



Evaluation of therapeutic potential of *Withania somnifera* root powder on oxidative stress and cartilage damage in collagen induced arthritis in rats

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ABSTRACT

Withania somnifera, commonly known as Ashwagandha, is a well-known herb in the field of alternative medicines. Its anti-inflammatory and immunomodulatory potentials have been authenticated well. The root powder of *W. somnifera* (600 and 800 mg/kg) was evaluated for its anti-oxidative and anti-cartilage damage potential in collagen induced arthritic (CIA) rats, using methotrexate (0.3 mg/kg) as a standard drug. Changes in biochemical and histopathological parameters in response to *W. somnifera* root powder were determined to assess its effect on CIA rats. *W. somnifera* root powder at 600 mg/kg resulted in a significant reduction in oxidative stress and cartilage damage and showed anti-oxidative and anti-cartilage damage potential in experimental CIA rat model.

Keywords: Anti-arthritis, Collagen induced arthritis, *Withania somnifera*, Oxidative stress

INTRODUCTION

Rheumatoid arthritis (RA) is considered a common chronic, inflammatory autoimmune disorder that causes the immune system to attack the joints. It is a disabling and painful inflammatory condition, which can lead to substantial loss of mobility due to pain and joint destruction. Generally, small joints are affected, but larger joints can also be involved; the pattern of joint involvement can differ from patient to patient [1]. It affects about 1% of the world population [2-4] having major impact on the significant increase in the lifetime cost and a decrease in quality of life [5]. The disease can occur at any age but it is most common among those aged between 40-70 years [6]. It affects women three times more often than men and can first develop at any age [7].

RA is characterized by chronic inflammation leading to joint destruction [8]. Although the pathophysiological basis of RA is not yet fully understood, oxidative stress caused by reactive oxygen species (ROS) have been suggested to be involved in its pathogenesis [9]. Thus, oxidative stress generated within the inflammatory joint can produce autoimmune phenomena and resulted in destruction of connective tissue in rheumatoid synovitis [10]. The presence of higher level of ROS

in RA patients than the healthy subjects causes oxidative stress in RA patients and as a consequence the antioxidant systems are impaired [11].

W. somnifera Dunal (*Ashwagandha*; Family-Solanaceae), popularly known as Indian ginseng, winter cherry and *ajagandha*, has been used for more than 2500 years in Ayurveda to treat several disorders like tumors, inflammation, arthritis, asthma, and hypertension [12]. It is generally regarded as the rich source of steroidal lactones, alkaloids, flavonoids, tannins and saponins etc. [13]. Roots of this plant are considered most effective for therapeutic purposes due to significant accumulation of its one of the active constituents, withanolides [14]. Keeping in view the pharmacological potential of this plant, present investigation was undertaken to assess the therapeutic potential of *W. Somnifera* root powder on oxidative stress and cartilage damage in collagen induced arthritis (CIA) in experimental rat model.

MATERIALS AND METHODS

Plant material and dose preparation: *W. somnifera* was collected from the Botanical garden, Banaras Hindu University, Varanasi, in October,

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2011 and was scientifically approved in the Department of Botany, Banaras Hindu University, Varanasi (identified by Prof. M.P. Singh, a well known plant taxonomist). The roots were collected, washed with sterile distilled water and dried at 80°C in an oven. The dried roots of *W. somnifera* were grinded with pestle and mortar into powdered form and used as oral feed for experimental rats post mixing with distilled water to get the desired concentration of 600 and 800 mg/kg, for the purpose of therapeutic treatment of experimental rats.

Experimental animals: Albino female rats (Wistar strain), 6-10 week of age were purchased from a central animal house facility, Banaras Hindu University, Varanasi and were acclimatized in the animal house conditions with 12:12 h light: dark schedule. The choice of the sex of the animals, i.e., females, was based on the findings that the autoimmune arthritis is mediated by sex hormones [15] and that female rats are more susceptible to arthritis as compared to the males [16]. Free access to food and water was given *ad libitum*. Six rats were used per study group. Rats were subdivided into the following groups: normal control rats; arthritic control rats; *W. somnifera* (600 mg/kg) treated arthritic rats; *W. somnifera* (800 mg/kg) treated arthritic rats and methotrexate (0.3 mg/kg) treated arthritic rats. All the experimental protocols were pre-approved by the animal ethical committee, Jiwaji University, Gwalior and Banaras Hindu University, Varanasi, India.

Induction of collagen induced arthritis: Collagen induced arthritis (CIA) in rats was developed according to Remmers *et al.* (2002) [17]. Collagen from bovine tracheal cartilage type II (CII) (obtained from Sigma Chemical Company St. Louis, Missouri, USA) was dissolved in cold 0.1N acetic acid (2 mg/ml) and was emulsified with an equal volume of freshly opened, cold Freund's adjuvant incomplete (IFA) (Sigma, USA). The emulsion was made by using three-way stopcock with syringe. Rats were injected intradermally at several sites on the back with a dose of 2 mg/kg of body weight. On the seventh day after primary immunization, the rats were re-immunized with 0.1 ml (100 µg) of similarly prepared collagen/IFA emulsion injected intradermally at the base of the tail.

Dose schedule: The water suspensions (1 ml) of *W. somnifera* (600 and 800 mg/kg) and methotrexate (0.3 mg/kg) were administered orally to the arthritic rats with the help of syringe cannula. *W. somnifera* (600 and 800 mg/kg) was administered at 10 am, daily. However, methotrexate (0.3 mg/kg) was given once a week, at the same time.

Sterile water was, however, given to the control as well as untreated arthritic control rats. The treatment of *W. somnifera* and methotrexate was started from day 20th post collagen immunization and continued up to 45th day.

Serum lipid peroxidation: Lipid peroxidation was measured following the method of Okhawa *et al.* [18]. In this method the released malondialdehyde (MDA) serves as an index of lipid peroxidation. For assessing serum lipid peroxidation, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA) aqueous solution were added to 0.2 ml of serum sample. Distilled water (0.6 ml) was added to make up the final volume 4ml and vortexed. The solution was kept in water bath at 100°C for 60 min and thereafter cooled in ice bath. To the solution, 1ml water and 5 ml n-butanol/pyridine mixture (15:1 v/v) were added and shaken vigorously. The content was centrifuged at 4,000 rpm for 10 min at 4°C and the absorbance of the organic upper layer was recorded at 532 nm against a reagent blank. The concentration of TBA reactive substance was expressed as nmoles MDA/ml of serum using 1,1,3,3-tetraethoxypropane (TEP) as the standard.

Serum glutamate pyruvate transaminase: Serum glutamate pyruvate transaminase (SGPT) in serum samples was assayed according to Reitman and Frenkel [19] by using kit (Span Diagnostics Ltd). The kit contains two reagents namely reagent 1 and reagent 2. Whereas reagent 1 contains L-alanine (200 mmol/l) and lactate dehydrogenase (1500 IU), reagent 2 is, however, made up of α -ketoglutarate (>35 mmol/l) and NADH (1.05 mmol/l). Reagent 1 (0.25 ml) was added in each tube. After incubation at 37°C for 5 min, 0.05ml of test serum was added to each tube and the content was mixed well followed by 30 min incubation at 37°C. After that 0.25 ml of reagent 2 was added, the content was mixed well and the tubes were incubated at room temperature for 20 min. At last 2.5 ml solution of NaOH (0.4 N) was added, the content was mixed well and the tubes were incubated at room temperature for 10 min. Absorbance of the brown color of the corresponding hydrazone developed in alkaline medium was measured spectrophotometrically at 505 nm using distilled water as a blank. The concentration of SGPT was expressed as IU/L of serum using OD of the test on Y-axis of the standard curve and extrapolated it to the corresponding enzyme activity on X-axis.

Histopathological study: For histopathological analysis, paw joints were collected at the end of experiment (45th day) and tissue samples of the joints (10 mm x 5 mm thick pieces) were fixed in

10% (v/v) neutral formalin. The formalin fixed tissues were cut into thin pieces (2-3 mm thick) and decalcified in 10% EDTA for 21 days. The decalcified tissues were dehydrated in ascending grades of alcohol, cleaned and embedded in high melting fresh paraffin. Sections (7 μ thick) were cut by using rotator microtome (Leica RM 2135). The sections were deparaffinized in xylene, rehydrated in descending grade of alcohol, washed, stained and counterstained with Harris haematoxylin and 1% eosin stain in 90% alcohol. The sections were kept in xylene for 25 min and mounted in DPX [20].

Histological score: The degree of cell infiltration in synovial fluid, destruction of cartilage and bone were investigated in haematoxylin and eosin stained slide of paw joints using Leica DM 6000 (Germany) equipped with digital camera and the images were captured. Joints were graded using a modified version of system adopted from Joosten *et al.* [21]. For each animal, both hind paws were examined.

The mean histological score was measured as the mean of score of all the three scores (synovial infiltration, bone destruction and cartilage destruction score) for individual group and the data were presented as six animals per group.

Synovial cell infiltration score: Synovial cell infiltration score was measured as mean score of two sections of six animals per group. The cell infiltration in synovial fluid was graded from 0 to 3 as follow: 0, no inflammatory cells in the joint cavity; 1, a few inflammatory cells in the joint cavity; 2, joint cavity partly filled with inflammatory cells; and 3, joint cavity totally filled with inflammatory cells.

Cartilage destruction score: Cartilage destruction score was measured as mean score of two sections of six animals per group. The destruction of cartilage was graded from 0 to 3 as follows: 0, normal appearance; 1, minor destruction of cartilage surface; 2, clear loss of cartilage; and 3, cartilage almost absent in the whole joint.

Bone destruction score: Bone destruction score was measured as mean score of two sections of six animals per group. The articular bone involvement was graded from 0 to 3 as follows: 0, normal appearance; 1, minor signs of destruction; 2, up to 30% destruction; and 3, more than 30% destruction.

Statistical analysis: The values were presented as means \pm SEM. Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test (all pair wise multiple comparison procedure)

(Sigma Stat 3.5, Systat Software Inc. USA). A value of * $P < 0.05$ was considered significant to arthritic control versus normal and treated groups.

RESULTS

Serum lipid peroxidation: A highly significant elevation in the serum lipid peroxidation (LPO) level (6.75 \pm 0.30 nmoles MDA/ml) was recorded in arthritic control rats with respect to their normal control counterparts having LPO level of 2.5 \pm 0.25 nmoles MDA/ml at 45th day. *W.somnifera* (600 and 800 mg/kg) and methotrexate (0.3 mg/kg) therapeutic treatments to the arthritic rats resulted in a significant decline in their serum LPO levels (3.5 \pm 0.33, 4.60 \pm 0.26 and 4.55 \pm 0.25 nmoles MDA/ml, respectively) at 45th day to that of the serum LPO levels of their arthritic control counterparts (Fig.1).

Serum glutamate pyruvate transaminase: A highly significant elevation in serum glutamate pyruvate transaminase (SGPT) activity (0.19 \pm 0.003 IU/L) was recorded in arthritic control rats with respect to their normal control counterparts having SGPT activity of 0.105 \pm 0.003 IU/L at 45th day. Arthritic rats treated with *W.somnifera* (600 and 800 mg/kg) and methotrexate (0.3 mg/kg) showed a significant decline in their SGPT activity (0.100 \pm 0.004, 0.12 \pm 0.003 and 0.15 \pm 0.002 IU/L, respectively) at 45th day to that of the SGPT activity of their arthritic control counterparts (Fig. 2).

Histopathological analysis of paw joints: Histology of joints of arthritic control rats showed vigorous proliferation of synovial cells, resulting in pannus formation and infiltration of mononuclear cells and neutrophils to the subsynovial region pannus formation, cartilage and bone destruction. Arthritic rats treated with *W. somnifera* (800 mg/kg) showed minimal to moderate synovial cell infiltration with less destruction of cartilage, however, they did not show any bone destruction. Whereas *W. somnifera* (600mg/kg) treated arthritic rats showed almost no sign of synovial cell infiltration, pannus formation, synovitis, cartilage and bone destruction (Fig. 3).

Mean histological score: A significant elevation in the mean histological score was recorded in arthritic control rats (1.78 \pm 0.22) to that of the mean histological score of their normal counterparts (0.06 \pm 0.07). *W. somnifera* (600 and 800 mg/kg) treatment to the arthritic rats resulted in a significant decline in their mean histological score of 0.72 \pm 0.16 and 1.17 \pm 0.19, respectively to that of the mean histological score of their arthritic control counterparts. Whereas, methotrexate (0.3 mg/kg) treatments to the arthritic rats also resulted in a

decline in their mean histological score, however, it did not significantly differ with the mean histological score of their arthritic control counterparts (Fig. 4).

Synovial cell infiltration score: A significant elevation in synovial cell infiltration score was observed in arthritic control rats (2.66 ± 0.21) to that of their normal counterparts (0.16 ± 0.16). Arthritic rats treated with *W. somnifera* (600 mg/kg) showed a significant decline in their synovial cell infiltration score (1.33 ± 0.21) when compared with their arthritic control counterpart. The arthritic rats treated with *W. somnifera* (800 mg/kg) also showed decline in their synovial cell infiltration score (2.16 ± 0.16) to that of its arthritic control counterpart, however, the difference was found to be statistically non-significant (Fig. 5).

Cartilage destruction score: Cartilage destruction score was measured after microscopic examination of joint histological slides. The cartilage destruction score was found to be higher in arthritic control rats (1.33 ± 0.21) as compared to the cartilage destruction score of arthritic rats treated with either 600 (0.66 ± 0.21) or 800 (1.16 ± 0.16) mg/kg of *W. somnifera* (Fig. 6).

Bone destruction score: Bone destruction score was measured after microscopic examination of joint histological slides. The bone destruction score was found to be higher in arthritic control (1 ± 0.36) as compared to the bone destruction score of arthritic rats treated with either 600 (0.16 ± 0.16) or 800 (0.5 ± 0.22) mg/kg *W. somnifera* (Fig. 7).

DISCUSSION

Disease-modifying anti-rheumatic drugs (DMARDs), the key therapeutic agents, reduce synovitis and systemic inflammation and improve function of the musculoskeletal system. Methotrexate, the drug used in the present study, is a leading DMARD which can be combined with other DMARDs [22, 23]. However, its use is also associated with a plethora of side effects including immunosuppression and emergence of opportunistic infections which can add to morbidity and mortality [24]. The last few years have witnessed rapid growth in popularity of complementary and alternative medicines (CAM) [25] with the prevalence of CAM usage by RA patients being anywhere in between 28 to 90% [26]. In recent years, due to its pharmacological properties, *W. somnifera* is gaining more attention for finding out an alternative medicine to treat an array of disorders. Oxidative stress plays a major role in the development of chronic and degenerative diseases such as cancer, arthritis, auto-immune disorders and neurodegenerative

diseases [27]. Increased oxidative stress is hypothesized to be important in the pathogenesis of RA and to both initiate and propagate inflammation [28]. In the present study, an elevated level of MDA, an end product of lipid peroxidation, was observed in the arthritic control rats. Elevated levels of lipid peroxidation or MDA in RA patients have been implicated to be due to an imbalance between the defence mechanism and free radical generation process [27]. CIA rats treated with *W. somnifera* (600 mg/kg) interestingly showed a significant decrease in their oxidative stress marker. *W. somnifera* has been known for its potent anti-oxidant and free radical quenching properties in various disease conditions [11]. Similar results on lowering of lipid peroxidation following *W. somnifera* administration have also been reported earlier [29, 30].

Serum SGPT has been reported to play a vital role in the formation of biologically active chemical mediators such as bradykinins in inflammatory process [31]. SGPT is considered as a good indicator of liver and kidney impairment and exhibited a significant increase in its activity in complete Freund's adjuvant (CFA) induced arthritic rats [32]. Thus, there is a positive correlation between the increased activity of serum SGPT and the disease activity in RA [33]. Elevated level of serum SGPT in arthritic rats can be correlated due to an increase in the liver fraction. In the present study, the level of SGPT in the arthritic rats was significantly higher than the arthritic rats treated with *W. somnifera* (600 mg/kg). Hepatoprotective and antioxidant potential of *W. somnifera* has also been well authenticated by several researchers [34, 35].

Histology of the joints of arthritic control rats showed vigorous proliferation of synovial cells, resulting in pannus formation and infiltration of mononuclear cells and neutrophils to the subsynovial region. Pannus destroyed the cartilage and bone. In contrast, control rats showed normal histology without any synovial infiltration, pannus formation, and cartilage and bone destruction. The joints of the control rats had moderate to severe synovitis and considerable inflammatory cell infiltration into mineralised and non-mineralised tissues. A significant elevation of synovial cell infiltration and mean histological score was observed in arthritic control rats when compared with the normal control rats. In arthritic rats treated with *W. somnifera* (600 mg/kg), synovitis and inflammatory cell influx were minimal, with no sign of cartilage, bone destruction and mean histological score. The significant decline in the histological symptoms and score in *W. somnifera* treated arthritic rats may be due to its widely documented anti-inflammatory and anti-oxidant

properties [36-38]. The results of the present study demonstrated that the oral administration of the root powder of *W.somnifera* effectively suppressed the oxidative stress and hence cartilage destruction in CIA in rats, as evidenced by the decrease in the oxidative stress markers and histopathological symptoms.

CONCLUSION

The results of the present study showed that the oral administration of the root powder of *W.somnifera* effectively suppressed the oxidative stress and hence cartilage destruction in CIA in

rats, as evidenced by the decrease in the oxidative stress markers and histopathological symptoms. This disease suppressing ability of the *W. somnifera* could be due to its anti-oxidant ability. Thus, *W. somnifera* treatment could be considered a potential therapeutic strategy for the treatment of RA.

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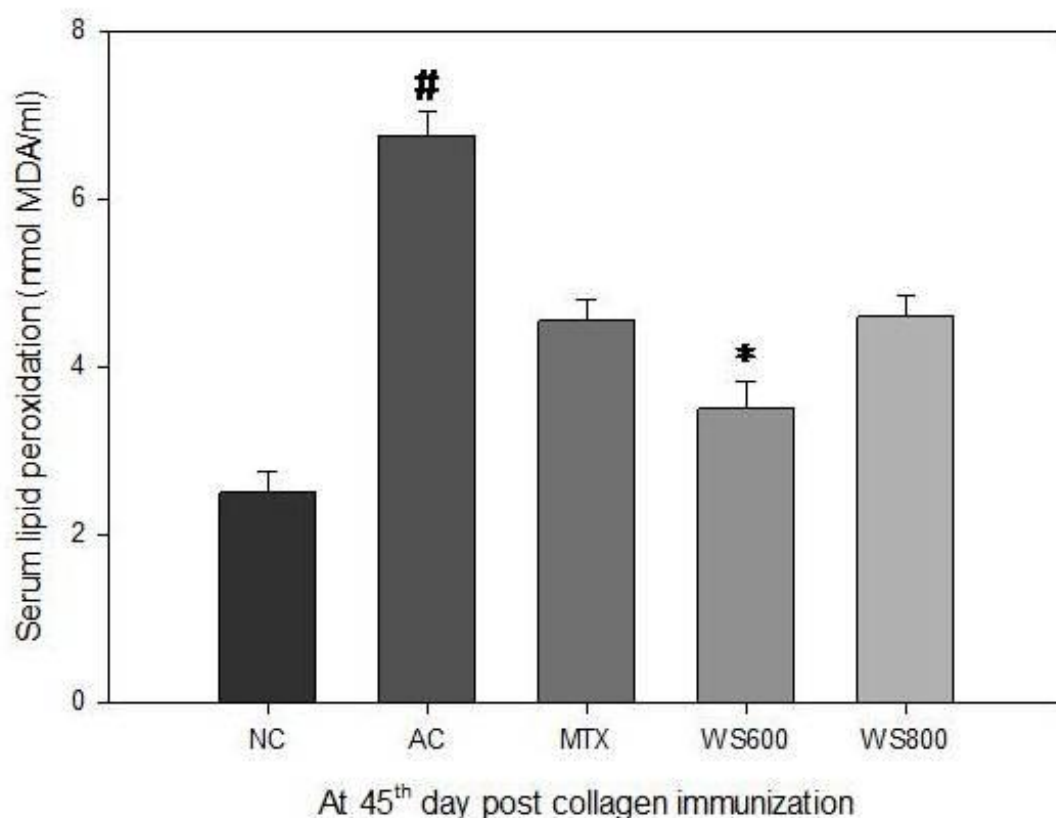


Fig. 1 Serum lipid peroxidation in rats at 45th day post collagen immunization. NC, normal control; AC, arthritic control; MTX, methotrexate (0.3 mg/kg¹); WS 600, *W. somnifera* (600 mg/kg); WS 800, *W. somnifera* (800 mg/kg) treated rats. The values are expressed as mean ± S.E.M., N=6. *P<0.05 vs AC, #P< 0.05 vs NC.

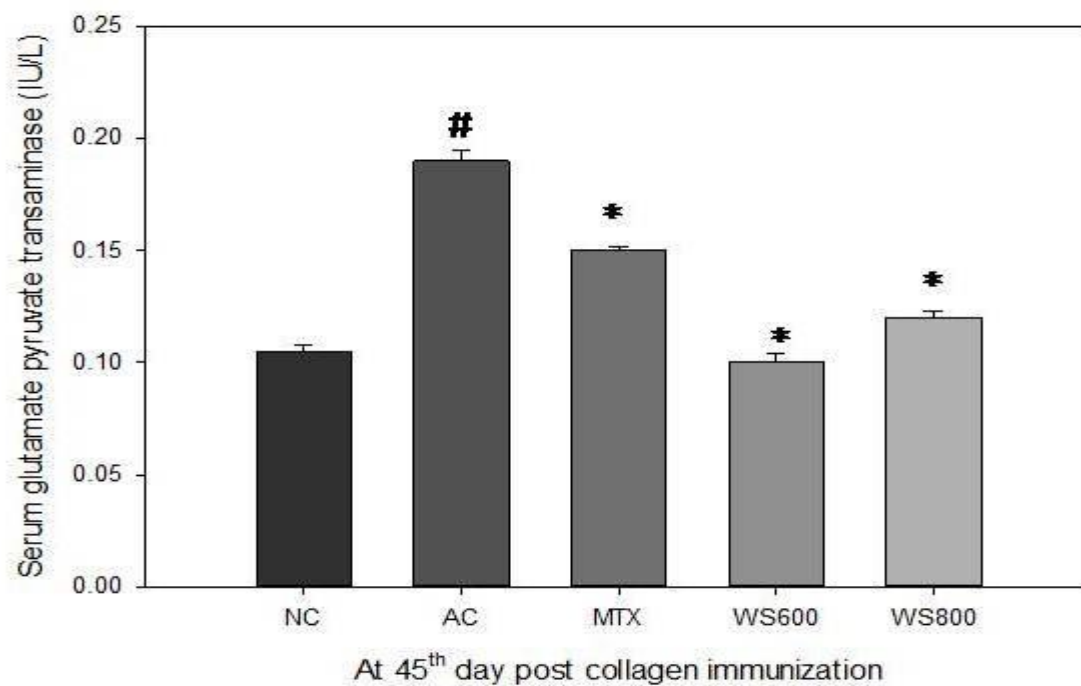


Fig. 2 Serum glutamate pyruvate transaminase (SGPT) level in rats at 45th day post collagen immunization. NC, normal control; AC, arthritic control; MTX, methotrexate (0.3 mg/kg); WS 600, *W. somnifera* (600 mg/kg); WS 800, *W. somnifera* (800 mg/kg) treated rats. The values are expressed as mean \pm S.E.M., N=6. *P \leq 0.05 vs AC, #P \leq 0.05 vs NC.

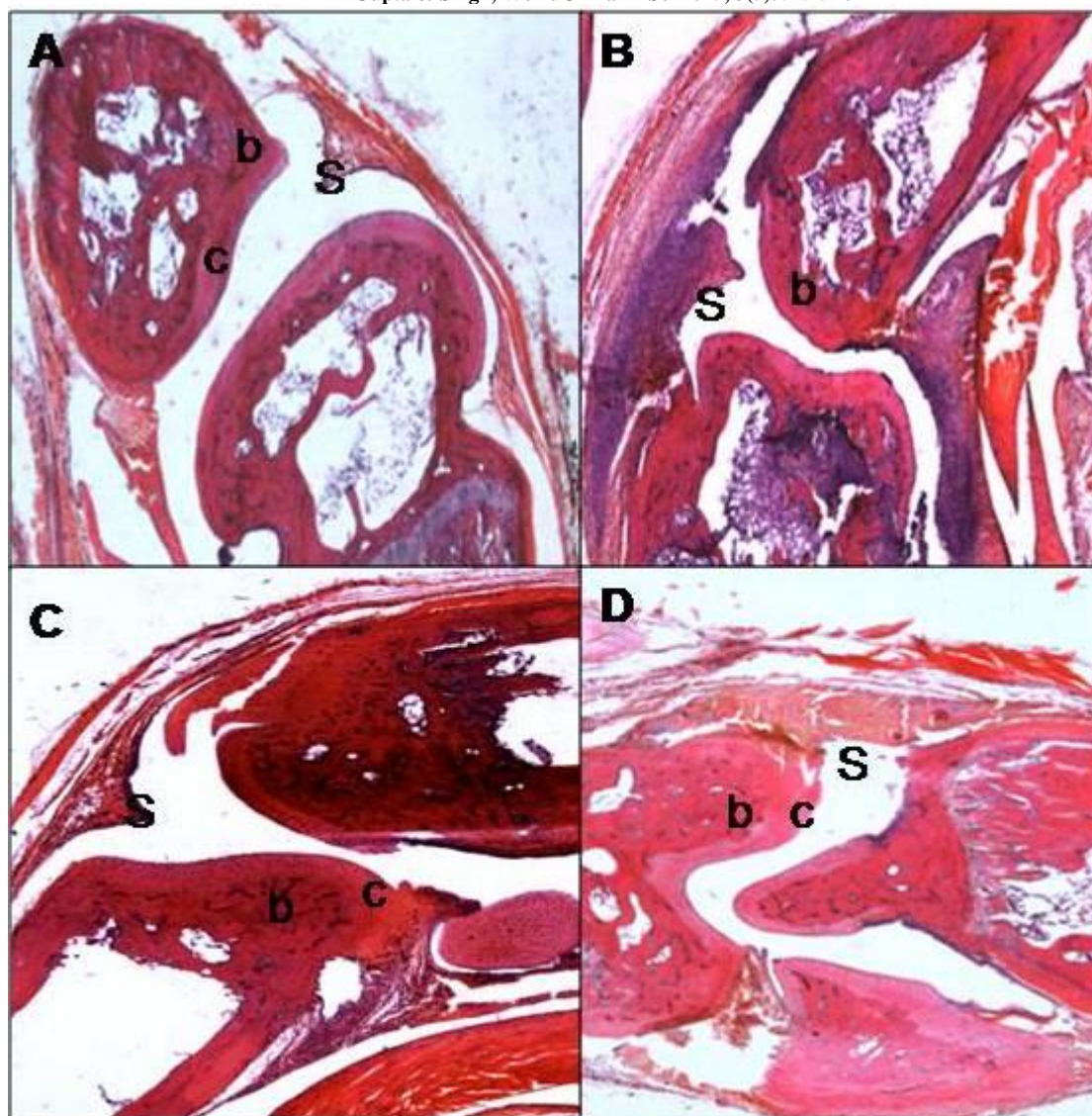


Fig. 3 Histological analysis of joint morphology. Light micrographs (25X) of rat phalangeal joint after hematoxylin and eosin staining. (A) Normal rat with normal synovial joint containing intact cartilage, bone and synovium, (B) Representative arthritic control rat with ulceration and damaging of cartilage, pronounced synovitis, cell infiltration and destruction bone compartment, (C) *W. somnifera* (600 mg/kg) treated with reduced synovitis, cell infiltration and cartilage ulceration, (D) *W. somnifera* (800 mg/kg) treated with almost no sign of synovitis or adherent cell to the cartilage and appear essentially normal. b, bone; c, cartilage; s, synovium. Pictures are representative of six distinct rats per group.

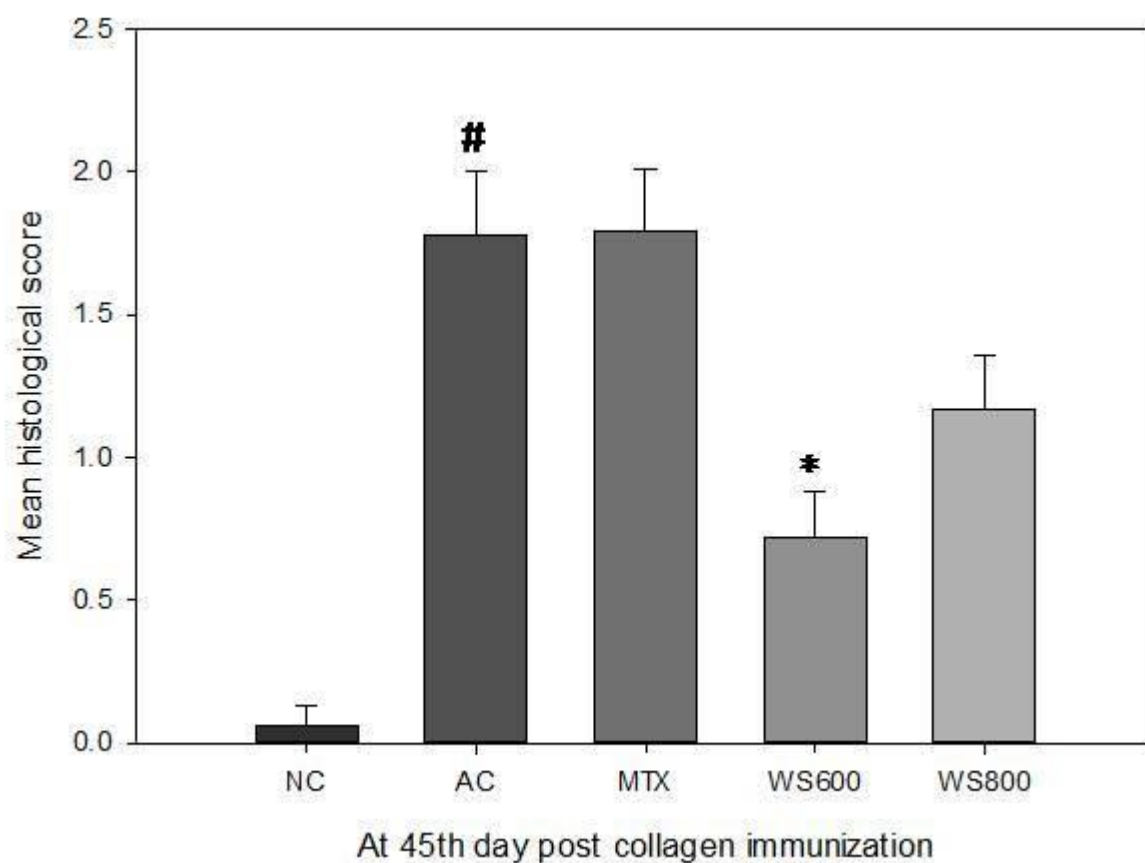


Fig. 4 Mean histological score at 45th day post collagen immunization. NC, normal control; AC, arthritic control; MTX, methotrexate (0.3 mg/kg); WS 600, *W. somnifera* (600 mg/kg); WS 800, *W.somnifera* (800 mg/kg) treated rats. The values are expressed as mean \pm S.E.M., N=6. *P \leq 0.05 vs AC, #P \leq 0.05 vs NC.

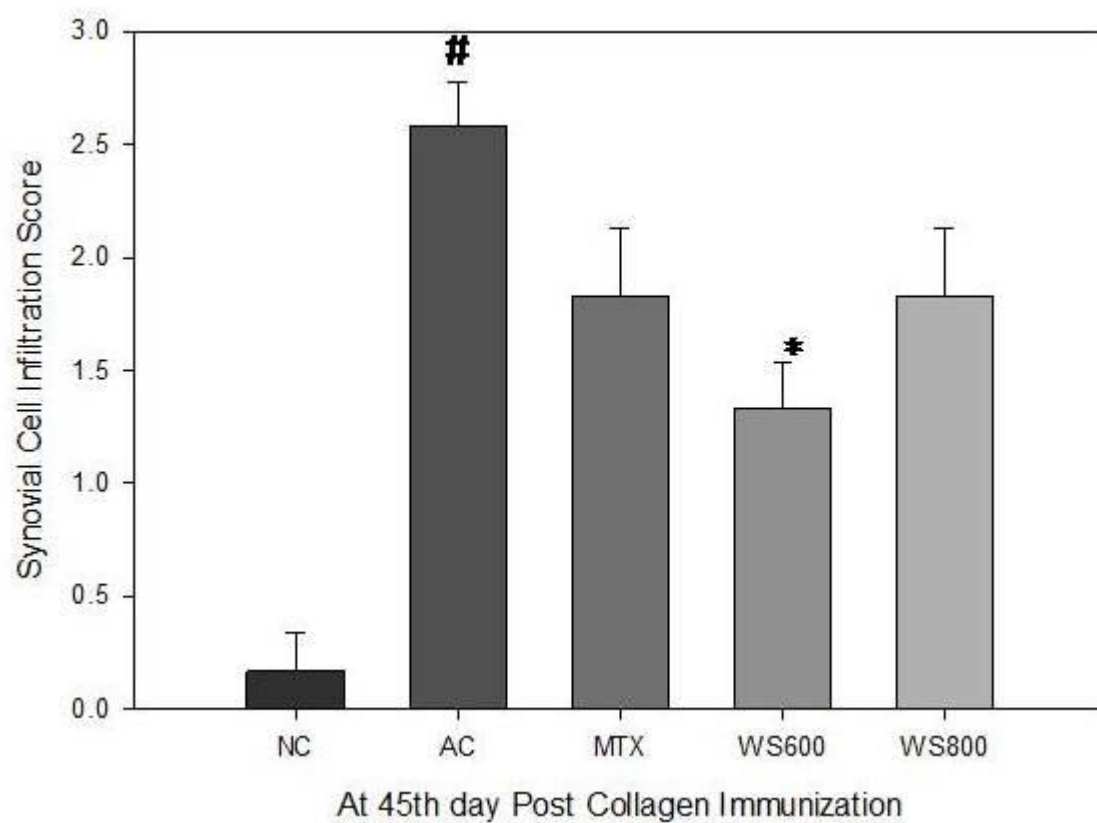


Fig. 5 Mean synovial cell infiltration score of rats from 0 to 45th day post collagen immunization. NC, normal control; AC, arthritic control; MTX, methotrexate (0.3 mg/kg); WS 600, *W. somnifera* (600 mg/kg); WS 800, *W. somnifera* (800mg/kg) treated rats. The values are expressed as mean \pm S.E.M., N=6. *P \leq 0.05 vs AC, #P \leq 0.05 vs NC.

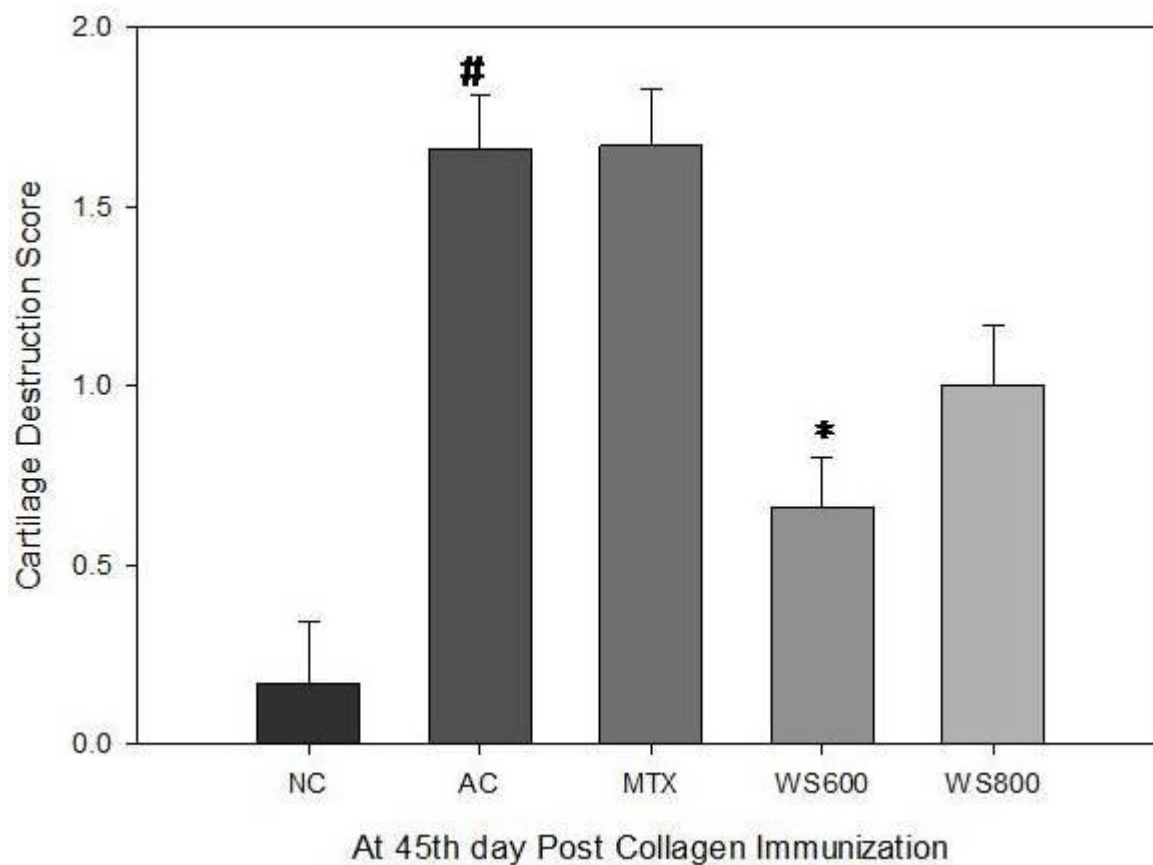


Fig. 6 Cartilage destruction score of rats at 45th day post collagen immunization. NC, normal control; AC, arthritic control; MTX, methotrexate (0.3 mg/kg); WS 600, *W. somnifera* (600 mg/kg), WS 800: *W. somnifera* (800 mg/kg) treated rats. The values are expressed as mean ± S.E.M., N=6. *P≤0.05 vs AC, #P≤ 0.05 vs NC.

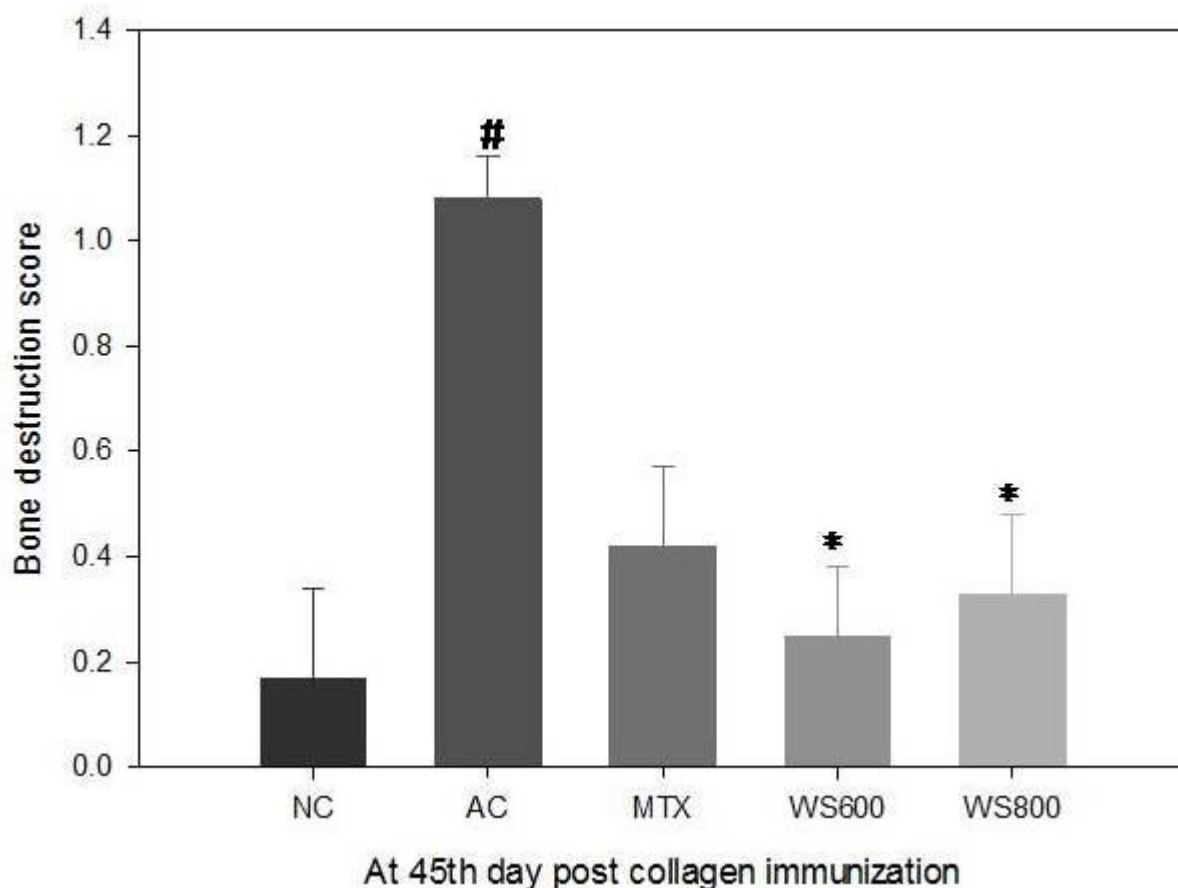


Fig. 7 Bone destruction score of rats at 45th day post collagen immunization. NC, normal control; AC, arthritic control; MTX, methotrexate (0.3 mg/kg); WS 600, *W. somnifera* (600 mg/kg); WS 800, *W. somnifera* (800 mg/kg¹) treated rats. The values are expressed as mean ± S.E.M., N=6. *P≤0.05 vs AC, #P≤ 0.05 vs NC.

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