The Investigation of The Network Pharmacology and Mechanism of Action of *Centella Asiatica* Extract on The Atopic Dermatitis Model

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ABSTRACT

Background: Atopic dermatitis (AD) is a chronic relapsing inflammatory skin condition which has a negative impact on children health. The well-known medicinal plant *Centella asiatica* extract (CE) is used in herbal skin care products to produce various pharmacological effects in dermatology. However, the molecular target of CE in suppressing inflammatory is largely unknown. **Objective**: the aim of this study was to examine anti-inflammatory properties and network pharmacology of CE in lipopolysaccharide (LPS)-induced AD *in vitro* model. Method: RAW264.7 cells were pre-treated with CE and then were stimulated with LPS and then were investigated cell viability, NO production, and the levels of pro-inflammatory mediators. In addition, the Search Tool for Retrieval of Interacting Genes (STRING), SwissTargetPrediction and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to construct the defined mechanism of action and network pharmacology. **Results**: CE showed the potent inhibitory effects on LPS-induced NO. In addition, CE significantly suppressed the expression of iNOS and COX-2, as well as the production of IL-2, IL-6, IL-10, and TNF-α. Furthermore, the network pharmacological analysis revealed the potential role of CE in biological processes such as regulating JAK/STATs pathway and inhibiting proinflammatory cytokines both of which were linked to AD pathogenesis. **Conclusion**: Our findings confirm our hypothesis that CE could be developed as a therapeutic therapy for atopic dermatitis due to its pharmacological action and signaling mechanism in the modulation of allergic skin inflammation.

Key words: Atopic dermatitis, *Centella asiatica*, Network pharmacology, Skin inflammation.

INTRODUCTION

Atopic dermatitis (AD), commonly referred to as atopic eczema, is a recurrent, chronic inflammation of the skin that affects around 10-20% of children with a particularly complex pathogenesis and caused by abnormal immune responses.[1] While the exact mechanism underlying AD is not fully understood, proinflammatory substances such tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-6 and chemokines may play a role in its induction.[2] Despite AD is not a life-threatening disorder, it can impact children's daily activities like play, sport, attendance at school, and sleep quality. As a result, AD has an adverse effect not only on the kid, but also on the caregiver, who may experience anxiety, depression, and a decline in self-esteem.[3] According to earlier studies, AD typically develops in low-income countries. The incidence rates in Thailand are 9.9% for children between the ages of 13 and 14 and 15.6% for children between the ages of 6-7.[4]

Macrophages contribute significantly to innate immunity by offering immediate defense against a variety of infections. Lipopolysaccharide (LPS), one of the most effective known activators, can activate macrophages *via* Toll-like receptor-4 (TLR-4) which mediated intracellular signaling cascades including nuclear factor-κB (NF-κB) and the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway.[5] The activation of these signaling pathways leads to in the production of inflammatory molecules

such as TNF-α, interleukin 6 (IL-6), COX-2 and iNOS, resulting in inflammation.[6] Previous studies discovered that JAK/STAT plays an essential role in regulating several immunological axes associated in the immunopathogenesis of AD. Furthermore, JAK/STAT is involved in the regulation of the epidermal barrier as well as the modulation of peripheral neurons linked to pruritus transduction.[7] Together, downregulating chemokines and targeting the JAK/STAT pathway may be a key strategy to treat AD.

Anti-inflammatory drugs, such as topical corticosteroids and topical calcineurin inhibitors are widely used in mild to moderate AD patients. Although these topical therapies can aid in reducing AD symptoms and inflammation through inhibition of cytokine production, they have long-term negative effects such as recurrence, skin atrophy, and bacterial/viral skin infections.[8,9] Additionally, it appears that few patients are satisfied with the treatment. As a result, there is increasing interest in the use of complementary and integrative medicine treatments, especially natural bioactive substances made from plant extracts, in AD patients.[10,11]

Centella asiatica, widely known as gotu kola, is a traditional medicinal herb that has been used to have pharmacological benefits for many diseases such as varicose ulcers, diarrhoea, fever, and eczema. The most significant bioactive triterpenes, asiaticoside, madecassoside.[12-14] In addition, asiaticoside are primarily responsible for the plant's therapeutic efficacy.[15,16] Previous study found that treatment with *Centella asiatica* inhibited inflammation cytokine including interleukin-6 (IL-6) and tumor

necrosis factor-α (TNF-α) in a dose-dependent manner.[17] In addition, C. asiatica cream could improve pigmentation in split- thickness skin grafts.[18] Although the beneficial effects of *Centella asiatica* have been described, the mechanism by which it modulates the progression of AD remains poorly understood.

Therefore, we aimed to unravel the cellular pathways affected by *Centella asiatica* treatment in RAW 264.7 macrophages, focusing on anti-inflammatory and mechanism of action. We firstly examined the underlying mechanism of *Centella asiatica* extract (CE) on the *in vitro* AD cell inflammation model induced by LPS stimulation, and then we used network pharmacology approach to investigate the role of active compounds *via* systems biology to explore the complex interactions between bioactive substances and their target proteins. These findings could aid in the development of this molecule for the treatment of AD, as well as the prediction of its important pharmacological activities based on its cellular mechanism of action.

MATERIALS AND METHODS

Chemicals and reagents

Centella asiatica extract (CE) was prepared by being extracted with 70% alcohol and was purchased from Thai-China flavors, and fragrances industry Co. Ltd. It found that CE contained asiaticoside 5.12% and madecassoside 5.1% as previously described.[18] High glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco), Fetal Bovine Serum (FBS, Gibco), Antibiotic-Antimycotic (Gibco), 4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, invitrogen), TRIzol™ reagent (invitrogen), and superScript™ III Reverse Transcriptase (invitrogen) were supplied by Thermo Fisher Scientific (MA, United States). Chemicals for Greiss reagent preparation i.e., sulfanilamide and N-(1-Naphthyl) ethylenediamine dihydrochloride were purchased from Sigma-Aldrich (Sigma-Aldrich, MO, USA) and phosphoric acid (H3PO4) was purchased from Fisher Scientific (Fisher Scientific, NH, USA). The iTaq Universal SYBR Green Supermix was purchased from Bio-rad (Bio-rad, CA, USA) and COX-2 and iNOS-2 primers were synthesized by Macrogen (Macrogen, Seoul, South Korea). The BD CBA Mouse Th1/Th2/Th17 Cytokine Kit was purchased from BD Bioscience (BD Bioscience, CA, USA).

Cell culture and ad cell model establishment

The RAW264.7 macrophage cell line was kindly provided by Dr. Sumet Kongkiatpaiboon. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated at 37°C with 5% CO_2 -humidified air environment. The cells were incubated for 24 h in medium supplemented with 10% FBS. Consequently, the cells were pre-incubated with or without the indicated concentrations of CE for 2 h in serum-free media, prior to the addition of LPS (0.05 µg/ml).

Cell viability assay

In this study, we used 3, [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay to determine cell viability. Firstly, RAW264.7 cells were seeded in a 96-well plate at a density of $5.0x10^4$ cell/well and incubated at 37°C for 24 h. Then, cells were treated with CE at the indicated concentrations for 24 h, followed by the addition of 5 mg/ml MTT solution to each well, and the plates were further incubated for 3 h at 37°C. After removing the supernatant, 100 µl DMSO was added to each well to solubilize formazan crystals. The absorbance at a wavelength of 590 nm was measured using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, MA, USA). The percentage of viable cells in comparison to untreated control cells was calculated.

Measurement of NO level

RAW264.7 cells were seeded and cultured in 96-well plates at 37°C for 24h. Then, cells were treated with CE for 2 h in serum-free medium, prior to the addition of LPS (0.05 µg/ml). NO was measured as the accumulation of NO, which were determined by using Griess reagent. The absorbance was measured at 545 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, MA, United States)

mRNA expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)

Real-time PCR was performed for the detection of the mRNA expression of iNOS and COX-2 genes. Briefly, the RAW264.7 cells were seeded at a density of $2.5x10^5$ cells/well in 12-well plates for overnight and then pre-incubated with CE concentrations 420, and 700 µg/mL for 2 h and stimulated with LPS (0.05 µg/ml) for 24 h. Total RNA was isolated from the cells using TRIzol™ reagent as recommended by the manufacturer. Subsequently, a reverse transcription reaction was performed using a superScript™ III Reverse Transcriptase kit for cDNA synthesis. PCR was carried out using specific forward and reverse primers: iNOS-2-F: 5'- GTT CTC AGC CCA ACA ATA CAA GA-3', iNOS-2-R: 5'-GTG GAC GGG TCG ATG TCA C-3'; COX-2-F: 5'-GAA GTC TTT GGT CTG GTG CGT G-3', COX-2-R: 5'-GTC TGC TGG TTT GGA ATA GTT GC-3' and GAPDH-F: 5'-GGA GAG TGT TTC CTC GTC CC -3', GAPDH-R: 5'-ATG AAG GGG TCG TTG ATG GC-3'.[19-21] The iTaq Universal SYBR Green Supermix was adopted for real-time PCR reagent according to the manufacturer's instructions. The cDNA template concentration between 1,000-1,400 ng/µl were amplified using the CFX96TM real-time PCR detection system (Bio-rad, CA, USA) under following PCR conditions: 94°C for 5 min (1 cycle); 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec (for 40 cycles); and a final extension phase at 72°C for 10 min. The expression of iNOS and COX-2 (target) genes was calculated by comparative Ct or delta-delta Ct (2–rrCt) method. The Ct of target genes were compared to housekeeping gene (GAPDH) and normalized with H0 sample. The relative gene expressions were presented from the ratios of test and untreated control samples.[22]

IL-2, IL-6, IL-10 and TNF- α assay

RAW 264.7 macrophages were pretreated with CE for 2 h and then induced with LPS (0.05 µg/ml) for 24 h. Cytokines accumulation in the samples were measured with BD CBA Mouse Cytokine Kit (BD Bioscience, CA, USA). Samples were measured on the BD FACSverse Flow Cytometer (BD Bioscience, CA, USA) and analyzed by FCAP Array™ Software (BD Bioscience, CA, USA). The operations were performed according to the manufacturer's instruction.

Network pharmacology analysis

The target genes were identified using SwissTargetPrediction (http://www.swisstargetprediction.ch/, accessed on 22 February 2023).[23] To construct Protein–Protein Interaction (PPI) Network, the Search Tool for the Retrieval of Interacting Genes (STRING) was used to identify the interaction between compound and their targets (https://string-db.org, assessed on 22 May 2023).[24,25] In addition, the pathway analysis was analyzed *via* Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway enrichment analyses (https://www.kegg.jp/kegg/pathway.html, accessed on 16 June 2023).[26]

Statistical analysis

In vitro data were presented as the mean ± standard error of the mean (SEM) from at least 3 independent experiments. Comparisons among groups were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. p-values < 0.05 were considered statistically significant.

RESULTS

Effect of CE on cell viability of RAW264.7 macrophages

In order to demonstrate the potential and effectiveness of CE, we first conducted an *in vitro* experiment to test the anti-inflammatory efficacy of CE in a laboratory setting. Macrophages cell are well established to be important in triggering and developing inflammatory responses.[27] To ensure that the concentration we employ is safe for these cells, we first investigated how this extract affected RAW264.7 macrophages. Cells were treated with various concentrations of CE for 24 and 48 h. Then, cell viability was evaluated by MTT assay. The results found that CE did not significantly reduce the viability of RAW264.7 macrophages cells at concentrations lower than 1750 µg/mL both 24 and 48 h (Figure 1A-B). For the following experiments, non-toxic concentrations (<880 µg/mL) of CE were used.

CE suppressed LPS-induced NO, iNOS, and COX-2 in RAW264.7 macrophages

One of the most potent inflammatory initiators, lipopolysaccharide (LPS) is a key component of the outer membrane of gram-negative bacteria and can stimulate monocytes and macrophages to create proinflammatory cytokines.[28,29] To confirm that the concentration of LPS and CE we use is safe for these cells, we firstly assess the viability of the cells. The results found that CE-treated cells with or without LPS exhibited no difference in cytotoxicity (Figure 2A).

Then, to characterize the effect of CE on LPS-induced NO production, we used a Griess reagent. The effectiveness of the inflammatory model was revealed by the considerable increase in NO release when cells were stimulated by LPS. The results found that LPS alone markedly stimulated NO production compared with the untreated control. Furthermore, the amount of NO levels reduced dramatically in a concentration-dependent manner, compared to the LPS-induced group (Figure 2B).

Proinflammatory mediators such as iNOS and COX-2 are involved in the upregulation of inflammatory responses and are primarily produced by activated macrophages. In addition, NO production is closely related iNOS and COX-2.[30,31] Accordingly, more research is needed to completely understand how CE inhibits inflammation in LPS-induced cells. We further investigated mRNA levels of iNOS and COX-2 by using qRT-PCR. The results found that LPS treatment significantly increased iNOS and COX-2 mRNA levels whereas CE significantly decreased mRNA levels of iNOS and COX-2 (Figure 2C-D).

Inhibitory effects of CE on LPS-induced production of IL-2, IL-6, IL-10 and TNF- α in RAW264.7 macrophages

In view of the relevance of pro-inflammatory cytokines in the inflammatory process, we examined the IL-2, IL-6, IL-10, and TNF-α production in LPS-induced RAW 264.7 macrophages pretreated with CE. Cells were pretreated with CE at various concentration (0, 420 and 700 µg/mL) for 2 h followed by stimulation with LPS for 24 h. The results found that pretreatment of CE was found to significantly suppressed LPS-induced production of IL-2, IL-6, IL-10 and TNF- α in concentration dependent manner (Figure 3A-D). Taken together, these results suggested that CE has anti-inflammatory activity by inhibiting the production of various LPS-induced pro-inflammatory mediators and cytokines.

Mechanisms of action of CE analyzed by the protein-protein interaction networks and signaling pathway

Computational approaches are widely used in drug discovery around for decades. The structure-activity connections of active substances can be used to predict their biological activity and pathway.[32] In addition, the network pharmacology could create complex relationships of interactions based on target molecules and biological processes, which can be used to thoroughly understand the molecular basis of natural compound.[33,34] As a result, we chose asiaticoside, a key ingredient of *Centella asiatica*, for the expected Network Pharmacology of CE.[35] Furthermore, when compared to other substances in CE, asiaticoside exhibited the greatest anti-inflammatory action.[35]

The asiaticoside chemical structure (Figure 4A) was created in ChemDraw and then changed to SMILES format. In the following step, we used SwissTargetPrediction, which higher accuracy in target prediction to predict the target genes of asiaticoside. The results found that most target class of asiaticoside were enzyme and transcription factor (Figure 4B). In addition, the top 15 targets were found as shown in Figure 4C. The potential role of asiaticoside in AD could be *via* stimulating or inhibiting these target genes.

To understand the regulation of CE on AD, we further analyzed the protein-protein interaction (PPI) network and signaling pathway. All proinflammatory mediator which investigated in previous experiment including iNOS, COX-2, IL-2, IL-6, IL-10 and TNF- α together with Top 15 target such as HSD11B2, BCL2L1, JUN, STAT3 were subjected to protein-protein interaction network analysis with the Search Tool for the Retrieval of Interacting Genes (STRING) in order to determine the significant kinase pathways. The results found that the GOterm
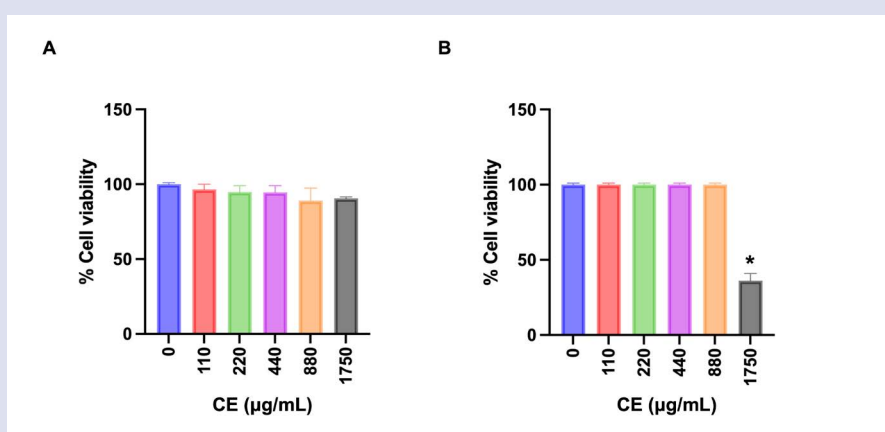

Figure 1: Effects of CE on the Viability of RAW 264.7 Cells. (A-B) Cytotoxicity of CE at various concentration (0-1750 µg/ml) on RAW 264.7 cells, followed by MTT assay at 24 and 48h, respectively. The data are presented as the means ± SEM of at least three independent experiments. * p < 0.05 (compared to the untreated group).
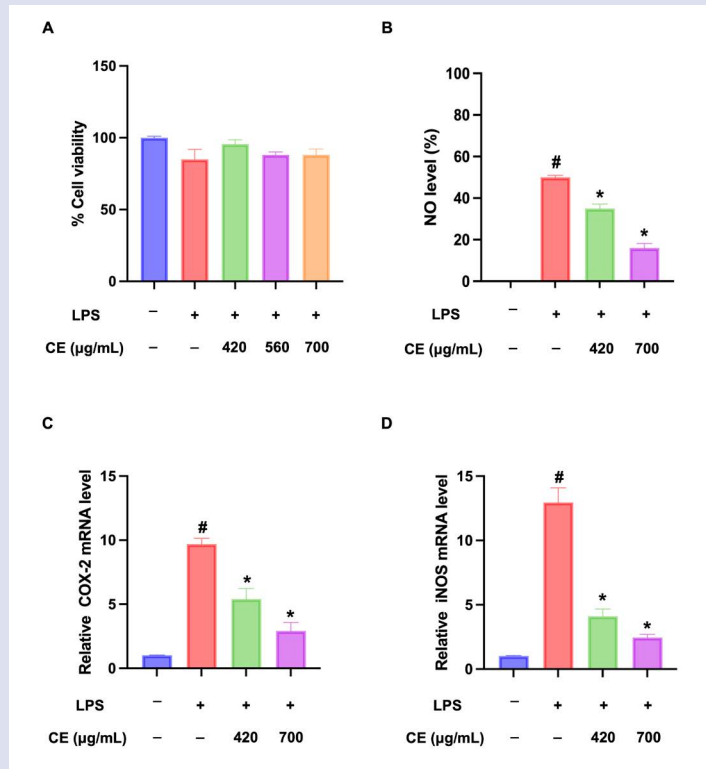
Figure 2: CE inhibits lipopolysaccharide (LPS)-induced pro-inflammatory mediators. (A) RAW264.7 cells were pre-treated with various concentration of CE for 2 h and then stimulated with LPS (0.05 μg/ml), followed by MTT assay. (B) RAW264.7 cells were pre-treated with CE for 2 h and then stimulated with LPS (0.05 μg/ml). After 24 h, the culture medium was collected, and the NO concentrations were measured by Griess assay. (C-D) RAW264.7 cells were pre-treated with CE for 2 h and then stimulated with LPS (0.05 μg/ml). After 24 h, cells were collected and were measured levels of iNOS and COX-2 by using qRT-PCR. The data are presented as the means ± SEM of at least three independent experiments. * $p < 0.05$ (compared with the cells treated with LPS alone) # $p < 0.05$ (compared with normal control group).
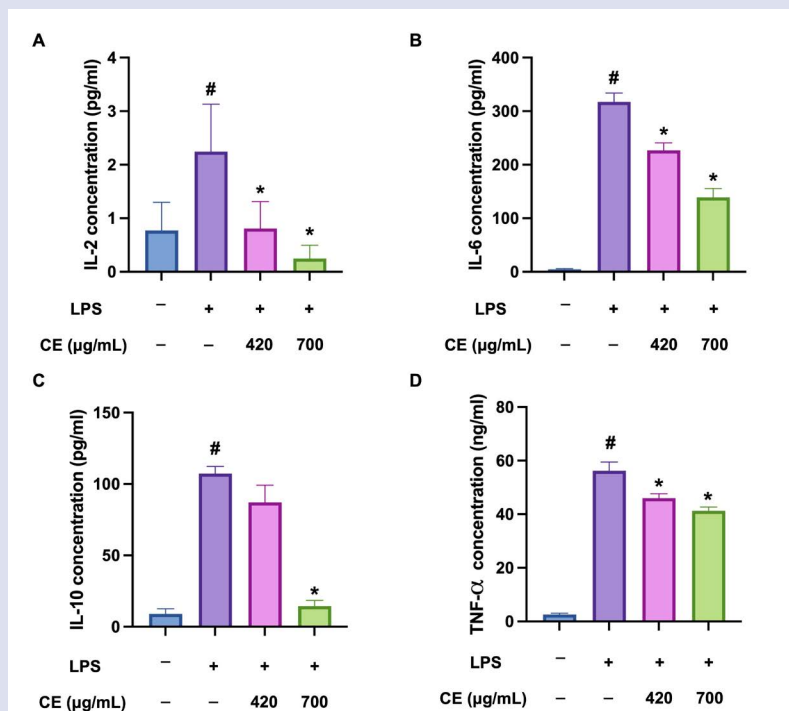

Figure 3: CE reduced LPS-induced IL-2, IL-6, IL-10 and TNF-α production in RAW264.7 macrophages. RAW 264.7 macrophages were pretreated with CE for 2 h and then induced with LPS (0.05 μg/ml) for 24 h. Cytokines accumulation in the samples were measured by using flow cytometer. The data are presented as the means ± SEM of at least three independent experiments. * $p < 0.05$ (compared with the cells treated with LPS alone) # $p < 0.05$ (compared with normal control group).
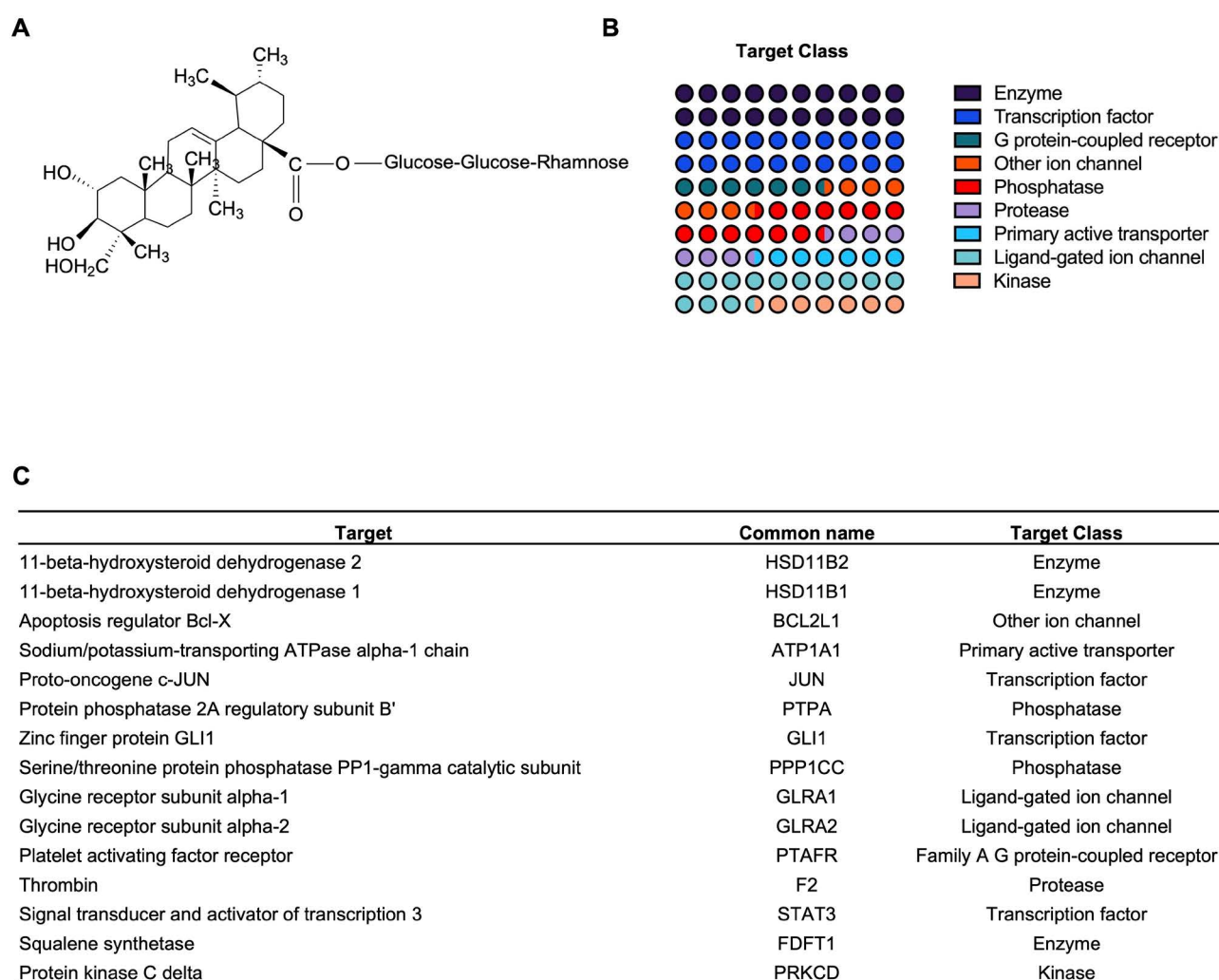
Figure 4: Molecular target of asiaticoside, which is major compound in CE. (A) Structure of asiaticoside (B-C) Target class and gene target which interact with asiaticoside using SwissTargetPrediction (http://www.swisstargetprediction.ch/, accessed on 22 February 2023).

in termed of biological processes were mostly associated with the regulation of inflammatory response. Interestingly, we found that CE may regulate *via* JAK/STAT pathway (Figure 5A). Moreover, the resulting PPI networks were presented in Figure 5B-D. The PPI network showed the nodes, and the edges represented the proteins, and the interaction diagram was created with genomic knowledge. These results suggested that the major mechanisms by which CE mediated AD was through the potent and sustained suppression of the JAK/STAT axis.

To further reveal the affected pathway of CE treatment, we utilized the KEGG mapper (https://www.kegg.jp/kegg/pathway.html) to construct the key signaling pathway affected by CE.[26] The results found that, consistent with the above analysis, the KEGG mapper also suggested that STAT3 was a vital player in the mechanism of action of CE in the protection against extracellular pathogens such as bacteria which associated with pathogenesis of AD (Figure 6).[36]

DISCUSSION

As topical corticosteroids have such potent anti-inflammatory and anti-allergic properties, they have been utilized topically to treat mild to moderate AD patients. However, prolonged treatment might result in adverse side effects such as immunological suppression and typical pathogenic infections.[8,9] For this reason, using natural products including herbs and plants are getting more and more popular for a variety of inflammatory disorders such as psoriasis and eczema.[37,38] In several countries, especially Thailand, have long been utilized natural product as medications.[18,39] For the first time, this study established the therapeutic efficacy, molecular mechanism, and network pharmacology of Centella extract in RAW264.7 macrophage cells, which serve as an atopic dermatitis model.

The inducible forms of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) found in activated macrophages have been linked to increased levels of nitric oxide (NO) and prostaglandins (PGs). By the activation of cellular inflammatory signaling, iNOS expression leads to NO overproduction, which impairs inflammatory reactions. Moreover, COX-2 expression is increased after cytokine stimulation during the immune reaction.[40-42] In addition, many studies have indicated that LPS could alter iNOS and COX-2 expression in RAW264.7 cells.[43,44] The reduction of iNOS and COX-2 expression in LPS-induced RAW264.7 is therefore considered to be an effective first step in determining the effectiveness of potential therapies for the treatment of inflammatory diseases, especially AD.
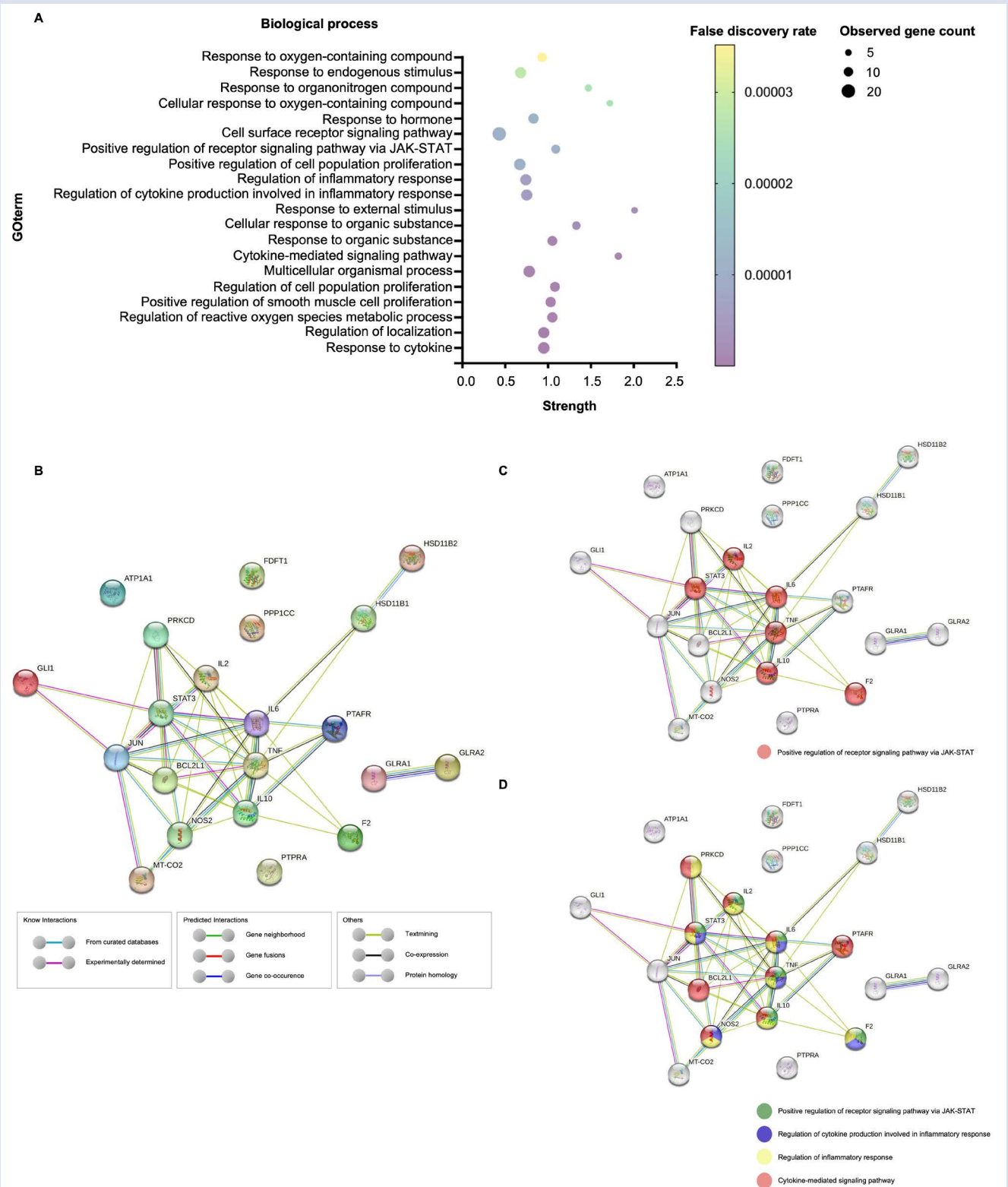
Figure 5: Network of functional protein-protein interactions of CE. (A) The 21-target genes/proteins were analyzed according to biological process and GOterm. (B-D) The Protein–protein interactions (PPI) network was constructed by using the STRING database to analyze the interactions (https://string-db.org, assessed on 22 May 2023).

In this study, we found that CE showed no toxic effects on RAW 264.7 cells (Figure 1). To support our study, previous studies found that an extract of C. *asiatica* at a concentration up to 1000 μg/mL showed cell viability more than 90% in macrophage cells.[45,46] Furthermore, CE significantly inhibited the production of NO in LPS-stimulated RAW264.7 cells (Figure 2B). In addition, the mRNA expression of iNOS and COX-2 were decreased in the LPS-stimulated RAW264.7 cells (Figure 2C-D). Furthermore, CE has anti-inflammatory effect by downregulating IL-2, IL-6, IL-10, and TNF-α, which are key cytokines that can activate multiple signaling pathways (Figure 3). Pre-treatment
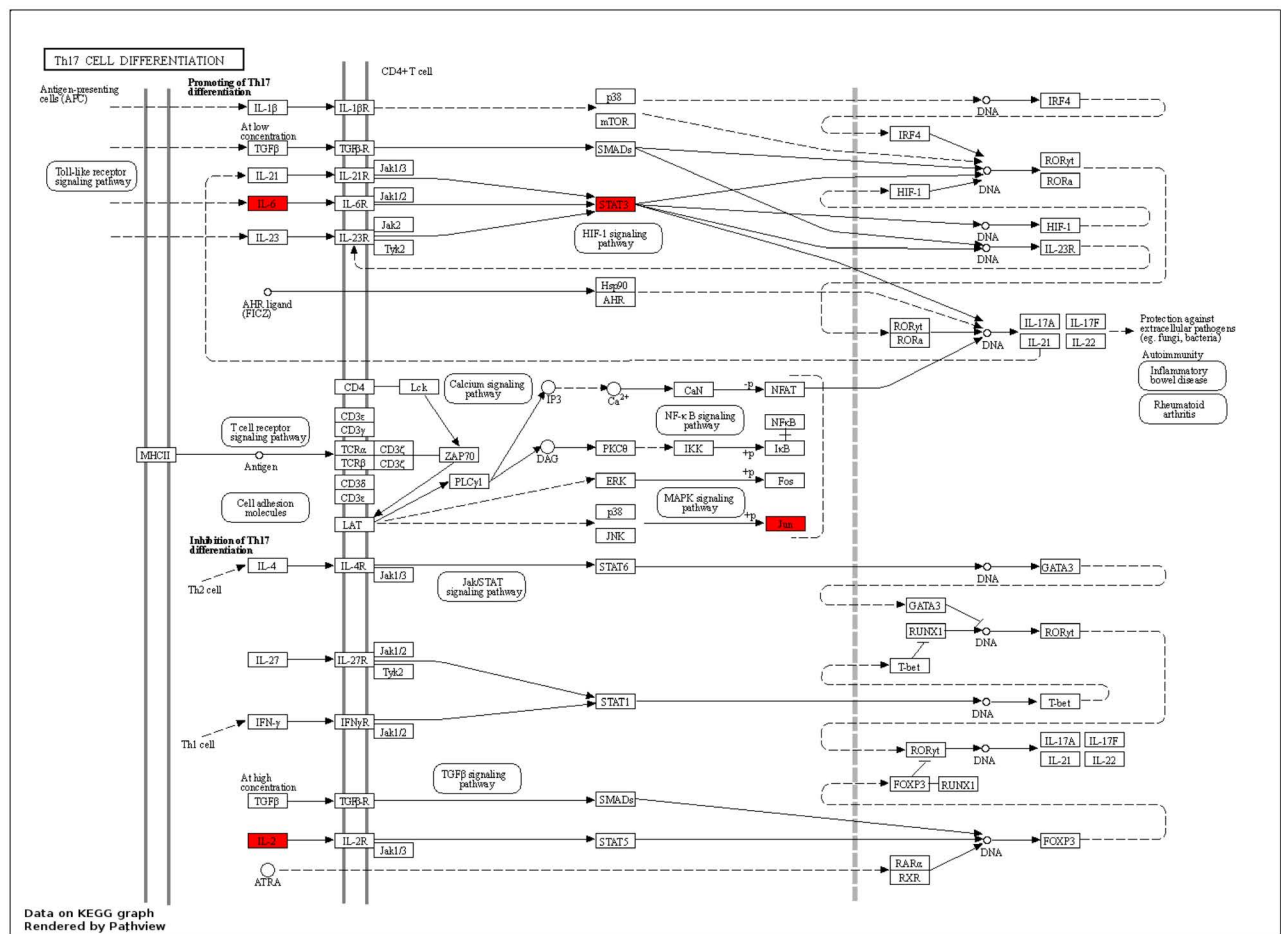
Figure 6: The JAK/STATs pathway is a target of CE. KEGG enrichment analysis was used to analyze the CE target obtained from KEGG Pathway Database and Pathview (https://www.kegg.jp/kegg/pathway.html, accessed on 16 June 2023). The red box represented all proteins considering they were actively defending against extracellular pathogens.

with CE could reverse this inflammatory phenomenon. According to these findings, CE may have protective and anti-inflammatory effects by preventing the production of pro-inflammatory components generated by LPS. Previous research found that Raw-Extract *Centella asiatica* (RECA) significantly suppressed the level of NO and TNF-α in RAW264.7 cells.[45] In addition, another study reported that *Centella asiatica* ethanol extract (CA) could suppress the levels of TNF-α, IL-4, IL-5, IL-6, IL-10, IL-17, iNOS and COX-2. As a result, we proposed that the mechanism of action was altered by the varying extraction method or ratio of substance in extract.[47]

To further explore the intracellular mechanism underlying the anti-inflammatory effect of CE, we firstly used network pharmacology analysis to further investigate for their biological effects and pathways. In the framework of traditional medicine, network pharmacology has emerged as a strong technique for investigating the complicated interactions between bioactive chemicals and their target proteins. The findings revealed that the main component of CE, asiaticoside, might interact with a wide range of target genes/proteins, as shown in Figure 4. Furthermore, we examined the protein-protein interaction by combining the *in vitro* results with the predicted target. Surprisingly, the data revealed that the key axis that regulates the effect of CE on AD was the JAK/STAT pathway (Figure 5 and 6). To support our data, previous research found that JAK/STATs pathway is crucial for cytokine triggered signaling in immune response, especially AD. Additionally,

prior studies discovered that a variety of natural compounds could decrease the inflammatory response in an atopic dermatitis model by targeting the JAK/STAT pathway.[48-50] We hypothesized that CE could inhibited the phosphorylation of STATs which in turn inhibited the transcription activation of target genes. Consistent with our finding, molecular docking revealed that asiaticoside had a high affinity with STAT3 for the treatment of the coronavirus disease 2019 (COVID-19).[51]

Altogether, these results demonstrate that CE exerts relevant anti-inflammatory properties *via* a cellular mechanism *via* inhibiting pro-inflammatory cytokines and JAK/STAT pathway in LPS-stimulated RAW 264.7 macrophages, a model for atopic dermatitis (Figure 7). Future studies employing specific inhibitors or gene knockdown approaches is needed to validate the proposed molecular mechanisms underlying the effects of CE in AD.

CONCLUSION AND RECOMMENDATIONS

With the use of an *in vitro* study and a computational technique, the investigation's findings confirmed the therapeutic effects of CE. In LPS-induced RAW 264.7 macrophage cells, CE suppressed the production of NO, iNOS, COX-2, IL-2, IL-6, IL-10, and TNF-α which are a characteristic of allergic reactions. In addition, this study provides evidence that CE might exhibit anti-inflammatory effect by targeting JAK/STATs pathway. Therefore, the findings of this study implied that CE might be a promising treatment for AD patients. However,
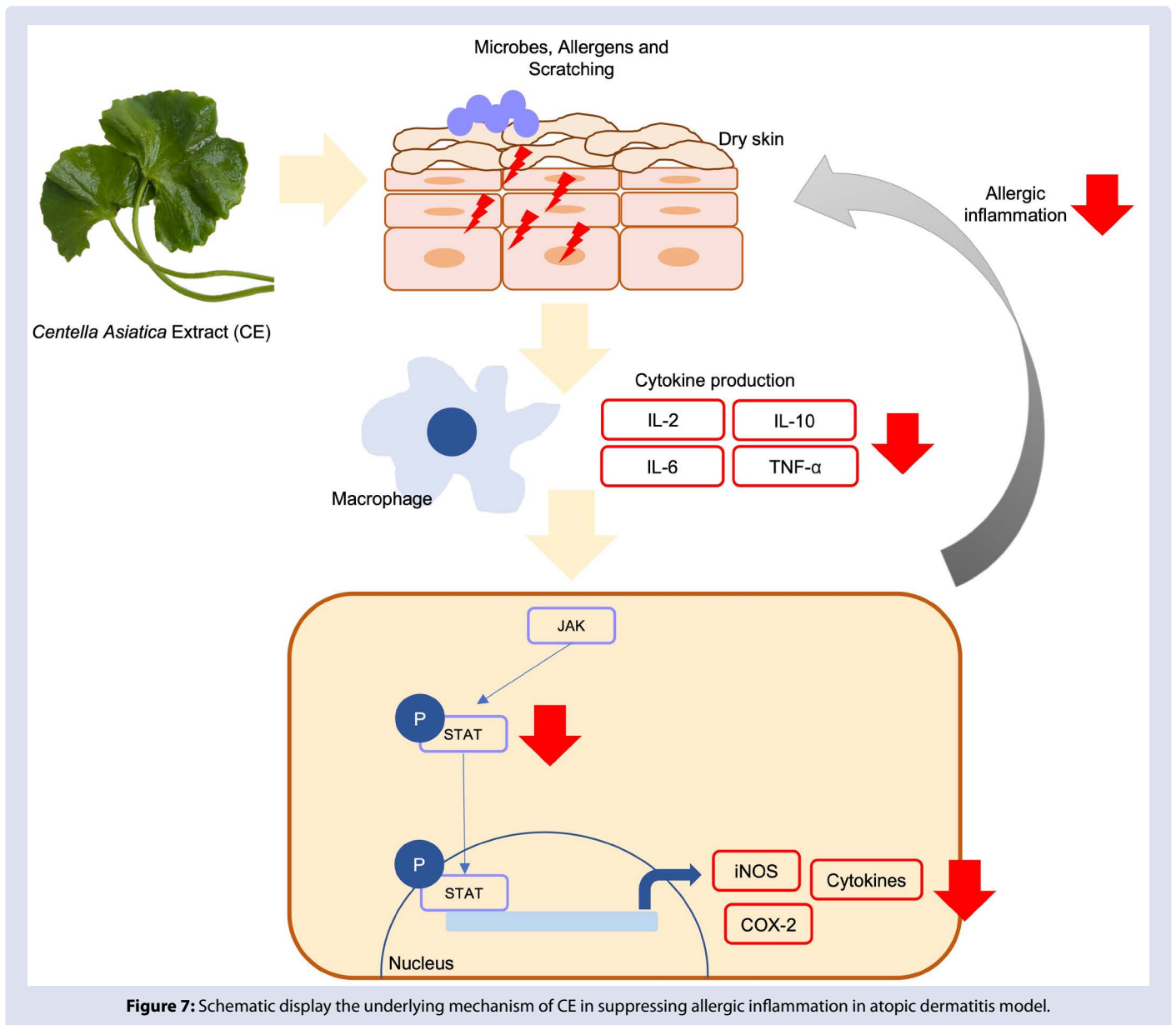
Figure 7: Schematic display the underlying mechanism of CE in suppressing allergic inflammation in atopic dermatitis model.

we still suggest further in-dept research study in both *in vitro* and *in vivo* model to provide inclusive scientific data of the pharmacological activity of CE in atopic dermatitis.

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DECLARATION OF COMPETING INTEREST

The authors declare "No conflicts of interest".

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