**Identification of Candidate Synovial Membrane Biomarkers after *Achyranthes aspera*
Treatment for Rheumatoid Arthritis**

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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, haematoxylin 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

Key words: *Achyranthes aspera*, Saponins, Rheumatoid arthritis, two-dimensional DIGE

ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease whose main symptom is a heightened inflammatory response in synovial tissues. To verify the anti-arthritis 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-glactopyranosyl (1 $\rightarrow$ 2)(O- β -D-glucopyranosyloxy)-oleanolate as main compounds in root of *Achyranthes 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* saponins 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 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* have 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 anti-inflammatory activity in the carrageenan-induced hind paw edema model [11]. It

1 has also been reported that using ethanol extracts of *A. aspera* in the same model results in
2 anti-inflammatory and anti-arthritic activities at doses ranging from 100-500 mg/kg [12, 13].

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

12 Mass spectrometric proteomic and protein biochip analyses of synovial fluid and plasma
13 from patients suffering from RA and OA investigated the inflammation profile behind
14 different rheumatic pathologies. Calgranulin B and MRP 8 were specific to the synovial fluid
15 collected from RA patients [16, 17]. Serum amyloid A protein was found solely in RA
16 patients' plasma and synovial fluid [16]. A two-step proteomic approach was used to identify
17 biomarkers of disease severity in the synovial fluid and serum of patients with RA. C-reactive
18 protein, calgranulin A, B, and C were identified as potential protein marker candidates for
19 prognosis of the erosive form of RA [18]. A proteomics approach was applied to analyze the
20 proteomic patterns of fibroblast-like synovial cells and peripheral blood mononuclear cells in
21 RA patients. Proteins BiP, colligin, HC gp-39 were characterized as potential autoantigens in
22 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 were increase while lymphoid enhancer binding factor decrease 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 saponins 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, Bellerica, MA, USA) equipped with a CH dual 5 ϕ probe. Samples were dissolved in 0.6 mL pyridine-d₅ 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 hrs 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 μ g 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. Normal group was injected with normal saline and given 0.5% sodium carboxymethylcellulose (CMC-Na) daily by gavage. 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

1 Tripterygium wilfordii Hook (TWH), or 0.3 mg/kg dexamethasone (DEX), respectively. Each
2 group was given continuous dosings at seventh days of the first immunization and lasted for
3 14 days in a standard volume of 1 mL/100 g.

4 Amount of hind paw swelling was measured during drug treatment. Volume of swelling
5 was determined using a MK-550 volume meter (Muromachi Kikai Co., Tokyo, Japan) before
6 the initial immunization and repeated every two days thereafter. The paw swelling (Δ mL)
7 was defined as the paw volume after immunization.

8 2.5 Histological Staining

9 Twenty days after the initial immunization, the hind paws of all rats were radio-graphed
10 using a high frequency X-ray camera (Perlove, PLX160A , Nanjing, China) with 40 kV and
11 22 mAs. The radiographs were exported by DRConsole (Perlove, PLX160A , Nanjing,
12 China). After examining the X-rays, rats were sacrificed by diethyl ether asphyxiation and
13 three rats of each group were used for histology examination. Hind paws were fixed in 10%
14 formalin for 24 hrs and then decalcified with RapidCal-Immuno™ (BBC Biochemical,
15 Seattle, WA, USA) for 48 hrs. Tissue samples were dehydrated, paraffin embedded, and
16 sectioned on a microtome (Microm HM-325; MICROM, Walldorf, Germany). A
17 4- μ m paraffin section was stained with haematoxylin and eosin (H&E). The stained slides
18 were viewed using an Olympus BX51 microscope (Tokyo, Japan) and digital high-resolution
19 microphotographs of the samples were captured for further analysis (Olympus DP 10,
20 Olympus, Tokyo, Japan).

21 2.6 Protein Preparation and 2D-DIGE

22 Synovium were dissected for the preparation of protein extraction. Four biological

1 replicates were used to identify the differentially expressed proteins. Equal amounts of normal,
2 vehicle, and achyranthes-treated (300 mg/kg) tissue (100 mg) were quick-frozen in liquid
3 nitrogen and ground-up into a fine powder using a mortar and pestle. All samples were
4 solubilized with DIGE lysis buffer (9 M urea, 4% CHAPS, every 100 mg tissue added 200 μ L
5 buffer) for 1 hr at 30 °C and the resulting solution was centrifuged twice at 15,000 g for 15
6 min to remove insoluble substance. Total protein levels were determined by a Bradford assay.
7 The pH of the extracts was then adjusted to 8.0 with a 50 mM NaOH solution. Each gel was
8 loaded with 50 μ g of protein that had been labeled with 400 pmol of CyDye™ DIGE
9 Fluor minimal dyes (Cy3, Cy5, or Cy2, GE Healthcare, Amersham Biosciences, Uppsala,
10 Sweden). Cy2 was used exclusively to label the internal standard and other samples were
11 labeled by Cy3 and Cy5 shown in Supplementary Table S1. Labeled proteins were kept on ice
12 for 30 min and then quenched with 1 μ L lysine (10 mM).

13 The volume of the samples was then adjusted to 450 μ L with lysis buffer (9 M urea, 4%
14 CHAPS, 1% IPG buffer, 1% DTT) and loaded onto 24 cm, 3-10 NL IPG strips (GE
15 Healthcare). The details of isoelectric focusing and second dimension electrophoresis were
16 described in Supplementary Method. Gels were scanned using a DIGE Imager Scanner
17 (Typhoon 9400, GE Healthcare) at a resolution of 100 μ m. Image analysis was performed and
18 included spot detection, gel overlay, and normalization based on the pooled internal standard.
19 We then quantified using Decyder2D software according to the manufacturer's instructions
20 (v7.0, GE Healthcare). Volumes of the detected spots were normalized by dividing the sum of
21 spot volumes for all detected spots, and then multiplied by 100 to obtain spot percentage
22 (normalized spot volume, vol %). Differences in spot intensities were statistically analyzed

1 using a Student's t-test. Protein spots that had an average of 50% variation ($P < 0.05$) were
2 considered as differentially expressed and chosen for further analysis.

3 2.7 Silver Staining and Protein Identification

4 Gels loaded with 200 μg of protein from each group were subjected to 2D-DIGE as
5 described above and silver stained as previously reported [21]. Images were scanned with an
6 ImageScanner (GE Healthcare) at a resolution of 300 dpi and at 256-gray level.

7 The selected gel spots were manually excised, destained, and washed sequentially with
8 100 μL 25 mM ammonium bicarbonate (NH_4HCO_3), 50% acetonitrile (ACN) in 25 mM
9 ammonium bicarbonate, and 100% ACN. Gels were then vacuum-dried, rehydrated in
10 digestion buffer (0.02 $\mu\text{g}/\mu\text{L}$ trypsin, in 10 % ACN/25 mM NH_4HCO_3), and covered with 20
11 μL 10 % ACN/ NH_4HCO_3 . After 16 h of digestion at 37 °C, the gels were extracted with 50 μL
12 5 % TFA in 67 % ACN. The digested solution and extracts were mixed and freeze-dried.

13 MALDI-TOF/TOF mass spectrometry was used in protein identification. Briefly, dried
14 digests were dissolved in 5 μL 0.1% TFA and mixed with the same volume of matrix solution
15 (α -cyano-4-hydroxycinnamic acid saturated with 50% ACN/1 % TFA). A 1 μL aliquot of this
16 mixture was deposited onto the MALDI target. Subsequent MS analyses were performed on
17 MALDI-TOF/TOF MS system (ABI 5800, Applied Biosystems, Foster city, CA, USA).
18 Peptides were analyzed in positive ion mode. The MS and MS/MS spectra were combined
19 and searched against the NCBI nr database using GPS Explorer (v3.6, Applied Biosystems)
20 and MASCOT (v2.3, Matrix Science). The search parameters were as follows: NCBI nr
21 database (20130413, 24,553,352 sequences, 8,469,922,479 residues), taxonomy of Rattus
22 (68,315 sequences), trypsin digest with one missing cleavage, fixed modifications of

1 carbamidomethyl (C), none fixed modifications of Acetyl (Protein N-term), deamidated (NQ),
2 dioxidation (W), and oxidation (M), MS tolerance of 100 ppm, MS/MS tolerance of 0.3 Da.
3 The protein ID was chosen by the highest score searched in the database and proteins with at
4 least two peptides were satisfied the requirements. Confidence interval of 95% ($P < 0.05$) was
5 set as the significance threshold for mascot protein score.

6 2.8 Immunoblotting

7 In order to verify changes in arthritis- and anti-inflammatory- associated proteins of
8 interest, we performed immunoblotting with antibodies (BIOSS, Beijing, China) against
9 selected proteins in synovium of hind paws. The method of protein extraction was the same as
10 described above. Three biological replicates were used for immunoblotting. The protocols
11 were described in detail in the Supplemental Method.

12 2.9 Statistical Analysis

13 Data were expressed as means $\pm$ standard deviations (SD) and analyzed using SPSS
14 software (version 16.0, Chicago, IL, USA). 2D-DIGE spot abundance was statistically
15 evaluated using Decyder2D software. Differences between experimental groups and vehicle
16 control group or between normal group and vehicle control group were assessed using paired
17 Student's t test. For all tests, $P < 0.05$ was considered significant.

19 3. Results

20 3.1 Identification of Anti-RA Composition in *A. aspera*

21 *Qualitative and quantitative of Important Compounds*

22 Four main compounds were isolated and structures were identified by nuclear magnetic

1 resonance (NMR) spectroscopy (Supplementary Figure S1). The NMR spectral data were
2 described in Supplementary NMR spectral data. ¹³C NMR spectral data of compound 3 and 4
3 are provide in a list in Supplementary Table S2.

4 Compound 1 was obtained as a colorless, needle-shaped crystal (methanol). The NMR
5 spectral data are consistent with CAS database identification of methyl caffeate (CAS
6 No.3843-74-1). Compound 2 was obtained as a white, needle-shaped crystal (methanol).
7 Spectral data are consistent with previous reports as 25-*S*-inokosterone [22]. Compound 3 was
8 obtained as a white powder (methanol). Spectral data are consistent with previous reports of
9 25-*S*-inokosterone β -D-Glucopyranosyl 3-(O- β -D-glucopyranosyloxy)-oleanolate [23].
10 Compound 4 was obtained as a white powder (methanol). Spectral data are consistent with
11 previous reports as β -D-Glucopyranosyl 3-(O- β -D-glactopyranosyl (1 $\rightarrow$ 2)(O- β -D-
12 glucopyranosyloxy)-oleanolate [23].

13 Two of these main compounds accounted for a large proportion of the saponin content.
14 The standards of β -D-Glucopyranosyl 3-(O- β -D- glucopyranosyloxy)-oleanolate (AA-1) and
15 β -D-Glucopyranosyl 3-(O- β -D- glactopyranosyl-(1 $\rightarrow$ 2)- (O- β -D-glucopyranosyloxy)-
16 oleanolate (AA-2) were purified in our lab. According to the calculation method of area
17 normalization, the purity of each AA-1 and AA-2 was more than 98%. These two compounds
18 were carefully weighed and dissolved as control samples in the mobile phase to 0.552 mg/mL
19 and 0.575 mg/mL, respectively. HPLC chromatogram of total saponins (Black), AA-1 (Green),
20 and AA-2 (Blue) are shown in Supplementary Figure S2. AA-1 and AA-2 were measured in
21 four separate total saponin extracts. The average contents were 17.067% (AA-1) and 17.50%
22 (AA-2) (Supplementary Table S3).

1 *Effect of Achyranthes Saponins on CIA in Rats*

2 CIA was induced with primary and booster injections of CII/CFA at an interval of 7 days.
3 Experimental groups began receiving treatment (e.g. *Achyranthes*) beginning on day 8.
4 Arthritis symptoms, such as edema and erythema, appeared ten days after primary
5 immunization. The vehicle control group reached maximum paw swelling 19 days after the
6 first immunization, at which point it gradually diminished (Figure 1A). The two hind paws
7 were not significantly different from each other.

8 The effects of different total saponin doses from *Achyranthes* on the symptoms of CIA
9 were the examined. Our results indicated that medium-dose (150 mg/kg) and high-dose (300
10 mg/kg) of total saponins resulted in a marked reduction on hind paw volume of arthritic rats
11 when compared to vehicle controls (* $P < 0.05$, ** $P < 0.01$), however, Low dose (75 mg/kg)
12 showed no significant change compared to vehicle controls. Compared to healthy rats, the
13 subjects' overall weight in both vehicle and experimental groups was significantly reduced
14 (Figure 1B). Notably, the DEX treatment group subjects had paw volumes that continued to
15 decline, even past vehicle control. The serious emaciation of DEX group indicated critical
16 damage of organism that showed superiority of TWH and *Achyranthes* saponins in keeping
17 weight of rats.

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

1

2 Radiographs were taken to exam the bone degradation in the hind paws of *Achyranthes*
3 saponins-treated arthritic rats. Representative hind paw photos and their corresponding
4 radiographs from normal, vehicle, and experimental rats are shown in Figure 2. These
5 representative photos show the characteristic severe soft tissue swelling and corresponding
6 radiographs reveal bone erosion in the joints of the vehicle arthritic rats when compared to
7 healthy controls. Importantly, a high dose of *Achyranthes* saponins (300 mg/kg) was found to

1 markedly inhibit the soft tissue swelling and bone erosion of arthritic rat joints.

Figure 2. Representative photos and their corresponding radiographs of the hind paws from normal, vehicle, and drug-treated rats at twenty days post-initial immunization. Rats received a daily, oral treatment that contained different doses of *Achyranthes* saponins, TWH, DEX, or vehicle. These treatments began on day 8 after the first immunization. The left hind paw of each group was used for photographs and radiographs.

2

3 A histopathological examination was performed to detect joint inflammation in the hind
 4 paws of *Achyranthes* saponins-treated arthritic rats. Representative histological sections of the
 5 ankle joints from normal, vehicle, and experimental rats are shown in Figure 3. The normal
 6 rat joint presented with sound structural integrity along with a clean joint space and a smooth
 7 articular cartilage surface. There were no detectable indicators of either synovial cell
 8 proliferation or synovial tissue edema (Figure 3A, B and C). In contrast, the synovial joint

1 space of vehicle rat was markedly narrower than normal subjects (Figure 3A). Furthermore,
2 the synovial joint showed varying degrees synovial hyperplasia (marked by black star in
3 Figure 3B), fiber tissue hyperplasia (shown as fibroblast proliferation in Figure 3B and C),
4 and inflammatory cellular infiltration (e.g. neutrophil and monocyte in Figure 3B and C) in
5 vehicle control and other drug treated groups. Pyocyte were observed in the joints of vehicle
6 control, *Achyranthes* 75 and 150 rats which presented severe inflammation. Finally, the joints
7 of vehicle control had obvious cartilage erosion and bone hyperplasia destruction (Figure 3A).
8 Notably, rats treated with high doses of *Achyranthes* saponins (300 mg/kg) had less joint
9 inflammation and synovial hyperplasia.

Figure 3. Histopathological changes of rat joints from normal, vehicle, and drug-treated groups twenty days after the first immunization. Rats received a daily, oral treatment with different doses of saponins, TWH, DEX, or vehicle, beginning on day 8 after the first immunization. The left hind paw of each group was taken to produce the histological sections. (A) The representative H&E stained histopathological sections of the ankle joints from normal, vehicle, three doses of *Achyranthes* saponins, TWH, and DEX arthritic rats are shown (original magnification $\times 40$). The scaleplate

represents 500 μm . (B) Representative H&E stained histopathological sections of joint capsule from normal, vehicle, three doses of *Achyranthes* saponins, TWH, and DEX arthritic rats are shown (original magnification $\times 400$). The scaleplate represents 50 μm . (C) The representative H&E stained histopathological sections of ankle joint articular surface from normal, vehicle, three doses of *Achyranthes* saponins, TWH, and DEX arthritic rats are shown (original magnification $\times 400$). The scaleplate represents 50 μm . Key: AC, articular cartilage; C, cartilage; JS, joint space; S, synovial; T, tibia.

1

2 3.2 Protein Profiling of 2D-DIGE in the Synovium of CIA Rats

3 2D-DIGE was used to understand both the molecular pathogenesis of RA and identify
4 possibly biomarkers resulting from *Achyranthes* saponins therapy. Normal, vehicle, and the
5 high dose of *Achyranthes* (300 mg/kg) groups were chosen for the proteomics study as this
6 dose showed the most anti-arthritis effect. All biological replicates DIGE images were
7 displayed in Supplementary Figure S3. Twenty-eight spots were selected that significantly
8 differential expressed ($P < 0.05$) between normal and vehicle groups , between vehicle and
9 the *Achyranthes* (300 mg/kg) groups, or between the *Achyranthes* (300 mg/kg) groups and
10 normal (Figure 4). Among the differentially expressed spots, 23 proteins were successfully
11 identified by MALDI-TOF/TOF MS. The qualitative analysis showed that 16 proteins were
12 significantly increased and six significantly decreased when comparing vehicle to controls.
13 Seven proteins were significantly increased and 14 significantly decreased in *Achyranthes*
14 experimental group when compared to vehicle. Additionally, 11 proteins were significantly
15 increased and 7 significantly decreased in *Achyranthes* experimental group when compared to

1 normal (Table 1 and Supplementary Table S4). GO annotations terms were extracted by
 2 molecular function, biological process, and cellular component in rats synovial tissues
 3 proteome annotation (Supplementary Figure S4).

Figure 4. The differential protein spots identified by MALDI-TOF/TOF MS. Twenty-five differentially expressed protein spots were marked by their respective number and arrows on the silver stained gel.

4 3.3 Immunoblotting Analysis of RA Pathogenesis and *Achyranthes* Treatment-Related Proteins

7 Immunoblot analysis was used to confirm the expression patterns of two candidate
 8 markers identified from the RA and drug treatment groups, protein disulfide-isomerase A3
 9 (PDIA3) and vimentin, which were changed significantly in vehicle group and recovered to
 10 normal group after drug treatment (Figure 5A). Our statistical analysis on relative expression
 11 indicated that both PDIA3 and vimentin were significantly increased in vehicle rats as
 12 compared to normal rats. Importantly, these two proteins decreased in rats receiving
 13 *Achyranthes* therapy as compared to vehicle (Figure 5B).

Figure 5. Immunoblotting analysis of selected candidate biomarkers for RA pathological process and therapy targets. (A) Representative Western blot of vimentin and protein disulfide-isomerase A3 (PDIA3) in normal, vehicle, and *Achyranthes*-treated groups. (B) Relative expression (mean $\pm$ SD) of vimentin and protein disulfide-isomerase A3 (PDIA3) in normal, vehicle, and *Achyranthes*-treated groups. The actin was used to normalize (** $P < 0.01$).

4. Discussion

A. aspera is one of the ethnomedicines used by the She ethnic minority in China. Its first known record is in “Draft of Materia Medica” (Chinese, “Ben cao tu jing”) during the Song Dynasty of China. As a member of TCMs, it is used in combination with Himalayan teasel root and chaenomeles to treat rheumatoid arthritis. These effects have been recapitulated in rodent models of RA. For instance, extracts from 95% ethanol of *A. aspera* inhibited carrageenan-induced paw edema in a model of Freund’s complete adjuvant-induced arthritis [12]. Quercetin and rhamnoglucoside were considered contributing factors to inhibit the edema produced by carrageenan [13]. Prior work has shown that a triterpene saponin isolated from TCMs application leads to pro-inflammatory cytokine and mediator regulation of RA in rats. Moreover, that TNF- α and COX-2 may be the effective targets in the serum and synovial

1 membrane of CIA rats [24]. A second saponin has also been shown to alter serum levels in
2 CIA-treated mice of anti-CII IgG2a antibody, prostaglandin E2, TNF- α , IL-1 β , and IL-6 [25].
3 However, there has been no work done on the details of what molecular mechanism is
4 responsible for the anti-inflammation and anti-arthritic activities of these saponins. In this
5 study, the total saponins extracted from *A. aspera* were shown to relieve RA symptoms in
6 CIA rats, including ankle joint edema (Figure 2), synovial cell hyperplasia (Figure 3B),
7 inflammatory cells infiltration (Figure 3B and C), and injury of articular cartilage (Figure 3A
8 and C). These results indicate that *A. aspera* is a promising candidate for arthritic therapy.
9 Moreover, that saponin is the effective compound that possesses the anti-inflammation and
10 anti-arthritic activities that have been previously reported.

11 Our histopathological result showed obvious fibroblast hyperplasia and inflammatory
12 cellular infiltration (e.g. neutrophil and monocyte) in joint capsule and articular surface
13 afflicted with RA (Figure 3). Correspondingly, our proteomics result showed that fibrinogen B
14 beta chain and the fibrinogen gamma chain were detected at higher levels in vehicle-treated as
15 compared to normal rats (2.43 and 1.73 times higher, respectively, $P < 0.05$). Moreover, these
16 two proteins decreased 3.95 and 1.71 times, respectively, in the *Achyranthes*-treated when
17 compared to vehicle-treated rats ($P < 0.05$) (Table 1). Fibrinogen has been postulated to be a
18 potent RA inducer and plasminogen has been reported to have opposing effects on paw and
19 knee joints in TNF- α -driven arthritic mice. Importantly, both the exacerbated arthritic
20 severity in the paws joints and the dramatically decreased arthritic symptoms within the knee
21 joints were mechanistically linked to fibrinogen [26]. This past work is further supported by
22 our fibrinogen expression results from the synovial tissue of CIA rats. Furthermore, we

1 speculate that the fibrinogen B beta chain is more sensitive to RA-derived inflammation and
2 also to subsequent saponin therapy.

3 The procollagen type I and type VI, apolipoprotein A-I (apoA-I), and PDIA3 had the
4 same expression pattern as fibrinogen in that they all had significantly increased levels in
5 synovial tissues of vehicle CIA rats. Additionally, they all had significantly decreased levels
6 in the synovial tissue derived from drug-treated rats. The post-translationally modified
7 (citrullinated) fibrinogen is highly capable of inducing arthritis. Prior work has shown that
8 there is IgG variability in response to particular fibrinogen-derived antigens from individual
9 mice [27]. Fibrinogen, collagen, and vimentin can induce anti-cyclic, citrullinated peptide
10 antibodies as autoantigens by rheumatoid factors [28]. Metalloproteinases, an interstitial
11 collagenase, plays a key part in the destruction of joints in rheumatoid arthritis and initiates
12 the degradation of type I and III collagens [29, 30]. The collagen type I alpha 1 increased over
13 two times in the RA synovium of rats (Table 1) implies metalloproteinase inhibition and less
14 destruction of joints in rheumatoid arthritis. It has been reported that apoA-I was up-regulated
15 in infectious bursal disease in virus-infected chicken embryo fibroblasts [31]. Furthermore,
16 that apo A-I was heavily secreted from chondrocytes in subjects' knees that had received
17 surgical depositions of amyloid [32]. Apo A-I has also been considered a valid biomarker for
18 the infliximab response to rheumatoid arthritis [33]. In this study, the promotion of apo A-I in
19 inflamed synovial tissue was presumed to be an autoimmune mechanism in the
20 monocyte-macrophages that had been activated by T lymphocytes. This caused an inhibition
21 of both TNF- α and IL-1 β production [34].

22 Protein disulfide isomerase is a key enzyme that catalyzes the disulfide formation and

1 isomerization in compact folding intermediates [35]. PDIA3 (also called thiol-oxidoreductase
2 ERp57, ER60, or GRP58) is one member of the protein disulfide isomerase superfamily and
3 is essential for the stable assembly of the peptide-loading complex and its export from
4 the endoplasmic reticulum to the cell surface [36]. PDIA3, superoxide dismutase (SOD), and
5 procollagen were robustly up-regulated in chondrocyte-like cells during chondrogenesis *in*
6 *vitro* [37]. According to our 2D DIGE results, both PDIA3 and SOD were both elevated more
7 than 1.5 times in the inflamed synovial tissue compared to normal. Our immunoblotting
8 results further confirmed the significant difference in expression of PDIA3 between
9 vehicle-treated and normal rats. Collectively, these results are in accordance with the
10 histopathology of articular cartilage proliferation in the progression of arthritis.

11 Immune complexes of IgG are mostly in plasma and, less frequently, in synovial fluid.
12 They are often pathogenic and work by activating complement and stimulating cytokine
13 synthesis [38]. In the synovial tissue, IgG-2a was detected at levels 1.7-fold higher in
14 inflamed synovial tissue compared to normal. It is a special autoantigen that did not have any
15 significant change during saponin therapy, thus indicating it as a negative therapy target of
16 *Achyranthes* saponin. The alpha-enolase, a glycolytic enzyme expressed in most tissues, had
17 the most similar expression pattern when compared to IgG. Its increasing expression has been
18 reported in monocytes and macrophages isolated from the synovium of RA patients and the
19 arthritic ankle tissues of mice with collagen-induced arthritis [39]. These past findings are
20 consistent with our current results.

21 Vimentin, a major cytoskeleton component, is detectable in synovium, fibroblast-like
22 synoviocytes, and vascular endothelial lining cells [40, 41]. Vimentin was reported as being

1 highly phosphorylated in tumors that do not overexpress epidermal growth factor receptor
2 tyrosine kinase and that it is associated with tumor growth, invasion, drug resistance, and poor
3 prognosis [42]. As confirmed by western blotting, the vimentin could have positive
4 correlation with inflammation invasion degree and sensitive to *Achyranthes* saponins
5 treatment in the synovium of CIA rats.

6 Alpha-1-antiproteinase (also known as alpha 1-antitrypsin, α AT), is a serpins
7 superfamily member and is considered inhibit a wide variety of proteases [43]. The secretion
8 of α AT has been shown to be associated with most cancer and inflammatory disease *in vitro*
9 and *in vivo*. Increasing levels of α AT in drug-treated rats may be associated with the inhibition
10 of inflammation and a negative correlation of the TNF- α concentration to levels of α 1AT
11 inactivation [44]. The manganese superoxide dismutase (MnSOD) is a mitochondrial matrix
12 protein that serves a protective function from reactive oxygen species. Over-expression of
13 extracellular superoxide dismutase in mouse synovial tissue contributed to the attenuation of
14 the inflammation and inhibition of IL-1 β , TNF- α , and matrix metalloproteinase in
15 fibroblast-like synoviocytes [45]. Moreover, MnSOD was found to decrease in osteoarthritic
16 chondrocytes and as a key factor in redox imbalance in osteoarthritis [46]. However, there
17 have been conflicting reports stating that MnSOD was induced by the chondroitin sulfate
18 treatment in osteoarthritis [47]. In our current results, MnSOD increased after *Achyranthes*
19 saponins treatment which was in line with the latter findings. It implied the protective
20 function of MnSOD from reactive oxygen species could benefit for RA therapy.

21 In the current study, *Achyranthes* saponins have been shown to be the active component
22 in slowing the progression of joint swelling, soft tissue inflammation, and bone erosion in

1 orally-treated, CIA rats. These results indicate that *Achyranthes aspera* is a potential
2 candidate for future clinical trials on RA patients. Alterations in fibrinogen, procollagen, and
3 apoA-I levels indicate pathology and mechanistic association with RA-induced inflammation.
4 Vimentin and PDIA3 could be the candidate biomarkers specific to *Achyranthes* saponins
5 therapy for rheumatoid arthritis in synovial membrane.

6

7 **Acknowledgments**

8 This work was supported by the Zhejiang Provincial Natural Science Foundation of China
9 (Grant No. Y2100765), the National Science Foundation of China (Grant No. U1303122),
10 Zhejiang Province Science and Technology Innovation team (Grant No. 201210044-10).

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Table 1. Differential proteins in synovium of different groups of rats identified by 2D-DIGE proteomics and MALDI-TOF/TOF mass spectrometry.

Spot ID ^a	Protein name	Accession No.	SC ^c (%)	Matched peptide number	M. Score ^d	Theor. Mr/pI ^e	Exper. Mr/pI ^f	Vehicle/Normal		Therapy/Vehicle		Therapy /Normal	
								Fold change ^g	P value	Fold change ^g	P value	Fold change ^g	P value
400	hemopexin precursor	gi 16758014	11	5	127	52000/7.58	91250/5.45	1.95	0.001	-1.29	0.011	1.51	0.010
441	serotransferrin precursor	gi 61556986	9	5	293	78512/7.14	87810/7.89	1.68	0.001	-1.72	0.000	-1.03	0.566
742	Protein disulfide-isomerase A3	gi 1352384	18	8	172	57044/5.88	63807/6.25	1.56	0.007	-2.1	0.009	-1.35	0.118
792	fibrinogen B beta chain	gi 455105	13	6	112	54896/7.89	61725/8.12	2.43	0.000	-3.95	0.000	-1.62	0.002
895	fibrinogen gamma chain	gi 122065184	9	5	94	51228/5.62	58049/5.77	1.73	0.001	-1.71	0.001	1.01	0.929
927	vimentin	gi 14389299	7	3	36	53757/5.06	56977/4.39	1.21	0.030	-1.58	0.014	-1.3	0.028
950	alpha-enolase	gi 158186649	11	4	150	47440/6.16	55793/7.10	1.68	0.000	-1.11	0.038	1.52	0.001

975	IgG-2a protein	gi 1220486	6	3	40	50977/7.52	54833/7.20	1.71	0.000	1	0.995	1.71	0.000
1130	lamin A	gi 453180	8	5	81	71884/6.20	49327/5.43	-1.68	0.001	-1.01	0.934	-1.69	0.001
1207	procollagen, type VI, alpha 3, isoform CRA_a	gi 149037629	3	5	53	240932/5.26	46914/5.16	1.07	0.037	-1.96	0.001	-1.84	0.001
1319	haptoglobin precursor	gi 60097941	14	5	84	39052/6.10	42765/4.82	1.66	0.000	-1.32	0.003	1.26	0.000
1328	haptoglobin precursor	gi 60097941	9	5	69	39052/6.10	42566/5.08	1.65	0.000	-1.17	0.035	1.41	0.000
1357	smooth-muscle alpha tropomyosin	gi 207350	22	6	288	32713/4.74	41623/4.17	-1.69	0.002	-1.55	0.046	-2.62	0.000
1437	procollagen, type 1, alpha 1, isoform CRA_a	gi 149053909	3	2	45	72112/5.95	38853/4.87	2.15	0.000	-1.54	0.001	1.39	0.003
1455	vimentin	gi 14389299	18	7	205	53757/5.06	38430/4.38	1.60	0.003	-1.69	0.018	-1.69	0.775
1568	Alpha-1-antiproteinase	gi 112889	8	3	51	46278/5.70	35102/5.56	1.25	0.045	2.45	0.002	3.07	0.002
1692	carbonic anhydrase 3	gi 31377484	31	6	209	29698/6.89	32155/8.02	-1.93	0.001	1.47	0.002	-1.31	0.014

1794	apolipoprotein A-I	gi 2145143	30	6	258	29869/5.51	30082/5.84	2.21	0.000	-1.74	0.000	1.27	0.003
1826	carbonic anhydrase III	gi 203225	13	4	76	29668/6.49	29524/7.32	-1.81	0.003	2.84	0.002	1.57	0.011
1918	superoxide dismutase [Mn]	gi 8394331	9	2	58	24887/8.96	27431/8.64	2.21	0.042	1.76	0.030	3.9	0.001
2236	mitochondrial ATP synthase, H ⁺ transporting F1 complex beta subunit	gi 89574017	10	2	105	37228/4.97	19717/4.22	-2.42	0.000	2.43	0.000	1.01	0.898
2451	Fatty acid-binding protein, adipocyte	gi 2494405	26	3	96	14813/7.71	16851/6.82	-2.17	0.002	1.34	0.006	-1.62	0.004
2647	hemoglobin, alpha 2	gi 60678292	50	5	227	15446/8.45	13353/8.39	1.04	0.699	2.72	0.003	2.82	0.003

^a Spot ID was created by the Decyder2D software and marked in Figure 4.

^b Sequence coverage.

^c Protein ID is the Mascot results of MALDI-TOF/TOF MS searched from the NCBI database .

^d The Mascot score reported after searching against the NCBI nr database. Protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits in the Mascot Search Results, individual ion scores > 43 indicate identity or extensive homology ($P < 0.05$).

^e Theoretical mass (kDa) and pI of identified proteins were retrieved from the protein database.

^f Experimental mass (kDa) and pI of identified proteins. Experimental values were calculated with Image Master 2D Platinum Software using standard molecular mass markers.

^g Average relative protein abundance change between two groups. Positive value indicates increase and negative value indicates decrease.