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Original Research Article

3-O-Caffeoylquinic acid in *Periploca forrestii* Schltr extract ameliorates collagen-induced arthritis by inducing IL17/IL23 cells in rats

Qiaoyi Ning[1,2], Xueming Yao[2], Ying Huang[2], Lei Hou[2], Daomin Lu[2], Yutao Yang[3], Yamei Zhan[4], Yiting He[5], Wukai Ma[2]*

[1]The 2nd Clinical School, Guangzhou University of Chinese Medicine, Guangzhou 510120, [2]Rheumatology and Immunology Department, The 2nd Hospital Affiliated with Guizhou university of Traditional Chinese Medicine, Guiyang 550003, [3]Physical Examination Center, The 2nd Hospital Affiliated With Guizhou University of Traditional Chinese Medicine, Guiyang 550003, [4]Department of Pharmacy, Guizhou Provincial People's Hospital, Guiyang 550002, [5]Intellectual Property Management and Transformation Center, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou 510120, China

For correspondence:* ***Email:* *w7n3kx@163.com;* ***Tel:*** *+86-0851-85556970*

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Abstract

Purpose: *To study the therapeutic effect of 3-O-caffeoylquinic acid (3-O-CQA) from Periploca forrestii extract (PFE) on collagen-mediated arthritis (CIA) in rats, as well as the potential underlying mechanism of action.*

Methods: *PFE and 3-O-CQA were successively and intragastrically administered to CIA rats. Paw swelling, arthritic scores and H & E staining were used to evaluate the therapeutic effect of 3-O-CQA. Moreover, to determine the effects of PFE and 3-O-CQA on fibroblast-resembling synoviocytes obtained from arthritic subjects (RAFLS), the viability of RAFLS cultured in vitro was measured with MMT, while apoptotic lesions were analyzed by flow cytometry. The levels of IL-6 in CIA and RAFLS were determined by enzyme-linked immunosorbent assay (ELISA), while quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunoblotting were used to assess their mRNA and polypeptide levels, respectively.*

Results: *PFE in 3-O-CQA ameliorated swelling and reduced arthritic scores in CIA rat model, and also decreased cytokine levels ($p < 0.05$). By decreasing mRNA and protein expressions, 3-O-CQA repressed the phosphorylation of STAT3 and JAK2 as well as the protein levels of IL-23 and RORγt ($p < 0.05$).*

Conclusion: *The results of this study show that CIA and RAFLS are ameliorated in rats by 3-O-CQA in PFE through regulation of IL17/ IL23 and Th17 cells. Thus, 3-O-CQA affords a therapeutic strategy for the management of collagen-induced arthritis.*

Keywords: *Arthritis, Periploca forrestii Schltr extract, 3-O-Caffeoylquinic acid, Interleukin (IL)-17, IL-23, Th17 cells*

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INTRODUCTION

It is recognized that RA is a persistent inflammatory ailment which usually leads to critical disability or even precocious death [1]. Thus, RA constitutes a severe public health burden [2]. Consequently, there is a very pressing need to identify treatment strategies

for RA. It has been reported that macrophages along with T cells and B cells induced the pathogenesis of RA through secreting a series of cytokines [3]. Recently, researchers discovered that immune abnormality induced by T cells (containing Th1 and Th17) was the chief pathogenetic mechanism of RA. It has been reported that several cytokines related to RA activated the JAK-STAT pathway [4]. Moreover, it has been predicted that RORγt is the transcriptional activator which enhanced IL-17 production by Th17 cells [5]. Previous studies have indicated that Th17 and its effector IL-17 play essential roles during the entire stage of RA development [6]. Thus, suppression of the JAK2/STAT3 route and regulation of differentiation of Th17 cells may be important steps for RA therapy. Currently, traditional Chinese medicine is receiving more and more attention for RA therapy due to its clear curative effect and fewer adverse reactions [7]. The traditional Chinese herb *P. forrestii* is used by local folks to relieve arthritis [8, 9]. One of the components of *Periploca forrestii* Schltr is 3-*O*-CQA. It has been reported that 3-*O*-CQA inhibited the effects of arthritis on the immune system of rats by decreasing inflammatory factors and improving antioxidant ability [10]. Thus, it is very important to verify whether 3-*O*-CQA and *Periploca forrestii* Schltr extract could relieve arthritis. In this study, the therapeutic effect of PFE was investigated, as well as the likely mechanism underlying CIA in rats and rheumatoid arthritic fibroblast-like synoviocytes (RAFLS).

EXPERIMENTAL

Plant sample and processing

Periploca forrestii Schltr was purchased from the 2nd Hospital Affiliated with Guizhou University of Traditional Chinese Medicine. Then, a high-speed Chinese medicine crusher was used to grind the drug into a powder which was sieved through 24-mesh sieve and 50-mesh sieve. Then, 50 g of the powder was weighed, and 50% ethanol was added at material-to-liquid ratio of 1:15. Reflux extraction was performed twice in a water bath at 80℃, with each extraction lasting 2 h. The extracts were filtered and the filtrates were combined. The combined filtrate was decompressed and dried in a rotary evaporator at 50℃. 3-O-Caffeoylquininc acid (3-*O*-CQA) was bought from Shanghai Yuanye Biotechnology.

Animals and cell culture

Six-to-eight-week-old Spraque-Dawley (SD) rats were purchased from the 2nd Hospital Affiliated with Guizhou University of Traditional Chinese Medicine. All rats were housed in a temperature-controlled room with a 12-h light/12-h dark cycle, with provision of tap water and standard chow. Moreover, RAFLS were obtained from RA patients following the protocol reported previously [11]. The research received approval from the ethical authority of The 2nd Hospital Affiliated with Guizhou University of Traditional Chinese Medicine (approval no. KYW2018003), and was performed in compliance with international guidelines for animal studies [12]. Signed written informed consents were obtained from the patients and/or guardians.

Collagen-mediated arthritis (CIA) model

The CIA model was established according to a previously published method [13]. A 4 mg/mL solution of CII was prepared in glacial ethanolic acid and deposited in refrigerator for 12 h at 4 °C. Thereafter, emulsification of the solution was done with CFA using a 1:1 volume ratio. The rats were subcutaneously injected in the tail roots with CII emulsion, followed 14 days later with secondary immunization through subcutaneous inoculation with CII emulsion of IFA (100 μL/rat) at the same location. The disease intensity was estimated using two indexes (paw oedema and arthritis rating) as previously described [14].

H&E staining

Samples of ankle joint of right-hand paw were collected after anesthesia, after which the endometriosis was subjected to routine histological processing and H&E staining. The stained sections were examined under a light microscope (Olympus, Tokyo, Japan).

Enzyme linked immunosorbent assay (ELISA)

Cytokine levels were determined with ELISA. The first and biotin-labeled second antibodies were incubated in sequence with the plate. Then, absorbance was read in a spectrophotometer at 450 nm.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The extraction of total RNA from RAFLS and Th17 cells was done with TRIzol, and reverse transcription (RT) of the RNA was done with Invitrogen RT kit, followed by RT-PCR. The relative levels gene expressions were quantified using the $2^{-\Delta\Delta CT}$ method, with GAPDH utilized as the control. Table 1 shows the primers used.

Western blotting

Total polypeptides were obtained from RAFLS and Th17 cells by lysing using RIPA buffer. The proteins were subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to a PVDF film which was sealed using non-fat milk, and subsequently incubated for 12 h with appropriate 1º antibodies at 4 ℃. Thereafter, the membrane was incubated with 2º immunoglobulin linked to horse radish peroxidase. The antibodies used in the study were anti-JAK2 (1:1000 dilution, cat: ab108596, Abcam, USA); anti-p-JAK2 (1:1000 dilution, cat: ab32101, Abcam, USA); anti-STAT3 (1:1000 dilution, cat: ab68153, Abcam, USA); anti-p-STAT3 (1:1000 dilution, cat: ab76315, Abcam, USA); anti-IL-23R (1:1000 dilution, cat: ab175072, Abcam, USA); anti-RORγt (1:1000 dilution, cat: ab111174, Abcam, USA), anti-Foxp3 (1:1000 dilution, cat: ab215206, Abcam, USA), and anti-GAPDH (1:1000 dilution, cat: 10494-1-AP, Proteintech, USA).

MTT assay

3-(4,5)-Dimethylthiahiazo(-z-y1)-3,5-di-phenyte-trazoliumromide (MTT) assay was carried out to determine the viability of RAFLS. Following 24-h treatment with PFE in 96-well plate, each well was incubated for 4 h with MTT solution (20 µL). Then, the resultant formazan crystals were solubilized with DMSO, and absorbance was read at 570 nm.

Flow cytometry

In the flow cytometry assay, 5 µL of PI and 5 µL of Annexin V were used to culture the RAFLS. Thereafter, apoptotic changes in RAFLS were analyzed using ACEA NovoCyteTM flow cytometer.

Statistical analysis

The SPSS software (25.0 version, SPSS, Inc., USA) and GraphPad (6.0 version) were used for statistical analysis. Results are expressed as mean ± SD (n = 3). Paired comparison was done with *t*-test, while multiple-group comparison was done with ANOVA. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of PFE on CIA in rats

Compared with the normal control group, the CIA model rats presented visible pathological features of RA (Figure 1 A). For instance, there were evidence of invasion by inflammation-inducing cells, disorder-cum-hyperplasia of synovial cells, increased number of blood vessels, and dilatation phenomenon. The CIA model had higher volume of hind foot swelling (Figure 1 B), greater arthritis index (AI) (Figure 1 C) and higher cytokine levels, i.e., TNF-α (Figure 1 D), IL-6 (Figure 1 E), and IL-17 (Figure 1 F). However, these pathological manifestations (volume of hind foot swelling, AI and cytokine levels) were mitigated by treatments with PFE and the positive drug leflunomide (LEF, Figure 1 A - F). Moreover, relative to control, treatment outcome in PFE group was similar to that in LEF group (Figure 1 A - F).

PFE inhibited viability and promoted apoptotic lesions in RAFLS

As shown in Figure 2 A, compared with control group, the inhibitory effect of PFE on RAFLS viability and its potential to induce cell apoptosis increased with increasing doses of PFE. The effects of PTE were close to those of the positive drug, LEF (Figure 2 B). Besides, after determining IL-17 cytokine level via ELISA (Figure 2 C).and the mRNA expressions of IL-17, IL-23, TNF-α and IL-6 through qRT-QCR assay (Figure 2 D), it was seen that the levels of cytokines and mRNA decreased with increasing doses of PFE, which was analogous to the effect of LEF. Moreover, results from Western blot assay of IL-23R, JAK2, p-JAK2, STAT3, and pSTAT3 showed that LEF and PFE inhibited the expression levels of these proteins (Figure 2 E). Thus, these results indicate that PFE inhibited multiplication and promoted apoptotic lesions of RAFLS.

Table 1: Sequences of primers used

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
IL-17	CTCAGACTACCTCAACCGTTCC	GTGCCTCCCAGATCACAGAAG
IL-23	CACCACTGGGAGACTCAACA	AGGATCTTGGACGGAGAAGA
TNF-α	ATCAATCGGCCCGACTATCTC	GCAATGATCCCAAAGTAGACCTG
IL-6	GGAGACTTGCCTGGTGAAAA	GTCAGGGGTGGTTATTGCAT
RORγt	CTGCACTGTGTGAAGGGTGA	GACAAGCCTTTTCTCCATCG
Foxp3	TTCACCTATGCCACCCTCAT	CTCACTCTCCACTCGCACAA
GAPDH	GAGAAGTATGACAACAGCCTC	ATGGACTGTGGTCATGAGTC

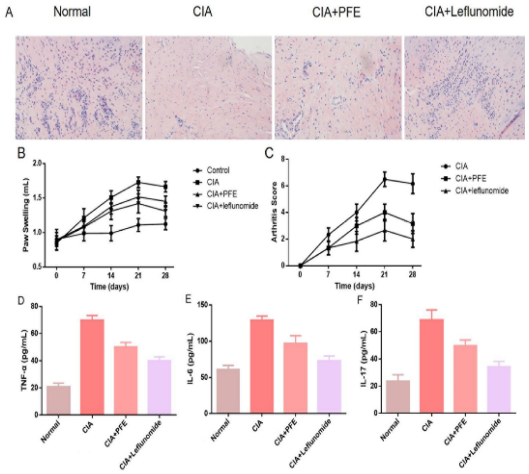

Figure 1: Effect of PFE on CIA in rats. (A) H&E-stained hind foot pathomorphology in each group. Measured values of hind foot swelling volume (B) and AI (C). Serum TNF-a (D), IL-6 (E), and IL-17 (F), as estimated via ELISA

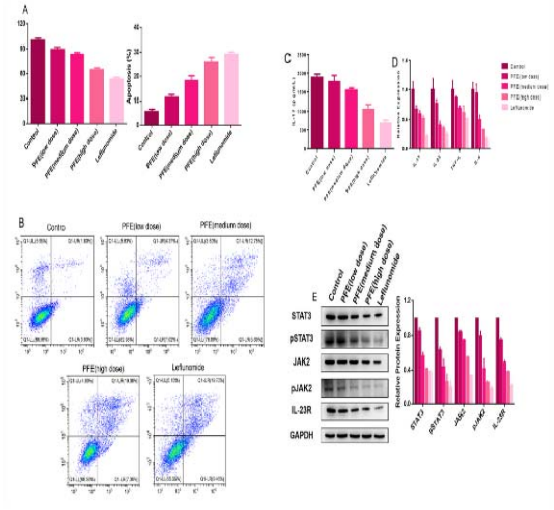

Figure 2: PFE inhibited proliferation and promoted apoptosis of RAFLS *in vitro*. (A) Cell apoptosis, as measured using FACS. (B) Proliferative potential, as analyzed via MTT method. (C) IL-17 level, as determined through ELISA. (D) Relative expression levels of IL-17, IL-23, TNF-a and IL-6 mRNA, as measured *via* qRT-PCR assay. (E) Relative protein expression levels of STAT3, pSTAT3, JAK2, pJAK2 and IL-6 determined *via* Western blot assay

Effect of PFE on differentiation of Th17 cells

Considering that Th17 cells are unlikely to be detected in RAFLS, relative levels of mRNA and polypeptide of Th17 cell-specific markers RORγT and FoxP3 were determined. These were aimed at ascertaining the effect of PFE on Th17 cell differentiation. Results showed that LEF and PFE decreased RORγt expression, but promoted mRNA and polypeptide levels of Foxp3, when compared with the control group (Figure 3 A and B).

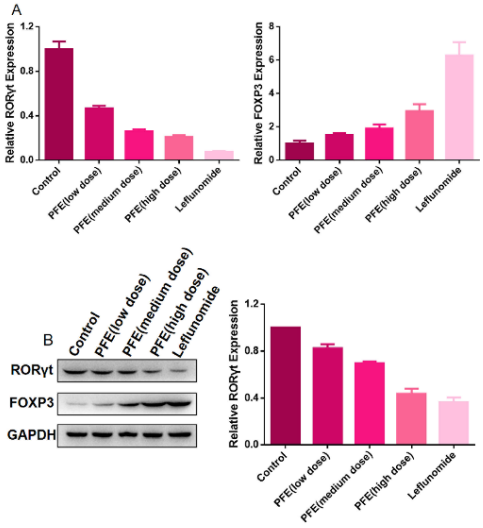

Figure 3: Effect of PFE on differentiation of Th17 cells in CIA rats. Relative mRNA (A) and protein (B) expression levels of RORγt and Foxp3

3-*O*-CQA inhibited proliferation and promoted apoptosis of RAFLS *in vitro*

The effect of 3-*O*-CQA on RAFLS was determined *in vitro*. As shown in Figures 4A and 4B, 3-*O*-CQA and LEF induced apoptosis in RAFLS and suppressed cell survival. Moreover, the inhibitory effect of 3-*O*-CQA on IL-17, IL-23, TNF-a and IL-6 and related proteins i.e., JAK2, p-JAK2, STAT3, and pSTAT3, were similar to those of LEF *in vitro* (Figures 4C-4E). Hence, these data show that 3-*O*-CQA in PFE inhibited the multiplication, but promoted apoptotic lesions of RAFLS.

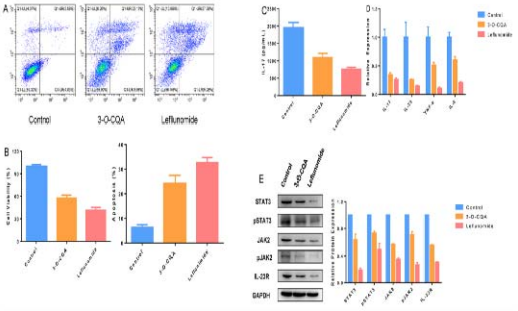

Figure 4: 3-*O*-CQA protected RASFs *in vitro*. (A) Cell apoptosis, as determined with FACS. (B) Proliferation, as determined via MTT method. (C) Levels of IL-17, as determined through ELISA. (D) Relative mRNA amounts of cytokines, as determined *via* qRT-PCR assay. (E) Relative protein expression levels of STAT3, pSTAT3, JAK2, pJAK2 and IL-6

Effect of 3-*O*-CQA on differentiation of Th17 cells

Figure 5A and B show that LEF and 3-*O*-CQA reduced the mRNA and protein levels of RORγt, while the mRNA and protein levels of Foxp3 were increased.

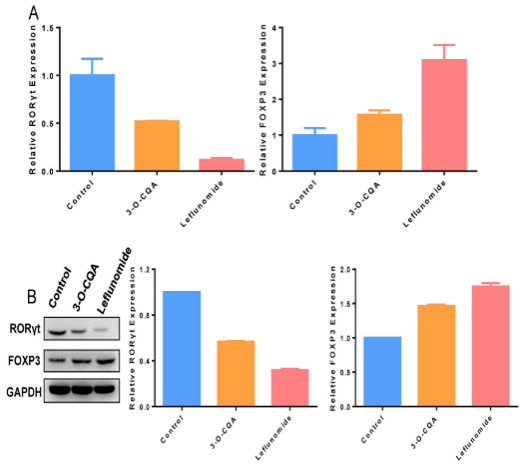

Figure 5: Effect of 3-O-CQA on differentiation of Th17 cells in CIA rats. (A) mRNA levels of RORγt and Foxp3. (B) RORγt and Foxp3 levels

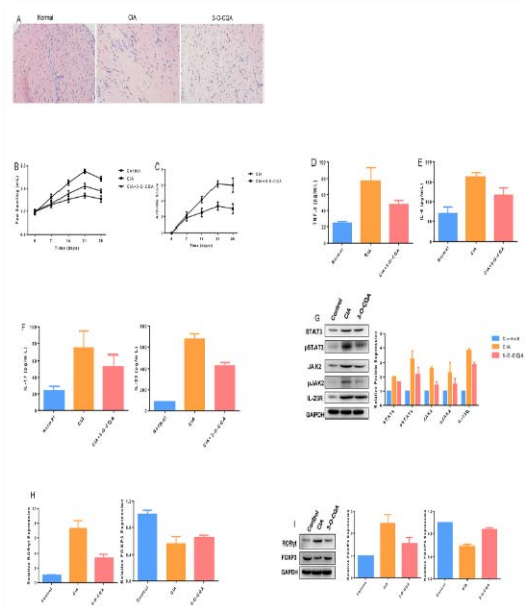

Figure 6: Effect of 3-O-CQA on CIA rats. (A) H&E-stained hind foot pathomorphology in each group. Volume of hind foot swelling (B) and AI (C), as measured. Serum TNF-a (D), IL-6 (E), and IL-17 (F), as estimated using ELISA. (G) Relative protein expression levels of STAT3, pSTAT3, JAK2, pJAK2 and IL-6, as measured via Western blot assay. (H) mRNA levels of RORγt and Foxp3. (I) Polypeptide levels of RORγt and Foxp3, as measured *via* immunoblot assay

Effect of 3-O-CQA on CIA rats

The CIA model was successfully established in rats, as shown in Figure 6 A. In contrast to the CIA model group, it was observed that the volume of hind foot swelling (Figure 6 B), arthritis index (AI) (Figure 6 C) and levels of cytokines i.e., TNF-α (Figure 6 D), IL-6 (Figure 6 E), and IL-17 and IL-23 (Figure 6 F) were down-regulated in the treated rats. Besides, the relative polypeptide amounts of JAK2, p-JAK2, STAT3, p-STAT3 and IL-23, as were determined via western blot assay are shown in Figure 6 G. Results displayed that 3-O-CQA significantly suppressed protein levels of p-STAT3, p-JAK2 and IL-23. Moreover, the relative amounts of Th17 cell-related markers RORγt and Foxp3 are presented in Figures 6 H and I. It is evident that 3-O-CQA down-regulated the mRNA and polypeptide levels of RORγt and up-regulated those of Foxp3, in contrast to CIA group (Figure 6 H and I). In summary, these results demonstrated that 3-O-CQA from PFE ameliorated collagen-induced arthritis via stimulation of the IL17/IL23 axis in the rat.

DISCUSSION

Most RA patients need long term treatment with NSAIDS, cDMARDs or/and GC [15]. However, it has been reported that these drugs are associated with some severe and adverse side effects such as injuries in liver, kidney and digestive tract [2]. Luckily, during clinical management, bDMARDs have been used successfully to treat RA. Moreover, bDMARDs offer different treatment choices for the RA patients [16-18]. Although these drugs have good therapeutic effects, many patients are unable to afford them due to the high treatment costs. Long-term active RA could bring disability, decreased work efficiency, financial burden and adverse effect on the quality of life [2]. Hence, there is need to find affordable candidate drugs for treating RA. In this study, it was shown that the traditional Chinese medicine *Periploca forrestii* Schltr and its bioactive component 3-*O*-CQA exerted anti-arthritic effects on CIA rats, and suppressed the cell viability of synovial fibroblasts.

There are many studies on how to choose trial animal models of RA. The results demonstrated that the rat models of CIA and adjuvant-induced arthritis (AIA) are appropriate models for finding out the pathological mechanisms underlying RA, as well as original and alternative drugs for RA. Comparatively speaking, the CIA model is much more like human arthritis than the AIA model. Moreover, the rat model of CIA shows the typical

damage to the joints, and the distinct inflammatory reactions in synovial membranes [19, 20]. Therefore, this study utilized CIA rats to determine the anti-arthritic effects *of Periploca forrestii* Schltr and 3-*O*-CQA. Moreover, after successful establishment of the CIA model in SD rats, arthritis index as well as the paw swelling volume were determined in the experimental phase. The results revealed that PFE and 3-*O*-CQA markedly reduced the arthritis index as well as the volume of paw edema in CIA rats, just like the positive drug leflunomide. Besides, through H&E histopathological examination, it was discovered that PFE and 3-*O*-CQA reduced hyperplasia of synovial membrane and decreased cartilage damage.

Synovial cells play a pivotal part in outbreak of RA. Therefore, regulation of the viability and death of synovial cells is likely to offer a lead to fresh drugs for medical treatment of RA [21, 22]. In this study, it was seen that PFE suppressed the multiplication of RAFLS and enhanced RAFLS apoptosis. Results of MTT and flow cytometric assays showed that PFE exerted anti-proliferative and pro-apoptosis effects on RAFLS *in vitro*. Based on these findings, the influence of 3-*O*-CQA on RAFLS was investigated, and results showed that it produced the same trend as PFE with respect to regulating RAFLS cell proliferation and apoptosis.

To investigate the mechanism associated with the control of CIA rat arthritis and RAFLS cell proliferation and apoptosis by PFE and 3-*O*-CQA, other experiments were carried out in this study. Various immune-related cells have been reported to participate in the progression of RA [23]. Moreover, macrophages and T cells induce the pathogenesis of RA through secretion of a series of cytokines [3]. Therefore, this study investigated levels of cytokines via ELISA, and the results indicated that PFE and 3-*O*-CQA down-regulated these cytokines *in vivo* and *in vitro*. Moreover, these results were confirmed with qPCR. Previous studies showed that IL-6 activated the STAT3 pathway, thereby accelerating the conversion of activated CD4+ T cells into Th17 cells which secreted more IL-17, and IL-17 activated the JAK-STAT pathway [4]. Therefore, this study analyzed the expressions of Th17-related proteins. The results indicated that PFE down-regulated IL-23R, JAK2, p-JAK2, STAT3, pSTAT3 and RORγt, and also promoted the expression of Foxp3. Moreover, the results revealed that PFE ameliorated collagen-induced arthritis via up-regulation of cytokine levels, and influenced Th17 cell differentiation. A similar trend in the amelioration of RA was observed with 3-*O*-CQA.

CONCLUSION

These results demonstrate that 3-*O*-CQA and *Periploca Forrestii* Schltr extract, when used together, ameliorate collagen-induced arthritis via up-regulation of cytokines related to IL17/IL23 axis, and influence Th17 cell differentiation. Thus, 3-*O*-CQA affords a therapeutic strategy for the management of collagen-induced arthritis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the

current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contributions of authors

We declare that this study was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qiaoyi Ning and Xueming Yao were responsible for animal experiments, cell experiments and data analysis, Ying Huang, Lei Hou, Daomin Lu, Yutao Yang, Yamei Zhan, Yiting He and Wukai Ma were responsible for animal experiments and some cell experiments, and Qiaoyi Ning was responsible for subject design, experimental quality control and paper writing. All the authors read and approved the final manuscript.

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