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Original Article

SYNTHESIS AND EVALUATION OF PHTHALATE ANALOGUE OF DICLOFENAC AGAINST FREUND'S COMPLETE ADJUVANT INDUCED ARTHRITIS IN RAT

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ABSTRACT

Objective: The objective of the present study is to evaluate the effect of Phthalate analogues of diclofenac in Freund's complete adjuvant (FCA) induced Arthritis in the rat.

Methods: Twenty four female albino wistar rats were enrolled in this study and are divided into 4 groups (six each). The groups were designed as follows: Group I: vehicle control, Group II: arthritic control, Group III: diclofenac treated, Group IV: phthalate analogue of diclofenac treated. Various assessments such as anti-arthritic activity, biochemical estimations, haematological parameters, ulcerogenesis, radiological and histopathological studies were evaluated.

Results: Arthritic control group exhibited significant increase in the level of paw volume, arthritic score ($p<0.0001$), Serum glutamic pyruvic transaminase (SGPT) ($p<0.001$), Serum glutamic oxaloacetic transaminase (SGOT) $p<0.01$), rheumatoid arthritis factor, C-reactive protein (CRP), White Blood Cells (WBC), Creatinine and uric acid and a significant decrease in Red Blood Cells (RBC). Increased swelling of joints, bony destruction and profound ulceration were observed in the Arthritic control group. All these conditions were reversed in diclofenac and phthalate analogue of diclofenac groups.

Conclusion: We conclude that phthalate analogue of diclofenac shows potent anti-arthritic activity with milder ulceration when compared to diclofenac treatment.

Keywords: Diclofenac, Freund's Complete Adjuvant (FCA), Phthalate moiety, Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease with the main clinical manifestation of systemic complications including synovial inflammation, joint lesion and bone damage [1]. In RA, pannus is rich in secretion of activated macrophages and other inflammatory mediators, resulting in the destruction of tissues when compared to normal synovial fluid, which is essentially a cellular [2]. One of the most important groups of mediators in RA is cytokines. The most prominent of these include TNF, IL-1, and IL-6, which are released in the synovial microenvironment [3, 4]. Rheumatoid arthritis is also characterized by the presence of autoantibodies known as rheumatoid factors (RF) and anti-citrullinated peptide antibodies (ACPA). Therefore RA synovial fluid is abundant in neutrophils, macrophages, T lymphocytes, autoantibodies and dendritic cells. Chondrocytes secrete proteolytic enzymes that lead to destruction of articular cartilage causing bone deformity and loss of joint function. It is thought that costimulation of both humoral and cellular immunity may contribute to the pathology of the disease [5].

RA affects all the races, where the female population is affected three times more than the male population. The cause of RA is still a mystery. The management of RA includes Nonsteroidal anti-inflammatory Drugs (NSAIDs), Glucocorticoids, Disease-Modifying Anti-Rheumatic Drugs (DMARDs) and Biologic agents [6]. Among the most popular NSAIDs worth mentioning is diclofenac ranked 30th among the top 200 drugs with respect to new prescriptions.

Diclofenac sodium has been demonstrated to have antipyretic activity and to be an effective analgesic in rats and mice and in therapeutic trials in patients with rheumatoid arthritis, osteoarthritis or pain of varying origin [7]. Despite the intensive research that has been aimed at the development of diclofenac, their clinical usefulness is still restricted by their gastrointestinal side effects like gastric irritation, ulceration, bleeding, perforation and also cardiovascular complications. Hence, the scope of the research widens for the newer, effective and safer alternative medicine.

Gastrointestinal (GI) toxicity is attributed to direct contact of free carboxylic acid (–COOH) moiety present on diclofenac sodium with GI mucosal cells [7]. Hence as an alternative approach, in order to avoid major GI side effects, we have focused our research work on Synthetic derivatization based upon chemical modification of diclofenac structure. Therefore we have attempted to design phthalate analogue of diclofenac by replacing free acidic group on diclofenac structure by the most potent less acidic heterocyclic ligand–phthalate moiety. The latter showed negligible GI toxicity, due to very good selective interactive energies with cyclooxygenase-2 (COX-2) when compared to that of diclofenac sodium [8]. Hence the present study was therefore aimed to synthesize and evaluate phthalate analogue of diclofenac and was designed to investigate whether the synthesized compound exerts an ameliorative effect on arthritis in rats exposed to Freund's complete adjuvant (FCA) and to ascertain its potential mechanism with less ulcerogenicity.

MATERIALS AND METHODS

Selection and synthetic scheme of the phthalate analogue of diclofenac

Diclofenac sodium was hydrolyzed using concentrated sulphuric acid to convert salt to acid. The obtained intermediate was refluxed for 22 h with absolute alcohol and concentrated sulphuric acid. The obtained ethyl ester reaction mixture was sodium bicarbonate solution and recrystalised with methanol. The recrystalised ester was refluxed with hydrazine hydrate along with absolute alcohol for 22 h. Finally the precipitated mixture was filter dried and recrystalised from methanol. Yield: 87.44%, mp: 112 °C.

Acute oral toxicity studies of phthalate analogue of diclofenac–OECD 423 guideline

Acute oral toxicity studies were performed using a phthalate analogue of diclofenac at the dose level of 300 mg/kg orally in mice.

Animals

Healthy adult female Albino Wister Rats (150-200 g: 24 rats) were obtained from Swamy Vivekananda College of Pharmacy, Tiruchengodu.

All animals used in this study were handled with Indian National Science Academy Guidelines and the animal experiments were performed according to the guidelines prescribed by CPCSEA. The study protocol was approved by the Institutional Animal Ethics Committee (Reg. No.889/PO/ac/05/CPCSEA/dated 9th November 2011).

Study design

24 Albino Wistar rats were used for this study. They were randomly divided into 4 groups of each of 6 rats. Thirty minutes after administration of vehicle/drug, arthritis was induced by sub plantar injection of0.1 ml of 0.5% Freund's complete adjuvant (FCA) into the tibia tarsal joint. Group-I animal receives only distilled water, Group-II animal receives 0.1 ml of 0.5% Freund's complete adjuvant, Group-III animal receives0.1 ml of 0.5% Freund's complete adjuvant along with Diclofenac 10 mg/kg/p. o for 21 d, Group-IV receives 0.1 ml of 0.5% Freund's complete adjuvant along with phthalate analogue of Diclofenac 30 mg/kg/p. o for 21 d [9].

Anti-arthritic activity of phthalate analogue of diclofenac was evaluated on paw volume, arthritis score on day 5, 10, 15 and day 21. Moreover body weights of animals were monitored regularly during the course of the experiment. On day 21, blood was withdrawn by a retro-orbital puncture for assessment of haematological parameters and animals were sacrificed under light ether anaesthesia to study histopathology of joints.

Arthritis score

Morphological feature of arthritis was monitored according to the extent of erythema and oedema of the joints, using the criteria as follows: normal paw = 0, mild swelling and erythema of digits = 1, moderate swelling and erythema of digits = 2, severe swelling and erythema of digits = 3, gross deformity and inability to use limbs = 4 [10, 11]. The scores for each paw were then added to get the total arthritic score.

Paw volume

The left hind paw volume of all animal was measured just before Freund's complete adjuvant injected at different time intervals till 21 d using Plethysmography. The change in paw volume was measured as the difference between the final and initial paw volumes [11, 12].

Haematological, biochemical, radiological studies

The haematological parameters like RBC, WBC, PCV and HB count were assessed using chesbrough and McArthur. The hemoglobin count was determined by acid haematin method [13]. On day 21, blood was withdrawn by retro-orbital puncture and serum was used for estimation of biochemical markers like SGOT, SGPT, ALP, RA factor, Creatinine and serum uric acid [14, 15]. Radiographs were taken for the hind paw of all groups and examined for soft tissue swelling, bony erosions and narrowing of spaces between the joints [16-18].

Determination of the ulcerogenic effect

The animals were sacrificed on 22nd day and the stomach is dissected out. The contents of the stomach are drained into a graduated centrifuge tube and their activity determined by titration with 0.1N NaoH. The stomach was opened along its greater curvature and its inner surface examined for ulceration with the binocular microscope [19].

The ulcer index is calculated and the ulcer severity graded as mentioned below:

0 = Normal coloured stomach, no ulcer

0.5 = Red colouration

1 = Spot ulcers

1.5 = Haemorrhagic streaks

2 = Ulcers

3 = Perforation

Histological analysis

The animals were sacrificed on day 21 by cervical dislocation. Ankle joints were separated from the hind paw, weighed and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 5-thickness. The sections were stained with haematoxylin and eosin and evaluated under a light microscope [20].

Statistical analysis

The statistical comparison was made between arthritic control and treated group. They were analyzed by one way ANOVA followed by Dunnet's comparison test. The level of significance was at $p<0.05.16$.

RESULTS

Effect of phthalate analogue of diclofenac changes in acute oral toxicity studies

The phthalate analogue of diclofenac does not produce any toxic symptoms or mortality up to dose level of 300 mg/kg orally in mice and hence the drug was considered safe for further pharmacological screening. As per OECD-423 1/10 (30 mg/kg) of phthalate analogue of diclofenac was used for future pharmacological screening. No lethal toxic reaction was observed till the end of 21 d.

Effect of phthalate analogue of diclofenac on an arthritic score

In this study, there was a significant increase in arthritic score in the arthritic control group when compared to vehicle control. On the day of 5th, 10th, 15th and 21st days, arthritic control group showed significant ($p<0.0001$) increase when compared to phthalate analogue of diclofenac and diclofenac group (table 1).

Effect of phthalate analogue of diclofenac on change in paw volume

There was a significant ($p<0.001$) increase in paw volume in all FCA (Freund's complete adjuvant) administered group compared to phthalate analogue of diclofenac and diclofenac treated group. This showed biphasic response where there was small change in decrease in paw volume from 10 to 15 d. Hence this change was no significant. Treated group (Group III and IV) significantly ($p<0.01$, $p<0.001$) showed decreased paw volume when observed till end of study. There was significant change in paw volume of phthalate analogue of diclofenac and diclofenac treated group (table 2)

Effect of phthalate analogue of diclofenac on haematology estimation

The haematological parameters were observed in which WBC and PCV showed significant ($p<0.001$) increase only group II (arthritic control) when compared to other groups. RBC and HB showed decreased ($p<0.0001$) value in group II. (table 3)

Effect of phthalate analogue of diclofenac on biochemical estimation

In this study, group II showed a significant ($p<0.0001$) increase when compared to group III and IV. SGPT and SGOT in group II is significantly increased ($p<0.001$, $p<0.01$) when compared to phthalate analogue of diclofenac and diclofenac treated group. ALP, CRP and RA factor were also increased in group II and showed the significant value ($p<0.001$) (table 4)

Effect of phthalate analogue of diclofenac on radiographic studies

In adjuvant-induced arthritic rat (group II), soft tissue swelling along with narrowing of joints spaces were observed which implies bony destruction in arthritic condition. The standard drug diclofenac 10 mg/kg treated group have prevented this bony destruction and also there is no swelling of joint (fig. 2: A, B, C, D).

Effect of phthalate analogue of diclofenac on anti-ulcer activity

Group I has showed normal colour stomach and scored as 0. Group II (negative control) showed major effect and the score is found to be 1. Diclofenac treated (Group III) group has showed gastric mucosal damage compared to group II and the score is 2. Group IV phthalate analogue of diclofenac with inducer has showed less ulcer activity compared to group II and the score is 3 (fig. 2: A, B. C, D)

Effect of phthalate analogue of diclofenac on histopathological study

The histopathological study has shown significant prevention against bony destruction by soft tissue swelling are narrowing of joint spaces when compared with an arthritic control group (fig. 3: A, B, C, D)

Table 1: Effect of phthalate analogue of diclofenac on arthritic score

Group	Arthritic score			
	5 days	10 days	15 days	21 days
Group I-Vehicle control	1.07±0.01	1.48±0.06	1.50±0.23	1.62±0.24
Group II-(FCA)	1.33±0.01	3.07±0.19a**	3.30±0.21a**	3.91±0.04a***
GroupIII(FCA+diclofenac)	1.56±0.17	2.98±0.07a***	2.83±0.13a***	2.67±0.22b***
GroupIV(FCA+phthalate analogueof diclofenac)	1.61±0.03	2.63±0.18b***	2.42±0.21b***	2.34±0.405b***

Values are expressed as mean±SEM, n=6 Symbols represent statistical significance: *** p<0.001, **p<0.01, *p<0.5: a-Group I Vs II, III and IV b-Group II Vs II, IV.

Table 2: Effect of phthalate analogue of diclofenac on change in paw volume

Group	Paw volume (ml)			
	5 days	10 days	15 days	21 days
Group I-Vehicle control	1.01±0.03	1.91±0.07	1.86±0.30	1.61±0.07
Group II (FCA)	1.23±0.22	3.44±0.28a*	3.77±0.19	3.83±0.16a**
GroupIII(FCA+diclofenac)	1.30±0.05	2.92±0.36b*	2.85±0.10	2.67±0.30
GroupIV(FCA+phthalateanalogueofdiclofenac)	1.44±0.23	2.65±0.167a***	2.40±0.30	2.33±0.20b**

Values are expressed as mean±SEM, n=6, Symbols represent statistical significance: *** p<0.001, **p<0.01, *p<0.5: a-Group I Vs II, III, IV. b-Group II Vs, III and IV

Table 3: Effect of phthalate analogue of diclofenac on Haematology parameters

Group	RBC (1x103/µl)	WBC (1x103/µl)	HB (g/dl)	PCV (%)
GroupI(Vehicle control)	5.05±0.21	7.460±0.27	14.91±0.81	40.07±0.98
Group II (FCA)	2.70±0.18a**	17.92±1.55a***	9.095±0.39a**	45.32±3.65a***
GroupIII(FCA+diclofenac)	4.37±0.14b***	11.97±0.83	15.48±0.17	38.43±2.75
GroupIV(FCA+phthalate analogue of Diclofenac)	4.150±0.24	10.35±0.53	14.42±0.88	39.47±1.033

Values are expressed as mean±SEM, n=6 Symbols represent statistical significance: *** p<0.001, **p<0.01, *p<0.5: a-Group I Vs II, III,IV. b-Group II Vs I, III and IV

Table 4: Effect of phthalate analogue of diclofenac on biochemical parameters

Group	Creatinine	Uric acid	SGPT	SGOT	ALP
Group I (Vehicle control)	0.393±0.09	1.500±0.18	25.50±0.76	33.67±3.49	68.33±16.02
Group II (FCA)	1.47±0.22a***	5.93±0.08a***	54.10±3.25a***	55.40±1.15a**	217.5±19.4a***
Group III (FCA+diclofenac)	0.270±0.06b***	5.300±0.09b**	26.23±0.92b***	35.00±2.22b**	86.50±11.76b*
Group IV (FCA+phthalate analogue of diclofenac)	0.500±0.30b***	4.25±0.52	23.83±2.24b***	33.67±2.07b**	70.33±8.65b**

Values are expressed as mean±SEM, n=6, Symbols represent statistical significance: *** p<0.001, **p<0.01, *p<0.5: a-Group I Vs II,III,IV. b-Group II Vs, III and IV

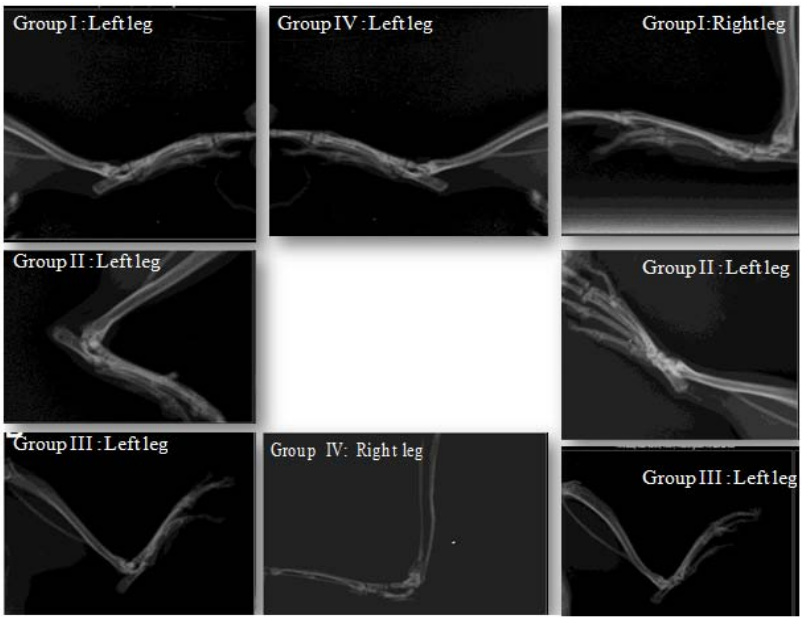

Fig. 1: Effect of phthalate analogues of diclofenac on radiography

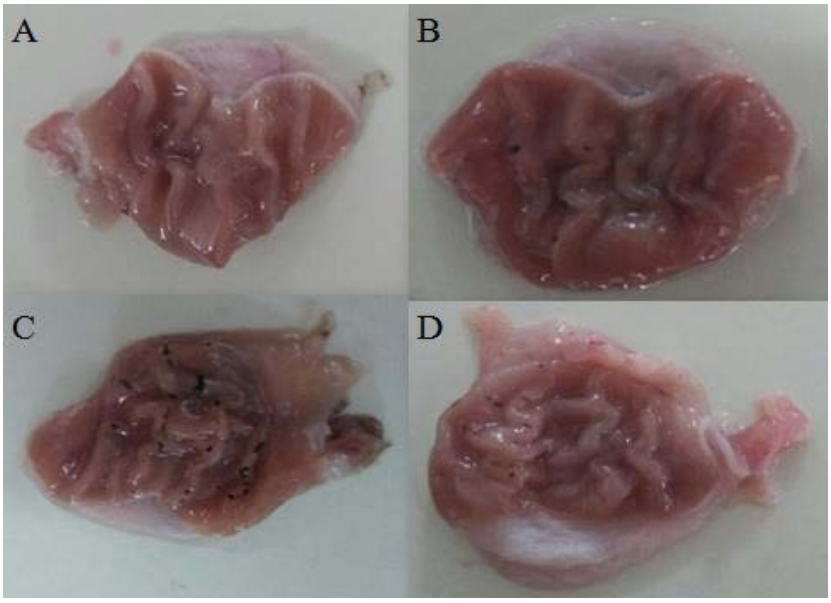

Fig. 2: Effect of phthalate analogue of diclofenac on anti-ulcer activity A-Group I, B-Group II, C-Group III and D-Group IV

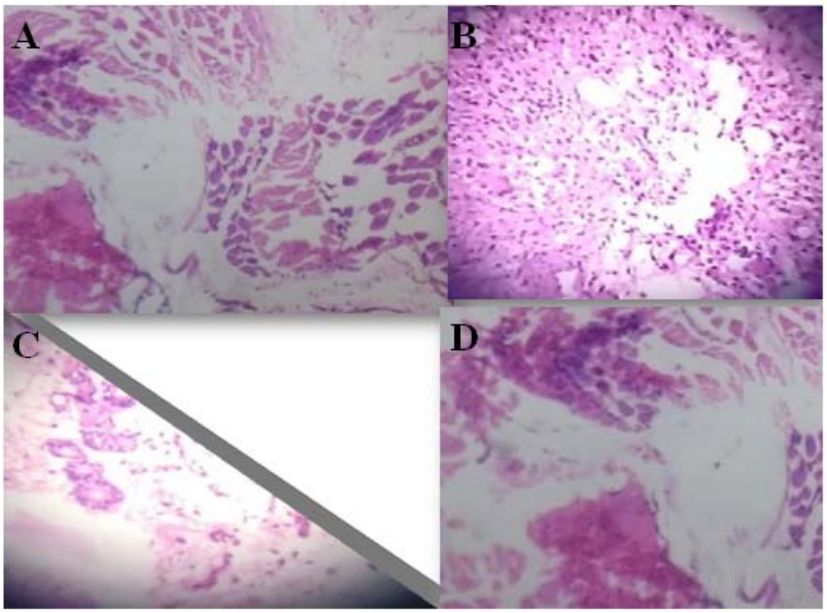

Fig. 3: Effect of diclofenac and phthalate analogue of diclofenac on histological analysis, A-Group I, B-Group II, C-Group III and D-Group IV

DISCUSSION

Non-steroidal anti-inflammatory drugs, often referred to as NSAIDs, are assumed to be well tolerated and are widely used as initial therapy for anti-arthritis. Everyone is familiar with these types of drugs with millions using them for pain. The gastrointestinal side effect associated with all traditional NSAID's is mainly due to the presence of free carboxylic acidic group. Hence in the present study the–COOH group of diclofenac was replaced by the substitution of phthalate group to reduce its side effect and to potentiate RA activity.

The phthalate analogue of diclofenac does not produce any toxic symptoms or mortality up to the dose level of 300 mg/kg orally in mice and hence, the drug was considered safe for further pharmacological screening. Therefore 1/10 of the dose (30 mg/kg) was *s*elected for *in vivo* study.

In the present study, Freund's complete a djuvant was used for the induction arthritis. This model is widely used to study the pathogenesis of rheumatoid arthritis for testing therapeutics and this model is characterized by a very rapid erosive disease. The bacterial peptidoglycan and muramyl dipeptide present in the FCA are responsible for the induction of adjuvant arthritis [21]. The determination of paw swelling is an apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and assessing of therapeutic effects of drugs. In the present study, the rat was selected as an animal model since they develop a chronic swelling in multiple joints with an influence of inflammatory cells and followed by erosion of cartilage in joints and destruction of bones. The rat model is a close resemblance to rheumatoid arthritis of human beings [22].

As one of the major factors in assessing the degree of inflammation and curative efficacy of drug is hind paw volume and this was measured using plethysmography in the present study. FCA group showed increased paw volume due to stimulation of inflammatory cytokines in cell-mediated immunity when compared control group. Phthalate analogue of diclofenac treatment group shows significantly inhibitory effect on hind paw swelling due to reduced inflammatory response when compared to diclofenac group [23].

In the present study, the reduction in RBC and haemoglobin level in the adjuvant induced arthritic control rats denotes the anaemic condition. These decreased levels of RBC, and HB (haemoglobin) was significantly increased by phthalate analogue of diclofenac and diclofenac treatment. The increased levels of WBC and packed cell volume (PCV) in the adjuvant induced arthritic control rats due to the stimulation of immune system against the invading pathogens. These increased levels of WBC and packed cell volume (PCV) was significantly decreased by phthalate analogue of diclofenac and diclofenac treatment. All these symptoms indicate an anemic condition, which is a common diagnostic feature in patients with chronic arthritis.

In this study, RA factor in FCA group shows significant increase due to formation of immune complexes that contribute to the progress of rheumatoid arthritis. The phthalate analogue of diclofenac and diclofenac treatment group showed significant decrease in RA factor when compared to FCA control (Mali SM *et al.*, 2011). And CRP level of FCA group is increased due to plasma level increase and tissue damage. The Phthalate analogue of diclofenac revert this increased level when compared to diclofenac group [24].

In this study, serum SGPT, SGOT and ALP was raised in Arthritic rat due to liver and spleen impairment, whereas in phthalate analogue of diclofenac the level is significantly lowered due to abnormal high level of plasma when compared to diclofenac group. From the obtained results it was shown that creatinine and uric acid levels were increased due to damage of renal tubules that leads to massive accumulation. The Phthalate analogue of diclofenac shows significant decrease in creatinine and uric acid levels when compared to diclofenac group.

The histopathology analysis identifies the ability of the bones to re-form upon treatment with diclofenac 10 mg/kg and Phthalate analogue of diclofenac 30 mg/kg p. o have that shows significant prevention against bony destruction, tissue swelling and narrowing of joint spaces when compared with complete Freund's adjuvant control group.

Radiography changes in rheumatoid arthritis condition are useful markers which indicate the severity of the disease. Soft tissue swelling is earlier radiographic sign, whereas prominent radiographic changes like bony erosion and narrowing of joint spaces can be observed only in the developed stages of arthritis. In adjuvant induced arthritic rat (group II), soft tissue swelling along with narrowing of the joint spaces were observed which implies the bony destruction in arthritic condition. The test compound phthalate analogue of diclofenac has prevented this bony destruction and also there is no swelling of the joint.

In ulcerogenecity of FCA Group shows mild ulceration with ulcer score-2 when compared to control group. Diclofenac group produce more ulceration with ulcer score-3 when compared to control. Phthalate analogue of diclofenac group shows very less incident of ulceration with ulcer score-1 when compared to control group.

The overall findings of the current study reveal that Phthalate analogue of diclofenac attenuates FCA induced arthritis in rats.

CONCLUSION

In conclusion, replacement of-COOH group of diclofenac and addition of phthalic anhydride group to produce phthalate analogue of diclofenac acts as potential ligand for anti-arthritis with less gastro intestinal toxicity. Further clinical data are required to explore this synthesised analogue of diclofenac as potential ligand for improving the status of RA patients.

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AUTHORS CONTRIBUTIONS

The corresponding author, P. Manimekalai prepared the manuscript, P. Sudhakar supervised the experimental work. KM Preetu Shukla has done the laboratory work and data collection. G. Murugananthan reviewed the manuscript and guided to improve the quality of the manuscript.

CONFLICT OF INTERESTS

We declare that there was no conflict of interest

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