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Identification of candidate synovial membrane biomarkers after *Achyranthes aspera* treatment for rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease whose main symptom is a heightened inflammatory response in synovial tissues. To verify the anti-arthritic activities of *Achyranthes aspera* and its possible therapy-related factors on the pathogenesis of RA, the saponins in *A. aspera* root were isolated and identified to treat the collagen-induced arthritis (CIA) rats. Phytochemical analysis isolated and identified methyl caffeate, 25-S-inokosterone, 25-S-inokosterone β-D-glucopyranosyl 3-(O-β-D-glucopyranosyloxy)-oleanolate, and β-D-glucopyranosyl 3-(O-β-D-galactopyranosyl (1 → 2)(O-β-D-glucopyranosyloxy)-oleanolate as main compounds in the root of *A. aspera*. Proteomics was performed to determine the differentially expressed proteins in either inflamed or drug-treated synovium of CIA rats. Treatment resulted in dramatically decreased paw swelling, proliferation of inflammatory cells, and bone degradation. Fibrinogen, procollagen, protein disulfide-isomerase A3, and apolipoprotein A-I were all increased in inflamed synovial tissues and were found to decrease when administered drug therapy. Furthermore, Alpha-1-antiproteinase and manganese superoxide dismutase were both increased in drug-treated synovial tissues. The inhibition of RA progression shows that *A. aspera* is a promising candidate for future treatment of human arthritis. Importantly, the total saponins found within *A. aspera* are the active component. Finally, autoantigens such as fibrinogen and collagen could act as inducers of RA due to their aggravation of inflammation. Given this, it is possible that the vimentin and PDIA3 could be the candidate biomarkers specific to *Achyranthes* saponin therapy for rheumatoid arthritis in synovial membrane.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder that attacks the joints, causing chronic and painful inflammation. There are currently several therapeutic options to treat RA [1]. Interestingly, many traditional Chinese medicines (TCMs) have been shown to have therapeutic effects on ameliorating arthritic symptoms for their immunosuppression, anti-inflammation, and/or antioxidant activity [2]. The She are one of China's ethnic minorities where they reside in the southern mountain areas, which have a humid climate that induces pain level of RA [3,4]. During the two thousand years that they have spent fighting the disease, the She people have found that a decoction of *Achyranthes aspera* L. (Amaranthaceae) has robust efficacy for use as an RA therapy, however, the clinical effect has not been proved in report yet.

A. aspera is a traditional medicinal plant used in many Asian countries. The non-alkaloid fraction of the *A. aspera* leaf possesses a pronounced anti-carcinogenic effect [5]. Moreover, the ecdysterone found in the seeds of *A. aspera* has shown both immunostimulatory and growth promoting properties in the larvae of the common carp *Cyprinus carpio* [6]. Extracts of *A. aspera* also possess in vivo wound healing activity [7]. They have also been used as folk remedies for reducing internal heat due to increased metabolism in the disease state as well as antipyretic, snake bite, and wound healing [8–10]. However, despite the wide and varied use of *A. aspera* extracts, their pharmaceutical and pharmacological mechanisms have yet to be elucidated.

Finally, the ethyl acetate fraction of the crude extract of *A. aspera* (200 mg/mL) has shown an anti-inflammatory activity in the

Abbreviations: 2D-DIGE, two-dimensional DIGE; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CII, bovine type II collagen; CMC-Na, sodium carboxymethylcellulose; DEX, dexamethasone; H&E, hematoxylin and eosin; NMR, nuclear magnetic resonance; OA, osteoarthritis; RA, rheumatoid arthritis; SpA, spondyloarthropathy; TCMs, traditional Chinese medicines; TWH, Tripterygium wilfordii Hook; RA, rheumatoid arthritis; CIA, collagen-induced arthritis; SpA, spondyloarthropathy; OA, osteoarthritis.

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carrageenan-induced hind paw edema model [11]. It has also been reported that using ethanol extracts of *A. aspera* in the same model results in anti-inflammatory and anti-arthritic activities at doses ranging from 100–500 mg/kg [12,13].

There is intense interest in employing proteomics technology to foster a better understanding of disease processes, develop new biomarkers for diagnoses, for use in the early detection of disease, and to accelerate drug development [14]. Recently, this technology has been used in the study of RA biomarkers found in blood and synovial tissue. The proteomes of different synovial tissues between RA, spondyloarthropathy (SpA), and osteoarthritis (OA) were analyzed. Fructose bisphosphate aldolase A and alpha-enolase were candidate biomarkers used to distinguish between SpA and OA. Calgranulin A myeloid related protein 8 (MRP 8) was also markedly higher in RA and SpA patients when compared to OA patients, with the latter having levels below the limits of detection [15].

Mass spectrometric proteomic and protein biochip analyses of synovial fluid and plasma from patients suffering from RA and OA investigated the inflammation profile behind different rheumatic pathologies. Calgranulin B and MRP 8 were specific to the synovial fluid collected from RA patients [16,17]. Serum amyloid A protein was found solely in RA patients' plasma and synovial fluid [16]. A two-step proteomic approach was used to identify biomarkers of disease severity in the synovial fluid and serum of patients with RA. C-reactive protein and calgranulins A, B, and C were identified as potential protein marker candidates for prognosis of the erosive form of RA [18]. A proteomics approach was applied to analyze the proteomic patterns of fibroblast-like synovial cells and peripheral blood mononuclear cells in RA patients. Proteins BiP, colligin, and HC gp-39 were characterized as potential autoantigens in RA [18,19]. Such patterns are useful helping the clinical diagnoses of RA. The proteome of the inflamed paws of mice with collagen-induced arthritis (CIA) showed that ferritin light chain and antioxidant protein increased while lymphoid enhancer binding factor decreased in inflamed paws which provided more detailed understanding of arthritic joints [20]. These results demonstrate the utility of a proteomics approach in understanding a complex disease like arthritis. Importantly, it has further utility in identifying possible molecular markers for future diagnoses.

In this study, active compounds from *A. aspera* root were isolated and identified. The CIA model was used in rats to clarify the pharmacological activity of these isolated compounds. To further establish potential biomarkers found during pathogenesis and therapy, a comparative proteomics analysis of synovial tissue in normal, vehicle control, and *A. aspera* therapy groups was performed using two-dimensional DIGE (2D-DIGE).

2. Materials and methods

2.1. Phytochemical analysis in A. aspera root

Fresh *A. aspera* root was identified by Lin Zhang, an associate professor at the College of Biomedical Engineering and Instrument Science at Zhejiang University. The extraction, isolation, and quantification of saponin protocols are described in detail in the Supplemental Method. All NMR spectra were recorded on a Bruker ARX-500 and ARX-125 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a CH dual 5φ probe. Samples were dissolved in 0.6 mL pyridine-d_5 and transferred into a 5-mm NMR tube. All chemical shifts are expressed as δ (ppm) relative to the internal standard TMS (δ = ppm) and scalar coupling constants are reported in Hz.

2.2. Animals

We used standard and specific pathogen-free (SPF)-Wistar male rats obtained from the Shanghai SLAC Laboratory Animal Co. Ltd. of the Chinese Academy of Sciences (Shanghai, China). All subjects weighed between 180–200 g. The animals were maintained in a temperature-controlled room in a 12 h light–dark cycle with free access to both standard rodent chow and water. All animal care and experimental procedures in accordance with the guidelines and regulations set forth by the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China. All work was approved by the Ethical Committee on Animal Experiments at University Zhejiang, Zhejiang, China (SYXK/Zhe/2007-0098).

2.3. Induction of type II CIA

The rats were immunized with 100 μg bovine type II collagen (CII) (Chondrex, Redmond, WA) in a 1:1 mixture of complete Freund's adjuvant (CFA) to collagen. Subjects received an injection subcutaneously into the base of the tail. A second booster injection was given seven days after the initial injection. The normal group was injected with normal saline at the same volume as experimental subjects.

2.4. Drug treatment and index measurement

The CIA rats were randomly divided into 6 groups of 10 subjects per group. The normal group was injected with normal saline and given 0.5% sodium carboxymethylcellulose (CMC-Na) daily by gavage. The vehicle control group was immunized with CII-CFA and given 0.5% CMC-Na daily by gavage. Drug treatment groups were immunized with CII-CFA and administered by gavage daily with 75, 150, or 300 mg/kg achyranthes extract, 40 mg/kg Tripterygium wilfordii Hook

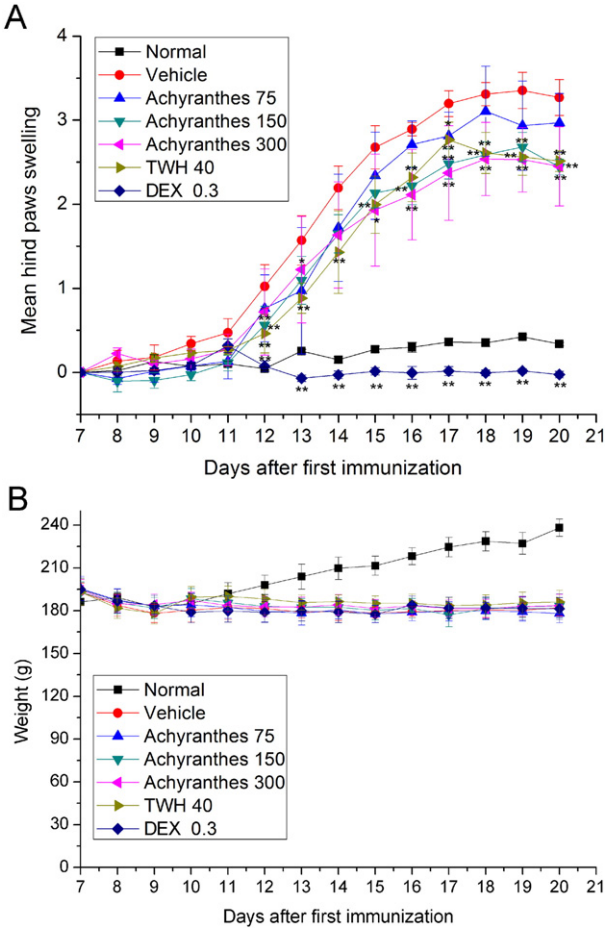

Fig. 1. Measurement of mean hind paw swelling and weight. (A) Mean hind paw swelling changed seven days after the first immunization. Data are expressed as mean ± SD (n = 10). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the vehicle arthritic rats. (B) Weight changes at seven days after first immunization.