

Contents lists available at [ScienceDirect](http://www.sciencedirect.com)

Journal of Nutrition & Intermediary Metabolism

journal homepage: <http://www.jnimonline.com/>

Protective effects of methanolic extract of *Adhatoda vasica* Nees leaf in collagen-induced arthritis by modulation of synovial toll-like receptor-2 expression and release of pro-inflammatory mediators

Rana Adhikary, Arnab Majhi, Sayantika Mahanti, Biswadev Bishayi*

Department of Physiology, Immunology and Microbiology Laboratory, University of Calcutta, University Colleges of Science and Technology, Calcutta, India

ARTICLE INFO

Article history:

Received 14 September 2015

Received in revised form

28 October 2015

Accepted 3 November 2015

Available online 26 November 2015

Keywords:

Adhatoda vasica

Chemokines

Inflammation

Oxidative stress

Rheumatoid arthritis

TLR-2

ABSTRACT

RA associated with oxidative stress and chronic inflammation has been a major health problem among the population worldwide. In this study protective effect of methanolic extract of *Adhatoda vasica* leaf (AVE) was evaluated on Collagen-induced arthritis in male Swiss albino mice. Post oral administration of AVE at 50, 100 and 200 mg/kg body weight doses decreased the arthritic index and footpad swelling. AVE administration diminished pro-inflammatory cytokines in serum and synovial tissues. Reduced chemokines and neutrophil infiltration in synovial tissues after AVE administration dictated its protective effect against RA. Decreased LPO content and SOD activity along with concomitant rise in GSH and CAT activities from liver, spleen and synovial tissues indicated regulation of oxidative stress by AVE. In addition decreased CRP in serum along with suppressed TLR-2 expression in CIA mice after AVE treatment was also observed. Protective effect of AVE in RA is further supported from histopathological studies which showed improvement during bone damage. In conclusion this study demonstrated *A. vasica* is capable of regulating oxidative stress during CIA and therefore down regulated local and systemic release of pro-inflammatory mediators, which might be linked to mechanism of decreasing synovial TLR-2 expression via downregulating release of its regular endogenous ligands like CRP.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Abbreviations: AVE, *Adhatoda vasica* extract; CAT, Catalase; CFA, Complete Freund's adjuvant; CIA, Collagen induced arthritis; COX-2, Cyclooxygenase-2; CRP, C-reactive protein; DTNB, 5, 5'-dithiobis 2-nitrobenzoic acid; ELISA, Enzyme linked immunosorbent assay; FcγRIIa, Fc-gamma receptor IIa; GSH, Reduced glutathione; IC, Immune complexes; IFA, Incomplete Freund's adjuvant; IFN-γ, Interferon gamma; IL, Interleukins; iNOS, Inducible isoform of nitric oxide synthase; IU, International unit; LPO, Lipid peroxides; MCP-1, Monocyte chemotactic protein-1; MPO, Myeloperoxidase; NADPH, Nicotinamide adenine dinucleotide phosphate; NF-κB, Nuclear factor -κB; NO, Nitric oxide; NSAIDS, Non-steroidal anti-inflammatory drugs; OD, Optical density; PBS, Phosphate buffer saline; PMN, Polymorphonuclear neutrophil; PVDF, Polyvinylidene difluorides; RA, Rheumatoid arthritis; RF, Rheumatoid factors; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; SAA, Serum amyloid A; SGOT, Serum glutamate oxaloacetate transferase; SGPT, Serum glutamate pyruvate transferase; SLE, Systemic lupus erythematosus; SOD, Superoxide dismutase; TBARS, Thiobarbituric acid reactive substrates; TCA, Trichloro acetic acid; TLR, Toll like receptor; TNF-α, Tumor necrosis factor - alpha; Tris-EDTA-HCl, Tris ethylene diamine hydrochloric acid.

* Corresponding author. Department of Physiology, Immunology and Microbiology Laboratory, University of Calcutta, University Colleges of Science and Technology, 92 APC Road, Kolkata 700 009, West Bengal, India.

E-mail address: biswadevbishayi4@gmail.com (B. Bishayi).

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease affecting about 1–2% of the world's population [1], characterized by systemic and irreversible destruction of the cartilage, tendons and bone initiated upon deposition of autoimmune complexes into synovial joint spaces. However the factors responsible for development of RA are not clear, genetic polymorphisms and environmental factors were mentioned to contribute in RA pathogenesis [2]. Chronic inflammation during RA is associated with severe oxidative stress and inflammatory reactions into joints and other tissues [3], therefore agents with anti-inflammatory and anti-oxidant potentials will possess obvious means of their use against RA [4].

Existing treatment options in RA includes targeting of different molecules on B-cells, e.g. rituximab, ocrelizumab and veltuzumab; targeting cytokines by means of specific monoclonal antibodies, but many of them has been banned for their disadvantageous effects [5]. A number of different anti-inflammatory drugs have also been introduced such as naproxen, salsalate, tolmetin and NSAIDS, blockers of COX-2, which also possess potential side effects [6]. Antioxidant supplement has been suggested as alternate

therapeutic approach that possesses lesser risk has been mentioned in many studies [7,8].

Application of natural products has been potential remedies in treatment of RA in the old practice of traditional medicinal system. *A. vasica* Nees, the acanthaceae is a medicinal herb that is being used in indigenous system for several hundred years and is considered as an official drug in Pharmacopoeia, India, 1994. Antioxidant properties in different parts of this plant have been well documented in a number of studies [9]. Anti-inflammatory potential of pyrroloquinazoline alkaloids from this plant has been shown against adjuvant-induced experimental arthritis in mice [10]. Since RA is a chronic inflammatory disease, associated with oxidative stress and inflammatory reactions initiated by a complex network of signaling, treatment with a natural extract containing an array of important bio-active molecules will obviously possess beneficial effect over the use of any isolated and purified compound.

Involvement of the components of innate immune system always have been interesting in the area of rheumatology and were investigated for their roles in rheumatoid arthritogenesis [11,12]. Toll like receptors (TLRs) are molecules of innate immune system, present on the surfaces of macrophages and dendritic cells that are critical for generation of B and T lymphocyte responses under conditions of pathogenic invasion [13]. In recent years, studies on the involvement of TLR-2 in many auto-immune diseases like SLE (Systemic lupus erythematosus) and RA has implicated crucial role of many TLRs in pathogenesis of these autoimmune diseases [14]. Among eleven different subtypes, expression of TLRs, -2, -3, -4 and -7 have been found in RA synovium from mice, indicating their possible involvement in pathogenesis of RA [15,16]. Apart from the microbial products which are conventional ligands for TLR-2, there are different endogenous molecules expressed within cells at the time of chronic inflammation under conditions of stress, apoptosis and tissue necrosis e.g., heat shock proteins -60 and -70, fibrinogen, fibronectin, hyaluronic acid, tenascin-C. Therefore whether AVE affects the TLR-2 expression in synovial tissues was an obvious question.

The aim of this study was to evaluate anti-arthritis potential of *A. vasica* against auto-immune arthritis in Swiss albino mice, using collagen-induced arthritis (CIA) model. Effect of the methanol extract of *A. vasica* in inflammatory response during CIA was studied by measuring CRP, different cytokines in serum and synovial tissue and by assessment of neutrophil accumulation in spleen and synovial tissues. Level of GSH, TBARS, NO and enzymatic activities of SOD and CAT in liver, synovial tissue and spleen was determined to assess the effect of the *A. vasica* extract in neutralizing oxidative stress during CIA. Immunoblotting experiment has been performed to determine whether AVE has any impact on the changes in TLR-2 expression in synovial tissue during CIA.

2. Materials and methods

2.1. Plant collection and preparation of extract

Whole *A. vasica* plants were collected from Singur in West Bengal, India. Botanical identification of the samples was confirmed taxonomically by The Botanical Survey of India, Ministry of Environment and Forest, Govt. of India, [Ref No. CNH/50/2014/Tech.II/103]. 10 g of dried, finely ground leaves were soaked into 30 ml of 70% methanol at 30 °C for 12 h with shaking and the methanol was then allowed to evaporate completely (under sterile conditions) and was filtered with Whatman's #1 filter paper. It was repeated thrice and finally the filtrate was centrifuged at 2000 rpm for 10 min. The supernatant collected was treated with charcoal for decolorization and was then air-dried to completeness under

sterile conditions. The yield was 7.1% (7.1 g powdered extract/100 g dried finely-ground leaf). The powdered form of the extract was dissolved into sterile PBS.

2.2. Experimental animals+

Male Swiss-Albino mice (20–22 g, 3–4 weeks of age) obtained from the Chittaranjan National Cancer Institute, Kolkata, India were used in this study. All the animals were housed in separate polystyrene cages in pathogen-free facilities maintained at 25 [±2] °C, with 50–60% relative humidity, and 12 h light: dark cycle. All mice had *ad libitum* access to normal laboratory diet (NLD) that consisted of 22.5% wheat flour, 60.0% roasted Bengal-gram flour, 5.0% skimmed milk powder, 4.0% casein, 4.0% refined groundnut oil, 4.0% salt mixture and 0.5% vitamin mixture, as recommended for mice, by the National Center for Laboratory Animal Sciences, National Institute of Nutrition, India and filtered tap water. All experiments involving animals were conducted according to the protocols approved by Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of CPCSEA [Approval# IAEC/IV/Proposal/BB-2/2014, dated 26-08-2014], Ministry of Environment and Forest, Government of India. The animals were divided into six experimental groups, each of which containing six mice (n = 36), i.e., NLD fed control group, AVE (100 mg/kg) fed group, CIA group, CIA + AVE (50 mg/kg) fed group, CIA + AVE (100 mg/kg) fed group and CIA + AVE (200 mg/kg) fed group.

2.3. Dosage of plant extracts

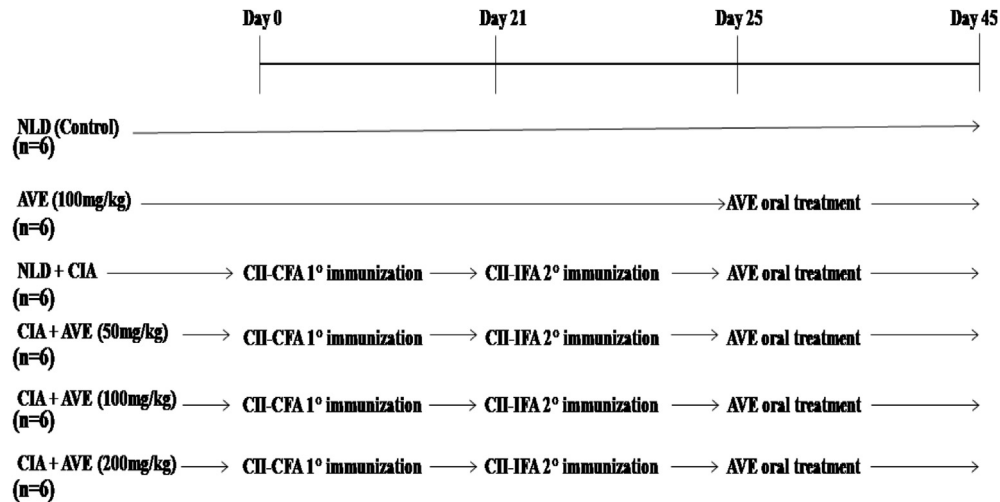
The doses of plant extract used in this study were determined from oral acute toxicity study. AVE at 500, 750, 1000, 1500, 2000, or 2500 mg/kg body weight doses were administered to mice *p.o.* Doses up to 2000 mg/kg did not cause death or behavioral changes upto 72 h. According to earlier studies 1/20th of this dose, i.e., 100 mg/kg, was considered as the safe dose for this study [17]. To bracket this value, doses of 50 and 200 mg/kg were also selected as the dose regimen in this study.

2.4. Preparation of type II collagen emulsion for immunization

Lyophilized bovine type II collagen (Sigma, St. Louis, MO) was dissolved in 0.05 M acetic acid solution at 2 mg/ml concentration. An equal volume of Freund's complete adjuvant was then added and the solution was emulsified slowly [18]. The booster dose was prepared by emulsifying type II collagen with equal volume of Freund's incomplete adjuvant.

2.5. Immunization and treatment

Mice were immunized subcutaneously at the base of the tail with 100 µl of type II collagen-CFA emulsion, and all the mice were provided normal laboratory diet for 20 days. At day 21 after the primary immunization, the mice were again immunized with 100 µl of type II collagen in IFA [18]. The mice were then carefully monitored for onset of early signs of arthritis, i.e., redness/deformities/swelling in the joints and/or toes, etc. After the secondary immunization AVE was administered *per orum* daily in a volume of 200 µl (animals from the control group received only sterile PBS) from the date of onset of first signs of arthritis (i.e., day 25 after primary immunization), routinely up to day 41 of the experiment (i.e., 20 days after secondary immunization). On day 45 after the primary immunization, all mice were euthanized using ether. The precise protocol by which animal experiment was conducted including administration of CIA, treatment with AVE and the whole



duration of the experiment was given below:

2.6. Assessment of arthritic scores and joint swelling

Mice were monitored for signs of arthritis for which severity scores were derived as: 0 = no signs of arthritis; 1 = swelling/redness in only one joint; 2 = swelling/redness in more than one joint; 3 = swelling/redness in entire paw; 4 = severe swelling of entire paw with deformity and/or ankylosis. The macroscopic arthritic score of each mouse was presented as the sum of each score of the four limbs, with the maximum score being 16 for all four limbs. Footpad swelling was evaluated by measuring the thickness of the two hind paws with an electric digital caliper every other day beginning on day 21 after primary immunization (i.e. day 4 after secondary booster immunization). On day 45 after the first immunization, all mice were euthanized for analysis.

2.7. Collection of blood and tissue samples

Immediately after being euthanized, blood samples were obtained from each mouse by cardiac puncture. The blood samples were then placed at 4 °C for 45 min and then centrifuged at 3000 rpm for 5 min at 4 °C. The serum was collected as the pale white yellow colored supernatant, aliquots were assessed for total protein levels using Bradford's method [19], and the remaining aliquots were stored at –20 °C for later analysis. Liver, spleen and synovial joints from the hind and fore limbs were then collected from each animal and stored at –20 °C for use in later experiments.

2.8. Histopathological analysis of synovial joints

Histopathological analyses of the isolated synovial joints were carried out [20]. Knee joints isolated at necropsy were fixed immediately for 2 h in 4% paraformaldehyde and then de-calcified with 10% EDTA at 4 °C. The samples were then embedded in paraffin, sections were prepared (at 6.0 μm thickness), and the tissues were then stained with hematoxylin and eosin prior to analysis using a light microscope.

2.9. Serum glutamate oxaloacetate transferase (SGOT) and glutamate pyruvate transferase (SGPT) activity measures

An aliquot (100 μl) of serum was mixed with 0.5 ml SGOT and

SGPT substrates and incubated with serum and were allowed to react with 2,4-DNPH solution and another incubation were followed by addition of 0.4 (N) NaOH solution [21]. The intensity of the developed color was read at 540 nm. The SGOT and SGPT activities were expressed in IU/ml of serum.

2.10. Assessment of serum CRP level

Concentrations of CRP in serum were determined using a commercial sandwich ELISA kit (My Biosource, San Diego, CA) according to manufacturer instructions, with final absorbance values being measured using a microplate reader (BioRad, Hercules, CA). Values of CRP in samples were extrapolated from a standard curve prepared in parallel. All values were reported as ng CRP/ml serum. The level of sensitivity of the kit was 2000 ng CRP/ml.

2.11. Assessment of TNF-α, IFN-γ, IL-1β, IL-6, IL-12, and IL-10 from serum and synovial joints

Synovial joints were lysed in lysis buffer (containing 300 mM NaCl, 15 mM Tris [pH 7.4], 2 mM MgCl₂, 2 mM Triton X-100, 20 ng pepstatin A/ml, 20 ng leupeptin/ml, and 20 ng aprotinin/ml) and then centrifuged at 2900 rpm for 15 min at 4 °C; the supernatants were kept at –20 °C until use. The homogenates and serum were normalized for protein content by Bradford's method. Concentrations of pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-6, and IL-12, as well as of anti-inflammatory IL-10, were determined using commercial kits (RayBiotech, Inc., Norcross, GA) according to manufacturer instructions. Levels for each cytokine in a sample were extrapolated from standard curves prepared in parallel. All values were reported as ng cytokine/ml serum. The levels of sensitivity of the kits were 60 pg TNFα, 5 pg IFNγ, 5 pg IL-1β, 2 pg IL-6, 5 pg IL-12, and 45 pg IL-10 respectively.

2.12. Assessment of chemokines like IL-8 and MCP-1 in synovial joints

Concentrations of IL-8 (My Bio Source) and MCP-1 (Ray Biotech Inc) were determined from synovial joint homogenates using commercial ELISA kits. The experiments were performed according to the manufacturer's instructions. The levels of sensitivity of these kits were 2 ng IL-8, and 3 pg MCP-1 respectively.

2.13. Determination of myeloperoxidase (MPO) activity in spleen and synovial joints

MPO activity was analyzed to reflect neutrophil (PMN) infiltration into tissues, as it is closely related to the number of PMN present. Spleen and synovial tissues isolated from mouse after sacrifice were homogenized with 20 mM Tris–EDTA–HCl (pH 7.0) buffer supplemented with sucrose and protease inhibitor cocktail. Homogenate was mixed with 10 mM potassium phosphate buffer, *o*-dianisidine dihydrochloride (0.167 mg/ml) and 0.005% H₂O₂; MPO activity was measured at 405 nm [22].

2.14. Assessment of anti-oxidant status in different tissues

Liver, spleen, and synovial tissues were separately homogenized on ice for 30 s using a Polytron homogenizer with ice cold 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 9000 rpm for 20 min at 4 °C, supernatants were collected and aliquots were used to assess the total protein content by Bradford method and anti-oxidant status. GSH was estimated as acid soluble sulfhydryl content, by its reaction with DTNB (Ellman's reagent) in μ moles/mg of tissue protein [23]. The tissue contents of lipid peroxidation products in tissue homogenate were determined as the generation of thio-barbituric reactive substances in n moles/mg of tissue protein [24]. SOD enzyme activity in tissue homogenate was measured as the amount of enzyme capable inhibiting 50% oxidation of pyrogallol [25]. CAT enzyme activity in tissue homogenate was measured as the decomposition of H₂O₂ in μ moles/min/mg of tissue protein [25].

2.15. Assessment of NO production in synovial joints

Nitrite concentrations in paw tissues were measured as an index reflecting local NO production. Synovial tissues were homogenized into ice cold 1 ml sterile PBS. After centrifugation at 12,000 rpm for 30 min at 4 °C, supernatants were collected and analyzed for NO production using a modified Griess method, as described earlier [26]. The amounts of NO produced were determined by extrapolation from a standard curve prepared in parallel using sodium nitrite.

2.16. Immunoblot analysis for TLR-2 expression in synovial tissue

Synovial joints isolated during euthanasia from different groups of mice were lysed with RIPA buffer, supplemented with Nonidet P-40. 60 μ g of tissue lysate was removed, denatured at 100 °C for 5 min, resolved by 10% SDS-PAGE, and then electro-transferred onto nitrocellulose membranes. After blocking for 2 h at 4 °C in TBST (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20), containing 5% BSA, the membrane was washed thrice in TBST and probed overnight at 4 °C with anti-mouse-TLR-2 antibody (1:400 dilution; Biorbyt Ltd., Cambridgeshire, UK) in TBST, supplemented with 1% BSA. Blots were then washed thrice in TBST, incubated for 2 h at 4 °C with appropriate HRP-conjugated secondary antibodies (1:5000 dilution; Biorbyt Ltd. UK). The final signal was developed using Super Signal-chemiluminescent-substrate (Thermo Scientific, USA). Each blot was then exposed to X-Omat BT films (Kodak, Windsor, CO) and bands were quantified using QUANTITY ONE software (Bio-Rad, Inc., Hercules, CA).

2.17. Statistical analysis

All data are reported as mean \pm SD. Assessment of significant differences between groups were performed using a 1-way Analysis of Variance (ANOVA). A *p*-value < 0.05 was considered

significant. A Scheffe's *F*-test *post-hoc* test for multiple comparisons of the different groups was done when significant *p*-values were obtained.

3. Results

3.1. Macroscopic arthritic scoring and footpad swelling in CIA mice treated with AVE

Arthritis was induced in mice \approx 3 weeks after primary immunization with collagen II-CFA, i.e. 4 days after booster immunization with collagen II-IFA. In the course of treatment with AVE, it was observed that daily administration of the three doses of AVE, i.e., 50, 100, or 200 mg/kg, significantly inhibited arthritic progression (Fig. 1A and B). Maximal inhibition in arthritic scoring and footpad swelling was observed at the day 12 after booster immunization in the different groups of mice.

3.2. Histopathological analysis of knee joints in CIA mice treated with AVE

Hematoxylin-eosin staining of arthritic joint sections isolated from the mice in the various experimental groups revealed detectable degradation in the joint synovial tissues, inflammation, PMN accumulation and necrosis after collagen administration (Fig. 2). These indices of tissue damage appeared to be attenuated by AVE treatment at a dose of 100 mg/kg in CIA induced mice.

3.3. Effect of AVE on SGOT and SGPT concentrations

Administration of collagen II significantly increased levels of SGOT and SGPT, the markers of bone erosion, in the serum in case of CIA mice as compared to the NLD-fed control and AVE (100 mg/kg)-fed mice. All the three doses of AVE significantly reduced the SGOT and SGPT (Table 1) and suggesting an ability of the extract to mitigate bone erosion at times of arthritic progression. The reduction in the serum concentration of these enzymes was maximum with the 200 mg/kg AVE oral administration in CIA mice.

3.4. Effect of AVE on serum CRP levels

A significant increase in serum concentrations of C-reactive protein (CRP) in the CIA mice challenged with type II collagen emulsion was observed (indicating acute tissue damage) in comparison to outcomes noted in the NLD-fed control and AVE (100 mg/kg)-fed mice (Table 2). Administration of the three different doses of AVE from the day of onset of arthritic symptoms significantly reduced the concentration of this acute phase protein in serum from CIA mice.

3.5. Effect of AVE on serum cytokine concentrations

The concentrations of pro-inflammatory TNF α , IL-1 β , IFN γ , IL-6, and IL-12 were elevated in the serum of the mice challenged with type II collagen (CIA group) as compared to the NLD and AVE-fed groups. Treatment with AVE in CIA induced mice significantly decreased these cytokines in serum (Fig. 3A–E). On the other hand, the concentrations of IL-10 (Fig. 3F), a major anti-inflammatory cytokine, was reduced in CIA mice as compared to the NLD and AVE-fed mice. Treatment with AVE have significantly restored IL-10 level in serum from CIA mice.

3.6. Effect of AVE on synovial cytokine/chemokine concentrations

Levels of pro-inflammatory TNF α , IL-1 β , IFN γ , IL-6, and IL-12 in

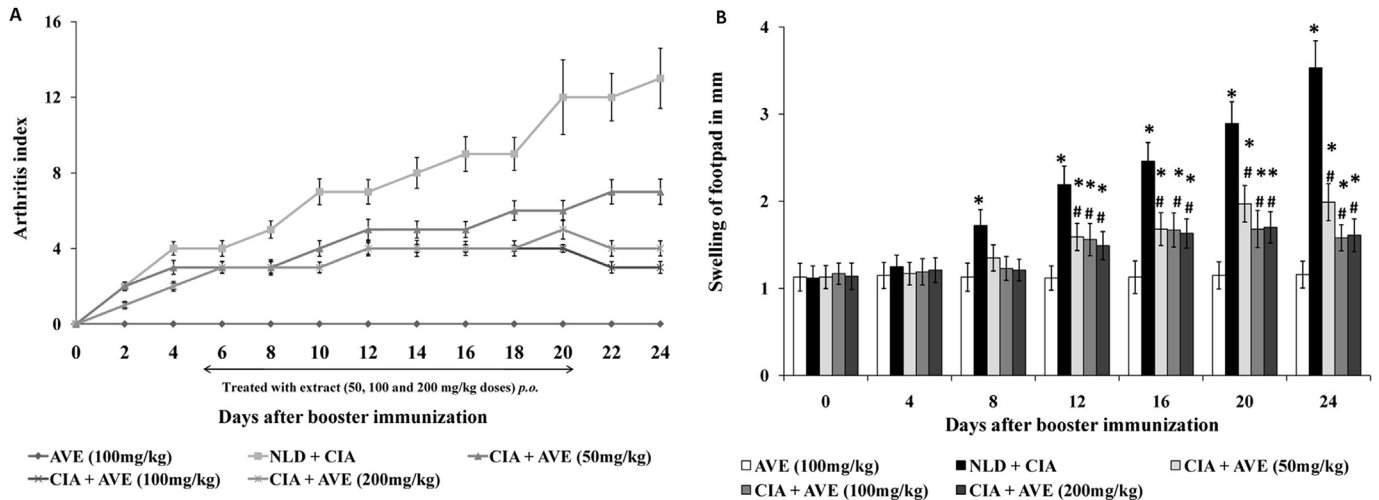


Fig. 1. Macroscopic arthritic scoring (A) and footpad swelling (B) in mice in the different treatment groups. The results were obtained from three repeated experiments; values are expressed as mean \pm SD (n = 6/group). *indicates significant difference in comparison to AVE (100 mg/kg)-fed mice ($p < 0.05$); #indicates significant difference in comparison to CIA mice ($p < 0.05$).

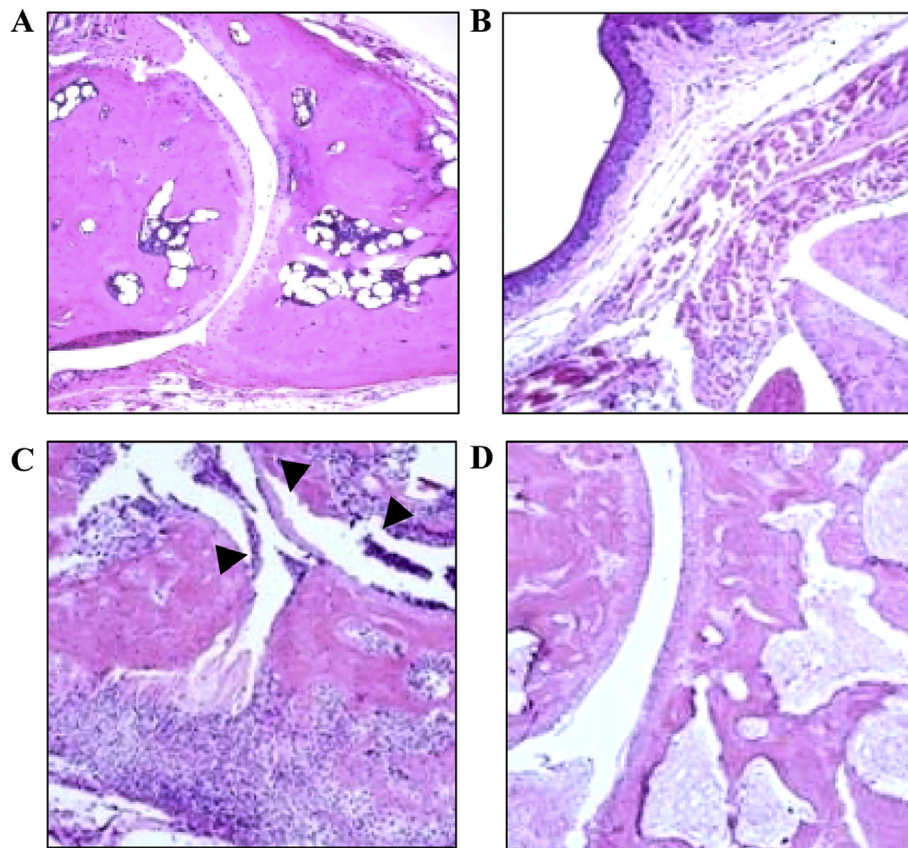


Fig. 2. Histopathological analysis of knee joints. Representative photographs of H&E-stained sections (magnification 100X) from knee joints of (A) control mice, (B) AVE (100 mg/kg)-only mice, (C) CIA mice, and (D) CIA mice treated with AVE (100 mg/kg). Cellular accumulation and epithelial damage is indicated in photographs. Arrows indicate damage in synovial lining and epithelia.

synovial tissue homogenates were also increased in the CIA mice in comparison to NLD and AVE-fed counterparts. The CIA mice also had suppressed synovial IL-10 levels as compared to other groups. Treatment with AVE significantly suppressed the increases in local concentration of the pro-inflammatory cytokines in synovial tissues (Fig. 4A–E), and significantly increased the level of IL-10

(Fig. 4F). In addition, CIA mice showed a significant elevation in tissue levels of IL-8 and MCP-1, two important chemokines in the joint synovium. A marked decrease in levels of these chemokines was also observed with treatment of AVE (Fig. 4G and H) in CIA mice.

Table 1
Serum concentration of SGOT (in IU/ml) and SGPT (in IU/ml).

Sl. No.	Groups	SGOT in IU/ml (mean \pm SD)	SGPT in IU/ml (mean \pm SD)
1.	NLD (control)	49.27 \pm 6.29	19.28 \pm 3.13
2.	AVE (100 mg/kg)	47.29 \pm 7.12	21.18 \pm 3.73
3.	NLD + CIA	101.92 \pm 15.29*#	58.27 \pm 9.21*#
4.	CIA + AVE (50 mg/kg)	69.21 \pm 9.87*#	34.19 \pm 5.17*#
5.	CIA + AVE (100 mg/kg)	59.27 \pm 8.29 [^]	23.31 \pm 3.22 [^]
6.	CIA + AVE (200 mg/kg)	53.19 \pm 7.33 [^]	20.19 \pm 2.98 [^]

Effects of AVE on SGOT and SGPT levels. Changes in the SGOT and SGPT activities in serum from the mice of all the six groups. The results were reproduced in three repeated experiments. Values are expressed as means \pm SD of six mice per group. P value less than 0.05 was considered as significant. *indicates significant (P < 0.05) difference in comparison to NLD (control), #indicates significant difference (P < 0.05) in comparison to AVE (100 mg/kg) fed mice and [^]indicates significant difference (P < 0.05) in comparison to CIA induced mice.

Table 2
Serum concentration of C-reactive protein (in ng/ml).

Sl. No.	Groups	Concentration of serum CRP in ng/ml (mean \pm SD)
1.	NLD (control)	124.26 \pm 13.91
2.	AVE (100 mg/kg)	119.32 \pm 12.34
3.	NLD + CIA	141.71 \pm 18.94*#
4.	CIA + AVE (50 mg/kg)	132.56 \pm 21.46 [^]
5.	CIA + AVE (100 mg/kg)	127.81 \pm 18.57 [^]
6.	CIA + AVE (200 mg/kg)	122.16 \pm 19.73 [^]

Effects of AVE on serum CRP levels. Concentrations of serum CRP (in ng/ml) measured from all the six groups using ELISA method. The results were reproduced in three repeated experiments. Values were expressed as means \pm SD of six mice per group. P value less than 0.05 was considered as significant. *indicates significant (P < 0.05) difference in comparison to NLD (control), #indicates significant difference (P < 0.05) in comparison to AVE (100 mg/kg) fed mice and [^]indicates significant difference (P < 0.05) in comparison to CIA induced mice.

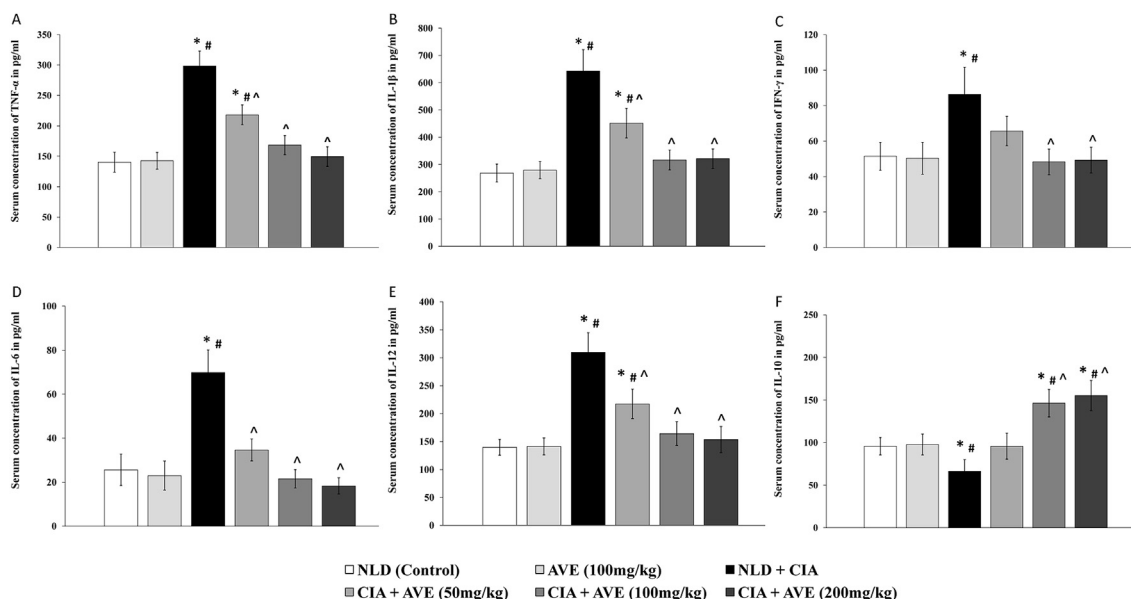


Fig. 3. Serum cytokine concentrations in mice. Levels of (A) TNF α , (B) IL-1 β , (C) IFN γ , (D) IL-6, (E) IL-12, and (F) IL-10 in serum from mice as determined by ELISA. Results were reproduced in three experiments. The results are obtained from three repeated experiments and the values are expressed as mean \pm SD; (n = 6/group). *indicates significant difference in comparison to NLD (control) (p < 0.05); #indicates significant difference in comparison to AVE (100 mg/kg)-fed mice (p < 0.05); and, [^]indicates significant difference in comparison to CIA mice (p < 0.05).

3.7. Effect of AVE on MPO activity in spleen and synovial tissue

Myeloperoxidase activity (MPO; measure of neutrophil activation in/infiltration into tissues) was assessed in the spleen and synovial tissues. The results indicated significant elevation of MPO in both tissues in the CIA mice compared to counterpart tissues from NLD and AVE-fed groups. Treatment with AVE significantly reduced MPO activity in both tissues from CIA mice, suggesting an inhibitory effect of AVE on tissue neutrophil accumulation (Fig. 5A and B).

3.8. Effect of AVE on anti-oxidant status in hepatic, splenic and synovial tissues

GSH content as well as CAT activities were significantly decreased in the liver, spleen and synovial tissue in CIA mice, along with concomitant increase in LPO content and SOD enzyme activities. Treatment with AVE at the different doses restored GSH levels, as well as CAT activities nearly those in the controls, indicating that AVE have potentially reduced ROS-mediated oxidation of

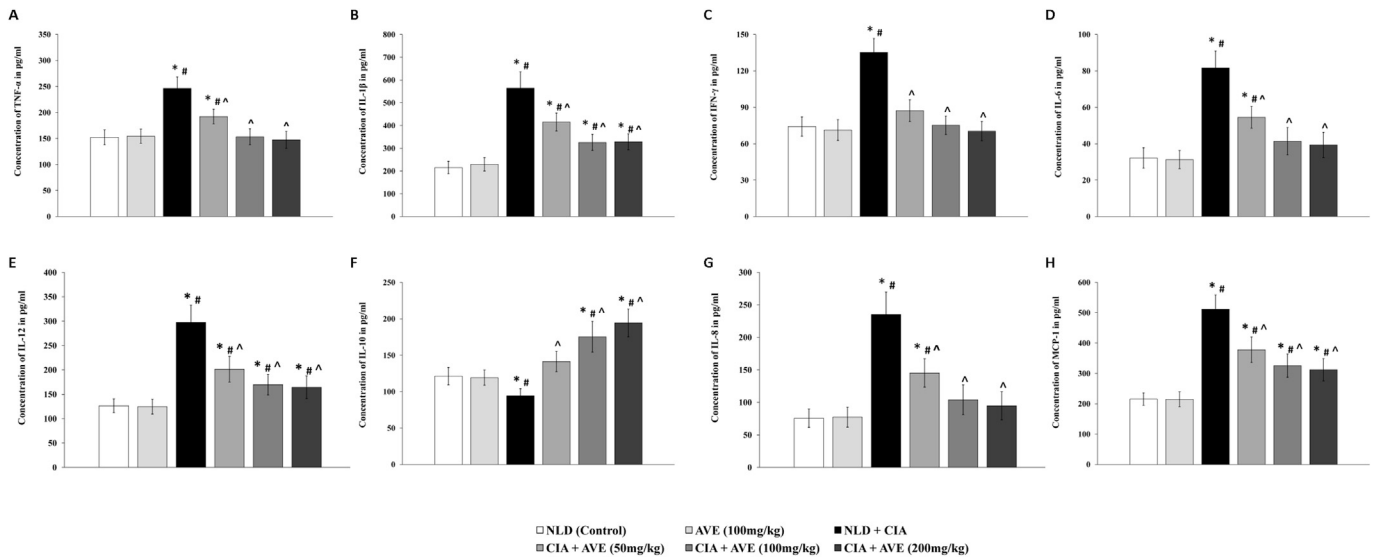


Fig. 4. Synovial cytokine and chemokine concentrations. Levels of (A) TNF α , (B) IL-1 β , (C) IFN γ , (D) IL-6, (E) IL-12, (F) IL-10, (G) IL-8, and (H) MCP-1 in synovial tissues from mice as determined by ELISA. Results were reproduced in three experiments. The results are obtained from three repeated experiments and the values are expressed as mean \pm SD; (n = 6/group). *indicates significant difference in comparison to NLD (control) ($p < 0.05$); #indicates significant difference in comparison to AVE (100 mg/kg)-fed mice ($p < 0.05$); and ^ indicates significant difference in comparison to CIA mice ($p < 0.05$).

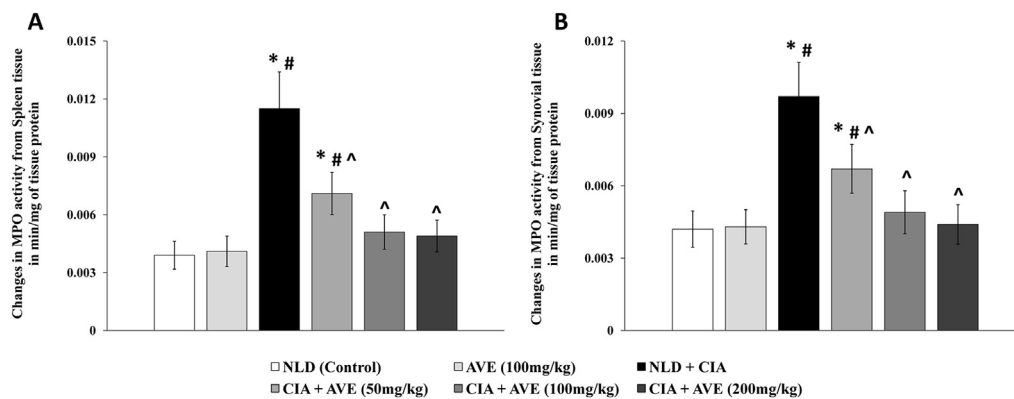


Fig. 5. MPO activity in spleen and synovial tissue. MPO activity was analyzed as an index of neutrophil infiltration into the (A) spleen and (B) synovial tissues. The results are obtained from three repeated experiments and the values are expressed as mean \pm SD; (n = 6/group). *indicates significant difference in comparison to NLD (control) ($p < 0.05$); #indicates significant difference in comparison to AVE (100 mg/kg)-fed mice ($p < 0.05$); and ^ indicates significant difference in comparison to CIA mice ($p < 0.05$).

glutathione and ROS generation in those tissues. AVE treatment also have attenuated the LPO content and SOD activities in liver, spleen and synovial tissues significantly (Figs. 6–8) in CIA mice.

3.9. Effect of AVE on NO production in synovial tissues

NO production in the synovial tissues in mice with AVE treatment was significantly reduced in CIA mice (Table 3), indicating a protective role of the AVE during chronic inflammation in synovial tissue.

3.10. AVE attenuated TLR-2 expression in synovial joints of CIA mice

Elevated TLR-2 expression in synovial tissues was observed in CIA mice (Fig. 9, Panel A [lane 3] and Panel B [lane 3]). AVE (100 mg/kg) inhibited this increase in TLR-2 expression in the CIA mice (Fig. 9, Panel A [lane 4]). AVE treatment at this level did not induce any changes in control animals (Fig. 9, Panel A [lane 2] and Panel B [lane 2]).

4. Discussion

RA in mice is almost exactly mimicked by collagen-induced arthritis, with neovascularization and pannus formation leading to synovitis type of inflammation [27], instead of the basic difference in initiation of pathogenesis, i.e. in case of RA the individual develops antibody against citrullinated peptides of vimentin and fibronectin, whereas in case of CIA, the antibody response is driven against type II collagen, a component of diarthrodial joint. In the present study we have shown that oral administration of AVE to mice provided protection against the progression of auto-immune arthritis as indicated from the results of arthritic scoring, following the booster injection with collagen-IFA which was observable from the day 12 after booster immunization. Histopathological study of joints also supported the fact that *A. vasica* provided significant resistance against bone damage during chronic inflammatory reactions, the fact further supported by attenuation of SGOT and SGPT by AVE, the clinical markers for arthritis.

Collagen type II in emulsion with CFA activates both the cell-mediated and humoral arms of the immune system that depends

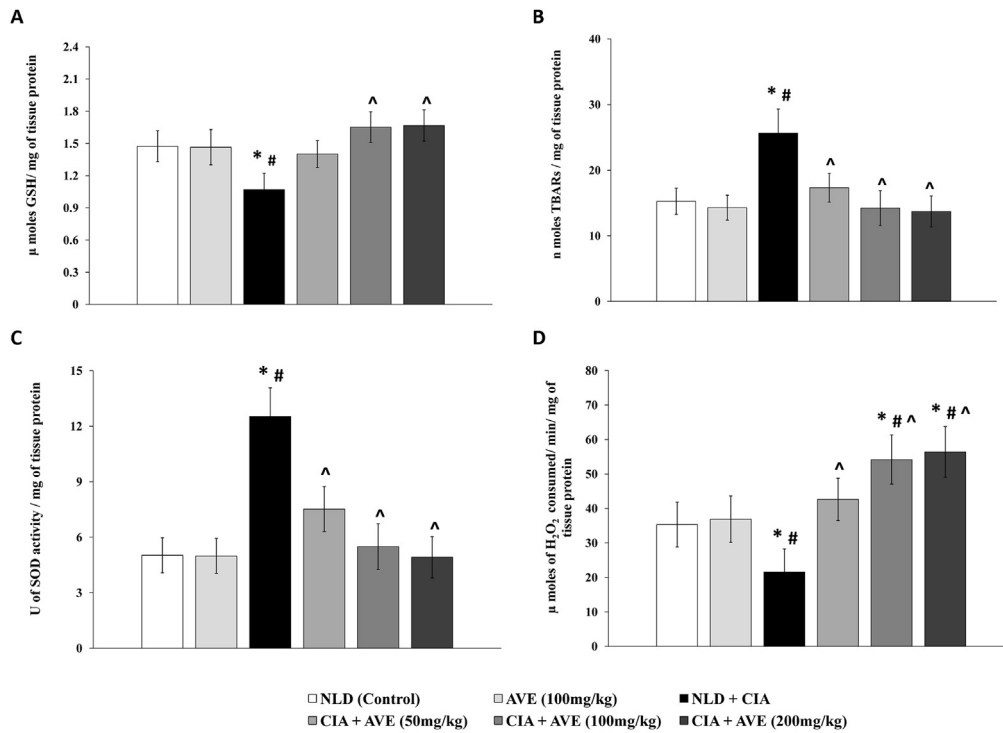


Fig. 6. Anti-oxidant status in liver tissues. Levels of (A) GSH (in μ mole/mg tissue protein), (B) LPO (in nmole/mg tissue protein), (C) SOD (U/mg tissue protein) and (D) catalase activity (in μ mole H_2O_2 consumed/min/mg tissue protein) in hepatic tissues of mice. The results are obtained from three repeated experiments and the values are expressed as mean \pm SD; (n = 6/group). *indicates significant difference in comparison to NLD (control) ($p < 0.05$); #indicates significant difference in comparison to AVE (100 mg/kg)-fed mice ($p < 0.05$); and, ^indicates significant difference in comparison to CIA mice ($p < 0.05$).

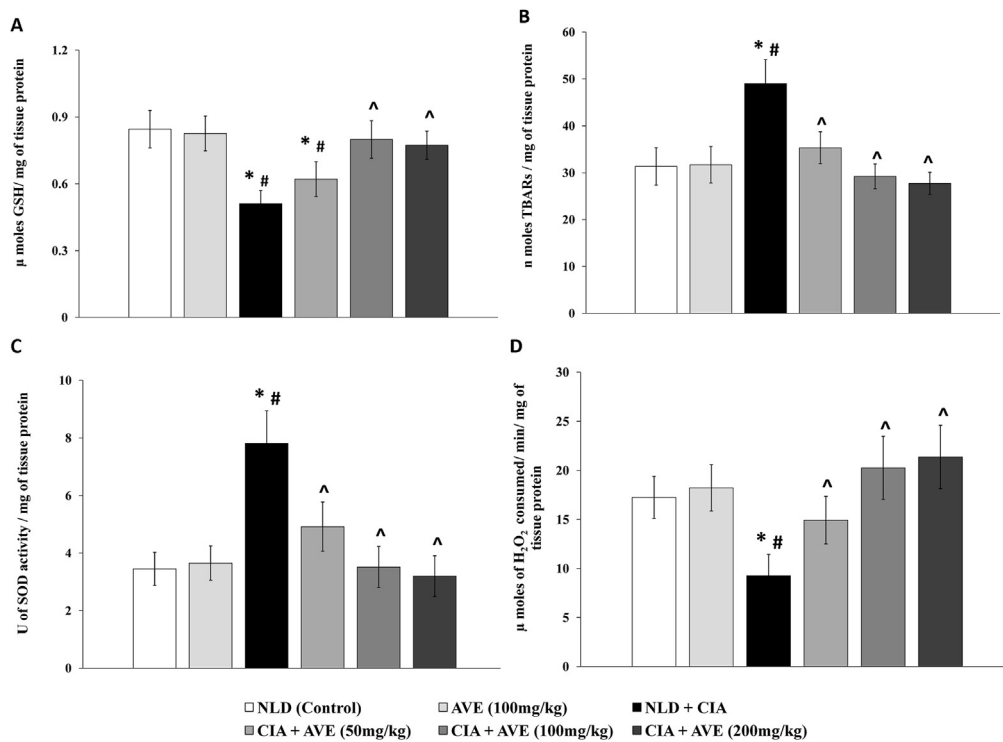


Fig. 7. Anti-oxidant status in spleen tissues. Levels of (A) GSH (in μ mole/mg tissue protein), (B) LPO (in nmole/mg tissue protein), (C) SOD (U/mg tissue protein) and (D) catalase activity (in μ mole H_2O_2 consumed/min/mg tissue protein) in splenic tissues from mice. The results are obtained from three repeated experiments and the values are expressed as mean \pm SD; (n = 6/group). *indicates significant difference in comparison to NLD (control) ($p < 0.05$); #indicates significant difference in comparison to AVE (100 mg/kg)-fed mice ($p < 0.05$); and, ^indicates significant difference in comparison to CIA mice ($p < 0.05$).

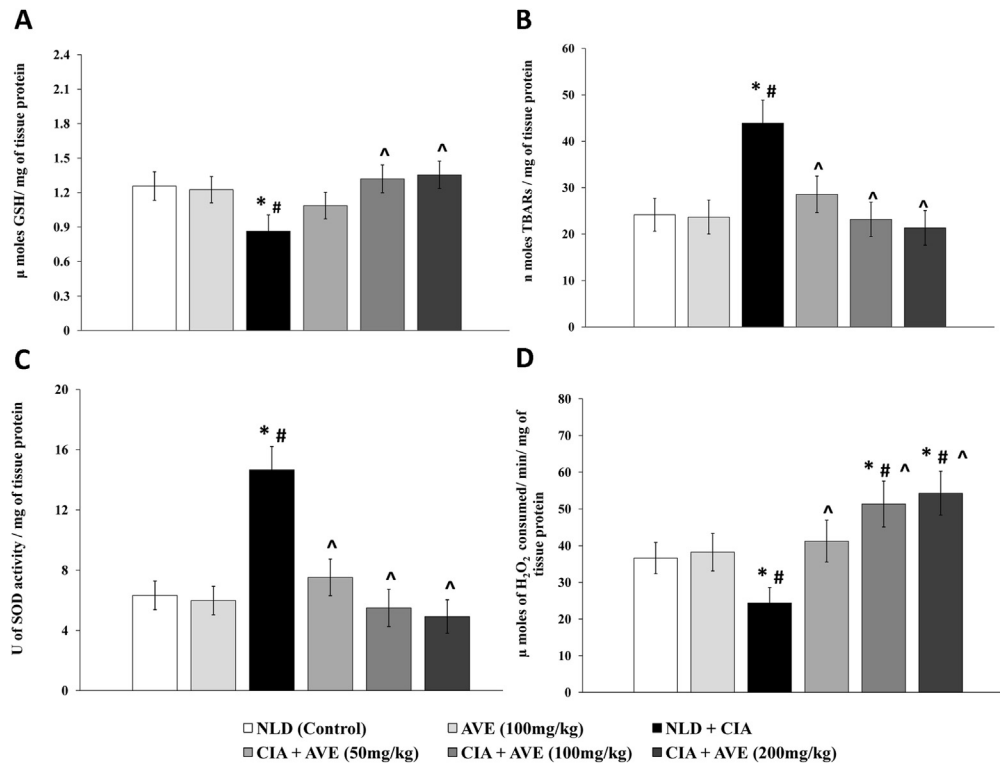


Fig. 8. Anti-oxidant status in synovial tissues. Levels of (A) GSH (in μ mole/mg tissue protein), (B) LPO (in nmole/mg tissue protein), (C) SOD (U/mg tissue protein) and (D) catalase activity (in μ mole H_2O_2 consumed/min/mg tissue protein) in synovial tissues from mice. The results are obtained from three repeated experiments and the values are expressed as mean \pm SD; (n = 6/group). *indicates significant difference in comparison to NLD (control) ($p < 0.05$); #indicates significant difference in comparison to AVE (100 mg/kg)-fed mice ($p < 0.05$); and, ^ indicates significant difference in comparison to CIA mice ($p < 0.05$).

Table 3
Synovial nitric oxide production (in μ g/g of synovial tissue).

Sl. No.	Groups	NO level (in μ g/g of tissue) (mean \pm SD)
1.	NLD (control)	19.61 \pm 3.65
2.	AVE (100 mg/kg)	21.39 \pm 3.98
3.	NLD + CIA	92.62 \pm 13.11*#
4.	CIA + AVE (50 mg/kg)	55.32 \pm 9.21*#
5.	CIA + AVE (100 mg/kg)	31.27 \pm 6.18 ^
6.	CIA + AVE (200 mg/kg)	26.29 \pm 5.19 ^

Effects of AVE on NO production in synovial tissues. Alterations in production of nitric oxide (μ g/g of tissue protein) in synovial tissues from all the six different groups of animals. The results were reproduced in three repeated experiments. Values are expressed as mean \pm SD of six mice per group. P value less than 0.05 was considered as significant. *indicates significant ($P < 0.05$) difference in comparison to NLD (control), #indicates significant difference ($P < 0.05$) in comparison to AVE (100 mg/kg) fed mice and ^ indicates significant difference ($P < 0.05$) in comparison to CIA induced mice.

on MHC presentation to the T-cells [28]. Activation of T-cells provide cytokines, i.e. IL-2, IFN- γ that are typically important for triggering sufficient inflammatory responses in the body. Immune complexes deposited into the synovial joints elevate pro-inflammatory cytokines in serum, through induction of mononuclear cells [29]. In circulation these immune complexes can stimulate peripheral PMNs and macrophages to secrete pro-inflammatory cytokines, like TNF- α , via Fc γ R1a on the macrophages [29]. In both ways, there is activation of synovial macrophages which ultimately results in increased production of TNF- α and IL-1 β , two key pro-inflammatory cytokines involved in RA pathogenesis [30]. Besides TNF- α and IL-1 β , other equally important cytokines like IL-6, IL-12 and IL-15 are also produced by the synovial macrophages, which are pro-inflammatory in nature.

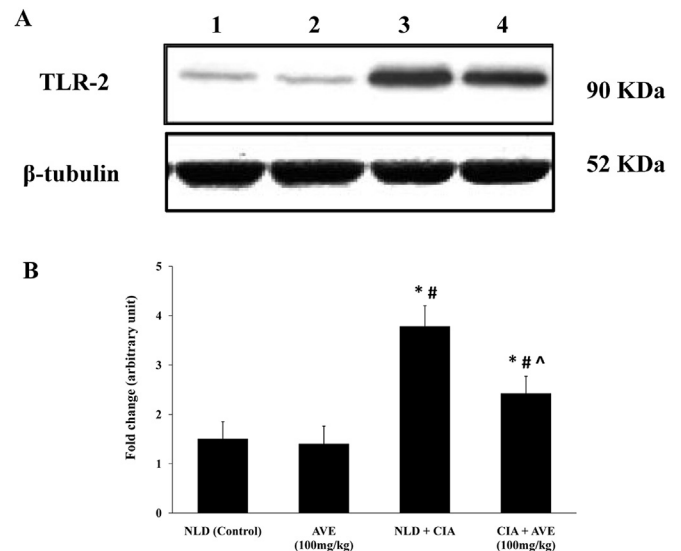


Fig. 9. TLR-2 expression in synovial joints of CIA mice. Synovial tissue homogenates were prepared for the analysis of TLR-2 expression by immunoblot assay for samples from CIA (A, upper panel) mice. Respective fold-changes in expression are shown in B and C. All samples were probed with β -tubulin to assure equal protein loading in gels occurred (A, lower panel). Expression of TLR-2 was measured in terms of fold-change over control. Results were reproduced in three experiments. Values shown are mean \pm SD; (n = 6/group). *indicates significant difference in comparison to NLD (control) ($p < 0.05$); #indicates significant difference in comparison to AVE (100 mg/kg)-fed mice ($p < 0.05$); and, ^ indicates significant difference in comparison to CIA mice ($p < 0.05$).

Among a number of different pro-inflammatory mediators, IL-6 in particular can induce acute phase inflammatory response in

hepatic tissues during CIA and results in an increment in the serum levels of CRP, SAA, pentraxins and other inflammatory mediators [31]. Serum CRP, a surrogate marker of disease severity that correlates with final outcome of arthritis [32,33] is also a potent endogenous ligand for TLR-2 present on the surfaces of synovial fibroblasts, PMNs and macrophages [34], and its transcription is regulated by pro-inflammatory cytokines including IL-6 [35]. Since regulation of TLR-2 expression has been correlated to protection in many diseases, treatment of arthritic mice with AVE that affect CRP and IL-6 could reasonably be expected to translate into clinical efficacy against RA [36].

Serum concentration of CRP, the most common type of acute phase proteins was tested in our experiments and was found to be significantly attenuated in case of CIA mice treated with AVE. This explains protective role of AVE against liver damage and inflammatory reactions in hepatic tissues during pathogenesis of CIA. TNF- α , IL-1 β , IL-6 and IL-12 were also found to be attenuated with AVE treatment when measured in serum as well as synovial tissues of CIA mice. IL-10 is the most potent anti-inflammatory cytokine and a chief regulator of arthritogenesis [37] which was also elevated in serum and synovial joints after AVE treatment in CIA mice. Elevation in myeloperoxidase activity from spleen and synovial joints with concert to the marked reduction in IL-8 and MCP-1, the two biologically important chemokines for PMNs and monocytes in the synovial tissues in CIA mice treated with AVE shows inhibitory potential of AVE against neutrophil and monocyte infiltration in synovial joints.

Restored GSH level and attenuation of the lipid peroxide generation along with modulated enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) in liver, spleen and synovial joints from CIA mice treated with AVE might dictate how the antioxidant or radical scavenging activity of AVE attenuates chronic inflammation in arthritic joints. TNF- α dependent NO production in inflamed synovium has been described as a major cause of synovial hyperplasia in RA through onset of hypoxia mediated angiogenesis [38] and the suppression of NO production in synovial joints in our study might have been resulted from decreased TNF- α and iNOS expression in CIA mice treated with AVE.

Amongst a number of different endogenous ligands, there are two such molecules that primarily activate TLR-2; gp96, a component of synovial lining and serum amyloid A, an acute phase protein released during liver inflammatory responses [39]. In our study we found suppression of TLR-2 expression in arthritic joints from CIA mice treated with AVE. To explain the mechanism how AVE dampened TLR-2 expression, we can speculate from the MPO activity and CRP level in CIA mice which shows a marked reduction after AVE treatment. MPO activity as a direct marker of neutrophil accumulation, along with local chemokines (IL-8 and MCP-1) clearly depicts a significant decrease in neutrophil in synovial joints from CIA mice treated with AVE. AVE, prevents oxidative stress in liver, and therefore is also expected to suppress the acute phase response in liver and CRP level in serum. Therefore, decreased PMN infiltration in synovial joints that is directly proportional to the amount of TLR-2 expressed in the synovial tissue along with decreased CRP, the potent activator of TLR-2 mediated signaling pathway can be correlated to the suppression of TLR-2 expression from arthritic joints in CIA mice treated with AVE. Therefore, suppression of TLR-2 expression in arthritic joints in collagen induced arthritic mice after treatment with AVE could be a therapeutic approach.

In an unpublished observation GC–MS study confirmed abundance of diterpene such as phytols, hexadecanoic acids, alkaloids such as 2'-(trimethylsiloxy)-2-methyl-7-phenylindole - 1-H - pyrrolo [2, 3- β] pyridine - 1- propionitrile and glucosinolates like desulphosinigrin which was identified in the AVE. However, the

effect shown by AVE that ameliorates CIA induced inflammation and progressive bone damage is an outcome of a cumulative effect of all these bioactive compounds, therefore further studies are warranted on these chemical constituents from methanol extract of leaves of *A. vasica* after purifying them and administering them separately in animal models with respect to collagen induced arthritis.

5. Conclusion

The present study has demonstrated that the administration of methanolic extract of *A. vasica* leaf improves against chronic inflammation during deleterious progression of RA by down-regulating synovial TLR-2 expression and pro-inflammatory mediator release and it can be used in pharmacological research for future drug discovery and development in the fields of rheumatology.

Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Acknowledgements

This work was supported and funded by Department of Science and Technology (DST), Govt. of West Bengal, Calcutta, India [Sanction number. 297(Sanc)/ST/P/S&T/9G-08/2012].

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jnimm.2015.11.001>.

References

- [1] A. Kaur, P. Nain, J. Nain, Herbal plants used in treatment of rheumatoid arthritis: a review, *Int. J. Pharm. Pharm. Sci.* 4 (2012) 44–57.
- [2] E. Choy, Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis, *Rheumatology* 51 (2012) 3–11.
- [3] R.K. Kim, T.Y. Chung, H. Shin, Red ginseng saponin extract attenuates murine collagen-induced arthritis by reducing pro-inflammatory responses and matrix metallo-proteinase-3 expression, *Biol. Pharm. Bull.* 33 (2010) 604–610.
- [4] D. Khanna, G. Sethi, K.S. Ahn, M.K. Pandey, A.B. Kunnumakkara, B. Sung, A. Aggarwal, B.B. Aggarwal, Natural products as a gold mine for arthritis treatment, *Curr. Opin. Pharmacol.* 7 (2007) 344–351.
- [5] G.R. Burmester, E. Feist, T. Dornier, Emerging cell and cytokine targets in rheumatoid arthritis, *Nat. Rev. Rheumatol.* 10 (2014) 77–88.
- [6] J.A. Hinson, D.W. Roberts, L.P. James, Mechanisms of acetaminophen-induced liver necrosis, in: *Handbook of Experimental Pharmacology*, Springer-Verlag Berlin Heidelberg, New York, 2010, pp. 369–405.
- [7] A. Mahajan, V.R. Tandon, Antioxidants and rheumatoid arthritis, *J. Ind. Rheumatol. Assoc.* 12 (2004) 139–142.
- [8] S. Jaswal, H.C. Mehta, A.K. Sood, J. Kaur, Antioxidant status in rheumatoid arthritis and role of antioxidant therapy, *Clin. Chim. Acta* 338 (2003) 123–139.
- [9] T. Jayakumar, M. Ilayaraja, M.P. Sridhar, S. Sivalokanathan, T.R. Prasad, M.P. Balasubramanian, Protective effect of *Adhatoda vasica* on D-galactosamine induced liver damage in rats with reference to lipid peroxidation and antioxidant status, *J. Natur. Remed* 9 (2009) 91–98.
- [10] B. Singh, R.A. Sharma, Anti-inflammatory and antimicrobial properties of pyrroloquinazoline alkaloids from *Adhatoda vasica* Nees, *Phytomedicine* 20 (2013) 441–445.
- [11] W.P. Arend, The innate immune system in rheumatoid arthritis, *Arth. Rheum.* 44 (2001) 2224–2234.
- [12] G. Falgarone, O. Jaen, M.C. Boissier, Role for innate immunity in rheumatoid arthritis, *Jt. Bone. Spine* 72 (2005) 17–25.
- [13] A. Iwasaki, R. Medzhitov, Regulation of adaptive immunity by the innate immune system, *Science* 327 (2010) 291–295.
- [14] Q. Huang, R.M. Pope, Toll-like receptor signaling: a potential link among rheumatoid arthritis, systemic lupus, and atherosclerosis, *J. Leuko. Biol.* 88 (2010) 253–262.
- [15] M. Iwahashi, M. Yamamura, T. Aita, A. Okamoto, A. Ueno, N. Ogawa, S. Akashi, K. Miyake, P.J. Godowski, H. Makino, Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid

- arthritis, *Arth. Rheum.* 50 (2004) 1457–1467.
- [16] L.K. Sorensen, A. Havemose-Poulsen, S.U. Sonder, K. Bendtzen, P. Holmstrup, Blood cell gene expression profiling in subjects with aggressive periodontitis and chronic arthritis, *J. Periodontol.* 79 (2008) 477–485.
- [17] H.C. Oliveira, M.P. Dos-Santos, R. Grigulo, L.L. Lima, Antidiabetic activity of *Vatairea macrocarpa* extract in rats, *J. Ethnopharmacol.* 115 (2008) 515–519.
- [18] D.D. Brand, K.A. Latham, E.F. Rosloniec, Collagen-induced arthritis, *Nat. Protoc.* 5 (2007) 1269–1275.
- [19] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [20] A. Majhi, K. Kundu, R. Adhikary, M. Banerjee, S. Mahanti, A. Basu, Combination therapy with ampicillin and azithromycin in an experimental pneumococcal pneumonia is bactericidal and effective in down regulating inflammation in mice, *J. Inflamm.* 11 (2014) 5–22.
- [21] S. Reitman, A. Frankel, Colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases, *Am. J. Clin. Pathol.* 28 (1957) 56–63.
- [22] S. Majumdar, K. Dutta, S.K. Manna, A. Basu, B. Bishayi, Possible protective role of chloramphenicol in TSST-1 and coagulase-positive *Staphylococcus aureus*-induced septic arthritis with altered levels of inflammatory mediators, *Inflammation* 34 (2011) 269–282.
- [23] J. Sedlak, R.H. Lindsay, Estimation of total, protein-bound, and non protein sulfhydryl groups in tissue with Ellman's reagent, *Anal. Biochem.* 25 (1968) 192–205.
- [24] P. Mal, D. Ghosh, D. Bandyopadhyay, K. Dutta, B. Bishayi, Ampicillin alone and in Combination with riboflavin modulates *Staphylococcus aureus* induced septic arthritis in mice, *Ind. J. Exp. Biol.* 50 (2012) 677–689.
- [25] K. Dutta, D. Nandi, B. Bishayi, Repeated systemic *Escherichia coli* infection enhances anti-oxidant response in hypercholesterolemic mice inducing cardiovascular inflammation, *Inflammation* 32 (2009) 89–98.
- [26] A. Majhi, R. Adhikary, A. Bhattacharyya, S. Mahanti, B. Bishayi, Levofloxacin-Ceftriaxone combination attenuates lung inflammation in a mouse model of bacteremic Pneumonia caused by multidrug-resistant *Streptococcus pneumoniae* via inhibition of cytolytic activities of pneumolysin and autolysin, *Antimicrob. Agents. Chemother.* 58 (2014) 5164–5180.
- [27] E. Lubberts, W.B. van den Berg, Cytokines in the pathogenesis of rheumatoid arthritis and collagen-induced arthritis, in: *Cytokines and Chemokines in Autoimmune Disease*, E-Publishing Inc, USA, 2003, pp. 194–203.
- [28] B. Catchpole, N.A. Staines, A.S. Hamblin, Antigen presentation of type II collagen in rats, *Clin. Exp. Immunol.* 125 (2001) 478–484.
- [29] L. Mathsson, J. Lampa, M. Mullazehi, J. Rönnelid, Immune complexes from rheumatoid arthritis synovial fluid induce FcγRIIa dependent and rheumatoid factor correlated production of tumour necrosis factor- α by peripheral blood mononuclear cells, *Arth. Res. Ther.* 8 (2006) 1–10.
- [30] R.O. Williams, L. Marinova-Mutafchieva, M. Feldmann, R.N. Maini, Evaluation of TNF-alpha and IL-1 blockade in collagen-induced arthritis and comparison with combined anti-TNF-alpha/anti-CD4 therapy, *J. Immunol.* 165 (2000) 7240–7245.
- [31] A.K. Abbas, A. Lichtman, S. Pillai, *Immunity to microbes*, in: *Cellular and Molecular Immunology*, Elsevier, Philadelphia, 2007, pp. 356–358.
- [32] T. van den Broek, J.R. Tesser, S. Albani, The evolution of biomarkers in rheumatoid arthritis: from clinical research to clinical care, *Exp. Opin. Biol. Ther.* 8 (2008) 1773–1785.
- [33] J. Devlin, A. Gough, A. Huissoon, P. Perkins, R. Holder, R. Reece, V. Arthur, P. Emery, The acute phase and function in early rheumatoid arthritis. C-reactive protein levels correlate with functional outcome, *J. Rheumatol.* 24 (1997) 9–13.
- [34] R.L. He, J. Zhou, Z.C. Hanson, J. Chen, N. Cheng, D.R. Ye, Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor-2, *Blood* 113 (2009) 429–437.
- [35] P.T. Lukey, H.C. Perry, S. Yang, S. Parry, M.C. Dickson, V.H. Norris, P.G. Russell, M. Watissée, I. Rioja, K.P. Ray, S. Crowe, M. Binks, Single doses of p38 MAP kinase inhibitors or prednisolone affect CRP and IL-6 in patients with active rheumatoid arthritis, *Open. J. Immunol.* 2 (2012) 85–97.
- [36] M.B. Pepys, G.M. Hirschfield, C-reactive protein: a critical update, *J. Clin. Invest.* 111 (2003) 1805–1812.
- [37] L.A. Joosten, E. Lubberts, P. Durez, M.M. Helsen, M.J. Jacobs, M. Goldman, W.B. van den Berg, Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction, *Arth. Rheum.* 40 (1997) 249–260.
- [38] F. Bao, P. Wu, N. Xiao, F. Qiu, Q.P. Zeng, Nitric oxide-driven hypoxia initiates synovial angiogenesis, hyperplasia, and inflammatory lesions in mice, *PLoS. One* 7 (2012) e34494.
- [39] N. Cheng, R. He, J. Tian, P.P. Ye, R.D. Ye, Cutting edge: TLR-2 is a functional receptor for acute-phase serum amyloid A, *J. Immunol.* 181 (2008) 22–26.