# Anti-inflammatory Activities of *Cassia alata* Leaf Extract in Complete Freund's Adjuvant Arthritis in Rats

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***ABSTRACT***

***Objective:*** *To investigate the anti-inflammatory effects of a hexane extract of* Cassia alata *leaves in complete Freund's adjuvant (CFA) arthritis in rats.*
***Method:*** *A hexane extract of* Cassia alata *leaves was administered by oral gavage to CFA arthritic rats (500 mg/kg, n = 6). Controls received corn oil (2 ml, n = 6). The CFA arthritic model was induced by the injection of 0.5 ml (CFA) into the synovial cavity of the right knee joint of the hind leg of rats. The ability of the plant extract to reduce swelling as a sign of arthritic inflammation was assessed by obtaining the circumference of the knee joint before and for twenty eight days post arthritis induction. Reduction of leukocyte infiltration into the blood and synovial cavity of the arthritic rats were assessed using automated counting and Wrights method. Protection against cartilage erosion was also assessed histologically.*
***Results:*** Cassia alata *extract significantly (*$p = 0.0032$*) reduced knee circumference (swelling) in the CFA arthritic rats. Total and differential leukocyte counts in both blood and synovial fluid from* Cassia alata *treated animals were significantly (*$p \leq 0.05$*) lower than in control animals. Protective effects against cartilage degradation on the femoral head of the knee joint were observed in* Cassia alata *treated animals, as normal cartilage structure and chondrocyte arrangement were maintained.*
***Conclusions:*** *The results suggest that* Cassia alata *exhibits anti-inflammatory activities that should be further examined and potentially exploited for anti-arthritic therapies.*

**Keywords:** Arthritis, anti-inflammatory, *Cassia alata,* swelling

# Actividades Anti-inflamatorias del Extracto de Hojas de *Cassia alata* en Artritis Inducida en Ratas Mediante Adyuvante Completo de Freund

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***RESUMEN***

***Objetivo:*** *Investigar los efectos anti-inflamatorios del extracto de hexano de hojas de* Cassia alata *en artritis inducida por adyuvante completo de Freund (CFA) en ratas.*
***Método:*** *Un extracto de hexano de hojas de* Cassia alata *fue administrado por gavage oral a ratas artríticas por CFA (500 mg/kg, n = 6). Los controles recibieron aceite de maíz (2 ml, n = 6). El modelo artrítico de CFA fue inducido inyectando 0.5 ml (CFA) en la cavidad sinovial de la rótula derecha de la pata trasera de las ratas. La capacidad del extracto de la planta en cuanto a reducir la inflamación como signo de la inflamación artrítica, fue evaluada obteniendo la circunferencia de la rótula antes y durante veintiocho días posterior a la inducción de la artritis. La reducción de la infiltración de leucocitos en la sangre y la cavidad sinovial de las ratas artríticas fue evaluada usando el conteo automatizado y el método de Wright. También se evaluó histológicamente la protección contra la erosión del cartílago.*
***Resultados:*** *El extracto de Cassia alata redujo significativamente (*$p = 0.0032$*) la circunferencia de la rodilla (inflamación) en las ratas artríticas por CFA. Los conteos totales y diferenciales de leucocitos tanto en la sangre como en el líquido sinovial de los animales tratados con* Cassia alata *fueron significativamente (*$p \leq 0.05$*) más bajos en los animales del control. Los efectos protectores contra la*

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*degradación del cartílago en la cabeza femoral de la rótula fueron observados en los animales tratados con* Cassia alata, *ya que se mantuvieron la estructura normal del cartílago y las disposición de los condrocitos.*

***Conclusiones:*** *Los resultados sugieren que la* Cassia alata *exhibe propiedades anti-inflamatorias que deben ser examinadas ulteriormente y explotadas potencialmente para las terapias anti-artríticas.*

**Palabras claves:** artritis, anti-inflamatorio, *Cassia alata*, inflamación

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## INTRODUCTION

Adjuvants are commonly used to elicit an immune response by the host animal to an antigen. Once the immune system is stimulated and recruited, the actions of adjuvants produce the characteristic features of inflammation which include swelling, polymorphonuclear infiltration, tissue destruction and pain at the affected site in the host animals (1, 2). Complete Freund's adjuvant (CFA) is an antigen solution of attenuated mycobacterium tuberculosis emulsified in mineral oil and mannide mono-oleate, a surfactant (1). Complete Freund's adjuvant arthritis is an established monoarthritic model of arthritis, producing the hallmark features of oedema, cartilage erosion and pain which are similar to those displayed in rheumatoid arthritis in humans (3). As such, CFA arthritis is often used to screen potential anti-arthritic agents (4).

The *Cassia alata* (*C alata*) plant, commonly known as Candlestick Senna, Wild Senna, Ringworm Cassia and King of the Forrest, belongs to the family Caesalpiniaceae (5). It has been used traditionally in herbal medicine for the treatment of conditions such as constipation and various skin diseases including *Pityriasis versicolor* (6). The plant is native to America and Africa and has bilateral leaves being symmetrically opposed and folding together at night (7, 8, 9). *C alata* has been previously reported to have anti-inflammatory activities in a carrageenan induced rat paw oedema model of acute inflammation (10). However, no study has been done to evaluate the effectiveness of *C alata* in a chronic model of inflammation such as arthritis.

Rheumatoid arthritis (RA) is a chronic autoimmune, inflammatory disease which destroys cartilage and eventually bone (11). There are several limitations to the current treatment modalities that are used to manage the degenerative processes and symptoms of RA. The frontline drugs for RA treatment include disease modifying anti-rheumatic drugs (DMARDs), salicylates, nonsteroidal anti-inflammatory drugs (NSAIDs), selective cyclo-oxygenase-2 (COX-2) inhibitors and TNF-α inhibitors (12, 13). The use of these agents is limited by the precipitation of undesirable effects such as gastrointestinal ulcers, haemorrhage, exacerbation of hypertension, myelosuppression and neutropenia (14, 15). It is of paramount importance therefore, that new therapeutic avenues for the management of RA that will offer safer profiles of treatment continue to be explored. The exploitation of indigenous medicine has been a consistent source of anti-inflammatory drugs for decades (16). It is therefore pertinent that we continue to explore the herbal pharmacopeia for its therapeutic potential in inflammatory arthritis such as RA. The current study is the first to evaluate the anti-inflammatory properties of *C alata* in a chronic inflammatory model. We examined the effects of a hexane extract of the leaves of *C alata* on swelling, leukocyte infiltration and cartilage morphology in CFA induced arthritis in rats.

## MATERIALS AND METHODS

Studies were conducted in female Sprague Dawley rats (~ 300 g) obtained from the animal house in the Department of Basic Medical Sciences at the University of the West Indies (UWI), Mona. Ethical approval was obtained from the Ethics Committee of the Faculty of Medical Sciences, UWI, Mona. The animals were housed in cages under conditions of constant temperature and a 12h/12h light-dark cycle with access to food (Purina Chow, USA) and water *ad libitum*.

The *Cassia alata* plant was obtained from Clarendon, Jamaica, and authenticated by Mr Patrick Lewis at the herbarium, UWI, Mona Campus. A voucher specimen (#35349) was deposited at the herbarium. The leaves of *C alata* were removed from the stems, washed with running water and allowed to drip dry. Leaves were homogenized in methanol (100 g leaves –1000 ml methanol). The extract was filtered and concentrated *in vacuo* using a rotary evaporator. The methanol (Pharmaco-AAPER, USA) fraction was then partitioned between hexane (Pharmaco-AAPER, USA) and water. The hexane fraction was then concentrated *in vacuo* then re-dissolved in corn oil (Villasenor, 2002).

Animals were anaesthetized with ethyl ether and arthritis induced by injecting 0.5 ml CFA (1 mg/ml; Sigma Aldrich, USA) into the synovial cavity of the right knee joint (18) using a 29-gauge needle. The point of injection was marked in order to maintain consistency in measurement of the knee circumference. Animals were classified as arthritic when notable changes in swelling and redness were observed.

All animals received treatment *via* oral gavage. There were three treatment groups for all protocols (n = 6, per group). Group one received *C alata* (500 mg/kg), Group 2 received diclofenac (25 mg/kg: Sigma Aldrich, USA) and Group 3 (controls) received corn oil (2 ml: ACH Food Company). Treatment in all groups commenced 30 minutes pre-arthritis induction and continued daily for the duration of the 28-day study.

The knee circumference of each animal was measured at a marked point using a tape measure. This method of measurement was a modification of the method previously used by Levy *et al* (18). Each measurement was done in triplicate and then averaged. The measurement of the knee circumference began a day before induction of CFA arthritis, and continued for the duration of the study. At the end of 28 days, animals were sacrificed and the arthritic knee removed for histological studies.

Animals used for leukocyte count were also induced with CFA arthritis thirty minutes after receiving their treatments. For 14 days, animals continued to receive their treatment, after which they were sacrificed. Blood was then removed from the heart *via* vacupuncture, and the synovial fluid was collected from the knee joint through aspiration, after the injection of 25 µls of saline into the synovial cavity. Total leukocyte count in blood was done using an automated cell counter (Beckman Coulter, USA), and the leukocyte count in the synovial fluid was assessed using the cytospin (Thermo Scientific, USA) to create slides and staining by Wright's method.

On day 28 of the study period, animals were sacrificed and total knee joints resected. The total knee joint was fixed for four days in 4% formaldehyde (Mallinckrodt Backer Inc, USA) followed by decalcification in formic acid (BDH Chemicals Ltd, UK). The knee joint was then dehydrated and embedded in paraffin. Longitudinal sections of 3µm each were cut and then stained with either haematoxylin (Hopkin and Williams Ltd, UK) and eosin or safranin O (BDH Chemicals Ltd UK). Sections were then examined with light microscopy for damage to the articular cartilage lining the femoral head.

Each value was expressed as mean ± the standard errors of the mean (SEM). The unpaired Student's *t* test was used to determine significant differences between the control group and test groups. A value of $p \leq 0.05$ was used to define statistically significant differences.

## RESULTS

### Effect of *C alata* on swelling in CFA treated rats

The injection of CFA into the knee joint resulted in increased knee circumference. For the *C alata* group, there was a peak increase of 24.67% ± 0.1706 (relative to normal pre-induction measurements) on day two following the injection of CFA. Over the 28-day study, *C alata* produced a gradual reduction in the knee circumference to a final value that was only 0.8% ± 0.0233 greater than the pre-induction measurements. This reduction was significantly greater ($p = 0.009$) than that of corn oil treated controls, which showed a value that was 9.87% ± 0.0845 greater than the pre-induction values on day 28 (Fig. 1). Diclofenac, the known anti-arthritic drug, also produced a significant ($p = 0.009$) decline in the knee circumference to a value that was only 2.28% ± 0.0826 higher than the pre-induction measurements.

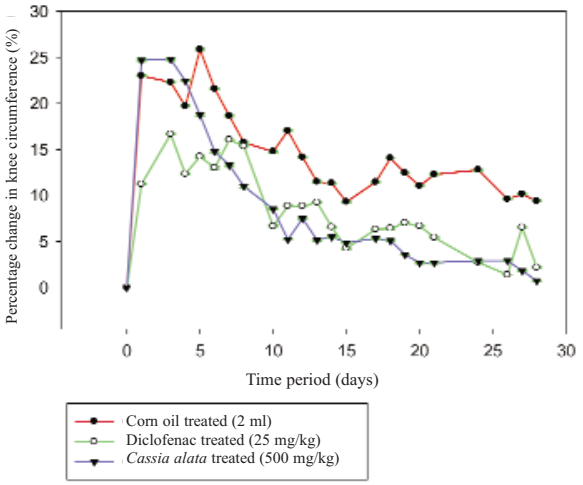


Fig. 1: Graph comparing percentage change in knee circumference of complete Freund's adjuvant (CFA) treated animals receiving *cassia alata* (500 mg/kg,) corn oil (2 ml) and diclofenac (25 mg/kg). Data are presented as means +/- SEM.

### Effects of *C alata* leaf extract on leukocyte count in the blood of CFA induced arthritic rats

Total blood leukocytes in CFA induced arthritic rats was determined on day 14 following induction. Figure 2 illustrates that the total leukocyte count ($61 \times 10^3 \pm 1 \times 10^3$) in the blood of animals treated with C *alata* leaf extract was lower than that of control animals ($101 \times 10^3 \pm 19 \times 10^3$). The difference, however, was not significant ($p = 0.125$). However, the total leukocyte count ($17 \times 10^3 \pm 17 \times 10^3$) in the blood of diclofenac treated animals was significantly (0.008) lower than that of corn oil treated animals (Fig. 2).

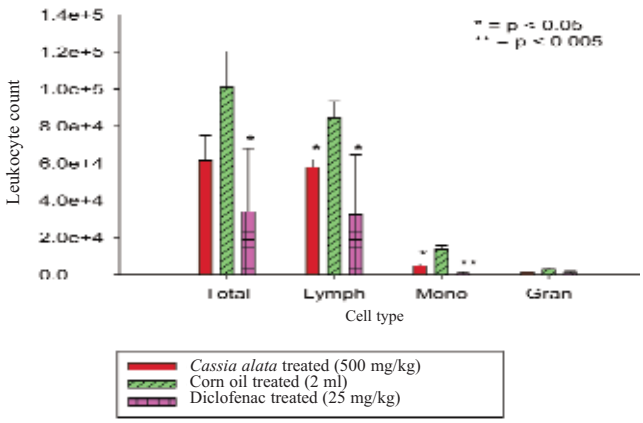


Fig. 2: Graph comparing the total differential leukocyte count in blood of CFA treated animals receiving *C alata* (500 mg/kg), corn oil (2 ml) and diclofenac (25 mg/kg). Asterisks (*) represent significant differences between the treated groups and corn oil treated controls Data are presented as means +/- SEM.

The differential lymphocyte ($56 \times 10^3 \pm 124$), monocytes ($4 \times 10^3 \pm 1 \times 10^3$), and granulocyte ($1 \times 10^3 \pm 307$) counts respectively, were significantly ($p = 0.013$, 0.002 and 0.053)

lower in the *C alata* treated animals (Fig. 2) when compared to the corn oil treated animals (lympho: mono: granulocyte).

**Effects of *C alata* leaf extract on leukocyte count in the synovial cavity of CFA induced arthritic rats**

Synovial fluid aspirates were analysed for leukocyte profiles on day 14 of the study. Figure 3 illustrates that the total leukocyte count (62.0 ± 22.0) in the synovial fluid of animals treated with *C alata* leaf extract was significantly (0.016) lower than that of the control animals (96 ± 4). By comparison, the total leukocyte count (0 ± 0) in the synovial fluid of diclofenac treated animals was significantly ($p$ = 0.008) lower than that of the corn oil treated animals (Fig. 3). The differential lymphocyte (16 ± 7), monocyte (2 ± 1), neutrophil (4 ± 2) and eosinophil (0 ± 0) counts respectively were also significantly ($p$ = 0.008, 0.002, 0.005 and 0.032) lower in the *C alata* treated animals when compared to those of the corn oil (lymphocyte 44 ± 2, monocyte 12 ± 1, neutrophil 25 ± 3 and eosinophil 14 ± 4) treated animals (Fig. 3). Similar results were obtained with diclofenac treatment (Fig. 3).

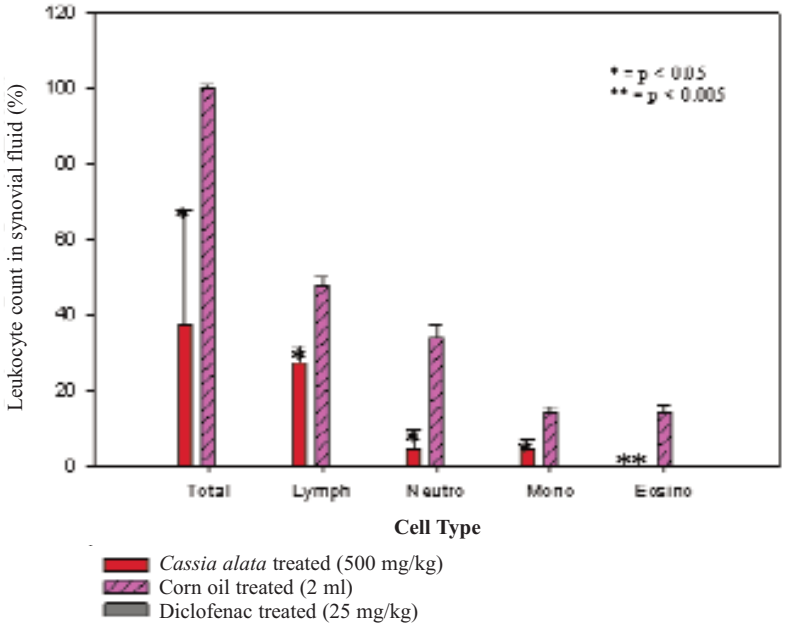


Fig. 3: Graph comparing the total and differential leukocyte count in synovial fluid of CFA treated animals receiving *C alata* (500 mg/kg corn oil (2 ml) and diclofenac (25 mg/kg). Asterisks (*) represent significant difference between the treated groups and corn oil treated controls. Data are presented as means +/- SEM.

**Effects of *C alata* leaf extract on femoral cartilage morphology of CFA induced arthritic rats**

Figure 4a shows cartilage lining the femoral head of the knee joint from a normal non-arthritic rat. The cartilage surface is smooth and undisturbed and normal chondrocyte morphology and arrangement in lacunae is depicted. Figure 4b shows cartilage from corn oil treated CFA arthritic animals. In these animals, there was significant erosion of the cartilage surface and there was disruption of normal chondrocyte arrangement. In CFA treated animals given *C alata* (Fig. 4c) and diclofenac (Fig. 4d), cartilage morphology and structure preserved as smooth articular surface as well as normal chondrocyte arrangement were observed.

**DISCUSSION**

Complete Freund's adjuvant is used extensively for the induction of experimental arthritis. Intra-articluar administration of the adjuvant results in the production of mono-arthritis within a few days. This mono-arthritis is usually less severe in its symptomatic manifestations and is more tolerable to the animal (19). Other modalities of injection of CFA which includes injection in the tail base results in a chronic arthritis involving multiple joints, promoting a widespread systemic disease that results in severe discomfort and distress (19). We therefore chose the intra-articular route of administration of CFA in our study.

Maximal oedematous swelling of the knee joints was observed in both treatment and control animals within two days of injecting 0.5 ml (1 mg/ml) CFA into the synovial cavity of the right knee joints. This swelling was indicative of the induction of the inflammatory response by CFA (20). The magnitude of swelling observed was consistent with results reported by Levy *et al* (18). The volume of CFA

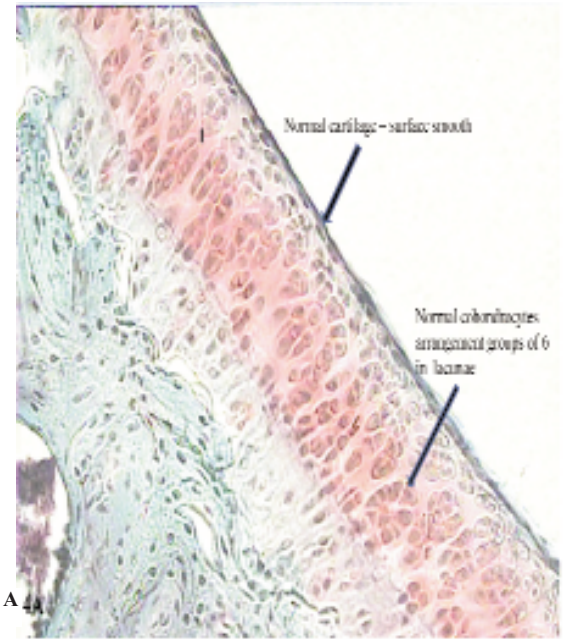


Fig. 4a: Representative micrograph of histological sections of cartilage lining the femur of a normal non-arthritic knee joint. Panel A illustrates normal articular cartilage lining the femur of a rat (mag $^{x}$ 10), scale bars = 50 µm.

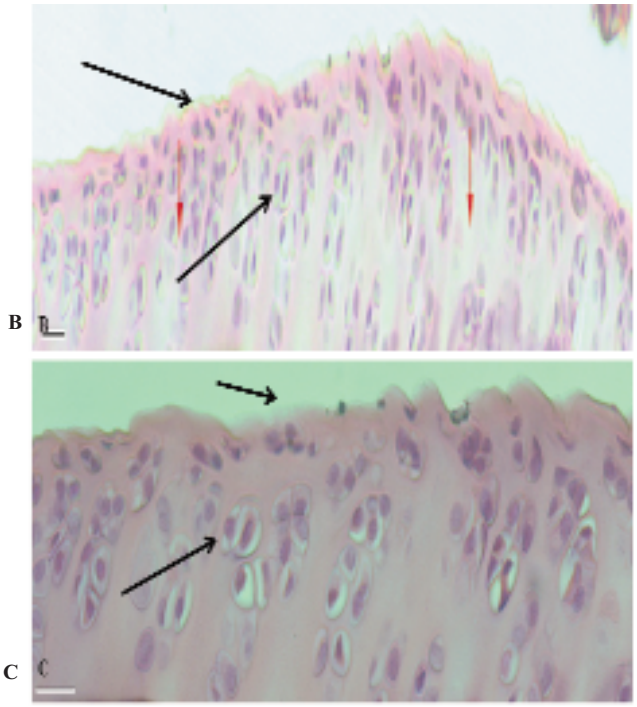


Fig. 4b: Representative micrograph of histological sections of cartilage lining the femur of the knee joint from CFA arthritic animals treated with corn oil (2 ml). Panels B and C illustrate damage to the articular cartilage lining the femur of a rat that was given a single dose of CFA (0.5 ml of 1 mg/ml) in the synovial cavity and a daily oral dose of (0.2 ml) of corn oil for 28 days. A (Mag $^{x}$ 10), B (Mag $^{x}$ 20), scale bars = 50 µm and 20 µm respectively. Arrows indicate areas of cartilage degradation and abnormal chondrocyte arrangement.

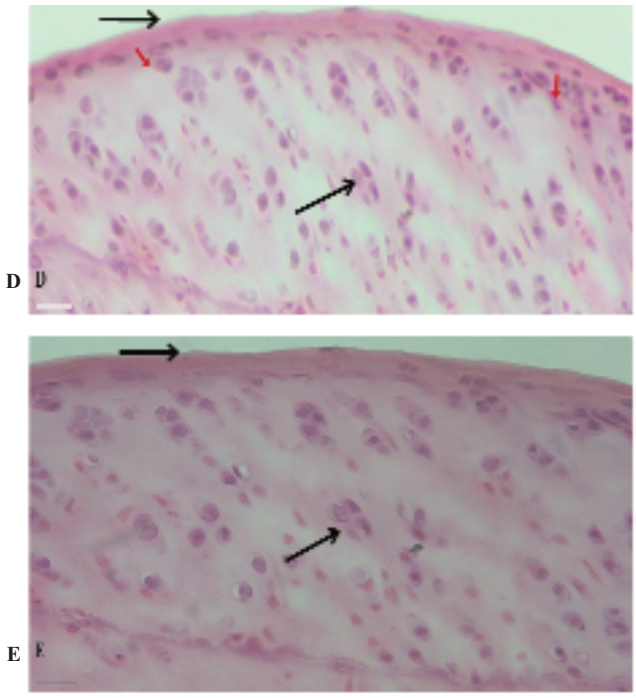


Fig. 4c: Representative micrograph of histological sections of cartilage lining the femur of the knee joint from CFA arthritic animals treated with *C alata* (500 mg/kg). Panels D and E show the articular cartilage lining the femur of a rat that was given a single dose of CFA (0.5 ml of 1 mg/ml) in the synovial cavity and a daily dose (500 mg/kg) of *C alata* for 28 days. C (Mag $^{x}$ 10), D (Mag $^{x}$ 20), scale bars = 50 µm and 20 µm respectively. Arrows indicate areas of smooth cartilage and normal chondrocyte arrangement.

injected into the knee was discounted from this response by using the knee circumference taken thirty minutes after the injection as the baseline from which the magnitude of oedematous swelling was calculated.

Succeeding the initial signs of oedema, both *C alata* and diclofenac progressively and significantly ($p < 0.05$) reduced the swelling in the knee joints to a final knee circumference on day 28 that was respectively 0.7% and 2 higher than the pre-induction values. Contrasting results were seen on the 28-day study period in the control animals that received corn oil. The final knee circumference value was 9.87% higher than the pre-induction measurement. The mag-nitude of swelling in *C alata* treated animals was maintained at significantly lower values than the corn oil controls for the duration of the study suggesting that the reduction of swell-ing was due to exposure of the animals to *C alata*. These findings also suggest that *C alata* (500 mg/kg/day) exhibits comparative anti-inflammatory potency to diclofenac (25 mg/kg/day), a main NSAID used in the pharmacotherapy of chronic arthritic conditions such as rheumatoid arthritis (21). The capability of diclofenac to reduce oedema is due to its potent inhibitory actions against prostaglandin $E_2$ [$PGE_2$] (22), and similar mechanistic routes for the anti-inflammatory actions of *C alata* will be explored

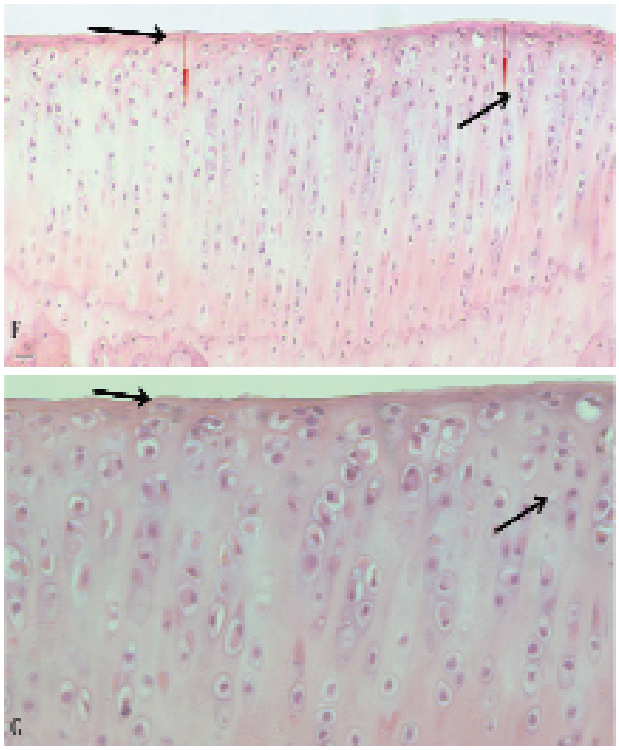

Fig. 4d: Representative micrograph of histological sections of cartilage lining the femur of the knee joint from CFA arthritic control animals that received diclofenac (25 mg/kg). Panels F and G show the articular cartilage lining of the femur of a rat that was given a single dose of CFA (0.5 ml of 1 mg/ml) in the synovial cavity and a daily dose (25 mg/kg) of diclofenac for 28 days. E (Mag $^{x}$ 10), F (Mag $^{x}$ 20), scale bars = 50 µm and 20 µm respectively. Arrows indicate areas of smooth cartilage and normal chondrocyte arrangement.

in future studies. Furthermore, anti-cyclo-oxygenase activity has been demonstrated for *C alata* in acute inflammatory studies (17), providing a strong basis that inhibition of COX might also be involved in the anti-inflammatory mechanisms exhibited by *C alata* in this chronic model.

In addition to reduction in swelling, significantly ($p < 0.05$) lower numbers of leukocytes were evident in the synovial fluid of animals treated with *C alata* when compared to the control group that received corn oil. This finding reflects the ability of *C alata* extract to reduce the infiltration of leukocytes into the inflamed synovial area, a hallmark sign of the active inflammatory process in CFA arthritis (18) and its human counterpart RA (23). This finding is suggestive of the anti-inflammatory mechanism of *C alata* exhibiting anti-chemotactic activity, as this directly affects cell migration (24). Further studies will therefore examine the effect of *C alata* extract on key mediators of leukocyte chemotaxis and migration such as TNF-alpha, IL-1 beta and vascular adhesion molecules (25). The differential cell counts of monocytes, lymphocytes, neutrocytes and eosinophils in synovial fluid of *C alata* treated animals was also significantly ($p < 0.05$) lower when compared to the controls. In particular, the monocyte count is of paramount interest as they are key players of the mononuclear phagocyte system (MPS). They develop from pro-monocytes in the bone marrow and migrate as monocytes through the blood to the peripheral tissues, where they replenish the pool of tissue and inflammatory macrophages (26).

Lower monocyte numbers therefore means lower macrophage numbers in *C alata* treated animals and suggests that the macrophage could be a major target for the anti-inflammatory activity of *C alata* extract. Macrophages are the major sources of pro-inflammatory mediators such as TNF-alpha, IL-1 beta (11), nitric oxide and reactive oxygen species (27) that promote pain, swelling and cartilage degradation during rheumatoid arthritis (28) and CFA arthritis (18). Here we suggest from the current findings that the reduction in swelling produced by *C alata* could have been due to a reduction in the numbers of macrophages and hence the production of pro-inflammatory mediators from these cells.

Analysis of leukocyte counts in the blood of *C alata* treated animals revealed no significant differences when compared to corn oil treated controls ($p = 0.125$). The differential counts of lymphocytes, monocytes and granulocytes, however, were significantly higher in controls when compared to *C alata* treated animals. Generally, the leukocyte count in blood of rheumatoid arthritic patients is within the normal range but leukocyte counts may be mildly elevated secondary to inflammation and may correlate with arthritis severity (29, 30). The cell type responsible for the elevated numbers usually varies (30) and there is little published data on the occurrence of leukocytosis in rheumatoid arthritic patients. The results suggest that *C alata* extract may influence white blood cell profiles in blood of CFA arthritic rats and this might have implications for further studies and its application to leukocytosis in rheumatoid arthritis.

Histopathological analysis of cartilage from *C alata* (Fig. 4c) and diclofenac (Fig. 4d) treated animals revealed that there was preservation of cartilage integrity and morphology. This was in contrast to observations for corn oil treated controls where there was significant erosion of the articular cartilage surface and loss of normal chondrocyte arrangement (Fig. 4b). The evidence points to a protective mechanism of *C alata* against the chondro-destructive chemical mediators that usually direct articular insult in inflammatory arthritis. Such mediators include prostaglandin $E_2$, nitric oxide, and the pro-inflammatory cytokines TNF-alpha and IL-1 beta. In particular, prostaglandin $E_2$ has been associated with collagen breakdown and proteoglycan loss in cartilage (31). Nitric oxide, TNF-alpha and IL-1 beta contribute to cartilage erosion by the activation of metalloproteinases and the inhibition of proteoglycan sysnthesis (32, 33). In further studies, we will evaluate the effect of *C alata* on the production of these mediators. Finally, the present study also reports a protective effect of diclofenac against

cartilage erosion that was of similar magnitude to that observed for *C alata*. This activity of diclofenac has been observed in arthritis and is linked to an inhibition of monocyte superoxide production and proteoglycan degradation (34, 35).

In conclusion, this study reports anti-inflammatory activity of *C alata* (500 mg/kg/day) in CFA arthritis that is characterized by reduced swelling, leukocyte infiltration into synovial fluid and cartilage degradation. The magnitude of the anti-inflammatory activity of *C alata* is comparable to that of diclofenac, a standard NSAID. These findings suggest that further studies should be conducted to evaluate the potential applicability of this extract in therapy for chronic arthritic conditions such as rheumatoid arthritis.

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