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RESEARCH ARTICLE

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Artemisia argyi extract ameliorates IL-17A-induced inflammatory response by regulation of NF-κB and Nrf2 expression in HIG-82 synoviocytes

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Abstract

Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease that results in joint destruction and disability in the adult population. RA is characterized by the accumulation and proliferation of fibroblast-like synoviocytes. Many pro-inflammatory mediators are associated with RA, such as interleukin (IL)-1β, IL-6, IL-17, cyclooxygenase-2 (COX-2), and nuclear factor kappa B (NF-κB). Furthermore, IL-17 upregulates the production of other pro-inflammatory mediators, including IL-1β and IL-6, and promotes the recruitment of neutrophils in RA. *Artemisia argyi*, a

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traditional Chinese herbal medicine, is used for the treatment of diseases associated with inflammation and microbial infections. In this study, synoviocytes (HIG-82) were treated with varying doses of *A. argyi* extract (AAE) following IL-17A stimulation. Proliferation of the IL-17A-stimulated cells was increased compared to that of the non-stimulated control cells. However, cell proliferation decreased significantly in a dose-dependent manner following AAE treatment. Treatment of IL-17A-stimulated cells with AAE resulted in decreased levels of phosphorylated (p)-NF-κB, p-IκB-α, and COX-2. Enzyme-linked immunosorbent assay results showed that IL-1β and IL-6 levels were increased in the IL-17A-stimulated group but decreased in the AAE treatment group. Additionally, we found that AAE facilitated nuclear factor erythroid 2-related factor 2 (Nrf2) expression and promoted its nuclear translocation, thereby inducing the expression of heme oxygenase-1. Moreover, AAE did not attenuate IL-17A-induced inflammatory mediator production in the presence of ML385, an Nrf2-specific inhibitor. These results suggest that the downregulation of expression of pro-inflammatory cytokines and the transcription factor NF-κB by AAE may be a potential therapeutic strategy for reducing inflammation associated with RA.

KEYWORDS
Artemisia argyi, inflammation, rheumatoid arthritis, synoviocytes

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, symmetrical, inflammatory autoimmune disease that initially affects small joints, progressing to larger joints, and eventually the skin, eyes, heart, kidneys, lungs, and other organs.[1] RA is often characterized by synovitis, peripheral joint inflammation, and progressive destruction of cartilage and bone.[2] Based on a recent statistic, nearly 1% of the world population[3] and 1%–2% of the Western population suffer from RA.[4,5]

Key inflammatory mediators that regulate the inflammatory process in RA include tumor necrosis factor (TNF)-α, interleukin-1 (IL)-1, and IL-6.[6] In addition, increased levels of IL-17 have been observed in patients with RA, and disease severity has been linked to the degree of increase in IL-17 levels.[3,6–8] This cytokine also plays an important role in chronic inflammation that occurs during the pathogenesis of autoimmune diseases and allergies such as RA in humans, for which a mouse model of collagen-induced arthritis (CIA) is available.[6,9,10] Thus, IL-17 strongly contributes to autoimmune diseases that are accompanied by inflammation.

Studies have indicated that the synovial compartment is a key site for the pathogenesis of RA.[11,12] With the help of chemokines, leukocytes migrate to the synovium, causing synovial inflammatory infiltration and proliferation of fibroblast-like synoviocytes (FLSs), which induce synovial hyperplasia.[11,13] In addition, immune cells within the synovial membrane release many inflammatory mediators, such as IL-1, IL-17, and TNF-α, which result in an inflammatory cascade. Finally, inflammatory mediators accumulate in the synovial fluid, leading to cartilage and bone damage.[9,13] The transcription factor nuclear factor kappa B (NF-κB) is intimately involved in the regulation of the expression of numerous genes in the setting of the inflammatory response,[14] and cyclooxygenase-2 (COX-2) expression in most tissues is highly regulated with rapid induction in response to inflammatory stimuli.[15] Furthermore, the expression of NF-κB and COX-2 has been found to be involved in the pathogenesis of RA.[14,16] Thus, the inflammatory mediators IL-17, NF-κB, and COX-2 play important roles in the inflammatory process of RA. Furthermore, inflammatory protein modifications may regulate by NF-κB during different signaling cascades, such as the nuclear factor erythroid 2-related factor (Nrf2) signaling pathway.[17–20] Nrf2 activation and absence may regulate the expression of NF-κB and pro-inflammatory cytokines.[20–22] Moreover, previous studies have shown that Nrf2 can protect cells by reducing inflammation through Nrf2 and heme oxygenase-1 (HO-1) pathway.[18–20] Thus, we assessed the related expression of the NF-κB, inflammatory mediators, Nrf2 and HO-1 in this study.

Traditional Chinese medicine (TCM), including Chinese herbal remedies, acupuncture, and manipulative therapies, are commonly used as adjunctive therapies for a variety of diseases in Asian countries.[23] In the Chinese population, TCM has been used as an adjunctive therapy for RA.[24–27] *Artemisia argyi*, a widely used TCM, is frequently used for the treatment of diseases such as malaria, hepatitis, cancer, inflammatory diseases, asthma, and infections caused by microbial infections[28–31]; however, research on the use of *A. argyi* in RA or FLSs is lacking.

Previous studies have reported that FLSs (such as HIG-82 synoviocytes) can be used as an in vitro model to evaluate the inflammatory processes in RA.[32,33] There is no substantial evidence supporting the anti-RA property of *A. argyi* as a herbal medicine, and the molecular mechanisms underlying the effects remain unclear; these factors

limit its application in clinical practice. Therefore, this study was performed to evaluate the anti-RA potential of *A. argyi extract* (AAE) and investigate the underlying protective effect of *A. argyi* in IL-17A-stimulated FLSs. Additionally, the potential role of IL-17 in the induction of inflammatory responses in FLSs was investigated.

2 | MATERIALS AND METHODS

2.1 | Preparation of AAE

Artemisia argyi was identified and deposited by the Department of Chinese Medicine, Hualien Tzu Chi Hospital (Hualien, Taiwan). The aerial parts (leaves) of *A. argyi* were washed in fast-flowing tap water to eliminate surface dirt. The plant material was dried to remove moisture and then cut and ground to powder form with the help of a mechanical blender. Dried powdered leaves of *A. argyi* (150 g) were extracted with 500 ml of 75% aqueous ethanol in an ultrasonicator for 24 h. The extract was then concentrated using a vacuum concentrator, and the obtained AAE was stored at 4°C prior to use. The extraction method was performed as previously described, with minor modifications.[34]

2.2 | Chemicals and antibodies

Antibodies against the following were used: phosphorylated (p)-NF-κB, p-inhibitor of NF-κB (p-IκB)-α, COX-2, HO-1, β-actin, and histone H1 (Santa Cruz Biotechnology, Dallas, TX). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Antibodies against p-Nrf2 were obtained from Bioss Antibodies (Woburn, MA). Recombinant rabbit IL-17A was purchased from Abcam (ab209282; MO) and dissolved in phosphate-buffered saline (PBS). ML385 was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). When used for treatment, the final concentrations included ≤0.1% DMSO.

2.3 | Cell culture

Rabbit synoviocytes cell line HIG-82 were obtained from the American Type Culture Collection (ATCC number: CRL-1832, Manassas, VA). Cells were cultured in Ham's F-12 nutrient mixture (Hyclone, Logan, UT) supplemented with penicillin–streptomycin (100 units/ml penicillin and 100 μg/ml streptomycin) and 10% fetal bovine serum (FBS). All cultures were maintained at 37°C in 5% CO_2 humidified air, as previously described.[35] Upon attainment of experimental conditions, synoviocytes were treated with various doses of AAE, as described below, following stimulation with the inflammatory agent IL-17A at 200 ng/ml. The cell culture media and cells were collected 24 h after stimulation for subsequent analyses.

2.4 | Cell viability and treatment

Cell viability was determined using the Cell Counting Kit 8 (WST-8/CCK8) (ab228554; Abcam, Cambridge, UK), as previously described.[36,37] To determine the concentration of AAE to be used for all experiments, synoviocytes were seeded in 96-well plates at a density of 1×10^3 cells/well and allowed to attach overnight at 37°C in a 5% CO_2 incubator. The cells were then treated with various doses of AAE (0–500 μg/ml) for 48 h to determine the lethal dose and cell viability. After the treatment period, 10 μl of CCK8 solution was added to each well, and the plates were incubated for 2 h at 37°C in a 5% CO_2 incubator. The optical density at 460 nm (OD_{460}) was recorded, and the results were expressed as the percentage of cell viability. On completion of the viability assay (data not shown), doses of 25, 50, and 100 μg/ml of AAE were selected for all subsequent experiments. The obtained results were normalized and calculated to the corresponding controls and displayed as percentages.

2.5 | Protein extraction and western blotting

Protein extraction and western blotting analysis were performed as previously described, with minor modifications.[38–41] Cells were plated in Petri dishes, treated with AAE, and stimulated with IL-17A, as described in cell culture section. Whole protein from the cells of each plate was harvested using cell lysis RIPA buffer (Thermo Fisher Scientific, Waltham, MA). Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA), followed by protein separation through sodium dodecyl sulfate polyacrylamide gel electrophoresis. The resolved proteins were transferred to a polyvinylidene fluoride membrane (Merck Millipore, Billerica, MA),

2.6 | Enzyme-linked immunosorbent assay

IL-1β and IL-6 released into the conditioned media were measured using the respective ELISA kits (ab273237 and ab277389, respectively; Abcam) according to the manufacturer's instructions. Determine the optical density of each well immediately, using a microplate reader according to wavelengths of 450 nm immediately.

2.7 | Immunofluorescence

Cells were first sub-cultured on a glass slide (Millicell® EZ Slice, Millipore Corporation). On the next day, the cells were washed with PBS (pH 7.4) three times before being fixed with 4% paraformaldehyde (PFA) for 15 min, followed by further washing with PBS three times. Subsequently, cells were permeabilized with 0.1% Triton X-100 for 15 min, washed with PBS three times, and coated with 5% goat serum; all these steps were performed at room temperature. Thereafter, cells were incubated with the primary antibody against p-Nrf2

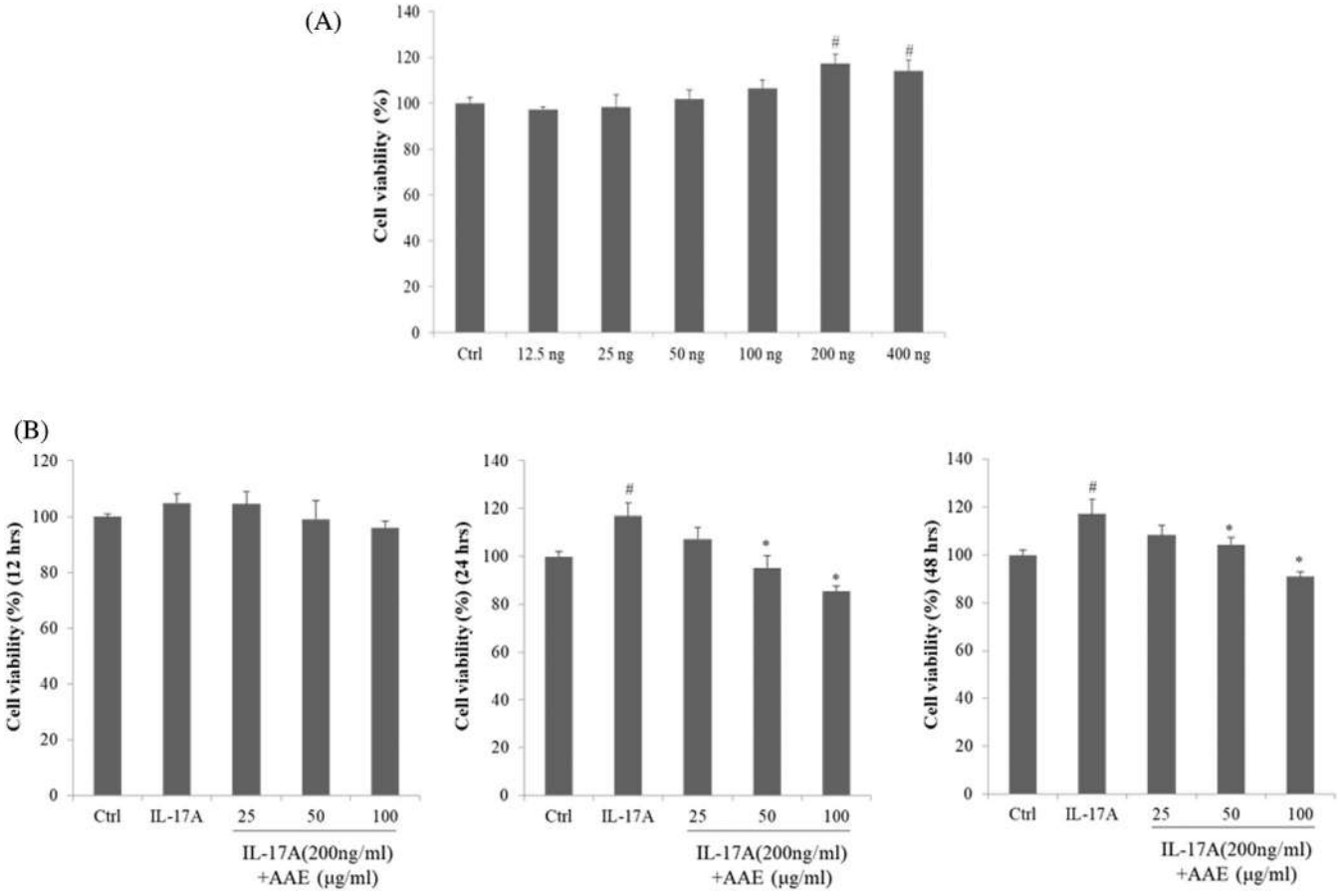

FIGURE 1 Effects of *Artemisia argyi* extract (AAE) on proliferation of synoviocytes. (A) HIG-82 rabbit synoviocytes were treated with a range of concentrations of IL-17A (12.5–400 ng/ml). (B) HIG-82 rabbit synoviocytes were treated with a range of concentrations of AAE (25–100 μg/ml), and cell proliferation viability was analyzed using a CCK-8 assay. # denotes statistical significance ($P < .05$) compared to the unstimulated control treatment. * denotes statistical significance ($P < .05$) compared to the IL-17A-stimulated treatment group.

(diluted in 1 ml goat serum to obtain a 1% concentration) at 4°C overnight. On the third day, cells were washed with PBS three times and incubated with the Alexa Fluor® 594 secondary antibody at room temperature for 2 h in the dark. For visualization of nuclei, the cells were washed three times, stained with 4,6-diamidino-2-phenylene (DAPI; diluted at a rate of 1:10 000), followed by incubation for 5 min and subsequent washing three times. Finally, the images were visualized using fluorescence microscopy (CKX53; Olympus, Tokyo, Japan).

2.8 | Nuclear/cytosolic protein fractionation

Nuclear and cytosolic fractions were obtained using the nuclear/cytosol fractionation kit (BioVision, Milpitas, CA), according to previously reported procedures[20,42] and manufacturer's instructions. Following treatment, cells were resuspended in chilled cytosolic protein extraction buffer and incubated at 4°C. The resulting suspension was centrifuged at 4°C, and the supernatant was processed to obtain the cytoplasmic fraction, whereas the pellets were processed with nuclear lysis buffer to yield the nuclear fraction. The fractions were analyzed through western blotting.

2.9 | Statistical analysis

All results were analyzed using standard statistical techniques. Prism 7.0 (GraphPad) was applied to the graphs. Data were analyzed using the Student's *t*-test and ANOVA. Quantitative data are presented as mean ± *SD*. Each experiment was repeated at least three times. Differences were considered statistically significant when *P*-value <.05, whereas *P*-value <.01 indicated high significance, *P*-value <.001 indicated tremendous significance.

3 | RESULTS

3.1 | Effect of AAE on the viability of IL-17A-challenged synoviocytes

To examine the proliferation of IL-17A-challenged synoviocytes, a CCK-8 assay was performed using synoviocytes treated with IL-17A at concentrations ranging from 12.5 to 400 ng/ml. The results showed that at 200 ng/ml, IL-17A remarkably induced synoviocytes proliferation (Figure 1A). Furthermore, synoviocytes were

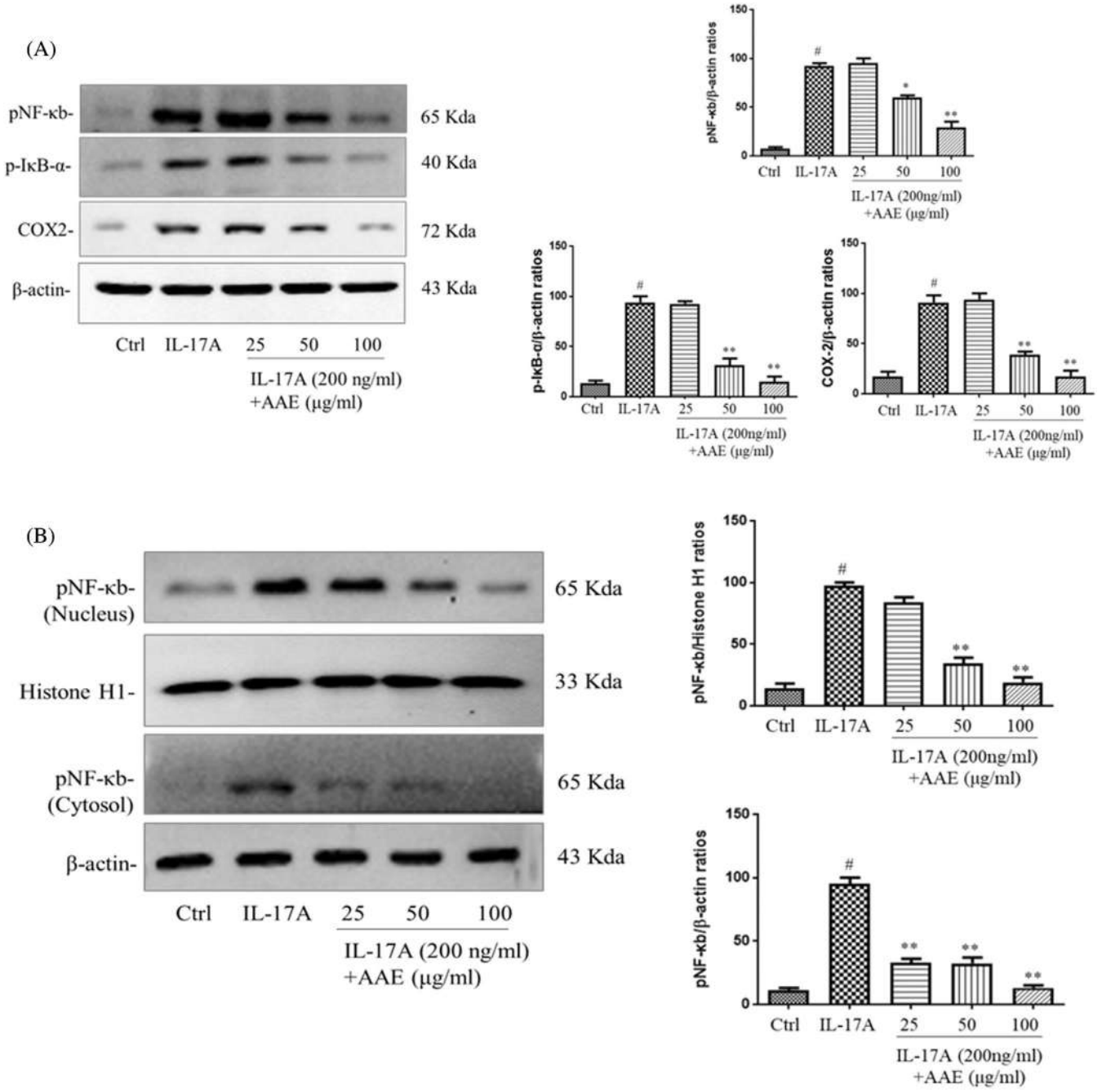

FIGURE 2 Effects of *Artemisia argyi* extract (AAE) on IL-17A-induced inflammatory mediators. (A) HIG-82 rabbit synoviocytes were treated with AAE following induction with IL-17A, and the expression levels of p-NF-κB, p-IκB-α, and COX2 were analyzed using western blotting. # denotes statistical significance ($P < .01$) in comparison with the unstimulated control treatment. * and ** denote statistical significance ($P < .05$ and $P < .01$, respectively) in comparison with the IL-17A-stimulated treatment group. (B) p-NF-κB expression levels in nucleus and cytoplasm were assessed by western blotting. # denotes statistical significance ($P < .01$) in comparison with the unstimulated control treatment. ** denotes statistical significance ($P < .01$) in comparison with the IL-17A-stimulated treatment group.

treated with IL-17A at 200 ng/ml and AAE at concentrations ranging from 25 to 100 μg/ml for 12, 24, and 48 h. It was found that IL-17A treatment at 200 ng/ml for 24 h significantly affected proliferation of synoviocytes. The effects of AAE on cell proliferation are shown in Figure 1B. Stimulation with IL-17A increased cell proliferation by 16.9% compared to that in unstimulated control cells. However, treatment with AAE decreased cell viability in a dose-dependent manner. Proliferation of cells subjected to AAE treatment at 100 μg/ml was significantly decreased by 29.9% when compared with that of the control cells stimulated with IL-17A (200 ng/ml) (Figure 1B). The results of three repeated and separate experiments were similar.

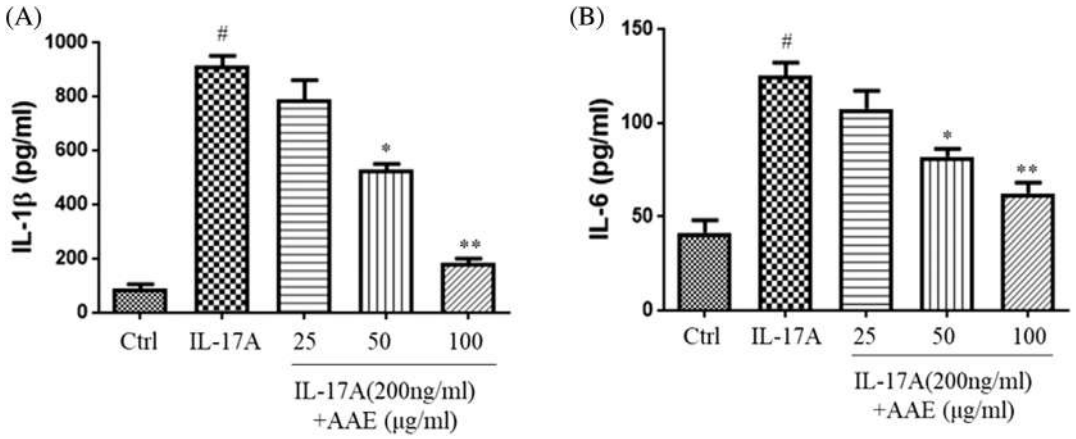

FIGURE 3 Effects of IL-1β and IL-6 expression in IL-17A-stimulated HIG-82 synoviocytes under *Artemisia argyi* extract (AAE) treatment. HIG-82 rabbit synoviocytes were treated with AAE following induction with IL-17A, and the expression levels of IL-1β (A) and IL-6 (B) were analyzed using ELISA. # denotes statistical significance ($P < .01$) compared to the unstimulated control treatment. * and ** denote statistical significance ($P < .05$ and $P < .01$, respectively) in comparison with the IL-17A-stimulated treatment group.

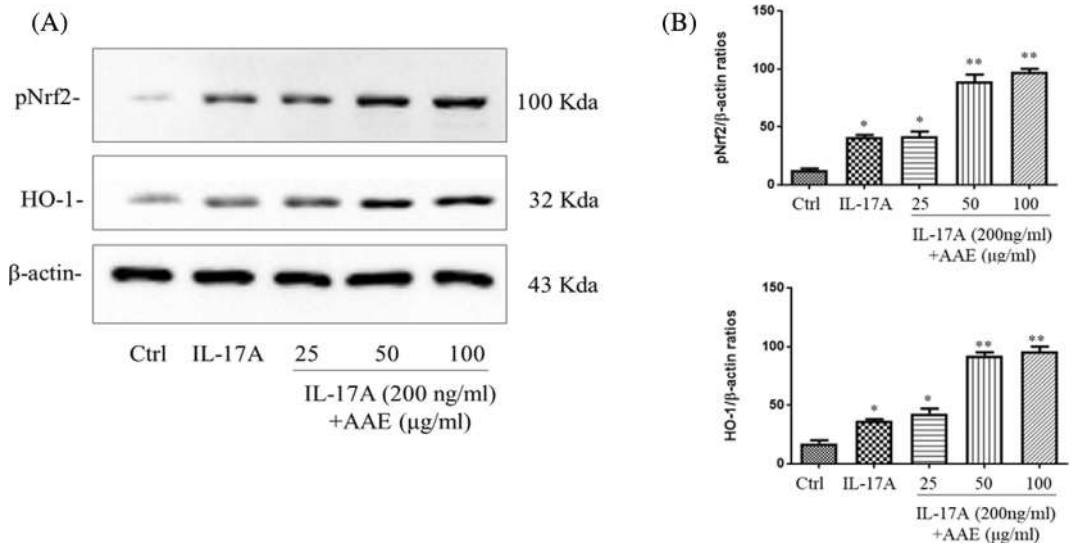

FIGURE 4 Effects of *Artemisia argyi* extract (AAE) on p-Nrf2 and HO-1 expression. HIG-82 rabbit synoviocytes were treated with AAE following induction with IL-17A, and the expression levels of p-Nrf2 and HO-1 were analyzed by western blotting. * and ** denote statistical significance ($P < .05$ and $P < .01$, respectively) in comparison with the unstimulated control treatment.

3.2 | Effects of AAE on inflammatory markers expression of IL-17A-challenged synoviocytes

NF-κB and COX-2 are intimately involved in the regulation of inflammatory responses. Furthermore, these two are involved in the pathogenesis of RA. Therefore, we examined the effect of AAE treatment on IL-17A-induced inflammatory proteins, including p-NF-κB, p-IκB-α, and COX-2. Stimulation with IL-17A increased p-NF-κB, p-IκB-α, and COX-2 levels compared to those in unstimulated control cells. However, AAE significantly decreased p-NF-κB, p-IκB-α, and COX-2 expression under inflammatory conditions compared to that in stimulated control cells (Figure 2A). Additionally, preparation of nuclear and cytoplasmic extracts from AAE-treated synoviocytes (Figure 2B) and western blotting analysis revealed that AAE significantly decreased the nuclear translocation of p-NF-κB following IL-17A challenge. These results indicated the upregulation of COX-2 expression following p-NF-κB activation by IL-17A. Furthermore, AAE significantly improved IL-17A-induced production of inflammatory mediators in synoviocytes (Figure 2). The results of three repeated and separate experiments were similar.

3.3 | Effects of AAE on IL-1β and IL-6 levels in IL-17A-challenged synoviocytes

Using specific ELISA kits for each cytokine, we measured the expression levels of the selected cytokines involved in the inflammatory response and known to be regulated by NF-κB activation in RA. IL-1β and IL-6 levels were significantly increased under IL-17A-challenged conditions

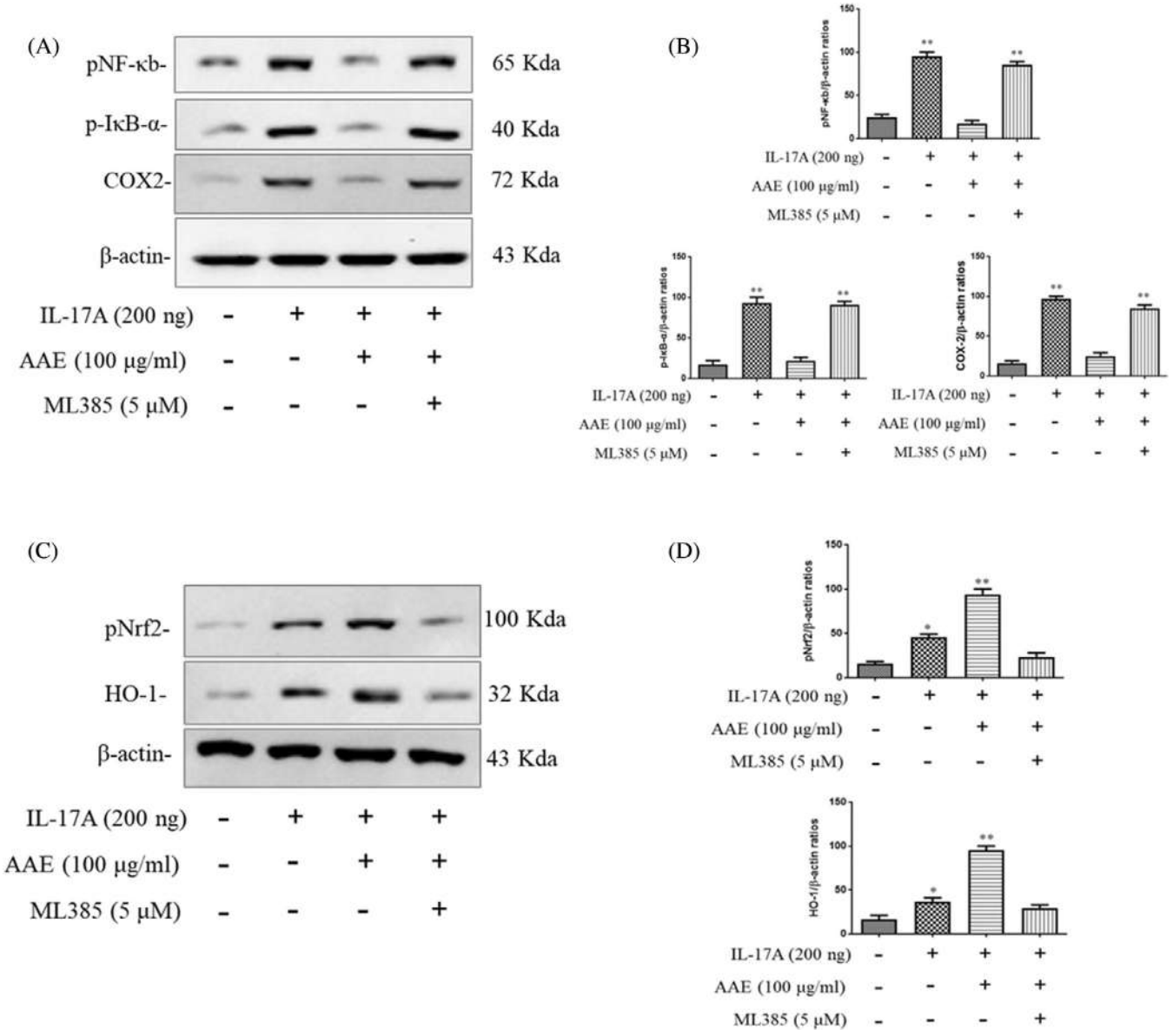

FIGURE 5 Effects of *Artemisia argyi* extract (AAE) or ML385 treatment on IL-17A-induced inflammatory mediators. HIG-82 rabbit synoviocytes were treated with ML385 for 24 h. The cells were then incubated with AAE for 24 h following treatment with IL-17A (24 h). (A) p-NF-κB, p-IκB-α, and COX2 levels were evaluated through western blotting. (B) HO-1 and p-Nrf2 levels were evaluated through western blotting. * and ** denote statistical significance ($P < .05$ and $P < .01$, respectively) in comparison with the unstimulated control treatment.

compared with those in control cells. In contrast, the significantly high levels of IL-1β and IL-6 in synoviocytes exposed to IL-17A were significantly decreased in a dose-dependent manner of AAE (Figure 3). The results of three repeated and separate experiments were similar.

3.4 | AAE upregulates HO-1 and p-Nrf2 levels in IL-17A-challenged synoviocytes

HO-1 is a cytoprotective enzyme that responds to oxidative and/or inflammatory stimuli. Therefore, we examined the effect of AAE on HO-1 levels in synoviocytes through western blotting analysis. We found that HO-1 levels increased significantly following AAE treatment (25–100 μg/ml). Additionally, quantification of altered p-Nrf2, a transcription factor responsible for the regulation of cellular antioxidant levels and anti-inflammatory activity in mammals, following AAE treatment revealed upregulated p-Nrf2 expression (Figure 4). The results of three repeated and separate experiments were similar.

3.5 | AAE treatment alters the expression of p-NF-κB, p-IκB-α, COX-2, HO-1, and p-Nrf2 in IL-17A-challenged synoviocytes under ML385 treatment

To investigate whether Nrf2 contributes to the neuroprotective effects of AAE, synoviocytes were incubated with ML385, an

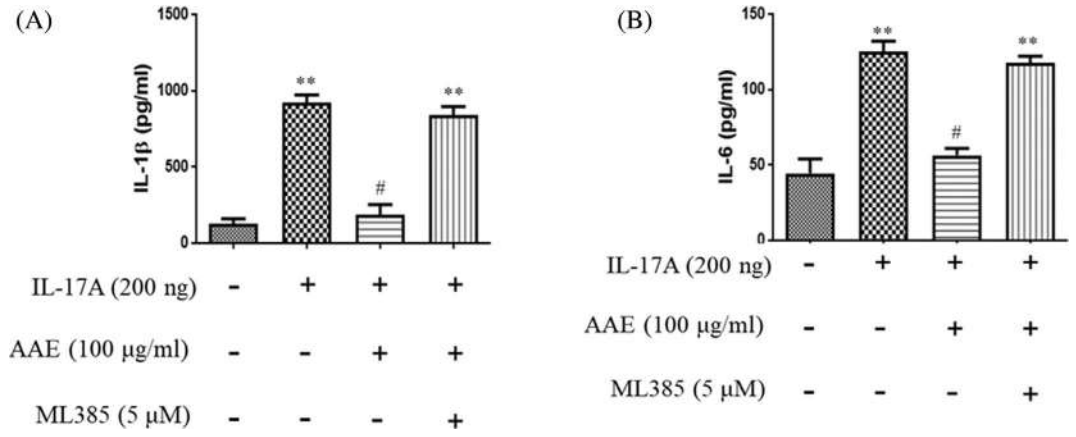

FIGURE 6 Effects on IL-1β and IL-6 expression in IL-17A-stimulated HIG-82 synoviocytes treated with *Artemisia argyi* extract (AAE) or ML385. HIG-82 rabbit synoviocytes were treated with ML385 for 24 h. The cells were then treated with IL-17A for 24 h and then incubated with AAE for 24 h. The levels of IL-1β (A) and IL-6 (B) were determined using ELISA. ** denote statistical significance ($P < .05$) in comparison with the unstimulated control treatment. # denotes statistical significance ($P < .01$) compared to the IL-17A-stimulated treatment group.

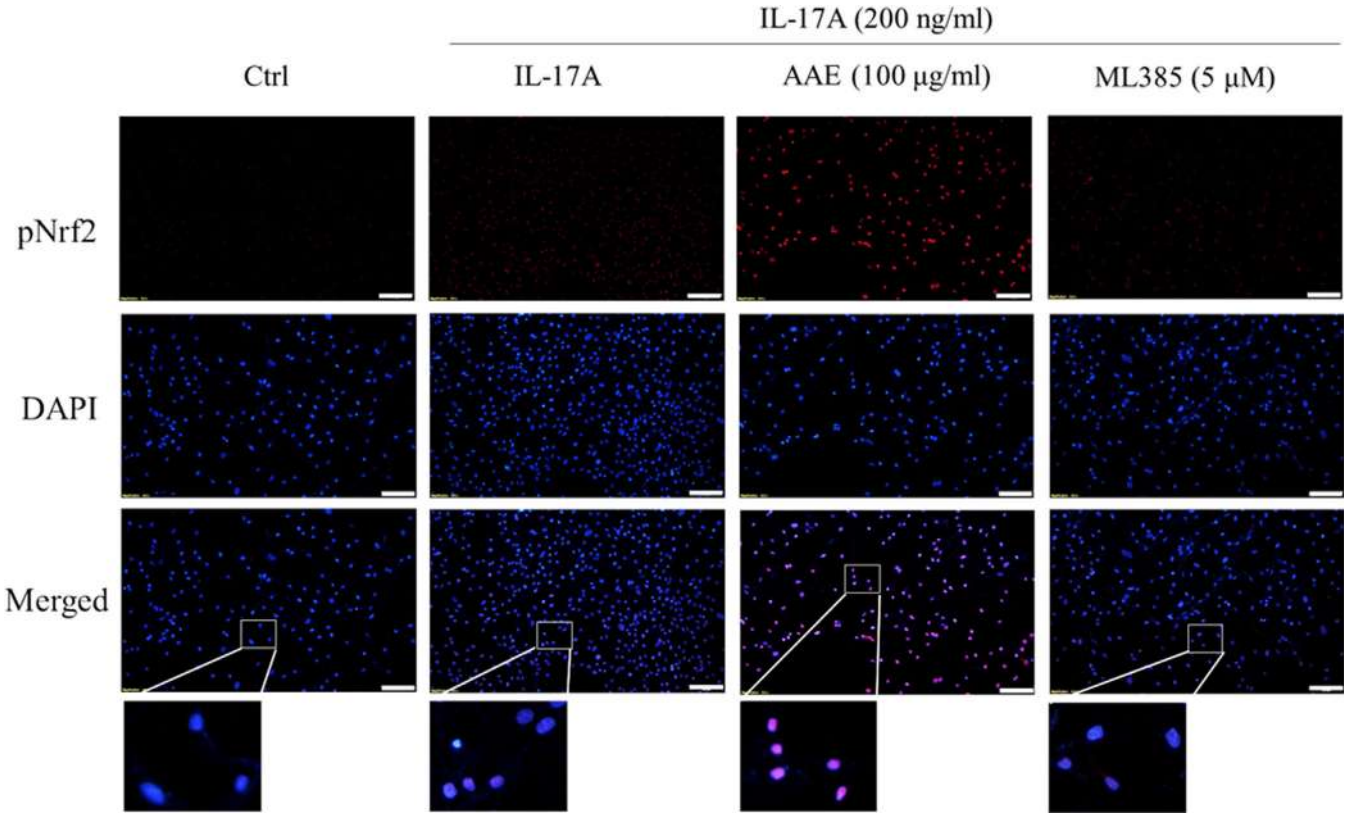

FIGURE 7 Immunofluorescence staining was performed to observe the effects of ML385 or *Artemisia argyi* extract (AAE) treatment on Nrf2 translocation. DAPI was used as a nuclear marker. The upper, middle, and bottom panels indicate p-Nrf2 (red), DAPI (blue), and merged images, respectively. Bars, 100 μm

Nrf2-specific inhibitor. The results showed that levels of p-NF-κB, p-IκB-α, and COX-2 were increased upon IL-17A challenge but decreased following AAE treatment; however, these effects were reversed after ML385 (5 μM) treatment (Figure 5A). Moreover, p-Nrf2 and HO-1 levels were slightly elevated following IL-17A treatment and significantly increased following AAE treatment compared to the levels observed in the control group and the group treated with IL-17A alone; however, these effects were reversed after ML385 (5 μM) treatment (Figure 5B). The results of three repeated and separate experiments were similar.

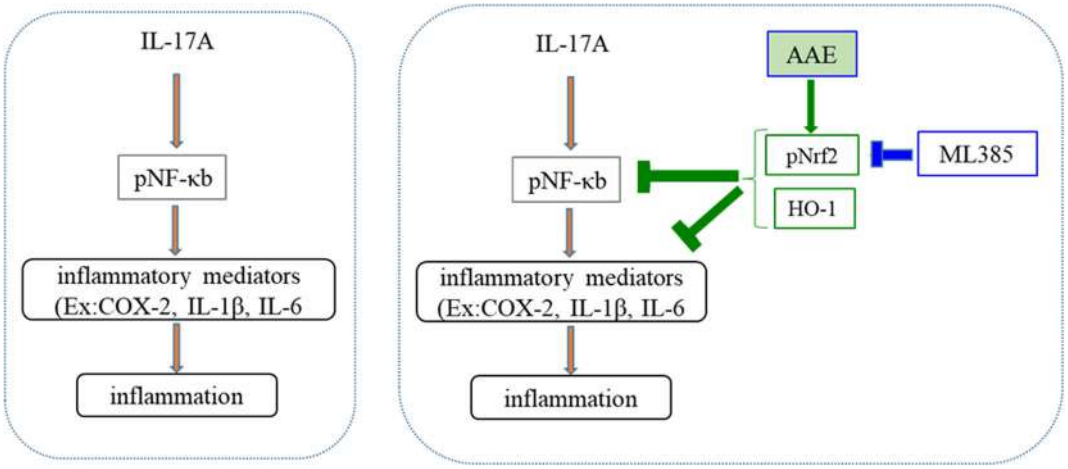

FIGURE 8 Schematic representation mode of *Artemisia argyi* extract (AAE) action in IL-17A-stimulated HIG-82 synoviocytes.

3.6 | Effects of AAE on IL-1β and IL-6 levels in IL-17A-challenged synoviocytes under ML385 treatment

IL-17A-mediated increase in IL-1β and IL-6 levels in the conditioned media of synoviocytes were significantly decreased following AAE treatment, whereas ML385 treatment dramatically abolished these AAE-mediated effects following IL-17A challenged (Figure 6). The results of three repeated and separate experiments were similar.

3.7 | Changes in Nrf2 nuclear translocation in IL-17A-challenged synoviocytes under AAE or ML385 treatment

To further confirm the beneficial effect of AAE on p-Nrf2 nuclear translocation, we performed immunofluorescence staining. As demonstrated by the results in Figure 7, AAE treatment after IL-17A exposure resulted in significant p-Nrf2 nuclear translocation compared to that in the other treatment groups. In contrast, these effects were reversed after ML385 (5 μM) treatment (Figure 7). And the schematic representation mode of AAE action in IL-17A-stimulated HIG-82 synoviocytes was shown in Figure 8.

4 | DISCUSSION

In this study, we explored the role of *A. argyi*, a traditional Chinese herbal medicine, in rabbit synoviocytes stimulated with IL-17A. The results showed that cell proliferation and inflammatory mediators were increased upon treatment with the pro-inflammatory cytokine IL-17A compared to that in the unstimulated group; however, this increase was inhibited dose-dependently by treatment with AAE. Additionally, we found that AAE treatment increased Nrf2/HO-1 levels in the rabbit synoviocytes cell line HIG-82 stimulated with IL-17A.

During infection, IL-17 is required to eliminate extracellular bacteria and fungi by inducing antimicrobial peptides, such as defensins.[6] In turn, IL-17 upregulates additional pro-inflammatory mediators, including IL-1β, IL-6, TNF-α, C-reactive protein, matrix metalloproteases and anti-microbial proteins, and is especially potent in the expansion and recruitment of neutrophils.[43] Furthermore, IL-17 plays an important role in inflammatory diseases such as RA and collagen-induced arthritis.[6,9,10] The pathogenesis of RA is multifactorial and often associated with the synovial compartment.[11,35] Interestingly, in this study, it was found that synoviocyte proliferation was increased following treatment with the pro-inflammatory cytokine IL-17A, whereas subsequent treatment with AAE attenuated synoviocyte proliferation and the production of pro-inflammatory mediators (IL-1β and IL-6). The synoviocyte proliferation rates in these experiments were consistent with those obtained in previous studies examining rabbit synoviocytes cell line HIG-82 stimulated with TNF-α or IL-1β.[35,44]

Artemisia argyi is a TCM that has been used to treat various disorders, such as oral ulcers,[45] dyspepsia, arthroncus, anaphylactic disease,[46] inflammatory diseases,[47,48] and cancer.[49] AAE has been found to suppress the proliferation of human primary T lymphocytes in vitro.[50] Additionally, Zhang et al. found that the 0.2 ml 100 mg/ml ethanol extract of *A. argyi* had an inhibitory effect on *Staphylococcus aureus* and could protect mice from death induced by *S. aureus* infection in vivo.[51] Furthermore, the ethanol extract of *A. argyi* can be used as a therapeutic agent for inflammatory skin diseases as well as for the inhibition of inflammatory mediator release from macrophages (50 μg/ml) and inflammatory cytokine production in inflamed tissues in vivo (300 μg).[47] Interestingly, in our study, AAE (50 and 100 μg/ml) attenuated synoviocytes proliferation and the production of pro-inflammatory mediators (such as IL-1β and IL-6) and ameliorated inflammatory mediator expression, including p-NF-κB and COX-2. Moreover, AAE prevented p-NF-κB nuclear translocation. Therefore, our findings indicate that *A. argyi* influences p-NF-κB activation and nuclear translocation and subsequently ameliorates inflammatory mediator expression in the progression of rabbit synoviocytes

stimulated with IL-17A, thereby suggesting that AAE influences inflammation by inhibiting specific markers of inflammation, such as NF-κB.

Previous studies have shown that p-NF-κB may regulate inflammatory protein modifications through different signaling cascades, such as the Nrf2 signaling pathway axis.[17–19] In astrocytes, the absence of Nrf2 may induce highly aggressive inflammation through the activation of NF-κB and downstream pro-inflammatory cytokines.[21] Additionally, the activation of Nrf2–antioxidant signaling attenuates the NF-κB inflammatory response.[22] Nrf2 is a transcription factor that regulates the expression of a variety of cytoprotective molecules, and HO-1 and Nrf2 downstream proteins can protect cells by reducing inflammation.[52–54] Our study demonstrated that p-Nrf2 and HO-1 levels were increased following IL-17A treatment and that AAE treatment further upregulated HO-1 and p-Nrf2 levels, and p-Nrf2 nuclear translocation, suggesting their critical roles in the progression of cytokine-mediated synoviocyte inflammation. To better understand the relationship between Nrf2 and the anti-inflammatory effects in cytokine-stimulated synoviocytes, we pretreated synoviocytes with ML385 (an Nrf2-specific inhibitor). The results indicated that when combined with AAE treatment, ML385 reversed AAE-induced upregulation of p-Nrf2 and HO-1 levels in IL-17A-challenged synoviocytes. These results suggest that AAE might act as an Nrf2 activator to increase HO-1 expression, thereby attenuating the p-NF-κB-mediated inflammatory response during IL-17A challenge in synoviocytes. In the future, we need to clarify the details about how Nrf2 and HO-1 affect the NF-κB activation.

The results of the present study provide a fundamental basis for future research on the effects of *A. argyi* in RA. Possible limitations of this study include the in vitro design, which limits the direct extrapolation of results to an in vivo model. In addition, further studies are needed to elucidate the active component in *A. argyi* that affects proliferation and inflammation. Cumulatively, our study highlights that AAE exhibits anti-inflammatory activities by inhibiting proliferation, decreasing inflammatory mediator expression, and modulating Nrf2 and HO-1 levels in synoviocytes stimulated with IL-17A. The results of our study suggest a potential role of AAE as a complementary or supplemental treatment modality against arthritic and inflammatory symptoms associated with RA.

AUTHOR CONTRIBUTIONS

Jhong-Kuei Chen, Chia-Hua Kuo, Wei-Wen Kuo, and Cheng-You Lu designed the experiments; Jhong-Kuei Chen, Cecilia Hsuan Day, and Tsung-Jung Ho performed the experiments; Tso-Fu Wang, Pi-Yu Lin, Shinn-Zong Lin, and Cheng-You Lu analyzed the data; Chih-Yang Huang, Cheng-Yen Shih, and Cheng-You Lu supervised the studies and contributed reagents/materials/analysis tools; Jhong-Kuei Chen and Cheng-You Lu prepared the initial draft of the manuscript. All the four authors approve the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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