

## Article

# *Carthamus tinctorius* Suppresses LPS-Induced Anti-Inflammatory Responses by Inhibiting the MAPKs/NF- $\kappa$ B Signaling Pathway in HaCaT Cells

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**Abstract:** This study aimed to elucidate the anti-inflammatory activity of *C. tinctorius* leaves by measuring inflammatory parameters such as nitric oxide (NO) production and mRNA expression of iNOS, interleukin-6 (IL-6), and IL-1 $\beta$  in lipopolysaccharide (LPS)-induced HaCaT cells. Further, the effect of *C. tinctorius* ethanol extract on the MAPKs/NF- $\kappa$ B signaling pathway was examined in HaCaT cells. The phytochemical profile of the ethanol extract of *C. tinctorius* leaves was determined using UPLC-QTOF-MS/MS. The results indicated that the ethanol extract of *C. tinctorius* effectively attenuated LPS-induced secretion of NO, IL-6, and IL-1 $\beta$  in HaCaT cells. Further, LPS-stimulated mRNA and protein expressions of iNOS were decreased by pre-treatment with *C. tinctorius* ethanol extract at the transcriptional level in HaCaT cells. Moreover, the ethanol extract of *C. tinctorius* suppressed NF- $\kappa$ B signaling in LPS-induced HaCaT cells. This suppression was mediated by MAPKs/NF- $\kappa$ B signaling, inhibiting the phosphorylation of p38 and p65 in HaCaT cells. However, there is no significant effect on the phosphorylation of JNK by the ethanol extract. The QTOF-MS/MS analysis revealed the identification of 27 components in the ethanol extract of *C. tinctorius* leaves. The data demonstrate that the ethanol extract of *C. tinctorius* leaves protects the LPS-induced HaCaT cells by inhibiting the expression of iNOS, IL-6, and IL-1 $\beta$  and suppressing the phosphorylation of the p38, p65, p-JNK via inactivation of MAPKs/NF- $\kappa$ B signaling pathway. These results demonstrate that *C. tinctorius* leaves may serve as a potential candidate to prevent inflammation-related diseases.

**Keywords:** anti-inflammatory; *Carthamus tinctorius*; HaCaT cells; iNOS; MAPKs/NF- $\kappa$ B



**Citation:** Kim, S.-Y.; Hong, M.; Deepa, P.; Sowndhararajan, K.; Park, S.J.; Park, S.; Kim, S. *Carthamus tinctorius* Suppresses LPS-Induced Anti-Inflammatory Responses by Inhibiting the MAPKs/NF- $\kappa$ B Signaling Pathway in HaCaT Cells. *Sci. Pharm.* **2023**, *91*, 14. <https://doi.org/10.3390/scipharm91010014>

Academic Editor: William A. Donaldson

Received: 8 February 2023

Revised: 25 February 2023

Accepted: 1 March 2023

Published: 6 March 2023



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## 1. Introduction

Inflammation is an essential part of the defense mechanism of the host activated by the invasion of pathogens, damaged cells, and toxic compounds. Although inflammation plays an important role in the healing process, it is important pathogenesis of various chronic diseases, such as cardiovascular, diabetes, arthritis, and cancer [1]. In particular, the overproduction of nitric oxide (NO) is involved in inflammatory conditions, so it is regarded as an important pro-inflammatory mediator. NO synthases (NOS) enzymes are primarily responsible for the production of NO in mammalian cells [2,3]. NOS consists of three members which are neuronal NOS, endothelial NOS, and inducible NOS (iNOS). In these, iNOS is highly involved in the production of NO [4,5]. The inflammatory response is distinguished by producing several pro-inflammatory cytokines, including IL-1 $\beta$ , IL-6, TNF- $\alpha$ , etc. [6]. Overproduction of IL-1 $\beta$  upregulates adhesion molecule expression in endothelial cells to activate the translocation of leukocytes, which is related to hyperalgesia and fever [7,8].

Bacterial lipopolysaccharide (LPS) has been exhibited to stimulate macrophages, resulting in the production of pro-inflammatory cytokines via activating NF- $\kappa$ B. NF- $\kappa$ B plays a major role in the regulation of gene expression that is associated with immune and inflammatory responses [9,10]. In this context, NF- $\kappa$ B response genes have been suggested as a promising strategy to treat different inflammatory diseases by inhibiting the production of inflammatory mediators [11,12]. MAPK and NF- $\kappa$ B, signaling molecules play a role in the Toll-like receptor (TLR) pathway [13,14]. Shao et al. [15] reported that the NF- $\kappa$ B signaling molecule regulates inflammatory responses by expressing inflammatory mediators as well as pro-inflammatory cytokines. Under non-induced conditions, the heterodimers of the NF- $\kappa$ B components, especially p50/p65, bind to the inactive form I $\kappa$ B protein in the cytoplasm.

However, NF- $\kappa$ B (p50/p65) is released via the degradation and phosphorylation of I $\kappa$ B by LPS stimulation. Consequently, NF- $\kappa$ B p65 plays an essential role in cellular inflammation response, mainly entering the nucleus and encoding the various chemokines and cytokines [16,17]. MAPKs refer to a special type of serine/thrombin kinase that responds to extracellular signals, including JNK, p38, and ERK1/2. Additionally, the MAPK signaling pathway is a response to LPS-stimulated iNOS expression in activated inflammatory cells [18]. Furthermore, MAPKs are mainly responsible for the activation of NF- $\kappa$ B [19]. Therefore, the potential therapeutic approach for inflammatory injury may be the inactivation of NF- $\kappa$ B and MAPK pathways [20].

Safflower (*Carthamus tinctorius* L.) belongs to the family of Asteraceae and is commercially cultivated to produce edible oils, dyes, and medicines from seeds and flowers [21]. *C. tinctorius* has been traditionally used to treat diseases of the cardiovascular system, connective tissues, blood circulation, and musculoskeletal system in Korea [22,23]. According to traditional Chinese medicine, *C. tinctorius* is used to regulate menstruation, alleviate pain, promote blood circulation, and remove blood stasis [24]. *C. tinctorius* also exhibited biological activities such as antimicrobial, antithrombotic, anticoagulant, antinociceptive, antitumor, and anti-inflammatory activity [24–26]. Previously, many studies reported on the phytochemical analysis and biological properties of florets of *C. tinctorius* [24]. However, scientific studies on the biological properties of leaves of *C. tinctorius* are meager.

Hence, this study aimed to evaluate the anti-inflammatory effect of the ethanol extract from the leaves of *C. tinctorius* via the inhibition of NO production and mRNA expression of iNOS, IL-6, and IL-1 $\beta$  in LPS-induced HaCaT cells. Further, the anti-inflammatory mechanisms of the ethanol extract of *C. tinctorius* leaves on MAPKs/NF- $\kappa$ B signaling pathway were examined in HaCaT cells. In addition, the phytochemical profile of ethanol extract of *C. tinctorius* leaves was evaluated by UPLC/QTOF-MS/MS.

## 2. Materials and Methods

### 2.1. Collection of Plant Sample

The leaves of *C. tinctorius* were collected during the flowering stage in Jeollabuk-do ARES Herb and Wild Plant Experimental Farm, Namwon (N 35°25'02.47 E 127°31'34.12), South Korea. The plant sample was authenticated by a plant taxonomist Dr. Jang Geun Jung at Kangwon National University and deposited in Kangwon National University Herbarium, Chuncheon, Republic of Korea, with voucher number KPNS-0952.

### 2.2. Preparation of Ethanol Extract

The shade-dried leaves of *C. tinctorius* were powdered using a grinder about the size of 0.6 mm. The powdered leaves (1 kg) were extracted twice with 95% ethanol (4 L) at temperature of 25  $\pm$  2 °C for 2 days. Then, the obtained filtrates were combined and concentrated using a rotary vacuum evaporator at 40 °C (EYELA NE-1101, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Then, the obtained extract was dried using a lyophilizer (FD5505, ILSHIN BIOBASE, Dongducheon, Republic of Korea). The ethanol extract of *C. tinctorius* was dissolved with ethanol (at 10 mg/mL) for further analysis.

### 2.3. Determination of Total Phenolic Content

The total phenolic content of ethanol extract of *C. tinctorius* leaves was determined by the modified Folin–Ciocalteu method [27].

### 2.4. Cell Culture

HaCaT cells (gifted by Prof. Lee OH, Food Chemistry laboratory, Kangwon National University) (CVCL-0038) were maintained in DMEM containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin at 37 °C with 5% CO<sub>2</sub> [28].

### 2.5. Cell Viability Assay

The MTT assay was performed to determine the cell viability of HaCaT cells. The cells were treated with *C. tinctorius* ethanol extract at 30–1000 µg/mL for 24 h. The cytotoxicity of ethanol extract of *C. tinctorius* in these cells was tested based on the method described by Kim et al. [29].

### 2.6. Nitric Oxide Production Assay

HaCaT cells were pretreated with the ethanol extract of *C. tinctorius* (12.5–100 µg/mL) for 1 h and then stimulated with LPS (1 µg/mL) for 24 h. The nitrite concentration in the medium was calculated from a sodium nitrite (NaNO<sub>2</sub>) standard curve [28,30].

### 2.7. Isolation of Total RNA and Real Time-Polymerase Chain Reaction (RT-PCR)

The mRNA expression of iNOS, IL-6, and IL-1β was determined by RT-PCR analysis using QuantStudio 3 (Applied Biosystems, Foster City, CA, USA) system with FG POWER SYBR Green PCR master mix and gene-specific primers (Table 1) [28]. Total RNA was isolated from HaCaT cells by RNAiso PLUS. Total RNA (1 µg) was used to generate cDNA by reverse transcription using All-in-One First-Strand cDNA Synthesis SuperMix [31].

**Table 1.** Primer sequences used for quantitative real-time PCR analysis.

| Target Gene |         | Primer Sequence                 | Accession Number |
|-------------|---------|---------------------------------|------------------|
| iNOS        | Forward | 5'-CATGCTACTGGAGGTGGGTG-3'      | NM_010927        |
|             | Reverse | 5'-CATTGATCTCCGTGACAGCC-3'      |                  |
| IL-6        | Forward | 5'-GAGGATACCACTCCCAACAGACC-3'   | NM_031168        |
|             | Reverse | 5'-AAGTGCATCATCGTTGTTTCATACA-3' |                  |
| IL-1β       | Forward | 5'-ACCTGCTGGTGTGTGACGTT-3'      | NM_008361        |
|             | Reverse | 5'-TCGTTGCTGGTTCCTTG-3'         |                  |
| β-actin     | Forward | 5'-ATCACTATTGGCAACGAGCG-3'      | NM_007393        |
|             | Reverse | 5'-TCAGCAATGCCTGGGTACAT-3'      |                  |

### 2.8. Enzyme-Linked Immunosorbent Assay (ELISA)

HaCaT cells were pretreated with ethanol extract of *C. tinctorius* at 12.5–100 µg/mL for 1 h and then treated with LPS at 1 µg/mL concentration for 24 h. The amount of IL-6 and IL-1β was measured from the culture supernatants of HaCaT cells using ELISA kits (Invitrogen, Carlsbad, CA, USA) [29].

### 2.9. Western Blot Analysis

For Western blot analysis, total proteins were extracted from HaCaT cells using a lysis buffer with cocktails of protein inhibitors [32]. Total cellular protein concentration was determined using the Bradford assay. A 20 µg protein per well was loaded to 10% SDS-PAGE and then transferred to PVDF membranes [33]. The membranes were blocked with 5% skimmed milk for 2 h and then incubated with primary antibodies against p-JNK, 1:1000; JNK, 1:1000; p-ERK, 1:1000; ERK, 1:1000; p-p38, 1:1000; p38, 1:1000; p-p65, 1:500; p65, 1:500; iNOS, 1:500; β-actin, 1:1000 at 4 °C overnight (Cell Signaling Technology, Danvers,

MA, USA). Then, the washed membranes were incubated with secondary antibodies (Cell Signaling, 1:1000) for 2 h at room temperature.

#### 2.10. Phytochemical Analysis of *C. tinctorius* by UPLC-QTOF-MS/MS

The LC/MS systems consisted of a Waters Acquity UPLC I-Class system (Waters Co., Milford, MA, USA) with Waters Xeo G2 QTOF MS (Waters MS Technologies, Manchester, UK). An amount of 1 mg of different fractions (hexane, dichloromethane, and ethyl acetate) obtained from the ethanol extract of *C. tinctorius* was dissolved in 10 mL of 70% ethanol for LC-MS analysis. Then, 2  $\mu$ L of diluted solution of *C. tinctorius* was injected into a Waters ACQUITY UPLC BEH C18 column (50  $\times$  2.1 mm, 1.7  $\mu$ m). The column conditions and the phytochemical characterization were followed based on the method described by Shin et al. [34]. The compounds in the ethanol extract of *C. tinctorius* were identified by UNIFI 1.8 (Waters, Milford, CT, USA). The traditional Chinese medicine library and the in-house library were used for the tentative identification of chemical components.

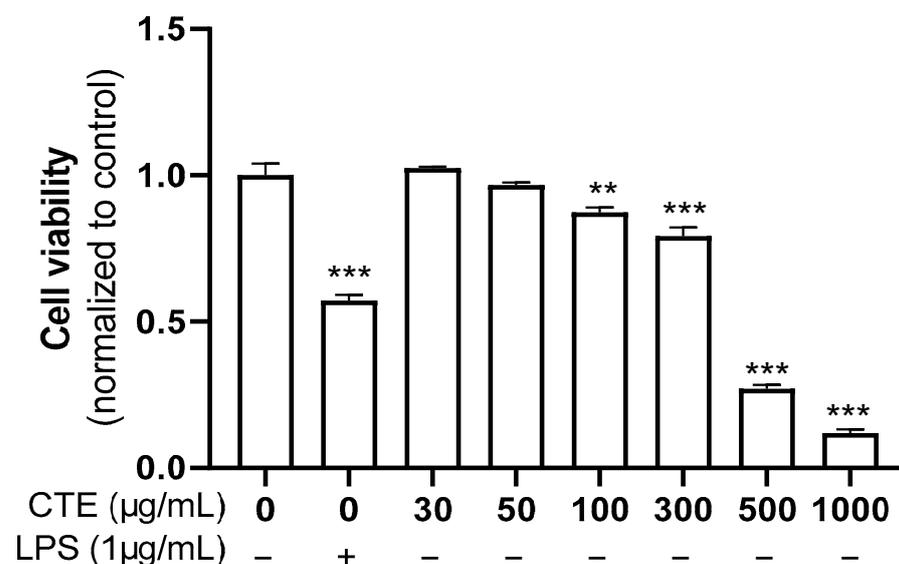
#### 2.11. Statistical Analysis

GraphPad Prism Version 8.0 (GraphPad, La Jolla, CA, USA) software was used for data analyses. The values are expressed as the mean of three independent determinations  $\pm$  SEM. Statistical differences were assessed using the Student–Newman–Keuls test for multiple comparisons after being analyzed with a one-way ANOVA. The *p* values < 0.05 were considered statistically significant.

### 3. Results

#### 3.1. The Effect of Ethanol Extract of *C. tinctorius* on the Viability of HaCaT Cells

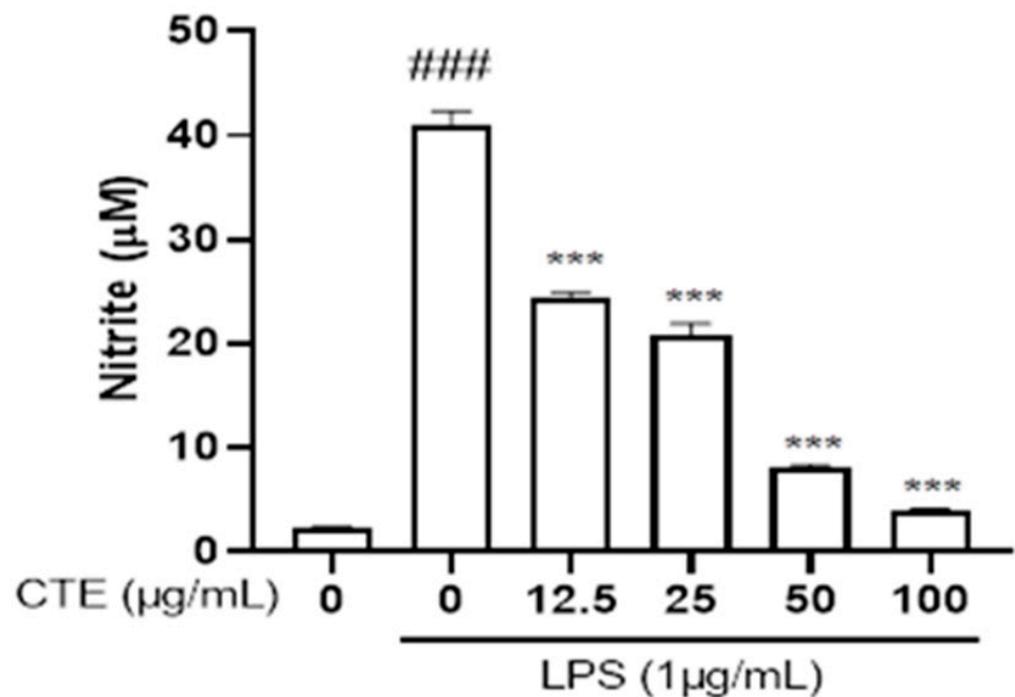
Previously, several studies reported atopic dermatitis-related inflammation in macrophages and keratinocytes [35–37]. Therefore, we investigated whether ethanol extract of *C. tinctorius* exhibits LPS-induced anti-inflammatory effects on HaCaT cells. For this purpose, the cytotoxicity of the ethanol extract of *C. tinctorius* was determined by MTT assays after incubating HaCaT cells with different concentrations of the ethanol extract of *C. tinctorius* (0–1000  $\mu$ g/mL) for 24 h. After the treatment, there was no significant effect observed in HaCaT with up to 100  $\mu$ g/mL concentration of the ethanol extract of *C. tinctorius* (cell viability > 90%) (Figure 1). Hence, we selected the concentration of *C. tinctorius* ethanol extract up to 100  $\mu$ g/mL for further experiment.



**Figure 1.** The effect of *C. tinctorius* ethanol extract on the cell viability of HaCaT cells. CTE, *C. tinctorius* ethanol extract (0–1000  $\mu$ g/mL). Values are expressed as mean (*n* = 5)  $\pm$  SEM. \*\* *p* < 0.01, \*\*\* *p* < 0.001 vs. 1% ethanol alone.

### 3.2. Effect of *C. tinctorius* Ethanol Extract on Nitric Oxide Production in LPS-Stimulated HaCaT Cells

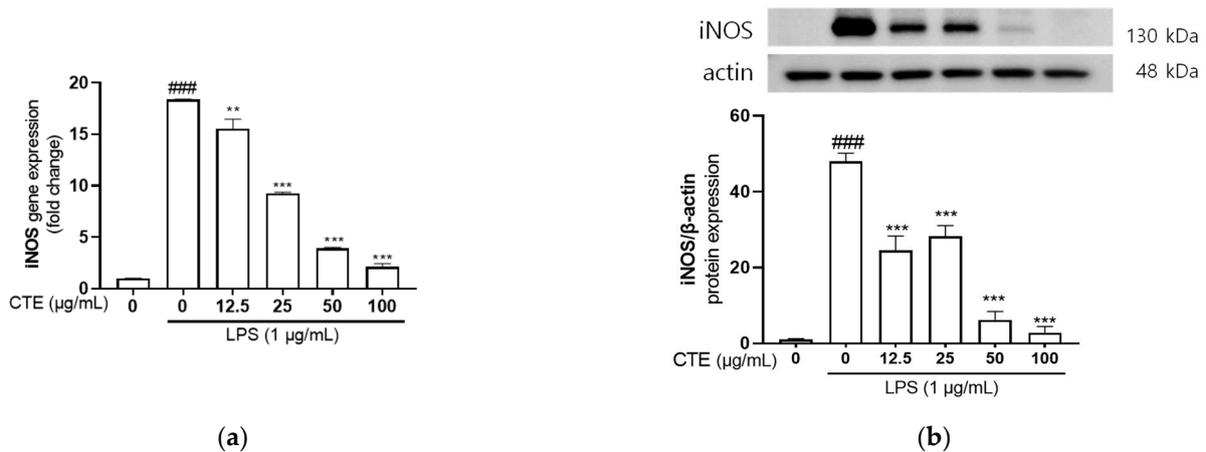
It is well known that LPS stimulation is responsible for the release of different inflammatory mediators, especially NO. NO is an important mediator that regulates the molecules with different biological functions including pathological processes in normal physiological conditions. However, excessive production of NO induces inflammation in abnormal physiological conditions [38,39]. Therefore, the inhibitory effect of *C. tinctorius* ethanol extract on NO production in LPS-induced HaCaT cells was evaluated (Figure 2). The NO production was significantly increased by accumulating a higher level of nitrite in HaCaT cells (40.97  $\mu\text{M}$ ). To investigate the effect of *C. tinctorius* extract on NO production, HaCaT cells were simultaneously treated with 1  $\mu\text{g}/\text{mL}$  LPS and various concentrations of extracts (12.5–100  $\mu\text{g}/\text{mL}$ ) separately for 24 h. When compared to the untreated control, HaCaT cells pre-treated with ethanol extract of *C. tinctorius* significantly ( $p < 0.001$ ) reduced the nitrite concentration in the medium to 4.02  $\mu\text{M}$  at the concentration of 100  $\mu\text{g}/\text{mL}$  (Figure 2).



**Figure 2.** Inhibitory effect of *C. tinctorius* ethanol extract on NO production by LPS-stimulated HaCaT cells. CTE, *Carthamus tinctorius* ethanol extract (0–100  $\mu\text{g}/\text{mL}$ ). Values are expressed as mean ( $n = 3$ )  $\pm$  SEM. ###  $p < 0.001$  vs. 1% ethanol alone, \*\*\*  $p < 0.001$  vs. LPS alone.

### 3.3. The Effect of Ethanol Extract of *C. tinctorius* on mRNA and Protein Expressions of iNOS in LPS-Stimulated HaCaT Cells

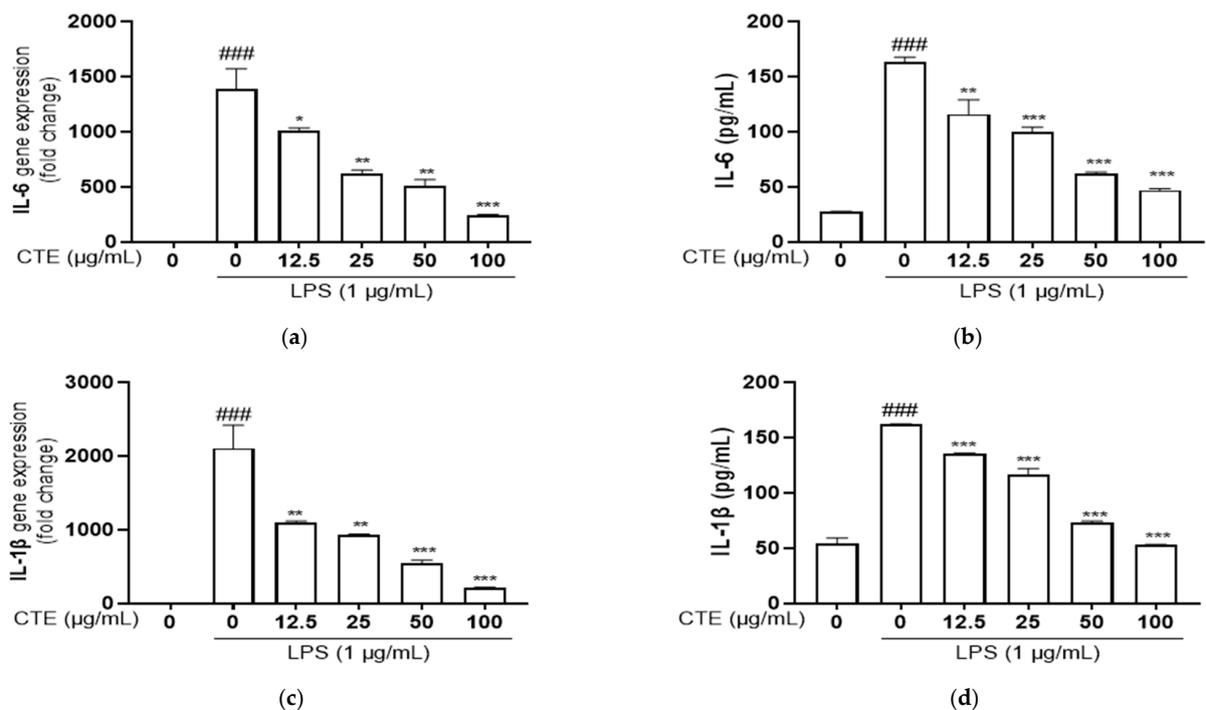
To determine the inhibitory action of *C. tinctorius* ethanol extract on NO production from HaCaT cells, we calculated the mRNA expression level of iNOS by RT-PCR and protein expression level by Western blot analysis. In response to LPS, the iNOS mRNA levels were significantly increased in HaCaT cells. However, the pre-treatment with ethanol extract of *C. tinctorius* drastically suppressed the mRNA expression of iNOS in HaCaT cells (Figure 3a). Western blot analysis also demonstrated that LPS-treated HaCaT cells markedly increased the protein expression of iNOS. However, pre-treatment with ethanol extract of *C. tinctorius* showed strong inhibitory activity against iNOS protein expression HaCaT cells (Figure 3b).



**Figure 3.** Effects of ethanol extract of *C. tinctorius* on mRNA and protein expressions of iNOS in LPS-stimulated HaCaT cells. (a) mRNA expression of iNOS in HaCaT cells; (b) protein expression of iNOS in HaCaT cells. Values are expressed as mean (n = 3) ± SEM. ### p < 0.001 vs. 1% ethanol alone, \*\* p < 0.01, \*\*\* p < 0.001 vs. LPS alone.

**3.4. The Effect of Ethanol Extract of *C. tinctorius* on Pro-Inflammatory Cytokines in LPS-Stimulated HaCaT Cells**

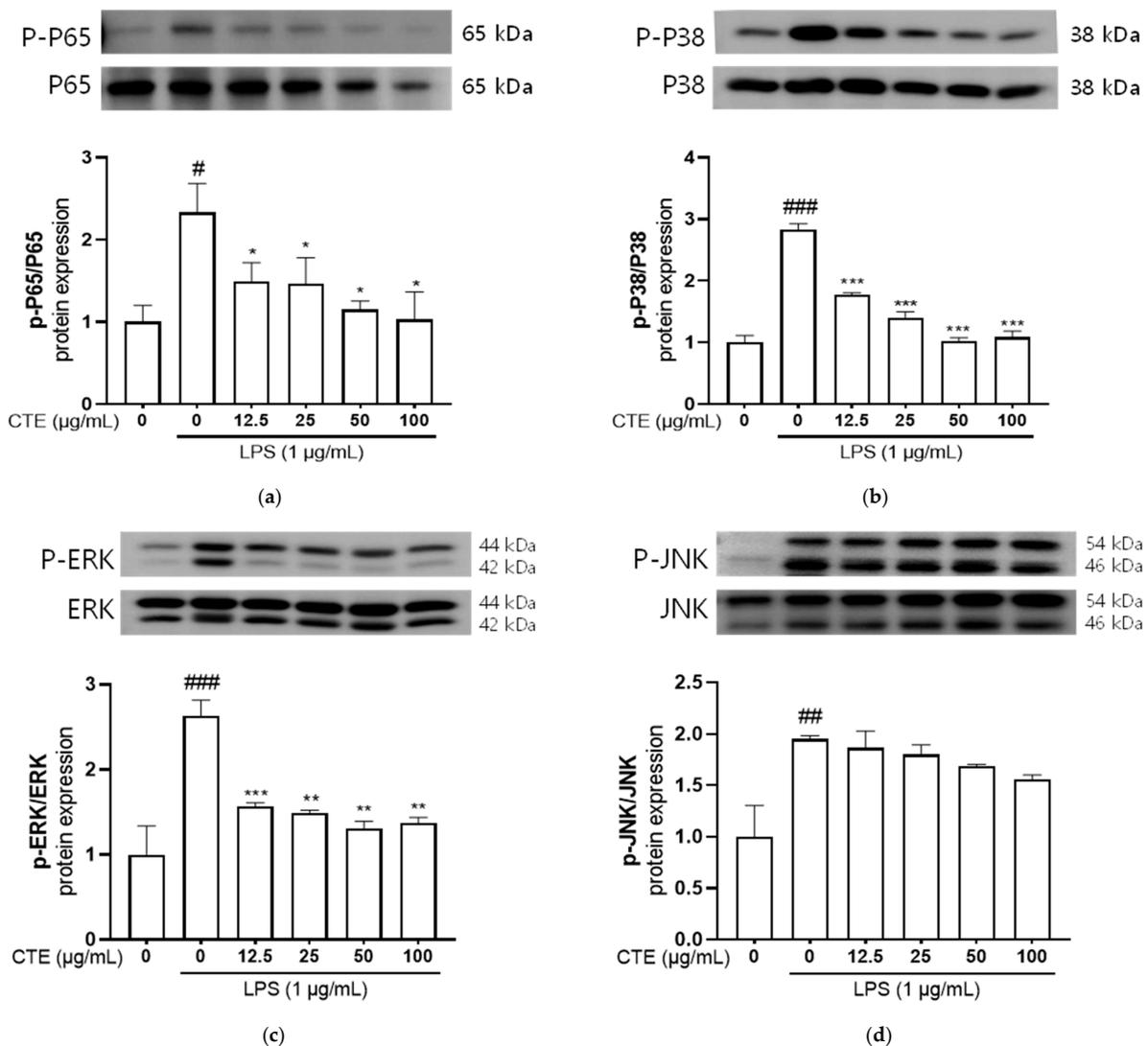
We also examined the inhibitory effects of *C. tinctorius* on the release of IL-6 and IL-1β in HaCaT cells induced by LPS. HaCaT cells were pretreated with different concentrations of *C. tinctorius* ethanol extract for 1 h and then stimulated with LPS (1 µg/mL) separately for 24 h. The mRNA expression level of IL-6 and IL-1β was determined in HaCaT cells by RT-PCR analysis. *C. tinctorius* ethanol extract significantly inhibited the mRNA expression levels of IL-6 and IL-1β in LPS-induced HaCaT cells (Figure 4) compared to that of the control.



**Figure 4.** Effects of *C. tinctorius* ethanol extract on pro-inflammatory cytokines in LPS-stimulated HaCaT cells. (a) mRNA expression of IL-6; (b) protein production of IL-6; (c) mRNA expression of IL-1β; (d) protein production of IL-1β. Values are expressed as mean (n = 3) ± SEM. ### p < 0.001 vs. 1% ethanol alone, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001 vs. LPS alone.

### 3.5. The Effect of *C. tinctorius* Ethanol Extract on MAPKs/NF- $\kappa$ B Activation in LPS-Stimulated HaCaT Cells

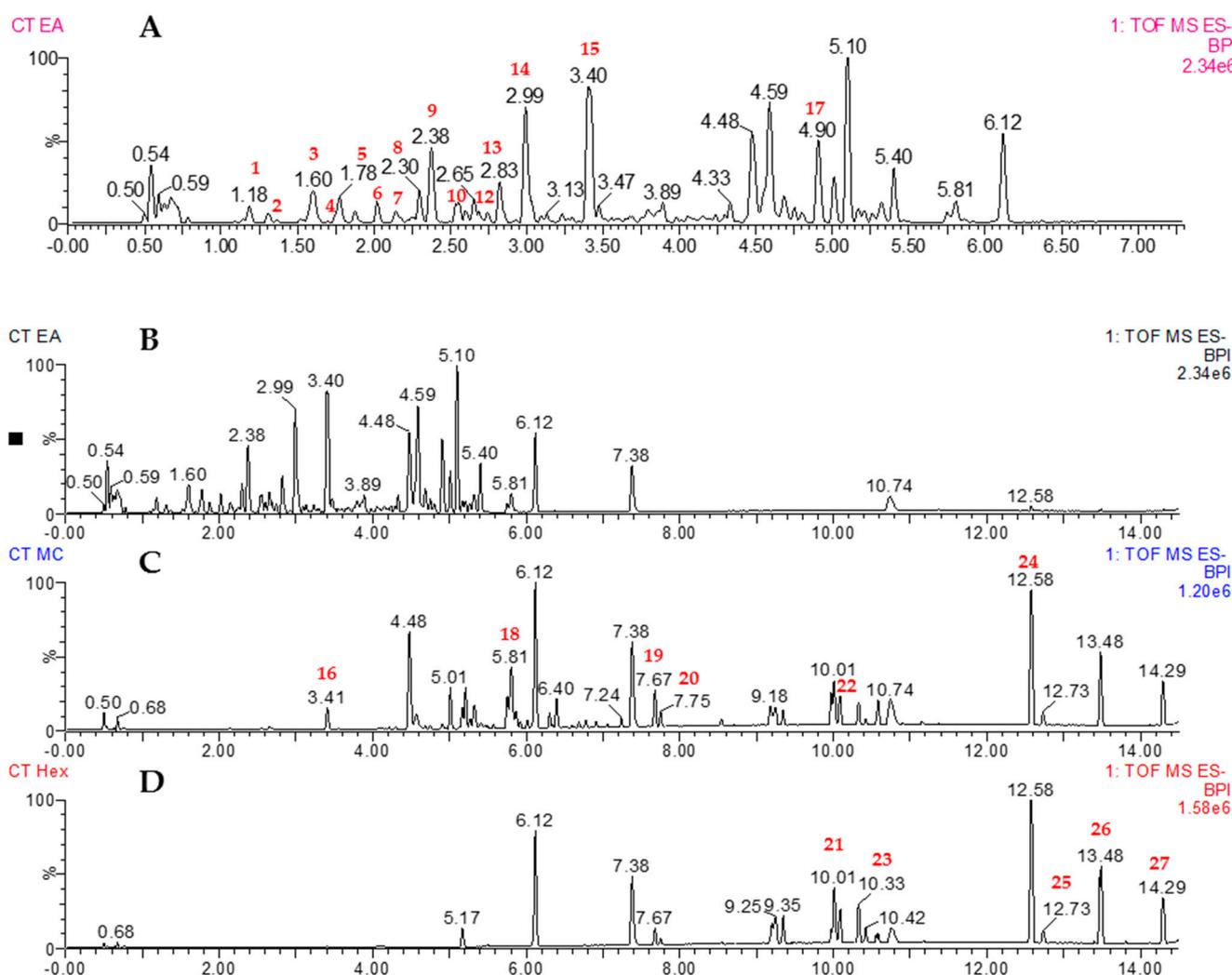
We investigated whether *C. tinctorius* ethanol extract inhibited the phosphorylation of p65 (a subunit of NF- $\kappa$ B) in HaCaT cells. The LPS treatment increased the phosphorylation of p65 in HaCaT cells, but *C. tinctorius* ethanol extract significantly attenuated the phosphorylation of P65 in LPS-stimulated HaCaT cells (Figure 5a). Subsequently, we identified which kinase was involved in the regulation of MAPKs/NF- $\kappa$ B activity in HaCaT cells. The results revealed that the phosphorylation of p38, ERK, and JNK (Figure 5b–d) was increased in HaCaT cells by LPS stimulation and the ethanol extract of *C. tinctorius* effectively inhibited the phosphorylation of p38 and ERK in HaCaT cells. However, the ethanol extract of *C. tinctorius* did not exhibit any effect on the phosphorylation of JNK in HaCaT cells. These results demonstrated that *C. tinctorius* ethanol extract exhibited anti-inflammatory activity on LPS-induced HaCaT cells by inactivating the MAPKs/NF- $\kappa$ B signaling pathway.



**Figure 5.** Effects of *C. tinctorius* on the phosphorylation of P65, P38, ERK, and JNK in LPS-stimulated HaCaT cells. (a) The immunoreactivity and quantitative analysis of p65 and phosphorylated p65 (p-p65); (b) p38 and p-p38; (c) ERK, and p-ERK; (d) JNK, and p-JNK. Values are expressed as mean (n = 3)  $\pm$  SEM. #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. 1% ethanol alone, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. LPS alone.

### 3.6. UPLC-QTOF-MS/MS Analysis of *C. tinctorius* Leaves

The total phenolic content of the ethanol extract of *C. tinctorius* leaves was  $18.27 \pm 0.13$  mg GAE/g. QTOF-MS/MS analysis was performed to detect the chemical profile in the ethanol extract of the leaves of *C. tinctorius*. For better separation of components, the ethanol extract was fractionated first with hexane, dichloromethane, and ethyl acetate successively to produce fractions of different polarities. Figure 6 shows the base peak intensity (BPI) chromatogram of different components from *C. tinctorius* leaves. The results revealed the presence of 27 different chemical components in the leaves of *C. tinctorius* (Table 2). The major components in the leaves of *C. tinctorius* are including protocatechuic acid ( $C_7H_6O_4$ ) caffeic acid ( $C_9H_8O_4$ ), coumaroylquinic acid ( $C_{16}H_{18}O_8$ ), coumaric acid ( $C_9H_8O_3$ ), quercetin hexoside ( $C_{21}H_{20}O_{12}$ ), kaempferol hexoside ( $C_{21}H_{20}O_{11}$ ), carthamine ( $C_{43}H_{42}O_{22}$ ) and kaempferol-3-O- $\beta$ -rutinoside ( $C_{27}H_{30}O_{11}$ ).



**Figure 6.** UPLC-QTOF-MS/MS analysis of different fractions obtained from ethanol extract of *C. tinctorius* leaves. (A) CT EA, ethyl acetate fraction (expanded chromatogram for 0.00 to 7.00 min); (B) CT EA, ethyl acetate fraction; (C) CT MC, dichloromethane fraction; (D) CT Hex, hexane fraction. Numerical in red font denotes the compound number listed in Table 2.

**Table 2.** The list of components identified in the ethanol extract of *C. tinctorius* leaves by UPLC-QTOF-MS/MS analysis.

| S. NO. | RT (min) | Tentative Identification                  | Formula   | Chemical Class          | m/z [M <sup>+</sup> H] | Mass Error (ppm) | Response | Fragmentation (m/z) | Reference |
|--------|----------|---|---|-------------------------|------------------------|------------------|----------|---------------------|-----------|
| 1      | 1.18     | Protocatechuic acid                       | C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>    | Phenolic acid           | 153.0192               | -0.9             | 259240   | 109.0297            | [40]      |
| 2      | 1.31     | Dihydrocaffeic acid                       | C <sub>9</sub> H <sub>10</sub> O <sub>4</sub>   | Phenylpropanoid         | 181.0503               | -1.6             | 148736   | 135.0448            | [41]      |
| 3      | 1.60     | Salicylic acid                            | C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>    | Beta hydroxy acid       | 137.0245               | 0.4              | 604343   | 93.0354             | [40]      |
| 4      | 1.76     | Esculetin                                 | C <sub>9</sub> H <sub>6</sub> O <sub>4</sub>    | Hydroxycoumarin         | 177.0191               | -1.1             | 150029   | 133.0295            | [42]      |
| 5      | 1.78     | Caffeic acid                              | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>    | Hydroxycinnamic acid    | 179.0347               | -1.4             | 415615   | 135.045             | [42]      |
| 6      | 1.89     | Coumaroylquinic acid                      | C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>  | Hydroxycinnamic acid    | 337.0930               | 0.3              | 86393    | 191.0563            | [42,43]   |
| 7      | 2.15     | Benzoic acid                              | C <sub>7</sub> H <sub>6</sub> O <sub>2</sub>    | Carboxylic acid         | 121.0298               | 2.6              | 226386   | 93.0344             | [42]      |
| 8      | 2.30     | 6-Hydroxykaempferol hexoside              | C <sub>21</sub> H <sub>20</sub> O <sub>12</sub> | Flavonoid               | 463.0883               | 0.3              | 299116   | 301.0359            | [44]      |
| 9      | 2.38     | Coumaric acid                             | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>    | Hydroxycinnamic acid    | 163.0399               | -1.1             | 1097613  | 119.0504            | [44]      |
| 10     | 2.50     | 3-(β-D-Glucopyranosyloxy)-5-octenoic acid | C <sub>14</sub> H <sub>24</sub> O <sub>8</sub>  | Fatty acid              | 319.1394               | -1.3             | 27071    | 157.0862            | Pubmed    |
| 11     | 2.61     | Quercetin hexoside                        | C <sub>21</sub> H <sub>20</sub> O <sub>12</sub> | Flavonoid               | 463.0884               | 0.4              | 127814   | 300.0288            | [44]      |
| 12     | 2.69     | Ferulic acid                              | C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>  | Hydroxycinnamic acid    | 193.0503               | -1.6             | 106615   | 134.0371, 149.060   | [40]      |
| 13     | 2.83     | Kaempferol-3-O-β-rutinoside               | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | Flavonoid               | 593.1518               | 1.1              | 334736   | 285.0413            | [44]      |
| 14     | 2.99     | Kaempferol hexoside                       | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub> | Flavonoid               | 447.0936               | 0.7              | 985035   | 285.0402            | [44]      |
| 15     | 3.40     | Azelaic acid                              | C <sub>9</sub> H <sub>16</sub> O <sub>4</sub>   | Dicarboxylic acid       | 187.0977               | 0.5              | 2328525  | 143.1076            | [40–42]   |
| 16     | 3.41     | Carthamine                                | C <sub>43</sub> H <sub>42</sub> O <sub>22</sub> | Hydroxycinnamic acid    | 449.1093               | 0.8              | 371603   | 287.0562            | [45,46]   |
| 17     | 4.90     | 4,6-Decadiyne-1-O-β-D-glucopyranoside     | C <sub>16</sub> H <sub>24</sub> O <sub>6</sub>  | Polyacetylene glycoside | 311.1503               | 0.9              | 878235   | -                   | [47]      |
| 18     | 5.81     | Trihydroxyoctadecenoic acid               | C <sub>18</sub> H <sub>34</sub> O <sub>5</sub>  | Fatty acid              | 329.2333               | -0.2             | 273521   | 211.1338            | [41,43]   |
| 19     | 7.67     | Hydroperoxyoctadecadienoic acid           | C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>  | Fatty acid              | 311.2225               | -0.7             | 281517   | 223.1670            | [41]      |
| 20     | 7.75     | 9-Octadecenedioic acid                    | C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>  | Fatty acid              | 311.2226               | -0.6             | 144857   | -                   | [41]      |
| 21     | 10.01    | Hydroxyoctadecadienoic acid               | C <sub>18</sub> H <sub>32</sub> O <sub>3</sub>  | Fatty acid              | 295.2274               | -1.4             | 376589   | 277.2175            | [41]      |
| 22     | 10.09    | Oxylipin                                  | C <sub>18</sub> H <sub>32</sub> O <sub>3</sub>  | Fatty acid              | 295.2275               | -1.1             | 249495   | 277.2176            | [41]      |
| 23     | 10.33    | Oxoctadecadienoic acid                    | C <sub>18</sub> H <sub>30</sub> O <sub>3</sub>  | Fatty acid              | 293.2115               | -2.4             | 227547   | 277.2175            | [41]      |
| 24     | 12.58    | Linolenic acid                            | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>  | Fatty acid              | 277.2175               | 0.7              | 1114693  | -                   | [41,42]   |
| 25     | 12.73    | n-Pentadecanal                            | C <sub>15</sub> H <sub>30</sub> O               | Fatty aldehyde          | 225.2221               | 0                | 149217   | 197.1912            | [43]      |
| 26     | 13.48    | Linoleic acid                             | C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>  | Fatty acid              | 279.2328               | -0.5             | 583977   | -                   | [42]      |
| 27     | 14.29    | Palmitic acid                             | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>  | Fatty acid              | 255.2325               | -1.8             | 182918   | -                   | [41,42]   |

#### 4. Discussion

*Carthamus tinctorius* L. is utilized to treat numerous ailments, including inflammation-mediated diseases in Asian countries. Inflammation is an essential part of our body’s defense mechanism that can be activated by various factors. However, the mechanisms of the anti-inflammatory activity of *C. tinctorius* leaves have not been evaluated. In this study, the LPS-stimulated anti-inflammatory activity of *C. tinctorius* ethanol extract in HaCaT cells was demonstrated. LPS can generate many pro-inflammatory cytokines, including NO. The excessive production of NO results in many inflammatory diseases and it is an important inflammatory mediator [48–50]. In the cytotoxicity study, *C. tinctorius* ethanol extract did not produce any effect on the viability of HaCaT cells at 100 µg/mL concentration (Figure 1). We evaluated the inhibitory effect of *C. tinctorius* on the NO production of HaCaT cells stimulated with LPS. The results demonstrated that *C. tinctorius* ethanol extract effectively inhibited the LPS-stimulated NO production in HaCaT cells (Figure 2) and downregulated mRNA and protein expressions of iNOS. The LPS-induced HaCaT cells produce a rapid inflammatory reaction that can release pro-inflammatory cytokines (IL-6 and IL1β) and inflammatory mediators (iNOS) [51].

Attracting circulatory immune function cells, especially, neutrophils to fight infection is beneficial [52]. Yet, the excessive inflammatory reaction can lead to injury of tissues and organs. Hence, during the inflammatory response, the production of inflammatory mediators and pro-inflammatory cytokines should be strictly controlled [53,54]. We revealed that *C. tinctorius* ethanol extract inhibited the mRNA and protein expressions of iNOS in HaCaT cells (Figure 3). In addition, we determined the inhibitory effects of *C. tinctorius* on the release of IL-6 and IL-1β in LPS-induced HaCaT cells. We found that *C. tinctorius* ethanol extract also inhibited IL-6 and IL-1β mRNA expression in HaCaT cells. A previous study reported that *C. tinctorius* has a significant effect on the downregulation of iNOS and IL-1β in LPS-activated RAW 264.7 cells [55]. NF-κB is a regulatory transcription factor and is mainly involved in cellular responses to stimuli for the expression of TNFα, IL-1β, IL-6, iNOS, and COX-2 [50].

Dimers of NF-κB (p50/p65) are released and phosphorylated by LPS stimulation, immediately enter the nucleus, and bind exclusively with DNA sequences for promoting the expression of target genes [17,56]. Therefore, the inhibitory effect of *C. tinctorius* ethanol extracts on LPS-stimulated NF-κB was evaluated. This study found that phosphorylation of p65 was significantly increased by LPS stimulation for 1 h, which means increased NF-κB activation. However, *C. tinctorius* ethanol extract reduced the phosphorylation of p65 in HaCaT cells, revealing a notable inhibitory effect on NF-κB activity. Consequently, *C. tinctorius* ethanol extract inhibited the inflammatory mediator and pro-inflammatory

cytokines expression in LPS-induced HaCaT cells by downregulating the NF- $\kappa$ B. A previous study reported that *C. tinctorius* aqueous extract proved to suppress bleomycin-stimulated mRNA levels of IL-1 $\beta$ , TNF- $\alpha$ , and TGF- $\beta$ 1 in lung homogenates. Furthermore, it inhibits the increased activity of NF- $\kappa$ B and p38 MAPK phosphorylation in lung tissues [57]. *C. tinctorius* methanol extract showed anti-inflammatory activity through increased HO-1 induction via Nrf-2 signals by inhibiting LPS-induced expression of iNOS and COX-2 and TNF $\alpha$ -mediated VCAM-1 upregulation in RAW 264.7 cells [58].

A recent study proved that *C. tinctorius* honey extract inhibited the NO production, suppressed iNOS, IL-1 $\beta$ , TNF- $\alpha$ , and MCP-1 expressions, decreased the phosphorylation of I $\kappa$ B $\alpha$ , inhibited the NF- $\kappa$ B-p65 protein, in LPS-induced RAW 264.7 cells through the activation of Nrf-2/HO-1 signaling pathway [59]. Moschamine isolated from *C. tinctorius* significantly suppressed mRNA and protein expression of COX-2, mPGES-1, iNOS, IL-6, and IL-1 $\beta$  by downregulating STAT1/3 activation in RAW 264.7 cells induced with LPS [60]. The florets of *C. tinctorius* contain a major active constituent called safflower yellow. This constituent inhibited inflammation in TNF- $\alpha$ -induced rat chondrocytes by regulating the NF- $\kappa$ B/SIRT1/AMPK pathways and endoplasmic reticulum stress [61]. Another study found that aqueous extracts from the petals of *C. tinctorius* and its main constituent safflower yellow prevented NO production and PGE2 in LPS-stimulated RAW264.7 cells by downregulating iNOS and COX-2 expressions [62]. Polyacetylene glucosides isolated from florets of *C. tinctorius* significantly inhibited NO production in LPS-stimulated RAW264.7 cells [27]. Hydroxysafflor yellow A from *C. tinctorius* L showed an anti-inflammatory effect in LPS-induced microglia activation by activating a nicotinamide adenine dinucleotide-dependent enzyme, SIRT1 [63]. In addition, dichloromethane extract and its water-ethanolic part of *Carthamus lanatus* aerial parts exhibited anti-inflammatory activity in activated human neutrophils [64].

The MAPK family, including p38, JNK, and ERK, play an essential role in the transcriptional regulation of the LPS-stimulated expression of iNOS [65]. MAPKs are the upstream activator of NF- $\kappa$ B via blocking the transcriptional activation of NF- $\kappa$ B by MAPK inhibitors [66,67]. In this study, p38, JNK, and ERK phosphorylation dramatically increased LPS-stimulated HaCaT cells, suggesting that LPS upregulated the MAPK pathway in HaCaT cells. *C. tinctorius* ethanol extract inhibited p38 and ERK phosphorylation in HaCaT cells. A previous study reported that schizandrin A showed a significant protective effect of LPS-induced inflammation in HaCaT cells by suppressing apoptosis, promoting cell viability, inhibiting the expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , downregulating the miR-127 expression, inactivating the p38MAPK/ERK and JNK pathways [68]. Liu et al. [69] demonstrated that sinomenine retards LPS-induced phosphorylation of p65, I $\kappa$ B $\alpha$ , and p38MAPK in HaCaT cells by downregulating the long non-coding RNA colon cancer-related transcript-1. Tanshinol showed a protective effect on LPS-induced HaCaT cells by downregulating the miR-122 and inhibiting the JNK and NF- $\kappa$ B pathways [70]. This study suggested that the anti-inflammatory effect of *C. tinctorius* ethanol extract was associated with the regulation of MAPKs/NF- $\kappa$ B signaling pathways in LPS-stimulated HaCaT cells.

In this study, 27 different components were identified from the dried leaves of *C. tinctorius* by UPLC-QTOF. Previous studies reported that derivatives of quinic acid, coumaric acid, quercetin, and kaempferol attenuated immune responses through various mechanisms [71–74]. In particular, kaempferol and quercetin effectively downregulated the activation of important transcription factors for iNOS (NF- $\kappa$ B and STAT-1) in LPS-stimulated macrophages [71]. In LPS-induced RAW 264.7 cells, ferulic acid and its derivatives inhibited the production of NO through the suppression of iNOS expression and decreasing the production of prostaglandin E2 and TNF- $\alpha$  [75]. Quinic acid derivatives from *Uncaria tomentosa* also inhibited NF- $\kappa$ B activation in TNF- $\alpha$ -stimulated type II alveolar epithelial-like, A549 cells [72]. In another study, Zhao et al. [76] reported that p-coumaric acid inhibited inflammatory cytokines production in LPS-stimulated RAW 264.7 cells by blocking NF- $\kappa$ B and MAPK signaling pathways. In HaCaT cells, caffeic acid and ferulic acid inhibited UVA-stimulated matrix metalloproteinase-1 by regulating antioxidant defense systems [77].

Flavonoids, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside isolated from flowers of *C. tinctorius* showed remarkable anti-nociceptive and anti-inflammatory activities [78]. Therefore, the presence of various bioactive metabolites in the ethanol extract of *C. tinctorius* leaves might be ascribed to its anti-inflammatory potential.

## 5. Conclusions

The results demonstrated that *C. tinctorius* ethanol extract inhibited LPS-stimulated NO, IL-1 $\beta$ , and IL-6 production as well as protein and mRNA expressions of iNOS in HaCaT cells. Further, the mechanistic study revealed that the anti-inflammatory effect of *C. tinctorius* ethanol extract is mediated via MAPKs/NF- $\kappa$ B by inhibiting the phosphorylation of p65, p38, and ERK in HaCaT cells. It could be concluded that the ethanol extract of *C. tinctorius* leaves can be utilized for the development of an anti-inflammatory agent.

**Author Contributions:** Conceptualization, S.K. and S.J.P.; methodology, S.K. and S.J.P.; formal analysis, M.H. and S.-Y.K.; investigation, M.H., S.-Y.K., and S.P.; resources, M.H. and S.K.; data curation, S.-Y.K., M.H., S.P. and P.D.; writing—original draft preparation, P.D. and K.S.; writing—review and editing, S.K. and K.S.; supervision, S.K. and S.J.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** This study was carried out with the support of the “Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01669705)”, Rural Development Administration, Republic of Korea.

**Conflicts of Interest:** The authors declare no conflict of interest.

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