

Beneficial roles of sustained release formulations of diacerein microspheres in comparison to free diacerein incomplete Freund's adjuvant (C.F.A.) induced arthritis model in rat

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ABSTRACT

Diacerein is an anti-inflammatory drug used to treat osteoarthritis. Available dosage forms of diacerein cannot reduce its main adverse effects like soft stool and diarrhea. The main aim of this work is to evaluate the antiarthritic effects of diacerein microsphere in the complete Freund's adjuvant (C.F.A.)-induced arthritis model in rats and on intestinal meal transit and peristaltic index. Diacerein microspheres were administered to experimental rats via oral routes. Five experimental groups of Sprague Dawley rats were considered: Group I (Negative control group), Group II (Positive control group), Group III or test group (Diacerein microsphere treated group), Group IV or Standard I group [Diacerein API (active pharmaceutical ingredient) treated group], Group V or Standard II group (Glucosamine treated group). Glucosamine was considered a standard drug because it has a similar mechanism of action to diacerein (IL-1 β inhibition), and various literature reviews showed that it has antiarthritic and cartilage protective effects. Various antiarthritic effects like paw volume, joint stiffness, arthritic index, gait test, body weight measurement, and X-ray of tibio-tarsal joints were evaluated in test group rats and compared to standard I standard II group. The Diacerein microsphere-treated group showed similar results compared to the standard I and II groups. A charcoal meal test was performed on fresh, experimental animals, and results showed that the diacerein microsphere causes the sustained release of diacerein so that diacerein-mediated diarrhea is reduced.

Keywords: Diacerein, Rheumatoid arthritis, Microsphere, Sprague Dawley rats.

INTRODUCTION

Rheumatoid arthritis (R.A.) is an autoimmune inflammatory disorder involving a plethora of cytokine networks that play a pivotal role in the origin and progression of the disease. Among the various IL-1 superfamily cytokines, IL-1 β plays an important role in the pathogenesis of R.A. It has been demonstrated in multiple studies that IL-1 β plays a variety of roles in the degeneration of articular cartilage. Chondrocytes primarily generate type-II collagen and aggrecan, two crucial structural proteins of the cartilage extracellular matrix. Type-II collagen and aggrecan synthesis are suppressed by IL-1 β , which prevents the creation of the extracellular matrix's structural constituents [1]. IL-1 β boosts the development of various matrix metalloproteinases (M.M.P.s), such as MMP-1, MMP-3, and MMP-13, which are primarily responsible for destroying cartilage [2]. IL-1 β stimulates

chondrocyte-mediated ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) metalloproteinase expression that causes degradation of aggrecan molecules [3]. Thus, inhibiting the IL-1 β signaling pathway is an important strategy for treating R.A.

Diacerein is an anthraquinone derivative that inhibits the enzyme IL-1 β converting enzyme (I.C.E.), which reduces the production of IL-1 β [4]. Thus, it can be hypothesized that IL-1 β mediated deleterious effects can be reduced by diacerein.

Long-term therapy of Rheumatoid Arthritis patients with disease-modifying antirheumatic drugs (DMARDs) and anti-TNF-monoclonal antibodies such as infliximab and adalimumab. Nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-II inhibitors are used occasionally to reduce pain. However, they are responsible for renal damage and cardiovascular complications due to

the inhibition of prostaglandin biosynthesis, which can be avoided by treatment with IL-1 β inhibitors like diacerein [4]. Various literature reviews revealed diacerein having a prophylactic effect in the C.F.A.-induced arthritis model in rats. Upadhyay *et al.*, 2021 investigated the prophylactic effects of diacerein in C.F.A. induced arthritis model in rats. Diacerein at 100 mg/kg dose significantly reduces paw volume in 21 days. Radiographic and histopathological improvements were seen at 100 mg/kg [5]. Louthrenoo *et al.*, 2019 investigated diacerein's effects on treating rheumatoid arthritis in methotrexate nonresponsive patients. Results indicated that diacerein significantly reduces joint pain and disease activity in methotrexate nonresponsive patients [6].

The main disadvantage of diacerein therapy is soft stool and diarrhea [7], which can be overcome by developing a sustained-release formulation of diacerein. In this research article, we have investigated the antiarthritic role of prepared diacerein microsphere compared to the marketed available formulation of the same drug in the complete Freund's adjuvant (C.F.A.) induced arthritis model in rats.

MATERIALS AND METHODS

Detailed procedure of diacerein microspheres was mentioned by Tathagata Roy *et al.* 2023 (In press) [8]. Evaluation parameters like % yield, swelling index, % entrapment efficiency, and invitro drug release studies were carried out, and an optimized batch was identified. Further, a scanning electron microscopy (S.E.M.) study of the optimized batch was also done. The optimized batch is then subjected to an antiarthritic study in a complete Freund's adjuvant (C.F.A.) induced arthritis model in rats.

Materials

C.F.A. was purchased from sigma Aldrich, U.S.A., whereas Emcure Pharmaceutical Ltd, India, and Panvo Organics Pvt provided diacerein and Glucosamine. Ltd, Chennai (India), as a gift sample. Charcoal was purchased from Global Chemie laboratory reagents and fine chemicals.

Instruments used

Medtronic M Series Cell Counter, MICROLAB-300, Plethysmograph (Inco Co, Ambala India), Microtome (HistoCore MULTI CUT - Semi-Automated Rotary Microtome).

Animals and diet

Male Sprague Dawley rats of 180gm-200gm body weight were used for the study. They were purchased from Saha Enterprise, 386/2, Nilachal bitrate, Kolkata-700051, West Bengal, and they were housed individually in animal cages (Tarsons). Animals were acclimated for one week in typical laboratory conditions like temperature $25 \pm 1^\circ\text{C}$ and humidity 50%-60%. Animals were exposed to a 12:12 h light/dark cycle with unrestricted access to both demineralized drinking water and commercially available rat chow diet throughout the study. Throughout the experiment, all animal-related procedures followed the "Institutional Animal Ethical Committee" recommendations for the care and use of laboratory animals. (CPCSEA Regn. No. 1938/P.O./Rc/S/17/CPCSEA).

1. Acute toxicity study of diacerein microspheres

For the Acute toxicity study, twenty-four Rats, i.e., 12 male and 12 healthy female Rats, were divided into two groups of 6 rats per sex. 12 rats were considered a control group, and 12 animals received a dose of 2000mg/kg body weight (according to OECD guidelines 420). Animals were allowed an acclimation period of 7 days to laboratory conditions before dosing. All the animals were observed daily for clinical signs and symptoms. The time of onset, intensity, and duration of these symptoms, if any, were recorded. All animals were observed twice daily for mortality during the study period. The weight of each mouse was recorded on day 0 and at weekly intervals throughout the study. The group's mean body weights were calculated.

The quantity of food consumed was recorded weekly, and the food consumption per rat was calculated.

2. *Subacute toxicity study of diacerein microsphere*

Dose selection criteria for sub-acute toxicity study:

The maximum Human Daily Dose of diacerein is 75mg twice daily. The dose conversion formula is bellowed:

Human Effective Dose = Animal dose (mg/kg) x Animal Km / Human Km [9]

For Dose selection of Rats:

Animal dose (mg/kg) = 150/60 kg Adult Human x 37/6 (Where 6 is rat Km factor and 37 is Human Km factor).

Rat Dose 15.4 mg/kg

We took the highest dose of 60 mg/kg, the middle dose of 30 mg/kg, and the lowest dose of 15 mg/kg body weight, as per guidelines (animal dose should be the multiplication of human therapeutic dose). Forty-eight healthy rats, i.e., 24 males and 24 females, were divided into four groups of 6 rats per sex, i.e., four dose groups receiving the dose of 0 mg/Kg, 15 mg/kg, 30 mg/Kg, and 60 mg/Kg. Animals were allowed an acclimatization period of 7 days to laboratory conditions before dosing. Every animal was checked daily for clinical signs and symptoms. The onset, intensity, and duration of these symptoms, if any, were all documented. Throughout the study, all animals were checked twice daily for mortality. Each rat's weight was recorded on day one and weekly intervals throughout the study. The mean body weights of the group were computed. The amount of food consumed by each group of six rats was recorded weekly, and the food consumption per rat was calculated for the control and dose groups.

After 28 day dosing period, all animals were sacrificed on the 29th day. Before sacrificing the animals' various laboratory investigations, like an estimation of various hematological and biochemical parameters, were performed. Blood samples were collected from the orbital sinus on the 29th day morning before sacrificing the animals using heparin as an anticoagulant.

Various hematological parameters like Hemoglobin (g/dL), Reticulocyte (%), Hematocrit (%), Platelets ($\times 10^3 / \mu\text{L}$), Neutrophils

(%), Lymphocytes (%), Monocytes (%), Mean Corpuscular Volume (μm^3), Mean Corpuscular Hemoglobin (pg), Mean Corpuscular Hemoglobin Concentration (g/dL), White Blood Corpuscles ($\times 10^3 / \mu\text{L}$), Eosinophils (%) were done using Medtronic M Series Cell Counter.

Various biochemical parameters like Total serum protein (g/dL), Blood Urea Nitrogen (mg/dL), Serum Glutamic Pyruvic Transaminase (IU/L), Serum Glutamic Oxaloacetic Transaminase (IU/L), Serum Alkaline Phosphatase (IU/L), Blood sugar (mg/dL), Creatinine (mg/dL), Total bilirubin (mg/dL) were studied using MICROLAB-300 semi-auto analyzer.

Necropsy of all animals was carried out on the 29th day, and the weights of the following organs were recorded: Liver, kidneys, and heart. The organ weights were recorded as absolute values, and their relative values (i.e., percent of the body weight) were calculated.

Tissue samples of heart, kidney, liver, lungs, and stomach organs from control and animals treated at the highest dose level of 60 mg/kg, were preserved in 10% formalin for histopathological examination to detect abnormality in these tissues.

3. *Induction of adjuvant-induced arthritis in rat*

Induction of arthritis was done by inducing 0.1 ml of complete Freund's adjuvant (C.F.A.) via a subcutaneous route into the subplanter region of the left hind paw in all the animals of the previously mentioned groups except for negative control. C.F.A. is a liquid paraffin suspension containing heat-killed *Mycobacterium butyricum* (10 mg/ml).

4. *Design of the experiment*

The dose of diacerein was calculated from a subacute toxicity study, whereas a dose of Glucosamine was selected based on a previously reported scientific article [10]. Animals were divided into 5 groups consisting of 6 animals each.

Group I (Negative control group): Nonarthritic group, only normal saline-injected group.

Group II (Positive control group): Arthritic rats with no drug treatment.

Group III (Test group): Arthritic rats treated with diacerein microspheres at an equivalent dose of 15.4mg/Kg body weight.

Group IV (Standard I group): Arthritic rats treated with diacerein at a dose of 15.4mg/Kg body weight.

Group V (Standard II group): Arthritic rats treated with Glucosamine at 300 mg/Kg body weight.

5. Measurement of Paw volume

A digital plethysmometer was used to measure experimental animals' swelling of the left hind paw. Paw volumes were measured weekly once (0 days, 7 days, 14 days, 21 days, and 28 days). The volume of the inflamed paw

can be calculated by subtracting the final paw volume from the initial paw volume [10].

6. Measurement of body weight

The body weight of all the animals was recorded weekly once up to 28 days, and the change in body weight of experimental animals of 5 different groups was compared with each other.

7. Scoring of arthritis index [11].

The arthritic index was calculated by examining the characteristics of swelling, erythema, redness, and the condition of the paws were assessed.

Scoring systems used to calculate the arthritis index is given in (Table 1):

Table (1): Scoring system of arthritis index.

Site of lesion	Nature of lesion	score
Ears	No nodules observed	0
	nodules observed	1
Nose	Lack of connective tissue swelling	0
	Presence of connective tissue swelling	1
Tails	No nodules observed	0
	Nodules observed	1
Fore paws	Inflammation is non-existent	0
	Inflammation exists in at least one joint	1
Hind Paws	Inflammation is non-existent	0
	Slight inflammation	1
	Moderate inflammation	2
	Severe inflammation	3

8. Joint stiffness study [12].

The study of joint stiffness was conducted on 28 days of drug therapy in experimental groups. The rat was grasped behind the back with the left palm, and the right fingers were used to bend and stretch the limbs in each direction.

Score 2: Both ankle flexure and extension mobility are restricted.

Score 1: either ankle bending or extension mobility restriction.

Score 0: The ankle movement is unrestricted.

9. Gait test [12]

At the end of the experiment, rats from each group were free to move around on a tabletop for a gait test.

Score 2: pulling the two hind limbs while moving on the two forelimbs (creeping behavior)

Score 1: non-active use of paw to support the body.

Score 0: animals using paws actively.

10. Haematological Analysis

Blood samples were collected from the animals via cardiac puncture after the drug treatment was completed on the 28th day. E.S.R., WBC, R.B.C., and hemoglobin contents were estimated.

11. X-ray analysis of joint injury in various experimental groups of animals

X-ray analysis of joint injury in various experimental groups of animals following the completion of drug treatment, animals from

all five groups were anesthetized with ketamine sulfate and placed on an X-ray plate the following day. All of the animals' left hind paw X-rays were taken and compared to animals in the control group.

12. Charcoal meal test [13]

This test was performed on rats to determine gastrointestinal motility. This study considered three groups of six fresh Sprague Dawley rats weighing 180-200 gm. The Control group received normal saline only, the test group received diacerein microspheres, and the standard group received diacerein orally. After one hour, all animals were orally given 1 mL of the charcoal meal (10% charcoal suspension in 5% gum acacia). All animals were sacrificed under diethyl ether anesthesia one hour after the charcoal meal was administered, and the length of the charcoal meal covered in the intestine from the pylorus to the caecum was assessed and expressed as a percentage of the total distance traveled.

The following formulae were used to calculate the peristaltic index and percentage inhibition/ acceleration of intestinal meal transit:

Peristalsis index = (length covered by charcoal meal /length of the small intestine) × 100. [14]

% of inhibition/acceleration of intestinal meal transit = mean distance traveled by the control - mean distance traveled by the test group/mean distance traveled by the control. [14]

STATISTICAL ANALYSIS

All data are presented as mean±SEM. GraphPad Prism 5.0 was employed to perform a statistical analysis of the data. One-factor analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test was used to test the statistical significance of differences between groups.

RESULTS AND DISCUSSIONS

1. Acute toxicity study of diacerein microsphere

a) Clinical signs: All 24 animals of the control and test groups were free of intoxicating signs throughout the dosing period of 14 days. There were no abnormalities in

clinical signs of intoxication due to the use of diacerein in any of the four dose groups of animals.

- b) Mortality: No fatality of the experimental animals was recorded under the circumstances of the current study.
- c) Body weight: In the case of both male and female animals, the control group animals gained normal body weight. During the 14-day dosing period, animals treated with high doses gained weight at a similar rate to the control group.
- d) Food Consumption: Food consumption of experimental animals during the acute toxicity study was similar to control groups.

2. Sub-Acute toxicity study

- a) Clinical signs: All 48 animals of the control and test groups were free of intoxicating signs throughout the dosing period of 28 days. No abnormalities were observed in clinical signs of intoxication due to the application of the said drug in any of the four dose groups of animals.
- b) Mortality: No fatality of the experimental animals was recorded under the circumstances of the current study.
- c) Body weight: Group Mean Body Weight of Low, Middle, and High dose groups of the animal show no drastic weight reduction during the dosing period and can be comparable with the mean body weight gain of the control group of animals
- d) Food consumption: For 28 days of the subchronic oral toxicity study, each group's regular monitoring of Food consumption (Food given – Leftover) was done every 7 days at intervals and recorded accordingly. The food consumption of low, middle, and high-dose groups of animals was satisfactory and did not show any abnormality or reduction in food intake.
- e) Terminal studies:
 - e.1. Laboratory investigations:
 - e.1.1. Haematological investigations: Haematological parameters of Low, Middle, and High dose group animals showed no abnormalities compared to the control

group. It may be concluded that no adverse reactions have occurred in the hematological parameters of any of the animals due to the application of the drug under investigation.

e.1.2. Biochemical investigations: Biochemical parameters of all 3 test groups are normal compared to the control group. From the results of the biochemical analysis of all 48 rats, it may be concluded that all the results are within the permissible limit, so the test product has no adverse effect on biochemical parameters.

e2. Necropsy: All the necropsy parameters of Low, Middle, and High dose group animals were within the normal range compared to the control group. It may be concluded that no adverse reactions have occurred in the animals' necropsy parameters due to the application of the drug under investigation.

Histopathological slides of various organs of animals are given in Figures 1A, 1B, and 1C.

No abnormality was observed in any of the tissue sections of the Liver, Kidneys, and Stomach of control and test groups of male and female animals.

The kidney section shows the histology of the normal kidney comprising glomeruli (G) and renal tubules (R.T.). No evidence of any pathological lesion was seen.

The stomach section shows histology of the normal stomach comprising the muscular layer (M), submucosal layer (S.M.), Lamina Propria (L.P.), and Mucus layer (M.U.). No evidence of any ulcerating or malignant process was seen.

The liver section shows hepatocytes (H), central vein (CV), and sinusoids (S) are intact. No evidence of any pathological lesion was seen.

e3. Histopathology:

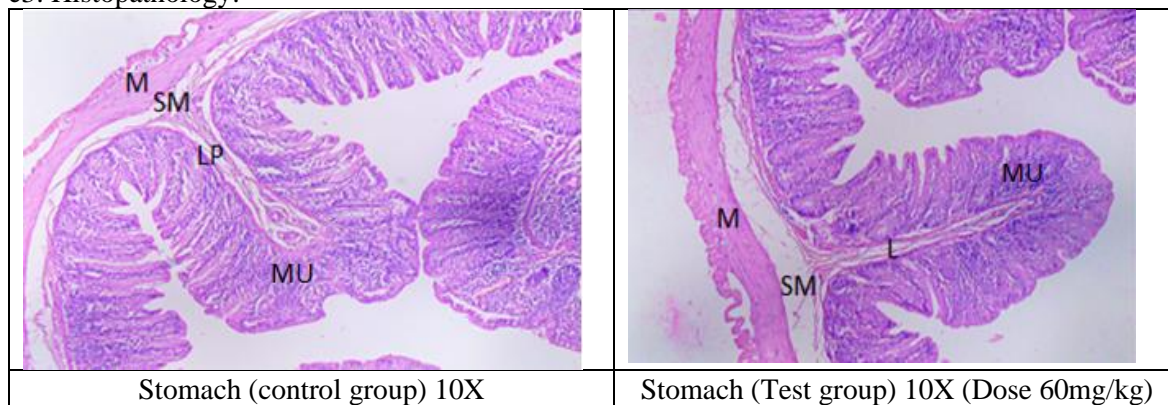


Figure (1A): Histopathology of the stomach of both control and test groups.

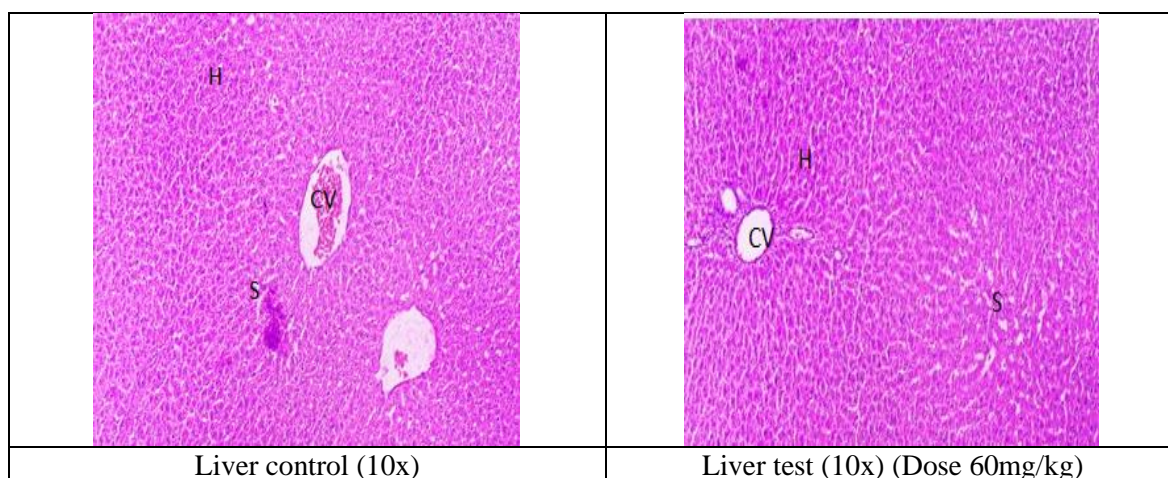


Figure (1B): Histopathology of the liver of both control and test groups.

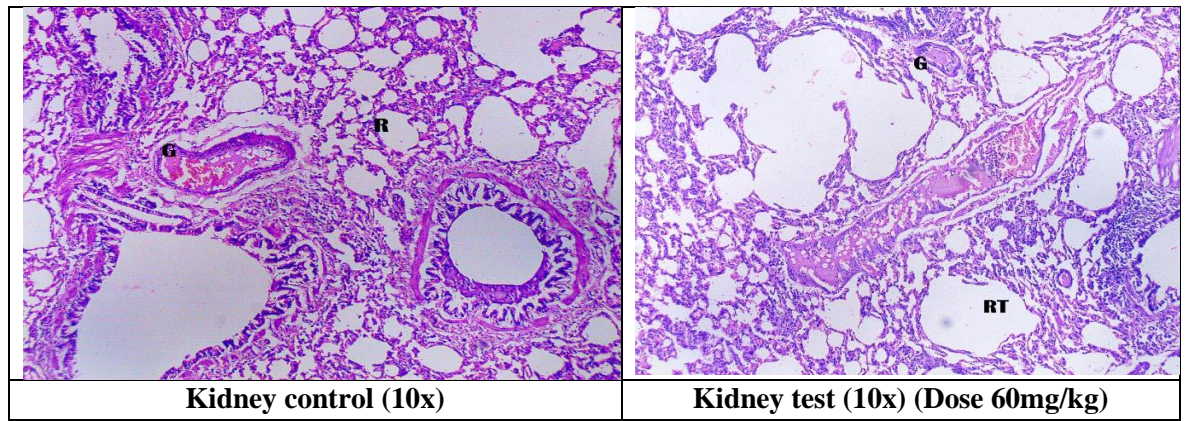


Figure (1C): Histopathology of the kidney of both control and test groups.

e) *Paw volume measurement*

Some pictures of the paw of different animal groups of animals are shown in figure 2.

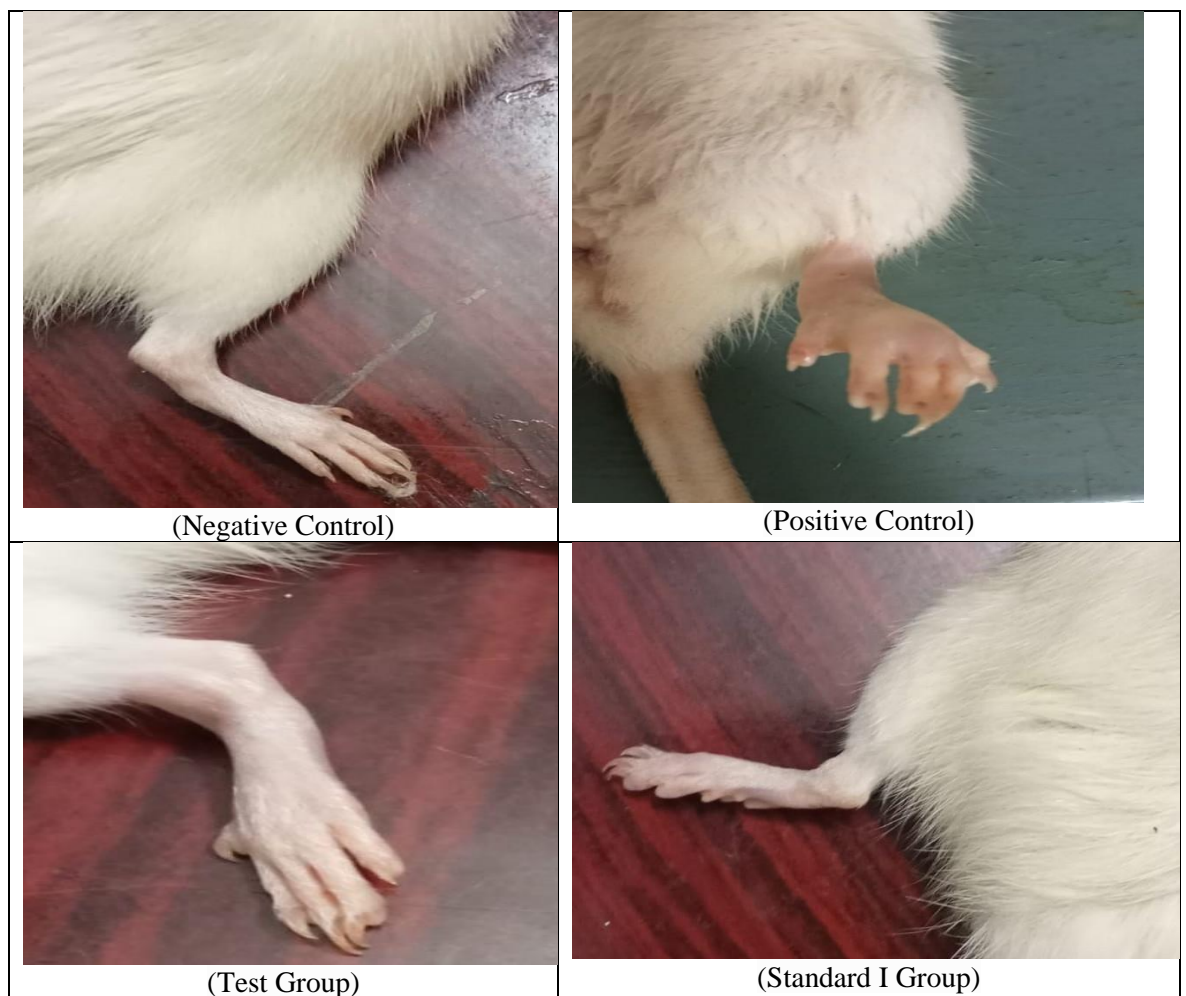




Figure (2): Paws of different groups of animals after 28 days of drug treatment. Throughout the experiment, CFA-induced arthritic rats significantly increased paw volume compared to negative control rats. Diacerein microsphere-treated group, Diacerein API treated group, and Glucosamine treated group showed a significant decrease in paw volume compared to the positive control group (Figure 2) (table 2). The test group showed a nonsignificant decrease in paw volume compared to the standard I group.

Table (2): Paw volume data of 5 groups of animals.

Days	Negative control	Positive control	Test Group	Standard 1 group	Standard 2 group
	(Mean paw volume \pm S.E.M.)				
0	1.21 \pm 0.02	1.650 \pm 0.026***	1.630 \pm 0.037###	1.66 \pm 0.02###	1.63 \pm 0.02###
7	1.21 \pm 0.03	1.870 \pm 0.022***	1.790 \pm 0.032###	1.83 \pm 0.02###	1.86 \pm 0.02###
14	1.21 \pm 0.02	2.2 \pm 0.022***	1.340 \pm 0.018###	1.42 \pm 0.03###	1.54 \pm 0.02###
21	1.21 \pm 0.02	2.370 \pm 0.024***	1.280 \pm 0.017###	1.32 \pm 0.02###	1.36 \pm 0.01###
28	1.21 \pm 0.02	2.490 \pm 0.036***	1.230 \pm 0.017###	1.25 \pm 0.02###	1.25 \pm 0.01###

Data represented as mean \pm S.E.M. ***P < 0.001 compared to a normal control group. ### P < 0.001 compared to a positive control

group. (One-way ANOVA followed by Turkey's multiple comparisons test).

Paw volume measurement

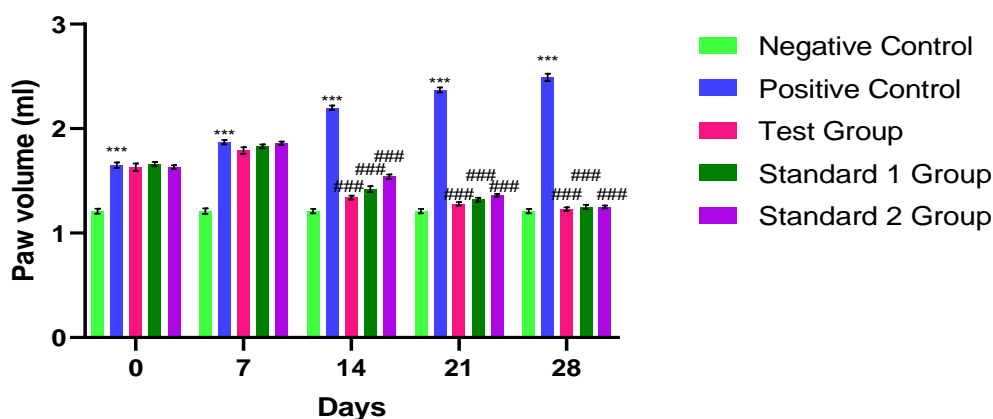


Figure (3): Paw volume measurement. [Figure 3: Paw volume measurement.

Data represented as mean \pm S.E.M. ***P < 0.001 compared to a normal control group. ### P < 0.001 compared to a positive control group. (One-way ANOVA followed by Turkey's multiple comparisons test)].

Paw swelling is a simple and sensitive parameter for evaluating the therapeutic effectiveness of numerous anti-inflammatory drugs [16]. An increase in paw volume was observed in the positive control group animals throughout the experiment, whereas all treatment groups showed a decrease in paw volume from 14 days of drug treatment. The test group showed nonsignificant alterations in paw volume compared to the standard I and standard II group indicating that the test formulation of diacerein is showing a similar effect to the standard formulation of diacerein and standard II drug (Glucosamine).

Major findings on paw volume measurement

a) Diacerein microspheres significantly reduce the paw volume in C.F.A. induced

arthritis model in rats, indicating its anti-inflammatory properties.

b) Diacerein microspheres showed a similar effect on paw volume measurement to the standard formulation of diacerein (Diacerein API) and standard II drug (Glucosamine), indicating similar efficiency of the test formulation with standard I formulation and Glucosamine.

4. Body weight measurement

During the experimental period, the body weight of all experimental animals was assessed on 0-day, 7-day, 14-day, 21-day, and 28-day. Throughout the experiment, the body weight of the negative control group rats, test group rats, and standard II rats increased steadily, but the body weight of positive control and standard I group rats decreased significantly, as shown in figure 4 and table 3.

Table (3): Body weight data in 5 groups of animals.

Days	Negative control	Positive control	Test Group	Standard 1 group	Standard 2 group
	(Mean body weight \pm S.E.M.)				
0	185.83 \pm 1.37	185.33 \pm 1.36	184.16 \pm 0.72	183.83 \pm 1.03	184.83 \pm 1.14
7	188 \pm 1.23	181.83 \pm 1.49**	186.17 \pm 0.79 ^{aa}	180 \pm 0.91	186.67 \pm 1.09 [#]
14	190.67 \pm 1.26	178.83 \pm 1.34***	188.87 \pm 0.96 ^{###, aaa}	176.83 \pm 0.95	190.33 \pm 0.87 ^{###}
21	194.33 \pm 0.93	174.66 \pm 0.80***	191.33 \pm 0.81 ^{###, aaa}	172 \pm 0.78	193.50 \pm 0.66 ^{###}
28	197.17 \pm 0.87	170.16 \pm 0.55***	194.17 \pm 0.79 ^{###, aa, bb}	167 \pm 0.53 [#]	197.67 \pm 0.61 ^{###}

Data represented as mean \pm S.E.M. ***P < 0.001, **P < 0.01 compared to a normal control group. #P < 0.05, ###P < 0.001 compared to positive control group. ^{aaa}P < 0.001, ^{aa}P < 0.01 compared to a standard I group. ^{bb}P < 0.01 compared to the standard II group. (One-way ANOVA followed by Turkey's multiple comparisons test).

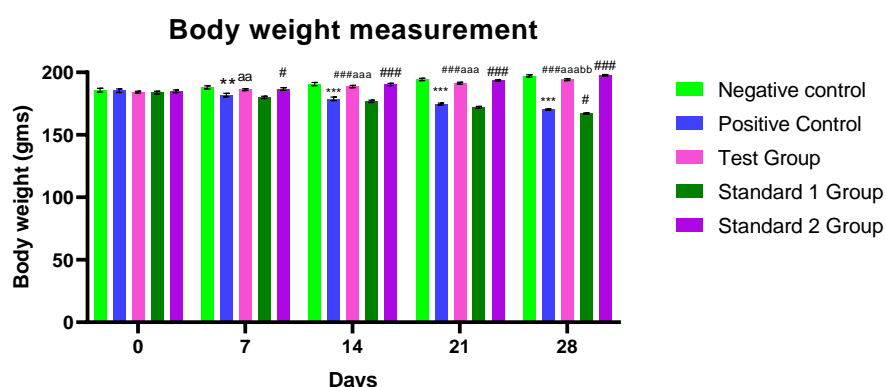


Figure (4): Body weight assessment of a different group of animals.

Figure 4: Effect of diacerein microsphere on body weight of CFA-induced arthritis model in the rat. Data represented as mean \pm S.E.M. ***P < 0.001, **P < 0.01 compared to a normal control group. #P < 0.05, ###P < 0.001 compared to positive control group. ^{aaa}P < 0.001, ^{aa}P < 0.01 compared to a standard I group. ^{bb}P < 0.01 compared to the standard II group. (One-way ANOVA followed by Turkey's multiple comparisons test).

Body weight assessment is an important parameter to be evaluated in the antiarthritic model. Various literature review reveals that CFA-induced arthritis in experimental animals significantly reduces body weight [16]. In this study, the positive control group also showed a significant decrease in body weight. The negative control group showed a significant increase in body weight throughout the experimental period. The Standard I group significantly decreased body weight as all the animals suffered from soft stool and diarrhea mediated by diacerein. The Standard II group also showed increased body weight throughout the experimental period. The test group also showed an increase in body weight as the sustained release formulation of diacerein releases the drug sustainably, so diarrhea and soft stool are not seen in this group.

Major findings on paw volume measurement:

- The Standard I group significantly decreased body weight as all the animals suffered from soft stool and diarrhea mediated by diacerein.
- The Standard II group also showed increased body weight throughout the experimental period.
- The test group also showed an increase in body weight as the sustained release formulation of diacerein releases the drug sustainably, so diarrhea and soft stool are not seen in this group.

5. Scoring of arthritis index

The arthritis index was scored after 28 days of drug treatment in all five groups of animals (Figure 5).

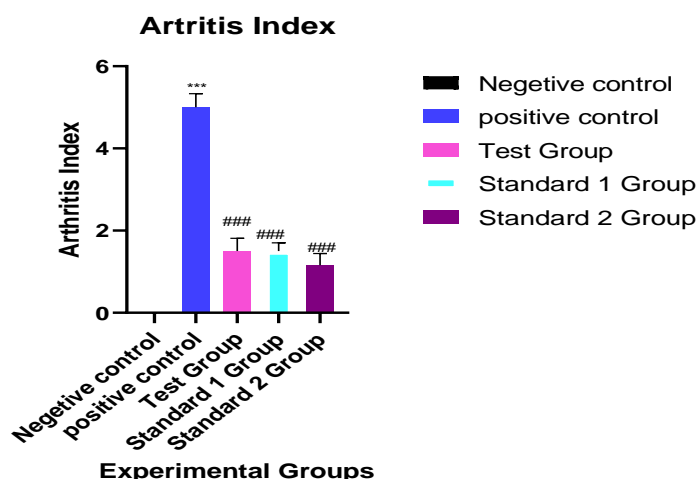


Figure (5): Arthritis index of all 5 groups of animals.

Figure 5: Effect of diacerein microspheres on the arthritic index in CFA-induced arthritis model in rat. Data represented as mean \pm S.E.M. ***P < 0.001 compared to a negative control group, ###P < 0.001 compared to a positive control group. (One-way ANOVA followed by Turkey's multiple comparisons test).

Functional parameter like the arthritis index significantly differs between positive control rats and negative control rats. Significant improvement in arthritic index occurred in the test, standard I, and standard II groups. When compared to standard I and standard II groups, the test group showed nonsignificant alterations in the arthritic index, which indicates the

same efficacy of the test formulation compared to standard I and standard II groups.

6. Joint stiffness study

Following the completion of drug treatment, all five groups of animals have undergone a joint stiffness study (Figure 6).

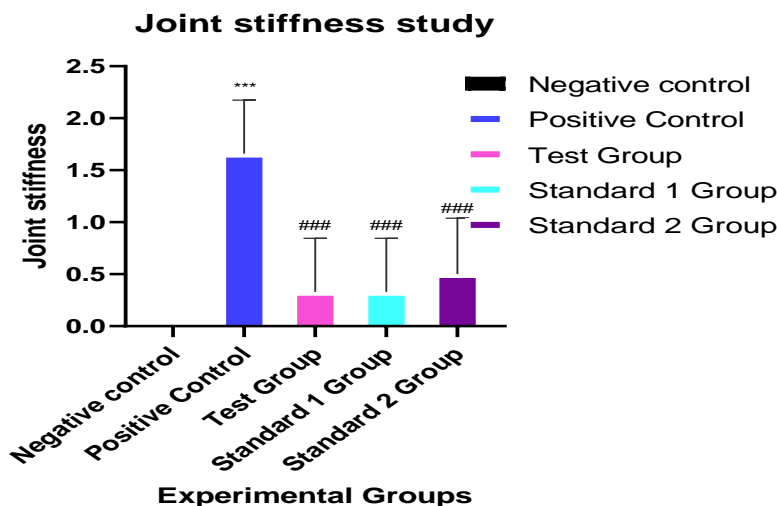


Figure (6): Joint stiffness study of all 5 groups of animals.

Figure 6: Effect of diacerein microspheres on joint stiffness study in C.F.A. induced arthritis model in rat. Data represented as mean \pm S.E.M. *** $P < 0.001$ compared to a negative control group, ### $P < 0.001$ compared to a positive control group. (One-way ANOVA followed by Turkey's multiple comparisons test).

Functional parameters like joint stiffness significantly differ between positive and negative control rats. Significant improvement in joint stiffness occurred in the test, standard I, and standard II groups. Compared to a standard I and standard II groups, the test group showed nonsignificant alterations in joint

stiffness, indicating the same efficacy as the test formulation compared to standard I and standard II groups.

7. Gait test

Following the completion of drug treatment, all five groups of animals have undergone a gait test study (Figure 7).

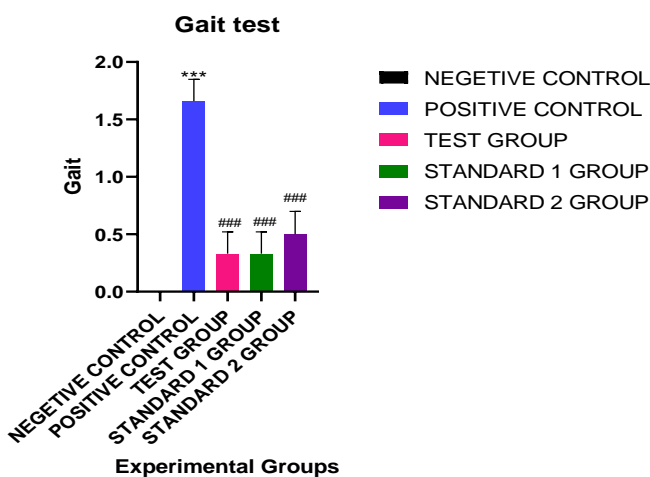


Figure (7): Gait test study of all 5 groups of animals.

Figure 7: Effect of diacerein microspheres on gait test study in C.F.A. induced arthritis model in rat. Data represented as mean \pm S.E.M. *** $P < 0.001$ compared to a negative control group, ### $P < 0.001$ compared to a positive control group. (One-way ANOVA followed by Turkey's multiple comparisons test).

Results of the gait test in the negative control and positive control groups differ significantly. In the test group, standard I and standard II groups, significant improvement was seen in experimental animals. Compared to

the standard I and standard II groups, the test group showed a nonsignificant change in the results of the gait test, indicating similar efficacy.

8. Haematological Analysis

Blood samples were collected from cardiac puncture after 28 days of drug treatment,

and hemoglobin count (gm/dl), W.B.C count, R.B.C count, and E.S.R. count were measured in 5 groups of animals were recorded and are illustrated in table 4 and figure 8, 9,10 and 11.

Table (4): Haematological data of 5 groups of animals.

Hematological parameters	Negative control	Positive control	Test Group	Standard 1 group	Standard 2 group
	(Mean values ± S.E.M.)				
Hemoglobin count	15.52±0.54	9.25 ± 0.04***	13.70±0.07###, aa	13.60±0.07###	12.49±0.09###
ESR count	5.26±0.06	13.38±0.16***	6.5±0.10###, aaa	6.49±0.09###	8.43±0.09###
WBC count	6.22±0.05	12.57±0.16***	7.42±0.1###, aaa	7.48±0.08###	8.38±0.08###
R.B.C. count	7.39±0.08	3.54±0.08***	5.5±0.1###	5.41±0.09###	5.29±0.07###

***P < 0.001 compared to negative control group, ###P < 0.001 compared to positive control group. aaP < 0.01 compared to the standard I group. aaaP < 0.001 compared to the standard II group. (One-way ANOVA followed by Turkey's multiple comparisons test).

Haemoglobin Count (gm/dL)

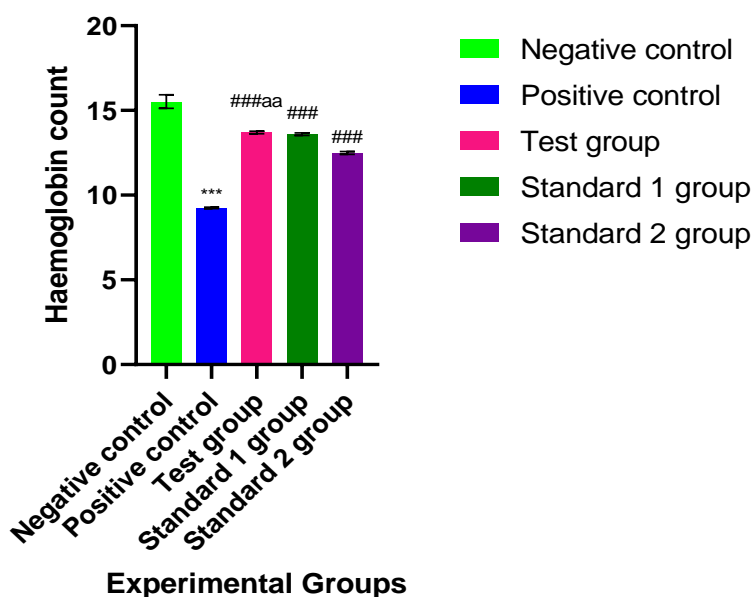


Figure (8): Haemoglobin count in 5 groups of animals (Positive control vs. negative control, test vs. positive control, standard I vs. positive control, standard II vs. positive control comparison).

Figure 8: Effect of diacerein microsphere on hemoglobin count in CFA-induced arthritis model in rat. Data represented as mean ± S.E.M. ***P < 0.001 compared to a negative control group, ###P < 0.001 compared to a positive control group, aaP < 0.01 compared to the standard II group. (One-way ANOVA followed by Turkey's multiple comparisons test)

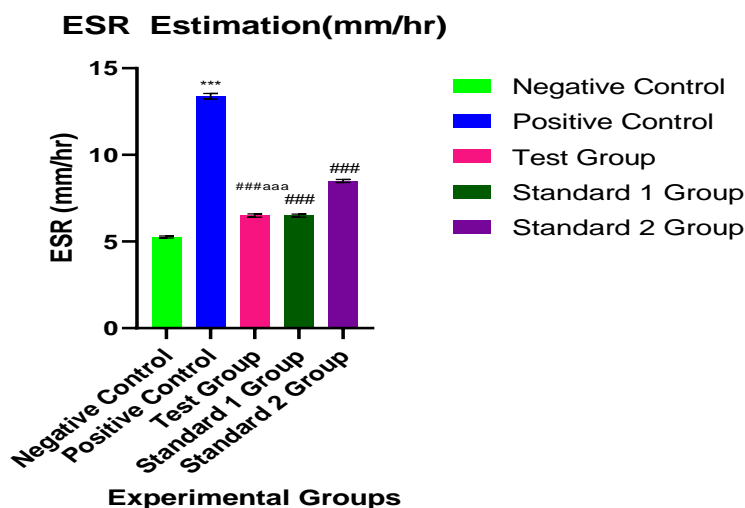


Figure (9): E.S.R. count in 5 groups of animals (Positive control vs. negative control, test vs. positive control, standard I vs. positive control, standard II vs. positive control comparison.)

Figure 9: Effect of diacerein microspheres on E.S.R. estimation in C.F.A.- induced arthritis model in rat. Data represented as mean \pm S.E.M. ***P < 0.001 compared to the negative control group, ###P < 0.001 compared to a positive control group, aaaP < 0.001 compared to the standard II group. (One-way ANOVA followed by Turkey's multiple comparisons test).

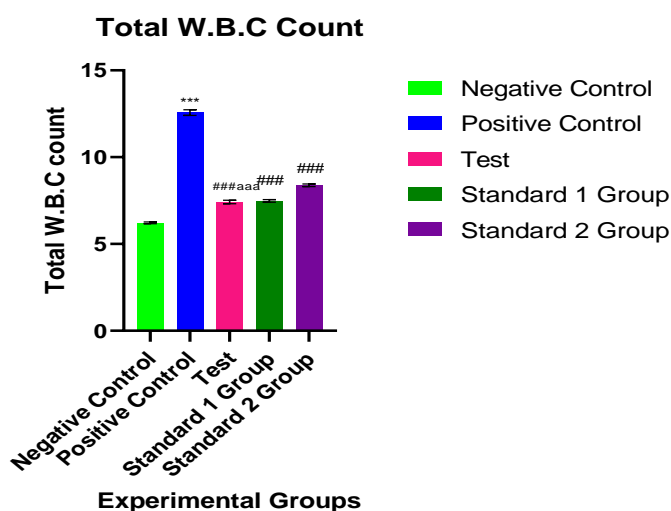


Figure (10): Total W.B.C count in 5 groups of animals (Positive control vs. negative control, test vs. positive control, standard I vs. positive control, standard II vs. positive control comparison).

Figure 10: Effect of diacerein microspheres on total W.B.C count in C.F.A. induced arthritis model in rat. Data represented as mean \pm S.E.M. ***P < 0.001 compared to a negative control group, ###P < 0.001 compared to a positive control group, aaaP < 0.001 compared to standard II group. (One-way ANOVA followed by Turkey's multiple comparisons test)

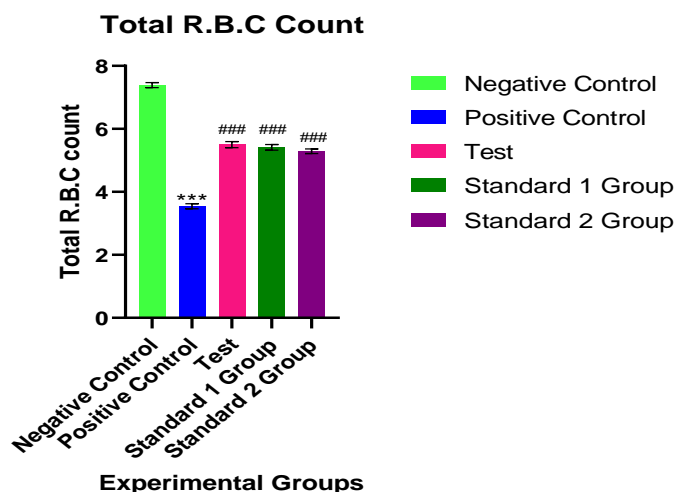


Figure (11): Total R.B.C. count in 5 groups of animals.

Fig 11: Effect of diacerein microsphere on total R.B.C count in C.F.A. induced arthritis model in rat. Data represented as mean \pm S.E.M. ***P < 0.001 compared to a negative control group, ###P < 0.001 compared to a positive control group. (One-way ANOVA followed by Turkey's multiple comparisons test).

Patients were suffering from active R.A. exhibit hematological changes such as chronic anemia, an increased number of W.B.C (leukocytosis), and an increase in E.S.R. [17]. IL-1 β mediates a moderate increase in WBC count [18]. CFA-induced arthritic rats also showed an increase in E.S.R. count and W.B.C count, whereas a decrease in R.B.C count and hemoglobin count compared to the negative control group. Researchers have reported similar hematological changes in arthritic rats [19, 14]. Diacerein microsphere treated group (test group), diacerein API formulation treated group (standard I group), and glucosamine formulation treated group (standard II group) showed significant

improvements in all hematological parameters compared to the positive control group (Figures 8, 9, 10 and 11). In all hematological studies, the test group showed nonsignificant alterations compared to the test 1 group. In hemoglobin count, E.S.R. count, and total W.B.C count, the test group showed significant improvement compared to the standard II group (Figure 8, 9, 10).

9. X-ray analysis of joint injury in various experimental groups of animals

After completion of drug treatment on the 28th day, the next day, animals were subjected to X-ray analysis of tibiotarsal joints. Some pictures of the X-ray analysis of the joints of 5 groups of animals are given in figure 12.



Figure 12A:(Negative Control)



Figure 12B: (Positive control)

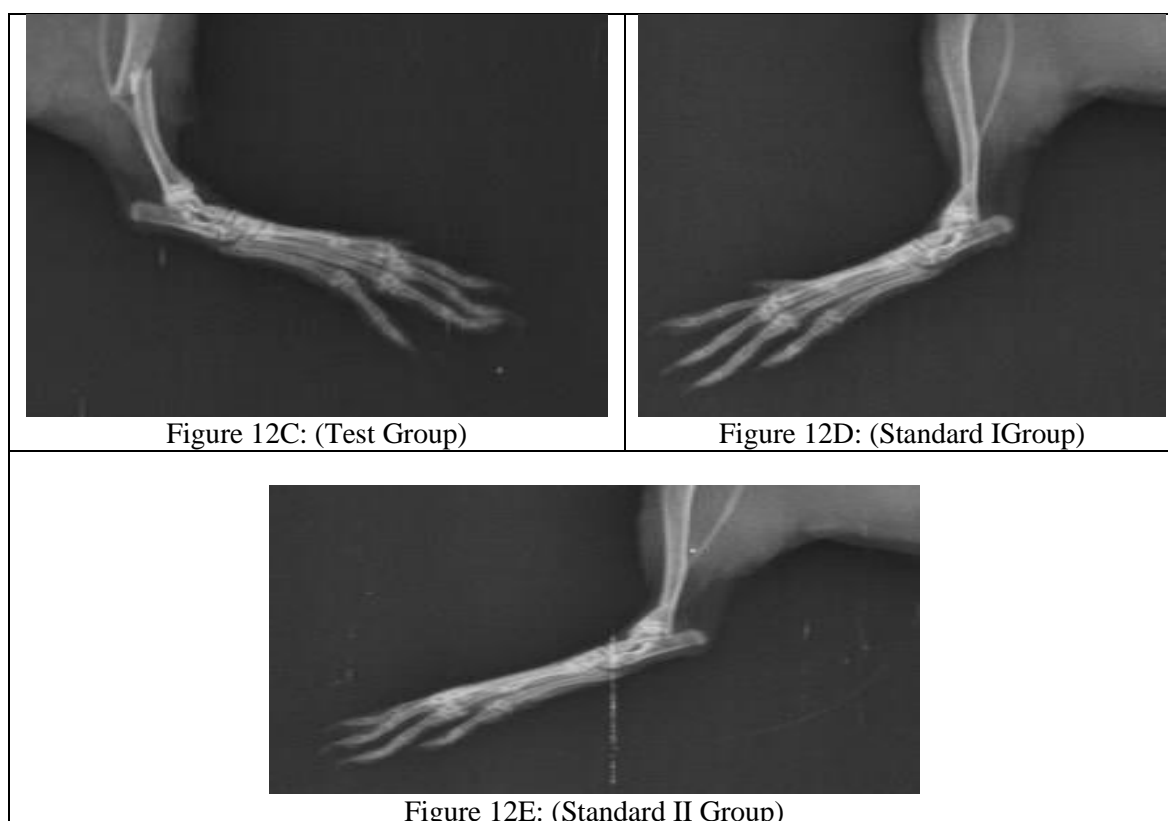


Figure (12): X-Ray analysis of tibiotarsal joints after completion of drug treatment.

Adjuvant-induced alterations in bone architecture include resorption of bone matrix, formation of osteophyte cells, and expansion of joint space with the erosion of bone and subsequent inflammation and swelling of soft tissue (Figure 12B). In contrast, no alterations in joint architecture were found in negative control groups (Figure 12A). In the test group (Figure 12C), Standard I group (Figure 12D), and standard II group (Figure 12E), a

reduction in joint space with no findings of joint swelling, inflammation, or bone deterioration was seen. In the test group (Figure 12C), Standard I group (Figure 12D), and standard II group (Figure 12E), a reduction in joint space with no findings of joint swelling, inflammation, or bone deterioration was seen.

10. Charcoal meal test

Data from the charcoal meal test is represented in Table 5.

Table (5): Charcoal meal test.

Group	the total length of the intestine (cm)	Distance traveled by marker (cm)	Peristalsis index	%inhibition/Acceleration
Negative control	80.31± 0.88	68.30± 0.48	85.16 ± 1.32	
Test Group	76.74 ±0.71	34.86 ± 0.62 ^{###}	45.46± 1.1 ^{###}	48.96
Standard Group	91.81± 1.61	90.87 ± 1.71 ^{***}	98.96 ± 0.2 ^{***}	-33.04 (Acceleration)

Data are expressed as mean ± S.E.M.; ***p < 0.001 versus the control group, ### p < 0.001 versus the standard group.

A charcoal meal test was used to assess gastrointestinal propulsion [20]. Diacerein is known to cause diarrhea mainly due to the inhibition of IL-1 β . Expression of IL-1 β significantly increased in diarrhea in pigs [21] and E.

coli-induced diarrhea [22]. The cytokine is thought to be a component of the system that triggers immune responses to infections in the gut. Inhibiting IL-1 β may thus make the diacerein-treated individual less capable of

fighting off common gut infections, resulting in a high prevalence of diarrhea. Apart from this, diacerein stimulates PGE2 production in the intestinal gut, so peristaltic activity increases, resulting in diarrhea [23].

The experimental data shows that the distance traveled by the marker (charcoal meal) and peristalsis index in the standard group significantly increased in the standard group compared to the normal control group. Acceleration of intestinal meal transit was also observed in the standard group. The test group showed a significant decrease in distance traveled by the marker (charcoal meal) and peristalsis index compared to the standard group due to the sustained release of diacerein from the microsphere. The test group also showed significant inhibition of intestinal meal transit compared to the standard group. From the data, it can be concluded that the diacerein microsphere significantly decreases peristaltic movement in rats; thus, diarrhea is reduced.

CONCLUSION

From this research, we can conclude that diacerein microsphere-treated rats significantly reduce arthritic parameters like paw volume, arthritic index, joint stiffness, and gait test, as well as improve various hematological parameters like WBC count, R.B.C. count, Haemoglobin count, and E.S.R. count. X-Ray analysis of tibiotarsal joints showed significant reductions in bone deformity, joint space, and joint swelling. Similar improvements were observed in the diacerein API-treated group and glucosamine-treated group. Nonsignificant alterations were observed among these groups indicating similar efficacy. Data from the charcoal meal test indicates lower peristaltic index and inhibition of gastrointestinal motility, suggesting that diacerein microspheres successfully release the drug sustainably so that diacerein-induced diarrhea is reduced and it can be considered as sustained release formulation.

ETHICS APPROVAL AND CONSENT TO PUBLICATION

Ethical approval

Ethical approval (Registration No. 1938/P.O./Rc/S/17/CPCSEA) was granted from Institutional Animal Ethics Committee,

TAAB Biostudy Services, 69 Ibrahimpur Road, Jadavpur, Kolkata – 700 032.

Consent for publication

All the authors have read and approved the manuscript for publication.

Availability of data and materials

Not applicable.

Author's contribution

Tathagata Roy: Involved in conceptualization, writing-original draft, data curation, formal analysis, methodology development, supervision, validation, visualization, and writing review & editing of the manuscript.
Dr. Tapan Kumar Chatterjee: Involved in conceptualization, formal analysis, and supervision of the research work.

Competing interest

The authors declare that there is no conflict of interest.

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