades mediated by MAPKs can be used as therapeutic agents against inflammatory diseases. In this study, pretreatment with DiCQAs significantly reduced the phosphorylation of ERK, JNK and p38 in LPS-induced RAW264.7 macrophage cells (Fig. 4E and G, Fig. S2B). These results collectively suggested that the anti-inflammatory property of DiCQAs was also associated with the blockage of the MAPKs signaling pathway, and by which suppressed the production of various inflammatory mediators via the inhibition of ERK, JNK and p38 phosphorylations in RAW264.7 macrophage cells. Similarly, a previous study reported that the polyphenols from *I. latifolia* Thunb suppressed LPS-induced COX-2 by the reduced phosphorylation of JNK and ERK (Zhang et al., 2018). From these data, DiCQAs inhibited the expression of LPS-induced iNOS, COX-2, TNF-α, IL-1β, and IL-6 in RAW264.7 cells, which might be mainly associated with the ability of DiCQAs to inhibit NF-κB and MAPKs signal pathway. As shown in Fig. 5, the potential molecular mechanisms of anti-inflammtory activity of DiCQAs in LPS-induced RAW264.7 cells through inhibiting the NF-κB and MAPKs signal pathways. DiCQAs decreases LPS-induced pro-inflammation cytokines IL-1β, IL-6 and TNF-α and pro-inflammation mediator NO and PGE_2 via the inhibition of IκBα degradation and ERK, JNK and p38 phosphorylations in RAW264.7 macrophage cells.

5. Conclusion

DiCQAs could suppress the expressions of pro-inflammatory mediators such as NO and PGE_2 as well as various cytokines (IL-1β, IL-6 and

TNF-α) in LPS-stimulated RAW264.7 macrophage cells through blocking NF-κB and MAPKs signaling pathways. The current results indicate that DiCQAs from *I. kudingcha* have effective anti-inflammatory activities. This study gives scientific evidences that DiCQAs from *I. kudingcha* has anti-inflammatory property, suggesting that DiCQA may be a promising prophylactic for inflammatory diseases. However, further research is needed to evaluate their anti-inflammatory activity *in vivo*.

Conflicts of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This work was supported by Grants-in-Aid for scientific research from the National Natural Science Foundation of China (31871761) and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.011.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.011.

References

Byun, E., Sung, N., Byun, E., Song, D., Kim, J., Park, J., Song, B., Park, S., Lee, J., Byun, M., 2013. The procyanidin trimer C1 inhibits LPS-induced MAPK and NF-κB signaling through TLR4 in macrophages. Int. Immunopharm. 15, 450–456.

Cargnello, M., Roux, P.P., 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol. Mol. Biol. Rev. 75, 50–83.

Chen, G., Xie, M., Dai, Z., Wan, P., Ye, H., Zeng, X.X., Sun, Y., 2018. Kudingcha and fuzhuan brick tea prevent obesity and modulate gut microbiota in high-fat diet fed mice. Mol. Nutr. Food Res. 62, 1700485.

Chen, L.F., Greene, W.C., 2004. Shaping the nuclear action of NF-κB. Nat. Rev. Mol. Cell Biol. 5, 392–401.

Chen, X., Miao, J., Wang, H., Zhao, F., Hu, J., Gao, P., Wang, Y., Zhang, L., Yan, M., 2015. The anti-inflammatory activities of Ainsliaea fragrans Champ. extract and its components in lipopolysaccharide-stimulated RAW264. 7 macrophages through inhibition of NF-κB pathway. J. Ethnopharmacol. 170, 72–80.

Chu, H., Tang, Q., Huang, H., Hao, W., Wei, X., 2016. Grape-seed proanthocyanidins inhibit the lipopolysaccharide-induced inflammatory mediator expression in RAW264. 7 macrophages by suppressing MAPK and NF-κB signal pathways. Environ. Toxicol. Pharmacol. 41, 159–166.

Dong, L., Yin, L., Zhang, Y., Fu, X., Lu, J., 2017. Anti-inflammatory effects of ononin on lipopolysaccharide-stimulated RAW 264.7 cells. Mol. Immunol. 83, 46–51.

Eiserich, J.P., Hristova, M., Cross, C.E., Jones, A.D., Freeman, B.A., Halliwell, B., Van Der Vliet, A., 1998. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 391, 393–397.

Fan, J., Wu, Z., Zhao, T., Sun, Y., Ye, H., Xu, R., Zeng, X.X., 2014. Characterization, antioxidant and hepatoprotective activities of polysaccharides from *Ilex latifolia* Thunb. Carbohydr. Polym. 101, 990–997.

Fan, S., Zhang, Y., Hu, N., Sun, Q., Ding, X., Li, G., Zheng, B., Gu, M., Huang, F., Sun, Y., 2012. Extract of Kuding tea prevents high-fat diet-induced metabolic disorders in C57BL/6 mice via liver X receptor (LXR) β antagonism. PLoS One 7, e51007. https://doi.org/10.1371/journal.pone.0051007.

Faro, M.L.L., Fox, B., Whatmore, J.L., Winyard, P.G., Whiteman, M., 2014. Hydrogen sulfide and nitric oxide interactions in inflammation. Nitric Oxide 41, 38–47.

Fontnieves, M., Sansfons, M.G., Gorina, R., Bonfillteixidor, E., Salasperdomo, A., Marquezkisinousky, L., Santalucia, T., Planas, A.M., 2012. Induction of COX-2 enzyme and down-regulation of COX-1 expression by lipopolysaccharide (LPS) control prostaglandin E2 production in astrocytes. J. Biol. Chem. 287, 6454–6468.

Gan, D., Zeng, X.X., Liu, R.H., Ye, H., 2015. Potential mechanism of mycelium polysaccharide from *Pholiota dinghuensis* Bi in regulating the proliferation and apoptosis of human breast cancer MCF-7 cells through p38/MAPK pathway. Journal of Functional Foods 12, 375–388.

Gandhi, J., Gaur, N., Khera, L., Kaul, R., Robertson, E.S., 2015. COX-2 induces lytic reactivation of EBV through PGE_2 by modulating the EP receptor signaling pathway. Virology 484, 1–14.

Hollenbach, E., Neumann, M., Vieth, M., Roessner, A., Malfertheiner, P., Naumann, M., 2004. Inhibition of p38 MAP kinase-and RICK/NF-κB-signaling suppresses inflammatory bowel disease. FASEB J. 18, 1550–1552.

Hommes, D., Van, Den, Blink, B., Plasse, T., Bartelsman, J., Xu, C., Macpherson, B., Tytgat, G., Peppelenbosch, M., Deventer, S.V., 2002. Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease. Gastroenterology 122, 7–14.

Hong, S., Joo, T., Jhoo, J., 2015. Antioxidant and anti-inflammatory activities of 3, 5-dicaffeoylquinic acid isolated from Ligularia fischeri leaves. Food Science and Biotechnology 24, 257–263.

Hotamisligil, G.K.S., 2006. Inflammation and metabolic disorders. Nature 444, 860–867.

Hou, R., Han, Y., Fei, Q., Gao, Y., Qi, R., Cai, R., Qi, Y., 2018. Dietary flavone tectochrysin exerts anti-inflammatory action by directly inhibiting MEK1/2 in LPS-pprimed macrophages. Mol. Nutr. Food Res. 62, 1700288.

Jiang, F., Guan, H., Liu, D., Wu, X., Fan, M., Han, J., 2017. Flavonoids from sea buckthorn inhibit the lipopolysaccharide-induced inflammatory response in RAW264. 7 macrophages through the MAPK and NF-κB pathways. Food & Function 8, 1313–1322.

Kaminska, B., 2005. MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits. Biochim. Biophys. Acta 1754, 253–262.

Kim, J.Y., Jeong, H.Y., Lee, H.K., Yoo, J.K., Bae, K., Seong, Y.H., 2011. Protective effect of *Ilex latifolia*, a major component of "kudingcha", against transient focal ischemia-induced neuronal damage in rats. J. Ethnopharmacol. 133, 558–564.

Kim, J., Na, H., Kim, C., Kim, J., Ha, K., Lee, H., Chung, H., Kwon, H.J., Kwon, Y., Kim, Y., 2008. The non-provitamin A carotenoid, lutein, inhibits NF-κB -dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-κB-inducing kinase pathways: role of H_2O_2 in NF-κB activation. Free Radic. Biol. Med. 45, 885–896.

Komatsu, W., Itoh, K., Akutsu, S., Kishi, H., Ohhira, S., 2017. Nasunin inhibits the lipopolysaccharide-induced pro-inflammatory mediator production in RAW 264 mouse macrophages by suppressing ROS-mediated activation of PI3 K/Akt/NF-κB and p38 signaling pathways. Biosc. Biotech. Biochem. 81, 1956–1966.

Kumar, S., Boehm, J., Lee, J.C., 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat. Rev. Drug Discov. 2, 717–726.

Lee, M., Kwon, M., Choi, J., Shin, T., No, H.K., Choi, J., Byun, D., Kim, J., Kim, H., 2012. Anti-inflammatory activities of an ethanol extract of Ecklonia stolonifera in lipopolysaccharide-stimulated RAW 264.7 murine macrophage cells. J. Agric. Food Chem. 60, 9120–9129.

Li, L., Xu, L.J., Ma, G.Z., Dong, Y.M., Peng, Y., Xiao, P.G., 2013. The large-leaved Kudingcha (*Ilex latifolia* Thunb and *Ilex kudingcha* CJ Tseng): a traditional Chinese tea with plentiful secondary metabolites and potential biological activities. J. Nat. Med. 67, 425–437.

Liu, L., Sun, Y., Laura, T., Liang, X., Ye, H., Zeng, X.X., 2009. Determination of polyphenolic content and antioxidant activity of kudingcha made from *Ilex kudingcha* CJ Tseng. Food Chem. 112, 35–41.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. Nature 454, 436–444.

Nathan, C., 2002. Points of control in inflammation. Nature 420, 846–852.

Nelson, D.E., Ihekwaba, A.E.C., Elliott, M., Johnson, J.R., Gibney, C.A., Foreman, B.E., Nelson, G., See, V., Horton, C.A., Spiller, D.G., Edwards, S.W., McDowell, H.P., Unitt, J.F., Sullivan, E., Grimley, R., Benson, N., Broomhead, D., Kell, D.B., White, M.R.H., 2004. Oscillations in NF-κB signaling control the dynamics of gene expression. Science 306, 704–708.

Nelson, G., Paraoan, L., Spiller, D.G., Wilde, G.J.C., Browne, M.A., Djali, P.K., Unitt, J.F., Sullivan, E., Floettmann, E., White, M.R.H., 2002. Multi-parameter analysis of the kinetics of NF-κB signalling and transcription in single living cells. J. Cell Sci. 115, 1137–1148.

Neuman, M.G., 2007. Immune dysfunction in inflammatory bowel disease. Transl. Res. 149, 173–186.

Perkins, N.D., 2006. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene 25, 6717–6730.

Perkins, N.D., 2007. Integrating cell-signalling pathways with NF-κB and IKK function. Nat. Rev. Mol. Cell Biol. 8, 49–62.

Puangpraphant, S., Berhow, M.A., Vermillion, K., Potts, G., Gonzalez De Mejia, E., 2011. Dicaffeoylquinic acids in Yerba mate (*Ilex paraguariensis* St. Hilaire) inhibit NF-κB nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and -3 in human colon cancer cells. Mol. Nutr. Food Res. 55, 1509–1522.

Samad, T.A., Moore, K.A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J.V., Woolf, C.J., 2001. Interleukin-1β-mediated induction of COX-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 410, 471–475.

Schaper, F., Rosejohn, S., 2015. Interleukin-6: biology, signaling and strategies of blockade. Cytokine Growth Factor Rev. 26, 475–487.

Shih, R., Wang, C., Yang, C., 2015. NF-kappaB signaling pathways in neurological inflammation: a mini review. Front. Mol. Neurosci. 8, 77. https://doi.org/10.3389/fnmol.2015.00077.

Song, J., Qian, Y., Li, G., Zhao, X., 2013. Anti-inflammatory effects of kudingcha methanol extract (*Ilex kudingcha* CJ Tseng) in dextran sulfate sodium-induced ulcerative colitis. Mol. Med. Rep. 8, 1256–1262.

Tak, P.P., Firestein, G.S., 2001. NF-κB: a key role in inflammatory diseases. J. Clin. Invest. 107, 7–11.

Wu, J., Liu, K., Shi, X., 2016. The anti-inflammatory activity of several flavonoids isolated from *Murraya paniculata* on murine macrophage cell line and gastric epithelial cell (GES-1). Pharmaceut. Biol. 54, 868–881.

Xie, M., Chen, G., Hu, B., Zhou, L., Ou, S., Zeng, X.X., Sun, Y., 2016. Hydrolysis of dicaffeoylquinic acids from *Ilex kudingcha* happens in the colon by intestinal microbiota. J. Agric. Food Chem. 64, 9624–9630.

Xu, D., Wang, Q., Zhang, W., Hu, B., Zhou, L., Zeng, X.X., Sun, Y., 2015. Inhibitory

activities of caffeoylquinic acid derivatives from *Ilex kudingcha* C.J. Tseng on α-glucosidase from *Saccharomyces cerevisiae*. J. Agric. Food Chem. 63, 3694–3703.
Yang, G., Ham, I., Choi, H., 2013. Anti-inflammatory effect of prunetin via the suppression of NF-κB pathway. Food Chem. Toxicol. 58, 124–132.
Zhang, T., Hu, T., Jiang, J., Zhao, J., Zhu, W., 2018. Antioxidant and anti-inflammatory effects of polyphenols extracted from *Ilex latifolia* Thunb. RSC Adv. 8, 7134–7141.
Zhao, X., Wang, Q., Qian, Y., Song, J., 2013. *Ilex kudingcha* CJ Tseng (Kudingcha) has *in vitro* anticancer activities in MCF-7 human breast adenocarcinoma cells and exerts anti-metastatic effects *in vivo*. Oncology Letters 5, 1744–1748.
Zhou, R., Shi, X., Gao, Y., Cai, N., Jiang, Z., Xu, X., 2015. Anti-inflammatory activity of guluronate oligosaccharides obtained by oxidative degradation from alginate in lipopolysaccharide-activated murine macrophage RAW 264.7 cells. J. Agric. Food Chem. 63, 160–168.
Zhu, K., Li, G., Sun, P., Wang, R., Qian, Y., Zhao, X., 2014. *In vitro* and *in vivo* anti-cancer activities of Kuding tea (*Ilex kudingcha* CJ Tseng) against oral cancer. Experimental and Therapeutic Medicine 7, 709–715.