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Anti-arthritic potential of *Biophytum sensitivum* in Freund's adjuvant induced arthritis in rats

Dr. Jigneshkumar I. Patel*[1], Dr. Dipen R. Bhimani[1]
1.Lecturer, B.K. Mody Government Pharmacy College, Polytechnic campus, Near Aji Dam Chokdi, Rajkot-360003, India

ABSTRACT
Rheumatoid arthritis (RA) is one of the most common inflammatory disorders affecting the population worldwide. Herbal treatments are gaining popularity as complementary alternative medications among patients due to their potential benefits and minimal side effects. *Biophytum sensitivum* is known traditionally to possess a wide spectrum of medicinal properties and used in various inflammatory diseases such as asthma, ulcerative colitis and arthritis. The methanolic extract of Biophytum *sensitivum* is reported to have inhibitory action on proinflamatory cytokines (TNF-α, IL-1b, IL-6) and NF-Kb. The methanolic extract of *Biophytum sensitivum* also Possess angiostatic property (angiogenesis, play a significant role in pathogenesis of reumatoid arthritis). On the basis of above mentioned facts, the present study is undertaken to evaluate the anti-arthritic potential of methanolic extract of whole plant of *Biophytum sensitivum* in Freund's adjuvant induced arthritis in rats. The methanolic extract of whole plant of *Biophytum sensitivum* (MeBS) was prepared. Rats were divided into six groups having six rats in each group. Group 1 served as vehicle control and received saline solution 2ml/kg. All the other groups had received 0.1 ml injection of Complete Freund's Adjuvant at sub plantar region of left hind paw of rat to induce arthritis experimentally. A time period of 7 days was given for induction of arthritis. After 7 days, rats were started on various drug regimens that were continued upto 3 weeks. Group 2 which is assigned as disease control had received only CFA injection (0.1ml) on day 1.Group3 which is standard group had received methotrexate (50µg/kg/week, p.o.). Group 4, 5 & 6 were treatment groups and had received MeBS (100,200& 400mg/kg/day, p.o.), respectively. Anti- arthritic potential of MeBS was evaluated by Complete Freund's adjuvant induced arthritis model. Assessment of body weight, paw thickness and paw volume in both hind limbs (i.e CFA injected and CFA non injected left and right hind paw respectively), arthritic swelling, inflammatory markers, radiological and histopathological examination were carried out. Methanolic extract of Biophytum sensitivum (MeBS) showed significant reduction ($p<0.05$) in paw thickness, paw volume of both hind limbs and in arthritic swelling. MeBS also showed significant reduction ($p<0.05$) in levels of inflammatory markers such as Rheumatoid factor, C-Reactive protein and Erythrocyte Sedimentation Rate. Radiological and histopathological examination supported the anti- arthritic effect of MeBS. The findings of present study reveals that MeBS (400mg/kg) shows beneficial effects against CFA induced arthritis model of rats.

Keywords: Rheumatoid arthritis, CFA, Methanolic extract of Biophytum sensitivum.

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INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common inflammatory disorders affecting the population worldwide. It is an autoimmune disease in which the body's immune system mistakenly attacks the synovial membrane of the joints resulting in swelling and pain in and around the joints.[1,2] Rheumatoid arthritis is a chronic, progressive, systemic, disease which not only affects the joints but a wide range of extra-articular organs. It is generally characterized by inflammation and swelling (hyperplasia) of synovial membrane, production of autoantibodies (rheumatoid factor and anti- cyclic citrullinated peptide), bone and cartilage and destruction, and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders.[1,3] Polyarthralgia with symmetrical, migratory or intermittent joint involvement, especially in the hands and feet are most seen clinical features of RA. Proximal interphalangeal and metacarpophalangeal joints of the fingers, interphalangeal joints of the thumbs and wrists, and metatarsophalangeal joints of the toes are typically affected during the early stages of the disease. [1,4] Other joints of the upper and lower limbs, such as the shoulders, elbows and knees are also affected. Morning stiffness which is one of the common clinical manifestations associated with rheumatoid arthritis, may last for 30 minutes to several hours, and usually reflects the severity of joint inflammation. [1]

RA is a potentially fatal disease with increased prevalence of other serious illnesses which can lead to an average decrease of life expectancy upto7–10 year and increases the mortality rate about two folds. The predominant conditions that lead to the increased rate of co- morbidity and mortality include, renal impairment, cardiovascular disease, infections and lymphomas. [1] Approximately 1% of world population is affected by rheumatoid arthritis with women being two to three times more commonly affected than men. Prevalence of RA increases with age in both sexes; it affects nearly 5% of women and 3% of men over the age of 65. The peak age of incidence is about 30–50 years in women and slightly older in men. [1,5] Rheumatoid arthritis also affects young children. The Prevalence of RA in India is 0.7%. [6]

Although the cause remains unclear, RA is considered to be due to faulty response of the immune system with genetic, hormonal, and environmental factors playing a key role. The environmental risk factors that have been associated with RA are mainly alcohol intake and smoking. It increases the risk up to 40 times as compared with non-exposed groups. Genetic factors contribute about 53–65% of the risk of developing this disease. These are linked with a series of genes that carry information related with RA mainly those that regulate the HLA major histocompatibility complex and some other factors such as T cell signaling genes, cytokine promoters, and many others. [7, 8]

Pathologically, rheumatoid arthritis is characterized by the infiltration of a variety of inflammatory cells into the joint. Normally the synovial membrane is acellular which now becomes hypertrophied and highly vascularized resulting into the formation of a so-called pannus (an abnormal tissue growth in joints which causes bone deterioration, cartilage destruction, inflammation and pain). There is proliferation of synovial fibroblasts and an increase in the number of inflammatory cells present within the joints. The inflammatory cells involved in rheumatoid arthritis include B-cells, T-cells (largely CD4 helper cells), plasma cells and macrophages. These cells release cytokines which causes the synovium to release proteolytic enzymes, which in turns results into the destruction of bone and cartilage. Interleukin-1(IL-1), Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF)-α, and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) are key cytokines involved in rheumatoid arthritis. These play a crucial role in the pro inflammatory reaction. [1]

The treatment strategy for RA aimed at relieving pain, improving mobility and arrest in articular damage.[9] Non-pharmacological management includes occupational therapy, physiotherapy, education and psychological support. Early treatment with aggressive DMARDs (disease-modifying anti-rheumatic drugs) should commence, on diagnosis of rheumatoid arthritis, to prevent joint destruction. Patients who do not respond to DMARDs treatment, cytokine inhibitors can be used. There are several therapeutic agents available which target different cytokines in the inflammatory pathway. For symptomatic relief NSAIDs (Non-steroidal anti-inflammatory drugs) may be considered and should be co-prescribed with a proton pump inhibitors. Steroids are useful as combination therapy with DMARDs in the early stages of DMARDs introduction for rapid relief in symptoms associated with rheumatoid arthritis. [1, 10]

Though these drug therapies including NSAIDs, DMARDs, corticosteroids and monoclonal antibodies are capable of reducing inflammation and cartilage degradation to a varied extent, their chronic use can lead to severe side effects including serious secondary complications and oxidative stress. [9, 10] Patients are in need of complementary and alternative medicines because of potential side effects associated with chronic use of allopathic medication used for the treatment of rheumatoid arthritis. In Ayurveda and other Indian literature many herbal plant description are mention for their different use. India has about 45,000 plant species and among them most of possesses medicinal properties.[11] There are number of herbal plants from the indigenous sources that are used for the treatment of rheumatoid arthritis. Some of them are Alpinia galangal, Coriandrum sativum, Mimosa pudica, Mangifera indica, Ocimum basilicum, Piper nigrum, Rubia cordifolia and Vitex negundo. [12,13] Biophytum sensitivum has traditionally been used in various

inflammatory diseases such as asthma, ulcerative colitis and arthritis. [14,15] But there is no reported evidence to prove its anti arthritic activity. So, an attempt was made to investigate and evaluate the anti- arthritic potential of *Biophytum sensitivum* in experimentally induced arthritis in rats.

MATERIALS AND METHOD

Plant collection and authentication

The whole plants of *Biophytum sensitivum* were purchased from AgroPack, Chenganur, Kerala. Plant authentication was done at School of Science, Department of botany, RK university, Rajkot.

Plant extraction [15]

Whole plants of *Biophytum sensitivum* were washed thoroughly with water and shade dried at room temperature. Dried plants were powdered and 100gm powder was extracted with 500ml of 80% methanol in a soxhlet apparatus. The solvent was evaporated to dryness using water bath. The final product obtained is weighed and stored in an airtight container.

Preliminary Phytochemical screening of methanolic extract of *Biophytum sensitivum* [16, 17]

Preliminary phytochemical screening of the methanolic extract of *Biophytum sensitivum* was performed for the presence of alkaloids, glycosides, tannins, flavanoids, saponins, steroids, carbohydrates and amino acid.

LC-MS analysis of Methanolic extract of *Biophytum sensitivum*.

An efficient liquid chromatography– electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) method was developed for identification and simultaneous determination of Bioflavonoid in methanolic extraxt of *Biophytum sensitivum*. Specification of LC-MS method: (1) Column: Gemini C18,(250mm x 4.6mm, 5µm), (2) Mobile Phase: 5mM Ammonium Acetate in water: Methanol,(30:70) (3) Flow rate : 1.00mL/min (4) Column oven temperature : 40±0.3˚C (5) Auto sampler temperature: 15±3˚C (6) Volume of injection: 20µl (7) Detector: PDA & Mass detector (8) Detection wavelength: 254 nm (9) Run time: 20.0 minutes (10) Ion Source: Electro spray ionization (11) Polarity: Positive & Negative.

Animal selection

Albino wistar rats of either sex was used. They were housed at temperature (22±10C), relative humidity (30-70%) and 12h/12h light dark cycle and provided pelleted diet and purified drinking water. The protocol of the experiment was approved by the Institutional Animal Ethics Committee (IAEC) as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Approved Protocol number was BKMGPC/IAEC22/RP49/2018.

Complete Freund's Adjuvant (CFA) induced arthritis model in rats. [18-20]

Rats were divided into six groups having six rats in each group. Group 1 served as vehicle control and received saline solution 2ml/kg. All the other groups had received 0.1 ml injection of Complete Freund's Adjuvant at sub plantar region of left hind paw of rat to induce arthritis experimentally as shown in table 1. A time period of 7 days was given for induction of arthritis. After 7 days, rats were started on various drug regimens that were continued up to 3 weeks. Group 2 which is assigned as disease control had received only CFA injection (0.1ml) on day 1. Group3 which is standard group had received methotrexate (50µg/kg/week,p.o.). Group 4, 5 & 6 were treatment groups and had received MeBS (100,200& 400mg/kg/day, p.o.), respectively as shown in table 1.

Table 1: Study design- Complete Freund's Adjuvant (CFA) induced arthritis model in rats.

Group (n=6)	Induction of arthritis	Treatment
Group 1. Normal control	---	Normal saline per oral from day 1 to day 28
Group 2. Disease control	0.1ml Complete Freund's Adjuvant (CFA) injected (subplantar injection) at left hind paw of rat on day 1	---
Group 3. Standard		Methotrexate(50µg/kg/week). per oral administered from day 8 to day 28
Group 4. Treatment (MeBS 100)		MeBS (100mg/kg) per oral, administered from day 8 to day 28
Group 5. Treatment (MeBS 200)		MeBS (200mg/kg) per oral administered from day 8 to day 28
Group 6. Treatment (MeBS 400)		MeBS (400mg/kg) per oral administered from day 8 to day 28

Evaluation parameters

Physical parameters [18, 20]

Body weight (gm), paw volume (ml) and paw thickness (mm) were measured on 0, 7th, 14th, 21th , 28th day after injection of CFA. Paw volume was measured using digital plethysmometer. The percentage reduction in paw volume was calculated by the following equation: % reduction in Paw volume = 100 [1-(Vt/Vc)] Where, Vc = paw volume in the control, Vt = paw volume in test.

Evaluation of arthritis swelling [21]

Joint swelling will be scored as per the arthritis index. A score of 0–4 will be assigned as follows:

0 = No evidence of hyperemia and/or inflammation

1 = Hyperemia with little or no paw swelling,

2 = Swelling confined predominantly to the ankle region, with modest hyperemia,

3 = Increased paw swelling and hyperemia of the ankle and metatarsal regions,

4 = Maximal paw swelling and hyperemia involving the ankle, metatarsal, and tarsal regions.

Estimation of Blood Parameters: RF, ESR and CRP [18,21]

After 28 days, rats were anaesthetized and blood was withdrawn through the retro-orbital vein puncture. ESR was estimated by Westergren method. CRP and RF were measured by agglutination test.

Radiographical study [22]

On 28th day of study, X-ray Radiographs of rats from each group were recorded on X-ray machine. Radiological visual evaluation was performed for the following conditions: i. Erosions ii. Joint space narrowing iii. Joint space destruction.

Histopathology of joints [22]

After the 28th day, animals were sacrificed; ankle joints were removed from left hind paw of rats and fixed for 4 days in 10% formaldehyde. After decalcification in 3% nitric acid, the tissues were processed for paraffin embedding and sections were taken using a microtome. The tissue sections (5µm thick) were stained with haematoxylin and eosin and were examined microscopically for histopathological changes.

Histopathological changes of arthritis damage were evaluated for following characteristics: Inflammatory cells in the synovial tissues, Destruction of articular cartilage, Bone and cartilage erosion and Cartilage and bone destruction by pannus formation and vascularity.

Statistical analysis

Data will be expressed as mean ± SEM. Significant differences will be detected using one-way analysis of variance (ANOVA) followed by post test using computer based fitting program (Graphpad prism 8.0) for multiple comparisions. $P < 0.05$ will be considered statistically significant.

RESULTS AND DISCUSSION

Plant extraction

The extraction of powdered plants of *Biophytum sensitivum* was carried out using 80% methanol at 40-45˚C by soxhlet extraction method. The final product obtained was dark green in colour. The percentage yield of the extract is 12 % W/W.

Preliminary phytochemical investigation

The Methanolic extract of *Biophytum sensitivum* was subjected to phytochemical investigation. The results revealed the presence of flavonoids, tannins (phenolic compounds), glycosides, saponins, steroids, carbohydrates and proteins.

LC- MS analysis of methanolic extract of *Biophytum sensitivum*

LC-MS data of methanolic extract of *Biophytum sensitivum* extract shows major ion product at m/z 537.1 for amentoflavone (Mw=354.35 g/mol) which confirmed the presence of bioactive

constituent amentoflavone in the methanolic extract of whole plant of *Biophytum sensitivum.*

Evaluation Parameters

Effect of MeBS on Body weight of animals

The average gain and loss in the body weight of animals on day 0, 7, 14, 21 and 28 as compared with the initial body weight in each treatment group has been given in Figure 1. The animals in the disease control group showed gradual decrease in body weight; however, the animals in other groups continue to grow normally.

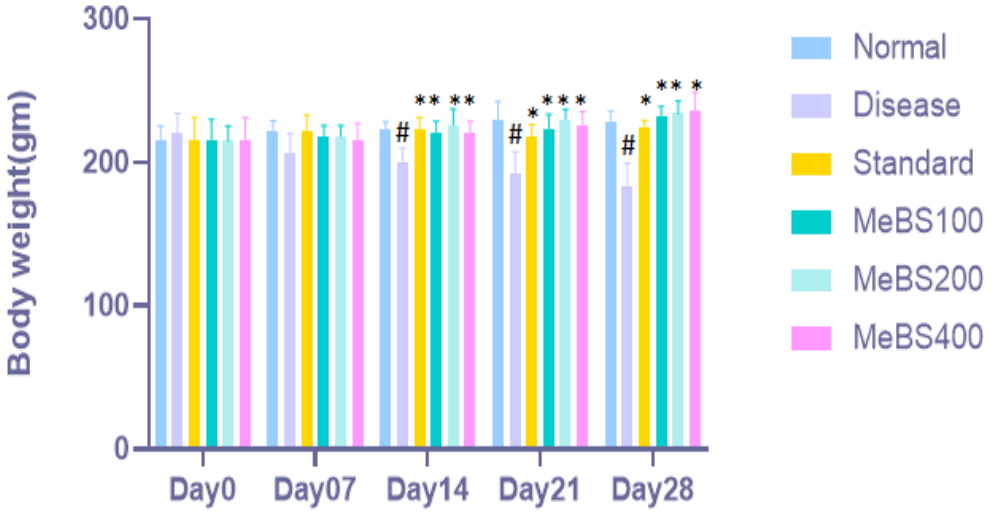


Figure 1. Effect of MeBS on body weight of animals

All values are expressed in Mean ± SEM; n = 6 for each group

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

Effect of MeBS on left hind paw thickness (paw injected with CFA) of animals

Thickness of left hind paw in animals of disease control group was significantly higher as compared to normal control group on day 14, 21 and 28. There was significant reduction in paw thickness in all treated groups as compared to that in disease control group on day 28 (P<0.05) as shown in figure 2.

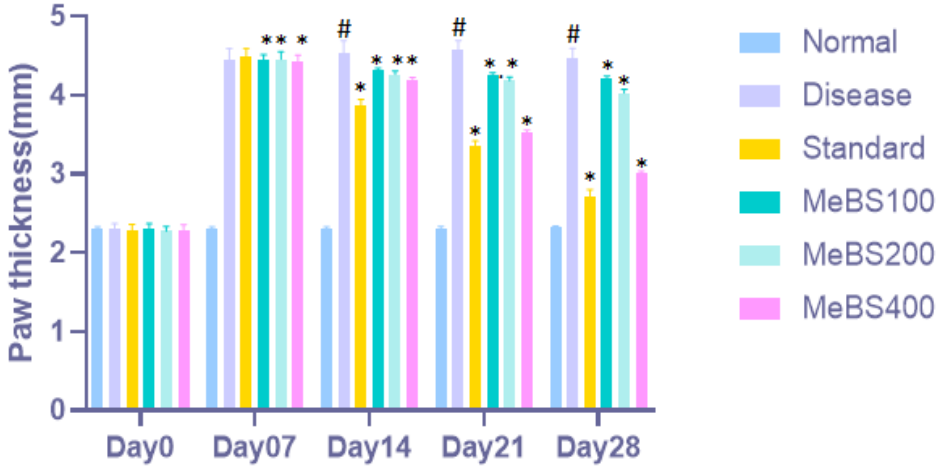


Figure 2. Effect of MeBS on left hind paw thickness (paw injected with CFA) of animals

All values are expressed in Mean ± SEM; n = 6 for each group

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

Effect of MeBS on left hind paws volume (paw injected with CFA) of animals

Volume of left hind paw in disease control animals was significantly higher as compared to normal control on day 14, 21 and 28. There was significant reduction in paw volume in all treated groups as compared to that in disease control group on day 28 (P<0.05) as shown in figure 3.

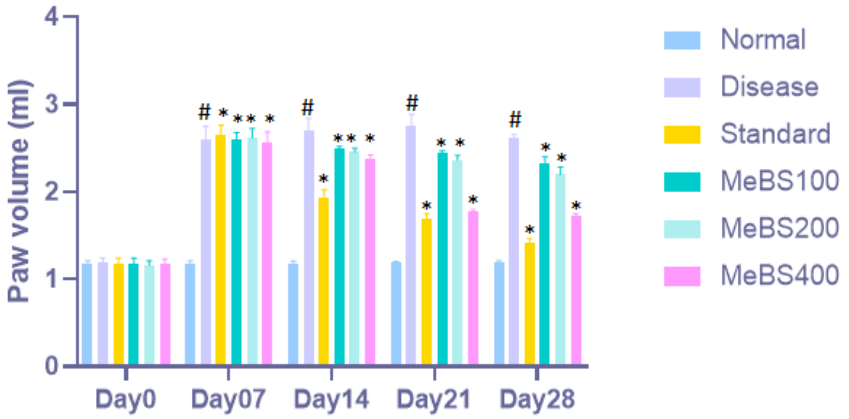


Figure 8. Effect of MeBS on left hind paw volume (paw injected with CFA) of animals

All values are expressed in Mean ± SEM; n = 6 for each group

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

Effect of MeBS on right hind paw volume (non-injected paw) of animals

The CFA-induced disease (arthritis) control group showed signs of arthritis development, as seen by the increase in the paw volume in both CFA-injected (left) and CFA-non-injected (right) hind

paws, which indicates primary and secondary arthritic lesions. There was significant reduction in right paw volume in all treated groups as compared to that in disease control group on day 28 (P<0.05) as shown in figure 4.

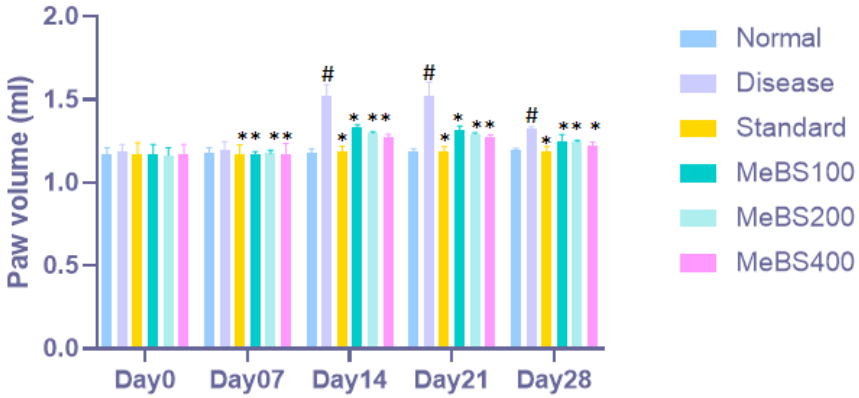


Figure 4. Effect of MeBS on right hind paw volume (non-injected paw) of animals

All values are expressed in Mean ± SEM; n = 6 for each group

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

Effect of MeBS on arthritic swelling.

Clinical evaluation of arthritic swelling in animal's left hind paw showed that the disease control group had the highest score - 4 which was significantly greater than that what observed in the normal control group. All treatment groups showed dose dependent reduction in clinical score of arthritic index. Group MeBS400 showed similar results as compared to the results observed in group treated with the standard drug as shown in figure 5.

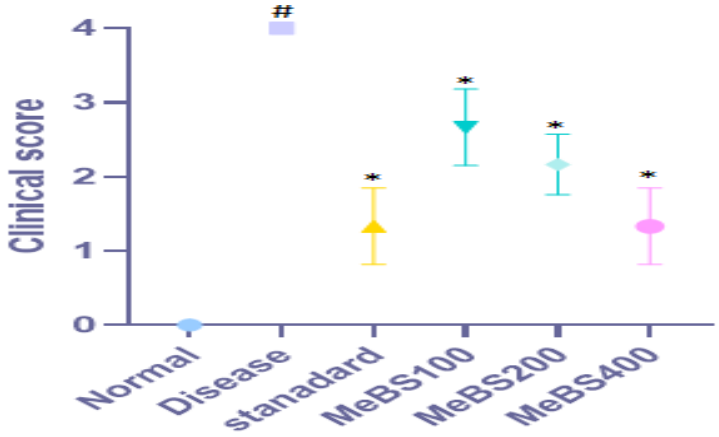


Figure 5. Effect of MeBS on arthritic swelling

All values are expressed in Mean ± SEM; n = 6 for each group

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

Effect of MeBS on blood parameters: Rheumatoid Factor (RF), C-Reactive Protein (CRP) and Erythrocyte Sedimentation Rate (ESR)

Detection of circulating autoantibody called rheumatoid factor (RF) against Fc portion of autologous IgG is reported in about 80% of cases of rheumatoid arthritis. RF antibodies are heterogeneous and consist of IgM and IgG class which causes inflammatory damage to synovium, small blood vessels and collagen. Increase in RF is a major indication of rheumatoid arthritis.[7]

Increase in the level of C- Reactive Protein (CRP) is an acute phase response occurs as a result of increasing concentration of IL-6, which is produced by the macrophages as well as adipocytes in response to inflammatory conditions such as bacterial, viral or fungal infections, rheumatic and other inflammatory diseases. Erythrocyte sedimentation rate (ESR) is the rate at which red blood cells in whole blood descend in a standardized tube in a period of one hour. It is a common hematology test and a non-specific measure of inflammation. The ESR is governed by the balance between the pro-sedimentation factors mainly fibrinogen, and those factors resisting sedimentation, namely the negative charge of the erythrocytes (zeta potential) during inflammation, the high proportion in blood causes red blood cells to stick to each other forming 'rouleaux' which settle faster, due to their high density. ESR is known to increase in inflammation and autoimmune disorders such as rheumatoid arthritis.[23,24]

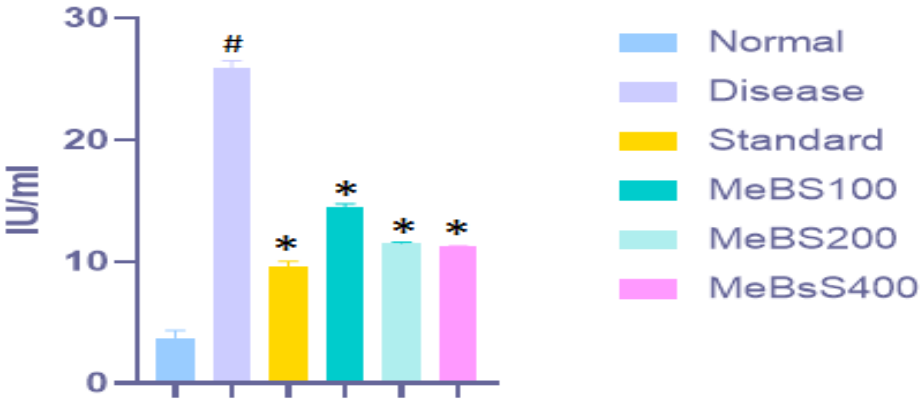


Figure 6: Effect of MeBS on rheumatoid factor

All values are expressed in Mean ± SEM; n = 6 for each group

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

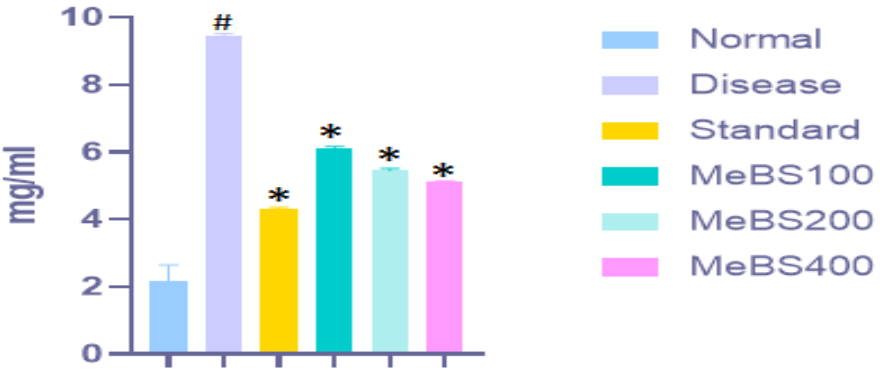


Figure 7:Effect of MeBS on C - Reactive Protein (CRP)

All values are expressed in Mean ± SEM; n = 6 for each group.

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

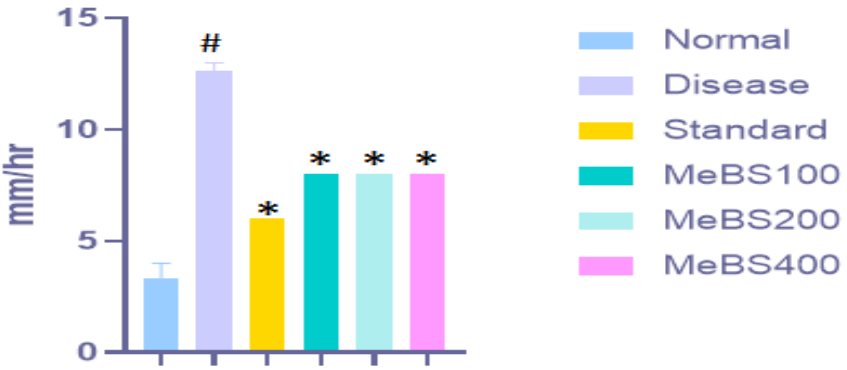


Figure 8. Effect of MeBS on erythrocyte sedimentation rate

All values are expressed in Mean ± SEM; n = 6 for each group

#indicates significant difference from Normal control at P<0.05

*indicates significant difference from Disease control at P<0.05

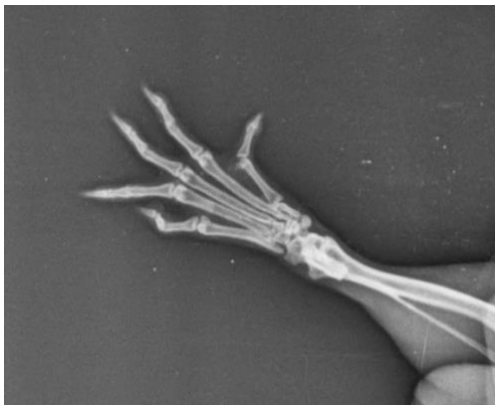
Inflammatory markers, viz. Rhematoid Factor (RF) and C – Reactive Protein (CRP) and Erythrocyte Sedimentation Rate (ESR) were significantly higher in animals of disease control group as compared to the animals of normal control group. Groups treated with standard drug showed marked reduction in levels of RF, CRP and ESR. All MeBS treated groups (MeBS100, MeBS200 & MeBS400) also showed significant decrease (P<0.05) in the levels of all inflammatory markers as shown in figure 6, 7 and 8.

Radiographical study

Bone destruction, which is a common feature of adjuvant arthritis, was examined by radiological analysis. Normal bone and joint construction can be seen in animals from normal group. Animals in CFA induced arthritis group had developed definite joint space narrowing of the intertarsal

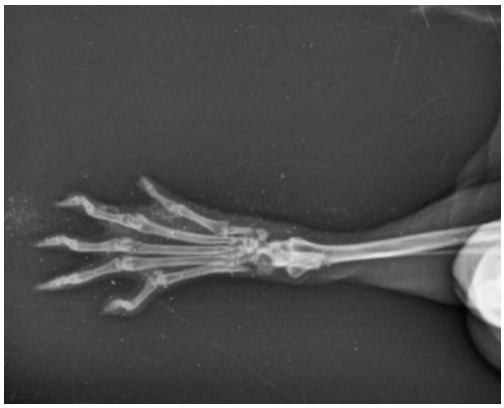
joints, diffuse soft tissue swelling that included the digits, diffuse demineralization of bone, marked periosteal thickening, and cystic enlargement of bone and extensive erosions produced narrowing or pseudo widening of all joint spaces.

In contrast, animals treated with the standard drug and MeBS attenuate abnormalities consisted of asymmetric soft tissue swelling and small erosions, periosteal thickening and minimal joint space narrowing, predominantly localized to the proximal areas of the paws as shown in figure 9.



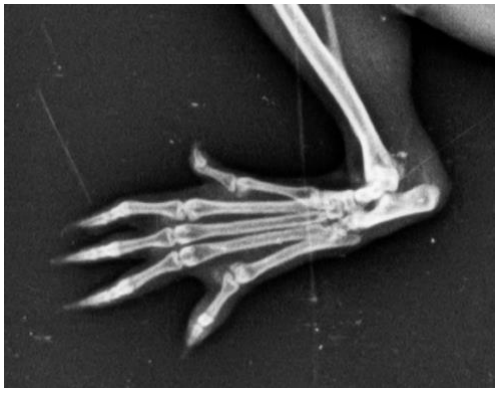
(A)

Normal bone and joints construction



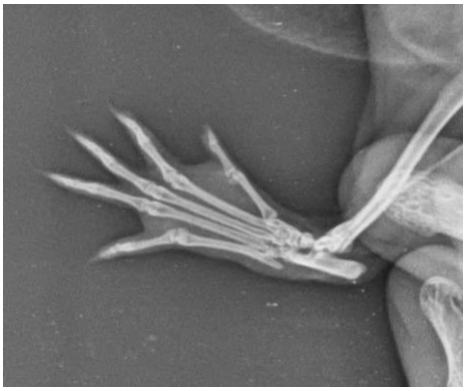
(B)

Soft tissue swelling, diffuse bone structure and erosion and joint space narrowing



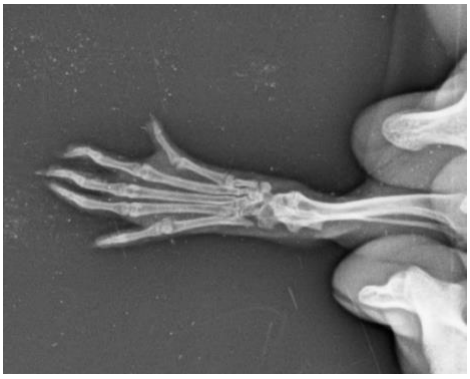
(C)

Normal joint spaces and no bone diffusion and erosion

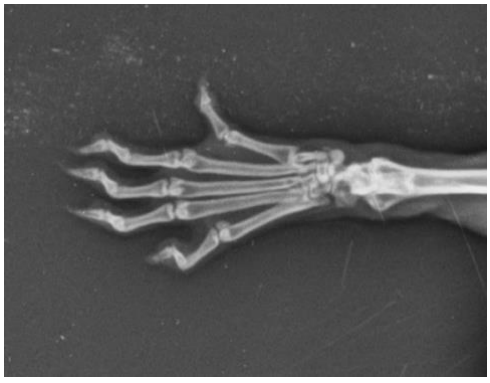


(D)

Narrow joint spaces, significant reduction in bone destruction



(E)



(F)

Less joint space narrowing and bone diffusion **Less soft tissue swelling, normal joint spaces and bones**

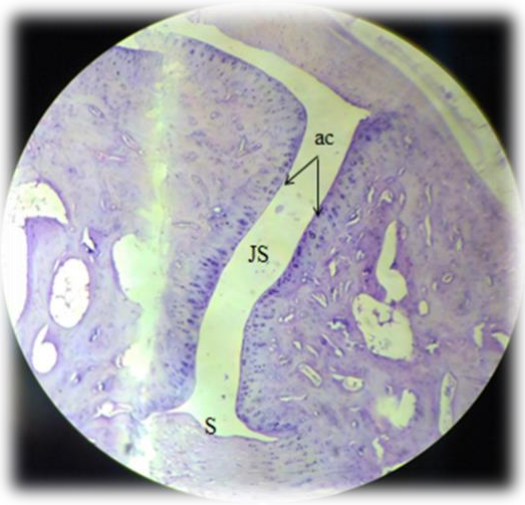
Figure 9. Radiology of ankle joints of rats

(A) Normal control (B) Disease control (C) Standard (D) MeBS100 (E) MeBS200 (F) MeBS400

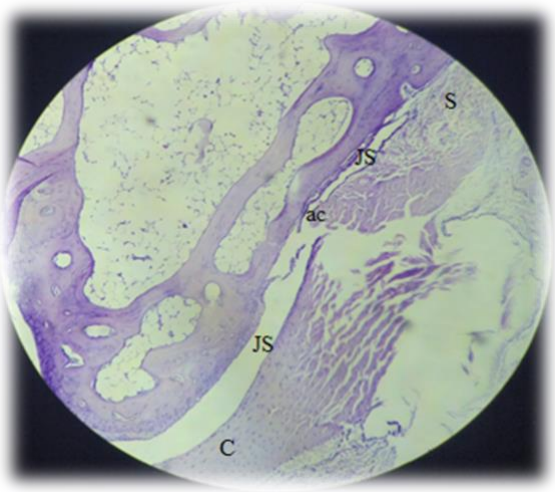
Histopathology

In normal control, animals showed intact articular cartilage (ac) and no vascularity formation into the joint space (JS) having normal synovial membrane (S). Arthritis control animals showed severe destructive lesions in articular cartilage, cellular infiltration and edematous synovial membrane. Vascularity formation was also observed into the joint space in disease control animals leading to marked narrowing of joint space.

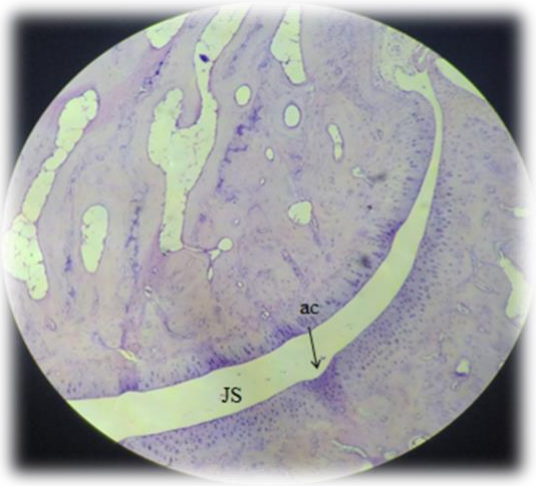
Animals treated with standard drug restored normal architecture of articular cartilage and joint space which can also be seen in animals in MeBS400 group. Varying degree of improvement in joint space narrowing and reduction in synovial proliferation and cellular infiltration can be observed in MeBS100 and MeBS200 groups as shown in figure 10.



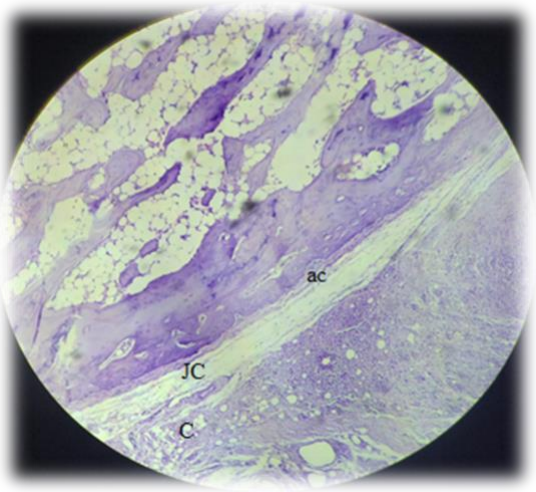
(A)
Intact articular cartilage, normal joint space, no synovial proliferation and cellular infiltration



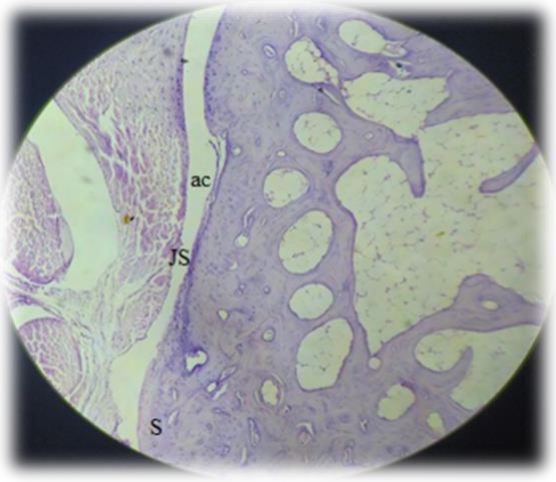
(B)
Damaged articular cartilage, narrow joint space, synovial proliferation and cellular infiltration



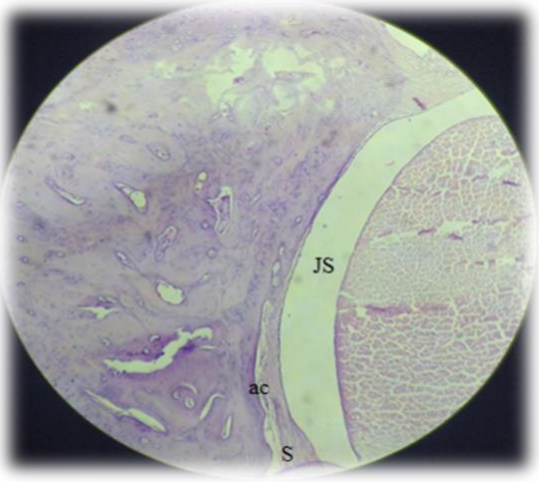
(C)
Normal joint space, profound reduction in articular lesion



(D)
Cellular infiltration, significant reduction in joint space narrowing and articular lesion



(E)
Less cellular infiltration and synovial proliferation, significant reduction in articular lesion



(F)
Normal joint space, significant reduction in articular damage and synovial proliferation

Figure 10. Histopathology of ankle joints of rats

(A) Normal control (B) Disease control (C) Standard (D) MeBS100 (E) MeBS200 (F) MeBS400

CONCLUSION

The findings of present study reveals that the methanolic extract of whole plant of *Biophytum sensitivum* (MeBS 400mg/kg) shows beneficial effects against CFA induced arthritis model of rats. The methanolic extract of whole plant of *Biophytum sensitivum* shows significant reduction in physical parameters like paw thickness, paw volume and arthritic swelling associated with rheumatoid arthritis. It also shows reduction in increased level of inflammatory markers such as Rheumatoid factor, C-Reactive Protein and Erythrocyte Sedimentation Rate. Above mentioned factors might have contributed the anti-inflammatory and anti-arthritic activity of MeBS in

mitigating the signs and symptoms associated with rheumatoid arthritis in rats. So in the light of above findings and discussion, it can be concluded that the Methanolic extract of *Biophytum Sensitivum* might possess anti-arthritic potential in treatment of rheumatoid arthritis. However further studies are require to characterize the actual mechanism of action of MeBS against rheumatoid arthritis.

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