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# 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

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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.



## 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

*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, Polyvinylidine 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.

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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-arthritic 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 from 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