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RESEARCH ARTICLE

Evaluating the Efficacy of a Polyherbal Formulation in Ameliorating Arthritis Induced by Freund's Complete Adjuvant

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Abstract

Background: Rheumatoid Arthritis (RA) is a persistent autoimmune condition associated with severe repercussions. The study assesses the potential antiarthritic effects of a Polyherbal Formulation (PHF) containing extracts from *Tinospora cordifolia, Rosa damescena*, and *Acacia leucoploea* in a rat model of RA, with a focus on evaluating its impact on joint inflammation, bone degradation, and cartilage preservation. **Methods:** Arthritis was induced using Freund's Complete Adjuvant (FCA), and animals were treated with PHF (200 and 400 mg/kg), prednisolone, or control treatments for 28 days. Various parameters were assessed in this study, including body weight, paw volume, arthritis severity score, haematological measurements, serum markers (creatinine, ALP, total proteins), cytokine levels (IL-1β, IL-6, IL-10, and TNF-α), and radiographic alterations. **Results:** FCA-treated rats exhibited significant body weight loss, paw oedema, increased arthritis severity scores, altered haematological parameters, and elevated serum markers compared to normal controls. PHF treatment at both doses mitigated body weight loss, reduced paw oedema, and improved arthritis severity scores. Hematological changes induced by FCA were also attenuated by PHF treatment. Serum creatinine, ALP, and total protein levels, elevated in FCA-treated rats, were significantly improved by PHF. Furthermore, PHF modulated cytokine levels, decreasing IL-1β, IL-6, and TNF-α while increasing IL-10. Radiographic analysis displayed reduced joint damage in PHF-treated rats compared to FCA controls. **Conclusion:** This comprehensive investigation highlights PHF's potential to mitigate the inflammatory processes associated with RA, as evidenced by improved clinical, haematological, and biochemical parameters. The study underscores the promise of traditional herbal compounds in managing RA and suggests PHFs as novel therapeutic options. Further mechanistic studies are warranted to elucidate the exact pathways involved.

Keywords: Arthritis, Formulation, Herbs, Radiographic Analysis, Traditional Medicine

1. Introduction

Millions of people worldwide suffer from Rheumatoid Arthritis (RA), a chronic autoimmune disease that manifests as an intricate interplay of inflammatory processes in the synovial membrane and joints[1]. Characterized by painful joint swelling, morning stiffness, and synovial inflammation, RA can lead to joint destruction, bone degradation, and substantial loss of function[2]. It poses a significant burden on both patients and healthcare systems due to its chronic nature and debilitating consequences. Environmental triggers

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and genetic predisposition both play a role in the complex aetiology of RA. An important environmental risk factor linked to the beginning of RA is tobacco use[3]. About 0.92% of Indian adults suffer from Rheumatoid Arthritis (RA). Each year, there are between 20–40 new cases per 100,000 people in Lac, with females being more frequently affected[4,5]. Increased expression of certain cytokines, such as Tumour Necrosis Factor (TNF)-α and Interleukins (ILs), is crucial in promoting the inflammatory and degenerative processes seen in RA[6,7]. Non-steroidal anti-inflammatory medicines (NSAIDs), Disease-Modifying Antirheumatic Drugs (DMARDs), and biologic therapies that target cytokines are frequently used in conventional therapy approaches for RA. However, these treatments may be limited by economic constraints, potential side effects, and variations in patient responses[8]. As a result, Complementary and Alternative Medicine (CAM) modalities have gained popularity among RA patients as adjunct or alternative treatments. *Ayurveda* and acupuncture, in particular, have demonstrated efficacy in providing relief for RA symptoms[9,10].

Within CAM, herbal medicine holds promise for addressing the complexities of RA. Centuries of traditional knowledge have yielded a repertoire of herbal remedies used to manage arthritis-related symptoms. Despite the long history of herbal medicine in arthritis treatment, scientific validation of the pharmacological properties of individual herbs and their combinations remains an ongoing pursuit[11]. Many plant-based compounds possess anti-inflammatory, immunomodulatory, and antioxidant properties that could contribute to RA symptom alleviation[12]. In this study, we embark on a comprehensive exploration of the antiarthritic potential of a Polyherbal Formulation (PHF) in an animal model of RA. Combining the principles of traditional medicine with modern scientific scrutiny, we formulated the PHF based on a selection of herbs known for their antiarthritic effects. We address the synergistic effects of *T. cordifolia* Miers, *A. leucoploea*, and *R. damascena*, each bringing distinct bioactive compounds to the formulation.

By evaluating the PHF's effects on joint inflammation, bone loss, and cartilage preservation, our work intends to address the knowledge gap between conventional wisdom and current research. We seek to elucidate the mechanism of action behind the possible antiarthritic effects of the PHF by employing a well-established preclinical model. With this strategy, we hope to advance the investigation of PHFs as novel therapeutic alternatives and add to the established body of research that supports the use of herbal chemicals in the treatment of RA.

2. Materials and Methods

2.1 Chemicals

Chemicals like prednisolone and Freund's Complete Adjuvant (FCA) were procured from Sigma Pvt. Ltd. India. Biochemical kits for ALP, ALT, and AST, were procured from ERBA UK. ELISA kits for IL-1β, IL-6, IL-10 and TNF-α, were obtained from Elabsciences USA.

2.2 Animals

Healthy Wister albino rats of female animals (150-200 gm) were utilized. Animals procured from Raghavendra Enterprises, Bangalore. The animals were housed individually in clean polypropylene cages in the air-conditioned room where the temperature was 22 ± 20°C with 50% ± 10% relative moisture with a 12 h dark and light cycle. All over the study, animals were maintained at normal laboratory circumstances and were given commercial laboratory animal feed and water with libitum. Before conducting experiments, ethical permission was taken from the Institutional Animal Ethics Committee, Sri Padmavathi School of Pharmacy, Tiruchanoor (SPSP/CPCSEA/IAEC-1016/a/2020/015).

2.3 Plant Material

Taxonomically identified mature fresh stems of *T. cardifolia* bark of *A. leucopholea* and petals of *R. damescena* Linn were purchased from a local vendor.

2.4 Preparation of Plant Extract

The coarse powder made from the shade-dried stem pieces of *T. cordifolia,* petals of *R. damescena* and bark of *A. leucopholea* were extracted by macerated for 72 hrs with ethanol at room temperature. Individual plant extracts were collected in conical flasks, and filtered, and the solvents were evaporated under a reduced pressure until the extracts were dry.

2.5 Preparation of Poly-herbal Formulation

According to the ED_{50} of individual herbs, a Polyherbal Formulation (PHF) was developed. ED_{50} of *T. cardifolia* (517.81 mg/kg)[13], *A. leucopholea* (200 mg/kg). *R. damascena* (600 mg/kg)[14]. The percent contents of PHF were calculated from individual ED_{50} of the plant extracts as, *T. cardifolia* (20%), *A. leucopholea* (20%), and *R. domescena* (60%). PHF at a dose of 200mg/kg and 400mg/kg were used for the present study according to ED_{50} values from previous studies.

2.6 Induction of Arthritis

On the 1st day, all the animals except normal control animals, Freund's Complete Adjuvant (FCA) (0.1ml) was injected into the sub plantar area of the left hind paw. FCA consists of dead *Mycobacterium butyricum* particles suspended in thick liquid paraffin oil.

2.7 Experimental Design

Twenty-four adult female Wistar rats with initial weights ranging from 150g to 200g were evenly distributed into five groups, each consisting of six rats. Rats in Groups I and II were given 0.5% sodium carboxy methyl cellulose (Na CMC) at a dosage of 3 ml/kg/day for a total of 28 days as the normal and FCA control groups, respectively. Group III received prednisolone as the standard control, with a dose of 0.885 mg/kg administered orally. Groups IV and V were subjected to treatments with PHF at doses of 200 mg/kg and 400 mg/kg, respectively. The test formulation and the standard drug were administered for 28 days.

2.8 Assessment of the Body Weight and Paw Volume

Body weight was taken on the first day and the final day. Hind paw volume was measured on the first day of FCA treatment and then every 7 days for the next 28 days. Paw volume was monitored using a digital plethysmometer every week to assess the level of paw oedema caused by FCA injection and suppressed by different therapies. To calculate the % inhibition of FCA-induced arthritis, the following formula was used[15].

$$\%\ \text{protection from paw oedema} = \frac{V_{control} - V_{treated}}{V_{control}} \times 100$$

where $V_{control}$ is the paw volume of the control animals and $V_{treated}$ is the paw volume of the treated animals.

2.9 Arthritis Severity Scoring

The severity of the arthritis was evaluated and assessed on a scale of 0 to 4. The severity of arthritis was categorized based on the following parameters:

- No oedema at all (grade 0)
- Minor swelling or erythema in a finger (grade 1)
- Diabetic finger swelling (grade 2)
- Ankle or wrist swelling (grade 3)
- Significant arthritic swelling in the fingers and wrist (grade 4)

2.10 Estimation of the Blood Parameter

On day 29, blood samples were collected and divided into two separate tubes, with one of the tubes containing the anticoagulant dipotassium EDTA. By puncturing the retro-orbital plexus, the blood was collected. A blood cell counter was used to estimate the haemoglobin, Red Blood Cells (RBC), and White Blood Cells (WBC) levels in the blood sample that was drawn into a tube containing the anticoagulant. The serum obtained from the blood sample without anticoagulant was subjected to centrifugation at 2,500 g for 10 minutes. Subsequently, the levels of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), and cytokines were quantified in the serum.

2.11 Estimation of Serum Creatinine, ALP and Total Proteins

Serum levels of serum creatinine, Alkaline Phosphatase, and total proteins were determined using a biochemical diagnostic kit, following the instructions provided by the kit manufacturer. An EM 360 (ERBA) autoanalyzer was used for this purpose. The results for serum creatinine, ALP, and total proteins were reported in units of U/L (units per litre) of the serum.

2.12 Estimation of Cytokines

ELISA kits were used to measure the levels of the pro-inflammatory cytokine IL-1, IL-6, and TNF-α, as well as the IL-10 an anti-inflammatory cytokine. Using standard curves, the concentrations were determined and reported in picograms per millilitre (pg/ml) of serum.

2.13 Radiographic Analysis

Rats were given FCA to induce arthritis, and X-rays of their knee joints were taken to verify and assess the severity

of the arthritis. All of the rats received intraperitoneal injections of sodium thiopental 40 mg/kg to produce anaesthesia following the study's conclusion. The rats were then placed on X-ray plates, and photographs of the left ankle joint were obtained. Criteria like calcification, erosion, and periosteal response, which can result in bone thickening and the destruction of bone structure, were looked at in the tarsometatarsal region.

2.14 Statistical Analysis

SPSS version 26 and ORIGIN software were used for the statistical analysis and data visualisation respectively. The Standard Error of the Mean (SEM) and mean values were displayed with the data. Group differences were assessed using Tukey's post hoc test and a one-way ANOVA. When grading the data, the non-parametric Kruskal-Wallis test was used. To establish statistical significance, a cutoff point of $p<0.05$ was employed.

3. Results

3.1 Body Weight

Animal body weights were similar across all groups at the time of grouping. When compared to the normal control animals, animals administered FCA lost 13.4% of their body weight, with the data being statistically significant ($p<0.001$). PHF 200 and PHF 400 administration caused a considerable increase in body weight in the FCA-treated rats (Figure 1), with growth rates exceeding those of the FCA-treated group by 4.8% and 8.7%, respectively ($p< 0.001$).

3.2 Paw Volume

Animals treated with FCA developed paw oedema, which was demonstrated by twice ($p<0.001$) the paw volumes that were measured on all days. When RA-induced animals were treated with PHF (200 and 400 mg/kg), the paw volume (Figure 2A) significantly decreased over all periods that were studied on days 14, 21, and 28 respectively.

3.3 Arthritis Severity Score

Arthritis severity was significantly exacerbated in rats treated with FCA when compared to untreated control rats. The severity score exhibited a significant increase on all the testing days ($p<0.001$). However, the rats that received PHF treatment (400 mg/kg) after the FCA treatment showed a notable improvement in their

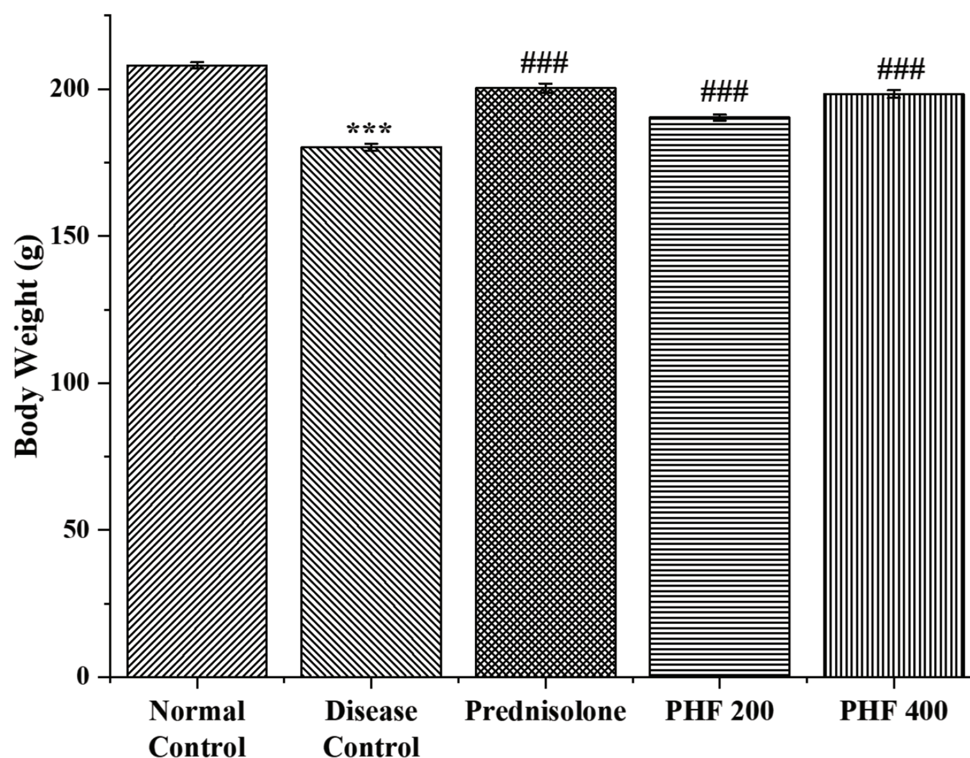

Figure 1. Impact of PHF on body weight of FCA induced RA rats. Results are shown as mean ± SEM. $^{###}p<0.001$ in comparison to disease control rats and $^{***}p<0.001$ in comparison to normal control rats.

arthritic conditions, resulting in lower scores compared to the FCA control group. The severity score on day 29 was significantly lower ($p<0.05$) than that of the FCA control group. Figure 2B-E illustrates the results of arthritis severity scores.

3.4 Blood Parameters

3.4.1 Haematological Parameters

The use of FCA led to a reduction in RBC and Hb levels ($p < 0.05$), along with an increase in ESR and WBC count ($p < 0.05$) when compared to the normal control group. However, both doses of PHF significantly ($p<0.01$) mitigated the haematological alterations caused by FCA. The findings for haematological parameters are displayed in Table 1.

3.4.2 Serum Creatinine, ALP and Total Proteins

Creatinine, ALP, and total protein serum levels were considerably ($p < 0.05$) increased in FCA-treated animals than in the normal control group. On the other hand, PHF at 200 and 400 mg/kg significantly decreased ($p<0.01$) the levels of total protein, ALP, and serum creatinine compared to the FCA control group. Table 2 contains the information for total protein, ALP and serum creatinine levels.

3.4.3 Effect of PHF on Cytokines

The pro-inflammatory cytokines IL-1, IL-6, and TNF-α were significantly ($p<0.001$) greater in rats treated with FCA than in normal control rats, whereas the anti-inflammatory cytokine IL-10 was significantly ($p<0.001$) lower in these same rats. When compared to the FCA control group, PHF at a dose of 400 mg/kg significantly elevated IL-10 levels ($p<0.01$) and decreased IL-6 ($p<0.01$) and IL-1β ($p<0.001$). In the same way, PHF in both doses increased TNF-α in FCA-treated rats ($p<0.01$ and $p<0.001$). The results of the cytokine estimations are shown in Figure 3.

3.4.4 Radiographic Analysis

The radiographic pictures of the knee joints in the various rat groups are shown in Figure 4. The radiographs unequivocally show that the adjuvant treatment-exposed rats exhibited traits such as periosteal response, uneven joint space, soft-tissue oedema, and a reduction in joint space. The joint spaces of the PHF-treated groups and the control group, in comparison, were normal, there was no periosteal reaction seen, and the joints looked healthy.

4. Discussion

RA is a chronic, progressive autoimmune condition that affects numerous joints, and causes deformity, disablement, and early mortality in most sufferers. A symbiotic network of cytokines, proteolytic enzymes, and prostanoids is involved in the complicated pathophysiology of RA[16]. Considering the potential side effects of the available conventional therapy, we have formulated polyherbal formulation with different plant extracts that have the property of antiarthritic activity. PHF consists of extracts from the stem of *T. cordifolia,* petals of *R. damescena* and bark of *A. leucopholea* in the ratio of 1:3:1. The stem extract of *T. cordifolia* was proven for its antiarthritic activity in FCA model[17,18]. The hydro-alcoholic extract of petals of *R. damascene* has been proven for its analgesic and anti-inflammatory activity[14]. Similarly, the bark extract had alleviated inflammation[19].

Rheumatoid arthritis pathogenesis is frequently studied using the adjuvant-induced arthritis model created by Complete Freund, which is typified by a rapidly erosive disease. FCA contains both muramyl

Table 1. Effect of PHF on haematological parameters in FCA-induced arthritis in rats

S. No.	Group	RBC ($\times 10^6$cells/μl)	Hb (g/dl)	ESR (mm/hr)	WBC ($\times 10^3$cells/μl)
1	Normal Control	5.35±0.09	12.6±0.14	3.5±0.12	8.43 ±0.45
2	Disease Control	3.08±0.05*	8.23±0.17*	8.02±0.23*	14.02±0.56*
3	Prednisolone	5.30±0.11##	12.7±0.26##	4.02±0.54##	9.12±0.03##
4	HPF 200	5.00±0.04##	11.4±0.16##	5.02±0.04##	10.13±0.11##
5	HPF 400	5.14±0.42##	12.3±0.22##	5.34±0.17##	9.02±0.71##

Results are shown as mean ± SEM. ##$p<0.001$ in comparison to disease control rats and *$p<0.05$ in comparison to normal control rats.

Table 2. Effect of PHF on haematological parameters in FCA-induced arthritis in rats

S. No.	Group	Serum Creatinine (mg/dl)	ALP (U/L)	Total Protein (g%)
1	Normal Control	5.49±0.32	50.54±0.02	3.62±0.14
2	Disease Control	15.90±0.45*	82.88±0.04*	8.66±0.96*
3	Prednisolone	5.28±0.34##	52.04±0.06##	4.02±0.24##
4	HPF 200	5.02±0.4##	50.42±0.03##	5.78±0.96##
5	HPF 400	4.15±0.32##	54.44±0.04##	4.45±0.72##

Results are shown as mean ± SEM. ##p<0.001 in comparison to disease control rats and *p<0.05 in comparison to normal control rats.

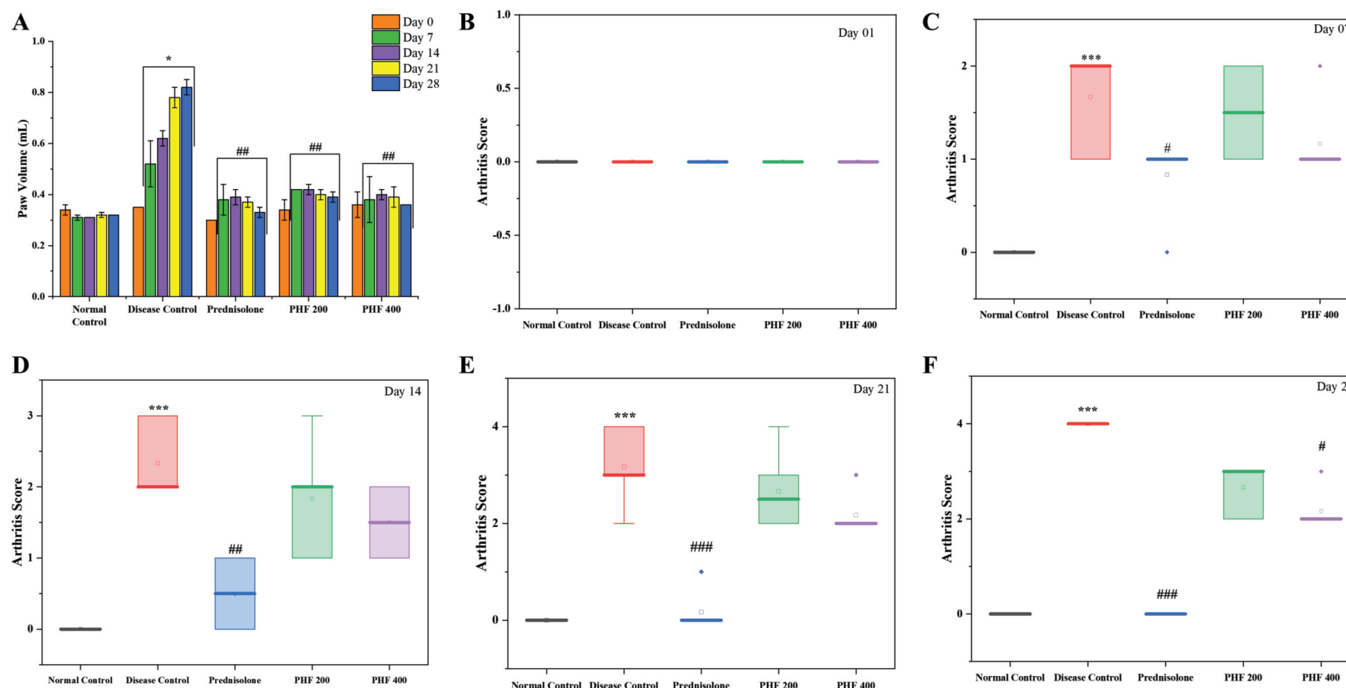

Figure 2. **(A).** Paw volume on 0, 7, 14, 21, and 29 days, **(B-E).** Arthritis severity score in FCA treated rats. Results are shown as mean ± SEM. *p<0.05, **P<0.01, ***P<0.001 in comparison to rats in the normal control group, and #p<0.05, ##p<0.01, ###p<0.001 in comparison to rats in the disease control group.

dipeptide and bacterial peptidoglycan, which cause adjuvant arthritis[20].

Rheumatoid cachexia, a condition associated with arthritis, is characterised by weight loss and loss of lean body mass[21]. The notable alterations in body weight observed in rats treated with FCA are consistent with findings from other research studies[22].

The intensity of arthritis is quantified through the arthritic score, which serves as a measure to evaluate joint inflammation[23]. The finding that PHF-treated rats had much lower arthritic ratings than FCA-treated rats suggests that PHF has anti-inflammatory properties. Paw oedema can be swiftly and sensitively assessed to assess the degree of inflammation and the therapeutic and curative effects of drugs. Rats receiving FCA had larger paws, which was consistent with other studies that had been published[24]. On the other hand, administering PHF to rats who had been injected with FCA reduced the oedema in their paws. This finding demonstrates PHF's ability to reduce inflammation brought on by FCA in the paw.

The arthritic rats in the current study displayed decreased RBC counts, reduced Hb levels, and elevated ESR. These manifestations collectively indicate the presence of anaemia, a common symptom in chronic arthritis patients. The relative concentrations of plasma proteins, particularly fibrinogen and globulins, as well as variables like the quantity and size of RBCs,

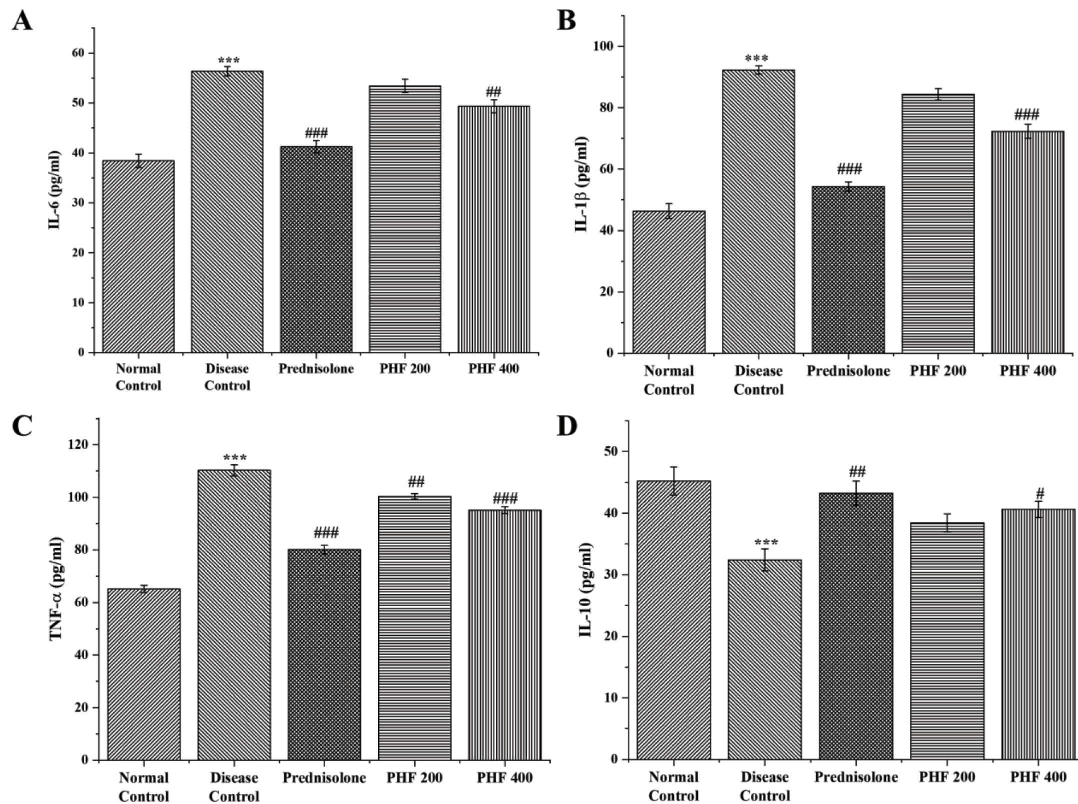

Figure 3. Impact of PHF on the levels of **(A).** IL-6 **(B).** IL-1β **(C).** TNF-α **(D).** IL-10. Results are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 in comparison to rats in the normal control group, and $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 in comparison to rats in the disease control group.

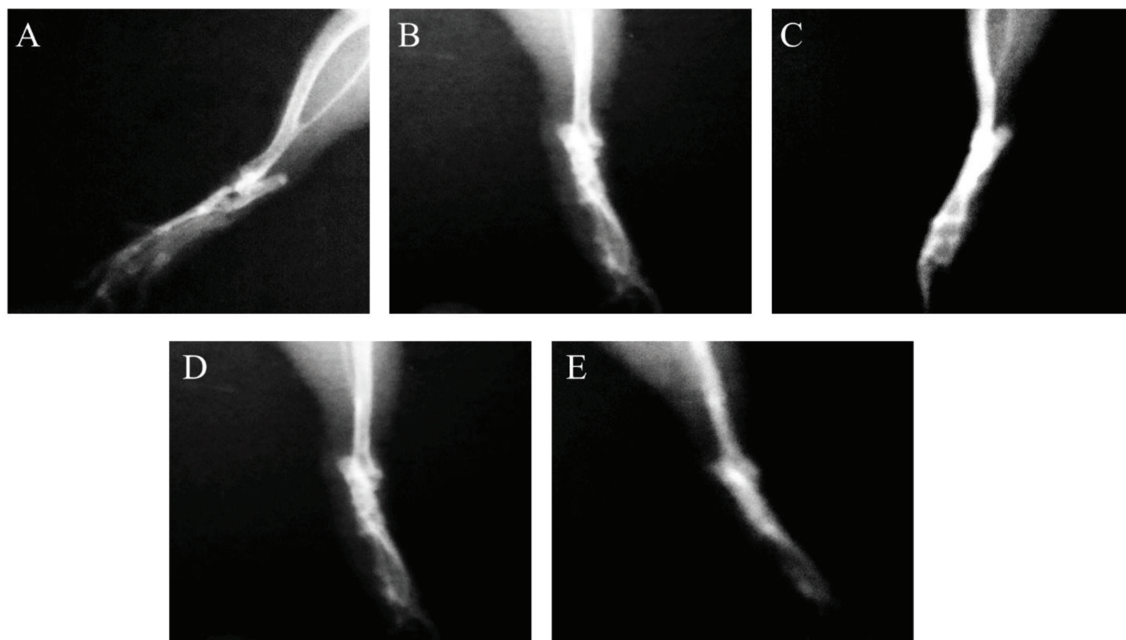

Figure 4. Radiographic assessment of PHF on **(A)**. Normal control, displaying a healthy knee bone structure, **(B).** FCA Control, showing characteristic abnormalities, **(C).** FCA-induced arthritis treated with prednisolone, **(D).** FCA-induced arthritis treated with PHF at a dose of 200 mg/kg, exhibiting reduced joint changes, **(E).** An animal with FCA-induced arthritis treated with PHF at a dose of 400 mg/kg, also displaying reduced joint alterations.

affect ESR. An increased ESR is a sign of active but difficult-to-identify illness processes. Similar to how injections, wounds, surgeries, or tissue necrosis cause inflammation, acute phase proteins in ESR do the same[25]. An elevated White Blood Cell (WBC) count serves as an indicator of infections and inflammatory conditions[26].

Chronic inflammation causes macrophage activation, which releases cytokines that have been linked to immunological arthritis. Inflammatory areas overexpress IL-6, which can be very important in chronic inflammation[6]. TNF-α, IL-1 and IL-6 boost the production of acute-phase proteins[24]. Furthermore, it triggers specific humoral and cellular immunological responses, such as T-cell activation and B-cell differentiation. Proinflammatory cytokines (IL-1β, IL-6, and TNF-α) were found to significantly increase in rats treated with FCA, whereas anti-inflammatory cytokine (IL-10) levels were found to significantly decrease. In rats with arthritis, PHF therapy led to a significant decrease in TNF-α, IL-6, and IL-1β levels, suggesting that PHF has an anti-inflammatory impact. In the hind paw bones of the arthritis-induced rats, radiological examinations regularly revealed a significant reduction or, nearly complete lack of joint gaps. The results show that PHF has antiarthritic activity possibly due to its effect on the immune and haematopoietic systems as evidenced by the modulation of inflammatory cytokines[11].

5. Conclusion

The current experimental investigation indicates that PHF possess both anti-inflammatory and anti-arthritic activities. The PHF brought about its effect in FCA-induced arthritic rats by decreasing paw volume, correcting haematological parameters creatinine, modulating cytokine levels and restoring knee joint arrangement. The precise mechanism of PHF and its usefulness in therapeutic contexts need to be determined through additional research.

6. Ethics Statement

The Institutional Animal Ethics Committee of Sri Padmavathi School of Pharmacy, Tiruchanoor had approved the protocol for conducting experiments on animals with the approval number SPSP/CPCSEA/IAEC-1016/a/2020/015.

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