Ethyl acetate fraction of *Curcuma longa* leaves suppresses IL-6-induced STAT3 activation via ERK signaling in Hep3B cells

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Abstract. The interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling pathway plays a pivotal role in regulating inflammation and tumorigenesis, making it a significant target for therapeutic intervention. Although the rhizome of *Curcuma longa* (*C. longa*) has been extensively investigated, the pharmacological potential of its aerial parts, such as the leaves, remains largely unexplored. The present study investigated the inhibitory effects of the ethyl acetate fraction of *C. longa* leaf extract (CL-E) on IL-6-induced STAT3 activation in Hep3B hepatoma cells *in vitro*. CL-E significantly suppressed IL-6-stimulated STAT3 transcriptional activity in a dose-dependent manner (IC_{50}, 33.9±3.7 μg/ml) without compromising cell viability (up to 60 μg/ml). Mechanistically, CL-E reduced STAT3 phosphorylation at Tyr705 while enhancing STAT3 phosphorylation at Ser727; this opposing pattern suggests an ERK-mediated rebalancing of STAT3 signaling that attenuates canonical Tyr705-dependent activation and augments Ser727-associated modulation. Additionally, CL-E inhibited STAT3 nuclear translocation and downregulated the mRNA expression of STAT3 target genes, including C-reactive protein and suppressor of cytokine signaling 3. Mechanistic investigations revealed that CL-E promoted extracellular signal-regulated kinase (ERK)1/2 phosphorylation, leading to increased STAT3 Ser727 phosphorylation. Notably, the inhibitory effect of CL-E on STAT3 Tyr705 phosphorylation was reversed by MEK1/2 (U0126) or PKC inhibitors (bisindolylmaleimide II), indicating that CL-E modulates STAT3 signaling through an ERK-mediated mechanism. Collectively, these *in vitro* findings identify *C. longa* leaves as an underutilized botanical resource with the potential to regulate IL-6/STAT3 signaling, warranting *in vivo* evaluation to establish its efficacy, safety and pharmacokinetics, and to define potential therapeutic utility in IL-6/STAT3-driven conditions.

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Abbreviations: CL-E, Curcuma longa leaf extract; CRP, C-reactive protein; IL-6, Interleukin-6; STAT3, signal transducer and activator of transcription 3; SOCS3, suppressor of cytokine signaling 3

Key words: aerial part, *Curcuma longa* L., IL-6, STAT3, ERK1/2, MAPK

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine integral to immune regulation, inflammatory signaling, cellular proliferation, and survival (1,2). Upon binding to its receptor complex, which includes gp130, IL-6 activates the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathway, resulting in the transcription of a wide array of target genes implicated in inflammatory and oncogenic processes (3-7). The activation of STAT3 is tightly regulated by phosphorylation at two key residues, Tyrosine705 and Serine727. Specifically, phosphorylation of STAT3 at Tyr705 is a critical event that promotes STAT3 dimerization, nuclear translocation, and subsequent transcriptional activation of downstream genes such as suppressor of cytokine signaling 3 (SOCS3) and C-reactive protein (CRP), which serve as important markers of IL-6-mediated responses. Aberrant or sustained activation of the IL-6/STAT3 signaling axis has been strongly associated with the pathogenesis of various chronic inflammatory diseases, including rheumatoid arthritis (RA) and

inflammatory bowel disease (IBD), as well as malignancies such as hepatocellular carcinoma, thus underscoring its significance as a therapeutic target (8-10).

Given the pivotal role of IL-6/STAT3 signaling in chronic inflammation and tumorigenesis, considerable research has focused on identifying pharmacological inhibitors that target this pathway. Natural products, particularly those derived from medicinal plants, have emerged as valuable sources of bioactive compounds capable of modulating cytokine signaling. *Curcuma longa* L. (Zingiberaceae), commonly known as turmeric, has long been used in traditional Asian medicine and is well known for its immunoregulatory, antioxidant, antihepatotoxic, hypocholesterolemic, and anticancer properties. These effects are primarily attributed to curcuminoids such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin, which are abundant in the rhizome (11-16). While the pharmacological activities of turmeric rhizome have been extensively investigated, the aerial parts of the plant, including the leaves, remain largely underexplored, despite reports of their antioxidant, antibacterial, and immuno-regulatory effects (17,18).

Recent studies have reported that *C. longa* leaf extracts (CL-E) modulate inflammatory signaling and host immunity. Phytochemical analyses of the aerial parts have identified flavonoids, tannins, and polyphenols that underlie the documented antioxidant and immunoregulatory activities (19-21). Nevertheless, the molecular basis by which these constituents affect cytokine-driven pathways remains unclear. In particular, it is unknown whether, and how, CL-E influences the IL-6/STAT3 pathway-a central regulator of acute-phase responses, inflammation, and tumorigenic signaling. Since STAT3 activation depends on site-specific phosphorylation at Tyr705 and Ser727 that governs dimerization, nuclear import, and transcriptional output, defining the impact of CL-E on these regulatory nodes addresses this key mechanistic gap.

In light of the ongoing need for novel natural inhibitors of the IL-6/STAT3 axis, we utilized a STAT3 luciferase reporter system to evaluate the effects of the ethyl acetate fraction of *C. longa* leaves on STAT3 activation, including downstream events such as STAT3 phosphorylation, nuclear translocation, and the expression of IL-6-responsive genes such as SOCS3 and CRP. To further elucidate the upstream regulatory mechanisms, we investigated the involvement of the MEK-ERK pathway employing specific pharmacological inhibitors, thereby characterizing the signaling cascade implicated in the CL-E mediated inhibitory effects on STAT3. Accumulating evidence suggests that protein kinase C (PKC) can activate the Raf-MEK-ERK cascade and that ERK-driven Ser727 phosphorylation can modulate- or in some contexts antagonize-Tyr705-dependent STAT3 activation (9). We therefore hypothesized that the ethyl acetate fraction of *C. longa* leaves (CL-E) attenuates IL-6/STAT3 signaling by engaging a PKC-MEK/ERK axis and shifting the Tyr705/Ser727 phosphorylation balance.

Collectively, our findings provide novel insights into the molecular mechanisms by which CL-E modulates IL-6/STAT3 signaling and suggest its potential as a natural therapeutic agent for inflammation-related disorders.

Materials and methods

Reagents and chemicals. Recombinant human IL-6 was procured from R&D Systems. Mouse anti-phospho STAT3 (Tyr705) IgG was obtained from Calbiochem. Rabbit anti-phospho Stat3 (Ser727) IgG, anti-total STAT3 IgG, anti-phospho ERK1/2 IgG, anti-total ERK1/2 IgG, β-actin and secondary antibody were purchased from Cell Signaling Technology. All other reagents, including genistein, U0126, and bisindolylmaleimide II, were acquired from Sigma-Aldrich.

Preparation of crude extracts. Aerial parts of *C. longa* L. were provided by the Rural Development Administration (RDA) of Korea. The plant materials were extracted using methanol solvents (5 lx2 times). Methanol extracts (1 g) were suspended in 50 ml of distilled water and successively partitioned with ethyl acetate (100 ml x 3), yielding the ethyl acetate-soluble fraction (227 mg, CL-E).

Cell line and cell culture. Human hepatoma Hep3B cells were obtained from the American Type Culture Collection (ATCC; no. HB-8064) and were maintained in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin at 37˚C in a humidified 5% CO_2 incubator. All cell culture reagents were obtained from GibcoBRL (Life Technologies).

Establishment of stable cell line expressing pStat3-Luc. As previously described, a stable Hep3B cell line expressing a STAT3-responsive luciferase reporter (pStat3-Luc) was established (22). Briefly, Hep3B cells were co-transfected with pStat3-Luc (containing a STAT3-binding site) and pcDNA3.1(+) carrying a hygromycin resistance gene (Clontech Laboratories) using Lipofectamine Plus (Invitrogen). After 48 h, cells were selected with 100 μg/ml hygromycin, and stable clones were expanded. Luciferase expression was confirmed by luciferase assay.

Luciferase assay. Stable pStat3-Luc-expressing Hep3B cells were seeded in 96-well plates at a density of $2x10^4$ cells/well. After 24 h, the cells were serum-starved for 12 h and subsequently treated with IL-6 (10 ng/ml) for 12 h in the presence or absence of test compounds. Luciferase activity was quantified using a commercial kit (Promega) according to the manufacturer's instructions.

Cell viability. Hep3B cells were seeded at a density of $1x10^4$ cells/well and incubated for 24 h. The medium was then replaced with serum-free DMEM containing varying concentrations of test samples. Following 48 h of treatment, MTT solution (0.5 mg/ml) was added to each well and incubated for 4 h. Subsequently, 100 μl of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 540 nm using a microplate reader, and cell viability was calculated relative to the untreated control.

Western blot analysis. Total protein lysates were prepared and analyzed using Western blot as previously described (22). Briefly, Cells were lysed in Cell Lysis Buffer (Cell Signaling

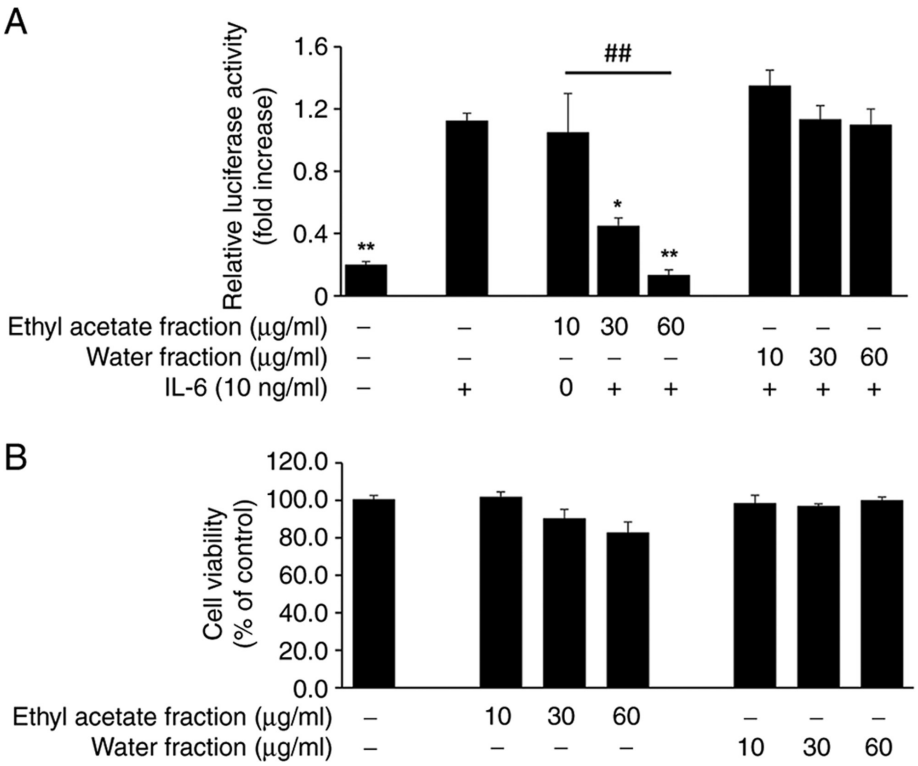

Figure 1. Ethyl acetate fraction of CL-E inhibits IL-6-induced STAT3 activation in Hep3B cells. (A) Hep3B cells stably expressing STAT3-responsive luciferase reporter (pSTAT3-Luc) were treated with IL-6 (10 ng/ml) for 12 h in the presence or absence of CL-E or an aqueous fraction of *C. longa* L. leaves extract at concentrations of 10, 30, and 60 μg/ml. Luciferase activity was measured according to the manufacturer's instructions. Data are presented as relative luciferase activity normalized to the untreated control. (B) Hep3B cells were seeded in 96-well plates and treated with each fraction for 24 h at the indicated concentrations. Cell viability was assessed using the MTT assay. Results are expressed as the percentage of viable cells relative to the untreated control. Data represent the mean ± SE (n≥3). *P<0.05 and **P<0.01 vs. the IL-6 alone group; $^{\#\#}$P<0.01 indicates a significant difference between the 10 and 60 μg/ml CL-E doses. CL-E, *Curcuma longa* L. extract; IL-6, interleukin 6; STAT3, signal transducer and activator of transcription 3.

Technology) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentrations were determined using the DC protein assay (Bio-Rad). Equal amount of whole cell lysates (20-40 μg per lane) were separated by SDS-PAGE on 4-12% gradient gels and transferred to PVDF membranes (Amersham Bioscience). Membranes were blocked with 5% (w/v) non-fat dry milk in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature, then incubated overnight at 4˚C with primary antibodies against p-STAT3 (Tyr705; 1:1,000; CST #9145), p-STAT3 (Ser727; 1:1,000; CST #9134), t-STAT3 (1:1,000; CST #4904), p-ERK (1:1,000; CST #4370), t-ERK (1:1,000; CST #4695) and β-actin (1:2,000; CST #4967). After washing in TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2,000; CST #7074) for 30 min. Signals were developed using an enhanced chemiluminescence (ECL) substrate (West-ZOL Plus kit; iNtRON Biotechnology) and recorded on X-ray film (Eastman Kodak Co.). All blots were reproduced in triplicate experiments.

Immunocytochemistry for STAT3 localization. Hep3B cells were cultured on 8-well Nunc Lab-Tek chamber slides and treated with IL-6 (10 ng/ml) for 4 h, in the presence or absence of CL-E. Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 100% cold methanol for 5 min, and blocked with 5% BSA for 30 min. Cells were incubated overnight at 4˚C with rabbit anti-STAT3 antibody (1:200), followed by FITC-conjugated goat anti-rabbit IgG (1:1,000) for 1 h. Slides were mounted using SlowFade Gold antifade reagent (Invitrogen), and fluorescence images were acquired using a Leica DM5000B microscope (Leica).

RNA isolation, cDNA synthesis, and real-time RT-PCR. Total RNA was extracted from Hep3B cells using the RNeasy MinElute Cleanup Kit (Qiagen), including on-column DNase treatment. RNA concentration and quality were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems), and real-time PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems). TaqMan primers for human CRP (Hs04183452_g1) and SOCS3 (Hs02330328_s1) were obtained from Applied Biosystems. 18S rRNA (Hs99999901_s1) was used as an endogenous control. Data were analyzed using the $2^{-\Delta\Delta CT}$ method, and gene expression levels were presented relative to those of untreated controls.

Data analysis. All experiments were performed in triplicate. Data are presented as the mean ± standard error (SE). Statistical analyses were conducted using GraphPad Prism 5 (GraphPad software Inc.). One-way ANOVA followed by Tukey's post hoc

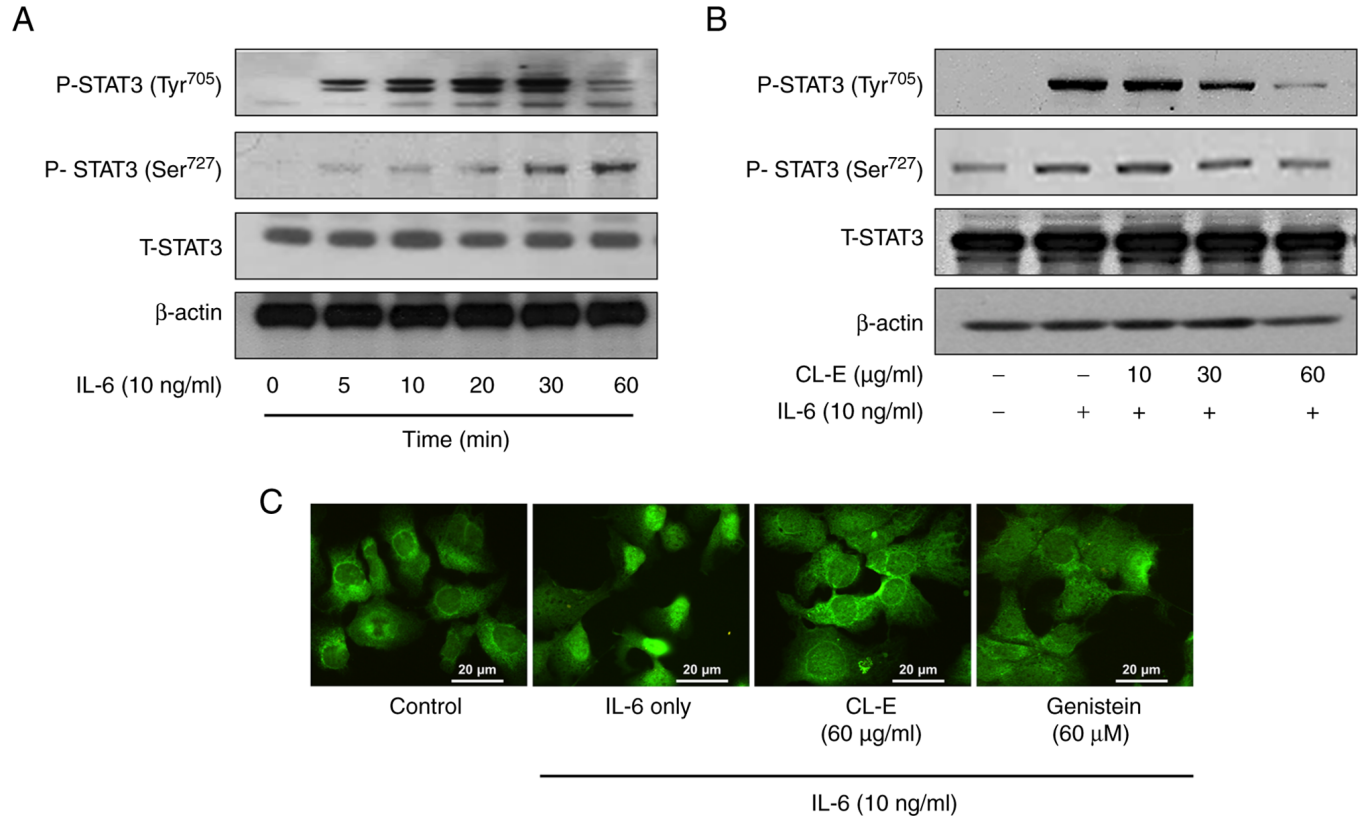

Figure 2. CL-E inhibits IL-6-induced STAT3 phosphorylation and nuclear translocation in Hep3B cells. (A) Hep3B cells were treated with IL-6 (10 ng/ml) for the indicated time periods (0-60 min). Total cell lysates were analyzed by western blotting using antibodies against P-STAT3 (Tyr705), P-STAT3 (Ser727) and total STAT3. (B) Cells were pretreated with the CL-E at 10, 30 and 60 μg/ml for 1 h and then stimulated with IL-6 (10 ng/ml) for 30 min. Proteins were extracted and analyzed by western blotting as described above. (C) STAT3 nuclear translocation was assessed by immunofluorescence microscopy. Hep3B cells were cultured on Nunc Lab-Tek II 8-well chamber slides and treated with IL-6 (10 ng/ml) for 4 h in the absence or presence of CL-E (60 μg/ml) or genistein (60 μM, positive control). Cells were fixed, permeabilized and stained with anti-STAT3 antibody followed by FITC-conjugated secondary antibody. Subcellular localization of STAT3 was visualized using fluorescence microscopy (Scale bar: 20 μm). CL-E, *Curcuma longa* L. extract; IL-6, interleukin 6; STAT3, signal transducer and activator of transcription 3; P-, phosphorylated.

test was used, and differences were considered statistically significant at $P<0.05$, $P<0.01$, and $P<0.001$.

Results

CL-E inhibits IL-6-induced STAT3 transcriptional activity without cytotoxicity. To explore natural products that inhibit IL-6-induced activation, a luciferase reporter assay was initially conducted using Hep3B cells stably transfected with a STAT3-responsive luciferase construct (pSTAT3-Luc) for screening purposes. During preliminary screening, methanol extracts of *C. longa* L leaves were found to suppress IL-6-stimulated STAT3-dependent activity (data not shown). Based on this observation, the methanol extract was subsequently partitioned into an ethyl acetate fraction (CL-E) and an aqueous fraction to isolate the active component. Both fractions were evaluated for their ability to inhibit IL-6-induced STAT3-dependent transcriptional activity. Cells were stimulated with IL-6 (10 ng/ml) for 12 h in the presence or absence of each fraction. As illustrated in Fig. 1A, CL-E significantly inhibited IL-6-induced luciferase activity in a dose-dependent manner (IC_{50}: 33.9±3.7 μg/ml), with a significant difference between the 10 and 60 μg/ml concentrations, whereas the aqueous fraction exhibited no significant inhibitory effect. Importantly, MTT assay results demonstrated that CL-E treatment up to 60 μg/ml did not induce cytotoxicity under the experimental conditions (Fig. 1B), indicating that the observed inhibition of STAT3 activity was not attributable to reduced cell viability.

CL-E suppresses STAT3 Tyr705 phosphorylation and nuclear translocation in IL-6-stimulated cells. To elucidate the mechanism underlying the inhibitory effect of CL-E on STAT3 transcriptional activity, we investigated its impact on STAT3 phosphorylation at two key regulatory residues, Tyr705 and Ser727, which are essential for STAT3 activation and function. Initially, a time-course analysis of STAT3 phosphorylation following IL-6 stimulation was conducted. Hep3B cells were treated with IL-6 (10 ng/ml) for varying durations (0, 5, 10, 20, 30, and 60 min), and cell lysates underwent Western blot analysis using phospho-specific antibodies against STAT3 phosphorylated at Tyr705 and Ser727, as well as total STAT3. As depicted in Fig. 2A, IL-6 treatment resulted in rapid, robust phosphorylation of STAT3 at Tyr705, peaking at approximately 20-30 min and declining thereafter. STAT3 phosphorylation at Ser727 also increased in response to IL-6 but appeared later and with less intensity compared to that at Tyr705. These results confirm

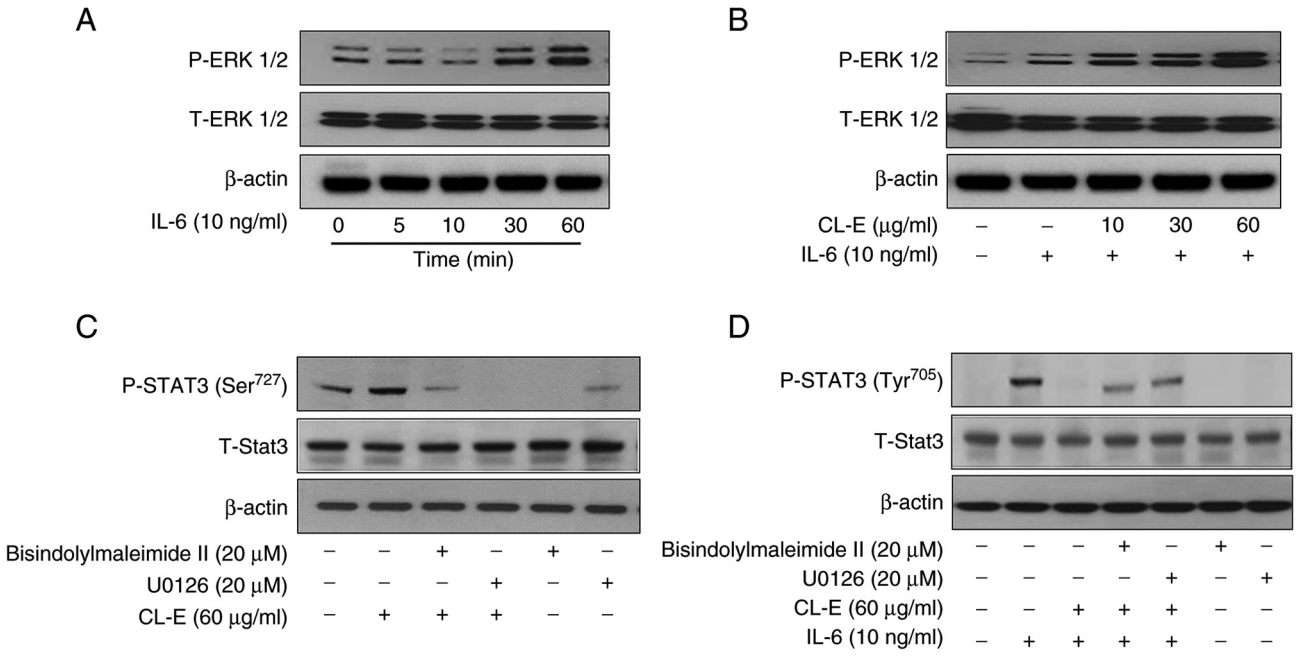

Figure 3. ERK signaling pathway contributes to CL-E-mediated regulation of IL-6-induced STAT3 activation. (A) Hep3B cells were stimulated with IL-6 (10 ng/ml) for the indicated times (0, 5, 10, 30 and 60 min). Protein lysates underwent western blotting analysis using antibodies against P-ERK1/2 and total ERK1/2 to evaluate time-dependent ERK1/2 activation. (B) Cells were pretreated with CL-E at 10, 30 and 60 μg/ml for 1 h, followed by IL-6 stimulation (10 ng/ml) for 30 min. ERK1/2 phosphorylation was analyzed by western blotting analysis. (C) To investigate the role of ERK in STAT3 Ser727 phosphorylation, cells were treated with CL-E (60 μg/ml), PKC inhibitor bisindolylmaleimide II (20 μM), and/or the MEK1/2 inhibitor U0126 (20 μM) for 1 h in the absence of IL-6. Phosphorylation of STAT3 (Ser727) and total STAT3 were examined by western blotting analysis. (D) To determine whether ERK activation contributes to regulation of STAT3 Tyr705 phosphorylation, cells were pretreated with CL-E, U0126 and/or bisindolylmaleimide II (20 μM) for 1 h before IL-6 stimulation (10 ng/ml, 30 min). STAT3 Tyr705 phosphorylation was analyzed by western blotting. CL-E, *Curcuma longa* L. extract; IL-6, interleukin 6; STAT3, signal transducer and activator of transcription 3; P-, phosphorylated.

activation of the canonical IL-6/STAT3 signaling pathway in Hep3B cells. To determine whether CL-E treatment affects IL-6-stimulated STAT3 phosphorylation, Hep3B cells were pretreated with CL-E (10, 30, and 60 μg/ml) for 1 h, followed by IL-6 stimulation (10 ng/ml for 30 min).

As illustrated in Fig. 2B, CL-E strongly inhibited IL-6-induced STAT3 Tyr705 phosphorylation in a dose-dependent manner, with maximal suppression at 60 μg/ml. Conversely, CL-E exerted no inhibitory effect on IL-6-stimulated STAT3 Ser727 phosphorylation. Total STAT3 levels remained constant, indicating that the observed effects were not due to changes in STAT3 protein expression. Give that phosphorylation at STAT3 Tyr705 promotes STAT3 dimerization and nuclear translocation, further examination of whether CL-E affects STAT3 nuclear localization was conducted using immunofluorescence microscopy. Hep3B cells cultured on chamber slides were pretreated with CL-E (60 μg/ml) or genistein [60 μM, a known STAT3 inhibitor (23)] for 1 h, followed by IL-6 stimulation for 4 h. Cells were fixed, permeabilized, and stained with an anti-STAT3 antibody, followed by FITC-conjugated secondary antibody. In cells exposed to IL-6, STAT3 was predominantly localized within the nucleus, indicative of its activated state. Conversely, in cells pretreated with CL-E or genistein, STAT3 was primarily cytoplasmic (Fig. 2C), suggesting that CL-E effectively inhibits STAT3 nuclear translocation, likely by suppressing Tyr705 phosphorylation.

These findings collectively demonstrate that CL-E inhibits IL-6/STAT3 signaling by suppressing STAT3 Tyr705 phosphorylation and preventing its nuclear translocation, thereby reducing its transcriptional activity.

CL-E enhances ERK1/2 phosphorylation and modulates STAT3 activation via the ERK signaling pathway. Previous studies have indicated that the activation of ERK1/2 can modulate IL-6-induced STAT3 activation (24-26). To investigate this possibility, we examined the involvement of the ERK1/2 pathway, known to regulate STAT3 phosphorylation at Ser727. Initially, we assessed ERK1/2 phosphorylation following IL-6 stimulation over time. Hep3B cells were treated with IL-6 (10 ng/ml) for 0, 5, 10, 30, and 60 min, and ERK1/2 phosphorylation was analyzed via Western blotting. As depicted in Fig. 3A, ERK1/2 phosphorylation remained low for the initial 10 min, began to increase at 30 min, and peaked at 60 min. Subsequently, we investigated whether ERK1/2 phosphorylation was affected by CL-E treatment in the presence of IL-6. Hep3B cells were pretreated with CL-E at concentrations of 10, 30, and 60 μg/ml for 1 h prior to IL-6 stimulation (10 ng/ml for 30 min).

As shown in Fig. 3B, CL-E significantly enhanced IL-6-induced ERK1/2 phosphorylation in a concentration-dependent manner, with the strongest effect observed at 60 μg/ml, suggesting that CL-E potentiates ERK activation under IL-6 stimulation. To determine whether ERK activation contributes to the regulation of STAT3 phosphorylation, we assessed STAT3 Ser727 phosphorylation in the absence of

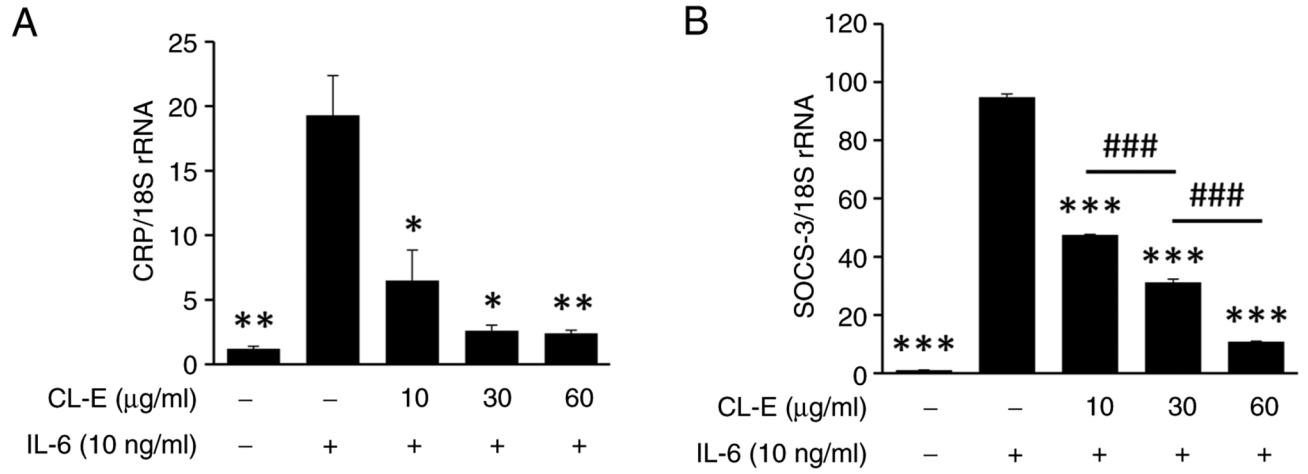

Figure 4. CL-E inhibits IL-6-induced expression of CRP and SOCS3 mRNA in Hep3B cells. Hep3B cells were pretreated with CL-E at concentrations of 10, 30, and 60 μg/ml for 1 h, followed by IL-6 stimulation (10 ng/ml) for 6 h. Total RNA was extracted, and the mRNA levels of (A) CRP and (B) SOCS3 were measured using quantitative real-time PCR. Gene expression levels were normalized to 18S rRNA and are presented as fold change relative to the untreated control. Data represent the mean ± SE (n≥3). *P<0.05, **P<0.01 and ***P<0.001 vs. the IL-6-treated group; $^{\#\#\#}$P<0.001. CL-E, *Curcuma longa* L. extract; IL-6, interleukin 6; CRP, C reactive protein; P-, phosphorylated; SOCS3, suppressor of cytokine signaling 3.

IL-6. Hep3B cells were treated with CL-E (60 μg/ml), either alone or in combination with the MEK1/2 inhibitor U0126 or the PKC inhibitor bisindolylmaleimide II. As shown in Fig. 3C, CL-E alone markedly increased STAT3 Ser727 phosphorylation. However, this effect was suppressed by co-treatment with U0126, suggesting that CL-E-induced Ser727 phosphorylation is mediated through the MEK/ERK pathway. Interestingly, bisindolylmaleimide II also attenuated Ser727 phosphorylation, indicating a possible role for PKC upstream of ERK in mediating the effect of CL-E.

Finally, to further ascertain whether ERK- and PKC-dependent signaling pathways are involved in the regulation of STAT3 Tyr705 phosphorylation, we examined the effects of these inhibitors under IL-6 stimulation. As depicted in Fig. 3D, CL-E pretreatment suppressed IL-6-induced STAT3 Tyr705 phosphorylation, an effect that was reversed by U0126. Similarly, co-treatment with bisindolylmaleimide II partially restored Tyr705 phosphorylation, further corroborating the involvement of PKC-ERK signaling in the modulation of IL-6/STAT3 activation by CL-E.

CL-E suppresses IL-6-induced expression of CRP and SOCS, downstream targets of STAT3 signaling. CL-E suppresses IL-6-induced expression of CRP and Suppressor of cytokine signaling 3 (SOCS3), downstream targets of STAT3 signaling. CRP is primarily produced by hepatocytes in response to IL-6 stimulation and can be transcriptionally induced via the JAK/STAT3 pathway or a redox-sensitive NF-κB pathway mediated by Rac1 activation (27-29). SOCS3 acts as a classical negative feedback regulator of IL-6-induced JAK/STAT3 signaling by binding to phosphorylated tyrosine residues on JAK kinases (9).

Given that SOCS3 is a direct transcriptional target of STAT3, the inhibition of STAT3 signaling results in decreased SOCS3 expression. To assess the downstream effects of CL-E-mediated STAT3 inhibition, we examined the expression of inflammation-related target genes regulated by IL-6/STAT3 signaling. We focused on CRP and SOCS3, both recognized as STAT3 target genes, serving as indicators of the inflammatory response and negative feedback regulation, respectively (30). Hep3B cells were pretreated with CL-E (10, 30, and 60 μg/ml) for 1 h, followed by IL-6 stimulation (10 ng/ml) for 6 h. Total RNA was extracted, and mRNA levels of CRP and SOCS3 were quantified via real-time PCR using 18S rRNA as the internal control. As illustrated in Fig. 4, IL-6 treatment significantly upregulated the expression of both CRP and SOCS3 mRNA levels compared to those of untreated cells. Pretreatment with CL-E significantly attenuated IL-6-induced expression of both genes, and a concentration-dependent reduction was observed for SOCS-3.

These findings indicate that CL-E effectively suppresses IL-6/STAT3-dependent transcriptional activation of both pro-inflammatory (*CRP*) and feedback inhibitor genes (*SOCS3*). This transcriptional repression aligns with the observed inhibition of STAT3 Tyr705 phosphorylation and nuclear translocation by CL-E (Fig. 2), further supporting its potential as an anti-inflammatory agent targeting the IL-6/STAT3 signaling axis.

Discussion

In this study, we demonstrated that CL-E effectively inhibits IL-6-induced STAT3 activation in Hep3B cells. CL-E suppressed STAT3-dependent transcriptional activity in a dose-dependent manner without inducing cytotoxicity. Mechanistically, CL-E inhibited phosphorylation of STAT3 at Tyr705, blocked its nuclear translocation, and reduced the expression of STAT3 downstream target genes such as *CRP* and *SOCS3*. Notably, CL-E enhanced ERK1/2 phosphorylation, which led to increased STAT3 Ser727 phosphorylation. Pharmacological inhibition of the ERK and PKC pathways reversed CL-E-mediated inhibition of STAT3 Tyr705 phosphorylation, suggesting a regulatory crosstalk between MAPK signaling and STAT3 activation. These findings are consistent with previous reports on natural product-derived STAT3 inhibitors. For example, manassantin A and B from *Saururus chinensis*, and Kansuinine A and

B from *Euphorbia kansui L.* have been shown to suppress IL-6-induced STAT3 phosphorylation and nuclear translocation by targeting both STAT3 Tyr705 and ERK1/2-mediated Ser727 phosphorylation (31,32). In agreement with previous reports, CL-E also appears to exert dual modulatory activity, suppressing canonical STAT3 activation via STAT3 Tyr705 phosphorylation while activating ERK signaling, possibly as part of a feedback or compensatory mechanism. These results suggest that CL-E may serve as a promising natural therapeutic candidate for targeting STAT3-mediated inflammation and cancer progression. The differential regulation of STAT3 phosphorylation at Tyr705 and Ser727 by CL-E is particularly noteworthy, given that these two phosphorylation sites serve distinct roles in STAT3 signaling. Tyr705 phosphorylation is critical for STAT3 dimerization, nuclear translocation, and DNA binding, and is typically considered a hallmark of canonical STAT3 activation in response to cytokines such as IL-6 (27,33). In contrast, Ser727 phosphorylation, which is often mediated by MAPK or PKC signaling, contributes to the modulation of STAT3's transcriptional activity, interaction with co-activators, and in certain contexts, non-canonical functions (34). To further investigate the modulation of STAT3 signaling by CL-E, we examined previous evidence implicating the ERK and PKC pathways in IL-6 signal transduction. It has been documented that PKC activation by phorbol esters, such as PMA, can influence IL-6 signaling through ERK activation (24). While ERK signaling has been shown to synergize with the JAK/STAT pathway in enhancing cytokine-induced gene expression in certain contexts (35), other studies suggest that ERK activation can also antagonize STAT3 signaling, contingent upon the cellular environment (34,36,37). Notably, SOCS3, a well-known negative feedback regulator of the JAK/STAT pathway, has been reported to be upregulated by PMA via ERK activation, and this induction was inhibited by MAPK inhibitors (38). Based on these findings, we hypothesized that the inhibitory effect of CL-E on STAT3 activation may involve the activation of PKC and ERK1/2. Indeed, our results demonstrated that CL-E significantly induced ERK1/2 phosphorylation, which was abolished by the MEK1/2 inhibitor U0126, indicating that the effect is dependent on the MAPK/ERK pathway.

Furthermore, CL-E enhanced STAT3 Ser727 phosphorylation even in the absence of IL-6, and this effect was diminished by co-treatment with the PKC inhibitor bisindolylmaleimide II, suggesting that PKC activity contributes to this mechanism. Given previous reports that ERK-mediated STAT3 Ser727 phosphorylation can interfere with STAT3 Tyr705 phosphorylation (34,36,37), our findings support a model in which CL-E-mediated ERK activation leads to a shift in STAT3 phosphorylation balance, thereby attenuating its activation. Collectively, these results highlight ERK and PKC signaling as key modulators involved in the suppression of IL-6/STAT3 signaling by CL-E. The ability of CL-E to suppress IL-6-induced expression of CRP and SOCS3 further supports its anti-inflammatory potential. CRP is a classical acute-phase protein synthesized in hepatocytes under IL-6/STAT3 regulation, and elevated CRP levels are widely used as biomarkers for systemic inflammation, cardiovascular risk, and cancer prognosis (39,40). In our study, CL-E treatment markedly reduced CRP mRNA expression in IL-6-stimulated Hep3B cells, suggesting that CL-E may attenuate systemic inflammatory responses at the transcriptional level. *SOCS3*, another STAT3 target gene, acts as a negative feedback regulator by binding to JAKs and preventing further cytokine signaling. Although SOCS3 is classically viewed as an inhibitor of STAT3 signaling, paradoxically, its overexpression in chronic inflammatory states can desensitize anti-inflammatory signals and contribute to persistent disease persistence (41,42). Our finding that CL-E downregulates SOCS3 expression suggests that it may restore homeostatic feedback mechanisms by reprogramming the inflammatory environment. These findings are consistent with previous studies on flavonoids and diarylheptanoids derived from natural products, which have demonstrated suppression of IL-6-stimulated inflammatory markers and STAT3 target genes (30,43). Collectively, our results highlight the therapeutic potential of CL-E as a botanical modulator of inflammatory signaling, particularly in diseases characterized by hyperactivation of the IL-6/STAT3 axis, such as rheumatoid arthritis, inflammatory bowel disease, metabolic syndrome, and hepatocellular carcinoma. The rhizome of *C. longa* has been extensively studied and commercialized for its curcuminoid content and associated pharmacological activities, including anti-inflammatory, antioxidant, and anticancer effects (8,44,45).

In contrast, the aerial parts, particularly the leaves, have been relatively understudied in scientific research. Nonetheless, recent investigations have begun to reveal that *C. longa* leaves contain bioactive compounds such as polyphenols, flavonoids, and tannins, which exhibit antioxidant and antimicrobial properties (19-21). The present study further corroborates the potential of CL-E as an effective modulator of IL-6/STAT3 signaling. Notably, CL-E was able to modulate the STAT3 signaling pathway without inducing cytotoxicity, underscoring its potential as a safe, mechanism-based botanical regulator of inflammatory responses. Furthermore, our data suggest that CL-E operates through ERK- and PKC-dependent pathways, offering new insights into how its phytochemicals may regulate the IL-6 signaling cascade. Future research should focus on isolating and structurally characterizing the active constituents of CL-E, and on evaluating their *in vivo* efficacy in various IL-6–driven animal models of rheumatoid arthritis and inflammatory bowel disease, such as collagen-induced arthritis (RA) and DSS-induced colitis (IBD). Additionally, comparative studies with turmeric rhizome extracts, regarding bioactivity and chemical composition, may elucidate whether CL-E offers distinct advantages in terms of potency, selectivity, or safety. From a resource utilization perspective, the use of *C. longa* aerial parts, which are often discarded during cultivation, could contribute to more sustainable and comprehensive utilization of this medicinal plant.

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Availability of data and materials

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

Authors' contributions

SWL and SL conceived and designed the study. HJJ, EJP and YK performed the experiments and analyzed the data. EP and WK contributed to the design of the experiments, and to the analysis and interpretation of the data, and, together with SWL and SL, supervised the overall research. HJJ and YK wrote the original manuscript. EP and WK, together with SWL and SL, revised the manuscript critically for important intellectual content and confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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