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Protective effects of phytocompounds from *Alpinia calcarata* (Haw.) Roscoe in Freund's adjuvant induced arthritis in rats

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Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder of unknown etiology which affects multiple joints and causes cartilage erosion. Due to the side effects associated with the conventional treatment strategies, alternate medicine using plant extracts is on the rise for the treatment of arthritic conditions. In the present study, we evaluated the anti-arthritic potential of two phytocompounds from *Alpinia calcarata* (Haw.) Roscoe, a potential medicinal herb, locally called 'Kolinji', used in traditional medicine. The selected compounds, fenchol and 2,4-di-tert-butylphenol, were evaluated for their efficacy in Freund's complete adjuvant induced arthritis in rats. Arthritis assessment was done by measuring the paw volume, paw size, arthritic score and body weight. Various hematological and biochemical parameters were assessed on the last day of the study. Histological and radiological analysis of the ankle joints was done. The compounds treated at both doses (50 and 100 mg/kg body wt.) showed a dose dependent decrease in paw volume, paw size and arthritic score following arthritis induction. Treatment with the standard drug (diclofenac) and phytocompounds were followed by favourable outcome in the altered hematological parameters and the level of liver enzymes. The radiological and histological analysis also confirmed the anti-arthritic potential of these compounds. The results revealed the potential of the plant for use in the therapeutic management of rheumatoid arthritis.

Keywords: 2,4-di-tert-Butylphenol, Fenchol, Freund's complete adjuvant, Lesser galangal, Snap Ginger

Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by the inflammation of synovium leading to destruction of bones and cartilage in various joints[1]. It is two to three times more frequent in women than in men and can start at any age, with a peak incidence between the fourth and sixth decade of life. Apart from the conventional treatment strategies using nonsteroidal anti-inflammatory drugs, disease modifying antirheumatic drugs and glucocorticoids, newer and safer drugs are continuously being searched, as long term usage of these drugs have resulted in adverse effects. Alternative medicine provides another approach for treatment of RA and currently a number of medicinal plants are under scientific evaluation to develop a novel drug. Various clinical and preclinical studies have reported the mechanism of action of herbal extracts and the role of phytoconstituents, such as flavonoids, terpenoids, alkaloids and sterols in attenuating the symptoms of RA by targeting the inflammatory biomarkers involved in the pathogenesis of RA[2].

The selected plant *Alpinia calcarata* (Haw.) Roscoe (Fam. Zingiberaceae), commonly called Snap ginger or Lesser galangal, and locally, Kolinji, is a rhizomatous perennial herb commonly used in the traditional medicinal systems and is cultivated in tropical countries including China, India, Srilanka and Malaysia. The rhizomes, the most important part of the plant, is known to possess a broad spectrum of medicinal and pharmacological properties. Decoction of the rhizomes is widely used in the treatment of bronchitis, cough, respiratory ailments, diabetes, asthma and arthritis[3]. Phytochemical analysis of the rhizomes has revealed the presence of major phytoconstituents such as polyphenols, tannins, flavonoids, steroid, glycosides and alkaloids[4]. Major phytoconstituents identified from *A. calcarata* include protocatechinic acid, quercetin, 4-O-methyl-syringic acid, vanillic acid, methyl cinnamate, 1,8-cineole, camphor, α-terpineol, β-pinene, α-pinene, camphene, endo-fenchyl acetate, etc[5].The anti-inflammatory activity of *A. calcarata* validated by the carrageenan-induced paw oedema model in rats concluded that inhibition of histamine and prostaglandin synthesis production was the probable mechanisms by which it mediates its anti-inflammatory action[6].

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Here, we selected two compounds, fenchol and 2,4-di-tert-butylphenol, from *Alpinia calcarata* for studying its antiarthritic potential using adjuvant induced arthritis model. Fenchol (2,2,4-trimethylbicyclo[2.2.1]heptan-3-ol) is a bicyclic monoterpenoid reported from various species of aromatic plants and is a commonly used volatile compound in fragrances and flavouring agents[7]; and 2,4-di-tert-butylphenol is a bioactive compound identified from several medicinal plants and lower organisms[8] that has been reported to possess various bioactivities including antioxidant[9], antifungal[10], antibacterial[11], herbicidal[12] and antibiofilm[13] activities.

Materials and Methods

Chemicals

Fenchol, 2,4-di-tert-butylphenol and diclofenac sodium were purchased from Sigma Aldrich (USA). Freund's complete adjuvant was obtained from Chondrex (WA). All other chemicals used were of highest quality available.

Experimental animals

Sprague Dawley (SD) rats (180-240 g) of both sexes obtained from the Small Animal Breeding Station, College of Veterinary and Animal Sciences, Thrissur were maintained in the animal house of Department of Biotechnology and Microbiology, Kannur University. The animals were maintained under standard environmental conditions with 12-h dark/light cycles and were fed with standard pellet diet (Krish Scientists Shoppe) and water ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee (KULS/IAEC/ 2013/08) and the guidelines of CPCSEA, Govt. of India were followed for the care and maintenance of animals.

Induction of Arthritis and treatment protocol

Animals were divided into 7 groups of 6 animals as follows: Gr. I, Normal rats; Gr. II, FCA induced arthritic rats; Gr.oup III, FCA + diclofenac (10 mg/kg body wt.); Gr. IV & V, FCA + DTBP (50 and 100 mg/kg body wt.); Gr. VI & VII, FCA + fenchol (50 and 100 mg/kg body wt.), respectively. Arthritis was induced by a single intradermal injection of 0.1 mL of Freund's complete adjuvant (10 mg/mL) into the plantar surface of the left hind paw of the animal under light ether anesthesia. The dosing of all the groups was started from day 9th once daily through oral route and the study was continued till day 24. Diclofenac (10 mg/kg bodywt.) was used as the standard drug in the study. The severity of arthritis was evaluated by the changes in in paw size using digital vernier calipers on the 3rd, 6th, 9th, 12th, 15th, 18th and 21st day after arthritis induction[14]. During the experimental period, the body weight was measured using a digital weighing balance every 3rd day after adjuvant injection. The mean changes in injected paw with respect to initial paw volume, were calculated on respective days and percentage inhibition of paw edema with respect to untreated group (control) was calculated using the formula:

$$Percentage\ inhibition = \left\{\left(1 - \frac{Mean\ changes\ in\ paw\ volume\ of\ treated\ rat}{Mean\ changes\ in\ paw\ volume\ of\ untreated\ rat}\right)\right\} X\ 100$$

On day 25th, the animals were euthanized and blood was collected for hematological and biochemical evaluation. Organs such as thymus spleen and liver were removed to determine the wet weight of organs. Ankle joints were separated for histological analysis

Visual arthritis scoring system

The morphological feature of the arthritis like swelling and erythema[15] was monitored by set of visual criteria as follows: normal paw = 0, mild swelling and erythema of digits = 1, swelling and erythema of the digits = 2, severe swelling and erythema = 3, gross deformity and inability to use the limb = 4 on respective days. Thus, the maximum possible score for both hind paws was 8[16].

Hematological and biochemical analysis

RBC, WBC, Hemoglobin, ESR and platelet count were estimated using Horiba 5 part Hematology analyser. serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP), Total proteins (TP) and C- reactive protein (CRP) were determined using standard diagnostic kits (Proton, Accurex) and analysed using Statfax autoanalyser.

Organ index

At the end of the experiment, rats were euthanized and liver spleen and thymus were promptly removed and weighed. The index of the organs was expressed as the percentage (%) wet weight of organ versus body weight[17]. The organ indexes were calculated using the following formula

$$Organ\ index = \left[\frac{Weight\ of\ organ(g)}{Body\ weight\ (g)}\right] X\ 100$$

Radiological analysis

Radiographic changes in RA conditions are useful diagnostic measures which indicate the severity of the

disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the developed stages (final stages) of arthritis[18]. X ray radiographs the hind paw of rats in each groups were taken using conventional X ray machine on day 24 and examined for radiological changes.

Histological analysis

Ankle joints were separated from the hind paw, weighed and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% Nitric acid, processed for paraffin embedding and sectioned at 3-5 μM thickness. The sections were stained with haematoxylin and eosin and evaluated under light microscope for the presence of hyperplasia of synovium, pannus formation and destruction of joint space.

Statistical analysis

All values were expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Post Hoc Tukey HSD using SPSS software version 20. A P value of <0.05 was considered statistically significant.

Results

Effect of 2,4-DTBP and Fenchol on paw edema in FCA induced arthritis

Intraplantar injection of FCA rapidly produced typical inflammatory swelling and redness in the injected left hind paw from day 1 which reached a peak on day 9. In arthritic control group, the paw swelling was maintained throughout the experimental period with gross deformity of the injected limb by day 24. The paw thickness of the injected paw were measured every 3rd day following FCA injection. The percentage inhibition of paw edema was calculated in each case and tabulated. Treatment with the standard drug Diclofenac showed a significant decrease in the edema from day 12 onwards when compared to control (P <0.05). Treatment with the compounds, DTBP and Fenchol, suppressed paw edema in a dose dependent manner. Treatment with the compounds at both doses exhibited a significant reduction in paw swelling when compared to control on day 24 (P <0.05) which was indicated by a decrease in the paw thickness measured (Fig. 1A).

Effect of 2,4-DTBP and Fenchol on arthritic score in FCA induced arthritis

All the groups of rat injected with FCA started showing signs of clinical inflammation indicated by swelling and redness of the left hind paw. The arthritic score was significantly increased from day 6 to 12 in arthritic control group which remained increased throughout the period of the study. Rats treated with Diclofenac showed significant decrease in arthritic score from day 12 till day 24. The arthritic score of the phytocompounds treated group showed a dose dependent decrease following treatment in a statistically significant manner when compared to control (Fig. 1B and Fig. 2).

Effect of 2,4-DTBP and Fenchol on body weight in FCA induced arthritis

The body weight of rats in all groups were monitored every 3rd day after adjuvant induction till the end of the experiment. It was observed that the normal rats gained weight progressively until the end

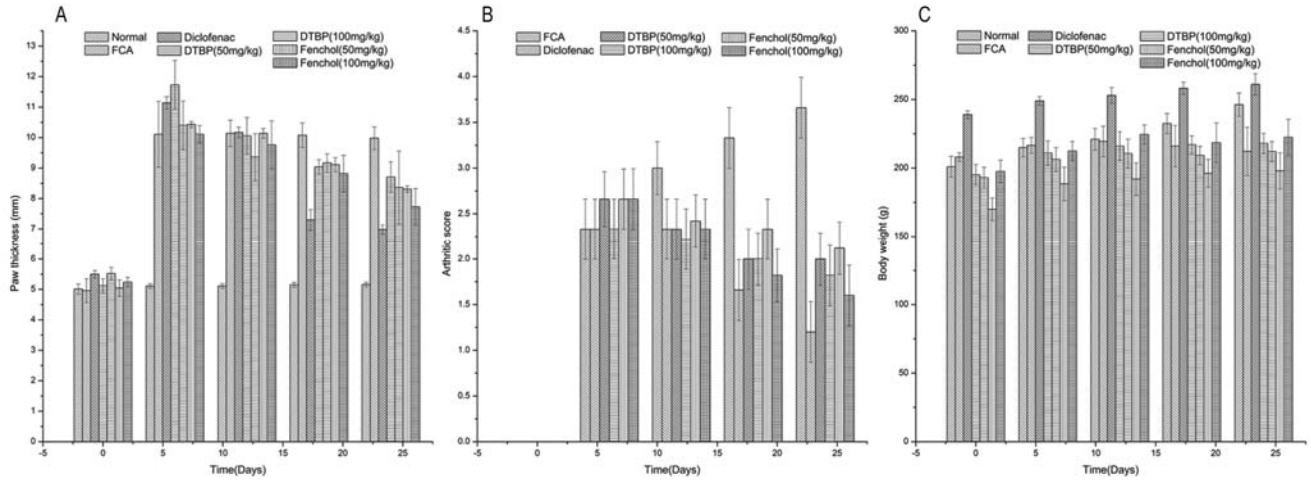

Fig. 1 — Effect of DTBP and Fenchol on (A) paw thickness; (B) arthritic score; and (C) body weight in FCA induced arthritis. [Values are expressed as mean ± SEM (n=6). [a] normal *vs*. other groups, [b] FCA versus other treatment groups. Symbols represent statistical significance *(P <0.05), #(P <0.001)]

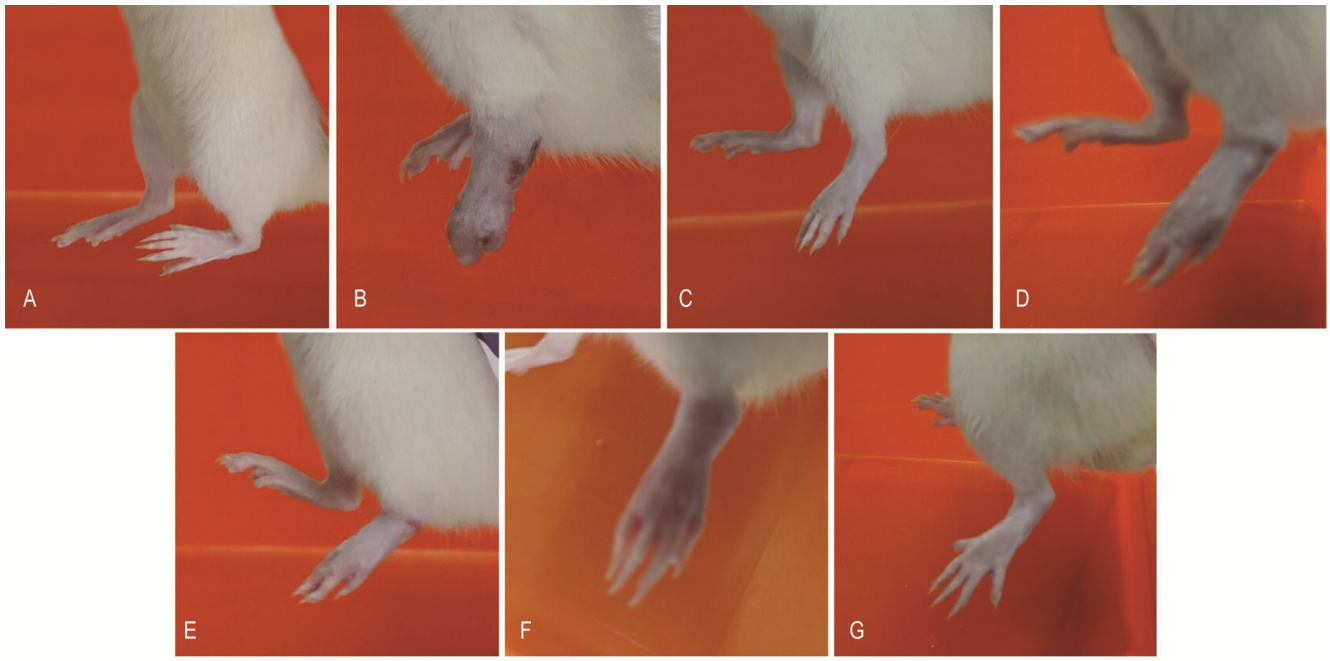

Fig. 2 — Effect of DTBP and Fenchol on the extent of paw swelling in FCA induced arthritis. (A) normal; (B) FCA group; (C) Diclofenac treated group; (D & E) DTBP (50 and 100 mg/kg) treated group; and (F & G) Fenchol (50 and 100 mg/kg) treated group

of the experimental period (24 days). Adjuvant arthritic rats also gained weight until the 10th day, but at lower rates than those of normal rats. After the 10th day, a gradual decrease was observed, so that the mean body weight did not differ significantly from the initial weight by day 24. The average gain in the body weight on day 24 as compared with the initial body weight in each group has been tabulated. Rats treated with Diclofenac showed a statistical significant increase in body weight gain (P <0.05) when compared to FCA group. Treatment with the phytocompounds showed a slight increase in body weight gain when compared to the arthritic group which was not much statistically significant (Fig. 1C).

Effect of 2,4-DTBP and Fenchol on hematological and biochemical parameters

There was a significant reduction in RBC count and haemoglobin in arthritic group when compared to normal (P <0.001). A significant increase in WBC count, ESR and platelet count was also observed in FCA group compared to normal group (P <0.05). Treatment with the standard drug and phytocompounds were followed by favorable outcome in the altered hematological parameters. The levels of liver enzymes serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP) was found to be elevated in FCA induced arthritic groups compared to normal rats. Treatment with diclofenac and phytocompounds at both doses (50 and 100 mg/kg) could significantly decrease the level of these enzymes indicating a beneficial effect. The elevated CRP level following arthritis induction was significantly reduced by treatment with diclofenac as well as phytocompounds (Tables 1 and 2).

Effect of 2, 4-DTBP and Fenchol on organ index in FCA induced arthritis

FCA induced arthritic group showed a significant increase in liver index when compared to normal rats (P <0.05). Treatment with Diclofenac, DTBP (50 mg/kg) and Fenchol (100 mg/kg) was accompanied by a significant decrease (P <0.05) in the liver index showing a beneficial effect. A slight increase in the weight of spleen was observed in FCA group compared to normal group, whereas no signification reduction of spleen index was observed in all treatment groups. The thymus index of the FCA group did not show any statistically significant change when compared to normal group or any other treatment groups (Table 3).

Radiological analysis

The radiographs of normal rats exhibited normal bone and cartilage morphology with no signs of soft tissue swelling, whereas those of the arthritis group

Table 1 — Effect of DTBP and Fenchol on hematological parameters in FCA induced arthritis. Values are expressed as mean ± SEM (n=6). [a] normal *vs*. other groups, [b]FCA versus other treatment groups. Symbols represent statistical significance *(P <0.05), #(P <0.001)

Group	Hb (g/dL)	RBC ($\times 10^6$ cells/mm^3)	WBC ($\times 10^3$ cells/mm^3)	ESR (mm/h)	Platelet (lakhs/Cumm)
Normal	13.86±0.20	6.96±0.37	9.23±1.62	4.26±0.40	3.36±1.19
FCA group	9.566±0.87$^{a\#}$	4.233±0.45$^{a\#}$	13.43±2.07^{a*}	13.51±2.02$^{a\#}$	10.58±1.01$^{a\#}$
Diclofenac	12.83±0.25^{a*b*}	6.80±0.40$^{b\#}$	9.96±0.57^{b*}	5.28±0.91$^{b\#}$	5.20±1.60$^{a*b\#}$
DTBP (50 mg/kg)	10.43±0.70$^{a\#}$	4.80±0.40$^{a\#}$	12.68±2.87^{a*}	9.32±1.64$^{a\#b*}$	10.25±0.83$^{a\#}$
DTBP (100 mg/kg)	12.58±0.60^{a*b*}	5.70±0.30^{a*b*}	10.06±1.01^{b*}	6.68±1.03$^{a*b\#}$	6.36±0.8.$^{a*b\#}$
Fenchol (50 mg/kg)	10.266±0.58$^{a\#}$	4.00±0.36$^{a\#}$	11.60±9.84	10.18±0.32$^{a\#b*}$	8.43±0.76$^{a\#b*}$
Fenchol (100 mg/kg)	12.93±0.25^{a*b*}	6.20±0.30$^{a*b\#}$	9.93±5.13^{b*}	6.39±1.04$^{a*b\#}$	5.63±0.70$^{a*b\#}$

Table 2 — Effect of DTBP and Fenchol on biochemical parameters in FCA induced arthritis. [Values are expressed as mean ± SEM (n=6). a- normal versus other groups, b- FCA versus other treatment groups. Symbols represent statistical significance *(P <0.05), # (P <0.001)]

Group	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Total Proteins (mg/L)	CRP (mg/L)
Normal	94.66±3.75	41.38±1.85	100.66±6.35	5.90±0.05	1.333±0.33
FCA group	322.66±6.48$^{a\#}$	73.66±4.90$^{a\#}$	394.00±18.19^{a*}	6.86±0.12$^{a\#}$	14.33±1.44$^{a\#}$
Diclofenac	214.33±22.02$^{a\#b\#}$	77.33±2.40$^{a\#}$	178.66±9.24$^{a\#b\#}$	6.86±0.08$^{a\#}$	4.33±0.69$^{a*b\#}$
DTBP (50 mg/kg)	250.66±4.40$^{a\#b*}$	62.00±5.12^{a*b*}	170.66±12.67$^{a*b\#}$	6.26±0.17^{b*}	9.00±2.07$^{a\#b*}$
DTBP (100 mg/kg)	141.66±21.16$^{a*b\#}$	54.66±5.68^{a*b*}	132.66±6.17$^{a*b\#}$	6.06±0.25^{b*}	4.66±0.87$^{a*b\#}$
Fenchol (50 mg/kg)	294.36±8.75$^{a\#}$	65.00±4.61$^{a\#}$	287.33±6.98$^{a\#b\#}$	6.4±0.25^{a*b*}	10.33±1.5$^{a\#b*}$
Fenchol (100 mg/kg)	254.33±10.26$^{a\#b*}$	54.66±0.87^{a*b*}	238.33±15.30$^{a\#b\#}$	6.00±0.11^{b*}	7.20±0.57^{a*b*}

presented with joint degradation, narrowing of joint spaces and soft tissue swelling. Diclofenac treatment effectively reduced the extent of pathological features of arthritis evident from the radiographs. Treatment with compounds also showed a dose dependent reduction in bone erosion and soft tissue swelling, suggesting a beneficial role in treatment (Fig. 3).

Table 3 — Effect of DTBP and Fenchol on organ index in FCA induced arthritis. [Values are expressed as mean ± SEM (n=6). [a] normal versus other groups, [b] FCA *vs*. other treatment groups. Symbols represent statistical significance *(P <0.05), #(P <0.001)]

Group	Organ index (%)		
	Liver	Spleen	Thymus
Normal	2.27±0.27	0.15±0.010	0.0260±0.001
FCA group	2.83±0.015^{a*}	0.1913±0.023	0.018±0.001
Diclofenac	1.90±0.26^{b*}	0.17±0.027	0.0243±0.007
DTBP (50 mg/kg)	2.15±0.085^{b*}	0.19±0.02	0.022±0.007
DTBP (100 mg/kg)	2.66±0.12	0.18±0.02	0.024±0.008
Fenchol (50 mg/kg)	2.40±0.065	0.19±0.008	0.027±0.10
Fenchol (100 mg/kg)	2.11±0.16^{b*}	0.18±0.007	0.025±0.002

Histological analysis

Histological analysis of the control group showed normal joint architecture and cell structure. The joint architecture was markedly abnormal in arthritic rats with massive infiltration of polymorphic neutrophils into the edematous synovium and joint cavity. Additional findings such as pannus formation, bone erosion, synovial hyperplasia were also evident. Treatment with Diclofenac significantly supressed the infiltration of inflammatory cells in synovial membrane and joint cavity. The rats treated with the phytocompounds showed significantly reduced pathological changes in a dose dependent manner compared to arthritic rats. Higher doses of the compounds showed a protective effect on the joint structure with marked reduction of articular cartilage destruction, bone erosion and inflammatory cell infiltration (Fig. 4).

Discussion

The use of animal models of RA has contributed greatly to the overall knowledge of processes/ mediators important in the generation of inflammation, cartilage destruction and bone resorption, thus leading to important advances in therapeutic intervention in this destructive disease[19].

Hind paw swelling is a characteristic feature of adjuvant induced arthritis which is monitored from day 9 (onset of disease) to 15 or greater depending on duration desired[20]. All rats showed an arthritic score >1indicating the incidence of arthritis before the initiation of treatment[21]. Several lines of studies have reported the efficacy of paw swelling as an index to measure arthritis severity in adjuvant induced arthritis model for the preclinical testing of both synthetic and herbal extract[22,23]. The changes in paw edema following arthritis induction and treatment analyzed by caliper measurements in the present study could clearly assess disease progression and remission.

A record of the weight changes which occur in treated and control rats during the development of the syndrome provides useful supplementary information for assessing the activity of the compounds examined[24]. It has been reported that during the development of the arthritic syndrome the rats

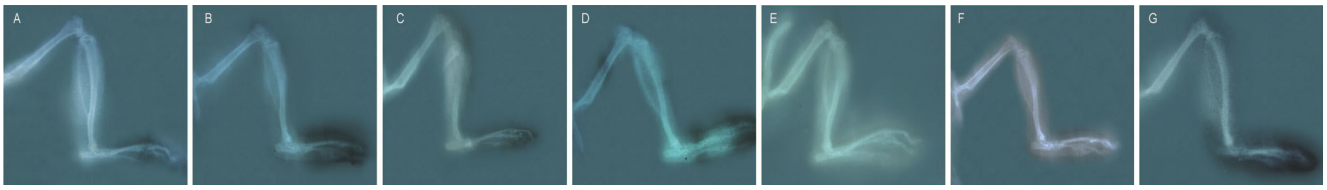

Fig. 3 — X-ray radiographs of rat paws. (A) normal; (B) FCA group; (C) Diclofenac treated group; D & E) DTBP (50 and 100 mg/kg) treated group; and (F & G) Fenchol (50 and 100 mg/kg) treated group

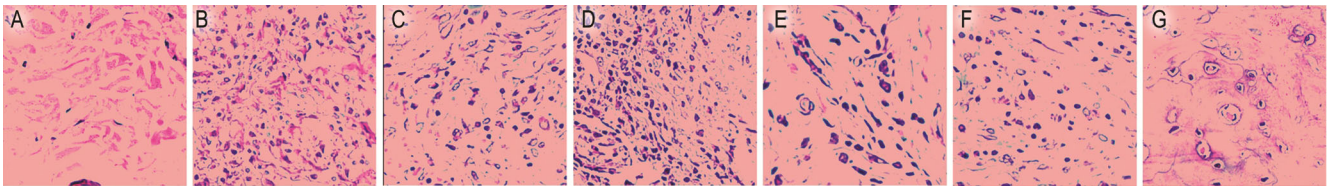

Fig. 4 — Histological analysis of joints. (A) normal; (B) FCA group; (C) Diclofenac treated group; D & E) DTBP (50 and 100 mg/kg) treated group; and (F & G) Fenchol (50 and 100 mg/kg) treated group

invariably lost weight. The heavier the rat at the time of injection, the greater was the weight loss in absolute terms[25]. The results of the changes in body weight from the present study are consistent with these findings[26].

The marked decrease in body weight of arthritic rats was associated with a marked increase in liver and spleen weights, results that coincide with the report of Bendele 2001, who stated that splenomegaly and hepatomegaly are the main extra-articular manifestations in the adjuvant arthritic model. The author also stated that the restoration of both organs is linked to agents that are active against adjuvant arthritis and that possess an anti-arthritic effect[18]. The present study was accompanied by an increase in liver weight which was beneficially affected by treatment, but no significant difference in the weight of spleen was noted in any of the treatment groups[27].

Patients with rheumatoid arthritis (RA) may present with haematological abnormalities either at the time of diagnosis, or during the course of their illness[28]. Anaemia, thrombocytosis and eosinophilia are observed commonly whereas neutropenia, thrombocytopenia, large granular lymphocytosis (LGL) and malignancies are uncommon[29]. A decrease in the level of hemoglobin and RBC, and increase in the level of WBC, platelet and ESR were noted in the present study in arthritic rats which were reverted towards normal in treatment groups[30]. Elevated liver enzymes in patients with rheumatoid arthritis may have various causes. These can range from the rheumatic disease itself, the anti-rheumatic medication or be the manifestation of an associated autoimmune disease[31]. It has been reported that there is a relationship between the severity of rheumatoid arthritis and the hepatic dysfunction indicated by certain liver function tests[32]. Estimation of CRP level is one of the most reliable and objective measure and a useful prognostic factor for disease progression to joint damage associated with RA[33]. As an inflammatory biomarker for RA, CRP correlates with disease activity, histological changes in the synovium, and radiological progression, responding very quickly to changes in disease activity[34]. The present study on biochemical parameters also confirmed a positive correlation between the level of liver enzymes and disease severity which were favorably affected by treatment. Similar was the results obtained in the case of CRP levels.

Radiographic analysis provides useful insights into the severity of arthritis by the degree of soft tissue swelling bony erosions and narrowing of joint spaces[35]. The major radiographic changed evident in the radiographs of present study was soft tissue swelling, joint degradation and narrowing of joint spaces which was beneficially affected by treatment[36]. The histological sections from the inflamed joints of RA patients exhibit infiltration of inflammatory cells and hyperplasia, which is responsible for the degradation of cartilage and bone[37]. The histological sections of joints from arthritic group were marked by the massive infiltration of neutrophils which was significantly decreased in treatment groups indicating a therapeutic potential of the tested compounds[38].

Conclusion

The results of the present study showed that both the selected compounds at the doses tested could

significantly affect the reduction in paw edema, alterations in hematological and biochemical parameters following arthritis induction. The significant reduction in the elevated liver enzymes following treatment with the compounds indicated that the tested compounds were safe in terms of hepatotoxicity. Taken together from the results presented, it can be concluded that the phytocompounds present in *Alpinia calcarata* possess significant anti-arthritic activity which supports the use of this plant in traditional medicine for arthritis treatment.

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Conflict of interest

Authors declare no conflict of interests.

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