# Evaluation of Curcumin, Piperine, and Ferrous Sulphate Combination Potential in the Adjuvant-induced Arthritis Model in Male Wistar Rats

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**ABSTRACT**
**Background:** Rheumatoid arthritis (RA) is an autoimmune disorder known for prolonged joint inflammation and deformity condition. Currently, Complementary and alternative medicine (CAM) treatment in RA patients is preferred. **Objectives:** The research focuses on exploring the combination (curcumin (200mg/kg), piperine (10mg/kg), and ferrous sulphate (0.1mg/kg) potential in the adjuvant-induced arthritis model (AIA) compared with disease control and prednisone acetate (standard 5 mg/kg) in the adjuvant-induced arthritis model (AIA). **Materials and Methods:** Male Wistar rats in each group was treated with standard, curcumin, piperine, and ferrous sulphate individually as well as in combination for 28 days after the induction of arthritis. Evaluation parameters were body weight, paw edema, mobility condition, and stair climbing test. Furthermore, Red Blood Cells (RBCs), White Blood Cells (WBCs), immune organ index (spleen and thymus) was elucidated. The study was concluded with histopathology, X-ray radiography, Tumour Necrosis Factor α (TNF α), and Interleukin-1beta (IL-β) examination. **Results:** The combination showed significance with the gradual increase in body weight and mobility (7th day), whereas the conditions of paw edema and stair climbing were found effective (28th day). RBC and WBC counts were found to be clinically significant. The combination was found to be highly significant in estimation of immune organ index. Combination showed significant change in infiltration of inflammatory cells, joint space, and minimal erosion in bone indicating satisfactory anti-arthritic effects. Furthermore, the combination showed improvement in joint radiodensity and narrowing in joint space. The level of TNF-α and IL-1β were found significant. **Conclusion:** The combination showed anti-inflammatory and anti-arthritic activity.
**Keywords:** Adjuvant-induced arthritis model (AIA), Complete Freud's adjuvant, Interleukin-1beta (IL-β), Thymus, Spleen, Rheumatoid arthritis.

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by prolonged joint inflammation, affecting at least twice as many women as men with peak incidence at the age of 50.[1] It is characterized by chronic inflammation of the synovium that leads to the destruction of articular cartilage. RA pathogenesis involves complex humoral and cellular reactions thereby leading to infiltration of lymphocytes, monocyte in the synovium.[2] It further leads to the release of autoantibodies such as anti-cyclic citrullinated peptide and increased C-reactive protein thereby leading to dysregulation of immune response in the pathogenesis of the disease.[3-4] 1% of the world population is affected by RA.[5] As RA is an inflammatory condition, nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are used as first-line therapy to reduce the pain and swelling caused due to RA.[6] NSAIDs are divided into groups based on their chemical structure and selectivity: acetylated salicylates, non-acetylated salicylates, propionic acids, enolic acids, anthranilic acids, and selective Cyclooxygenase receptor-2 (COX-2) inhibitors.[7] Corticosteroids at a mean dose of 8 mg daily were widely used in the 1.4% of the patients over 54 years for the treatment of RA.[8] Furthermore, Disease-modifying antirheumatic drugs (DMARDs) act as second-line therapy for the treatment of RA.[9] Biologic DMARDs are target specific and are prescribed after the failure of conventional DMARD therapy.[10] Tissue damage occurrence in RA is treated by DMARD's which work on the principle of reducing inflammatory response and levels of cytokines.[11]

Complementary and alternative medicine (CAM) has become popular in patients with RA and has now begun to take the adjunct treatment option for chronic diseases like RA. Natural plant products account for the predominant

section for inflammatory and immune disorders. The herbal CAM belongs to a diverse traditional system of medicine and is found to be beneficial as a treatment option for RA.[12-13] India has a rich variety of medicinal plants and more than 2000 plants of medicinal value have been mentioned in ancient Tibbi, Unani and Ayurvedic systems of medicine.[14] Turmeric (Curcuma longa) is a bright yellow color curry spice that is native to Southeast Asia primarily India. Curcumin (1, 7-bis (4-hydroxy-3- methoxyphenyl)-1, 6-heptadiene-3,5-dione), is the active constituent extracted from the rhizomes of turmeric.[15] Scientific studies on Curcumin show its immunomodulatory property to support its ethnopharmacological uses. Furthermore, previous studies have also shown that curcumin has a therapeutic role in the treatment of RA synovitis.[16]

Curcumin modifies Nuclear factor-kappa B (NF-$\kappa$B) signaling, phospholipase A2 and proinflammatory cytokines such as interleukin production.[17] Although curcumin possesses immunomodulatory action the poor oral bioavailability and low absorption by the small intestine restricts its use for treatment.[18] Although curcumin possesses immunomodulatory action the poor oral bioavailability and low absorption by the small intestine restricts its use for treatment.[19] Curcumin and piperine synergistically increase the Transforming growth factor (TGF-$\beta$) level and significantly improve collagen repair thereby preventing the progression of RA.[20-21] Anaemia of chronic inflammation and iron-deficient anemia has been commonly diagnosed in RA patients.[22] Though the literature has witnessed and evaluated with clear evidence of individual effects of curcumin, piperine, and ferrous sulphate in adjuvant-induced arthritis (AIA) model, nevertheless strategic combination (curcumin (200mg/kg), piperine (10mg/kg), and ferrous sulphate (0.1mg/kg) treatment for RA is not evaluated and studied. The research work highlighted in this article elucidates the drug combination has potential to reduce the severity of RA in the adjuvant-induced arthritis model (AIA).

## MATERIALS AND METHODS

### Drugs and chemicals

Complete Freud's adjuvant (CFA) was procured from Sigma-Aldrich, USA. Prednisolone acetate and Ferrous sulphate were obtained as a gift sample from Avik Pharmaceuticals Ltd and Agrawal life sciences Pvt. Ltd, India respectively. Curcumin and piperine were obtained as gift samples from Plant lipids, India. All other reagents used throughout the experimentation were of analytical grade. ELISA kits for the detection of serum concentration levels were procured from Krishgen Biosystems, India.

### Animals

Male Wistar rats having a weight range of 150–180 g were procured from the National Institute of Biosciences, Pune, India. Rats were housed under standard conditions (Temperature 22±2°C, Humidity 75±5%, 12 hr light, and 12 hr dark cycle) in a registered animal house. They received a basal nutritional diet (Nutrivet life sciences, Pune, India) and purified water was provided *ad libitum*. Rats were allowed to acclimatize for 7 days before the experimentation. The experimental design (CPCSEA/IAEC/BNCP/P-33/2020) was approved by the Institutional Animal Ethics Committee (IAEC) which was constituted under the norms of the (CPCSEA), Committee for Control and Supervision of Experiments on Animals, India. All experimental procedures were performed on rats as per National Institutes of Health (NIH) guidelines for handling experimental animals.

### Experimental design

CFA 0.1 ml was injected in the subplantar region into the left hind paw on day 0 to all the groups except the normal control group which received 0.1 ml of paraffin oil. Curcumin and Piperine were suspended in corn oil, whereas ferrous sulphate and Prednisolone acetate were suspended in Carboxy Methyl Cellulose (CMC). All the rats in each group were treated orally for 28 days. The experimental design is as follows:

Group I: Normal (corn oil)

Group II: Disease control (RA induced without treatment)

Group III: Standard (Prednisolone 5 mg/kg)

Group IV: Curcumin (200mg/kg)

Group V: Piperine (10mg/kg)

Group VI: Ferrous sulphate (0.1mg/kg)

Group VII: Combination (Curcumin 200 mg/kg, Piperine 10 mg/kg and Ferrous sulphate 0.1 mg/kg)

## Evaluation Parameters
### Evaluation of Body Weight

On day 0, before the CFA injection, the initial body weight was recorded. The body weight was recorded for the next 3 days post-induction after which the readings were analyzed on weekly basis till the 28th day.

### Evaluation of Paw Edema in Arthritis

Mitutoyo's Digimatic caliper was used for determining the severity of paw edema in experimental rats. Before induction, the edema on the left paw was recorded, after which the recording was done for the next 3 days and further on weekly basis till the 28th day.[23-24]

### Evaluation of Mobility

Male Wistar rats were trained for seven days before CFA administration. They were placed for 5 min in a wooden cage of $100 \times 50 \times 50$ cm with a climbing chamber. The cage was slightly illuminated with the floor covered with food shavings and a staircase at the corner with steps 2 and 3 with water and food respectively. Observations were recorded individually for each rat on day 0 and 21st day. The following scale was adopted to score the observations: 0 -The rat lies down, 1- The rat crawls, 2- The rat walks, 3- The rat climbs, 4- The rat runs and climbs with some difficulty, 5- The rat runs and climbs well. The effect of the drugs on mobility was evaluated.[23-24]

### Stair Climbing Ability Test

Male Wistar rats were first acclimatized for 7 days before the onset of the study in a wooden observation box of 30 cm height that comprised of 3 steps of 5, 10, 15 cm respectively. Rats were trained to climb the staircase, water in a petri dish was placed on step 2, and food pellets on step 3. Rats were kept for 6 hr fasting and the score was calculated based on the following scale.

Score 0: The male Wistar rat does not climb.

Score 1: The male Wistar rat climbs one stair

Score 2: The male Wistar rat climbs stairs one and two.

Score 3: The male Wistar rat climbs stairs one, two, and three.

The scoring index for the stair-climbing test was recorded and evaluated on days 1st,3rd,7th,14th,21st, 28th day of the study.[23-24]

### Evaluation of RBC and WBC count

On 0, 7th, 14th, 21st, and 28th-day blood was collected by the retro-orbital puncture and was immediately analyzed for WBC and RBC count in Nihon Kohden Mek-6550k and expressed in mm/cube.

## Immune Organ Index

Mature T lymphocytes are produced by the thymus which also secretes the thymic hormone, is principally involved in cellular immunity. Though there is a higher proportion of B lymphocytes in the spleen compared to T-lymphocytes and macrophages. Hence, the spleen is associated with humoral immunity. Indices of the thymus or spleen are expressed as the weight of the thymus or spleen (mg) per 10 g body weight. The immune organ index of the thymus and spleen depends on the proliferation of lymphocytes which reflects immune function. On the 28th day, male Wistar rats were sacrificed by the CO2 asphyxiation method. Spleen and thymus were isolated and wet-weighed to calculate the immune organ index. The formula used was:

*Organ weight of the rats = (Wet weight of thymus or spleen) / Bodyweight of rats (gm)*

## Histopathological Examination of Paw and Liver

At the end of the arthritic model study, the rats were sacrificed by the $CO_2$ asphyxiation method. The hind paws of the rats were isolated, fixed with neutral buffered 10% formalin, decalcified for 10 days with EDTA, and embedded in paraffin for histological examination. Similarly, the liver was fixed with neutral buffered 10% formalin and immersed in paraffin for histological studies. The paraffin sections were stained with hematoxylin and eosin. The sections were evaluated in a light microscope at 400X regarding cell infiltration, cartilage damage, and bone erosion.

## X-ray Radiography

The rats were sacrificed on the 28th day and the hind paw was removed. X-ray radiography technique was used to check joint and bone deformation severity and blindly scored according to the extent of joint spaces, osteophytes, and joint structure on a scale of 0-4.[25-27] Table 1 illustrates the classification criteria for X-ray radiography based on the severity of the disease.

## Measurement of Serum Cytokines Concentration

On the 28th day, retro-orbital puncture technique was used to collect the blood sample and the plasma was utilized for the measurement of Tumour Necrosis Factor-alpha (TNF- α) and Interleukin-1beta (IL-β) using the manufacturer's instructions as provided in the kits by Krishgen Biosystems, India.[28]

## Statistical Analysis

The statistical evaluation was performed with the help of GraphPad Prism 8 for 64-bit Windows. All the evaluation data were compared to assess the statistical significance using One-way ANOVA (Analysis of Variance) followed Dunnett's post hoc test. The data were represented as mean ± SEM values. For all comparisons, the significant level was set at *$p$ (<0.05), **$p$ (<0.01), ***$p$ (<0.001).

# RESULTS

## Evaluation of Body Weight

The body weight of the disease control group was markedly decreased when compared with the control group of male Wistar rats. 0 and 1st days did not show significant effect with treated groups. On the 3rd day, a significant rise was observed with the ferrous sulphate group. 7th-day significance with the combination group was observed when compared with the disease control. On 14th, 21st, and 28th significant change was noticed by the ferrous sulphate group when they were compared with the disease control. The results are depicted in Figure 1A.

## Evaluation of Paw Edema

Sub plantar injection of CFA in the left hind paw causes the induction of arthritis which reached peak edema on day 1. On 3rd day, the piperine group noticed a significant reduction in paw thickness with the disease control group. Standard and combination groups showed a significant reduction in paw thickness compared to the disease control group on the 7th, 14th, and 21st days. Also, the ferrous sulphate group observed a significant reduction in paw thickness on the 14th day. On the 21st day, standard, piperine, ferrous sulphate, and combination noticed a significant reduction in paw thickness whereas on the 28th day was highly significant in comparison to disease control. Figure 1B reveals all the results related to paw thickness in the AIA model.

## Evaluation of Mobility

There was no significant notice on the 0 and 1st days in the treated groups. On the 7th day, Curcumin was moderately significant whereas piperine and ferrous sulphate showed minimum significance with regards to an increase in mobility. On the 7th day, the combination showed the highest significance with regards to increase in mobility whereas the 14th, 21st, and 28th days were significant in treated groups. Figure 1C shows evaluation with regards to mobility parameters in the AIA model.

## Stair Climbing Ability Test

No significant change was observed in treated groups on the 0, 1st, and 3rd days of the study. On the 7th day, Standard, curcumin, and combination groups were significant whereas standard, piperine, and combination noticed a significant change on the 14th day. Curcumin and piperine groups were significant on the 21st day. All treated groups have shown significant effect on the 28th day which revealed an increase in stair climbing ability of male Wistar rats as indicated in Figure 1D.

### Evaluation of RBC and WBC count

There was no significant change observed in RBC count on days 0,14th, 21st as compared with the disease control. 28th-day ferrous sulphate group noticed a significant change in comparison to the disease control group (Table 2). Standard, curcumin, piperine, ferrous sulphate, and combination groups were highly significant in decreasing the WBC count with the disease control on the 14th, 21st, and 28th days (Table 3).

**Table 1: Classification criteria for X-ray radiography based on the severity of the disease.**

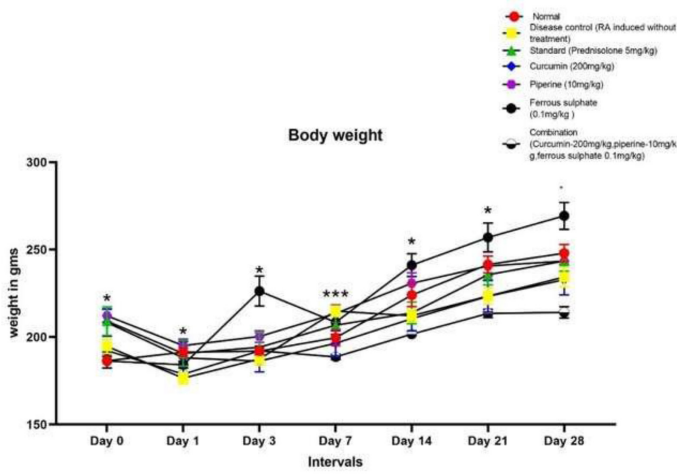| | | | Classification criteria | | |
|---|---|---|---|---|---|
| **Radiographic Grade** | **0** | **1** | **2** | **3** | **4** |
| Classification | Normal | Doubtful | Mild | Moderate | Severe |
| Description | No Feature of Osteoarthritis (OA) | Minute Osteophytes | Definite Osteophytes | Multiple Osteophytes | Large Osteophytes |
| | | Doubtful Joint space narrowing (JSN) | Mild Joint Space Reduction | Moderate Joint Space Reduction | Joint Space Greatly Reduced and Subchondral Sclerosis |

**Figure 1A:** Effects on body weight in AIA model.
Data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.
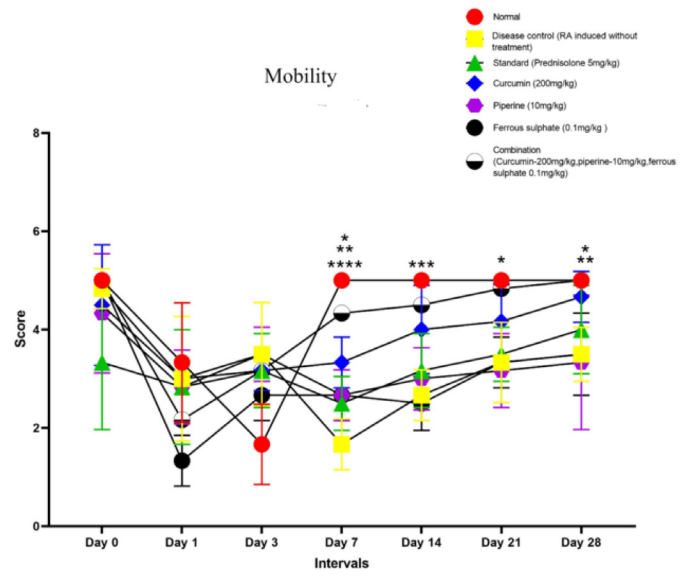


**Figure 1C:** Evaluation of mobility in the AIA model.
Data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.
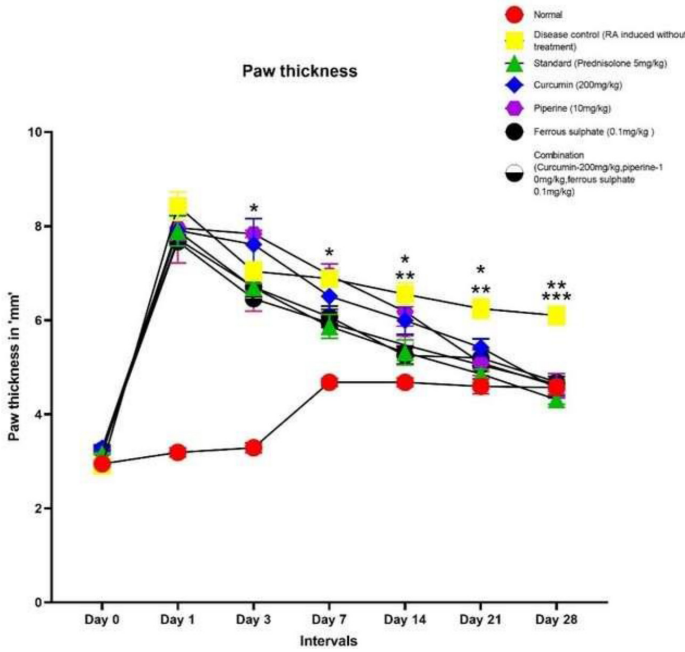


**Figure 1B:** Indication of the paw thickness in the AIA model.
Data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.
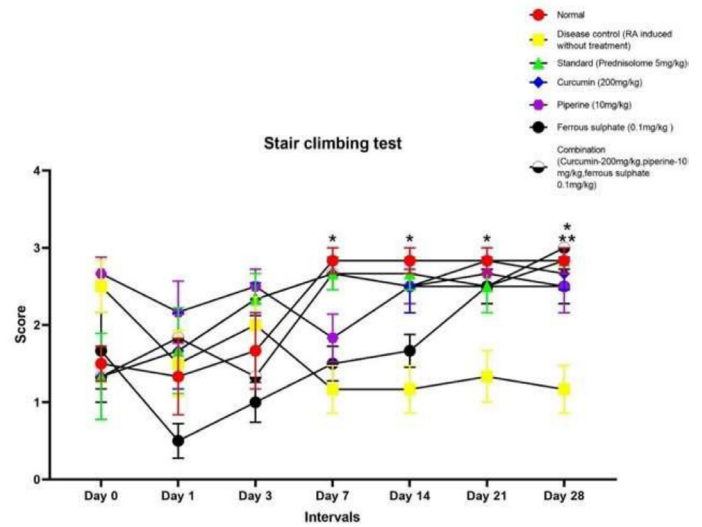


**Figure 1D:** Evaluation of the stair climbing ability in the AIA model.
Data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.

**Table 2: RBC count in AIA model.**

| RBC | 0 day | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|---|
| Normal | 6.15±0.32 | 7.52±0.28 | 6.26±0.122 | 7.91±0.07 | 8.40±0.37 |
| Disease control (RA induced without treatment) | 6.71±0.18 | 7.14±0.10 | 7±0.23 | 7.19±0.30 | 7.95±0.18 |
| Standard (Prednisolone 5 mg/kg) | 5.87±1.32 | 5.56±0.43 | 7.53±0.47 | 7.57±0.47 | 7.95±0.53 |
| Curcumin (200mg/kg) | 6.28±0.61 | 6.71±0.14 | 6.93±0.26 | 8.59±0.68 | 7.91±0.57 |
| Piperine (10mg/kg) | 6.31±2.1 | 8.40±0.28 | 7.22±0.12 | 7.66±0.31 | 8.90±0.26 |
| Ferrous sulphate (0.1mg/kg) | 5.23±1.62 | 5.98±0.83 | 6.90±0.34 | 8.73±0.36 | 10.26±0.12** |
| Combination (Curcumin 200 mg/kg, Piperine 10 mg/kg, and Ferrous sulphate 0.1 mg/kg) | 5.76±0.92 | 7.45±0.32 | 7.14±0.04 | 7.39±0.12 | 8.48±0.06 |

All the data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.

**Table 3: WBC count in AIA model.**

| WBC | 0 day | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|---|
| Normal | 8.28±1.34 | 21.13±1.14 | 11.36±0.52 | 15.23±0.14 | 16.35±0.155 |
| Disease control (RA induced without treatment) | 8.15±0.21 | 32.9±0.36 | 49.53±3.21 | 45.1±3.86 | 39.83±2.79 |
| Standard (Prednisolone 5 mg/kg) | 8.97±2.01 | 11.96±2.86*** | 15.83±4.05*** | 16.10±2.27*** | 6.13±0.56*** |
| Curcumin (200mg/kg) | 8.04±1.37 | 13.5±0.70*** | 14.66±2.16*** | 24.4±2.15*** | 12.96±1.20*** |
| Piperine (10mg/kg) | 7.21±2.98 | 29.56±3.8 | 13.26±0.86*** | 17.7±12.5*** | 14.36±0.50*** |
| Ferrous sulphate (0.1mg/kg) | 7.73±0.91 | 11.66±2.89*** | 10.86±1.41*** | 10.93±0.82*** | 11.96±0.78*** |
| Combination (Curcumin 200 mg/kg, Piperine 10 mg/kg, and Ferrous sulphate 0.1 mg/kg) | 8.34±1.72 | 10.3±2.46*** | 15±0.55*** | 15.8±10.4*** | 18.53±1.29*** |

All the data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.
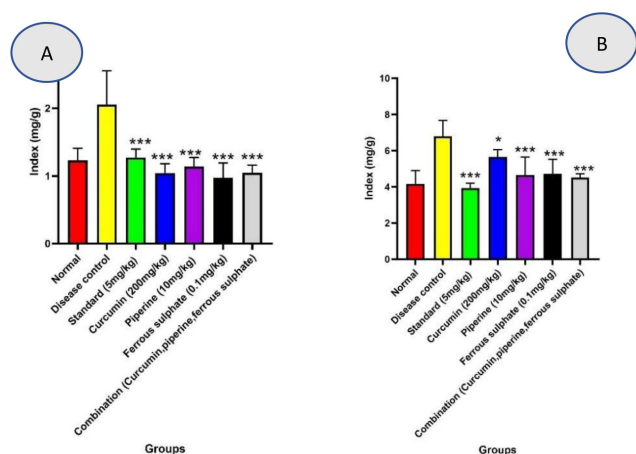


**Figure 2:** Effects on the organ weight index of the thymus (A) and spleen (B) on 28th day in AIA model.
Data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.



**Figure 3:** Evaluation of microscopic examination of the paw in AIA model.



**Figure 4:** Evaluation of microscopic examination of the liver in AIA model.

### Immune organ index

Standard, Curcumin, Piperine, Ferrous sulphate and combination groups were highly significant in decreasing the organ weight index of the thymus whereas the curcumin group was least significant in comparison to other groups concerning spleen. Figure 2 reveals the organ weight index of the thymus (A) and spleen (B) on the 28th day in the AIA model.

### Histopathological examination of paw and liver

The microscopic examination of the paw showed normal bone tissue in the normal group. Furthermore, disease control showed inflammatory changes such as infiltration of inflammatory cells, decreased joint space, and minimal erosion in bone. Besides, groups treated with standard, curcumin, piperine, ferrous sulphate, and combination showed a decrease in the severity and incidence of changes indicating anti-arthritic effects. The histopathological examination is observed with changes in Figure 3 in the AIA model.

The microscopic examination of the liver from the control group did not show any abnormality of pathological significance. However, disease control showed hepatocellular infiltration of inflammatory cells. Whereas, it was noticed a reduction in incidence and severity of lesions in the groups treated with standard, curcumin, piperine, ferrous sulphate suggesting protective effect in RA condition. The liver examination has been shown in Figure 4 in the AIA model.
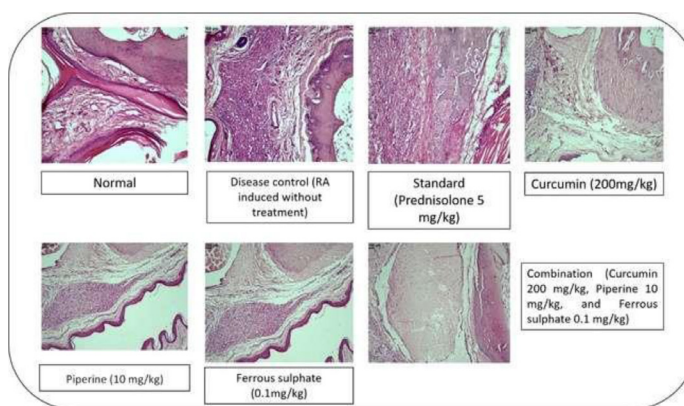
### X-ray radiography examination

Radiographic examination of the paw from the control group did not reveal any change in joint distension and joint space radiodensity. In the case of the disease control group osteoarthritic changes such as increased joint radiodensity and narrowing in joint space were observed. However, the severity and intensity were reduced in the standard, curcumin, piperine, ferrous sulphate groups, and combination groups. The results indicate anti-arthritic potential in the treated groups as shown in Figure 5. Table 4 shows the scoring pattern of the X-ray assessment of the paw based on Kellgren and Lawrence's (K&L) classification criteria.

*Measurement of serum cytokines concentration*

It was observed that TNF-α levels and IL-1β were moderately significant in treated groups as compared to disease control which was highly significant as in Figure 6 A and 6 B.

## DISCUSSION

AIA is a chronic inflammatory disease in rats, which is linked to a reduction in body weight.[29] The weight loss noticed in arthritic rats may be due to the decrease in absorption of glucose and leucine in the rat intestine. Previous studies conducted with the AIA model reveal that there is a gradual increase in body weight with curcumin and piperine.[30-31] The daily body weight gain in the disease control group was significantly less than that observed in the normal group throughout the entire experiment. Also, in treatment groups (Standard, curcumin, piperine, ferrous sulphate, and combination groups), it was observed that the bodyweight of male Wistar rats was gradually kept on increasing till the 28th day. However, a significant increase was observed with the ferrous sulphate group showing the drug was effective in reducing the effect of AIA on body weight. In combination, the increase in body weight indicates that the intestine's absorption capability has been restored as discussed in the results.

CFA is an important component of the bacterial cell membrane acts as a common arthritogenic factor that leads to inflammation in the paw that further progresses to RA in male Wistar rats. The greatest sign of experimental arthritis is swelling in the hind paws of rats.[32] Previous studies showed that consistent measurement of arthritic score post-treatment with curcumin and piperine delayed the onset of arthritis symptoms.[33-34] The studies showed that prolonged treatment of 28 days with curcumin and piperine moderately reduction in paw edema. However, groups treated with ferrous sulphate and combination showed significant change on the 28th day as compared to the disease control group.

The mobility and stair-climbing ability are drastically affected in patients suffering from RA due to inflammation of the synovium. Increased production of inflammatory mediators in the synovium during the early stages of RA enhances catabolic activity, accelerating cartilage destruction.[35] Previous studies conducted showed that curcumin is beneficial in improving the mobility and stair-climbing ability of patients suffering from RA.[36] It is proven that piperine improves the bioavailability of curcumin, therefore curcumin and piperine in synergism can help in improving the mobility and stair-climbing ability in RA.[37] The study revealed that the combination group improves the mobility and stair-climbing ability as compared to the disease control group in the AIA model.

Decreased iron absorption leading to anaemia was found in patients suffering from RA. Previous studies showed that acute administration of bioavailable curcumin alongside Ferrous Sulphate supplements does not impair iron absorption thereby helping in tackling anaemia in RA patients.[38] The RBC count performed on 0, 1st, 3rd, 7th, 14th, 21st, 28th days suggests that ferrous sulphate was efficient in increasing the RBC count of the male Wistar rats. Also, the combination group showed a
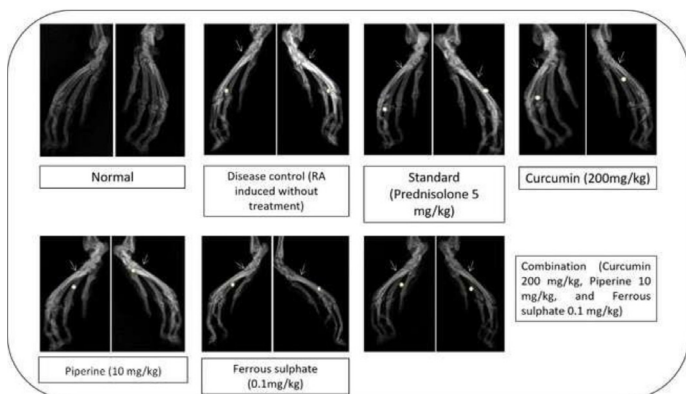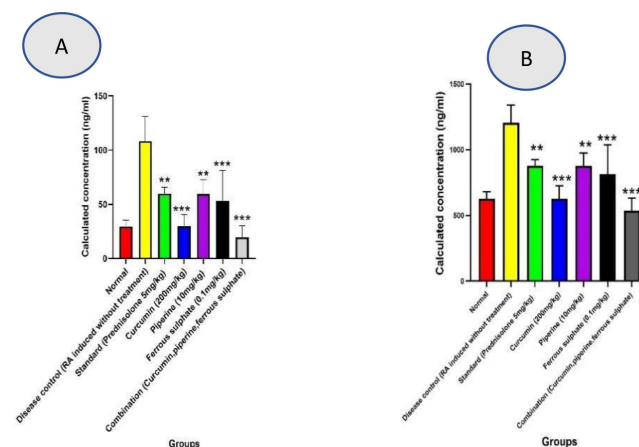
**Figure 6 A and 6 B:** Evaluation of serum cytokine concentrations of TNF-α (A) and IL-1β (B) on the 28th day in the AIA model.
Data were represented as Mean + SEM with *n*=6 in each group. Dunnett's post hoc test was performed. "*" depicts *P*<0.05, "***" depicts *P*<0.001.



**Figure 5:** Evaluation of X-ray radiography in AIA model.

**Table 4: Scoring pattern of the X-ray assessment of the paw based on Kellgren and Lawrence's (K&L) classification criteria.**

| | | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| **Radiographic Grade** | | **Normal** | **Doubtful** | **Mild** | **Moderate** | **Severe** |
| **Groups** | **No of rats** | | | | | |
| Normal | 3 | 0 | 0 | 0 | 0 | 0 |
| Disease control | 3 | 0 | 0 | 2 | 0 | 0 |
| Standard (Prednisolone 5 mg/kg) | 3 | 0 | 0 | 0 | 0 | 0 |
| Curcumin (200mg/kg) | 3 | 0 | 1 | 0 | 0 | 0 |
| Piperine (5mg/kg) | 3 | 0 | 1 | 0 | 0 | 0 |
| Ferrous sulphate (0.1mg/kg) | 3 | 0 | 1 | 0 | 0 | 0 |
| Combination (Curcumin 200 mg/kg, Piperine 10 mg/kg, and Ferrous sulphate 0.1 mg/kg) | 3 | 0 | 0 | 0 | 0 | 0 |

The header spanning row above Radiographic Grade reads: **Kellgren and Lawrence (K&L) classification criteria**

comparatively increase RBC count than the standard suggests that it could be used for better management of RBC levels in the blood of arthritic patients.

Elevated levels of WBC are found in patients suffering from RA due to a cascade of reactions that begins as a result of inflammation.[39] Previous studies have shown that curcumin in combination with piperine helps in reducing the WBC count of patients suffering from inflammation.[40] The WBC count on the 28th day showed that the treatment groups are highly significant in reducing the WBC levels in the blood. On comparing it was observed that combination was as significant as the standard for reduction of WBC count in male Wistar rats.

The thymus and spleen, in particular, have been shown to regulate the immune response in individuals.[41] Previous studies show reduced organ index weight of thymus and spleen post-treatment with curcumin and piperine.[42-43] All the treatment groups were a significant change in reducing the organ weight index of the thymus and spleen. Histopathology studies showed changes in tissue associated with the disease. Histopathological analysis shows the direct measure of comparison between normal and disease groups and indicates the effect of the drug on the tissue.[44] Previous studies show curcumin and piperine combination reduced gross and histopathological liver changes.[45] Histological staining showed that curcumin and piperine significantly reduced the inflammatory area in the ankle joints.[33-34] Histopathological examination of the paw in all treatment groups showed a decrease in the severity of edema indicating anti-arthritic potential. Liver histopathological examination showed a decreased severity of lesions in treated groups as compared to the disease control group.

Previous studies illustrates X-ray radiographic analysis of curcumin and piperine showed that the drugs restored joint architecture and reduced swelling of joints in RA.[46] X-ray analysis of the paw showed that all treatment groups were efficient in reducing the bone erosion of the joint. Furthermore, the combination group was significantly reduced bone erosions as compared to the disease control group. Previous studies on ELISA reading of curcumin and piperine for TNF-α and IL-1β suggest that treatment groups were significant in reducing the levels of cytokines thereby reducing the inflammation.[47-48] ELISA readings showed piperine, ferrous sulphate, standard, and combination groups were highly significant for reducing the levels of TNF-α and IL-1β as compared to the disease control group.

## CONCLUSION

The research study performed shows that the combination group is as potent as the standard for reducing the severity of the RA. The side effects associated with long-term damage caused by steroids can be reduced by the usage of a combination of drugs. Thus, future pharmacotherapeutic agents from natural origin can provide a breakthrough in RA treatment strategy. However, more preclinical and clinical evidences are needed to be performed to support therapy related to RA.

## ACKNOWLEDGEMENT

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**RA:** Rheumatoid arthritis; **NSAIDs:** Nonsteroidal anti-inflammatory drugs; **COX:** Cyclooxygenase receptor; **DMARDs:** Disease-modifying antirheumatic drugs; **CAM:** Complementary and alternative medicine; **NF-κB:** Nuclear factor-kappa B; **TGF:** Transforming growth factor; **AIA:** Adjuvant-induced arthritis; **CFA:** Complete Freud's adjuvant; **DMSO:** Dimethyl sulfoxide; **CPCSEA:** Control and Supervision of Experiments on Animals; **CMC:** Carboxy Methyl Cellulose; **EDTA:** Ethylenediamine tetraacetic acid; **OA:** Osteoarthritis; **JSN:** Joint space narrowing; **TNF-α:** Tumour Necrosis Factor-alpha; **IL-β:** Interleukin-1beta.

## Animal studies

The experimental design (CPCSEA/IAEC/BNCP/P-33/2020) was approved by the Institutional Animal Ethics Committee (IAEC), which was constituted under the norms of the (CPCSEA), Committee for Control and Supervision of Experiments on Animals, India.
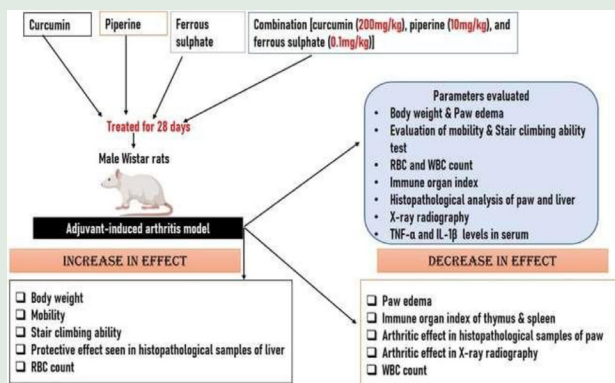
## Author Contributions

Conceptualization of the research studies in the article – AT and GD; Data curation for the entire study- AT, NB, and GD; Formal analysis; Investigation; Methodology; Project administration; Resources; Software related to the study- AT, NB, and GD; Supervision- GD; Validation- AT, GD, Visualization of the study; Roles/Writing - original draft- AT, NB and GD, Writing - review and editing- AT, NB, and GD.

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## GRAPHICAL ABSTRACT

## SUMMARY

- The current research article is on combination (curcumin (200mg/kg), piperine (10mg/kg), and ferrous sulphate (0.1mg/kg) potential in the adjuvant-induced arthritis model.
- The parameters evaluated were body weight, paw edema, mobility condition, and stair climbing test, estimation of RBCs, WBCs, immune organ index (spleen and thymus).
- The study was supported with histopathology, X-ray radiography, Tumour Necrosis Factor α (TNF α), and Interleukin-1beta (IL-β) examination.
- The combination revealed anti-arthritic activity.

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